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THE EFFECTS OF EARLY LIFE NUTRIENT RESTRICTION ON THE CARDIOVASCULAR SYSTEM OF THE ADULT SHEEP

by

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DM thesis

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ABSTRACT
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DM thesis

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There is now strong epidemiological and animal research showing that undernutrition in gestation and early postnatal life is linked with a higher incidence of cardiovascular disease in adulthood. The physiological processes involved are not yet clear. The aim of this thesis was to investigate how aspects of the cardiovascular system in the adult sheep are affected by early life periods of undernutrition, and to investigate to concept that mismatches in these periods may influence these responses.

Welsh Mountain ewes received 100% of global nutritional requirements at all times (C) except from minus 30 to day of conception (B), from minus 15 to 15 days after conception (A), or from day 1 of gestation to 31 days gestation (U) when they received 50% of total nutrient requirements. Offspring of groups C & U were then fed ad libitum (CC & UC) or at a level that reduced body weight to 85% of individual target weight from 12 to 25 weeks postnatal age (CU & UU). The adult sheep cardiovascular function was studied at 2.5 years and 3.3 years.

At 2.5 years the UC males showed an increased interventricular wall thickness without loss in function. These effects were not seen if early postnatal restriction was also received. In contrast, females subject in the gestational undernutrition (UC) showed a dampened heart rate response to a stressor, which was not seen when combined with a postnatal challenge (UU). Basal adrenaline was elevated in male and female singletons exposed to the postnatal challenge (CU & UU). The stressor produced an enhanced adrenaline response in the females in the postnatally challenged group (CU). This effect was attenuated when combined with a gestational challenge (UU).

Thus early life undernutrition alters adult cardiovascular physiology and may have consequences for cardiovascular function and disease in later life. These effects are sex-specific. The cardiovascular system is affected by the mismatch between gestation and early postnatal nutrition.

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DECLARATION OF THE AUTHOR

I, …**Julian Boullin**…declare that the thesis entitled

The effects of early life nutrient restriction on the cardiovascular system of the adult sheep

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:

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Proceedings of the National Academy of Science of the USA, May 2007;104(22):9529-33
Mismatched pre- and postnatal nutrition leads to cardiovascular dysfunction and altered renal function in adulthood

Boullin JP, Green LR, Khan OA, Morgan JM, Hanson MA
European Heart Journal (Abstract Book) Sept 2005
The effect of undernutrition on cardiac morphology in adult male sheep

Boullin JP, Morgan JM
Heart 2005 Jul;91(7):874-5
The Development of Cardiac Rhythm

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Signed:……

Date:……

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Sammy – my dearest wife without whom none of this could be possible. I'm a lucky man.

ABBREVIATIONS

2D	two dimensional
ACE	angiotensin converting enzyme
ACh	acetylcholine
ACTH	adrenocorticotrophic hormone
Ang I	angiotensin I
Ang II	angiotensin II
ANOVA	analysis of variance
AUC	area under the curve
AV	atrioventricluar
BCL	basic cycle length
BCS	body condition score
BMI	body mass index
bpm	beats per minute
C	control diet
Ca^{2+}	calcium
CAD	coronary artery disease
CCS	cardiac conduction system
CHD	coronary heart disease
Cl^-	chloride
CRF	
CRH	corticotrophin releasing hormone
cSNRT	corrected sinus node recovery time
CVD	cardiovascular disease
CVS	cardiovascular system
d	day
dBP	diastole blood pressure
dGA	days gestational age
DNA	deoxyribose nucleic acid
DOHaD	developmental origins of health and disease
ECG	electrocardiogram
Echo	echocardiography
EDTA	ethylenediaminetetraacetic acid
ERP	effective refractory period
FS	fractional shortening
GFR	glomerular filtration rate
HPA	hypothalamic-pituitary-adrenal
HR	heart rate
HRV	heart rate variability
IUGR	intrauterine growth restriction
i.v.	intravenous
IVS	interventricular septum
IVSd	interventricular septum in diastole
IVSs	interventricular septum in systole
K^+	potassium
kg	kilogram's
O_2	oxygen
p	probability
PAR	predictive adaptive response
pCO_2	partial pressure of carbon dioxide
pO_2	partial pressure of oxygen
PWd	posterior wall in diastole
PWs	posterior wall in systole
LDL	low density lipoprotein
LV	left ventricle
LVH	left ventricular hypertrophy
LVIDd	left ventricular internal dimension in diasystole
LVIDs	left ventricular internal dimension in systole
LVM	left ventricular mass
MAP	mean arterial pressure
mmHG	millimetres of mercury
M-mode	motion mode
mRNA	messenger ribonucleic acid
ms	milliseconds
MWT	mean wall thickness
Na^+	sodium
RA	right atrium
RAS	renin angiotensin system
RNA	ribonucleic acid
RV	right ventricle
RVC	Royal Veterinary College
RWT	relative wall thickness
SA	sinoatrial
sBP	systolic blood pressure
SD	standard deviation
SEM	standard error mean
SNRT	sinus node recovery time
TI	transport and isolation
UK	United Kingdom
USA	United States of America
VF	ventricular fibrillation
VT	ventricular tachycardia
vs.	versus
WCL	wenkebach cycle length
WHO	World Health Organisation
VCP	ventricular conduction pathway

Chapter 1 - General Introduction

1.1 The Developmental Origins of Health and Disease

1.1.1 Overview

Since the late 1980's numerous epidemiological studies have shown an association between impaired fetal growth and disease in adult life such as coronary heart disease and metabolic syndrome. This led to the fetal origins of adult disease hypothesis (Barker, 1995), which proposes that insults in fetal life cause in utero adaptations which in turn "programmes" for disease in adult hood.

This introduces an additional factor to the development of disease in addition to the well recognised contributions of genetic make up and adult life style factors. It has since been shown that the window for potential insults covers a much wider time period from preconception through to infancy. The role of undernutrition has been shown to play an important role in these early life adaptations, and many animal models have been developed to study this. At present there is little information as to the underlying processes that link critical early life developmental periods to cardiovascular disease in later life. This thesis will investigate the physiological mechanisms that may be involved in this programming, and how this leads to cardiovascular dysfunction in adult life.

1.1.2 Epidemiology of DOHaD

Epidemiological research has shown that coronary heart disease (CHD) had been steadily increasing over the last century; this has been followed by a gradual decrease over the last thirty years (The World Health Report, 1999). However, there have been no parallel changes in adult lifestyle of the long established CHD risk factors such as smoking and obesity. Biochemical and physiological measurements in adult life, including serum cholesterol and blood pressure have been linked to CHD (Keys, 1980). Yet even when combined with adult lifestyle these risk factors have limited ability to predict CHD (Rose & Marmot, 1981) leaving this changing incidence unexplained. These findings were followed by geographical studies which showed a correlation in CHD mortality in England and Wales from 1968 to 1978 and previous infant mortality (less than one year old) (Barker & Osmond, 1986a). The concentration of low mortality from CHD in the south and east contrasted with the high mortality in the northern industrial towns, and the poorer rural areas in the north and west of the country. No links could be drawn with adult lifestyles such as cigarette smoking and dietary fat consumption (Office of Population Censuses and Surveys, 1990). These findings led to the suggestion that CHD was associated with poor childhood living conditions (overcrowded housing and recurrent exposure to infections), and poor nutrition in early life.

Findings from other studies supported the general hypothesis that CHD is linked with adverse influences in early life. Forsdahl was the first to report that atherosclerotic heart disease correlated with past infant mortality in his Norwegian studies (Forsdahl, 1977). He suggested that a poor standard of living in childhood and adolescence was a risk factor for heart disease.

One large study of 15,726 people in middle and late life born during 1911 to 1930 in Hertfordshire, UK illustrates clearly the association between birth weight and CHD (Figure 1.1). This revealed that death rates from coronary heart disease inversely correlated with birth weight, even within the normal range (Barker *et al.*, 1993b).

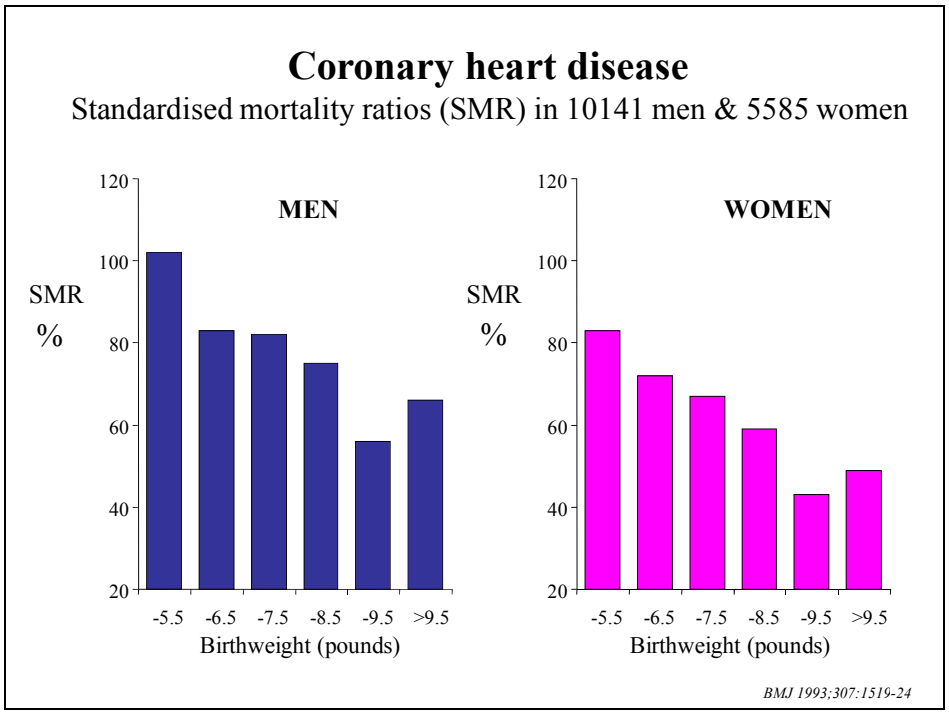


Figure 1.1: Standardised mortality ratios for CHD in men and women.
(Barker et al., 1993b)

There are now many epidemiological studies which demonstrate the association of impaired fetal growth with various other chronic diseases such as hypertension (Barker, 1996), and metabolic syndrome (Reaven, 1988) and chronic bronchitis (Barker *et al.*, 1991;Shaheen & Barker, 1994). This association has now been shown in both developed

and developing countries in diverse populations in terms of race, gender and age (Godfrey & Barker, 2001;Huxley *et al.*, 2000;Stein *et al.*, 1996).

These studies led to Barker's hypothesis that an adverse environment *in utero* and during infancy permanently changes the body's structure, physiology, and metabolism and leads to CHD in adult life. The "developmental origins of health and disease" model proposes that risk of developing chronic disease in adulthood is related not only to predetermined genetic factors but also by environmental factors acting in early life. This is due to the process of programming, whereby a stimulus or insult during a critical period of development results in lasting or lifelong adaptations (Lucas, 1994).

Programming seems to occur in organs when they are growing rapidly. Outside these critical periods the system does not have such plasticity to respond to its environment. The timing of the insult determines which tissues and systems are involved. These adaptations occur in order to promote short-term survival under conditions of malnutrition, stress of other deprivation even though they are detrimental to health in later life when the environment is less challenging (Barker, 1998a). The programmed effects may not be evident until much later in life due when the early life adaptations are combined with the postnatal risk factors for disease such as poor diet or smoking. For example, one study showed the highest incidence of type 2 diabetes to be in people who had a low birth weight and were then obese in adulthood (Hales *et al.*, 1991). This process will be discussed later in the "Predictive Adaptive Response" – Section 1.1.9.

1.1.3 Disease vulnerability

There are well known risk factors for cardiovascular disease, derived from statistical associations between factors and disease prevalence. These impart risk for cardiovascular disease and include cigarette smoking, obesity, hyperlipidaemia, diabetes mellitus and hypertension. Conventionally these risk factors are thought to act in adult life to accelerate destructive processes such as atheroma, rise in blood pressure and loss of glucose tolerance. Now that these have been identified it has enabled primary prevention programmes including patient education to be set up in most western countries. There is some evidence that the decrease in incidence of cardiovascular disease over the past few decades has been related to risk factor modification such as decreases in cigarette smoking (Rosamund, 2008). However, the currently accepted risk factors do not explain differences in "vulnerability" for cardiovascular events. There are still many unexplained discrepancies in disease incidence. For example, on one hand a life-long smoker with hypertension and hypercholesterolaemia can live into his 80's without signs of cardiovascular disease; this is in contrast to the 30 year old with no identified risk factors who suffers a myocardial infarction.

At present, it is not known why some individuals are much more vulnerable to cardiovascular disease than others. These differences in vulnerability are often attributed to a genetic predisposition, even when this cannot be demonstrated in the patient's family history. There are now recognised specific gene defects (Arnett *et al.*, 2004). However, the role of genetic diversity in affected cardiovascular pathology within human populations has not yet been substantiated. Furthermore, the epidemiological evidence described in Section 1.1.2 suggests that an adverse early life environment imparts a powerful influence on the

cardiovascular system and leads to vulnerability for cardiovascular disease in a significant proportion of the population.

This physiological process which leads to vulnerability has been termed programming. It is this principle which I wish to investigate, by analysis of potential mechanisms involved in the early life development, which then leads on to programming of cardiovascular disease in later life.

1.1.4 Growth measures

Weight is the conventional "growth measure" used. However, many different patterns of early growth have been studied in humans, using various measures of body proportions. At birth increased cranial to abdominal circumference, which is indicative of fetal growth impairment with brain sparing has been shown to be a strong predictor for subsequent hypertension (Law *et al.*, 1992;Martyn & Greenwald, 1997). Other associated measures include low ponderal index (birthweight [kg]/length $[m]^3$), low body mass index (weight [kg]/height $[m]^2$), and reduced birth weight to placental weight ratio (Law *et al.*, 1992). Thinness at birth is strongly associated with low placental weight, which is in turn associated with increased prevalence of CHD. This was illustrated in a study of births in Preston between 1935 and 1943 which showed that a greater placental weight is an important factor in fetal growth. A decrease in placental weight is associated an increase in the ratio of length to head circumference and that blood pressures were higher in those adults who had been small babies with large placentas (Barker *et al.*, 1990).

These findings have all been independent of the length of gestation and indeed the associations are strongest in term babies, strengthening the belief that disease in adult life is linked to low rates of fetal growth and not simply premature birth.

1.1.5 Disproportionality

Animal studies have shown that blood pressure and metabolism can be permanently changed by levels of undernutrition that do not influence measured growth. Preliminary observations point to similar effects in humans. In the Hertfordshire study death rates from coronary heart disease were inversely correlated with birth weight within the normal range (5.5lb/2500g to 9.5lb/4310g; Barker *et al.*, 1993). Furthermore, human fetal metabolism can be permanently altered by undernutrition without affecting the size of the baby at birth as seen in the Dutch famine (Rizzoni *et al.*, 1998). In view of this birth weight should be seen as a crude measure of fetal growth that may not be sensitive to mild alterations. Indeed, birth weight may underestimate the contribution of intrauterine nutrient restriction in later life. If the maternal dietary impairment is not severe, fetal and placental compensatory mechanisms may prevent a reduction in birth weight.

The cardiovascular system is an integral part of the fetal compensation and may be adversely affected by *in utero* and early life challenges at levels that do not influence fetal, birth, or childhood weight.

1.1.6 Undernutrition

The association of impaired fetal growth and development with risk of cardiovascular disease in adulthood has lead researchers to question what processes are involved in this causing this insult. As discussed, it has long been known that certain diseases are a result of undernutrition at a critical stage of early life resulting in persisting changes in the body's structure (Forsdahl, 1977). The undernutrition models in sheep, rat and guinea pig have provided strong evidence for this (Davis & Hohimer, 1991;Langley-Evans *et al.*, 1996c;Lingas & Matthews, 2001).

It is well established that smallness at birth or disproportionate growth in utero is linked to increased risk of cardiovascular disease in adulthood. The early studies that looked at this association were based on retrospective human populations. Since then prospective animal studies have been used to provide evidence to support the developmental origins of adult disease hypothesis. These studies have centred on the use of undernutritional challenges in early life. There can be many ways in which the supply chain from mother to fetus can be compromised. The inadequate consumption of macronutrients appears to have a gestation-specific effect. Reduction in either maternal or fetal placental blood flow can impair transplacental nutrient exchanges (Godfrey, 2002). Finally, the placenta itself can have transport defects along with abnormalities in endocrine function, metabolism or vascular structure and thereby prevent optimal nutrient exchange (Langley-Evans *et al.*, 1996b).

There have been many different approaches to the nutritional challenge used and indeed there are many different perturbations possible, I have grouped these into three, as follows,

i) the type of nutrient restriction,

ii) the severity of the challenge, and

iii) the timing of the challenge.

1.1.7 Type of nutrient restriction

Previous studies have restricted different nutrient components within the diet. Calorific reduction and altered macronutrients such as protein have been studied in the animal model. In relation to cardiovascular disease it has been reported that a 50% reduction of dietary protein intake (Gardner *et al.*, 1997;Langley & Jackson, 1994;Langley-Evans & Jackson, 1995) or severe calorie restriction (Woodall *et al.*, 1996b) leads to elevated blood pressure in adult offspring. Severe protein restriction will enable us to draw comparisons with developing countries. The largest programming effects of nutritional manipulation on the development of hypertension are observed when the mammalian diet is modified to produce an imbalance in the nutrient supply. For example, the feeding of low-protein diets in rat pregnancy generates offspring that have a blood pressure that is 15-30mmHg above that of the control animals by the age of weaning (Langley-Evans *et al.*, 1996c). Similarly, low iron diets (Gambling *et al.*, 2003) and high-saturated-fat diets (Khan *et al.*, 2003) in rat pregnancy produce large effects on the blood pressure of the offspring. Where the diet is manipulated in such a way that the intakes of the nutrients are reduced in

a balanced manner during pregnancy the impact on blood pressure of the subsequent offspring is still observed, but tends to be of a lower magnitude (Woodall *et al.*, 1996b).

For ruminants such as the sheep a total nutrient restriction has usually been used. This is because a significant proportion of the dietary nitrogen is supplied by the gut commensals making it difficult to manipulate the proportion of protein received in this micro-environment. Moderate global dietary restriction has also been studied previously. One group reported rats fed a 30% reduction of total diet from day 0 to day 18 of gestation induced hypertension in the adult offspring (Ozaki *et al.*, 2001). Another investigation used a sheep model where lambs from ewes subjected to only 15% dietary restriction during gestation were hypertensive (Hawkins *et al.*, 1999).

Despite the variation in the magnitude of the effects produced by different dietary protocols, there does appear to be a general consistency in the type of physiological changes that result. As I will describe latter in the Chapter 2 – General Methods, I have also used a global dietary restriction in my studies in this thesis.

1.1.8 Severity of challenge

It is well established that severe maternal undernutrition during pregnancy causes intrauterine growth retardation. The effects on the growth retarded offspring's postnatal development has also been demonstrated in the (Woodall *et al.*, 1996a). Reducing the diet by 75% was shown to lead to elevated blood pressure. However other studies have used varying and less severe degrees of reduction in nutrition. It is of interest that in some of

these an alteration in the homeostatic development was produced even in the absence of altered body growth. It appears that it is the timing of the insult within the critical windows of development that is more important than the severity, with insults occurring earlier having greater effects on cardiovascular development in the offspring (Hoet & Hanson, 1999).

1.1.9 Timing of the challenge

1.1.9.1 The pre- and peri-conceptional period

This period of mammalian development has long been recognised as an early "developmental window" during which environmental conditions may influence the pattern of future growth and physiology (Fleming, 2006). There is now a growing appreciation of the impact that pre- and periconceptional environment has on the health of the fetus. The effect of the maternal nutrient status immediately prior to pregnancy would seem logically to impact on early development. Indeed, a woman's nutritional status prior to pregnancy, measured by pre-pregnancy weight, has been significantly associated with intrauterine growth retardation (weight for gestational age at birth $<10^{th}$ percentile of a reference population) and low birth weight (WHO, 1995). Research into this critical "window" promises to be a developing area of interest within the developmental origins of health and disease field. A growing volume of literature has demonstrated in vivo that the embryonic environment and particularly maternal diet can modulate future development.

In rats, maternal low-protein diet administered exclusively during the pre-implantation period caused abnormal postnatal growth and organ size and the onset of

elevated blood pressure in a sex-specific manner (Kwong *et al.*, 2000). Periconceptional undernutrition in sheep also disturbs later fetal development and physiology, resulting in an increase in fetal mean arterial pressure in late fetal life (Edwards & McMillen, 2002), and similar effects have been proposed for poor fetal growth and low birthweight in the human (Wynn & Wynn, 1988).

Recent studies in sheep have shown that a periconceptionally restricted supply of vitamin B_{12}, folate and methionine, within normal physiological ranges have effects on the offspring in adulthood (Sinclair *et al.*, 2007). The period of dietary micronutrient restriction was from 8 weeks preceding until 6 days after conception. Whilst this did not result in changes in establishment of pregnancy nor birth weight, it did lead to adult offspring which were both heavier and fatter. The offspring's cardiovascular function was also assessed at 23 months of age. In the challenged group they reported an increase in mean arterial pressure in the males (a sex specific effect). This group also showed increased adiposity, insulin resistance, altered immune function. The researchers hypothesised that this was due to epigenetic modifications to the DNA methylation in the preovulation oocyte and/or preimplanation embryo, with the consequent long term health implications for the offspring.

In a recent human study poor periconceptional blood glucose control in diabetic mothers led to myocardial hypertrophy and reduced fetal ventricular cardiac function (Gardiner *et al.*, 2006). These long term alterations in phenotype relating to early pre- and peri-conceptional periods suggest an important role for maternal-embryonic interactions and cues in the setting of fetal growth and development.

These and many other studies have demonstrated how the early embryo appear to "perceive" the maternal nutrient supply and so predict what it is likely to be later on in gestation. Thus, it converts this information into mechanisms controlling its own development (Fleming, 2006;Wynn & Wynn, 1988). This illustrates how developmental plasticity can lead from maternal physiology through to a "selected" embryo with an appropriate phenotype for fetal development.

1.1.9.2 Prenatal development

There is a vast amount of valuable evidence relating to timing of challenges and their effects on humans from epidemiological studies of famines associated with the Second World War. This includes the Leningrad siege (Stanner & Yudkin, 2001) and the Dutch Hunger Winter (Roseboom *et al.*, 2001c). These wartime famines subjected large populations to periods of severe undernutrition. However, children continued to be conceived and born under harsh conditions. Follow-up studies of these individuals, now in their 60's have given important clues about the impact of prenatal stressors on human development and long-term health. In the case of the Dutch Hunger Winter it is clear that whilst prenatal undernutrition had only a small impact on fetal growth, in the long term it programmed for an increased risk of coronary heart disease (Roseboom *et al.*, 2000a), obesity (Ravelli *et al.*, 1999), renal dysfunction (Painter *et al.*, 2005), and type 2 diabetes (Ravelli *et al.*, 2000). With this population it has been possible to distinguish the effects of famine during discrete periods of development. It appears that exposure to undernutrition in the first trimester of pregnancy is the strongest predictor of cardiovascular disease in adulthood.

Animal studies provide critical data that supports the DOHaD hypothesis and enables an understanding of the mechanisms that may link prenatal challenges to the functional capacity of the organs and systems in the mature state. It has been consistently noted in both large (sheep) and small animals (mice, rats and guinea-pigs) that prenatal fetal exposure to any form of undernutrition produces elevated blood pressure (Gopalakrishnan *et al.*, 2005;Langley-Evans *et al.*, 1994). An elevation is systolic blood pressure has been consistently shown in both male and female offspring (Gardner *et al.*, 1998) even in the absence of alteration in birthweight (Langley-Evans *et al.*, 1996a).

1.1.9.3 Postnatal development

The early postnatal environment has been implicated as an important determinant of adult health status, for example socioeconomic status in early life has been associated with risk of heart disease in adult life (Wannamethee *et al.*, 1996). Another study has shown that in men the risk of coronary heart disease from reduced birth weight is increased further by low weight gain in infancy (by 1 year old; Barker *et al.*, 1989). Eriksson and colleagues have reported on the childhood trajectories with increased risk of coronary heart disease for both men and women associated with lower infant weight gain (Forsen *et al.*, 2004a;Forsen *et al.*, 2004b). Irrespective of size at birth, low weight gain during infancy was associated with increased risk of coronary heart disease.

1.1.9.4 Mechanisms of programming

Experimental studies of programming using animal models have provided not only major support for the developmental origins of disease hypothesis but also insights into the mechanisms involved. The association between altered growth and cardiovascular disease can be broadly divided into four possible mechanistic processes:-

1. Developmental plasticity,
2. Materno-fetal endocrine exchange,
3. Epigenetic mechanisms, and
4. Gene expression.

1.1.9.4.1 Developmental plasticity

The first of these, "developmental plasticity", is the phenomenon whereby one genotype gives rise to a range of different physiological or morphological states in response to different environmental conditions during development (West-Eberhard, 1986). This is a simple and immediately intuitive mechanism whereby developmental insults exert permanent effects on physiology, metabolism and health through alteration of the body. This has been demonstrated by numerous experiments showing that minor alterations in the diets of pregnant animals can produce permanent changes in the offspring's physiology and metabolism even with no change in body size at birth. Changes to the number of cells or the type of cells present could have profound effects on organ function. Factors that limit nephrons formation may impair renal function, and raise local and systemic blood pressure

which may in turn promote cardiovascular disease. In the rat a prenatal nutritional insult although has no effect on kidney size results a reduction in nephrons number by up to 30% (Marchand & Langley-Evans, 2001). This form of remodelling could occur as a result of disruption of cell proliferation or differentiation at key developmental stages.

1.1.9.4.2 Materno-fetal endocrine exchange

The placenta is not only a conduit for the exchange of nutrients, and waste products between the mother and fetus. Endocrine signals between placenta and fetus and between mother and placenta play a critical role in the regulation of fetal development and nutrient partitioning (Godfrey, 2002).

Glucocorticoids have the capacity to move freely across the placenta. They are powerful modulators of gene expression, and studies have shown that they accelerate fetal organ maturation. The enzyme 11ß-hydroxsteroid dehydrogenase type 2 in the placenta maintains the levels of steroids in the fetus, stopping inappropriately high concentrations of active corticosteroids and thereby ensuring the appropriate development of the fetal hypothalamic-pituitary-adrenal axis.

A role of overexposure of the fetus to glucocorticoids from the mother has been proposed as a mechanism for nutritional programming. Dodic *et al* have shown that administration of dexamethasone in the early prenatal life of sheep results in elevated blood pressure, left ventricular hypertrophy and reduced cardiac functional reserve in adult life (Dodic *et al.*, 2001e).

The feeding of low protein diets in rat pregnancy reduces both the activity and the mRNA expression of placental 11ß-hydroxsteroid dehydrogenase type 2 (Bertram *et al.*, 2001). Moreover, blockade of the maternal glucocorticoid synthesis through pharmacological adrenalectomy prevents the programming of hypertension in the offspring of rats fed low protein diets in pregnancy, demonstrating the glucocorticoid dependence of the nutritional effect (McMullen & Langley-Evans, 2005). The down-regulation of 11ß-hydroxsteroid dehydrogenase type 2 by undernutrition may be the common pathway through which a broad range of nutritional insults produce a narrow and similar range of programmed responses.

1.1.9.4.3 Epigenetic mechanisms

This proposes that epigenetic modification/gene packaging in early life alters gene expression with long-term physiological effects (Razin & Cedar, 1994). Mechanisms such as DNA methylation or histone acetylation effectively silence gene expression. Recent animal studies have shown that the effects of nutrition can be relatively selective, giving rise to variation in the provision of potential methyl donors in the diet during pregnancy impacting on the expression of transposable elements in the Agouti locus (Waterland & Jirtle, 2003). The critical window for this epigenetic modification of gene expression appears to extend far beyond the embryonic period, which was originally thought to be the only time that this form of gene silencing might be sensitive to the environment. An example of this occurring in the early postnatal period was shown when stress induced behaviours of rat mothers during lactation lead to altered DNA methylation in their suckling young (Weaver *et al.*, 2004).

1.1.9.4.4 Gene expression

There are now many reports of changes in gene expression following a prenatal or early postnatal nutritional insult. Evidence of gene-nutrient interactions on the genesis of type 2 diabetes has been shown (Eriksson, 2002, Diabetes 51, 2321-2324). The effects of a polymorphism of the gene encoding peroxisome proliferators-activated receptor γ2 (PARG2) depends on birth weight, which serves as a marker for intrauterine nutrition. It has been suggested that the Pro12Ala polymorphism of the gene increases tissue sensitivity to insulin and thereby protects against type 2 diabetes. The Pro12Ala polymorphism has been shown to only influence fasting plasma insulin concentrations in men and women that had a low birth weight. It is well known that low birthweight is associated with raised plasma insulin concentrations, indicating insulin resistance. However, this difference was confined to people with the Pro12APro polymorphism and the Pro12Ala polymorphism protected against the effect. The interaction between the effects of the gene and the birth weight was statistically significant.

A fundamental problem with this theoretical mechanism is that we cannot be certain in ascertaining whether the changes in gene expression are a cause of abnormal physiology and disease or whether they are a consequence. Furthermore it is not clear whether the changes in gene expression are related to the effects of developmental plasticity, the effects of endocrine balance or even epigenetic modification. For example it has been shown that the expression of the angiotensin II AT_2 receptor is modified by feeding a low-protein diet *in utero* (McMullen *et al.*, 2004). Furthermore, DNA microarray studies have shown that the genes in the kidneys are modified by the intrauterine protein restriction (Langley-Evans

et al., 2005). However, it is also known that the AT_2 receptor expression is controlled by glucocorticoids and that levels of expression both determine the development of specialised structures within the kidney and are regulated by the presence of those specialised structures.

1.1.9.5 Predictive adaptive response model

As previously discussed, programming in early life can lead to chronic disease in later life. Disease risk appears to be partly dependant on the interaction of environmental processes in adulthood. The inter-relation of a combination of early life and later life challenges poses further questions as to how the body responds. For example, incidence in CHD is higher in those born smaller who become relatively obese as adolescents or adults (Eriksson *et al.*, 2001). When undernutrition during development is followed by improved nutrition many animals stage accelerated or "compensatory" growth which can be measured in body weight or length. This restores the animals body size but might have long-term cost, such as increased cardiovascular disease. Babies who are thin at birth lack muscle, a deficiency that will persist because of the crucial period for muscle growth is ~30 weeks *in utero*, and there is little cell replication after birth (Widdowson *et al.*, 1972). If they then gain weight rapidly in childhood, they are liable to put on fat rather than muscle, leading to disproportionately high fat mass in later life. This might be associated with the development of insulin resistance because children and adults who had low birth weights but overweight later in life have a high incidence of insulin resistance (Barker *et al.*, 1993a).

Another mechanism linking retarded early growth followed by compensatory growth with later disease is through the effect of growth on the kidney. Small babies have reduced numbers of nephrons (Mackenzie & Brenner, 1995;Merlet-Benichou *et al.*, 1994). It has been suggested that this leads to hyperperfusion of each nephron and consequent glomerular sclerosis. Rapid childhood growth is thought to then increase the hyperperfusion. Aging brings nephron death and a cycle of increasing blood pressure, glomerular sclerosis and further nephron loss, leading to the development of hypertension. This mechanism proposes that essential hypertension is a disorder of growth with two separate mechanisms, i.e. a growth-promoting process in childhood and a self-perpetuating mechanism which has its effects later in adult life. This would explain the small effects of birth size on blood pressure levels in the normal population, but its large effects on the risk of hypertension (Barker *et al.*, 2002).

Gluckman and Hanson (Gluckman & Hanson, 2004d) have termed this process of fetal programming the "predictive adaptive response" (PAR), and defined its theoretical developmental concept. This model proposes that environmental factors acting in early life induce changes in fetal development. These fetal developments result in a permanent change in the physiology or morphology of the individual. The changes seen are not responses to the underlying environment at the time of exposure, but rather are based on the fetus's prediction of its extra-uterine environment. It is proposed that these changes are therefore designed to optimise survival in its predicted environment, and therefore define a range of environments within the individual can optimally thrive until and through its reproductive phase. However, should the actual postnatal environment fall out with this

predetermined range, then conversely the individual is maladapted and therefore predisposed to disease.

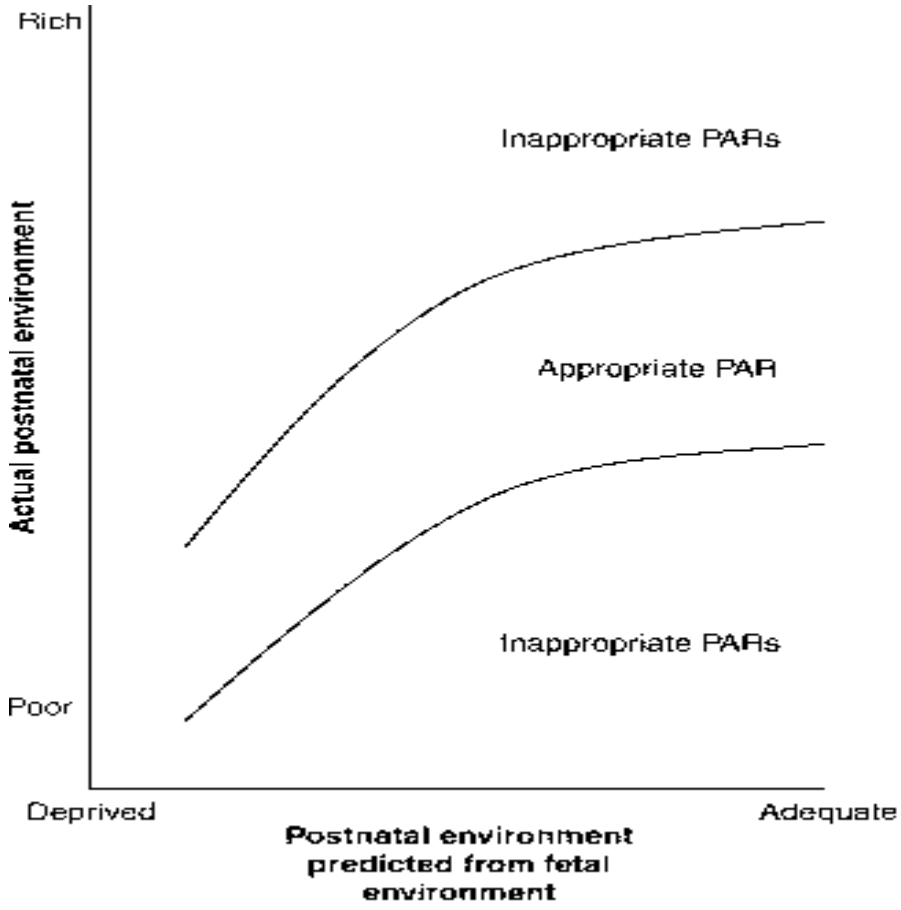


Figure 1.2: Schematic summary of the predictive adaptive response model (adapted from (Gluckman & Hanson, 2004d).

Figure 1.2 illustrates this model showing the horizontal axis representing the range of the postnatal environment as predicted from the environment to which the fetus is exposed. The vertical axis represents the range of environment to which the individual is actually exposed during reproductive adult life. The curves show the upper and lower limits of the combination of fetal prediction and postnatal environment for achieving health.

One advantage of the PAR model is that it explains the importance of the post natal environment and in particular how mismatches in the pre- and post natal environment amplify the risk of disease in later life. Another strength of the PAR model is that it emphasises the role of the maternal environment as a mediator of health as well as disease. An implicit assumption of all previous models is the concept of a "trade off "– i.e. in response to a hostile intra-uterine environment, the developing organism makes immediate adaptive responses by reducing either body growth or growth of individual organs to promote its chances of immediate survival. It then has to cope throughout its life with the consequences of the irreversible components of this prenatal adaptive strategy (Hales & Barker, 2001). By contrast, the PAR model emphasises the importance of maternal environment not only in the development of disease, but as a protective adaptive mechanism.

If the prenatal and postnatal environments match, the physiological settings achieved through the processes of developmental plasticity will leave the organism well prepared for the postnatal environment. Conversely, mismatches between the prenatal and postnatal environment may be pathogenic.

The PAR model emphasises the importance of mismatches between the pre- and postnatal environment in determining cardiovascular function. There have to date been few studies assessing this in the animal model. Part of this thesis will test this hypothesis when looking at the cohort of animals that had both prenatal and early postnatal nutritional challenges.

1.2 Cardiovascular Disease

1.2.1 Definition

Cardiovascular disease (CVD) is a broad term encompassing a wide range of disease pathologies, including ischaemic heart disease, stroke, and sudden cardiac death. At different stages of this thesis I will focus on the various disease states and the underlying physiology involved.

In the early 1900's diseases of the heart and circulatory system accounted for less than 10 % of all deaths worldwide. By the end of the century cardiovascular disease was the number one cause of death in the UK and the rest of the western world, accounting for nearly half of all deaths in the developed world and a growing proportion of 25 per cent in the developing world (The World Health Report, 1999). In 2005 in the UK alone there were 208,000 deaths from CVD with more than one in three (36%) people dying from cardiovascular disease. In the UK cardiovascular disease is the main cause of premature death (death before the age of 75 years; Office for National Statistics, 2005). About half (48%) of all deaths from cardiovascular disease are from coronary heart disease, about a quarter (28%) are from stroke and approximately 4% due to arrhythmias. Although CVD rates are declining in the western world, they are increasing in almost every other region of the world. From a worldwide perspective the global burden of CVD is accelerating, reflecting the change in the developing countries' economies. By 2020 it is predicted that CVD will surpass infectious disease as the world's number cause of death and disability.

I will now give a brief overview of the cardiovascular disease processes relevant to my thesis.

1.2.2 Hypertension

This is a term used to describe chronic elevation of arterial blood pressure. In humans this is currently defined as systolic or diastolic pressures equal to or greater than 140mmHg or 90mmHg respectively. Hypertension has been shown to be a significant risk factor for cardiovascular disease. In 90 to 95 % of patients diagnosed with hypertension no known cause is found and this is termed "essential" or primary hypertension. Therefore it is only the remaining 5 to 10% where an underlying cause is found. Despite extensive research no unifying hypothesis accounts for the pathogenesis of essential hypertension. However, a natural progression of this disease suggests that early elevations in blood volume and cardiac output may precede and then initiate subsequent increases in systemic vascular resistance. Untreated hypertension can lead to left ventricular dysfunction, stroke, ischaemic heart disease, peripheral vascular disease, renal dysfunction and retinopathy. Nearly 1 in 3 adults in the USA have hypertension (Fields *et al.*, 2004). Understanding of this disease process is therefore essential for detection, treatment and importantly prevention in order to limit the subsequent disease burden.

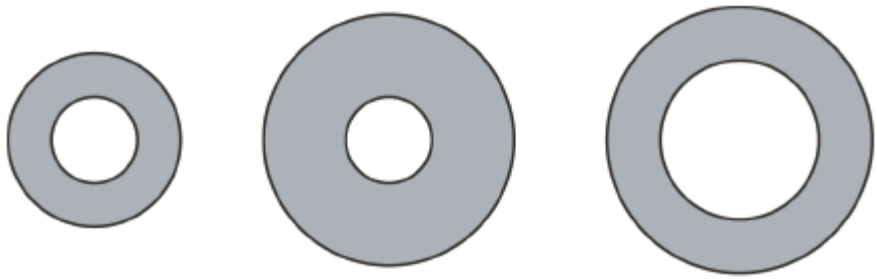
1.2.3 Coronary heart disease

This relates to the disease processes that affect the coronary arterial circulation with consequences for the heart and its function. Hence myocardial ischaemia occurs when there is an imbalance between the supply of oxygen and other essential nutrients and the myocardial demand for these substances. This most commonly occurs as a result of obstructive coronary heart disease in the form if coronary atherosclerosis. Coronary atherosclerosis is a complex process characterised by accumulation of lipid, macrophages and smooth muscle cells in intimal plaques in the large and medium sized epicardial coronary arteries. This eventually leads to a reduction in luminal diameter that will cause a haemodynamically significant stenosis. At this stage smaller distal intramyocardial arteries and arterioles are maximally dilated and any increase in myocardial oxygen demand provokes ischaemia. Disease can manifest itself also by plaque rupture. This can then also lead to myocardial ischaemia due to a critical stenosis, or alternatively complete occlusion of the vessel leading to myocardial infarction.

1.2.4 Left ventricular dysfunction and heart failure

Left ventricular hypertrophy is defined as an increase in the mass of the ventricle, which can be secondary to an increase in wall thickness, and increase in cavity size, or both. This occurs as the ventricle adapts to increased stress, such as chronically increased volume load or increased pressure load (as in hypertension). Although hypertrophy is a physiological response to increased stress, the response can become pathological and ultimately lead to deterioration in function. For example, hypertrophy is a normal

physiological adaptation to exercise training that enables the ventricle to enhance its pumping capacity. This type of physiologic hypertrophy is reversible and non-pathological. In contrast, chronic hypertension causes pathologic ventricular hypertrophy. This response enables the heart to develop greater pressure and to maintain a normal stroke volume despite the increase in afterload. However, over time pathologic changes occur in the heart that can lead to heart failure. In the case of chronic pressure overload the inside radius of the chamber may not change; however, the wall thickness greatly increases as new sarcomeres (the myocytes basic contractile unit) are added in parallel to existing sarcomeres. This is termed concentric hypertrophy (Figure 1.3b). This type of ventricle is capable of generating greater forces and higher pressures, while the increased wall thickness maintains normal wall stress. A hypertrophied ventricle, however, becomes "stiff" (compliance is reduced) which impairs filling, reduces stroke volume and leads to a large increase in end-diastolic pressure. Changes in end-systolic volume depend upon changes in afterload and inotropy. Hence concentric hypertrophy can cause diastolic dysfunction. On the other hand, if the precipitating stress is volume overload, the ventricle responds by adding new sarcomeres in series with existing sarcomeres. This results in ventricular dilation while maintaining normal sarcomere lengths. The wall thickness normally increases in proportion to the increase in chamber radius. This type of hypertrophy is termed eccentric hypertrophy (Figure 1.3c), and often accompanies systolic dysfunction.



a) Normal b) Concentric hypertrophy c) Eccentric hypertrophy

Figure 1.3: Illustration of concentric versus eccentric hypertrophy compared to normal

The term heart failure is used when the heart is unable to supply adequate blood flow and therefore oxygen delivery to peripheral tissues or organs. Heart failure most commonly involves the left ventricle. Right ventricular failure, although sometimes found alone or in association with pulmonary disease, most often occurs secondary to left ventricular failure. Data from the US Framingham Heart Study indicate that heart failure incidence approaches 10 per 1,000 population after age 65 (Lloyd-Jones *et al.*, 2002). The numbers are rapidly increasing owing to an aging population. Despite many new advances in drug therapy and implantable devices the morbidity and mortality for chronic heart failure remains high.

1.2.5 Cardiac Arrhythmias and Sudden Cardiac Death

Sudden cardiac death is defined as an unexpected death due to a cardiac cause within one hour of symptoms (Myerburg & Castellanos A, 1986), is not necessarily arrhythmia induced. However, a malignant ventricular arrhythmia has been estimated to be causative in up to 80% of such deaths (Bayes de *et al.*, 1989). Sudden cardiac deaths

account for more than 250,000 deaths per year in the United States (Thom *et al.*, 2006). The incidence of sudden cardiac death increases with increasing age. This is not surprising since cardiac arrhythmias are commonly associated with underlying cardiac pathology such as coronary heart disease and heart failure (Davies, 1981). However, this can occur in an otherwise healthy heart owing to inherited mutations in genes encoding ion channel function.

1.3 The Development of Heart

In order to study cardiovascular pathophysiology in the context of this thesis and the developmental origins of disease hypothesis it is first necessary to understand its development and function.

The heart is the first organ to form and function in the embryo, and all subsequent events in the life of the organism depend on the hearts ability to match its output with the demands for oxygen and nutrients. The formation of the heart involves a number of complex processes which include cell division, cell enlargement, cell death, and cell migration. By these processes, the heart is formed from an undifferentiated mass of cells in the early embryo to a fully formed heart with a full complement of cardiac cells in the late embryo.

This development of the human heart is described in detail in this section. There is very little equivalent literature specific to the sheep. This human data can then be extrapolated to the sheep model.

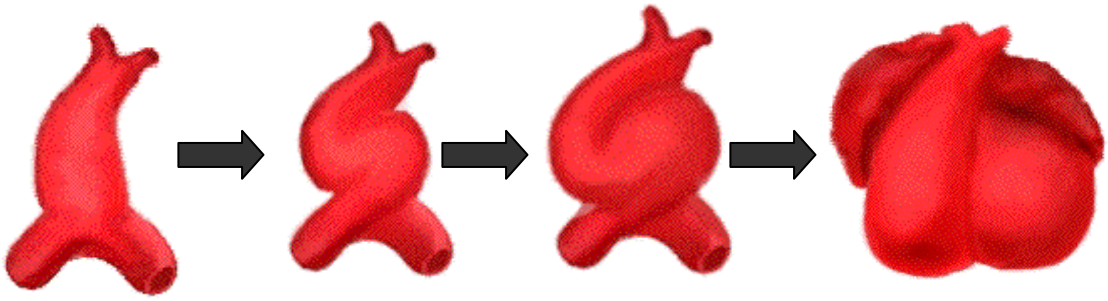
1.3.1 Heart tube formation

As early as 15 days after conception cells derived from the mesoderm become localized in the developing embryo. At days 18 to 20 these angiogenic cell clusters coalesce to form the left and right endocardial tubes. They then move towards each other and by day 22 the endocardial tubes have fused to form a single heart tube. The first pulsations of the primitive circulatory system start approximately 22 days after fertilization. At this point, the embryonic heart is a tube divided at each end (Fig 1.4a). By 24 days its pulsations are capable of generating blood flow.

1.3.2 Looping and Septation

As the primitive heart continues to develop, it begins to lengthen. From day 22 to day 24, the heart tube doubles in length. The ends of the tube are fixed, therefore it is forced to bulge and twist within the pericardial sac (Figure 1.4b). This "looping stage" is characterised by a rightward bulging of the middle of the heart, which continues to bend until a C-shaped loop is formed (Fig 1.4c; (Kramer & Yost, 2002). Between days 25 and 28 dilatation of the ventricle occurs forming a bulboventricular loop (Lotze *et al.*, 1999). This transforms the fetal heart from a pulsatile tube into the hollow single chambered pump. "Septation" then occurs, which involves the formation of the atrial septum, interventricular septum and the atrioventricular septum, partitioning the heart into four chambers (Fig 1.4d). This multichambered heart enables the right (pulmonary) and left (systemic) ventricular and atrial chambers of the heart to create a dual circulation supplying the lungs and body. At the end of the 7^{th} week the human heart has reached its final stage of development.

Because the fetus does not use its lungs, most of the blood is diverted to the systemic circulation. This is accomplished by a right to left shunting of blood that occurs between the two atria.



a) **b)** **c)** **d)**

Figure 1.4: Illustration of the fetal development of the heart
(modified from Paul Bauer, Animated Cardiac Development, 2002 RnCeus Interactive)

1.3.3 Heart Valves and Arterial Trunks

Formation of the cardiac valves essential for unidirectional blood flow, arise from localised swellings of the endocardium (endocardial cushions). Early in the 8th week neural crest cells are important in the formation and patterning of the outflow tract and great arteries.

1.3.4 Cardiac Conduction System

Each heartbeat with cardiac contraction and relaxation is controlled by a specialised cluster of cells in the right atrium, called the sinoatrial node, which functions as the

pacemaker. Electrical impulses are propagated through the heart by the cardiac conduction system. The embryonic lineage that form the cardiac conduction system have been unclear until recently, when it was shown that Purkinje cells are derived from a subpopulation of the ventricular cardiomyocytes in response to signalling by endothelin-1 and neuregulins (Gourdie *et al.*, 1998;Pennisi *et al.*, 2002).

1.3.4.1 The Development of the Cardiac Conducting System

The cardiovascular system is the first organ system to form and function in the developing embryo. In the early stages the heart is a slow conducting, single tubular structure generating an even peristaltic contraction. During embryogenesis it then develops into a four chambered heart with synchronous contraction for a dual circulation. These synchronous contractions of the atrial and ventricular chambers are dependent on the development of high conduction velocities resulting in alternating slow and fast conducting segments. In the early embryonic heart tube, an electrocardiogram similar to that of an adult has been recorded. This indicates the electrical activity of sequentially activated heart chambers (Van Mierop, 1967), so demonstrating the functioning of the cardiac conduction system (Uysalel *et al.*, 1996), before any morphological development can be distinguished. Throughout the developmental process the heart must maintain its rhythmical contractions through coordinated activation of the myocardium.

The components of this system include the sinoatrial (SA) node, the atrioventricular (AV) node, and the ventricular conduction pathway consisting of the bundle of His, the right bundle branch, the left bundle branch and the Purkinje fibres.

1.3.4.2 The Sinoatrial node

In the adult the SA node is found at the junction of the right common cardinal vein and the wall of the right atrium, within the terminal groove. These cells have the most rapid inherent rhythm so setting the rate for the rest of the myocardium, and thereby function as the dominant pacemaker. This pacemaker activity has been illustrated in chick embryos at an age approximately equivalent to 20 days in the human embryo (Hirota *et al.*, 1979). The pacemaker activity develops in the inflow tract of the primary heart tube and is the first element to function in the CCS. Action potentials spread from the posterior inflow tract to the anterior outflow tract of the heart generating a wave of contraction (Kamino *et al.*, 1981). However it is not until approximately 35 days of human development that a morphologically identifiable SA node can be seen (Viragh & Challice, 1980). Little is known about mechanisms that initiate and maintain the growth of the SA node. The impulse is then conducted via the atrial myocardium to the AV node.

1.3.4.3 The Atrioventricular node

When formed, the AV node is found at the base of the interatrial septum close to the endocardium, at the apex of the triangle of Koch. It delays the impulses passage from atria to ventricular myocardium. The AV node like the SA node performs this function before being morphologically identifiable (Moorman *et al.*, 1998), and is first recognisable when the looping heart divides into atrial and ventricular components from ~5 weeks of human development onwards (de Jong *et al.*, 1992;Lieberman & Paes de, 1967;Pennisi *et al.*, 2002).

1.3.4.4 The Origin of Nodal Cells

The nodal cells of the AV and SA node are similar to embryonic cardiomyocytes. They are small with a poorly developed sarcoplasmic reticulum and lack a functional contractile unit due to poorly organised actin and myosin filaments. Advances in molecular markers have recently enabled us to trace the development of these nodes. In a murine model the GATA-6 reporter gene has marked an atrioventricular ring that subsequently integrates into the atrioventricular conduction system. This ring shows predominant staining in the wall to the right side of atrioventricular canal, thought to be the developing AV node during the process of chamber formation

1.3.4.5 Ventricular Conduction Pathway

The ventricular conduction pathway (VCP) enables the rapid passage of impulses into the contractile ventricular myocardium. This is facilitated by organised high-conductance gap junction proteins. This is essential for activation of the ventricles from apex to base, resulting in the efficient ejection of blood from the ventricles into the outflow tracts at the base of the heart. It can be divided into sections. The bundle of His emanates from the AV node at the posterior right atrial wall near the atrial septum above the atrioventricular groove. This runs through the upper margin of the ventricular septum before bifurcating becoming the left and right bundle branches which descend on either side of the septum. These end by dividing, to give rise to the Purkinje fibres. These fibres branch to form a terminal network that lies just underneath the cardiac endothelial surface.

1.3.4.6 Origin of the Ventricular Conduction Pathway Cells

Fate map studies using chick embryos have found that cells from three distinct embryonic origins, namely the cardiogenic mesoderm, the neural crest and the proepicardial organ constitute the cell lineage of the heart. It had previously been suggested that the CCS originated from the neural crest. Indeed cells of the CCS do express markers common to neuronal cells such as HNK-1 and some neurofilament proteins (Gorza *et al.*, 1988). However it has since been shown that the cells of the VCP are derived from a subset of embryonic myocytes. Retroviral lineage studies on chick embryo cells from each of the three cell lines were tagged using replication incompetent vectors (Cheng *et al.*, 1999;Gourdie *et al.*, 1995). Tagged Purkinje fibre cells were found exclusively in the myocytes clones, and no conduction cells were produced from cardiac neural crest or primordial epicardial cells. These studies also went on to demonstrate that cells of the CCS were recruited locally rather than by "outgrowth" and branching of the early framework.

1.3.4.7 Purkinje Fibre Differentiation

In the maturing chick embryonic heart Purkinje fibres develop in the subendocardium along coronary artery branches, suggesting a role of arteriogenesis in the differentiation of Purkinje cells (Davies, 1930;Takebayashi-Suzuki *et al.*, 2000;Vassall-Adams, 1982). This led to the belief that embryonic myocytes may be induced to form Purkinje fibres by receiving paracrine signals originating from arterial vascular tissues. It has now been demonstrated that cultured embryonic myocytes convert to a Purkinje cell

phenotype after exposure to one such paracrine factor, endothelin (Gourdie *et al.*, 1998). Furthermore, this inductive response declined with the progression of development suggesting that the responsiveness of myocytes to endothelin is a distinct developmental process. The potent vasoconstrictor endothelin is a shear stress-induced cytokine abundant in the arterial system, and endothelin receptors are present in all myocytes (Molenaar *et al.*, 1993). This may be of particular importance in helping us to understand how the development of the CCS can be affected by environmental factors, such as shear stress or pressure. It should be noted that these studies used avian models. Optical mapping of cardiac electrical activity using a voltage-sensitive dye in the murine CCS has demonstrated that a functional His-Purkinje system exists surprisingly early, even before septation has begun. Therefore in the murine model a functioning network of conducting cells exists prior to the formation of the coronary vessels, so questioning the arteriogenesis theory (Rentschler *et al.*, 2001). It may be that a basic structure is initially laid down following which cardiomyocytes continue be recruited in order to expand the conductive cell network. This will require further lineage analysis studies.

1.3.5 Development of the Cardiomyocyte

Growth of the human heart during and after these early developmental periods is primarily through hyperplasia (cell division). While the fetal heart grows by myocyte enlargement and proliferation, myocytes lose their capacity for proliferation in the perinatal period after terminal differentiation (Smolich *et al.*, 1989). Unlike humans small mammals (rat and rabbit) myocytes continue to mature after birth (Klitzner, 1991). Large mammals

such as sheep tend to have young that are more mature than rodents after birth as in humans (Oparil *et al.*, 1984). In the fetal sheep, as in many other species, terminal differentiation is marked by the appearance of two nuclei in the cardiac myocyte that were formerly mononucleated. The rate of proliferation during intrauterine life and the occurrence of terminal differentiation in the perinatal period together determine to maximum number of myocytes in the heart for life (Jonker *et al.*, 2007). Hence, after birth the postnatal heart grows almost exclusively by cellular enlargement (Majamaa-Voltti *et al.*, 2002).

Thus, loss of cardiac myocytes after myocardial damage results in irreparable damage to the adult heart, and so increased likelihood of eventual heart failure and early death.

Intrauterine conditions affect cardiac myocyte proliferation, enlargement, and terminal differentiation, but myocyte responses to abnormal conditions depend on the level of maturity of the heart. For example increased systolic or diastolic haemodynamic loads in near-term fetal sheep have been shown to increase myocardial mass (Davis & Hohimer, 1991). It is not known if the growth of these loaded hearts occurs by increased myocyte proliferation or by myocyte enlargement. Adult hearts typically respond to increases in haemodynamic load by a combination of myocyte enlargement and ventricular remodelling to reduce myocardial systolic wall stress (Grossman *et al.*, 1975).

1.3.6 The Fetal Circulation

The fetal circulation differs from the adult circulation by having an umbilical circulation and vascular shunts (the foramen ovale, the ductus arteriosus and the ductus venosus).

The umbilical vein returns from the placenta carrying oxygenated blood and nutrients from the mother. This supplies the fetal tissues with oxygen and nutrients such as glucose, lactate and amino acids.

Blood flows from the umbilical vein into the porta hepatis where it is joined by the portal vein. Blood flow in the portal sinus then divides to branches of the left and right lobes of the liver, or through the ductus venosus, which connects to the inferior vena cava, allowing umbilical venous blood to bypass the hepatic circulation (Rudolph, 1985). In the adult, blood entering the heart through the vena cava would enter the right atrium, pass through to the right ventricle and be ejected through the pulmonary artery to the pulmonary circulation. In the fetus the lungs do not function as organs of gas exchange, and thus blood flow is shunted away from the pulmonary circulation by means of the two vascular shunts, the foramen ovale and ductus arteriosus. In the fetus, blood flow in the vena cava can enter the right atrium or pass through the foramen ovale to the left atrium. Blood ejected from the right ventricle can pass to the lungs via the pulmonary artery, but the majority passes through the ductus arteriosus and enters the systemic circulation along with blood ejected by the left ventricle (Ramsey, 1985). Cardiac output at this stage is therefore determined by summing both the left and right ventricular outputs and described as the combined ventricular output.

1.3.7 Transition at Birth

At birth, important changes occur in the cardiovascular system which involve removal of the placenta, the closure of the vascular shunts and changes in the regional blood flow including increased flow to the lungs (Walker, 1993).

The fetal circulation function is replaced by two distinct circulations that function in series. This is achieved by closure of the ductus venosus, the foramen ovale and the ductus arteriosus. The umbilical circulation supplies 95% of the ductus venosus blood flow. When this is cut out at birth there is a large fall in flow followed by permanent closure of the ductus venosus by proliferation of connective tissue into the lumen (Meyer & Lind, 1965). At birth the increased blood flow to the pulmonary circulation leads to increased pulmonary return and a concurrent reduction in blood flow to the inferior vena cava due to removal of the umbilical circulation. This pressure change leads to closure in the foramen ovale. The oxygen tension at birth produces vasoconstriction of the smooth muscle of the ductus arteriosus leading to its closure (Clyman, 1987). The increased oxygen levels produced by breathing are vital for cardiovascular adjustment at birth, including vasodilatation and decreased vascular resistance in the pulmonary circulation. The decrease in pulmonary vascular resistance results in a decrease in the afterload exerted on the right ventricle, and this in addition to the increased venous return to the left ventricle and the surge in catecholamines at birth contributes to the elevation of cardiac output at birth (Berning *et al.*, 1997).

1.4 Adult Cardiovascular Physiology

1.4.1 The Circulatory System

It simple terms this is made up of two primary components, the heart and the blood vessels. The heart can be viewed functionally as two pumps with the pulmonary and systemic circulations situated between the two pumps (Fig. 1.5). In this way the right side of the heart with the pulmonary circulation, and the left side of the heart with the systemic circulation are arranged in series.

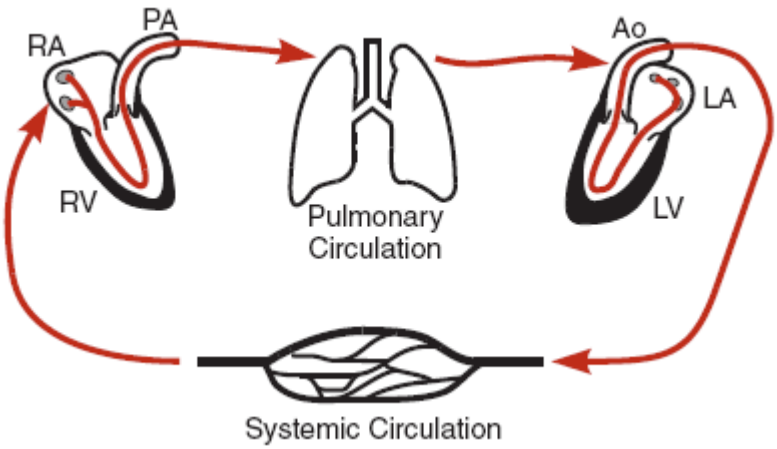


Figure 1.5: Diagram of the overview of the cardiovascular system.
RA, right atrium; V, right ventricle; PA, pulmonary artery; Ao, aorta; LA, left atrium; LV, left ventricle (Abbas et al., 2005;Levy & Pappano, 2006).

Therefore, all of the blood that is pumped from the right ventricle enters into the pulmonary circulation and then into the left side of the heart from where it is pumped into the systemic circulation before returning to the heart. This in-series relationship of the two sides of the heart and the pulmonary and systemic circulations requires that the output of

each side of the heart closely matches the output of the other so that there are no major blood volume shifts between the pulmonary and systemic circulations.

The pulmonary circulation is the blood flow within the lungs that is involved in the exchange of gases between the blood and alveoli. The systemic circulation is comprised of all the blood vessels within and outside of organs excluding the lungs. The right side of the heart comprises the right atrium and the right ventricle. The right atrium receives venous blood from the systemic circulation and the right ventricle pumps it into the pulmonary circulation where oxygen and carbon dioxide are exchanged between the blood and alveolar gases. The left side of the heart comprises the left atrium and the left ventricle. The blood leaving the lungs enters the left atrium by way of the pulmonary veins. Blood then flows from the left atrium into the left ventricle. The left ventricle ejects the blood into the aorta, which then distributes the blood to all the organs via the arterial system.

The pumping activity of the heart is usually expressed in terms of its cardiac output, which is the amount of blood ejected with each contraction (i.e., stroke volume) multiplied by the heart rate. Any factor that alters heart rate or stroke volume will alter the cardiac output. The heart rate is determined by groups of cells within the heart that act as electrical pacemakers (see Section 1.3.4), and their activity is increased or decreased by autonomic nerves and hormones (see Section 1.4.2). The action potentials generated by these pacemaker cells are conducted throughout the heart and trigger contraction of cardiac myocytes. This results in ventricular contraction and ejection of blood. The force of ventricular contraction, and therefore stroke volume, is regulated by mechanisms intrinsic to the heart, by autonomic nerves and hormones.

1.4.1.1 Echocardiography

This is the use of ultrasound to examine the heart and record information in the form of echoes i.e. reflected sonic waves (Calsen, 1975;Feigenbaum, 1994). The technique I used in this research is the transthoracic approach. Here the ultrasonic transducer is placed on the surface of the thorax and the ultrasonic beam is directed towards the part of the heart being examined. In this way the cardiac walls can be defined and seen to move with the cardiac cycle inscribing a greyscale signal, whilst blood-filled cavities are relatively echo free (appears black).

Acoustic windows are transducer positions that allow ultrasound access to the cardiac structures, which is limited by the bony thoracic cage and the adjacent air-filled lung. Each echocardiographic image is defined by its acoustic window i.e. the position of the transducer, and the view i.e. the image plane. In this way a two dimensional echocardiographic image of the heart can be obtained. There are many different imaging modalities such as M-mode which uses a focused ultrasonic beam with a high sampling rate (1800 frames per second vs. 30 to 60 frames per second for 2D imaging). This enables identification of thin and continuously moving structures such as the ventricular endocardium more accurate and reproducible (Fortuin & Pawsey, 1977). However, the potential disadvantage of M-mode data is that a non-perpendicular orientation of the structure may be obtained. This can be avoided by using the 2D imaging to position the M-mode sampling plane (Abbasi *et al.*, 1973).

An M-mode image perpendicular to the long axis of and through the centre of the left ventricle at the papillary muscle level provides standard measurements of systolic and diastolic wall thickness and chamber dimensions. These measurements only represent a single line through the left ventricle and thus do not accurately describe the left ventricle when regional wall abnormalities arise, such as after a myocardial infarction, when they must be used with caution (Sahn *et al.*, 1978a).

1.4.2 The Autonomic Nervous System

The autonomic nervous system is an important regulator of cardiovascular function. In addition to autonomic nerves, many circulating factors (humoral substances) exist that affect cardiac and vascular function. The humoral factors include circulating catecholamines, epinephrine (adrenaline) and norepinephrine (noradrenaline). These originate from two sources:-

i) The adrenal medulla releases catecholamines (80% epinephrine, 20% norepinephrine) when preganglionic sympathetic nerves innervating this tissue are activated. This occurs during times of stress.

ii) Sympathetic nervous system (SNS) is another source of circulating catecholamines, principally norepinephrine.

Endogenous norepinephrine functions primarily as local neurotransmitter at sympathetic postganglionic receptors. Epinephrine may stimulate the same receptor sites but reaches that site as a blood-borne hormone from the adrenal medulla, together with

medullary norepinephrine. Stress studies have confirmed the concept of the hormonal nature of epinephrine and the local neurotransmitter nature of norepinephrine (Chernow *et al.*, 1982). Normally, most of the norepinephrine released by the sympathetic nerves is taken back up by the nerves and metabolized. However, a small amount of released norepinephrine diffuses into the blood and circulates through-out the body. At times of high levels of sympathetic nerve activation, the amount of norepinephrine spilling over into the blood can increase dramatically.

Stress causes activation of the hypothalamic-pituitary-adrenocortical axis and the SNS, with responses that include increases in body temperature, blood pressure, heart rate, and plasma catecholamine and glucocorticoid concentration.

Circulating epinephrine has several direct cardiovascular actions that depend upon the relative distribution of adrenergic receptors in the different organs and the relative affinities of the different receptors for epinephrine. Epinephrine has a much greater affinity for ß-adrenoceptors than α-adrenoceptors. Therefore at low to moderate circulating levels epinephrine preferentially binds to ß-adrenoceptors, resulting in stimulation of heart rate, inotropy and dromotropy (primarily ß1-adrenoceptor mediated). At low concentrations epinephrine also binds to ß2-adrenoceptors located on small arteries and arterioles (particularly in skeletal muscle) and causes vasodilatation. The net result is an increase in heart rate. Although it increases systolic pressure it also decreases diastolic pressure due to vasodilatation and a consequent decrease in systemic vascular resistance with very little change in the mean arterial pressure (Figure 5.1).

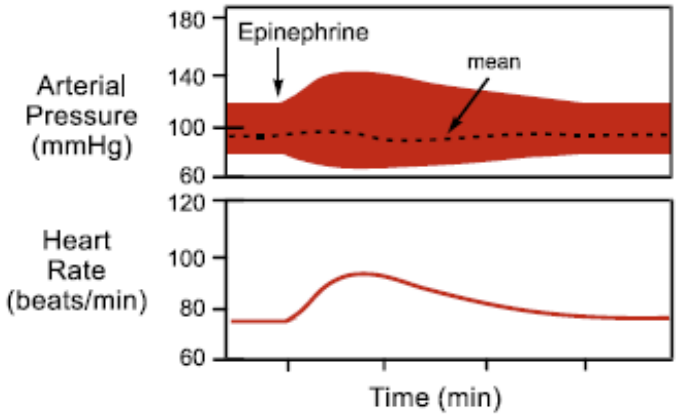


Figure 1.6: Effects of intravenous low dose epinephrine on blood pressure and heart rate (adapted from Cardiovascular Physiology Concepts - Klabunde)

With increasing concentrations of epinephrine there is further cardiac stimulation along with α-adrenoceptor mediated activation of vascular smooth muscle leading to vasoconstriction. This increase in both cardiac output and systemic vascular resistance therefore leads to an increase in arterial blood pressure.

Circulating norepinephrine has a high affinity the ß1 and α1-adrenoceptors, and so affects the cardiovascular system predominately through ß1 and α1-adrenoceptors mediated affects. This results in an increase in mean arterial blood pressure and pulse pressure. There is initially a transient increase in heart rate due to norepinephrine binding to the ß1-adrenoceptors in the sinoatrial node followed by a secondary bradycardia due to a vagal-medially baroreceptor reflex (in response to the increase in arterial pressure).

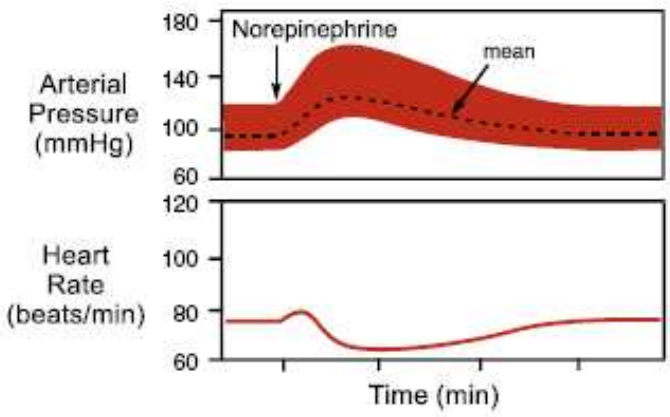


Figure 1.7: Effects of intravenous norepinephrine on blood pressure and heart rate (adapted from Cardiovascular Physiology Concepts - Klabunde)

It is important to remember that most norepinephrine released by the sympathetic nerves is taken up and metabolised locally. It is only an "overspill" that will be allowed to diffuse into the blood circulation and therefore have a systemic affect.

The stress response is largely mediated by the hypothalamic-pituitary-adrenal (HPA) axis and the sympathoadrenomedullary (SA) axis. Activation of the HPA axis increases the secretion of corticotrophin-releasing factor (CRF), which stimulates ACTH and leads to glucocorticoid release from the adrenal cortex. Activation of the SA axis, however, increases the release of epinephrine and norepinephrine from the adrenal medulla and stimulates the sympathetic norepinergic nerves, increasing norepinephrine secretion.

Previous research has looked at differential stressor effects (psychological and physical) on the concentration of plasma catecholamines in animals. Psychological stress can evoke hypertension, tachycardia, and arrhythmia (Morimoto *et al.*, 1993;Nakamori *et al.*, 1993). Isolation is recognised as a psychological stress in social ungulates/herd animals such as sheep. A previous study looked stress responses in sheep transferred in a trolley a

"short distance" from the holding barn into an "isolation hut" (Parrott *et al.*, 1994) as well as other stressors such as motor transport stimulation and standing in water. The "transfer and isolation" resulted in the most significant response with a large and sustained increase in epinephrine release within 10 minutes of initiating the stressor. There was also an increase in the plasma norepinephrine within the first 10 minutes, although this trend did not reach statistical significance ($P<0.08$). Isolation has been shown to increase plasma norepinephrine levels in goats (Carbonaro *et al.*, 1992).

The association between low birth weight and increased incidence of cardiovascular and metabolic disease in later life may be due to hyperactivity or resetting of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathoadrenomedullary axis in response to stress in prenatal and early postnatal life. Both the HPA and sympathoadrenomedullary axis is functional *in utero* and has been shown to influence metabolism and cardiovascular function in the fetus during late gestation (Tangalakis *et al.*, 1992b). Low birth weight pigs have been shown to have altered cardiovascular function with elevated blood pressure at 3 months (Poore *et al.*, 2002), and elevated adrenal responsiveness to insulin induced hypoglycaemia at 1 year of age (Poore & Fowden, 2003b).

1.4.2.1 Analysis of heart rate response

In order to analyse heart rate response an electrogram must be recorded. Implantable loop recorders have developed to permit cardiac monitoring to capture the cardiac electrogram without the need for external monitoring. The Reveal device is one

such implantable loop recorder which has the ability to "freeze" the current and preceding rhythm for up to 40 minutes after activation. The ECG signal is stored in a circular buffer capable of retaining recorded rhythm. It is implanted subcutaneously over the thoracic cavity. It measures 6.1 x 1.9 x 0.8cm, and weighs 17 grammes. It has two bipolar sensing leads 3.7cm apart within the shell of the device (Kenny & Krahn, 1999).

The use of an implantable device is essential to this experiment. It limits problems with artefact which is particularly problematic when transporting a large animal in a crate. It also minimises human contact potentially confounding the stress response being measured.

1.4.3 The Adult Cardiac Conduction System

Propagation of electrical impulses throughout the heart is through the specialized cardiac conduction system, which includes the sinoatrial node; atrioventricular (AV) node; His bundle; right and left bundle branches; and the Purkinje system.

Both the ordinary myocardium (atrial and ventricular) and the specialized cardiac conduction system allow conduction of electrical impulses. Most cells in the cardiac conduction system also depolarize spontaneously, which enables these cells to function as cardiac pacemakers. The inherent spontaneous rate of depolarization is progressively slower from the sinus node down to the Purkinje fibres. The sinus node is therefore the dominant pacemaker.

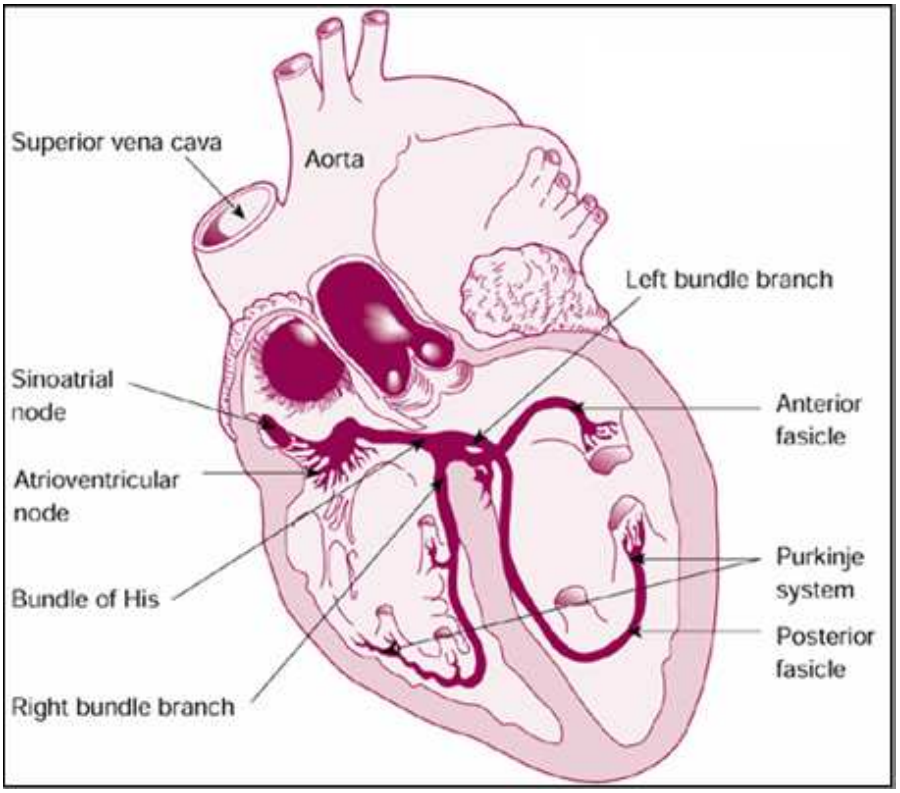


Figure 1.8: Illustration of the adult human heart and its normal cardiac conduction system (adapted from (Camm, 1998)

Sinus node automaticity is not recorded on the surface ECG. Sinus impulse activates the atrial myocardium. Activation of the atrial myocardium produces the P wave on the surface ECG. During sinus rhythm, the initial part of the P wave represents right atrial activation, and the terminal part of the P wave represents activation of the left atrium with some overlap in the middle. The impulse then depolarizes the AV node, the His bundle, the bundle branches, the Purkinje network and the ventricular myocardium. Propagation of impulse through the AV node, His bundle branch-Purkinje system is also not recorded on the surface ECG and occurs during the isoelectric PR segment. Ventricular muscle depolarization produces the QRS complex. Depolarization of the atrial and ventricular myocardium triggers the corresponding atrial and ventricular contraction. Atrial

depolarization is followed by atrial repolarization, but is generally not discernible on the surface ECG. However, recovery of the ventricular myocardium, which follows the QRS complex, is clearly recorded as a T wave on the surface ECG.

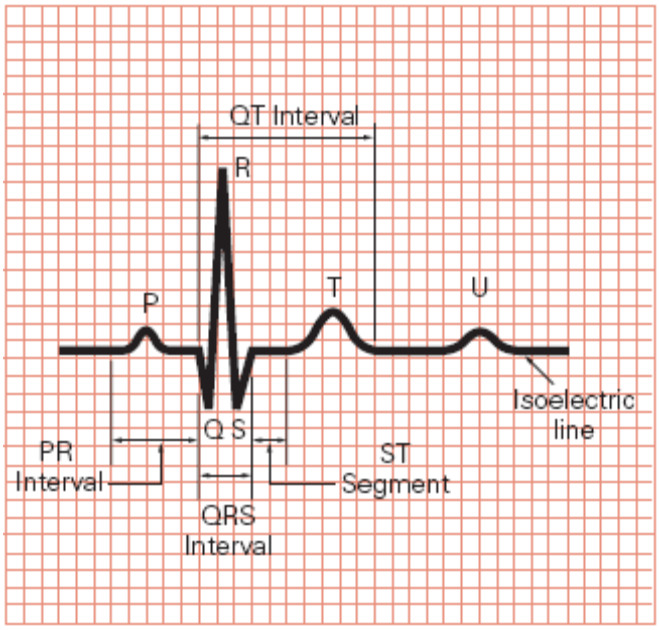


Figure 1.9:- Showing a waveform of the surface electrocardiogram

(adapted from Camm, 1998)

1.4.3.1 Evaluation of the Electrophysiological Properties of the Heart

The study of the electrical system of the heart is called cardiac electrophysiology. Various techniques can be used to evaluate the cardiac conduction system, its potential for arrhythmia and the underlying mechanisms. An understanding of the anatomy of the

cardiac conduction system and its normal electrophysiology is required, to then understand the principles of its assessment.

1.4.3.1.1 Refractory Periods

Once a cell has been depolarized, it can not be depolarized again until the ionic fluxes that occur during depolarization are reversed i.e. repolarized. The refractory period is a measure of repolarization of the cardiac tissue.

The refractory period is measured in vivo, by analyzing the tissues response to premature paced impulses.

1.4.3.1.2 Effective Refractory Period (ERP)

When introducing a premature impulse, that impulse will fail to propagate through tissue that is refractory. The ERP of a tissue is the longest coupling interval (time between the last normal impulse and the premature impulse) that fails to propagate through that tissue. This means that a premature impulse any later will propagate the impulse since the tissue has recovered and is no longer refractory.

1.4.3.1.3 Automaticity

Cardiac electrophysiology can assess the normal automaticity of the SA node, due to a phenomenon known as overdrive suppression. The myocardium is paced at a rate more

rapid than the SA node, thereby depolarizing the SA node faster than it can be depolarized by its own intrinsic automaticity. When the overdrive pacing is then stopped there is then a relative pause before the SA node recovers and begins depolarizing spontaneously again.

1.4.3.1.4 Sinus node recovery time (SNRT)

Measurement of the SNRT is based on the principle of overdrive suppression, where a pause is induced by an automatic focus using temporary overdrive pacing.

An electrode catheter is placed in the high right atrium (near the SA node). Pacing is initiated at a rate slightly faster than the basic sinus rate. Pacing is then continued at a constant rate for a fixed duration of 30 seconds and abruptly stopped. The recovery time is the interval from the last paced atrial complex to the first spontaneous SA nodal depolarization. After the initial recovery interval there is a gradual return of the sinus rate to the baseline rate. However in SA nodal disease, marked pauses can occur in the initial recovery interval or just after this (secondary pauses). A series of pacing sequences are used with gradually shortening of the drive trains paced interval down to 300msec (200 beats/min). This results in several recovery intervals determined at each paced interval. In addition the presence of secondary pauses can be recorded. The longest interval observed of either the recovery intervals or the secondary pauses can then be taken as the SNRT.

The SNRT is dependant on the basic sinus cycle length i.e. rate dependant. Sinus node recovery time therefore must be corrected (cSNRT) for the spontaneous cycle length by calculating the difference between both i.e. cSNRT = SNRT – BCL.

1.4.3.1.5 Wenckebach cycle length (WCL)

Incremental pacing can also be used to evaluate the AV conduction system. Long drive trains of atrial impulses at a constant cycle length must first be introduced. Pacing sequences slightly faster than the basic cycle length are initially used, and gradually increased (i.e. incremental pacing). At a point the AV node's refractory period will be reached. At this time, only intermittent conduction occurs through the AV node (Mobitz type I second-degree heart block). The atrial pacing rate at which this occurs is called the Wenckebach cycle length.

1.4.3.1.6 Evaluation of Susceptibility to Ventricular Tachycardia

Ventricular arrhythmias, regardless of proximate cause or ultimate mechanism are the final common pathway of sudden cardiac death. Through ventricular stimulation studies it is now understood that most patients with ventricular tachycardia have re-entrant foci as a source of their arrhythmias (Wellens *et al.*, 1976), although not exclusively (Murin & Cagan, 1995). The ability to induce VT with programmed stimulation is indicative of a re-entrant arrhythmia (Josephson *et al.*, 1978). For successful induction two prerequisites must be present. The first is an anatomical circuit with appropriate electrophysiological characteristics. The second is a critically timed premature impulse to the re-entrant circuit (Hartzler & Maloney, 1977). Delivering a premature impulse to a re-entrant circuit can in practise be challenging. It is influenced by the distance of the pacing electrode to the re-

entrant circuit, as well as the intervening tissues refractory characteristics and conduction velocity.

The principle of a VT stimulation study is based on the fact that faster pacing rates decrease the refractory period and thereby increase the conduction velocity through the ventricular myocardium. This allows premature impulses to arrive earlier at a re-entrant circuit (if present) increasing the chances of initiating re-entry. Stimulation protocols use two basic techniques to increase the likelihood of this by decreasing the refractory period. These are by coupling impulses together (typically up to three programmed extrastimuli) and pacing incrementally at rapid rates (Mann *et al.*, 1983;Rosenfeld *et al.*, 1986). The former technique will be employed in Chapter 6.

1.5 The sheep model

Firstly the animal model is crucial in further advancing our current knowledge. Animal studies provide critical data that supports the DOHaD hypothesis and enables an understanding of the mechanisms that may link nutritional factors in the early life to the functional capacity of the organs and systems in the mature state. Animal models allow the examination of specific effects of a simple dietary change, independently of the confounding factors that are inevitable associated with the long-term epidemiological studies, such a the psychological stress of a famine, or wartime environment.

The sheep model is an excellent animal model to test the developmental origins of health and disease hypothesis, and is used in my thesis. It is a docile animal and tolerant of

chronic instrumentation. It is comparable to humans in developmental aspects due to its relatively long period of gestation (~147 days). This also has the advantage that a long gestation allows a specific focused period of challenge during *in utero* development to be pin-pointed. Sheep also have a high proportion of singleton pregnancies (unlike many other animal models such as rodents and pigs). For this reason the Welsh Mountain sheep breed was used which have a particular tendency to produce singleton offspring.

It is easy to manipulate the total nutrient intake of the sheep through identification by a tag, meaning nutrition can be based on individual monitoring rather than each group as a whole. Their temperament, larger size and longer lifespan compared for example with the rodent means that regular blood samples and growth measurements are possible.

Another advantage of the sheep model is that they have a similar trajectory of organ maturation to humans. For example, as in humans cardiomyocyte and renal glomeruli cell division is completed by birth (Mackenzie & Brenner, 1995;Moritz & Wintour, 1999), unlike many other animals such as the rat where development continues for ~10 days after birth. The large size of the sheep meant that relatively large volumes of blood (<10% total blood volume) can be sampled, often necessary in studies with multiple time intervals where both venous and arterial samples as well as duplicate sample analysis may be required.

In general terms the sheep has the advantage of being well studied in developmental programming of later life pathology and has proved a robust animal model. When required the mother and fetus can be catheterised to measure fetal endocrine status, nutrient uptakes and fetal metabolism. This has been important in characterising the placental-fetal

interactions. In sheep, varying degrees of fetal growth restriction can be induced by maternal nutrient restriction, placental embolization with microspheres, surgical limitation of placental implantation sites, umbilical artery ligation, and administration of corticosteroids (Anthony *et al.*, 2003).

1.6 Overview and aims

1.6.1 Overview

The primary focus of this work is to investigate the effects of early life global undernutrition on cardiovascular function of the adult sheep. Within this I will study the effects of the combination of early gestation and early postnatal undernutrition on the adult sheep and whether this leads to an altered cardiovascular response. My thesis will analyze particular aspects of *in vivo* cardiovascular physiology incorporating cardiac morphology and function, cardiac electrophysiology, and the autonomic systems response to stress. Early life nutrient restriction may cause programmed alterations within the cardiovascular system, i.e. altered settings or sensitivity. In adulthood this may then lead to physiological responses such as hypertension, which may then in turn cause an increased risk of cardiovascular disease in later life.

In this thesis I have examined the following general hypothesis:

- **Early life global nutrient restriction affects the cardiovascular physiology of the adult sheep, and alters the response to a subsequent nutrient challenge**

1.6.2 Aims

Chapter 3

- To investigate the effect of nutrient restriction during early gestation and early postnatal life on cardiac morphology and function in adult sheep, and to determine whether early postnatal life nutrient restriction alters the effects on the heart when combined with an early gestation nutrient restriction.

Chapter 4

- To investigate the effect of nutrient restriction during preconceptional and periconceptional life on the cardiac morphology and function in the adult male sheep.

Chapter 5

- To investigate the effect of nutrient restriction during early gestation and early postnatal life on the heart rate response to stress in the adult sheep, and to determine whether the gestation nutrient restriction alters the response to a postnatal challenge.

Chapter 6

- To investigate the effect of nutrient restriction during preconceptional and periconceptional life on the cardiac electrophysiology of the adult male sheep.

Chapter 2 - General Methods

2.1 Sheep husbandry

2.1.1 Ewes

All procedures contained in this thesis were approved by the Home Office and were conducted in accordance with the UK 1986 Animals (Scientific Procedures) Act 1986. Welsh Mountain sheep were used as they are relatively small (30-50kgs), adapted to harsh highland environments and produce a higher proportion of singleton fetuses compared to other breeds.

Fertile ewes in their second or third parity and of uniform good body condition (~3 on a scale of 1–5; (Russel *et al.*, 1969) were brought to The Royal Veterinary College (North Mymms, Hertfordshire), ear tagged and then group housed in open barns on straw. They were allowed to acclimatize, for at least 1 week prior to conception, to a complete pelleted diet (GFW Titmus, Hertfordshire, UK) that provided 100% of their nutritional requirements, as per standard guidelines (Agricultural and Food Research Council, 1993). The diet consisted of barley, wheat, micronised full fat soya, grass meal, molasses, chopped straw, calcium carbonate, dicalcium phosphate salt and sheep vitamin/mineral supplement. As fed, it provided 9.6 MJ/kg (metabolisable energy) and 14.75% crude protein.

All routine vaccinations, anthelmintic treatments, and foot care were performed as necessary. The health and welfare of the animals was checked throughout the study and any

signs of ill health or disease were reported to a veterinary surgeon who advised treatment according to standard veterinary practice.

2.1.2 Mating

All ewes oestrous cycle were synchronised by the use of medroxyprogesterone acetate impregnated sponges (Veramix, Upjohn Ltd, Crawley, UK). Sponges were removed on Study Day "minus 2". One of three raddled Welsh Mountain ram was then introduced for three days. Day 0 of gestation was taken as the first day at which an obvious raddle mark was observed. Pregnancy was confirmed by measuring plasma progesterone concentrations at day 16 of gestation (Ridgeway Science Ltd, Avington, UK). On Day 60 each ewe underwent a uterine ultrasound scan to confirm pregnancy. Any remaining non-pregnant ewes were withdrawn from the study.

2.1.3 Maternal Dietary Manipulation

Ewes were individually housed on straw from 7 days before conception to 37 days of gestation, and thereafter group housed with animals at a similar gestational age. They were allowed to acclimatize to an *ad libitum* complete pelleted diet for the 7 days before conception and were fed once a day (MacFadyen *et al.*, 1997). During tupping animals had group access to an *ad-libitum* complete diet. The diet consisted of barley, wheat, micronised full fat soya, grass meal, molasses, chopped straw, calcium carbonate, dicalcium phosphate salt and sheep vitamin/mineral supplement (10.8 MJ/kg metabolisable

energy, 14.98 g/kg crude protein; P316-RVC EXP Sheep Nuts GJW Titmus, Hertfordshire, UK). The diet ration was adjusted according to body weight, as per standard guidelines (Agricultural and Food Research Council, 1993). Water was provided *ad-libitum* at all times.

Before conception ewes were randomly assigned to a control group or a dietary restricted group by randomly selecting a ewe an ear tag that was coded with a coloured flag to indicate treatment group. From minus 30 days conception to 31 days gestation, ewes received either 100% (control; group C, $n = 52$) or one of three dietary regimes, described below.

All nutritional manipulations were carried out at the RVC by technicians on site. Maternal body weight and body condition score (BCS) were measured weekly and any animal that lost more than 15% of its original body weight whilst on the 50% restricted diet was offered diet *ad libitum* from the next day.

2.1.3.1 Pre-conceptional undernourished group (Group B)

From minus 30 days gestation to the first day of gestation the ewe was fed 50% of global nutritional requirements (50% reduction in standard pellet ration; group B, $n = 28$). For the remainder of gestation the ewe received 100% of nutritional requirements, as did the offspring up to time of study.

2.1.3.2 Peri-conceptional undernourished group (Group A)

From minus 15 days gestation to 15 days gestation the ewe was fed 50% of global nutritional requirements (50% reduction in standard pellet ration; group A, *n* = 33). For the remainder of gestation the ewe received 100% of global nutritional requirements, as did the offspring up to time of study.

2.1.3.3 Early gestation undernourished group (Groups UC &UU)

From day 1 to day 31 of gestation the pregnant ewe was fed 50% of global nutritional requirements (50% reduction in standard pellet ration; group U, *n* = 42), followed by 100% of requirements for the remainder of gestation.

2.1.4 Postnatal nutrient restriction (Groups CU & UU)

Between 12 and 25 weeks of age, lambs were grouped with lambs of similar body weight and were fed 100% of global nutritional requirements.

However, a subset of the control (CU, n=17) and early gestation nutrient restricted groups (UU, n=22) were fed at an intake level that reduced body weight to 85% of their target weight, as predicted from each animal's individual growth trajectory between birth and 12 weeks of age. This weight reduction was achieved by removing the pelleted diet but maintaining free access to hay. Lambs in this restricted group were individually monitored to keep body weight on the desired trajectory, with feed adjusted according.

At 25 weeks of age postnatally nutrient restricted lambs were returned to larger group housing and received 100% of nutritional requirements in the form of creep pellets (~0.5kg each). At approximately 32 weeks of age, lambs were transferred onto a standard ration of an adult complete pelleted diet (Ewbol 18 nuts, BOCM Pauls LTD, Loughborough, UK), according to body weight, as per standard guidelines (Agricultural and Food Research Council, 1993) plus *ad libitum* hay and water. Each group contained approximately equal numbers of males and females and twin lambs were divided so that one was assigned to low nutrient and one to the control diet.

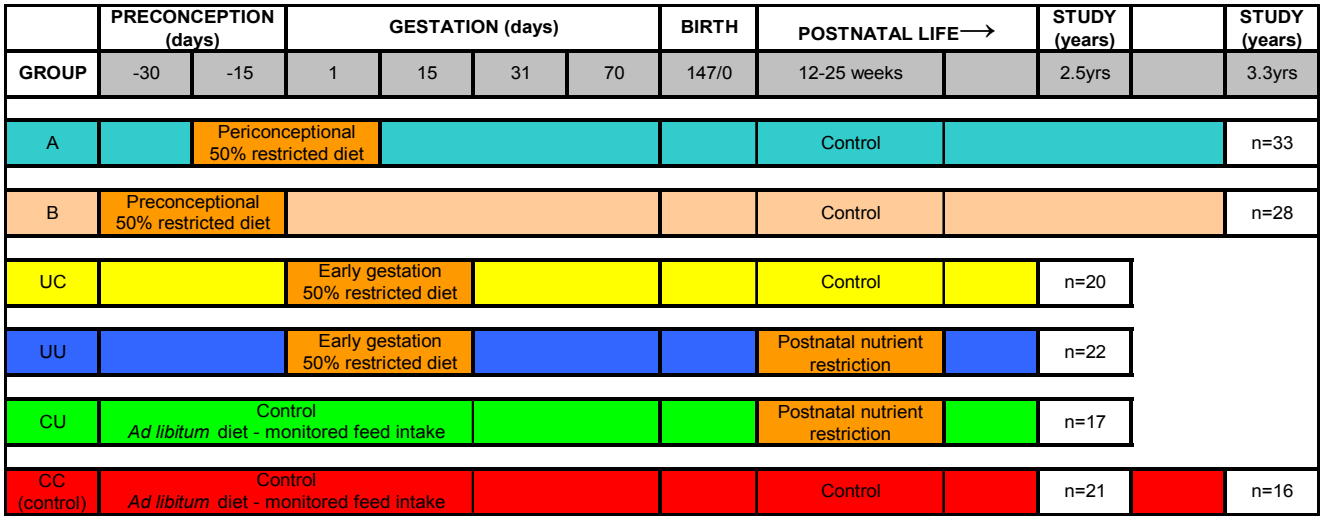


Figure 1.10: Diagrammatic representation of lamb nutritional protocol (timeline not to scale).

Orange boxes represent periods of undernutrition with 100% nutrient requirements at all other times. Group numbers are comprised of males and females.

2.1.5 Lamb husbandry

Ewes delivered and suckled their lambs naturally until lambs were weaned at 12 weeks of age. All ewes lambed in individual pens and returned to the group housing when the lambs were old enough. Each lamb was identified with a uniquely numbered ear tag and a microchip. At birth, all lambs were weighed and the following measurements were taken: crown rump length, abdominal circumference, femur length and biparietal diameter. Lambs were weighed again at 4, 8 and 12 weeks of age and every week thereafter. They had blood samples taken at monthly intervals and the blood taken was 5% of calculated blood volume divided into aliquots in the ratio 3 plasma: 2 serum: 2 EDTA: 1 trace metal: 1 fluoride. When the lambs had a blood volume large enough to allow 50ml blood sampling they went onto the same blood protocol as was used with the ewes. This analysis was performed by another researcher and does not form part of this thesis.

The post-weaning lamb diet consisted of free access to water and hay and each morning and afternoon creep pellets were provided to the group such that each lamb had access to a standard ration according to body weight, as per standard guidelines (Agricultural and Food Research Council, 1993). Creep pellets provide 12.22 MJ/kg metabolisable energy, 18% crude protein and contain 70% dry matter (Prestige Lamb Pellets + Decox, BOCM Pauls, Loughborough, UK).

Males were castrated at 1.5 years of age.

2.1.6 Twins and Singletons

Each group contained approximately equal numbers of males and females and the ratio of singleton to twin lambs was also approximately equal.

	Male		Female		Total	
	Singletons	**Twins**	**Singletons**	**Twins**	**Singletons**	**Twins**
A	4	8			4	8
B	10	4			10	4
control for A/B	3	5			3	5
UC	4	5	2	8	6	13
UU	5	6	4	7	9	13
CU	3	5	4	5	7	10
control for C/D	5	8	2	7	7	15

Figure 1.11: Group animal numbers according to sex and offspring number.

2.1.7 Response to Nutritional Challenges

The effects of nutritional challenges are not covered by this body of work. This data is included in the Appendices 4.1 and 5.

2.2 Surgical procedures

2.2.1 Preparation and anaesthesia

24 hours prior to surgery lambs were penned in a small group with free access to water. Food was withheld from the lamb 16 hours prior to surgery, but water was allowed *ad libitum*. This was to avoid bloating and the regurgitation of rumen contents. The lamb was brought into the surgery and the neck area shaved. The head was held back and the jugular vein identified. Anaesthesia was induced with thiopentone sodium BP (10mg/kg, 0.5mg/ml, Link Pharmaceuticals, Horsham, West Sussex, UK) via the jugular vein. The animal was placed on its back on the operating table with its head extended back. Using a large animal vetinary laryngoscope the sheep was then intubated with a cuffed endotracheal tube (8mm Portex, Hythe, Kent, UK), with the aid of a flexible bougie introducer. The endotracheal tube was then secured with a tie around the back of the head. This was then connected to the anaesthetic machine, and the animal was stabilised and fixed with a neck support bag and leg ties. Anaesthesia was maintained with 2% halothane (Vetothane Halothane PhEur, Virbac Ltd, Cambridge, UK) in 2 litres/minute of oxygen within a closed circuit system. Carbon dioxide was removed by a soda-lime filter system. Throughout the procedure heart rate, blood pressure, respiratory rate and oxygen saturations were monitored and anaesthesia was confirmed by the absence of an eye blinking response.

All surgical areas were prepared with shaving and then cleaning with iodine surgical scrub (Videne, Adams Healthcare, Leeds, UK). The area was then prepared with an iodine solution (Pevidine, BK veterinary products Ltd., UK) to achieve aseptic working conditions. All surgeons scrubbed their hands and wore sterile gowns and gloves.

2.2.2 The REVEAL loop recorder

2.2.2.1 Implantation of the loop recorder

At 2.5 years old (130 ± 1 week) a cardiac loop recorder (REVEAL plus, Medtronic, Inc., Minneapolis, USA; Figure 2.1) was placed subcutaneously in the left axillary region. Surgical equipment was heat sterilised in an autoclave or cold-sterilized using Novasapa (Fort Dodge Animal Health Ltd., Southampton, UK).

The sheep was prepared the sheep for the procedure, using an aseptic technique. The surgical site and the surrounding area was first shaved then cleaned with an iodine cleaning solution. Drapes were then used to establish a sterile field. Local anaesthetic was injected into the skin and subcutaneous tissues. A single incision (2 cm in length) was made down to the subcutaneous fat. Scissors were used for blunt dissection, so creating a subcutaneous pocket 2 x 7 cm in size, so as to be slightly smaller than the size and shape of the REVEAL device for a tight fit. Haemostasis was maintained as required; if necessary bleeding vessels were tied off with 3.0 absorbable sutures (Coated Vicryl, Ethicon, Belgium). The device was then inserted with electrodes facing the skin.

Figure 1.12: Photo of a REVEAL loop recorder (to scale)

The device was sutured secure to adjacent underlying tissue using 3.0 non-absorbable (Mersilk, Ethicon, Belgium) sutures through the two suture holes on the device. Suturing helped to minimize the potential for post-implant rotation and migration of the device through animal movement. The incision was closed with subcuticular absorbable sutures and cutaneous absorbable suture. Finally the wound was sprayed with oxytetracycline hydrochloride (Terramicin, Pfizer, UK).

2.2.2.2 Programming the implanted loop recorder

To programme the device the programmer header was first be placed over the skin surface of the pocket. One green light on the header showed to confirm successful communication. The ECG could then be viewed on the programmer screen. The gain settings were first adjusted to optimise the signal amplitude, so as to achieve optimal R-wave sensing. The storage mode was then set to a single 42 minute manual device activation.

2.2.2.3 Activating the implanted loop recorder

To trigger the REVEAL to record a hand held activator was help over the pocket of the implanted device. To confirm a recoding a green LED light on the activator flashed 5 times. In this way the REVEAL device recorded a 42 minute single channel electrogram. This is a “looped” 40 minute portion prior to activation and a 2 minute recording following device activation. The sampling rate was 100Hz (*i.e.* 50 samples per second).

2.2.2.4 Downloading the telemetry from the loop recorder

This was done after the post-mortem and removal of the device from the animal. The programmer header was first placed over the device. One green light on the header showed to confirm successful communication. The appropriate instructions were then followed on the programmer in order to interrogate the device. Once the download was complete the recording was written to a floppy disc.

2.2.2.5 Loop recorder data analysis

The raw data was first decompressed. The activation point was logged on the data, allowing precise time measurement of initiation of stressor. Minute averaged heart rates were then calculated over the course of the recording.

2.3 Physiological monitoring

2.3.1 Blood gas analyser

A blood gas analyser (ABL700, Radiometer, Copenhagen, Denmark) was used to monitor changes in gas exchange, metabolism, pH and electrolyte balance in arterial blood (0.5ml) samples taken during experiments and daily samples taken to assist in evaluation of the animals health. The blood was collected in heparinised (1ml) syringes as required by equipment protocol. The analyser measured the following parameters: pH, pCO_2, pO_2, Na^+, K^+, Ca^{2+}, Cl^-, glucose, lactate, haematocrit (Hct), and haemoglobin (Hb).

2.3.2 Blood samples

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 and then stored at -80 °C for later analysis.

2.4 Experimental protocol

2.4.1 Cardiovascular assessment of lambs at 2.5 and 3.3 years

At approximately 2.5 and 3.3 years of age, animals were transported to Southampton for assessment of cardiovascular physiology. These procedures will be described in the relevant chapters.

2.4.2 Transport and isolation experiment at 2.5 years

At approximately 2.5 years of age, animals were transported to Southampton for assessment of heart rate and catecholamine response to stress in the adult sheep. These procedures will be described in the relevant chapters.

2.5 Post-mortem

Following these *in vivo* experiments a post mortem was carried out and tissue and blood vessels collected for subsequent analysis.

All drapes, trays and surgical instruments were sterilized by autoclave. A set of instruments was also cold sterilized using Novasapa (Pharmaceutical Manufacturing Co., Bolton, Lancashire, U.K.) for the initial sterile dissection through the skin surface.

The animals were then killed by an overdose of a barbiturate, Phenobarbitone Sodium (0.8ml/kg i.v., 200mg/ml, Animalcare Ltd, York, U.K.). It was then measured for crown-rump length, abdominal circumference, femur length, biparietal diameter and shoulder height. It was then secured to the post-mortem table with leg ties and large open laparotomy and sternotomy incisions were made. These areas were then covered with drapes to give a sterile field under which the organs were removed according to the post-mortem sheet (Appendix 2). The organs were weighed in sterile trays and the relevant pieces selected according to the post-mortem sheet. There were then processed by one of three methods:

1. Fast frozen in liquid nitrogen,

2. Slow frozen in liquid nitrogen,

3. Embedded in optimal cutting temperature (OCT) compound, and slow frozen.

These tissues are stored at -80 °C until required for future work for molecular analysis.

2.6 Data Analysis

2.6.1 Power calculations

When setting up a study, a power calculation is used to calculate the appropriate sample size needed to detect a statistically significant difference of a given magnitude (Altman & Bland, 1996). In order to carry out the calculation in a study with two independent groups of continuous data the following equation is used:

$$\boldsymbol{n = (2\{z_{1-(\alpha/2)} + z_{1-\beta}\}^2\ \sigma^2) / \delta^2}$$

$z_{1-(\alpha/2)}$ and $z_{1-\beta}$ are critical values in determining a difference between the two means, which can be simplified using the following values:

The most commonly used value for significance (α) is 0.05, giving $z_{1-(\alpha/2)} = 1.96$

The most commomly used value for power (ß) is 80%, giving $z_{1-\beta} = 0.84$

Therefore:

$$\boldsymbol{n = 16\ \sigma^2 / \delta^2}$$

This means that a sample size of **n** in each group will have 80% power to detect a difference in means of **δ**, assuming that the common standard deviation is **σ**, and that the test is performed at the 5% significance level (two-sided).

Therefore in order to calculate the sample size for the current study the expected magnitude of change (**δ**) and standard deviation (**σ**) need to be determined. These can be

obtained from preliminary studies, the first numbers through the study, or the results of a similar study.

To determine the sample size required to detect alterations in left ventricular function on transthoracic echocardiography in adult sheep due to fetal life challenges a study by (Dodic *et al.*, 2001b) was used. This studied the differences in left ventricular size and function in adult sheep that had been subject to a dexamethasone challenges at 27 days of gestation with those that had not. There were no studies that had studied echocardiographic left ventricular size and function and the effects of early life nutrient restriction in the adult sheep.

$\delta = 14$

$\sigma = 7$

$n = 16\ \sigma^2 / \delta^2$

$n = 4$

Hence sample size should be > 4

To determine the sample size required to detect alterations in coronary flow in adult sheep due to fetal life challenges a study by (Davis *et al.*, 2003b) was used. This looked at coronary flow in adult sheep comparing controls with those made anaemic in fetal life. Similarly, there were no studies that have studied coronary flow and the effects of early life nutrient restriction in the adult sheep.

$\delta = 5.1$

$\sigma = 1.8$

$n = 16\ \sigma^2 / \delta^2$

$n = 1.99$

Hence sample size should be ≥ 2.

I have also performed retrospective power calculations in the General Discussions Chapter – Section 7.2.2.

2.6.2 Summary measures

This study yields a series of measurements taken from each individual subject at specific time points. These are referred to as serial measurements (Altman, 1995). If independent analysis is applied at each time point it becomes difficult to interpret the findings as there may be a difference at one time point but not at any of the others and no explanation as to why this difference has occurred as it does not relate to a specific component of the experiment. Also subjects with missing values cannot be analysed. The analysis of serial measurements is therefore simplified by reducing each subject's data to a certain feature of particular interest common to the group as a whole, such as the mean value. The advantage of a summary measures approach is that it allows the analysis of data sets with missing value, handles variation in timing of observations and the results are more easily interpreted.

2.6.3 Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Bartletts's test for equal variance and a normality test (GraphPad Prism version 3.00, GraphPad Software, San Diego, California, USA) were applied to all data sets. All data were normally distributed and groups were of equal variance, therefore this allowed the use of parametric statistics throughout the thesis.

The C & D group data (including both male and females) were significantly different between sexes and were therefore split accordingly. In all groups subgroup analyses of the effects of twinning were also tested for.

Data were analysed by ANOVA (SPSS Inc, Illinois, USA). Change in heart rate, epinephrine and norepinephrine in response to transport and isolation were assessed by calculating the area under the response curve (AUC) and the maximum change (GraphPad Prism version 3.00, GraphPad Software, San Diego, California, USA). For all statistical tests, significance was accepted at $P < 0.05$.

2.6.4 Multiple comparisons

Performing multiple comparisons on a pair of data sets increases the probability of finding a significant difference just by chance, as within each test there is a 5% chance of a false positive result. Thus, in this thesis, this approach has been avoided where possible. However, when it was necessary to perform multiple comparisons, *P* values were adjusted using the Bonferroni method. The *P* value obtained from each test was multiplied by the

number of comparisons. The disadvantage of this model is that for a large number of comparisons it is highly conservative. Thus, comparisons were limited to those that were specifically relevant to the research objective.

2.6.5 Analysis of Variance

Analysis of variance is a statistical test which can be used to compare means between groups. The method of this test is based on assessing how much the overall variation in the data is attributable to differences between the group means, and comparing this with the amount attributable to the differences between individuals within the same group. The null hypothesis is that the variance between groups is not different from the variance within the groups.

When the group means to be compared are defined by one factor, the method is termed one-way analysis of variance. Although not used in my thesis the unpaired t test would be appropriate to use when comparing between only two groups. Since I have compared 3 or more groups one-way analysis of variance is the appropriate statistical test, rather than repeated comparisons using unpaired t tests. In some experiments in this thesis, the groups can be defined by two or more factors. For example, the catecholamine response data in Chapter 5 can be defined by group (A, B and D) and sample time. In these instances multi-factorial analysis of variance has been used. Using this method, the total variation in the catecholamine response can be divided into that due to differences between groups, differences between sample times, and differences within groups at each sample time. In addition it is possible to calculate the interaction between group and sample time, i.e. to

examine if differences between groups are dependent on sample time or vice versa. Thus, two-way analysis/multi-factorial of variance can identify if there are significant effects of the main factors, which in the above example are group and time. If a significant effect of group is shown, it is possible to compare the means at each time point post-hoc with a Bonferroni correction (Altman, 1995).

Chapter 3 - Effects of moderate early gestation undernutrition with or without undernutrition in early postnatal life on cardiac morphology and function in the adult sheep

3.1 Introduction

As previously discussed Barker's original "fetal origins of health and disease" hypothesis arose from epidemiological studies showing an increase in death from ischaemic heart disease was associated with low birth weight in a large cohort of adult men and women (Barker & Osmond, 1986b). Since this seminal work much research has confirmed these associations. Exposure to malnutrition during the Dutch famine, especially during the first gestational trimester, led to a greater occurrence of coronary heart disease in adults (Roseboom *et al.*, 2000a). Much effort has also been made to establish the cause of these findings and to understand the underlying physiological mechanisms involved.

In animal and human models, impaired fetal development is associated with an increased incidence of hypertension (Langley-Evans *et al.*, 1994). In animals, many adverse environmental influences have been shown to link impaired fetal development to hypertension, including maternal malnutrition, hypoxia and anaemia, placental malfunction, and excess exposure to stress hormones.

Another step in understanding the cardiovascular mechanisms relating to fetal programming has come from epidemiological studies showing increased left ventricular mass has been associated with reduced infant growth. In this study of human subjects

between 8 and 24 years of age, increased left ventricular mass (adjusted for gender, present age, weight, and height) was found on echocardiography to be associated with infants with low postnatal weights at 9 months and 2 years of age (Zureik *et al.*, 1996). Similar changes have been reproduced in the animal studies. Pregnant ewes were challenged with a very brief exposure of high levels of dexamethasone early in gestation (27 days gestation). This gave rise to hypertensive female offspring, associated with left ventricular hypertrophy (LVH) and reduced cardiac functional reserve at seven years of age (Dodic *et al.*, 2001a).

It is well recognised that there are gender differences in the incidence of cardiovascular disease, with women having a reduced risk during reproductive years but an increased risk post-menopause compared with men. Likewise, programming of hypertension is well demonstrated in male animal models, but often fails to be produced in female offspring (Woods *et al.*, 2005).

In summary, both the human epidemiological data and animal work has indicated that both the gestation period and the early postnatal period of development are critical windows for the programming of cardiovascular function (Roseboom *et al.*, 2000a). However, no study has investigated the long term effects of fetal undernutrition during these critical periods on the cardiac structure and function, their interactions, and whether any changes seen are sex specific.

3.1.1 Aims

The aims of chapter 3 were:-

1. **To investigate the effect of early gestation nutrient restriction on cardiac structure and function in the adult sheep.**

2. **To investigate the effect of early postnatal nutrient restriction on cardiac structure and function in the adult sheep.**

3. **To investigate whether early gestation undernutrition influences how early postnatal nutrient restriction affects cardiac structure and function.**

4. **To investigate whether any effects of undernutrition in early life on cardiac structure and function in the adult sheep are sex specific.**

3.2 Methods

3.2.1 Diets

As described in "Maternal Dietary Manipulation" (Section 2.1.3), and "Postnatal Nutrient Restriction" (Section 2.1.4).

Summary for the dietary challenges is as follows:-

Group CC - had no maternal dietary restriction i.e. **the control** (*n* = 22).

Group CU - had a period of **postnatal undernutrition** where the offsprings dietary restriction was 12 to 25 weeks of age (*n* = 17).

Group UC – had a period of **early gestational undernutrition** where the dietary restriction was from day 0 to 30 days gestation (*n* = 20).

Group UU - had a **combined early gestational undernutrition and postnatal undernutrition** (*n* = 22).

3.2.2 Echocardiographic Studies

At 2.5 years left ventricular structure and function was determined by transthoracic echocardiography under general anaesthesia (Lehot *et al.*, 1991). Data (mean ± S.E.M.) were analysed by ANOVA and a Bonferroni post-hoc test.

To avoid inducing confounding stress responses from restraining a conscious animal all echocardiograms were performed under general anaesthesia. Induction was with 5% sodium thiopentone (10mg/kg, 0.5mg/ml, Link Pharmaceuticals, Horsham, West Sussex, UK) via the jugular vein, an endotracheal tube was inserted, and general anaesthesia was maintained with a 2% halothane/oxygen mixture.

The area for probe placement was shaved and cleaned. Ultrasound contact gel was applied to improve the contact between the skin and transducer. A Hewlett Packard Sonus 2500 system (Ishii *et al.*, 1996) was used equipped with a 3.5-Mhz transducer (Hewlett Packard). Two dimensional transthoracic echocardiography was performed with the animal in the left lateral decubitus position. Left parasternal views were orientated to obtain standard short-axis views of the left ventricle (LV) at the level of the papillary muscles. Assessment of wall thickness was then performed by M-mode scanning at the level of the papillary muscle, perpendicular to the septum. Only frames with optimal visualization of interfaces that simultaneously showed the interventricular septum, LV internal diameter and LV posterior wall were used for readings. At least 5 cardiac cycles were averaged for each data point. The images were stored on Super VHS magnetic tapes.

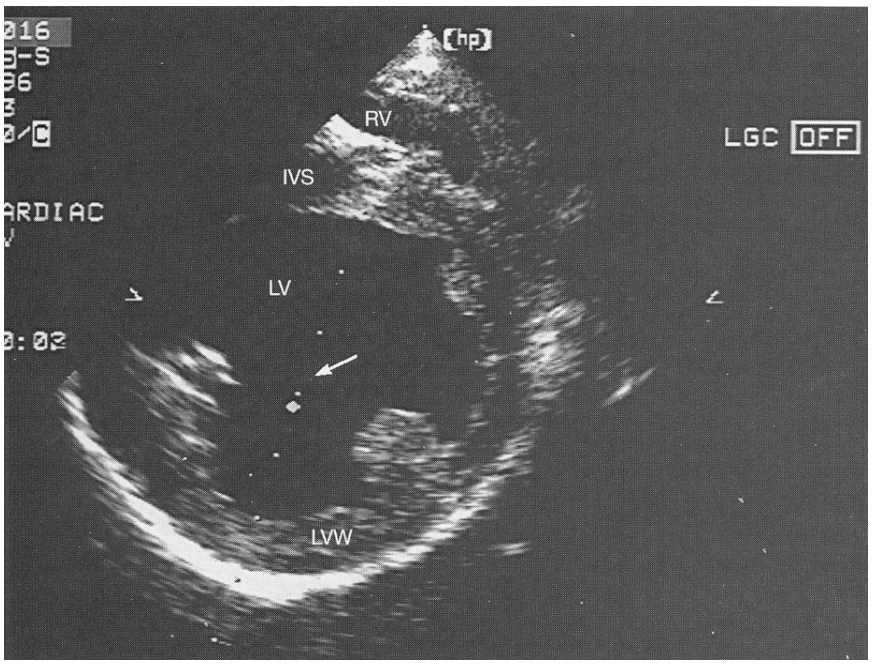


Figure 3.1: Example of 2-D echocardiogram of the parasternal short axis.

The following measurements were made both in systole (s) (defined by the frame exhibiting the smallest left ventricular cavity dimension) and diastole (d) (defined by the frame exhibiting the largest left ventricular cavity dimension) using the American Society for Echocardiography leading edge method (Sahn *et al.*, 1978b):

i) Interventricular septal wall thickness (IVSs and IVSd).

ii) LV posterior wall thickness (PWs and PWd).

iii) LV internal diameter (LVIDs and LVIDd).

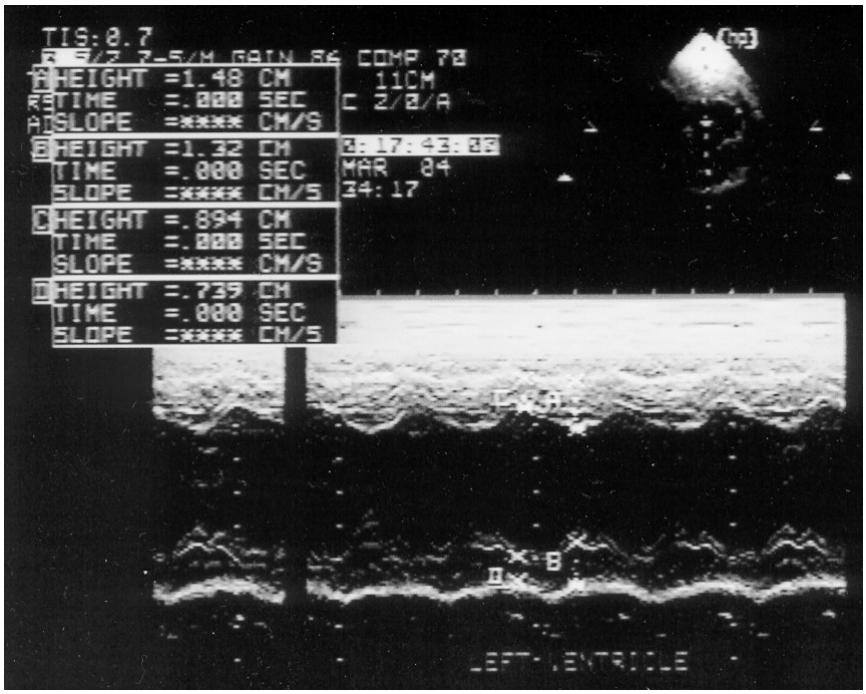


Figure 3.2: Example of M-mode echocardiogram in the parasternal short axis, for LV measurements.

The following measurements were then calculated:-

a) Left ventricular mass (LVM): $1.04([LVIDd+PWd+IVSd]^3-[LVIDd]^3)-13.6g$

This is the Penn conversion formula, which uses the standard cube formula, so assuming a spherical LV geometry, and where 1.04 is the specific gravity of muscle (Devereux & Reichek, 1977).

b) Fractional shortening (FS): [(LVIDd – LVIDs) / LVIDd] x 100

c) Relative wall thickness (RWT): (IVSd + PWd) / LVID

d) Mean LV wall thickness (MWT): (IVSd + PWd) / 2

All results are presented as the mean of three consecutive recordings of each measurement, which were stored on Super-VHS analogue tape. Tracings were read by 2 experienced observers and, interobserver coefficients of variation were calculated for interventricular septum, the posterior wall, the LV internal diameter, and for LV mass. All data was corrected for animal body weight, although not graphically represented as such unless differences seen, in order to preserve perceivable data.

3.2.3 Post-mortem

At conclusion of all in vivo experiments, animals were killed humanely with an overdose of sodium phenobarbitone (160 mg/kg i.v.) for tissue collection.

At post mortem the whole heart was dissected out for weighing following removal of the great vessels and the pericardium. The left ventricle with interventricular septum, and the right ventricle were then dissected out. The gross epicardial fat was then removed, and the resulting myocardium was weighed.

3.2.4 Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Parametric statistics were used as data was normally distributed and groups were of equal variance. Data were analysed by factorial ANOVA with a post hoc Bonferroni test (GraphPad Prism, and SPSS Inc.). A probability of < 0.05 was considered significant.

A database was set up containing 71 subject rows with experimental and postmortem variables and indicators for early gestation diet, postnatal diet, twinning and sex (SPSS Inc, Illinois, USA). A univariate analysis of variance model was fitted to the data with early gestation diet, postnatal diet, sex and twinning as the main factors. When there was an interaction between sex and either of the diets the data were split according to sex and a univariate analysis of variance model was fitted to the data with early gestation diet, postnatal diet and twinning as the main factors.

I consulted a statistician at the Medical Research Council (Southampton General Hospital) and they initially analysed it including the effect of dam identity. This was felt to over-complicate the analysis as it split the data into groups where the maximum number was 2 (*i.e.* twins). This was then compared with data from methods described above and the results were the same therefore it was decided to proceed with the factorial statistical method described above.

3.3 Results

3.3.1 Left ventricular wall thickness

Male sheep had a greater left IVSd, PWd and MWT than females ($P < 0.01$) and a tendency for a greater RWT (Figure 3.4a $P = 0.08$). In males only, UC had a greater IVSd (Figure 3.3a) and MWT (Figure 3.4b, $P < 0.01$) than CC. This effect was not seen in UU compared to CU.

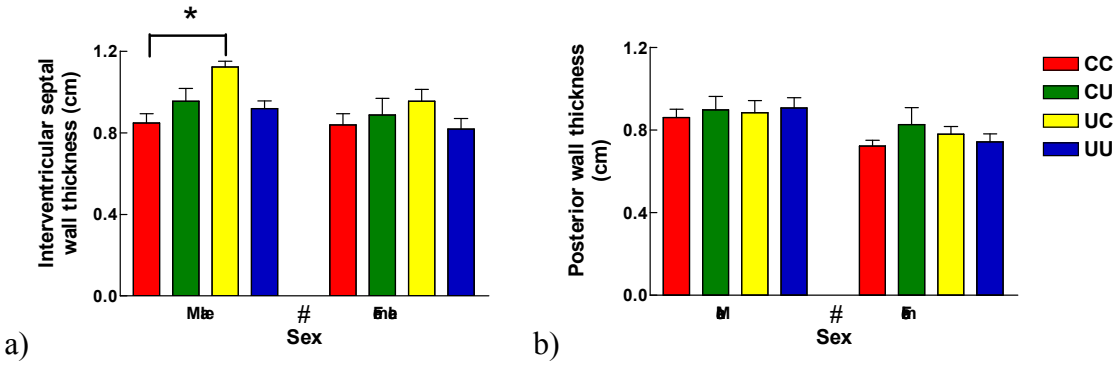


Figure 3.3: LV wall thickness for male and female sheep in the IVSd (a) and PWd (b). Values are mean ± S.E.M. * $P < 0.01$, UC significantly different to CC. $^{\#}$ $P < 0.01$, male sheep significantly different to female sheep.

However, there was no corresponding increase in posterior wall thickness. Therefore, the males in the early gestation undernutrition group alone have asymmetrical thickening of the interventricular septum, compared to the control group. This is a sex-specific finding.

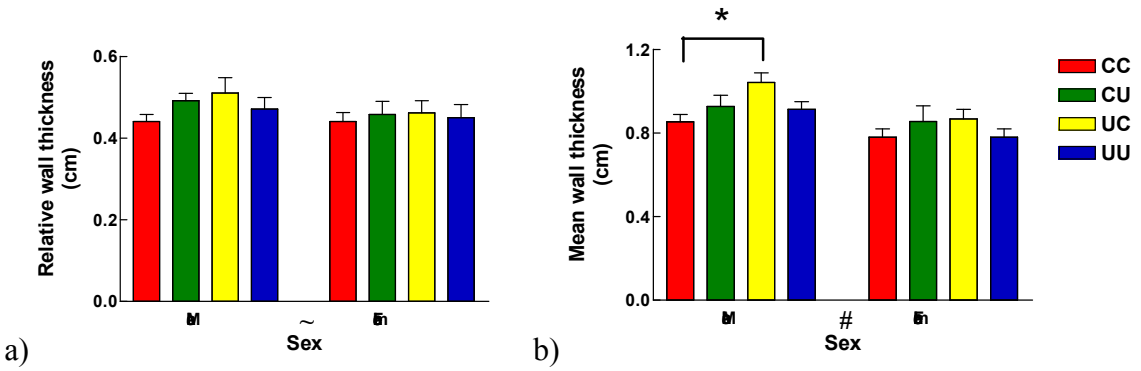


Figure 3.4: Calculated RWT (a) and MWT (b) for male and female sheep. Values are mean ± S.E.M.

* $P < 0.01$, UC significantly different to CC. # $P < 0.01$, male sheep significantly different to female sheep. ~ $P = 0.08$, males different to females.

3.3.2 Left ventricular cavity size

There was no effect of sex, early gestation or postnatal nutrition on left ventricular cavity size in end-systole and end-diastole.

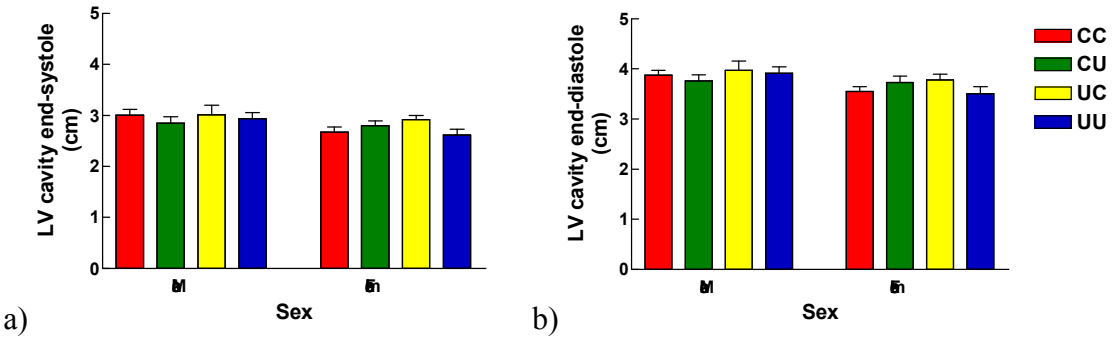


Figure 3.5: LV cavity size in end-systole (a), and end-diastole (b), in male and female sheep. Values are mean ± S.E.M.

3.3.3 Left ventricular functional assessment

Male sheep had a greater left ventricular mass than females ($P < 0.001$). There was no effect of early gestation or postnatal nutrition on fractional shortening or LV mass.

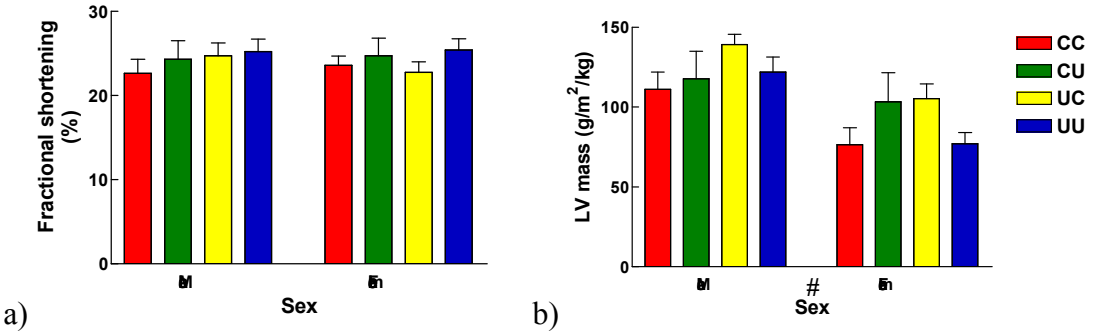


Figure 3.6: Fractional shortening (a), and left ventricular mass (b), in male and female sheep. Values are mean ± S.E.M. $^{\#}$ $P < 0.01$, males significantly different to female sheep

3.3.4 Mean arterial pressure

There was no effect of sex, early gestation or postnatal nutrition on mean arterial blood pressure, and no associated trends in the male groups corresponding to the differences in wall thickness were identified.

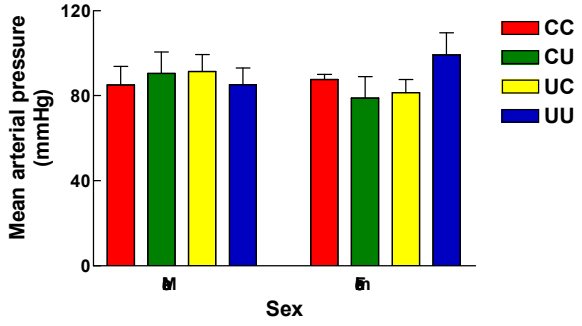
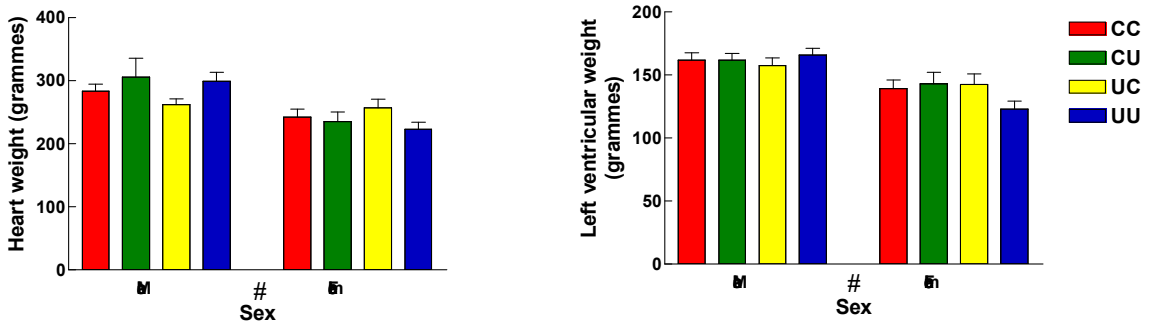


Figure 3.7: Mean arterial BP in male and female sheep. Values are mean ± S.E.M.

3.3.5 Heart weights

Male sheep had a greater heart weight and left ventricular weight than females ($P < 0.001$).



a) b)

Figure 3.8: Graphs showing total heart weights (a) and left ventricular weights (b) in male and female sheep. Values are mean ± S.E.M. $^{\#}P < 0.01$, male sheep significantly different to female sheep.

3.3.6 Male growth data correlated against interventricular septal wall thickness

There was a significant positive correlation between interventricular septal wall thickness and growth rates during the postnatal challenge in the early gestation restricted males (Figure 3.9), that was not seen in the early gestation control males ($P = 0.12$, R^2=0.21, data not shown).

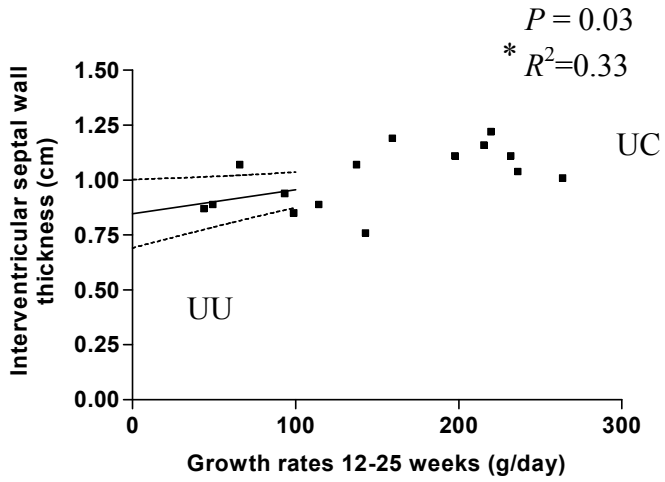


Figure 3.9: The relationship between interventricular wall thickness and growth rate during the postnatal nutritional challenge for early gestation restricted male sheep (Groups UU & UC). Dotted lines represent 95% confidence interval. * $P < 0.05$, indicates a significant correlation.

3.3.7 Variability

Tracings were read by 2 experienced observers. Interobserver coefficients of variation in the study were 4.2% for interventricular septum, 4.8% for posterior wall, 1.8% for LV internal diameter, and 6.3% for LV mass.

3.4 Discussion

3.4.1 Early gestation undernutrition and LV wall thickness

This study has shown undernutrition during early gestation results in an increase in interventricular septal wall thickness and mean LV wall thickness in adult male sheep. However, increase in thickness is not seen in the posterior LV wall of this group. The difference in the interventricular septum is therefore diluted when the interventricular wall thickness and posterior wall thicknesses are averaged together. When relative wall thickness was analysed this trend is still seen but statistical significance is lost (P=0.32) since the combined wall thicknesses are represented as a proportion on the LV cavity size in end-diastole. Once again this effect was not seen with male offspring exposed to both periods of undernutrition (UU).

There were also no differences seen in the LV cavity sizes. No differences were seen in the post-mortem left ventricular weights nor the calculated left ventricular mass, which is not unexpected since the observed differences are seen in a specific region of the left ventricle alone.

Similar asymmetrical wall thickening of the interventricular septum has previously been seen in horses following exercise training (Stadler *et al.*, 1993). This may therefore represent a species specific LV wall thickening. However concentric hypertrophy has also been shown in other adult sheep studies in response to an early life challenge (Dodic *et al.*, 2001d). It may be that this represents the early stages of development of hypertrophy, where mild initial thickening is evident only in the interventricular septum. Indeed, the interventricular septal wall thickness has been found to have the best reproducibility and is

therefore the most accurately measured parameter (Lim *et al.*, 2001). This may be a physiological adaptation which precedes any myopathic process, since no functional impairment has been demonstrated in association with the increased left ventricular wall thickness.

Parallels can also be made with a recognised pattern of hypertrophy observed in the late gestation fetuses of diabetic mothers with increased periconceptual maternal glycated haemoglobin, which is a measure of poor diabetic control (Gardiner *et al.*, 2006). In this instance the hypertrophy is characterised by thickening of the interventricular septum, and to a lesser extent the ventricular free walls. Similarly, this asymmetry has been proposed as an early adaptation, prior to the development of more marked symmetrical left ventricular hypertrophy with thickening of the posterior LV wall. The mechanism of this development is speculative, but is believed to occur as a consequence of both fetal hyperinsulinaemia and the normally increased expression and affinity of insulin receptors which leads to the proliferation and hypertrophy of cardiac myocytes (Gardiner *et al.*, 2006).

3.4.2 Early gestation undernutrition combined with early postnatal undernutrition

When early gestational undernutrition is combined with an early postnatal challenge there is no increase in the interventricular septal wall thickness.

Growth data previously reported (Cleal *et al.*, 2007a) in this cohort of animals has shown that the postnatal nutrient restriction produced a clear reduction in fractional growth during this period (12-25 weeks of age). After the postnatal nutrient challenge (25-35

weeks of age), UU has a greater fractional growth rate than UC, but CU did not have a significantly greater growth rate than CC. Thus, early gestational undernutrition enhanced both early postnatal growth rates (i.e. 0-12 weeks) and the recovery from a period of postnatal undernutrition. These findings indicate that these animals develop strategies aimed at protection of body weight from an anticipated period of undernutrition.

Thus, postnatal undernutrition, which stalls this accelerated growth following early gestation undernutrition (*i.e.* group UU), appears to prevent the increases in left ventricular wall thickness. This suggests that matching of pre-and post-natal nutrition is beneficial, whereas a mis-match is detrimental to the heart in the long-term. This is in keeping with the predictive adaptive response hypothesis (Gluckman & Hanson, 2004d) which proposes that when prenatal and postnatal insults match an individual is better prepared for the postnatal environment. However, as illustrated in this study, if these predictions are not met then the adult will be mal-adapted and may be at a greater risk of disease.

3.4.3 Sex differences

The absence of any differences seen within adult female sheep suggests that either this is a male sex specific adaptation or conversely that there is a female sex specific protective mechanism thereby preventing the adaptation.

It is possible the absence of this effect in the female adult offspring may reflect different growth rates and susceptibility between the sexes in early gestation. This suggests that male offspring may be more vulnerable to early gestation undernutrition because they

grow more rapidly *in utero* than females (Pedersen, 1980). Male lambs also show accelerated early postnatal growth in response to early gestation undernutrition, which in humans is associated with increased risk of developing cardiovascular disease (Eriksson *et al.*, 2001). This therefore proposes that the sex specific changes seen relate to differences in growth rates which in turn lead to sex specific critical windows of development.

A sex specific protective mechanism preventing the adaptation also provides a possible explanation for these sex differences. All animals at the time these studies were sexually mature and fertile. It is well established in humans that the incidence of cardiovascular disease in pre-menopausal women is lower than in age-matched men (Schwartz *et al.*, 1995). An increased incidence in systemic hypertension has been documented in post-menopausal women (Reckelhoff, 2001b). Consequently it has been proposed that female sex hormones represent an intrinsic cardioprotective mechanism. In spontaneously hypertensive rats ovariectomy causes hypertrophy and an unfavourable myocardial remodelling (Santos *et al.*, 2004). The precise mechanism whereby menopause favours the development of hypertension, hypertrophy and ventricular remodelling remains undefined.

3.4.4 Effects of twinning

At 2.5 years of age there was no difference in weight between twin and singleton lambs (Cleal *et al.*, 2007a – Appendix 4.1), and twinning had no effect on cardiac morphology and function in the adult sheep.

3.4.5 Mechanisms

The development of hypertension and left ventricular hypertrophy has previously been seen in adult sheep in response an alternative early life challenge (Dodic *et al.*, 2001d). This group used a brief but relatively severe early gestational exposure to dexamethasone. They showed this was associated with left ventricular hypertrophy and reduced cardiac functional reserve. The group hypothesised chronic hypertension as the underlying aetiology.

Since no changes were seen in resting mean arterial blood pressure in my studies, this may suggest that the mechanism for interventricular wall increased thickness is not in response to an increased afterload imposed on the LV by an elevated arterial blood pressure. Other possible remodelling stimuli include direct neuroendocrine activation which induces a combination of molecular and cellular events. This includes hypertrophy of cardiac myocytes, changes in gene expression with a re-expression of fetal programs and decreased expression of adult programs, changes in the quantity and nature of the interstitial matrix, and cell death. In this way epigenetic regulation of gene transcription provides a strong candidate mechanism for fetal programming. This can occur through persistent alteration of gene transcription, such as through DNA methylation, which is largely established in utero (Lillycrop *et al.*, 2005). These events lead to changes in the structure of the ventricle first manifesting as left ventricular hypertrophy, and later affecting the function of the ventricle, which may result in further pump dysfunction and increased wall stresses, thereby promoting further pathological remodelling and cardiac failure.

However, while no increase in basal blood pressure at 2.5 years old was observed, data published by my research group on this cohort of sheep at the same time point has shown differences in blood pressure responsiveness in the early gestation undernourished group. A bolus of Angiotensin II resulted in an increased blood pressure response in CU *vs.* CC. Also a similar increased blood pressure as well as an increased overnight urine output was seen in response to frusemide in group CU (Cleal *et al.*, 2007a). This suggests an altered vascular responsiveness with chronic stress-induced hypertension as an underlying process in the mechanism of the observed increase in left ventricular wall thickness.

In this study an increase in interventricular septal wall thickness and mean wall thickness was seen without a corresponding increase in the LV cavity size. This can be seen in the early stages of hypertrophy, without functional impairment. In the pathological context this pattern is called "concentric" hypertrophy, usually seen in response to chronic hypertension, whereby the wall thickness increases while the inside radius of the chamber does not change. This response enables the heart to develop greater pressure and to maintain a normal stroke volume despite the increase in afterload. This type of ventricle is then capable of generating greater forces and higher pressures, while the increased wall thickness maintains normal wall stress. A hypertrophied ventricle however, will ultimately become stiff with reduced compliance, which impairs filling, reduces stroke volume and leads to a large increase in end-diastolic pressure. Changes in end-systolic volume depend upon changes in afterload and inotropy. In this way concentric hypertrophy causes diastolic dysfunction which can to turn lead to pulmonary congestion and oedema.

3.5 Conclusion

This study shows early gestation undernutrition leads to an increase in left ventricular wall thickness in adult male sheep. These adaptations are sex specific. However, this difference is not seen when early gestation undernutrition is combined with early postnatal undernutrition. Hence a mis-matching of pre- and post-natal environments induces increased left ventricular wall thickness in adult male sheep that was not seen when environments were similar. The underlying mechanism implicated in these cardiovascular adaptations is proposed to be chronic stress-induced hypertension.

Chapter 4 - Effects of pre- and periconceptional undernutrition on cardiac morphology and function in the adult male sheep

4.1 Introduction

I have presented data in chapter 3 showing that early gestation dietary challenge gave rise to an increase in interventricular wall thickness and mean LV wall thickness in the adult male sheep. This early gestational period of undernutrition was from the day after conception to 31 days gestation. In this chapter I wish to further examine the precise critical period of development during which this programming has occurred. It may be that the programming seen in Chapter 3 occurs due to effects within a more focused time period in the early gestational window. It is also possible that the critical window precedes or extends beyond the early gestational challenge. Recent studies in sheep have shown that a periconceptionally restricted supply of vitamin B_{12}, folate and methionine have the effect of elevating the blood pressure in the offspring's adulthood (Sinclair *et al.*, 2007). The period of dietary micronutrient restriction was from 8 weeks preceding until 6 days after conception. At 23 months of age the offspring in the challenged group lead to an increase in mean arterial pressure in the males (a sex specific effect). This work is therefore in keeping with the theory that the critical window precedes the early gestational challenge, or is focused within the first 6 days of gestation, and forms the basis of this hypothesis studied in this chapter. In this chapter the challenges were exerted earlier as illustrated in Figure 4.1 below.

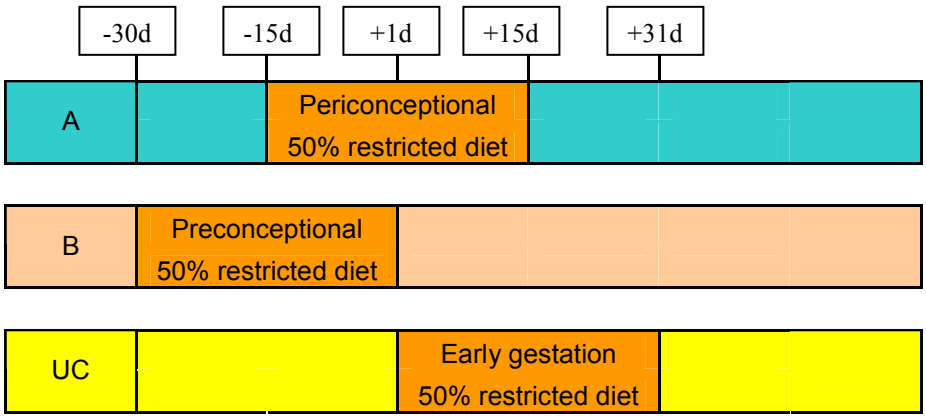


Figure 4.1**:** Timeline showing the overlapping periods of nutrient challenge in this chapter and the previous chapter 3 ("d" - represents days).

The first challenge is in the "periconceptional" period where the fertile ewe is restricted to 50% global dietary restriction but from minus 15 days to 15 days after conception (group A). This period therefore overlaps with the previous window where programming was seen. The second challenge occurs earlier still, in "preconception". The ewe is again restricted to 50% global dietary restriction but from minus 30 days to the day of conception (group B). This may help to establish exactly how early programming of the cardiovascular system can occur.

4.1.1 Aims

The aims of chapter 4 were:-

1. **To investigate the effect of periconceptional nutrient restriction on the cardiac structure and function of the adult male sheep.**

2. **To investigate the effect of preconceptional nutrient restriction on the cardiac structure and function of the adult male sheep.**

4.2 Methods

4.2.1 Dietary challenge

The nutritional dietary manipulation has been described in section 2.1.3. Only male offspring were studied. To summarize the 3 groups are made up of:-

"A"- Periconceptional undernourished group (-15 to +15 days of conception; n=12);

"B" - Preconceptional undernourished group (-30 to the day of conception; n=14); and

"Control" group (n=8).

4.2.2 Echocardiographic Studies

At 3.3 years left ventricular structure and function was determined by two dimensional transthoracic echocardiography under general anaesthesia (Lehot *et al.*, 1991), using methods described in chapter 3.

4.2.3 Post-mortem

As described in Section 3.2.3.

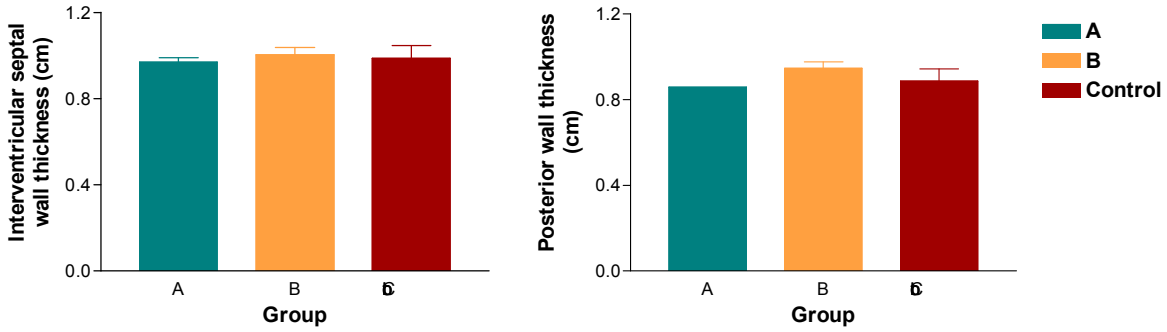
4.2.4 Statistical analysis

As described in Section 3.2.4.

4.3 Results

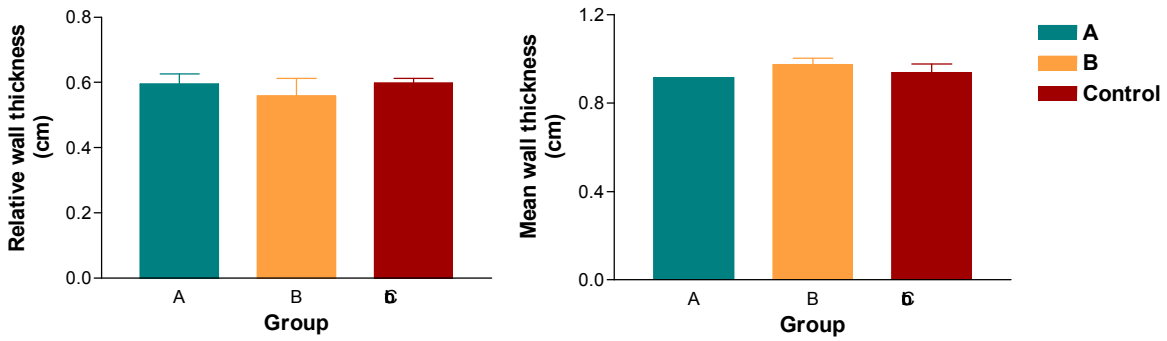
All results are expressed as mean ± standard error of the mean (SEM). Parametric statistics were used as data was normally distributed and groups were of equal variance. Data were analysed by 2 way ANOVA with a post hoc Bonferroni test (GraphPad Prism, and SPSS Inc.). A probability of < 0.05 was considered significant.

4.3.1 Left ventricular wall thickness



a) b)

Figure 4.2: Interventricular septal wall thickness (a) and LV posterior wall thickness (b)

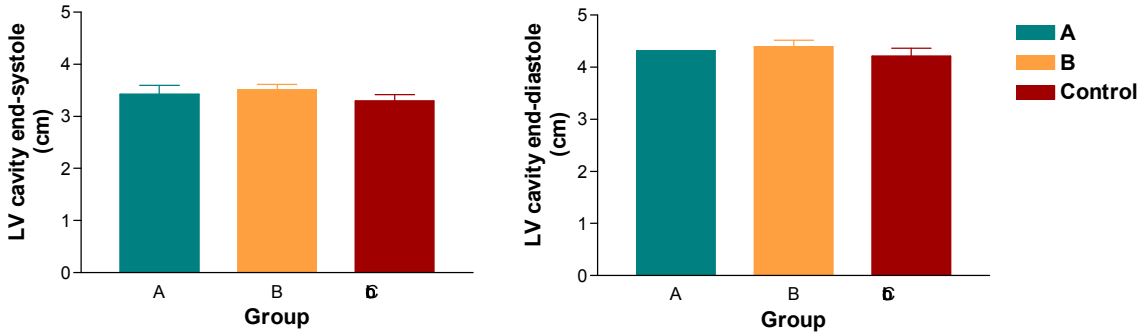


a) b)

Figure 4.3: Calculated LV relative wall thickness (a) and LV mean wall thickness (b)

No differences were seen the individual LV wall thicknesses, or in the relative and mean LV wall thicknesses.

4.3.2 Left ventricular cavity size

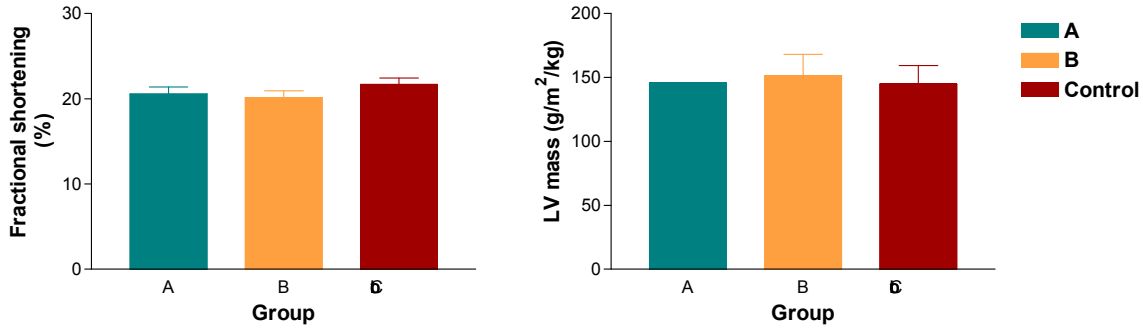


a) b)

Figure 4.4: LV cavity size in end-systole (a) and end-diastolic (b)

No differences were seen the LV end systolic or end diastolic cavity sizes.

4.3.3 LV functional assessment



a) b)

Figure 4.5: Fractional shortening (a) and left ventricular mass (b)

No differences were seen the calculated LV fractional shortening or in the LV mass.

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4.3.4 Mean arterial pressure

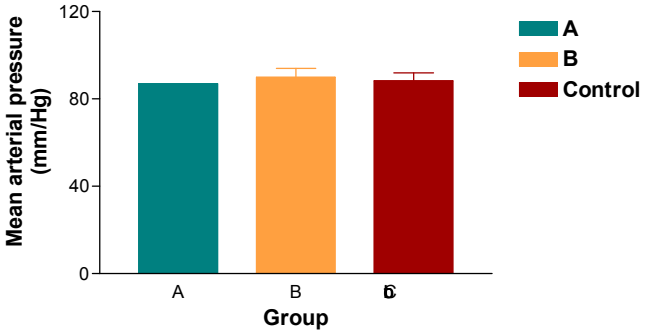
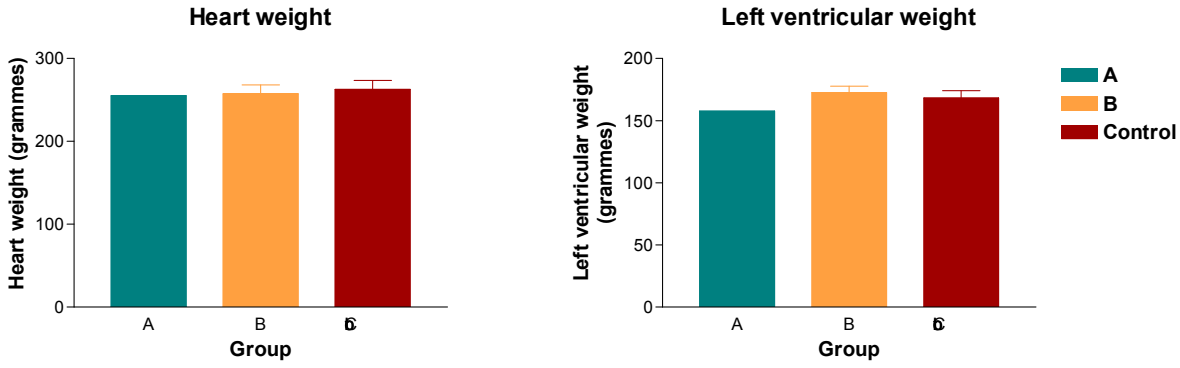


Figure 4.6: Mean arterial blood pressure
(values are mean ± S.E.M.)

No changes were seen in mean arterial blood pressure between the groups.

4.3.5 Heart weights



a) b)

Figure 4.7: Graphs showing whole heart weights (a), and left ventricular weights (b) (values are mean ± S.E.M.).

No differences were seen in the whole heart weights nor the left ventricular weights of these male groups.

4.4 Discussion

No differences were seen in left ventricular wall thickness, cavity size, heart weight or functional assessments. Analysis of offspring number and correlation to animal weight likewise did not draw out any differences between the 3 groups.

In the previous chapter we saw a sex specific effect of undernutrition in early gestation in the male adult sheep with an increase in LV interventricular septal wall and mean LV wall thicknesses. This effect relates to a period of 50% global nutrient restriction

from day 1 to day 31 of gestation. We have looked at a similar nutrient challenge in Group A, which were subject to 50% globally restricted diet from 15 days prior to conception to 15 days into gestation. Therefore there is an overlap in the dietary challenge, from day 1 to day 15 of gestation (as illustrated in Figure 4.1). It is possible that the critical window of development in the process that leads to the increase in LV wall thickness lies between 15 and 31 days gestation.

This is still very early in the development of the primitive heart. The first 30 days coincides with the stage of the embryonic heart tube development, before looping or septation has occurred. Although the heart tube is being developed it the first 15 days of gestation, the primitive circulatory system is unlikely to have developed the ability to generate a pulse until at least 20 days after fertilization. It may be that this has a determining factor on the susceptibility to the nutrient challenge.

Alternatively it may be that studying the effects of the nutrient challenges in adult sheep 10 months older (3.3 years as opposed to 2.5 years of age) has led to an attenuation or compensation in any changes to the cardiac structure that may have been present earlier in life. It is possible that the increases in left ventricular wall thickness could lead to increased wall stress and thereby pathological remodelling and associated wall thinning. However if this were to occur it would be indicative of fairly advanced cardiac failure and we would expect to see an increase in left ventricular cavity size and a reduction in left ventricular systolic function which was not seen. If left ventricular diastolic function alone were affected this would not have been identified with the echocardiographic parameters used. However, similarly if a proposed reduction in left ventricular wall thickness was related to compensatory left ventricular remodelling it would not be possible to have

isolated diastolic LV dysfunction without any other abnormality, such as LV cavity dilatation, which would expected to precede remodelling.

Another consideration is that all echocardiographic measurements have been of the left ventricle. This is the most important chamber in the adult heart with abnormalities in structure and function associated with the vast majority of pathological disease. However, in both the human and sheep fetus the right ventricle is both larger (in cavity size) and has a higher cardiac output (Anderson *et al.*, 1981;Rasanen *et al.*, 1996), due to the demands from supplying blood to the placenta. Challenges in fetal life more therefore have a more significant impact on the right ventricular function. Whilst right ventricular function can be technically more challenging to assess, it would be useful to study this in the future. Applying fetal cardiac physiology, flow from the right ventricle carries desaturated blood directly to the placenta for oxygenation, while the left ventricle carries saturated blood to the organs including the heart and brain. Any changes in right ventricular flow in the fetus would affect the balance of placental flow and reoxygenation. This could in turn potentially affect many other developmental processes during the critical period in fetal development, significant to the developmental origins of disease hypothesis.

4.5 Conclusion

No differences were seen in left ventricular wall thickness, cavity size, heart weights or functional assessments of the preconceptional and periconceptional undernourished groups. In the previous chapter we saw a sex specific effect of undernutrition in early gestation in the male adult sheep with an increase in LV

interventricular wall and mean LV wall thicknesses. Due to the overlap in the nutritional challenges it is possible that the critical window of development in the process that leads to the increase in LV wall thickness lies between 15 and 31 days gestation.

Chapter 5 - Effects of moderate early gestation undernutrition with or without undernutrition in early postnatal life on the stress response in the adult sheep

5.1 Introduction

Increased plasma catecholamines and sympathetic neuronal traffic has been shown to lead to myocardial hypertrophy (Rostrup *et al.*, 1994;Volpe *et al.*, 1984). This can then lead to a perpetuating cycle where left ventricular hypertrophy leads to increased wall stresses which in turn can activate remodelling stimuli such as increased mechanical wall stress and neuroendocrine activation, leading to myocyte hypertrophy and thereby promoting further hypertrophy and pathological remodelling. In chapter 3 the effects of early gestational undernutrition were shown to lead to increased left ventricular wall thickness. In this chapter I will look at stress response in the adult sheep, focusing on the sympathetic nervous system. This will help to assess any sympathetic involvement that may be seen in association with the increased left ventricular wall thickness found in the early gestational undernourished group.

An important way in which the early environment can have long-term effects is by resetting key hormonal systems that control growth and development, which in turn can influence the predisposition to adult CVD and metabolic disease. Several neuroendocrine systems appear to be involved (Phillips, 2002) but of particular importance are the hormonal systems which mediate the stress response, including the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. There is good evidence from

animal studies that the HPA axis is highly susceptible to programming during development (Poore & Fowden, 2003a), and studies in humans have also reported alterations in the HPA axis in association with low birthweight (Ward *et al.*, 2004). The autonomic nervous system is also known to be susceptible to the influence of environmental factors during development (Young, 2002). Changes in the density of sympathetic innervation or in the functional state of the sympathetic nerves and the adrenal medulla, at rest or in response to specific exposures, can be altered by fetal or neonatal exposures. Preterm birth and fetal growth restriction has been shown to be associated with increased sympathoadrenal activity in childhood, as indicated by stress-induced increases in heart rate and urinary catecholamines (Johansson *et al.*, 2007). These changes have been shown to persist into adulthood (Jones *et al.*, 2007). There has been considerable difficultly in understanding the underlying mechanisms because the autonomic nervous system, which is comprised of multiple function-specific units, forms just one part of the neurohumoral control of the heart and circulation.

I have studied two end-points of the stress response in the form of heart rate response and catecholamine levels. Differences seen in this work may help to unravel the complex mechanisms involved in why adults who were small babies have an enhanced stress response.

5.1.1 Aims and Objectives

The aims of Chapter 5 were:-

1. **To investigate the effect of early gestation nutrient restriction on the stress response in the adult sheep.**

2. **To investigate the effect of early postnatal nutrient restriction on the stress response in the adult sheep.**

3. **To investigate whether early gestation undernutrition influences how early postnatal nutrient restriction affects the stress response.**

4. **To investigate whether any effects of undernutrition in early life on the stress response in the adult sheep are sex specific.**

5.2 Methods

At 132 ± 1.0 weeks of age the sheep stress response to transport and isolation was studied. This was done using the implanted loop recorder and blood sampling (Sections 2.2.2 and 2.3.2). Group numbers for males: CC, n = 11; CU, n = 5; UC, n = 6; UU, n = 6; and females: CC, n = 6; CU, n = 5; UC, n = 7; UU, n = 7.

5.2.1 Stress exposure

Sheep were restrained in a metabolic cage for 3 days acclimatisation following any previous physiological experimentation. Sheep were kept in small groups (2-7 animals) in a room, housed in individual crates. At a set time on the morning of the stress exposure experiment (to minimise confounding physiological effects of the circadian rhythm) the sheep was taken from its room, and wheeled still in its crate (~2 minutes in duration) to a separate room approximately 200 meters away. In this way the sheep was transferred and isolated from its herd community. There was no human interaction on the day, prior to the study.

5.2.2 Measurement of heart rate response

The implanted loop recorder (REVEAL plus, Medtronic, Inc., Minneapolis, USA) was activated 30 minutes after the stressor. This was done using the activator by holding in over the surface of the skin directly over the device. This would thereby record an electrogram of the 40 minutes prior to activation and for 2 minutes after activation.

5.2.3 Blood Sampling

10mL venous blood samples were taken from an in-dwelling jugular venous catheter (See *2.3.2*). Note catecholamines are rapidly degraded and therefore samples were centrifuged immediately (10 min at 4 ºC). Plasma was then stored in multiple aliquots at –20 ºC. This ensured that the plasma was drawn off and frozen within 30 minutes.

Two baseline samples were taken 15 and 5 minutes prior to transporting the animal (time 0). Three further samples were taken at 10 minute intervals, at 10, 20 and 30 minutes after transporting the animal, whilst still in isolation.

At all 5 time-points 1mL arterial blood was taken from the in-dwelling carotid arterial catheter and processed immediately in the blood gas analyser (See *2.3.1*).

5.2.4 Catecholamine analysis

Plasma norepinephrine and epinephrine concentrations were measured from the same aliquot using a commercially available radioimmunoassay kit (2 CAT RIA, Labor Diagnostika Nord, Nordham, Germany), validated in the use of large animals (Boylan & Susa, 1985;Ricchiuti *et al.*, 2002). The sensitivity of the assay for norepinephrine was 12.5 pg/ml and the interassay coefficient of variation for the assay was 21.1% at a value of 1179 pg/ml. The sensitivity of the assay for epinephrine was 2.5 pg/ml and the interassay coefficient of variation for the assay was 14.2% at a value of 218 pg/ml.

5.2.5 Data analysis

Basal norepinephrine and epinephrine concentrations were derived from the average pre-stress concentrations (Touboul *et al.*, 2006). Calculations for each animal determined the maximum norepinephrine and epinephrine concentration (Δ peak) and the area under the response curves (AUC) provided that an increase was observed.

All results are expressed as mean ± standard error of the mean (SEM). Parametric statistics were used as data was normally distributed and groups were of equal variance. Data were analysed by 2 way ANOVA with a post hoc Bonferroni test (GraphPad Prism, and SPSS Inc.). A probability of < 0.05 was considered significant.

5.3 Results

5.3.1 Heart rate response

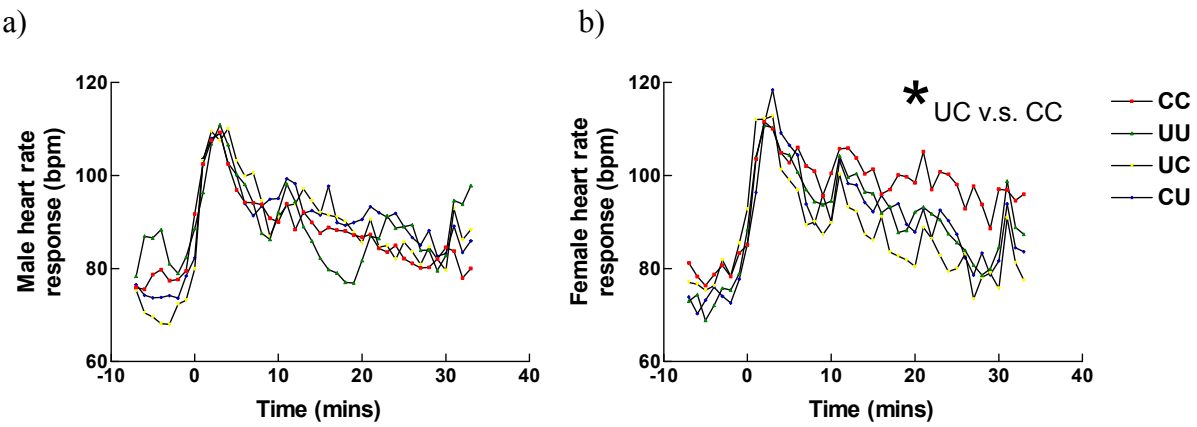


Figure 5.1: The course of heart rate (beats/min; mean ± SEM) in a) male and b) female adult sheep groups before and during transfer and isolation stress. * P<0.001, UC different to CC.

The transfer and isolation stressor resulted in a significant increase in heart rate in all groups (P<0.001). No differences were seen in the baseline values (males 76 ± 3 bpm, 81 ± 4 bpm, 69 ± 3 bpm and 72 ± 4 bpm; females 77 ± 4 bpm, 73 ± 2 bpm, 76 ± 3 bpm and 72 ± 4 bpm for groups CC, CU, UC and UU respectively). The females heart rate response

to the stressor was lower in the group that had a period of early gestational undernutrition when compared to the control group (CC, 96.3 ± 1.4 bpm; UC, 87.1 ± 1.6 bpm; $p<0.001$). Further analysis showed there was no difference between these two groups peak levels (CC, 115 ± 5 bpm; UC, 119 ± 5 bpm) nor the maximal change in heart rate (CC, 37.5 ± 4.5 bpm; UC, 44.9 ± 4.7 bpm). The area under the curve between these two groups showed a trend for decreased area under the curve in the early gestation undernourished group compared to the control group (CC, 744.2 ± 120.3 pg/min/ml, *vs.* UC, 465.9 ± 105.6 pg/min/ml; $P<0.1$). Analysis of the different time periods revealed that this difference related to a lower heart rate response from 10 to 30 minutes post commencement of stressor in the early gestation undernourished group compared to control.

5.3.2 Catecholamine response

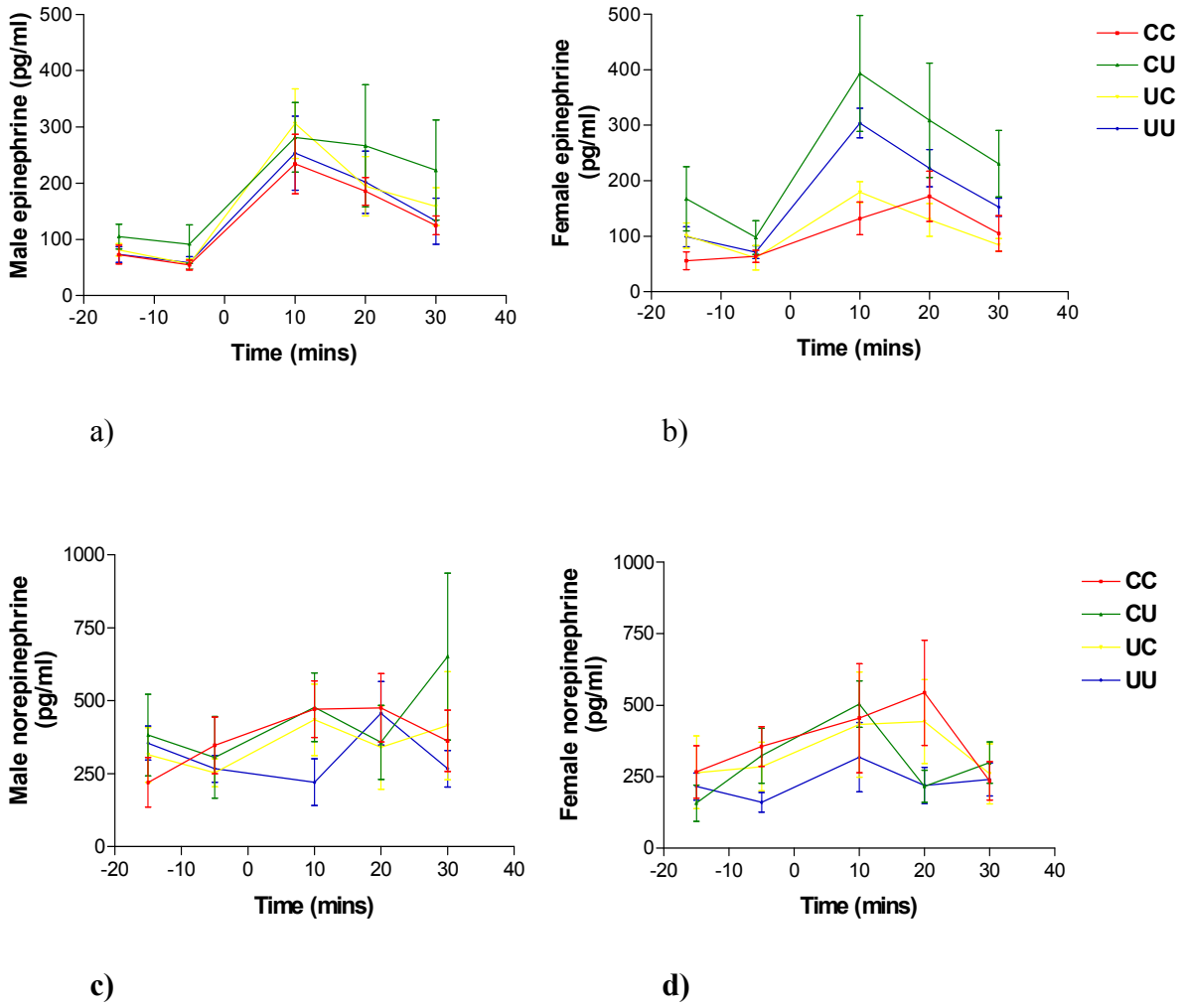


Figure 5.2: Plasma epinephrine concentrations (mean ± S.E.M.) in a) males and b) females and plasma norepinephrine concentrations in c) males and d) females before and after transfer and isolation stress.

The transfer and isolation stress resulted in increases in epinephrine concentrations in all groups ($P<0.001$). However, norepinephrine concentrations did not increase with the transfer and isolation stress. Therefore, no further analysis of norepinephrine response was made.

5.3.2.1 Basal catecholamine results

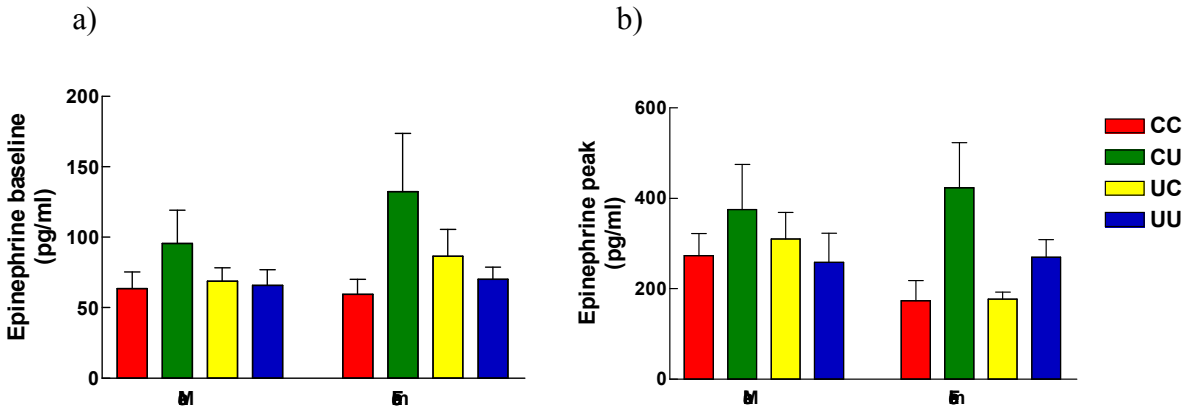


Figure 5.3: Plasma epinephrine (mean ± S.E.M.) at a) baseline, and b) peak plasma concentration.

Analysis of basal epinephrine concentrations showed an interaction ($P < 0.05$) between CC and CU groups, and offspring number. Further analyses revealed that, in singletons, basal epinephrine was increased ($P < 0.05$) by postnatal undernutrition alone (CC, 66.5 ± 21.2 pg/ml, $n = 6$ *vs.* CU, 138.3 ± 22.4 pg/ml, $n = 4$) but not those that received gestational undernutrition followed by postnatal undernutrition (UU, 65.7 ± 7.4 pg/ml, $n = 7$ *vs.* UC, 94.4 ± 23.8 pg/ml, $n = 5$). This effect was not influenced by sex.

5.3.2.2 *Responses to Transfer and Isolation stressor*

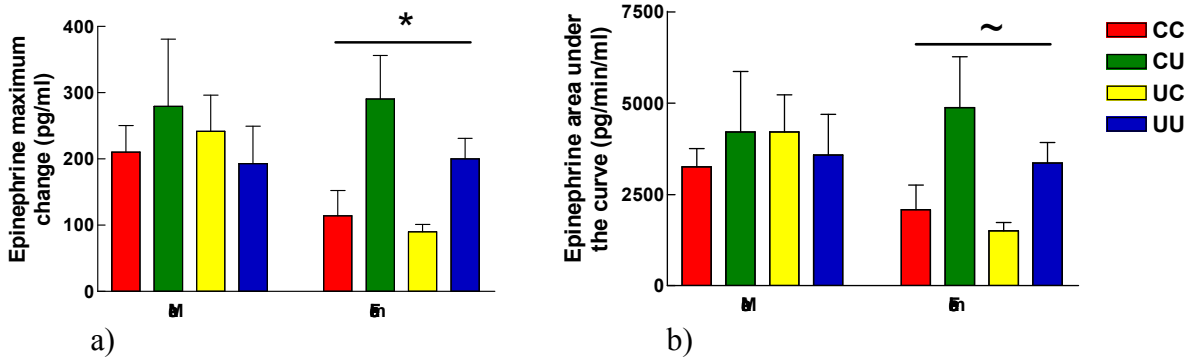


Figure 5.4: Plasma epinephrine concentrations (mean ± S.E.M.) a) maximum change, b) area under the curve.

* P<0.001, CU & UU different to CC & UC. ~ P<0.005, CU & UU different to CC & UU.

In females, there was a significant interaction ($P < 0.001$) between time during the TI test and postnatal nutritional regime for plasma epinephrine concentrations, indicating an enhanced response to stress in postnatally undernourished females, regardless of the prenatal nutritional regime. This was supported by increased maximal change and AUC for epinephrine concentrations in CU and UU females (CU, 290 ± 65 pg/ml and 4867 ± 1397 pg.min.ml^{-1}; UU, 188 ± 33 pg/ml and 2980 ± 555 pg.min.ml^{-1}, respectively; p<0.005) compared to those that received control postnatal nutrition (CC: 116 ± 37 pg/ml and 2052 ± 685 pg.min.ml^{-1}, respectively; UC: 91 ± 10 pg/ml and 1349 ± 226 pg.min.ml^{-1}, respectively). However this effect appears to be attenuated in the group that had both early gestational and early postnatal periods of undernutrition.

5.4 Discussion

5.4.1 Heart rate response

This has shown that undernutrition in early gestational life leads to a suppressed heart rate response to a psychological stressor in the adult female sheep. However, these effects were not seen in combination with a postnatal challenge. This appears to be a sex specific effect occurring only in females. In the early gestational undernourished females no corresponding differences were seen in epinephrine levels at baseline or in response to stress. This suggests that an alternative mechanism to sympathetic stimulation is responsible for this suppressed heart rate response.

It has previously been demonstrated that the effects of sympathetic denervation on heart rate are actually relatively small. Sympathetic tone is relatively high in most organ circulations producing significant vasodilatory and blood pressure effects. In contrast, the heart rate is under a relatively high level of vagal (parasympathetic) tone. It may be that this difference therefore relates to altered parasympathetic stimulation. The absence of a significant norepinephrine rise or indeed increased rise in response to the stress challenge does not rule out the possibility of a parasympathetic mediated mechanism. Higher centres within the cerebral cortex connect with the hypothalamus modulating medullary neuronal activity. Parasympathetic vagal fibres innervating the heart originate from cell bodies within the medulla of the brainstem. Efferent vagal fibres exit the medulla and travel to the heart within the vagus nerve. These fibres innervate specific tissue sites, through the release of neurotransmitter acetylcholine. This then binds to muscarinic receptors in the heart

which then decrease chronotropy. Acetylcholine does also bind at the sympathetic nerve terminal stimulating norepinephrine release which then acts on the adrenoceptors of the heart. However, most of the norepinephrine released by sympathetic nerves is taken back up by the nerves and metabolised. Only a small amount of released norepinephrine diffuses into the blood to circulate throughout the body. Hence, parasympathetic modulation of heart rate response in the female group subject to prenatal undernutrition remains a possible mechanism.

5.4.2 Norepinephrine levels

Other stress response studies using a sheep model have shown a significant acute rise in epinephrine but not norepinephrine (Linares *et al.*, 2007). As previously discussed most norepinephrine released by the sympathetic nerves is taken up and metabolised locally. It is only an "overspill" that will be allowed to diffuse into the blood circulation and therefore have a systemic affect. If the norepinephrine response is not great enough to reach this critical threshold no corresponding rise will be seen in the plasma samples measuring circulatory levels.

5.4.3 Epinephrine levels

In singletons, basal epinephrine was increased by postnatal undernutrition alone, but not those that received both gestational undernutrition followed by postnatal undernutrition.

This was not a sex specific effect. However, the weight of this effect comes predominantly from the female groups as illustrated in figure 5.3a.

This study has also shown that undernutrition in early postnatal life leads to an enhanced response in epinephrine to a stressor in the adult female sheep. This is a sex specific effect, occurring only in females. The effects of this discrete period of undernutrition occurred regardless of prenatal challenge, although the effect did appear to be attenuated when combined with an early gestational period of undernutrition.

When considering these changes seen it is important to note that circulating catecholamines form one part the total neurohumoral control mechanisms that all interact together to ensure cardiovascular homeostasis. Therefore, the autonomic nervous system as well as the other humoral factors including the renin-angiotensin-aldosterone system, atrial natriuretic peptide, and antidiuretic hormone (vasopressin) may need to be considered in the underlying mechanisms involved in the change seen. I have assessed one part neurohumoral control, the acute phase response to the stress, through activation of the sympathetic-adrenomedullary nervous system, which responds to short-term stress through the production of catecholamines. This is in contrast to the hypothalamic–pituitary–adrenocortical system which involves an increase in plasma cortisol levels, modulating the response to long-term stress in sheep (Mellor *et al.*, 2002) in much the same way as in humans.

It is important to note that although elevated baseline epinephrine levels and an enhanced response in epinephrine to a stressor were seen, no corresponding elevation in heart rate was seen. An enhanced response in epinephrine to a stressor may be due to a

significant down-regulation of the ß-adrenergic receptors and perhaps other components of those signal transduction pathways. Sympathetic overactivity with elevated catecholamines has been reported in the early stages of essential hypertension and has been involved in the pathogenesis of left ventricular hypertrophy in essential hypertension. This increased sympathetic outflow thought to be due to a reduced beta2-adrenoceptor density and function which results in the loss of adrenergic responsiveness to changes in the activity of the autonomic nervous system (Calls *et al.*, 2000;Witte *et al.*, 2004).

This has been recognised in conditions associated with cardiac overload and congestive heart failure (Homcy *et al.*, 1991). This leads to elevated catecholamine levels with no corresponding increased heart rate response. Indeed in the later stages of disease decreased heart rate variability is now a commonly recognised poor outcome measure in this group (Bonaduce *et al.*, 1999).

Numerous investigations in humans and experimental animals have demonstrated elevated plasma epinephrine levels in conditions associated with cardiac overload and congestive heart failure (Bristow *et al.*, 1990). Increased plasma catecholamines and sympathetic neuronal traffic have also been considered to contribute to exercise-induced myocardial hypertrophy (Ostman-Smith, 1981). Previous studies in dogs have described cardiac hypertrophy developing from chronic infusion of "subhypertensive" doses of catecholamines. Left and right ventricular hypertrophy was demonstrated despite the absence of sustained haemodynamic changes (Patel *et al.*, 1991). It is therefore possible that increased catecholamine responsiveness as seen in this study could lead to subsequent maladaptive myocardial hypertrophy and subsequent cardiac failure.

Increased efferent sympathetic drive on the myocardium has also been shown to affect other aspects of the cardiovascular system. It can interfere with cardiac electrophysiology, and has been shown to alter ventricular fibrillation thresholds in vulnerable patients (Frasure-Smith *et al.*, 1995). It may also increase the likelihood of vasospasm in damaged and partially occluded coronary arteries, thus leading to an increasing risk of myocardial ischaemia possibly leading to angina or even myocardial infarction due to both increased myocardial demand and diminished supply (Krittayaphong *et al.*, 1997).

5.5 Conclusion

This has shown that undernutrition in early gestational life leads to a suppressed heart rate response to a psychological stressor in the adult female sheep. Undernutrition in early postnatal life conversely leads to alterations in the epinephrine levels. Basal epinephrine was elevated in male and female singletons exposed to the postnatal challenge. The transport and isolation stressor produced an enhanced epinephrine response in the female sheep in the postnatally challenged group. While this was also observed in the female group subject to both prenatal and postnatal undernutrition the effect was less marked.

Possible underlying mechanisms have been discussed. However, this is complex homeostatic system. The difficultly in understanding the underlying mechanisms relating to the stress response is that the autonomic nervous system is comprised of multiple function-

specific units and forms just one part of this hormonal system. I have studied two end-points of the stress response in the form of heart rate response and catecholamine levels.

The stress response is composed of multiple inter-related function-specific subunits. It is therefore difficult to tease out each subdivision and its part to play in programming changes in the stress response in adulthood. The development of the major hormonal responses which mediate the stress response (including the autonomic nervous system) occurs on a regional rather than a global basis. It is likely that each subdivision of the stress response responds to a different set of environmental variables, and that each subdivision may have a different "critical window" of development. The end-result is multiple perturbations which does then pose considerable difficulties in understanding of mechanisms underlying the programming of the stress response. Whilst my work has helped to unravel one chain in the complex mechanisms involved, there is still some way to go before we are able to understand why adults who were small babies go on to have an enhanced stress response.

Chapter 6 - Effects of pre- and periconceptional undernutrition on cardiac electrophysiology in the adult male sheep

6.1 Introduction

Sudden death is the most common form of death in the western world. The worldwide incidence (per year) is estimated at 3 million and 400,000 in Western Europe. The survival is estimated at less than 5% (de Vreede-Swagemakers *et al.*, 1997). The vast majority of these deaths are due to electrical disturbances of the heart, namely ventricular tachycardia or ventricular fibrillation. Preventative therapy is now available for those at high risk in the form of implantable defibrillators. Therefore is an ever growing interest in identifying those at risk.

The importance of cardiac arrhythmias as an underlying mechanism for increased cardiovascular death in adulthood following undernutrition in early life is not yet known. Chapter 3 of my work revealed an increase in left ventricular wall thickness in the male sheep subject to an early gestational undernutrition challenge. Left ventricular hypertrophy has long been recognised as a risk factor in arrhythmias and sudden cardiac death (Myerburg *et al.*, 1992). In Chapter 5 I demonstrated elevated epinephrine levels in response in early postnatal life nutrient challenges. Increased efferent sympathetic drive on the myocardium has also been shown to effect the cardiovascular electrophysiology, and may alter ventricular fibrillation thresholds in vulnerable patients (Frasure-Smith *et al.*, 1995). Patients that have recovered from unexpected ventricular tachycardia or ventricular fibrillation have been shown to have markedly increased cardiac sympathetic activity

compared with appropriate reference groups (Esler, 1992). These clinical findings support a role for cardiac autonomic dysfunction, specifically sympathetic activation and vagal withdrawal, in arrhythmogenesis.

It is important to note the groups studied are the periconceptional and preconceptional challenged sheep with a control group. At the time of planning this work only the data from the chapter 3 relating to the increase in left ventricular wall thickness in the early gestational undernourished group was available. This was also part of a larger initiative of trials over a prolonged time period, with study planning, maternal challenges and maturation of offspring requiring approximately 5 years. There were no remaining animals from the early gestation and postnatal challenged groups. However, it is proposed that the discussed possible mechanistic relationships may still apply in the remaining cohort of animals which had an overlap with the early gestational challenged group. It was therefore felt to be of valve studying the effects of pre- and periconceptional undernutrition on cardiac electrophysiology in the adult male sheep. Any alterations in the cardiac conduction properties as a sequel to these challenges may suggest a potential for arrhythmias and sudden cardiac death.

6.1.1 Aims and Objectives

The aims of chapter 6 were:-

1. **To investigate the effect of periconceptional nutrient restriction on the cardiac structure and function of the adult male sheep.**

2. **To investigate the effect of preconceptional nutrient restriction on the cardiac structure and function of the adult male sheep.**

6.2 Methods

6.2.1 Dietary methods

The nutritional dietary manipulation has been described in section 2.1.3. To summarize the 3 groups are made up of:-

"A" - periconceptional undernourished group (-15 to +15 days of conception; n=12);

"B" - preconceptional undernourished group (-30 to the day of conception; n=14); and

"Control" group (n=8).

6.2.2 Electrophysiology methods

Animals were studied at 172 ± 1 weeks (approximately 3.3 years old) under general anaesthesia as detailed in the General methods preparation and general anaesthesia section 2.2.1.

6.2.2.1 Initial set up

The anaesthetised sheep's trunk and groin areas were sheared. It was then placed in dorsal recumbent position, and secured with leg ties to the operating table. Defibrillation pads were then applied antero-posteriorly to the left side of the sternum and back. 10 surface ECG (4 limb and 6 chest) electrodes were placed in the conventional positions used in human clinical practice. These electrodes were then secured with sutures in order to prevent dislodgement. A baseline 12 lead ECG was then recorded, ensuring any artefact and interference was minimised and gain settings appropriate.

Transcutaneous right femoral venous access was then obtained. This was done using a modified Seldinger technique. A needle with 10ml syringe was advanced, just below the skin crease in the right groin area, medial to the arterial pulsation at a 45 degree angle. When flash back into the syringe was seen, good flow was first confirmed on drawing back with the syringe. The syringe was then removed and a soft tipped wire advanced through the needle into the femoral vein. The needle was then removed. A "6 French" sheath and dilator set (Daig Corp., Minnetonka, Minnesota, USA) was then advanced over the wire up to the hilt. The dilator was then removed and once again blood was drawn back via the side port on the sheath to ensure a good position within the femoral vein. The sheath was then flushed via the side port with 0.9% normal saline, and secured with a skin suture. A bolus of 5,000 units of heparin was given intravenously followed by 1,000 units of heparin intravenously every hour, to help prevent thrombus formation on the intravascular equipment during the experiment.

A haemostatic valve on the sheath allowed introduction and passage of a bipolar catheter electrode into high right apex (HRA) and right ventricle (RV) apex under fluoroscopy.

Baseline pulse and intra-arterial blood pressure recordings are made prior to initiation of programmed stimulation.

An “8 French” percutaneous vascular sheath (Daig Corp., Minnetonka, Minnesota, USA) was inserted percutaneously into the right femoral artery using the modified Seldinger technique as described above. A coronary guiding catheter (Baxter Healthcare Corp., Irvine, GA, USA) was advanced under fluoroscopic guidance into the ascending aorta.

6.2.2.2 Electrocardiogram measurements

The ECG is recorded at a paper speed of 25 mm/sec. In the vertical direction, the amplitude of ECG signals is measured in millivolts with standardization of 1mV. Amplitude of 1 mV is equivalent 10 mm of ECG paper.

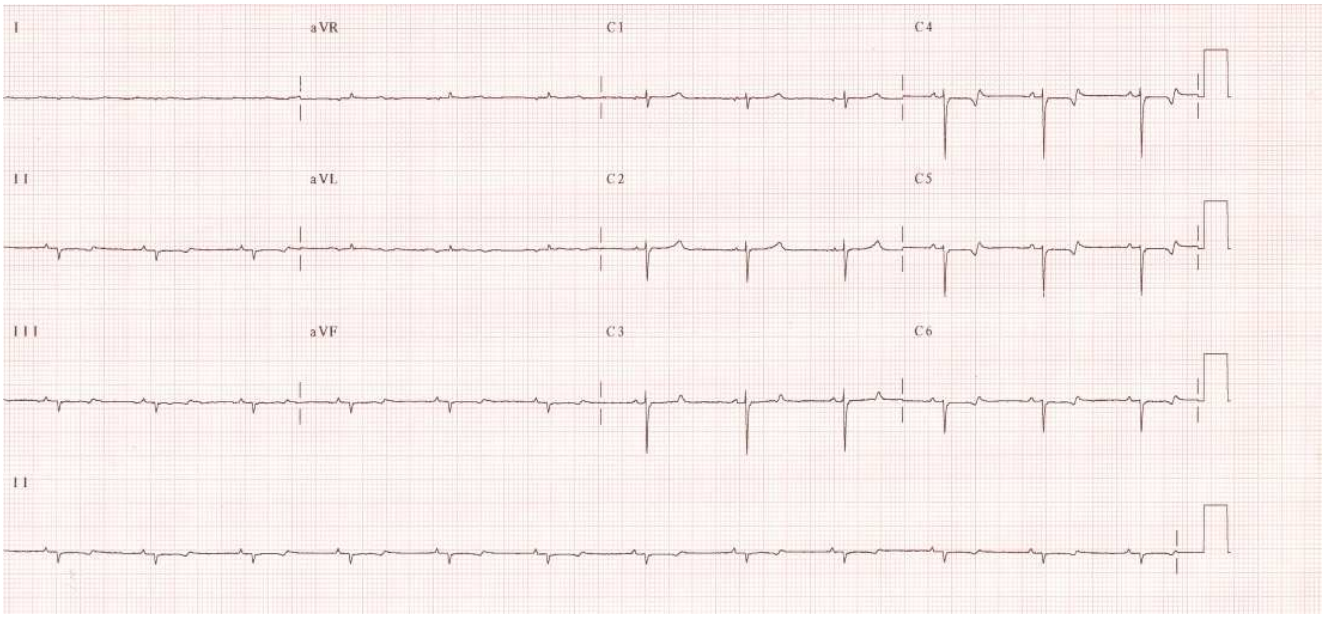


Figure 6.1: Example of a 12 lead electrocardiogram in an adult male sheep.

Measurements were taken as follows:-

- The PR interval was measured from beginning of the P wave to onset of the QRS complex.

- The QRS interval was measured where the first downward deflection is taken as the Q wave, the first upward deflection the R wave, the second downward deflection the S wave, and any second upward deflection another R wave.

- The QT interval was measured from onset of the QRS complex to the end of the T wave (which includes ventricular activation and repolarization). Since the overall QT interval depends on the heart rate, this was then corrected (QTc). The standard clinical correction was performed using Bazett's formula:- $QTc = QT/\sqrt{RR}$.

- The RR interval was taken measured the time interval from the start of an R wave to the start of the next R wave.

6.2.2.2.1 Right atrial measurement of Wenckebach cycle length

The bipolar catheter electrode was placed in the high right atrium to allowing pacing in the right atrium and measurement of the Wenckebach cycle length. This is done by incremental pacing, where the RA is paced at a constant cycle length with gradual shortening until the onset of AV nodal Wenckebach's phenomenon (a physiologic response at faster pacing rates).

6.2.2.2.2 Right atrial measurement of Sinus node recovery time (SNRT)

An electrode catheter was placed in the high right atrium (so as to be near the SA node). Pacing was initiated at a rate slightly faster than the basic sinus rate (~10bpm faster, rounded up to the nearest 10 beat interval). Pacing was then continued at a constant rate for a fixed duration of 30 seconds and then abruptly stopped. The recovery time is the interval from the last paced atrial complex to the first spontaneous SA nodal depolarization.

A series of pacing sequences were used with gradually shortening of the drive trains paced interval down to 300msec (200 beats/min). Hence several recovery intervals for each animal determined at each paced interval. The presence of a “secondary pause” was recorded if seen. The longest interval observed of the recovery intervals were then taken as the SNRT for that animal.

The SNRT is dependent on the basic sinus cycle length i.e. rate dependent. Sinus node recovery time was therefore corrected (cSNRT) for the spontaneous cycle length by calculating the difference between both i.e. cSNRT = SNRT – BCL.

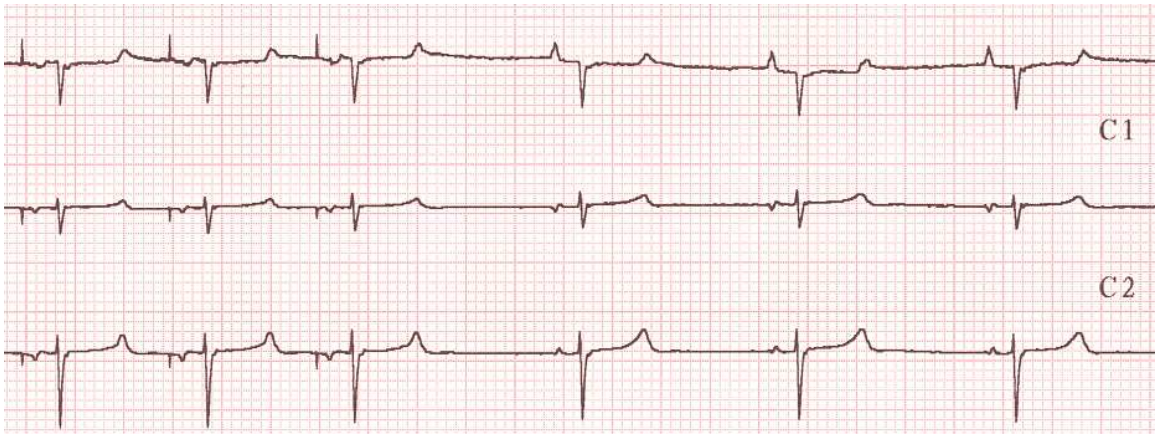


Figure 6.2: Electrocardiogram example of assessment of SNRT

6.2.2.2.3 Determine diastolic pacing threshold and effective refractory period (ERP)

For the study of this physiologic phenomenon, "the effective refractory period", a single extra stimulus is applied after a series of beats with a constant cycle length (Lee *et al.*, 1998). The scanning is initiated late during electrical diastole, and the coupling interval is progressively decreased until the ventricular muscle is refractory (i.e. can not be depolarised). The ERP was taken as the longest coupling interval for which a premature extra stimulus failed to propagate through the tissue, so initiating a paced beat.

6.2.2.2.4 VT induction at RV & LV apex

For ventricular stimulation, the pacing sites used were the right and left ventricular apex. The right ventricular apex was first used to assess for stimulation of ventricular tachyarrhythmias. A 10 beat drive train was used at 350ms cycle length. The stimuli were of 2 msecs duration at twice the diastolic threshold, then repeated at 5 volts, and finally at 10 volts. The 4 extrastimuli used were at 280ms, 270ms, 260ms, 250ms consecutive intervals with all extrastimuli shortened by 10 ms simultaneously until S2 refractoriness or ventricular arrhythmia (VT or VF) induction (See Figure 6.3). In order to reduce the episodes of polymorphic VT and VF very short coupling intervals of <200 ms were not used. This protocol was then repeated with the pacing catheter in the left ventricular apex, assessing its propensity for ventricular tachyarrhythmias.

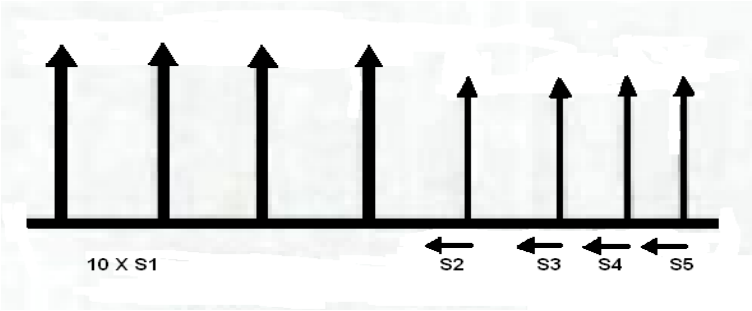


Figure 6.3: Diagram of the VT stimulation protocol used.
S1= stimuli of the basic drive train; S2-S5 = extrastimuli.

Sustained VT was defined as monomorphic VT lasting for more than 30 seconds. Different episodes of VT were considered as the "same morphology" if the axis in the limb

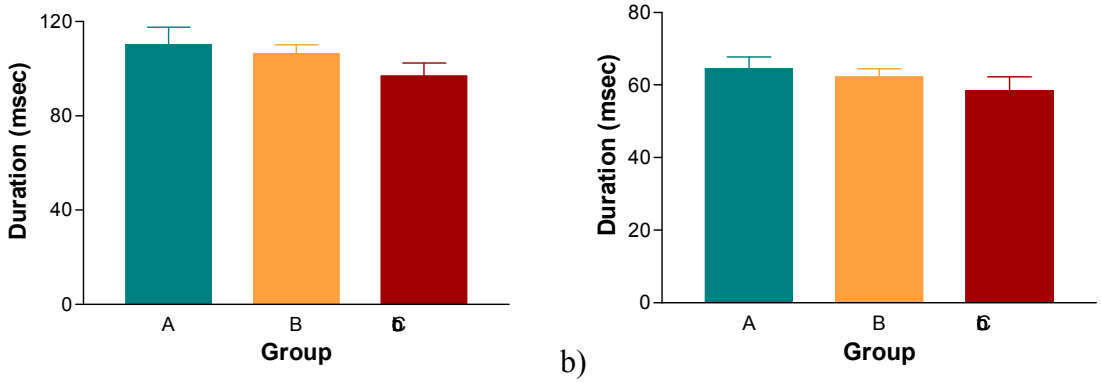
leads did not differ more than 30° and the cycle length differed less than 20 ms. VT termination was attempted using burst pacing at five times diastolic pacing threshold or if unsuccessful by extrathoracic cardioversion. Reproducible VT Induction was defined as three inductions during five successive induction attempts at one stimulation site using the same stimulation protocol. Induction of VF was regarded as a non-specific finding.

6.2.3 Data analysis

All results are expressed as mean ± standard error of the mean (SEM). Parametric statistics were used as data was normally distributed and groups were of equal variance. Data were analysed by 2 way ANOVA with a post hoc Bonferroni test (GraphPad Prism, and SPSS Inc.). A probability of < 0.05 was considered significant.

6.3 Results

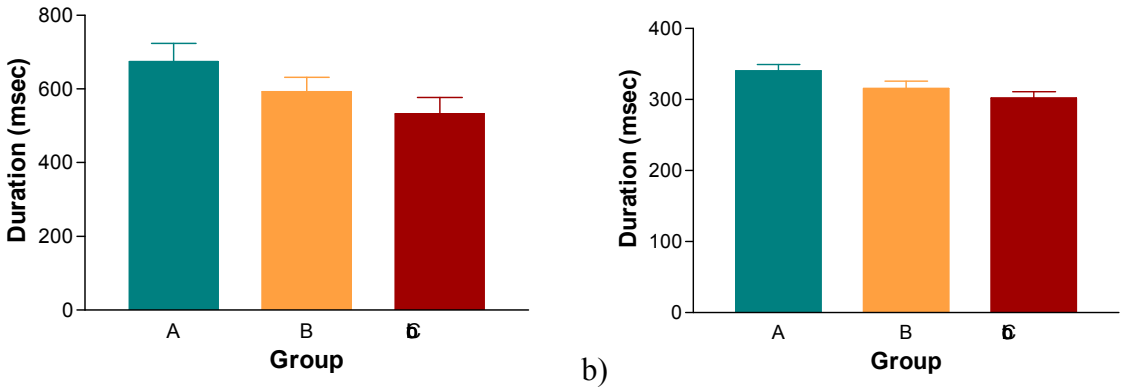
All results are expressed as mean ± SEM. The data was analysed using a univariate analysis of variance model using diet as the main factor. Significance was accepted at $P < 0.05$.



a) b)

Figure 6.4: Graphs showing a) PR interval, and b) QRS interval

There was no difference in PR interval (110.1 ± 7.5msec, 106.4 ± 3.7msec, 96.85 ± 5.5msec) or QRS interval (64.4 ± 3.4msec, 62.2 ± 2.4 msec, 58.4 ± 3.9 msec) between the periconceptional, the preconceptional and the control groups.



a) b)

Figure 6.5: Graphs showing a) RR interval, and b) Corrected QT interval

There was no difference between the periconceptional, the preconceptional and the control groups in RR interval (674.3 ± 49.4 msec, 592.4 ± 39.1 msec, 532.3 ± 44.5 msec), or the corrected QT interval (415.0 ± 6.8msec, 413.0 ± 6.6msec, 417.8 ± 5.9msec).

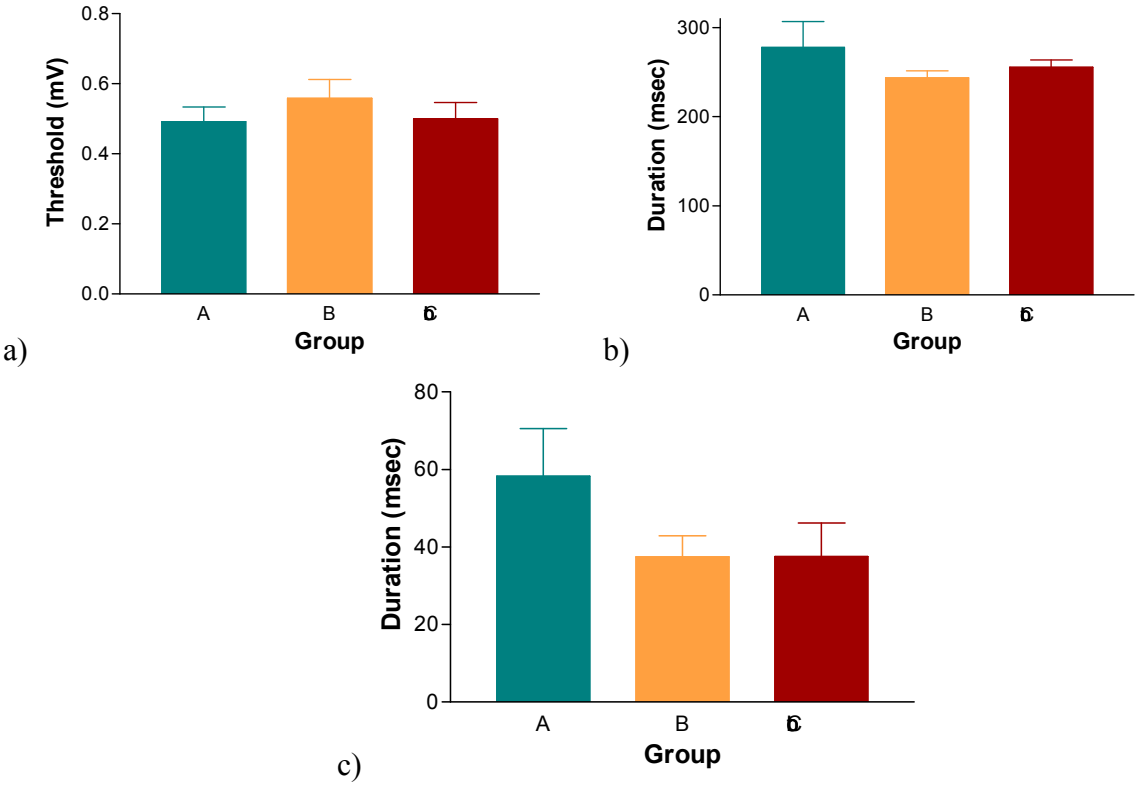


Figure 6.6: Graphs showing a) Right atrial threshold, b) Wenckebach cycle length, and c) Corrected sinus node recovery time

There were no differences in the right atrial threshold (0.4917 ± 0.0417mV, 0.5583 ± 0.0543mV, 0.5000 ± 0.0463mV), the Wenckebach cycle length (278 ± 3msec, 244 ± 8msec, 256 ± 8msec) or the corrected sinus node recovery time interval (1.458 ± 0.31sec, 1.009 ± 0.13sec, 1.036 ± 0.22sec) between the periconceptional, the preconceptional and the control groups.

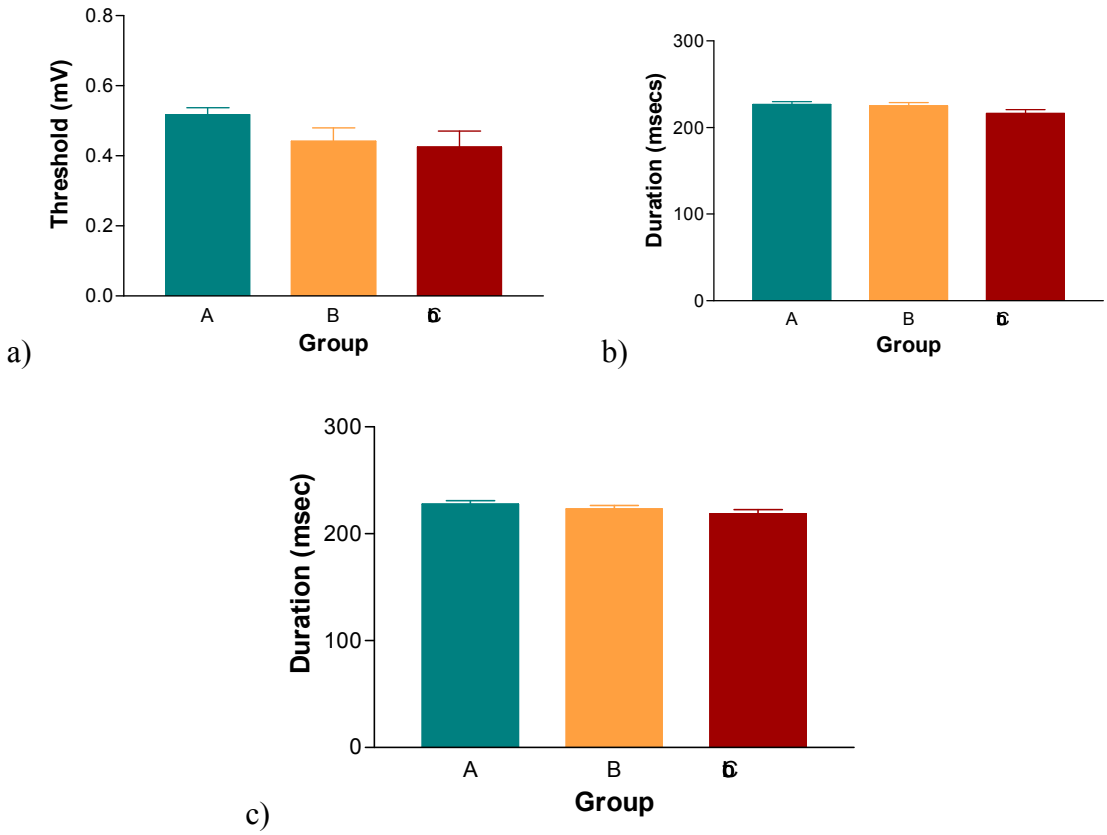


Figure 6.7: Graphs showing a) Right ventricular thresholds, b) Right ventricular effective refractory period, and c) the extrastimuli cycle length in the right ventricle for VT stimulation

There was no difference between the periconceptional, the preconceptional and the control groups in the right ventricular threshold (0.5167 ± 0.0207mV, 0.4417 ± 0.0379mV, 0.4250 ± 0.0453mV), the right ventricular effective refractory period (226.7 ± 3.6msec, 225.0 ± 4.0msec, 216.3 ± 4.6msec), or the extrastimuli cycle length in the left ventricle for VT stimulation.

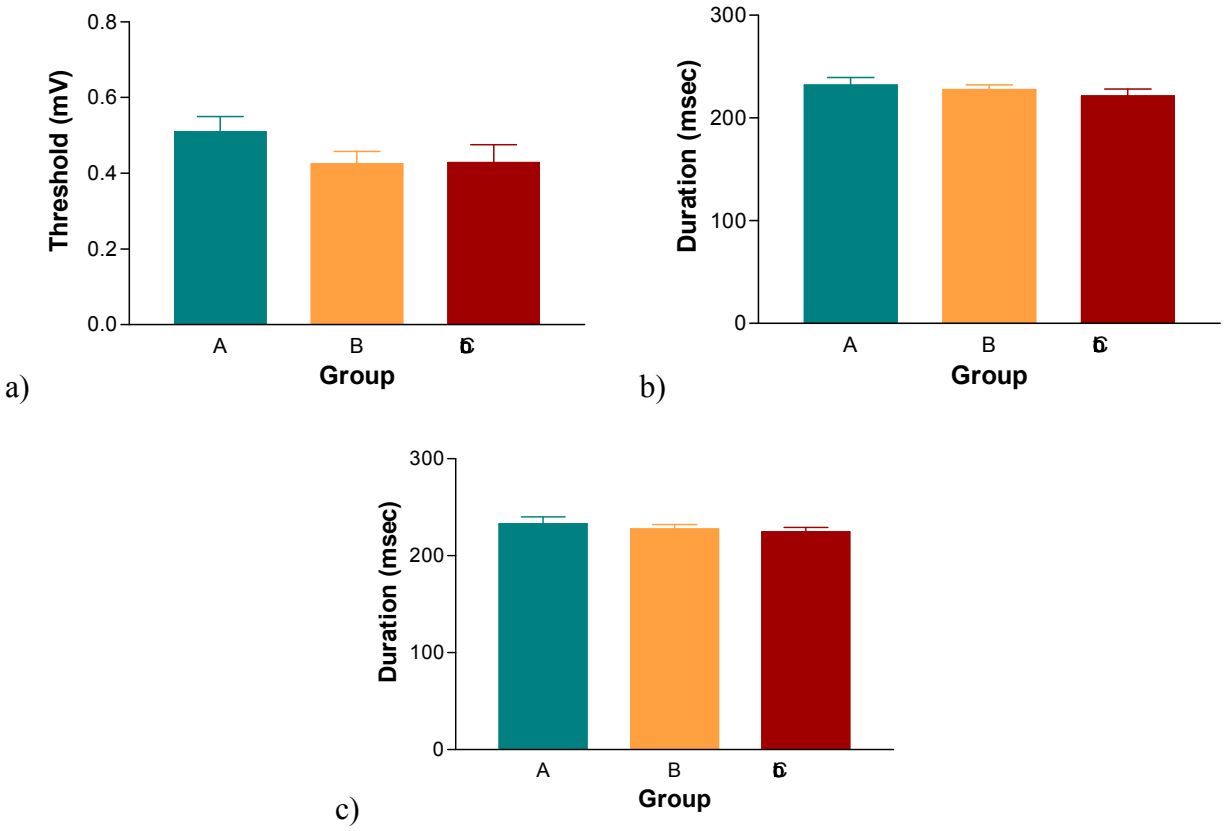


Figure 6.8: Graphs showing a) left ventricular threshold, b) left ventricular effective refactory period, and c) the extrastimuli cycle length in the left ventricle for VT stimulation

There was no differences in the left ventricular threshold (0.5091 ± 0.0415mV, 0.4250 ± 0.0329mV, 0.4286 ± 0.0474mV), the left ventricular effective refractory period (231.8 ± 7.5msec, 227.5 ± 4.6msec, 221.4 ± 6.7msec), or the extrastimuli cycle length in the left ventricle for VT stimulation between the periconceptional, the preconceptional and the control groups.

6.4 Discussion

6.4.1 Evaluation of the Sinoatrial node

The sinus node recovery time was used to assess the automaticity of the sinoatrial node. No previous data is available for sheep. Recovery intervals were shorter than would be expected in the humans. Studies in normal adult humans have suggested cSNRT of 270 ± 113msec (mean ± SD), with a range of 66 - 508msec acknowledging that there is wide variability in normal humans (Breithardt *et al.*, 1977). Certainly all values were within the normal range for humans, at all pacing rates, and no secondary intervals were seen.

It should be considered that the sinus node is sensitive to changes in the autonomic system. As we have shown in Chapter 5, the adult sheep are sensitive to physical stressors. These increases in sympathetic tone and circulating catecholamines may lead to increases the sinus rate at the time of our studies. Conversely, the anaesthetic agents used, particularly halothane used for maintenance of anaesthesia increases parasympathetic tone. If any abnormalities were found it could be questioned as to whether or not this was due to dysfunction of the intrinsic sinoatrial node function or whether it was related to autonomic tone. However since these studies have shown normal conduction this can not be questioned. It may however be an area of concern for future work.

6.4.2 Evaluation of the Atrioventricular Conduction

The atrioventricular conduction intervals were obtained from the resting ECG. Lengthening of the PR interval and atrioventricular block can occur with any abnormality in which conduction of sinoatrial impulses to the ventricle is delayed or interrupted. The PR interval was measured within normal range, and no AV block was seen in our study. The QRS durations were all within normal range and there were no differences between the groups. This suggests that the infranodal distal conducting system (His bundle to the Purkinje ramifications) has not been affected by the early like nutritional challenges.

The Wenckebach cycle length was measured using incremental pacing. This stresses the AV conducting system with increasingly rapid atrial pacing to test the weak link in the AV conducting system. At faster and faster atrial pacing eventually the refractory period of the AV node is reached. At this point second degree AV block was seen. In all cases the initial pattern of block seen was Mobitz type I, which is the normal expected phenomenon with rapid atrial pacing. The normal Wenckebach cycle length in humans is usually accepted as ≤ 450msecs. Once again in our adult sheep there were no differences seen between the 3 groups, and the range for all animals was 200 – 360msec.

All parameters measures showed no differences between the 3 groups. This would suggest that the sinus node, the AV node, the His bundle, the left and right bundle branches and the Purkinje ramifications are of normal position and function.

6.4.3 The Ventricular conduction system

No monomorphic ventricular tachycardia was triggered during the VT stimulation studies. There was ventricular fibrillation seen is some of the animals. The significance of this will be discussed. I would first like to consider the potential risk factors for ventricular tachyarrhythmia, and therefore the possible mechanisms of ventricular tachyarrhythmia in my groups. This will allow me to then interpret the significance of these results.

Most ventricular arrhythmias are due to either abnormal re-entry or automaticity. Re-entrant circuits are usually formed following a heart disease that results in scar deposition in the ventricular myocardium. This most commonly occurs in cardiomyopathy and ischaemia heart disease in humans. The re-entrant circuits most often arise during the healing process, in the border zone between scar tissue and normal myocardium. Once a re-entrant circuit is formed it is always present and can therefore generate a ventricular tachyarrhythmia at any time. This also means that re-entrant arrhythmias can be induced with the programmed pacing techniques used in this study (Myerburg & Castellanos A, 1986). However from the results of the effects on cardiac morphology and function in this same cohort of sheep we found no evidence of structural heart disease or cardiomyopathy. As previously discussed ischaemic heart disease has not been identified in sheep. This would suggest that the induction of a re-entrant ventricular tachyarrhythmia would seem unlikely.

Secondly the mechanisms for automatic ventricular arrhythmia, which account for a minority of ventricular arrhythmias in humans, is usually associated with acute medical conditions such as:-

a) Acute myocardial infarction or ischaemia;

b) Electrolyte and acid-base disturbances, hypoxemia;

c) Increased sympathetic tone.

We have assessed and reasonably excluded electrolyte, acid-base disturbances, hypoxaemia and ischaemic heart disease. The only mechanism that it would seem likely to be affected is differences in sympathetic tone between our groups. This is not a sensitive method for identifying differences in sympathetic tone. The first reason for this is that this mechanistic subset accounts for such a small proportion of all ventricular arrhythmias. Secondly automatic ventricular arrhythmias are often not inducible through electrophysiological studies. Therefore if differences do exist in ventricular arrhythmias due to differences in sympathetic tone these electrophysiological studies are unlikely to be sensitive enough to identify differences in this subset. Further direct analysis of sympathetic tone is needed.

The sensitivity of pacing protocols is directly related to the number of extra stimuli utilized. This occurs, however, at the expense of specificity when polymorphic VT/VF can be induced at very short coupling intervals by using multiple extra stimuli. Regardless of the pacing protocol, the induction of sustained monomorphic VT constitutes a specific response which is seldom induced in subjects not prone to such arrhythmias clinically. In

contrast, the induction of polymorphic VT/VF with four extra stimuli at short coupling intervals can be non-specific and does not provide a reliable guide for serial testing (Morady *et al.*, 1984).

Therefore in view of the discussed mechanisms of ventricular tachyarrhythmia's, in this cohort of sheep with a structurally normal heart with no risk factors for ischaemic heart disease I would expect the propensity for ventricular arrhythmias to be very low. The results of this study are in keeping with this.

6.4.4 Conclusion

This chapter has shown that neither pre-conceptional nor peri-conceptional nutrient restriction affects the cardiac electrophysiological properties in adult male sheep.

In our cohort of sheep we know from studies in Chapter 5 that there were no structural heart abnormalities found on echocardiography and there have been no other indicators of cardiac disease. Coronary artery disease would not be expected in an otherwise healthy ruminant of this age. While it would therefore be unexpected to draw out any differences in our groups, it is never-the-less valuable to show that it does not occur in adult sheep that have been subject to pre-conceptional and periconceptional undernutrition, as using this animal model may give insight into early pathophysiological processes in humans.

Generally, increasing age is accompanied by increasing fibrosis of the conduction systems. This process would arguably exaggerate any pre-existing variation. This raises the

possibility that by studying an older cohort of animals would increase the likelihood of finding cardiac electrophysiological differences between the groups.

Chapter 7 - General Discussion

7.1 Overview

This main aim of this thesis was to determine if early life undernutrition altered the cardiovascular physiology of the adult sheep. To investigate this, five different nutritional challenges were used including a combined challenge. A broad assessment of cardiovascular physiology was studied, included many different techniques. The main points to emerge from this work will be summarised in the following sections.

7.1.1 Chapter 3

This has shown undernutrition during early gestation causes a sex specific increase in interventricular septal wall thickness and mean LV wall thickness in adult male sheep. The increased left ventricular wall thickness did not result in functional loss, and it is proposed that this may represent the early stages of hypertrophy, preceding the myopathic process. The data suggest a critical window of development from day 1 to day 31 of gestation.

Left ventricular hypertrophy has been shown in other animal models, e.g. following a brief early gestational exposure to glucocorticoids. It is possible that the observed phenotypic changes seen in the early gestation undernourished group are as a result of remodelling stimuli through neuroendocrine activation. Epigenetic regulation of gene transcription provides a strong candidate mechanism for fetal programming. This can occur

through persistent alteration of gene transcription, such as through DNA methylation or histone modification, which is largely established in utero. Whilst resting blood pressure is unchanged in this group, these animals have been found to be hypertensive during stress (Cleal *et al.*, 2007a). This raises the possibility of my favoured mechanism of to an altered vascular responsiveness with chronic stress induced hypertension leading to the increase in left ventricular wall thickness.

When early gestational undernutrition is combined with an early postnatal challenge no changes where seen in the left ventricular wall thicknesses. This suggests that matching of pre-and post-natal nutrition is beneficial, whereas a mismatch is detrimental to the heart in the long-term. Hence the underlying mechanism responsible for the changes following the early gestation undernutrition must be at least partially reversible.

7.1.2 Chapter 4

This chapter studied male adult sheep and did not include a combination challenge. No differences were seen in left ventricular wall thickness, cavity size or functional assessments between the preconceptional, periconceptional and control groups.

The periconceptional group's dietary challenge overlaps with the early gestational challenged group (minus 15 to 15 days gestation *vs*. day 1 to day 31 gestation respectively. This suggests that the critical window of development in the process that leads to the increase in left ventricular wall thickness lies between 15 and 30 days gestation, and further work is needed to explore this.

7.1.3 Chapter 5

This has shown that undernutrition in early gestational life leads to a suppressed heart rate response to a psychological stressor in the adult female sheep. Undernutrition in early postnatal life conversely leads to alterations in the adrenaline levels. Basal adrenaline was elevated in male and female singletons exposed to the postnatal challenge. The stressor produced an enhanced adrenaline response in the female sheep in the postnatal challenged group. While this was also observed in the female group subject to both prenatal and postnatal undernutrition the effect was less marked. No corresponding changes in heart rate response. I have proposed the mechanism for this increased sympathetic outflow is due to a reduced adrenoceptor density and function, which results in the loss of adrenergic responsiveness.

7.1.4 Chapter 6

This chapter has describes the results of invasive cardiac electrophysiological studies on the male adult sheep exposed to either peri-conceptional or pre-conceptional nutrient restriction. It has shown no effect on the cardiac electrophysiological properties of these challenged sheep compared with the controls. In view of previous results seen in other groups showing effects on left ventricular wall thickness and adrenaline levels it has been proposed that these groups may have had an increased susceptibility to arrhythmogenesis. However, in the absence of any structural heart abnormalities, impairment in left ventricular function (Chapter 4) nor any expectation of coronary artery

disease in healthy relatively young ruminants, the absence of any changes in cardiac electrophysiology was not entirely unexpected.

7.2 Reflections on my research

7.2.1 The sheep model

The sheep have a relatively long gestation period (~147 days); they also have a relatively long live span (puberty commences by 30-50 weeks of age, (Foster & Karsch, 1975). This type of study therefore requires a long period of time from conception to nutrition challenges and then to maturation into adulthood prior to physiological study. As discussed in Section 1.5 this does have advantages such as being more accurate and comparable to human gestation. However, animals with a shorter gestation and life-span, such rodents and other small animals can be studied over a much shorter time interval and enables the researcher to develop and focus a hypothesis from repeated experiments over the course of a period of research for a thesis. I was in part able to do this due to the staged nature of our work, with the first 2 groups being studied at ~2.5 years of age and the second set of groups at 3.3 years of age. These did however involve animals which had received different nutritional challenges.

Since the sheep is a herd animal, all animals were keep in groups so as to avoid the stress of isolation except during experimentation. Whilst my work focuses on the cardiovascular system, simultaneous work was performed on the animals to assess growth, the renin-aldosterone system and hypothalamus-pituitary-adrenal system in this set of

animals has been reported elsewhere (Cleal *et al.*, 2007a;Cleal *et al.*, 2007b;Poore *et al.*, 2006). This required a group of researchers (usually at least four) working long hours. A strict standardised protocol was followed throughout all experiments by skilled scientists thus minimising the occurrence of operator errors. This had the advantage of allowing large amounts of data to be collected. One disadvantage that came from this was the time constraints available for my cardiovascular studies. For example, it would have been useful to perform more detailed echocardiographic studies of these animals including assessments of diastolic function as well as responses to a stress challenge (such as an intravenous fluid bolus). However, this was not possible due to the surgical procedures which immediately followed my studies and the time constraints required to limit the duration of general anaesthesia as well as the sheer volume of procedures performed in a day.

Another limitation of my work links into the points previously mentioned. The restrictions bound by the Home Office animal welfare regulations meant that I was not able to test my protocols prior to experimenting on the nutritionally challenged groups. This did mean that it was not possible to collect some data in the early experiments. For example to my knowledge no previous studies have been done measuring many of the electrophysiological parameters in my work. To this end in was impossible to "rehearse" my *in vivo* studies are therefore very difficult to ensure an infallible protocol. Indeed some of the protocols did alter in the early stages of the electrophysiological studies. The lower limit for the ventricular stimulation studies extrastimuli was initially 180 milliseconds. However, it soon became apparent that at this level the majority of animals were induced into ventricular fibrillation. This was of no predictive value in assessing susceptibility for arrhythmogenesis, but was potentially detrimental to ongoing experimentation where

prolonged ventricular fibrillation caused the animal to become “unwell” with altered physiological parameters or indeed its demise.

7.2.2 Statistical analysis

A power calculation for my data was made for the echocardiographic and hormone assay analysis. No similar studies have been published to enable a power calculation for the electrophysiological studies. It is of interest that all these observations were non-significant. As discussed, this is in keeping with correlated findings in the human field. However, it does raise the possibility of type II error, where a difference is not identified due to the sample size being too small to identify it.

I have therefore performed the following retrospective power calculations:-

1) Based on results from echocardiographic measurements of interventricular septal wall thickness (IVSd):-

   $n = 16\ \sigma^2 / \delta^2$

   $n = 16 \times (0.11)^2 / (0.7)^2$

   $n = 3.95$ *i.e.* $n > 4$, suggesting an adequately power study.

2) Based on results from electrophysiological studies

   $n = 16\ \sigma^2 / \delta^2$

   $n = 16 \times (0.02)^2 / (0.002)^2$

$n = 0.0064/0.000004$ i.e. $n > 1600$, suggesting an under-powered study.

This retrospective power calculation may therefore suggest that differences in the electrophysiology studies were not significant because the study was underpowered, implying that the difference would have been significant if a more appropriately powerful study had been done. However, many would regard this is a misuse of power and should be avoided, arguing that post-hoc power analysis does not add information beyond that of the *p* value, because it is derived by just solving the sample size equation for power after plugging in the observed values from the study.

7.2.3 Time constraints

One strength of this work was the broad cardiovascular aspects that were investigated. These were largely predetermined drawing on both my cardiological expertise and the resources, available to me. However, it soon became apparent once my experiments were underway that novel findings would ideally need to be investigated further, in order to probe further into the mechanisms underlying the changes seen. For example, the finding of increased left ventricular wall thickness in the early gestational challenged group then posed the question of whether this was due to cellular hypertrophy or hyperplasia. I would have liked to have investigated this further with histological analysis. Due to the time constraints on my period of research I was not able to do this. Relevant tissue samples have been stored, and I hope that this is possible for other researchers within our department to undertake in the future.

7.2.4 Sex specific effects

Attempts were made to avoid confounding variables from inherent differences between the sexes. All animals were studies after puberty which commences by 30-50 weeks of age in both males and females (Karsch & Foster, 1975). No physiological studies were carried out during the breeding season, which runs from October to May (Santiago-Moreno *et al.*, 2001). Finally all animals were studied at the same age in order to limit the developmental differences in hormone status within the same sex group. It was still deemed necessary to analyse separately males and females due to differences in growth, body composition and size influencing the physiological response to undernutrition.

It is well known that the incidence of cardiovascular events and hypertension in pre-menopausal women is lower than that of age matched men. After the menopause, cardiovascular morbidity and mortality in women become similar, if not higher, than that in men, indicating that female sex hormones play an important protective role upon the maintenance of the vasculature (Rappelli, 2002). However, gender differences in renin-angiotensin system components also seem to exist and may play a central role in the hypertension and cardiovascular disease. For example, in normotensive populations, the plasma renin activity is significantly higher in men than that in women, and it is higher in postmenopausal vs. pre-menopausal women (Fisman *et al.*, 2002). Oestrogen is protective against hypertension, possibly by modulating the balance of the angiotensin subtypes (Brosnihan *et al.*, 1997). In spontaneously hypertensive rats, ovariectomy has been shown

to cause cardiac hypertrophy and an unfavourable myocardial remodelling (Santos *et al.*, 2004).

Alternatively, the fact that the male sex is a risk for the development of hypertension and cardiovascular disease may relate to the adverse effects of the male sex hormone. Indeed, absence of testosterone in experimental models abolishes hypertension (Reckelhoff, 2001a), most likely through interaction with the renin-aldosterone system. These observations suggest both male and female sex hormones, via interaction with regulatory pathways may contribute to the development of hypertension and cardiovascular disease.

Understanding the possible mechanisms of the sex differences in cardiovascular disease is vital to our understanding of its potential impact on the developmental origins of cardiovascular disease. This suggests any differences seen in the left ventricular thickness in our male group may have been attenuated in the respective female group due to either the protective effects of oestrogen or the gender differences in renin-angiotensin system.

An alternative hypothesis may be directly attributed to the mechanisms underlying the developmental of origins of health and disease hypothesis. It is well established that males and females grow at different rates due to differences in male and female hormones (Robert *et al.*, 1999). Indeed in this cohort of animals the early growth rates were greater in the males than females (Cleal *et al.*, 2007c). Fetal demand is, in part, determined by the fetal growth trajectory, with faster growing fetuses being more vulnerable to impaired fetal nutrition (Godfrey, 1998;Godfrey & Barker, 2001;Harding, 2001). Therefore, with male fetuses growing on average at a faster rate than female fetuses, the male offspring may be

more vulnerable to prenatal undernutrition because they grow more rapidly *in utero* than females.

In this way sex differences may relate to the predictive-adaptive response concept, whereby males and females have different biological roles. For example the male may be driven to achieve early maximal body size in order to ensure reproductive success, whereas the female strives to ensure extended fertility. It may be that the sex differences seen in my studies relate to a more complex combination of influences involving interactions of the above proposed mechanisms to varying degrees. Understanding these mechanisms may lead to the development of novel, potentially sex-specific strategies for prevention and treatment of hypertension and cardiovascular disease.

7.2.5 Twinning effects

Attempts were made to limit the number of multiple pregnancies. For this reason the sheep model was used and the Welsh Mountain breed was used (as discussed in Section 1.5). However, it is possible that keeping a hardy mountain bred in the relatively comfortable environment of an open barn possibly led to a greater number of twin pregnancies than expected.

It would be expected that during periods of undernutrition the ewe would spend more of her own body's reserves to fuel the metabolism and growth of two fetuses or suckling lambs. Nutrient challenges would be expected to have a different grade of effect in twin compared to singleton pregnancies. In humans there are distinct fetal growth patterns

in singleton and twin pregnancies (Blickstein, 2004) and even when a twin is lost in early gestation the birth weight of the remaining twin does not match that of a singleton (Depp *et al.*, 1996).

However no differences were seen between twins and singletons in the early gestation undernourished group. The response to gestational undernutrition in terms of weight was greater in ewes bearing twins. Also twin fetuses had a higher fetus to placenta ratio and were smaller than singletons at birth. This variable does introduce a possible confounding variable to my studies. It should be noted this sub-group analysis of twins versus singletons does inevitably reduce group sizes and therefore potentially dilute effects of one sub-group and introduce type II error.

7.2.6 Nutritional challenges

As discussed in Section 1.1.7 in our sheep model a total nutrient restriction challenge was used. This is because a significant proportion of the dietary nitrogen is supplied by the gut commensals making it difficult to manipulate the proportion of protein received in this micro-environment. These challenges may not resemble the current human dietary inadequacies seen in the western world where maternal dietary deprivation due to poverty, lack of education or social issues is often related to altered micronutrient intake with a disproportionately high intake of saturated fat. However, malnutrition in the western world does still involve a moderate global reduction of all dietary constituents, and when considering the global context of malnutrition and disease this is still the prevalent dietary inadequacy worldwide.

7.2.7 The predictive adaptive response model

The first cohort of animals in these experiments included a control, a prenatally and a postnatally undernourished group as well as a fourth group which had been subject to both of these challenges. The animals studied in these experiments have been valuable in providing evidence in favour of the predictive adaptive response model. This has been demonstrated in the male which following an early prenatal period of undernutrition developed an increased left ventricular wall thickness, which was not seen in the group that also had the matched postnatal challenge. In the females both postnatally undernourished groups had an elevated adrenaline levels in response to a stressor, however, this effect was dampened in the group subject to both pre-and postnatal challenges.

Hence this thesis has shown evidence of a predictive adaptive response at two critical periods of development, on different components of the cardiovascular system in both male and female adult sheep. If the prenatal prediction is not reflected by the postnatal environment LV hypertrophy is induced. Conversely, the effects of postnatal undernutrition leading to elevated adrenaline stress responses are protected against by prenatal undernutrition. A mismatch of pre and postnatal environments leads to altered cardiovascular physiology in adulthood which can be linked with human cardiovascular disease.

7.3 Conclusion

This thesis has shown that whilst pre- and periconceptual nutrient restriction has shown no effect, early gestation and early postnatal nutrient restriction independently cause sex-specific changes in the cardiovascular system in adult life. However the most significant novel findings of this body of work are that the effects of a nutrient environment mismatch seems to have a greater impact than combined (i.e. matched) pre- and postnatal challenges. This supports the concept that adult cardiovascular function is determined in part by developmental responses to intrauterine nutrition made in expectation of the postnatal nutritional environment. If these predictions are not met then the adult will be mal-adapted and may be at greater risk of disease. This highlights the importance of nutrition in early life in determining later cardiovascular health, and has specific implications for devising preventative strategies to reduce the impact of a mismatch in nutrition in human populations undergoing rapid economic transition.

7.4 Future work

The findings reported in this thesis have demonstrated the importance of investigating the effects of early life undernutrition in adulthood. These studies have generated many possible avenues for future research.

In view of the changes seen in left ventricular wall thickness, further echocardiographic studies such as assessment of diastolic function would be of value. Further analysis of the myocardial tissue would also be of interest. Through histological studies it could be possible to cell count thereby answering the question of wall thickness relating to hypertrophy or hyperplasia. However this may not be possible due to the fast freezing and storage of the myocardium which will introduce error through shrinkage. Other analysis to consider is collagen content and typing. This may give an indication of the cause of the left ventricular hypertrophy. Increased collagen levels would suggest a pathological process as opposed to physiological hypertrophy such as that seen in human athletes.

The stress response study has also led to further questions as to the precise mechanism underlying the changes we have shown. Myocyte adrenergic receptors could also be analysed to see is there is indeed a down-regulation in adrenergic receptors, which would confirm or indeed refute my proposed mechanism.

No differences were seen in the cardiac electrophysiology of the peri- and pre-conceptional nutrient restricted groups. However, in view of the LV wall thickness and stress response changes seen in the early gestational and postnatally restricted groups it

would be of value to assess the cardiac electrophysiology in these groups (as discussed in chapter 6).

It is clear that more detailed animal studies are needed to further analysis the mechanisms of nutritional programming. This must first be done in order to enable us to explore the possible parallels in human disease states. At some point in the future there will then need to be a human intervention study, timed in pregnancy and early life, aimed at the prevention of cardiovascular disease, in order to determine the exact nature of the nutrient balance which determine early life growth patterns and long-term health. At this time I hope that the developmental origins of disease hypothesis can be conclusively substantiated and go on contribute to preventive strategies that will be of utmost importance to the health of the nation.

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Appendix 1 – Sheep Diets

APPENDIX 1.1: MATERNAL DIET

G J W TITMUS, New Mill, Hamer Lane, Wheathampsted, Hertfordshire

P316 – RVC EXP Sheep Nuts: a complementary feeding stuff for feeding to sheep.

Protein	14.75%
Fibre	16.80%
MER	10.80 MJ/kg
Vitamin D3	2000 iu/kg
Vitamin E	10mg/kg (alpha tocoperal)
Oil (A)	2.50%
Ash	8.20%
Vitamin A	10 000 iu/kg

Raw Materials:
Treated straw, Grass, Hipro soya E, Micronized wheat, Wheat, Barley, Full fat soya, Molasses, Limestone, Calcium carbonate, Dicalcium phosphate salt, Sheep vitamin/mineral supplement.

APPENDIX 1.2: EWBOL PRESTIGE LAMB PELLETS + DECOX

BOCM PAULS LTD, Lindum Mill, Shepshed, Loughborough, Leics., LE12 9BS

This is a complementary feeding stuff for feeding, with forage, to growing and fattening lambs up to 70% of dry matter.

Oil	4.00 %	Vitamin A-retinol	8000 iu/kg
Protein	18.00 %	Vitamin D3-cholecalciferol	2500 iu/kg
Fibre	12.00 %	Vitamin E-alpha tocopherol	30 iu/kg
Ash	9.50 %	Sodium Selenite-selenium	0.30 mg/kg
Moisture	13.80 %		

Contains the following ingredients in descending order by weight:
Barley, Sunflower ext, Copr exp, Soyabean hulls, Molasses, Malt culms, Peas, Palm kernel exp, Brazilian toasted soya, Bakery by-product, Calcium carbonate, Low glucosinolate rape seed ext, Salt, Vegetable oils.

This medicated feedstuff:
Contains DECCOX (V.m 13997/4009) at a concentration of 100mg/kg DECOQUINATE BP as directed by the veterinary surgeon.

APPENDIX 1.3: EWBOL 18 NUTS

BOCM PAULS LTD, Lindum Mill, Shepshed, Loughborough, Leics., LE12 9BS

This is a complementary feedingstuff for feeding, with forage, to ewes up to 70% of dietary dry matter intake.
In the preparation of this diet precautions have been taken to minimise the potential risk for those sheep prone to copper toxicity. However no guarantee can be given due to the known differences in both breed and management systems. This feedstuff is not recommended for feeding to commercially milked ewes.

Oil	5.00 %	Vitamin A-retinol	8000 iu/kg
Protein	18.00 %	Vitamin D3-cholecalciferol	2500 iu/kg
Fibre	9.90 %	Vitamin E-alpha tocopherol	150 iu/kg
Ash	9.50 %	Sodium Selenite-selenium	0.60 mg/kg
Moisture	13.80 %		
Magnesium	0.53 %		

This product contains 7.1g of cal mag in 1.42 kgs of feed. Animals should not be fed more than 15g cal mag per day.

Contains the following ingredients in descending order by weight:
Sunflower ext, Malt culms, Wheatfeed, Wheat, Palm kernel exp, Low glucosinolate rape seed ext, Molasses, Cocoa hulls, Maize gluten feed, Beans, Cat feed, Calcium carbonate, Vegetable oils, Salt, Magnesium oxide.

This product contains Addarome to optimise dry matter intake.
This product contains Selenomathionine for enhanced health.

Appendix 2 – Daily record sheet

ADULT HN3-1 DAILY RECORD SHEET (PPL 30/ 1858)

Animal ID: _____Sex: male / female (Lumbers & Yu, 1999)
(delete as appropriate)

Protocol: 19b4: Sheep nutritional/metabolic studies **Research start date:**

Date	Comments (e.g. check defecating, incision sites, eating, appearance, flushed caths, Abx given)	Feeding				
		Time	Type	Refusal (g)	New (g/day)	Initial
Thur		9am	Ewbol		500 g	
Fri		9am	Ewbol		500 g	
Sat		9am	Ewbol		500 g	
Sun		9am	Ewbol		500 g	
Mon		9am	Ewbol		500 g	
Tue		9am	Ewbol		500 g	
Wed		9am	Ewbol		500 g	
Thur		9am	Ewbol		500 g	
Fri		9am	Ewbol		500 g	

Feeding regime: Ewbol 18 nuts 500g/day (all am) + hay and water ad libitum

Appendix 3 – Results sheet

ADULT HN3-1 STUDY RESULTS SHEET (PPL 30/ 1858)

ANIMAL NUMBER:
DATE:

ECHOCARDIOLOGY			
	1	2	3
LVES			
LVED			
EF			
FS			
IVSs			
IVSd			
PWs			
PWd			

CORONARY FLOW RESERVE			
	1	2	3
HR			
Art BP			
Cath BP			
Ao BP			
RR			
Temp			
O2 Sats			
Hb			

ELECTROPHYSIOLOGY							
HRA				RV		LV	
THRESHOLD			THRESHOLD				
OUTPUT			OUTPUT				
SNR	600		ERP				
	500		VT STIM	mA	S_2	mA	S_2
	400						
	350			2.0		2.0	
WCL	Wky			5.0		5.0	
	2:1 at		NOTES				

Appendix 4 – Presentations & publications

APPENDIX 4.1: Proceedings of the National Academy of Science of the USA 104, 9529-9533 (2007).

Mismatched pre- and postnatal nutrition leads to cardiovascular dysfunction and altered renal function in adulthood

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The authors declare no conflict of interest.
This article is a PNAS Direct Submission.
Abbreviations: CV, cardiovascular; PAR, predictive adaptive response; C, control; U, undernutrition; RAS, renin–angiotensin system; Ang II, angiotensin II; MLCK, myosin light chain kinase; MAP, mean arterial blood pressure.
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www.pnas.org_cgi_doi_10.1073_pnas.0610373104 PNAS **May 29, 2007** vol. 104 no. 22 **9529–9533**

The early life environment has long-term implications for the risk of developing cardiovascular (CV) disease in adulthood. Fetal responses to changes in maternal nutrition may be of immediate benefit to the fetus, but the long-term effects of these adaptations may prove detrimental if nutrition in postnatal life does not match that predicted by the fetus on the basis of its prenatal environment. We tested this predictive adaptive response hypothesis with respect to CV function in sheep. We observed that a mismatch between pre- and postnatal nutrient environments induced an altered CV function in adult male sheep that was not seen when environments were similar. Sheep that received postnatal undernutrition alone had altered growth, CV function, and basal hypothalamo–pituitary–adrenal axis activity in adulthood. Prenatal undernutrition induced greater weight gain by weaning compared with the prenatal control diet, which may provide a reserve in the face of a predicted poor diet in later life. In an adequate postnatal nutrient environment (i.e., relatively mismatched), these offspring exhibited cardiac hypertrophy and altered CV function in adulthood. These data support the concept that adult CV function can be determined by developmental responses to intrauterine nutrition made in expectation of the postnatal nutritional environment, and that if these predictions are not met, the adult may be maladapted and at greater risk of CV disease. Our findings have substantial implications for devising strategies to reduce the impact of a mismatch in nutrition levels in humans undergoing rapid socio-economic transitions in both developing and developed societies.

Epidemiological studies have shown that the environment in early life may have long-term effects on the risk of adult onset diseases such as hypertension and coronary heart disease (reviewed in ref. 1). Studies of adults who were *in utero* at the time of the Dutch Famine (November 1944 to May 1945) provide direct evidence that maternal undernutrition during early gestation, when the nutrient demands of the conceptus are minimal, leads to increased incidence of coronary heart disease in adult offspring (2). Animal studies support this observation; for example, in sheep, maternal undernutrition in the period after conception influences cardiovascular (CV) function in late gestation in the absence of any changes in birth weight (3, 4). An association between perturbations of the peri-implantation environment and altered postnatal CV function has been confirmed in both rats (5) and sheep (6). The pattern of postnatal growth may also influence later health, as studies in humans have shown that reduced size at birth and accelerated childhood growth confer an increased risk of CV disease in adulthood (7, 8).

Recently, it has been suggested that although responding to changes in maternal nutrition may be of immediate benefit to the fetus, the long-term effects of these adaptations may prove detrimental if nutrition in postnatal life does not match that predicted by the fetus on the basis of its prenatal environment (1). Thus, the biological response to aspects of

the prenatal environment conveys fitness in a similar postnatal environment, and such prenatal responses may have arisen by selection for optimal postnatal fitness (9). Such responses are not only manifest in terms of prenatal survival but also reproductive success. Such ''predictive adaptive responses'' (PARs) are similar to the environmentally induced phenotypes in invertebrates such as the gregarious vs. solitary forms in locusts (10), queens vs. workers in honey bees (11), and seasonal polyphenisms in moths (12). Mammalian examples include seasonal changes in coat thickness in meadow voles (13). If the developmental prediction is incorrect, e.g., a mismatch between pre- and postnatal environment occurs, the phenotype induced by PARs may be poorly adapted to the postnatal environment and result in decreased fitness. Thus in humans, PARs made in response to a suboptimal intrauterine nutrient environment may be inappropriate if a substantial increase in postnatal nutrition arises after economic development or migration. PARs may therefore play a role in the rising incidence of CV and metabolic disease in developing countries. Indeed, Indian children who were small at birth but heavy at 8 years of age, indicating changing nutritional status, have increased risk factors for CV disease, including insulin resistance and increased plasma LDL cholesterol (14). The PARs concept may also apply to placental insufficiency, with or without fetal growth restriction, as opposed to maternal dietary deficiency, when the fetus experiences nutrient deprivation and prepares mistakenly for a life of dietary deprivation. Such mismatches between the pre- and postnatal nutrient environment might, by virtue of inappropriate PARs, therefore underlie the increasing prevalence of CV dysfunction in adulthood in both developed and developing countries.

To investigate the PARs hypothesis in a species with a developmental trajectory comparable to humans, we manipulated separately the pre- and postnatal nutrient environment of sheep during periods equivalent to those identified as critical in determining human CV health. Hypertension associated with low birth weight in rats is predominantly in male offspring (15), and in both rats and humans it is well established that hypertension with renal failure is more likely to occur in males (16), thus we only used male offspring in this study.

Results and Discussion

Ewe and Preweaning Lamb Weight in Response to Early Gestation Undernutrition.

In studies of the famine of the Dutch Hunger Winter, early gestation undernutrition is associated with increased prevalence of CV disease (2); therefore, in this study, ewes received either 100% (C) or 50% (U) of total nutrient intake over the first 31 of 147 days of gestation. During the first 31 days of gestation, U ewes gained less weight than C ewes (Fig. 1*a*). This reduction in weight gain is comparable to that in normal human pregnancies in which there is a mild reduction in calorific intake, rather than that of severe starvation as in the Dutch Hunger Winter (17). Such a reduction in weight gain during pregnancy is also seen in adolescent pregnancies (18) and when high physical activity is carried out while pregnant (19). The nutritional challenge had no effect on their offspring in terms of birth weight (3.89 ± 0.17 vs. 3.62 ± 0.18 kg) or biometry (data not shown), but U lambs showed greater preweaning growth than C lambs and were heavier at 12 weeks (Fig. 1*b*). After birth, the composition and quantity of the milk consumed by the lamb will determine early postnatal growth rate (20, 21), although this was not examined in this study. The lambs could also have altered metabolism or appetite, as studies in rat have shown that undernutrition during fetal life leads to a preference for high-fat foods in the offspring (22) and changes in the hypothalamic regulation of food intake (23).

Postweaning Lamb Growth After Pre- and Postnatal Undernutrition.

Epidemiological data indicate that early postnatal growth is associated with altered adult CV function (24). We therefore subdivided both C and U groups to receive postnatal nutrient restriction (a level that reduced body weight to 85% of individual target weight predicted from the 0–12 week growth trajectory) (CU and UU) or adequate nutrition (CC and UC) between 12 and 25 weeks of age (immediately postweaning). The nutrient restriction produced a clear reduction in fractional growth rate during this period (Fig. 1*c*). After the postnatal nutrient challenge (25–35 weeks), UU had a greater fractional growth rate than UC, but CU did not have a significantly greater fractional growth rate than CC (Fig. 1*d*). Thus, early gestation undernutrition enhanced both early postnatal growth rate (i.e., 0–12 weeks) and recovery from a period of postnatal undernutrition. These findings indicate that these animals develop strategies aimed at protection of body weight from an anticipated period of undernutrition.

CV Dysfunction in Adult Sheep at 1.5 Years of Age After Mismatch of Pre- and Postnatal Nutrition.

We then assessed CV function in the 1.5-year-old lambs, focusing on the responsiveness of the renin–angiotensin system (RAS) because previous studies have implicated the RAS as a candidate mechanism linking reduced intrauterine nutrition to altered CV function in adulthood (25). The loop diuretic frusemide was used to stimulate the RAS via reduced distal tubular sodium and volume, leading to increased renin and angiotensin II (Ang II) release (26). An initial increase in blood pressure was observed followed by a gradual decrease in blood pressure due to volume depletion. Both nutritionally mismatched groups (UC and CU), but not the matched group (UU), had an increased blood pressure response to frusemide compared with CC (Fig. 2*f*). The increased blood pressure response to frusemide was not associated with altered basal plasma angiotensinogen (Fig. 2*a*), plasma renin activity (Fig. 2*b*), and Ang II (Fig. 2*c*), nor with an altered plasma renin activity

(Fig. 2*d*) and plasma Ang II (Fig. 2*e*) response to frusemide. Also, the baroreflex was unaltered by either of the nutritional challenges (unpublished observations). The results therefore suggest either a greater vascular responsiveness to Ang II or an altered blood pressure decrease in response to volume depletion.

CV/Renal Dysfunction in Adult Sheep at 2.5 Years of Age After Postnatal Undernutrition.
In the same animals at 2.5 years old, we found a similar increased blood pressure response to frusemide in CU (Fig. 3*a*), but the effect had disappeared in UC. CU also had an increased overnight urine output (Fig. 3*e*), with no change in sodium concentration (data not shown) after the challenge, suggesting a prolonged response to frusemide. Vascular reactivity was investigated *ex vivo* in the renal artery by using myography (27). We found that postnatal undernutrition increased the contractile response to phenylephrine in sheep that received prenatal control nutrition (CU vs. CC) but not in those that received prenatal undernutrition (UU vs. UC, Fig. 3*f*). Also, an increased blood pressure response to a bolus of Ang II was observed in postnatally undernourished sheep with a control prenatal diet (CU vs. CC) but not those with a restricted prenatal diet (UU vs. UC, Fig. 3*c*). The enhancement of the blood pressure response to frusemide and the exaggerated diuresis was blocked by prior administration of the angiotensin-converting enzyme inhibitor captopril (Fig. 3*b*). Because
captopril blocks Ang II production, this finding suggests that the postnatal nutrient restriction affects the Ang II component of the frusemide response rather than the diuresis component. Cortisol is known to up-regulate ovine arterial blood pressure responses to Ang II (28), and we found that CU vs. CC, but not UU vs. UC, had increased basal plasma cortisol (Fig. 3*d*). Thus, altered hypothalamo–pituitary–adrenal axis activity may underlie the observed phenotypic changes (29). For all parameters there was no statistical difference between groups CC and UU.

CV Dysfunction in Adult Sheep at 2.5 Years of Age After Prenatal Undernutrition.
Early gestation nutrient restriction thus appears to confer advantageous phenotypic changes if the animal is faced with a poor postnatal environment. However, if the postnatal environment is mismatched, a disadvantageous phenotype may be observed. Consistent with this observation are findings that in 2.5-year-old sheep, prenatal undernutrition with a control postnatal diet resulted in increased interventricular septal wall thickness (Fig. 4*b*) and increased mean left ventricular wall thickness (UC, 10.4 ± 0.5 mm vs. CC, 8.5 ± 0.4 mm). However, these effects were not observed after prenatal undernutrition combined with postnatal undernutrition (UU vs. CU). Although we observed no difference between groups in basal blood pressure at 2.5 years of age (CC, 90.4 ± 1.5 mmHg; CU, 87.5 ± 3.5 mmHg; UC, 94.4 ± 1.8 mmHg; UU, 90.8 ± 2.0 mmHg), the altered cardiac wall thickness could be a precursor of longer-term CV dysfunction, because in both sheep (30) and humans (31), left ventricular hypertrophy is associated with hypertension. For all of the above parameters, there was no statistical difference between groups CC and UU. Compared with control lambs (CC), prenatal undernutrition alone (UC), but not when combined with postnatal undernutrition (UU), resulted in increased basal tone and sensitivity to phenylephrine in the left internal thoracic artery (32) and increased constriction ($_pEC_{50}$) to acetylcholine in isolated coronary arteries at 2.5 years of age (Fig. 4*d*). These observations may indicate longer-term CV dysfunction such as hypertension in UC sheep. Indeed, in the male offspring of protein-restricted pregnant rats (33, 34), altered vascular reactivity is associated with hypertension. A number of potential cellular signaling changes could be hypothesized to underlie the altered vascular reactivity. To investigate this possibility, we focused on myosin light chain kinase (MLCK), a key component of smooth muscle signalling pathways, and acetylcholine receptors (M3) on smooth muscle cells (35). We found that MLCK mRNA levels in the coronary artery were increased in UC, but not UU, compared with CC (Fig. 4*c*), whereas M3 mRNA levels were unchanged (data not shown). Insufficient PCR and myography data were obtained from CU animals because of difficulty in the dissection of such small vessels. Increased MLCK activity is associated with proliferation and migration of smooth muscle cells (36), and in the coronary artery the resultant wall thickening is associated with increased arterial stiffness (37), which therefore affects vascular function. Increased MLCK is associated with increased fibrogenesis in the smooth muscle (38) and in the present study is consistent with the increased growth rate in response to the early gestation undernutrition. Postnatal undernutrition, which stalls the accelerated postnatal growth induced by early gestation undernutrition (UU), appears to prevent these outcomes, indicating that matching of pre- and postnatal nutrition is beneficial whereas a mismatch is detrimental to long-term CV function.

Conclusion

We have shown that modest nutrient restriction in early gestation produces phenotypic changes in the offspring of a species comparable to humans in terms of maturity at birth. These effects occur without changes in birth weight. They could constitute a postnatal survival strategy by enhancing postnatal growth and also a predictive response to promote growth recovery after an anticipated postnatal nutritional challenge. If the prenatal prediction is not reflected in the postnatal environment, left ventricular hypertrophy, increased coronary artery vascular reactivity, and MLCK mRNA expression
are induced in adult life. Conversely, the effects of postnatal undernutrition (increased vasoconstrictor responsiveness and urine output in response to frusemide and elevated basal plasma cortisol) are prevented by prior early gestation undernutrition.

Our data are consistent with the PARs concept (9). However, the PAR induced may not be complete because the responses of the two matched groups (CC and UU) are not always identical. Although we cannot exclude the possibility that the responses can be explained simply as an inappropriate developmental outcome of an appropriate response to an adverse insult in early pregnancy, there were no obvious signs of disruption of development. Our concept of nutritionally induced PARs is supported by other animal studies in which the coronary atherosclerotic (39) or endothelial function (40) effects of a high-fat diet were prevented by prior feeding of a similar diet to the pregnant mother and those in which reduced longevity after a postnatal cafeteria diet was prevented if growth was restricted by nutritional restriction at suckling (41).

The biological response to poor prenatal nutrition induces a phenotype best suited to a similar poor postnatal nutrition. Poor prenatal nutrition followed by adequate postnatal nutrition or adequate prenatal nutrition followed by poor postnatal nutrition (mismatch) leads to adult phenotypes similar to those in human CV and metabolic disease, such as endothelial dysfunction and cardiac hypertrophy, and altered vascular tone, blood pressure control and renal function, and weight gain. This concept may be particularly important in populations in which the mismatch of pre- and postnatal nutritional environments is exaggerated from generation to generation by a rapid socio-economic transition. A nutritional mismatch may also occur in Western society when maternal dietary intake during pregnancy does not meet the energy demands of the conceptus because of high physical activity or dieting prior or during the early stages of pregnancy, or in adolescent pregnancies. Alternatively, the fetal prediction of postnatal environment may be inappropriate because of maternal or placental disease or the greater maternal constraint associated with small stature or primiparous pregnancy. Lastly, neonatal conditions such as feeding high-fat and -calorie infant formula or weaning onto inappropriate foods can exacerbate the mismatch between developmental prediction and later nutrition.

Although the role of environmental mismatch in producing pathophysiology is supported by experimental data, it is now important to elucidate the mechanisms underlying such long-term physiological changes. Evidence is emerging of a role for epigenetic changes to DNA, affecting the expression of both imprinted and non-imprinted genes and induced by nutritional or hormonal factors (42, 43). Indeed, feeding a reduced protein diet to pregnant rats induces elevated blood pressure and endothelial dysfunction in the offspring and is associated with permanently increased expression in the liver and heart of genes such as the glucocorticoid receptor (GR) and PPAR due to hypomethylation of their respective promoters and associated changes in histone acetylation and methylation (44). The identification of markers for such phenotypic changes in early life will be important for interventions aimed at reducing the substantial global burden of CV morbidity and metabolic disease.

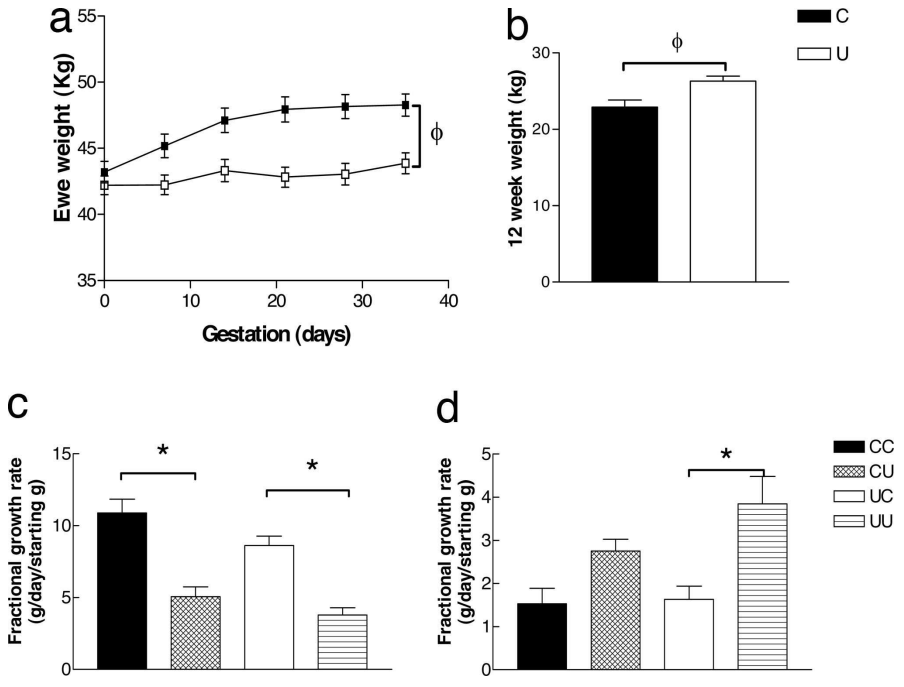

Fig. 1. Size and growth of ewes and male offspring. (*a*) Weight between 0 and 35 days of gestation in C (n = 21) and U (n = 26) ewes. (*b*) Weight at 12 weeks of age in C (n = 24) and U (n = 28) lambs. Fractional growth rate at 12–25 weeks (*c*) and 25–35 weeks (*d*) in CC (n = 14), CU (n = 10), UC (n = 14), and UU (n = 14) lambs. □ $P < 0.05$, early gestation nutrient-restricted group significantly different from early gestation control group; *, $P < 0.05$, postnatal nutrient-restricted group significantly different from postnatal control group (by two-way ANOVA). Values are mean ± SEM.

Methods

Experimental Model.

Welsh Mountain ewes (U.K. Animals Scientific Procedures Act 1986) in their second or third parity received 100% (C, n = 21) or 50% (U, n = 26) of total nutrient requirements between days 1 and 31 of gestation and 100% thereafter. The diet consisted of barley, wheat, micronized full-fat soya, grass meal, molasses, chopped straw, calcium carbonate, dicalcium phosphate salt, and sheep vitamin/mineral supplement. As fed, the diet provided 9.6 MJ/kg (metabolizable energy) and 14.75 g of crude protein. Ewes delivered and suckled their lambs naturally until weaning at 12 weeks of age (C, n = 24 [9 single (s), 15 twin (t)] and U, n = 28 [14 s, 14 t] lambs). All lambs were weighed at birth and at 12 weeks of age. Offspring were fed ad libitum [CC, n = 14 (6 s, 8 t) and UC, n = 14 (7 s, 7 t)] or at a level that reduced body weight to 85% of individual target weight (predicted from the 0–12 week growth trajectory) from 12 to 25 weeks postnatal age and ad libitum thereafter [CU, n = 10 (3 s, 7 t) and UU, n = 14 (7 s, 7 t)]. All lambs received 100% of nutritional requirements from 25 weeks of age onwards. The diet consisted of free access to water and hay, and creep pellets (Prestige Lamb Pellets _ Decox; BOCM Pauls Ltd., Loughborough, U.K.) were provided each morning and afternoon. As fed, creep pellets provided 10.51 MJ/kg metabolizable energy) and 18% crude protein. At 32 weeks of age, lambs were transferred onto a standard ration of an adult complete pelleted diet (Ewbol 18; BOCM Pauls Ltd.) plus ad libitum hay.

Surgical Procedures.

At 9.9 ± 0.1 months of age, all lambs were vasectomized, and carotid artery loops (externalization of artery within a flap of skin) were created under general anesthesia (3% halothane/O_2) to allow temporary catheters to be implanted easily at a later date. At 1.5 (16.5 ± 0.1 months) and 2.5 (29.6 ± 0.2 months) years of age, catheters were inserted into the carotid artery and jugular vein under general anesthesia (3% halothane/O_2) using sterile techniques.

Experimental Procedures.

RAS function was assessed at 1.5 and 2.5 years of age using frusemide (5 mg/kg i.v. bolus) and at 2.5 years using captopril (500 microg/kg per h i.v. infusion), angiotensin I (0.05 microg/kg i.v. bolus), and Ang II (0.05 microg/kg i.v. bolus). Mean arterial blood pressure (MAP) was monitored via the carotid artery catheter by using a physiological pressure transducer. At 2.5 years, cardiac morphology and left ventricular function was determined by transthoracic echocardiography under general anesthesia (3% halothane/O_2).

Hormone Analysis.

Basal plasma renin and angiotensinogen were measured in duplicate using RIA as described (45). Plasma Ang II was measured in duplicate using a sensitive and specific competitive protein-binding RIA (46). Cortisol was measured (single measurement) in EDTA plasma by using an Immulite analyzer (DPC, Llanberis, U.K.).

Isolated Vascular Assessment.

Vessels were dissected clean of connective tissue, and 2-mm segments were mounted on the Mulvany–Halpern wire myograph (27). Concentration–response curves to acetylcholine (1 nM to 1 mM) in the left anterior interventricular artery and phenylephrine (10 nM to 100 _M) in the right renal artery were carried out as described (47).

Molecular Biology.

Total RNA was extracted from the ovine left anterior interventricular coronary artery by using TRIzol (Sigma, Poole, U.K.) and was reverse transcribed into cDNA. Muscarinic M3 receptor and MLCK mRNA levels were analyzed relative to 18S ribosomal RNA (Applied Biosystems, Warrington, U.K.) by using real-time PCR (48) (*Taq*man; Applied Biosystems ABL Prism 7700 Sequence Detection System). Coefficient of variation was <15%. Using a geNorm normalizing kit (Primer Design, Southampton, U.K.), we established that 18S is one of the most stable genes with a normalization factor of 0.82 (this must be <1.5) (Hollis, Anthony, M.A.H, and L.R.G, unpublished observations).

Data Analysis.

Data are expressed as mean ± SEM, and a significant difference was accepted at $P < 0.05$. Vascular contraction in response to phenylephrine or acetylcholine was expressed as percentage of the maximum contraction in response to physiological saline solution with equimolar substitution of K_ for Na_ (125 mM). The pEC_{50} was calculated by using Prism (Graphpad Software Inc., San Diego, CA) and compared by one-way analysis of variance. For each frusemide experiment, the area under the MAP response curve was calculated (-30 to 120 min). For each Ang II experiment, the maximum MAP response was calculated. Growth and blood pressure data were analyzed by using multifactorial analyses of variance (three-way ANOVA), which tested the effects of prenatal diet, postnatal diet, and number of offspring per pregnancy. Where significant effects of any factor or a significant interaction were found, further analyses were performed having split the data by that factor/s. Statistical analyses were performed by using SPSS version 8 (SPSS, Chicago, IL). Unpaired Student's *t* test was used to identify differences between two factors. Statistical tests were subject to Bonferroni multiple-comparison correction where appropriate.

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APPENDIX 4.3: International Society for Heart Research conference, 2006

The Effect of Pre- and Peri-conceptional Undernutrition on Cardiac Electrophysiology in Adult Male Sheep Offspring

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Introduction
Size at birth weight is associated with cardiovascular disease. The mechanisms underlying this association have not been determined. The induction of ventricular tachycardia (VT) and dispersion of effective refractory period (ERP) is linked to cardiac arrhythmogenesis and sudden cardiac death. We used a sheep model to investigate whether the timing of nutritional challenges around conception subsequently influences the cardiac electrophysiology (EP) in adulthood.

Methods
Ewes were assigned to dietary groups prior to mating and fed 100% total nutrient requirements (Control, n=8) or 50% total nutrient requirements in the 30 days prior to conception (PRE, n=10) or for 15 days before and after conception (PERI, n=12), and 100% thereafter. Male offspring were weaned at 13 weeks and then fed standard diet *ad libatum*. At 3.3 years, EP studies were conducted under general anaesthesia. By pacing in the right ventricular (RV) and left ventricular (LV) apex we measured the dispersion of ERP and susceptibility to ventricular tachycardia (VT) induction. VT induction was negative if not stimulated following a predefined intracardiac pacing protocol. Data (mean ± S.E.M.) were analysed by ANOVA and a Bonferroni post-hoc test.

Results

	VT induction from RV	VT induction from LV	Dispersion of ERP
Control	0	0	15.71±3.69
PRE	0	0	10.83±2.60
PERI	0	0	15.45±5.62
P value			0.64

Conclusion
VT was not induced with intracardiac electrical stimulation and no differences were seen in the dispersion of refractoriness between dietary groups. This suggests that pre- and peri-conceptional undernutrition is not associated with latent ventricular arrhythmogenesis or sudden cardiac death at this age. Other aetiological and developmental determinants of cardiovascular disease need to be studied.

APPENDIX 4.4: International Congress for DOHaD, 2006

The Effect of Pre- and Peri-conceptional Undernutrition on Coronary Artery Velocity in Adult Male Sheep Offspring

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Introduction
It is well recognised that size at birth is linked to cardiovascular disease (Barker, 1998b). In sheep that were made anaemic *in utero* coronary flow is increased in adulthood (Davis *et al.*, 2003a). We used a sheep model to investigate whether nutritional challenges around time of conception influences the coronary artery velocity (CAR) in adult male sheep.

Methods
Welsh Mountain ewes (UK Animals (Scientific Procedures) Act 1986) were assigned to dietary groups prior to mating and fed 100% total nutrient requirements (Control, n=6) or 50% total nutrient requirements 30 days prior to conception (PRE, n=8) or for 15 days before and after conception (PERI, n=10) and 100% thereafter. Male offspring were weaned at 13 weeks and then fed standard diet *ad libatum*. In male offspring at 3.3 years under general anaesthesia (Lehot *et al.*, 1991), resting and maximal coronary velocity (adenosine induced hyperaemia) and coronary artery velocity reserve (CAVR) were measured with an intravascular doppler guide wire in the left anterior descending coronary artery (absolute CAVR=maximal CAV-resting CAV; relative CAVR=maximal CAV/resting CAV). Data (mean ±S.E.M.) were analysed by ANOVA and a Bonferroni post-hoc test.

Results

	Resting CAV	Maximal CAV	Absolute CAVR	Relative CAVR
CONTROL	8.87±0.83	31.56±1.01	22.69±1.45	3.71±0.35
PRE	9.93±0.88	34.72±1.43	24.79±1.05	3.69±0.27
PERI	9.34±0.62	37.33±1.48*	27.98±1.43*	4.10±0.27

**P<0.05, significantly different from control group*

Conclusion
This data shows that periconceptional, but not preconceptional, undernutrition increases maximal CAV and absolute CAVR. This is the first study to show that coronary circulation is influenced by undernutrition in the periconceptional period and that the effects persist into adulthood. The adaptive consequences of this effect are not known.

APPENDIX 4.5: Physiology Society Bristol, 2005
***Proceedings of J Physiology* July 2005; 567P: C3**

Sex differences in cardiac morphology of adult sheep following moderate early gestation undernutrition with or without undernutrition in early postnatal

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Undernutrition is implicated in linking *in utero* and postnatal growth to the development of cardiovascular disease in later life (Eriksson *et al.*, 1999; Roseboom *et al.*, 2000). We therefore investigated the effects of early gestation and postnatal undernutrition on cardiac morphology and left ventricular function in adulthood.

Welsh Mountain ewes (Animals (Scientific Procedures) Act 1986) received 100% (group C, n=25) or 50% of global nutrient requirements (group U, n=26) from conception to day 30 of gestation, and 100% thereafter. Offspring were then fed either *ad libitum* (CC, n=15 and UC, n=13) or at a level that reduced body weight to 85% of individual target weight (predicted from 0-12 wk growth trajectory) from 12 to 25 weeks postnatal age and *ad libitum* thereafter (CU, n=10 and UU, n=13). Each group contained approximately equal numbers of males (n=27) and females (n=24). At 2.5 years cardiac morphology and left ventricular function was determined by transthoracic echocardiography under general anaesthesia (Lehot *et al.*, 1991). Data (mean ±S.E.M.) were analysed by ANOVA and a Bonferroni post-hoc test.

In male but not female offspring an increase was seen in the interventricular septal wall thickness (UC, 11.2 ±0.3mm vs. CC, 8.5 ±0.5mm, $p<0.01$) in the early gestation undernutrition group (UC) compared to the control group (CC), and also in the mean left ventricular wall thickness (UC, 10.4 ±0.5mm vs. CC, 8.5 ±0.4mm, $p<0.05$). This effect was not seen with exposure to both periods of undernutrition (UU). These results were independent of blood pressure. No changes seen in left ventricular fractional shortening.

This study suggests that the increase in left ventricular wall thickness following early gestation undernutrition is sex specific. Mismatches between the *in utero* and postnatal environment may have important consequences for adult cardiac morphology. The absence of this phenomenon in the female adult offspring may reflect different growth rates and susceptibility between the sexes in early gestation.

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APPENDIX 4.6: International Congress for DOHaD, Nov 2005

The Effect of Pre- and Peri-conceptional Undernutrition on Cardiac Electrophysiology in Adult Male Sheep Offspring

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Introduction

Size at birth weight is associated with cardiovascular disease (Barker *et al.*, 1989). However the mechanisms underlying this association have not been determined. The induction of ventricular tachycardia (VT) and dispersion of effective refractory period (ERP) is linked to cardiac arrhythmogenesis and sudden cardiac death. We used an animal model to investigate whether the timing of nutritional challenges around time of conception influences the cardiac electrophysiology (EP) in adult male sheep.

Methods

Welsh Mountain ewes (UK Animals (Scientific Procedures) Act 1986) were assigned to dietary groups prior to mating and fed 100% total nutrient requirements (Control, n=8) or 50% total nutrient requirements 30 days prior to conception (PRE, n=12) or for 15 days before and after conception(PERI, n=12), and 100% thereafter. Male offspring were weaned at 13 weeks and then fed standard diet *ad libatum*. At 3.3 years, EP studies were conducted under general anaesthesia (Lehot *et al.*, 1991). By pacing in the right ventricular (RV) and left ventricular (LV) apex we measured the dispersion of ERP (difference in ERP between RV and LV apex) and susceptibility to ventricular tachycardia (VT) induction. VT induction was negative if not stimulated following a predefined intracardiac pacing protocol. Data (mean ±S.E.M.) were analysed by ANOVA and a Bonferroni post-hoc test.

Results

	VT induction from RV	VT induction from LV	Dispersion of ERP
Control	0	0	15.71±3.69
PRE	0	0	10.83±2.60
PERI	0	0	15.45±5.62
P value			0.64

Conclusion

VT was not induced with intracardiac electrical stimulation and no differences were seen in the dispersion of refractoriness between dietary groups. This suggests that pre- and peri-conceptional undernutrition in not associated with latent ventricular arrhythmogenesis or sudden cardiac death at this age. Other aetiological and developmental determinants of cardiovascular disease need to be studied.

APPENDIX 4.7: ESC Congress, Stokholm, Sweden (September 2005)

The effect of undernutrition on cardiac morphology in adult male sheep

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Introduction: Epidemiological studies have suggested that maternal undernutrition during gestation causes increased risk of coronary heart disease for offspring in later life. The mechanisms underlying this association are not clear. We investigated the effects of global prenatal undernutrition on blood pressure and cardiac morphology and function in adult male sheep.

Methods: Welsh Mountain ewes were fed *ad libitum* (control diet, n=8), or a global prenatal nutrient restricted diet (50% total requirements, 0-30 days gestation, term=147 days, n=7). Their male offspring were fed *ad libitum* from birth onwards. At 2.5 years cardiac morphology and function was determined by transthoracic echocardiography and blood pressure was measured while under general anaesthesia. Data were analysed by ANOVA and a Bonferroni *post-hoc* test.

Results: Mean left ventricular wall thickness (LVWT) was greater in the early gestation undernutrition group compared to the control. No differences were seen in fractional shortening (FS), left ventricular mass (LVM) and mean arterial pressure (MAP).

	Control	**Prenatal**	**p value**
LVWT (mm)	11.6 ± 0.3	13.6 ± 0.6	0.04
FS	23.1 ± 1.8	26.3 ± 2.0	0.59
LVM (mg/kg)	1.56 ± 0.2	1.78 ± 0.2	0.80
MAP	85.1 ± 8.7	91.4 ± 7.9	0.93

Conclusion: Prenatal nutrient restriction is associated with increased LV wall thickness, independent of blood pressure. This supports the hypothesis that undernutrition in utero leads to altered cardiac development, and suggests a mechanistic basis for the link between the prenatal environment and risk of coronary heart disease in later life.

APPENDIX 4.8: ***British Journal of Obstetrics and Gynaecology*** **2005;112(4):511**

The effect of moderate early gestation undernutrition with or without undernutrition in early postnatal life on cardiac morphology in adult male sheep

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Undernutrition is implicated in linking in utero and postnatal growth to the development of cardiovascular disease in later life. We investigated the effects of early gestation and postnatal undernutrition on cardiac morphology and function in adult male offspring. Welsh Mountain sheep received a control (CC, n=8), or global nutrient restricted diet early gestation (50% total requirements, 0-30 days gestation, term=147 days, UC, n=7) or postnatal (12-25 weeks, CU, n=5) nutrient challenge or both (UU, n=8). At 2.5 years cardiac morphology and function was determined by transthoracic echocardiography. Data were analysed by ANOVA and a Bonferroni *post-hoc* test. Systolic interventricular septal wall thickness was greater in UC compared to the control (CC, $p<0.01$). However, this effect was not seen with exposure to both periods of undernutrition (UU). Ventricular remodelling following early gestation undernutrition is absent when combined with an early postnatal challenge. Thus mismatches between the in utero and postnatal environment may have important consequences for cardiac morphology.

Appendix 5 – Growth data