UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

The Impact of Diet in Pregnancy on Fetal Renal and Cardiovascular Development

by

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ABSTRACT

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THE IMPACT OF DIET IN PREGNANCY ON FETAL RENAL AND CARDIOVASCULAR DEVELOPMENT

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The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that alterations in fetal nutrition results in developmental adaptations that permanently change structure, physiology and metabolism of the offspring to enable survival. These adaptations during early development may determine an individual's susceptibility to a variety of health problems including cardiovascular (CV) disease and related disorders in adult life. It has been proposed that this association may result from impaired prenatal kidney growth and lower nephron endowment at birth induced by suboptimal *in utero* nutrition. However, the effects of maternal nutrient restriction on fetal kidney development have not been investigated as yet. The aim of this thesis was to investigate if maternal nutrition restriction during gestation results in altered renal or CV development.

In the first study ewes received either 100% (C) or 50% (R) of total nutrient requirements for the first 31 days of gestation (dGA), and 100% requirements thereafter. In the second study ewes received either 100% (C) of total nutrient requirements throughout gestation, 40% (E) from 1–31 dGA or 50% (L) from 104 dGA onwards, at all other times ewes were fed 100% requirements. In late gestation, fetuses were surgically instrumented and basal CV and renal parameters, and responses to a number of stimuli were measured.

The first study found that a 50% maternal nutrient restriction did not alter fetal body or organ weights, kidney biometry, basal CV function or baroreflex during late gestation. 50% maternal nutrient restriction had no effect on fetal renin angiotensin system (RAS) in terms of CV or renal response to frusemide. However, the responsiveness to Angiotensin II (Ang II) was blunted in the maternal restricted fetuses. The second study found that neither a more severe peri-implantation nor a late gestation maternal nutrient restriction altered any of the fetal parameters measured in the first study. Maternal nutrient restriction did not alter fetal nephron number in late gestation. There was no difference between the groups in the overall CV response to hypoxia.

These findings suggest that poor *in utero* nutrition does not alter renal development and function, basal CV control, baroreflex or chemoreflex in fetal life. Peri-implantation restriction blunted the fetal mean arterial pressure response to Ang II, dependent on intensity of challenge, which may indicate altered Ang II receptors populations in the peripheral vasculature. In conclusion reduced maternal nutrition during peri-implantation and late gestation, periods previously implicated as critical periods of development, appear to have no effect on fetal renal development.

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Abbreviations

11βHSD2	11β-hydroxysteroid dehydrogenase
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotrophic hormone
AFRC	Agriculture and Food Research Council
Ang I	Angiotensin I
Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
Aogen	Angiotensinogen
AT1	Angiotensin II type 1 receptor
AT2	Angiotensin II type 2 receptor
AUC	Area under the curve
AVP	Arginine vasopressin
BCS	Body condition score
CHD	Coronary heart disease
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CVD	Cardiovascular disease
CVO	Combined ventricular output
dGA	Days of gestation
DOHaD	Developmental origins of health and disease
ECoG	Electrocorticogram
EGF	Epidermal growth factor
FFA	Free fatty acids
FGF	Fibroblast growth factor
GFR	Glomerular filtration rate
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
IUGR	Intrauterine growth restriction
MAP	Mean arterial pressure
mRNA	Messenger ribonucleic acid
PAR	Predictive adaptive response
pCO_2	Carbon dioxide partial pressure
PDGF	Platelet-derived growth factor
PNMT	phenylethanolamine N-methyltransferase
pO_2	Oxygen partial pressure
RAS	Renin angiotensin system
rRNA	Ribosomal ribonucleic acid
RVC	Royal Veterinary College
TGF	Transforming growth factor
wGA	Week of gestation

Declaration of the Author

I, Lucinda Braddick declare that the thesis entitled 'The impact of diet in pregnancy on fetal renal and cardiovascular development' and the work presented in it are my own. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this university or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this has always been clearly attributed;
- where I have quoted the work of others, the source is always given. With the exception of such quotations this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as:

Braddick L, Burrage D, Noakes DE, Hanson MA, & Green LR (2005). The effect of early gestation nutrient restriction on late gestation fetal cardiovascular responses to angiotensin II in sheep. *Journal of the Society for Gynecologic Investigation* **12**, 113A.

Braddick L, Burrage D, Cleal JK, Noakes DE, Hanson MA, & Green LR (2006). Moderate early gestation undernutrition has no effect on kidney size and function in late gestation sheep. *Journal of the Society for Gynecologic Investigation* **13**, 207A.

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Finally, thanks to my family for being so supportive always, especially the terrible twins...

1. Introduction

1.1 Chronic disease

Cardiovascular disease (CVD) is the biggest cause of death in the UK, accounting for about 40% of all deaths (BHF, 2004). Globally, nearly 30% of mortalities can be attributed to CVD (WHO, 2003). The two main diseases in this category are coronary heart disease (CHD) and stroke, which are both caused by blockage in an artery. Although mortality from CHD is falling rapidly it still causes about one in five deaths in men and about one in six deaths in women each year in the UK (BHF, 2004). A rapid increase in CVD has been observed in developing countries, where it is predicted to become the leading cause of death by 2010 (WHO, 2003). The speed of the increase of instances of CVD excludes a purely genetic effect and suggests that other factors must play a major role.

1.2 Epidemiology

In Britain the highest rates of heart disease were found to be in the lowest income groups and poorest areas, and these areas also displayed the highest infant mortality rates in the 1920's (Barker & Osmond, 1986). These observations led to retrospective epidemiological studies in Britain, which showed that low birth weights within the normal range were associated with an increased risk of CHD in adult life (Barker & Osmond, 1986; Barker *et al.*, 1990; Barker *et al.*, 1993). Birth weight on its own is a crude measurement of growth, and where body proportions were recorded at birth, associations have been seen between CHD risk and several measurements including overall small head circumference, shortness and thinness, which may reflect altered fetal growth (Barker *et al.*, 1990; Barker *et al.*, 1993). The association between CHD and birth weight parallel associations between birth weight and hypertension and diabetes, which are both major risk factors for heart disease (Barker *et al.*, 1989; Hales *et al.*, 1991).

Raised blood pressure in adult life has been associated with low birth weight in a number of studies around the world (see (Law & Shiell, 1996) for a systematic review). This effect is observed within the normal distribution of birth weights, and not just those delivered pre-term or classified as clinically growth restricted. Alcohol consumption is linked with increased blood pressure (Barker *et al.*, 1990), but the link with birth weight appears to be independent of this (Barker *et al.*, 1990). Increased adult body mass index has also been connected with raised blood pressure, and the highest pressures seem to occur in those individuals who are small at birth and become obese as adults (Eriksson *et al.*, 2001). Therefore it has been hypothesised that a stimulus in the early environment can lead to changes in growth *in utero* which can be exacerbated by obesity in later life, and ultimately leads to an increased instance of raised blood pressure and CVD (figure 1.1).

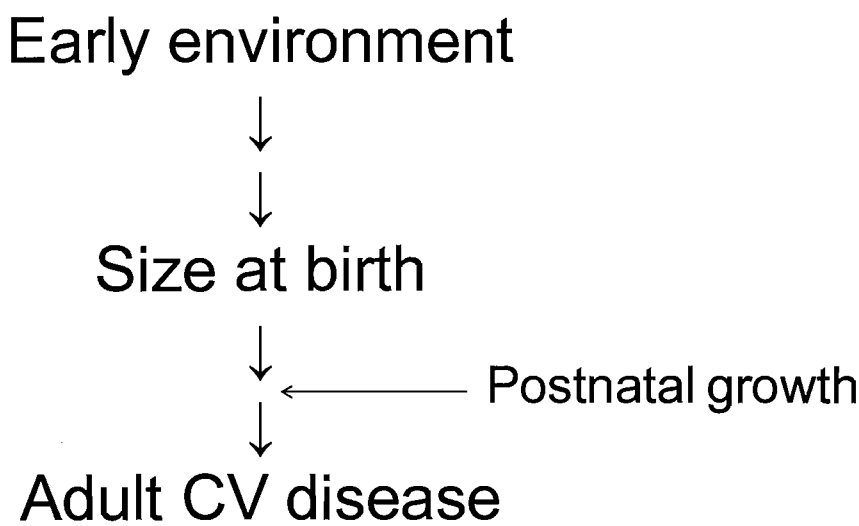

Figure 1.1 Summary of hypothesis founded on evidence of epidemiological studies.

1.3 Developmental Origins of Health and Disease (DOHaD)

The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that alterations in fetal nutrition result in developmental adaptations that permanently change structure, physiology and metabolism to enable immediate survival and/or promote suitability of an individual for its future environment. It is thought that these adaptations during early development affect an individual's susceptibility to a variety of health problems including hypertension, heart disease and related disorders in adult life (Barker, 1998).

There are several types of response to early environment that may occur. The response may be developmentally disruptive with no adaptive value, such as the responses to environmental teratogens (Finnell *et al.*, 2002), or it could confer immediate survival advantages, as is the case when alterations in regional blood flow ensure that no damage occurs to vital organs such as the brain during reduced fetal oxygen supply (Gluckman & Hanson, 2004a). A third type of response, termed a predictive adaptive response (PAR), has been proposed that appears to have mainly future adaptive value. One example of a PAR is the coat of the meadow vole which is thicker at birth if the offspring is born at a time of decreasing day length therefore, thus preparing the offspring *in utero* for winter (Gluckman & Hanson, 2004b). These PARs are believed to allow the developing organism to set its postnatal physiological phenotype to match its predicted postnatal environment to give optimal chance of survival (Gluckman & Hanson, 2004c). Where a mismatch occurs between the predicted and actual postnatal environment it may lead to an increased risk of disease (Gluckman & Hanson, 2004d).

A number of animal studies have been used to investigate the effects of poor intrauterine environment on the mechanisms which may predispose to raised blood pressure and CVD in later life. These have revealed that the severity and the duration of the challenge are key in determining the level of impact on the developing fetus (McMillen & Robinson, 2005e). The physiological effects of reduced nutrition in pregnancy are not necessarily a result of reduced fetal growth, as there is a graded relationship between size at birth (within the normal range) and raised blood pressure in epidemiology and animal models. This raises the possibility that

placental or fetal compensatory mechanisms that occur in response to reduced maternal nutrition preserve gross fetal growth and hence birth weight, but nonetheless have physiological effects with consequences in later life.

1.4 Nutritional requirements during pregnancy

It is important that nutrient supply during pregnancy exceeds the basic metabolic requirements of the fetus to enable growth. These nutrients are required to provide the energy for growth, the accretion of new tissues and fuel reserves also to sustain metabolic needs basally, and for breathing and body movements in late gestation. The fetus gains nutrition from a number of sources, primarily those provided by the mother but also placental and endogenous sources (figure 1.2). In early and mid gestation the placenta also has high demands for its growth and maintenance, and its growth rate is greater than that of the fetus (figure 1.3) (Schneider, 1996a). The period of early to mid gestation coincides with the period of maximal placental growth.

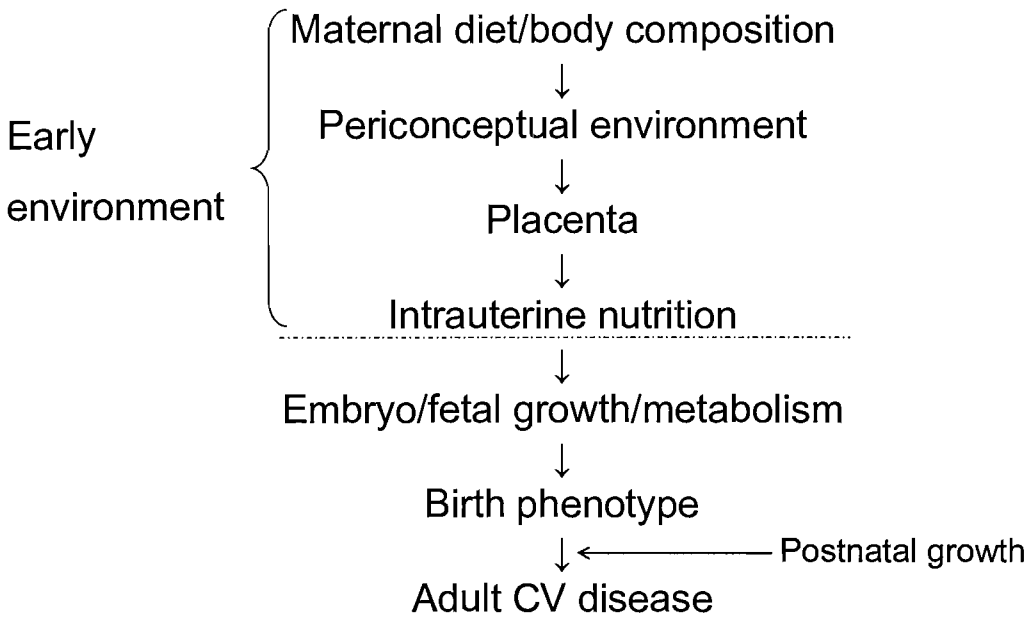

Figure 1.2 Factors in early environment may affect adult phenotype and disease risk.

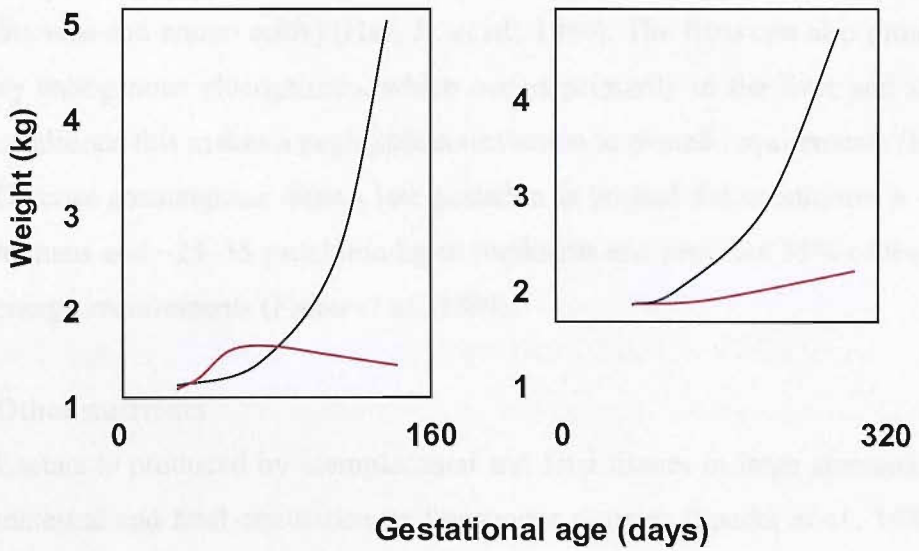

Figure 1.3 Growth rates of the fetus (black) and placenta (red) in the sheep (left) and human (right) adapted from (Schneider, 1996b).

Oxygen

Oxygen crosses the placenta down the concentration gradient by passive diffusion from maternal to fetal blood (pO_2 maternal aorta ~95 mm Hg, pO_2 fetus ~25 mm Hg) (Silver, 1984b). The majority of this oxygen is utilised by uteroplacental tissues before it reaches the fetus. Oxygen is the only nutrient that the fetus is unable to synthesise or store therefore its supply is vital. Oxygen consumption by the fetus in late gestation is ~350 μmol/min/kg fetal body weight in humans and about ~315μmol/min/kg fetal body weight in sheep (Hanson *et al.*, 1995).

Glucose

Glucose is transported across the placenta by facilitated diffusion and therefore requires transporters and a concentration gradient (Johnson & Smith, 1980). The gradient for transport of glucose is determined by maternal and fetal blood glucose concentrations. In humans, fetal levels are normally 60–70% of maternal values (>4.0 mmol/l) (Silver, 1984a). In ruminants glucose levels are lower, fetal sheep levels are 25–30% of maternal values (2.5–3.5 mmol/l) (Hay, Jr. *et al.*, 1984). In mid- and late gestation the rate of facilitated glucose transport across the placenta increases in both humans and sheep (Hay, Jr., 1991). Uteroplacental tissues consume a significant proportion of the glucose before it reaches the fetus for oxidation or non-oxidation purposes (such as lipogenesis, glycogenesis, conversion to lactate,

fructose and amino acids) (Hay, Jr. *et al.*, 1984). The fetus can also produce its own glucose by endogenous glucogenesis, which occurs primarily in the liver and kidneys, but in basal conditions this makes a negligible contribution to overall requirements (Hay, Jr. *et al.*, 1984). Glucose consumption during late gestation in normal fed conditions is ~45 μmol/min/kg in humans and ~23–35 μmol/min/kg in ruminants and provides 35% of the carbon and 30% of energy requirements (Fisher *et al.*, 1980).

Other nutrients

Lactate is produced by uteroplacental and fetal tissues in large amounts and is released into maternal and fetal circulation by transporter proteins (Sparks *et al.*, 1982). Amino acids are essential for oxidation, protein synthesis and as a source of carbon and nitrogen for other metabolic processes. These can be supplied either transplacentally or by synthesis within fetoplacental tissues (Carter *et al.*, 1991). Essential amino acids that cannot be synthesised *de novo* must be maternally derived. There are a number of different forms of lipid found in the fetus including triglycerides, phospholipids and free fatty acids (FFA). The amounts vary between species and nutritional states. In the fetus lipids are essential for growth but appear to have a less easily defined role in fetal oxidative metabolism. Fetal FFA can come from the mother by placental transfer, from synthesis in the fetus or from triglyceride and phospholipids break down (Fowden, 1995).

1.5 Fetal growth

Genetically programmed developmental patterns are intrinsic to each tissue and control fetal growth. These patterns can be modified by circulating hormones and other factors. Fetal growth follows a strict sequence, and the impact of any changes in nutrient levels is therefore dependent on the stage of development. Organogenesis starts in early gestation whereas most fetal increase in size occurs in the last third of gestation, but each tissue has its own growth and maturation timetable and may be more affected by adverse conditions when their metabolic demands are high.

The fetal period is a phase of tissue maturation and rapid growth due to cell division. In humans and sheep fetal weight follows a sigmoid growth curve, it is slow for the first few

weeks and then increased exponentially until near term (Schneider, 1996c)(figure 1.3). Human fetuses grow from 8 g at 8 weeks to about 3,400 g at birth, a 425-fold increase (Marsal *et al.*, 1996). The fetus grows in length mainly in the second trimester (4–6 months) and most of the weight is put on in the third trimester (7–9 months). The fetal growth trajectory is influences by the number of fetuses carried per pregnancy, maternal size, age, placental size and parity. Studies in sheep have shown that fetal growth rate remains constant until about 111 dGA and then gradually decreases for the remainder of gestation (Mellor & Murray, 1981). Most of fetal growth therefore occurs in the second half of gestation (70–147 dGA in sheep) and is controlled by the interaction of genome with the availability of oxygen and glucose and the endocrine responses to variation in their supply.

In humans the first organ system to start developing is the nervous system which begins to form about 18 day of gestation (dGA) (Bolender & Kaplan, 2004). At 22 dGA the heart starts beating and begins weak circulatory movement of fluids by the following day (Bolender & Kaplan, 2004). A functional circulatory system is necessary for growth beyond this stage as simple diffusion can not supply sufficient oxygen to support cell metabolism (Bolender & Kaplan, 2004). During the third week of gestation (wGA) development lung buds arise from the endoderm of the foregut and branching morphogenesis is initiated (Wert SE, 2004). The liver bud arises from an outpouch of the distal foregut from four wGA (Bolender & Kaplan, 2004). The pronephros is the first of the embryonic kidneys and begins development from about three wGA (Hinchliffe *et al.*, 1992).

The kidneys of both humans and sheep are fully developed by birth (figure 1.4). In humans nephrogenesis is completed by the 36^{th} wGA (term: 40 wGA) and in sheep by 120 dGA (where term: 145 dGA) (Gimonet *et al.*, 1998). The development of the mammalian kidney (metanephros) can only occur after it has been preceded by the formation and involution of two embryonic kidneys known as the pronephros and the mesonephros (Brace *et al.*, 1998). In humans the pronephros is rudimentary and non functioning, and appears during the third wGA and regresses by the fifth wGA (Brace *et al.*, 1998). The mesonephros appears at about three to four wGA, when they develop a glomerulus like filtering system. These organs then begin to degenerate from the fifth to the twelfth wGA (Brace *et al.*, 1998). The ureteric bud is

formed at week 5 and goes through many divisions, beginning with the development of the metanephros from week 6 and continuing with the growth of the individual nephrons. Approximately two thirds of the nephrons develop during the last trimester of gestation (Hinchliffe *et al.*, 1992).

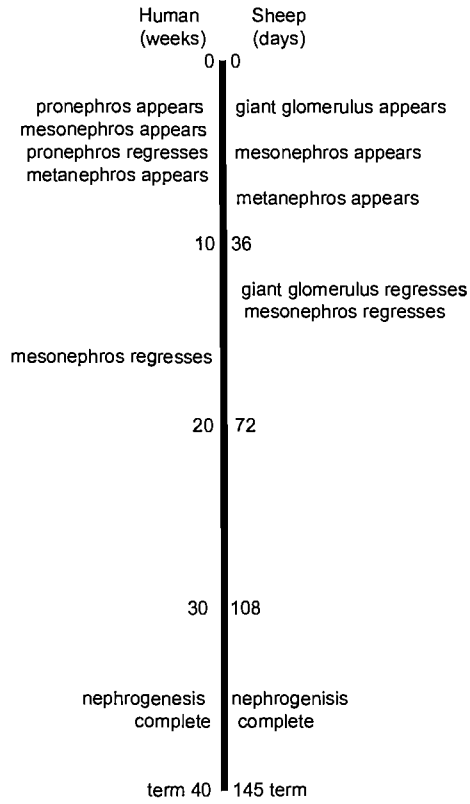

Figure 1.4 Kidney development in the fetus, left: human, right: sheep (Davies & Davies, 1950; Wintour *et al.*, 1998).

In sheep the pronephros never develops as such, however there is a 'giant glomerulus' that develops instead, which is comprised of about 15 fused glomeruli in an irregular mass (Brace *et al.*, 1998). The mesonephros is first observed at 15 dGA, and unlike the human mesonephros it functions and the filtrate it produces makes up the early allantoic fluid (Brace *et al.*, 1998). At about 30 dGA the mesonephros reaches its maximal size and then slowly regresses (Brace *et al.*, 1998). The ureteric bud is formed at 27 dGA and develops in a similar way as human metanephros, and nephrogenesis is completed by 10 dGA. A number of studies have shown that fetal nephrogenesis is susceptible to disruption by challenges at specific time points, which is discussed in section 1.7.

1.5.1 Regulation of fetal growth

As well as this internal timetable, each organ is also reliant on various external factors, including hormones which modify growth rates and trigger maturation. Hormones found in the fetal circulation are derived from the maternal circulation, uteroplacental tissue, fetal endocrine gland secretion and para/autocrine signalling. Maternal hormones have varying abilities to cross the placenta, which acts as a barrier to the passage of some molecules from mother to fetus.

Hormones may be secreted by the endocrine glands of the fetus, which are functional from early gestation and become more responsive as gestation progresses. In humans the hypothalamus differentiates between 6–12 wGA and neurohormones can be detected between 8–16 wGA (Thorburn & Harding, 1994). Pituitary hormones can be detected in fetal plasma between 7 and 14 wGA (Thorburn & Harding, 1994). Rudimentary adrenal glands which are able to secrete steroids are present from the eighth wGA (Mesiano & Jaffe, 1997). The thyroid gland becomes progressively more responsive to thyrotrophin releasing hormone during the last half of gestation (Thorburn & Harding, 1994), whereas the parathyroid glands are able to secrete parathyroid hormone from as early as 10 wGA (Thorburn & Harding, 1994). The testis can synthesise testosterone from 6–7 wGA, and although the ovaries are capable of secreting androgens and oestrogen from early development, there is no evidence that they do (Thorburn & Harding, 1994). Locally acting hormones can also be released from cells to adjacent cells in a paracrine fashion (Thorburn & Harding, 1994).

Insulin has a fundamental role as a mitogen in the early embryo, and its absence results in severe growth retardation (Hill, 1995). The main drivers of fetal growth are peptide growth factors including insulin like growth factors I and II, fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF) and transforming growth factors (TGF) α and β (Hill, 1995). Gene manipulation experiments have shown that peptide growth factors are essential not only for overall growth but also for specific events during the formation and differentiation of tissues and organs in the embryo (Hill, 1995). Maternal nutrient restriction can reduce growth factor abundance, which would be expected

to compromise fetal organ development depending on the timing and duration of the nutrition restriction (Bauer *et al.*, 1995).

Cortisol maintains fetal blood pressure, and increasing levels of this hormone near birth (cortisol surge) play a role in the maturation of organs in preparation for postnatal life. For the majority of gestation glucocorticoids are low in concentration in the fetus and are derived from the mother via a maternal-fetal concentration gradient. Placental 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) catalyses the rapid metabolism of cortisol to inert forms and maintains the concentration gradient (Murphy *et al.*, 1974; Bernal *et al.*, 1980). Placental 11βHSD2 activity is regulated by nutritional and endocrine factors and varies widely between species (Seckl, 2001; Clarke *et al.*, 2002). This variation in activity parallels the magnitude of the maternal-fetal cortisol concentration gradient (Fowden & Forhead, 2004a). Overexposure to synthetic glucocorticoids via maternal administration slows fetal growth in rats, sheep, monkeys and humans (Seckl, 2001), and can have various effects on the differentiation of a number of organs and tissues including kidneys, lungs, muscle and fat (Fowden *et al.*, 1998).

Angiotensin II (Ang II) has also been implicated in the regulation of cellular growth, and is known to mediate cell growth in cardiac myocytes, fibroblasts and in vascular smooth muscle cells via the Ang II type 1 receptor (AT1) pathway (Dinh *et al.*, 2001e). Ang II can induce the expression and release of various endogenous growth factors including FGF, TGF-β_1 and PDGF (Dinh *et al.*, 2001d), and is thought to potentiate the mitogenic effects of EGF and the proto-onco-genes c-*fos*, c-*myc* and c-*jun* (Grady *et al.*, 1991). The predominant fetal Ang II receptor subtype is the Ang II type 2 receptor (AT2), and predominance switches to the AT1 receptor postnatally in rats (Grady *et al.*, 1991). The AT2 receptor counteracts several of the growth responses initiated by AT1 and growth factor receptors. AT1 mRNA expression increases towards term concurrent with the progressive rise in circulating levels of cortisol and consistent with glucocorticoid induction of AT1 expression (Whorwood *et al.*, 2001). Infusion of exogenous cortisol can up- or down- regulate AT1 expression in a tissue specific manner in fetal sheep (Robillard *et al.*, 1995).

Thyroid hormones present in fetal plasma are derived primarily from fetal sources in sheep, although in other species such as man and rabbits they can also have a maternal origin (Fowden & Forhead, 2004b). Fetal thyroid hormone concentrations are not related to metabolite concentrations during normal conditions but are reduced during hypoxaemic conditions associated with IUGR (Fowden & Silver, 1995). Fetal hypothyroidism leads to an asymmetrical type of IUGR with a reduction in muscle mass (Fowden & Silver, 1995). It also alters the development of the fetal nervous system, skeleton, skin, lungs and skeletal muscle. Therefore, thyroid hormones affect both tissue accretion and differentiation. These processes are stimulated via modulation of IGF production and by metabolic actions, which increase fetal O_2 consumption. Thus, thyroid hormones promote fetal development and act as signals of energy availability (Fowden & Forhead, 2004c).

Concentrations of hormones in the fetal circulation change during development and in response to various stimuli. Towards term there are specific changes in the concentrations of key hormones including cortisol which act as a maturation signal to the fetus. These induce changes in tissue function and morphology that prepare the fetus for extrauterine life. Nutritional state can also cause changes in hormone concentrations and can cause a precocious fetal cortisol surge resulting in preterm birth (Bloomfield *et al.*, 2003b). Reduced nutrient availability can lower anabolic hormones such as insulin and increase catabolic hormones such as growth hormones (Bauer *et al.*, 1995; Woodall *et al.*, 1996a; Oliver *et al.*, 2005). The types of changes in hormones are dependent on the size, duration and nature of the insult (Roseboom *et al.*, 2001a; Oliver *et al.*, 2002a; Bloomfield *et al.*, 2003a).

1.6 Cardiovascular control

Control of the cardiovascular system is maintained by the integrated effects of the autonomic nervous system and a variety of endocrine mechanisms (figure 1.5). Short term (seconds to minutes) control of the cardiovascular system is primarily achieved by changes in heart rate and vascular resistance, while long term (days to weeks) control requires regulation of fluid balance.

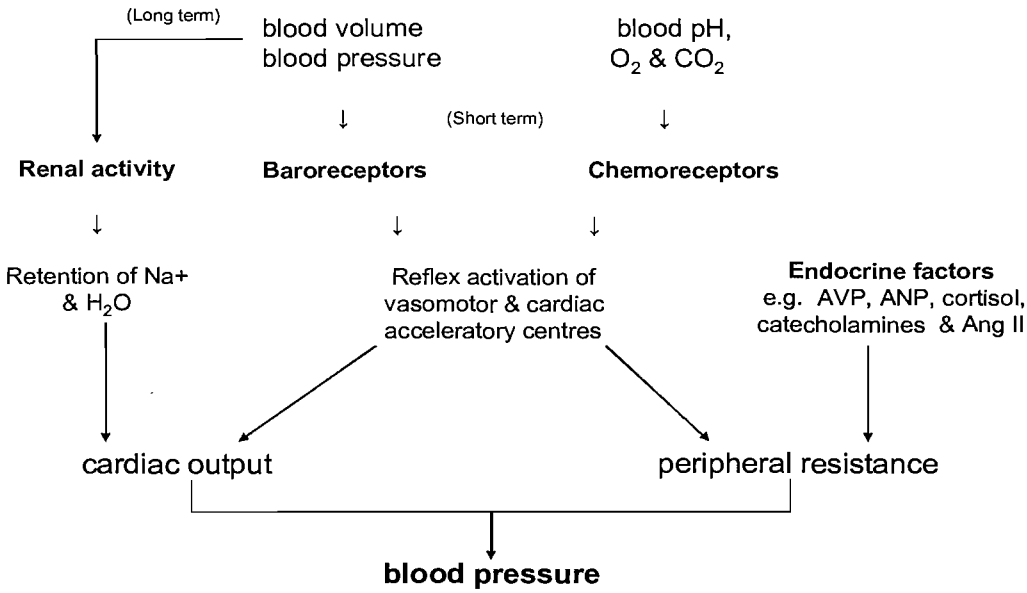

Figure 1.5 Summary of the main factors of cardiovascular control.

1.6.1 Fetal Circulation

The fetal circulation is arranged so that blood bypasses the lungs to a large extent and perfuses the placenta, which is achieved through several shunts within the fetal cardiovascular system (figure 1.6).

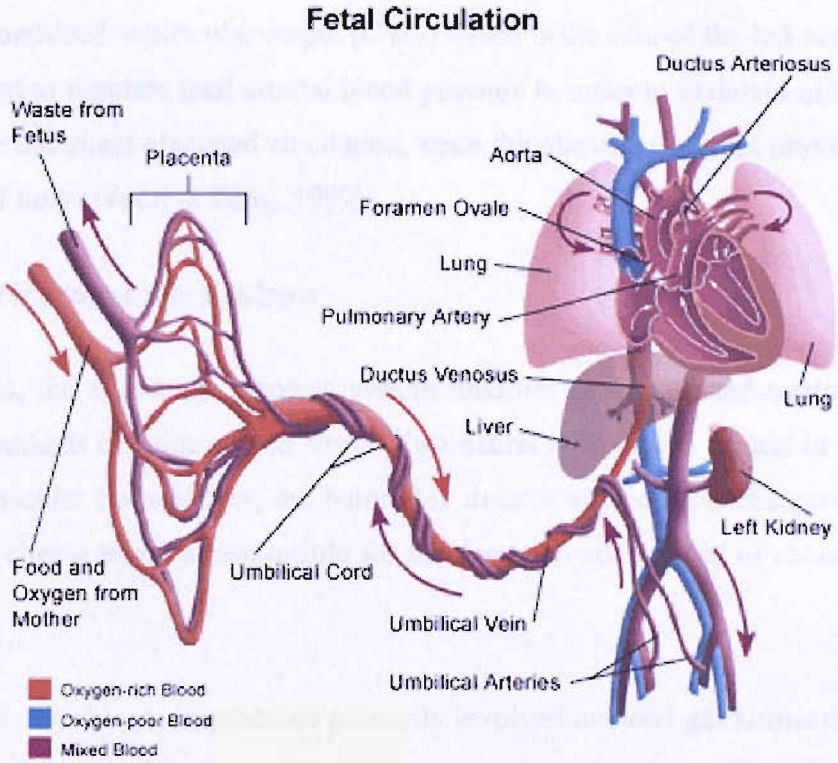

Figure 1.6 Fetal circulation (from www.health.uab.edu).

The umbilical vein transports blood rich in nutrients and O_2 to the fetal body from the placenta. About 30–40% of the blood from the umbilical vein bypasses the hepatic circulation and joins the inferior vena cava via the ductus venosus. In the fetus the lungs do not function as gas exchange organs, therefore blood is shunted away from the pulmonary circulation by the other vascular shunts. Blood flow from the vena cava can enter the right atrium or pass through the foramen ovale, a distinct channel connecting the inferior vena cava to the left atrium. The portocaval pressure gradient causes blood to accelerate as it enters the ductus venosus, this accelerated blood flow causes 'preferential streaming' of the highly oxygenated blood towards the foramen ovale. From the left atrium enters the left

ventricle, then the ascending aorta and vessels to supply the head and upper body. If output from the left ventricle falls, then the aortic arch is fed by blood from the ductus arteriosus in a reversed fashion through the isthmus aortae (Kiserud & Acharya, 2004). The majority of blood ejected from the right ventricle goes through the ductus arteriosus and enters the systemic circulation, along with blood ejected from the left ventricle (Rudolph, 1985), where it can supply the fetal tissues with oxygen and nutrients. Cardiac output is therefore described as the combined ventricular output (CVO) which is the sum of the left and right outputs. It is important to regulate fetal arterial blood pressure in order to maintain efficient gas exchange with the umbilical-placental circulation, since the placenta does not provide much regulation of blood flow (Wood & Tong, 1999).

1.6.2 Neuronal mechanisms

In adults, the autonomic nervous system modifies heart rate and contractility to meet the daily demands of exercise and stress. Two neural reflexes are central in the maintenance of cardiovascular homeostasis; the baroreflex detects and corrects changes in blood pressure, and the chemoreflex is responsible for the detection and control of chemical changes in the blood.

In adults, the chemoreceptors are primarily involved in blood gas homeostasis but also play a role in cardiovascular control. These organs are located peripherally, in the carotid and aortic bodies, and also centrally in the medulla (Mitchell *et al.*, 1963). Chemoreceptors are activated by decreases in arterial pO_2 and increases in pCO_2 or H^+. The detection is rapid as each carotid or aortic body has a plentiful blood supply ensuring the chemoreceptors are always in close contact with the arterial blood. If the blood supply to the chemoreceptors is diminished, the ensuing reduction of O_2 and build up of excess CO_2 and H^+ also activates the chemoreceptors. Activation of the chemoreceptor results in stimulation of the medulla vagal centre, resulting in decreased heart rate. The inspiratory centre in the medulla is also stimulated, which sends nervous impulses to increase breathing rate and the volume of the lungs during inhalation. This causes a secondary response via the pronounced pulmonary reflex response and hypocapnia which inhibit the medullary vagal centre which attenuates the decrease in heart rate and may increase it (Marshall, 1998). If the pulmonary response to

chemoreceptor is blocked the heart rate response may be greatly exaggerated (Marshall, 1998).

There is evidence for chemoreceptors function *in utero* from direct recording as early as 90 dGA (Blanco *et al.*, 1984) but they appear to have little or no role in the control of fetal breathing movements (Dawes *et al.*, 1983; Moore *et al.*, 1989). This should not be surprising as fetal gaseous exchange is dependent on placental exchange, umbilical blood flow and distribution of blood flow to organs. In the fetus acute hypoxia results in an initial bradycardia, a gradual increase in blood pressure (Boddy *et al.*, 1974; Giussani *et al.*, 1993) and a redistribution of blood flow away from the periphery in favour of the heart, brain and adrenals (Boddy *et al.*, 1974; Cohn *et al.*, 1974; Rudolph, 1984; Giussani *et al.*, 1993). The initial bradycardia and increase in peripheral resistance results from carotid chemoreceptor activation (Itskovitz *et al.*, 1991; Giussani *et al.*, 1993; Bartelds *et al.*, 1993).

The arterial baroreflex is the most important mechanism providing short term regulation of arterial blood pressure postnatally. Baroreceptors are spray type nerve endings which are activated by stretch. They are found in the walls of most large arteries of the thoracic and neck region, but are concentrated in two areas known as the aortic and carotid sinus baroreceptors, which are located at the arch of the aorta and at the bifurcation of the common carotid artery respectively. Increases in arterial pressure are detected by these receptors and lead to increased activation of their afferent fibres, through which signals are transmitted to the central nervous system (CNS). Increased stimulation results in activation of the sympathetic inhibitory and parasympathetic pathways and inhibition of the sympathetic excitatory pathways. This produces an overall decrease in heart rate and peripheral resistance and thereby lowers arterial pressure. Decreased baroreceptor activation due to low arterial pressure produces the inverse efferent response, but if blood pressure falls below the lower threshold (~70 mmHg) then baroreceptors no longer respond and chemoreceptor activation comes into play. The baroreceptors respond only to rapid changes in pressure, if the change in pressure is maintained over days or weeks then the baroreflex operating point changes (McCubbin *et al.*, 1956). Therefore long term changes in blood pressure following insults during gestation can be investigated by testing the baroreflex operating point.

The afferent limb of the baroreflex is in existence before birth and can be elicited by in response to experimental stimuli from as early as 88 dGA in fetal sheep (Blanco *et al.*, 1988). Baroreceptor activity is phasic, in synchrony with the arterial pulse pressure (Blanco *et al.*, 1988). Increases in blood pressure applied directly by compression of the abdominal aorta or injection of exogenous vasoconstrictors or vasodilators that produce changes in arterial pressure, result in increased or decreased discharge from baroreceptor afferents (Maloney *et al.*, 1977; Blanco *et al.*, 1988). The resulting change in heart rate demonstrates that the baroreflex is functional in the fetus in late gestation and may help to maintain blood pressure homeostasis. The importance of the baroreflex in maintaining blood pressure in the fetus is unclear as the fetal mean arterial pressure (MAP) is lower than that of the neonate or adult and thus may be below the threshold pressure for eliciting the reflex. However, denervation of the baroreceptor afferent outputs led to increased arterial pressure variability in fetal sheep (Itskovitz *et al.*, 1983), indicating that arterial baroreceptors do help regulate variability of arterial pressure and heart rate in fetal life. The sensitivity of the reflex in fetuses is reset as arterial pressure increases throughout the last third of gestation so the baroreflex sensitivity decreases and threshold increases with gestational age (Blanco *et al.*, 1988).

1.6.3 Fluid balance

In adults, the main system through which long term arterial pressure is controlled is the renal control of blood volume. Increases in extracellular fluid volume cause increased blood volume, which results in an elevation of preload at the heart. According to Starling's Law, this increases cardiac output, which causes an increase in MAP. Increased cardiac output causes an increase in blood flow through peripheral tissues and results in peripheral vasoconstriction via the myogenic response to the increase in stretch (Schubert & Mulvany, 1999). This secondary effect of an increase in peripheral resistance also contributes to an increase in MAP. Therefore, changes in MAP are tightly linked to changes in extracellular fluid volume. Increased renal blood flow in association with raised MAP leads to increased renal output of water and salt through pressure diuresis and naturesis, thereby restoring extracellular fluid volume to normal levels and leading to normalisation of MAP (figure 1.7). Regulation of blood flow in the renal medulla is thought to be less effective than in other vascular beds. Pressure natriuresis-diuresis occurs as a result of increases of medullary blood flow, whereby elevations of vasa recta capillary pressure and the imbalance of Starling forces result in an increase of renal interstitial hydrostatic pressure. This increase of interstitial fluid pressure is transmitted throughout the entire encapsulated kidney and provides a global signal to inhibit tubular sodium reabsorption and initiate the pressure-natriuresis process.

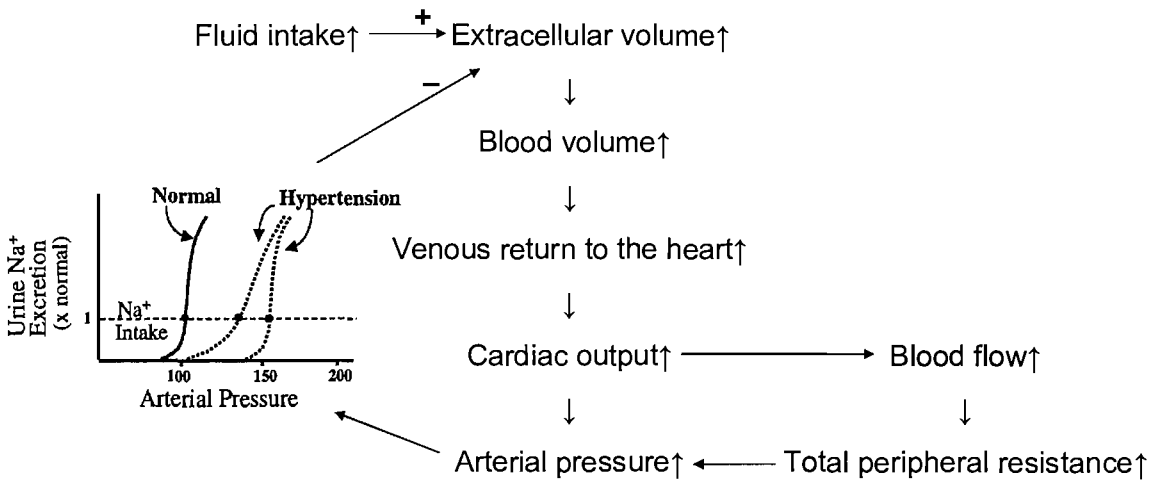

Figure 1.7 Basic renal-body fluid feedback mechanism for long term regulation of blood pressure and body fluid volume, adapted from (Hall, 2003b).

In all cases of chronic hypertension, sodium excretion is maintained at a normal rate (equal to intake) despite the high blood pressure, which is indicative of a resetting of pressure naturesis (Hall, 2003). It is unclear if this impaired pressure naturesis plays a primary role in causing hypertension or occurs secondarily to increased pressure (review (Hall, 2003a)).

The dynamics and regulation of fluid balance in the fetus is different from adults. The fetus is surrounded by fluid and the fetal body contains a much higher proportion of water than that of the adult. Also, the rate of fluid movement within the fetal body is 5–10 times higher than that of the adult, and there are fluid pathways that do not exist in adults (Brace, 1994). In early gestation the human fetus is 95% water, and this percentage decreases throughout gestation to about 70% at term, which is still much higher than in the adult (~57%) (Brace, 1994). In addition to the fluid compartments seen in adults (intracellular, extracellular, blood, plasma, and interstitial space), the fetus has fluid filled lungs, amniotic fluid and in sheep and ungulates there is also an allantoic fluid sac (Wintour *et al.*, 1986). Each fluid movement within the fetus is either actively and/or passively regulated, and control of fetal fluid movement maintains fetal blood volume so that cardiac output is sufficient to enable adequate blood perfusion of tissues (Brace, 1994). Therefore, in response to a number of circumstances (such as haemorrhage, hypoxia and changes in maternal osmolarity) simultaneous changes in fetal swallowing, urinary output, transcapillary flow, lymph flow, tracheal flow and intramembranous flow occur to keep blood volume within normal limits (Brace, 1995). The autonomic nervous and endocrine systems in combination with passive responses to alterations in vascular pressure or osmolarity are responsible for producing these responses (Brace, 1994).

Glomerular filtration rate (GFR) is the volume of fluid filtered from the renal glomerular capillaries into the Bowman's capsule per unit time. Clinically, this is often measured to determine renal function. In humans the average GFR at 20-29 years of age is ~116 mL/min/1.73 m^2 this is adjusted to a typical body size (body surface area = 1.73 m^2) (Coresh *et al.*, 2003). GFR falls with increasing age and over 70 years of age the average GRF is ~75 mL/min/1.73 m^2 (Coresh *et al.*, 2003). In comparison to these levels GFR is low in fetal life in humans and sheep, but increases with gestational age to ~2.66 ml/min at term (Kurjak *et*

al., 1981; Gibson & Lumbers, 1995). The GFR in sheep increases 3-fold within the first 24 hours following birth and then increases progressively, reaching adult levels by 2 years of age (Nakamura *et al.*, 1987; Robillard *et al.*, 1994). Fetal urine is hypoosmotic in comparison to fetal plasma; in fetal sheep plasma osmolarity is about 290 mOsm/kg, and urine osmolarity varies from 100 to 250 mOsm/kg H_2O (Weitzman *et al.*, 1978). Kidneys are unable to concentrate urine to adult levels until after birth (Robillard *et al.*, 1994). This may be due to the immaturity of the renal medulla or the lower sensitivity to arginine vasopressin (AVP, see below 1.6.3). Fetal urinary output is much higher than in adults; in late gestation it is approximately 0.4 ml/min, which is equivalent to about 25–30% of fetal body weight over 24 hours (Kurjak *et al.*, 1981). Therefore, relatively small changes in fetal urinary output can have large effects on fetal fluid balance over time (Brace, 1994).

Aside from the capability to control arterial pressure through changes in extracellular volume, the kidneys also have another powerful mechanism for controlling pressure. This is the renin angiotensin system (RAS) discussed further in 1.6.4.

1.6.4 Endocrine mechanisms

Hormones such as AVP, atrial natriuretic peptide (ANP), glucocorticoids, catecholamines, and the RAS contribute to cardiovascular control.

Arginine vasopressin

AVP is synthesised as large precursor molecules by the posterior pituitary. This hormone is involved in the regulation of fluid balance and acts by causing the kidneys to retain water. It is also a potent vasoconstrictor in high concentrations, thereby increasing blood pressure. AVP is released in response to increased osmolarity in extracellular fluids that are sensed by osmoreceptors located in or near the hypothalamus. In the sheep fetus, AVP is actively synthesised by 70 dGA. Fetal sheep plasma concentrations have been reported to be ~1.9 μU/ml at 101–120 dGA, but significantly lower (~0.77 μU/ml) at 121–141 dGA (Weitzman *et al.*, 1978). In fetal sheep, increased plasma osmolarity caused by infusion of hypertonic saline stimulates secretion of AVP by 101 dGA (Weitzman *et al.*, 1978; Robillard *et al.*, 1994). The sensitivity of the kidney to AVP in the fetus is lower than that of the adult

(Robillard *et al.*, 1994).

Atrial natriuretic peptide

ANP is synthesised and stored in the atria of the heart and is released in response to distension of the atrial wall caused by increased blood pressure, hyperosmolarity and in response to other factors including Ang II, AVP and acetylcholine (Smith *et al.*, 1989a). It regulates blood pressures by combined effects on intravascular volume, vasorelaxation, natriuresis, and diuresis. ANP reduces intravascular volume by increasing microvascular permeability by mechanisms that are as yet unknown. In the adult kidney ANP acts on natriuretic peptide receptor A to modulate renal function in a number of ways (Potter *et al.*, 2006). ANP increases GFR by regulating the tone of glomerular afferent and efferent blood vessels differentially (Potter *et al.*, 2006). ANP also decreases Na^+ reabsorption in the proximal tubules and collecting duct and decreases renin secretion from the juxtaglomerular cells (Potter *et al.*, 2006). Together, these processes result in a reduction in natriuresis, diuresis, and renin secretion (Potter *et al.*, 2006). ANP causes vascular smooth muscle relaxation through natriuretic peptide receptor A. Concentrations of ANP in the fetus are higher than those of the mother suggesting that ANP is synthesised by the fetus. In the sheep fetus infusion of ANP can cause hypotension and administration of antiserum to neutralise endogenous ANP causes a rise in MAP for the duration of the infusion (Brace *et al.*, 1989; Cheung, 1991). Therefore ANP may also have a physiological role in regulation of MAP and fluid balance in fetal life (Smith *et al.*, 1989b).

Hypothalamic-pituitary-adrenal axis

Activation of the HPA axis and release of mineralocorticoids and glucocorticoids from the adrenal gland plays an important role in the regulation of blood pressure. The mineralocorticoid aldosterone is released from the adrenal cortex in response to increased plasma Ang II, ACTH or potassium levels or decreased sodium levels or blood volume. Aldosterone stimulates sodium reabsorption and water retention thus raising blood pressure. The steroid hormones, glucocorticoids alter blood pressure by direct vasoconstriction, up-regulation of adrenoreceptors (Walker & Williams, 1992) and up regulation of components of the RAS system (Forhead *et al.*, 2000b). Throughout most of fetal life cortisol and

adrenocorticotrophic hormone (ACTH) levels are lower than maternal levels. In late gestation concentration of both slowly rises, until a surge of cortisol over the last 24 - 48 hours prior to birth which initiates parturition in sheep (Challis & Brooks, 1989). This is concurrent with the rise in MAP and fall in heart rate in the lead up until birth. Indeed cortisol has been shown to increase MAP in fetal sheep (Tangalakis *et al.*, 1992).

Catecholamines

Catecholamines are secreted primarily from the adrenal medulla under conditions of sympathetic activation (during stress or exercise). Adrenaline and noradrenaline circulate and cause increases in fetal heart rate and combined ventricular output (CVO) by activating their receptors in the heart and the vasculature. Smooth muscle contracts in response to stimulation of the α_1-adrenoceptors by noradrenaline. This constriction causes a decreased vascular compliance and increased peripheral resistance which contribute to an increase in systolic and diastolic arterial pressure. Noradrenaline also causes increased activity of the heart. Adrenaline has almost the same effects as noradrenaline but also has a greater stimulating effect on the β-adrenoreceptors, and therefore has a greater effect on cardiac stimulation with much less vasoconstriction than caused by noradrenaline. The fetal adrenal medulla does not secrete significant amounts of adrenaline until late in gestation, after cortisol causes the induction of phenylethanolamine n-methyltransferase (PNMT) which converts noradrenaline to adrenaline (Walker, 1994). Fetal plasma noradrenaline levels are about 1 ng/ml under normal conditions, and adrenaline levels are lower, normally below 0.05 ng/ml (Derks *et al.*, 1997). In late gestation, hypoxia, asphyxia, haemorrhage and hypoglycaemia can increase plasma catecholamine levels (Walker, 1994).

Renin Angiotensin System

The RAS is a hormone system that is vital in the regulation of both short and long term blood pressure and blood volume in the body and is a focus of this thesis and therefore will be discussed in detail here.

In adults reduced perfusion of the juxtaglomerular apparatus in the kidneys, causes the juxtaglomerular cells to release the enzyme renin. Renin cleaves an inactive peptide called angiotensinogen (Aogen), converting it into angiotensin I (Ang I). Ang I is then converted to Ang II by angiotensin converting enzyme (ACE) and Ang II stimulates angiotensin receptors (figure 1.8) (Lavoie & Sigmund, 2003a).

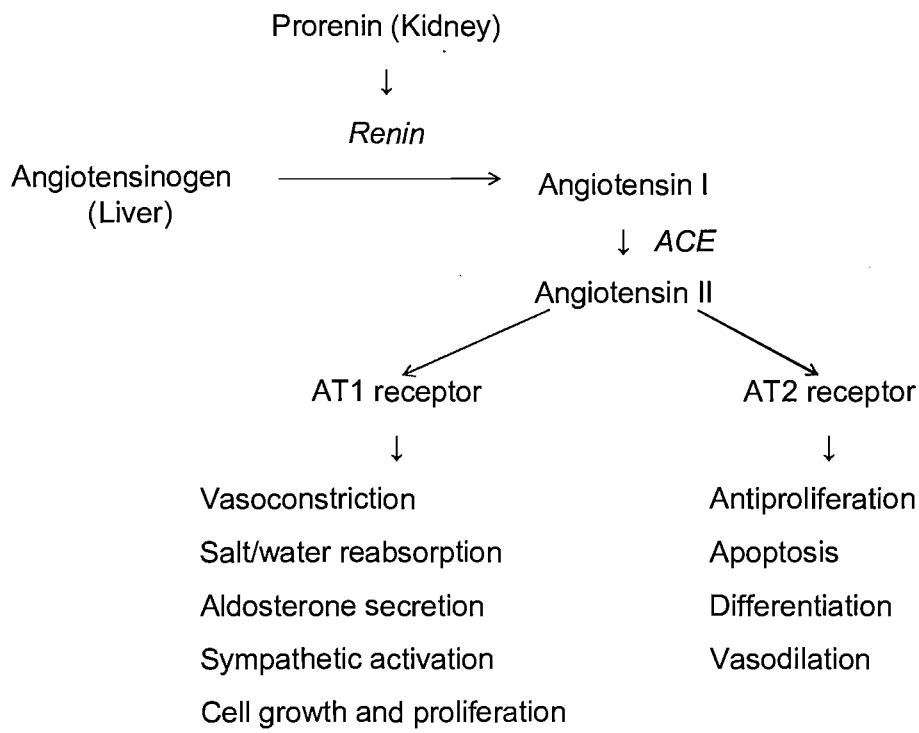

Figure 1.8 Basic renin angiotensin cascade.

Ang II has a variety of effects on the body in addition to its effects as a potent vasoconstrictor (figure 1.8). In the kidneys Ang II reduces renal blood flow, maintains GFR through efferent arteriolar vasoconstriction (Lumbers *et al.*, 2003), and causes retention of water and salt, thus increasing the extracellular fluid volume and slowly increasing arterial pressure. Ang II also

has a direct effect on tubular sodium transport, enhancing proximal tubule Na+/H+ exchanger activities decreasing sodium excretion (Carey & Siragy, 2003). Ang II stimulates the posterior pituitary to release AVP which increases fluid retention in the kidneys (Lavoie & Sigmund, 2003b). Ang II also acts on the adrenal cortex causing the release of aldosterone, which acts on tubules (such as the distal convoluted tubules and the cortical collecting ducts) in the kidneys, causing them to reabsorb more Na^+ and water from the urine. Aldosterone also acts on the CNS to increase a person's appetite for salt, and to make them feel thirsty. These effects act to increase the amount of fluid in the blood, making up for a loss in volume, and to increase blood pressure.

Two main Ang II receptors have been identified and other atypical receptors have been described (Dinh *et al.*, 2001c). The AT1 receptor mediates most of the biological actions of Ang II whereas the AT2 receptor is suggested to act by mediating anti-proliferation, apoptosis, differentiation and possibly vasodilatation (Dinh *et al.*, 2001b). The AT1 receptor is predominant in adults and in umbilical arteries, and the AT2 receptor is predominant in the fetus but rapidly declines at birth (Dinh *et al.*, 2001a). The RAS is often manipulated clinically to treat high blood pressure. Inhibitors of ACE (such as captopril, enalapril, lisinopril and ramipril) are often used to reduce the formation of the more potent Ang II from Ang I (Bicket, 2002). Alternatively, angiotensin receptor blockers (such as candesartan, losartan and valsartan) can be used to prevent Ang II from acting on angiotensin receptors (Sica, 2006).

In sheep, the RAS is active in fetal life and plays a role in regulation of fetal blood pressure, as has been shown by blockade of fetal RAS by saralasin an Ang II antagonist (Lumbers & Stevens, 1987). Although fetal renin levels are high, levels of Ang II are similar to maternal levels (Lumbers, 1995). ACE is found mainly in the pulmonary circulation; in fetal life pulmonary blood flow is low, therefore ACE may not be able to exert its maximum effect. The fetal RAS maintains GFR and renal excretion of sodium and water (Lumbers, 1995). Unlike in adult life physiological levels of Ang II do not stimulate either proximal tubular reabsorption nor aldosterone secretion (Lumbers, 1995).

1.7 Effect of reduced nutrition on fetal growth and cardiovascular development

When fetal nutritional demands are not met the fetus can respond in a number of ways, depending on the timing and the severity of the challenge; it can reduce nutrient demand by decreased growth and metabolism, mobilise stores, increase placental uptake and it can change the distribution of nutrients by altering cardiovascular distribution in favour of essential organs (such as the heart, brain and adrenal) at the expense of the periphery.

1.7.1 Evidence for undernutrition as the cause of poor fetal growth and adult cardiovascular disease

The Dutch famine of 1944–1945 provides evidence that poor nutrition *in utero* is associated with later CVD in a population that was previously and subsequently relatively well nourished (Painter *et al.*, 2005b). Official daily rations for adults fell from ~1400 calories in October to below 1000 calories in late November 1944, and between December and April of the following year they were further reduced to between 400 and 800 calories (Painter *et al.*, 2005b). The rations rose back to over 2000 calories a day by June 1945 after liberation of the Netherlands (Painter *et al.*, 2005b). These rations would have been supplemented by the black market, soup kitchens, church organisations and foraging trips to the countryside, but it is thought that official rations largely reflect the variation over time of total food available (Painter *et al.*, 2005b). Studies of the Dutch famine birth cohort have split individuals exposed to famine during gestation into three groups: those exposed in early, mid or late gestation (Painter *et al.*, 2005b). The three exposed groups have been compared with each other, with individuals born prior to the famine then exposed as infants, and with those conceived after the famine (Painter *et al.*, 2005b).

Babies exposed to famine in late or mid gestation were lighter, shorter, thinner and had smaller head circumference for gestational age than those conceived after the famine (Painter *et al.*, 2005b). In contrast, those exposed to famine in early gestation were slightly heavier and larger than babies conceived after the famine (Painter *et al.*, 2005b). Individuals were

investigated at 50 years of age. Exposure to famine in early gestation has been associated with increased incidence of CHD (Roseboom *et al.*, 2000b), raised lipids (Roseboom *et al.*, 2000a), altered clotting (Roseboom *et al.*, 2000c) and more obesity (Ravelli *et al.*, 1998) compared to those conceived after the famine. Individuals exposed during mid gestation were found to have increased instances of obstructive airways disease (Lopuhaa *et al.*, 2000) and microalbuminuria, which is indicative of glomeruli damage (Painter *et al.*, 2005a). Those individuals exposed to famine in late gestation had decreased glucose tolerance (Ravelli *et al.*, 1998). These are striking associations, especially since the group exposed in early gestation, which appear to be most affected by the famine, were no smaller than those conceived after the famine. Thus, the timing of reduced nutrition is clearly important in determining its effects. These studies are of a relatively small cohort, partly due to impaired fertility during the famine (Stein & Susser, 1975), and high rates of infant mortality during the famine (Roseboom *et al.*, 2001a). This also means only the fittest, those who have managed to survive to adult life, are included in the study. It is also difficult to define the exact timings of famine exposure due to partial overlap between the groups, and also the degree of exposure as supplement food was available.

1.7.2 Animal studies of reduced nutrition

To peruse the human observations in greater detail, a variety of animal models have been utilised to study the effects of reduced maternal nutrient during various stages of gestation.

Effects of reduced nutrition in rodent models

Studies in rats showed that a total global nutrient restriction (70%) during the whole of pregnancy led to offspring with low birth weight that go on to develop hypertension (Langley & Jackson, 1994; Woodall *et al.*, 1996b). Hypertension has been observed in these models as early as four weeks after birth and persists throughout life despite any further manipulations of the mother or offspring (Woodall *et al.*, 1996b). Elevated blood pressure in postnatal life has also been seen in rat offspring as a result of a relatively mild (30%) global nutrient restriction (Ozaki *et al.*, 2004). The balance of nutrients is also important as is revealed by low protein diets where the overall calories in the diet are maintained. A 50% reduction in protein (to 9%) intake was associated with a reduced birth weight to placental weight ratio and caused an increase in blood pressure that was still apparent at 44 weeks (Langley-Evans *et al.*, 1999a). Reduction in protein has also been found to decrease circulating threonine and increases in methylation of DNA which may alter gene expression throughout life (Rees *et al.*, 2000). A low protein diet fed only in the preimplantation period 0 – 4.5 dGA also results in hypertension, altered birth weight, postnatal growth and organ/body weight ratio (Kwong *et al.*, 2000). Overall these studies reveal the importance of adequate balanced nutrition throughout gestation for fetal growth and cardiovascular development and highlight the preimplantation period as critical.

Result of reduced nutrition in sheep model

Fetal adaptations to undernutrition have been investigated in sheep as they are a comparable model for human pregnancies. They have a high incidence of singleton pregnancies and a full complement of cardiomyocytes and renal glomeruli are formed prenatally (Hanson *et al.*, 1993; Brace *et al.*, 1998). Fetal sheep have a high tolerance for surgery, which allows chronic instrumentation for several weeks with vascular catheters and electrodes, thus their growth and development has been well documented (Edwards *et al.*, 2001a). A variety of intrauterine

nutritional challenges have been studied in the sheep which have revealed that the nutritional status and body composition of the mother at time of mating (Osgerby *et al.*, 2003) and also the timing of the challenge (Edwards *et al.*, 2001b) are important in the nature of the response.

For ruminants a total nutrient restriction (as a percentage of control diet) has most often been applied, as a significant proportion of the dietary nitrogen is supplied by the gut fauna of micro-organisms making it difficult to manipulate the proportion of protein received. It is the digestive system of the sheep that provides the greatest contrast to man (Hecker & Wenham, 1983). The herbivorous sheep has four voluminous stomachs and a considerable bulk of intestine. Food passes into the rumen, where bacteria and protozoa break down the cellulose wall to begin the release of nutrients. Small parcels or 'cuds' of food are regurgitated back into the mouth for maceration via chewing and mixing with saliva. The next stomach is the reticulum which passes food back to the rumen, and then from the reticulum it passes to the omasum, which involves more bacterial breakdown. The final stomach is the abomasum or true stomach which is similar to that of the monogastric animal. Here, digestive enzymes break down the plant proteins and fats and kill the bacteria. Therefore, although the nutrient uptake involves mechanisms different to those of humans, the nutrients that pass into the metabolic pathways are exactly the same and facilitate the same processes of growth and development.

Undernutrition in the first half of pregnancy can alter placental morphology with no effect on fetal size, therefore fetal growth may be successfully maintained as a result of placental adaptations (Steyn *et al.*, 2001). Maternal nutrient restriction in early gestation can have a variety of long lasting effects. Hawkins *et al.* showed that a 15% reduction in maternal global dietary intake for the first half of gestation resulted in reduced baseline blood pressure in late gestation and elevated baseline blood pressure postnatally with no changes seen in fetal or placental weight (Hawkins *et al.*, 2000a). This challenge also resulted in a lower baroreflex operating point in late gestation (Hawkins *et al.*, 2000c). Furthermore, a 50% reduction in maternal nutrition during the first 30 dGA has been shown to result in increased pulse pressure, a reduced rate pressure product and a leftward shift in baroreflex curve when

assessed in the offspring at 1 year of age (Gardner *et al.*, 2004). The baroreflex sensitivity to Ang II infusion was also blunted relative to the control group at 1 year of age (Gardner *et al.*, 2004). A previous study in our group has found that a 50% maternal nutrient restriction for the first 31 dGA increased blood pressure response to RAS stimulation (by frusemide) in 1.5 but not in 2.5 year old offspring, however, there was no change in baroreflex response (Cleal *et al.*, 2007a). Therefore, a mild nutritional challenge during the first half of gestation can alter cardiovascular control in fetal life and has persistent effects in postnatal life, and a shorter nutritional challenge can affect cardiovascular control in early adulthood. **There is little known as yet about the effects of a short early gestation challenge spanning the implantation period on cardiovascular control in the fetus.**

There have been fewer studies that investigate the effects of nutrient restriction during the period of rapid fetal growth which occurs in late gestation, and these have found conflicting results. The response of the fetus to undernutrition during late gestation is dependent on its growth rate; only in a rapidly growing fetus does growth rate decrease during nutrient restriction, slower growing fetus do not reduce growth rates (Harding, 1997). Slower growing fetuses may have adapted to an earlier gestational insult, and have thus protected themselves from challenges later in gestation (Harding, 1997; Oliver *et al.*, 2005). Therefore, unless fetal growth rate prior to any nutritional challenge is measured and taken into consideration, any results may be obscured. Considering the most severe challenge first, a 95% maternal nutrient restriction from 105–125 dGA, resulted in reduced fetal body, liver, heart and weight at 125 dGA and reduced body weight at birth (Oliver *et al.*, 2001). Whereas if this nutrient restriction (95%) only lasted 10 days (105–115 dGA) no change in fetal body or organ weight was seen (Oliver *et al.*, 2001). This severe challenge did not affect the blood pressure of the offspring when measured at 5 or 30 months of age (Oliver *et al.*, 2002b). A 75% maternal nutrient restriction from 100–124 dGA did not significantly reduce fetal weight but did reduce thymus and liver weight when measured at 124 dGA (Bauer *et al.*, 1995). Edwards and McMillen found that a 50% maternal nutrient restriction from 115–145 dGA resulted in no difference in fetal body, organ or placental weight at 145 dGA (Edwards & McMillen, 2001). This challenge resulted in an increased fetal blood pressure and responsiveness to increasing doses of Ang II in singleton fetuses (Edwards & McMillen,

2001). A previous study in this group found that a 50% maternal nutrient restriction from 118–140 dGA resulted in no change in fetal body weight, but a reduction in absolute kidney weight and an increase in adrenal weight as % fetal body weight (Burrage, 2006). The differences in these findings are probably due to the variation in timing, duration and intensity of the challenges used in these studies, which also makes them difficult to compare directly. **Further investigation into the effects of a short maternal nutritional challenge in late gestation on cardiovascular control in the fetus is required.**

1.7.3 Mechanisms of altered fetal growth/cardiovascular control following undernutrition in utero

As discussed in 1.3, the mechanisms that underlie the link between poor nutrition *in utero* and altered fetal growth and cardiovascular control have yet to be fully elucidated. The HPA axis (Matthews, 2002), peripheral vasculature (Brawley *et al.*, 2003a), kidney function (Wintour *et al.*, 2003a) and the RAS (Langley-Evans *et al.*, 1999a) have been proposed as potential candidates (figure 1.9).

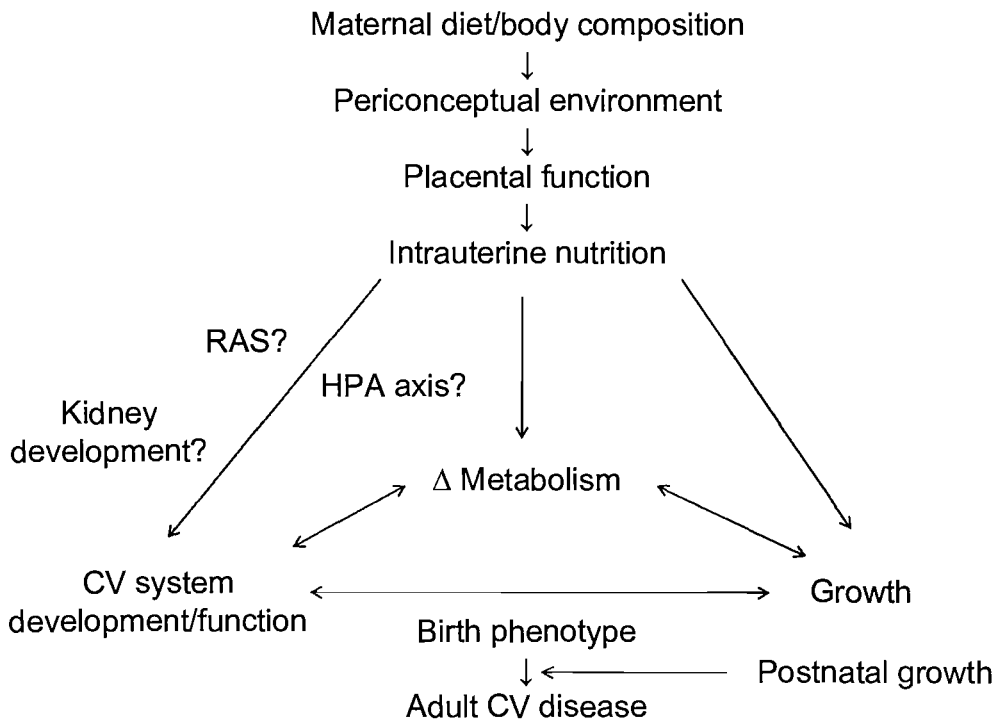

Figure 1.9 Possible mechanisms involved in the DOHaD hypothesis, adapted from (Cleal, 2005).

The effect of nutrient restriction on the hypothalamic-pituitary-adrenal (HPA) axis

The HPA axis (figure 1.10) is part of the endocrine system and it is involved in the regulation of metabolism, growth and cardiovascular function. The hypothalamus controls pituitary-adrenal activity by secretion of corticotrophin-releasing hormone (CRH) and AVP, which then stimulates ACTH release from the anterior pituitary gland. ACTH then initiates the synthesis and secretion of cortisol from the adrenal cortex. Glucocorticoids act at multiple locations within the body to maintain homeostasis but can also act in the CNS to modify behaviour and learning (Matthews, 2002). Glucocorticoids aid in the regulation of blood pressure via direct vasoconstriction actions in vasculature, regulation of catecholamine synthesis, Aogen and NO, actions in the CNS and regulation of water excretion and normal GFR in the kidney (Hammer & Stewart, 2006). The HPA axis is highly regulated via negative feedback mechanisms since over exposure to glucocorticoids is very damaging. In fetal life it can cause premature birth (Bloomfield *et al.*, 2003c) and in adult life it is associated with atherosclerosis, immunosuppression, depression and cognitive impairment as well as raised cholesterol levels (Matthews, 2002).

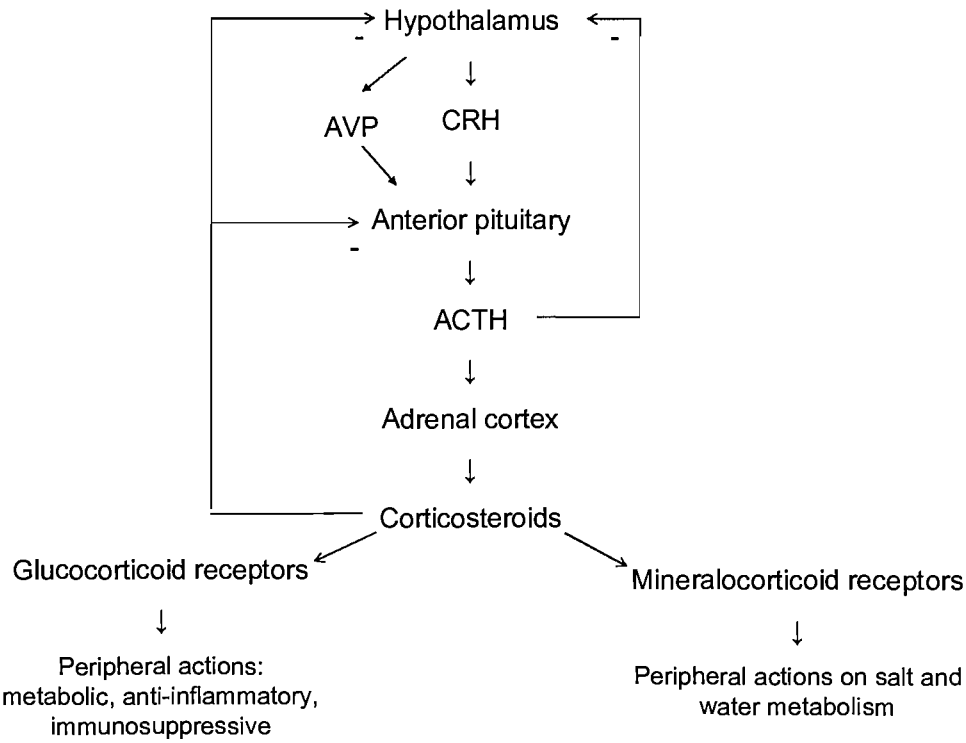

Figure 1.10 The HPA axis. CRH: corticotrophin releasing hormone, AVP: arginine vasopressin, ACTH: adrenocorticotrophic hormone.

It has been proposed that increased fetal exposure to glucocorticoids is the mechanism behind the associations between low birth weight and increased risk of cardiovascular and metabolic disorders (Seckl *et al.*, 1999). A stressful *in utero* environment such as placental insufficiencies, undernutrition or hypoxia can expose the fetus to raised levels of cortisol via raised maternal cortisol, altered 11βHSD2 activity, activation of the fetal HPA axis or alterations in glucocorticoid receptors (GR) (Edwards & McMillen, 2001). Raised glucocorticoid exposure can alter fetal growth, affect cellular differentiation and development, and alter the timing of organ maturation. Indeed exogenous administration of glucocorticoids during pregnancy retards fetal growth resulting in low birth weight in humans and other mammals, and also elevates the blood pressure of offspring in rat and sheep models (Reinisch *et al.*, 1978; Benediktsson *et al.*, 1993; Dodic *et al.*, 1998).

In rats, undernutrition during late gestation induces growth retardation and the fetuses are subjected to overexposure of maternal corticosterone which disturbs the development of the HPA axis (Lesage *et al.*, 2001). Rats exposed to a low protein diet *in utero* (9%) had normal corticosterone levels but a blunted diurnal pattern in plasma ACTH concentrations (Langley-Evans *et al.*, 1996a). But this diet has been found to increase GR protein and mRNA expression in the kidney, liver, lung and brain that persisted into adulthood (Bertram *et al.*, 2001). Altered prenatal nutrition has also been shown to result in altered mRNA expression and methylation of GR genes in rat pups, which may result in altered GR levels and glucocorticoid sensitivity (Lillycrop *et al.*, 2005) .

In sheep, maternal undernutrition during late gestation activates the fetal HPA axis and can alter adult HPA function (Edwards *et al.*, 2001c; Bloomfield *et al.*, 2003a). Numerous studies have led to the proposal that maternal undernutrition during the peri-conception and/or the preimplantation period may act to alter the functional sensitivity of the HPA axis and has been suggested to lead to premature birth (Bloomfield *et al.*, 2003d). The degree of impact of altered nutrition on the HPA axis seems to be dependent on the severity and the duration of the challenge (McMillen & Robinson, 2005d). The impact of maternal hormonal and metabolic responses to undernutrition on the epigenetic regulation of gene expression in the

developing embryo could underlie changes in the HPA axis (Edwards & McMillen, 2002).

The effect of nutrient restriction on the peripheral vasculature

Peripheral resistance plays a key role in overall cardiovascular control (section 1.6). The ability of the baroreceptors, chemoreceptors and various endocrine factors to exert effects on the peripheral resistance is dependent on the vasoreactivity of the peripheral vasculature. Peripheral vascular dysfunction is associated with and often precedes the development of hypertension, atherosclerosis and type II diabetes (Brawley *et al.*, 2003b). The provision of nutrients to the fetus and placenta depends partly on uterine blood flow. Uterine blood flow is increased during normal pregnancy because of vasodilation and vascular remodelling (Reynolds & Redmer, 1995). Therefore abnormalities in the maternal or fetal vasculature as a result of maternal dietary manipulations may underlie the alterations of cardiovascular control.

The vasoreactivity of small resistance and large conduit arteries can be assessed using the *in vitro* techniques of wire myography and organ bath respectively to measure changes in tension exerted by isolated vessels (Brawley *et al.*, 2003c). A maternal low protein diet impairs endothelial function in the uterine and mesenteric arteries in the pregnant rat (Itoh *et al.*, 2002), but supplementation with glycine (Brawley *et al.*, 2004) or folate (Torrens *et al.*, 2006) reverses this effect. Also the same maternal low protein diet has been found to alter vascular responses in the male offspring, female offspring when pregnant, and in second generation male offspring (Brawley *et al.*, 2003d). In sheep a 50% maternal global nutrient restriction for the first half of gestation blunts systemic small artery function in late gestation fetuses (Ozaki *et al.*, 2000). Also a 30% global or protein restriction for the first half of gestation blunts relaxation in systemic artery isolated from mid-gestation fetuses (Nishina *et al.*, 2003). A previous study in our group found that a 50% peri-implantation nutrient restriction increased constriction to acetylcholine in coronary arteries but had no effect on response to phenylephrine response in renal artery from 2.5 year old sheep (Cleal *et al.*, 2007a). It could be that these changes may underlie the alterations in postnatal cardiovascular control.

The responsiveness of the peripheral vasculature *in vivo* can be assessed using vasoactive drugs such as Ang II, noradrenaline and captopril, although this is not as sensitive as *in vitro* measurements (Endemann & Schiffrin, 2004). In sheep a 50% maternal nutrient restriction for the first 95 dGA has been found to have no effect on the response to noradrenaline, Ang II or captopril in three year old offspring (Gopalakrishnan *et al.*, 2004). Although, a 50% maternal nutrient restriction for the first 30 dGA has been found to blunt response to Ang II in one year old offspring (Gardner *et al.*, 2004). Whereas, maternal nutrient restriction from 115 dGA increases response to Ang II in late gestation fetuses (Edwards & McMillen, 2001). A previous study in our group found that a 50% peri-implantation nutrient restriction had an increased blood pressure response to RAS activation (by frusemide) in 1.5 year old offspring, this may indicate greater vascular responsiveness to Ang II (Cleal *et al.*, 2007a). **Therefore it is important to investigate the effect of a peri-implantation nutrient restriction on vascular responsiveness in fetal sheep *in vivo*.**

The effect of nutrient restriction on chemoreflex

Chemoreflex plays an important role in cardiovascular control (section 1.6). Models of hypoxia have been used to investigate chemoreflex and other mechanisms of late gestation fetal cardiovascular control. Fetal hypoxia can be induced by several methods; in this section the studies referred to are in late gestation fetal sheep with acute hypoxia induced by reduced maternal inspired oxygen unless otherwise specified. Hypoxia produces an initial marked decrease in heart rate which is rapid in onset and then gradually returns to control values. This bradycardia is accompanied by a gradual increase in blood pressure which is maintained throughout the hypoxic period (Boddy *et al.*, 1974; Cohn *et al.*, 1974; Giussani *et al.*, 1993). Hypoxia causes a redistribution of blood flow; this is achieved by changes in blood flow patterns. This redistribution maintains oxygen delivery to the brain and heart at the expense of the liver and periphery (Cohn *et al.*, 1974). The cardiovascular responses to hypoxia are controlled by a combination of the mechanisms. The rapid initial fetal blood flow and heart rate responses are primarily chemoreflexly mediated (Giussani *et al.*, 1993). The secondary responses are mediated by several endocrine factors including catecholamines, AVP, cortisol and Ang II (Green, 2001).

A hypoxia challenge in late gestation may reveal changes in cardiovascular control not seen in basal conditions. Prevailing hypoxaemia but not acidemia or hypoglycaemia enhanced chemoreflex function during acute hypoxia (Gardner *et al.*, 2002). Previous studies in sheep have shown that a 15% maternal nutrient restriction for the first half of gestation can increase femoral artery vascular resistance during hypoxia in late gestation suggesting that the chemoreflex is augmented (Hawkins *et al.*, 2000b). No change was seen in the heart rate, blood pressure or femoral blood flow response to hypoxia in this model (Hawkins *et al.*, 2000b). Therefore there may be changes in chemoreflex that may underlie the alterations in postnatal cardiovascular control. **It is important to investigate the effect of maternal nutrient restriction during the peri-implantation and late gestation period on fetal chemoreflex.**

The effect of nutrient restriction on the kidney

As described in section 1.6 the kidney has an important role in cardiovascular control, and disrupted kidney development could therefore lead to cardiovascular impairment in the adult.

Reduction in kidney nephron number may lead to hyperperfusion of individual nephrons causing a cycle of glomerular sclerosis, more nephron death, and increasing blood pressure (Mackenzie & Brenner, 1995). Keller *et al* compared the number and volume of glomeruli in victims killed in accidents, and found that those with hypertension had significantly fewer glomeruli per kidney than matched normotensive controls (Keller *et al.*, 2003). As no new nephrons are formed after birth it may be that individuals born with fewer nephrons are at a higher risk of developing hypertension. Reduced glomerular number and increased glomerular size in adults and children have been seen to be associated with low birth weight (Hughson *et al.*, 2003). Small for gestational age babies have been found to have reduced renal growth between 26 and 34 weeks of gestation and kidneys that are relatively narrow for their length (sausage-shaped kidneys) (Konje *et al.*, 1996). The Southampton Women's Survey has also found that thinner mothers with lower triceps skin fold thickness tend to have fetuses that have sausage-shaped kidneys (Mukherjee *et al.*, 2005). Previously sausage-shaped kidneys have been associated with hypertension (Raman *et al.*, 1998).

Uteroplacental insufficiency has been shown to cause up to a 30% reduction in nephron number in fetal rabbits (Bassan *et al.*, 2000), newborn piglets (Bauer *et al.*, 2002) and 2 week old rats (Merlet-Benichou *et al.*, 1994). This reduction in nephron number is associated with a reduction in renal function in the rat (Merlet-Benichou *et al.*, 1994) and pig (Bauer *et al.*, 2002) offspring. A maternal low protein diet throughout pregnancy in rats also produces a significant deficiency in nephron number in offspring in postnatal life (Langley-Evans *et al.*, 1996b; Woods *et al.*, 2001a; Welham *et al.*, 2002), and in adult life a decrease in renal function (Nwagwu *et al.*, 2000). However there is little information to date regarding maternal diet induced effects on fetal kidney development. Sheep have a full complement of glomeruli formed prenatally, as in humans (Brace *et al.*, 1998). Twins, an example of increased constraint *in utero*, were found to have fewer nephrons than singletons, but the relative kidney weight was not different to those of singleton (Mitchell *et al.*, 2004). Early gestation seems to be a critical window in kidney development in sheep as exogenous glucocorticoid treatment at 28 dGA has been found to decrease nephron number and glomerular volume in adult life (Wintour *et al.*, 2003c). A 50% maternal nutrient restriction from 28–78 dGA resulted in fetuses with reduced kidney weight, smaller glomerular size, and a thinner nephrogenic zone but also a greater density of mature glomeruli at 78 dGA (Gilbert *et al.*, 2003). But to date the effect of undernutrition has not been studied in the peri-implantation period. **It is now important to investigate the effect of a peri-implantation nutrient restriction on kidney growth and renal function in fetal sheep.**

Determinant of nephron number

The mechanisms that regulate nephron number are starting to be investigated. Cell death is an important part of normal kidney development; defects in cell death may cause developmental abnormalities of the kidneys (Coles *et al.*, 1993). Intrauterine growth restriction (IUGR) induced by ligation of the uterine arteries in of rats late gestation (19 dGA where term = 21.5 dGA) caused an increase in apoptosis, significantly reducing glomeruli number when measured at 21 days postnatally (Pham *et al.*, 2003a) (figure 1.11). The increase in apoptosis observed was due to an increase in caspase-3 activity brought about by reduced expression of the anti-apoptotic protein Bcl-2 and a corresponding increase in expression of the pro-apoptotic protein Bax (Pham *et al.*, 2003b).

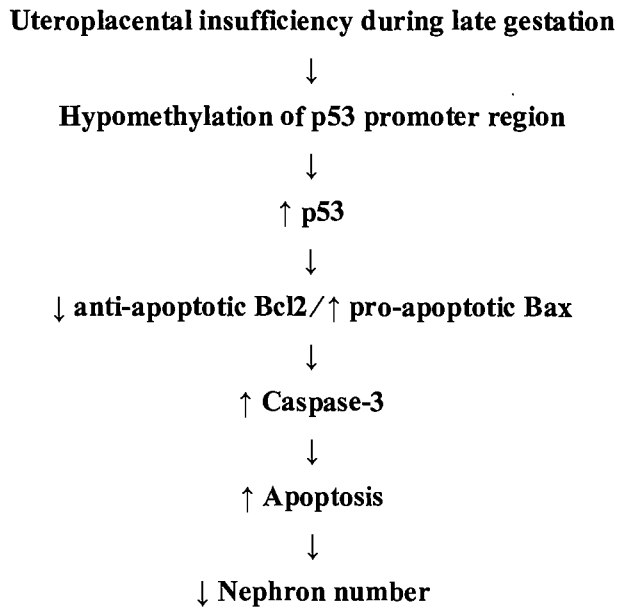

Figure 1.11 A potential mechanism by which placental insufficiency induced from 19 dGA may cause decreased nephron numbers in rats, adapted from (McMillen & Robinson, 2005c).

All components of the RAS are expressed from very early in gestation in the rat (Yosipiv & el Dahr, 1996), sheep (Wintour *et al.*, 1996; Butkus *et al.*, 1997) and human (Schutz *et al.*, 1996) meso- and metanephros and the intrarenal RAS plays an important role in the development of the kidneys (Guron & Friberg, 2000b). Rat offspring of dams fed a low protein diet throughout pregnancy had reduced renal renin mRNA, renin concentration and tissue Ang II levels in postnatal life (Woods *et al.*, 2001b). In sheep, placental insufficiency resulted in reduced body weight, kidney weight and the ratios of renin/actin mRNA and angiotensin mRNA/18s rRNA in the kidneys (Zhang *et al.*, 2000). It was proposed that the suppression of fetal renal renin and Aogen expression could alter the activity of the intrarenal RAS and so affect the growth and development of the kidney (Zhang *et al.*, 2000). Maternal nutrient restriction (50%) in sheep from 28 to 77 dGA results in lambs with raised MAP, fewer renal glomeruli, and increased expression of ACE and AT2 receptor protein in the kidney at 9 months of age (Gilbert *et al.*, 2005). The same challenge (50% nutrient restriction, 28 – 78 dGA) resulted in an increase in AT1 receptor expression in the kidney, liver, lung and adrenals of neonatal offspring (144 - 146 dGA) with an increase in GR mRNA abundance and a decreased 11βHSD2 mRNA expression (Whorwood *et al.*, 2001). These results indicate that undernutrition increases the peripheral tissue sensitivity to

glucocorticoids causing an increase in AT1 receptor expression. Indeed, treatment with exogenous glucocorticoids in early gestation have been suggested to cause a premature maturation of the kidney with an increase in renal Aogen and AT1 and AT2 receptor mRNA when studied in late gestation (130 dGA) (Moritz *et al.*, 2002) (summary figure 1.12).

Low protein diet Rat Throughout gestation (Woods *et al.*, 2001c)	→	↓Renal Ang II & renin ↓Renal renin mRNA Newborn offspring	→	↓Nephrogenesis?	→	↓Nephron number
Maternal UN Sheep Mid-gestation (Whorwood *et al.*, 2001)	→	↑GC & AT1 mRNA in kidney & other tissues Neonate offspring	→	Premature completion of nephrogenesis?	→	↓Nephron number
Maternal UN Sheep Mid-gestation (Gilbert *et al.*, 2005)	→	↑Renal ACE ↑Renal AT2 receptor 9 month old offspring	→	Premature completion of nephrogenesis?	→	↓Nephron number
GC administration Sheep Early gestation (Moritz *et al.*, 2002)	→	↑Renal Aogen mRNA ↑AT1 & 2 receptor mRNA 130 dGA fetus	→	Premature completion of nephrogenesis?	→	↓Nephron number

Figure 1.12 Summary of the impact of maternal low protein diet, undernutrition and glucocorticoid (GC) administration on the intrarenal RAS and possible consequences for nephrogenesis during critical windows of development in rats and sheep, adapted from (McMillen & Robinson, 2005b).

Peripheral RAS

The intrarenal and peripheral RAS do not necessarily change in parallel in response to changes in intrauterine environment. As discussed above, a low protein diet during pregnancy decreases intrarenal RAS activity in rats (Woods *et al.*, 2001d). Whereas a similar low protein diet appears to increase circulating RAS activity, in so far as rats exposed to a low protein diet *in utero* have a greater and more prolonged pressor response to increasing doses of Ang II (Langley-Evans, 2001). Moreover, treatment with ACE inhibitors (captopril

or enalapril) lowered blood pressure reversibly in mature adult rats exposed to a low protein diet *in utero* (Langley-Evans & Jackson, 1995; Manning & Vehaskari, 2001). Treatment with captopril from 2 to 4 weeks after birth produced an irreversible decrease in blood pressure in protein restricted offspring (Sherman & Langley-Evans, 1998). This may be due to up-regulation of AT1 receptors as losartan (a specific AT1 receptor inhibitor) also reduced blood pressure irreversibly in the offspring of protein restricted dams when administered from 2 – 4 weeks of age (Sherman & Langley-Evans, 2000). The up-regulation of AT1 receptors may be in the kidney, brain, heart or vasculature and glucocorticoids are known to increase AT1 receptor expression at different peripheral sites including the vasculature (Sato *et al.*, 1994).

Experimental restriction of placenta growth results in fetuses that have a greater decrease in MAP in response to infusion of the ACE inhibitor captopril than control fetuses in late gestation (Edwards *et al.*, 1999). This result suggests that RAS plays a greater role in the regulation of arterial blood pressure in placentally restricted fetuses in late gestation (Edwards *et al.*, 1999). Maternal undernutrition (50%) during the last 30 dGA also results in an increase in maternal plasma concentrations of cortisol with an associated increase in fetal arterial blood pressure and an augmented pressor response to increasing doses of Ang II (Edwards *et al.*, 2001c). Therefore, increased exposure to cortisol during fetal life may result in an increase in sensitivity to Ang II vasoconstrictor actions through an increase in AT1 receptor expression or changes in downstream actions within the vascular smooth muscle (McMillen & Robinson, 2005a). Glucocorticoid treatment for 48 hours in early gestation causes a rightward shift in baroreflex response to phenylephrine infusion (when measured at 40 months), but there were no change in vascular sensitivity to Ang II infusion (measured at 4, 10 & 19 months) (Dodic *et al.*, 1998; Dodic *et al.*, 1999). Glucocorticoid administration in late gestation can cause an increase in femoral vascular resistance, arterial blood pressure (Derks *et al.*, 1997) and augment systolic and diastolic blood pressure responses to increasing doses of Ang II but not noradrenaline (Tangalakis *et al.*, 1992). Fetal cortisol infusion also causes an increase in AT1 receptor mRNA within the fetal heart (Segar *et al.*, 1995) and a greater hypotensive effect after blockade of AT1 receptor (Forhead *et al.*, 2000b). **No one has yet determined the effect of a peri-implantation nutrient restriction on the peripheral RAS in sheep.**

1.8 Hypotheses

Maternal nutrient restriction either in peri-implantation or late gestation will:

- Lead to a redistribution of resources that reduces fetal growth and alters organ size in a tissue specific manner.
- Alter fetal renal function and RAS in late gestation, which may result in altered cardiovascular control.
- Alter the development of the kidney resulting in fewer nephrons in late gestation.

1.9 Aims

This thesis aimed to investigate the effects on the fetal cardiovascular system, kidney, RAS and fetal growth following either peri-implantation or late gestation maternal nutrient restriction. This thesis concentrates primarily on the role of the RAS and the kidney although other mechanisms of cardiovascular control were also studied.

Small size at birth is associated with increased risk of diseases in later life, including CVD and related disorders in adult life (Barker, 1998). Poor *in utero* nutrition may lead to altered fetal growth and cardiovascular development. This could be a direct effect of diet or via exposure to stress hormones.

The aim of chapter 3 was to investigate the effect of peri-implantation maternal nutrient restriction on ewe body composition and weight, and on the growth and organ size of the fetus.

Altered cardiovascular control in adult sheep offspring has previously been observed following maternal nutrient restriction in early gestation (Gardner *et al.*, 2004; Cleal, 2005). It is not clear as yet what mechanisms underlie these changes in cardiovascular control nor is it yet known whether these changes in cardiovascular control are already present in the fetus.

The aim of chapter 4 was to investigate the effect of peri-implantation maternal nutrient restriction on fetal renal and peripheral vascular mechanisms of cardiovascular control in late gestation.

The effects on the fetus of any nutritional challenge are dependent on the severity and timing of the particular challenge.

The aim of chapters 5 was to investigate the effects of either a more severe peri-implantation or a late gestation maternal nutrient restriction on the ewe weight and body composition, and on the growth of the fetus.

The aim of chapter 6 was to investigate the effects of either a more severe peri-implantation or a late gestation maternal nutrient restriction on fetal renal and peripheral vascular mechanisms of cardiovascular control in late gestation.

One candidate mechanism linking poor *in utero* nutrition and high blood pressure in later life is the kidney. There is a degree of functional redundancy in the fetal kidney and so the impact of changes in structure may only become apparent in later life. Organ weight is a crude indicator of structure and function, therefore it is important to investigate if maternal nutrient restriction results in any change in organ structure.

Thus the aim of chapter 7 was to investigate the effects of the more severe peri-implantation or a late gestation maternal nutrient restriction on fetal kidney nephron number in late gestation.

2 General Methods

All procedures contained in this thesis were approved by the Home Office and were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.1 Sheep Husbandry

2.1.1 Ewes

Welsh Mountain sheep were used as they are small (35 – 70 kg), adapted to harsh highland environments and produce a higher proportion of singleton fetuses compared to other breeds. Ewes in their 1st parity (2003/4 n = 48, 2004/5 n = 52) were brought in and maintained according to normal sheep husbandry at the Royal Veterinary College (RVC) (North Mymms, Hertfordshire). Prior to study enrolment the ewes were kept at grass. All ewes in the study were weighed and body condition scored over the 3rd lumbar vertebra (Russell, 1991) (John Thompson, RVC) as a means of assessing the fatness of the animal (appendix 1). Muscle and fat depth were measured from ultrasound (Aloka SSD 210 DX11, BCF Technology, Livingstone, UK) images of the 3rd lumbar region, using the internal callipers of the ultrasound machine (figure 2.1).

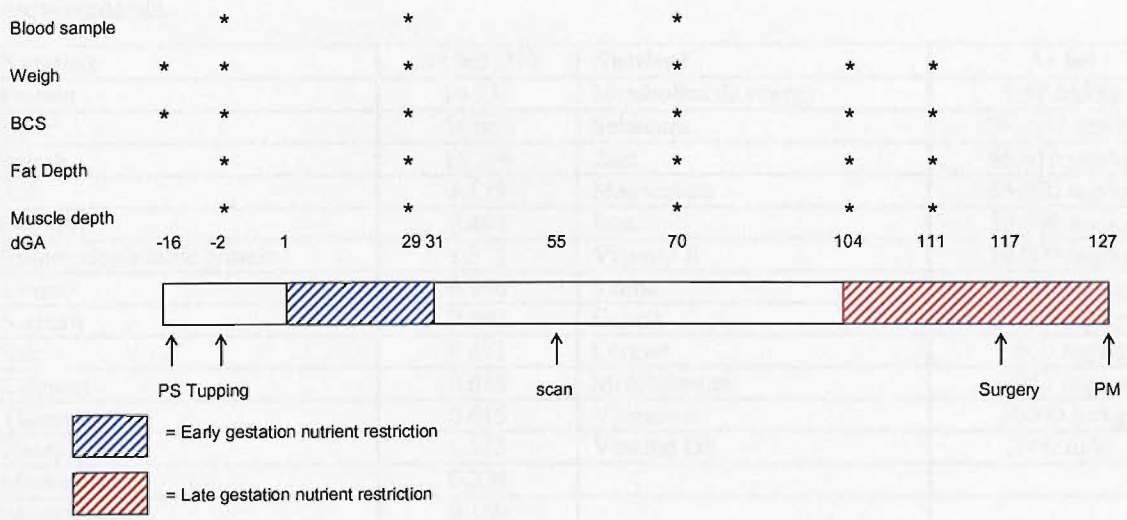

Figure 2.1 Overall Timeline. * denotes blood samples and measurement of weight, body condition score (BCS), fat depth and muscle depth. PS = progesterone sponge, PM = post mortem. Term = 145 dGA.

2.1.2 Mating

Ewes with a condition score of 2.5 or above were enrolled over the sheep breeding season (Oct-Feb) in batches of 3-4. The ewes had a progesterone sponge inserted vaginally to synchronise oestrous, the progesterone sponge was removed at -2 dGA and a raddled ram was introduced. Day 0 of gestation was taken as the first date at which an obvious raddle mark was observed (figure 2.1).

2.1.3 Nutrition regime

Ewes were individually housed on straw and nutritional requirements were calculated according to ewe start weight (measured at -16 dGA) and adjusted for fetal gestational age according to Agriculture and Food Research Council (AFRC) nutritional guidelines 1993, (RUMNUT (Ruminant Nutrition) version 5 for sheep software A T Chamberlain, Hampshire).

In 2003/04 (Chapter 3 and 4) ewes received either 50% of their total nutrient requirements from day 1 - 31 (Restricted (R), $n = 20$) or 100% nutrient requirements (Control (C), $n = 27$) using a complete pellet diet (table 2.1). At all other times animals were fed 100% nutrient requirements.

Nutrient	As fed (%)	Nutrient	As fed
Protein	14.750	Metabolisable energy	9.57 MJ/kg
Fibre	16.653	Selenium	200.037 mg/kg
Starch	15.200	Zinc	65.610 mg/kg
Ash	8.175	Magnesium	65.050 mg/kg
OIL (A)	2.493	Iron	39.500 mg/kg
Rumen degradable protein	1.375	Vitamin E	10.000 mg/kg
Lysine	0.766	Iodine	4.086 mg/kg
Sodium	0.692	Cobalt	2.033 mg/kg
Salt	0.691	Copper	1.500 mg/kg
Calcium	0.688	Molybdenum	0.053 mg/kg
Threonine	0.615	Vitamin A	10000 iu/kg
Phosphorus	0.313	Vitamin D3	2000 iu/kg
Methionine	0.224		
Magnesium	0.150		
Chloride	0.086		

Table 2.1 Nutritional composition of diet (P316, Charnwood Milling Co. Ltd, Suffolk, UK) fed to ewes at RVC and Southampton.

In 2004/05 (Chapter 5, 6 and 7) ewes received either 40% of their total nutrient requirements from 1 - 31 dGA (Early (E), $n = 20$), 50% of their total nutrient requirements from 104 – 130 dGA (Late (L), $n = 12$) or 100% of nutrient requirements throughout (C, $n = 25$). At all other times animals were fed 100% nutrient requirements.

2.1.4 Maternal monitoring

Pregnancy was confirmed using a progesterone assay (ELISA, Ridgeway Science, UK) on plasma collected directly from the jugular vein into a chilled lithium heparin blood collection tubes at 18 dGA. Ewes were ultrasound scanned at ~55 dGA and any ewes that were non-pregnant or carrying twins were removed from the trial. Maternal blood samples were taken prior to conception at -2 dGA, then at 29 and 70 dGA for subsequent analysis.

At enrolment (-16 dGA) weight and body condition score (BCS) were recorded. Weight, BCS, fat depth and muscle depth were recorded at -2, 29, 70, and either 104 or 111 dGA when ewes were transported to Southampton. Those ewes which were weighed at 104 dGA were weighed again at 111 dGA in Southampton.

2.2 Surgery

2.2.1 Materials for instrumentation

Catheters

All catheters (Portex translucent PVC tubing, Portex Ltd, UK) were assembled at University of Southampton and identified by coloured markings at each end (details table 2.2). The amniotic, tracheal, bladder and maternal catheters included small holes in the sides near their internal ends to avoid blockage problems. The bladder catheter had a 5 mm length of vinyl tubing (ID 2 mm, OD 3 mm) attached to the proximal end to allow anchoring (figure 2.2).

Catheter	Length (m)	ID (mm)	OD (mm)	Distance inserted (cm)
Carotid artery	1.5	1	2	5
Femoral artery	1.5	1	2	8
Femoral vein	1.5	1	2	8
Bladder	1.5	1	2	1
Trachea	1.5	1	2	3
Amniotic	1.5	1	2	n/a
Maternal jugular vein	1.2	2	3	30

Table 2.2 Details of catheters used.

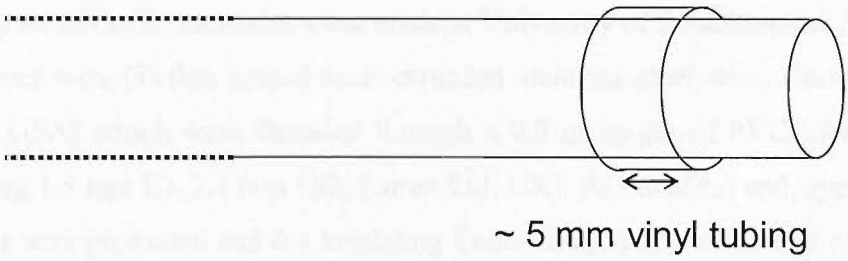

Figure 2.2 Proximal end of bladder catheter

18 G blunt needles (TYCO healthcare group, LP, MA, USA) were placed into the distal ends of the fetal catheters, and 14 G blunt needles (VetTech, UK) were placed into the distal ends of maternal catheters. These provided a female port to enable connection to a male to male connector (SIMS Portex Ltd, UK) and then a three way tap (VYGON, Ecouven, France).

Flow probes

Blood flow in the femoral and carotid arteries was measured using Transonic ® flow probes (Transonic Systems Inc, Ithaca, USA). The probes are made up of a body, containing two ultrasonic transducers, and a fixed acoustic reflector (in this study a U-bend) (figure 2.3). The downstream transducer emits an ultrasound signal through the vessel, which is reflected by the on the other side of the vessel, to the upstream transducer for detection. An ultrasound signal is then emitted by the upstream transducer, and the sequence is reversed. Blood-flow inside the vessel affects the time taken for the beam to pass between transducers, and the two opposing ultrasound transit-times can be used to calculate blood flow within the vessel (Drost, 1978).

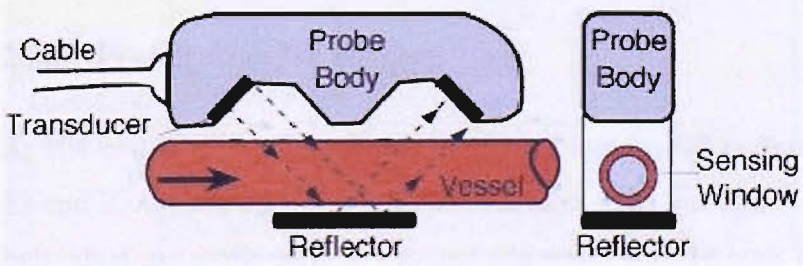

Figure 2.3 Transonic flow probe.

Electrocorticogram

Electrocorticogram (ECoG) electrodes were made at University of Southampton. Three 1.1m lengths of Cooner wire (Teflon coated multi-stranded stainless steel wire, Cooner Wire & Cable co, CA, USA) which were threaded through a 0.9 m length of PVC tubing (Portex clear PVC tubing 1.5 mm ID, 2.1 mm OD, Portex Ltd, UK). At the distal end, approximately 8 cm of Cooner wire protruded and the insulating Teflon from 1 cm of the end of each wire was removed with a soldering iron. At the proximal end approximately 12 cm of each Cooner wire protruded and one wire was chosen as the earth, knotted and a section (~1 cm) bared below the knot (the distal end was also marked with a knot). The two remaining wires had rubber discs threaded onto them, and then were knotted 4 cm from the tubing, the wire below the knots (~6 cm) was bared and rolled into balls (figure 2.4). Silicon sealant (RS components, UK) was used to seal both ends to help prevent infection. A 2 mm brass pin (NL972, Digitimer Ltd, UK) was soldered to the distal end of one of the recording wires, and 1 mm stacking plugs (NL970, Digitimer, UK) were soldered to the distal ends of the other recording wire and the earth.

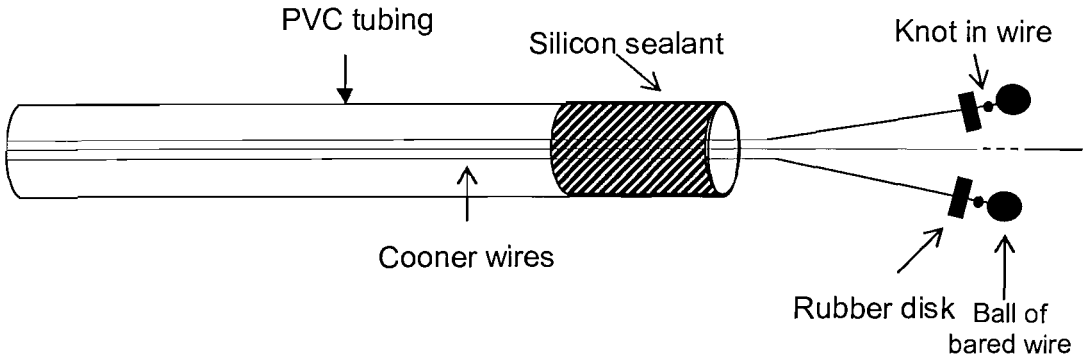

Figure 2.4 Proximal end of an ECOG electrode. 3 strands of Cooner wire protruding from polyvinyl tubing end filled with silicon sealant. Earth wire (centre) with bared portion (---), and recording wires with knots above rubber disks and balls of bared wire.

2.2.2 Preparation for surgery

At 104 or 111 dGA the pregnant ewes were transported to Southampton (2003/2004: C, n = 13 and R, n = 10, 2004/2005: C, n = 12, R, n = 12 and L, n = 11) where they were put into individual metabolic carts in a monitoring room with 12 hour light/dark cycle to acclimatise for at least 5 days. The ewes were fed 100% nutritional requirements calculated according to body weight at enrolment (-16 dGA) and adjusted for gestational age. Ewes also had access

to bags of wheat straw to maintain rumen function. The ewes were starved for 24 hours prior to surgery to reduce the chance of ruminal tympany which is caused by a build up of gas produced by the bacterial flora in the ruminant stomach and causes impaired breathing and possibly subsequent death. Starvation also reduces the chance of regurgitation, which can choke intubated animals.

Surgery took place at 116-118 dGA. Anaesthesia was induced by intravenous (i.v.) administration of aqueous sodium thiopentone (750 mg in 10 ml saline, LINK Pharmaceuticals Ltd, UK) to the jugular vein. The ewe was then transferred to the operating table, its neck extended, supported, intubated with a cuffed endotracheal tube (7.0mm ID, SIMS Portex Ltd, UK) using a laryngoscope and the cuff of the endotracheal tube was inflated. Anaesthesia was maintained with 2% halothane (halothane BP 100% v/v, Rhodia Organique Fine Ltd, UK) in oxygen using a closed circuit system (Aestiva/5 anaesthetic machine, Datex-Ohmeda Ltd, UK). Maternal ocular reflexes, heart rate and O_2 saturation were monitoring throughout to verify the depth of anaesthesia. The first incision was made once the anaesthetist confirmed the animal was fully unconscious. Every 15 minutes cardiovascular and airway gas parameters were recorded.

Using electric clippers, the neck (see 2.2.5), abdomen, and half way up one flank (for exteriorisation of catheters) were shaved. These areas were scrubbed three times with water soaked cotton wool and iodine based scrub solution (Videne, Adams Healthcare, UK), drying with paper towels between and after. They were then thoroughly covered with organic iodine preparation (Pevidine, Novartis Animal Health Ltd, UK).

All surgery procedures were carried out under strict aseptic conditions as the fetus lacks a developed immune system, which makes it susceptible to infections. Prior to surgery, all surgeons washed nails, hands, forearms and elbows with surgical scrub (Hydrex surgical scrub, Adams Healthcare, UK) and a sterile brush for several minutes before rinsing, this protocol was repeated twice more finally drying thoroughly with a sterile towel. Surgeons wore hats and masks and sterile surgical gowns and gloves and took care to maintain sterility at all times using surgical drapes and operating equipment.

2.2.3 Exteriorisation of fetus

A midline incision approximately 10 cm long was made by scalpel and diathermy in the lower abdominal wall in 2 stages (skin then muscle) avoiding piercing the gut. The uterus was palpated to determine the fetal number and position and the area containing the fetal hindquarters was exteriorised. A small (approximately 5 cm) incision was made in the uterine wall at the site of fetal tail attachment avoiding large visible blood vessels and cotyledons. The corners of the incision were clamped with babcocks and the fetal hindquarters were exteriorised from the uterus to the required extent whilst minimising loss of amniotic fluid. After checking the umbilical cord was in a suitable position and not snagged, the uterus was clamped around the fetus with babcocks to form a watertight seal. The hind quarters of the fetus were then positioned and anchored to provide a suitable operating area. Once instrumentation was completed the fetus was returned to the uterus and the uterine incision was closed using two layers of suturing. The fetal head was then located and a second incision was made for exteriorisation in the same way as for the hind quarters.

2.2.4 Fetal instrumentation

Catheters

To enable implantation of the femoral vein catheter an incision (approx. 5-6 cm long) was made in the right inguinal skin crease. The femoral vein (located near to the psoas muscle, identified by colour and lack of pulse) was exposed and a 3 cm length was cleared of connective tissue. The distal end of the vessel was ligated with 2/0 silk suture (Pearsalls Ltd, UK), the proximal end was supported with 2/0 silk suture, a small incision was made and the catheter fed in 8 cm. After checking the patency of the line with a syringe of saline (Baxter Healthcare Ltd, UK) the catheter was secured at both ends of the exposed vessel using 2/0 silk suture.

The same procedure was carried out at the left inguinal skin crease, and the femoral artery (located near to the psoas muscle, identified by having a pulse) was exposed taking care not to damage the femoral nerve. Catheterisation took place as for the femoral vein.

A suprapubic incision was made on the left ventral surface to expose the bladder. The bladder was catheterised via puncture incision, and the catheter secured by a purse string suture (3/0 silk suture).

An incision (approx. 8 cm) was made in the midline of the fetal neck and the right carotid artery was located by pulse. About 3 cm of the artery was dissected free of connective tissue and separated carefully from the vagus nerve. Catheterisation took place as for the femoral vein with the catheter being inserted 5-6 cm.

The trachea was located through the same incision as the carotid artery, and a suture was tied loosely around it. The tracheal catheter was inserted into a hole made using a curved needle between two rings of cartilage and was secured using the tied suture.

The amniotic catheter was anchored to the back of the fetal neck by suturing through two of the holes made in the proximal end of the catheter.

Flow probes

After the right femoral vein and the right carotid artery had been catheterised (described above), the same incisions were used to expose about 2 cm of the right femoral and left carotid artery and clear them of connective tissue and nerves. The U-bend of the flow probe was placed around the vessel and the body of the probe was attached with 2 screws. When the femoral artery probe had an integral mesh reinforced silicone flange, this was sutured to surrounding tissue to anchor the probe and prevent twisting.

ECoG electrodes

The fetal skull was exposed from the parietal bone to the occipital bone. At two points symmetrical about the midline suture, a hand drill was used to make holes in the skull taking care not to pierce the dura. The balls of bared Cooner wire and knots were fed into the holes, and the rubber discs attached to the skull with superglue (RS electronics, Northants, UK). The earth wire was sewn into the subcutaneous layer with the bared portion remaining inside the skin.

Post-instrumentation

After instrumentation was complete at each site, the catheters were checked for patency using a syringe of heparinised (heparin 50 iμ/ml, Leo Laboratories Ltd, UK) saline, and the incision was closed. The ECoG electrode, catheters and flow probes were secured in two places on the fetal exterior, making sure they would remain unkinked and would not interfere with fetal movement. The fetal abdominal circumference, biparietal diameter and femur length were measured at surgery.

The catheters, flow probes and electrodes were tunnelled through the maternal flank and secured to the ewe's back. The incision in the flank was closed in two layers using a purse string stitch with 2/0 silk suture. The abdominal incision was closed in two layers, the muscle layer was closed with a nonabsorbable monofilament suture (Ethilon, Johnson & Johnson Intl, BE-1932 St-Stevens-Woluwe, Belgium) and the skin was sutured with 2/0 silk suture.

2.2.5 Maternal instrumentation

After location by venous occlusion, skin was cut to expose a 10 cm section of maternal jugular vein was exposed and it was cleared of connective tissue. The catheter was implanted as for the fetal femoral vein (see 2.2.4), but the catheter was inserted 20 cm. A 14 G angiocatheter (Abbocath-T, Abbott Laboratories, UK) was inserted into the right maternal jugular vein via a small hole made with a size 11 scalpel blade. A more concentrated heparinised saline (100 U/ml) was used for flushing maternal catheters. The incision was closed using 2/0 silk suture and the catheters and angiocatheter were secured with 2/0 silk suture in two places on the maternal neck.

2.2.6 Post-surgery care

Ewe and fetus received antibiotics towards the end of surgery (table 2.3). During surgery catheters were filled with heparinised saline and then flushed daily to maintain their patency. At least four days post-operative recovery was allowed before experimentation, during which a daily antibiotic regime (table 2.3) was followed and blood samples were collected from the ewe and the fetus for blood gas analysis (section 2.3.7).

Antibiotic	Target	Surgery	Recovery days (x No. of days)
Betamox	Ewe (i.m.)	150 mg/kg	NA
Crystapen	Ewe (i.v.)	600 mg	300 mg (x = 5)
Gentamycin	Ewe (i.v)	40 mg	40 mg (x= 2)
Crystapen	Amnion (i.a.)	300 mg	150 mg (x = 5)
Gentamycin	Amnion (i.a.)	40 mg	40 mg (x = 2)
Crystapen	Fetus (i.v.)	300 mg	150 mg (x = 5)

Table 2.3 Antibiotic regime, Gentamycin (40 mg/ml, Faulding Pharmaceuticals Plc, UK), Crystapen (150 mg/ml Benzylpenicillin sodium, Britannia Pharmaceuticals, UK), Betamox long acting (150 mg/ml Amoxycillin, Norbrook Laboratories Ltd, UK).

2.3 Physiological monitoring

2.3.1 Blood flow

Flow probes were connected via an extension lead to a Transonic transit time perivascular flowmeter (Transonic TS420). The flowmeter was housed in a Transonic double flowmeter console (Transonic TS402) and the filters were set to 40 Hz.

2.3.2 Blood pressure and heart rate

The distal ends of the fetal carotid artery, tracheal and amniotic catheters were connected via three way taps to saline filled sterile pressure domes (Menscap 844-22, Skoppum, Norway). Gold plated pressure transducers (Menscap 844-26) were attached to the domes, and then connected via an extension lead to the pressure amplifiers (NL108, Digitimer Ltd, UK). The gain of the amplifier was set to 1.0 V which is appropriate for blood pressure measurements as 1.0 V at the output corresponds to 100 mmHg pressure at the transducer. To correct for the difference between fetal heart height and transducer height, amniotic pressure was subtracted from arterial pressure using a differential amplifier (NL143, Digitimer Ltd, UK). To enable a raw recording of amniotic pressure at the same time as the correction describe above the output was split into two outputs using a divider (LEMOUK Ltd, UK).

2.3.3 ECoG activity

The ECoG pins were plugged into a head stage (NL100AK, Digitimer Ltd, UK). The head stage was connected to an AC coupled differential amplifier (NL104, Digitimer Ltd, UK) with the gain set at 1K, the low frequency cut off at 0.1 Hz. The balance was set to reduce interference from external sources and match settings to those of the head stage. The NL104 was then connected to a filter module (NL125, Digitimer Ltd, UK) with the filter windows set between 50-100 Hz and 300-400 Hz. All the Neurolog (NL) units were housed in a NL900D case (Digitimer Ltd, UK).

2.3.4 Data acquisition

Outputs from the flow modules, pressure modules, and ECoG were connected to a Maclab/8 (AD Instruments Ltd, UK), then to a computer running Chart4 for Windows software (AD Instruments Ltd, UK), where the data were recorded at 20 samples per second (100 samples per second during phenylephrine experiment). Flows were calibrated using the zero and scale functions of the TS420 when first connected. Pressures were calibrated daily, between 0 and 100 mmHg, using a sphygmomanometer connected at the pressure dome.

Data were collected with Chart4 software and collated using the 'data pad' function. Mean arterial pressure was calculated from the standard formula (1/3 systolic blood pressure + 2/3 diastolic blood pressure). Heart rate was calculated from the peaks (above a noise level of 10%) on the blood pressure trace. Blood pressures, flows and heart rate were averaged over one minute periods apart from during the baroreflex. Fetal ECoG state was assessed by visual discrimination (Walker & Pratt, 1998). Vascular resistance was calculated for the carotid and femoral artery by dividing MAP by blood flow (Green *et al.*, 1998).

When experimenters entered the animal room or other events occurred which may have disturbed the animals, such as unexpected loud noises, it was noted on the chart recording. When the data were analysed these periods and the next 5 minutes were excluded to remove effects of maternal stress on fetal cardiovascular parameters. The arterial pressure recording

failed on a few occasions due to temporary catheter occlusion. This could be observed by the loss of pulse from the trace. Blood pressure data during these periods was excluded and heart rate data were calculated from the carotid or femoral flow recording instead.

2.3.5 Blood samples

During pregnancy (-2, 29 and 70 dGA) 45 ml blood samples were taken (table 2.4) directly from the ewe jugular using a syringe and needle for subsequent analysis.

Blood tube	Volume of blood	Volume of plasma	Number of aliquots
Lithium heparin	15 ml	7 ml	5
Clotted blood/serum	8 ml	3 ml	2
EDTA (K3)	12 ml	5 ml	5
Fluoride tube	1 ml	200 µl	1

Table 2.4 Maternal blood collection

At the time of experimentation fetal arterial blood samples were taken from the carotid artery catheter using a syringe after removal of dead space (saline) from within the catheter. The dead space was returned to the catheter and flushed through with 3ml of heparinised saline to avoid blood clotting within the catheter. All blood samples were placed immediately into chilled EDTA or lithium heparin blood collection tubes. Serum samples were allowed to clot at room temperature before centrifuging. The samples were centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was removed and decanted into approximately 1 ml aliquots of plasma/serum then stored at -80°C for measurement of plasma hormones.

2.3.6 Urine samples

Urine samples were collected by opening the bladder catheter and allowing the bladder to draining under gravity into a sterile container for 30 minutes. The volume was recorded and 2 ml samples were centrifuged at 3000 rpm for 10 minutes. The supernatant was decanted into 1 ml aliquots and stored at -80°C for subsequent analysis. The remaining urine was sterilised through a syringe end filter (0.2 µm pore size, Sartorius Ministart, Fisher Scientific, UK)) and returned to the amniotic cavity.

2.3.7 Blood gas analysis

After surgery a blood gas analyser (ABL700, Radiometer, Copenhagen, Denmark) was used to monitor maternal and fetal pH, blood gases, oximetry, electrolyte, glucose, lactate and bilirubin in blood and fetal urine (maternal 0.5 ml, fetal 0.2 ml) samples. The blood was collected into chilled 1 ml heparinised syringes to avoid clot formation. A maximum of 22 ml of fetal blood was removed in any one protocol, less that 10% of predicted blood volume for size (~250ml).

The analyser measures pH, oxygen partial pressure (pO_2), carbon dioxide partial pressure (pCO_2), K^+, Na^+, Ca^{2+}, Cl^-, glucose, lactate, bilirubin, haematocrit, and haemoglobin. The analyser calculates O_2 saturation (sO_2), fraction of oxyhaemoglobin (FO_2Hb), fraction of carboxyhaemoglobin (FCO_2Hb), fraction of deoxyhaemoglobin (FHb), fraction of methaemoglobin (FMetHb), base excess and bicarbonate. The pH, pO_2 and pCO_2 were corrected for temperature (fetal; 39.5°C, maternal; 39°C).

2.3.8 Assays

Immulite validation

The use of the immulite system with sheep plasma was validated by: comparing a set of results with those of the same samples analysed using a radioimmunoassay developed specifically for sheep plasma; assaying a known sheep sample under various dilutions to show linearity; and spiking a sheep sample with a known concentration of human cortisol to show that the expected results were achieved, with no interference due to sheep plasma.

Adrenocorticotrophic hormone (ACTH)

Plasma ACTH (1-39) levels were measured (single measurement) using a sequential immunometric assay (LKAC5, DPC Ltd, Gwynedd, UK) run on an Immulite analyser (DPC, Ltd). The intra and inter assay coefficient of variation was 1.1% and 3.2% respectively.

Cortisol

Total plasma cortisol levels were measured (single measurement) using a solid-phase, competitive chemiluminescent enzyme immunoassay (LKC05, DPC Ltd, Gwynedd, UK) run on an Immulite analyser (DPC, Ltd). The intra and inter-assay coefficient of variation were 4.6% and 8.2% respectively.

Angiotensinogen

This work was carried out by Peter Marsters (Obstetrics and Gynaecology, Queen's Medical Centre, University of Nottingham).

Basal plasma angiotensinogen was measured in duplicate using RIA developed by Professor Broughton Pipkin (University of Nottingham), as described previously (Tetlow & Broughton, 1983). Plasma angiotensinogen was determined by the direct measurement of the maximum Ang I generation in plasma incubated at pH 7.5 and 37 °C in the presence of excess exogenous human renin and angiotensinase inhibitors.

Plasma renin activity

This work was carried out by Peter Marsters (Obstetrics and Gynaecology, Queen's Medical Centre, University of Nottingham).

Plasma renin activity was measured in duplicate using RIA developed by Professor Broughton Pipkin (University of Nottingham), as described previously (Tetlow & Broughton, 1983). Plasma renin was measured as the rate of generation of Ang I (ng/ml/h) in the presence of added substrate at pH 7.5 and 37 °C.

Angiotensin converting enzyme activity

This work was carried out with the assistance of Dr Jane Cleal and Rachel Henke (Centre for DOHaD, University of Southampton, UK). Basal plasma ACE concentrations were measured in duplicate by an enzyme assay developed by Dr A Forhead (The University of Cambridge) (Forhead *et al.*, 2000a). This assay was adapted from (Raimbach & Thomas, 1990) and (Hurst & Lovell-Smith, 1981) and has been validated in both adult and fetal sheep plasma

and tissue for both plasma/extraction quality and incubation length by Dr A Forhead. The intra and inter-assay coefficients of variation were 8.98% and 8.09%, respectively.

Angiotensin II

Plasma Ang II levels were measured in duplicate using a commercially available RIA (Euria-angiotensin II; ImmunoDiagnostic Systems Ltd, Tyne and Wear, UK) following its separation from plasma proteins using chromatography columns (C18 sep-pak; Waters Corporation, Massachusetts, USA). The intra and inter-assay coefficients of variation were 8.0% and 7.5% respectively.

Creatinine

Creatinine was measured in duplicate in plasma and urine using the commercially available Infinity^{TM} Creatinine Liquid Stable reagent (Thermo Electron Corporation, Australia). Creatinine reacts with alkaline picrate to produce a reddish colour complex (Jaffe reaction) (Husdan & Rapoport, 1968). The red colour formed is directly proportional to the creatinine concentration and is measured spectrophotometrically at 500 nm. The intra and interassay coefficients of variation were 4.9% and 8.5% respectively.

Osmolarity

Osmolarity of 100 µl of urine was measured in duplicate using a Roebling Micro-Osmometer (Borolabs, UK). The principle of calculating osmolarity is that the freezing point of an aqueous solution is measured, and the reduction below that of pure water is a direct measure of the osmotic concentration. The intra and interassay coefficients of variation were 5.3% and 7.4% respectively.

2.4 Experimental protocols

Fetuses (2004; C, n = 10, R, n = 10, 2005; C, n = 9, E, n = 9, L, n = 6) were studied on 2 successive days between 123-126 dGA (figure 2.5) following at least 5 days recovery from surgery. Continuous recordings were made of amniotic, tracheal and arterial pressure, fetal heart rate, ECoG, femoral and arterial blood flow throughout the experimental protocols. The responses to a number of challenges were assessed see details below.

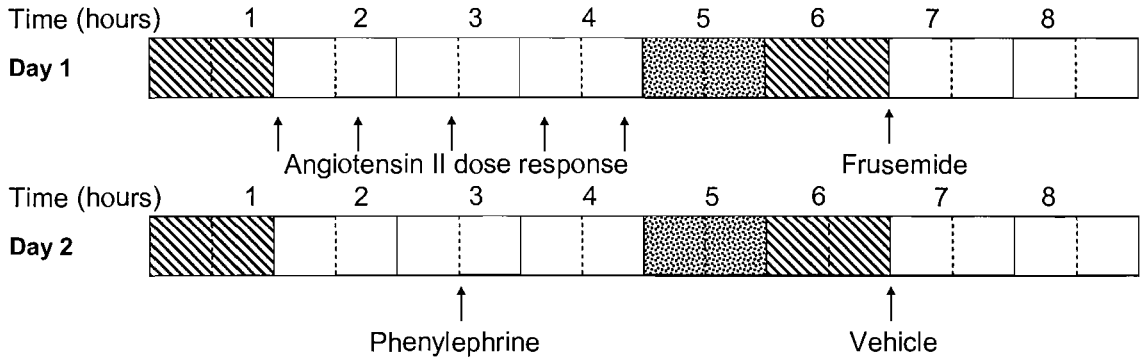

Figure 2.5 Experiment timeline. Baseline ▨, recovery ▩.

2.4.1 Angiotensin dose response

Vascular sensitivity to Ang II was assessed using increasing doses of Ang II (0 μg, 1 μg, 2.5 μg, 5 μg, and 10 μg, Sigma-Aldrich Co, UK). In each fetus bolus i.v. doses of Ang II (Sigma-Aldrich Co, UK), dissolved in sterile saline (2 ml) were administered every 30 minutes. Blood pressure was allowed to return to baseline levels between doses. All doses were given whilst the fetus was in high voltage ECoG activity.

2.4.2 Frusemide protocol

Kidney function and the function of the RAS were assessed using a bolus of saline vehicle or frusemide (20mg, Phoenix, Pharmacia, UK). The ewe was made to stand for the duration of the experiment, and nuts and water were removed but access to wheat straw was allowed. The fetal bladder catheter was opened and allowed to drain under gravity into a sterile container from time point -90 to -60 to minimise variability in the bladder volume at onset of

protocol (figure 2.6). The urine was then collected at 30 minute intervals for the duration of the experiment and the volume was recorded. 2 ml of urine was retained from each 30 minute period for subsequent analysis (pH, electrolyte, creatinine & osmolarity, see section 2.3 for details). The remaining urine was sterilized through a syringe end filter (0.2 μm pore size, Sartorius Ministart, Fisher Scientific, UK) and returned to the amniotic cavity. A basal fetal blood sample was taken at -45 min for blood gas and hormone analysis (figure 2.6 and table 2.4). At 0 minutes a 20 mg bolus of frusemide (or vehicle) was given directly to the fetus iv. At 15 min, 45 min and 75 min further blood samples were taken for blood gas and hormone analysis (figure 2.6 and table 2.4).

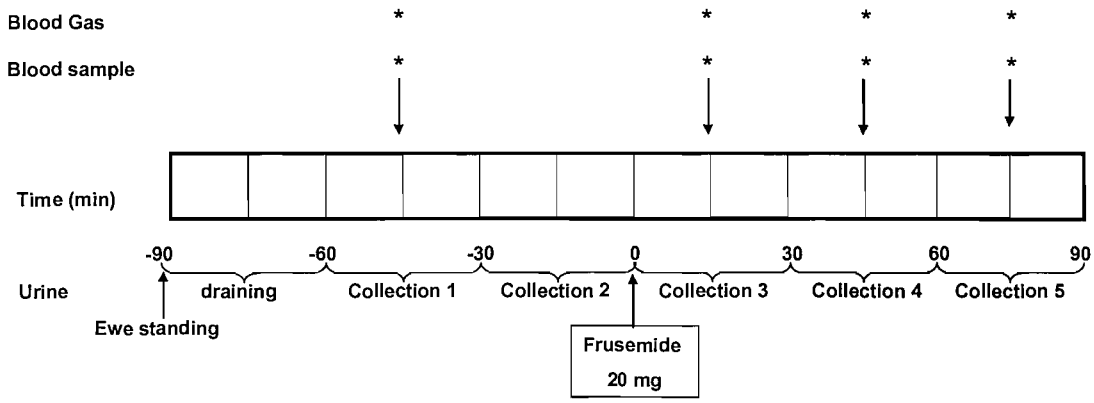

Figure 2.6 Frusemide protocol, * denotes where blood gas and samples were taken.

Time point (min)	Blood tube	Blood volume (ml)	Plasma volume (ml)	Assays
-45	EDTA	5	3	Renin, cortisol, ACTH & Ang II
	L-Hep	2	1	Creatinine, ACE & Aogen
15	EDTA	2	1.5	Renin
45	EDTA	5	3	Renin, cortisol, ACTH & Ang II
	L-Hep	1	0.5	Creatinine
75	EDTA	5	3	Renin, cortisol, ACTH & Ang II
	L-Hep	1	0.5	Creatinine

Table 2.5 Blood samples taken for analysis.

2.4.3 Baroreflex

After a one hour baseline period the fetal baroreflex response was examined. While the fetus was in high voltage ECoG activity a 75 μg i.v. bolus of phenylephrine (Sovereign Medical,

Essex, UK) dissolved in sterile saline (2 ml) was given. For description of calculation of response see appendix 4.

2.4.4 Hypoxia

In the second year only, a hypoxia challenge was used to assess the fetal cardiovascular response to reduced oxygenation (figure 2.7).

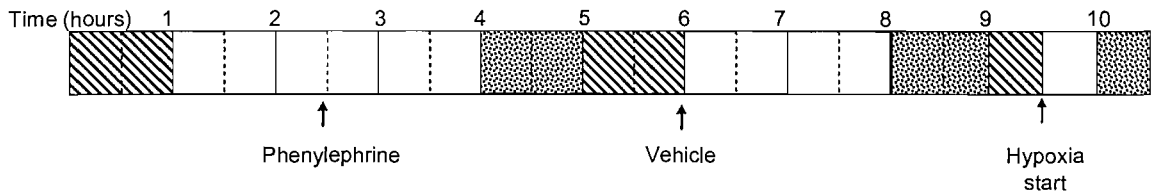

Figure 2.7 Experiment timeline. Baseline ▨, recovery ▩

The ewe was made to stand for the duration of the experiment, and nuts, water and wheat straw were removed. The bladder catheter was opened and allowed to drain under gravity into a sterile container from -60 to -30 minutes to minimise variability in the bladder volume at onset of protocol. The urine was then collected at 30 minute intervals for the duration of the protocol (figure 2.8). Hypoxia was induced by manipulating maternal inspired gases (Giussani *et al.*, 1993). A customised translucent polythene bag (457 x 762 mm, Macfarlands Group) was placed over the ewe's head and secured loosely. Known concentrations of medical air, N_2 and CO_2 were piped into the bag from an external gas supply. Following a 1 hour normoxic control period (44 l/min air), fetal hypoxia (PO_2 to ~ 11 mmHg) was induced for 30 minutes by reducing maternal F_IO_2 (14 l/min air, 22 l/min N_2, 1.2 l/min CO_2). The gas mixture was then returned to normoxic conditions (44 l/min air) for a 30 minute recovery period. Fetal arterial blood was collected into a heparinised syringe (section 2.3.7) at 15 minutes before and 5, 15, 30 and 60 minutes after hypoxia was started for blood gas analysis to ensure a suitable level of hypoxia was achieved and maintained. Blood samples (section 2.3.5) were taken at -15, 15, 30 and 60 minutes for hormone analysis (figure 2.8 and table 2.6).

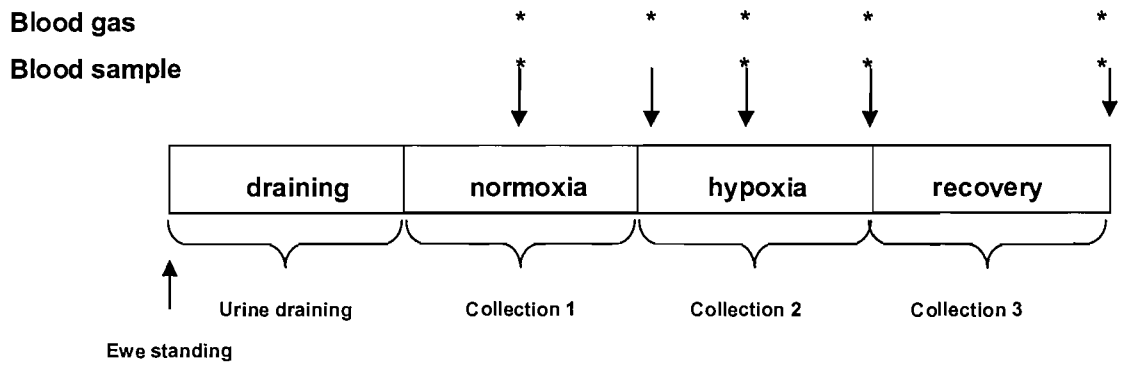

Figure 2.8 Hypoxia time line, * denotes where blood gas and samples were taken.

Time point	Blood tube	Volume of blood (ml)	Volume of plasma (ml)	Assays
-15	EDTA	6	3.5	Renin, cortisol & Ang II
15	EDTA	3	2	Ang II
30	EDTA	6	3.5	Renin, cortisol & Ang II
60	EDTA	6	3.5	Renin, cortisol & Ang II

Table 2.6 Blood sample for analysis.

2.4.5 Additional protocols

In addition to experiments detailed in this thesis all the fetuses underwent two days of monitoring and experimental protocols including insulin induced maternal hypoglycaemia and blood flew assessment using fluorescent microspheres for full details see (Burrage, 2006).

2.5 Post mortem

2.5.1 Fetal post mortem

All ewes and fetuses were killed with an overdose of sodium pentobarbitone to the ewe i.v. at 127 dGA. The abdomen was opened and the uterus removed at the cervix. Under sterile conditions the uterus was then opened and the fetus was removed and the umbilical cord cut. Fetal position, body weight, crown rump length, abdominal circumference, femur length, biparietal diameter and degree of meconium staining were recorded. Correct placement of catheters, flow probes and electrodes was confirmed. Flow probes were dissected out. The fetal brain, heart, lungs, liver, kidneys and adrenals were removed and weighed. The

cotyledons were removed from the placenta, sorted into types (A, B, C and D) and weighed. Tissues were fast frozen in liquid nitrogen, slow frozen on dry ice or fixed in formalin (10% neutral buffered, Surgipath, UK) for molecular analysis or histology (details appendix 2). In addition the whole uterus was weighed in 2004/05.

2.5.2 Kidneys

At post mortem the left fetal renal artery was catheterised (Portex translucent PVC tubing, I.D 0.58 mm, O.D 0.96 mm, Portex Ltd, UK,) and the kidney was removed. Heparin (1 ml, 5000IU/ml, Leo Laboratories Ltd, UK) and then papaverine hydrochloride (1ml, 0.24 mg) were injected into the renal artery. The kidney was then perfused (at a physiological pressure, 50 mmHg) with 50 ml of heparinised saline and then 50 ml of formalin. The perfused kidney was cut in half longitudinally and immersion-fixed in formalin, then dehydrated in 70% alcohol ready for embedding in glycol methacrylate (JB-4 Embedding Kit, Polysciences, Germany) for histological study.

2.6 Analysis strategy

Linear relationships between variables were investigated by linear regression analysis. Differences between group means were analysed by Student's unpaired t-test when simple comparison of two sets of single figures were involved. More complex comparisons necessitated the use of analysis of variance (ANOVA), where the explained (between group, whole data set) variability is compared to the unexplained (within group, in each individual group) variability. For comparison of more than one experimental group with a control group, a 1 way ANOVA is used. Where two factors may affect one outcome, a 2 way ANOVA determines both the main effect of each factor and detects interactions between the factors (whether one effect is modified by another effect).

For changes in parameters over time during a treatment, the presence of a statistically significant response was investigated using repeated measures ANOVA, as a standard ANOVA assumes that each observation is independent of the last. Where more than one experimental group is involved, it is necessary to employ a two way approach to take account

of possible interactions between the effects of the treatment and the experimental group. These 'between-subjects' tests will show if different groups have a substantially different type of response (for example, one increases in blood pressure during the treatment while another falls), but more subtle differences between the profile of responses may not be detectable with this analysis. For this reason a 'summary of measures' approach is recommended for medical statistical analysis of the magnitude and profile of a response between groups (Matthews *et al.*, 1990b). In this thesis maximum response and time to maximum response were used.

All data were tested for normality prior to analysis using Prism which tests for deviations from Gaussian distribution using the Kolmogorov-Smirnov (KS) test. It is hard to tell whether or not the values came from a Gaussian distribution by looking at the distribution of a small sample of data. A small sample size is defined as less than 12 and therefore should not represent a problem in this thesis (Altman, 1991). Where normality was not confirmed data were analysed using appropriate non-parametric tests.

2.7 Data analysis

In all cases statistical analysis was performed using SPSS 12.0 for Windows (2003) or GraphPAD (GraphPAD Software Inc., Dan Diego, USA) and significance accepted if $p<0.05$.

2.7.1 Power calculations

A power calculation was performed using a nomogram (Altman, 1991) (appendix 3). This involves calculating the standardised difference (standardised difference = difference of interest / standard deviation of each group). This can be used with the nonogram to calculate the required sample size and power.

The primary outcome was taken to be fetal MAP. A difference of approximately 2 mmHg MAP following early gestation maternal nutrient restriction was previously observed as significant (Hawkins *et al.*, 2000c). The average standard deviation was 1.7 mmHg which

gives a standardised difference of 1.18. Therefore, to achieve a power of 70% at a significance level of 0.05, 18 animals are required i.e. 9 per group.

2.7.2 Ewe biometry

Maternal parameters were assessed at several time points throughout gestation (see 2.1.5). The last time point was either 104 or 111 dGA depending on when delivery to Southampton occurred, and data from these points were analysed together. Even so the data for the last time point (104/111) is incomplete (n = 16 out of 20). All values are expressed as mean ± SEM. Weights, BCS, fat and muscle depth of the ewes were compared between the groups by repeated measures ANOVA. Change from pre-challenge measurements were calculated and analysed in a similar manner. Where significant effects were found by ANOVA a Bonferroni *post-hoc* test was used to identify where significant differences were.

2.7.3 Fetal biometry

All values are expressed as mean ± SEM. Change from surgery, ponderal index, organ size as % of body weight and organ ratios were calculated. Fetal body proportions, organ weights, and calculated parameters were compared between diets by unpaired Student's t-test (chapter 3) and 1 way ANOVA (chapter 5).

2.7.4 Baroreflex

Arterial pressure was acquired by chart (see section 2.3 for details) for the two minutes before the phenylephrine injection until after the maximum change in pressure. Arterial pressure and R-R interval were determined on a beat-by-beat basis. R - R interval the time elapsing between two consecutive R waves in the electrocardiogram was plotted against preceding systolic pressure (details appendix 4). The linear portion of the curve was used to determine the gradient and operating point of the response. The gradient is a measure of the sensitivity of the baroreflex response and the operating point (equivalent to the EC50 in pharmacology) is the pressure value at which the half-maximal RR interval response is seen. The dose per kg fetus was calculated after post mortem. All parameters were compared by unpaired Student's t-test (chapter 4) and 1 way ANOVA (chapter 6).

2.7.5 Angiotensin II response

Systolic, diastolic and mean arterial pressure, heart rate, carotid and femoral flow were acquired by Chart (details section 2.3). The peak response to Ang II was recorded for each dose, compared between the groups by repeated measures ANOVA and a Bonferroni *post-hoc* test were used to identify where significant differences occurred. Individual animal's response to Ang II was expressed as area under the curve (AUC) and compared between diets by unpaired Student's t-test (chapter 4) and 1 way ANOVA (chapter 6).

2.7.6 Frusemide/vehicle

Systolic, diastolic and mean arterial pressure, heart rate, carotid and femoral flow were acquired by Chart (details section 2.3) and were averaged over consecutive 10 minute periods during frusemide and vehicle experiments. Individual animal cardiovascular response to frusemide/vehicle were reduced to summary measures (Matthews *et al.*, 1990a) (maximum response and time to maximum response) and compared between groups by unpaired Student's t-test (chapter 4) and 1 way ANOVA (chapter 6).

2.7.7 Renal function

Urine flow, osmolarity and Na^+ concentration were measured. Glomerular filtration rate (GFR) (ml/min) was calculated as urine creatinine (g/l) X (urine volume (ml/min) / plasma creatinine (g/l)) (Adzick *et al.*, 1985). These parameters were analysed by repeated measures ANOVA, and a Bonferroni *post-hoc* test were used to identify where significant differences occurred.

3 The effect of 50% peri-implantation maternal nutrient restriction on fetal growth and organ size

3.1 Introduction

Low birth weight has been associated with high blood pressure and greater risk of CVD in later life (Barker & Osmond, 1986). The Dutch famine revealed that the effects on birth weight, the occurrences of risk factors and diseases in later life are dependent on the timing of periods of undernutrition (Roseboom *et al.*, 2001b; Painter *et al.*, 2005b).

The impact of any period of nutrient restriction is dependent on the timing of the insult and the organs and systems developing during that critical window. A variety of intensities of maternal nutrient restriction have been investigated in sheep and some of these have been found to alter cardiovascular function in fetal (Hawkins *et al.*, 2000b; Gardner *et al.*, 2004) and adult (Gardner *et al.*, 2004) offspring. These changes are observed in the absence of any growth retardation, therefore compensatory mechanisms may maintain growth at the expense of fetal cardiovascular function. **However, no one has yet investigated how a peri-implantation nutrient restriction affects fetal body weight or organ weight in late gestation.**

Nutrient restriction for short time periods during very early windows of development can have long term consequences (Kwong *et al.*, 2000). Small for gestational age babies (Konje *et al.*, 1996) and fetuses of thinner mothers (Mukherjee *et al.*, 2005) have narrower sausage shaped kidneys. Altered kidney development may reduce glomerular number, which has been associated with low birth weight (Hughson *et al.*, 2003) and hypertension (Keller *et al.*, 2003). Therefore, there may be changes in fetal regional growth, e.g. of kidneys, which may impact on kidney function and result in altered cardiovascular control. **However, no one has yet investigated how peri-implantation nutrition affects fetal kidney dimensions in late gestation.**

The mechanisms behind the association of low birth weight with increased risk of

hypertension and CVD have not yet been elucidated, but may be the result of a signal such as glucocorticoids rather than a direct consequence of altered nutrients. Exposure to excess glucocorticoids restricts fetal growth and leads to postnatal hypertension in rats (Benediktsson *et al.*, 1993; Seckl, 2001) and sheep (Fowden *et al.*, 1996; Dodic *et al.*, 1998; Jensen *et al.*, 2002). Maternal stress such as 50% maternal nutrient restriction in late gestation can increase maternal cortisol levels in sheep, although this did not alter fetal cortisol levels (Edwards & McMillen, 2001). The fetus may be exposed to raised glucocorticoids through mechanisms other than direct exposure via increased maternal levels, such as altered 11βHSD2 activity, activation of the fetal HPA axis or alterations in GR. The severity of this challenge was designed to produce effects on fetal development without causing maternal stress, but without measuring cortisol and ACTH there is no way of confirming that this is the case. **Therefore it is important to investigate if peri-implantation nutrient restriction alters maternal cortisol or ACTH during gestation.**

3.2 Hypothesis

50% peri-implantation nutrient restriction will lead to a redistribution of resources that reduces fetal growth and organ size in a tissue specific manner.

3.3 Aims

To determine the effect of a 50% nutrient restriction during the peri-implantation period on:

1. Maternal weight, BCS and fat and muscle depth during gestation.
2. Maternal hormones (cortisol and ACTH) during gestation.
3. Fetal body measurements, organ weights and kidney dimensions in late gestation.

3.4 Methods

3.4.1 Diet and surgery

Pregnant Welsh mountain ewes (Animals (Scientific Procedures) Act 1986) were maintained according to normal practices at the RVC (details section 2.1). They were penned individually on straw and received either a 50% of their total nutrient requirements for 30 days peri-implantation (R, n = 10) or 100% nutrient requirements (C, n = 10) for the first 30 days of pregnancy (details section 2.1.3). Both groups were then fed 100% nutrient requirement for the rest of gestation. Blood samples were taken prior to conception at -2 dGA then at 29 and 70 dGA to be analysed for levels of maternal hormones (details section 2.3). At enrolment (-16 dGA) weight and BCS were recorded. Weight, BCS, fat depth and muscle depth were recorded at -2, 29, 70, and either 104 or 111 dGA (figure 3.1). At ~117 dGA the fetuses were surgically instrumented as described in general methods (details section 2.2) and the femur length, biparietal diameter and abdominal circumference were measured. At 127 dGA post mortem took place and fetal body measurements and organ weights were measured (details section 2.5).

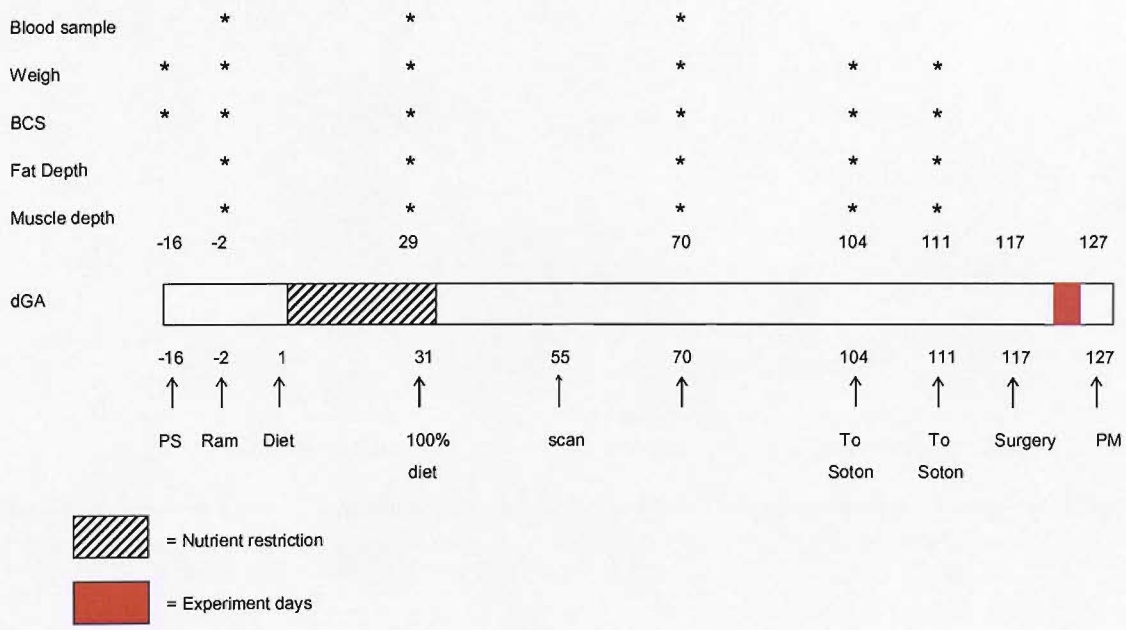

Figure 3.1 Timeline. * denotes blood samples, and measurement of weight, BCS and fat and muscle depth. PS = progesterone sponge, PM = post mortem. Term = 145 dGA

3.4.2 Analysis and statistics

For a general description of the analysis strategy, and the power calculation and statistics packages used, see section 2.6.

Male and female fetuses were analysed together due to the small sample size of some of the groups when divided according to sex. No significant differences were found between males and females in body weight, any organ weight, when analysed by Students's t test.

Where serial measurements were made during an experiment, e.g. maternal weight, these were compared by repeated measures 2 way ANOVA, over time and between dietary groups to identify any differences between the groups. When the result was significant over time, the times at which parameters differed significantly from baseline were identified by Bonferroni *post-hoc* tests.

Fetal biometry and organ weights were compared between groups by Student's t-test.

3.5 Results

3.5.1 Ewe biometry

When all animals were considered together, ewe weights decreased between −16 and −2 dGA and increased between −2, 70 and 111 dGA, BCS increased between −2 and 70 dGA, fat depth decreased between −2 and 29 dGA, muscle depth did not change significantly during gestation (figure 3.2). There was no significant effect of maternal nutrient restriction on ewe weight, BCS, fat or muscle depth.

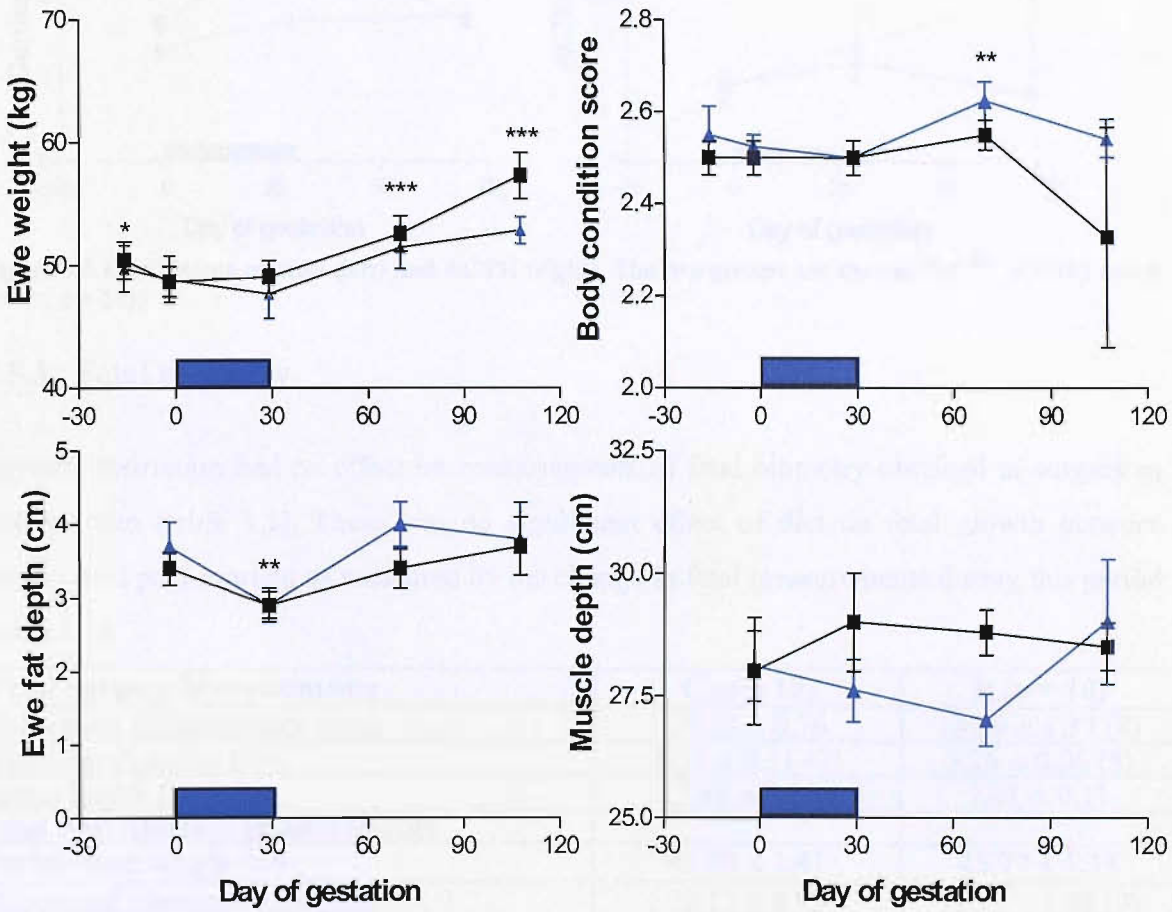

Figure 3.2 Ewe weight (top left), BCS (top right), fat (bottom left) and muscle depth (bottom right). The two groups are shown; C (■, $n = 10$) and R (▲, $n = 10$, final time point, $n = 6$). * $p < 0.05$, ** $p < 0.01$ and $p < 0.001$ significantly different from -2 dGA, all animals.

3.5.2 Ewe plasma hormones

When all animals were considered together, there was no significant change in ewe plasma cortisol or ACTH over time (figure 3.3). There was no significant difference in plasma cortisol or ACTH between control and restricted ewes in the first 75 dGA (figure 3.3).

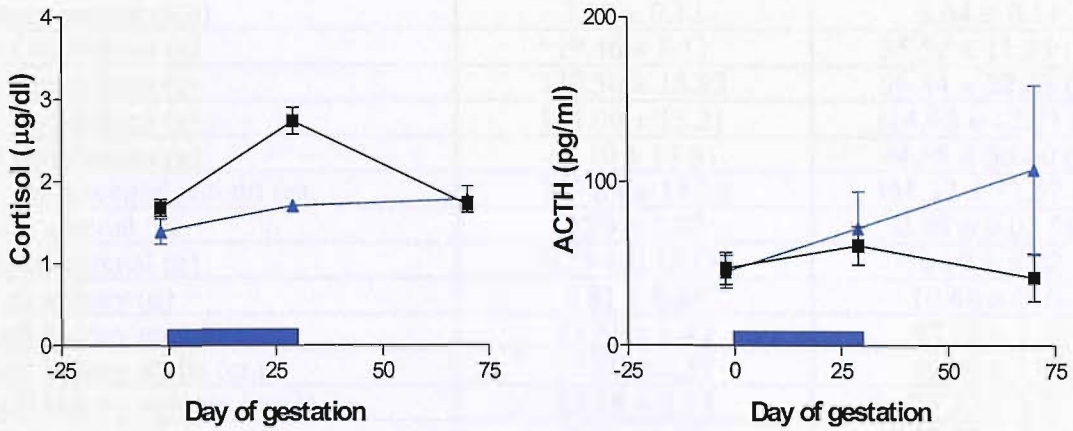

Figure 3.3 Ewe plasma cortisol (left) and ACTH (right). The two groups are shown; C (—■—, $n = 10$) and R (—▲—, $n = 10$).

3.5.3 Fetal biometry

Nutrient restriction had no effect on measurements of fetal biometry obtained at surgery or post-mortem (table 3.1). There was no significant effect of diet on fetal growth between surgery and post-mortem as measured by the change in fetal measurements during this period (table 3.1).

Fetal Surgery Measurements	**C ($n = 10$)**	**R ($n = 10$)**
Abdominal circumference (cm)	19.82 ± 0.76	18.89 ± 1.37 (8)
Biparietal diameter (cm)	5.31 ± 0.11 (7)	5.26 ± 0.06 (8)
Femur length (cm)	7.88 ± 0.21	7.61 ± 0.11
Fetal Post Mortem Measurements		
Crown-rump length (cm)	45.00 ± 1.41	45.77 ± 1.13
Abdominal circumference (cm)	32.13 ± 0.95	33.92 ± 1.08 (9)
Biparietal diameter (cm)	5.50 ± 0.05	5.57 ± 0.10
Femur length (cm)	8.37 ± 0.22	8.23 ± 0.17
Shoulder height (cm)	33.17 ± 0.64	33.96 ± 1.10
Change from surgery to PM		
Abdominal circumference (cm)	12.31 ± 0.96	15.51± 1.42 (7)
Biparietal diameter (cm)	0.21 ± 0.09 (7)	0.31 ± 0.13 (8)
Femur length (cm)	0.49 ± 0.32	0.62 ± 0.21

Table 3.1 Fetal measurements at surgery (117 dGA) and post-mortem (127 dGA), mean ± SEM. C: control R: restricted. Bracketed numbers indicate n different to that given in the column heading.

Early gestation maternal nutrient restriction had no effect on fetal body or organ weight as measured at post mortem (127 dGA), on kidney length, width or volume or on organ weight when calculated as percentage of body weight (table 3.2).

Post-mortem parameters	**C (*n* = 10)**	**R (*n* = 10)**
Male : Female	3 : 7	1 : 1
Body weight (Kg)	3.02 ± 0.12	3.04 ± 0.11
A Cotyledons (g)	19.46 ± 8.11	35.59 ± 11.45 (9)
B Cotyledons (g)	140.50 ± 18.92	99.34 ± 22.23 (9)
C Cotyledons (g)	134.00 ± 15.21	114.90 ± 12.21 (9)
D Cotyledons (g)	61.10 ± 17.81	94.55 ± 56.40 (9)
Total placental weight (g)	355.06 ± 15.54	344.37 ± 33.57 (9)
Left adrenal (g)	0.24 ± 0.02	0.20 ± 0.01 (8)
Right adrenal (g)	0.35 ± 0.15 (9)	0.19 ± 0.02
Left kidney (g)	9.81 ± 0.84	10.68 ± 0.70
Left kidney length (cm)	35.50 ± 1.42	37.95 ± 1.60
Left kidney width (cm)	17.80 ± 0.59	19.10 ± 0.60
Left kidney volume (cm2)	22.88 ± 2.24	23.23 ± 1.70
Right kidney (g)	9.81 ± 0.82	10.23 ± 0.64
Liver (g)	105.66 ± 5.35	112.47 ± 8.74
Heart (g)	19.88 ± 1.23	19.12 ± 0.93
Lung (g)	68.36 ± 3.86	72.00 ± 2.18
Brain (g)	35.81 ± 0.58	35.39 ± 1.27
Pituitary (g)	0.09 ± 0.01 (7)	0.11 ± 0.01 (6)
Spleen (g)	4.74 ± 0.46 (8)	5.17 ± 0.79 (3)
as % of body weight		
Total placental weight	0.12 ± 0.009	0.12 ± 0.01 (9)
Left adrenal	0.01 ± 0.001	0.007 ± 0.001 (8)
Right adrenal	0.01 ± 0.005 (9)	0.007 ± 0.001
Right kidney	0.32 ± 0.02	0.35 ± 0.01
Liver	3.49 ± 0.08	3.84 ± 0.29
Heart	0.66 ± 0.02	0.65 ± 0.01
Lung	2.25 ± 0.07	2.48 ± 0.09
Brain	1.20 ± 0.05	1.21 ± 0.04
Pituitary	0.003 ± 0.000 (7)	0.004 ± 0.001 (6)
Spleen	0.15 ± 0.01 (8)	0.18 ± 0.03 (3)

Table 3.2 Fetal body weight and organ weights at post-mortem (127 dGA), mean ± SEM. C: control R: restricted. Bracketed numbers indicate *n* different to that given in the column heading.

3.6 Discussion

In this chapter it was found that a 50% peri-implantation nutrient restriction had no significant effect on maternal weight, BCS, fat or muscle depth. A 15% nutrient restriction for the first half of gestation results in a significantly greater percentage change from baseline in ewe body weight and BCS compared to control ewes (Hawkins *et al.*, 2000a). Ewe weight and BCS fell in the restricted ewes, however ewe weight increased and BCS was maintained in control ewes from as early as 14 days into the challenge (Hawkins *et al.*, 2000a).This conflicts with both the findings in this chapter and those of Gardner *et al.* (2004) that early gestation maternal nutrient restriction (50%, 1-30 dGA) had no significant effect on maternal weight gain or BCS. These conflicting findings may be due to the different duration and intensity of the challenges, as the Hawkins *et al.* (2000) study used a milder maternal nutrient restriction (15% vs. 50%), but it lasted more than twice as long as both the challenge used in the present study and that of Gardner *et al.* (2004). Therefore it would affect developing systems differently as it continues past the peri-implantation stage, through different critical periods for organ development. The embryo communicates directly with the uterine environment during the pre-implantation period, but after this time the placenta affects fetal growth (Thorburn & Harding, 1994). The findings of the Hawkins *et al.* (2000) study may have been due to increased demands following implantation required for the growth of the placenta and fetus. Therefore it appears that the ewes were able to maintain normal weight gain during pregnancy despite undergoing a 50% nutrient restriction during the peri-implantation period.

In this chapter early gestation maternal nutrient restriction had no effect on maternal plasma cortisol or ACTH levels for the first 75 dGA. Gardner *et al.* (2006) found no change in maternal plasma cortisol levels at 2 or 30 dGA during a 50% maternal nutrient restriction from 1–30 dGA, which agrees with the findings in this chapter. Whereas a maternal nutrient restriction that reduces maternal weight by 15% from 60 days prior to conception to 30 dGA reduced maternal plasma cortisol and ACTH levels during (Bloomfield et al., 2004; Jaquiery et al., 2006) and following (Jaquiery et al., 2006) the nutrient restriction. These conflicting findings may be due to the different intensity and duration of the challenges. In both the

Bloomfield *et al.* (2004) and the Jaquiery *et al.* (2006) studies the reduction in maternal body weight was achieved by 48 hours of starvation, and then feeding concentrate at 1–2% control of body weight per day to maintain this reduced weight, whereas control ewes received concentrate at 3–4% of body weight per day throughout. This was more severe than the maternal nutrient restriction used in the present study. The duration of the challenge in both the Bloomfield *et al.* (2004) and the Jaquiery *et al.* (2006) studies was three times as long as the one used in this chapter, starting 60 days prior to conception. It has been found previously that maternal body condition at the time of conception is as important as the level of nutrient intake during early pregnancy in relation to later cardiovascular function (Gopalakrishnan et al., 2005). So a challenge spanning both periods (such as those of Bloomfield *et al.* (2004) and Jaquiery *et al.* (2006)) is likely to have an additive effect and therefore greater consequences for the ewe and offspring.

The finding of no significant difference in fetal body or organ weights are consistent with a number of previous investigations including those of our group where a 50% nutrient restriction for the first 31 dGA did not result in any changes in fetal measurements at 70 dGA or birth weight of singletons (Cleal, 2005). Gardner *et al.* (2004) also found no differences in offspring birth weights, growth rates in the first year of life or organ weights at 1 year following a 50% nutrient reduction for the first 30 dGA. The studies of both Gardner *et al.* (2004) and Cleal *et al.* (2007) found that an insult of similar intensity altered cardiovascular control postnatally, therefore there may be altered cardiovascular control in the present fetal model. Therefore there may be no effect of a 50% peri-implantation nutrition restriction on fetal growth, or compensatory mechanisms may maintain growth at the expense of other physiological processes, or there may be altered regional growth, which affects the development of organ structure, e.g. the kidney, which may impact on function and alter cardiovascular control.

Birth weight has been used as an indicator of poor nutrition and of possible cardiovascular dysfunction in adult life in epidemiological studies. Birth weight is a crude measurement without body proportions and may underestimate how much poor nutrition *in utero* contributes to later disease. Similarly, organ weight is a crude indicator of function therefore

it is also important to investigate if there are any changes in organ structure and function. In the present study 50% peri-implantation nutrient restriction did not change kidney length, weight or volume, to the best of my knowledge this has not been previously studied in sheep. In humans small for gestational age babies (Konje et al., 1996) and fetuses of thinner mothers (Mukherjee et al., 2005) have narrower sausage shaped kidneys. The kidneys play a major role in overall cardiovascular control and therefore any alterations in kidney structure such as altered nephron number or volume could have long lasting effects on blood pressure and cardiovascular control. Reduced nephron number has previously been associated with low birth weight in adults and children (Hughson et al., 2003), and hypertension (Keller et al., 2003). Studies in animal models have observed reduced nephron number, such as early gestation glucocorticoid treatment in sheep (Wintour *et al.*, 2003c), and low protein diet throughout gestation in rats (Langley-Evans *et al.*, 1999b). Therefore impaired kidney development and reduced nephron number have been suggested as a possible mechanism linking poor *in utero* nutrition and increased risk of hypertension and CVD in adult life (Wintour *et al.*, 2003b).

3.7 Conclusion

A 50% peri-implantation maternal nutrient restriction had no significant effect on maternal body parameters. Therefore it appears that the ewes were able to maintain appropriate for gestational age weight gain even with a 50% nutrient restriction during the peri-implantation period. There was no significant difference in maternal plasma cortisol or ACTH when measured during gestation, although the fetus may still be exposed to raised glucocorticoids via other changes. No effect of a 50% peri-implantation nutrition restriction on fetal growth or organ weight was observed when studied at 127 dGA. Therefore either there was no change in fetal development or there may have been transient decreases in growth followed by compensatory growth, or there may have been altered regional growth, which affected the development of organ structure, such as the kidney, which may have impacted on renal function and altered cardiovascular control. Therefore it is important to investigate the effect of a peri-implantation maternal nutrient restriction on fetal cardiovascular control and renal function.

4 Impact of 50% maternal restriction during the peri-implantation period on late gestation fetal cardiovascular control and renal function

4.1 Introduction

In chapter 3, no difference was found between ewes that had been exposed to a 50% peri-implantation nutrient restriction and control ewes with respect to maternal weight gain, plasma cortisol or ACTH levels during gestation. The 50% peri-implantation maternal nutrient restriction had no effect on fetal size, kidney dimensions or organ weights in late gestation compared to the control fetuses. However, it still remains possible that the peri-implantation nutrient restriction may have caused altered organ structure, a transient decrease in growth followed by compensatory growth or altered cardiovascular control may have maintained growth. Any change in kidney structure may impact on renal function and alter cardiovascular control.

There is an association between maternal nutrition during pregnancy and altered cardiovascular control in adult life (Langley & Jackson, 1994; Woodall *et al.*, 1996b; Hawkins *et al.*, 2000c; Roseboom *et al.*, 2001a). Poor *in utero* nutrition during the peri-implantation period can result in altered postnatal cardiovascular control (Gardner *et al.*, 2004; Cleal *et al.*, 2007a). **Therefore, it is important to investigate whether altered cardiovascular control is manifest in fetal life.**

The arterial baroreflex is the most important mechanism providing short term regulation of arterial blood pressure. In fetal sheep the baroreflex is functioning from as early as 88 dGA (Blanco *et al.*, 1988), and can be altered by insults during gestation such as maternal dexamethasone treatment (Dodic *et al.*, 1999; Fletcher *et al.*, 2002). Nutritional insults in early gestation have been found to alter baroreflex in fetal (Hawkins *et al.*, 2000c) and postnatal life (Gardner *et al.*, 2004). **No one has yet investigated whether peri-implantation nutrient restriction affects fetal baroreflex function.**

Cardiovascular control may also be altered by changes in a number of other mechanisms including the RAS. A 50% maternal nutrition restriction for the first 30 dGA resulted in a blunted sensitivity to Ang II infusion at one year of age (Gardner *et al.*, 2004). As well as alterations in vascular responsiveness to Ang II there may also be changes in other components of the RAS resulting in altered activation of Ang II. Previous studies in our group have found that a peri-implantation maternal nutrient restriction (50%, 1-31 dGA) resulted in an increased blood pressure response to a bolus of frusemide at 1.5 years but not at 2.5 years of age (Cleal *et al.*, 2007a). **It is important to investigate if this alteration in renal RAS is already manifest in fetal life following a peri-implantation nutrient restriction.**

Renal control of blood volume is the main system through which long term arterial pressure is controlled. 48 hours of glucocorticoid infusion at 27 dGA impairs nephrogenesis, resulting in reduced nephron number and increases in blood pressure in adult life (Wintour *et al.*, 2003c). Changes in nutrient supply to the fetus have also resulted in changes in nephrogenesis and renal function (Merlet-Benichou *et al.*, 1994; Bassan *et al.*, 2000; Bauer *et al.*, 2002). It has been suggested that a reduction in nephron number may lead to hyperperfusion of individual nephrons causing a cycle of glomerular sclerosis, more nephron death, reduced renal function and increasing blood pressure. **No one has yet investigated how a peri-implantation nutrient restriction affects fetal renal function in late gestation.**

4.2 Hypothesis

- 50% Peri-implantation nutrient restriction alters fetal renal function and RAS in late gestation, which may result in altered cardiovascular control.

4.3 Aims

To carry out a 50% maternal nutrient restriction during peri-implantation and monitor its effect on fetal cardiovascular and renal function and renal RAS in late gestation by:

1. Monitoring basal cardiovascular parameters.
2. Testing the baroreflex response.
3. Testing the response to increasing doses of Ang II.
4. Measuring blood gases, and plasma hormone levels.
5. Measuring fetal cardiovascular, renal and RAS responses to frusemide.

4.4 Methods

Welsh mountain ewes (Animals (Scientific Procedures) Act 1986) were housed, fed, mated and surgically instrumented as described in chapter 2. Fetuses (control (C), n = 9; restricted (R), n = 9) were studied on 2 successive days between 123–124 dGA (figure 4.1). Continuous recordings were made of amniotic, tracheal and arterial pressure, fetal heart rate, ECoG, femoral and arterial blood flow throughout the experimental protocols. Vascular sensitivity to Ang II was assessed using increasing doses of Ang II (0 μg, 1 μg, 2.5 μg, 5 μg, and 10 μg, Sigma-Aldrich Co, UK). Kidney function and the function of the RAS were also assessed using a bolus of saline vehicle or frusemide (20mg, Phoenix, Pharmacia, UK). Baroreflex sensitivity was assessed using phenylephrine (75μg, i.v. bolus, Sovereign Medical, Essex, UK).

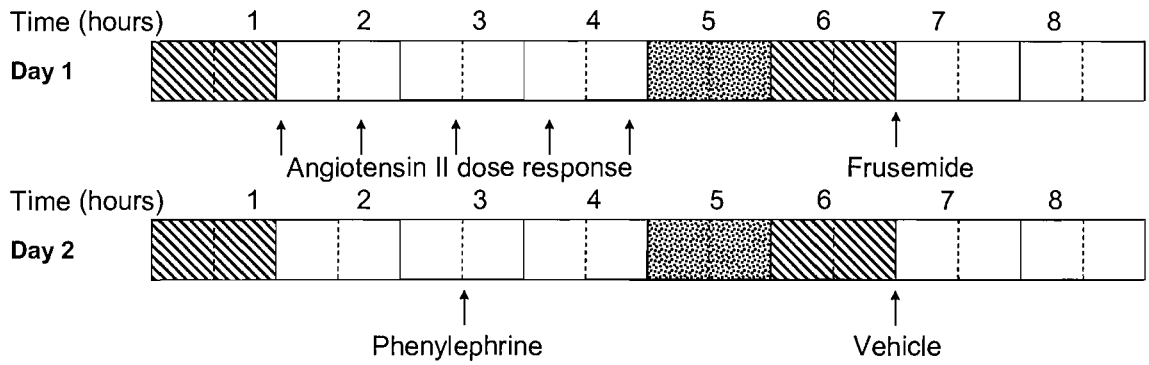

Figure 4.1 Experiment timeline. Baseline ▨, recovery ▩.

4.4.1 Data analysis

For a description of the analysis strategy, the power calculation and the statistics packages used, see section 2.6.

Sex

Male and female fetuses were analysed together due to the small size of some groups when divided according to sex. No significant differences were found between males and females in body weight, nor any organ weight, when analysed by Student's t test.

Where serial measurements were made (such as cardiovascular or response to stimuli) during an experiment, data were analysed by repeated measures 2 way ANOVA, over time and between dietary group in order to identify any substantial difference in the profile of a response between the groups. Where the result of the analysis was significantly different over time, the times at which parameters differed significantly were identified by a Bonferroni *post-hoc* test. Significance values are stated for *post-hoc* test results.

For other factors, where only one measurement is made or measurements were expressed as a 'summary of measures' (such as AUC, maximum response or time to maximum response), data were compared between groups by Student's t-test.

4.5 Results

4.5.1 Cardiovascular parameters

There was no difference in basal systolic, diastolic or mean arterial blood pressure, femoral or carotid arterial flow or heart rate between the C and R groups in late gestation (table 4.1).

123 dGA	**C (*n* = 9)**	**R (*n* = 9)**
MAP (mm Hg)	44.68 ± 0.83	47.45 ± 2.66
Systolic pressure (mm Hg)	61.18 ± 2.36	65.40 ± 2.57
Diastolic pressure (mm Hg)	33.67 ± 0.70	35.19 ± 2.62
Carotid flow (ml/min)	87.41 ± 11.62	63.23 ± 5.18
Femoral flow (ml/min)	33.36 ± 4.98	22.51 ± 4.46
Heart rate (BPM)	166.70 ± 5.27	172.70 ± 4.80
124 dGA		
MAP (mm Hg)	43.74 ± 0.76	43.46 ± 1.22
Systolic pressure (mm Hg)	58.74 ± 1.76	57.83 ± 1.34
Diastolic pressure (mm Hg)	36.25 ± 0.66	36.28 ± 1.35
Carotid flow (ml/min)	70.49 ± 13.52	60.22 ± 5.76
Femoral flow (ml/min)	26.72 ± 5.78	21.79 ± 4.18
Heart rate (BPM)	168.00 ± 4.56	172.80 ± 6.50

Table 4.1 Basal cardiovascular measurements, mean ± SEM. C: control R: restricted.

Baroreflex

There was no difference between the C and R fetuses in the sensitivity or operating point of the baroreflex or dose of phenylephrine per kg body weight (table 4.2). In all fetuses, MAP increased and fetal heart rate, carotid and femoral blood flows fell in response to phenylephrine administration however, there was no significant difference in the response between the two groups (table 4.2).

Baroreflex	**C (*n* = 9)**	**R (*n* = 9)**
Phenylephrine dose (μg/kg)	25.50 ± 1.20	25.35 ± 0.99
Sensitivity (ΔRR / ΔSBP)	0.0079 ± 0.0013	0.0110 ± 0.0023
Operating point (mm Hg)	75.94 ± 2.52	70.95 ± 1.76
Maximum response		
MAP (Δ mm Hg)	32.98 ± 2.27	28.92 ± 3.11
Heart rate (Δ BPM)	51.56 ± 5.54	57.92 ± 6.73
Carotid flow (Δ ml/min)	29.79 ± 5.06	22.84 ± 2.73
Femoral flow (Δ ml/min)	22.25 ± 3.71	17.03 ± 3.17 (7)

Table 4.2 Response to phenylephrine, mean ± SEM. Bracketed numbers indicate *n* different to that given in the column heading.

Response to angiotensin II

The MAP response to Ang II was significantly blunted in R compared with C fetuses (AUC: C = 286.7 ± 13.37; R = 230.3 ± 18.42 mm Hg/μg, $p<0.05$). Further analysis showed that it was the responses to the highest doses of Ang II that were blunted in the restricted group (5 μg, $p = 0.037$; 10 μg, $p = 0.046$), but no significant difference was found between the two groups in response to the three lowest doses (0 μg, $p = 0.459$, 1 μg, $p = 0.361$, 2.5 μg, $p = 0.190$) (figure 4.2 & table 4.3). Fetal heart rate and femoral and carotid artery blood flow fell in response to Ang II administration. There was no significant difference in the change from baseline in heart rate or arterial blood flows between the R and C groups (table 4.3).

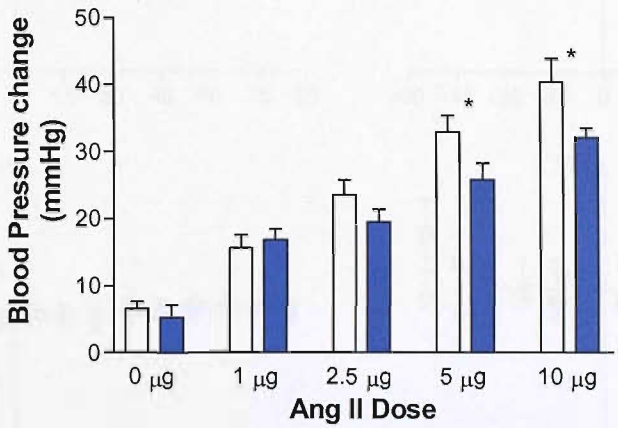

Figure 4.2 Maximum change in fetal MAP in response to Ang II mean ± SEM. Control □, $n = 9$ and nutrient restricted ■, $n = 9$. * $p < 0.05$ C vs. R.

MAP response (Δ mm Hg)	C ($n = 9$)	R ($n = 9$)
0 μg Ang II	6.62 ± 1.05	5.24 ± 1.86
1 μg Ang II	15.66 ± 1.93	16.82 ± 1.62
2.5 μg Ang II	23.51 ± 2.25	19.55 ± 1.84
5 μg Ang II	32.94 ± 2.49	25.83 ± 2.44 *
10 μg Ang II	40.43 ± 3.56	32.22 ± 1.29 *
Maximum response to 10 μg Ang II		
Heart rate (Δ BPM)	55.95 ± 9.02	58.86 ± 9.48
Carotid flow (Δ ml/min)	14.46 ± 4.36	15.62 ± 3.15
Femoral flow (Δ ml/min)	20.07 ± 2.87	14.55 ± 3.15 (7)

Table 4.3 Maximum cardiovascular response to Ang II, mean ± SEM. Bracketed numbers indicate n different to that given in the column heading. * $p < 0.05$ C vs. R.

Response to frusemide

When all animals were considered together a 20 mg bolus of frusemide administered to the fetus did not lead to a significant change from baseline in blood pressure or heart rate (figure 4.3).

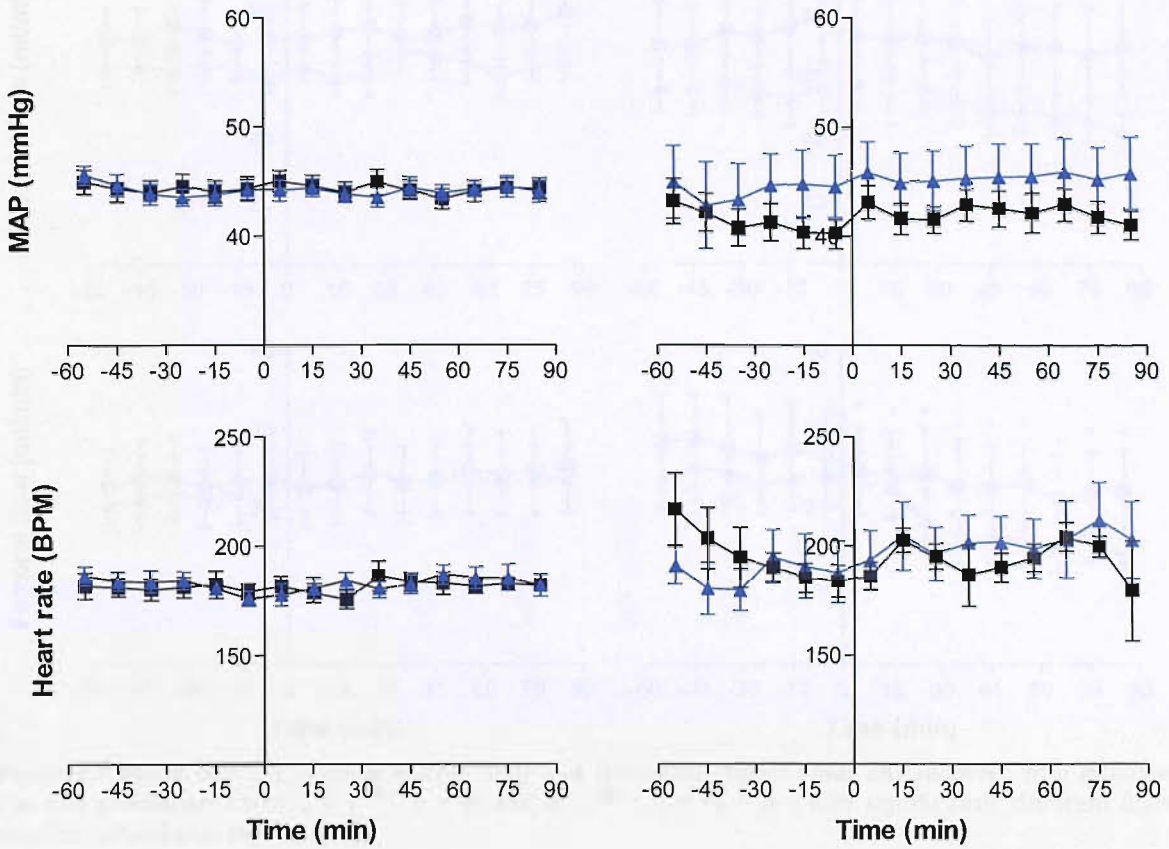

Figure 4.3 MAP (top) and heart rate (bottom) response to vehicle (left) and frusemide (right) bolus administered at 0 minutes. The two groups are shown; C (■, 100% of nutrient requirements throughout gestation, n = 9) and R (▲, 50% of nutrient requirements from 1-31 dGA, 100% requirements thereafter, n = 9).

When all animals were considered together a 20 mg bolus of frusemide administered to the fetus did not lead to a significant change from baseline in carotid artery blood flow, although femoral artery blood flow decreased significantly (figure 4.4).

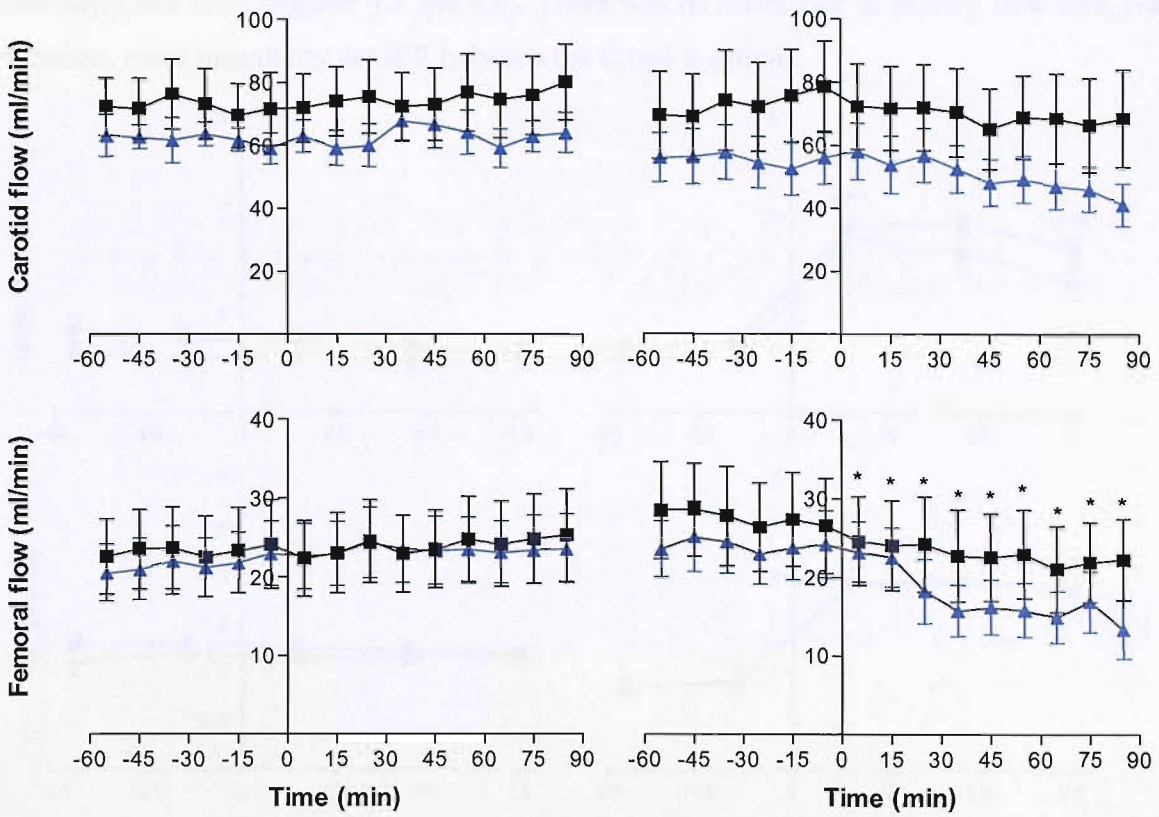

Figure 4.4 Blood flow response to vehicle (left) and frusemide (right) bolus administered at 0 minutes. The two groups are shown; C (■, n = 9) and R (▲, n = 9). * $p < 0.05$ significantly different from baseline (all animals together).

4.5.2 Renal function

Basal function

There was no difference in basal GFR, urine flow rate, Na^+ excretion or osmolarity between C and R fetuses in late gestation (table 4.4).

123 dGA	C (n = 9)	R (n = 9)
GFR (ml/min)	4.14 ± 0.37	3.68 ± 0.37
Flow rate (ml/min)	0.62 ± 0.07	0.56 ± 0.08
Na^+ excretion (mmol)	53.67 ± 5.75	53.06 ± 4.75
Osmolarity (mosmol)	142.1 ± 10.86	136.3 ± 7.94
124 dGA		
GFR (ml/min)	3.59 ± 0.54	3.52 ± 0.49
Flow rate (ml/min)	0.80 ± 0.14	0.58 ± 0.10
Na^+ excretion (mmol)	78.94 ± 5.91	72.44 ± 4.25
Osmolarity (mosmol)	186.9 ± 12.65	180.9 ± 11.45

Table 4.4 Basal renal measurements, mean ± SEM.

Response to frusemide

Frusemide administration significantly increased fetal urinary flow rate, Na^+ excretion, urine osmolarity and GFR (figures 4.5 and 4.6). There was no difference in urinary flow rate, Na^+ excretion, urine osmolarity or GFR between the C and R groups.

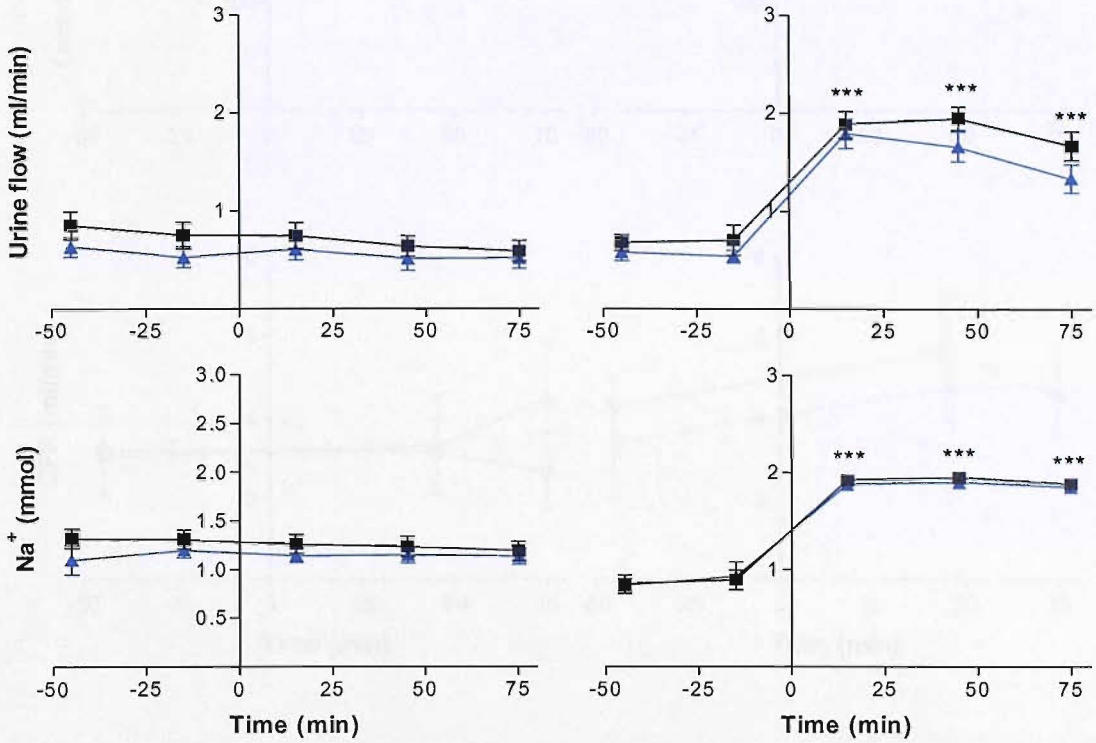

Figure 4.5 Urine flow rate (top) and Na^+ excretion (bottom) following administration of saline (left) and frusemide (right) administered at 0 minutes. The two groups are shown; C (■, n = 9) and R (▲, n = 9). *** $p < 0.001$ significantly different from baseline (all animals together).

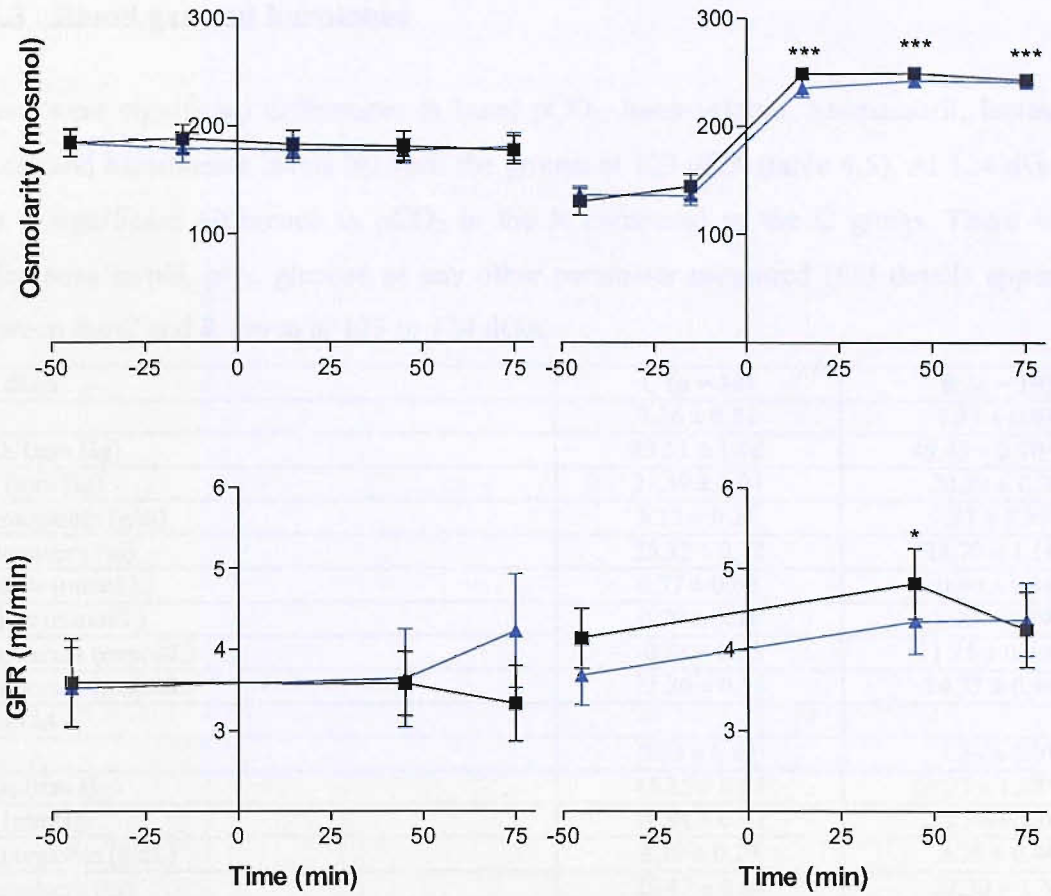

Figure 4.6 Osmolarity (top) and GFR (bottom) following administration of saline (left) and frusemide (right) administered at 0 minutes. The two groups are shown; C (■, $n = 9$) and R (▲, $n = 9$). * $p < 0.01$, *** $p < 0.001$ significantly different from baseline (all animals together).

4.5.3 Blood gas and hormones

There were significant differences in basal pCO_2, haemoglobin, haematocrit, lactate, base excess and bicarbonate levels between the groups at 123 dGA (table 4.5). At 124 dGA there was a significant difference in pCO_2 in the R compared to the C group. There were no differences in pH, pO_2, glucose or any other parameter measured (full details appendix 5) between the C and R group at 123 or 124 dGA.

123 dGA	**C (n = 10)**	**R (n = 10)**
pH	7.36 ± 0.01	7.35 ± 0.01
pCO_2 (mm Hg)	43.51 ± 1.08	49.45 ± 0.70***
pO_2 (mm Hg)	21.39 ± 0.94	20.20 ± 0.90
Haemoglobin (g/dl)	8.13 ± 0.26	9.27 ± 0.39*
Haematocrit (%)	25.32 ± 0.78	28.70 ± 1.16*
Glucose (mmol/L)	0.77 ± 0.05	0.80 ± 0.06
Lactate (mmol/L)	0.79 ± 0.04	1.00 ± 0.09*
Base excess (mmol/L)	-0.84 ± 0.65	1.25 ± 0.49*
Bicarbonate (mmol/L)	23.26 ± 0.50	24.73 ± 0.44*
124 dGA		
pH	7.35 ± 0.01	7.33 ± 0.01
pCO_2 (mm Hg)	45.25 ± 0.68	50.37 ± 1.28***
pO_2 (mmHg)	19.94 ± 0.92	22.96 ± 2.05
Haemoglobin (g/dL)	8.19 ± 0.29	8.79 ± 0.44
Haematocrit (%)	25.47 ± 0.87	27.30 ± 1.33
Glucose (mmol/L)	0.86 ± 0.04	0.77 ± 0.06
Lactate (mmol/L)	0.94 ± 0.08	1.10 ± 0.11
Base excess (mmol/L)	-0.71 ± 0.62	0.24 ± 0.87
Bicarbonate (mmol/L)	23.28 ± 0.52	23.83 ± 0.74

Table 4.5 Basal blood gas measurements, mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ C vs. R.

There were no differences in basal plasma hormone measurement between C and R fetuses. (table 4.6). The fetal plasma renin activity was raised at 124 dGA compared to 123 dGA (table 4.6)

123 dGA	**C (n = 10)**	**R (n = 10)**
Cortisol (μg/dl)	1.47 ± 0.23	1.40 ± 0.15
ACTH (pg/ml)	60.56 ± 11.47	44.79 ± 8.39
Aogen (μg Ang I/ml)	0.38 ± 0.07	0.32 ± 0.09 (9)
PRA (ng/ml/hr)	6.20 ± 1.10	10.55 ± 3.34 (9)
ACE (nmoles/ml/min)	22.16 ± 1.47	20.72 ± 1.89
Ang II (pmol/l)	9.66 ± 4.36	13.16 ± 4.70
124 dGA		
Cortisol (μg/dl)	2.01 ± 1.40	1.60 ± 0.15
ACTH (pg/ml)	36.25 ± 9.55	27.08 ± 1.97
Aogen (μg Ang I/ml)	0.38 ± 0.06	0.39 ± 0.07 (9)
PRA (ng/ml/hr)	24.73 ± 5.29	29.40 ± 5.19 (9)
ACE (nmoles/ml/min)	21.71 ± 2.02	22.83 ± 2.29

Table 4.6 Basal plasma hormone measurements, mean ± SEM. Plasma renin activity (PRA),

Response to frusemide

A 20 mg bolus of frusemide administered to the fetus led to a significant increase in fetal plasma Ang II levels, but there was no significant difference in Ang II response to frusemide between the groups (figure 4.7).

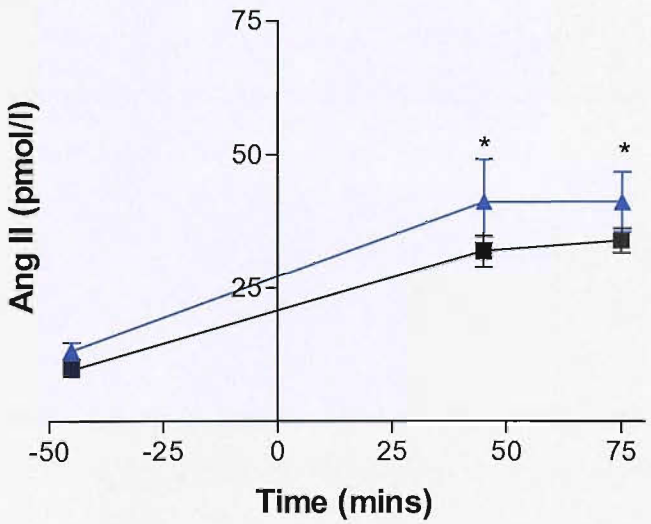

Figure 4.7 Fetal plasma Ang II following administration of frusemide at 0 minutes. The two groups are shown; C (■, n = 10) and R (▲, n = 9). * $p < 0.05$ significantly different from baseline (all animals together).

A 20 mg bolus of frusemide administered to the fetus led to a significant increase in fetal PRA, but there was no significant difference in PRA response to frusemide between the groups (figure 4.8).

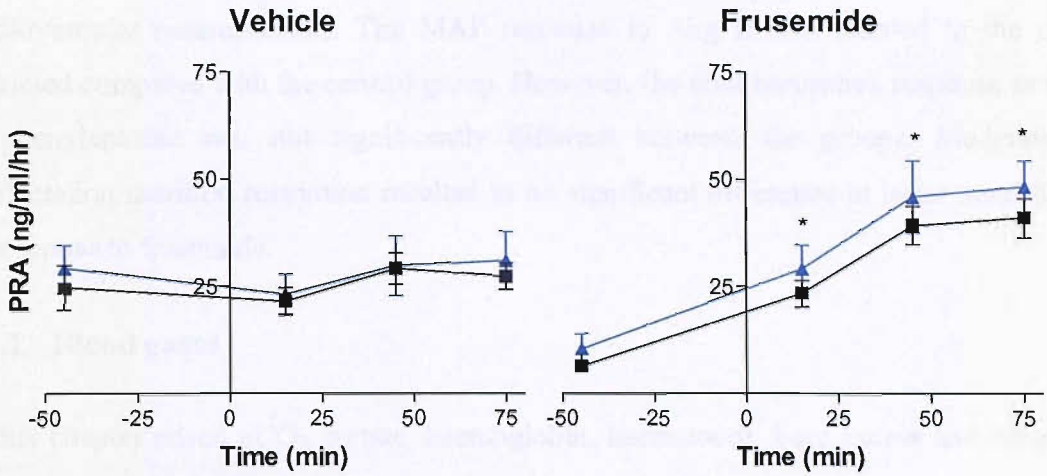

Figure 4.8 Fetal plasma renin activity (PRA) following administration of saline vehicle or frusemide at 0 minutes. The two groups are shown; C (, $n = 10$) and R (, $n = 9$). * $p < 0.05$ significantly different from baseline (all animals together).

4.6 Discussion

In this chapter, no significant difference was found between control fetuses and those that had been exposed to a 50% peri-implantation maternal nutrient restriction in basal cardiovascular measurements. The MAP response to Ang II was blunted in the nutrient restricted compared with the control group. However, the fetal baroreflex response to a bolus of phenylephrine was not significantly different between the groups. Moderate peri-implantation nutrition restriction resulted in no significant difference in basal renal function or response to frusemide.

4.6.1 Blood gases

In this chapter raised pCO_2, lactate, haemoglobin, haematocrit, base excess and bicarbonate have been observed in late gestation in response to reduced maternal nutrition in peri-implantation with no alterations any other blood gas parameter measured. Although these parameters were increased relative to the control group, all the parameters were within levels previously recorded in late gestation fetal sheep following surgery (Gardner *et al.*, 2002). Increased fetal pCO_2 and lactate levels have been observed following maternal nutrition restriction (reduce ewe weight by 15%, from -61 – 30 dGA), although they found no change in haemoglobin levels and did not report haematocrit, base excess or bicarbonate levels (Oliver *et al.*, 2005). The raised CO_2 and lactate levels were in conjunction with lower pH and pO_2 levels which have not been observed in this study, and were proposed to be due to altered placenta function (Oliver *et al.*, 2005). The lower pH could be due to the raised pCO_2 and lactate levels in comparison to the fetuses in the present study, possibly as a result of the increased severity and duration of the maternal challenge. A less severe nutrient restriction 15% for the first half of gestation had no effect on blood gas parameters in late gestation (Hawkins *et al.*, 1999; Hawkins *et al.*, 2000a; Hawkins *et al.*, 2000b; Hawkins *et al.*, 2000c). In the present study haemoglobin and haematocrit levels were higher in the nutrient restricted compared to the control fetuses, this would appear to indicate a greater potential for oxygen transport. However, no difference was seen in the key parameters of oxygen status (pO_2, arterial concentration of O_2, or O_2 tension at 50% saturation) therefore overall oxygen

transport appears not to have been affected. The increase in pCO_2, lactate, base excess and bicarbonate are all indicative of altered acid base balance. Reduced fetal oxygen delivery to tissues results in lactate acidemia and increased pCO_2 and could therefore be the reason that lactate and pCO_2 was increased in the nutrient restricted group, although no change was observed in fetal pO_2. Altered nutrition can lead to a redistribution of blood flow similar to the response to hypoxia (Crandell *et al.*, 1985) and may have resulted in altered O_2 supply to specific tissues which may have been the cause of these changes.

4.6.2 Hypothalamic-pituitary-adrenal axis

No change in basal fetal plasma cortisol or ACTH was found in late gestation following a 50% peri-implantation nutrient restriction. This agrees with the study of Gardner *et al* who reported no change in plasma cortisol or ACTH at one year of age following a 50% peri-implantation nutrient restriction (Gardner *et al.*, 2006). Also those of Hawkins *et al* who reported no change in plasma cortisol or ACTH in late gestation or postnatal life following a 15% maternal nutrient restriction for the first half of gestation (Hawkins *et al.*, 1999; Hawkins *et al.*, 2000a; Hawkins *et al.*, 2000b). Although Bloomfield *et al.* (2004) have reported an increase in fetal plasma cortisol following maternal nutrient restriction to decrease maternal weight by 15% from -60 to 30 dGA. The challenge in the Bloomfield study was more severe and lasts for a longer duration than the challenges used in all the other studies discussed here which may explain the conflicting results.

4.6.3 Cardiovascular parameters

Peri-implantation nutrient restriction has no effect on basal blood pressure, heart rate or blood flow. In sheep maternal challenges have been found to have a variety of effects on basal cardiovascular parameters when measured in late gestation and in postnatal life. A 15% reduction in maternal nutrition for the first half of gestation caused baseline blood pressure to be reduced in late gestation (Hawkins *et al.*, 2000c) and elevated in postnatal life (Hawkins *et al.*, 2000b; Hawkins *et al.*, 2004). This study conflicts with the findings in this chapter, however, the changes in fetal blood pressure have not been found consistently following such a challenge (Hawkins *et al.*, 2000b; Hawkins *et al.*, 2004). A 50% nutrient restriction from 0–

30 dGA caused no difference in heart rate or systolic, diastolic or mean arterial pressure at 1 year of age (Gardner *et al.*, 2004), but elevated blood pressure and heart rate prior to feeding at 3 years of age (Gopalakrishnan *et al.*, 2004). This agrees with the finding of this chapter and suggests that changes in basal cardiovascular function only become apparent in later life in this model.

4.6.4 Baroreflex

No difference in fetal baroreflex response to a bolus of phenylephrine in terms of sensitivity, operating point or maximum response was observed between control and restricted groups in this chapter. This indicates that there was no difference in baroreflex responsiveness or the α_1-adreno-receptor population following peri-implantation nutrient restriction. This finding conflicts with data from both dexamethasone treatment and maternal nutrient restriction studies in sheep which found altered baroreflex responses in fetal and postnatal life. Two days of dexamethasone treatment at 27 or 64 dGA increased baroreflex operation point at ~3 years of age (Dodic *et al.*, 1999). Whereas, 50% maternal nutrition restriction from 0 – 30 dGA lowered baroreflex operating point at 1 year of age (Gardner *et al.*, 2004). Also a 15% reduction in maternal nutrition for the first half of gestation lowered baroreflex operating point in late gestation fetuses (Hawkins *et al.*, 2000c). We may not have seen an effect as our nutritional challenge was for a shorter period of time than that of Hawkins *et al.* (2000). Gardner *et al.* (2004) used a similar length and severity of challenge as used in this chapter, and they found postnatal changes in baroreflex. Therefore, changes may not become apparent in this model until postnatal life. The transition from fetus to newborn life is associated with numerous hemodynamic adjustments, including changes in heart rate and peripheral vascular resistance and a redistribution of blood flow. There is an immediate postnatal increase in resting heart rate, MAP, and resetting of the baroreflex curve (Segar *et al.*, 1994). Therefore, any changes in baroreflex response may only become apparent when it is operating in this postnatal setting.

4.6.5 RAS

The MAP response to Ang II was found to be blunted in the nutrient restricted compared to the control group in the present study. These findings agree with those of Gardener *et al.* (2004) who found a blunted response to Ang II infusion at one year of age relative to the control group using a model similar to the one used in this study (50% reduction from 0 - 30 dGA). A 50% maternal nutrient restriction from 0 - 95 dGA resulted in no difference in response to increasing doses of Ang II at three years of age (Gopalakrishnan *et al.*, 2004). Previous studies in our group have found that there was a greater MAP response to Ang II at 2.5 years of age in males that had received a nutritional challenge from 12–25 weeks of age (Cleal, 2005). The conflicting data on responsiveness to Ang II may be due to the variety of challenges used and the ages at which responses have been tested. The appearance of AT1 receptors in systemic blood vessels is developmentally regulated and different in different regions of the fetal cardiovascular system (Burrell *et al.*, 2001). The AT2 receptors are predominant in fetal life but decrease during development; their actions are not well defined but they appear to have opposing actions to AT1 receptors (Burrell *et al.*, 2001). Challenges at different times in development may alter the ratio of angiotensin receptors. Maternal nutrient restriction from 28–77 dGA significantly increased AT1 expression in adrenal, kidney, liver and lung tissue at both 77 and 144-146 dGA, but no change was seen in AT2 expression (Whorwood *et al.*, 2001). Therefore nutrition restriction can change the expression of angiotensin receptors, which may have caused altered Ang II response. However, there may also be differences in the circulating levels and activity of RAS components i.e. Aogen, renin, Ang II and ACE. Ang II can act via AT1 and AT2 receptors to regulate cellular growth and is important in ensuring normal renal development and function. Therefore any change in Ang II receptors could not only alter the responsiveness to Ang II altering the well documented functions of the RAS, but also alter normal renal development and function.

In adults the reduction in plasma sodium levels caused by frusemide causes release of renin and thereby activation of the RAS. The rise in plasma renin activity causes an increase in blood pressure in sheep via increased conversion of Aogen to Ang II and activation of Ang II

receptors (Patel & Smith, 1997), and in the present study we have reported that frusemide increased fetal plasma rennin activity and Ang II. The response to frusemide can be used to indicate changes in any of the components of the RAS or kidney function. Fetal blood pressure responses to frusemide have not been studied in much detail and have produced conflicting results. This chapter showed that a bolus of frusemide in late gestation caused a decrease in femoral artery flow with no change in blood pressure, heart rate or carotid blood flow. A 6 mg iv bolus of frusemide at 124 – 142 dGA resulted in no change in fetal arterial blood pressure, whereas a 4 hour infusion of frusemide (10 mg/hr) resulted in an increase in fetal arterial pressure and heart rate (Lumbers & Stevens, 1987; Kelly *et al.*, 1993). To the best of my knowledge the femoral blood flow response to frusemide has not been reported previously. The reduction in femoral blood flow observed here, may enable maintenance of blood flow to the essential organs during a period of reduced blood volume, similar to the mechanism induced in response to hypoxia or haemorrhage (Thorburn & Harding, 1994).

Peri-implantation nutrient restriction resulted in no change in the cardiovascular response to frusemide in late gestation. Previous studies in our group have found that a 50% nutrient restriction for the first 31 days of pregnancy increased blood pressure response to frusemide at 1.5 years of age, but not at 2.5 years of age compared with the control group (Cleal *et al.*, 2007a). If the offspring underwent a postnatal nutrition restriction in addition to the peri-implantation challenge the altered blood pressure response to frusemide was no longer seen, this implies that it is the result of an early effect that may be advantageous in a poor postnatal environment (Cleal *et al.*, 2007a). The previous studies in our group included twins as well as singletons; this would increase the degree of constraint on the fetus during the challenge. In the light of the postnatal effects seen by Cleal *et al.* (2007) in this model, it may be that the changes in fetal physiology do not cause any changes in RAS mediated cardiovascular control until later in life, or it may be that the frusemide challenge itself has not revealed underlying changes in the RAS.

4.6.6 Renal function

In this study a moderate peri-implantation nutrient restriction was found to have no effect on basal renal function when measured in late gestation. This contradicts findings from other

studies where changes in renal function have been observed following alterations to *in utero* nutrition supply. Uteroplacental insufficiency, induced by partial ligation of the utero-placental vessels during the last third of gestation, reduces postnatal GFR in rats (Merlet-Benichou *et al.*, 1994). Maternal low protein diet throughout gestation in rats also produces a decrease in renal function, and an increase in plasma Na^+ concentration in postnatal life (Langley-Evans *et al.*, 1996b; Nwagwu *et al.*, 2000; Vehaskari *et al.*, 2001; Woods *et al.*, 2001e). The conflicting results are probably due to differences in species; rats are litter bearing species and therefore have the added constraint of multiple littermates competing for the available nutrients and space. Organ development takes place at varying rates in different species. Nephron induction is complete before the end of gestation in humans and sheep, whereas it continues postnatally in rats. A previous study in this group found no change in basal renal function in adult life following a similar peri-implantation challenge (Cleal, 2005).

Frusemide resulted in a marked diuresis and natriuresis, and an increase in urine osmolarity and GFR. These findings back up those of previous studies, including those in fetal sheep (Lumbers & Stevens, 1987; Kelly *et al.*, 1993). Moderate peri-implantation nutrient restriction resulted in no significant difference in any of these responses to frusemide in late gestation. This was in agreement with previous studies in our group which have found that 50% nutrient restriction for the first 31 dGA resulted in no changes in renal response (urine output) to frusemide when measured at 1.5 or 2.5 years (Cleal *et al.*, 2007a). While maternal nutrition restriction appears to have no effect on renal response to frusemide there may be changes in renal function that are not revealed by the frusemide challenge. At 2.5 years of age sheep are fully mature, but as they can live up to 15 years they are still relatively young at 2.5 years. Therefore, there may be changes in nephron number that only cause changes in renal function after further damage caused by hyperperfusion of individual nephrons causing a cycle of glomerular sclerosis, and more nephron death. Alternatively, there may be no effect with this intensity of peri-implantation challenge.

4.7 Conclusion

The data in this chapter refute the hypothesis that moderate peri-implantation maternal undernutrition alters fetal cardiovascular control in late gestation, as there was no change in basal blood pressure or baroreflex. The fetal RAS does appear to be altered, as the blood pressure response to Ang II was blunted following peri-implantation nutrient restriction. However no change in renal function or circulating RAS was observed. It is possible that the dietary challenge in this study only has effects on the peripheral RAS. In the light of the postnatal effects of increased blood pressure response to frusemide at 1.5 years of age seen by Cleal *et al.* (2007) in this model, it may be that the changes in fetal physiology do not cause any changes in renal function or RAS mediated cardiovascular control until later in life. Alternatively, the frusemide challenge may no reveal underlying changes in the RAS. In the light of these observations it is now important to investigate the effects of a more severe peri-implantation or a late gestation maternal nutrient restriction.

5 The effect of 60% peri-implantation or 50% late gestation maternal nutrient restriction on fetal growth and organ size

5.1 Introduction

In Chapter 3 it was found than 50% peri-implantation nutrient restriction resulted in no change in maternal body parameters, plasma cortisol or ACTH levels. The maternal nutrient restriction also had no effect on fetal body or organ weights when measured in late gestation. The nutrient restriction was moderate, and ewe weight gain and BCS were not significantly affected. The duration of the nutrient restriction was 30 days (about one fifth of gestation) spanning the implantation period. This is very early in gestation when the nutritional demands on the ewe by the embryo are relatively low. Thus, the lack of effect seen in this study could be due to the intensity, duration and/or the timing of the challenge.

Epidemiological studies have found associations between low birth weight and high blood pressure and a greater risk of CVD in later life (Barker & Osmond, 1986). The Dutch famine of 1944–1945 revealed the importance of the timing of any maternal nutrient restriction in relation to both weight and body parameters at birth and instances of risk factors and diseases in later life (Ravelli *et al.*, 1998; Roseboom *et al.*, 2000b; Painter *et al.*, 2005b). The present study found no differences in fetal weight in late gestation following a 50% peri-implantation nutrient restriction (chapter 3); this agrees with other studies which have investigated challenges of a similar intensity and duration during this peri-implantation period (Gardner *et al.*, 2004; Cleal *et al.*, 2007a). **It is now important to investigate the effects of a more severe challenge in this peri-implantation period.**

There have been fewer studies that investigate the effects of maternal nutrient restriction during the period of rapid fetal growth which occurs in late gestation, and these have found different effects on fetal body and organ weights (Bauer *et al.*, 1995; Edwards & McMillen, 2001; Oliver *et al.*, 2001; Burrage, 2006). Kidney development can be impaired by nutrient restriction in late gestation (Oliver *et al.*, 2001; Burrage, 2006), which may impact on kidney function and result in altered cardiovascular control. The differences between the studies are

probably due to the variation in timing, duration and intensity of the challenges used, and this also makes them difficult to compare directly. **Further investigation into the effects of maternal nutritional challenge in late gestation on fetal body and organ weights and kidney dimensions in late gestation is required.**

5.2 Hypothesis

60% peri-implantation or 50% late gestation maternal nutrient restriction will lead to a redistribution of resources that reduces fetal growth and alter organ size in an organ specific manner.

5.3 Aims

To carry out 60% maternal nutrient restriction during peri-implantation or 50% maternal nutrient restriction in late gestation and monitor its effects on:

1. Maternal weight, BCS and fat and muscle depth during gestation.
2. Maternal hormones (cortisol and ACTH) during gestation.
3. Fetal body measurements and organ weights and kidney dimensions in late gestation.

5.4 Methods

5.4.1 Diet and surgery

Pregnant Welsh mountain ewes (Animals (Scientific Procedures) Act 1986) were maintained according to normal practices at the RVC. They were penned individually on straw and received either a 60% reduction of their total nutrient requirements from 1 - 31 days (E), 50% reduction of their total nutrient requirements for 23 days in late gestation starting at 104 dGA (L) or 100% of nutrient requirements throughout (C). At all other times animals in all groups were fed 100% nutrient requirements. Blood samples were taken prior to conception at -2 dGA then at 29 and 70 dGA to be analysed for levels of maternal hormones. At enrolment (-16 dGA) weight and BCS were recorded. Weight, BCS, fat depth and muscle depth were recorded at -2, 29, 70, and either 104 or 111 dGA (figure 5.1). At ~117 dGA the fetuses were instrumented as described in general methods (see 2.4.3) and the femur length, biparietal diameter and abdominal circumference were measured.

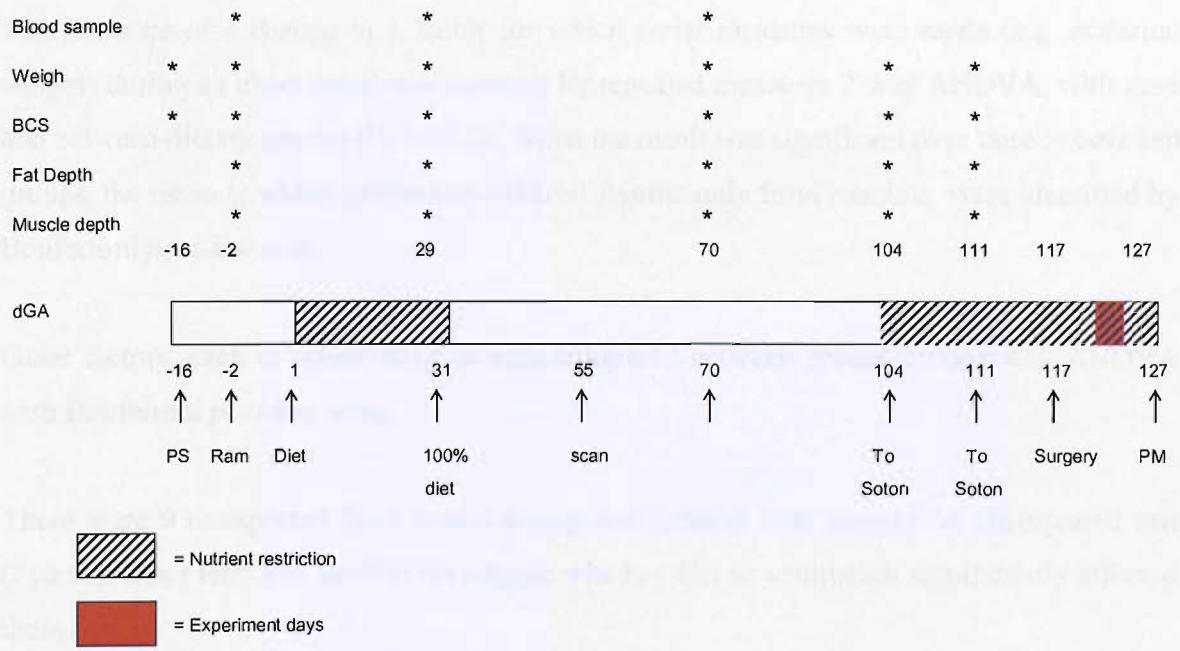

Figure 5.1 Timeline. * denotes blood samples, and measurement of weight, BCS and fat and muscle depth (PS = progesterone sponge, PM = post mortem).

5.4.2 Post mortem

At 127–130 dGA post mortem took place as described in 2.5 and fetal body measurements and organ weights were measured. The dimensions of the left kidney were determined as described in 2.5.

5.4.3 Analysis and statistics

For a general description of the analysis strategy, and the power calculation and statistics packages uses, see section 2.6.

Sex

Male and female fetuses were analysed together due to the small size of some groups when divided according to sex. No significant differences were found between males and females in body weight, or any organ weight, when analysed by Student's t test.

The presence of a change in a factor for which serial measures were made (e.g. maternal weight) during an experiment was assessed by repeated measures 2 way ANOVA, with time and between dietary groups (C, E or L). When the result was significant over time or between groups, the times at which parameters differed significantly from baseline were identified by Bonferroni *post-hoc* tests.

Other factors, such as organ weights were compared between groups by one way ANOVA with Bonferroni *post-hoc* tests.

There were 9 unexpected fetal deaths during and straight after surgery. A chi-squared test (Fisher's exact test) was used to investigate whether diet or ventilation significantly affected these deaths.

5.5 Results

5.5.1 Ewe biometry

When all animals were considered together, the ewe weight at the start and end of the two dietary periods were not different between the groups. Ewe weight increased throughout gestation until 111 dGA, and then fell between 111 and 127 dGA (figure 5.2). When all animals were considered together, ewe BCS did not change significantly between -16 and 104 dGA, although between 104 and 125 dGA ewe BCS fell (figure 5.2). There was no significant difference in BCS between the dietary groups (figure 5.2).

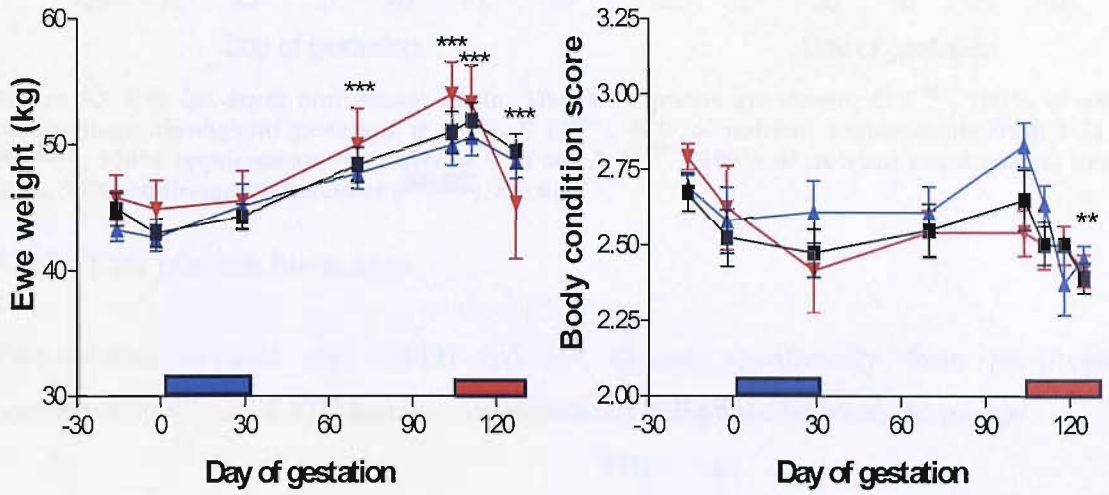

Figure 5.2 Ewe weight and body condition score. The three groups are shown; C (■, 100% of nutrient requirements throughout gestation, n = 10), E (▲, 40% of nutrient requirements from 1-31 dGA (▬), 100% requirements thereafter, n = 9) and L (▼, 100% of nutrient requirements until 104 dGA, 50% requirements thereafter (▬), n = 6). ** $p < 0.01$ and *** $p < 0.001$ significantly different from -2 dGA, all animals.

Neither ewe fat nor muscle depth changed significantly from pre-pregnancy measurements during gestation. There was no significant effect of diet on either of these parameters (figure 5.3). No measurements were obtained after 104 dGA.

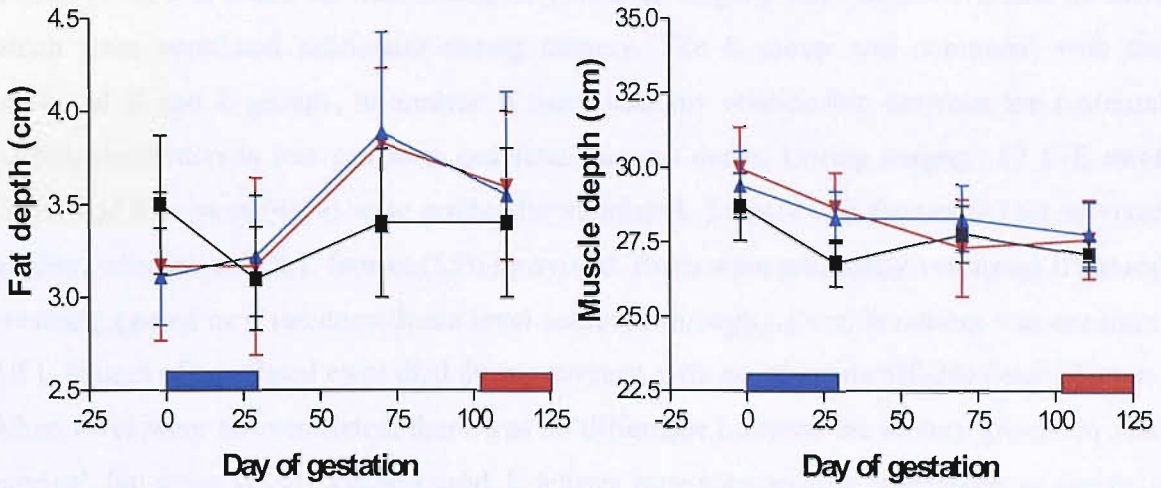

Figure 5.3 Ewe fat depth and muscle depth. The three groups are shown; C (, 100% of nutrient requirements throughout gestation, *n* = 10), E (, 40% of nutrient requirements from 1-31 dGA (), 100% requirements thereafter, *n* = 9) and L (, 100% of nutrient requirements until 104 dGA, 50% requirements thereafter (), *n* = 6).

5.5.2 Ewe plasma hormones

Ewe plasma cortisol and ACTH did not change significantly from pre-pregnancy concentration (figure 5.4). There was no significant difference between the groups.

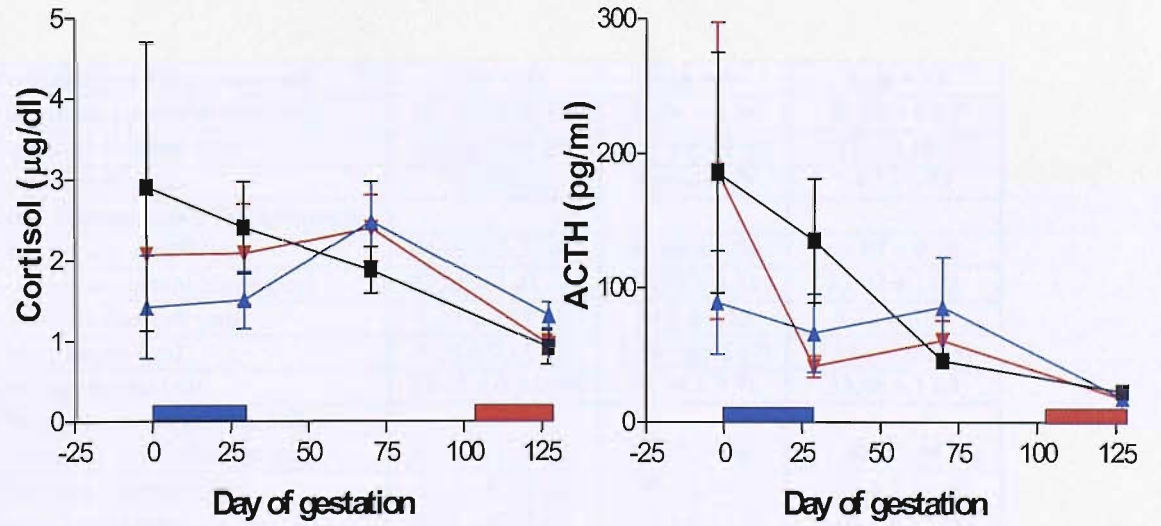

Figure 5.4 Maternal cortisol and ACTH levels. The three groups are shown; C (, 100% of nutrient requirements throughout gestation, *n* = 10), E (, 40% of nutrient requirements from 1-31 dGA (), 100% requirements thereafter, *n* = 9) and L (, 100% of nutrient requirements until 104 dGA, 50% requirements thereafter (), *n* = 6).

5.5.3 Surgery survival

Nine fetuses did not survive the surgery. One fetus (C) died the day following surgery, and 8 fetuses (1 C, 2 E and 5 L) died during or just after surgery. All but one (E) had mothers which were ventilated artificially during surgery. The L group was compared with the combined C and E groups, to analyse if there was any relationship between the maternal nutrient restriction in late gestation and fetal surgical death. During surgery, 12 C/E ewes (52%) and 5 L ewes (45%) were artificially ventilated. Twenty C/E fetuses (87%) survived surgery, whereas only 6 L fetuses (55%) survived. Ewes were artificially ventilated if natural breathing ceased or if the anaesthesia level achieved through natural breathing was too light. All L fetuses of ventilated ewes died during surgery, with no other identifiable causal factors. When ewes were not ventilated, there was no difference between the dietary groups in fetal survival, but when ventilation was used, L fetuses were substantially more likely to perish ($p < 0.001$ chi-squared test).

5.5.4 Fetal biometry

Nutrient restriction had no effect on measurements obtained at surgery or post mortem (table 5.1). There was no significant effect of diet on fetal growth between surgery and post mortem as measured by the change in fetal measurements during this period (table 5.1).

Fetal Surgery Measurements	**C (n = 9)**	**E (n = 9)**	**L (n = 6)**
Abdominal circumference (cm)	21.18 ± 0.28 (8)	21.04 ± 0.06	20.03 ± 0.67
Biparietal diameter (cm)	5.35 ± 0.06 (8)	5.38 ± 0.06 (6)	5.32 ± 0.10 (5)
Femur length (cm)	7.53 ± 0.22	7.60 ± 0.23	7.47 ± 0.23
Fetal Postmortem Measurements			
Crown-rump length (cm)	44.40 ± 0.74 (8)	43.54 ± 1.04	43.67 ± 0.78
Abdominal circumference (cm)	31.01 ± 1.41 (8)	32.94 ± 0.59	32.02 ± 1.02
Biparietal diameter (cm)	5.70 ± 0.17 (8)	5.63 ± 0.12	5.72 ± 0.14
Femur length (cm)	8.18 ± 0.11 (6)	8.06 ± 0.15 (7)	7.98 ± 0.23 (5)
Shoulder height (cm)	33.65 ± 0.84 (8)	33.74 ± 0.71	33.48 ± 1.03
Change from surgery to PM			
Abdominal circumference (cm)	10.42 ± 1.19 (8)	12.12 ± 0.44	11.40 ± 2.54 (5)
Biparietal diameter (cm)	0.22 ± 0.18 (6)	0.30 ± 0.14 (8)	0.20 ± 0.10 (4)
Femur length (cm)	0.53 ± 0.29 (6)	0.78 ± 0.11 (8)	0.68 ± 0.13 (4)

Table 5.1 Fetal measurements at surgery (117 dGA) and post mortem (127 dGA), mean ± SEM. C: control, E: early gestation restricted and L: late gestation restricted. Bracketed numbers indicate n different to that given in the column heading.

Maternal nutrient restriction had no effect on body weight or organ weight measured at post mortem, or on organ weight when calculated as percentage of body weight (table 5.2).

Post mortem parameters	**C (n = 8)**	**E (n = 9)**	**L (n = 6)**
Male : female	1 : 1	5 : 4	2 : 1
Ewe weight (Kg)	49.47 ± 1.36 (7)	48.71 ± 1.35 (7)	45.34 ± 4.41 (4)
Conceptus weight (Kg)	4.22 ± 0.09 (7)	4.21 ± 0.16 (7)	3.96 ± 0.12 (4)
Body weight (Kg)	2.91 ± 0.08	2.92 ± 0.12	2.80 ± 0.16
A + B Cotyledons (g)	248.44 ± 24.36	204.46 ± 39.10	298.13 ± 48.90
C + D Cotyledons (g)	96.53 ± 32.75	103.27 ± 37.17	39.46 ± 21.64
Total placental weight (g)	344.97 ± 20.79	307.73 ± 14.50	337.59 ± 28.51
Left adrenal (g)	0.22 ± 0.04 (7)	0.21 ± 0.01 (8)	0.23 ± 0.02
Right adrenal (g)	0.16 ± 0.02	0.17 ± 0.01	0.21 ± 0.02 (5)
Left kidney (g)	11.65 ± 0.56	11.33 ± 0.56	12.08 ± 0.68
Left kidney length (cm)	3.62 ± 0.09 (7)	3.56 ± 0.07	3.69 ± 0.10
Left kidney width (cm)	1.91 ± 0.06 (7)	2.03 ± 0.11	2.00 ± 0.07
Left kidney volume (cm^2)	25.50 ± 1.38	25.47 ± 1.13	23.29 ± 4.85
Right kidney (g)	9.66 ± 0.61 (7)	9.80 ± 0.40 (8)	9.66 ± 0.42
Liver (g)	101.06 ± 6.21 (7)	101.14 ± 3.89	102.59 ± 11.05
Heart (g)	18.99 ± 0.63	19.40 ± 0.56	17.44 ± 1.10
Lung (g)	79.30 ± 3.38	76.82 ± 6.11	71.33 ± 4.27
Brain (g)	36.40 ± 0.99 (7)	33.90 ± 1.00	35.93 ± 1.01
Pituitary (g)	0.10 ± 0.01 (7)	0.10 ± 0.01 (8)	0.09 ± 0.01 (5)
as % of body weight			
Total placental weight	11.85 ± 0.64	10.57 ± 0.43	12.03 ± 0.71
Left adrenal	0.01 ± 0.001 (7)	0.01 ± 0.0005 (8)	0.01 ± 0.0008
Right adrenal	0.01 ± 0.0006	0.01 ± 0.0005	0.01 ± 0.0007 (5)
Right kidney	0.34 ± 0.02 (7)	0.34 ± 0.02 (8)	0.35 ± 0.02
Left kidney	0.40 ± 0.02	0.39 ± 0.03	0.44 ± 0.03
Liver	3.43 ± 0.18 (7)	3.48 ± 0.12	3.63 ± 0.18
Heart	0.65 ± 0.02	0.67 ± 0.03	0.62 ± 0.03
Lung	2.72 ± 0.09	2.62 ± 0.15	2.56 ± 0.13
Brain	1.24 ± 0.04 (7)	1.17 ± 0.04	1.30 ± 0.06
Pituitary	0.004 ± 0.0002 (7)	0.004 ± 0.0003 (8)	0.003 ± 0.0005 (5)

Table 5.2 Fetal body weight and organ weights at post mortem (127 dGA), mean ± SEM. C: control, E: early gestation restricted and L: late gestation restricted. Bracketed numbers indicate n different to that given in the column heading.

5.6 Discussion

In this chapter it was found that fetal growth and organ size in late gestation were not altered by either of the maternal nutrition restrictions. Neither of the nutritional challenges significantly changed maternal biometry, or maternal plasma cortisol or ACTH levels at any time point measured. An unrelated and unexpected observation was that when ewes were ventilated late gestation restricted fetuses were substantially more likely to perish during or immediately following surgery when compared to the control and peri-implantation restricted group.

5.6.1 Intensity

There was no significant change in maternal weight gain following a 60% peri-implantation maternal nutrient restriction. The peri-implantation nutrient restriction resulted in no change in maternal plasma cortisol or ACTH levels at any time point measured. Therefore the ewe is able to maintain normal weight gain during this more severe peri-implantation nutrient restriction. With challenges that have aimed to reduce maternal weight by 10 or 15% of control values, rather than a global restriction of nutrient, the pregnant ewes were starved for 2 days, then fed concentrate at 25–50% of control levels to maintain the reduction in ewe weight (Harding, 1997; Bloomfield *et al.*, 2004; Jaquiery *et al.*, 2006). This reveals that the level of the reduction in ewe nutrients needs to be greater than used in this study to reduce ewe weight. Where maternal cortisol levels were measured in these studies it was reported to be lower both during and following the challenge (Bloomfield *et al.*, 2004; Jaquiery *et al.*, 2006). The severity of the diets used in this thesis were designed to produce effects on fetal cardiovascular and renal development without causing a pathological state or maternal stress (Gardner *et al.*, 2006; Cleal *et al.*, 2007b). The maternal cortisol and ACTH levels were measured to confirm that this is the case with this more severe nutrient restriction.

The peri-implantation maternal nutrient restriction resulted in no significant difference in fetal body or organ weights in late gestation, despite the increased intensity from the challenge used in chapter 3. This is the first time that the effect of a 60% maternal nutrient

restriction during the peri-implantation period has been investigated. Birth weight has been used as an indicator of poor nutrition, and linked to increased risk of CVD in adult life in epidemiological studies. Therefore there may be no effect of a 60% peri-implantation nutrient restriction, or compensatory growth, or there may be altered regional growth, which affects the development of organ structure, e.g. the kidney, which may impact on function and alter cardiovascular control.

5.6.2 Timing

There was increased incidence of fetal death during surgery in the late gestation nutrient restriction group, especially in fetuses of artificially ventilated ewes. In control and peri-implantation nutrient restriction ewes artificial ventilation of the ewe did not affect fetal survival, whereas in ewes undergoing the late gestation nutrient restriction, artificial ventilation during surgery was lethal to the fetus. Ewes were artificially ventilated if spontaneous breathing ceased or anaesthesia of sufficient depth was not reached by spontaneous breathing, as ventilation increases the amount of halothane absorbed. Halothane anaesthesia has been reported to have different effects on the fetal cardiovascular system. In sheep halothane anaesthesia decreases fetal cardiac output and placental perfusion (Sabik *et al.*, 1993), whereas another study found reduced fetal blood pressure (Biehl *et al.*, 1983). Halothane anaesthesia may result in fetal death due to depressed cardiac function and/or decreased placental perfusion. In the Dutch famine infant mortality was also highest in those exposed to famine in late gestation (Stein & Susser, 1975). Nutrient restricted in late gestation can alter cardiovascular control in sheep (Edwards & McMillen, 2001), therefore when the surgery occurs during the nutrient restriction the altered cardiovascular control may alter the ability to survive exposure to halothane. Previous studies in sheep have found that fetuses of ewes that underwent a 50% maternal nutrient restriction from 115 dGA following surgery were significantly less likely to survive experiments to post mortem (144 – 147 dGA) than control fetuses (Edwards & McMillen, 2001; Edwards *et al.*, 2001c). These results suggest that late gestation dietary restriction can affect fetal cardiovascular control and survival rates, and thus the ability to survive exposure to a high dose of halothane.

In this study we found no significant difference in maternal plasma cortisol or ACTH or

maternal weight gain during the late gestation nutrient restriction. To the best of my knowledge there is only one previous study that reported maternal cortisol levels in response to a late gestation nutrient restriction. They reported that a 50% maternal nutrient restriction from 115 dGA increased maternal plasma cortisol concentrations compared with controls during the first 10 days of the challenge (Edwards & McMillen, 2001). However, there was no difference in maternal cortisol levels when measured after 125 dGA (Edwards & McMillen, 2001). As maternal plasma cortisol levels were not measured between the start of the nutrient restriction and 126 dGA in this study we will not have measured any transient increase in cortisol that may have occurred.

No significant difference was found in fetal or organ weight following a late gestation maternal nutrient restriction. A 50% maternal nutrient restriction from 115 dGA resulted in no difference in fetal body weight, or in placental weight (Edwards & McMillen, 2001). Also a 25% maternal nutrient restriction from 100 dGA reduced birth weight but not significantly ($p < 0.07$) (Bauer *et al.*, 1995). Other studies have found reductions in fetal weight following a range of late gestation maternal nutrient restriction. For example a 95% nutrient restriction for 20 days from 105 dGA reduced weight at 125 dGA and at birth, although this is a more severe challenge than the one used in the present study (Oliver *et al.*, 2001). A 30% maternal nutrient restriction from 100 dGA reduced twin birth weight, but twinning is an additional challenge in terms of both constraint and competition for the available nutrients (Borwick *et al.*, 2003). A 50% maternal nutrient restriction from 118 dGA reduced kidney weight, increased adrenal weight but had no effect on body weight at 140 dGA; this was a similar severity and duration to the challenge used in the present study but took place later in gestation (118–140 dGA) (Burrage, 2006). Therefore, the variation in the challenges used in terms of exact timing intensity and duration and the time point at which effects were investigated may explain these conflicting findings.

5.7 Conclusion

Thus far, no changes in fetal body or organ weights, including kidney measurements, following 60% peri-implantation or a 50% late gestation nutrient restriction have been found. The increased incidences of death during surgery in the late gestation nutrient restricted group may indicate altered cardiovascular control. Also the high rate of fetal deaths in the late gestation group may have altered the distribution of the group, as the fetuses most affected by the nutritional challenge may well have been to those least likely to survive surgery. This would result in the least affected fetuses surviving to post mortem and being included in our analysis of birth and organ weight and therefore may not be a true reflection of the effect of the diet. Therefore it is important to investigate the effect of both the more severe (60%) peri–implantation and the late gestation maternal nutrient restrictions on fetal cardiovascular control and renal function.

6 Fetal cardiovascular and renal function following either a 60% peri-implantation or a 50% late gestation nutrient restriction

6.1 Introduction

6.1.1 Intensity

Raised blood pressure in adult life is associated with low birth weight, even within the normal distribution of birth weights. Maternal nutrient restriction can alter cardiovascular parameters in fetal and postnatal life even in the absence of changes in fetal body or birth weight (Hawkins *et al.*, 2000b; Hawkins *et al.*, 2000c; Gardner *et al.*, 2004). **As yet no one has investigated whether a more severe nutrition restriction in the peri-implantation period alters cardiovascular parameters or the RAS in fetal life.**

Models of hypoxia have been used to investigate the mechanisms of cardiovascular control and may reveal changes in cardiovascular control not seen in basal conditions. Previous studies in sheep have shown that a 15% maternal nutrient restriction for the first half of gestation can increase femoral artery vascular resistance during hypoxia in late gestation suggesting that the chemoreflex is augmented (Hawkins *et al.*, 2000b). No change was seen in the heart rate, blood pressure or femoral blood flow response to hypoxia in this model (Hawkins *et al.*, 2000b). **No one has yet studied how a peri-implantation maternal nutrient restriction affects the well described fetal cardiovascular response to hypoxia in late gestation.**

6.1.2 Timing

Late gestation is a period of rapid fetal growth, the demands on the mother are high, and maternal constraint starts to exert effects to limit fetal growth. Poor *in utero* nutrition during the last trimester of gestation reduces glucose tolerance in humans in later life (Ravelli *et al.*, 1998). This period is critical in kidney development as nephrogenesis is greatest in late gestation, and is complete at around 120 dGA in sheep, therefore challenges during this period may reduce nephron endowment. The RAS is also maturing, and the proportion of

AT1 receptors are increasing (Gimonet *et al.*, 1998). Few animal studies have investigated the effects of maternal nutrient restriction in late gestation, and they have found conflicting results (Bauer *et al.*, 1995; Edwards & McMillen, 2001; Oliver *et al.*, 2001; Oliver *et al.*, 2002c; Burrage, 2006). **As yet no one has investigated whether a 50% maternal nutrient restriction in late gestation (from 104 dGA) alters RAS, renal function or cardiovascular control in late gestation fetal sheep.**

A hypoxia challenge in late gestation may reveal changes in cardiovascular control not seen in basal conditions. Prevailing hypoglycaemia (blood glucose consistently ≤ 0.6 mmol/l) increased the femoral vascular resistance response to hypoxia in late gestation fetuses suggesting that the chemoreflex is augmented (Gardner *et al.*, 2002). Although no change was seen in the heart rate, blood pressure or femoral blood flow response to hypoxia in this model, this is similar to the altered hypoxia response following maternal nutrient restriction for the first half of gestation. **No one has yet looked at how a concurrent late gestation maternal nutrient restriction affects the well described fetal cardiovascular response to hypoxia in late gestation.**

6.2 Hypothesis

- A more severe (60%) peri-implantation or a late gestation maternal nutrient restriction alters fetal renal function or RAS in late gestation resulting in altered cardiovascular control.

6.3 Aims

To carry out either a 60% peri-implantation or a 50% late gestation maternal nutrient restriction and monitor its effects on fetal cardiovascular and renal function and renal RAS in late gestation by:

1. Monitoring basal cardiovascular parameters.
2. Testing the baroreflex response.
3. Testing the response to increasing doses of Ang II.
4. Monitoring renal function.
5. Measuring blood gases, plasma hormone and RAS components level.
6. Measuring fetal cardiovascular, renal and RAS responses to frusemide.
7. Measuring fetal cardiovascular, renal and hormonal response to a hypoxic insult.

6.4 Methods

Welsh mountain ewes (Animals (Scientific Procedures) Act 1986) were housed, fed, mated and surgically instrumented as described as in chapter 2. Fetuses (control (C), $n = 9$, early restricted (E), $n = 9$, and late restricted (L), $n = 6$) were studied on 2 successive days between 125-126 dGA (figure 6.1). Continuous recordings were made of amniotic, tracheal and arterial pressure, fetal heart rate, ECoG, femoral and arterial blood flow throughout the experimental protocols. Vascular sensitivity to Ang II was assessed using increasing doses of Ang II (0 μg, 1 μg, 2.5 μg, 5 μg, and 10 μg, Sigma-Aldrich Co, UK). Kidney function and the function of the RAS were also assessed using a bolus of saline vehicle or frusemide (20 mg, Phoenix, Pharmacia, UK). Baroreflex sensitivity was assessed using phenylephrine (75 μg, i.v. bolus, Sovereign Medical, Essex, UK). A hypoxia challenge which reduced the maternal FiO_2 (14 l/min air, 22 l/min N_2, 1.2 l/min CO_2) was used to assess the fetal cardiovascular response to reduced oxygenation. For full details of experimental protocols see section 2.4.

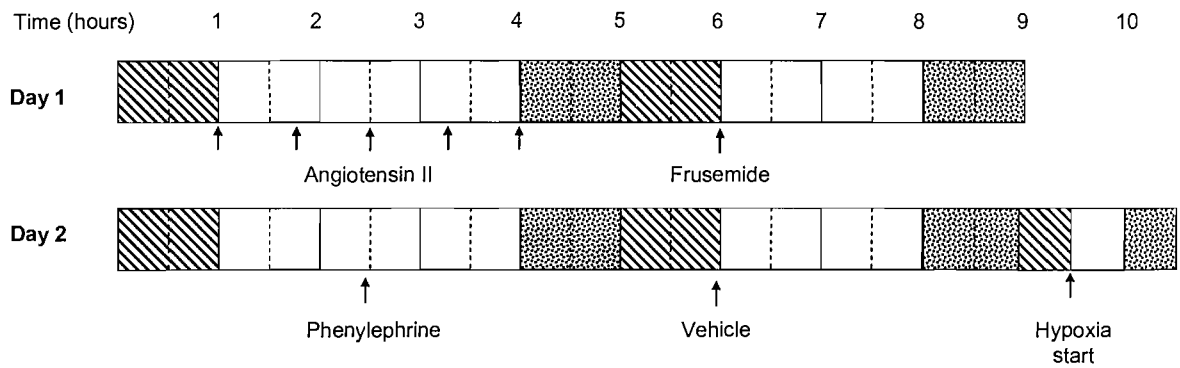

Figure 6.1 Experiment timeline. Baseline ▨, recovery ▩.

6.4.1 Data analysis

For a general description of the analysis, and the power calculation and statistics packages used, see section 2.6.

Male and female fetuses were analysed together due to the small size of some groups when divided according to sex. No significant differences were found between males and females

in body weight or any organ weight when analysed by Student's t test.

Where serial measurements were made (such as cardiovascular or response to stimuli), data were analysed by repeated measures 2 way ANOVA, with time and dietary group as factors. Where the result of the analysis was significantly different over time or between groups, where the differences occurred were identified by a Bonferroni *post-hoc* test.

Where only one measurement was made or measurements were expressed as a 'summary of measures' (such as AUC, maximum response or time to maximum response), data were compared between groups by 1 way ANOVA. If parameters differed significantly a Bonferroni *post-hoc* test was used to identify which groups differed significantly.

Values shown are mean ± SEM, and significance values are stated for *post-hoc* test results. Significance was accepted when $p < 0.05$.

6.5 Results

6.5.1 Cardiovascular parameters

Basal

There was no difference between groups in basal systolic, diastolic or mean arterial blood pressures, femoral or carotid arterial flow or heart rate in late gestation (table 6.1).

125 dGA	**C (*n* = 9)**	**E (*n* = 9)**	**L (*n* = 6)**
Mean arterial pressure (mm Hg)	43.6 ± 1.9	43.1 ± 0.9	44.3 ± 0.9
Systolic pressure (mm Hg)	56.0 ± 3.1	54.5 ± 1.6	56.3 ± 2.5
Diastolic pressure (mm Hg)	37.3 ± 1.4	37.4 ± 1.0	38.4 ± 1.1
Carotid flow (ml/min)	86.8 ± 7.8	81.7 ± 12.7 (8)	82.6 ± 15.0
Femoral flow (ml/min)	36.0 ± 2.9	35.8 ± 4.5	37.3 ± 9.2 (5)
Heart rate (BPM)	166.3 ± 4.7	169.7 ± 8.7	153.7 ± 22.5
126 dGA			
Mean arterial pressure (mm Hg)	40.8 ± 2.1	40.9 ± 2.5	42.6 ± 0.7
Systolic pressure (mm Hg)	51.2 ± 3.4	45.6 ± 4.3	55.5 ± 1.2
Diastolic pressure (mm Hg)	35.7 ± 1.7	35.9 ± 2.4	36.2 ± 1.0
Carotid flow (ml/min)	75.5 ± 8.7	69.5 ± 10.2	74.9 ± 9.3
Femoral flow (ml/min)	29.0 ± 4.1 (8)	29.1 ± 3.6 (8)	29.9 ± 6.8 (5)
Heart rate (BPM)	144.3 ± 14.9	160.7 ± 14.5	168.9 ± 7.9

Table 6.1 Basal cardiovascular measurements, mean ± SEM. Bracketed numbers indicate *n* different to that given in the column heading.

Baroreflex

There was no difference between the groups in the sensitivity or operating point of the baroreflex or dose of phenylephrine per kg body weight (table 6.2). MAP increased and fetal heart rate, carotid and femoral blood flows fell in response to phenylephrine, but there was no significant difference in the response between the groups (table 6.2).

Baroreflex	**C (*n* = 9)**	**E (*n* = 9)**	**L (*n* = 6)**
Phenylephrine dose (μg/kg)	25.60 ± 0.71 (7)	25.90 ± 1.13 (8)	27.29 ± 1.81 (5)
Sensitivity (ΔRR / ΔSBP)	0.008 ± 0.001 (8)	0.007 ± 0.001 (8)	0.008 ± 0.001 (5)
Operating point (mm Hg)	68.87 ± 4.43 (8)	68.73 ± 3.43 (8)	68.62 ± 4.21 (5)
Maximum response			
MAP (Δ mm Hg)	17.03 ± 1.41	18.42 ± 1.96	17.75 ± 1.50
Heart rate (Δ BPM)	54.88 ± 9.90	65.19 ± 6.98	41.54 ± 6.79
Carotid flow (Δ ml/min)	27.12 ± 2.85	28.29 ± 4.51 (8)	31.86 ± 3.12
Femoral flow (Δ ml/min)	19.35 ± 2.51	23.35 ± 2.55	22.57 ± 5.52 (5)

Table 6.2 Response to phenylephrine, mean ± SEM. Bracketed numbers indicate *n* different to that given in the column heading.

Response to angiotensin II

MAP increased and heart rate, femoral and carotid flow fell in response to Ang II. There was no significant difference in the MAP, heart rate or blood flow response between the groups (figure 6.2 & table 6.3).

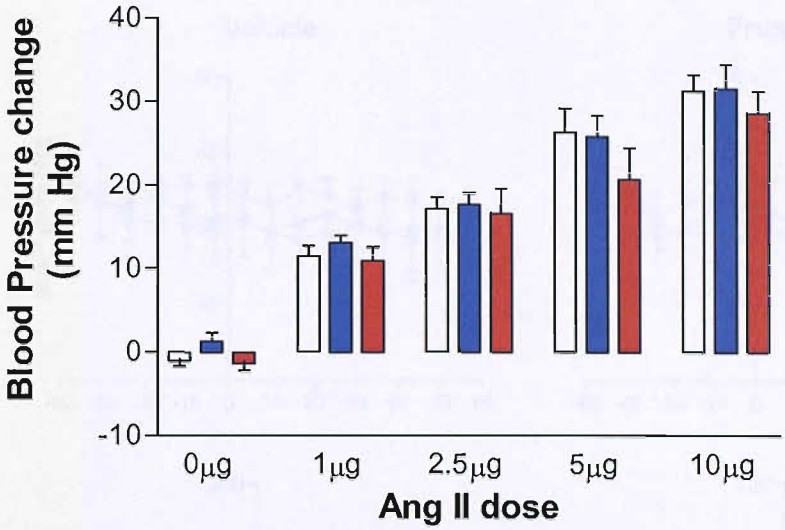

Figure 6.2 Maximum change in fetal MAP in response to Ang II, mean ± SEM. C (□, *n* = 9), E (■, *n* = 9) and L (■, *n* = 5).

MAP response (Δ mm Hg)	C (*n* = 9)	E (*n* = 9)	L (*n* = 6)
0 μg Ang II	-1.07 ± 0.61	1.29 ± 0.99	-1.27 ± 0.93 (5)
1 μg Ang II	11.42 ± 1.29	13.17 ± 0.84	10.98 ± 1.55 (5)
2.5 μg Ang II	17.12 ± 1.45	17.77 ± 1.34	16.70 ± 2.84 (5)
5 μg Ang II	26.26 ± 2.92	24.94 ± 2.43	20.82 ± 3.60 (5)
10 μg Ang II	31.26 ± 1.98	31.68 ± 2.78	28.69 ± 2.61 (5)
Maximum response to 10 μg Ang II			
Heart rate (Δ BPM)	77.42 ± 8.77	54.74 ± 9.20	51.60 ± 9.39
Carotid flow (Δ ml/min)	23.59 ± 6.30	16.27 ± 10.36 (8)	23.15 ± 6.97
Femoral flow (Δ ml/min)	25.15 ± 2.59	19.91 ± 4.90	17.02 ± 4.70 (5)

Table 6.3 Maximum cardiovascular response to Ang II, mean ± SEM. Bracketed numbers indicate *n* different to that given in the column heading.

Response to frusemide

When all groups were analysed together a 20 mg bolus of frusemide caused a rise in heart rate which was significant between 60 and 80 minutes, but no change in blood pressure (figure 6.3). A 2 ml vehicle infusion caused no response in these cardiovascular parameters.

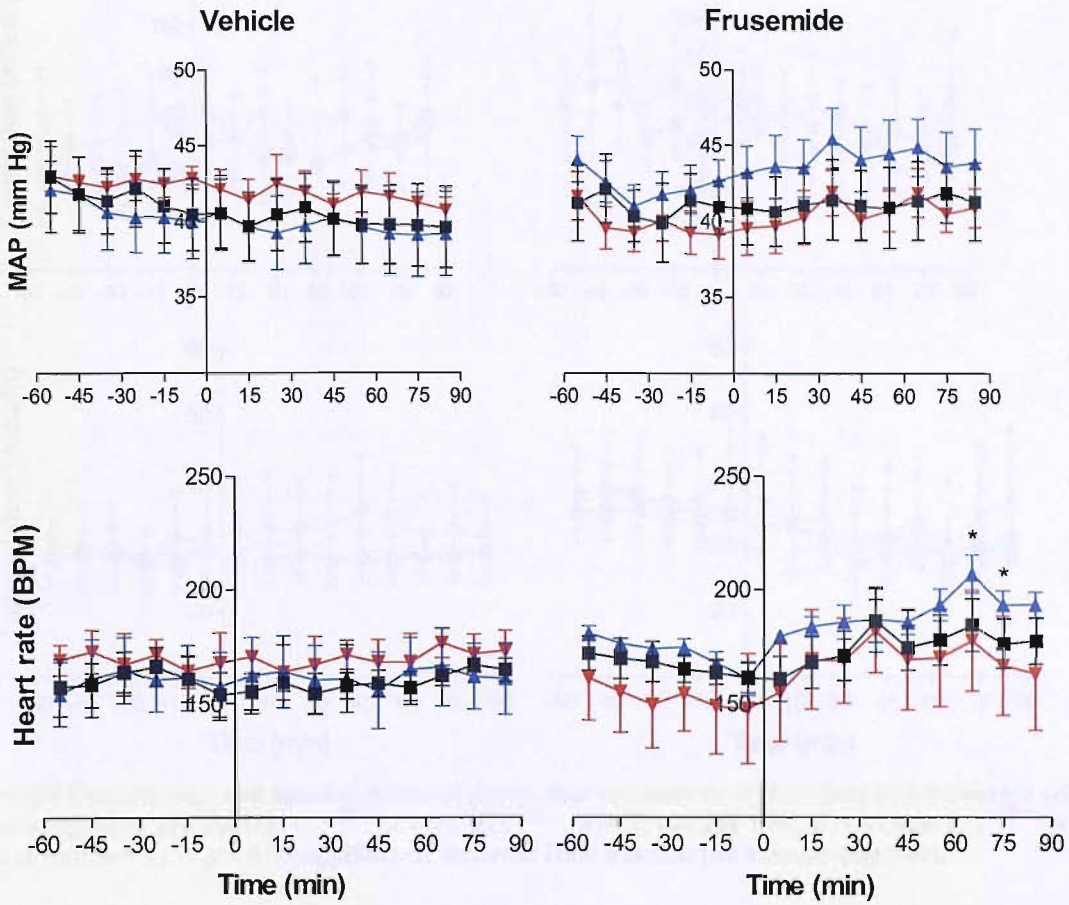

Figure 6.3 MAP (top) and heart rate (bottom) response to vehicle (left) and frusemide (right). The three groups are shown; C (■, $n = 9$), E (▲, $n = 9$,) and L (▼, $n = 6$). * $p < 0.05$ significantly different from baseline (all animals together).

When all groups were analysed together a 20 mg bolus of frusemide caused a decrease in femoral artery blood flow for the entire protocol, but no change in carotid artery blood flow (figure 6.4). A 2 ml vehicle infusion caused no response in blood flow.

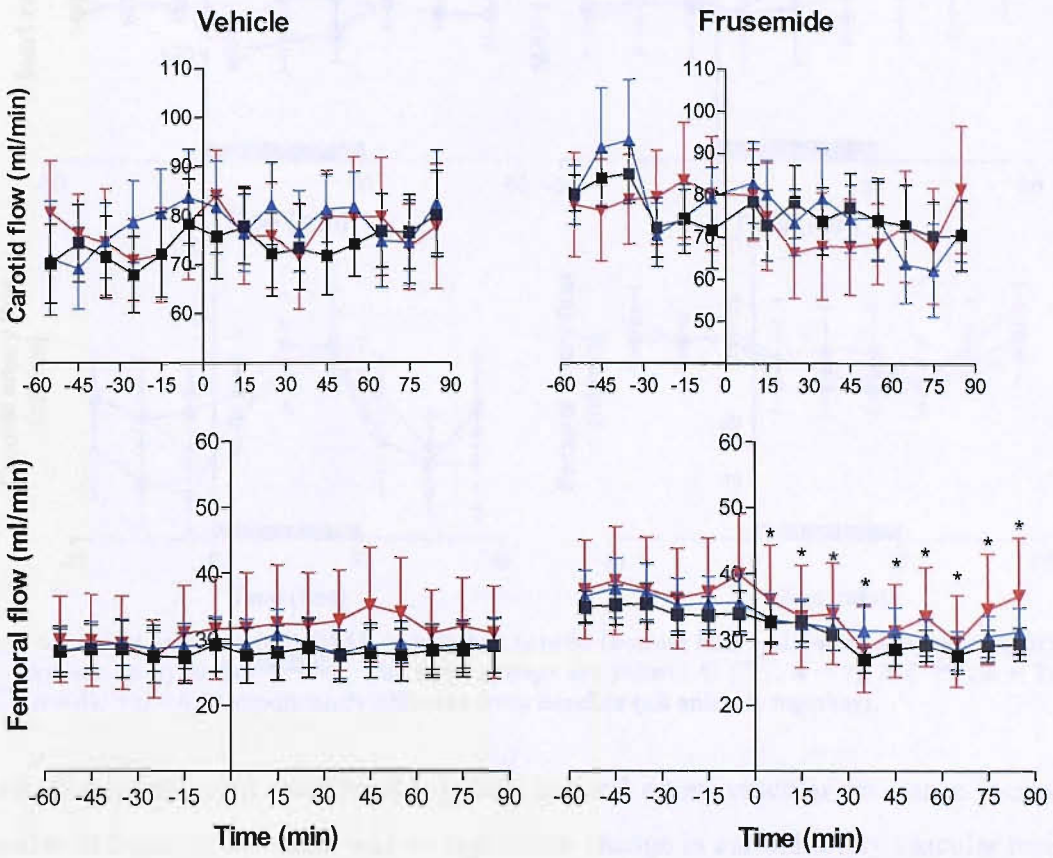

Figure 6.4 Carotid (top) and femoral (bottom) artery flow response to vehicle (left) and frusemide (right). The three groups are shown; C (■, n = 9), E (▲, n = 9, carotid flow, n = 8) and L (▼, n = 6, femoral flow, n = 5). * $p < 0.05$ significantly different from baseline (all animals together).

Response to hypoxia

When all groups were analysed together hypoxia caused a fall in heart rate within 5 minutes of onset. This was followed by a rise in blood pressure, which became significant by 35 minutes. Carotid blood flow increased and femoral blood flow decreased in response to hypoxia, and both reached significance by 5 minutes (figure 6.5). There was no difference in overall cardiovascular response to hypoxia between the groups.

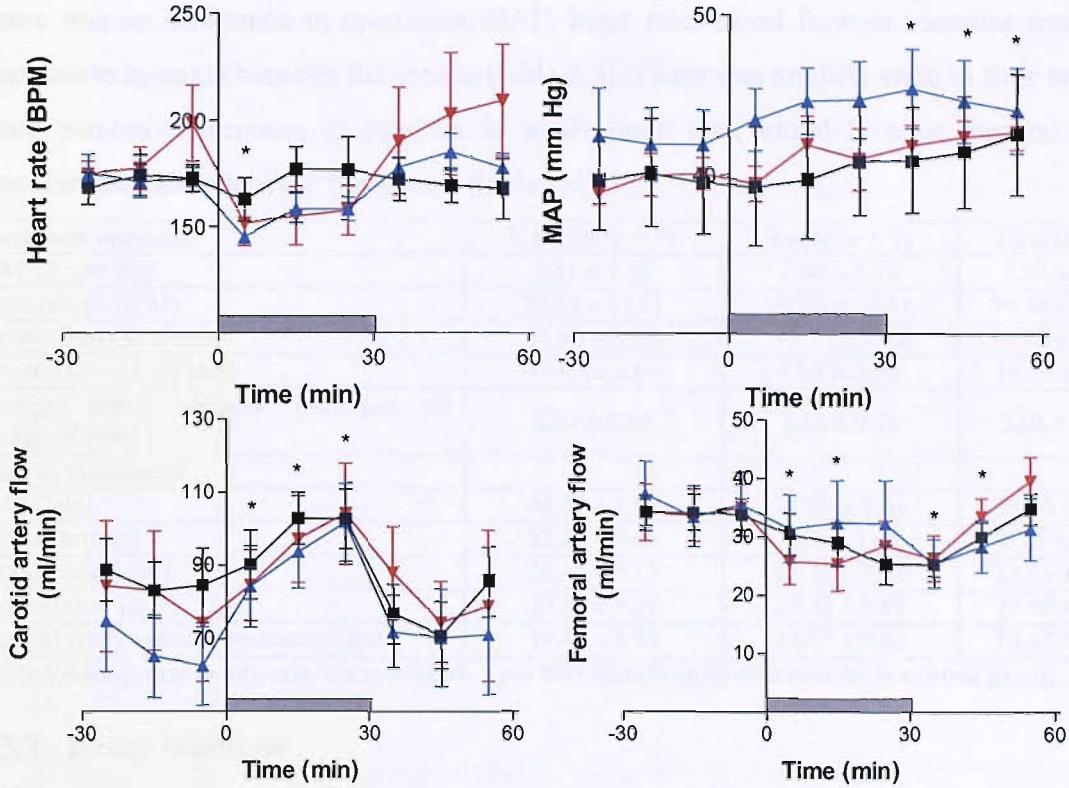

Figure 6.5 Heart rate (top left), MAP (top right), carotid (bottom left) and femoral (bottom right) artery flow response to hypoxia ( ). The three groups are shown; C (, $n = 7$), E (, $n = 7$) and L (, $n = 4$). * $p < 0.05$ significantly different from baseline (all animals together).

When all animals were considered together, femoral artery vascular resistance increased in response to hypoxia, but there was no significant change in carotid artery vascular resistance (figure 6.6). There was no difference in vascular resistance response to hypoxia between the groups.

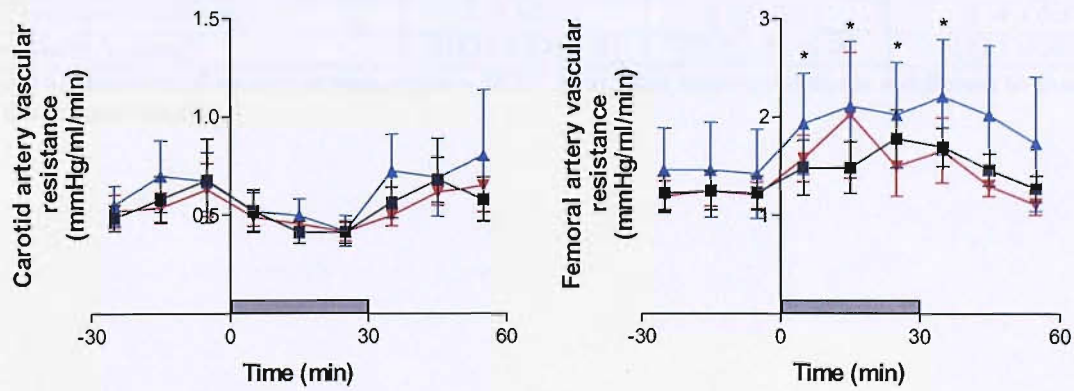

Figure 6.6 Carotid (left) and femoral (right) artery vascular resistance response to hypoxia ( ). The three groups are shown; C (, $n = 7$), E (, $n = 7$) and L (, $n = 4$). * $p < 0.05$ significantly different from baseline (all animals together).

There was no difference in maximum MAP, heart rate, blood flow or vascular resistance response to hypoxia between the groups (table 6.5). There was no difference in time taken to reach maximum response to hypoxia in MAP, heart rate, blood flow or femoral artery vascular resistance between the groups (table 6.4).

Maximum response	**Control (n = 7)**	**Early (n = 7)**	**Late (n = 4)**
MAP (Δ mm Hg)	5.31 ± 1.78	7.48 ± 1.78	7.55 ± 2.35
Heart rate (Δ BPM)	36.59 ± 13.11	64.09 ± 13.11	56.38 ± 17.35
Carotid flow (Δ ml/min)	21.43 ± 8.56	51.17 ± 8.56	34.22 ± 11.32
Femoral flow (Δ ml/min)	16.05 ± 2.60	15.8 ± 2.60	16.34 ± 3.96
Femoral artery vascular resistance (Δ mmHg/ml/min)	2.67 ± 0.66	3.16 ± 0.72	3.08 ± 1.02
Time to maximum			
MAP (min)	44.57 ± 4.95	27.00 ± 5.64	33.50 ± 8.69
Heart rate (min)	12.14 ± 5.44	4.75 ± 1.06	6.25 ± 1.55
Carotid flow (min)	28.14 ± 5.13	23.25 ± 2.68	27.75 ± 5.45
Femoral flow (min)	20.29 ± 4.92	22.88 ± 4.42	25.50 ± 8.41
Femoral artery vascular resistance (min)	19.41 ± 3.93	13.17 ± 4.45	10.67 ± 6.01

Table 6.4 Response to hypoxia, mean ± SEM. * $p < 0.05$ significantly different from control group.

6.5.2 Renal function

Basal

There was no significant difference in basal GFR, urine flow rate, Na^+ excretion or osmolarity between the groups (table 6.5).

125 dGA	**C (n = 9)**	**E (n = 9)**	**L (n = 6)**
GFR (ml/min)	4.65 ± 0.63 (8)	3.76 ± 0.54	3.37 ± 0.26
Flow rate (ml/min)	0.48 ± 0.13 (8)	0.57 ± 0.12	0.50 ± 0.11
Na^+ excretion (mmol/min)	1.7 ± 0.3	1.77 ± 0.38	1.43 ± 0.2
Osmolarity (mosmol)	172 ± 14.68	177.88 ± 29.55 (8)	129.33 ± 13.69
126 dGA			
GFR (ml/min)	3.54 ± 0.28	3.40 ± 0.65	2.89 ± 0.44 (5)
Flow rate (ml/min)	0.49 ± 0.09	0.66 ± 0.15	0.47 ± 0.12 (5)
Na^+ excretion (mmol/min)	2.28 ± 0.35	2.53 ± 0.41	2.34 ± 0.37
Osmolarity (mosmol)	202.13 ± 19.53 (8)	225.0 ± 20.03 (8)	182.5 ± 20.27

Table 6.5 Basal renal measurements, mean ± SEM. Bracketed numbers indicate n different to that given in the column heading.

Response to frusemide

When all the groups were analysed together, frusemide significantly increased urine flow rate, Na^+ excretion (figure 6.7) and urine osmolarity (figure 6.8) for the entire protocol. GFR was significantly increased by 45 minutes but had returned to basal levels by 75 minutes (figure 6.8). There was no significant difference in renal response to frusemide between the groups.

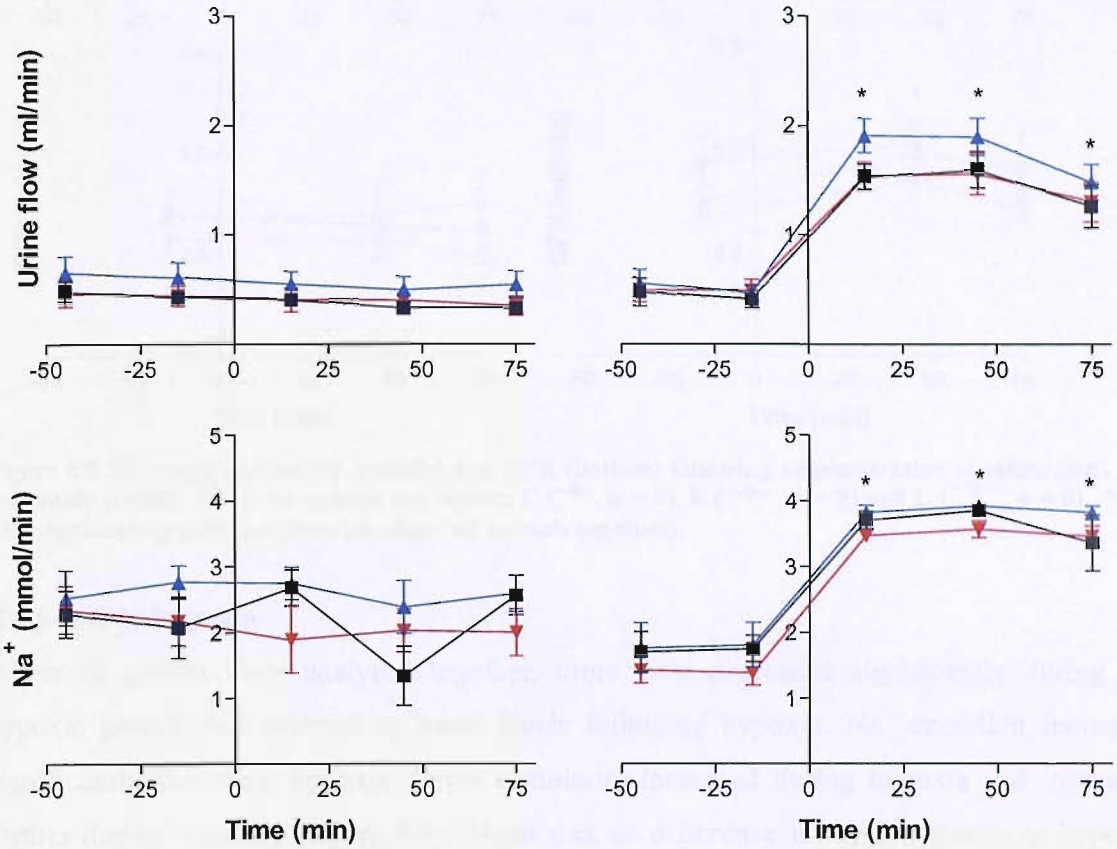

Figure 6.7 Urine flow and Na+ excretion following administration of saline (left) and frusemide (right). The three groups are shown; C (■, $n = 9$), E (▲, $n = 9$) and L (▼, $n = 6$). * $p < 0.05$ significantly different from baseline (all animals together).

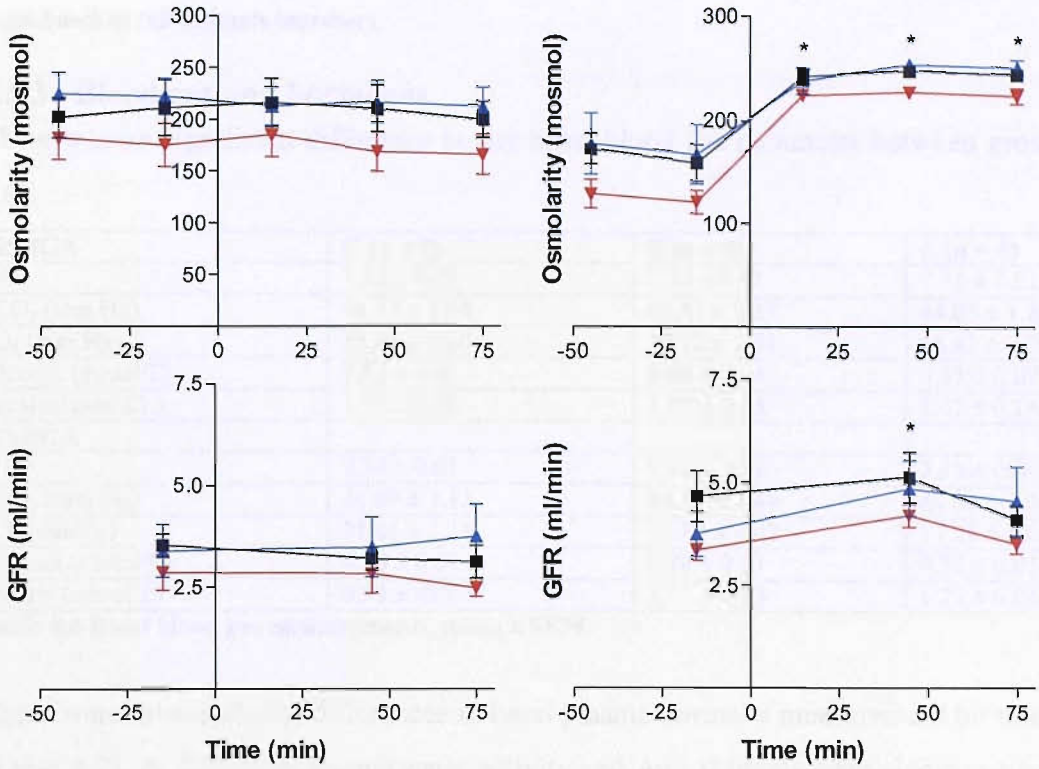

Figure 6.8 Na^+ (top), osmolarity (middle) and GFR (bottom) following administration of saline (left) and frusemide (right). The three groups are shown; C (■, $n = 9$), E (▲, $n = 9$) and L (▼, $n = 6$). * $p < 0.05$ significantly different from baseline (all animals together).

Response to hypoxia

When all groups were analysed together, urine flow decreased significantly during the hypoxic period, but returned to basal levels following hypoxia. Na^+ excretion increased significantly following hypoxia. Urine osmolarity increased during hypoxia and increased further during recovery (figure 6.9). There was no difference in renal response to hypoxia between the groups.

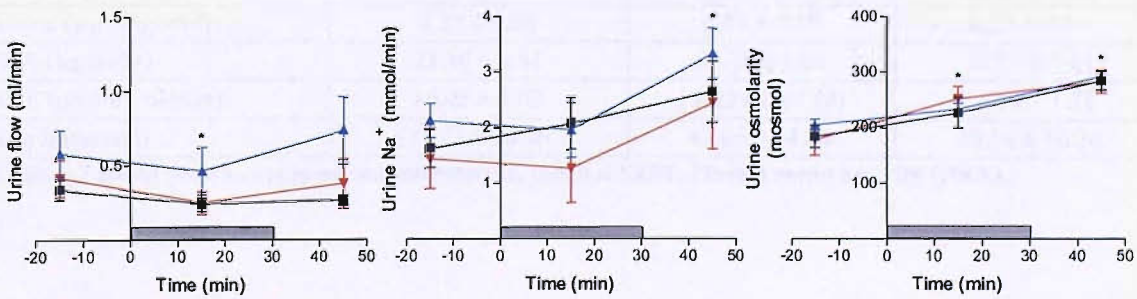

Figure 6.9 Urine flow (left), Na^+ (middle) and osmolarity (right) during hypoxia (▬). The three groups are shown; C (■, $n = 7$), E (▲, $n = 8$) and L (▼, $n = 5$). * $p < 0.05$ significantly different

from baseline (all animals together).

6.5.3 Blood gas and hormones

There was no significant difference in any basal blood gas parameter between groups (table 6.6).

125 dGA	**C (*n* = 9)**	**E (*n* = 9)**	**L (*n* = 6)**
pH	7.32 ± 0.01	7.32 ± 0.01	7.34 ± 0.01
pCO_2 (mm Hg)	44.13 ± 1.64	45.51 ± 1.17	44.05 ± 1.84
pO_2 (mm Hg)	18.83 ± 0.60	18.12 ± 1.51	16.43 ± 2.01
Glucose (mmol/L)	0.82 ± 0.05	0.88 ± 0.04	0.83 ± 0.07
Lactate (mmol/L)	1.03 ± 0.08	1.22 ± 0.15	1.42 ± 0.16
126 dGA			
pH	7.34 ± 0.01	7.33 ± 0.01	7.35 ± 0.00
pCO_2 (mm Hg)	44.09 ± 1.13	45.77 ± 1.49	44.09 ± 1.62
pO_2 (mmHg)	21.01 ± 1.16	19.19 ± 1.47	17.23 ± 1.05
Glucose (mmol/L)	0.76 ± 0.07	0.79 ± 0.03	0.72 ± 0.05
Lactate (mmol/L)	0.93 ± 0.06	1.13 ± 0.13	1.22 ± 0.06

Table 6.6 Basal blood gas measurements, mean ± SEM.

There were no significant differences in basal plasma hormone measurement between groups (Table 6.7). At 126 dGA plasma renin activity and Ang II levels were significantly elevated compared to 125 dGA.

125 dGA	**C (*n* = 9)**	**E (*n* = 9)**	**L (*n* = 6)**
Cortisol (μg/dl)	1.12 ± 0.22	1.83 ± 0.57 (8)	1.37 ± 0.92
ACTH (pg/ml)	38.96 ± 6.23	39.79 ± 7.71	23.53 ± 3.72
Aogen (μg Ang I/ml)	0.35 ± 0.13	0.43 ± 0.10	0.32 ± 0.07
PRA (ng/ml/hr)	10.33 ± 3.00	9.14 ± 2.48	10.16 ± 1.77
ACE (nmoles/ml/min)	14.75 ± 0.65	15.01 ± 0.93	15.02 ± 0.68
Ang II (pmol/l)	15.86 ± 9.56	14.74 ± 6.32 (8)	16.26 ± 6.52 (5)
126 dGA			
Cortisol (μg/dl)	2.29 ± 1.13	2.72 ± 1.21 (8)	1.88 ± 0.42
ACTH (pg/ml)	49.08 ± 21.30	39.80 ± 9.89 (8)	30.67 ± 8.36
Aogen (μg Ang I/ml)	0.22 ± 0.08	0.41 ± 0.10	0.37 ± 0.16
PRA (ng/ml/hr)	23.36 ± 6.81	35.28 ± 5.04	33.75 ± 7.01
ACE (nmoles/ml/min)	16.03 ± 1.02	15.80 ± 0.81 (8)	16.64 ± 1.28
Ang II (pmol/l)	37.73 ± 16.26	43.05 ± 14.74	38.34 ± 16.36

Table 6.7 Basal plasma hormone measurements, mean ± SEM. Plasma renin activity (PRA).

Response to frusemide

Frusemide significantly increased plasma Ang II levels (figure 6.10). There was no significant difference in plasma Ang II response to frusemide between groups.

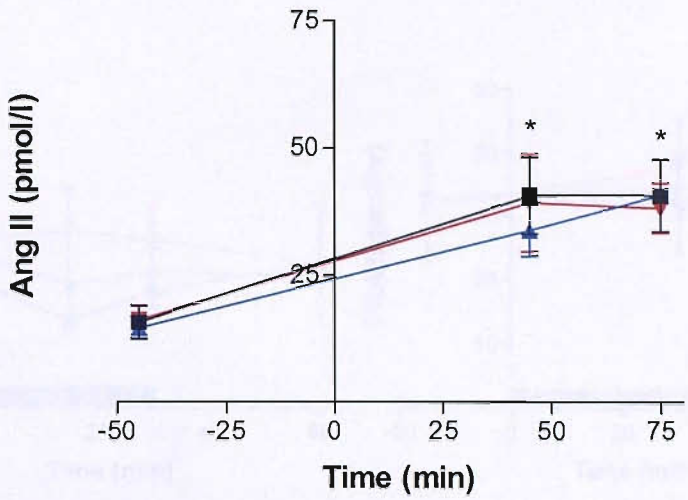

Figure 6.10 Effect of frusemide on fetal plasma Ang II levels. The three groups are shown; C (■, $n = 9$), E (▲, $n = 9$) and L (▼, $n = 6$). * $p < 0.05$ significantly different from baseline (all animals together).

Frusemide significantly increases PRA (figure 6.11). There was no significant difference in plasma Ang II response to frusemide between groups.

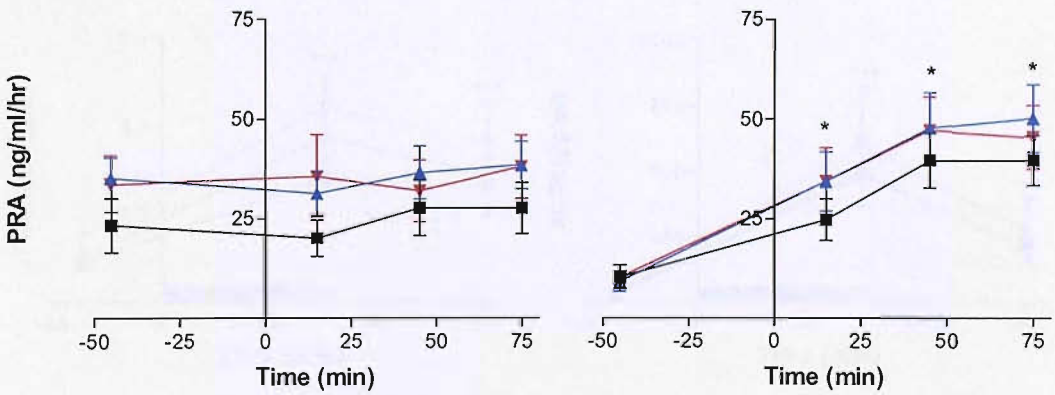

Figure 6.11 Effect of saline (left) or frusemide (right) on fetal plasma renin activity (PRA). The three groups are shown; C (■, $n = 9$), E (▲, $n = 9$) and L (▼, $n = 6$). * $p < 0.05$ significantly different from baseline (all animals together).

Response to hypoxia

Hypoxia had no effect on fetal plasma Ang II levels or plasma renin activity (figure 6.12). There was no significant difference in fetal plasma Ang II response to hypoxia between groups.

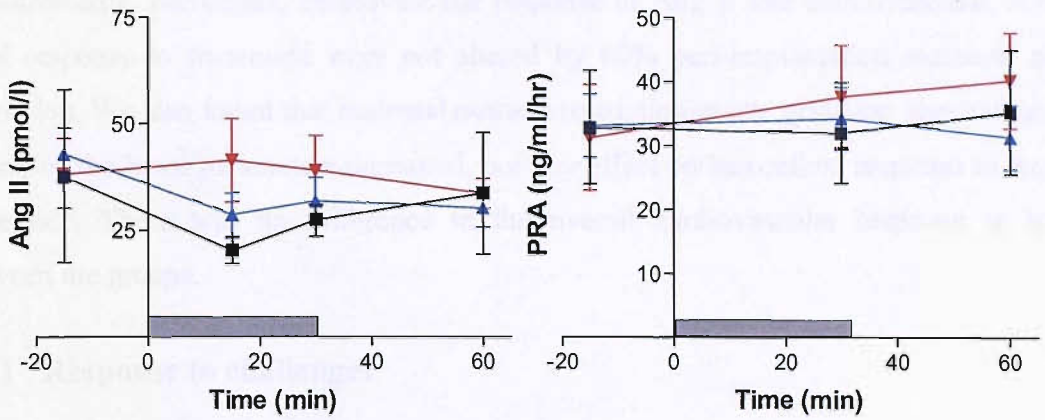

Figure 6.12 Effect of hypoxia ( ) on fetal plasma Ang II levels and plasma renin activity (PRA). The three groups are shown; C (, $n = 6$), E (, $n = 6$) and L (, $n = 4$).

Hypoxia significantly increased fetal plasma cortisol and ACTH levels and they remained raised during recovery (figure 6.13). There was no significant difference in fetal plasma cortisol or ACTH response to hypoxia between the groups.

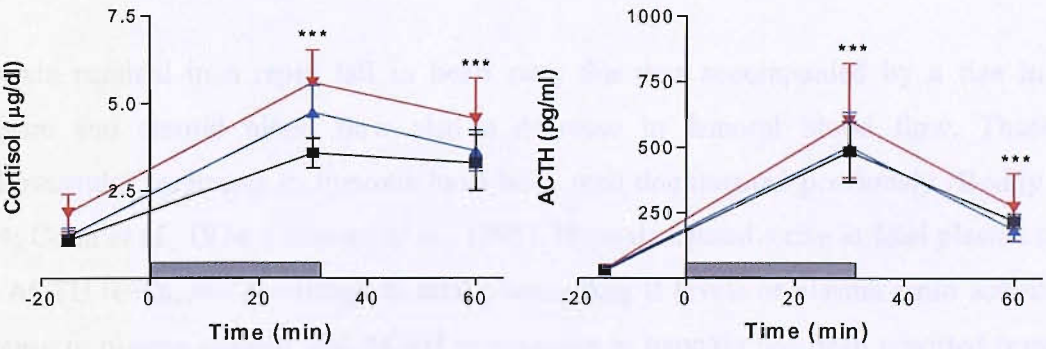

Figure 6.13 Effect of hypoxia ( ) on fetal plasma cortisol (left) and ACTH (right) levels. The three groups are shown; C (, $n = 7$), E (, $n = 8$) and L (, $n = 5$). * $p < 0.05$ significantly different from baseline (all animals together).

6.6 Discussion

In the present chapter it was found that a 60% peri-implantation maternal nutrient restriction had no effect on any basal renal function, blood gases, hormone or cardiovascular measurements. Baroreflex, cardiovascular response to Ang II and cardiovascular, RAS and renal response to frusemide were not altered by 60% peri-implantation maternal nutrient restriction. We also found that maternal nutrient restriction in late gestation also had no effect on any of the basal parameters measured, nor any effect on baroreflex, response to Ang II or frusemide. There was no difference in the overall cardiovascular response to hypoxia between the groups.

6.6.1 Response to challenges

The response to frusemide of no change in blood pressure or carotid artery femoral flow, an increase in heart rate, plasma Ang II levels and plasma rennin activity and decrease in femoral blood flow were similar to the findings in chapter 4. Frusemide resulted in a marked diuresis and naturesis and an increase in urine osmolarity and an increase in GFR similar to the findings of chapter 4 and those from previous studies including those in fetal sheep (Lumbers & Stevens 1987, Kelly et al 1993).

Hypoxia resulted in a rapid fall in heart rate; this was accompanied by a rise in blood pressure and carotid blood flow also a decrease in femoral blood flow. These fetal cardiovascular responses to hypoxia have been well documented previously (Boddy *et al.*, 1974; Cohn *et al.*, 1974; Giussani *et al.*, 1993). Hypoxia caused a rise in fetal plasma cortisol and ACTH levels, but no change in fetal plasma Ang II levels or plasma renin activity. The increase in plasma cortisol and ACTH in response to hypoxia has been reported previously (Akagi & Challis, 1990; Giussani *et al.*, 1994; Gardner *et al.*, 2001). Previous studies have found that hypoxia can increase Ang II in fetal life (Broughton *et al.*, 1974; Robillard *et al.*, 1984; Green *et al.*, 1998) although this is not seen in all studies (Wood *et al.*, 1990) and has not always reached significance by 30 minutes (Green *et al.*, 1998). The hypoxic challenge in this study only lasts 30 minutes which may explain why no rise in Ang II has been observed.

Hypoxia caused urine flow to decrease, urine osmolarity and Na^+ excretion to increase. A previous study has found a similar increase in fetal urine osmolarity and Na^+ excretion, although no change in urine flow in response to maternal induced hypoxia (Robillard *et al.*, 1981).

6.6.2 Effect of 60% peri-implantation restriction

In the present chapter it was found that a 60% peri-implantation nutrient restriction had no significant effect on fetal basal cortisol, ACTH, ACE or Ang II concentrations, basal cardiovascular parameters or baroreflex. The fetal response to frusemide in late gestation in terms of cardiovascular parameters, renal function and plasma Ang II concentration were not affected by the maternal nutrition restriction. All these parameters have been thoroughly discussed in chapter 4. Therefore this discussion will concentrate on areas where differences in the responses following the increased intensity were found. The hypoxia challenge will be discussed separately.

Blood gases

In the present chapter a 60% peri-implantation nutrient restriction resulted in no alterations any basal fetal blood gas parameter measured when measured at 125 or 126 dGA. This differs with the findings in chapter 4 of raised pCO_2, lactate, haemoglobin, haematocrit, base excess and bicarbonate following a milder (50%) peri-implantation nutrient restriction. In chapter 4 the fetuses had 6 ± 1 days of recovery from surgery when the changes in blood gases were observed. On the 2nd day of experiments, after 7 ± 1 days of recovery, only the pCO_2 remained raised. In this chapter there were 8 ± 1 days of recovery before the first experimental day. Therefore these contradictory results may be due to difference in the time of recovery given after surgery before starting the experiments.

Response to Angiotensin II

Our findings that a 60% peri-implantation nutrient restriction had no effect on the fetal cardiovascular response to Ang II when compared to the control group, conflicts the findings in chapter 4 of a blunted fetal MAP response to Ang II following a 50% peri-implantation restriction. This also conflicts with the findings of Gardener *et al.* (2004) of blunted MAP

response to Ang II at 1 year of age following a 50% peri-implantation nutrient restriction. Following the findings in chapter 4 and of Gardner *et al.* (2004) it would be expected that this more severe peri-implantation challenge would have similar or greater effects on the fetal cardiovascular response to Ang II. The levels of AT1 receptors determine the biological efficiency of Ang II. Therefore it may be that the mild challenge leads to a down regulation of the AT1 receptor as an adaptive mechanism, but the more severe challenge causes secondary adaptive mechanisms resulting in up regulation of Ang II receptors. Many agonists are known to regulate AT1 receptor expression in vascular cells, for example, angiotensin II, growth factors, low-density lipoprotein cholesterol, insulin, glucose, estrogen, progesterone, reactive oxygen species, cytokines, nitric oxide, and many others (Wassmann & Nickenig, 2006). Therefore changes in the levels of one or more of these factors in response to the nutritional challenge could alter AT1 expression. In fetal life particularly there is also the role of the AT2 population to consider, as Ang II acts at the AT2 receptor which results in vasodilation in addition to reducing the bioavalibility of Ang II. The AT1 to AT2 ratio changes throughout gestation with a decrease in the AT2 and an increase in the AT1 receptor population towards term. It may be that the more severe (60%) maternal restriction results in a delay in the increase of the AT1 population therefore there is no increased responsiveness to Ang II.

6.6.3 Effect of Timing

Blood gases

In the present chapter a 50% maternal nutrient restriction from 104 dGA resulted in no alterations any basal fetal blood gas parameter measured when measured at 125 or 126 dGA. Reduced fetal glucose levels has been observed following a late gestation nutrient restriction (Bauer *et al.*, 1995; Edwards *et al.*, 2001c), but no change in pO_2, pCO_2 or pH has been observed in these studies. The reduced level of fetal glucose conflicts with the findings in the present chapter. Edwards *et al.* (2001) used a similar intensity of challenge (50%) starting later in gestation (115 dGA), whereas Bauer *et al.* (1995) used a more severe challenge (75%) starting at 100 dGA. It is perhaps surprising that we have not observed any difference in fetal glucose levels as the maternal nutrient restriction was still ongoing whilst these experiments took place. A 50% reduction in the maternal intake during this period of rapid fetal growth would be predicted to alter the supply of nutrients available to the fetus and thereby alter the fetal blood glucose levels. Maternal glucose concentration is the main determinant of transplacental concentration gradient in normal conditions, although fetal glucose levels can rise independently of the maternal concentrations in adverse conditions (Hay, Jr., 1991) and this may be why no change in fetal glucose was observed in the present study.

Hypothalamic-pituitary-adrenal axis

In the present chapter it was found that a late gestation maternal nutrient restriction had no significant effect on basal fetal plasma cortisol or ACTH. This agrees with previous studies which reported no change in fetal plasma cortisol or ACTH following a 50% maternal nutrient restriction from 115 dGA (Edwards & McMillen, 2001; Edwards *et al.*, 2001c). Bloomfield *et al.* (2003) have reported a decrease in plasma cortisol in adult offspring following a 10 day period of severe maternal nutrient restriction in late gestation, although this decrease was not seen if the restriction lasted for 20 days. The results in the Bloomfield study seem counter intuitive as the longer period of severe maternal nutrient restriction has no effect on the offspring cortisol levels. It has been suggested that the brief challenge leads to a down regulation of the HPA axis as an adaptive mechanism. The more prolonged challenge may persist beyond some threshold and results in a secondary adaptive

mechanisms resulting in up regulation of the HPA axis, as the fetus prepares itself for delivery in a hostile environment (Bloomfield *et al.*, 2003a). The HPA axis is regulated by a number of factors including circadian inputs, sleep, food intake and physiological stresses including hypoglycaemia, infection and hypotension, also cortisol has negative feedback effects on CRH and ACTH secretion. If there were any transient changes in cortisol or ACTH levels they may not have been picked up in the current study.

Renin angiotensin system

In the present chapter it was found that a late gestation maternal nutrient restriction had no significant effect on basal fetal plasma angiotensinogen, PRA, ACE or Ang II measurements in late gestation. Ang II levels in 3 hour old sheep were raised following intramuscular injection of betamethasone either 0.5 mg/kg directly to the fetus or 0.2 or 0.5 mg/kg to the mother at 126/127 dGA, fetuses were delivered by caesarean section 24 hours after treatment (Ervin *et al.*, 2000). Our group has measured Ang II levels at 1.5 years of age and found no changes following a peri-implantation or postnatal nutrient restriction (Cleal *et al.*, 2007a). Low protein diet reduces serum ACE activity in rat offspring at 13 weeks of age (Langley-Evans & Jackson, 1995). No one has previously investigated the effects of late gestation nutrient restriction on circulating angiotensinogen, Ang II, PRA or ACE levels in fetal sheep.

Cardiovascular parameters

In the present chapter we found no change in fetal basal cardiovascular parameters following a late gestation maternal nutrient restriction. Although this conflicts with a previous sheep study which reported that 50% maternal nutrient restriction from 115 dGA increased blood pressure in late gestation fetuses (Edwards & McMillen, 2001). These conflicting findings may be due to the different timing of the nutrient restriction, the time that these parameters were measured or the breed of sheep. Our challenge started at 104 dGA (prior to surgery), whereas the challenge used by Edwards and McMillen (2001) started at 115 dGA (after surgery 110–113 dGA). We determined the basal blood pressure at 125 and 126 dGA, whereas Edwards and McMillen determined the basal blood pressure both prior to (115–125 dGA) and following (135–145 dGA) this time point. The present study used Welsh mountain ewes whereas Edwards and McMillen (2001) used Border-Leicester cross Merino ewes,

which makes it difficult to compare. The Welsh Mountain breed is well adapted to the often harsh environmental conditions and is smaller than many other breeds, therefore may be more able to withstand periods of maternal restriction.

Late gestation maternal nutrient restriction had no effect on fetal baroreflex sensitivity, maximum response or operating point (the pressure value at which the half-maximal RRI response is seen). This indicates that there is no difference in baroreflex responsiveness or the α_1-adreno-receptor population as a result of the late gestation challenge. Dexamethasone treatment in late gestation increased the operating point of fetal baroreflex (Fletcher *et al.*, 2002), showing that the baroreflex response can be altered by challenges during this period of gestation. To the best of my knowledge the effect of late gestation maternal nutrient restriction on fetal baroreflex has not previously been investigated therefore these finding are novel. This indicates that maternal nutrient restriction in late gestation had no effect of fetal baroreflex.

Late gestation maternal nutrient restriction resulted in no change in the fetal cardiovascular response to Ang II when compared to the control group. A 50% late gestation maternal nutrient restriction from 115 dGA increased responsiveness to Ang II in late gestation fetuses (Edwards & McMillen, 2001). This study also found increased basal blood pressure; these conflicting findings may be due to the different timing of the nutrient restriction, the time that these parameters were measured or the breed of sheep and have been discussed in more detail above. 50% late gestation maternal nutrient restriction had no effect on fetal cardiovascular or Ang II response to frusemide in late gestation. No one has previously investigated the effects of a late gestation maternal nutrient restriction on fetal cardiovascular response to frusemide. As we observed no changes it may be that there are no effects on fetal renal RAS cardiovascular control.

6.6.4 Response to hypoxia

Neither a 60% peri-implantation nor a 50% late gestation maternal nutrient restriction affected fetal blood pressure, heart rate, blood flow or femoral artery vascular resistance response to hypoxia. A 15% maternal nutrient restriction for the first half of gestation

increased femoral vascular resistance during hypoxia and recovery, but there were no changes in MAP, heart rate or femoral blood flow responses to hypoxia when studied in late gestation (Hawkins *et al.*, 2000b; Hawkins *et al.*, 2000c). Prevailing hypoglycaemia (<0.6 mmol/l) increased fetal femoral vascular resistance response to hypoxia (Gardner *et al.*, 2002). The finding of increased femoral vascular resistance in response to hypoxia in both these studies conflicts with the finding of this study, however, this may be due to both these studies using a 60 minute period of hypoxia, whereas only 30 minutes of hypoxia was used in the present study. The chemoreflex control of the fetal circulation in terms of heart rate control was not altered by maternal nutrient restriction.

Maternal nutrient restriction during the peri-implantation period or late gestation had no effect on the Ang II or plasma renin activity response to hypoxia. To the best of my knowledge this has not been investigated previously. In the present chapter we found that neither peri-implantation nor late gestation maternal nutrient restriction affected fetal plasma cortisol or ACTH response to hypoxia. Hawkins *et al.* (2000a) found that a 15% maternal nutrient restriction for the first half of gestation decreased the fetal plasma cortisol and ACTH response to hypoxia at 126–129 dGA, although this decreased response was not seen earlier in gestation (114–115 or 120–123 dGA). Prevailing adverse conditions in late gestation (hypoxaemia, academia and hypoglycaemia) increased the plasma cortisol response during and following an acute hypoxic challenge at 129–131 dGA (Gardner *et al.*, 2002). These findings conflicts with the present study, but as mentioned previously both Hawkins *et al.* (2000) and Gardner *et al.* (2002) used a 60 minute period of hypoxia, whereas only 30 minutes of hypoxia was used in the present study. The fetal HPA axis matures in late gestation therefore the difference in findings may be due to the relative maturity of the HPA axis at the time of the hypoxia challenge.

Regulation of fetal blood pressure is controlled by multiple mechanisms and previous studies have discovered that there is a level of functional redundancy (Green *et al.*, 1998; Edwards *et al.*, 1999). When the carotid chemoreflex mechanisms are removed in carotid sinus denervated (CSD) fetuses the rapid initial fall in fetal heart rate and rise in femoral vascular resistance are attenuated, but Ang II levels rise to a similar level as intact fetuses (Green *et*

al., 1998). ACE inhibition (by captopril) prevents the rise Ang II in response to hypoxia in both CSD and intact fetuses but the rise in MAP and femoral vascular resistance, and the fall in femoral blood flow are only blocked in CSD fetuses (Green *et al.*, 1998). Therefore the role of Ang II in regulation of MAP and periphery blood flow during hypoxia is only revealed in the absence of the carotid reflex mechanism. Captopril also reduces blood pressure in placentally growth restricted but not control fetuses (Edwards *et al.*, 1999). These studies indicate there is a layering of mechanisms and suggest that in adverse conditions the role of the RAS may become more important in the regulation of fetal blood pressure. Thus, any alterations in the control of fetal blood pressure may only become apparent with blockade of the RAS or in the absence of other control mechanisms such as carotid chemoreflex.

6.7 Conclusion

In the present chapter no change was seen in late gestation fetal cardiovascular or renal function following either a 60% peri-implantation or a 50% late gestation nutrient restriction in basal conditions. The fetal cardiovascular response to boluses of phenylephrine, Ang II, frusemide and hypoxia were not different between the groups. Overall this suggests that there are no alterations in late gestation baroreflex, chemoreflex or RAS mediated control of the cardiovascular system following either of the nutritional challenges. However, there might be changes in fetal physiology that are only revealed in postnatal life when adult circulation has been established and mechanisms of cardiovascular control are working in the postnatal environment. Thus far we have seen no change in fetal kidney size or renal function in fetal life. However, there may be altered regional growth or there may be changes in the renal RAS, both of which may impact on the development of renal structure and function and alter cardiovascular control in postnatal life. Therefore it is important to investigate the effect of more severe (60%) peri–implantation and 50% late gestation maternal nutrient restrictions on fetal nephron number.

7 Fetal glomerular number following either a peri-implantation or late gestation maternal nutrient restriction

7.1 Introduction

In chapters 5 and 6 data showed that neither a 60% peri-implantation nor a 50% late gestation maternal nutrient restriction had any effect on fetal growth, organ weight, kidney dimensions, cardiovascular or renal function when studied in late gestation. In chapter 4 data showed altered blood pressure responses to Ang II after a more mild (50%) peri-implantation nutrient restriction. In addition the 50% peri-implantation challenge has previously been reported to alter RAS mediated cardiovascular control in adult sheep offspring (Cleal *et al.*, 2007a). The mechanisms behind these alterations have yet to be revealed but it has been proposed that it may be the result of impaired kidney development and reduced nephron endowment and if so the functional implications might not be expected to become apparent until postnatal life.

The kidney plays a key role in blood pressure regulation, and is known to be involved in the development of hypertension. Cross transplantation studies have found that hypertension may travel with the kidney, inducing high blood pressure in the recipient (Rettig *et al.*, 1989). Neonatal uninephrectomy caused hypertension in adult rats (Woods, 1999). It has been proposed that a reduced number of nephrons will make a person more susceptible to renal disease and hypertension. A recent study has used three-dimensional non-biased stereological analysis to study the kidney of 10 healthy and 10 hypertensive people who died in accidents (Keller *et al.*, 2003). Compared to healthy individuals the kidneys of hypertensive people had significantly fewer glomeruli but of a greater volume. They proposed that the increased glomerular volume was due to overwork or hyperfiltration. There was no sign of obsolescent glomeruli, which would suggest ongoing nephron loss, therefore there may have been fewer nephrons originally.

Therefore the raised blood pressure associated with *in utero* nutrient restriction could be due to a reduction in nephron number and there is evidence that supports this hypothesis. Reduced glomerular number and increased glomerular size in adults and children have been

associated with low birth weight (Hughson *et al.*, 2003). The Southampton Women's Survey found that thinner mothers with lower triceps skin fold thickness tend to have fetuses with sausage-shaped kidneys (Mukherjee *et al.*, 2005). Previously this has been associated with hypertension (Raman *et al.*, 1998) and may be indicative of impaired renal development and possibly altered nephron number (Brenner *et al.*, 1988).

Animal studies have been used to investigate the susceptibility of nephrogenesis to challenges and have revealed that the effect on nephrogenesis is dependent on timing and severity. Unbiased counting technique has been used to show a reduced nephron number in seven year old hypertensive offspring of ewes that received glucocorticoid administration at 27 dGA (Wintour *et al.*, 2003c). This suggests that the peri-implantation period, at the beginning of nephrogenesis, is a critical period for kidney development. Mitchell *et al.* (2004) found reduced nephron number in twin sheep fetuses at 140 dGA. Twinning is a natural challenge that encompasses the whole of gestation, and on top of reduced nutrient availability there is the consideration of constraint of fetal growth, which would become more severe as gestation progresses. Nutrient restriction in mid-gestation resulted in reduced nephron number in offspring in the first year of postnatal life, as assessed by the acid maceration method (Gilbert *et al.*, 2005; Gopalakrishnan *et al.*, 2005). This may indicate that nephrogenesis may also be affected by challenges later in gestation.

Renal glomeruli, as a measure of nephron number, have been counted for over a hundred years by three main techniques: serial sections, independent sections and acid maceration (reviewed by (Bendtsen & Nyengaard, 1989)). All methods that deduce numbers from observations on independent sections are inefficient because the estimates are indirect, the stereological transformations are statistically unstable, and are biased by model assumptions about size, shape and distribution (Nyengaard & Benediktsson, 1990). There is a problem with trying to quantify a three dimensional tissue in a two dimensional histological plane. The likelihood of a three dimensional object appearing in a planar or histological section is not equal to the frequency of a particle in three dimensional space. This is classified as the size and shape and reference space problems. Processing of tissue leads to dimensional changes, particularly with paraffin embedding (~50% shrinkage) whereas if glycol

methacrylate is used the shrinkage is less (~5%) (Miller & Meyer, 1990). Isolation and counting of glomeruli by maceration in hydrochloric acid (Larsson *et al.*, 1980) is biased by over maceration and the cutting up of glomeruli. This has led to the development of a non-biased stereological counting technique called the disector method (Bertram, 1995).

This involves counting the glomeruli in a known fraction of the kidney (Gundersen *et al.*, 1988) and applying the disector counting technique (Sterio, 1984). The disector is a three-dimensional probe that samples particles uniformly, using two sections a known distance apart, and compares the number of particles in the two sections. Particles within an unbiased counting frame in the first section (the sample section) that are not present in the second section (the look up section) are counted. Therefore you are only counting the samples that disappear. All particles have an equal chance of being counted no matter if they are small or large, long or round, and can only be counted once.

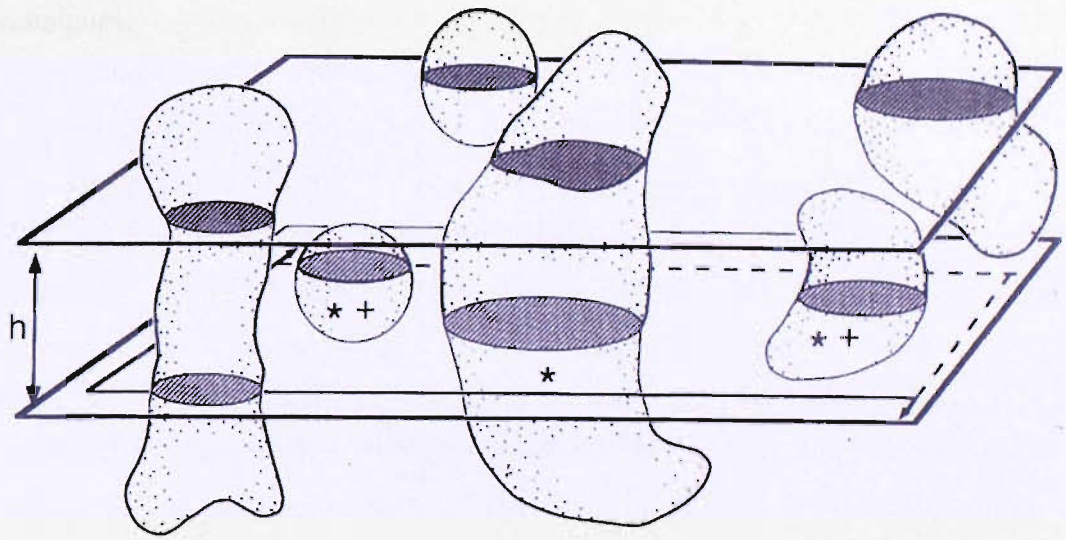

Figure 7.1 The disector technique (Bertram, 1995). The bottom plane is the sample section with inclusion (dashed) and exclusion (solid) lines. The top plane is the look up section, with *h* distance between them. Particles indicated by (*) are sampled by the counting frame, but only two of these are counted (+) as they are not present in the look up section.

No one has yet used these unbiased stereological techniques to determine the effects *in utero* nutrient restriction on nephron number in late gestation when the full complement of nephrons is already formed.

Nephron numbers were correlated to a number of factors that would be predicted to affect renal growth (maternal weight change during the peri-implantation period, renal blood flow and Ang II levels). Blood pressure, urine output and GFR may all be affected by nephron number and were also correlated to nephron number.

7.2 Hypothesis

In utero nutrition restriction will result in a reduction of glomerular number as measured in late gestation.

7.3 Aims

The aim of the current study was to determine if fetal kidney structure is altered by the 60% peri-implantation or late gestation maternal nutrition restriction using unbiased stereological techniques.

7.4 Methods

7.4.1 Experimental model

Ewes received either a 60% reduction of their total nutrient requirements from 1 - 31 days (E, n = 20), a 50% reduction of their total nutrient requirements for 23 days in late gestation starting at 104 dGA (L, n = 12) or 100% of nutrient requirements throughout (C, n = 25). At all other times animals in all groups were fed 100% nutrient requirements.

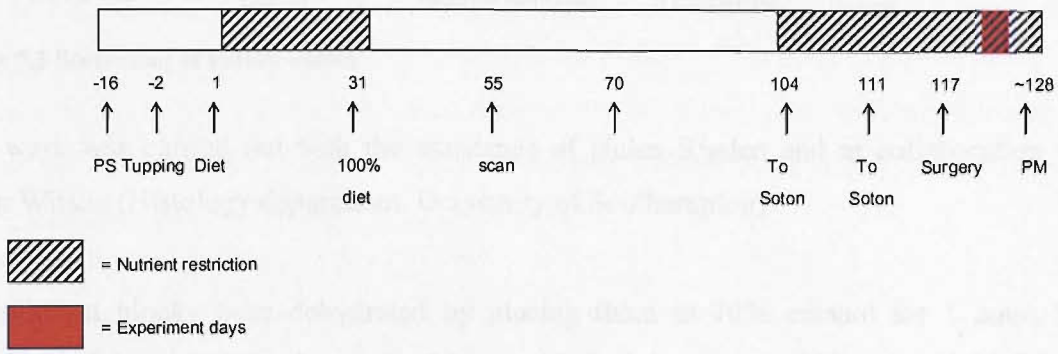

Figure 7.2 Experimental models.

7.4.2 Post mortem

At ~128 dGA the ewes and fetuses were killed with an overdose of sodium pentobarbitone to the ewe i.v. The left fetal renal artery was catheterised (Portex translucent PVC tubing, I.D 0.58 mm, O.D 0.96 mm, Portex Ltd, UK,) and the kidney was removed. Heparin (1 ml, 5000IU/ml, Leo Laboratories Ltd, UK) then papaverine hydrochloride (1ml, 0.24 mg) was injected into the renal artery. The kidney was then perfused with 50 ml of heparinised saline and then perfusion-fixed with 50 ml of formalin.

7.4.3 Fixing and embedding

The perfused kidney was cut in half longitudinally and immersion-fixed for 3 days in formalin, then transferred to 70% alcohol. Both halves were weighed and measured then cut into 5mm slices. Every other slice was selected with the starting slice selected randomly from the first two. The selected slices had the medulla removed leaving a small rim next to the cortex to prevent damage. The cortex was then cut into 5x5x2mm blocks (figure 7.3) and

these were arranged in the order they were cut. Every 10th block was selected with the starting block selected at random from the first 10.

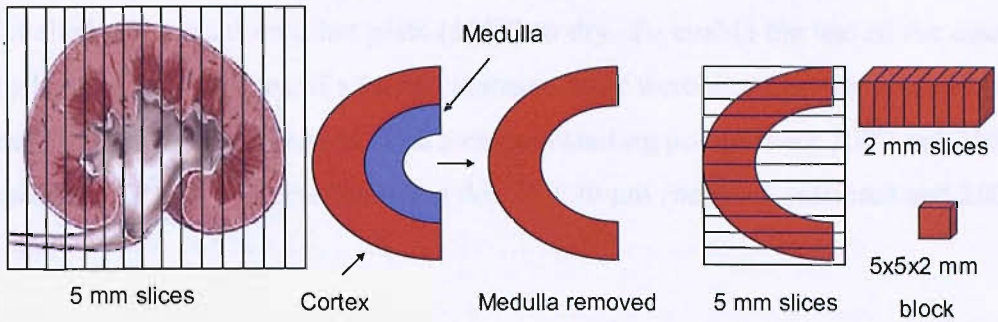

Figure 7.3 Sectioning of kidney cortex

This work was carried out with the assistance of Helen Rigden and in collaboration with Susan Wilson (Histology department, University of Southampton).

The selected blocks were dehydrated by placing them in 70% ethanol for 1 hour, 90% ethanol for 1 hour, 100% ethanol for 1 hour with 2 changes and 100% ethanol for 2 hours with 2 changes (all at 37°C). They were then placed in the embedding mould with the 5 x 5 mm plane facing upward to allow sectioning through the 2 mm plane and embedded in glycol methacrylate (GMA) (JB-4 embedding kit, Polysciences, Germany). This involved placing the blocks in three, 3 ml changes of infiltration solution (50ml JB-4 solution A; 0.625 g Benzoyl peroxide, plasticized), at 4°C. The first two infiltrations were for 3 hours each and the third infiltration was overnight. Next 2ml of JB-4 solution B was added to 50 ml of fresh infiltration solution and 1 ml of this was placed onto each block in a labelled mould. The samples were stored at 4°C for 24 hours to allow polymerization, and then they were stored at -20°C.

7.4.4 Cutting and staining

This work was carried out with the technical assistance of Helen Rigden, advice on methodology from Jane Cleal and in collaboration with Susan Wilson (Histology department, University of Southampton).

Half of the blocks embedded in GMA were removed from their moulds, trimmed and then sectioned extensively at 2 μm with a rotary microtome (R.Jung, Heidelberg). Once cut sections were collected and floated in a water bath, then picked up on uncoated slides, labelled and placed on a hot plate (50°C) to dry. To enable the use of the disector counting technique, two sections of a known distance apart were mounted per slide. As the block was cut each section was counted, with a random starting point. Every 200^{th} and 210^{th} section was mounted, giving a disector counting depth of 20 μm (between sections) and 200 μm between pairs.

The sections were stained with 1% Toluidine Blue in Borax (BHD, Poole, UK) for 30 seconds on a hot plate (50 °C) and then rinsed with hot running water. The sections were then dried firstly with blotting paper, then placed on a hot plate and finally mounted with DPX mountant (BDH, Poole, UK) and a coverslip.

7.4.5 Counting

The number of glomeruli (as a representative of nephron number) in the cortex of the kidney was counted by means of the physical disector technique, using a light microscope (Leica DMBL, Milton Keynes, UK; magnification X 10). Glomeruli were counted in four sampling fields per pair of sections and a counting frame was applied so that glomeruli within the frame and touching inclusion lines were counted and those outside the frame or touching the exclusion lines were not counted. The image viewed in each field was drawn onto an acetate sheet by the use of an arm attached to the microscope. The counting frame, landmarks and glomeruli for the first section were drawn onto the acetate. The landmarks drawn on the acetate were lined up with those of the second section so that it matched the first section. In a different colour pen the glomeruli present in the second section were drawn on the acetate. A glomerular was counted only if it was present in the first section but not the second ($\mathbf{Q^-}$).

7.4.6 Estimating total glomerular number

The total glomerular number (**N**) was estimated by the physical disector-fractionator method (Nyengaard & Benediktsson, 1990). The following equation was used:

$\mathbf{N = 2 \times 10 \times 2 \times 200 \times P_S/2P_F \times Q-}$

Where **2** was the inverse of the first sampling fraction (1/2 of the slices see 7.4.3), **10** was the inverse of the second sampling fraction (1/10 of the blocks see 7.4.3), **200** was the inverse of the section sampling fraction (1/200 of the sections see 7.4.3), $\mathbf{P_S}$ was the total area of the kidney sections, $\mathbf{P_F}$ was the fraction of the section area using for counting glomeruli (2 refers to the fact that counting was performed in both directions to double the counting efficiency), and **Q-** was the actual number of glomeruli counted. Approximately 150 - 400 glomeruli were counted for each kidney. One kidney was counted three times by 1 individual to obtain a coefficient of variation for intra-observer error.

7.5 Renal blood flow

Dr D Burrage measured renal blood flow to right kidney of this cohort of fetuses using fluorescent microspheres as part of another study (Burrage, 2006). The resulting data was used in this chapter.

7.6 Data analysis

For a description of the analysis strategy, the power calculation and the statistics packages used, see section 2.6.

Nephron numbers were compared between the groups by 1 way ANOVA and Bonferroni *post-hoc* test.

Nephron number was correlated with other parameters (e.g. maternal weight change during challenge, basal Ang II, renal blood flow, blood pressure, urine output and GFR) by linear regression.

7.7 Results

There was no significant difference in kidney glomeruli number between the groups (figure 7.4).

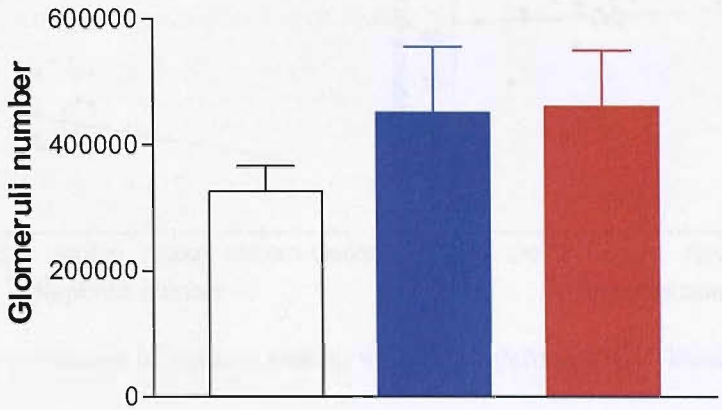

Figure 7.4 Kidney glomeruli number, mean ± SEM. C (□, *n* = 8), E (■, *n* = 9) and L (■, *n* = 5).

There was no correlation between nephron number and maternal weight change during challenge (-2–29 dGA) or right renal blood flow (figure 7.5).

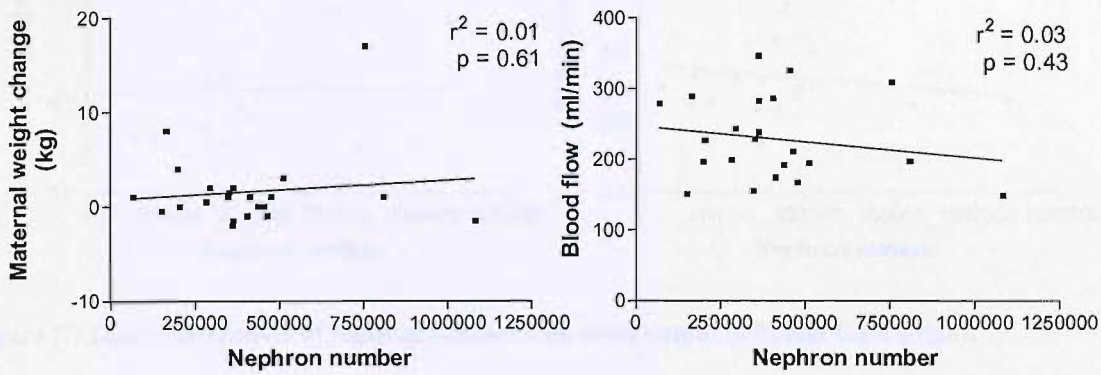

Figure 7.5 Linear correlation of nephron number with maternal weight change (left) and renal blood flow (right).

There was no correlation between nephron number and plasma Ang II levels or basal blood pressure (figure 7.6).

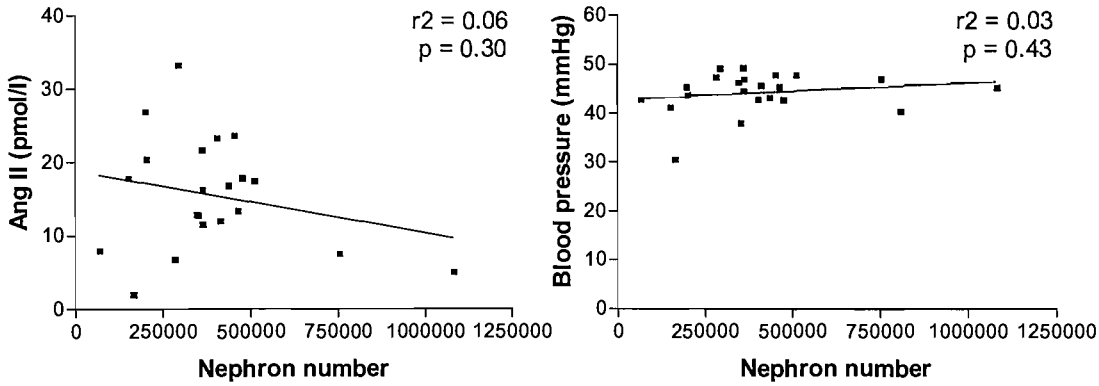

Figure 7.6 Linear correlation of nephron number with Ang II (left) and basal blood flow (right).

There was no correlation between nephron number and basal urine output or GFR (figure 7.7).

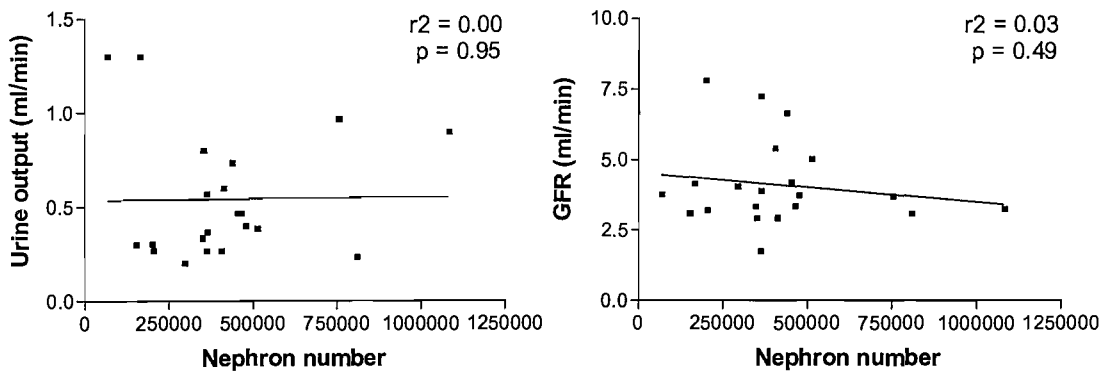

Figure 7.7 Linear correlation of nephron number with urine output (left) and GFR (right).

7.8 Discussion

In this chapter it was found that neither maternal nutrient restriction during peri-implantation nor late gestation had an effect on kidney glomeruli number in late gestation. There is some support for these findings, as a previous study in our group found a 50% peri-implantation nutrient restriction had no significant effect on fetal sheep nephron number at 70 dGA using non biased stereological technique on paraffin embedded samples (Cleal, 2005). Brennan *et al.* (2005) found that 50% under nutrition from 0–30, 31–65, 66–110 or 0–110 dGA had no significant effect on fetal sheep nephron number at 110 dGA using the acid maceration technique. Also umbilical placental embolization in late gestation sheep had no effect on nephron number at 140 dGA, using unbiased stereology (Mitchell *et al.*, 2004). Other studies have measured nephron numbers in sheep and have found changes following a variety of challenges. Gopalakrishnan *et al.* (2005) found that that both a 50% nutrient restriction from 28–80 dGA or a low maternal BCS reduced nephron number in 6 month old offspring. Gilbert *et al.* (2005) has found reduced nephron number following a 50% maternal nutrient restriction from 28–78 dGA at 245 days postnatally, but no significant difference in nephron number was found at 78 or 135 dGA (Gilbert *et al.*, 2007). These studies all used the acid maceration method (discussed in section 7.1) and are therefore biased by over maceration and the cutting up of glomeruli.

To the best of my knowledge the only studies that have found changes in nephron number using unbiased stereological techniques are those of Wintour *et al.* (2003) and Mitchell *et al.* (2004). Wintour et al. (2003) found that 48 hours of dexamethasone from 26–28 dGA reduced nephron number in seven year old sheep. This dexamethasone challenge is a different type of challenge than the maternal undernutrition used in the current study but does target the end of the peri-implantation period of gestation. Mitchell *et al.* (2004) have found reduced nephron number in twin sheep fetuses at 140 dGA. Twinning is a natural challenge that encompasses the whole of gestation, and on top of reduced nutrient availability there is the consideration of constraint of fetal growth. Therefore the inconsistency of these results may be due to the differences in the duration and severity of the challenges.

Ewe weight change during the peri-implantation nutrient restriction was not correlated with fetal nephron number. Maternal nutrient restriction would be predicted to affect individual ewes differentially due to factors such as the initial weight and BCS. There were no significant differences between initial ewe weight and BCS between the groups (Chapter 5). Ewe weight change during the peri-implantation period can be used as a measure of the effect of the nutrient restriction. There was no correlation between nephron number and ewe weight change, this supports the finding no change in nephron number following peri-implantation nutrient restriction. These findings indicate that maternal nutrition during this peri-implantation period has no effect on nephron number in late gestation.

Renal blood flow in late gestation was not correlated with fetal nephron number at 127 dGA. Renal blood flow in late gestation fetuses in this cohort fetuses was not altered by either peri-implantation or late gestation maternal nutrient restriction (Burrage, 2006). Adverse conditions such as hypoxia can lead to a redistribution of fetal circulation in favour of the vital organs (such as the heart and brain), this can result in altered regional growth. The findings of this study indicate that renal blood flow has no effect on fetal nephron number. However, due to the requirements of the techniques used to investigate these parameters the renal blood flow of the right kidney and kidney number in the left kidney were measured, therefore we would not necessarily expect to find a correlation.

Plasma Ang II levels in late gestation were not correlated with fetal nephron number. Inhibition of RAS using ACE inhibition, AT1 antagonism and targeted disruption of genes (Aogen and ACE) disrupts renal development, revealing that an intact RAS is a requirement for normal renal development (Guron & Friberg, 2000a). In the present study we found that neither peri-implantation nor late gestation maternal nutrient restriction had an effect on fetal plasma Ang II levels (Chapter 6). Plasma Ang II levels in late gestation have no relationship with fetal nephron number.

Neither basal blood pressure nor renal function (in terms of urine output and GFR) were correlated with nephron number in late gestation fetal sheep. Data have suggested that the complement of nephrons at birth may influence the development of hypertension in

adulthood, but this has not been reported in early life (Brenner *et al.*, 1988; Keller *et al.*, 2003). Therefore we would not necessarily expect a correlation between fetal blood pressure and nephron number. In newborn piglets nephron number and GFR have been found to be positive correlated (Bauer *et al.*, 2002). No one has previously looked at whether there is a correlation with fetal renal function and nephron number; the finding of this study suggests that in sheep no such correlation exists.

7.9 Conclusion

In this chapter no changes were seen in late gestation nephron number following either a 60% peri-implantation or a 50% late gestation nutrient restriction. This indicates that this level of nutrient restriction *in utero* has no effect on nephron development. The relationships between low birth weight and renal disease could be mediated in part through their mutual link to other conditions (such as hypertension, the metabolic syndrome and CVD), independent of nephron number (Hoy *et al.*, 2005). It remains possible that there might be changes in fetal renal physiology (such as nephron volume, structure and filtering capabilities) that leave them more susceptible to damage in later life.

8 General Discussion

8.1 Summary of results

Overall it was found that neither a 50% nor 60% peri-implantation nor a 50% late gestation maternal nutrient restriction had any effect on any of the maternal parameters measured: weight, BCS, fat, muscle depth or on fetal body or organ weight. Therefore it appears that the ewes were able to maintain appropriate for gestational age weight gain and maintain fetal growth in the face of these challenges. But the late gestation maternal nutrient restriction reduced the chance of fetal survival of surgery when ewes were artificially ventilated. The increased mortality may have skewed this group, as the fetuses most affected by the nutritional challenge may well have also been those least likely to survive surgery. This would result in the least affected fetuses surviving to post-mortem and being included in our analysis may therefore not be a true reflection of the effect of the diet. The lack of effect on maternal cortisol and ACTH indicates that the ewes were not stressed by the nutritional manipulations.

It was found that there were no significant differences in basal fetal renal function, blood gases, hormones or cardiovascular parameters following either a 50 or 60% peri-implantation or a 50% late gestation nutrient restriction. Therefore it appears that the fetus appears to be able to maintain cardiovascular control and renal function following this level of maternal nutrient restriction in respect of basal cardiovascular and renal function. The fetal baroreflex response to a bolus of phenylephrine was not significantly different between the groups. None of the challenges had an effect on cardiovascular, RAS or renal response to frusemide. However, the MAP response to Ang II was blunted in only the 50% peri-implantation nutrient restricted group and may indicate altered expression of Ang II receptors in the peripheral vasculature. The hypoxia challenge revealed that the chemoreflex control of the fetal circulation in terms of heart rate control was not altered by maternal nutrient restriction in peri-implantation or late gestation.

Neither a 60% peri-implantation nor a 50% late gestation maternal nutrient restriction had an

effect on fetal kidney glomeruli number in late gestation. Impaired nephrons endowment has previously been suggested at the mechanism linking hypertension with poor *in utero* nutrition (Brenner *et al.*, 1988). Therefore the finding of no change in nephron number implies that there may be some other mechanism responsible for the association.

8.2 The sheep model

The model animal used for this study was the sheep. This animal is docile and of appropriate size for the surgery and instrumentation carried out, and several of its organs including the kidney are thought to develop at a similar rate to the human (section 1.5), making it a good model for human development.

The size and resilience of the fetal sheep model did allow the instrumentation surgeries to be successful in the majority of cases, with the exception of the late gestation nutrient restricted group in chapter 5 and 6. The increased incidence of death in these fetuses revealed that the surgery was a major challenge to this group of fetuses. The weight loss in ewes in all groups following surgery revealed that the surgery is also a major challenge to the ewe. These surgeries therefore may well impact on the results of this experiment. Ideally, experiments should not introduce any confounding superimposed challenges. Less invasive ways of observing the fetus have been developed, such as Doppler ultrasound which allows observation of fetal cardiovascular parameters (Kiserud *et al.*, 2001). However, the ewe would have to be anaesthetised during the procedure which would have compounding effects on the cardiovascular system (Biehl *et al.*, 1983; Sabik *et al.*, 1993). Fetal ECG can be monitored non-invasively (Carter *et al.*, 1980; Camps-Valls *et al.*, 2004), although this does not enable monitoring of other fetal parameters such as blood pressure or flow. Instrumentation is currently the only method that enables the monitoring of cardiovascular, endocrine and metabolic status of the fetus.

Maternal dietary restriction was the nutritional challenge used in this thesis, and the level of challenge to the fetus, in terms of nutrient availability and hormones, depends on the maternal and placental responses (post-implantation) (Hay, Jr. *et al.*, 1984). Maternal nutrient

restriction had no effect on maternal plasma cortisol levels at any point measured in this thesis -2, 29 or 70 dGA (chapter 3 and 5). However there may be transient increases in cortisol, in response to nutrient restriction, as seen in previous studies (Edwards & McMillen, 2001). Moreover, even in the absence of changes in maternal plasma cortisol concentrations the fetus could still be exposed to raised glucocorticoids via altered 11βHSD2 activity, activation of the fetal HPA axis or alterations in GR, all of which have been reported previously in offspring following maternal nutrient restriction (Whorwood *et al.*, 2001; Bertram *et al.*, 2001; Bloomfield *et al.*, 2004; Jaquiery *et al.*, 2006). With the early gestation challenge which spans the peri-implantation period, the fetus may have been affected by maternal hormones and other environmental factors in the uterine milieu (McKiernan *et al.*, 1995). Therefore it is beyond the scope of this thesis to identify the precise signal to the fetus during maternal under nutrition, and it is likely that it is a combination of a number of factors.

Two factors were taken into consideration when calculating the timing of the maternal nutrition restriction. Firstly, by limiting the challenge to a particular stage of development the cause of any response can be identified more easily. Secondly, to build on previous observations and to enable comparison it was advantageous to use periods that had been used in our previous studies. The same 50% peri-implantation challenge was used in a series of studies on postnatal sheep at 1.5 and 2.5 years (Poore *et al.*, 2007; Cleal *et al.*, 2007a; Cleal *et al.*, 2007b). The severity of the challenges was designed to produce effects on fetal cardiovascular and renal development without causing a pathological state, which would have confounded the results. There was no change in fetal body or placental weight which indicates that the challenges did not produce obvious pathology and maternal cortisol was not elevated indicating that the ewes were not stressed by the dietary restriction. The effects of the peri-implantation restriction on later fetal cardiovascular responses Ang II and the decreased tolerance to surgery of the late gestation restricted fetuses suggest that alterations in the fetal cardiovascular system were achieved.

Statistical power is an issue in any study using animals due to the need to keep numbers to a minimum in line with the reduction recommended by the '3 Rs' (see Flecknell, 2002 for a

review). The power calculation indicated that 9 animals per group (see 2.6.1) were required. This was achieved for all groups except the late gestation nutrient restricted group (see 5.6.2 for discussion). Therefore the assessment of the differences between the late gestation and control group were slightly underpowered which may have possibly led to type 1 (false positive) or type 2 (false negative) errors. The practical value of power calculations is difficult to judge as they require knowledge of the 'difference of interest', which can only be estimated from previous studies in this type of experiment and there is a paucity of such data.

8.3 Does nutrient restriction alter fetal renal or cardiovascular development?

The main hypothesis of this thesis was that the fetus responds to poor *in utero* nutrition with a redistribution of resources that could affect fetal renal and cardiovascular development, and this will result in altered renal function and cardiovascular control in late gestation.

8.3.1 Does nutrient restriction alter the distribution of resources?

The findings of chapter 3 and 5 that peri-implantation nutrient restriction does not result in gross organ growth changes, imply that there is no alteration of distribution of resources. The peri-implantation challenge commences at day 1 of gestation and continues until day 31; this is when the size of the embryo is small and therefore demands on the ewe are relatively low. In the sheep implantation occurs at around 14–16 dGA (Imakawa *et al.*, 2004; Spencer *et al.*, 2004). If environmental factors at this early stage were to affect renal and cardiovascular development, it could involve alterations in the expression of genes that would be involved in renal and cardiovascular development.

The findings in chapter 5 that late gestation nutrient restriction does not result in any gross organ growth changes this provide no evidence of any redistribution of resources. The late gestation challenge starts at 104 dGA and continues until post-mortem at 127 dGA; this is a period of rapid fetal growth, where the demands on the ewe are much greater. The increased mortality at surgery suggests that these fetuses were compromised and the group may be biased as the fetuses most likely to survive surgery were probably least affected by the

nutrient restriction. As discussed previously this reveals the difficulty of interpreting data when the surgery an addition challenge is imposed at the same time as the nutrient challenge.

8.3.2 Does nutrient restriction alter RAS or cardiovascular control?

The data in chapters 4 and 6 refute the hypothesis that maternal undernutrition alters fetal cardiovascular control in late gestation, as there was no change in basal blood pressure or baroreflex. There does appear to be altered fetal RAS, as the blood pressure response to Ang II was blunted following a 50% peri-implantation nutrient restriction (chapter 4), but change in circulating RAS was observed. However, this blunted response was not seen following a more severe peri-implantation restriction (chapter 6) and may suggest that the change seen in chapter 4 was due to a type 1 (false positive) error. However, it may be that a mild challenge leads to a down regulation of the Ang II receptor as an adaptive mechanism, but the more severe challenge causes secondary adaptive mechanisms resulting in up regulation. There was no difference in the magnitude of cardiovascular response to hypoxia between the groups. Cleal *et al.* (2007) have found increased blood pressure response to frusemide and left ventricular hypertrophy in postnatally, following a 50% peri-implantation challenge. Therefore there may be changes in fetal physiology that have not been detected here, that cause changes in function in later life, when adult circulation has been established and mechanisms of cardiovascular control are working in the postnatal environment.

8.3.3 Does nutrient restriction alter renal development?

The data in chapter 7 revealed no change in late gestation nephron number following either a 60% peri-implantation or a 50% late gestation nutrient restriction. This seems to indicate that nutrient restriction *in utero* had no effect on nephron development. The peri-implantation challenge from 1–31 dGA is at the very beginning of kidney development (section 1.5) and any changes in renal development occurring during this period would be expected to have long lasting consequences. Wintour *et al.* (2003) found that exogenous glucocorticoid treatment at 28 dGA decreased nephron number in adult life, suggesting that kidneys are susceptible to challenges in this period. The late gestation challenge from 104–127 dGA spans the end of the period of rapid nephrogenesis (which is complete at around 120 dGA in

sheep). This suggests that maternal nutrient restriction in peri-implantation or late gestation do not affect fetal renal development in this model. It is still possible that there are changes in fetal renal physiology, such as nephron volume, that may leave them more susceptible to damage in later life.

8.4 Future work

There are several areas which are beyond the scope of this thesis but would be interesting to investigate in the light of its results. The basis of the change in blood pressure responsiveness to Ang II observed in chapter 4 requires further investigation both, *in vivo* and *in vitro*. Assessing the response to Ang II *in vivo* following a 50% peri-implantation restriction in a larger cohort of animals would rule out any possibility of a type 1 error. The vasoreactivity of small resistance arteries to Ang II could be assessed using the *in vitro* techniques of wire myography to measure changes in tension exerted by isolated vessels (Brawley *et al.*, 2003e). Analysis of the expression and distribution of the Ang II receptors AT1 and AT2 in tissues and/or vessels would reveal if altered angiotensin receptor population is responsible for altered responsiveness to Ang II.

It would be interesting to investigate the fetal renal physiology in more detail for example nephron volume, structure and filtering capabilities, to see if there are changes that may make them more susceptible to damage in later life.

8.5 Final conclusion

There are several possible interpretations of these results. There may be no major changes in fetal physiology as the dietary challenges were too moderate and optimum fetal growth and development could be maintained. In the light of previous work using this intensity of challenge, this seems to dispute the role of the decreased nephron endowment in linking early nutrient environment to later disease. There may be underlying changes in fetal endocrine systems e.g. the HPA axis or peripheral vasculature that are capable of maintaining normal cardiovascular and renal control in fetal life, and these changes only become apparent in adult life when there is an accumulation of damage as has been suggested for kidney function. The drugs we have used in this study to test fetal renal and cardiovascular control may not reveal any alterations, as there are many different integrated systems involved (figure 8.1) and only a few have been tested in this study.

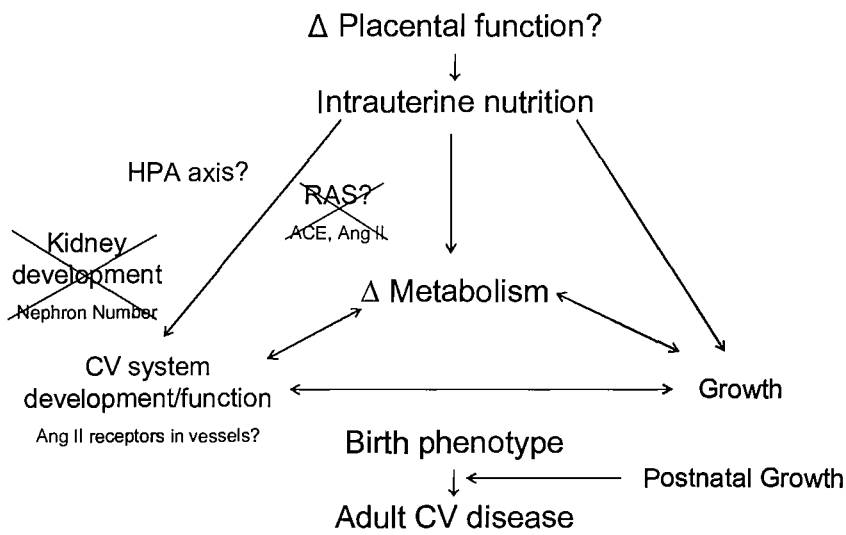

Figure 8.1 Summary of the main finding of this thesis.

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Appendix 1 Body condition scoring

Farmers use the sheep body condition score system to assess the condition of their animals. The animal is awarded a score between 0 – 5, with 0 being extremely emaciated and 5 being very fat. Intermediate half scores are often used when an animal's condition is not clear, and in this thesis quarter scores have been used, as placing an exact score is not as important as being able to assign a relative score.

Body condition score estimates the condition of muscling and fat development. The scoring is based on feeling vertebrae in the loin region and assessing the level of muscling and fat deposition over and around it (Figures 1-3). Loin vertebrae have a vertical bone protrusion (spinous process) and a short horizontal protrusion on each side (transverse process) in addition to the central spinal column. These protrusions are felt and used to assess an individual body condition score.

In this thesis the scores range from 2 to 4, a BCS of 2 (thin) is defined as sharp and prominent spinous processes, little fat cover, full muscle cover and rounded transverse processes. A BCS of 3 (average) has smooth and rounded spinous processes, smooth and well covered transverse processes, medium fat and full muscle cover. A BCS of 4 (fat) has a spine which can only be detected as a hard line, transverse processes which cannot be felt and a thick fat cover. Full details of BCS criteria can be found in Russell, 1991 and Khan *et al.*, 1992.

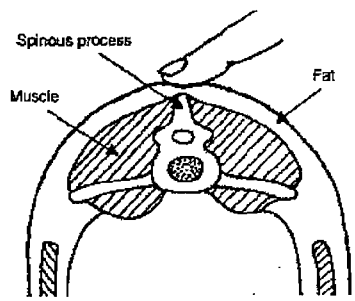

Figure A. 1.Feel for the spine in the center of the sheep's back, behind its last rib and in front of its hip bone.

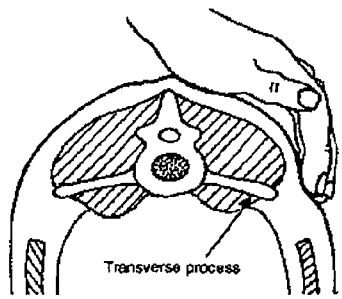

Figure A. 2. Feel for the tips of the transverse processes.

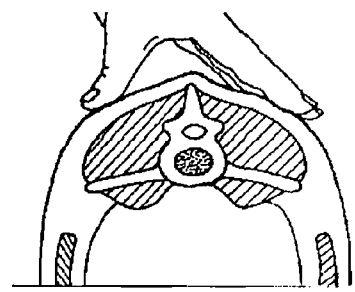

Figure A. 3. Feel for fullness of muscle and fat cover.

Appendix 2 Post mortem

Organ	Part sampled	Treatment			
		Fast freeze	Slow freeze	Fix	Other
Cotyledons	A	*			
	B	*			
	C	*			
	D	*			
Peri-renal fat		*			
Adrenal	left		½	½	
Kidney	left			*	Perfusion fix
Adrenal	right				
Kidney	right cortex	bottom ½			
	right medulla	bottom ½			
Liver	left lobe	*			
	right lobe	*			
Heart	left ventricle	*			
	right ventricle	*			
Thoracic aorta					
Lung	left	*			
	right	*			
Skeletal muscle	right soleus	½	½		
	right FDL	½	½		
Brain	front cortex	*			
	hypothalamus		*		
	pituitary		*		
	hipocampus	*			

Table A. 1. Treatment of samples taken from fetuses. Fixed tissues were fixed in formalin overnight, frozen tissues were stored at -80°C.

Appendix 3 Nomogram

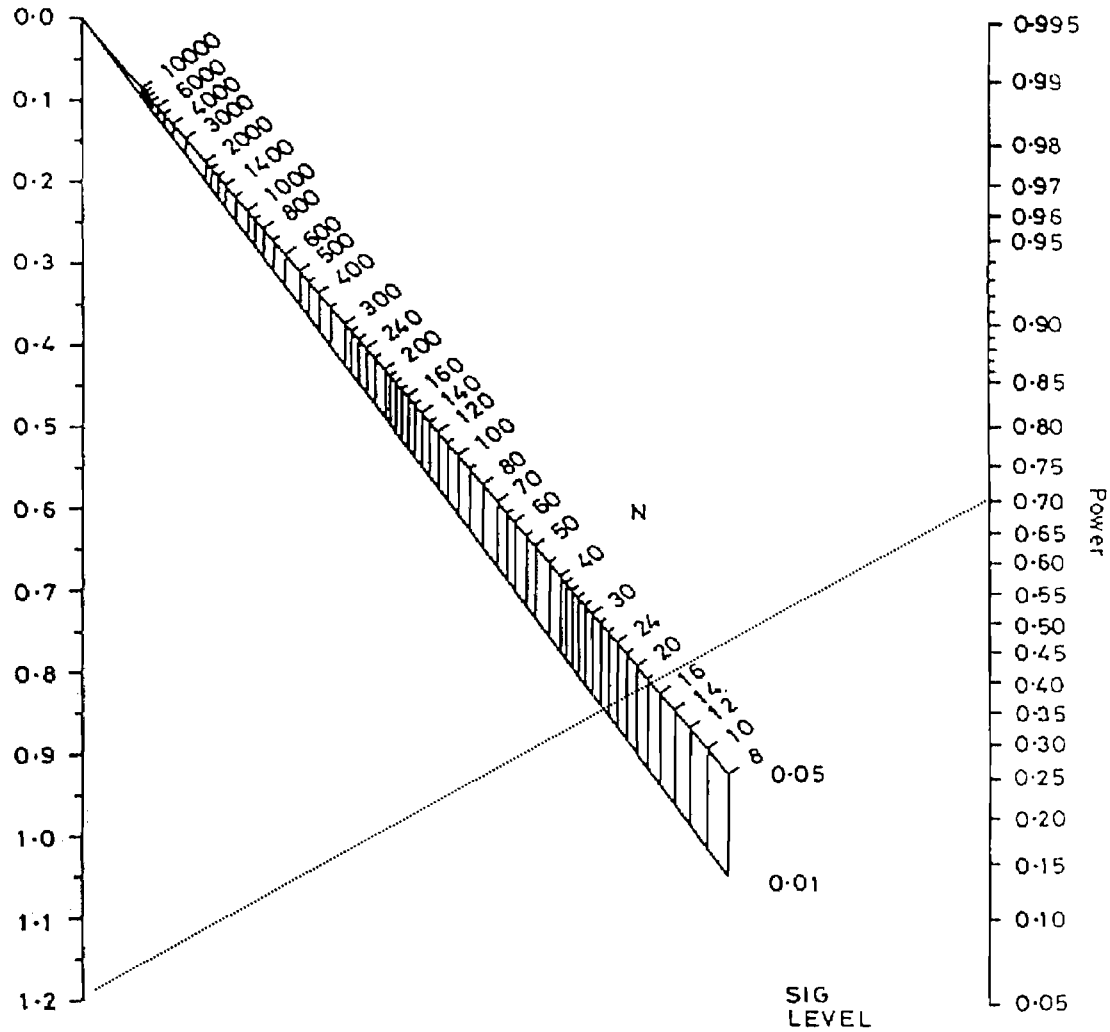

Figure A. 4. Nomogram for calculating sample size or power reproduced from Altman, 1991. Showing the sample size calculation for a standardized difference of 1.58 and 70 % power as used in this thesis.

Appendix 4 Baroreflex

Baroreflex sensitivity was tested using boluses of phenylephrine (PE) which causes vasoconstriction which rapidly raises blood pressure. To calculate the baroreflex sensitivity the time between two consecutive R waves in the electrocardiogram (R-R interval) is plotted against the proceeding systolic blood pressure when blood pressure is increasing. A linear regression is constructed, the slope is the measure of baroreflex sensitivity and expressed in ms/mm Hg. The operating point (equivalent to the EC50 in pharmacology), is the pressure value at which the half-maximal RRI response is seen.

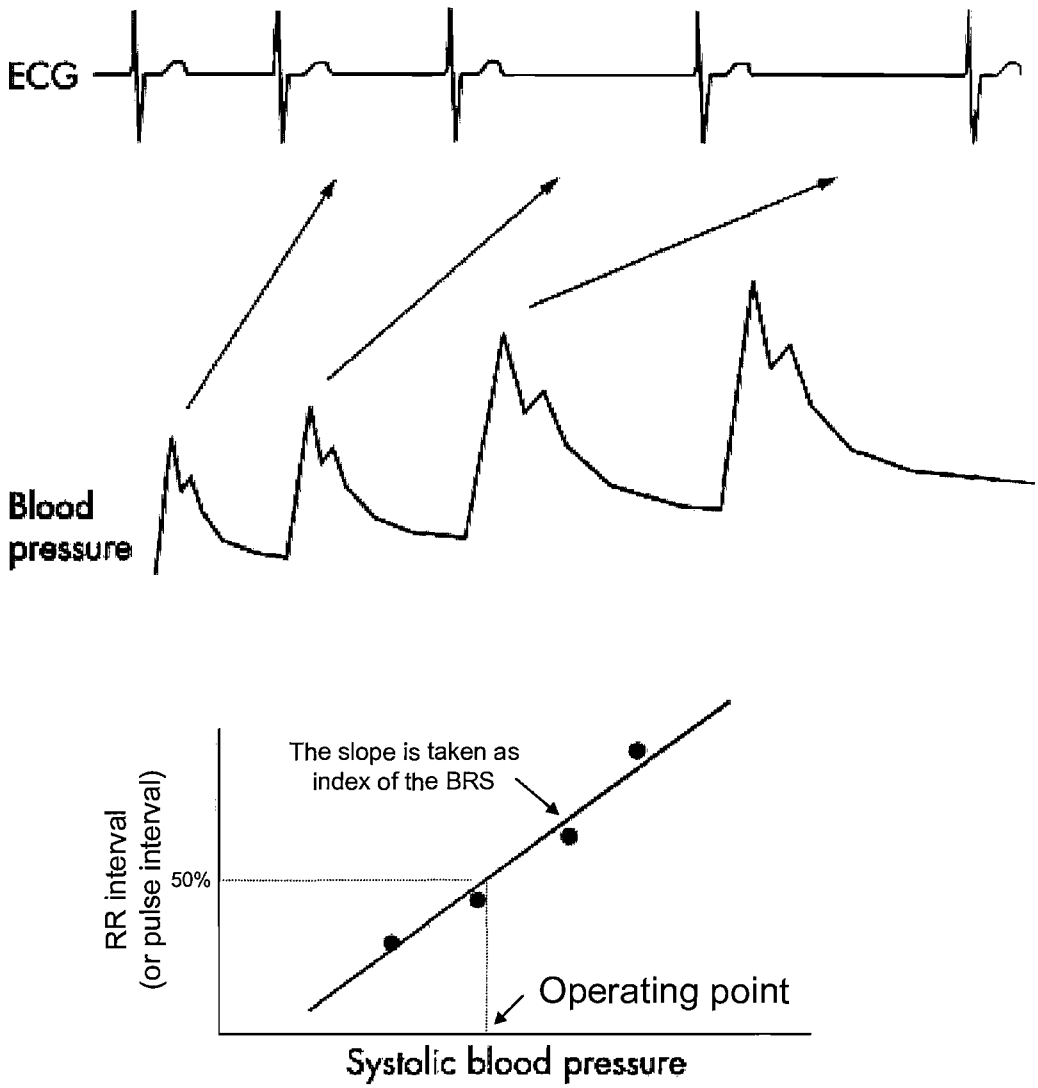

Figure A. 5. After a bolus injection of phenylephrine there is a progressive increase in blood pressure and a reflex slowing of heart rate. Baroreflex sensitivity (BRS) is calculated as the slope of the relation between systolic blood pressure and the subsequent RR interval during this ramp increase in blood pressure. Reproduced from Persson PB, *et al. J Hypertens* 2001;19:1699–705.

Appendix 5 Basal blood gas status (2004)

123 dGA	Control (n = 10)	Restricted (n = 10)
pH	7.36 ± 0.01	7.35 ± 0.01
pO_2 (mm Hg)	21.39 ± 0.94	20.20 ± 0.90
pCO_2 (mm Hg)	43.51 ± 1.08	49.45 ± 0.70 **
Haemoglobin (g/dL)	8.13 ± 0.26	9.27 ± 0.39 *
Haematocrit (%)	25.32 ± 0.78	28.70 ± 1.16 *
O_2 saturation (%)	60.58 ± 2.02	55.13 ± 2.82
Fraction of oxyhaemoglobin (%)	58.32 ± 1.94	52.97 ± 2.67
Fraction of carboxyhaemoglobin (%)	3.33 ± 0.14	3.37 ± 0.15
Fraction of deoxyhaemoglobin (%)	37.94 ± 1.93	43.16 ± 2.75
Fraction of methaemoglobin (%)	0.41 ± 0.04	0.50 ± 0.05
K^+ (mmol/L)	3.20 ± 0.10	3.36 ± 0.13
Na^+ (mmol/L)	140.50 ± 2.52	141.90 ± 0.46
Ca^{2+} (mmol/L)	1.20 ± 0.03	1.25 ± 0.04
Cl^- (mmol/L)	116.70 ± 0.93	113.50 ± 1.22
Glucose (mmol/L)	0.77 ± 0.04	0.80 ± 0.06
Lactate (mmol/L)	0.79 ± 0.04	1.00 ± 0.09 *
Bilirubin (µmol/L)	14.40 ± 1.66	14.20 ± 2.31
Arterial concentration of O_2 (Vol%)	6.62 ± 0.20	6.82 ± 0.36
O_2 tension at 50% saturation (mm Hg)	14.75 ± 0.31	15.29 ± 0.26
Base excess (mmol/L)	-0.84 ± 0.65	1.25 ± 0.49 *
Bicarbonate (mmol/L)	23.26 ± 0.50	24.73 ± 0.44 *
124 dGA		
pH	7.35 ± 0.01	7.33 ± 0.01
pO_2 (mm Hg)	19.94 ± 0.92	20.00 ± 1.23
pCO_2 (mm Hg) *	45.25 ± 0.68	50.62 ± 1.20
Haemoglobin (g/dL)	8.19 ± 0.29	8.79 ± 0.44
Haematocrit (%)	25.47 ± 0.87	27.30 ± 1.33
O_2 saturation (%)	56.97 ± 3.39	52.14 ± 3.11
Fraction of oxyhaemoglobin (%)	54.85 ± 3.22	49.17 ± 3.19
Fraction of carboxyhaemoglobin (%)	3.21 ± 0.17	3.10 ± 0.20
Fraction of deoxyhaemoglobin (%)	41.45 ± 3.31	47.09 ± 3.31
Fraction of methaemoglobin (%)	0.49 ± 0.05	0.64 ± 0.07
K^+ (mmol/L)	3.15 ± 0.08	3.15 ± 0.17
Na^+ (mmol/L)	143.20 ± 0.77	143.20 ± 0.57
Ca^{2+} (mmol/L)	1.26 ± 0.03	1.19 ± 0.04
Cl^- (mmol/L)	114.80 ± 0.96	114.30 ± 1.71
Glucose (mmol/L)	0.86 ± 0.04	0.75 ± 0.06
Lactate (mmol/L)	0.94 ± 0.07	1.11 ± 0.10
Bilirubin (µmol/L)	13.70 ± 1.69	13.50 ± 2.31
Arterial concentration of O_2 (Vol%)	6.24 ± 0.33	5.98 ± 0.38
O_2 tension at 50% saturation (mm Hg) *	14.61 ± 0.34	16.19 ± 0.53
Base excess (mmol/L)	-0.71 ± 0.62	0.24 ± 0.87
Bicarbonate (mmol/L)	23.28 ± 0.52	23.83 ± 0.74

Table A. 2. Basal blood gas parameters (mean ± SEM). Analysed by unpaired Student's t-test. * $p < 0.05$, ** $p < 0.01$ control vs restricted.

Appendix 6 Basal blood gas status (2005)

123 dGA	Control (n = 9)	Early (n = 9)	Late (n = 6)
pH	7.32 ± 0.01	7.32 ± 0.01	7.34 ± 0.01
pO_2 (mm Hg)	18.83 ± 0.60	18.12 ± 1.51	16.43 ± 2.01
pCO_2 (mm Hg)	44.13 ± 1.64	45.51 ± 1.17	44.05 ± 1.84
Haemoglobin (g/dL)	7.83 ± 0.56	9.07 ± 0.47	9.00 ± 0.50
Haematocrit (%)	24.42 ± 1.67	28.14 ± 1.41	27.85 ± 1.53
O_2 saturation (%)	55.76 ± 2.85	49.62 ± 4.65	45.67 ± 4.59
Fraction of oxyhaemoglobin (%)	53.79 ± 2.72	47.81 ± 4.48	43.97 ± 4.44
Fraction of carboxyhaemoglobin (%)	2.90 ± 0.17	2.91 ± 0.13	3.03 ± 0.19
Fraction of deoxyhaemoglobin (%)	42.71 ± 2.77	48.55 ± 4.49	52.28 ± 4.40
Fraction of methaemoglobin (%)	0.60 ± 0.07	0.73 ± 0.08	0.72 ± 0.12
K^+ (mmol/L)	2.84 ± 0.17	3.24 ± 0.17	2.80 ± 0.10
Na^+ (mmol/L)	143.44 ± 0.80	142.33 ± 0.73	143.67 ± 0.33
Ca^{2+} (mmol/L)	1.09 ± 0.03	1.17 ± 0.04	1.13 ± 0.04
Cl^- (mmol/L)	115.33 ± 1.89	113.89 ± 1.37	114.67 ± 1.52
Glucose (mmol/L)	0.82 ± 0.05	0.88 ± 0.04	0.83 ± 0.07
Lactate (mmol/L)	1.03 ± 0.08	1.22 ± 0.15	1.32 ± 0.17
Bilirubin (µmol/L)	11.22 ± 1.82	10.67 ± 1.62	11.33 ± 2.42
Arterial concentration of O_2 (Vol%)	5.76 ± 0.17	5.91 ± 0.40	5.47 ± 0.48
O_2 tension at 50% saturation (mm Hg)	14.23 ± 0.57	15.17 ± 1.15	14.46 ± 0.94
Base excess (mmol/L)	-3.31 ± 0.90	-2.79 ± 0.83	-2.30 ± 0.92
Bicarbonate (mmol/L)	21.07 ± 0.67	21.34 ± 0.69	21.73 ± 0.70
124 dGA			
pH	7.34 ± 0.01	7.33 ± 0.01	7.35 ± 0.00
pO_2 (mm Hg)	21.01 ± 1.16	19.19 ± 1.47	17.23 10.50
pCO_2 (mm Hg)	44.09 ± 1.13	45.77 ± 1.49	44.09 ± 1.62
Haemoglobin (g/dL)	8.50 ± 0.45	8.99 ± 0.42	9.45 ± 0.56
Haematocrit (%)	26.42 ± 1.37	27.87 ± 1.26	29.22 ± 1.65
O_2 saturation (%)	58.32 ± 2.67	54.41 ± 4.08	43.63 ± 6.99
Fraction of oxyhaemoglobin (%)	56.04 ± 2.59	52.04 ± 3.82	48.18 ± 1.67
Fraction of carboxyhaemoglobin (%)	3.27 ± 0.16	3.42 ± 0.27	3.40 ± 0.19
Fraction of deoxyhaemoglobin (%)	40.02 ± 2.55	43.73 ± 4.02	47.67 ± 1.72
Fraction of methaemoglobin (%)	0.67 ± 0.14	0.80 ± 0.12	0.75 ± 0.08
K^+ (mmol/L)	2.83 ± 0.15	3.26 ± 0.15	3.00 ± 0.13
Na^+ (mmol/L)	143.56 ± 0.90	141.78 ± 0.83	142.50 ± 0.56
Ca^{2+} (mmol/L)	1.07 ± 0.04	1.14 ± 0.04	1.11 ± 0.01
Cl^- (mmol/L)	113.22 ± 1.54	111.67 ± 1.76	112.67 ± 1.23
Glucose (mmol/L)	0.76 ± 0.07	0.79 ± 0.03	0.72 ± 0.05
Lactate (mmol/L)	0.93 ± 0.06	1.13 ± 0.013	1.22 ± 0.06
Bilirubin (µmol/L)	9.33 ± 2.93	10.11 ± 2.13	10.17 ± 1.35
Arterial concentration of O_2 (Vol%)	6.60 ± 0.24	6.51 ± 0.49	6.33 ± 0.35
O_2 tension at 50% saturation (mm Hg)	15.28 ± 1.15	14.73 ± 0.96	14.40 ± 1.14
Base excess (mmol/L)	-1.93 ± 0.81	-2.14 ± 0.96	-1.50 ± 0.86
Bicarbonate (mmol/L)	22.27 ± 0.67	21.98 ± 0.78	22.43 ± 0.63

Table A. 3. Basal blood gas parameters (mean ± SEM). Analysed by unpaired Student's t-test.

Appendix 7 Abstract 1

The effect of early gestation nutrient restriction on late gestation fetal cardiovascular responses to angiotensin II in sheep.

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Introduction: The renin-angiotensin system (RAS) is a candidate mechanism linking altered nutrition during pregnancy to cardiovascular disease and high blood pressure in later life. In young adult sheep, renal and cardiovascular control mechanisms are altered by an early gestation nutrient restriction (Cleal *et al*, J Physiol, 2004; 55P: C92*)*, suggesting fetal adaptations in the renal RAS. In this study we investigated the effects of moderate early gestation maternal nutrient restriction on the fetal cardiovascular response to angiotensin II (Ang II) in late gestation.

Methods: Pregnant Welsh Mountain ewes of uniform body weight were housed individually and received either 100% (C, n=9) or 50% (R, n=9) of their total nutrient requirements for the first 30 days of gestation (dGA) and 100% of requirement thereafter. At ~117dGA (term ~ 147dGA) under general halothane anaesthesia the fetus was surgically prepared with vascular catheters, flow probes (Transonic) around the femoral and carotid arteries, and electrocorticogram (ECoG) electrodes placed on the parietal cortex. After four days post-operative recovery, fetal mean arterial pressure (MAP), heart rate, ECoG and femoral and carotid artery blood flows were monitored continuously during intravenous administration of Ang II (0μg, 1μg, 2.5μg, 5μg, 10μg) and phenylepherine (baroreflex, 75μg) IV to the fetus. The area under the curve (AUC) response to Ang II and the baroreflex set point (IC50) and sensitivity (gradient) were analysed by unpaired Student's t-test.

Results: There were no significant differences between C and R fetuses in basal heart rate or MAP. Moreover there were no significant differences between the groups in baroreflex or maximal blood pressure response to phenylephrine. The MAP response to Ang II was significantly blunted in R compared to C fetuses (AUC C=286.7 ± 13.37, R=230.3 ± 18.42 mm Hg.μg, $p<0.05$).

Conclusion: Our observation of blunted late gestation fetal responses to Ang II, but not to phenylephrine, following early gestation nutrient restriction may indicate altered expression of angiotensin receptors in the peripheral vasculature.

Supported by the BBSRC

Appendix 8 Abstract 2

Moderate early gestation undernutrition has no effect on kidney size and function in late gestation sheep.

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Introduction: Poor *in utero* nutrition is implicated in the association between low birth weight and the development of hypertension in adulthood. Exposure to dexamethasone in early gestation altered late gestation renal function and produced hypertension in adult sheep (Moritz *et al*, Endo, 2002; 143:4455). Moderate undernutrition from 1 to 31 days gestation (dGA) altered renal and cardiovascular control in adult male sheep offspring (Cleal *et al*, J Physiol, 2004; 55P:C92*)*. This was associated with altered blood pressure (BP) responses to angiotensin II in late gestation fetuses (Braddick *et al, JSGI,* 2005; 12:88), however we do not know if the early gestation undernutrition altered fetal kidney function.(Moritz *et al.*, 2002)

Methods: Pregnant Welsh Mountain ewes were housed individually and fed 100 % (C, *n*=9) or 50 % (R, *n*=9) of their total nutrient requirements between 1 and 31 dGA, and 100 % thereafter. At ~117 dGA (term~147 dGA) under general halothane anaesthesia fetuses were surgically prepared with vascular and bladder catheters. At ~124 dGA fetal BP was monitored continuously and renal function was assessed before and at 30 min intervals after an i.v. bolus of frusemide (20 mg) or saline vehicle. At post-mortem (127 dGA) kidney weight and dimensions were calculated. Data are mean ± SEM and were analysed by ANOVA with Bonferroni post-hoc tests.

Results: Basal glomerular filtration rate (GFR. C= 4.1±0.4; R=3.7±0.4 ml/min), urine flow rate (C=0.6±0.1; R=0.6±0.1 ml/min), Na^+ excretion (C=1.8±0.3; R=1.7±0.1 mmol/L/min), osmolarity (C=139.3±10.4; R=130.3±6.4 mosmol) and BP (C=44.4±1.1, R=44.3±0.8 mmHg) were similar in C and R fetuses. There was no significant GFR (C=4.8±0.4; R= 4.4±0.4 ml/min) or BP (C=44.3±0.9; R=44.1±0.8 mmHg) response to frusemide in either dietary group. Urinary flow rate (C,$^{\Delta}$=1.2±0.1; R,$^{\Delta}$=1.2±0.1 ml/min), Na^+ excretion (C,$^{\Delta}$=2.4±0.2; R,$^{\Delta}$=2.0±0.1 mmol/L/min) and osmolarity (C,$^{\Delta}$=104.9±8.4; R,$^{\Delta}$=108.2±2.9 mosmol) increased in response to frusemide and to a similar extent in both groups. There was no difference in kidney biometry between groups.

Conclusion: Unlike dexamethasone exposure, a moderate early gestation nutrient restriction that alters adult cardiovascular control has no effect on fetal renal function at ~0.8 gestation. Thus if early gestation diet has programming effects on the kidney it may involve regulatory systems (e.g. renin-angiotensin-aldosterone) that do not mature until later in development.

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Appendix 9 Abstract 3

The effect of a moderate early gestation undernutrition on kidney glomerular number in fetal sheep.

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Introduction: Poor *in utero* nutrition is implicated in the association between low birth weight and the development of hypertension in adulthood (Barker *et al.*, 1989). It has been proposed that this association may result from impaired prenatal kidney growth and lower nephron endowment induced by suboptimal *in utero* nutrition (Brenner *et al.*, 1988). In addition thinner mothers, which may indicate relatively poorer nutrition, tend to have fetuses that have kidneys that are relatively narrow for their length (Mukherjee *et al.*, 2005). A 50% nutrient restriction from 1 to 31 days gestation (dGA, term ~147 dGA) alters renal and cardiovascular control in adult male sheep offspring (Cleal *et al.*, 2004). This challenge is associated with altered blood pressure (BP) responses to angiotensin II but no change in kidney size or function in late gestation fetuses (Braddick *et al.*, 2005; Braddick *et al.*, 2006). The aim of the current study was to determine if fetal kidney structure is altered by early gestation undernutrition.

Methods: Pregnant ewes were housed individually and fed 100% (C, n = 8) or 40% (R, n = 8) of their total nutrient requirements between 1 and 31 dGA, and 100% thereafter. All fetuses were singletons, the sex ratio was 1:1 in C and 5:3 in R groups. At post-mortem (127 dGA) the left kidney was perfusion fixed with formalin. Each kidney was cut in half and cut into 5 mm slices. Systematic sampling was used to select every 10^{th} piece which was embedded in glycomethacylate resin. The selected blocks were sectioned at 2 μm, and every 200^{th} and 210^{th} pair were collected and stained with toluidine blue. The physical dissector technique was used to count the number of glomeruli in the cortex by light microscopy. Data are mean ± SEM and were analysed by Student's t-test.

Results: Fetal body (C = 2.91 ± 0.08; R = 2.92 ± 0.12 kg) and kidney weight (Right; C = 9.66 ± 0.61; R = 9.80 ± 0.40 g; Left; C = 11.65 ± 0.56; R = 11.33 ± 0.56 g) were similar in both groups. There was no significant difference in fetal glomerular number in the left kidney in R compared to C fetuses (C = 326,100 ± 40,850; R = 457,300 ± 118,700).

Conclusion: Early gestation nutrition restriction did not alter fetal or organ growth or kidney development in late gestation sheep. Therefore altered renal and cardiovascular control in adult sheep following early gestation nutrient restriction is not due to altered glomerular number.

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