A serial ultrasound investigation of the effects of moderate preterm birth on the cardiovascular system and kidneys of sheep from birth to adulthood.

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Abstract

Introduction

Moderately preterm babies (delivered at 32 to 36 weeks gestation) make up the overwhelming majority of the circa 15 million premature babies born worldwide each year. Individuals born prematurely are at increased risk of developing high blood pressure and cardiovascular disease in adulthood, with males at greater risk than females. Structural and functional mal-adaptations of the heart, major arteries and kidneys in response to preterm birth are believed to contribute to the adult onset of disease, but the specific mechanisms remain unclear. Ultrasound imaging is ideal to perform non-invasive, *in vivo*, long term serial studies in a clinically relevant ovine model to demonstrate the effects of moderately preterm birth. Our aim was to use ultrasound imaging to compare the cardiovascular system and kidneys of moderately preterm sheep to term sheep from shortly after birth to adulthood.

Methods

Three separate cohorts all including male and female sheep were evaluated using ultrasound imaging. One short term cohort was comprised of male and female moderately preterm lambs that were compared at two hours after birth. A second short term cohort comprised moderately preterm sheep and term controls that were compared while under sedation at two days of age. A long term cohort was comprised of moderately preterm sheep and term controls that were compared at two days, two weeks, three, six and twelve months of age and then again under sedation at 14 months of age. Ultrasound measurements of the wall thickness, chamber diameter and percentage fractional shortening of the left ventricle, diameter and blood flow in the proximal ascending aorta, main, right and left pulmonary arteries, both common carotid arteries and dimensions of the kidneys and renal artery resistive indices were recorded, corrected for bodyweight where appropriate and compared.

Results

Ultrasound analyses demonstrated little evidence of significant structural or functional mal-adaptations of the cardiovascular system and kidneys from moderately preterm birth to early adulthood in our sheep model. Initially, moderately preterm sheep had significantly greater relative to bodyweight measurements of the left ventricle, major arteries and kidneys compared to term controls. These differences were essentially no longer evident by three months of age, subsequent to a substantial increase in bodyweight of the preterm sheep. There was no evidence that males have an increased cardiovascular risk compared to females as a result of moderate preterm birth that was independent of any maternal or fetal co-morbidities.

Conclusion

Our results demonstrate moderate preterm birth *per se* has few significant effects up until early adulthood in a clinically relevant large animal model and suggests a shift in focus to the contribution of co-morbidities to cardiovascular risk in future ultrasound serial studies of moderately preterm birth is warranted. The results of this thesis will help inform the management of individuals born moderately preterm and aid in the development of future serial non-invasive *in vivo* surveillance strategies for cardiovascular disease, with the objective of identifying risk in the growing population of those born prematurely.

Declaration

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

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Lombardo P, Schneider ME, De Matteo R, Harding R, Nguyen V, Polglase GR and Black MJ. “A serial ultrasound evaluation of cardiac structure and function from two days to six months of age in preterm lambs.” *World Heart Federation World Congress of Cardiology.* Melbourne, Australia. 4 - 7 May 2014. Poster.

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I dedicate this thesis to my late brother Joe. Thanks for introducing me to the world of science.

Contents

Abbreviations

AGA	appropriate for gestational age
ANOVA	analysis of variance
AT	adventitia thickness
B-mode	brightness mode
CCA	common carotid artery
cm	centimetre
CT	computer tomography
D	end diastolic velocity
%FS	percentage fractional shortening
IMT	intima-media thickness
IUGR	intra-uterine growth restriction
i.v.	intravenous
IVS	interventricular septum
IVSd	interventricular septum in diastole
IVSs	interventricular septum in systole
kg	kilogram
kHz	kilohertz
LV	left ventricle or left ventricular
LVAWd	LV anterior wall thickness (mm) in diastole
LVAWs	LV anterior wall thickness (mm) in systole
LVPWd	LV posterior wall thickness (mm) in diastole
LVPWs	LV posterior wall thickness (mm) in systole
LVIDd	LV internal short axis chamber diameter (mm) in diastole
LVIDs	LV internal short axis chamber diameter (mm) in systole
mg	milligram

MHz	megahertz
mm	millimetre
ml	millilitre
M-mode	time-motion mode
MRI	magnetic resonance imaging
NH&MRC	National health and medical research council
PI	pulsatility index
pO_2	partial pressure of oxygen
PPROM	preterm pre-labour rupture of the amniotic membranes
RI	resistive index
S	peak systolic velocity
s	second
SEM	standard error of the mean
SGA	small for gestational age
TEA	term equivalent age
‘V-drive’	‘V-Express’ sheep handler
USA	United States of America
2-d	two dimensional
%	percentage

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Chapter 1 Literature Review.

1.1. Introduction.

An average of 11.1% of all live births worldwide were premature (<37 weeks gestation) in 2010. This corresponds to an estimated 14.9 million babies (Blencowe et al. 2012). Preterm birth is second only to pneumonia as the biggest cause of death in children under five years of age worldwide, and is associated with 33% of child blindness, almost 50% of cerebral palsy and accounts for more than two thirds of perinatal deaths (Blencowe et al. 2012; Grobman 2012; Tracy et al. 2007). Further, preterm birth is associated with lifelong health problems including increased risk of chronic disease, such as type 2 diabetes (Kajantie et al. 2010) and metabolic syndrome (Gluckman & Hanson 2004) in adulthood. It has been demonstrated that preterm birth can affect the development of major arteries (Bonamy et al. 2005), kidneys (Rodriguez-Soriano et al. 2005; Vanpée et al. 1992) and is associated with the development of hypertension and an increased risk of cardiovascular disease in mid-life (Bayman, Drake & Piyasena 2014; Cooper, Atherton & Power 2009; Gubhaju, Sutherland & Black 2011). Even moderately preterm newborns (born at 32 to 36 weeks gestation) that do not require medical intervention are at increased risk of morbidity and mortality relative to full-term newborns. The risk of higher neonatal morbidity, mortality, neonatal health care requirements and associated costs increases as the gestational age at birth decreases. (Moutquin 2003; Petrou et al. 2006). The reasons for the increased risk are uncertain (Engle, Tomashek & Wallman 2007). Preterm birth is more common in males than in females (Zeitlin et al. 2002). Males born prematurely also appear to have a higher rate of morbidity and mortality than preterm females, but the underlying causes of potential sex-specific effects of preterm birth remain unclear (Ingemarsson 2003).

In addition to causing significant morbidity and mortality, preterm birth is also financially costly. In 2011 it contributed an estimated $26 billion in health care costs to the health care system in the United States of America (USA) alone (McCormick et al. 2011). As the worldwide rate of preterm birth increases, so too do the associated health care costs. (Tracy et al. 2007; VanderWeele, Lantos & Lauderdale 2012).

As preterm birth significantly affects health, health care delivery and financial costs, it is crucial that research be undertaken to understand the consequences of preterm birth and to facilitate prevention and/or management of the condition. In particular, there is a need to carry out investigations in order to better understand the long term health effects of moderate preterm birth (since most preterm births are moderately preterm), including the causes underpinning the physiological differences between sexes with regard to cardiovascular function (Pulver et al. 2009; Saigal & Doyle 2008).

Ethical restraints limit the study of human pregnancy and pregnancy outcomes when invasive interventions such as induction of preterm birth are required as part of the investigation. This necessitates the use of animal models. The sheep model has been used successfully to investigate adaptations to physiological perturbations, such as preterm birth, and to assess resultant effects on cardiovascular structure and function into adulthood (Bensley, J et al. 2010; Jonker, Sonnet et al. 2007; Vuguin 2007). In addition, serial follow up from preterm birth into adulthood can be achieved within a relatively short period of time (12 months) using the ovine model of pregnancy (De Matteo et al. 2010).

Longitudinal animal studies can be challenging because they are conducted over an extended time period, are expensive and may also require the culling of specific animals to obtain the required data at specific time points during the study (Yao et al. 2008). Diagnostic imaging can be used as a non-invasive method to monitor anatomical and physiological information in the same animal over an extended period of time, which may reduce or avoid the need to cull individual animals in order to obtain serial longitudinal data.

Ultrasound imaging is potentially an excellent modality for serial studies tracking the development of disease in animals over time as it is accurate and reliable (albeit more operator dependent than other imaging modalities such as Computer Tomography), non-invasive, portable, involves no ionising radiation and has a wide range of fetal and adult applications (Cowie 2011; Nelson, BP, Melnick & Li 2011; Yagel et al. 2009). It is routinely used in humans to evaluate cardiac, vascular and renal structure and function at all ages including newborns. However, to date there are few reports of longitudinal studies utilising serial ultrasound in animal models from birth to adulthood. Given the widespread use of animal models in medical research, there is a need to establish reliable and robust ultrasound techniques and protocols for conducting such studies.

1.2. Definition of preterm birth.

A term birth occurs between 37 to 42 weeks of gestation. A preterm birth is defined as birth occurring prior to 37 completed weeks of gestation (Martin, Joyce et al. 2008; WHO

1977). Preterm can be stratified by age of gestation at birth into moderate or late preterm (32 to 36 completed weeks), very preterm (28 to 31 completed weeks) and extremely preterm (< 27 completed weeks) (Li, Z et al. 2013; Tracy et al. 2007; WHO. 2004). It is useful to stratify preterm births into these subsets rather than consider all preterm births as an aggregate, in order to increase the prospect of detecting stronger associations with risk factors and hence facilitate prevention or intervention (Savitz 2008).

1.3. Incidence of preterm birth.

In 2010, preterm birth rates recorded worldwide varied from 5% of all live births in Northern Europe to 18.1% of all live births in Malawi. (Blencowe et al. 2012). Rates were generally highest (> 15%) in low income (developing) countries (mostly sub-Saharan) and lowest (< 9.3%) in high income (developed) countries, (mostly Europe). However, there were notable exceptions including the United States of America that recorded a preterm birth rate of 12.0% (Blencowe et al. 2012). The rate of preterm birth remained stable in Latin America, but increased in all other countries where reliable data could be obtained over the 1990 to 2010 year period, including in Europe and North America (Blencowe et al. 2012). Factors contributing to the increase in preterm birth include greater use of assisted reproduction techniques, an increasing number of multiple births and more obstetric intervention (Tucker & McGuire 2004).

Preterm birth rates differ substantially not only between countries but also within countries both regionally and temporally (Slattery & Morrison 2002; Tracy et al. 2007; VanderWeele, Lantos & Lauderdale 2012). Subsets of the overall preterm birth rate can be calculated according to specific outcomes or variables, such as gestational age, (Savitz 2008) and various relevant demographic characteristics including maternal age, (Martin, Joyce et al. 2008) parity, (Tracy et al. 2007) and ethnicity (Blencowe et al. 2012). For example, recent trend data shows that preterm birth rates have actually decreased each year in the USA from a peak of 12.8% (n = 4,265,555) for all live births in 2006, to 11.6% (n = 3,952,841) in 2012. However, the data can be further explored by looking at more specific sub-categories of interest. For example, the preterm birth rate declined for non-Hispanic white newborns over the same period from 11.7% (n = 2,308,640) to 10.3% (n = 2,134,044), for non-Hispanic black newborns from 18.4% (n = 615,247) to 16.5% (n = 583,489), and for Hispanic newborns from 12.2% (n = 1,039,077) to 11.6% (n = 907,677) (Martin, J et al. 2013; Martin, J et al. 2009; Martin, Joyce et al. 2008). Clearly, the more specific data demonstrates a stronger association with the non-Hispanic black cohort and a high preterm birth rate, than the aggregated preterm birth data alone would have suggested.

The most recent trend data for Australia shows that the overall rate of preterm birth remained relatively stable at around 8.3% of all births during 2009 to 2011, and then increased to 8.6% in 2013/14 (Table 1-1). This rise was almost entirely due to an increase in the number of moderate preterm births from a stable 6.6% in 2010/11 to 6.9% of all births in 2013/14. The number of post-term births has steadily declined from 0.9% to 0.5% of all births over the same time period (2009 to 2014). The preterm birth rate for Aboriginal and Torres Strait Islanders, however, is much higher than the rate for non-Indigenous women and has gradually increased from 13.1% in 2009 to 14.0% in 2014 (Table 1-1) (AIHW 2015, 2016; Hilder et al. 2014; Li, Z et al. 2011; Li, Z et al. 2012; Li, Z et al. 2013).

Table 1-1: Gestational age at birth in Australia 2009 to 2014.

Gestational age at birth (weeks)	Percentage of all births in Australia					
	2009 *n* = 299,220	2010 *n* = 299,563	2011 *n* = 301,810	2012 *n* =312,153	2013 *n* =309,489	2014 *n* =312,548
Extremely preterm < 27	0.9	0.9	0.9	0.8	0.9	1.7*
Very preterm 28 - 31	0.8	0.8	0.8	0.8	0.8	
Moderate preterm 32 - 36	6.5	6.6	6.6	6.9	6.9	6.9
Preterm < 37	8.2	8.3	8.3	8.5	8.6	8.6
Preterm < 37 (Indigenous)	13.1	13.5	13.8	14.3	14.0	14.0
Term 37 - 41	90.8	90.9	91	90.9	90.9	90.9
Post-term > 42	0.9	0.8	0.7	0.6	0.5	0.5

Note; gestational age not stated in 0.1% of births in 2009. *Only the total number of extremely and very preterm births available in 2014. (AIHW 2015, 2016; Hilder et al. 2014; Li, Z et al. 2011; Li, Z et al. 2012; Li, Z et al. 2013).

1.4. Causes and risk factors associated with preterm birth.

The major causes of preterm birth are spontaneous unexplained preterm labour, preterm pre-labour rupture of the amniotic membranes (PPROM) and multiple pregnancy. Other causes include pregnancy associated hypertension, cervical incompetence/uterine malformation, antepartum haemorrhage and intra-uterine growth restriction (IUGR) (Goldenberg et al. 2008; Gubhaju et al. 2014; Tucker & McGuire 2004).

The major risk factors for preterm birth include maternal infections (Goldenberg, Hauth & Andrews 2000), poor maternal nutritional status (Hendler et al. 2005), young maternal age (Akinbami, Schoendorf & Kiely 2000), substance abuse including tobacco smoking (Andres & Day 2000), previous preterm delivery and/or short inter-pregnancy interval (Hsieh et al. 2005), use of assisted reproduction techniques (Pandian, Templeton & Bhattacharya 2004), ethnicity (MacDorman 2011; Schaaf et al. 2013) and low economic status (Thompson et al. 2006).

1.5. Mortality and morbidity associated with preterm birth.

Preterm birth is a major source of neonatal mortality. An estimated one million neonatal deaths worldwide were attributed to preterm birth in 2000 (Lawn, Wilczynska-Ketende & Cousens 2006) with no significant change ten years later (Blencowe et al. 2012). A review conducted in 2009 of survival rates to discharge for extremely preterm infants in 14 high income countries (including Europe, North America and Australia), concluded that viability of infants born at ≤ 22 weeks gestation was unlikely, but survival improved significantly with each extra week of gestational age at birth (Dani et al. 2009). A review of 4446 extremely preterm newborns in the USA found that if they had received intensive care, or antenatal corticosteroids, or were female, or a singleton birth, the risk of neonatal death was reduced by the equivalent of a 1 week increase in gestational age at birth for each variable (Tyson et al. 2008). High income countries have significantly increased the likelihood of preterm newborns - particularly extremely preterm - surviving to discharge by continuing to improve neonatal intensive care (Vohr & Allen 2005). Infants born in the USA at < 24 weeks gestation who were previously considered ‘pre-viable’ or ‘borderline’ (Higgins, Delivoria-Papadopoulos & Raju 2005), now have survival rates to discharge of 30-70% (Salihu et al. 2013). However, in low income countries where neonatal intensive care is generally not available, less than 50% of very preterm newborns survive and those born extremely preterm are considered non-viable (Dimes et al. 2012; Katz et al. 2013).

The mortality rate for all preterm infants is higher compared to those born at full-term gestation. Even moderately preterm newborns are at least three times more likely than full-term newborns to die in the neonatal period (Kramer, Demissie & Yang 2000; Pulver et al. 2009). Unlike infants born very or extremely preterm, severe handicap (such as respiratory distress) is uncommon in moderately preterm infants (Schellenberg 2006). Nevertheless, as moderately preterm infants make up around 75% of all preterm births, they have the greatest impact on total infant mortality through weight of numbers (Kramer, Demissie & Yang 2000; Laws & Sullivan 2009).

Preterm birth is a major cause of morbidity including lung disease, brain injury, retinopathy of prematurity, necrotising enterocolitis and nosocomial infections (Cooke 1996; Goldenberg et al. 2008; Hack & Fanaroff 1999; Petrou et al. 2006). Despite moderately preterm infants being the size and weight of some full-term infants, they are not yet physiologically mature and have limited capacity to deal with the extra-uterine environment (Engle, Tomashek & Wallman 2007). They are four times more likely than full-term infants to have medical conditions such as temperature instability, jaundice, hypoglycaemia and respiratory distress diagnosed in the immediate post-natal period (Wang et al. 2004). Premature babies are at greater risk of developing neurological and developmental disabilities (Wood, NS et al. 2000) and behavioural problems (Hille et al. 2001; Tyson et al. 2008) than babies born at term. The occurrence of severe handicap is inversely related to gestational age even with high level care (Dani et al. 2009; Hack & Fanaroff 1999). Preterm birth is also associated with an increased risk of chronic conditions emerging in adulthood including cardiovascular and renal disease (Bayman, Drake & Piyasena 2014; Cooper, Atherton & Power 2009; Grobman 2012; Gubhaju, Sutherland & Black 2011; Keijzer-Veen, M et al. 2010; Kerkhof et al. 2012; Saigal & Doyle 2008). The consequences of moderate preterm birth on long term health are not well understood and merit further investigation (Bayman, Drake & Piyasena 2014; Engle, Tomashek & Wallman 2007; Schlesinger et al. 1987).

1.6. Differences in preterm birth outcome between males and females.

Among many different populations, male newborns appear to have a slightly higher (0.5 to 1.0%) incidence of prematurity and make up 55% of all preterm births (Cooperstock & Campbell 1996; Ingemarsson 2003; Zeitlin et al. 2002). Male fetuses and newborns experience higher morbidity and mortality rates than females (Elsmen, Steen & Hellstrom-Westas 2004; Jehan et al. 2009). This has been coined as the ‘male disadvantage' (Naeye et al. 1971). It is widely accepted that 'male disadvantage' exists, but the exact mechanisms underlying the disadvantage are not well understood (Hintz et al. 2006; Kirchengast & Hartmann 2009) and may include sex-specific mechanisms related to preterm birth. For example, preterm females have significantly greater catecholamine levels than males during labour, which may give them an advantage in coping with an hypoxic event (Ingemarsson 2003).

The 'male disadvantage' appears to continue into childhood, with prematurely delivered males being at greater risk for Sudden Infant Death Syndrome (Mage & Donner 2004), asthma, intellectual disability, behavioural disorders (Gissler et al. 1999), accidents (Kirchengast & Hartmann 2009), cardiac disease, cerebral palsy, neurological disorders and gastroenteritis (Elsmen, Steen & Hellstrom-Westas 2004). Sex-specific differences in the fetal programming of hypertension that appear in adulthood have also been demonstrated in various animal models including sheep (Lima et al. 2002; Rabbia & Valpreda 2003). The sex-specific mechanisms involved remain unclear, but include the effects of sex hormones, gestational age at birth, nutrition of the fetus and the timing of insults *in utero* (Berry, M et al. 2013; Schlesinger et al. 1987).

1.7. Developmental origins of disease.

There is mounting epidemiological and experimental evidence supporting the hypothesis that adult disease can have developmental origins (McMillen & Robinson 2005). David Barker and colleagues were the first to suggest that prenatal environmental factors such as poor nutrition can result in immediate mal-development of the fetus or induce changes in the form of adaptive responses that may be of benefit to the fetus in the short term, but predispose it to disease such as hypertension and type 2 diabetes later in life (Barker et al. 1989; Gillman 2005). In particular, there is an association between low birth weight and increased risk of developing cardiovascular disease in adulthood (Barker 2001; Roseboom et al. 2001; Skilton et al. 2011). Low birth weight can result from intrauterine growth restriction and/or preterm birth (Bensley, JG et al. 2016). There is also emerging evidence of a direct association between preterm birth and the adult onset of cardiovascular disease. (Bayman, Drake & Piyasena 2014; Cooper, Atherton & Power 2009; Keijzer-Veen, M et al. 2010; Kerkhof et al. 2012).

1.7.1. Preterm birth and the haemodynamic transition from the fetal to the newborn cardiopulmonary circulation.

The fetal cardiopulmonary circulation is structured to supply the fetus with oxygenated blood via the placenta. Blood returning from the placenta via the umbilical vein is well oxygenated (85 to 90%) (Stopfkuchen 1987). Approximately 50% of the umbilical venous blood passes through the left lobe of the liver and the rest by-passes the liver and connects directly with the inferior vena cava via the ductus venosus (Moore, Persaud & Torchia 2013). The well oxygenated (62%) blood from the ductus venosus and left lobe of the liver is mostly deflected by the valve of the inferior vena cava through the foramen ovale and directly into the left atrium (Sadler 2010). By comparison, most of the blood within the right ventricle originates from the superior vena cava and has an oxygen saturation of around 52% (Stopfkuchen 1987). Ninety percent of the right ventricular output is shunted from the pulmonary artery to the descending aorta via the ductus arteriosus to supply the lower body and the placenta (via the umbilical arteries) where re-oxygenation occurs (Weichert, Hartge & Axt-Fliedner 2010). The ductus arteriosus equalizes the blood pressure in the aorta and pulmonary artery, resulting in low blood flow to the lungs due to high pulmonary vascular resistance and high blood flow to the placenta due to its relatively lower vascular resistance (Rudolph 1970, 1979; Schmidt et al. 2005). During fetal life, the blood pressure in the right side of the heart is higher than the left side of the heart, with the right ventricle delivering 60 to 70% of the total cardiac output (Rychick 2004). As a consequence, the right ventricle develops a thicker wall compared to the left ventricle at birth as it has to work harder to forcibly eject blood until the haemodynamic transition occurs post-natally (Oparil 1985).

Breathing and the cessation of placental blood flow causes significant alterations to the circulatory system shortly after birth (Schmidt et al. 2005; Walther, FJ, Benders & Leighton 1993). The low resistance placental circulation is eliminated which abruptly increases the vascular resistance of the systemic circulation. Inflation of the lungs and exposure of the pulmonary vessels to higher arterial pO_2 concentration due to breathing reduces the pulmonary vascular resistance to below that of the systemic circulation, significantly increasing blood flow through the lungs (Rudolph 1970). The resultant increase in blood return to the left atrium via the pulmonary veins, in addition to the increased systemic vascular resistance, causes the left atrial pressure to rise above the right atrial pressure, functionally closing the foramen ovale immediately after birth (Sadler 2010).

Increased circulating arterial pO_2 and decreased levels of prostaglandin E2 cause an initial functional closure of the ductus arteriosus within 24 hrs in 90% of neonates born at term, and in all neonates within 96 hrs of birth (Evans 2005; Gardin et al. 1984; Moore, Persaud & Torchia 2013). Anatomical closure of the ductus arteriosus usually occurs at two to three weeks after birth (Weichert, Hartge & Axt-Fliedner 2010). While the ductus arteriosus remains patent after birth, there is reversal of the fetal right-to-left blood flow through the ductus which increases the blood flow to the pulmonary circulation and ultimately to the left atrium. In response to the increased blood flow to the left atrium (preload) and increased systemic blood pressure (afterload), the left ventricular wall thickness increases rapidly over that of the right ventricle after birth (Huether & McCance 2004; Moore, Persaud & Torchia 2013).

A persistent patent ductus arteriosus, PDA, is a common complication of preterm birth with around a third of neonates born at less than 30 weeks gestation requiring treatment for the condition (Koskinen et al. 2009; Kupinski 2013). Towards the end of a normal term gestation the ductal wall grows more muscular and less sensitive to the dilating effects of prostaglandin E2 and more sensitive to the vasoconstricting effect of circulating arterial oxygen (Evans 2005). However, neonates born preterm are likely to experience hypoxia due to inadequate lung function. In addition, the wall of the ductus arteriosus is structurally immature at birth - thereby remaining sensitive to the effects of prostaglandin E2 (Heuchan & Clyman 2014). As a result, the incidence of PDA is higher in preterm neonates than those born at term and is inversely related to gestational age at birth (Clyman & Roman 2007; Sivanandan et al. 2013).

PDA is associated with undesirable cardiovascular systemic effects including significant left-to-right shunting through the ductus which overloads the pulmonary circulation leading to pulmonary oedema and haemorrhage, cardiac failure, impaired renal perfusion and increased mortality in preterm infants (Koskinen et al. 2009; Raitakari et al. 2003; Touboul et al. 2007).

Once born and breathing, preterm neonates have to cope with the haemodynamic transition of the circulatory system and the *ex utero* environment regardless of the stage of development of the heart and vessels at birth. Short term adaptations of the structurally immature cardiovascular system are required to facilitate survival in the immediate post-natal period and may predispose the newborns to adverse consequences in later life.

1.7.2. Preterm birth and the heart and major arteries.

Prenatally and for a short time after birth, the myocardium in humans and sheep grows by increasing the number (hyperplasia) of mononucleated cardiomyocytes until they are terminally differentiated (Alexander 2005; Mayhew et al. 1997; Tare et al. 2014). Thereafter, myocardial growth is achieved mainly by hypertrophy of the terminally differentiated (binucleated) cardiomyocytes and non-muscle cell hyperplasia (Bubb et al. 2007; Burrell et al. 2003; Rudolph 2000). Premature birth disturbs the normal development of the heart by shortening the time available for cardiomyocyte hyperplasia prenatally which may lead to a reduced cardiomyocyte endowment (Burrell et al. 2003).

The cardiac wall grows in proportion to the increased workload imposed on the ventricle (as shown by the left ventricular wall which thickens rapidly after birth in response to blood pressure overload) (Li, L et al. 2001; Oparil 1985). Left ventricular hypertrophy is a fundamental indicator of cardiovascular disease (Foppa, Duncan & Rohde 2005). In preterm birth the left ventricle may not be structurally mature enough to cope with the abrupt and ongoing increase in blood pressure which can lead to mal-adaptive remodelling of the myocardium (Bayman, Drake & Piyasena 2014). Bensley et al (2010) demonstrated that moderately preterm birth resulted in cardiomyocyte hypertrophy, increased collagen deposition and altered cardiomyocyte maturation (including induction of polyploidy associated with irreversible stress related DNA changes) in lambs at 11 weeks postnatal age. The findings were attributed to the altered development of the structurally unprepared heart in response to the haemodynamic transition at preterm birth which would likely program for cardiac vulnerability in later life.

The vascular system.

The vascular system is created by two different mechanisms. Vasculogenesis is the formation of new blood vessels arising *de novo* from endothelial progenitor cells (Swift & Weinstein 2009). The primitive heart and primary blood vessels formed within the embryo are generated by vasculogenesis (Käßmeyer et al. 2009). Angiogenesis is the formation of new blood vessels that sprout from pre-existing capillaries (Patan 2000). Vasculogenesis was thought to be restricted to the pre-natal period, but since the early 2000s both vasculogenesis and angiogenesis have been shown to occur concurrently throughout life (Käßmeyer et al. 2009). Vessel walls are composed of three layers, the tunica intima consisting of a layer of endothelial cells lining the vessel lumen, the tunica media consisting of layers of smooth muscle cells, elastin and collagen, and the tunica adventitia - the outermost layer - comprising mainly collagen fibres encompassing perivascular nerves and tiny nutrient arteries known as the vasa vasorum (Craft et al. 2015; Seidelmann, Lighthouse & Greif 2014). The tunica media regulates vascular tone in the larger arteries with elastin functioning primarily to allow the vessel to stretch and distend during the cardiac cycle, thereby transferring the stress of cyclical blood pressure changes over the entire wall and evenly onto the collagen fibres (Kelleher, McLean & Mecham 2004).

Preterm birth leads to an alteration of normal vascular development resulting in impaired endothelial function and a reduction in microvascular density, arterial dimensions and arterial elasticity (Ligi et al. 2010). Elastin is deposited in the arterial wall mostly during late gestation (Martyn & Greenwald 1997). In order to successfully adapt to the increased blood pressure associated with the transition from a fetal to an extra-uterine circulation, large vessels such as the aorta, pulmonary and carotid arteries require an adequate amount of elastin in the vessel wall (Abitbol & Rodriguez 2012). An inadequate deposit of elastin due to premature delivery may be irreversible as the ability to accumulate elastin is severely limited after birth (Martyn & Greenwald 1997). Collagen makes up around 20% of the arterial wall, stretches much less than elastin and limits distention of the vessel at raised blood pressure levels (Tauzin 2015). Collagen progressively replaces elastin as a normal part of ageing and when elastin fibres are damaged due to mechanical stress or vascular disease such as atherosclerosis (Abitbol & Rodriguez 2012). A reduced elastin/collagen ratio is associated with a diminished vascular compliance of the large arteries and is a marker for hypertension and cardiovascular disease in adults (Bonamy et al. 2005; Ligi et al. 2010; Martyn & Greenwald 1997).

The various layers of the arterial wall can be visualised using B-mode ultrasound (Figure 2-7). Increased intima-media thickness, IMT, is a well-established marker for subclinical atherosclerosis and reduced arterial endothelial function in early adulthood (Skilton et al. 2011), as well as hypertension and cardiovascular disease such as myocardial infarct and stroke in later life (O'Leary et al. 1999; Raitakari et al. 2003). Preterm birth has been associated with increased IMT of the aorta in preschool children (Shimizu et al. 2014) and thickened intima-media of the carotid artery in children (Lee et al. 2014) and young adults (Lazdam et al. 2010). Adventitia thickness, AT, is significantly correlated with IMT and has been independently associated with endothelial dysfunction in coronary arteries and hypertension in animal studies (Herrmann et al. 2001; Kuwahara et al. 2002) as well as hypertension and stroke in humans (Kazmierski et al. 2009; Skilton et al. 2009). Ultrasound measurement of AT, in addition to IMT is warranted to obtain a more comprehensive understanding of vessel wall structure and associated atherosclerotic risk factors than measuring IMT in isolation. (Skilton et al. 2009).

As described in section 1.7.2. preterm birth has been shown to alter the structural development of the myocardium in sheep (Bensley, J et al. 2010). In a further study, Bensley et al (2012) demonstrated that preterm birth can also affect the structural development of the aorta and pulmonary artery. Eleven weeks after birth, the aorta of lambs born moderately preterm demonstrated a significantly narrower lumen and a thicker wall that had reduced smooth muscle content and areas of increased elastin deposition compared to controls. Unlike the aorta, the pulmonary artery did not display signs of injury but did demonstrate a significant increase in elastin content (Bensley, J et al. 2012). The findings are attributed to a mal-adaptive response to the haemodynamic transition at birth by the structurally unprepared aorta and pulmonary artery. Vascular wall changes that result in arterial narrowing are likely to predispose premature newborns to increased cardiovascular risk (i.e. coronary heart disease, stroke, heart failure and arteriosclerosis) by mid-life (Burke et al. 1995; Cooper, Atherton & Power 2009).

1.7.3. Preterm birth and the kidneys.

Development of the permanent kidney in humans begins with the emergence of the metanephros from the Wolffian duct and the metanephric mesenchyme in week five of gestation (Kett & Denton 2011; Shah et al. 2004). The ureteric bud arises from the Wolffian duct and invades the metanephric mesenchyme via elongation and iterative branching (Shah et al. 2004). The tips of the ureteric branches transform from comma-shaped to S-shaped bodies to form the renal tubules and the glomerulus (Faa et al. 2012). Nephron endowment is mainly determined by the initial branching of the ureteric bud tips, but nephrons can subsequently be formed around the stem of elongating ureteric branches (arcades) and via lateral branching (Shah et al. 2004). The normal nephron endowment can vary widely from 300,000 to 1,800,000 per kidney (Georgas et al. 2009; Nyengaard & Bendtsen 1992).

Nephrogenesis is completed by 34 to 36 weeks gestational age and no new nephrons are formed after that time in infants born at term gestation (Bagby 2009; Hinchliffe et al. 1992; Huang et al. 2007; Kandasamy, Smith & Wright 2012; Kett & Denton 2011). Preterm birth mostly occurs during late gestation, interrupting nephrogenesis and resulting in a reduced nephron endowment at birth (Hinchliffe et al. 1991; Rodríguez et al. 2004; Rudolph 2000). Reduced nephron number has been associated with an increased risk of chronic kidney disease and systemic hypertension in later life (Franke et al. 2010; Hoy et al. 2006; Keller et al. 2003). Although nephrogenesis has been shown to be ongoing in infants born preterm, the nephrons formed *ex utero* demonstrate an elevated number of morphological abnormalities. This is most likely in response to the haemodynamic factors in the post-natal environment such as a marked increase in systemic blood pressure, renal blood flow and glomerular filtration (Arant 1987; Black et al. 2013; Gubhaju et al. 2009; Sutherland et al. 2011). Any adverse occurrence that disturbs the completion of nephrogenesis is likely to compromise normal renal growth and subsequent kidney function (Gubhaju et al. 2014; Kandasamy, Smith & Wright 2012; Saint-Faust, Boubred & Simeoni 2014).

The period of nephrogenesis in sheep is similar to that in humans. Metanephric development begins in sheep at 0.2 of term gestation (0.13 in the human) and nephrogenesis is completed by 0.9 of term gestation in both sheep and humans (Singh, RR, Cuffe & Moritz 2012). Low nephron endowment in sheep has been associated with increased cardiovascular and renal risk in later life (Gilbert et al. 2005; Moritz et al. 2005; Singh, R et al. 2009; Singh, R et al. 2012). However, the precise role that nephron deficit and altered renal function plays in the mediation of this increased risk remains unclear (Alexander 2005). Further research is necessary to clarify the relationship between preterm birth and the adult onset of hypertension and cardiovascular disease. Serial studies extending from birth to adulthood using animal models of preterm birth that mirror human pathophysiology (such as the sheep model) should be utilised to conduct these investigations.

1.8. Using the sheep model to study the effects of preterm birth.

1.8.1. The sheep model of pregnancy.

Ethical considerations constrain experimental studies of pregnancy and preterm birth in humans, necessitating the use of animal models (Stopfkuchen 1987). A number of mammalian animal models have been utilised including rodents, domestic ruminants and non-human primates. Rodent models (including mice, rats, rabbits) are limited by altricial offspring born with underdeveloped brain and endocrine systems, significant post-natal organ maturation and the possibility of variable nutrient supply among multiple fetuses from the same litter (Vuguin 2007). Non-human primates (including monkeys, baboons, macaques) are the species that closely mirror human anatomy and pathophysiology, but ethical considerations, lifespan and expensive housing relative to other animal models restrict their use (Korcarz et al. 1997; Teitelbaum 2003; Vuguin 2007).

The ovine model has been well established and contributed significantly to the understanding of parturition and fetal physiology (Mitchell & Taggart 2009). Pregnant sheep and fetuses are large enough to tolerate invasive procedures such as general anaesthesia and surgery that are required as part of functional studies (Dickinson et al. 2016). Sheep deliver precocial young that develop for an extended period *in utero* and weigh around the same as human newborns at term (Carter 2007). The stages of organ development relative to birth, (in particular that of the heart and kidneys) and fetal vascular structures are also comparable in sheep and humans (Bensley, J et al. 2010; De Matteo et al. 2010; Singh, RR, Cuffe & Moritz 2012; Vonnahme et al. 2003). The sheep model has previously been used successfully to investigate fetal adaptation to physiological interventions, including preterm birth and to assess resultant effects on cardiovascular structure and function at birth and in adulthood (Bensley, J et al. 2010; Jobe et al. 1983; Jonker, Sonnet et al. 2007; Vuguin 2007). Sheep exhibit similar physiological consequences of prematurity as humans (Bennet et al. 2007).

'Male disadvantage' also appears to exist in sheep. Studies have shown that prematurely delivered male lambs suffer from a range of conditions including impaired haemodynamic responses to asphyxia (Bennet et al. 2007) and reduced post-natal lung function compared to female sheep (Kovar et al. 2001; Willet et al. 1997). A review of 82 preterm births from 1999 to 2008 by De Matteo et al (2010) in a sheep model of moderately preterm birth found that the male survival rate at 2 weeks of age was 44% (18/41), which was significantly lower than the 76% (31/41) survival for females. The majority of deaths were attributed to respiratory insufficiency, but it remained unclear why males were more likely to die.

The sheep model does vary from humans in some aspects, for example, endocrine control of parturition. Sheep maternal plasma progesterone concentration must fall, the so called 'progesterone withdrawal', to initiate the onset of labour. Conversely, humans maintain high concentrations of progesterone during labour (Challis et al. 2000). Another difference is the contribution of the fetus to the timing of delivery. For example, fetal death in sheep leads directly to delivery, whereas fetal death in humans does not directly affect the length of gestation (Mitchell & Taggart 2009). Sheep also have different placentation to humans. The human placenta is comprised of a single large discoid shaped attachment that invades the endometrium. In sheep, the placenta has numerous discreet attachments to specialised non-glandular vascular regions of the endometrium known as caruncles. However, both human and sheep placentas have cotyledons comprising a structurally similar villous tree within the placentome (Lea et al. 2005). Overall, the structure and function of human and sheep placentas are sufficiently alike for the sheep model to be utilised for a wide variety of investigations involving the placenta (Barry & Anthony 2008).

Another point of difference between the sheep model and humans is that twinning is more common in sheep. The twinning rate in Border Leicester Cross sheep is reported as 17.4% (Scaramuzzi, Hoskinson & Cognié 1993) compared to approximately 3.0% for humans (De Matteo et al. 2008; Laws & Sullivan 2009; Webbink, Roeleveld & Visscher 2006). While no animal model can perfectly replicate the human fetus and pregnancy, the sheep model is a long established, well researched, widely utilised and practical model that is suitable for the investigation of preterm birth effects.

1.8.2. The moderately preterm sheep model.

A full-term gestation for a Border Leicester Cross ewe is 147 (± 4) days after mating (De Matteo et al. 2008; Louey et al. 2000; Mitchell & Taggart 2009; Tare et al. 2014). The earliest a preterm lamb can be born and still survive without medical intervention, such as respiratory assistance, is 90% of a full-term gestation which is 132 to 134 days after mating. This corresponds to the moderate preterm period in sheep (De Matteo et al. 2009). This is equivalent to approximately 32 weeks gestation in humans, which is at the lower limit of the moderate preterm period. Studying the effects of preterm birth in lambs earlier than 132 days gestation has the potential to be confounded by the effects of mechanical ventilation, use of hyperoxic gases and other interventions that would most likely be required to maintain life. As sheep approach adulthood around 12 months of age, conducting long-term follow up of lambs to investigate the effects of preterm birth into adulthood is also feasible (De Matteo et al. 2010). A moderately preterm sheep model allows structural and physiological, short- and long-term analysis of the effect of preterm birth in the absence of confounding medical interventions that are required if the lambs are delivered earlier. In addition, moderate preterm birth is induced in a healthy ewe with a normal fetus and an otherwise uneventful pregnancy that would have delivered normally at term gestation. Therefore, any potential effects of moderate preterm birth that are revealed using this model would be free of the potentially confounding effects of maternal and fetal co-morbidity (Berry, M et al. 2013).

1.9. Overview of ultrasound imaging.

Ultrasound imaging is a diagnostic procedure that uses high frequency sound waves that are beyond the audible range for humans (above 20 kHz) to record anatomical structures and physiological data (Fausti et al. 1981; Langer et al. 2001; Lieu 2010). It is known by several names including medical ultrasound, sonography and ultrasonography. Specialist ultrasound techniques may be known by their application, for example, cardiac ultrasound is also known as echocardiography (Flachskampf & Daniel 2010) and several vascular applications (spectral Doppler, colour Doppler and power Doppler) may be known collectively as simply ‘Doppler’ ultrasound (Porta et al. 2012).

Ultrasound machines generally consist of a mobile system comprising a computer, a console for the controls, display monitor, various probes, recording devices and a printer (Hedrick, Hykes & Starchman 1995; Kremkau, F 2011). All commercially available ultrasound systems for human applications are small and light enough to be readily transportable (for example, can be pushed by one operator from the imaging department to the patient bedside within a typical hospital setting). Some ultrasound systems are small enough to be totally hand-held and are extremely useful for point-of-care situations outside conventional imaging departments, such as in emergency departments and neonatal intensive care units as well as in military and even aerospace applications (Kluckow, M & Evans 2016; Langer et al. 2001; Trinquart et al. 2009).

The ultrasound waves are transmitted into the body from a hand-held probe connected to the ultrasound machine. Ultrasound is almost totally reflected by air, so to ensure that the ultrasound beam emitted by the probe penetrates to the area of interest, acoustic coupling gel is placed on the probe and surface of the body to produce an airtight connection during scanning (Lieu 2010). The ultrasound beam emitted by the probe is directed into the body towards the structures of interest. The sound waves are reflected by the structures they encounter within the body and these echoes are then detected by the same probe operating in receive mode. The operator, known as a sonographer, can adjust various technical parameters including frequency of the ultrasound, power, selective amplification of the echoes, image depth and magnification to list a few, in order to optimise the image quality (Lieu 2010). In addition, the sonographer can insonate the area of interest from different angles by scanning in multiple planes, moving the body (or internal structures if mobile) into different positions and utilising different stages of the respiratory or cardiac cycle to optimise visualisation of the required structures. The ultrasound machine then constructs an image or other representation (for example, a graphical display of blood flow velocity) of the insonated structures using the received echo information. Images are produced in real-time, that is, they are acquired, processed and displayed so quickly that they appear to be created instantaneously. As such, the desired image is usually acquired, then ‘frozen’ and annotated before a hard copy is recorded as a static image and stored. A real-time image has the advantage of being able to demonstrate movement including transient events that may otherwise be missed by static imaging alone (Platts et al. 2010). In addition to recording static images, real-time images can also be recorded as short video clips (known as cineloops), or the complete real-time ultrasound examination can be recorded if required (Koski et al. 2006; Nankivell, Chapman & Gruenewald 2002). The stored images can be reviewed during the ultrasound examination and after the scanning has been completed (known as off-line processing) (Aldrich 2007).

One of the major strengths of ultrasound imaging is that it is considered a safer modality compared to x-ray imaging (including computerized tomography, CT) that utilizes ionizing radiation, or Magnetic Resonance Imaging, MRI, which can be hazardous in the presence of metals such as those found in implants like pacemakers (Flachskampf & Daniel 2010; Herfarth & Palmer 2009). To date, there has been no verified documented evidence of adverse biological effects caused by clinical ultrasound exposure to patients or machine operators since the widespread availability of ultrasound imaging was established in the late 1970s (Nelson, TR et al. 2009; WFUMB. 1998a; Ziskin & Petitti 1988). Nevertheless, biological effects caused by ultrasound have been demonstrated *in vitro* and in animal studies (Abramowicz et al. 2008; Church et al. 2008; Schneider-Kolsky et al. 2009) but how those findings relate to humans, if at all, is not known.

Ultrasound bio-effects can be categorised into two main types; thermal effects that result in tissue heating and mechanical effects that result in physical structural changes to the tissue cells (the most important of which is cavitation) (Nelson, TR et al. 2009). Limited information regarding possible bio-effects as a result of clinical exposures in humans, together with increasing acoustic power outputs from modern ultrasound machines, makes it prudent to use the minimum ultrasound exposure commensurate with obtaining the required diagnostic information (Duck & Henderson 1998). Recommendations on the safe use of ultrasound have been established by the World Federation for Ultrasound in Medicine and Biology to minimize any potential risk (WFUMB 1998; WFUMB. 1998a). These recommendations have been widely adopted as practice guidelines by national professional groups, such as the Australasian Society for Ultrasound in Medicine (ASUM 2012).

Despite the potential bio-effects of ultrasound imaging, the benefits to patients outweigh the risks, if any, when imaging is medically indicated and conducted by trained operators (Nelson, TR et al. 2009; WFUMB. 1998a). Indeed, ultrasound imaging is considered so safe that every pregnant woman in Australia is offered at least one ultrasound examination as part of routine antenatal care, generally to assess fetal and placental morphology at 18 to 20 weeks gestation (RANZCOG 2009). There is ongoing debate as to whether a further ultrasound scan, currently conducted around 11 to 14 weeks gestation to assess the risk of chromosomal abnormalities, be routinely extended to include more fetal morphology assessment to help determine patient specific risk for a range of complications including miscarriage, preterm delivery and fetal abnormalities (Nicolaides 2011; Wye & Benzie 2009).

Another advantage of ultrasound imaging is that it is more widely available and less expensive than CT or MRI (Flachskampf & Daniel 2010; Zanetti & Hodler 2000). Ultrasound imaging can be used to investigate a wide range of organs and body systems and has the capacity to resolve small structures down to 0.2 mm in size (Gindes et al. 2012; Sipos 2009; Zanetti & Hodler 2000). Ultrasound examinations are generally painless and non-invasive (Goyal et al. 2009). Images are produced in real-time which permit the assessment of transient events and moving structures including blood flow (Groenenberg, Hop & Wladimiroff 1991; Koski et al. 2006; Lieu 2010).

Despite these strengths, ultrasound imaging suffers from two significant disadvantages compared to other imaging modalities. Ultrasound beams cannot penetrate through air or dense bone (Aldrich 2007; Kremkau, F 2011). This limits the application of ultrasound imaging in some structures, for example, the lungs (need to traverse air) or the adult brain (need to traverse dense skull bone). Even when the ultrasound beam does not encounter air or dense bone, having to travel long distances through tissue - for example, in obese patients - may be problematic because the sound beam gets too attenuated by the tissues and cannot penetrate deeply enough to the area of interest. Consequently, even modern high quality ultrasound systems still have difficulty demonstrating structures such as the liver in a very large or obese person (Herfarth & Palmer 2009). The other significant disadvantage of ultrasound imaging is that it is highly operator dependent compared to other modalities. The quality of ultrasound imaging is heavily reliant on the technical knowledge, practical eye-hand co-ordination and image interpretation skills of the sonographer to optimize the image/data collection (Herfarth & Palmer 2009; Kim, MJ et al. 2009; Spyridopoulos et al. 2010).

A range of ultrasound modalities can be employed to acquire different structural and functional information.

1.9.1. B-mode (brightness mode) ultrasound.

Conventional (B-mode) ultrasound displays images of the insonated structures as thin 2-dimensional cross-sectional representations of the body in real-time. The structures that are demonstrated are determined by the sonographer placing the ultrasound probe over the area of interest and insonating the organ in question. Multiple planes can be demonstrated by simply sliding the probe across the body and changing the interrogated cross-section of anatomy. The echoes that return from the structures are detected and mapped into a gray-scale image (typically comprising up to 256 shades of gray from white to black) with the stronger echoes (more echogenic) appearing whiter, and the weaker echoes (less echogenic) appearing darker (Langer et al. 2001; Singh, J, Adams & Pierson 2003). An example of a B-mode image is demonstrated in Figure 1-1.

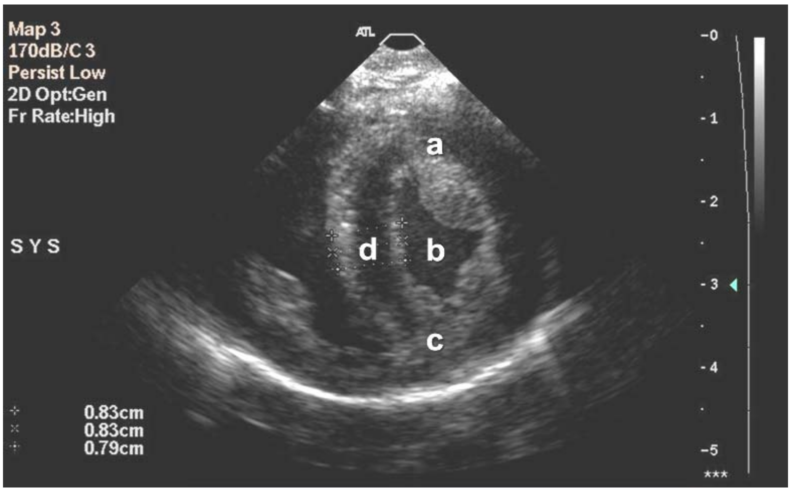

Figure 1-1: B-mode image demonstrating a short axis cross-section of the left ventricle of a sheep.
a-anterior wall, b-ventricle chamber, c-posterior wall, d-interventricular septum.

1.9.2. M-mode (time-motion mode).

Unlike B-mode imaging that displays a 2-dimensional cross-section of tissues that were insonated, M-mode imaging only displays the echoes that are detected from a single beam, or 'line-of-sight'. As such only 1 dimension is demonstrated, effectively as a line of dots in gray-scale that represent the strength of echoes along the single beam path. This line of information is then stepped sideways across the display screen and replaced by a new line of information. The lines summate over time to construct a graphical display of echoes from a single beam comprising depth in the y-axis versus time in the x-axis. An example of an M-mode image is demonstrated in Figure 1-2.

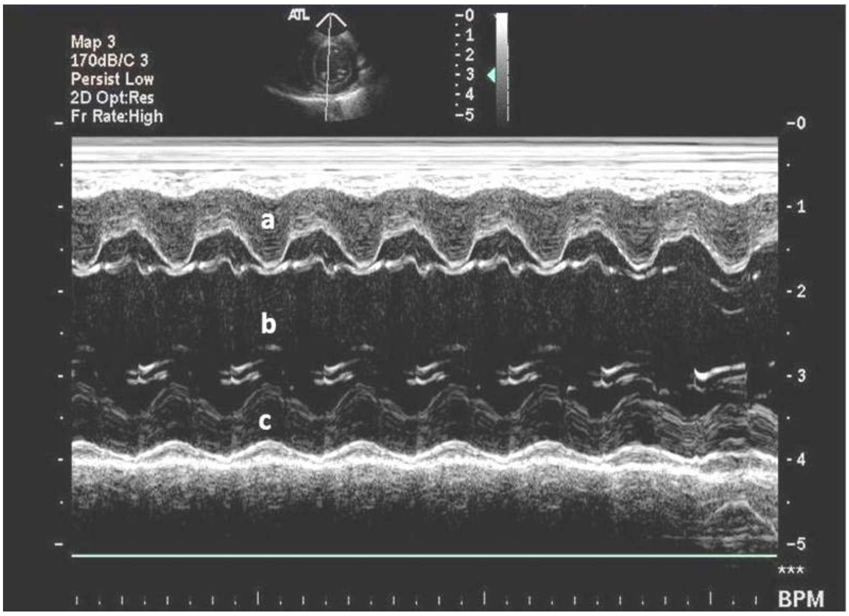

Figure 1-2: M-mode image of the left ventricle of a sheep demonstrating depth in the y-axis versus time in the x-axis.
a-anterior wall, b-ventricle chamber, c-posterior wall. Note the B-mode image located at top demonstrating the line of sight.

The graphical display can be halted, with the movement of any structures being recorded in the resultant static image. M-mode is employed to assess and record dynamic events, such as tissue movement, and its major application is in demonstrating heart structure and function in echocardiography (Ebeling Barbier et al. 2011; Whittingham 2007). The major drawback of M-mode imaging is that the data produced is one-dimensional and lacks spatial information, although that is overcome by simultaneously scanning in B-mode so that the observer can see what structures are being interrogated along the M-mode line of sight (Anderson 2007).

Blood flow in the cardiovascular system can be demonstrated using mainly three variations of Doppler ultrasound.

1.9.3. Colour Doppler Imaging.

In colour Doppler imaging, colour coded real-time blood flow information in a sonographer-selected region of interest (known as the colour box) is superimposed over the B-mode image. The resultant image displays the mean velocity of blood flow that exists at all points within the box by assigning a colour to the direction of blood flow relative to the ultrasound probe (Kim, MJ et al. 2009). Hues are added to the colours to represent different velocities, for example, a lighter hue demonstrates higher velocity (Torp-Pedersen & Terslev 2008). If no blood flow is detected, then no colour is assigned or superimposed on the gray-scale B-mode image.

However, blood flow is only detected if movement is relative to the ultrasound probe. This is because the ultrasound system is looking for Doppler shifts in the ultrasound echo frequency (compared to the original insonating ultrasound frequency) that have been caused by interaction with blood cells moving towards or away from the probe. Therefore, blood flow that is perpendicular to the ultrasound beam (i.e. flowing neither towards or away from the probe) is not detected because there are no mean Doppler frequency shifts. This may result in imaging artifacts, such as no apparent flow that distort the true blood flow characteristics (Hergum et al. 2010). A number of other factors including the frequency of ultrasound used, blood flow velocity, angle of insonation, tissue movement and various instrument settings may also affect the accuracy of colour Doppler imaging (Rubin et al. 1994; Singh, J, Adams & Pierson 2003; Stewart 2001).
An example of a colour Doppler image is demonstrated in Figure 1-3.

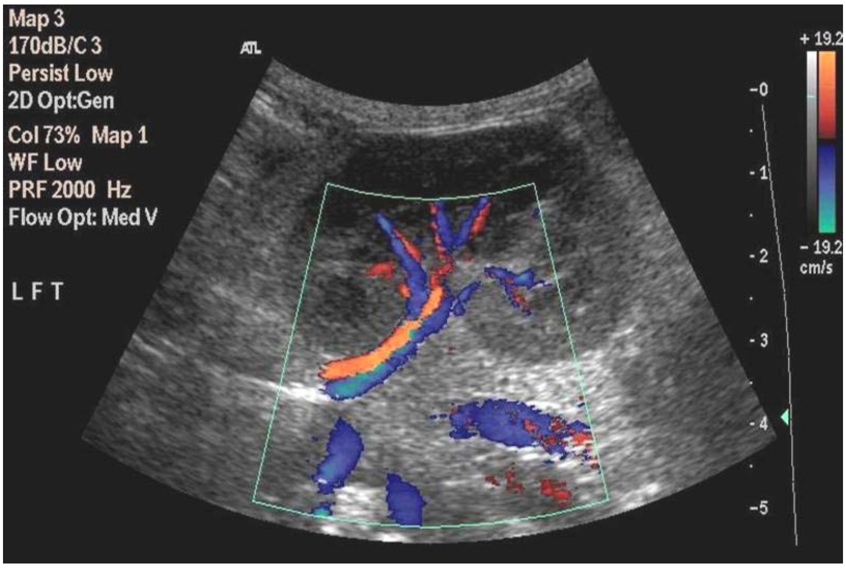

Figure 1-3: Colour Doppler image of a left kidney of a sheep.

Flow towards the probe (towards the top of image) assigned red (corresponds with the renal artery), flow away from probe assigned blue (corresponds with the renal vein.) The velocity colour bar is shown on right hand side.

1.9.4. Power Doppler Imaging.

Power Doppler is similar to colour Doppler in that it demonstrates blood perfusion as colour coded real-time blood flow information in a colour box superimposed over the B-mode image. However, power Doppler displays the strength (energy) of the Doppler shift caused principally by the concentration of blood cells, rather than the mean Doppler shift caused by blood flow as in colour Doppler imaging. The resultant power Doppler image displays a map of the blood perfusion as a single colour as it does not demonstrate speed or direction of blood flow like colour Doppler. Power Doppler has some advantages over colour Doppler in that it suffers less from image artifacts such as aliasing and is relatively independent of the insonation angle, but suffers more than colour Doppler from 'flash' artifacts caused by moving tissue (Allan et al. 2006; Rubin et al. 1994). In theory, power Doppler imaging should be better at detecting slow blood flow than colour Doppler imaging (Aso et al. 2005; Kremkau, F. 2011a; Porta et al. 2012). However, since the mid-2000s high quality ultrasound systems have developed colour Doppler technology that is at least as sensitive, if not better, than power Doppler in detecting slow blood flow (Jain et al. 1991; Torp-Pedersen & Terslev 2008). An example of a power Doppler image is demonstrated in Figure 1-4.

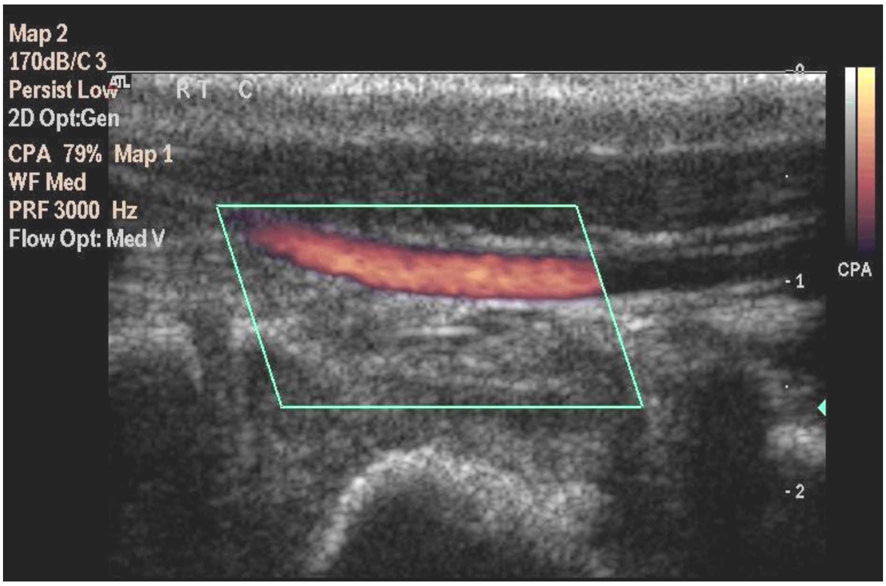

Figure 1-4: Power Doppler image of a long axis view of the right common carotid artery of a sheep.

Blood perfusion is represented by the amber colour in the artery, with no information about the direction or speed of blood flow.

1.9.5. Spectral Doppler Imaging.

Unlike colour or power Doppler imaging that has a colour box that can be as large as the entire B-mode image, spectral Doppler collects blood flow information from just a small sample volume, known as the sample 'gate'. The sonographer uses the B-mode image (and possibly a colour or power image as well) to locate the gate in areas of interest, for example, at the site of a vessel stenosis. The angle of the blood flow in the vessel to the insonating single line-of-sight spectral Doppler beam (which is superimposed over the B-mode image) is measured by the sonographer using an angle correction cursor on the image. This angle is known as the Doppler angle. The Doppler angle must be known for the ultrasound system to accurately calculate flow velocity when the blood flow is not parallel to the insonating spectral Doppler beam, which is usually the case (Whittingham 2007).

Spectral Doppler imaging displays blood flow from within the sample volume graphically, usually as a plot of the range of blood flow velocities (y-axis) versus time (x-axis), commonly referred to as a flow velocity waveform (Thuring, Malcus & Maršál 2011; Wood, MMR et al. 2010). The sample volume can then be relocated to another area of interest to obtain more blood flow information at another site. An example of a spectral Doppler image is demonstrated in Figure 1-5. Unlike colour Doppler imaging that provides average velocity values over a large area of interest, spectral Doppler provides much more quantitative information, such as the maximum, minimum, average and variance of blood flow, albeit, over a small volume. This quantitative information is required to accurately determine the blood flow characteristics and to assist clinical decision making (Wood, MMR et al. 2010). The spectral Doppler velocity information will be inaccurate if the Doppler angle is greater than 60 degrees, due to errors associated with a cos function in the Doppler equation used to make the calculation. To get around this problem, a number of indices have been defined involving ratios of velocities that are independent of the Doppler angle (Burns, P. 1993). They include S/D ratio (peak systolic velocity divided by end diastolic velocity), also known as A/B ratio, Resistive Index, RI, (peak systolic velocity minus end diastolic velocity divided by peak systolic velocity), also known as the Pourcelot Index, and Pulsatility Index (maximum velocity excursion between systolic and diastolic velocity, divided by the mean velocity) (Allan et al. 2006; Burns, P 1987).

The various Doppler modes complement each other and can be used together to provide the most accurate and clinically useful information as efficiently as possible. It can be tedious to scan through a large section of vessel using spectral Doppler alone to slowly build up a picture of the blood flow characteristics within the vessel. There is also the risk that a significant feature may be overlooked. In practice, the clinically relevant areas of a vessel such as areas of stenosis or turbulent blood flow, are quickly identified using B-mode and colour or power Doppler, and then those areas are selectively interrogated in greater detail using spectral Doppler (Burns, P. 1993). When real-time B-mode imaging is simultaneously combined with spectral Doppler it is known as duplex imaging. When a third mode, such as colour or power Doppler is also utilised simultaneously, it is known as triplex imaging.

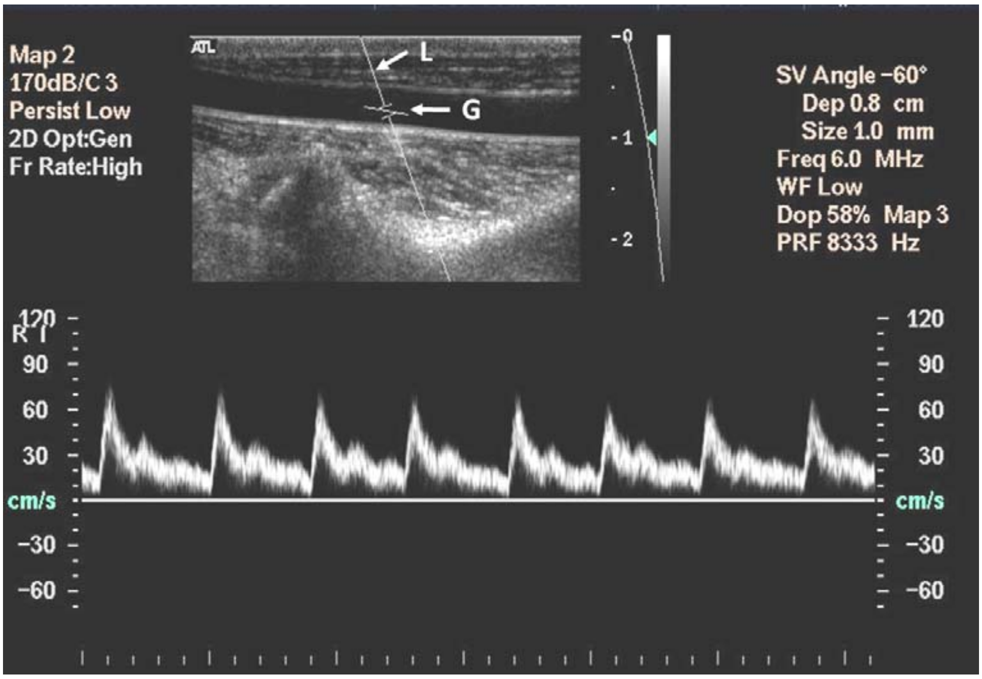

Figure 1-5: Spectral Doppler image of the right common carotid artery of a sheep.

B-mode image at top demonstrates a cross-section of the insonated structures, together with the spectral Doppler interrogation line of sight (long white line - L). The sample gate (double short lines along the line of sight - G) is located within the artery. The angle correction cursor has been aligned to the presumed direction of blood flow parallel to the artery wall. The flow velocity waveform in the lower panel demonstrates blood flow velocity varying over time with each heartbeat.

It is clear that preterm birth affects the development of the cardiovascular system and kidneys, leads to hypertension and chronic diseases in later life and affects males more than females. However, the specific mechanisms remain unclear and there is a need to carry out longitudinal studies in animal models to explore the effects of preterm birth into adulthood using non-invasive methods. Ultrasound imaging is an ideal modality for this purpose as it is portable, cheap and can be carried out repeatedly without the need for general anaesthesia. Despite this, there are few reports in the literature of studies investigating preterm birth utilising serial ultrasound imaging to track the development of disease from birth to adulthood. In this thesis, we report on the findings of cardiovascular and renal structure and function from immediately after moderately preterm birth into early adulthood as well as the establishment of ultrasound protocols for conducting the study.

1.10. Hypotheses and aims.

1.10.1. Hypotheses.

1. Serial ultrasound imaging can be used successfully to evaluate the cardiovascular system and kidneys of sheep from moderately preterm birth to adulthood.
2. Moderately preterm birth results in structural and functional mal-adaptations of the heart, major arteries and kidneys that contribute to the adult onset of disease.
3. Males are more likely to exhibit structural and functional mal-adaptations of the heart, major arteries and kidneys than females.

1.10.2. Aims.

1. To establish ultrasound imaging protocols for the evaluation of the left ventricle, proximal ascending aorta, main, right and left pulmonary, renal and common carotid arteries and the kidneys in sheep from moderately preterm birth to adulthood.
2. To use serial ultrasound imaging of male and female sheep from moderately preterm birth to adulthood to investigate the
   a) structure and function of the left ventricle.
   b) structure and blood flow within the proximal ascending aorta, main, right and left pulmonary arteries and the common carotid arteries.
   c) structure of the kidneys and blood flow within the renal arteries.
3. To use serial ultrasound imaging to demonstrate any significant sex-specific differences in the effects of moderately preterm birth on the morphology and function of the cardiovascular system and kidneys in sheep from birth to early adulthood.

Chapter 2 General methods.

2.1. Experimental outline.

This PhD project was comprised of four separate ultrasound evaluations that investigated the short and long term effects of moderate preterm birth on the cardiovascular system and kidneys of sheep (Figure 2-1).

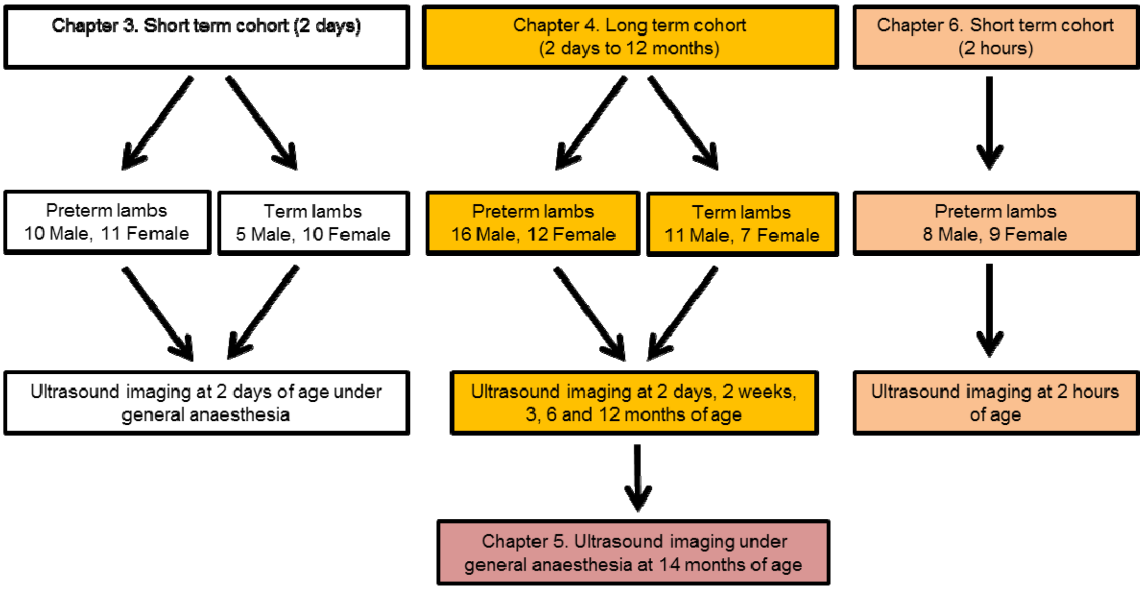

Figure 2-1: Schematic of sheep cohorts and ultrasound scanning schedule.

There were four separate investigations on three cohorts of sheep. A short term (2 days) cohort of lambs was evaluated in chapter 3. A long term (2 days to 12 months) cohort of sheep was evaluated in chapter 4. In chapter 5, ultrasound imaging was used to evaluate the long term sheep cohort at 14 months of age. A short term (2 hours) lamb cohort was evaluated in chapter 6.

In chapter three an ultrasound evaluation of the effects of preterm birth on the cardiovascular system and kidneys of anaesthetised two day old lambs was conducted on a short term (2 day) cohort. This cohort consisted of 21 preterm lambs (10 male, 11 female) born at 132 ± 1 days gestation, and 15 term lambs (5 male, 10 female) born at 147 ± 1 days gestation.

The aims of chapter three were:

1. To establish ultrasound imaging protocols for evaluation of the left ventricle, proximal ascending aorta, pulmonary, renal and common carotid arteries and the kidneys in anaesthetised moderately preterm and term lambs at two days after birth

2. To use ultrasound imaging to investigate the structure and function of the left ventricle in male and female moderately preterm and term lambs at two days after birth
3. To use ultrasound imaging to investigate the structure and blood flow within major arteries including the proximal ascending aorta, main, right and left pulmonary arteries and the common carotid arteries in male and female moderately preterm and term lambs at two days after birth
4. To use ultrasound imaging to investigate the structure of the kidneys and blood flow in the renal arteries of male and female moderately preterm and term lambs at two days after birth

In chapter four, a serial ultrasound evaluation of the effects of moderately preterm birth on the cardiovascular system and kidneys of lambs from birth to early adulthood (12 months of age) was conducted on a long term (12 months) cohort. Sheep underwent serial ultrasound imaging at two days, two weeks, three months, six months and twelve months of age. This cohort consisted of 28 preterm lambs (16 males, 12 females) born at 132 ± 1 days gestation, and 18 term lambs (11 males, 7 females) born at 147 ± 1 days gestation (Figure 2-1).

The aims of chapter four were:

1. To establish ultrasound imaging protocols for evaluation of the left ventricle, proximal ascending aorta, pulmonary, renal and common carotid arteries and the kidneys in moderately preterm sheep from two days to twelve months of age
2. To use ultrasound imaging to serially investigate the structure and function of the left ventricle in male and female sheep from two days to twelve months of age
3. To use ultrasound imaging to serially investigate the structure and blood flow within major arteries including the proximal ascending aorta, main, right and left pulmonary arteries and the common carotid arteries in male and female sheep from two days to twelve months of age
4. To use ultrasound imaging to serially investigate the structure of the kidneys and blood flow in the renal arteries of male and female sheep from two days to twelve months of age

In chapter five, an ultrasound evaluation of the effects of moderately preterm birth on the cardiovascular system and kidneys of sheep was conducted on the long term cohort of sheep (from chapter four), while under general anaesthesia at 14 months of age immediately prior to being euthanased for necropsy. The data from the ultrasound evaluation in chapter five were not included as part of the serial study in chapter four because the sheep were under general anaesthesia during ultrasound imaging at 14 months of age.

The aims of chapter five were to use ultrasound imaging of male and female anaesthetised moderately preterm and term sheep at 14 months of age to investigate and compare the:

1. structure and function of the left ventricle
2. structure and blood flow within major arteries including the proximal ascending aorta and main, right and left pulmonary arteries and the common carotid arteries
3. structure of the right kidney and blood flow in the right renal artery

The ultrasound evaluations in chapters three, four and five were conducted concurrently with the PhD project of Dr Vivian Nguyen who was under the supervision of Professor Jane Black and Associate Professor Graeme Polglase, Monash University. Dr Nguyen performed a morphometric and physiological evaluation of the sheep including bodyweight, stature, heart rate and arterial pressure measurements from birth to 2 days of age (chapter 3) and from birth to fourteen months of age (chapters four and five). Once the ultrasound evaluations in chapters three and five had been concluded, a histological analyses of the heart, aorta, carotid arteries and renal tissues were performed after necropsy by Dr Nguyen. The outcomes of Dr Nguyen's PhD research are supplementary to this project but the intention is to collaborate on all the findings with a view to possible joint publication of the results.

In chapter six, an ultrasound evaluation of the cardiovascular system and kidneys was conducted on a short term (2 hours) cohort of moderately preterm lambs while they were still undergoing the haemodynamic transition associated with birth (Figure 2-1). The cohort consisted of eight male and nine female lambs born at 132 ± 1 days gestation (Figure 2-1).

The aims of chapter six were to use ultrasound imaging to compare the:

1. structure and function of the left ventricle
2. structure and blood flow within major arteries including the proximal ascending aorta and main, right and left pulmonary arteries
3. structure of both kidneys and blood flow in the renal arteries

between male and female moderately preterm lambs during the haemodynamic transition at two hours after birth.

The ultrasound analyses performed in this chapter provides important *in vivo* data of the structure and function of the heart, kidneys and major arteries and was conducted concurrently with the PhD project of Dr Noreen Ishak (a PhD student under the supervision of Professor Richard Harding, Dr Robert De Matteo and Dr Foula Sozo, Monash University). Dr Ishak was evaluating lung structure and function in male and female preterm lambs during the haemodynamic transition from a fetal vascular circulation to an extra-uterine circulation in the eight hours immediately after birth. The preparation, delivery and post-natal care of all the lambs evaluated in this chapter were performed and/or supervised by Dr Noreen Ishak as part of her project. The morphological and physiological analyses conducted by Dr Noreen Ishak are supplementary to the ultrasound evaluation. The intention is to collaborate on all the findings when completed with a view to possible joint publication of the results.

2.2. Ethics approval.

All animal procedures were approved by the Monash University Animal Ethics Committee (approval MMCA-2011/01). Monash University adheres to the Australian National Health and Medical Research Council (NH&MRC) code for the care and use of animals for scientific purposes.

2.3. A modified sheep model of moderately preterm birth.

The model of preterm birth used in the short term (2 days) and long term studies (chapters 3 to 5) was based on the well-established protocols for inducing preterm and term birth, lamb delivery and post-natal care developed in Professor Richard Harding's laboratory at Monash University (De Matteo et al. 2010). However, in contrast to the previously published studies of De Matteo et al (2010) the ewes assigned to deliver preterm were administered a clinically relevant dose of antenatal corticosteroids. In using this approach we have mimicked the current treatment of women at high risk of delivering preterm and therefore have used a clinically relevant experimental paradigm (Berry, M et al. 2013).

Preterm birth was induced in time-mated crossbred ewes (Border Leicester X White Suffolk) by administering Epostane (2a, 4a, 17-4, 5-epoxy-17-hydroxy-4, 17-dimethyl1-3-ketoandrostane-2-carbonitrile, Sanofi-Synthelabo, Australia), via intravenous injection in the jugular vein (50 mg in 2 ml of ethanol). Epostane inhibits progesterone synthesis and subsequently induced labour approximately 48 hours after administration. In ewes assigned to deliver preterm, Epostane administration was timed to induce delivery at

90% of full term gestation at 132 ± 1 days (two weeks premature). For ewes assigned to deliver at full term, Epostane administration was timed to induce delivery at 147 ± 1 days gestation.

In addition, two separate clinically relevant doses (2 times 11.4 mg) of betamethasone (Celestone Chronodose, Schering-Plough, Australia) were administered antenatally via intramuscular injection in the rump/thigh of the ewes assigned to deliver preterm. The two doses were administered 24 hours apart with the first injection given approximately five hours prior to the Epostane injection. The dose of betamethasone was identical to a single course of antenatal corticosteroids currently recommended for women at risk of preterm delivery in Australia and New Zealand (Berry, M et al. 2013; Liggins Institute 2015). It is estimated that 70-80% of pregnancies that deliver at 24 to 34 weeks gestation in the developed world receive corticosteroid treatment (Henry, Shand & Welsh 2013). Betamethasone enhances survival after preterm delivery by accelerating fetal lung maturation without inducing parturition (Berry, L et al. 1997; Roberts & Dalziel 2013).

The model of preterm birth used in the short term (2 hours) evaluation (chapter 6) was based on previously published protocols (Ishak et al. 2012) that differed from the protocols used in the other studies of this thesis (chapters 3 to 5). Pressure catheters were chronically implanted in the pleura, carotid artery, jugular vein and amniotic sac of fetal sheep *in utero* at 125 days gestational age. A vascular occluder was placed around the umbilical cord so that blood flow in the cord could be blocked during delivery preventing anaesthetic agents administered to the ewe from entering the fetal circulation. Pressure measurements of the fetal sheep were recorded at 131 days gestational age, followed by administration of betamethasone (5.7 mg i.m., betamethasone (Celestone Chronodose, Schering-Plough, Australia) to the pregnant ewes. Two days later the moderately preterm un-anaesthetised lambs and their catheters were delivered by Caesarean section. Lambs were not mechanically ventilated but received supplemental oxygen to maintain an arterial oxygen saturation (SO_2) above 80% if required. Blood gases, arterial and intra-pleural pressures were monitored for eight hours after birth.

The induction, delivery and post-natal care of all lambs were performed and/or supervised by Dr Noreen Ishak and Dr Vivian Nguyen as part of their PhD projects. I have subsequently conducted comprehensive ultrasound analyses of the cardiovascular system and kidneys in the preterm and term sheep that were generated from those animal studies. This investigation was able to perform studies on the same animal cohorts used in the other PhD projects because ultrasound imaging is non-invasive, analyses could be performed without affecting outcomes in the other projects and ultrasound scanning could be scheduled without disrupting any of the investigations. The findings from these large and expensive series of experiments in a clinically relevant large animal model were maximised by conducting the additional ultrasound analyses. Utilising the same animals for more than one study made it possible to reduce the overall number of animals required to obtain scientifically valid data, as well as minimise animal stress and discomfort in keeping with the principles of 'replacement, reduction and refinement' relating to humane experimentation involving animals (Tannenbaum & Bennett 2015).

2.4. Ultrasound imaging of sheep.

Ultrasound equipment.

A conventional clinical ultrasound system, Philips ATL 5000 SonoCT® (Philips, Bothell, USA), with B-mode, Colour Doppler, Power Doppler, Spectral Doppler and M-mode (Barella 1999), was used in this study to acquire images of the heart, major arteries and kidneys (Figure 2-2).

Figure 2-2: Philips ATL SonoCT® ultrasound system set up next to a table for scanning lambs up to two weeks of age.

A heat pad or hot water bottle was placed under a towel on the table to keep newborn lambs warm.

The ultrasound probes and system application settings were varied depending on the area under investigation and the size/age of the sheep. Phased array cardiac probes (P12-5 with a 'pediatric cardiac/neonatal' application setting or a P5-3 or P4-2 probe with a 'pediatric cardiac/general' application setting) were used for imaging the heart, proximal ascending aorta and pulmonary arteries. A linear array small parts probe (L12-5 with a 'cardiovascular/carotid' application setting) was used for scanning the common carotid arteries. Curvilinear array abdominal probes (C7-4 with a 'pediatric/pediatric renal' application setting or C5-2 with an 'abdomen/renal' application setting) were used for imaging the kidneys and renal arteries. The lower frequency ultrasound probes were used in the older/larger animals in order to gain adequate ultrasound beam penetration to the structures of interest (Table 2-1).

Table 2-1: Ultrasound probes used in scanning sheep at two days to twelve months of age.

LV = left ventricle, IVS = Interventricular septum.

Structures	Ultrasound probe used for scans performed at 2 days and 2 weeks of age	Ultrasound probes used for scans performed at 3, 6 and 12 months of age
LV wall, IVS, proximal ascending aorta, main, right and left pulmonary arteries	P12-5	P5-3 or P4-2
Common carotid arteries	L12-5	L12-5
Kidneys and renal arteries	C7-4	C7-4 or C5-2

Lambs grow in size rapidly from two weeks to three months of age requiring lower frequency ultrasound to penetrate the chest and abdomen compared to newborn lambs. A P4-2 phased array cardiac probe operating at 4-2 MHz is required to penetrate deeply into the larger body of a grown sheep compared to a smaller younger lamb that only requires a P12-5 probe operating at 12-5 MHz to demonstrate the required structures.

Preparing sheep for ultrasound examination.

All sheep were weighed (kg) just prior to ultrasound examination in order for a number of the measurements to be corrected for bodyweight. The wool located over the area to be scanned was clipped as short as possible immediately prior to the ultrasound scan and a liberal amount of ultrasound transmission gel, Aquasonic 100® (Parker laboratories, NJ, USA), was spread on the skin to ensure that intimate contact with the ultrasound probe could be maintained during insonation. This optimised the transmission of ultrasound energy across the probe/skin interface by minimising the presence of air and also provided a slippery contact point for the probe so it could be slid easily across the surface of the skin during scanning.

It was necessary for sheep to be immobilised during the ultrasound examinations. There was no requirement to restrain the sheep during the ultrasound scanning conducted in chapter three, or during the final ultrasound examinations on the sheep in chapter five because they were already under general anaesthesia. However, the ultrasound examinations conducted on conscious sheep in chapter four and conscious lambs in chapter six required some form of gentle immobilisation. General anaesthesia or sedation for all animals were considered, but these options involved administration of medications (De Matteo et al. 2008) and mechanical ventilation (Quaedackers et al. 2004) and would introduce potentially confounding variables to any findings.

The general scanning set up was to have the ultrasound machine next to a platform (such as a table) that raised the animals up to approximately waist height, so the sonographer could reach both the sheep and the ultrasound machine console without having to extend their arms greatly or to bend over excessively (Figure 2-2). The size of the animal determined the restraining protocol that was used. More specific information about the restraining protocol used in the ultrasound imaging of animals in each study is provided in the experimental chapters.

Ultrasound scanning techniques.

The general approach to ultrasound scanning was to acquire the required images to visualise, record and take measurements of the heart, major arteries and kidneys while keeping the ultrasound scanning time, and therefore sheep handling, to a minimum. Measurements, such as bipolar renal length, that could be completed quickly using the ultrasound machine electronic callipers were performed during the course of the examination. However, a number of measurements, such as left ventricle dimensions that took a long time to perform, were completed off-line after the sheep had been scanned to reduce sheep handling time. At the end of each examination, the recorded images for the sheep were transferred from the ultrasound machine to a personal computer, (HP Elitebook®, Hewlett-Packard, Palo Alto, CA, USA) and ImageJ image processing software (version 1.47a, W Rasband, National Institutes of Health, USA) was used to perform the off-line measurements later on the transferred images. A summary of the measurements conducted during the ultrasound scan and those completed off-line after the examination had been completed is provided in Table 2-2.

Table 2-2: Summary of measurements conducted during the ultrasound scan and those completed off-line after the examination.

Measurement	During the ultrasound scan	After the ultrasound scan (off-line)
Left ventricle anterior wall thickness		✓
Left ventricle posterior wall thickness		✓
Left ventricle internal chamber diameter		✓
Interventricular septum thickness	✓	
Percentage fractional shortening of Left ventricle		✓
Ascending proximal aorta diameter	✓	
Pulmonary artery diameter	✓	
Left pulmonary artery diameter	✓	
Right pulmonary artery diameter	✓	
Maximum systolic blood flow in the aorta, pulmonary and renal arteries		✓
Right and left kidney length, width, thickness	✓	
Right and left kidney volume		✓
All common carotid artery measurements		✓
Resistive indices for renal and common carotid arteries		✓

Ultrasound scanning protocol for the heart.

The sheep were generally positioned on their back, or slightly on their right side and gently restrained in various ways depending on their size/age at the time of scanning. B-mode imaging of the heart was performed first to obtain a two-dimensional left parasternal short axis view of the left ventricle at the level of the papillary muscles (Moritz et al. 2005). An M-mode trace to demonstrate the thickness of the anterior and posterior wall of the left ventricle (LV) and the internal short axis chamber diameter over at least five consecutive cardiac cycles was then recorded for later off-line analysis (Figure 1-2).

The following left ventricular structures were measured in five consecutive cardiac pulses from the recorded M-mode trace (Figure 2-3) (Brown et al. 2002):

- LV anterior wall thickness (mm) in systole, LVAWs, and diastole, LVAWd.
- LV posterior wall thickness (mm) in systole, LVPWs, and diastole, LVPWd.

- LV internal short axis chamber diameter (mm) in systole, LVIDs, and diastole, LVIDd.

The measurements of each of the structures listed above were averaged and corrected for bodyweight (mm/kg). Fractional shortening of the left ventricle is an index of cardiac contractility and a surrogate for left ventricular function (Godfrey et al. 2012; Lang et al. 2005; Moritz et al. 2005). The percentage fractional shortening (%FS) of the left ventricle was calculated from the acquired measurements using the formula:
%FS= (LVIDd-LVIDs)/LVIDd x100. (Singh, R et al. 2009).

Left ventricular wall stress is an indicator of adaptive growth of the myocardium in response to haemodynamic changes such as hypertension (Jonker, S et al. 2010; Wikstrand 1984). The LV peak systolic wall stress was estimated from the acquired measurements using the Laplacian relationship formula: S = PR/(2h), where S = LV peak systolic wall stress (mm Hg), P = peak arterial blood pressure (mm Hg), R = radius of curvature of the LV short axis in systole (mm) Note; R = 0.5 x LV internal diameter in systole, and h = LV posterior wall thickness (mm) in systole (Moriarty 1980). The peak arterial blood pressure measurements were kindly provided by Dr V Nguyen who recorded the measurements as part of her PhD thesis. Arterial pressure was measured using a sphygmomanometer (cuff on fore-limb) attached to an Advisor® Vital Signs Monitor (SurgiVet®; Smiths Medical PM, Massachusetts, USA) (Nguyen, V. 2016).

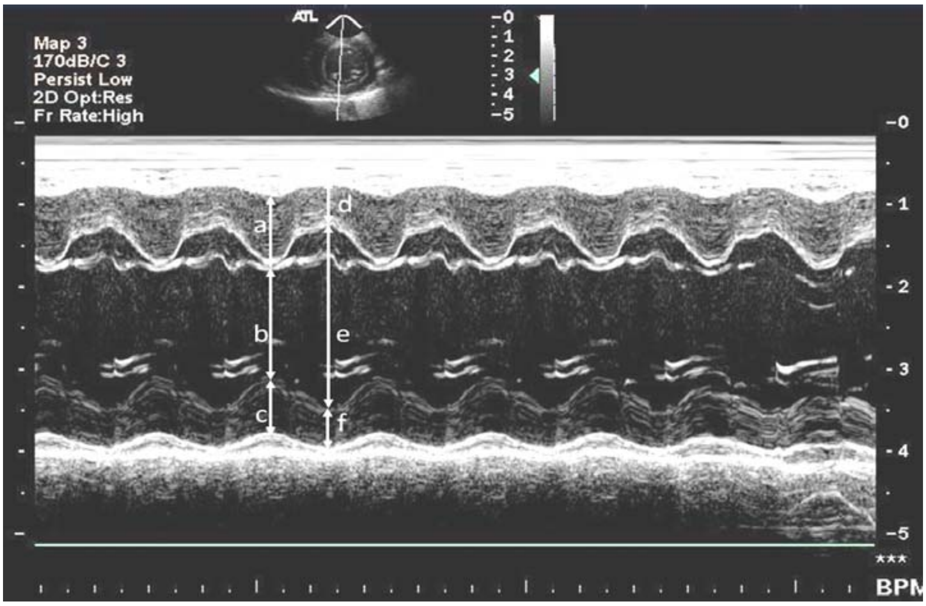

Figure 2-3: M-mode image demonstrating the thickness of the left ventricular structures of a sheep varying over time (8 cardiac cycles).

Anterior wall in systole, a, and diastole, d. Posterior wall in systole, c, and diastole, f. Internal short-axis chamber diameter in systole, b, and diastole, e.

It was not possible to accurately record the interventricular septum (IVS) thickness using M-mode imaging. Sheep have a keel shaped chest that makes it difficult to direct the M-mode interrogation beam (i.e. line of sight) perpendicularly to the IVS wall, and that would result in an over-estimation of the wall thickness. Two-dimensional B-mode imaging was used to record the IVS thickness in systole, IVSs, defined by the frame demonstrating the smallest left ventricle short axis internal chamber diameter and in diastole, IVSd, defined by the frame demonstrating the largest left ventricle short axis internal chamber diameter (Figure 2-4) (Dodic et al. 2001). The average thickness (mm) in systole and diastole was calculated from three separate measurements of the IVS in a plane perpendicular to the wall using the electronic callipers of the ultrasound machine during the examination. The average of these three measurements was also corrected for bodyweight (mm/kg).

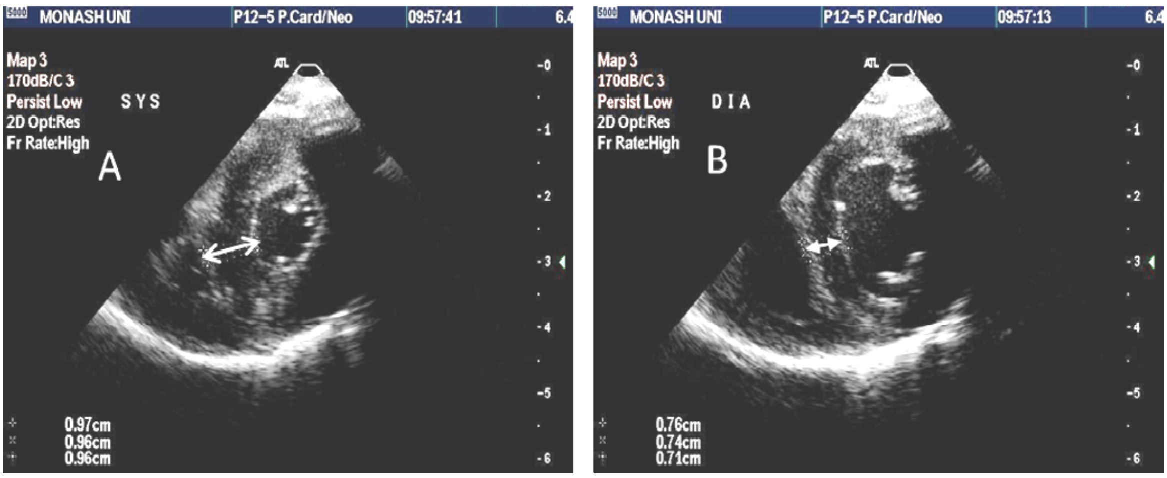

Figure 2-4: B-mode images of the interventricular septum (IVS) of a sheep.

Image A demonstrates the IVS thickness (open arrowheads) at systole, and image B demonstrates the IVS thickness (closed arrowheads) at end diastole.

Ultrasound examination of the proximal ascending aorta and pulmonary arteries.

Two-dimensional B-mode imaging was used to visualise the right ventricular outflow tract and pulmonary artery in the left parasternal short axis view at the level of the base of the heart (Rudski et al. 2010). The pulmonary artery will be referred to as the main pulmonary artery in order to clearly distinguish it from the right and left pulmonary arteries. The main pulmonary artery was then followed to its bifurcation into proximal right and left pulmonary arteries (all vessels shown in long axis) and B-mode images of these structures were recorded. The maximum internal diameter (lumen) of the proximal main, right and left pulmonary arteries were measured (mm) three consecutive times using the electronic callipers of the ultrasound machine and the mean internal diameter of each vessel was calculated. The mean internal diameter of each vessel were corrected for bodyweight (mm/kg).

The proximal ascending aorta (shown in short axis) at the level just distal to the aortic valve was demonstrated anterior to the proximal right pulmonary artery (Anderson 2007). The maximum internal diameter (lumen) of the proximal ascending aorta was measured (mm) in two perpendicular planes at this level using the electronic callipers and the average was calculated from the two measurements (Figure 2-5). The mean internal diameter of the proximal ascending aorta was corrected for bodyweight (mm/kg).

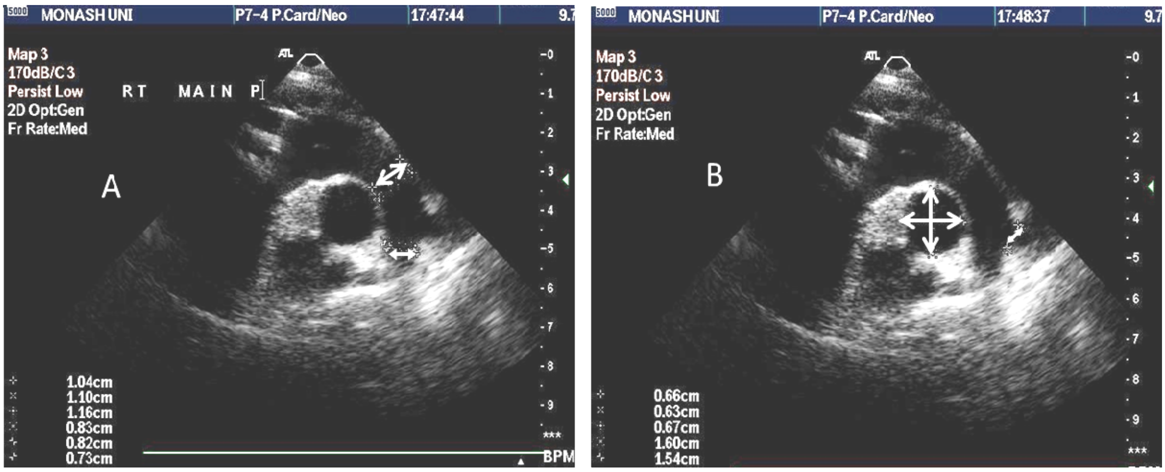

Figure 2-5: B-mode images of the main, right and left pulmonary arteries and proximal ascending aorta of a sheep.

Image A demonstrates the maximum internal diameters of the main pulmonary artery (open arrowheads) and right pulmonary artery (closed arrowheads). Image B demonstrates the maximum internal diameter of the proximal ascending aorta (open arrowheads) and the maximum internal diameter of the left pulmonary artery (closed arrowheads).

Blood flow velocity waveforms of the pulmonary arteries were obtained using spectral Doppler imaging. A spectral Doppler sample gate of 1.5 to 2.5 mm with an insonation angle less than 60 degrees was placed, in turn, centrally into the proximal main, right and left pulmonary arteries and a spectral trace of the blood flow within each artery containing at least four consecutive cardiac cycles was recorded for later off-line analysis (Figure 2-6). To obtain a blood flow velocity waveform of the proximal ascending aorta, the imaging plane was rotated 90 degrees from the left parasternal short axis view, to the left parasternal long axis view which shows the proximal ascending aorta in long axis. A spectral Doppler sample gate of 1.5 to 2.5 mm with an insonation angle less than 60 degrees was placed centrally in the proximal ascending aorta and a spectral trace of the blood flow containing at least four consecutive cardiac cycles was recorded for later off-line analysis.

The peak systolic velocity (cm/s) of four consecutive cardiac cycles in the blood flow velocity waveforms of each of the proximal ascending aorta, main, right and left pulmonary arteries were measured and averaged from the recorded images off-line (Figure 2-6). (Azpurua et al. 2010).

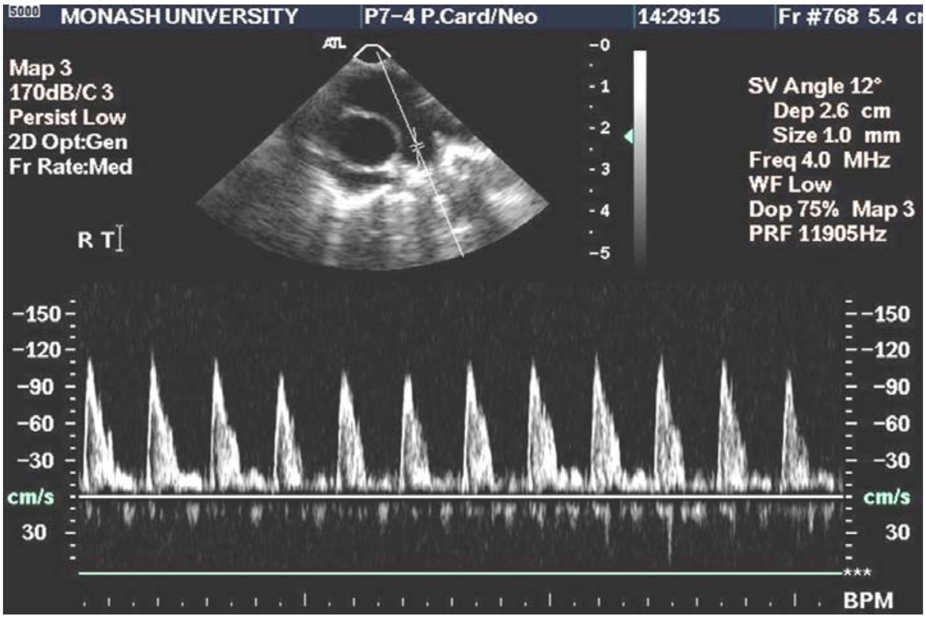

Figure 2-6: Duplex Doppler imaging of the right pulmonary artery of a sheep.

B-mode image at the top demonstrates the Doppler sample gate (double short lines along the line of sight) positioned centrally in the right pulmonary artery. The flow velocity waveform at the bottom demonstrates the blood flow velocity within the artery over time (12 heartbeats).

Ultrasound examination of the common carotid arteries.

The lambs were positioned on their back for ultrasound imaging as previously described, with their heads gently and securely turned approximately 45 degrees away from the side being examined (Vanoli et al. 2013). The cervical segment of the right common carotid artery was located using 2 dimensional B-mode imaging via a right antero-lateral neck approach (Kupinski 2013; Lima et al. 2002; Touboul et al. 2007). The common carotid artery was examined carefully using slightly different planes to identify its longest straight section in long axis. The maximum internal vessel diameter at a level at least 1 cm proximal to the carotid bifurcation was identified (Koskinen et al. 2009; Raitakari et al. 2003; Skilton et al. 2009). The common carotid artery was kept as perpendicular to the incident ultrasound beam as possible to optimise reflection and therefore maximise visualisation of the vessel wall structures. The image was then recorded for later off-line analysis (Touboul et al. 2012).

Spectral Doppler was used to obtain a blood flow velocity waveform of the right common carotid artery utilising the same view of the common carotid artery as in the B-mode image. A spectral Doppler sample gate of 1.0 to 1.5 mm utilising an insonation angle less than 60 degrees was placed centrally in the distal common carotid artery at least 1cm proximal to the bifurcation and a spectral trace of the blood flow containing at least three consecutive cardiac cycles was recorded for later off-line analysis (Figure 1-5).

Ultrasound imaging of the contralateral (left) common carotid artery was performed via a left antero-lateral neck approach using the equivalent protocol to the right common carotid artery to obtain the relevant measurements.

All common carotid artery measurements were completed off-line. Structural parameters (described below) of both common carotid arteries were measured three times from the recorded B-mode images (Figure 2-7). (Frauchiger et al. 2001; Koskinen et al. 2009; Touboul et al. 2012):

- Maximum external diameter (mm). The external diameter was defined as the distance between the adventitia-periadventitia interfaces of the near and far wall of the artery.
- Maximum internal diameter (mm). The internal diameter (lumen) was defined as the distance between the lumen-intima interfaces of the near and far wall of the artery.
- Maximum intima-media thickness, IMT, (mm) and adventitia thickness, AT, (mm) at the same point on the far wall of the common carotid artery. The IMT was defined as the distance between the lumen-intima interface and the media-adventitia interface (Roy et al.). The AT was defined as the distance between the leading edge of media-adventitia interface and the adventitia-periadventitia interface (Kazmierski et al. 2009).

The mean was calculated by averaging three measurements of each common carotid artery dimension. The average measurements were corrected for bodyweight (mm/kg).

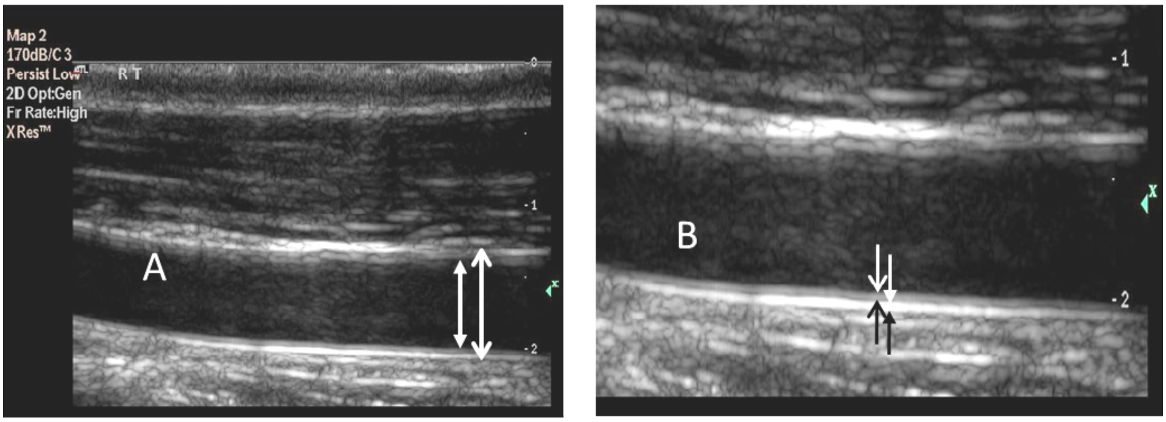

Figure 2-7: B-mode images of the right common carotid artery of a sheep.
Image A demonstrates the maximum external diameter (open arrowheads) and maximum internal diameter, or lumen (closed arrowheads). Image B demonstrates the maximum intima-media thickness (open arrowheads – visualised as a double line pattern of mid and low level bright stripes) and the maximum adventitia thickness (closed arrowheads – visualised as one highly bright stripe).

The maximum systolic, S, and end diastolic, D, blood flow velocity (cm/s) of three consecutive cardiac cycles in the recorded blood flow velocity waveform were measured and averaged for each common carotid artery. The resistive index, RI, which is a measure of the downstream resistance (i.e. intra-cerebral arterial impedence to blood flow) was calculated (Lima et al. 2002).

Ultrasound examination of the kidneys.

The sheep were generally positioned on their back for ultrasound imaging but some of the sheep were positioned slightly onto their left side when examining the right kidney and vice versa for the left kidney. The right kidney was located using 2 dimensional B-mode imaging via a right subcostal approach (Coombs 2004; Kadioglu 2010). The right kidney was examined carefully in the coronal plane from slightly different angles to identify the maximum bipolar length and the image was recorded. Longitudinal measurement of the maximum bipolar length (mm) and maximum renal width (mm), which was measured perpendicular to the bipolar length axis at the level of the hilum, were performed using the electronic callipers. To demonstrate the thickness of the right kidney, the imaging plane was rotated 90 degrees from the coronal plane into the transverse plane. The kidney was carefully scanned using slightly different planes to demonstrate its maximum thickness (mm) at the level of the hilum, and the thickness was then measured using the electronic callipers (Figure 2-8). Mean renal length (L) width (W) and thickness (T) values were calculated from three separate measurements of each dimension. The mean values were corrected for bodyweight (mm/kg) (Hricak & Lieto 1983; Schmidt et al. 2005). The volume (V) of each kidney was calculated using the equation for a prolate ellipsoid, V = L x W x T x 0.5233 (Kim, J-H et al. 2013; Weitz et al. 2013).

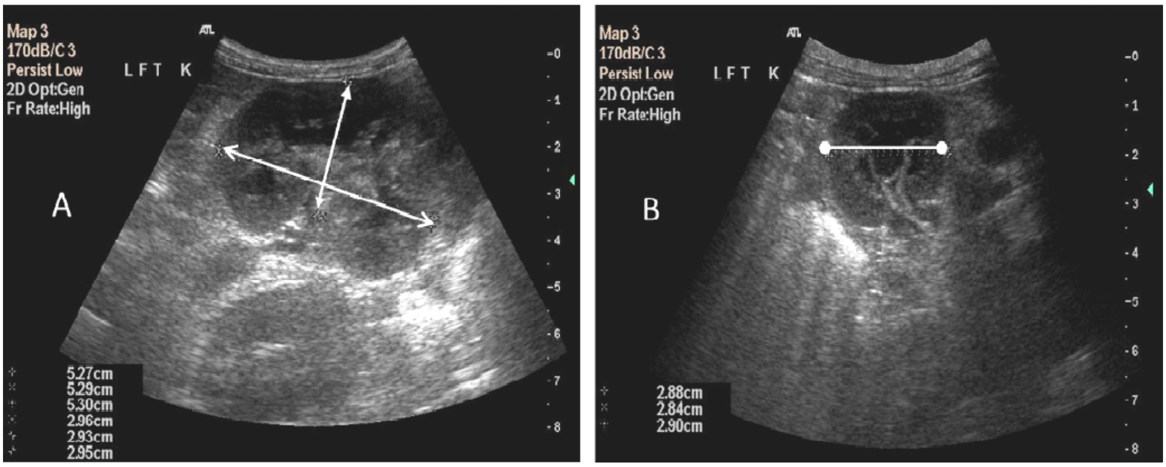

Figure 2-8: B-mode images of the left kidney of a sheep.
Image A demonstrates the maximum bipolar length (open arrowheads) and maximum width (closed arrowheads). Image B demonstrates the maximum kidney thickness (round arrowheads).

Following renal size measurement, the right renal artery was located using colour or power Doppler imaging via the same subcostal approach. Scanning was performed in different planes in order to visualise the longest straight section of the distal main renal artery and still maintain an insonation angle of less than 60 degrees for the spectral Doppler imaging to follow. Once the most successful view of the artery was obtained, a spectral Doppler sample gate of 1.0 to 2.5 mm with an insonation angle less than 60 degrees was placed centrally in the distal main renal artery as close as possible to the renal hilum and a spectral trace of the blood flow containing at least three consecutive cardiac cycles was recorded for later off-line analysis (Figure 2-9). The maximum systolic, S, and end diastolic, D, blood flow velocity (cm/s) of three consecutive cardiac cycles in the recorded blood flow velocity waveform of the right renal artery were measured and averaged from the recorded images off-line. The resistive index, RI, which is a measure of the downstream resistance (i.e. intra-renal arterial impedence to blood flow) was calculated using the formula: RI = (S-D)/S. (Allan et al. 2006; Rabbia & Valpreda 2003).

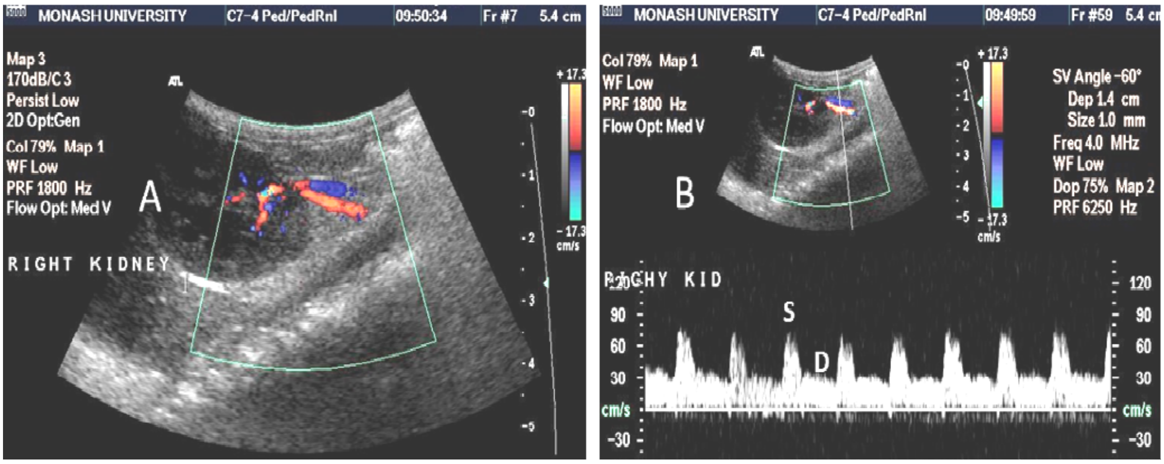

Figure 2-9: Colour and spectral Doppler images of the renal artery of a sheep. Image A is a B-mode image demonstrating the longest straight section of the distal right main renal artery (central red line). The renal vein is shown in blue above the artery. Image B shows a B-mode image at the top demonstrating the Doppler sample gate positioned centrally in the right main renal artery. The flow velocity waveform at the bottom demonstrates the blood flow velocity within the artery over time (8 heartbeats). The peak systolic velocity, S, and end diastolic velocity, D, for one of the cardiac cycles has been labelled.

Ultrasound imaging of the contralateral (left) kidney was performed via a left subcostal approach using the equivalent protocol to the right kidney to obtain the relevant measurements.

2.5. Statistical analysis.

Statistical analyses were performed using GraphPad Prism software (version 6.04; GraphPad software, San Diego, CA, USA). Data were analysed using a two-way analysis of variance (ANOVA), with gestational age at birth (preterm vs term) and sex (male vs female) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different. Data are presented as mean ± standard error of the mean (SEM), and statistical significance was accepted at $p < 0.05$.

All ultrasound examinations and measurements were carried out by a single operator (author) to avoid inter-observer errors (Huang et al. 2007; Riccabona et al. 2005; Schlesinger et al. 1987).

Chapter 3 Ultrasound evaluation of the effects of moderately preterm birth on the cardiovascular system and kidneys of anaesthetised two day old lambs.

3.1. Introduction.

Preterm birth is defined as delivery prior to 37 weeks gestation and accounted for 8.6% of all births in Australia in 2014 (AIHW 2016; Martin, Joyce et al. 2008). Approximately 75% of preterm births are classified as moderately preterm (32 to 36 weeks gestation) (Li, Z et al. 2013). Preterm birth is associated with increased morbidity and mortality not just in the neonatal period, but throughout life (Grobman 2012; Kerkhof et al. 2012; Kramer, Demissie & Yang 2000; Wang et al. 2004). Premature males are at increased risk compared to females - commonly referred to as the 'male disadvantage' (Ingemarsson 2003). Preterm birth is associated with the development of hypertension in adulthood which is a major risk factor for cardiovascular disease (Bayman, Drake & Piyasena 2014). However, the specific mechanisms remain unclear (Engle, Tomashek & Wallman 2007) and there is a need for non-invasive animal studies to determine what these mechanisms are (Pulver et al. 2009; Saigal & Doyle 2008). An ovine model of moderate preterm birth has been widely reported in the literature and is well established at Monash University where a number of researchers have investigated the effects of preterm birth on a wide range of organs and body systems including the heart, vascular system and kidneys (Barry & Anthony 2008; Bensley, J et al. 2012; Cock, ML et al. 2001; De Matteo et al. 2010; Jonker, Sonnet et al. 2007; Singh, R et al. 2012).

Ultrasound imaging is an ideal modality to conduct serial animal studies *in vivo* as it is reliable, portable, relatively cheap compared to other imaging modalities and involves no ionising radiation (Cowie 2011; Nelson, BP, Melnick & Li 2011; Yagel et al. 2009). However, to date there have been few reports of longitudinal studies utilising serial ultrasound in animal models tracking the development of disease from birth to adulthood. Ultrasound imaging could be used to evaluate changes in the cardiovascular system and kidneys due to preterm birth in a sheep model. However, ultrasound imaging techniques need to be adapted from those used in clinical practice to successfully evaluate sheep.

In this chapter, ultrasound imaging was conducted on a short term (2 day) cohort of anaesthetised moderately preterm lambs and term controls at two days after birth.

The aims of this chapter were:

1. To establish ultrasound imaging protocols for evaluation of the left ventricle, proximal ascending aorta, pulmonary, renal and common carotid arteries and the kidneys in anaesthetised moderately preterm and term lambs at two days of age.
2. To use ultrasound imaging to investigate the structure and function of the left ventricle in male and female moderately preterm and term lambs at two days of age.
3. To use ultrasound imaging to investigate the structure and blood flow within major arteries including the proximal ascending aorta, main, right and left pulmonary arteries and the common carotid arteries in male and female moderately preterm and term lambs at two days of age.
4. To use ultrasound imaging to investigate the structure of the kidneys and blood flow in the renal arteries of male and female moderately preterm and term lambs at two days of age.

This ultrasound investigation was conducted concurrently with another PhD project by Dr Vivian Nguyen (a student under the supervision of Professor Jane Black and Associate Professor Graeme Polglase) who performed a morphometric and physiological evaluation of the lambs that involved euthanasing the lambs after they had undergone ultrasound examination so that dissection and histological analysis of cardiovascular structures could be performed. This provided an opportunity to compare the ultrasound imaging findings with the morphometric investigation. The outcomes of Dr Nguyen's PhD research are supplementary to this project and the intention is to collaborate on all the findings when completed with a view to possible joint publication of the results.

3.2. Methods.

3.2.1. Animal studies.

A short term (2 day) lamb cohort was assembled by assigning time-mated ewes carrying singleton fetuses to deliver moderately premature (132 ± 1 days gestation, (~0.9 of term gestation); n = 21) or at full term (147 ± 1 days of gestation; n = 15). All ewes assigned to deliver preterm were administered a clinically relevant dose of betamethasone prior to delivery. The induction of delivery and antenatal betamethasone treatment has been described in detail in chapter 2 (section 2.3).

All preterm lambs were delivered vaginally and kept with their mother indoors in individual pens at the Monash Medical Centre Animal Facility in Clayton. Ewes were fed lucerne hay twice a day and water *ad libitum*. After birth, the preterm lambs were kept warm with heat lamps and hot water bottles and placed in an open plastic box lined with straw to reduce the likelihood of them being trampled.

The preterm lambs were bottle-fed colostrum from the mother once they were stable (normally within two hours of being born). The preterm lambs were fed at four to six hourly intervals (80 ml/kg/day) until they were able to stand unsupported and feed from the mother unassisted (usually within 24 hours after birth).

Two days after birth the lambs were anaesthetised with 1 ml/kg Sodium Thiopentone (50 mg/ml; Jurox, Rutherford, NSW, Australia) via intravenous injection in the jugular vein and general anaesthesia was maintained by inhalation of 2% Isoflurane (Isoflo; Abbott, Sydney, NSW, Australia). Ultrasound imaging was conducted on the heart, proximal ascending aorta, kidneys and the pulmonary, renal and common carotid arteries after the lambs were anaesthetised. A catheter was then inserted into the femoral artery of the lambs and the mean arterial pressure, oxygen saturation, temperature and heart rate were continuously recorded (Advisor® Vital Signs Monitor; SurgiVet®, Smiths Medical PM, Massachusetts, USA) for at least 20 mins. The lambs were euthanised after the data collection with 0.5 to 1.0 ml of undiluted Lethabarb (325 mg/ml; Pentobarbitone, Verbac Animal Health, NSW, Australia) via the femoral catheter. Other than the ultrasound imaging, the induction of labour, delivery, post-natal care, anaesthetisation, recording of the physiological data and euthanasia of the lambs were performed and/or supervised by Dr Vivian Nguyen as part of her PhD project.

3.2.2. Ultrasound imaging.

The ultrasound equipment and protocol for preparing the two day old lambs for scanning are described in chapter 2 (section 2.4). Specifically, a P12-5 MHz phased array cardiac probe with a 'pediatric cardiac/neonatal' application setting was used for scanning the heart, aorta and pulmonary arteries in this lamb cohort. A L12-5 MHz linear array small parts probe with a 'cardiovascular/carotid' application setting was used for scanning the carotid arteries. A C7-4 MHz curvilinear array abdominal probe with a 'pediatric/pediatric renal' application setting was used for scanning the kidneys and renal arteries (Table 2-1).

Ultrasound imaging was conducted on all lambs at two days of age. The lambs were under general anaesthesia throughout the ultrasound examination and therefore did not require any further immobilisation. The ultrasound evaluation of the heart, proximal ascending aorta, pulmonary and common carotid arteries, kidneys and renal arteries was performed as previously described in Chapter 2 (section 2.4). A summary of the measurements are as follows:

Heart

- Left ventricle anterior wall thickness (mm) in systole and diastole.
- Left ventricle posterior wall thickness (mm) in systole and diastole.
- Interventricular septum wall thickness (mm) in systole and diastole.
- Left ventricle internal short axis chamber diameter (mm) in systole and diastole.

The measurements of each of the structures were corrected for bodyweight (mm/kg). The percentage fractional shortening of the left ventricle was calculated from the acquired measurements (Singh, R et al. 2009).

Proximal ascending aorta, main, right and left pulmonary arteries

- The maximum internal diameter (mm) of the proximal ascending aorta, main, right and left pulmonary arteries were measured. The measurements were corrected for bodyweight (mm/kg).
- The peak systolic velocity (cm/s) of four consecutive cardiac cycles in each of the proximal ascending aorta, main, right and left pulmonary arteries were measured and averaged.

Common carotid arteries

- Maximum external and internal diameter (mm).
- Maximum intima-media thickness, IMT, (mm) and adventitia thickness, AT, (mm) were measured and corrected for bodyweight (mm/kg).
- The maximum systolic and end diastolic blood flow velocity (cm/s) of three consecutive cardiac cycles were measured and averaged for each common carotid artery and the resistive index, RI, was calculated.

Kidneys

- The maximum bipolar length (mm), width (mm) and thickness (mm) of each kidney were measured and corrected for bodyweight (mm/kg). Volume measurements were calculated based on the length, width and thickness measurements (mm^3).
- The maximum systolic and end diastolic blood flow velocity (cm/s) of three consecutive cardiac cycles were measured and averaged for each renal artery and the resistive index, RI, was calculated.

3.2.3. Statistical analysis.

Statistical analyses were performed using GraphPad Prism software (version 6.04; GraphPad software, San Diego, CA, USA). Data were analysed using a two-way analysis of variance (ANOVA), with gestational age at birth (preterm versus term) and sex (male versus female) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different. Data are presented as mean ± standard error of the mean (SEM). Comparison of survival rates was conducted using a Log-rank (Mantel-Cox) test. Statistical significance was accepted at $p < 0.05$.

3.3. Results.

3.3.1. Survival rates and ultrasound imaging.

Twenty one preterm lambs (10 male, 11 female) and 15 term lambs (5 male, 10 female) were delivered. The overall survival rate to two days of age was 13/21 (62%) for the preterm lambs comprising 6/10, (60%) male and 7/11 (64%) female lambs. All the term lambs survived to two days of age. One female preterm lamb and one female term lamb were not scanned due to scheduling issues. All the remaining lambs in the study underwent ultrasound imaging and were included in the analyses. There were no difficulties encountered in preparing the lambs for ultrasound examination or handling them during the imaging. Images of the heart, kidneys, major arteries and blood flow velocity data were successfully acquired using standard clinical equipment and the ultrasound scanning techniques for two day old lambs as previously described in chapter 2 (section 2.4). No gross congenital abnormalities or pathophysiology of the left ventricular structures, kidneys or major arteries were evident in the ultrasound images.

Lamb bodyweight

All lambs were weighed immediately prior to the ultrasound examination which was performed two days after birth. The average bodyweight of the preterm lambs was significantly lower than the average bodyweight of the lambs born at term (Figure 3-1).

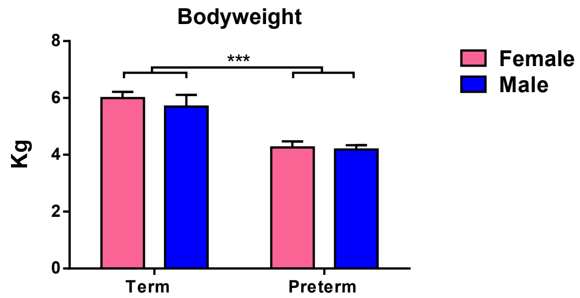

Figure 3-1: Mean bodyweight of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. ***p value ≤ 0.001 following Tukey's post-hoc analysis.

3.3.2. Ultrasound measurements.

Structural analysis of the left ventricle

There was no significant difference in the mean thickness of the anterior wall of the left ventricle measured in systole and diastole in preterm lambs compared to controls. However relative to bodyweight, the mean thickness of the left ventricle anterior wall in both systole and diastole was significantly higher in the preterm lambs (Table 3-1, Figure 3-2).

Table 3-1: Mean left ventricular (LV) measurements of preterm and term lambs at two days of age.

Mean heart dimension	Absolute value (mm)					Relative to bodyweight (mm/kg)				
	Preterm		Term		P Value	Preterm		Term		P Value
	Females n = 6	Males n = 6	Females n = 9	Males n = 5		Females n = 6	Males n = 6	Females n = 9	Males n = 5	
LV anterior wall systolic thickness	9.1 ± 0.73	8.7 ± 0.79	8.3 ± 0.48	7.6 ± 0.47	NS	2.2 ± 0.16	2.1 ± 0.19	1.4 ± 0.07	1.4 ± 0.16	Pg < 0.0001
LV anterior wall diastolic thickness	6.1 ± 0.52	6.3 ± 0.56	5.2 ± 0.23	4.6 ± 0.33	NS	1.4 ± 0.06	1.5 ± 0.14	0.9 ± 0.03	0.8 ± 0.10	Pg < 0.0001
LV posterior wall systolic thickness	6.7 ± 0.39	7.3 ± 0.46	7.9 ± 0.30	7.4 ± 0.40	NS	1.6 ± 0.17	1.8 ± 0.07	1.3 ± 0.07	1.3 ± 0.08	Pg = 0.0009
LV posterior wall diastolic thickness	4.7 ± 0.25	5.1 ± 0.27	5.6 ± 0.24	4.8 ± 0.17	NS	1.1 ± 0.12	1.2 ± 0.09	0.9 ± 0.05	0.9 ± 0.03	Pg = 0.0001
IVS systolic thickness	7.8 ± 0.77	8.2 ± 0.88	8.0 ± 0.44	9.0 ± 0.40	NS	1.9 ± 0.19	2.0 ± 0.18	1.3 ± 0.07	1.6 ± 0.07	Pg = 0.0039
IVS diastolic thickness	6.1 ± 0.55	6.3 ± 0.30	6.0 ± 0.41	6.9 ± 0.18	NS	1.4 ± 0.12	1.5 ± 0.08	1.0 ± 0.06	1.2 ± 0.08	Pg = 0.0003
LV systolic internal chamber diameter	11.8 ± 1.35	11.2 ± 1.28	13.6 ± 1.00	14.6 ± 0.92	Pg = 0.0411	2.8 ± 0.35	2.7 ± 0.35	2.3 ± 0.17	2.6 ± 0.07	NS
LV diastolic internal chamber diameter	18.5 ± 1.21	17.2 ± 0.80	21.0 ± 1.06	22.9 ± 0.73	Pg = 0.0011	4.4 ± 0.39	4.1 ± 0.20	3.5 ± 0.16	4.1 ± 0.19	NS

IVS = interventricular septum. Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. Tukey's post-hoc test was used to determine which data points of the factors were significantly different. NS = not significant ($p \geq 0.05$).

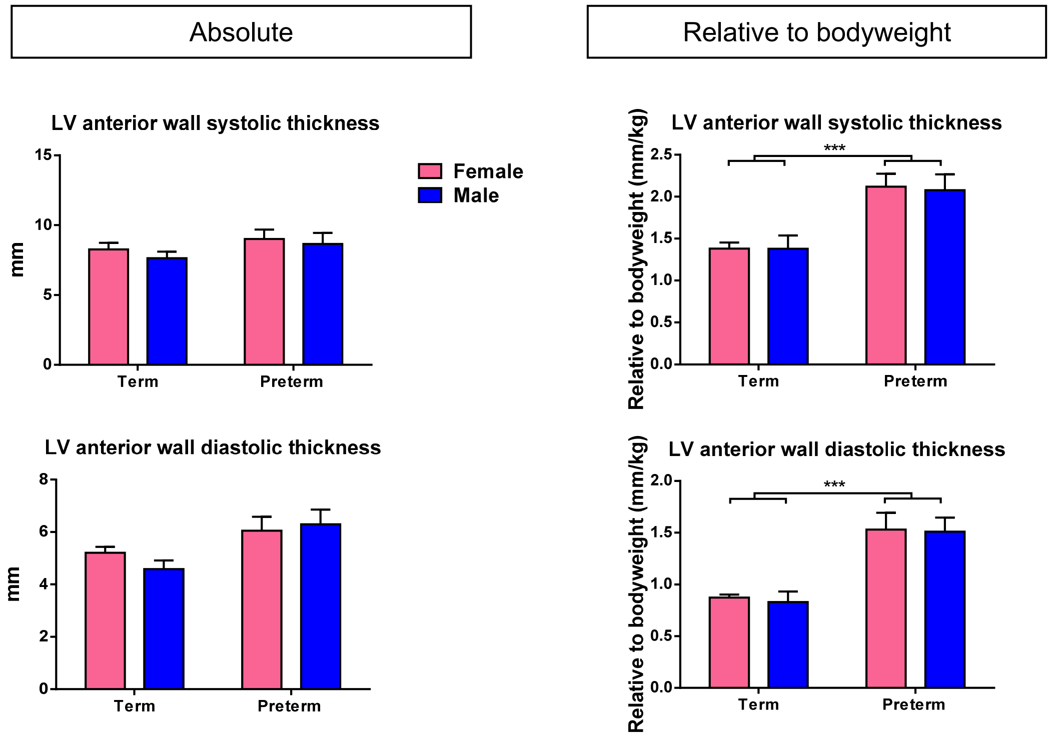

Figure 3-2: Mean anterior wall thickness (and relative to bodyweight) of the left ventricle (LV) of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. ***p value ≤ 0.001 following Tukey's post-hoc analysis.

No significant difference in the mean thickness of the posterior wall of the left ventricle measured in systole and diastole was found in preterm lambs compared to controls. Relative to bodyweight, the preterm lambs demonstrated a significantly higher mean thickness of the left ventricle posterior wall in both systole and diastole compared to lambs born at term (Table 3-1, Figure 3-3).

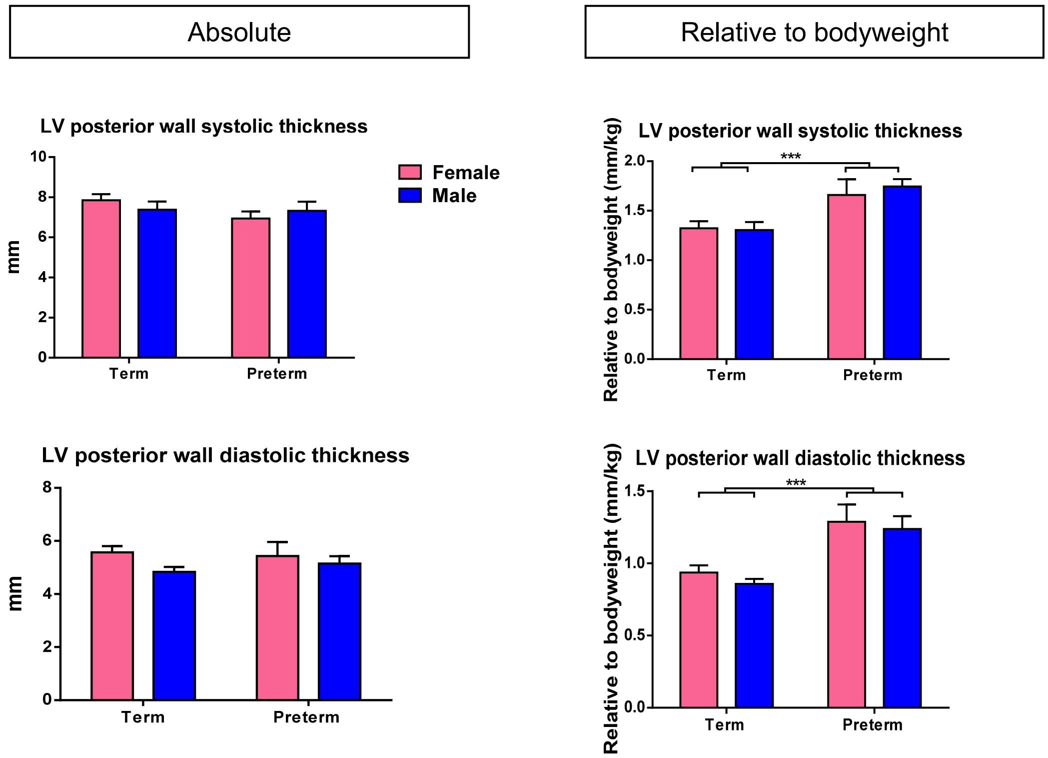

Figure 3-3: Mean posterior wall thickness (and relative to bodyweight) of the left ventricle (LV) of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. ***p value ≤ 0.001 following Tukey's post-hoc analysis.

There was no significant difference in the mean thickness of the interventricular septum measured in systole and diastole in preterm lambs compared to controls. Relative to bodyweight, the preterm lambs had a significantly higher mean thickness of the interventricular septum in both systole and diastole compared to lambs born at term (Table 3-1, Figure 3-4).

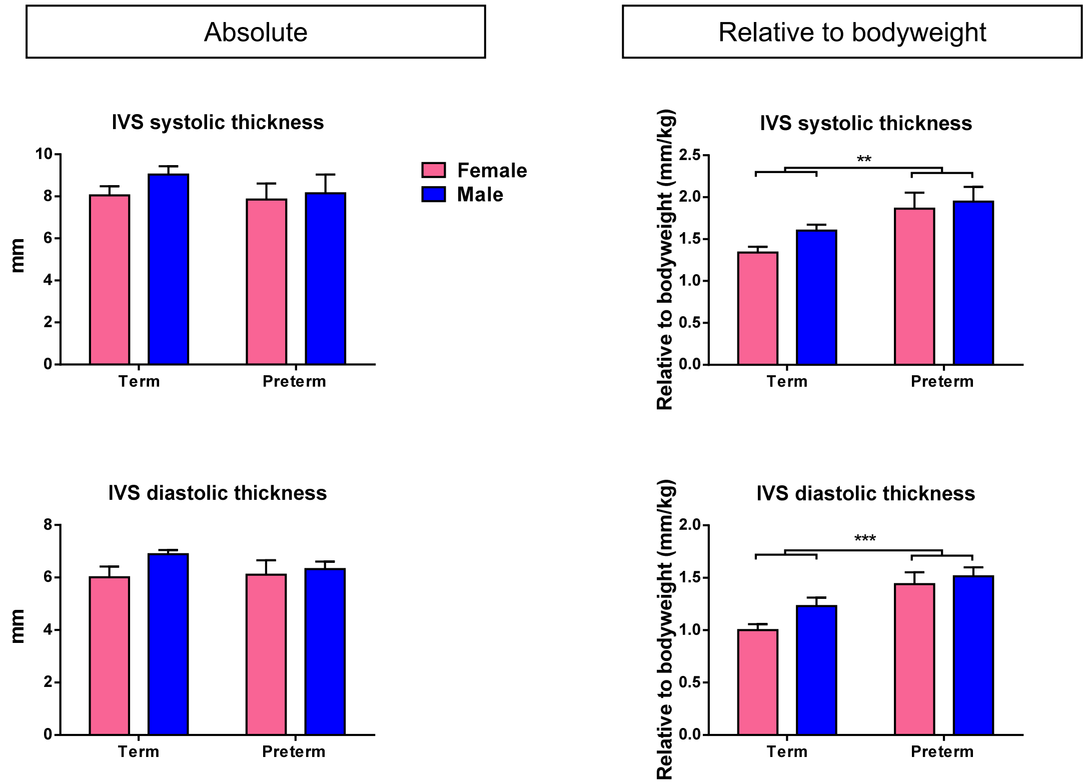

Figure 3-4: Mean thickness (and relative to bodyweight) of the interventricular septum (IVS) of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. $**p \leq 0.01$, $***p \leq 0.001$ following Tukey's post-hoc analysis.

The average left ventricle internal chamber diameter of preterm lambs in both systole and diastole was significantly lower than in lambs born at term. However, relative to bodyweight, there was no significant difference in the average internal chamber diameter of the left ventricle in systole and diastole in preterm lambs compared to controls. (Table 3-1, Figure 3-5).

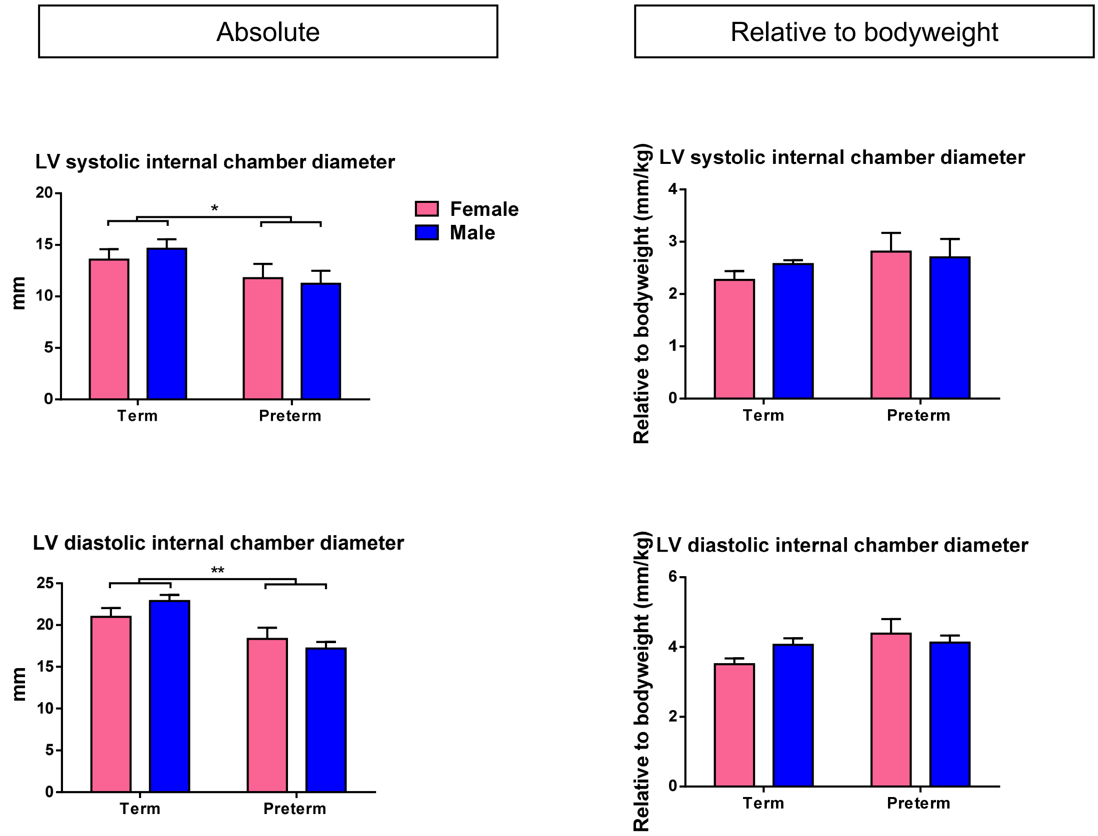

Figure 3-5: Mean left ventricle (LV) internal chamber diameter (and relative to bodyweight) of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. *$p < 0.05$, **$p \leq 0.01$ following Tukey's post-hoc analysis.

Functional analysis of the left ventricle

Percentage fractional shortening is a measure of the left ventricle wall contractility during systole and is an indicator of left ventricular function. Compared to controls, the average percentage fractional shortening of the left ventricle was not significantly different in preterm lambs at two days of age (Figure 3-6).

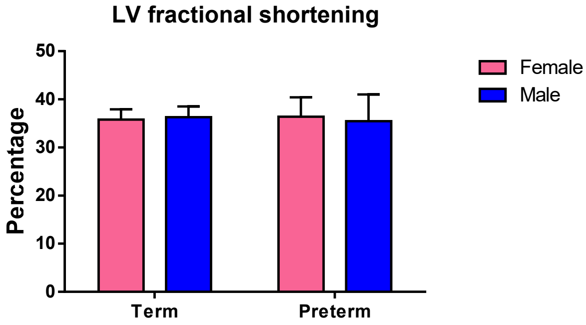

Figure 3-6: Average percentage fractional shortening of the left ventricle (LV) of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different.

There were no significant sex-specific differences in any of the parameters measured in the left ventricle in the preterm and term lambs.

Evaluation of the major arteries

Structural analysis of the proximal ascending aorta

The mean internal diameter of the proximal ascending aorta in preterm and term lambs at two days of age is shown in Table 3-2.

There was no significant difference in the mean internal diameter of the proximal ascending aorta in preterm lambs compared to controls. However, relative to bodyweight, the mean internal diameter of the proximal ascending aorta in the preterm lambs was greater than in the controls (Figure 3-7).

Table 3-2: Mean arterial measurements of preterm and term lambs at two days of age.

Mean vessel dimension	Absolute value (mm)					Relative to bodyweight (mm/kg)				
	Preterm		Term		P Value	Preterm		Term		P Value
	Females n = 6	Males n = 6	Females n = 9	Males n = 5		Females n = 6	Males n = 6	Females n = 9	Males n = 5	
Proximal ascending aorta internal diameter	10.4 ± 0.57	9.8 ± 0.36	10.8 ± 0.45	11.2 ± 0.45	NS	2.5 ± 0.19	2.3 ± 0.09	1.8 ± 0.08	2.0 ± 0.10	Pg = 0.0004
Main pulmonary artery internal diameter	7.8 ± 0.76	7.4 ± 0.23	8.1 ± 0.39	8.0 ± 0.59	NS	1.9 ± 0.17	1.8 ± 0.06	1.4 ± 0.07	1.4 ± 0.08	Pg = 0.0004
Left pulmonary artery internal diameter	4.2 ± 0.53	4.0 ± 0.53	4.2 ± 0.22	5.4 ± 0.55	NS	1.0 ± 0.13	0.9 ± 0.11	0.7 ± 0.03	1.0 ± 0.09	NS
Right pulmonary artery internal diameter	5.2 ± 0.34	5.2 ± 0.45	5.4 ± 0.28	6.2 ± 0.79	NS	1.2 ± 0.11	1.2 ± 0.12	0.9 ± 0.05	1.1 ± 0.08	Pg = 0.0124
Right CCA external diameter	3.0 ± 0.28	3.0 ± 0.16	3.5 ± 0.10	3.9 ± 0.23	Pg = 0.0017	0.7 ± 0.06	0.7 ± 0.05	0.6 ± 0.03	0.7 ± 0.02	Pg = 0.0395
Right CCA internal diameter	1.9 ± 0.24	1.7 ± 0.19	2.2 ± 0.11	2.6 ± 0.18	Pg = 0.0040	0.5 ± 0.04	0.4 ± 0.05	0.4 ± 0.03	0.5 ± 0.02	NS
Right CCA IMT	0.24 ± 0.03	0.29 ± 0.03	0.28 ± 0.02	0.29 ± 0.02	NS	0.06 ± 0.01	0.07 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	Pg = 0.0301
Right CCA AT	0.31 ± 0.01	0.33 ± 0.01	0.30 ± 0.01	0.33 ± 0.02	NS	0.08 ± 0.01	0.08 ± 0.01	0.05 ± 0.00	0.06 ± 0.00	Pg = 0.0003
Left CCA external diameter	2.9 ± 0.23	3.0 ± 0.24	3.2 ± 0.17	4.0 ± 0.25	Pg = 0.0110	0.7 ± 0.03	0.7 ± 0.06	0.5 ± 0.03	0.7 ± 0.03	Pg = 0.0447
Left CCA internal diameter	1.7 ± 0.18	1.8 ± 0.25	2.1 ± 0.11	2.7 ± 0.18	Pg = 0.0038	0.4 ± 0.02	0.4 ± 0.06	0.4 ± 0.02	0.5 ± 0.03	NS
Left CCA IMT	0.23 ± 0.02	0.29 ± 0.03	0.25 ± 0.02	0.27 ± 0.02	NS	0.06 ± 0.01	0.07 ± 0.01	0.04 ± 0.00	0.05 ± 0.01	Pg = 0.0075
Left CCA AT	0.28 ± 0.04	0.31 ± 0.02	0.30 ± 0.02	0.31 ± 0.03	NS	0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	Pg = 0.0094

CCA = common carotid artery, IMT = intima-media thickness, AT = adventitia thickness. Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different. NS = not significant ($p \geq 0.05$).

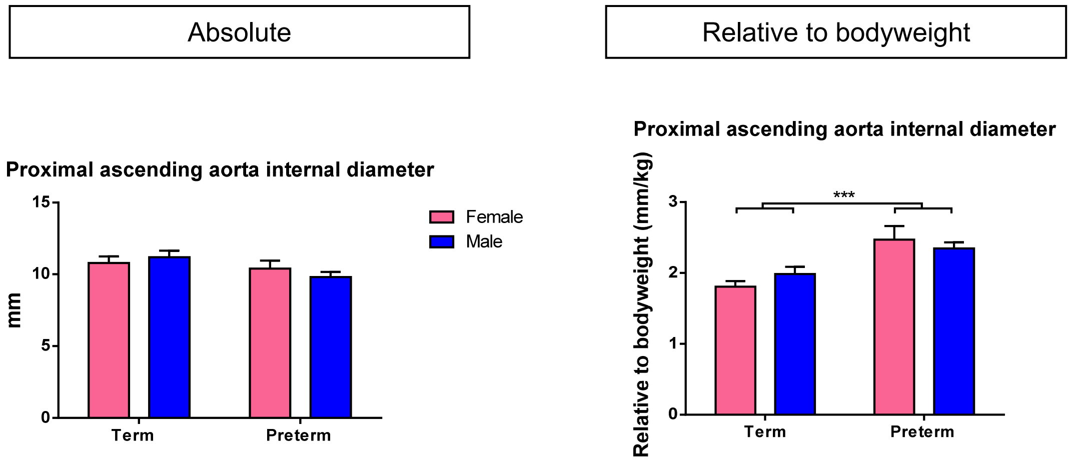

Figure 3-7: Mean internal diameter (and relative to bodyweight) of the proximal ascending aorta of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. ***p value ≤ 0.001 following Tukey's post-hoc analysis.

Blood flow analysis of the proximal ascending aorta

The average peak systolic blood flow velocity in the proximal ascending aorta of preterm and term lambs is shown in Table 3-3.

Table 3-3: Mean blood flow measurements in the major arteries of preterm and term lambs at two days of age.

Average peak systolic blood flow (cm/s)	Preterm		Term		P Value
	Females n = 6	Males n = 6	Females n = 9	Males n = 5	
Proximal ascending aorta	67.2 ± 10.5	73.2 ± 11.42	77.2 ± 6.53	100.4 ± 5.53	Pg = 0.0492
Main pulmonary artery	113.5 ± 7.47	93.5 ± 11.46	109.7 ± 10.06	113.9 ± 11.70	NS
Left pulmonary artery	121.2 ± 18.81	83.3 ± 10.71	114.5 ± 19.43	101.3 ± 15.45	NS
Right pulmonary artery	120.5 ± 20.31	73.6 ± 9.70	116.2 ± 10.27	123.8 ± 25.53	NS
Mean resistive indices					
Right renal artery	0.76 ± 0.06	0.84 ± 0.02	0.76 ± 0.03	0.75 ± 0.04	NS
Left renal artery	0.71 ± 0.05	0.84 ± 0.04	0.76 ± 0.04	0.79 ± 0.06	NS
Right common carotid artery	0.78 ± 0.08	0.81 ± 0.05	0.81 ± 0.02	0.80 ± 0.04	NS
Left common carotid artery	0.78 ± 0.07	0.75 ± 0.04	0.80 ± 0.02	0.80 ± 0.04	NS

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different. NS = not significant (p ≥ 0.05).

The average peak systolic blood flow velocity within the proximal ascending aorta was significantly lower (p = 0.0492) in preterm lambs compared to those born at term (Figure 3-8).

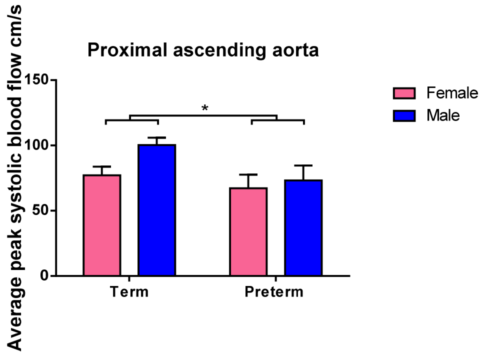

Figure 3-8: Average peak systolic blood flow velocity in the proximal ascending aorta of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. *p value < 0.05 following Tukey's post-hoc analysis.

Structural analysis of the main, right and left pulmonary arteries

The mean internal diameter of the main, right and left pulmonary arteries of preterm and term lambs at two days of age are shown in Table 3-2.

There were no significant differences in the mean internal diameters of the main and right pulmonary arteries in preterm lambs compared to controls. However, relative to bodyweight, the mean internal diameters of the main and right pulmonary arteries in the preterm lambs were greater than in the lambs born at term (Figure 3-9).

The mean internal diameter of the left pulmonary artery, including relative to bodyweight, was not significantly different in preterm lambs compared to controls (Figure 3-9).

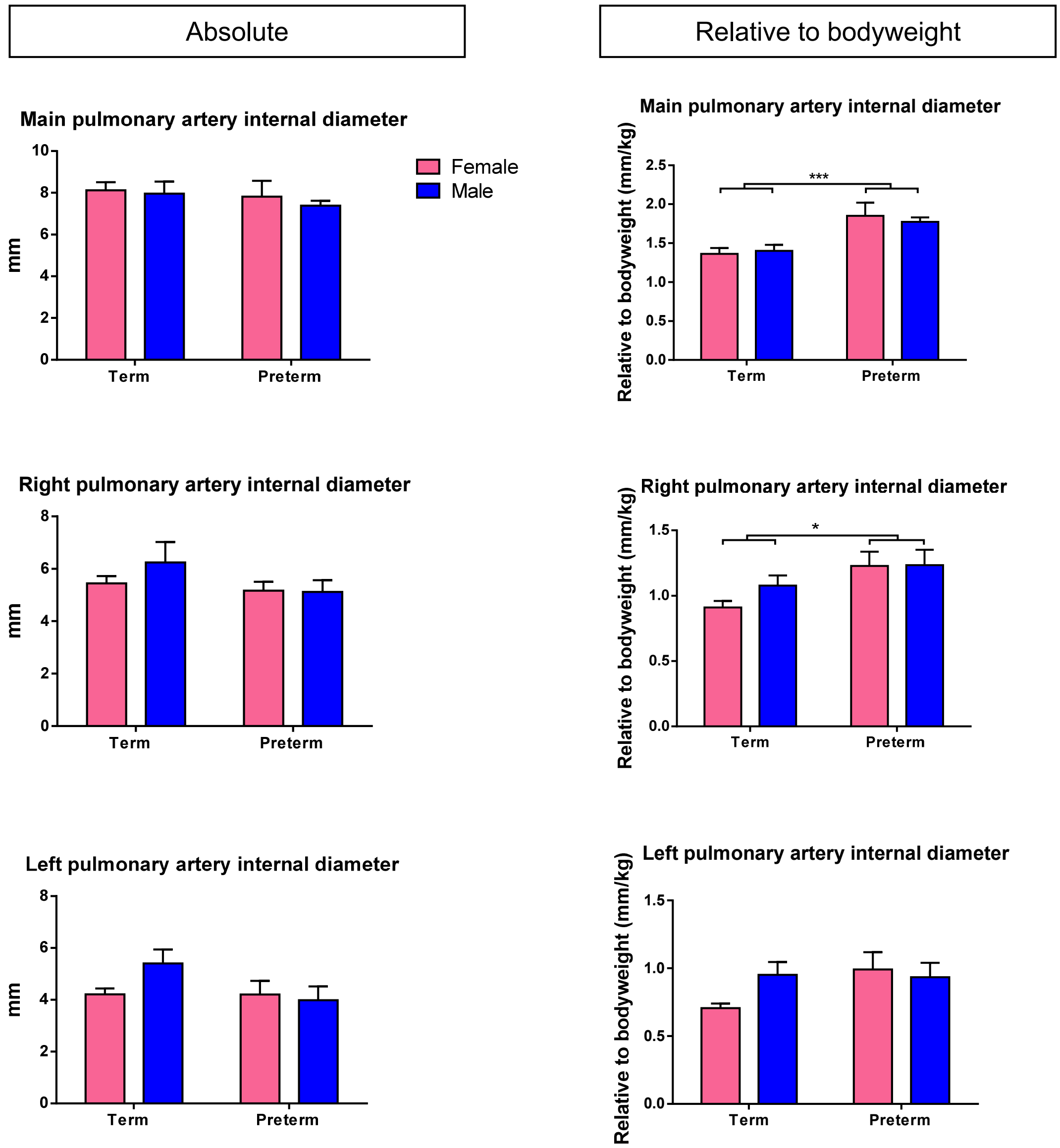

Figure 3-9: Mean internal diameter (and relative to bodyweight) of the main, right and left pulmonary arteries of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. *$p < 0.05$, ***p value ≤ 0.001 following Tukey's post-hoc analysis.

Blood flow analysis of the main, right and left pulmonary arteries

The average peak systolic blood flow velocity in the pulmonary arteries of preterm and term lambs is shown in Table 3-3. There were no significant differences between preterm and term lambs in the average peak blood flow velocity within the main, right and left pulmonary arteries.

There were no significant sex-specific differences in any of the parameters measured in the proximal ascending aorta or main, right and left pulmonary arteries.

Structural analysis of the common carotid arteries

A comparison of the mean internal and external vessel diameters, intima-media thickness (IMT) and adventitia thickness (AT) of both common carotid arteries are shown in Table 3-2.

The mean internal and external vessel diameters of both the right and left common carotid arteries were significantly lower in preterm lambs compared to lambs born at term.

Relative to bodyweight, the mean external diameter of both right and left common carotid arteries in the preterm lambs were greater than in the controls (Figures 3-10, 3-11). However, there were no significant difference between preterm and term lambs in the mean internal vessel diameter relative to bodyweight of the right and left common carotid arteries.

The mean IMT and AT of right and left common carotid arteries were not significantly different in preterm lambs compared to controls, however, relative to bodyweight the mean IMT and AT of the preterm lambs were greater than lambs born at term (Figures 3.10, 3-11).

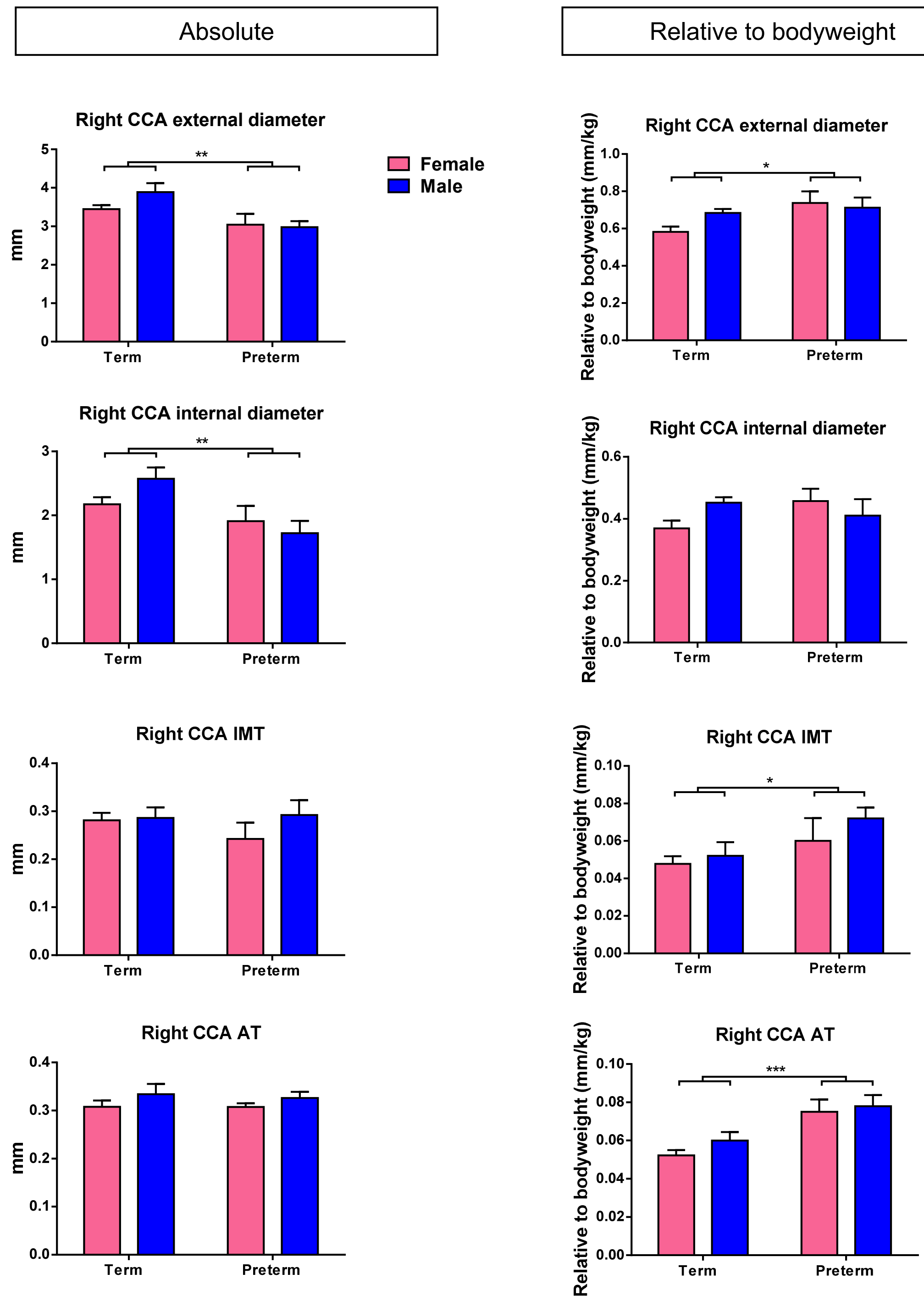

Figure 3-10: Right common carotid artery (CCA) diameter and wall thickness (and relative to bodyweight) of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 following Tukey's post-hoc analysis. IMT = intima-media thickness, AT = adventitia thickness.

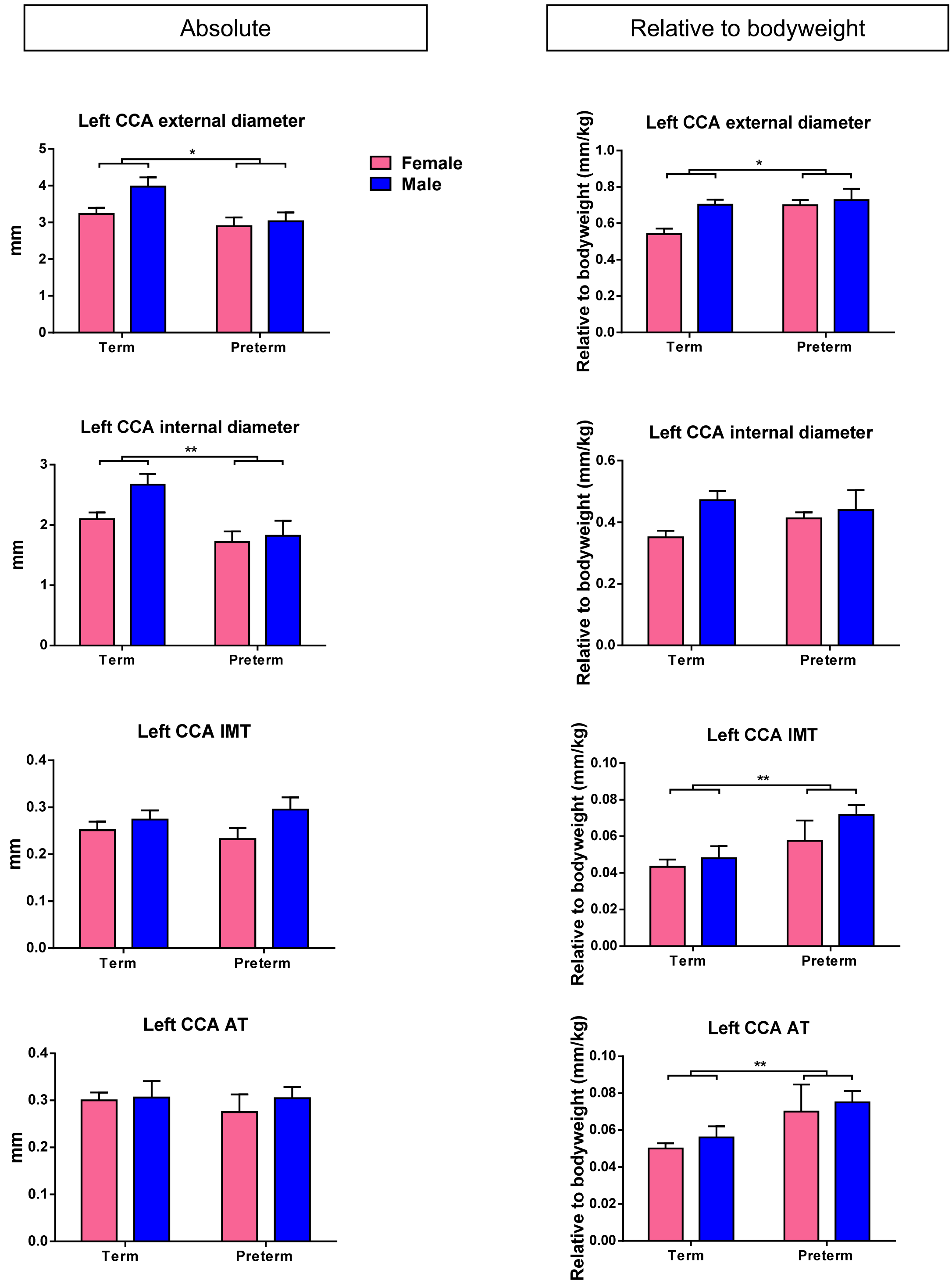

Figure 3-11: Left common carotid artery (CCA) diameter and wall thickness (and relative to bodyweight) of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. $^*p < 0.05$, $^{**}p \leq 0.01$ following Tukey's post-hoc analysis. IMT = intima-media thickness, AT = adventitia thickness.

Blood flow analysis of the common carotid arteries

There were no significant differences in the mean resistive indices of the right or left common carotid arteries in preterm lambs compared to controls. (Table 3-3).

There were no significant sex-specific differences in any of the parameters measured in the common carotid arteries.

Structural analysis of the kidneys

The average dimensions of the right and left kidneys in preterm and term lambs at two days of age are shown in Table 3-4.

The mean length and width of the right kidney and the mean length and thickness of the left kidney were significantly lower in preterm lambs than those born at term. However, relative to bodyweight, the mean length, width and thickness of the right and left kidneys were significantly greater in preterm lambs compared to controls (Figures 3-12, 3-13).

The mean volume of the right and left kidneys were significantly lower in preterm lambs, but relative to bodyweight there was no significant difference in the mean volume of either the right or left kidney compared to lambs born at term (Figure 3-14).

Table 3-4: Mean kidney measurements of preterm and term lambs at two days of age.

Mean kidney dimension	Absolute value (mm)					Relative to bodyweight (mm/kg)				
	Preterm		Term		P Value	Preterm		Term		P Value
	Females n = 6	Males n = 6	Females n = 9	Males n = 5		Females n = 6	Males n = 6	Females n = 9	Males n = 5	
Right kidney length	43.5 ± 1.50	44.7 ± 0.89	49.4 ± 1.04	46.4 ± 1.62	Pg = 0.0077	10.4 ± 0.50	10.7 ± 0.30	8.3 ± 0.32	8.2 ± 0.38	Pg < 0.0001
Right kidney width	18.0 ± 0.18	17.6 ± 0.61	21.2 ± 0.68	20.5 ± 0.88	Pg = 0.0003	4.3 ± 0.24	4.2 ± 0.15	3.6 ± 0.17	3.7 ± 0.28	Pg = 0.0055
Right kidney thickness	24.8 ± 0.53	25.1 ± 0.67	26.8 ± 0.48	25.5 ± 1.03	NS	5.9 ± 0.31	6.0 ± 0.26	4.5 ± 0.02	4.6 ± 0.28	Pg < 0.0001
Left kidney length	43.1 ± 2.03	44.2 ± 0.65	47.9 ± 0.71	47.1 ± 1.39	Pg = 0.0032	10.3 ± 0.48	10.6 ± 0.29	8.1 ± 0.24	8.4 ± 0.38	Pg < 0.0001
Left kidney width	17.4 ± 1.08	16.8 ± 0.92	20.0 ± 1.4	17.1 ± 1.04	NS	4.2 ± 0.31	4.0 ± 0.25	3.4 ± 0.23	3.0 ± 0.23	Pg = 0.0028
Left kidney thickness	23.1 ± 0.57	22.4 ± 0.80	25.8 ± 0.42	25.8 ± 1.08	Pg = 0.0004	5.5 ± 0.25	5.4 ± 0.30	4.3 ± 0.14	4.6 ± 0.21	Pg = 0.0002
Mean kidney volume	**Absolute value (cm3)**					**Relative to bodyweight (cm3/kg)**				
Right kidney	10.19 ± 0.62	10.37 ± 0.61	14.72 ± 0.68	12.77 ± 1.07	Pg = 0.0143	2.42 ± 0.11	2.48 ± 0.14	2.49 ± 0.16	2.25 ± 0.16	NS
Left kidney	9.20 ± 1.23	8.68 ± 0.55	13.05 ± 1.15	10.95 ± 1.20	Pg = 0.0002	2.17 ± 0.25	2.09 ± 0.14	2.18 ± 0.18	1.92 ± 0.14	NS

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different.
NS = not significant (p ≥ 0.05).

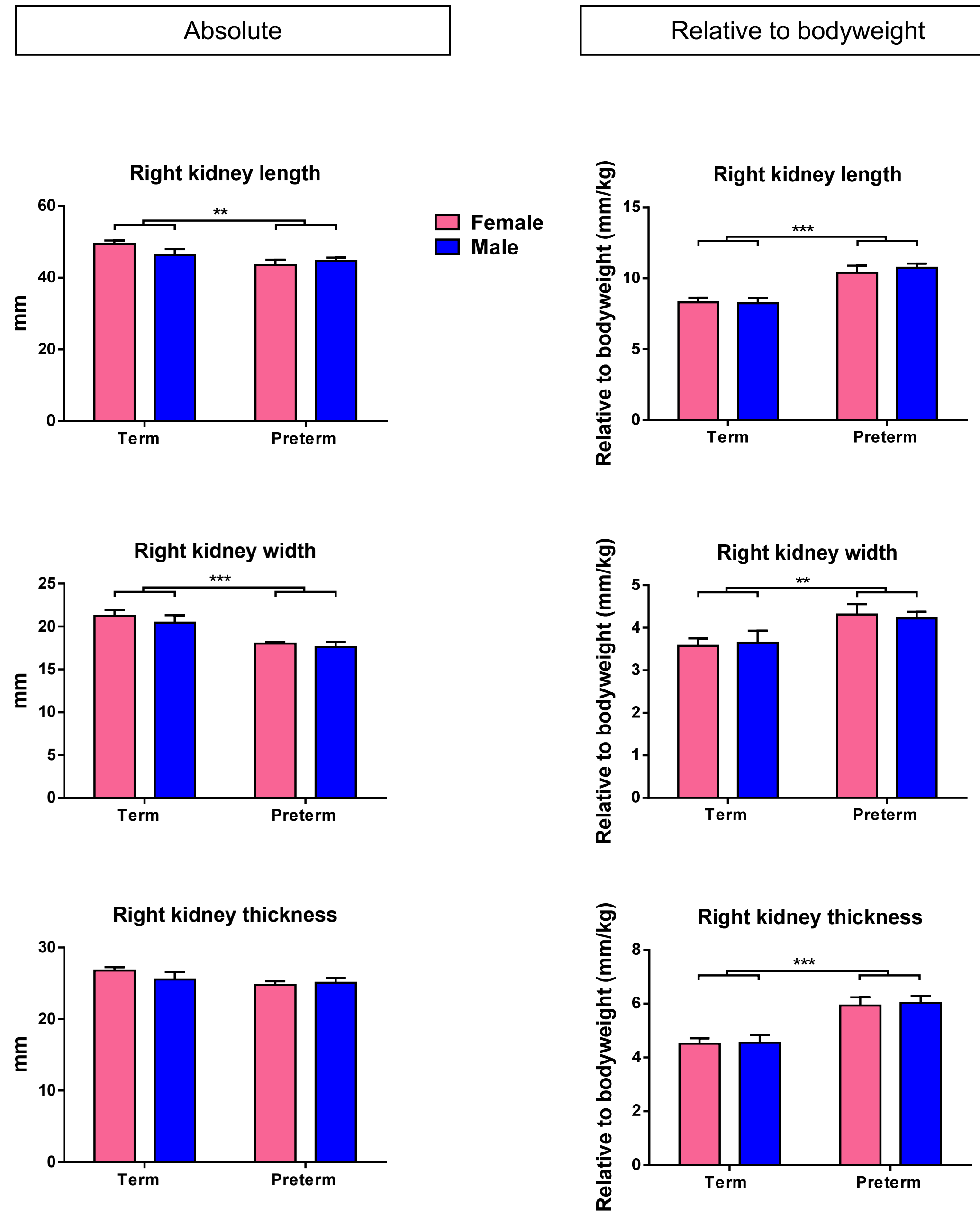

Figure 3-12: Mean right kidney dimensions (and relative to bodyweight) of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. $^{**}p \leq 0.01$, $^{***}p$ value ≤ 0.001 following Tukey's post-hoc analysis.

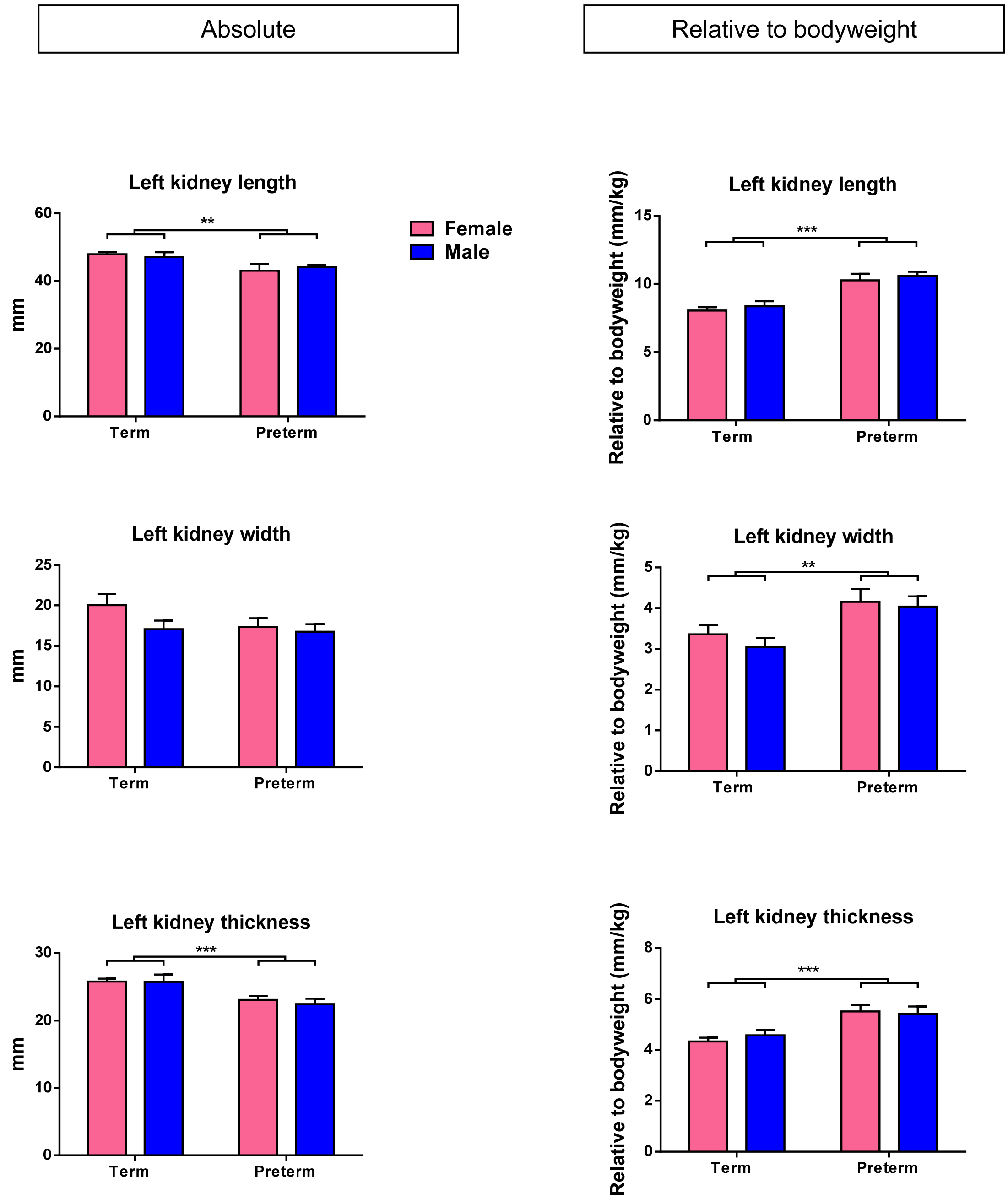

Figure 3-13: Mean left kidney dimensions (and relative to bodyweight) of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. **p ≤ 0.01, ***p value ≤ 0.001 following Tukey's post-hoc analysis.

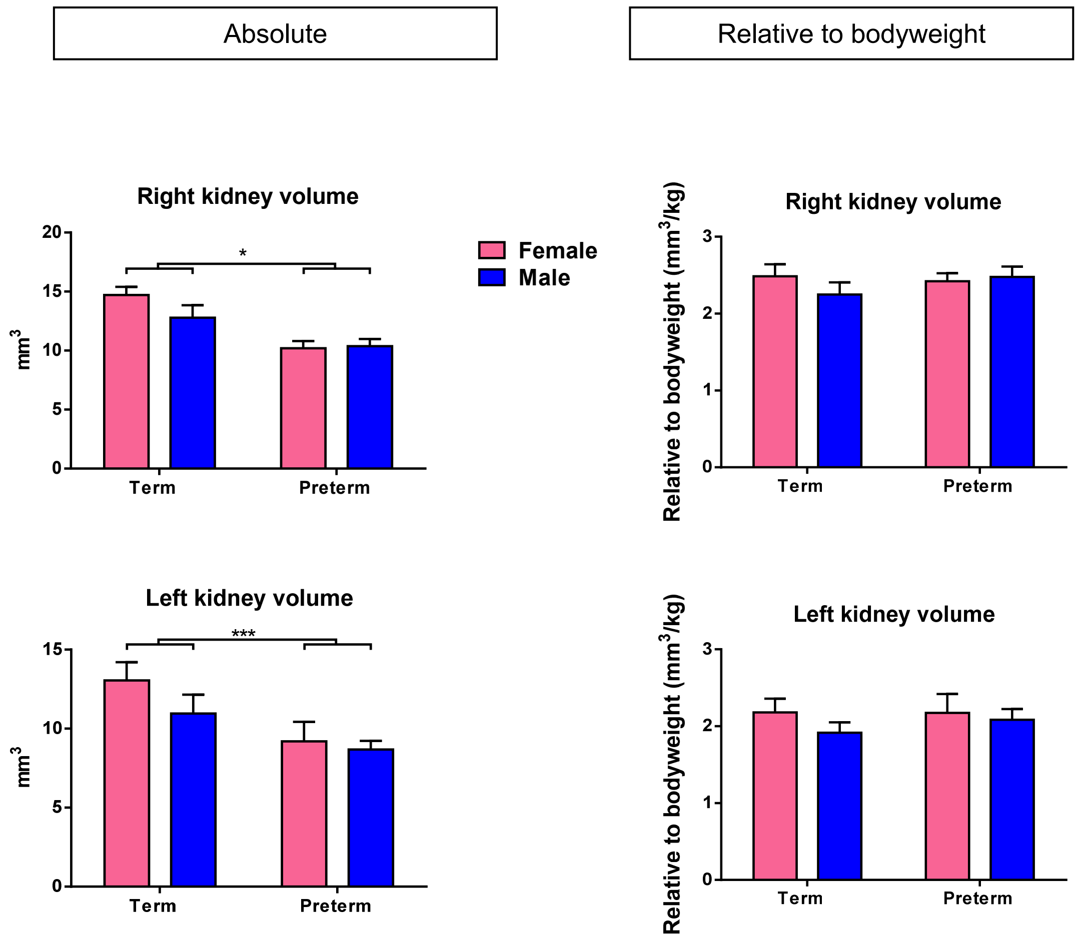

Figure 3-14: Mean right and left kidney volumes (and relative to bodyweight) of preterm and term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. $^{*}p < 0.05$, $^{***}p$ value ≤ 0.001 following Tukey's post-hoc analysis.

The size of the right kidney was compared to the left kidney in preterm lambs. There was no significant difference in mean bipolar length or mean kidney volume of the right kidney compared to the left (Figure 3-15).

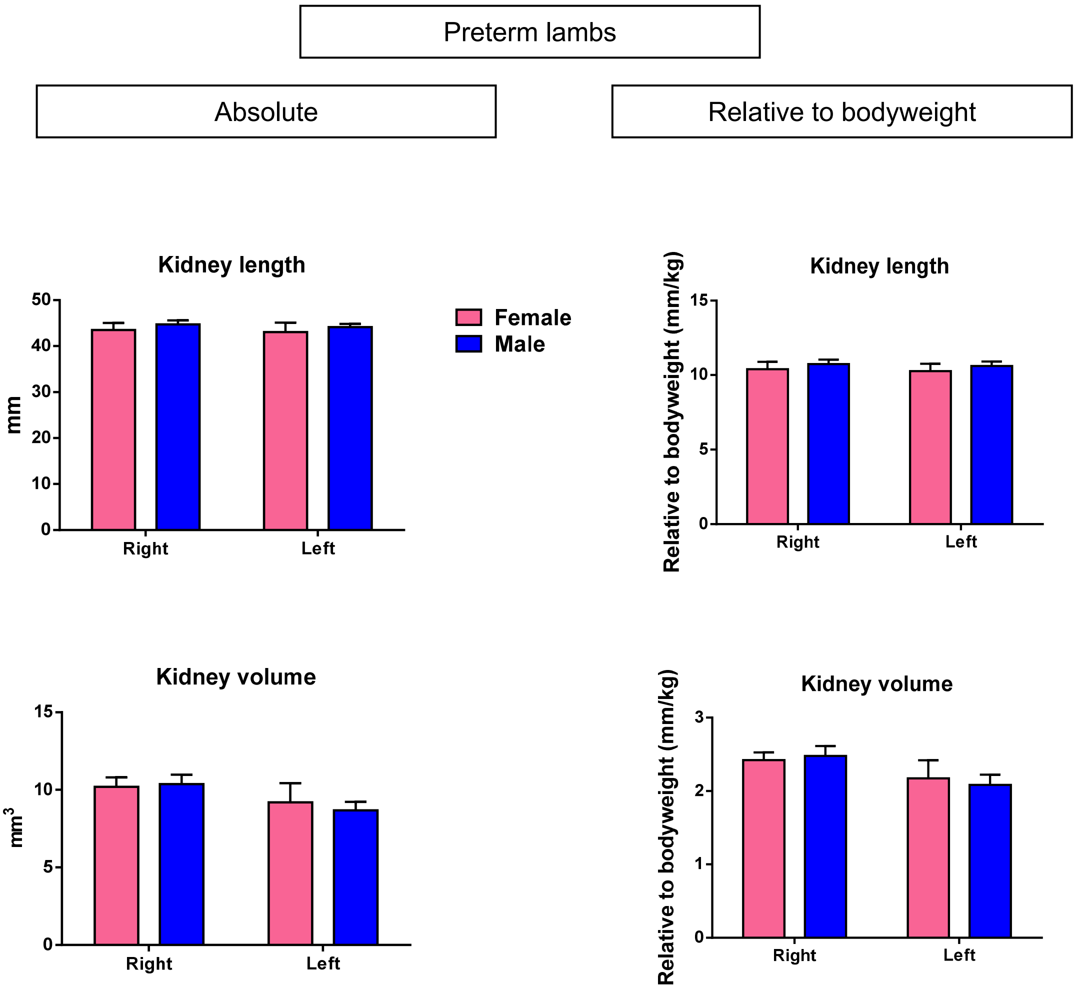

Figure 3-15: A comparison of the right and left kidney mean bipolar length and volume (and relative to bodyweight) of preterm lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with right versus left kidney (Pk) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different.

The size of the right kidney was compared to the left kidney in term lambs. There was no significant difference in mean bipolar length or mean kidney volume of the right kidney compared to the left (Figure 3-16).

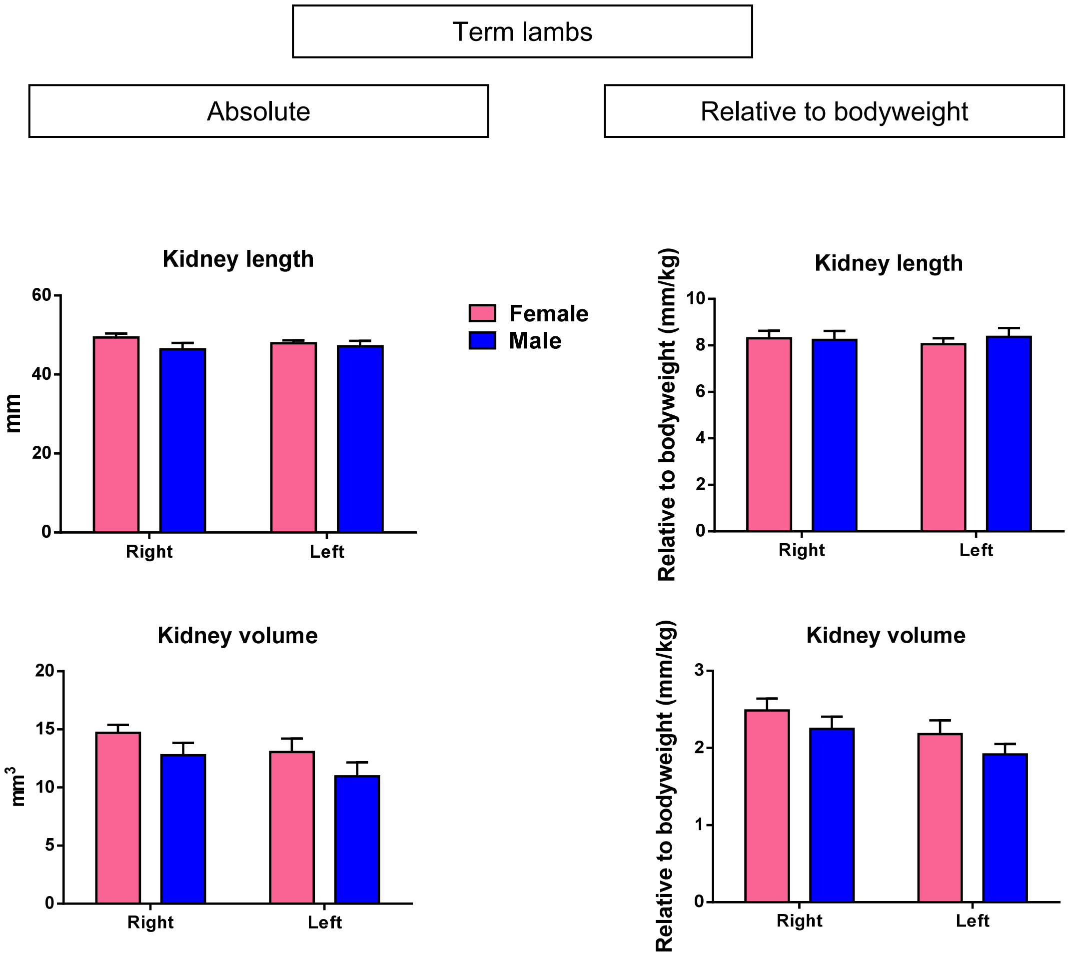

Figure 3-16: A comparison of the right and left kidney mean bipolar length and volume (and relative to bodyweight) of term lambs at two days of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with right versus left kidney (Pk) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different.

Blood flow analysis of the renal arteries

There was no significant difference in the mean resistive indices of the right or left renal arteries in preterm lambs compared to controls (Table 3-3).

There was no significant sex-specific difference in any of the parameters measured in the kidneys and renal arteries.

3.4. Discussion.

Ultrasound imaging technique for newborn lambs at two days of age

The structure and function of the LV, major arteries and both kidneys in newborn lambs was readily visualised using ultrasound scanning protocols adapted from those employed in clinical practice for human neonates. Conventional clinical ultrasound equipment was sufficient to obtain the required data and no specialised or animal specific apparatus was necessary other than a pair of clippers to trim the wool. There was no requirement to restrain the lambs during the ultrasound examination as they were under general anaesthesia. The only difficulty encountered in the ultrasound scanning technique was the inability to direct a trans-thoracic M-mode interrogation beam (i.e. line of sight) perpendicularly to the IVS wall due to the lambs having a keel shaped chest. This problem was overcome by using 2 dimensional B-mode imaging instead of M-mode imaging as previously described in section 2.4.

Effects of preterm birth on LV structure and function at two days of age

At two days after birth the average bodyweight of preterm lambs was significantly lower than the average bodyweight of lambs born at term. This was due to the preterm lambs missing out on the significant skeletal growth and abdominal fat deposition that occurs during the final two weeks of a term gestation (Poissonnet, LaVelle & Burdi 1988; Pope, Budge & Symonds 2014). Abdominal fat, particularly brown adipose tissue is required by sheep and infants to provide adequate thermoregulation and prevent hypothermia induced by the *ex utero* environment (Symonds et al. 2003). Abdominal fat stores can constitute up to two percent of total birth weight in term lambs and infants (Symonds & Lomax 1992). When comparing preterm to term lambs at two days of age some correction needed to be made to address the growth difference between the two groups. Therefore, we corrected size measurements for bodyweight to take into account the smaller growth of the preterm lambs (Cole et al. 1997; De Matteo et al. 2010; Singh, R et al. 2009).

An alternative to correcting for bodyweight is to correct for gestational age by comparing preterm lambs at term equivalent age (TEA). However, correcting for gestational age introduces potentially confounding variables. For example, the preterm lambs in this study could have been given an extra two weeks after birth to reach a gestational age 'equivalent' to term before being compared to term lambs. Although the preterm lambs would have increased in size after birth as they approached TEA, the drawback would be that the growth would not have taken place *in utero*. Compared to controls, the preterm lambs would have an extra two weeks to mature in, and adapt to the *ex utero* environment albeit with a structurally immature cardiovascular and renal system. Conversely, the term lambs would have structurally mature cardiovascular and renal systems at birth, but would still be adjusting to the haemodynamic transition associated with *ex utero* life when compared to preterm lambs at two days post-natal age. Nevertheless, correcting for gestational age by comparing preterm lambs at TEA to term lambs is widely accepted (Burrell et al. 2003; Mayhew et al. 1997; Sozo et al. 2006) and will be addressed in chapter 4.

Despite the difference in birth weights, the absolute thickness of the LV wall and IVS were not significantly different between preterm and term lambs. When corrected for bodyweight the thickness of the LV wall and IVS were significantly greater in the preterm lambs, but this was primarily due to the considerably lower bodyweight of preterm lambs rather than differences in the actual heart wall thickness. It is evident that the LV measured in lambs born at 132 days gestation had reached structural development comparable to that observed in lambs born at term. The percentage fractional shortening of the LV was similar in preterm lambs and controls with no significant difference in LV function demonstrated in the early post-natal period.

The potential effects of preterm birth on the heart structure at the cellular level, such as increased collagen deposition in the myocardium and altered cardiomyocyte maturation (Bensley, J et al. 2010), did not result in macroscopic changes to the LV wall thickness other than when adjusted for bodyweight. Further serial follow up of the lambs is necessary to determine if cardiac structural and functional changes in response to moderate preterm birth become evident after two days post-natal age and this will be addressed in chapter 4.

Effects of preterm birth on vessel structure and blood flow at two days of age

The absolute mean internal diameters of the ascending aorta, main, right and left pulmonary arteries were not significantly different between preterm and term lambs. This indicates that significant *in utero* growth in the size of these vessels was mostly complete at least two weeks prior to term. However, relative to bodyweight the diameters of the vessels (except for the left pulmonary artery) were significantly greater in the preterm lambs. This was mostly due to the considerably lower bodyweight of preterm lambs rather than differences in actual vessel size compared to term lambs. The internal diameter of the artery is an indicator of how much the vessel can distend in an *ex utero* blood circulation environment. Preterm birth is associated with altered vascular structural development and has been shown to reduce arterial wall elasticity and decrease vessel diameter in children (Ligi et al. 2010) and nine week old sheep (Bensley, J et al. 2012). However, reduced vessel diameter was not demonstrated in the proximal ascending aorta, main, right and left pulmonary arteries in our two day old moderately premature lambs using ultrasound imaging.

The absolute mean internal and external diameters of the right and left common carotid arteries were significantly lower in preterm lambs compared to controls. This indicates that significant *in utero* growth in the size of these vessels occurs towards the end of a normal term gestation. When corrected for bodyweight the external diameter of the vessels were significantly greater in the preterm lambs, although the internal diameters were not significantly different to controls. The absolute mean IMT and AT of the right and left common carotid arteries of preterm lambs were not significantly different to term lambs at two days of age. When corrected for bodyweight, the mean IMT and AT of the right and left common carotid arteries of preterm lambs were significantly greater than controls, again primarily due to the much lower bodyweight of preterm lambs rather than actual differences in vessel wall thickness. The IMT results are in agreement with studies in newborns (Schubert et al. 2013) and adolescents (Hovi et al. 2011) that found no significant difference in absolute common carotid artery IMT values between preterm and controls. However, there are inconsistencies in the literature and it is unclear why other studies have shown a significantly increased common carotid artery IMT in children (Lee et al. 2014) and young adults (Lazdam et al. 2010).

With the exception of the average peak systolic blood flow in the proximal ascending aorta, the blood flow in the main, right and left pulmonary arteries and both common carotid arteries of preterm lambs were not significantly different to term lambs at two days after birth. The peak systolic blood flow in the ascending proximal aorta was significantly higher in term lambs, but this result was marginal as the p-value just made the significance cut-off at 0.0492. Compared to controls, the mean resistive indices of the right and left common carotid arteries at two days of age were not significantly different indicating the resistance to blood flow through the brain via these vessels was not affected by regional microvascular changes associated with preterm birth.

Other than when corrected for bodyweight, no effects of moderately preterm birth on the arterial development, vessel wall structure and subsequent adaptation to the haemodynamic transition after birth were demonstrated except in the common carotid arteries. Serial evaluation of lambs is required to determine if changes to vascular structure and blood flow in response to moderate preterm birth can be demonstrated using ultrasound imaging after the initial post-natal period and into adulthood. This will be addressed in chapter 4.

Effects of preterm birth on the kidneys at two days of age

Renal growth disturbances and other kidney disorders are associated with changes to normal renal size (Kadioglu 2010). Renal length is the simplest and most clinically relevant measure of kidney size (Rosenbaum, Korngold & Teele 1984; Sargent & Wilson 1992). Renal volume is also used, but less frequently because it requires multiple measurements and has increased observer error compared to renal length measurement (Alexander 2005). However, renal volume has been shown to correlate better with renal function than renal length (Kent et al. 2010).

The absolute mean bipolar length and volume of the right and left kidneys were significantly lower in moderately preterm lambs compared to controls. It appears that the preterm lambs have to cope with the haemodynamic changes following birth with structurally immature kidneys and that may affect renal function. When corrected for bodyweight, the mean bipolar length of the right and left kidneys were significantly greater in the preterm lambs compared to controls, but this is entirely due to the much lower bodyweight of the preterm lambs. Kidney volumes corrected for bodyweight were not significantly different between preterm lambs and controls.

There was no significant difference in the growth of the right kidney compared to the left kidney in either the preterm lambs or controls. This is consistent with previous studies involving preterm infants which have demonstrated no significant difference in mean kidney bipolar length or volume between right and left kidneys and no sex- specific differences (Chiara et al. 1989; Schlesinger et al. 1987). However, other studies of low birth weight children have shown the mean bipolar length (Spencer, Wang & Hoy 2001) and volume (Schmidt et al. 2005) of the left kidney to be significantly larger than the right kidney, and larger kidneys in males compared to females. Studies of morphologically normal kidneys in children have also shown the left kidney to be significantly larger in mean bipolar length and volume compared to the right kidney, together with no sex-specific differences (Kadioglu 2010; Kim, J-H et al. 2013).

Compared to controls, the mean resistive indices of the right and left renal arteries were not significantly different, indicating that resistance to blood flow through the kidneys was not affected by renal microvascular changes associated with moderately preterm birth.

However, the immediate post-natal period may be too soon for adaptations of the renal vascular structures in response to moderately preterm birth to become evident as altered renal artery blood flow. Further serial follow up of the kidneys is required beyond two days of age to evaluate possible changes as a consequence of moderate preterm birth and this will be addressed in chapter 4.

Preterm birth and sex-specific differences

Males are at significantly increased risk of mortality and morbidity following preterm birth compared to females (Bennet et al. 2007). The sex-specific mechanisms underlying this observation remain uncertain (Berry, M et al. 2013). The male and female lambs were compared for all the parameters measured in this study and no significant sex-specific differences were found. The two day survival rate for the male preterm lambs was 6/10, (60%) and not significantly different to the survival rate of the female preterm lambs 7/11, (64%) ($p = 0.867$). By comparison, the survival rate to 14 days post-natal age for preterm lambs in a previous study using a similar moderately preterm birth model was significantly lower for males 18/41, (44%) than females 31/41, (76%) with 74% of the male lambs that died doing so within two days of birth (De Matteo et al. 2010). The clinically relevant dose of betamethasone (2 times 11.4 mg i.m.) administered to the ewes in our study may have increased male survival by progressing lung maturation more effectively compared to the non-clinical doses of 3 mg or 5.7 mg i.m. used by De Matteo. The benefits of glucocorticoid treatment for preterm delivery have been well documented but it has also been shown to adversely affect the development of various organs including the heart, blood vessels and kidneys depending on the type of glucocorticoid, dose and timing of delivery (Molnar et al. 2002; Moss et al. 2005). Although there was no evidence of male disadvantage in this study, further comparison of male versus female in a longer term study is required to evaluate possible sex-specific differences as a consequence of moderately preterm birth and this will be addressed in chapter 4.

Preterm birth and anaesthesia

Isoflurane is a commonly used anaesthetic agent in medicine and animal research (Martin, L et al. 2014). Isoflurane anaesthesia can reduce cardiac output, mean systemic arterial pressure and heart rate in newborn lambs. The cardiovascular system is still capable of maintaining adequate oxygen levels in response to hypoxemia during anaesthesia. (Brett et al. 1989; Sawyer 1973). General anaesthesia can alter regional vascular resistances in the coronary and renal vascular beds, however, regional blood flow may be only slightly affected (Vatner 1978). No significant difference in the resistance to blood flow through the renal vascular beds was demonstrated in the preterm lambs compared to controls. The effect of general anaesthesia on blood perfusion in other regional vascular beds such as the pulmonary vascular system has previously been shown to be insignificant in sheep (Walther, S et al. 1997). Our findings show no significant difference in the left ventricular function or blood flow velocity within the pulmonary arteries in the moderately preterm lambs compared to term controls. Our study compared anaesthetised preterm lambs to anaesthetised term controls. However, a comparison of anaesthetised preterm lambs to unanaesthetised preterm lambs may serve as a better evaluation of the effects of anaesthesia on left ventricular function and major arterial blood flow and this will be addressed in chapter 4.

3.5. Conclusion.

Ultrasound imaging of the left ventricle, proximal ascending aorta, kidneys, pulmonary, renal and common carotid arteries was effectively performed in newborn lambs. Moderately preterm lambs have structurally immature organs at birth that are ill prepared to cope in the *ex utero* environment. Responses to the immediate post-natal conditions including the haemodynamic transition after birth may lead to mal-adaptive changes that increase the risk of cardiovascular disease in later life. Other than when corrected for bodyweight, there were generally no significant differences demonstrated in the left ventricle, proximal ascending aorta, kidneys, pulmonary, renal and common carotid arteries between preterm lambs and controls using ultrasound imaging. There was no male disadvantage, sex-specific differences or effects of anaesthesia evident in the results. However, two days after birth may be too early for the effects of preterm birth to become evident using ultrasound imaging. Serial evaluation of lambs into adulthood is warranted to determine if mal-adaptations in response to moderately preterm birth become apparent after the immediate post-natal period and into later life and this will be addressed in chapter 4.

Chapter 4 A serial ultrasound evaluation of the effects of moderately preterm birth on the cardiovascular system and kidneys of lambs from birth to early adulthood (12 months of age).

4.1. Introduction.

In the previous chapter ultrasound imaging did not generally demonstrate significant differences in the structure of the heart, major blood vessels and kidneys of moderately preterm lambs at two days of age compared to controls. However, significant structural differences were apparent when adjusted for bodyweight, indicating that the changes in the preterm lambs were attributed to their smaller body size when compared to term lambs. Preterm lambs are exposed to haemodynamic changes at birth that includes an approximate 39% increase in mean arterial blood pressure (Louey et al. 2000). It is likely that the immature heart, major arteries and kidneys of moderately preterm sheep have to undergo adaptive remodelling in the post-natal period to accommodate the rapid increase in arterial pressure and functional demands following birth.

In previous studies at nine weeks post-term equivalent age, when examined microscopically, there was marked structural remodelling of the myocardium and the walls of the thoracic aorta and pulmonary artery of lambs that were born moderately preterm (Bensley, J et al. 2012; Bensley, J et al. 2010). In support of this idea there have been studies conducted in neonates using ultrasound imaging (Kozák-Bárány et al. 2001) and more recently in young adults using MRI (Lewandowski et al. 2013) that show an altered growth trajectory of the hearts in individuals that were born preterm.

Other studies of individuals born prematurely have shown markedly abnormal nephrons and renal function in the neonatal period (Huang et al. 2007; Sutherland et al. 2011) and reduced kidney size in early adulthood (Keijzer-Veen, M et al. 2010).

It is likely that cardiac, vascular and renal structural adaptations as a response to preterm birth, although beneficial in the short term, may predispose the lambs to hypertension and cardiovascular disease in later life (Bayman, Drake & Piyasena 2014; Grobman 2012). Two days after birth may be too early to detect adaptive structural or functional effects as a result of moderate preterm birth using ultrasound imaging. Hence, in this chapter it was hypothesised that overt structural and functional changes would be observed using ultrasound imaging of the cardiovascular system and kidneys of moderately preterm lambs as they grow to adulthood. A serial ultrasound evaluation was conducted from two days of age through to early adulthood to determine the longitudinal effects of moderate preterm birth on the structure and function of the heart, major arteries and kidneys.

The aims of this chapter were:

1. To establish ultrasound imaging protocols for evaluation of the left ventricle, proximal ascending aorta, pulmonary, renal and common carotid arteries and the kidneys in sheep from moderately preterm birth to adulthood.
2. To use ultrasound imaging to serially investigate the structure and function of the left ventricle in male and female sheep from two days to twelve months of age.
3. To use ultrasound imaging to serially investigate the structure and blood flow within major arteries including the proximal ascending aorta, main, right and left pulmonary arteries and the common carotid arteries in male and female sheep from two days to twelve months of age.
4. To use ultrasound imaging to serially investigate the structure of the kidneys and blood flow in the renal arteries of male and female sheep from two days to twelve months of age.

The ultrasound investigation in this chapter was conducted concurrently with another PhD project by Dr Vivian Nguyen who was under the supervision of Professor Jane Black and Associate Professor Graeme Polglase. Dr Nguyen performed a morphometric and physiological evaluation of the sheep including bodyweight, stature, heart rate and arterial pressure measurements from birth to twelve months of age (Nguyen, V et al. 2016). The outcomes of Dr Nguyen’s PhD research are supplementary to this project and the intention is to collaborate on the findings when completed with a view to have joint publication of the results.

4.2. Methods.

4.2.1. Animal studies.

Time-mated ewes carrying singleton foetuses were assigned to deliver moderately preterm (132 ± 1 days gestation, (~0.9 of term gestation); n = 28) or at full term (147 ± 1 days of gestation; n = 18) (Figure 2-1). All ewes assigned to deliver preterm were administered a clinically relevant dose of betamethasone prior to delivery. The induction of delivery and antenatal betamethasone treatment is described in detail in chapter 2 (section 2.3). The induction of labour, delivery and post-natal care of the lambs was performed and/or supervised by Dr Vivian Nguyen as part of her PhD project.

All preterm lambs were delivered vaginally and kept with their mother in individual pens in a large shed at the Monash University Animal Research Facility, Gippsland Field Station. Ewes were fed lucerne hay twice a day and water *ad libitum*. After birth, the preterm lambs were kept warm with heat lamps and hot water bottles and placed in an open plastic box lined with straw to reduce the likelihood of them being trampled (Figure 4-1). The preterm lambs were bottle-fed colostrum from the mother once they were stable (normally within two hours of being born). The preterm lambs were fed at four to six hourly intervals (80 ml/kg/day) until they were able to stand unsupported and feed from the mother unassisted (usually within 24 hours after birth).

Figure 4-1: Newborn preterm lamb kept with the mother in an individual pen for four to seven days after birth.

The preterm lambs remained with their mothers in individual pens for four to seven days, and then the warming devices were removed and from this point the lambs were self-sufficient enough to be reared by their mothers without any further assistance. The preterm lambs and mothers were then moved into a common pen in the shed for a further four weeks before being transferred to a grass paddock. Feed in the paddock was supplemented by lucerne hay and the animals had access to unlimited water at all times.

All term lambs were delivered vaginally, kept with their mother and reared unassisted in individual pens in the same shed as the preterm lambs. After a few days the term lambs and their mothers were moved into a common pen with the preterm lambs and their mothers for a further four weeks before being transferred to a grass paddock. Feed in the paddock was supplemented by lucerne hay and the animals had access to unlimited water at all times.

All lambs were weaned and separated from the ewes at 12 weeks post-natal age. Males were not castrated and after weaning were kept apart from the females in a separate paddock. All sheep remained at the Monash University Animal Research Facility, Gippsland Field Station until twelve months of age. The sheep were then transferred to the Monash Animal Services facility at Monash University in Clayton where they were housed for approximately six weeks before being euthanised. Histological analyses of the heart, aorta, pulmonary arteries and kidney tissues harvested at necropsy were then performed by Dr Vivian Nguyen as part of her PhD project.

Restraining protocols

Different restraining protocols for the lambs/sheep were required depending on the size of the animal at the time of ultrasound imaging. Lambs/sheep had to be immobilised in a way that was secure (animals under control at all times), safe (minimal risk of the animals, handlers or sonographer being injured) and feasible (inexpensive, utilising readily available equipment and producing the required data in minimal time). Non-sedated animals and in particular newborn lambs had to be handled securely, but gently, and managed in a way that minimised stress to the animals - such as reducing the ultrasound scanning time to a minimum and providing a quiet and calm environment throughout the examination.

Lambs up to two weeks of age were small, feeble and easily restrained by one person (handler) while the sonographer conducted the ultrasound examination. Lambs were simply positioned on their back or slightly onto one side depending on the area being investigated, and held gently and securely in position on a table next to the ultrasound machine (Figures 4-2, 4-3). Devices such as an overhead heat lamp and a hot water bottle or heat pad wrapped in a towel were placed under newborn preterm lambs to keep them warm during scanning.

Figure 4-2: Restrained two week old term lamb ready for scanning.
A two week old term lamb being gently but securely held in place.

Figure 4-3: Restrained two week old term lamb undergoing an ultrasound examination of the left kidney.
The probe is insonating the left kidney through the shaved patch of skin. Note the skin was shaved over the chest for insonation of the heart.

Three month old lambs were more active and therefore more difficult to handle than newborns. However, once newborn lambs (and adult sheep for that matter) were positioned on their backs or sides, they usually relaxed and no longer resisted being held. The equipment setup for the older lambs was essentially the same as for the two week old lambs, except a hammock type sling was used to provide a soft and comfortable platform for the heavier lambs to lie on (Figure 4-4). The lambs were positioned on their back or slightly onto one side in order to access the areas of interest (such as heart or kidneys) during the ultrasound scanning and held in position by two handlers - one holding the front legs and the other the hind legs. The lambs were still physically small and light enough to be easily lifted on and off the sling and held in position.

Figure 4-4: The hammock type sling platform used to provide a comfortable platform for lambs during ultrasound imaging.

Sheep were physically large and heavy at six months (approximately 35 kg) and twelve months of age (approximately 50 kg), so manual lifting of the sheep needed to be minimised to reduce the risk of injury to both handlers and sheep. A technique was developed that allowed for sheep to be easily manoeuvred into and out of position for ultrasound scanning, but did not involve manual lifting of unsupported sheep onto a raised platform. This was achieved by using a conveyor sheep handling device known as a ‘V-Express’ sheep handler (Arrow Farmquip, Tamworth, Australia), or known more simply as a ‘V-drive.’ (Figure 4-5)

Figure 4-5: V-drive sheep handler and set up for scanning sheep from six months of age.
Sheep enter the V-drive via a race and are transported via rubber belts to the end of the conveyor (foreground) where they were positioned and secured for examination next to the ultrasound machine.

Sheep were herded from a pen/holding area along a race onto the conveyor of the V-drive where they were picked up and held above the ground by a pair of long rubber belts. The weight of the sheep kept them securely restrained by the belts (Figure 4-6).

Figure 4-6: An adult sheep on a V-drive being held securely above the ground by rubber belts that can transport the sheep forward and backwards.
(*http://www.arrowfarmquip.com.au/products/v-express-sheep-handler-237.html)*

The rubber belts were then activated by a handler to transport the sheep along the conveyor and position them alongside the ultrasound machine and a second handler. The sheep were then easily rocked onto their back by the handlers holding the sheep under the neck/chest and gently flipping the sheep as the belts moved them along the conveyor. The sheep were then held in position for ultrasound examination by the two handlers - one holding the front legs and the other the hind legs. A small sack filled with straw was used to fill the gap between the rubber belts of the conveyor and support the sheep's neck and upper back during scanning (Figure 4-7).

Figure 4-7: A six month old lamb restrained by two handlers on a V-drive ready for scanning.

After the ultrasound examination was completed, the sheep were gently flipped back onto their belly, returning their legs to the initial suspended position between the rubber belts. The belts were then activated to move forward so the sheep could be transported off the drive and then herded along a race back to the pen/holding area. This technique did not require handlers to manually lift sheep on or off the conveyor. All manual handling of the sheep occurred after they were securely positioned and supported on the V-drive. In addition, the sheep were positioned on the V-drive at a height that allowed the sonographer to comfortably reach both the sheep and the ultrasound machine controls simultaneously without having to bend over or overextend. On average, the ultrasound examinations took approximately 35 to 45 minutes per animal to complete.

4.2.2. Ultrasound imaging.

As described in chapter 3, ultrasound imaging of the left ventricle, major arteries and kidneys can be readily performed in anaesthetised newborn lambs. Imaging protocols were created to perform serial ultrasound evaluations of sheep at two days, two weeks, three months, six months and twelve months of age. The schedule for the ultrasound examinations was chosen to correspond with developmental milestones from birth to adulthood. At two days after birth the lambs underwent ultrasound examination during the immediate post-natal period but without general anaesthesia as was used in chapter 3. The second ultrasound examination was performed two weeks after birth following the completion of the haemodynamic transition from a fetal to an *ex utero* circulation (Moore, Persaud & Torchia 2013; Sadler 2010). At two weeks after the birth, the preterm lambs born at 132 days gestational age had also reached term equivalent age (147 days from conception). The third ultrasound examination was scheduled to coincide with the lambs being weaned at three months of age. Lambs underwent the fourth ultrasound examination at six months of age after they had reached adolescence. The fifth ultrasound examination was performed at twelve months of age after the lambs had reached early adulthood.

The ultrasound equipment and protocol for preparing the sheep for scanning are described in chapter 2 (section 2.4). Imaging conscious sheep up to twelve months of age introduced challenges related to the safe handling of the animals and efficient data acquisition during ultrasound scanning. The equipment and restraining protocol required to perform the ultrasound examinations changed as the lambs grew larger over the course of the year. Lambs grow in size rapidly from two weeks to three months of age requiring lower frequency ultrasound to penetrate the chest and abdomen compared to newborn lambs. The equipment required for ultrasound imaging of the heart, kidneys, proximal ascending aorta, pulmonary, renal and common carotid arteries at two days after birth was the same as described in chapter three for the two day old lambs.

At two weeks of age a P12-5 MHz phased array cardiac probe with a ‘pediatric cardiac/neonatal’ application setting was used for scanning the heart, proximal ascending aorta and pulmonary arteries. A L12-5 MHz linear array small parts probe with a ‘cardiovascular/carotid’ application setting was used for scanning the common carotid arteries. A C7-4 MHz curvilinear array abdominal probe with a ‘pediatric/pediatric renal’ application setting was used for scanning the kidneys and renal arteries (Table 2-1).

At three to twelve months of age, P5-3 or P4-2 phased array cardiac probes with a ‘pediatric cardiac/general’ application setting were used for imaging the heart, proximal ascending aorta and pulmonary arteries. Curvilinear array abdominal probes (C7-4 with a ‘pediatric/pediatric renal’ application setting or C5-2 with an ‘abdomen/renal’ application setting) were used for imaging the kidneys and renal arteries. A L12-5 MHz linear array small parts probe with a 'cardiovascular/carotid' application setting was used for scanning the common carotid arteries (Table 2-1).

Ultrasound imaging was conducted on all animals in the long term cohort at two days, two weeks, three, six and twelve months after birth. At each time point, ultrasound evaluation of the heart, kidneys, proximal ascending aorta, pulmonary, renal and common carotid arteries were performed as described in Chapter 2 (section 2.4). A summary of the measurements are as follows:

Cardiac measurements:

- Left ventricle anterior wall thickness (mm) in systole and diastole.
- Left ventricle posterior wall thickness (mm) in systole and diastole.
- Interventricular septum wall thickness (mm) in systole and diastole.
- Left ventricle internal short axis chamber diameter (mm) in systole and diastole.

The measurements of each of the structures were corrected for bodyweight (mm/kg). The percentage fractional shortening of the left ventricle was calculated from the acquired ultrasound measurements as previously described in section 2.4 (Singh, R et al. 2009). The LV peak systolic wall stress was estimated from the acquired ultrasound measurements (and the blood pressure measurements obtained by Dr Nguyen as part of her PhD project) as previously described in section 2.4 (Moriarty 1980).

Measurements of the major arteries:

Proximal ascending aorta, main, right and left pulmonary artery

- The maximum internal diameter (mm) of the proximal ascending aorta, main, right and left pulmonary arteries were measured. The measurements were corrected for bodyweight (mm/kg).
- The peak systolic velocity (cm/s) of four consecutive cardiac cycles in each of the proximal ascending aorta, main, right and left pulmonary arteries were measured and averaged.

Common carotid arteries

- Maximum external and internal diameter (mm).
- Maximum intima-media thickness, IMT, (mm) and adventitia thickness, AT, (mm) were measured and corrected for bodyweight (mm/kg).

- The maximum systolic and end diastolic blood flow velocity (cm/s) of three consecutive cardiac cycles were measured and averaged for each common carotid artery. The resistive index, RI, was calculated as previously described in section 2.4 (Allan et al. 2006).

Renal measurements:

- The maximum bipolar length (mm), width (mm) and thickness (mm) of each kidney were measured and corrected for bodyweight (mm/kg). Volume measurements were calculated based on the length, width and thickness measurements (mm^3) as previously described in section 2.4 (Weitz et al. 2013).
- The maximum systolic and end diastolic blood flow velocity (cm/s) of three consecutive cardiac cycles were measured and averaged for each renal artery and the resistive index, RI, was calculated as previously described in section 2.4 (Rabbia & Valpreda 2003).

4.2.3. Statistical analysis.

Analyses were performed only on the data obtained from animals that survived to the end of the experimental period (12 months of age). This was done in order to minimise the potential effects of variables associated with ill health (for example, due to accidental trauma or medical conditions) on the final results.

Statistical analyses were performed using GraphPad Prism software (version 6.04; GraphPad software, San Diego, CA, USA). Comparison of survival rates was conducted using a Log-rank (Mantel-Cox) test. Data for each group according to sex was analysed separately using a two-way analysis of variance (ANOVA), with gestational age at birth (preterm versus term) and postnatal age (2 days, 2 weeks, 3, 6 and 12 months) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different.

A one-way analysis of variance (ANOVA) with a Tukey's multiple comparisons test was performed to compare data between preterm lambs at two days after birth, and preterm lambs at two weeks after birth (term equivalent age), as well as term lambs at two days after birth. Data are presented as mean ± standard error of the mean (SEM). Statistical significance was accepted at $p < 0.05$.

4.3. Results.

4.3.1. Survival rates.

Preterm cohort

Twenty eight preterm lambs (16 male, 12 female) were delivered. Five of the preterm lambs (3 male and 2 female) died shortly after delivery and before the first ultrasound examination that was scheduled at two days after birth. One male preterm lamb died two days after the first ultrasound examination. All these deaths were attributed to respiratory insufficiency. One female preterm lamb died 11 days after the initial ultrasound examination after being trampled in the holding pen. Survival rates for preterm lambs in the immediate post-natal period (to two weeks after birth when the second ultrasound examination was scheduled) was 21/28 (75%) overall, comprising 12/16 (75%) males and 9/12 (75%) females.

In the period between the second (two weeks after birth) and the third (three months after birth) scheduled ultrasound examinations, one female preterm lamb died (24 days after the second ultrasound scan) and three preterm males died (31, 38 and 63 days after the second ultrasound scan). The results of post-mortem pathology tests indicated that the four lambs were suffering from white muscle disease. Another female preterm lamb was euthanised 49 days following the fourth ultrasound examination (conducted at 6 months after birth) after it was discovered to have a broken leg. Survival rates for preterm lambs to the end of the study period were 16/28 (57.1%) overall, comprising 9/16 (56%) males and 7/12 (58%) females. There was no significant difference in the survival of male and female preterm lambs ($p = 0.903$).

Term cohort

Eighteen term lambs (11 male, 7 female) were delivered. One of the female term lambs had a complicated delivery (breech presentation) and died during birth. The survival rates for term lambs to the end of the study was 17/18 (94.4%) overall, comprising 11/11 (100%) males and 6/7 (85.7%) females. There was no significant difference in the survival of male and female term lambs ($p = 0.210$). Over the study period, significantly more term lambs survived than preterm lambs ($p = 0.009$).

As mentioned in the methods section 4.2.3, only the ultrasound examinations that were performed on lambs surviving to the end of the study (12 months of age) were included in the data analyses. This was done to reduce the potential confounding effects of variables such as respiratory insufficiency (1 male preterm lamb), accidental trauma (2 female preterm lambs) or white muscle disease (3 male and 1 female preterm lambs) on the results.

Sheep bodyweight

The animals were weighed immediately prior to each ultrasound examination. The average bodyweight of the female (Figure 4-8A) and male (Figure 4-8B) preterm lambs were significantly lower than the average bodyweight of the control lambs over the period from two days to twelve months post-natal age.

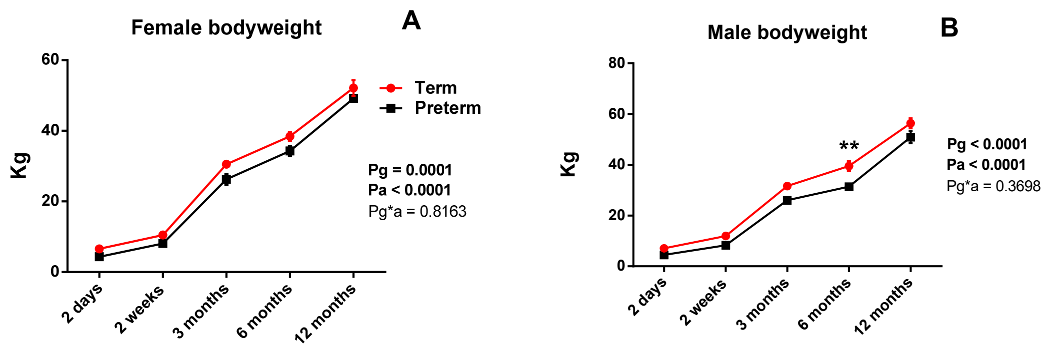

Figure 4-8: Bodyweights of female and male sheep at 2 days to 12 months of age
Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. **p ≤ 0.01 following Tukey's post-hoc analysis.

4.3.2. Ultrasound imaging.

There were no difficulties encountered in preparing the lambs for ultrasound examination or handling them during the ultrasound imaging at any of the time points. Images of the heart, kidneys, major arteries and blood flow velocity data were successfully acquired on most occasions (except for the left kidney) using conventional clinical ultrasound equipment coupled with ultrasound scanning protocols adapted from those employed in clinical practice for humans, together with the relevant immobilisation techniques for the lambs/sheep at each examination time point as previously described in section 4.2.2).

No gross congenital abnormalities or pathophysiology of the left ventricular structures, kidneys or major arteries were evident in any of the ultrasound images at any time points during the study.

4.3.3. Ultrasound measurements.

Structural analysis of the left ventricle from two days to twelve months of age.

Anterior wall of the left ventricle

The anterior and posterior walls of the left ventricle in systole and diastole were visualised during ultrasound examinations at all time points. It was not possible to direct a trans-thoracic M-mode interrogation beam (i.e. line of sight) perpendicularly to the IVS wall due to lambs/sheep having a keel shaped chest. This problem was overcome by using 2 dimensional B-mode imaging instead of M-mode imaging as previously described in section 2.4.

There was no significant difference in the mean systolic and diastolic thickness of the anterior wall of the left ventricle in female preterm lambs compared to female term lambs (Figures 4-9A, 4-9C). The mean systolic thickness of the anterior wall of the left ventricle in male preterm lambs was also not significantly different to controls (Figure 4-9E). However, the anterior wall in diastole was significantly thinner ($p = 0.0005$) in male preterm lambs compared to males born at term (Figure 4-9G).

Relative to bodyweight, the mean thickness of the left ventricle anterior wall was significantly greater in both female (systole $p < 0.0001$, diastole $p = 0.0001$) and male (systole $p < 0.0001$, diastole $p = 0.0220$) preterm lambs compared to controls. The larger relative thicknesses of the LV anterior wall compared to controls in the preterm lambs were evident until three months of age (Figures 4-9B, 4-9D, 4-9F, 4-9H).

Posterior wall of the left ventricle

There was no significant difference in the mean systolic and diastolic thickness of the posterior wall of the left ventricle in female preterm lambs compared to female term lambs (Figures 4-10A, 4-10C). The mean systolic thickness of the posterior wall of the left ventricle in male preterm lambs was also not significantly different to controls (Figure 4-10E). However, the posterior wall in diastole was significantly thinner ($p = 0.0001$) in male preterm lambs compared to males born at term (Figures 4-10G).

Relative to bodyweight, the mean thickness of the left ventricle posterior wall was significantly greater in both female (systole $p < 0.0001$, diastole $p < 0.0001$) and male (systole $p < 0.0001$, diastole $p < 0.0001$) preterm lambs compared to controls. The larger relative thicknesses of the LV posterior wall compared to controls were evident until two weeks after birth in preterm females and until three months after birth in preterm males (Figures 4-10B, 4-10D, 4-10F, 4-10H).

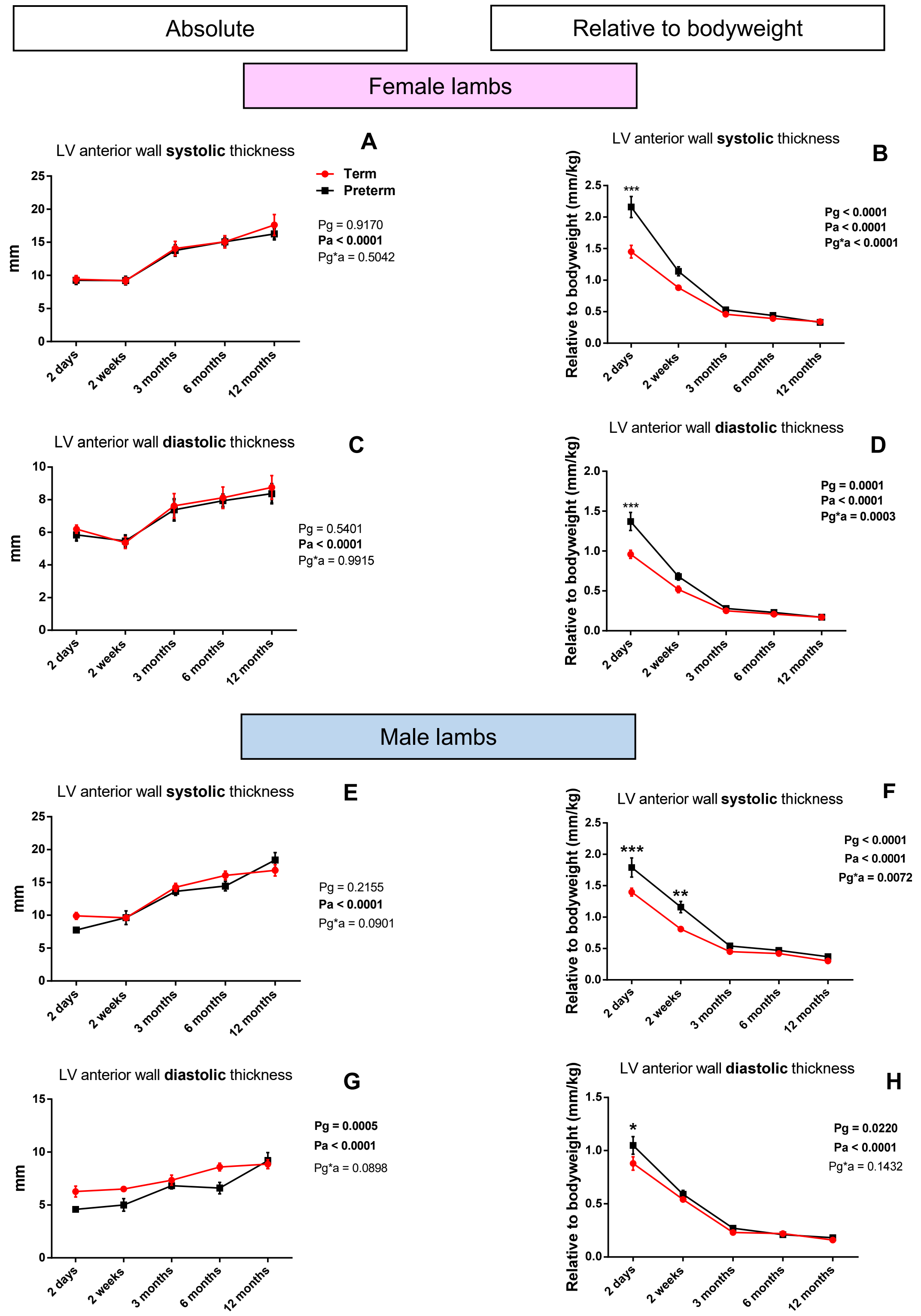

Figure 4-9: Mean anterior wall thickness (and relative to bodyweight) of the left ventricle (LV) of female and male lambs at 2 days to 12 months of age.
Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 following Tukey's post-hoc analysis.

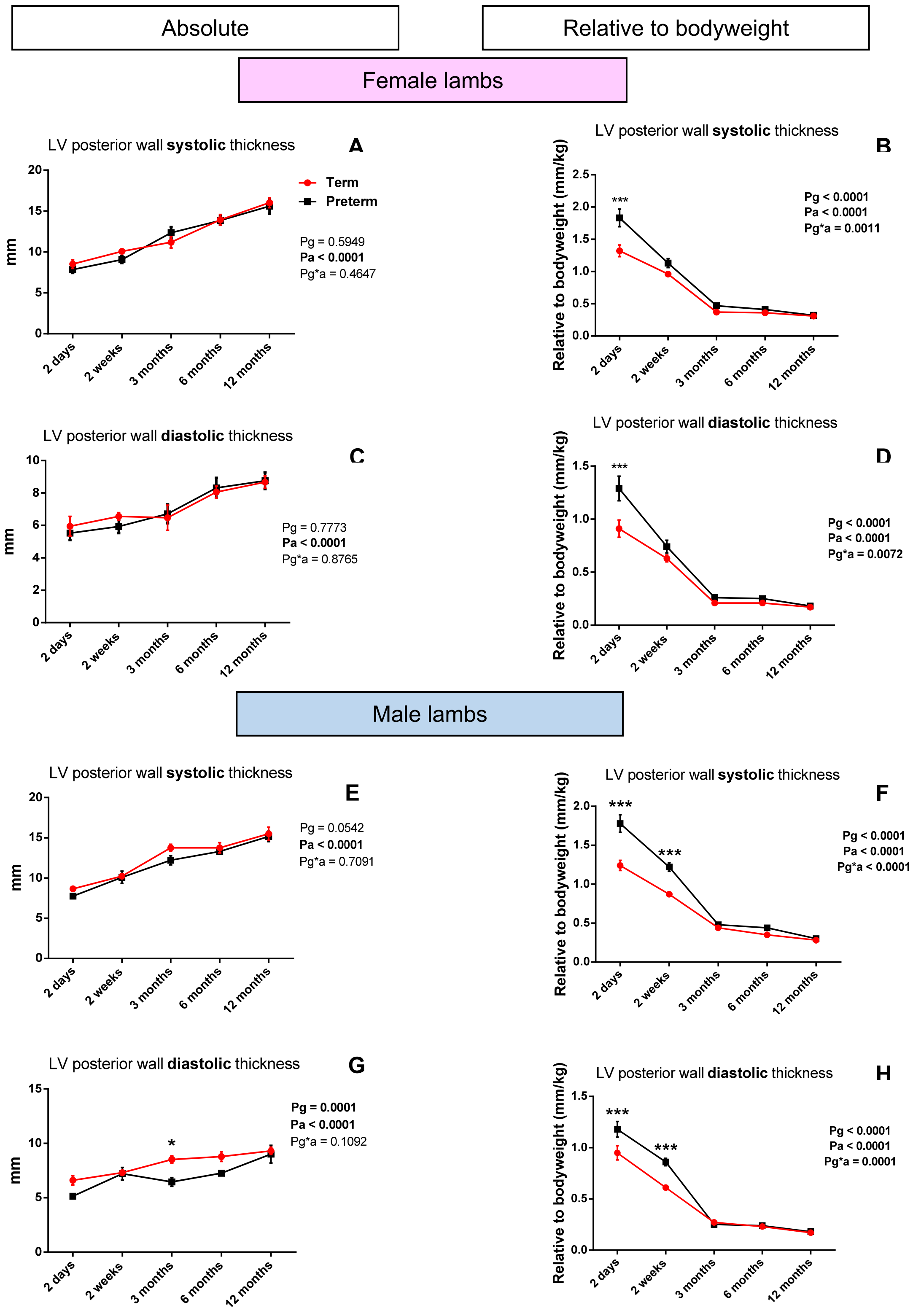

Figure 4-10: Mean posterior wall thickness (and relative to bodyweight) of the left ventricle (LV) of female and male lambs at 2 days to 12 months of age.
Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. *p < 0.05, ***p ≤ 0.001 following Tukey's post-hoc analysis.

Interventricular septum

Compared to controls, the mean systolic thickness of the IVS was not significantly different in the female preterm lambs (Figure 4-11A), however, the mean diastolic thickness was significantly lower ($p = 0.0078$) (Figure 4-11C). Male preterm lambs had a significantly thinner IVS compared to male term lambs in both systole ($p < 0.0001$) and diastole ($p < 0.0001$) (Figures 4-11E, 4-11G).

Relative to bodyweight, the IVS was significantly thicker in both female (systole $p = 0.0004$, diastole $p = 0.0009$) and male (systole $p < 0.0001$, diastole $p < 0.0001$) preterm lambs compared to controls. The larger relative thicknesses of the IVS compared to controls in the moderately preterm lambs were evident until three months after birth (Figures 4-11B, 4-11D, 4-11F, 4-11H).

Left ventricle internal chamber diameter

The mean systolic internal chamber diameter of the left ventricle in female preterm lambs was not significantly different to controls (Figure 4-12A). However, in diastole the internal chamber diameter was found to be significantly smaller ($p = 0.0219$) in the preterm females (Figure 4-12C). There was no significant difference in the mean internal chamber diameter of the left ventricle in male preterm lambs compared to male term lambs in systole or diastole (Figure 4-12E, 4-12G).

Relative to bodyweight, female preterm lambs had a significantly larger mean internal chamber diameter in diastole ($p = 0.0205$) (Figure 4-12D), but there was no significant difference in systole compared to controls (Figure 4-12B). The relative mean internal chamber diameter of the left ventricle was significantly larger in preterm males in both systole ($p = 0.0008$) and diastole ($p < 0.0001$) compared to controls (Figure 4-12F, 4-12H). The larger relative mean internal chamber diameters of the left ventricle compared to controls were evident until two weeks after birth in both preterm females and males.

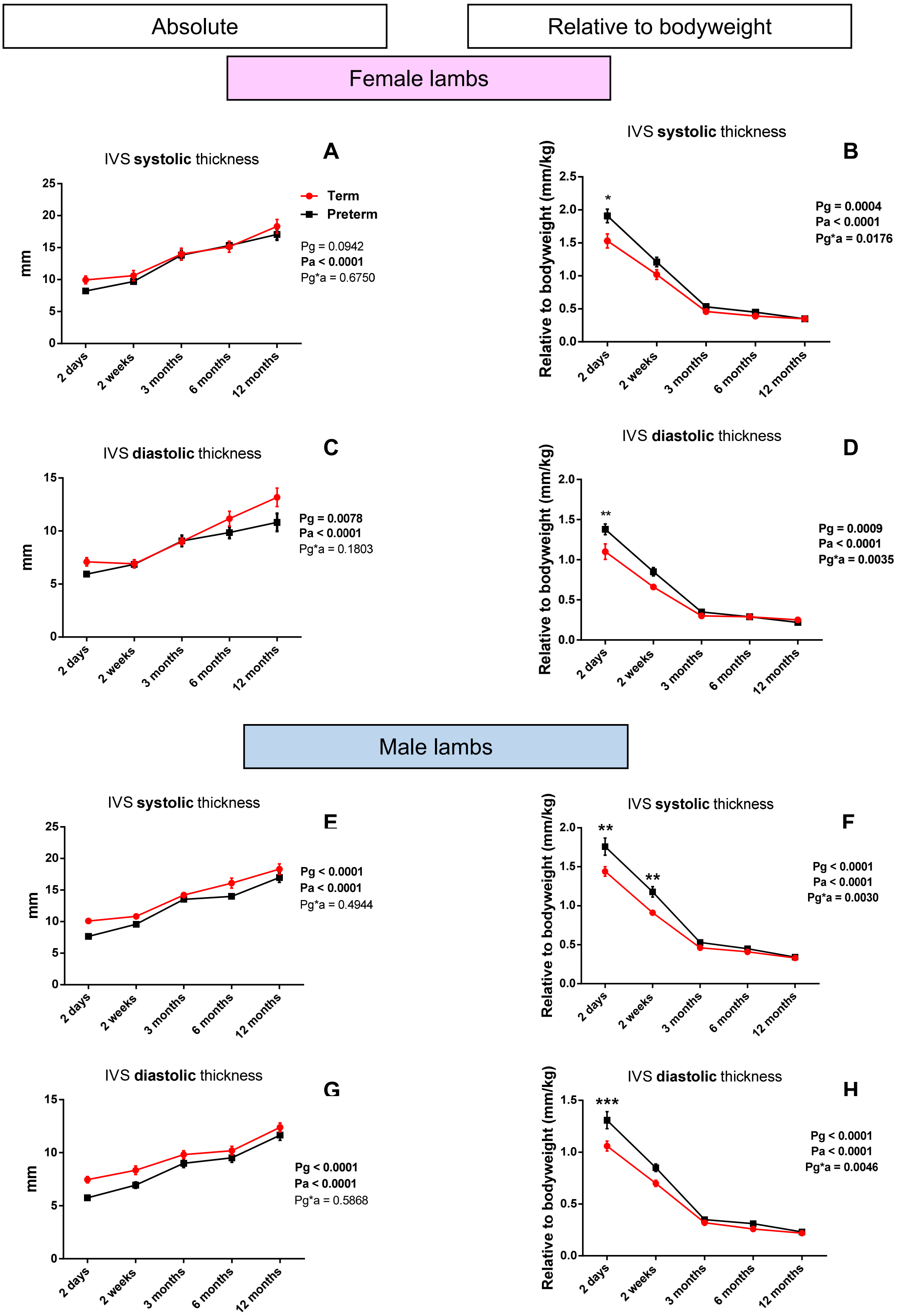

Figure 4-11: Mean thickness (and relative to bodyweight) of the interventricular septum (IVS) of female and male lambs at 2 days to 12 months of age.
Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 following Tukey's post-hoc analysis.

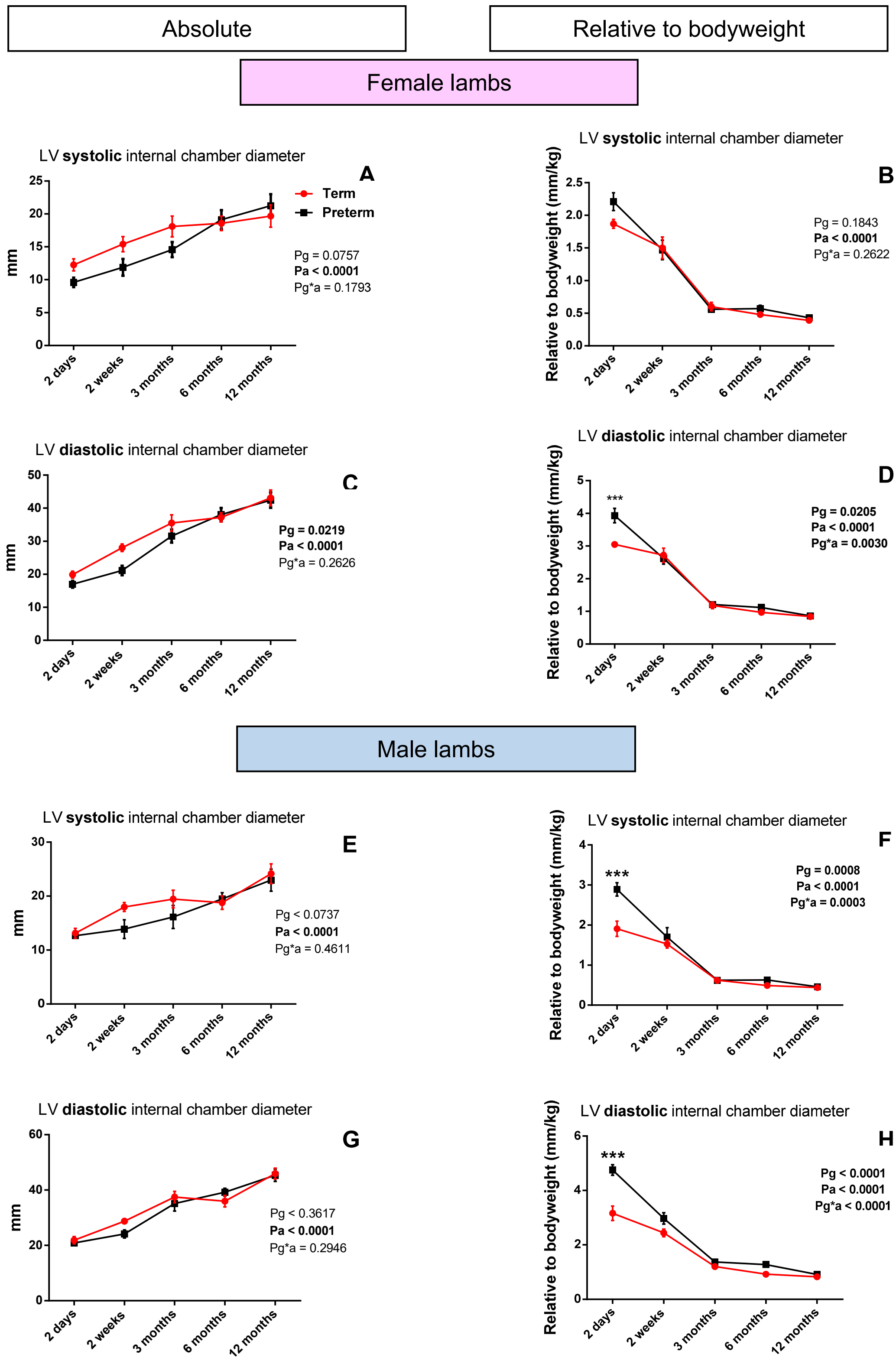

Figure 4-12: Mean left ventricle (LV) internal chamber diameter (and relative to bodyweight) of female and male lambs at 2 days to 12 months of age.
Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. ***p ≤ 0.001 following Tukey's post-hoc analysis.

Functional analysis of the left ventricle from two days to twelve months of age

The average percentage fractional shortening of the left ventricle of the female preterm lambs was not significantly different compared to controls (Figure 4-13A). However, the average percentage fractional shortening of the left ventricle of the male preterm lambs was significantly greater than male term lambs (Figure 4-13B). The increased fractional shortening compared to controls was most evident from two weeks to six months of age.

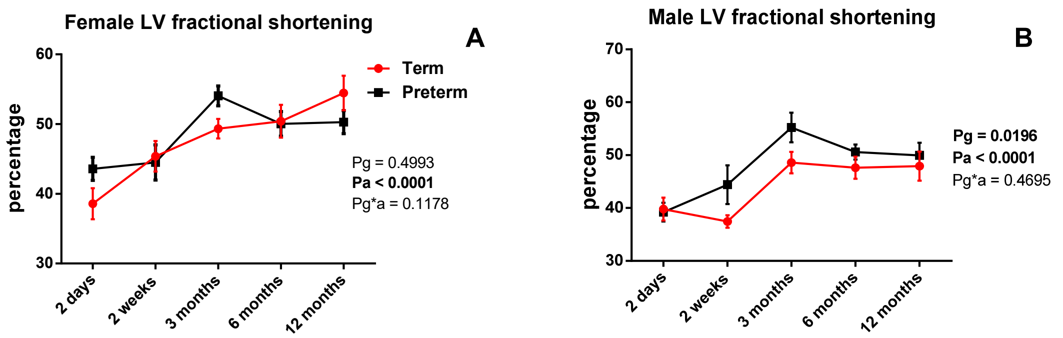

Figure 4-13: Left ventricle (LV) percentage fractional shortening of female and male lambs at 2 days to 12 months of age.

Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different.

Left ventricular peak systolic wall stress from two days to twelve months of age

The female, but not the male, preterm sheep exhibited significantly decreased systolic arterial blood pressure (p = 0.0157) compared to term sheep when evaluated over the 12 month study period from birth to early adulthood (Nguyen, V. 2016), (Figure 4-14). The left ventricular peak systolic wall stress was significantly lower (p = 0.0078) in the female preterm lambs, but not in the males, compared to term sheep over the study period (Figure 4-14). However, the lower LV peak systolic wall stress in the female preterm sheep is evident up till six months of age, after which time the LV wall stress normalises to that of the control sheep.

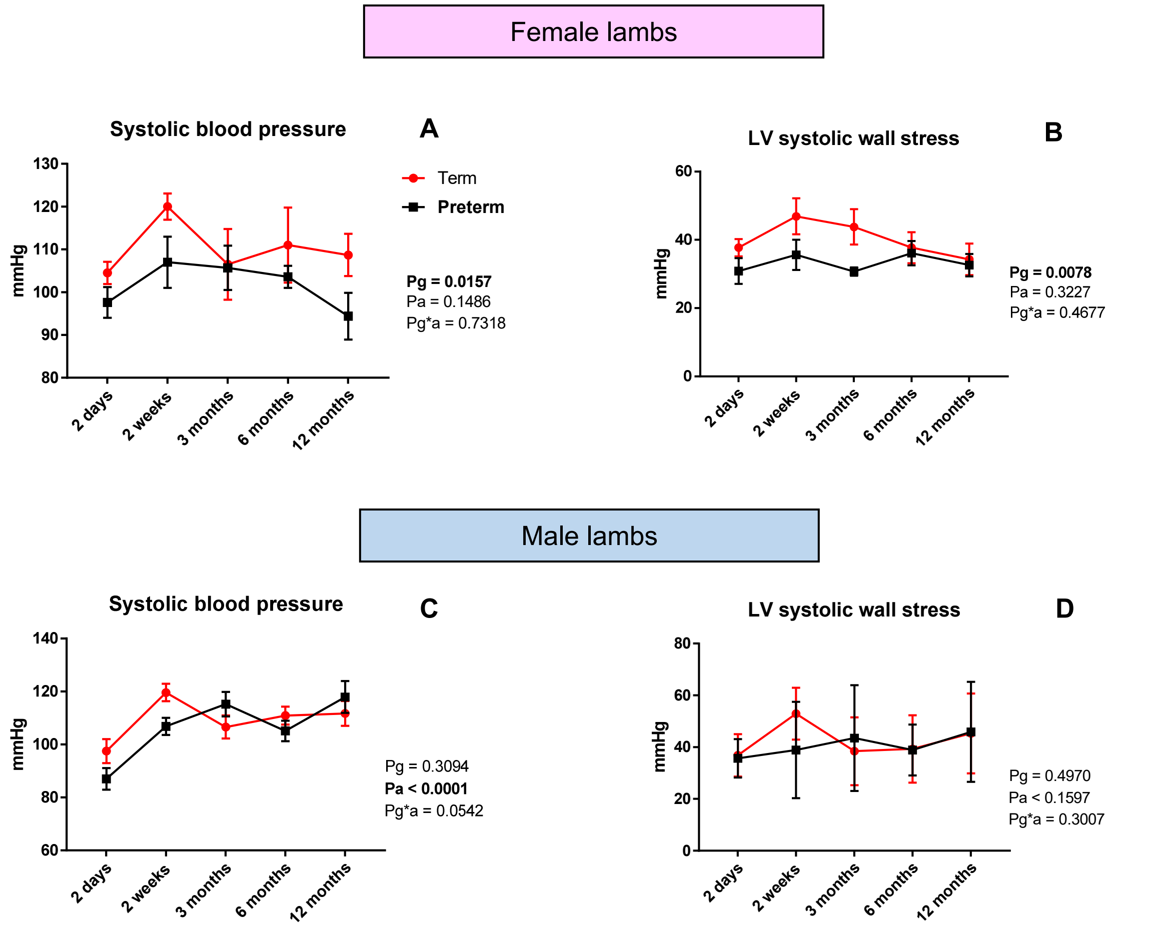

Figure 4-14: Systolic blood pressure and left ventricular peak systolic wall stress estimates of female and male sheep at 2 days to 12 months of age.
Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) and Tukey's post-hoc analysis with gestational age at birth (Pg) and postnatal age (Pa) as factors. (Blood pressure data courtesy of Dr Vivian Nguyen).

Structural analysis of the major arteries from two days to twelve months of age

Visualisation of the major arteries in ultrasound images

The proximal ascending aorta was visualised in ultrasound images at all time points. The main, right and left pulmonary arteries were visualised in all lambs at the two day and two week ultrasound examinations. At the three month ultrasound examination, the main, right and left pulmonary arteries were not visualised in 5/34 (14.7%) lambs. At the six month ultrasound examination, the main and right pulmonary arteries were not visualised in 4/34 (11.8%) sheep and the left pulmonary artery was not visualised in 8/34 (23.5%) sheep. At the twelve month ultrasound examination, the main and right pulmonary arteries were not visualised in 5/32 (15.6%) sheep and the left pulmonary artery was not visualised in 12/32 (37.5%) sheep. Failure to visualise these structures was due to an inadequate acoustic window as a consequence of air in the lungs or bony anatomy (such as ribs) intervening between the ultrasound probe and the structures.

Proximal ascending aorta

The mean internal vessel diameter of the proximal ascending aorta was significantly smaller in female ($p < 0.0001$) and male ($p < 0.0001$) preterm lambs compared to controls (Figures 4-14A, 4-14E).

Relative to bodyweight, the internal vessel diameter was significantly greater in both female ($p < 0.0001$) and male ($p < 0.0001$) preterm lambs compared to term lambs. The larger relative diameter compared to controls was evident from two days to two weeks of age in the female preterm lambs and to three months of age in the male preterm lambs (Figures 4-14B, 4-14F).

Main pulmonary artery

The mean internal vessel diameter of the main pulmonary artery in female preterm lambs was significantly smaller ($p = 0.0462$) compared to female term lambs (Figure 4-14C). There was no significant difference in the mean internal vessel diameter of the main pulmonary artery in male preterm lambs compared to controls. (Figure 4-14G).

Relative to bodyweight, the mean internal vessel diameter was significantly greater in both female ($p = 0.0023$) and male ($p < 0.0001$) preterm lambs compared to controls. The larger relative mean internal vessel diameter of the main pulmonary artery compared to controls was evident until two weeks after birth in preterm females and until three months of age in preterm males (Figures 4-14D, 4-14H).

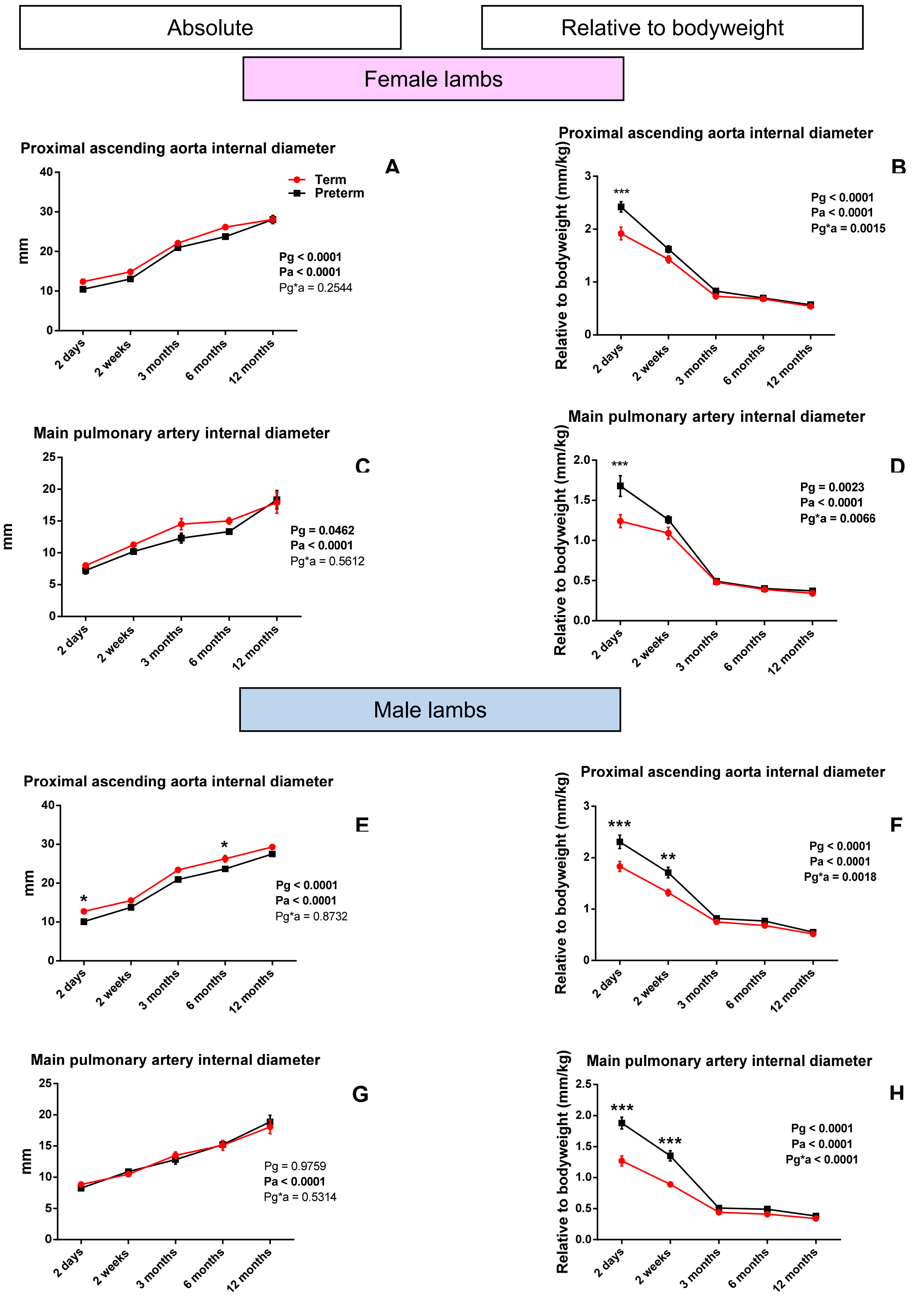

Figure 4-155: Mean internal diameter (and relative to bodyweight) of the proximal ascending aorta and main pulmonary artery of female and male lambs at 2 days to 12 months of age.

Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 following Tukey's post-hoc analysis.

Right pulmonary artery

The mean internal vessel diameter of the right pulmonary artery in female preterm lambs was not significantly different to controls (Figure 4-15A). However, the internal diameter was found to be significantly smaller ($p = 0.0483$) in male preterm lambs compared to male term lambs (Figure 4-15E).

Relative to bodyweight, the internal right pulmonary artery diameter was significantly greater in both female ($p < 0.0001$) and male ($p < 0.0001$) preterm lambs compared to controls. The larger relative diameter compared to controls was evident until three months of age in both the female and male preterm lambs (Figures 4-15B, 4-15F).

Left pulmonary artery

The mean internal vessel diameter of the left pulmonary artery in female preterm lambs was not significantly different to controls (Figure 4-15C). However, the internal diameter was found to be significantly smaller ($p = 0.0023$) in male preterm lambs compared to male term lambs (Figure 4-15G).

Relative to bodyweight, the internal left pulmonary artery diameter was significantly greater in both female ($p < 0.0001$) and male ($p < 0.0001$) preterm lambs compared to term lambs. The larger relative diameter compared to controls was evident until three months of age in both the female and male preterm lambs (Figures 4-15D, 4-15H).

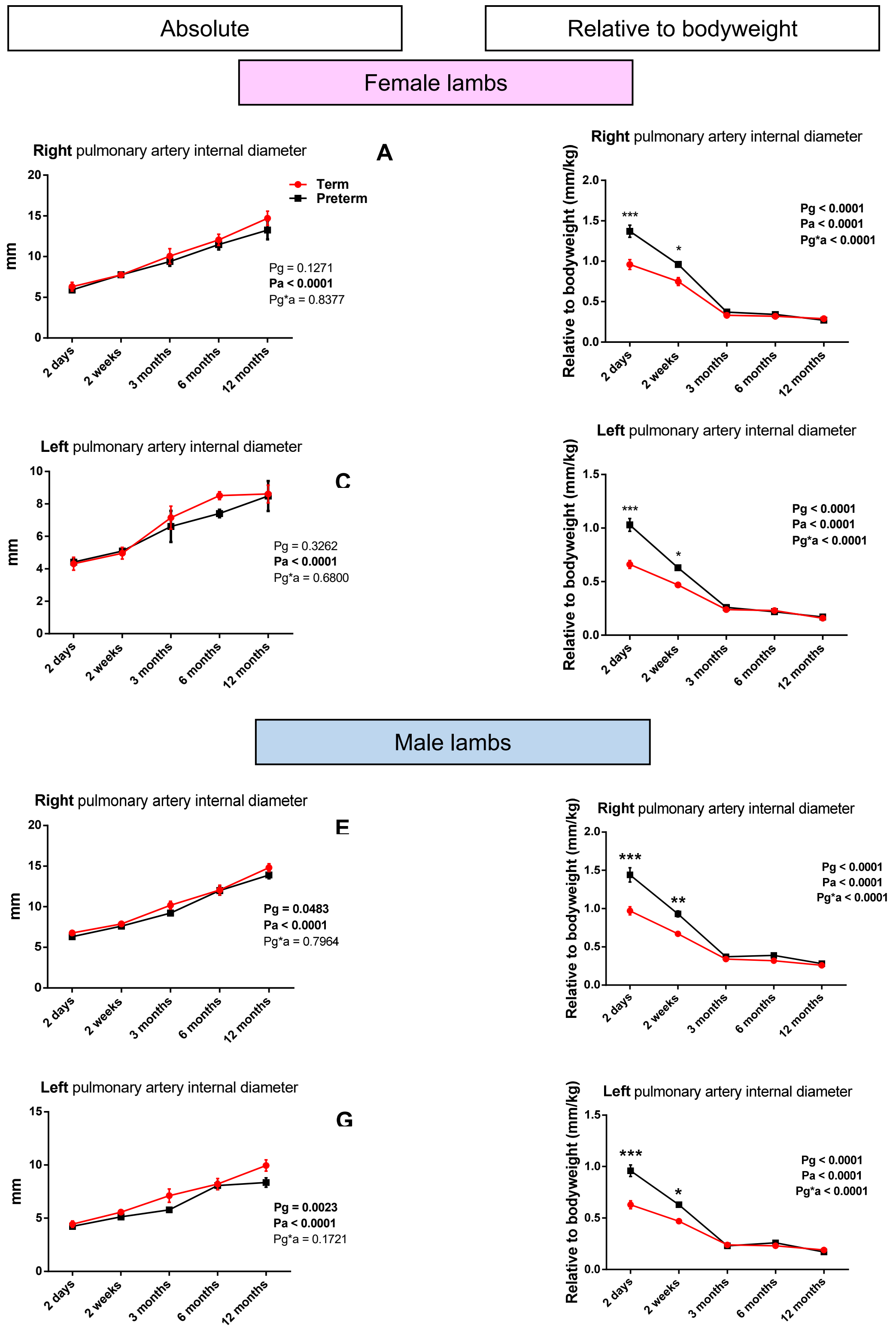

Figure 4-166: Mean internal diameter (and relative to bodyweight) of the right and left pulmonary arteries of female and male lambs at 2 days to 12 months of age. Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 following Tukey's post-hoc analysis.

Common carotid arteries: external vessel diameter

Both common carotid arteries were visualised on ultrasound images at all time points. The mean external vessel diameter of the right common carotid artery in female preterm lambs was not significantly different to controls (Figure 4-16A). However, the mean external diameter of the right common carotid artery was found to be significantly smaller ($p < 0.0001$) in male preterm lambs compared to male term lambs (Figure 4-16E). Relative to bodyweight, the mean external diameter of the right common carotid artery diameter was significantly greater in both female ($p < 0.0001$) and male ($p < 0.0001$) preterm lambs compared to term lambs. The larger relative diameter compared to controls was evident until three months of age in both the female and male preterm lambs (Figures 4-16B, 4-16F).

The mean external vessel diameter of the left common carotid artery in female ($p = 0.0107$) and male ($p < 0.0001$) preterm lambs were significantly smaller than in term lambs (Figures 4-16C, 4-16G). Relative to bodyweight, the mean external diameter of the left common carotid artery was significantly greater in both female ($p = 0.0005$) and male ($p < 0.0001$) preterm lambs compared to term lambs. The larger relative diameter compared to controls was evident until two weeks of age in the female and three months of age in male preterm lambs (Figures 4-16D, 4-16H).

Common carotid arteries: internal vessel diameter

The mean internal vessel diameter of the right common carotid artery in female preterm lambs was not significantly different to controls (Figure 14-17A). However, the mean internal diameter of the right common carotid artery was found to be significantly smaller ($p < 0.0001$) in male preterm lambs compared to male term lambs (Figure 4-17E). Relative to bodyweight, the mean internal diameter of the right common carotid artery diameter was significantly greater in both female ($p < 0.0001$) and male ($p < 0.0001$) preterm lambs compared to term lambs. The larger relative diameter compared to controls was evident until three months of age in both the female and male preterm lambs (Figures 4-17B, 4-17F).

The mean internal vessel diameter of the left common carotid artery in female ($p = 0.0096$) and male ($p < 0.0001$) preterm lambs were significantly smaller than in term lambs (Figures 4-17C, 4-17G). Relative to bodyweight, the mean internal diameter of the left common carotid artery was significantly greater in both female ($p = 0.0029$) and male ($p < 0.0001$) preterm lambs compared to controls (Figures 4-17D, 4-17H).

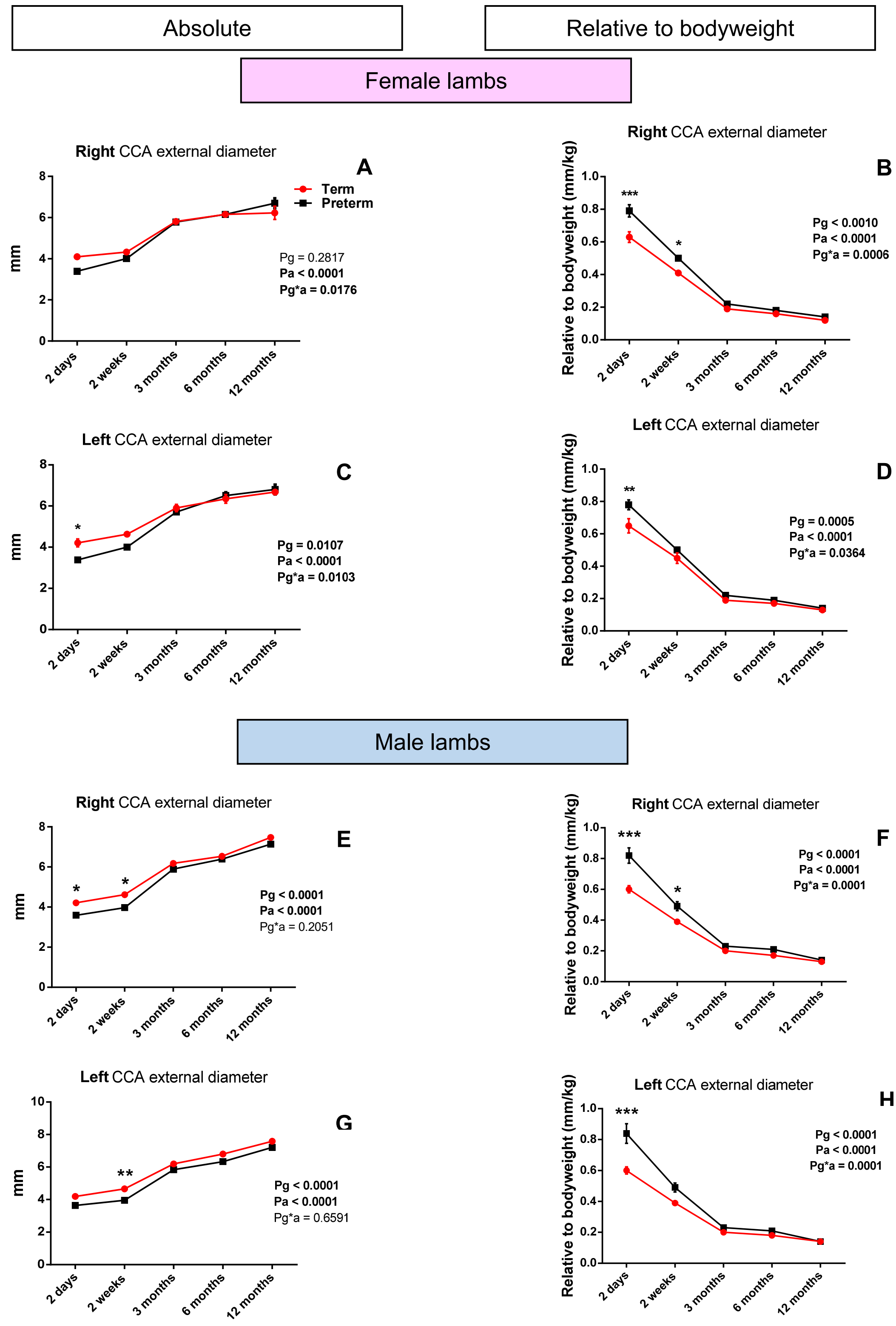

Figure 4-177: Mean external diameter (and relative to bodyweight) of the right and left common carotid arteries (CCA) of female and male lambs at 2 days to 12 months of age.

Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 following Tukey's post-hoc analysis.

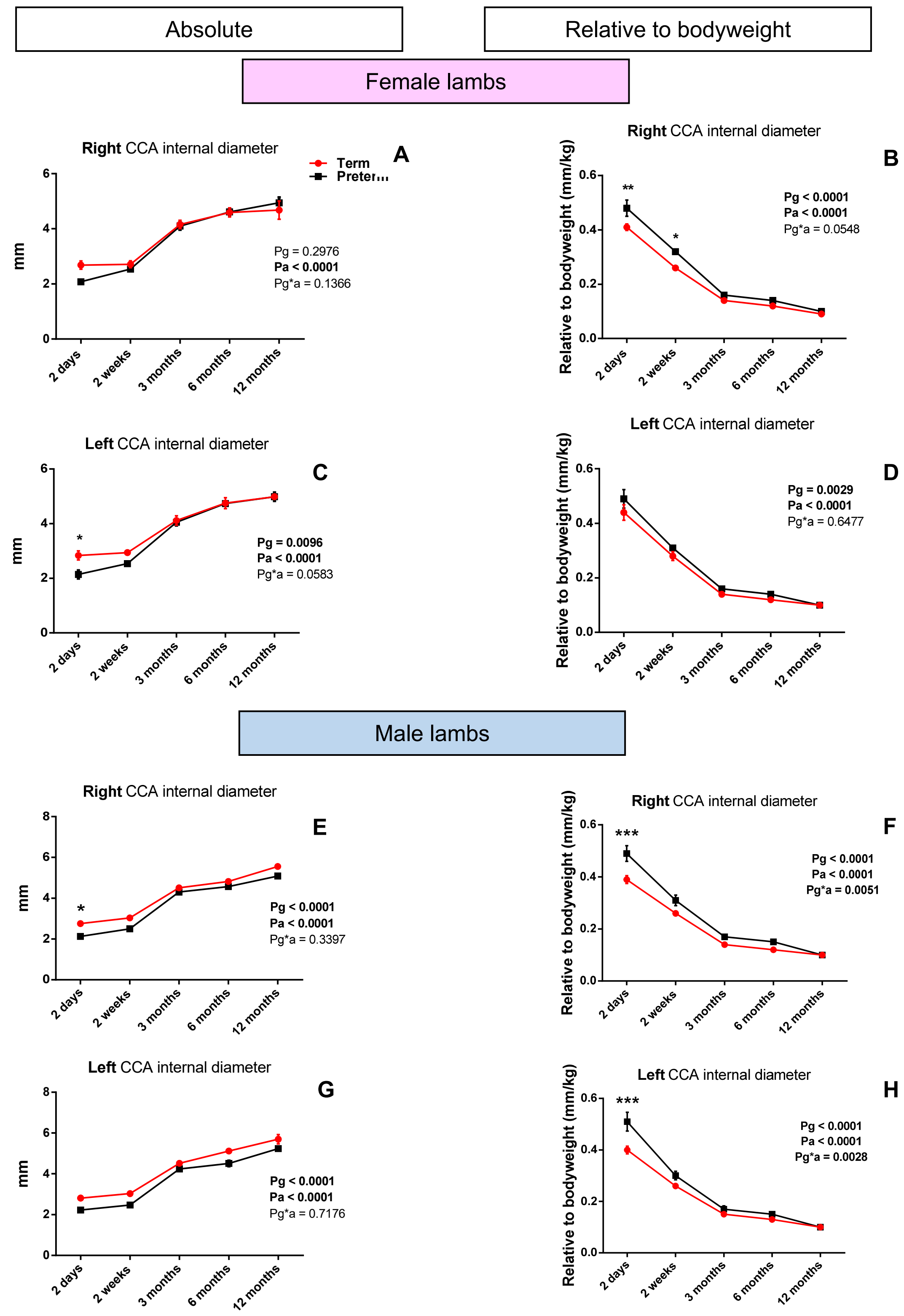

Figure 4-188: Mean internal diameter (and relative to bodyweight) of the right and left common carotid arteries (CCA) of female and male lambs at 2 days to 12 months of age.

Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. *p < 0.05, **p ≤ 0.01, ***p value ≤ 0.001 following Tukey's post-hoc analysis.

Common carotid arteries: intima-media thickness

The mean intima-media thickness of the right common carotid arteries in female and male preterm lambs was not significantly different to controls (Figures 4-18A, 4-18E). Relative to bodyweight, the mean intima-media thickness of the right common carotid artery was significantly greater in both female ($p = 0.0048$) and male ($p < 0.0001$) preterm lambs compared to term lambs. The larger relative thickness compared to controls was evident until three months of age in the female lambs and up to six months of age in the male lambs (Figures 4-18B, 4-18F). The mean intima-media thickness of the left common carotid artery in female preterm lambs was significantly smaller ($p = 0.0481$) than in female term lambs (Figure 4-18C). There was no significant difference in the mean intima-media thickness of the left common carotid artery in male preterm lambs compared to controls (Figure 4-18G). Relative to bodyweight, the mean intima-media thickness of the left common carotid artery was significantly greater in both female ($p = 0.0139$) and male ($p < 0.0001$) preterm lambs compared to controls. The larger relative thickness compared to term lambs was evident until two weeks of age in the female, and six months of age in the male preterm lambs (Figures 4-18D, 4-18H).

Common carotid arteries: adventitia thickness

The mean adventitia thickness of the right common carotid artery in female preterm lambs was not significantly different to female term lambs (Figure 4-19A). The mean adventitia thickness of the right common carotid artery was found to be significantly smaller ($p = 0.0100$) in male preterm lambs compared to controls (Figure 4-19E). Relative to bodyweight, the mean adventitia thickness of the right common carotid artery was significantly greater in both female ($p = 0.0026$) and male ($p < 0.0001$) preterm lambs compared to controls. The larger relative thickness compared to controls was evident until six months of age in the female lambs and to three months of age in the male preterm lambs (Figures 4-19B, 4-19F). The mean adventitia thickness of the left common carotid artery in female preterm lambs was significantly smaller ($p = 0.0007$) than in controls (Figure 4-19C). There was no significant difference in the mean adventitia thickness of the left common carotid artery in male preterm lambs compared to controls (Figure 4-19G). Relative to bodyweight, the mean adventitia thickness of the left common carotid artery was significantly greater in female ($p = 0.0043$) and male ($p < 0.0001$) preterm lambs compared to term lambs. The larger relative thickness was evident until three months of age in the female lambs and to two weeks of age in the male preterm lambs (Figures 4-19D, 4-19H).

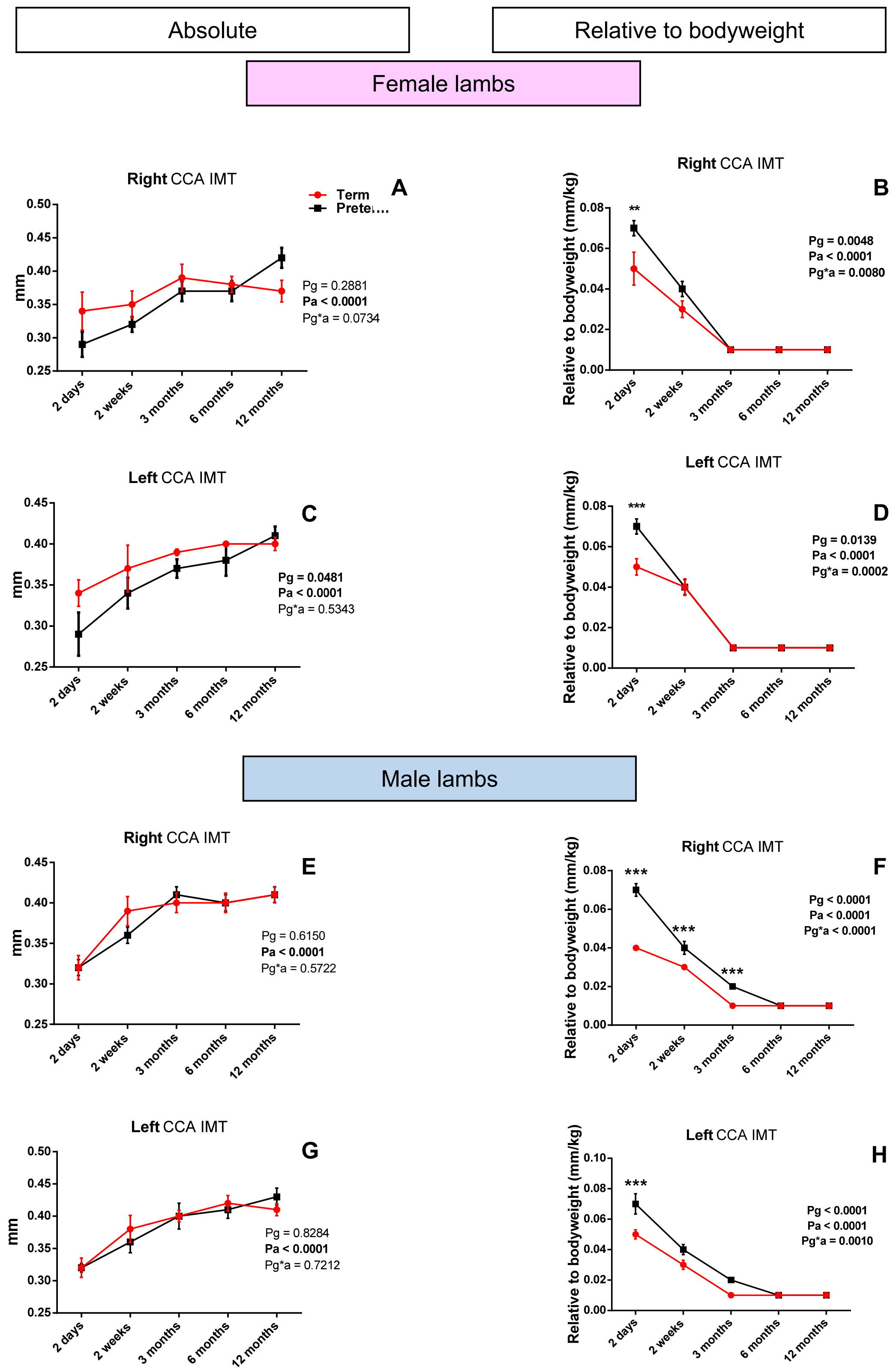

Figure 4-199: Mean intima-media thickness (IMT) (and relative to bodyweight) of the right and left common carotid arteries (CCA) of female and male lambs at 2 days to 12 months of age.
Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. **p ≤ 0.01, ***p ≤ 0.001 following Tukey's post-hoc analysis.

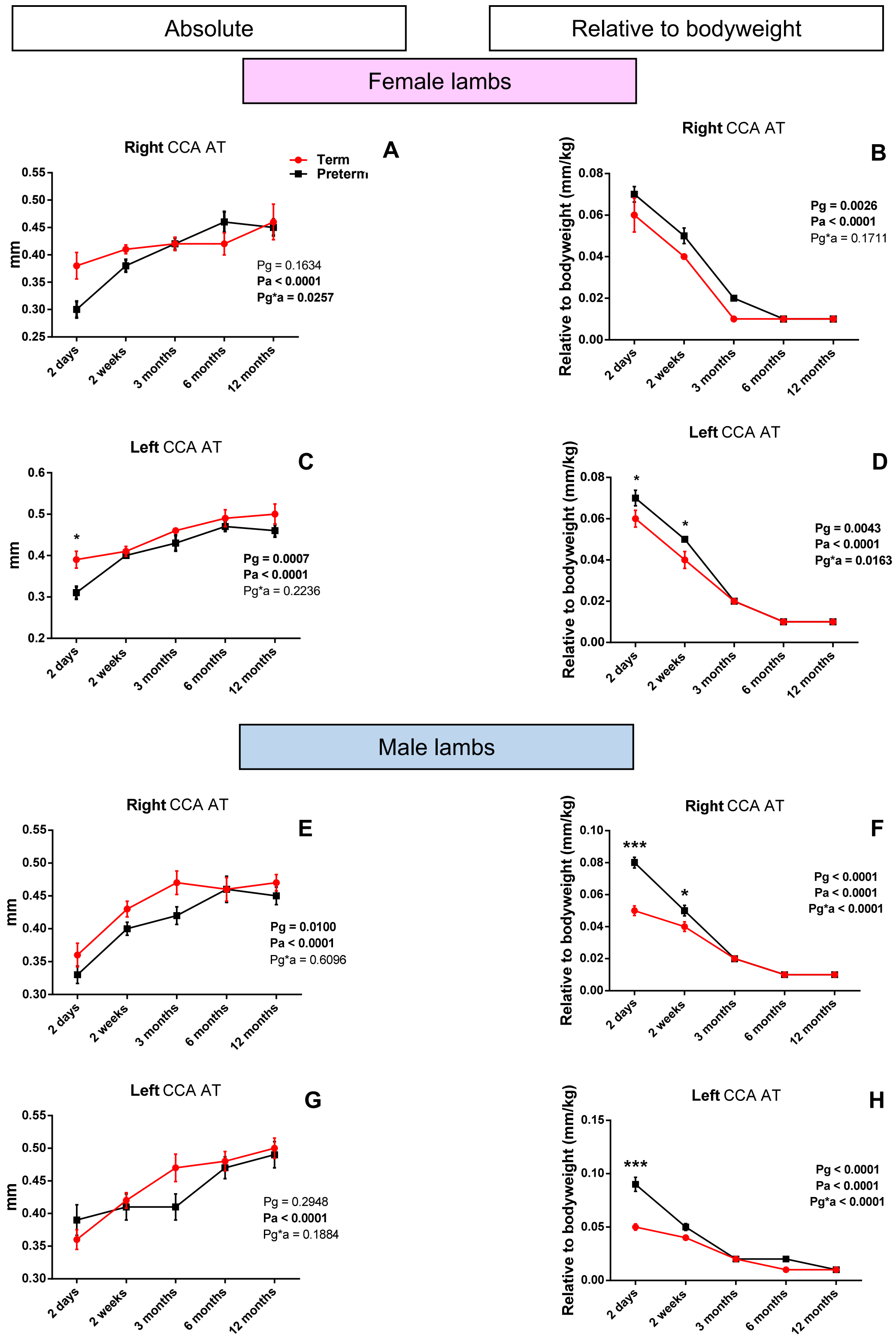

Figure 4-20: Mean adventitia thickness (AT) (and relative to bodyweight) of the right and left common carotid arteries (CCA) of female and male lambs at 2 days to 12 months of age.

Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. *p < 0.05, **p ≤ 0.01, ***p value ≤ 0.001 following Tukey's post-hoc analysis.

Blood flow analysis of the major arteries from two days to twelve months of age

A comparison of the average peak systolic blood flow velocity in the proximal ascending aorta, main, left and right pulmonary arteries of preterm lambs to term lambs are shown in Table 4-1. The blood flow velocities in the female preterm lambs were not significantly different to those recorded in the female term lambs. The average peak systolic blood flow velocity in the proximal ascending aorta and right pulmonary artery in the male preterm lambs were also not significantly different to controls. However, the blood flow velocities in the main ($p = 0.0143$) and left ($p = 0.0006$) pulmonary arteries were significantly lower in the male preterm lambs compared to controls.

The resistive index of the right renal artery of female preterm lambs was significantly higher compared to the female term lambs. The resistive index of the right renal artery of male preterm lambs was also raised compared to controls but not significantly (Table 4-1). The left renal artery resistive index was not calculated as the data obtained was unreliable or incomplete after two weeks post-natal age as discussed in section 4.3.2.

Compared to term lambs, there were no significant differences in the resistive indices of the right or left common carotid arteries of female or male preterm lambs (Table 4-1).

Table 4-1: Comparison of blood flow measurements in the major arteries of preterm and term lambs at 2 days, 2 weeks, 3, 6 & 12 months after birth.

Average peak systolic blood flow (cm/s)	Female preterm/term lambs p value	Male preterm/term lambs p value
Proximal ascending aorta	NS	NS
Main pulmonary artery	NS	0.0143
Left pulmonary artery	NS	0.0006
Right pulmonary artery	NS	NS
Mean resistive indices		
Right renal artery	0.0092	NS
Right common carotid artery	NS	NS
Left common carotid artery	NS	NS

Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different. NS = $p \geq 0.05$. The left renal artery resistive index was not calculated as the data obtained were unreliable or incomplete after two weeks post-natal age as discussed in section 4.3.2.

Structural analysis of the right kidney from two days to twelve months of age

Visualisation of the renal structures

The right kidney was visualised in all lambs/sheep in ultrasound examinations at all time points. The right renal artery was visualised at all time points except at the 12 month ultrasound examination where it could not be demonstrated well enough to record a flow velocity waveform to calculate a resistive index in 5/9 (55.6%) male term sheep and 2/6 (33.3%) female term sheep. The left kidney and left renal artery were visualised in all lambs at the two day and two week ultrasound examinations. At the three month ultrasound examination only 13/34 (38.2%) of the left kidneys and 9/34 (26.5%) of left renal arteries could be visualised in the lambs. The left kidney and left renal artery could not be reliably visualised in any sheep from six months of age. Non-visualisation of these structures was entirely due to gas within the overlying bowel preventing the ultrasound beam from reaching the areas under investigation. Only data from the right kidney was included in the analyses as data from the left kidney was not reliably obtained throughout the experimental period (2 days to 12 months of age).

Mean renal bipolar length

There was no significant difference in the mean bipolar length of the right kidney of female preterm lambs compared to controls (Figure 4-20A). The bipolar kidney length of the male preterm lambs were significantly smaller ($p < 0.0001$) than the male term lambs (Figure 4-20E).

Relative to bodyweight, the mean bipolar length of the right kidney was significantly greater in both female ($p < 0.0001$) and male ($p < 0.0001$) preterm lambs compared to term lambs. The larger relative length compared to controls was evident until three months of age in both the female and male preterm lambs (Figures 4-20B, 4-20F).

Mean renal width

There was no significant difference in the mean right kidney width of female preterm lambs compared to controls (Figure 4-20C). The right kidney width of the male preterm lambs was significantly smaller ($p = 0.0003$) than the male term lambs (Figure 4-20G).

Relative to bodyweight, the mean right kidney width was significantly greater in both female ($p < 0.0001$) and male ($p < 0.0001$) preterm lambs compared to term lambs. The greater renal width compared to controls was evident until three months of age in both the female and male preterm lambs (Figures 4-20D, 4-20H).

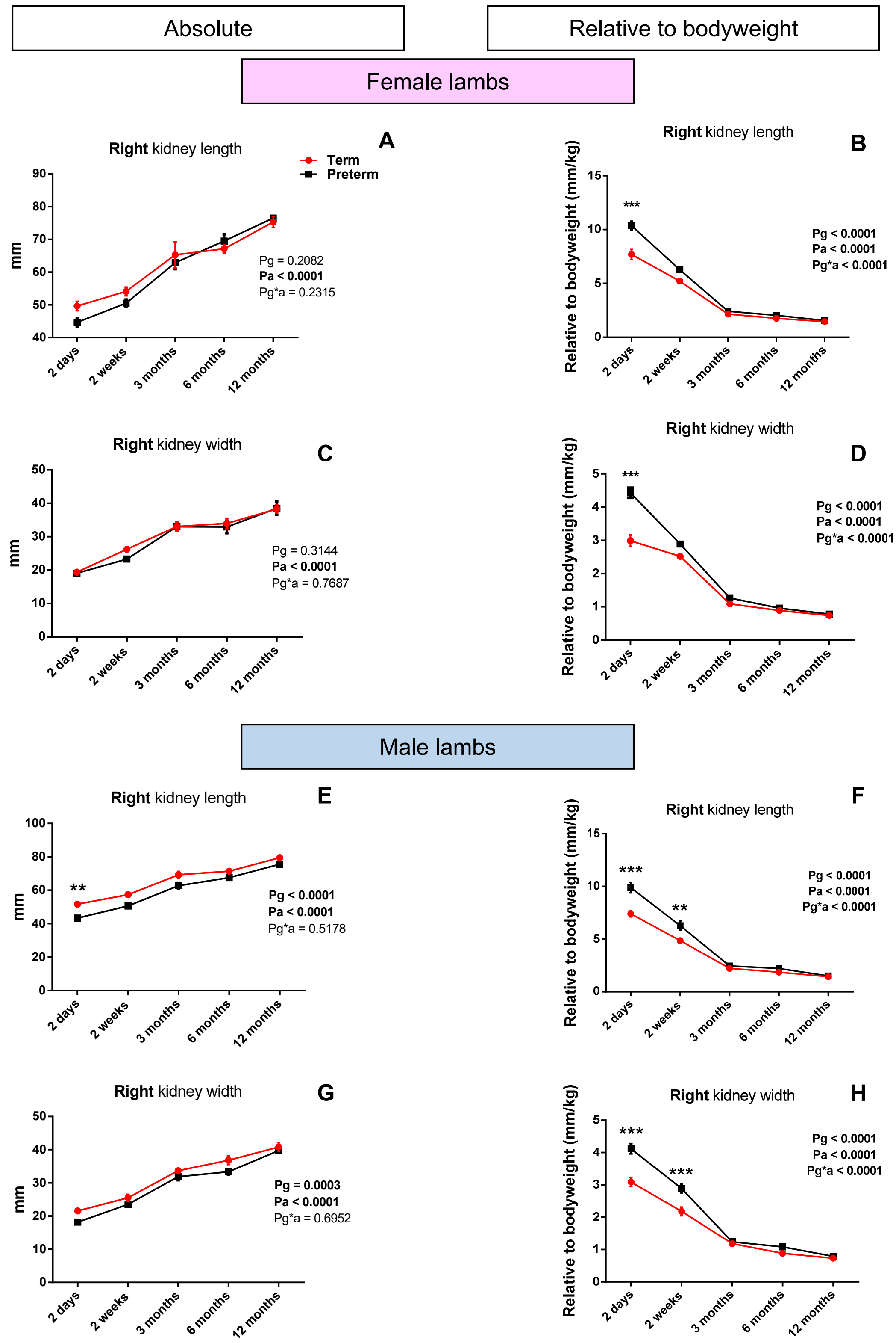

Figure 4-21: Mean bipolar length and width (and relative to bodyweight) of the right kidney of female and male lambs at 2 days to 12 months of age.
Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001 following Tukey's post-hoc analysis.

Mean renal thickness

There was no significant difference in the mean thickness of the right kidney of female or male preterm lambs compared to controls (Figures 4-21A, 4-21E).

Relative to bodyweight, the mean thickness of the right kidney was significantly greater in both female ($p < 0.0001$) and male ($p < 0.0001$) preterm lambs compared to term lambs. The greater renal thickness compared to controls was evident until three months of age in both the female and male preterm lambs (Figures 4-21B, 4-21F).

Mean renal volume

There was no significant difference in the mean right kidney volume of female preterm lambs compared to controls (Figure 4-21C). However, the right kidney volume of the male preterm lambs was significantly smaller ($p = 0.0002$) than the male term lambs (Figure 4-21G).

Relative to bodyweight, the mean right kidney volume was significantly greater in the female ($p = 0.0041$) preterm lambs compared to term lambs (Figure 4-21D). There was no significant difference in the relative right kidney volume of male preterm lambs compared to controls (Figure 4-21H).

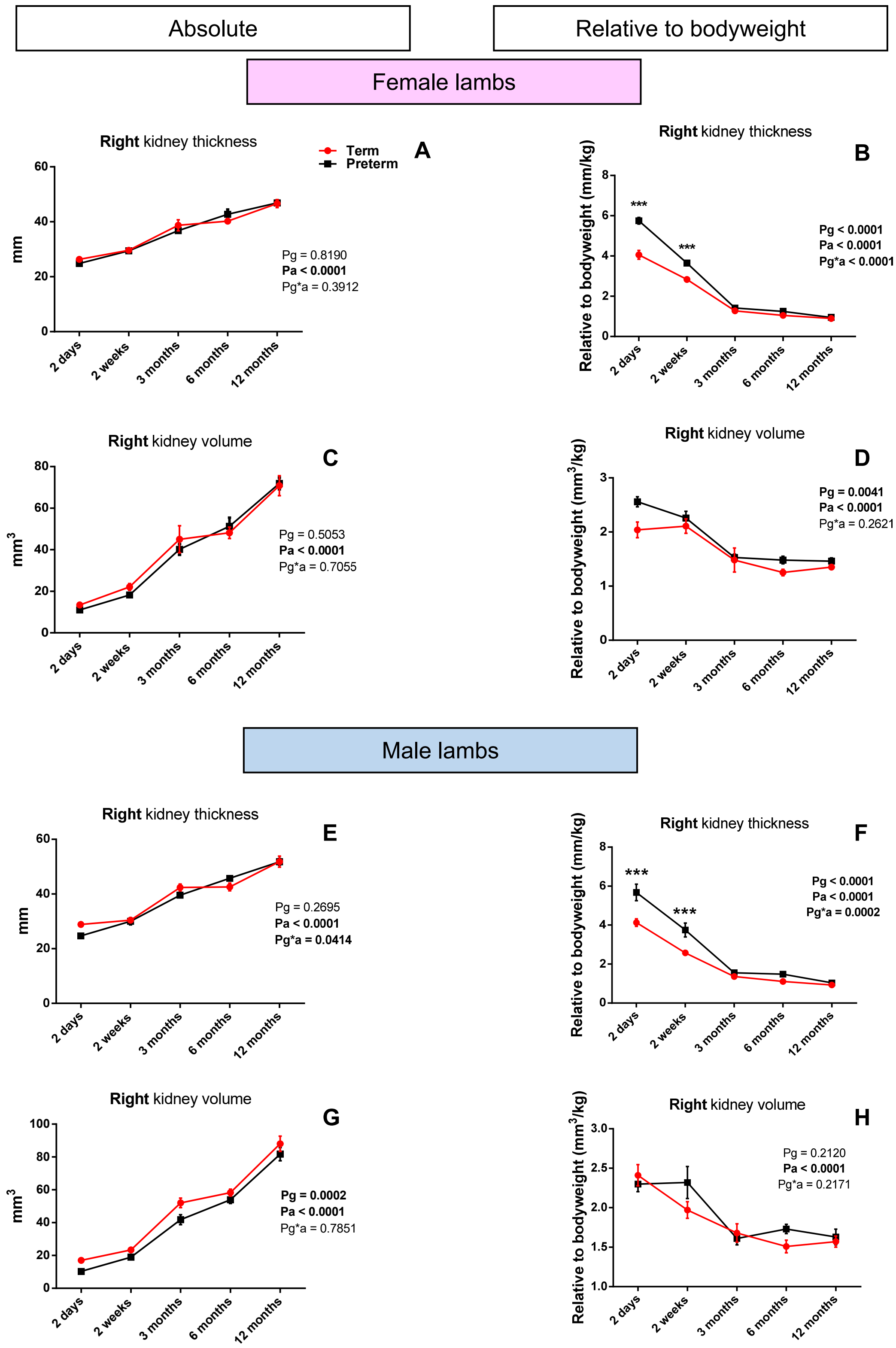

Figure 4-22: Mean thickness and volume (and relative to bodyweight) of the right kidney of female and male lambs at 2 days to 12 months of age.
Data represented as mean ± SEM. Data were analysed using a two way analysis of variance (ANOVA) with gestational age at birth (Pg) and postnatal age (Pa) as factors. *p < 0.05, ***p ≤ 0.001 following Tukey's post-hoc analysis.

Preterm lambs at term equivalent age (TEA) compared to control lambs at term

As discussed in chapter 3, the disparity in growth between lambs at birth due to gestational age can be addressed by comparing the preterm lambs at term equivalent age (TEA) to control lambs at two days after term birth, rather than correcting for bodyweight. The bodyweight and ultrasound measurements (absolute values) of preterm lambs at two weeks after birth (TEA) were compared to control lambs at two days after term birth (i.e. both groups compared around 149 days post conception).

Bodyweight

The birthweight of female and male preterm lambs was significantly lower than the birthweight of their counterpart control lambs at term. However, at TEA the bodyweight of the preterm lambs were significantly greater (female p = 0.0024 and male p = 0.0477) compared to the controls at term (Figure 4-22).

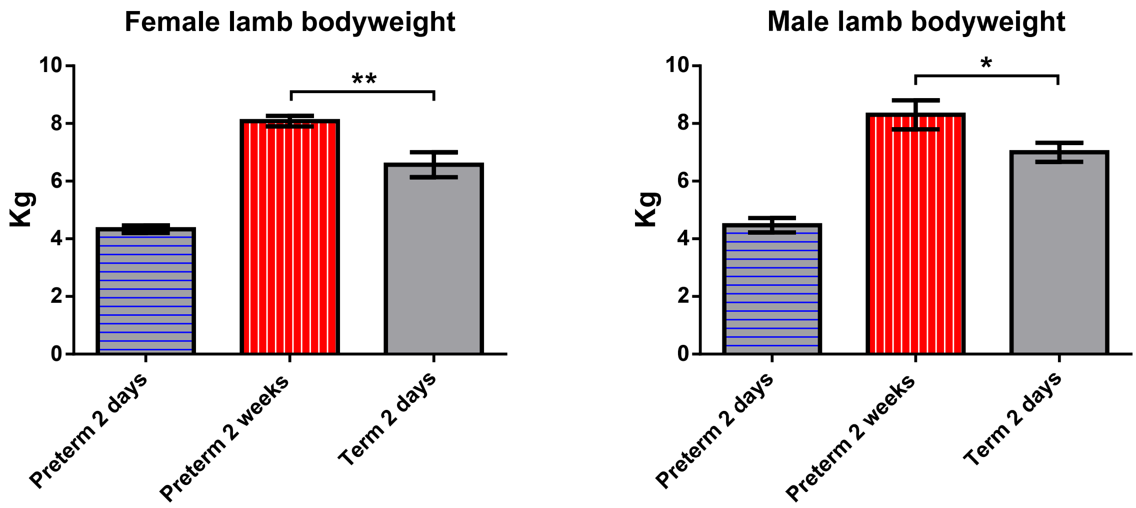

Figure 4-23: Comparison of preterm lamb bodyweight at two days and two weeks (TEA) after birth, to term lambs at two days after birth.
Data represented as mean ± SEM. Data were analysed using a one-way analysis of variance (ANOVA). *p < 0.05, **p ≤ 0.01 following Tukey's post-hoc analysis.

Left ventricle structure and function

There were no significant differences in the thickness of the left ventricle anterior and posterior walls, IVS, internal chamber diameter and percentage fractional shortening of the left ventricle of male or female preterm lambs at TEA compared to two day old controls.

Structural analysis of the major arteries

Compared to control lambs at term, no significant differences were demonstrated in the mean internal diameters of the proximal ascending aorta and left pulmonary arteries of male and female preterm lambs, or the right pulmonary artery of male preterm lambs at TEA. The mean internal diameter of the right pulmonary artery in female TEA preterm lambs was significantly larger than controls at term ($p = 0.0324$). The mean internal diameter of the main pulmonary artery was significantly larger in male ($p = 0.0008$) and female ($p = 0.0103$) TEA preterm lambs compared to controls at term.

The mean internal diameter of the left common carotid artery in male preterm lambs was found to be significantly smaller at TEA compared to control lambs at term ($p = 0.0203$). However, no other significant differences were demonstrated in external or internal diameter, IMT or AT of the right and left common carotid arteries in male or female lambs at TEA compared to controls.

Blood flow analysis of the major arteries

The peak systolic flow in the left pulmonary artery was significantly lower in the male ($p = 0.0184$) and female ($p = 0.0100$) TEA preterm lambs compared to controls at term. No other significant differences in peak systolic flow were demonstrated in the proximal ascending aorta, main and right pulmonary arteries between preterm lambs at TEA and control lambs at term.

The mean resistive indices of the right ($p = 0.0033$) and left ($p = 0.0442$) common carotid arteries at TEA were significantly lower in the male, but not female preterm lambs compared to term controls (Figure 4-23).

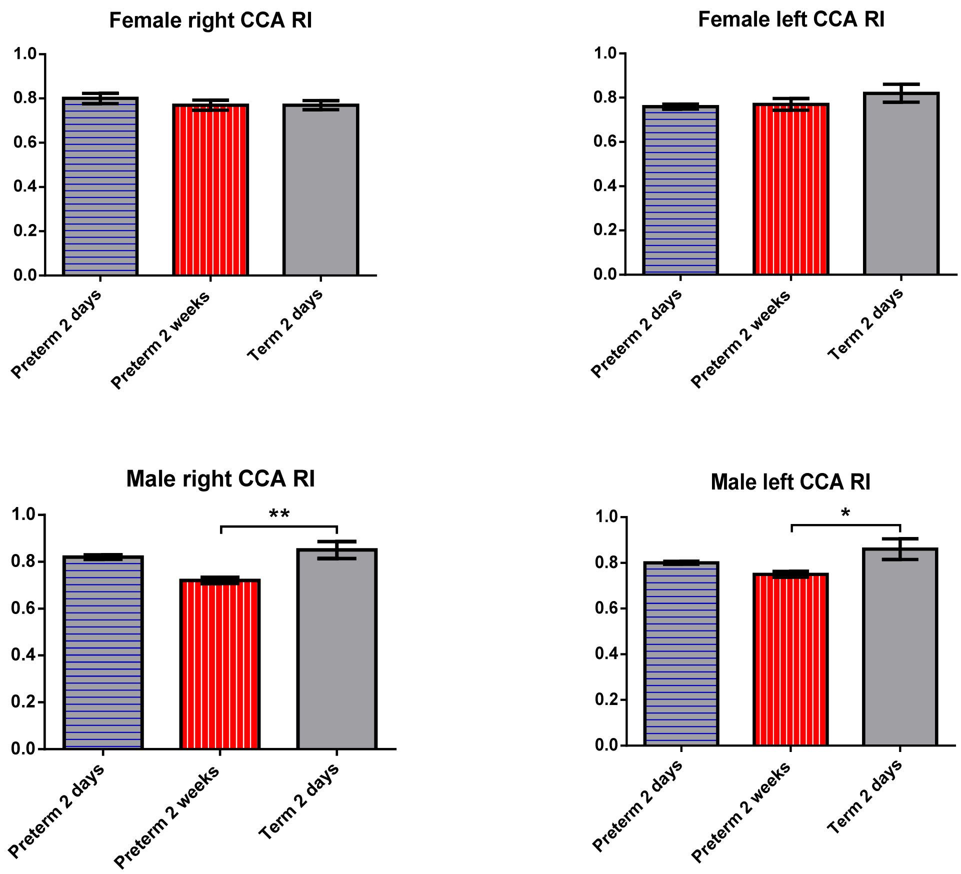

Figure 4-24: The resistive indices (RI) of the right and left common carotid arteries (CCA) of preterm lambs at two days and two weeks (TEA) after birth compared to term lambs at two days after birth.
Data represented as mean ± SEM. Data were analysed using a one-way analysis of variance (ANOVA). $*p < 0.05$, $**p \leq 0.01$ following Tukey's post-hoc analysis.

No significant differences in the resistive indices of the right and left renal arteries of male and female preterm lambs at TEA were demonstrated relative to control lambs at term.

Structural analysis of right and left kidneys

No significant differences in the bipolar length of the right and left kidneys of female and male preterm lambs at TEA were demonstrated relative to control lambs at term (Figure 4-24).

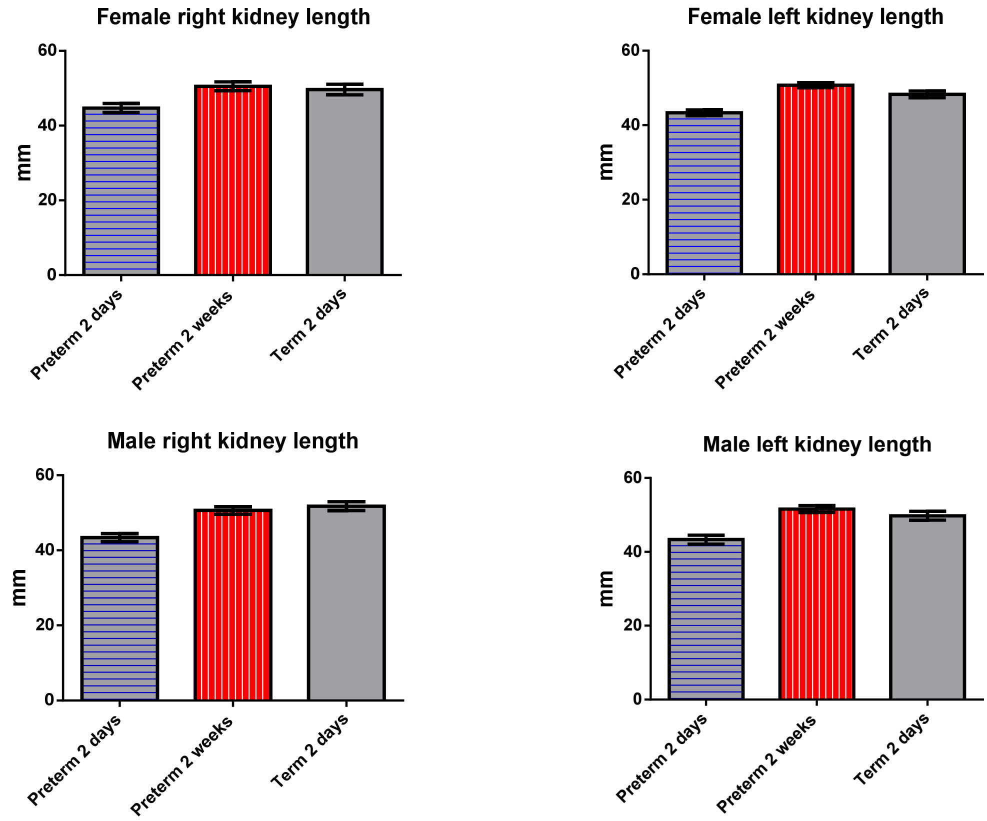

Figure 4-25: The bipolar length of the right and left kidneys of preterm lambs at two days and two weeks (TEA) after birth compared to term lambs at two days after birth.
Data represented as mean ± SEM. Data were analysed using a one-way analysis of variance (ANOVA). A Tukey's post-hoc test was used to determine which data points were significantly different.

The right kidney width ($p = 0.0037$), thickness ($p = 0.0378$) and volume ($p = 0.0106$) at TEA were significantly greater in the female, but not in male preterm lambs compared to term controls (Figure 4-25).

The female left kidney width (p = 0.0345) and volume (p = 0.0126) and male left kidney width (p = 0.0065) and volume (p = 0.0164) were greater in the preterm lambs at TEA than control lambs at term (Figure 4-25). There was no significant difference in the left kidney thickness of female or male preterm lambs at TEA when compared to lambs at term.

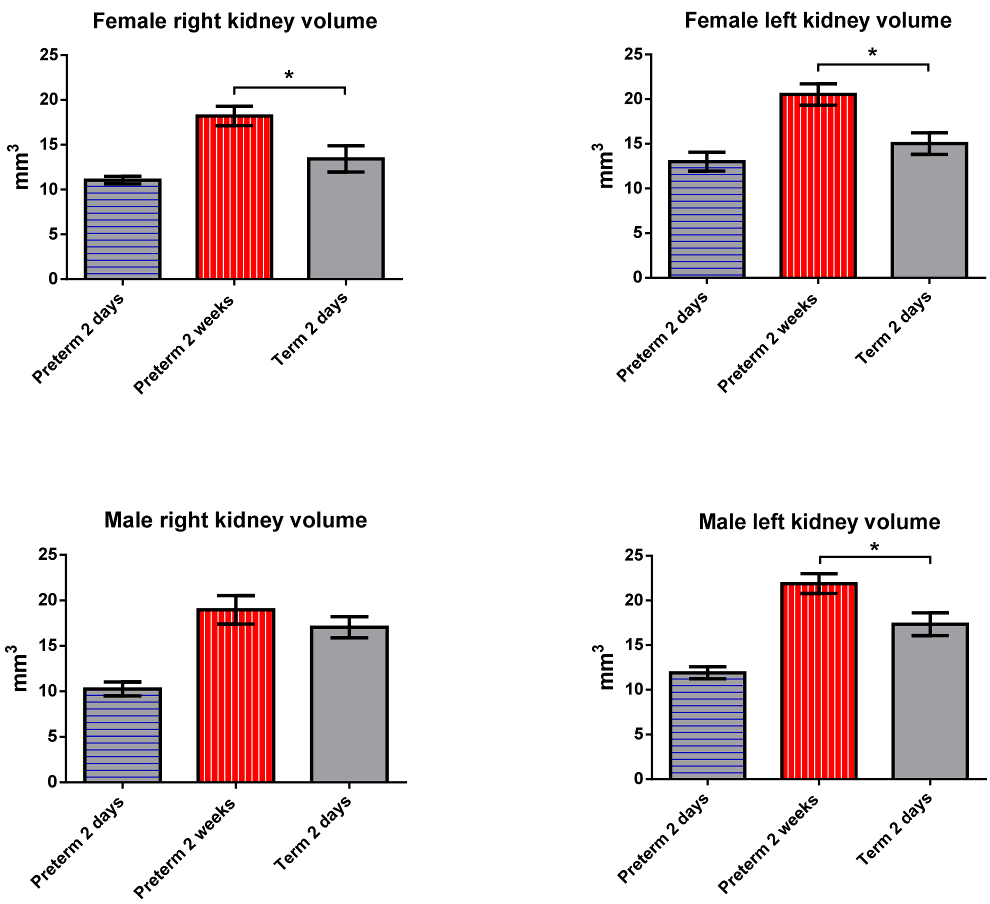

Figure 4-26: The volume of the right and left kidneys of preterm lambs at two days and two weeks (TEA) after birth compared to term lambs at two days after birth
Data represented as mean ± SEM. Data were analysed using a one-way analysis of variance (ANOVA). *p < 0.05 following Tukey's post-hoc analysis.

4.4. Discussion.

Ultrasound imaging of the left ventricle, major arteries and kidneys of sheep was successfully conducted at two days after birth through to adulthood without the need to anaesthetise the animals. Relative to bodyweight, significant structural differences of the left ventricle, major arteries and kidneys of preterm lambs were demonstrated compared to controls in the early post-natal period. However, none of these differences were still apparent six months after birth, with almost all differences normalised by three months of age. The relative structural differences in the initial post-natal period are most likely due to a combination of rapid post-natal weight gain in the preterm lambs, together with adaptation of an immature cardiovascular and renal system to the haemodynamic transition after birth. There was no evidence that moderate preterm birth resulted in adverse effects in early adulthood. This is an encouraging finding given that the overwhelming majority of premature births are moderately preterm (Hilder et al. 2014).

Evaluation of the structure and function of the left ventricle

There was little difference found in the absolute measures of the LV and IVS wall thickness or LV chamber diameter between preterm sheep and controls. This could be due to the growth in heart size being mostly completed by late gestation, in addition to structural alterations that occurred in response to the haemodynamic changes at birth (Bayman, Drake & Piyasena 2014; Tare et al. 2014). Moderate preterm birth has previously been shown to adversely affect the heart wall structure in sheep by significantly increasing collagen deposition in the myocardium, altering cardiomyocyte maturation and resulting in cardiomyocyte hypertrophy (Bensley, J et al. 2010). Those findings were in line with human studies that showed altered myocardial structure, increased LV mass and decreased fractional shortening compared to controls in one month old neonates born moderately preterm (Kozák-Bárány et al. 2001) and LV dysfunction in six month old infants born very preterm (Schmitz et al. 2004). More recently, young adults born very preterm were found to have increased LV mass and adverse ventricular structure and function compared to controls born at term (Lewandowski et al. 2013). An increased LV mass is an independent predictor of cardiovascular disease in adulthood (de Simone et al. 2002; Levy et al. 1990). Although Lewandowski found that the severity of the prematurity was associated with a greater risk of cardiovascular disease, it remains unclear whether individuals born moderately preterm are at significant risk of cardiac events in later life and follow up research has been suggested (Lewandowski et al. 2013; Norman 2013).

The evidence from previous studies clearly shows that preterm birth leads to adverse structural remodelling of the heart wall. However, these structural adaptations were not large enough to be revealed using ultrasound imaging in our study of moderately preterm sheep from shortly after birth to early adulthood. Compared to term sheep, gross changes to the LV wall thickness and LV chamber diameter were only demonstrated in the moderately preterm sheep when corrected for bodyweight, and those differences were all normalised by three months of age. Given that the absolute values of these measures were not significantly different, these results are most likely due to the lower relative bodyweight of the preterm lambs at birth and subsequent increase in growth rather than remodelling of the cardiac structures. The percentage fractional shortening of the LV of female preterm sheep was not significantly different to term sheep. However, the male preterm sheep had a significantly higher LV percentage shortening compared to controls over the 12 month study period. Preterm birth leads to increased collagen deposition in the myocardium that can result in stiffer LV walls and decreased contractility (Brower et al. 2006; Weber 1989). It is unclear why a LV that probably contains high levels of collagen in its walls would contract more than normal in males, but not females. Follow up studies including a morphological analysis of the ventricular wall in addition to LV function are warranted. The intention is to compare our ultrasound findings with morphological results obtained by Dr Vivian Nguyen as part of her concurrent PhD project on this cohort of sheep in the future. This will require further ultrasound imaging immediately before necropsy and that will be addressed in chapter 5.

Left ventricular wall stress can be used an indicator of adaptive structural changes of the myocardium in situations where there is abnormal loading of the heart, for example, in hypertension (Jonker, S et al. 2010; Yin 1981). According to the Laplacian relationship, left ventricular wall stress is proportional to both blood pressure and the size of the LV chamber and inversely proportional to the LV wall thickness (Moriarty 1980). Therefore, an increased thickness of the LV wall and/or a reduction in the size of the LV chamber is required in response to increased load (raised blood pressure), in order to normalise LV wall stress and preserve LV function (Burchfield, Xie & Hill 2013; Nadruz 2015; Wikstrand 1984).

Increased left ventricular mass has been demonstrated previously in infants (Aye et al. 2017) and in young adults (together with geometric changes) born moderately premature (Lewandowski et al. 2013) compared to term born controls. A study of sheep at birth that had been anaemic since 0.8 of gestation (very premature) found they had increased cardiac mass compared to controls, but their LV wall stress was normal, indicating that adaptive remodelling of the fetal heart had occurred *in utero* to maintain LV function (Jonker, S et al. 2010).

There were no significant differences in the systolic blood pressure, LV wall thickness, chamber size or wall stress of our male preterm sheep, compared to term controls, over the 12 month study period. By contrast, the female preterm sheep demonstrated significantly lower LV wall stress compared to the controls. This was likely due to the significantly lower systolic blood pressure over the 12 month study period in the female preterm sheep, given there was little difference in the absolute measures of the LV wall thickness or chamber diameter compared to term controls. The significantly lower LV wall stress was mostly evident soon after birth but had normalised to that of controls by six months of age, suggesting adaptation of the heart to the lower blood pressure had occurred by that time (Figure 4-14). Young adulthood (12 months of age) may be too early for any mal-adaptations of LV structure and function to become evident in the sheep. Further serial studies are recommended to ascertain if the LV structure and wall stress remain normalised in female sheep into mid and later life.

Evaluation of the structure and blood flow within the major arteries

In chapter three the absolute internal diameter measurements of the proximal ascending aorta, main, right and left pulmonary arteries were not significantly different between preterm and term lambs at two days after birth. Ho
.wever, serial evaluation over a 12 month period has shown that the internal diameter of the proximal ascending aorta was significantly decreased in all preterm sheep compared to controls. The internal diameter of the main pulmonary artery was significantly decreased in female preterm sheep and the right and left pulmonary artery diameters were significantly decreased in male preterm sheep compared to controls. These results are in line with previous studies that have demonstrated decreased arterial diameters in children (Jiang et al. 2006) and adolescent girls (Bonamy et al. 2005) born preterm and adverse aortic wall remodelling with a decreased lumen in 11 week old lambs born moderately preterm (Bensley, J et al. 2012). It is likely that these structural differences are a mal-adaptive response by the immature cardiovascular system to the increased systemic arterial blood pressure following preterm birth (Bensley, J et al. 2012; Sadler 2010). When corrected for bodyweight, the lumen of the proximal ascending aorta, main, right and left pulmonary arteries were all greater in preterm sheep compared to controls. These differences were all normalised by three months of age and were clearly due to the initial lower relative bodyweight of the preterm lambs given the absolute value of the vessel diameters were actually less in the sheep born preterm.

Consistent with the aorta and pulmonary arteries, the diameters of the right and left common carotid arteries were also significantly smaller in male and female preterm sheep compared to controls. Relative to bodyweight, the diameters were all greater in preterm sheep compared to controls. These relative differences also normalised by three months of age and were due to the initial lower relative bodyweight of the preterm lambs given the absolute value of the vessel diameters were smaller in the preterm sheep.

The absolute IMT and AT of the right and left common carotid arteries of preterm sheep were not significantly different to term sheep over the study period. When corrected for bodyweight, the IMT and AT of the right and left common carotid arteries of preterm sheep were significantly greater than controls, normalised by six months of age and were primarily due to the much lower bodyweight of preterm sheep rather than actual differences in vessel wall thickness. Our IMT results are in agreement with previous studies that demonstrated no association between carotid artery IMT and very preterm birth in newborns (Schubert et al. 2013) or birthweight and carotid artery IMT in middle-aged adults (Tilling et al. 2004). Nevertheless, there have been other studies that have shown a significantly increased common carotid artery IMT in children born very preterm (Lee et al. 2014) and in young adults born prematurely (Lazdam et al. 2010) or with very low birthweight (Hovi et al. 2011) compared to controls. Further investigation is required to determine the reason for the inconsistency especially given that an increased IMT is a marker for subclinical atherosclerosis in early adulthood (Skilton et al. 2011) and hypertension in later life (Raitakari et al. 2003).

With the exception of the peak systolic blood flow velocity in the main and left pulmonary arteries of male preterm sheep, there were no significant differences in the blood flow velocities in the major arteries of preterm sheep compared to controls over the study period. The reason for the blood flow velocity being significantly lower in these vessels is unclear, but the overall trend shows that preterm birth is not associated with changes in blood flow velocity. Compared to controls, the resistive indices of the right and left common carotid arteries over the 12 month period were not significantly different indicating the resistance to blood flow through the brain was not affected by moderate preterm birth.

The two day old anaesthetised preterm lambs in chapter three were compared to the two day old unanaesthetised preterm lambs in this chapter to determine if there were any effects of general anaesthesia on LV function and arterial blood flow. The only significant difference found was a lower peak systolic blood flow velocity in the proximal ascending aorta in both the male and female anaesthetised lambs. This result was in line with previous studies that have reported a reduction in aortic blood flow in healthy infants (Gueugniaud, P. et al. 1998b) and healthy adults (Gueugniaud, P et al. 1998) undergoing general anaesthesia using isoflurane. The LV percentage fractional shortening and peak systolic blood flow velocities of the main, right and left pulmonary arteries were also reduced in the anaesthetised lambs but not significantly. There were no significant differences between anaesthetised and conscious lambs with regard to the resistive indices of the right and left renal arteries, or the right and left common carotid arteries. This suggests that the resistance to blood flow through the brain and kidneys were not significantly affected by general anaesthesia which is in line with previous findings (Vatner 1978; Walther, S et al. 1997).

Evaluation of the right kidney and blood flow within the renal artery

There was no significant difference in the size (length, width, thickness and volume) of the right kidney in preterm female sheep compared to controls. However, in male preterm sheep the size of the right kidney was significantly smaller in all dimensions (except for thickness) compared to term controls. Moderate preterm birth disturbs nephrogenesis resulting in a reduced nephron endowment (Rodríguez et al. 2004) that may compromise subsequent renal growth and function (Gubhaju et al. 2014). Reduced ultrasound measurements of renal length and volume have been correlated to decreased renal function (Takata et al. 2016). Previous studies have found that preterm birth is associated with reduced kidney size and volume in infants (Schmidt et al. 2005) and young adults (Keijzer-Veen, M et al. 2010). The reason male, but not female, preterm sheep demonstrated a reduced kidney size is unclear and warrants further investigation. Relative to bodyweight, the size of the right kidney was significantly greater in both male and female preterm sheep compared to controls. The relative differences were normalised by three months of age and was more likely to have been influenced by the lower initial bodyweight and subsequent catch-up of the preterm lambs rather than adverse kidney growth.

Preterm lambs at term equivalent age (TEA) compared to control lambs at term

As previously discussed in chapter three, the disparity in growth between preterm lambs and control lambs at birth due to their different gestational ages was addressed by correcting for bodyweight. In this chapter it was possible to address the difference in growth in the immediate post-natal period by correcting for gestational age. This was achieved by comparing the preterm lambs at two weeks after birth (TEA) to term lambs at two days after birth (i.e. both groups compared around 149 days post conception).

It should be noted that despite having a lower birthweight, preterm lambs had significantly higher bodyweight at TEA compared to their counterpart controls at term (female $p = 0.0024$ and male $p = 0.0477$). Preterm lambs gained more weight in the first two weeks *ex utero* than term controls gained *in utero* during the corresponding two weeks. This is likely due to lambs born moderately preterm receiving better nutrition and oxygenation *ex utero* compared to lambs *in utero* during the final two weeks of a term gestation (Cock, M et al. 2005).

Comparing preterm lambs at TEA to control lambs at term has been widely reported in the literature (Burrell et al. 2003; Mayhew et al. 1997; Sozo et al. 2006). The purpose of the correction is to address the disparity in growth and development between preterm and term lambs at birth. The moderately preterm lambs in this study needed to mature a further two weeks after birth in order to reach the gestational age that was 'equivalent' to the lambs born at term. Comparisons with term lambs were then made at the same conceptional age (~147 days) so all the animals had the same period of time to mature and grow. However, correcting for gestational age does not take into account that the growth of the preterm lambs after birth takes place in an *ex utero* environment where they have to exist coping with structurally immature cardiovascular and renal systems.

At TEA, ultrasound imaging did not demonstrate any significant differences in the structure or function of the left ventricle of male and female preterm lambs compared to control lambs. Two weeks after birth, the growth of the left ventricle of the preterm lambs had caught up to the control lambs at term. Similarly, with the possible exception of the main pulmonary artery diameter which was greater in male and female preterm lambs, the structure of the major arteries of preterm lambs at TEA were equivalent to those of term lambs. The kidney dimensions were generally greater in the TEA lambs, although it was the female lambs that demonstrated significantly bigger kidneys compared to controls. This may be due to the female preterm lambs growing substantially during the first two weeks after birth (birthweight 4.33 ± 0.34 kg to 8.08 ± 0.49 kg at TEA, compared to 6.57 ± 1.06 kg for control lambs at term).

Post-natal lamb survival

Without the use of medical intervention or mechanical ventilation in the immediate post-natal period, the lambs born in our sheep model of preterm birth were at the limit of viability (De Matteo et al. 2010). Therefore, it was not surprising that six preterm lambs died of respiratory insufficiency associated with immature lung development and inadequate lung function within two weeks of birth. Two preterm lambs died during the study of accidental causes (trampled, broken leg) as a result of unexpected and acute physical trauma as distinct from disease. Surprisingly, four preterm lambs died of white muscle disease before reaching three months of age. White muscle disease, also known as ‘stiff muscle disease’ occurs mostly in newborn lambs up to three months of age (Robson 2007). It is associated with an inadequate intake of selenium or vitamin E (Beytut & Karatas 2002). White muscle disease causes hyaline degeneration and necrosis in the heart and skeletal muscles of lambs (Van Vleet & Ferrans 1986). The most obvious gross cardiac changes seen in white muscle disease is a chalky-white appearance of the endocardium of the right ventricle and chalky-white plaques in the endocardium of the left ventricle and interventricular septum (Cheema & Gilani 1978). The clinical signs of white muscle disease include muscle wasting leading to weakness, stiffness and difficulty in standing and walking (Deger et al. 2008). These clinical signs were observed in some of the preterm lambs as they approached three months of age. None of the term lambs showed any symptoms of the disease. The surviving preterm lambs were tested and found to have a low level of vitamin E in the blood. In addition, it is likely that the lambs had eaten feed pellets specifically formulated for adult sheep that contained substances that could induce ionophore toxicity in lambs (Jones 2001). After white muscle disease was confirmed in the sick lambs by a veterinarian, all preterm and term lambs were treated with a vitamin E supplement and provided feed pellets in their diet specifically formulated for lambs.

The four lambs that died from white muscle disease appeared healthy leading up to and during their last ultrasound examination. Death secondary to acute heart failure usually occurs within a few days once lambs exhibit the major clinical symptoms of white muscle disease (Gunes et al. 2010; Robson 2007). All four lambs died 24 to 63 days after their last ultrasound scan. The extent to which the lambs had white muscle disease at their last ultrasound examination remains unknown, as are any effects on the cardiovascular system and kidneys caused by the condition at that time. No gross abnormalities of the heart, major vessels and kidneys were visualised in any of the ultrasound examinations. Nevertheless, only the lambs that survived to the end of the study period were included in the analyses to reduce the possibility of confounding effects on the results.

Potential effects of Betamethasone

The two week post-natal survival rate for the preterm lamb cohort was 12/16 (75%) for males and 9/12 (75%) for females. By comparison, the survival rate to 14 days post-natal age for preterm lambs in a previous study using a similar model of preterm birth was significantly lower for males 18/41, (44%) than females 31/41, (76%) with 74% of the male deaths occurring within two days of birth (De Matteo et al. 2010). The clinically relevant dose of betamethasone (2 x 11.4 mg i.m.) administered to the ewes in our study may have increased male survival by progressing lung maturation more effectively compared to the non-clinical doses of 3 mg or 5.7 mg i.m. used by De Matteo. The benefits of glucocorticoid treatment for preterm delivery have been well documented but it has also been shown to adversely affect the development of various organs including the heart, blood vessels and kidneys depending on the type of glucocorticoid, dose and timing of delivery (Molnar et al. 2002; Moss et al. 2005).

Ultrasound imaging limitations

The scanning protocols and immobilisation techniques developed for the ultrasound examinations were sufficient to acquire all the required data except for visualisation of the left kidney from three months of age. The sheep stomach is comprised of four compartments - the rumen, reticulum, omasum and abomasum (Harfoot 1978). Young lambs with a diet restricted to milk have a collapsed, non-functioning rumen and reticulum (Ishihara, Ichikawa & Hotta 1985). When lambs begin to eat dry feed (around two to three months of age), various micro-organisms colonise the rumen and reticulum. These compartments then expand by a factor of four to occupy most of the abdominal cavity (Ishihara, Ichikawa & Hotta 1985). The rumen acts a large fermentation vat, digesting feed such as plant material and producing vast quantities of gas in the process (Harfoot 1978).

Ultrasound imaging cannot demonstrate structures that are located deep to an overlying gas filled bowel. Hence, we were not able to reliably demonstrate the left kidney in lambs from three months of age as they had been weaned by that stage and had a functioning rumen. An attempt was made to scan the left kidney in these older lambs via a posterior window (rather than laterally, or anteriorly), but having to penetrate the thick back musculature resulted in inadequate visualisation of the kidney. Conversely, there were no problems encountered in visualising the left kidney of young lambs that were still on a milk only diet and did not have an expanded and functioning rumen.

4.5. Conclusion.

It is well established that preterm birth results in adverse changes to cardiovascular and renal structures at the cellular level. Our serial ultrasound evaluation demonstrated few significant mal-adaptations of the cardiovascular system and kidneys in response to moderate preterm birth into early adulthood. It may be that the structural alterations due to moderate preterm birth are not gross enough to cause macroscopic changes and be displayed using ultrasound imaging. In addition, early adulthood may be too early for detrimental long term effects of preterm birth to become manifest. Further follow up evaluation of preterm sheep into later adulthood is warranted. Immediately prior to necropsy, there was an opportunity to conduct a further ultrasound evaluation on the cohort of sheep evaluated in this chapter, albeit under general anaesthesia. Therefore, another ultrasound assessment was performed on the sheep at around 14 months of age and the outcomes are reported in chapter five.

Chapter 5 Ultrasound evaluation of the effects of moderately preterm birth on the cardiovascular system and right kidney of anaesthetised sheep at 14 months of age.

5.1. Introduction.

In the previous chapter, serial ultrasound imaging was conducted in a cohort of sheep born at term or moderately preterm from birth to 12 months of age. The ultrasound investigation was conducted concurrently with another PhD project by Dr Vivian Nguyen (a student under the supervision of Professor Jane Black and Associate Professor Graeme Polglase) who performed a morphometric and physiological evaluation of the sheep (Nguyen, V et al. 2016). There was an opportunity to conduct a further ultrasound evaluation on these term and preterm sheep at 14 months of age, while they were under general anaesthesia, immediately prior to being euthanased for necropsy. The ultrasound analyses obtained at 14 months will complement the histological and physiological measurements obtained by Dr Nguyen and provide an opportunity to compare findings. The outcomes of Dr Nguyen's PhD research are supplementary to this project but the intention is to collaborate on all the findings once completed with a view to joint publication of the results. The data from this ultrasound evaluation at 14 months were not included as part of the serial study in chapter four because the sheep were under a general anaesthesia during ultrasound imaging. Whereas in the previous chapter all ultrasound measurements were performed on un-anaesthetised sheep.

The aims of this chapter were to use ultrasound imaging of male and female anaesthetised moderately preterm and term delivered sheep at 14 months of age to investigate and compare the:

1. structure and function of the left ventricle.
2. structure and blood flow within major arteries including the proximal ascending aorta and main, right and left pulmonary arteries and the common carotid arteries.
3. structure of the right kidney and blood flow in the right renal artery.

5.2. Methods.

5.2.1 Animal studies.

The long term cohort of sheep described in chapter four were transported from the Gippsland Field Station (where they were housed in paddocks) to the Monash Medical Centre Animal Facility in Clayton at approximately thirteen months of age. Further biometry (full body dual-energy x-ray absorptiometry - DEXA) and physiological measurements of the cardiovascular system (blood pressure/flow via catheters in the femoral artery and vein) were conducted on the animals by Dr Vivian Nguyen as part of her PhD project. The final physiological measurements were obtained by Dr Nguyen with the sheep under general anaesthesia (Isoflurane 2% in O_2 administered via endotracheal tube via a positive pressure ventilator) at 14 months ± 2 weeks of age. Before the sheep were euthanased for necropsy, an ultrasound evaluation of the left ventricle, major arteries and right kidney was performed.

5.2.2 Ultrasound imaging.

The ultrasound equipment and protocol for preparing the sheep for scanning are described in chapter 2 (section 2.4). No modification to the preparation and equipment required for the ultrasound examinations conducted at 12 months of age (chapter four) were required for the ultrasound examinations conducted at 14 months of age.

A P5-3 or P4-2 phased array cardiac probe with a ‘pediatric cardiac/general’ application setting were used for imaging the heart, proximal ascending aorta and pulmonary arteries. Curvilinear array abdominal probes (C7-4 with a ‘pediatric/pediatric renal’ application setting or C5-2 with an ‘abdomen/renal’ application setting) were used for imaging the right kidney and renal artery. A L12-5 MHz linear array small parts probe with a ‘cardiovascular/carotid’ application setting was used for scanning the common carotid arteries (Table 2-1).

The sheep were under general anaesthesia throughout the ultrasound examination and therefore did not require any immobilisation other than to have some strapping to secure them to the tabletop. The sheep were lying on their back on the examination table which was located next to the ventilator and ultrasound machine. The area on the left side of the chest over the heart, and anterior to the neck were both clipped immediately prior to the ultrasound scan in order to remove the wool (Figure 5.1).

Figure 5-1: An anaesthetised 14 month old sheep positioned for ultrasound examination.
The anaesthetised sheep was positioned on its back and the left side of the chest and anterior neck were shaved. The ventilator and ultrasound machine were located next to the table near the sheep's head.

Ultrasound evaluation of the LV, right kidney, proximal ascending aorta, main, right and left pulmonary arteries, right renal and both common carotid arteries were performed as described in Chapter 2 (section 2.4). A summary of the measurements are as follows:

Cardiac measurements:

- Left ventricle anterior wall thickness (mm) in systole and diastole.
- Left ventricle posterior wall thickness (mm) in systole and diastole.
- Interventricular septum wall thickness (mm) in systole and diastole.
- Left ventricle internal short axis chamber diameter (mm) in systole and diastole.

The measurements of each of the structures were corrected for bodyweight (mm/kg). The percentage fractional shortening of the left ventricle was calculated from the acquired ultrasound measurements as previously described in section 2.4 (Singh, R et al. 2009).

Measurements of the major arteries:

Proximal ascending aorta, main, right and left pulmonary arteries

- The maximum internal diameter (mm) of the proximal ascending aorta, main, right and left pulmonary arteries were measured. The measurements were corrected for bodyweight (mm/kg).
- The peak systolic velocity (cm/s) of four consecutive cardiac cycles in each of the proximal ascending aorta, main, right and left pulmonary arteries were measured and averaged.

Common carotid arteries

- Maximum external and internal diameter (mm).
- Maximum intima-media thickness, IMT, (mm) and adventitia thickness, AT, (mm) were measured and corrected for bodyweight (mm/kg).
- The maximum systolic and end diastolic blood flow velocity (cm/s) of three consecutive cardiac cycles were measured and averaged for each common carotid artery. The resistive index, RI, was calculated as previously described in section 2.4 (Allan et al. 2006).

Right kidney measurements:

- The maximum bipolar length (mm), width (mm) and thickness (mm) of the right kidney were measured and corrected for bodyweight (mm/kg). Volume measurements were calculated based on the length, width and thickness measurements (mm^3) as previously described in section 2.4 (Weitz et al. 2013).
- The maximum systolic and end diastolic blood flow velocity (cm/s) of three consecutive cardiac cycles were measured and averaged for the right renal artery and the resistive index, RI, was calculated as previously described in section 2.4 (Rabbia & Valpreda 2003).

5.3.3 Statistical analysis.

Statistical analyses were performed using GraphPad Prism software (version 6.04; GraphPad software, San Diego, CA, USA). Data were analysed using a two-way analysis of variance (ANOVA), with gestational age at birth (preterm versus term) and sex (male versus female) as factors. A Tukey's post-hoc test was used to determine which data points were significantly different. Data are presented as mean ± standard error of the mean (SEM). Statistical significance was accepted at $p < 0.05$.

5.3. Results.

Sheep bodyweight

All the sheep were weighed immediately prior to the ultrasound examination. There was no significant difference in the average bodyweight of the preterm sheep compared to term sheep at 14 months of age.

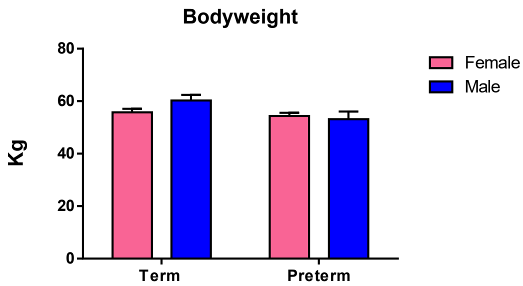

Figure 5-2: Average bodyweight of preterm sheep compared to term sheep at 14 months of age.
Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different.

5.3.1 Ultrasound imaging.

There were no difficulties encountered in preparing the sheep or handling them during the ultrasound examination as they were under general anaesthesia. Images of the heart, right kidney, major arteries and blood flow velocity data were successfully acquired for all the sheep. No gross abnormalities or pathophysiology of the left ventricular structures, right kidney or major arteries were evident in any of the ultrasound images obtained.

5.3.2 Ultrasound measurements.

Structural and functional analysis of the left ventricle at 14 months of age

There were no significant differences in the measurements of the left ventricle between preterm and term sheep at 14 months of age. Specifically, the mean systolic and diastolic thickness of the anterior and posterior walls of the LV, IVS and the mean systolic and diastolic internal chamber diameter of the LV in preterm sheep were not significantly different to those of sheep born at term (Figures 5-3, 5-4).

When corrected for bodyweight, there were no significant differences in the LV parameters except for the mean diastolic thickness of the LV anterior wall which was found to be significantly thicker ($p = 0.0303$) in the preterm sheep (Figures 5-3, 5-4).

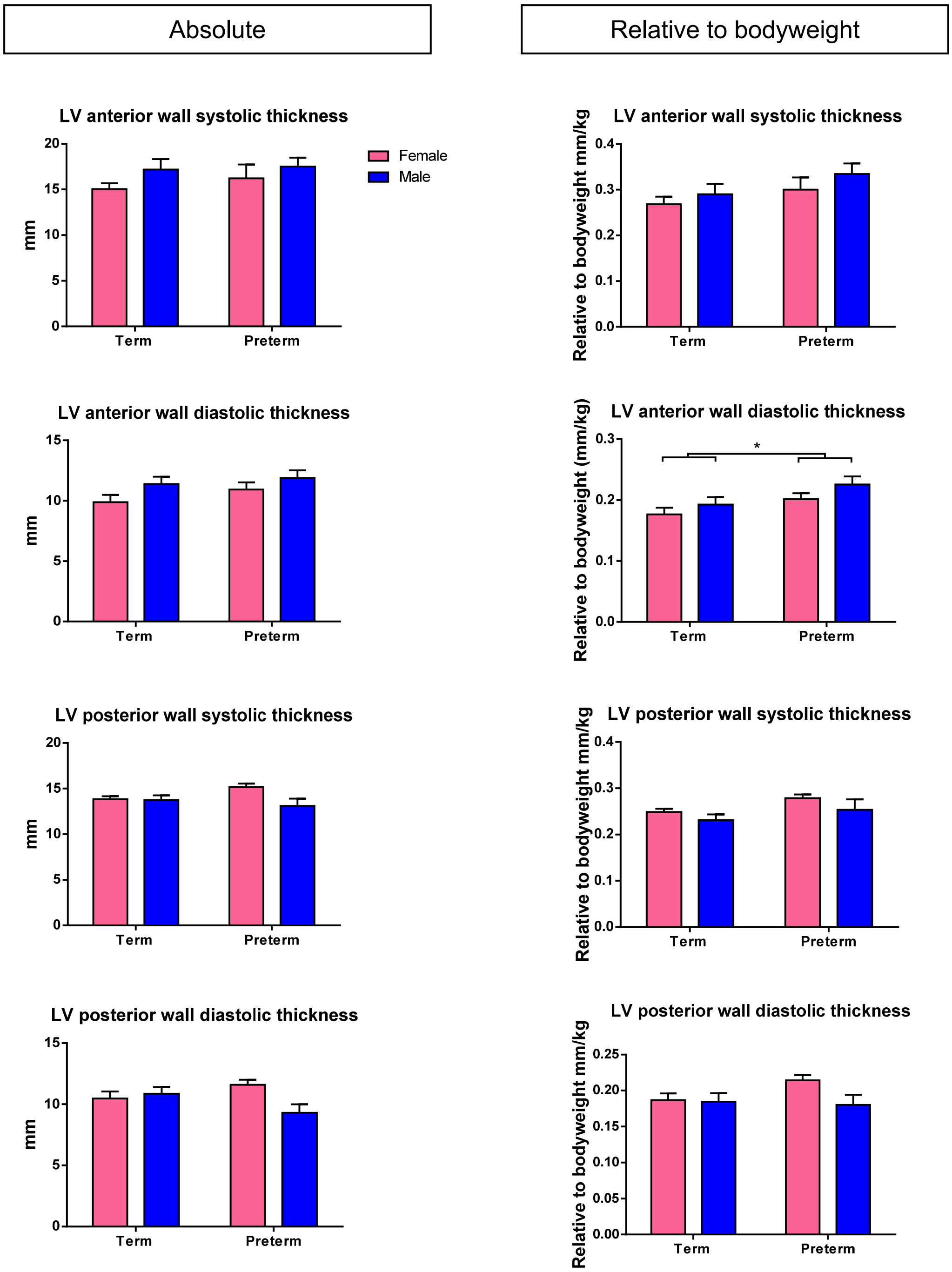

Figure 5-3: Mean systolic and diastolic left ventricle (LV) anterior and posterior wall thickness (absolute and relative to bodyweight) of preterm and term sheep at 14 months of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. *$p < 0.05$ following Tukey's post-hoc analysis.

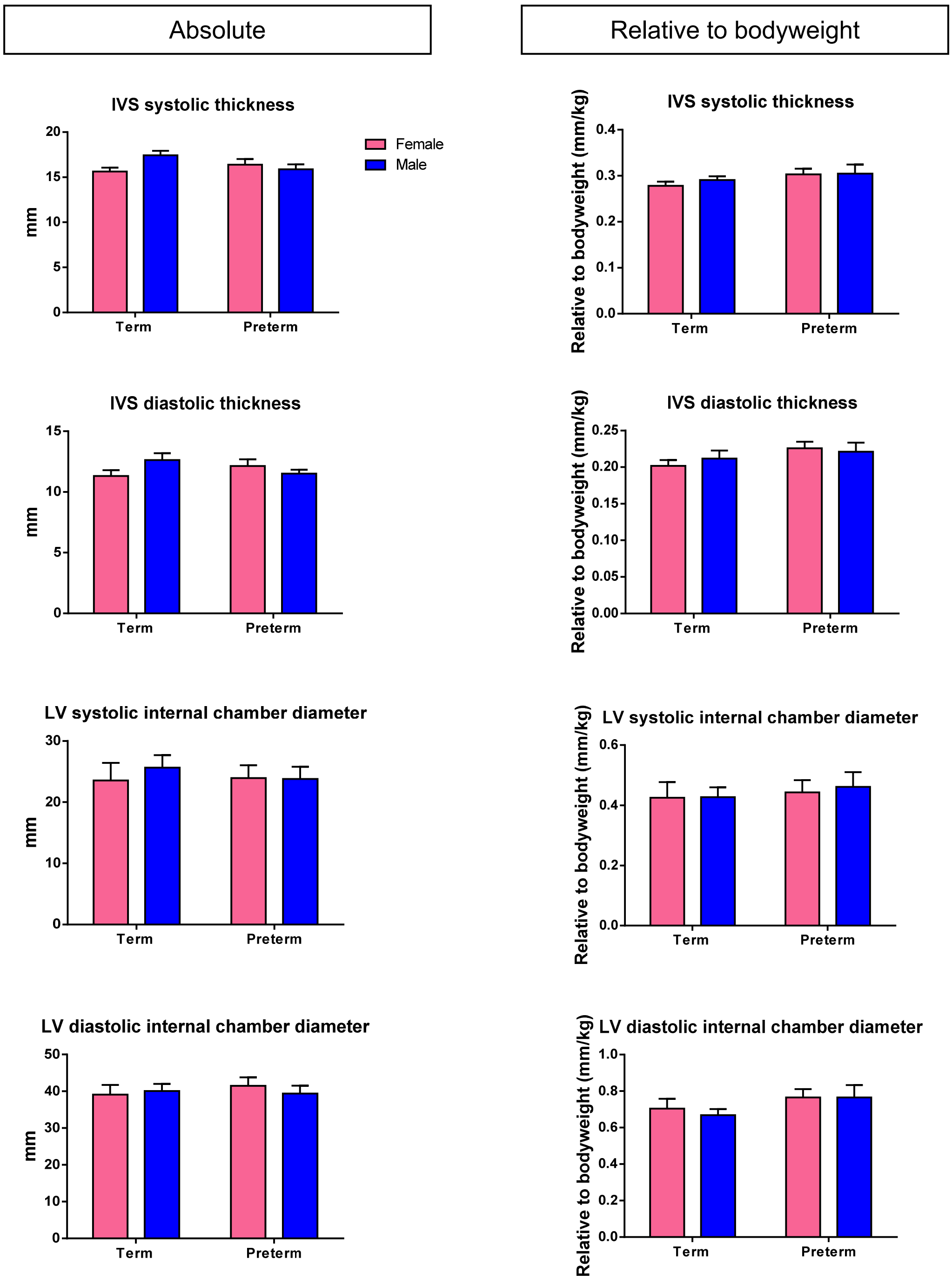

Figure 5-4: Mean systolic and diastolic interventricular septum (IVS) thickness and internal chamber diameter (and relative to bodyweight) of preterm and term sheep at 14 months of age.
Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different.

There was no significant difference in the average percentage fractional shortening of the left ventricle of preterm sheep compared to controls (Figure 5.5).

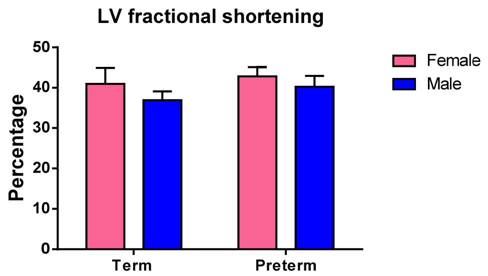

Figure 5-5: Mean left ventricle (LV) percentage fractional shortening of preterm and term sheep at 14 months of age.
Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different.

No significant sex-specific differences were demonstrated in any of the parameters measured in the left ventricle of preterm and term sheep.

Structural and blood flow analysis of the proximal ascending aorta and main, right and left pulmonary arteries at 14 months of age

There were no significant differences in the mean internal diameters of the proximal ascending aorta or main, right and left pulmonary arteries of 14 month old sheep born moderately preterm compared to those born at term.

No significant differences between preterm and term sheep were found when these diameter measurements were corrected for bodyweight, except for the mean internal diameter of the proximal ascending aorta which was significantly greater ($p = 0.0159$) in the moderately preterm sheep (Figure 5-6).

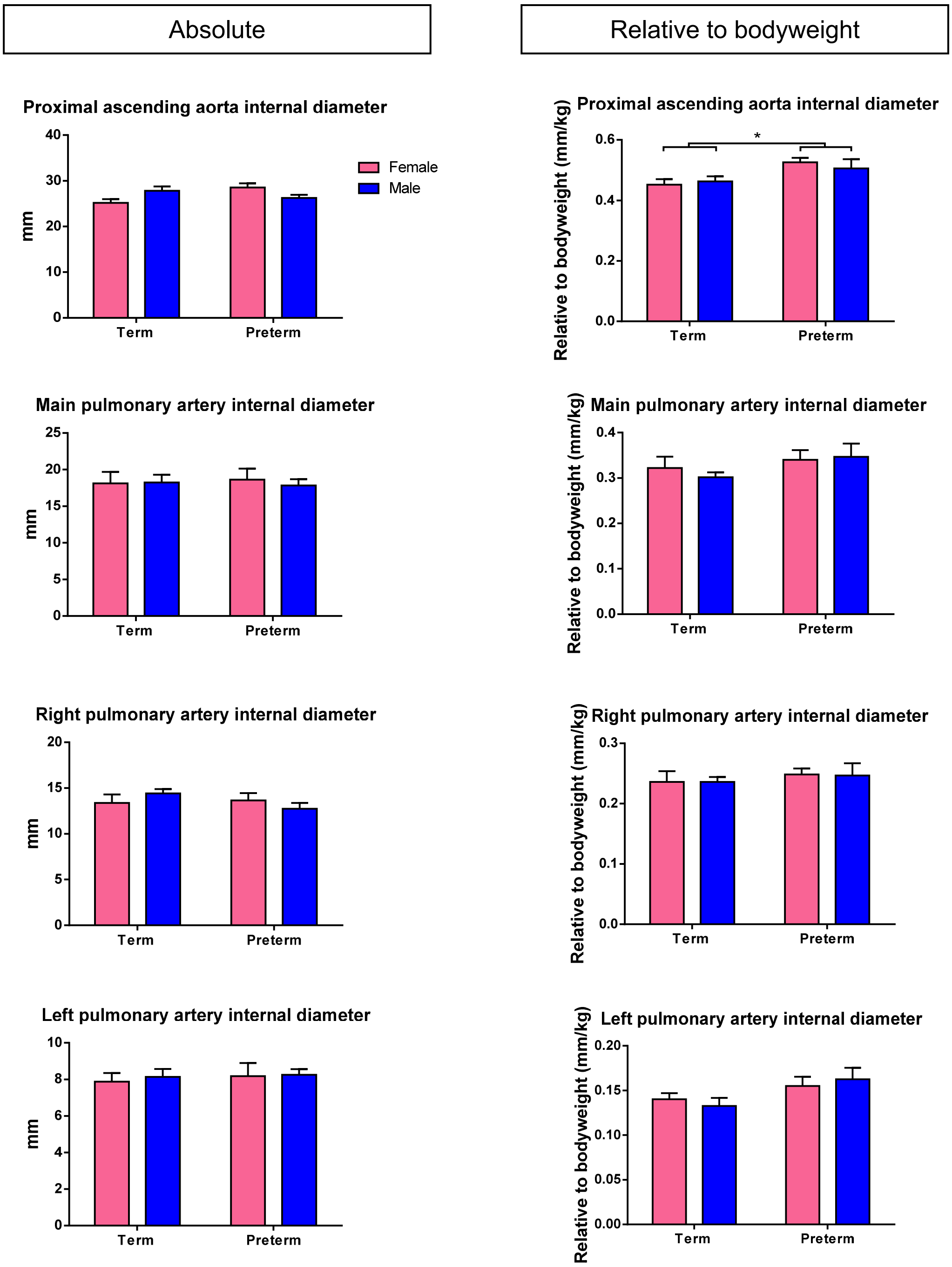

Figure 5-6: Mean internal diameter (and relative to bodyweight) of the proximal ascending aorta, main, right and left pulmonary arteries of preterm and term sheep at 14 months of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. $^{*}p < 0.05$ following Tukey's post-hoc analysis.

The average peak blood flow velocities in the proximal ascending aorta, main, right and left pulmonary arteries were not significantly different in moderately preterm sheep compared to sheep born at term (Figure 5-7).

There were no significant sex-specific differences in any of the measured parameters of the aorta and pulmonary arteries in moderately preterm and term sheep.

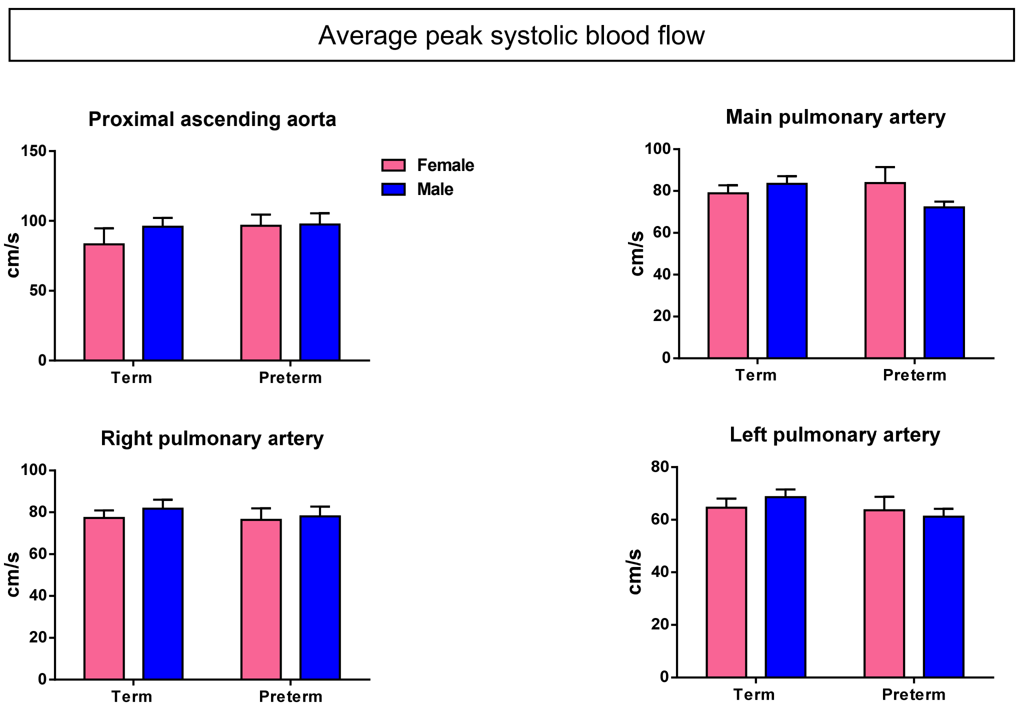

Figure 5-7: Average peak systolic blood flow velocities of the proximal ascending aorta, main, right and left pulmonary arteries of preterm and term sheep at 14 months of age.

Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different.

Structural and blood flow analysis of the common carotid arteries at 14 months of age

With the exception of the AT of the left common carotid artery, there were no significant differences in the mean external or internal diameters, IMT and AT measurements of the right and left common carotid arteries of 14 month old moderately preterm sheep compared to controls. The AT of the left common carotid artery of moderately preterm sheep was significantly smaller ($p = 0.0285$) than the AT of the term sheep (Figures 5-8, 5-9).

No significant differences between preterm and term sheep were found in the vessel diameters or wall thickness measurements when corrected for bodyweight, other than the mean internal diameter of the right common carotid artery which was significantly greater ($p = 0.0278$) in the preterm sheep compared to controls (Figure 5-8). Although not statistically significant ($p = 0.1790$), the mean internal diameter of the left common carotid artery was larger in the preterm compared to term sheep, possibly indicating a trend towards a larger internal common carotid artery diameter relative to bodyweight in moderately preterm sheep.

Sex-specific differences were demonstrated with male sheep having significantly greater mean external diameters (right $p = 0.0002$, left $p = 0.0004$) and internal diameters (right $p < 0.0001$, left $p = 0.0007$) of the common carotid arteries compared to female sheep (Figure 5-8). When corrected for bodyweight, male sheep had a significantly greater mean external diameter of the right CCA ($p = 0.0039$), and greater internal diameters of the right CCA ($p < 0.0005$) and left CCA ($p = 0.0161$) compared to female sheep (Figure 5-8). Relative to bodyweight, there was no difference in the left common carotid artery mean external diameter between male and female sheep (Figure 5-8).

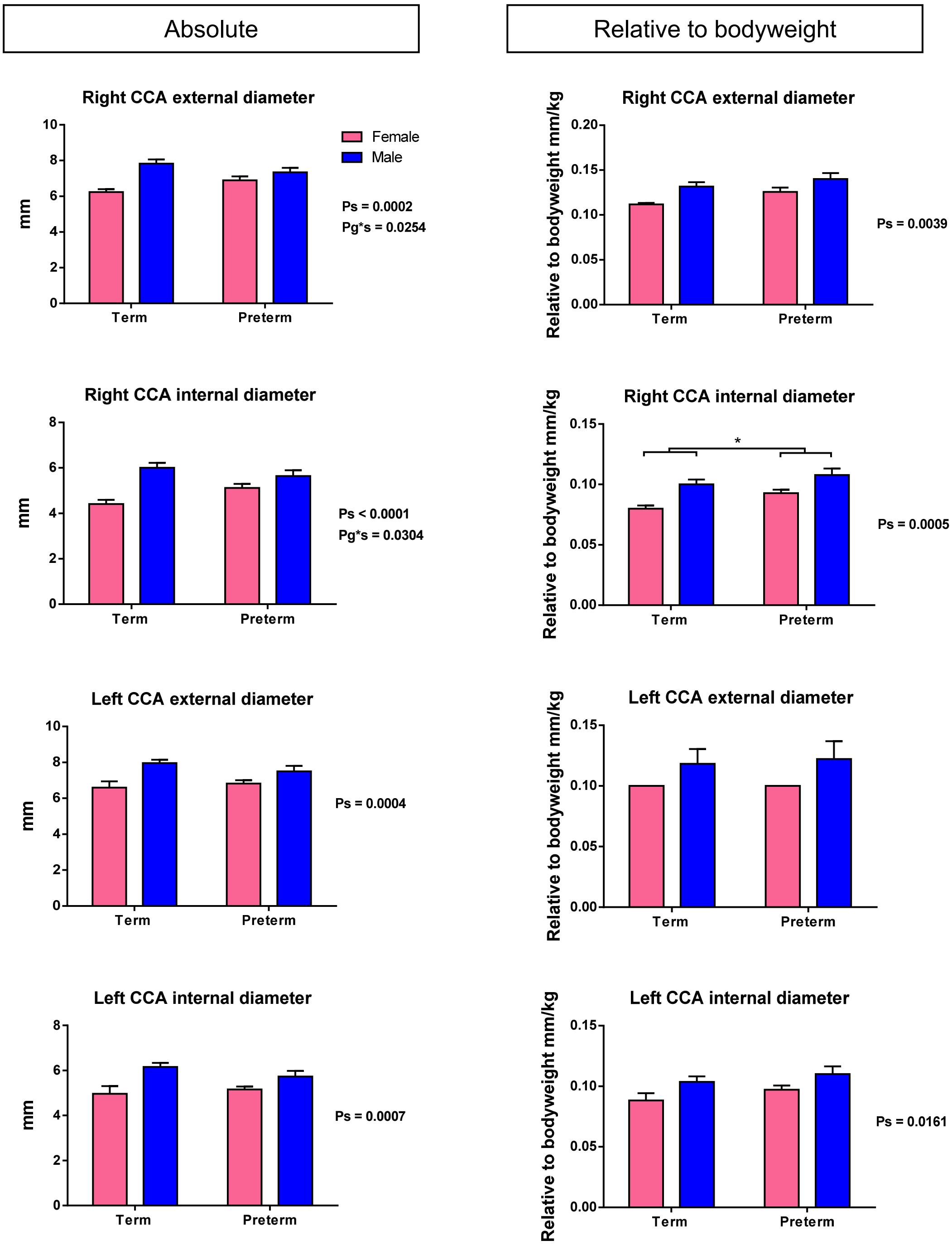

Figure 5-8: Mean external and internal diameter (and relative to bodyweight) of the right and left common carotid arteries (CCA) of preterm and term sheep at 14 months of age.
Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. *p < 0.05 following Tukey's post-hoc analysis.

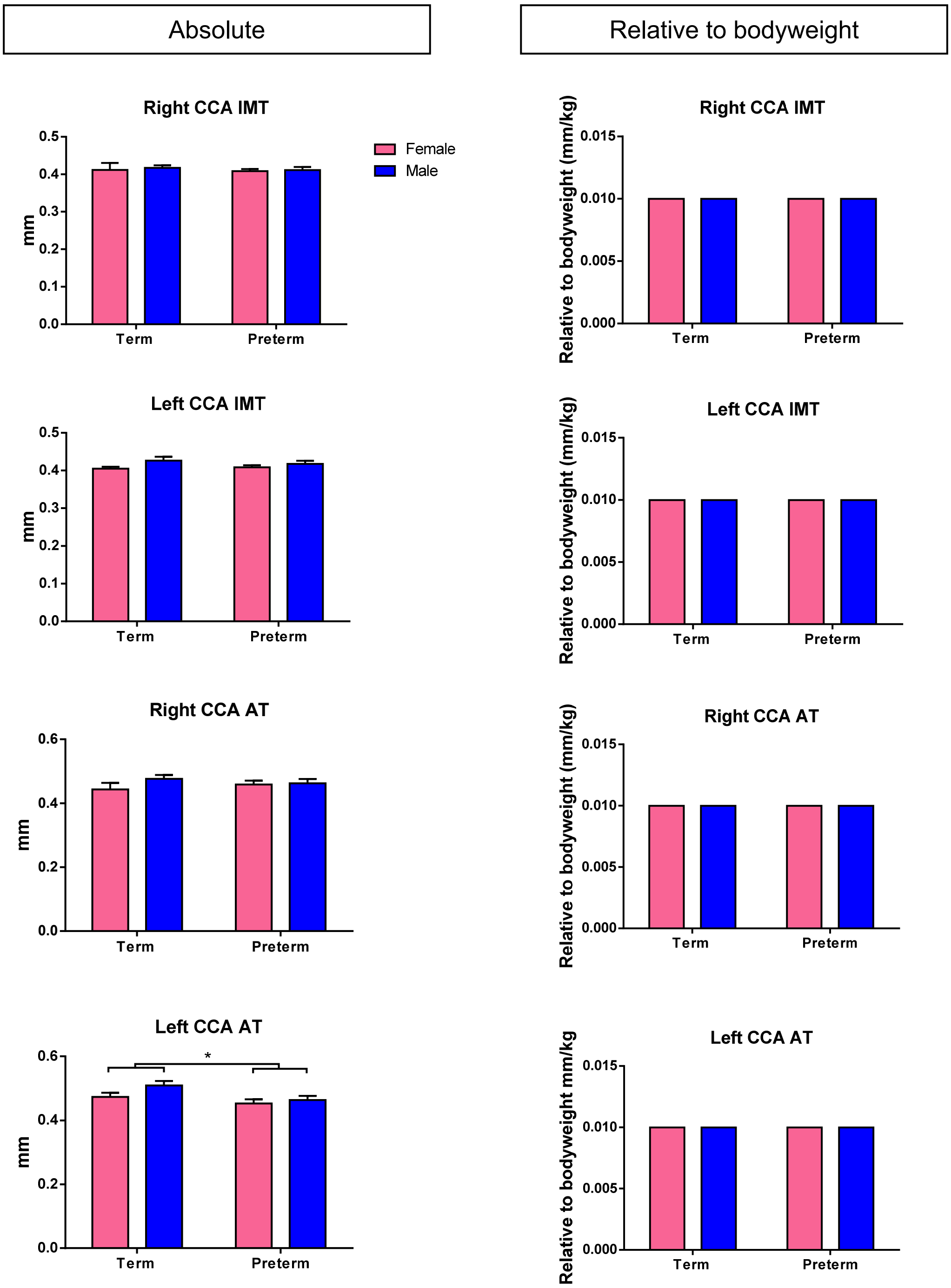

Figure 5-9: Mean intima-media thickness (IMT) and adventitia thickness (AT) (and relative to bodyweight) of the right and left common carotid arteries (CCA) of preterm and term sheep at 14 months of age.
Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. *$p < 0.05$ following Tukey's post-hoc analysis.

The resistive indices of the right and left common carotid arteries were not significantly different in moderately preterm sheep compared to term sheep at 14 months of age. However, male sheep had significantly higher resistive indices in both the right and left common carotid arteries compared to female sheep (Figure 5-10).

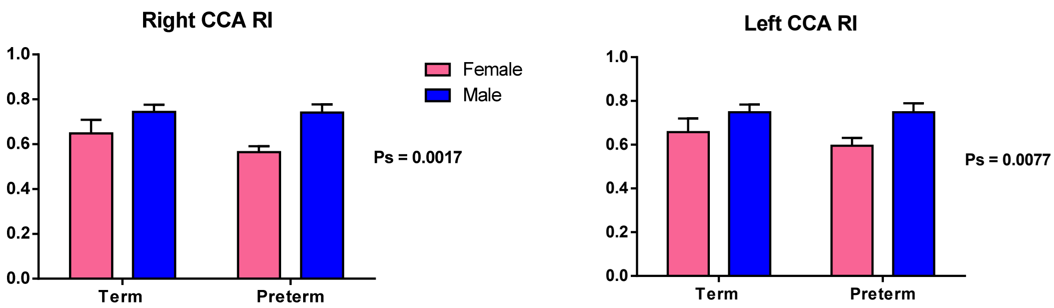

Figure 5-10: Resistive indices (RI) of the right and left common carotid arteries (CCA) of preterm and term sheep at 14 months of age.
Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. A Tukey's post-hoc test was used to determine which data points of the factors were significantly different.

Analysis of right kidney size and right renal artery blood flow at 14 months of age

There were no significant differences in mean bipolar length, width, thickness or volume of the right kidney of moderately preterm sheep compared to controls. No significant differences between preterm and term sheep were found when these measurements were corrected for bodyweight, except for the mean kidney bipolar length which was significantly greater ($p = 0.0403$) in the moderately preterm sheep compared to term sheep (Figure 5-11).

Male sheep had significantly greater right kidney volume ($p = 0.0463$) than female sheep but there was no significant difference when kidney volume was corrected for bodyweight (Figure 5-11).

There was no significant difference between moderately preterm sheep and controls, or between male and female sheep, in the RI of the right renal artery at 14 months of age.

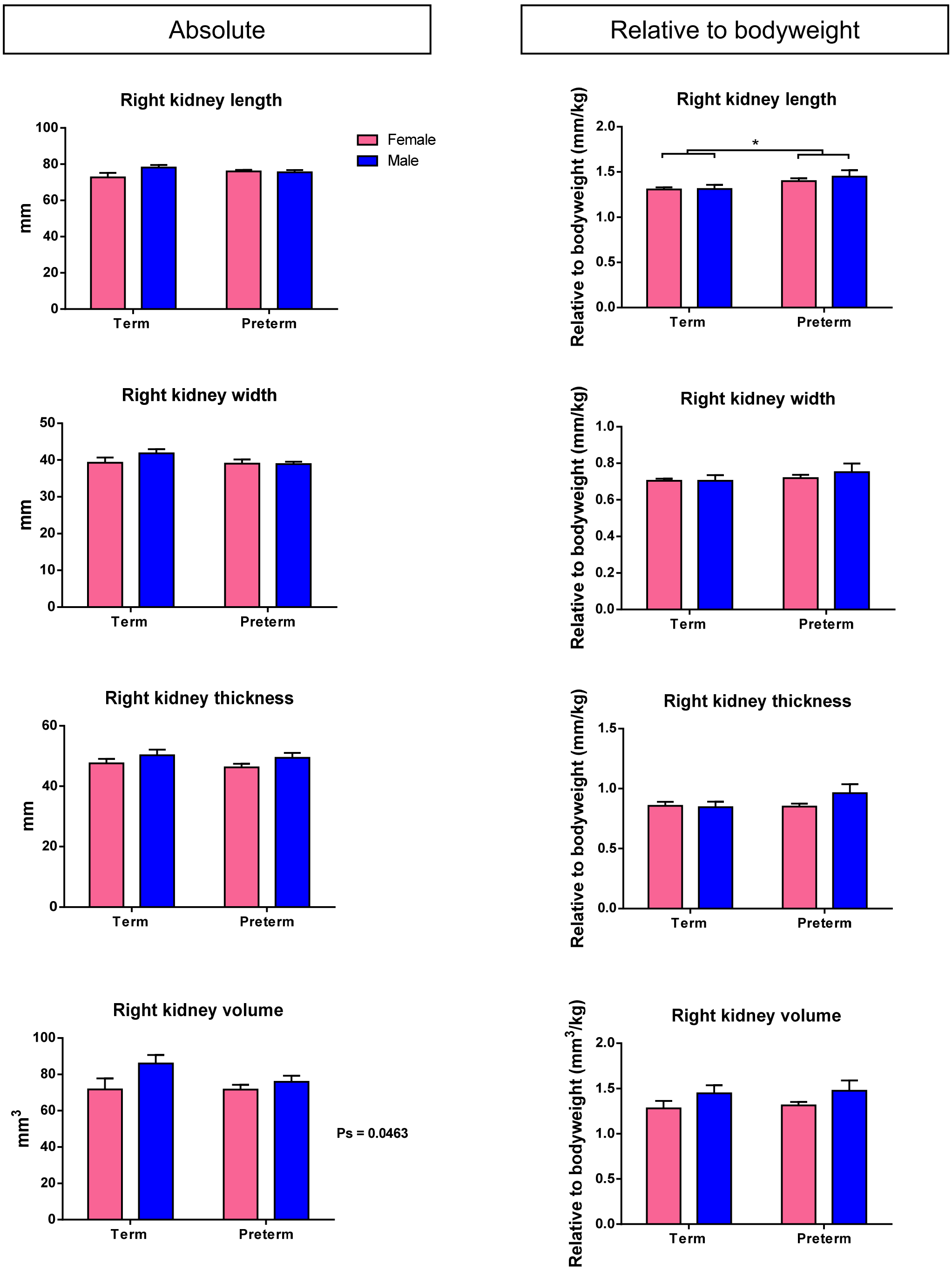

Figure 5-11: Right kidney dimensions (and relative to bodyweight) of preterm and term sheep at 14 months of age.
Data represented as mean ± SEM. Data were analysed using a two-way analysis of variance (ANOVA) with gestational age at birth (Pg) and sex (Ps) as factors. *p < 0.05 following Tukey's post-hoc analysis.

5.4. Discussion.

As expected, imaging of the left ventricle, major arteries and the right kidney in anaesthetised 14 month old sheep was relatively straightforward and easy to perform, when compared to the scanning of 12 month old conscious sheep that required restraining during the ultrasound examination (Chapter 4).

In this study there were very few differences in the structure and function of the left ventricle, major arteries and the right kidney at 14 months of age in sheep born moderately preterm when compared to those born at term. When adjusted for body weight, there were significant increases in relative LV anterior wall thickness in diastole, internal diameter of the proximal ascending aorta and right common carotid artery and in the length of the right kidney. All other measurements (absolute or relative) were not different, except for a significant decrease in the left common carotid artery AT thickness (but this was not different when adjusted for bodyweight). Interestingly, significant differences between the sexes were detected in the diameter and blood flow within the common carotid arteries and in the volume of the right kidney, yet these sex-specific differences were not apparent when the sheep were evaluated at 12 months of age. There was no difference in the structural or physiological responses to moderate preterm birth between the sexes. Overall, the findings of this study, conducted in a relevant large animal pre-clinical model are encouraging in the clinical context, considering that the majority of infants that are born prematurely are born moderately preterm.

Evaluation of the structure and function of the left ventricle

Based on previous studies in humans, we expected that by 14 months of age, when sheep are sexually mature and in early adulthood, there would be evidence of some anatomical and/or functional adaptations of the heart. Indeed, maladaptive remodelling of the heart wall and impaired cardiac function subsequent to preterm birth has previously been demonstrated in a cohort of 102 young adults (aged 20 to 39 years) using magnetic resonance imaging (MRI) (Lewandowski et al. 2013; Lewandowski et al. 2013b). In those studies, preterm birth was associated with an increase in LV wall mass, decreased internal chamber diameter, altered wall geometry and reduced systolic and diastolic function (Lewandowski et al. 2013). These effects were even more pronounced in the right ventricle (Lewandowski et al. 2013b). The difference in findings between the current study conducted in adult sheep born moderately preterm and the MRI studies in humans may relate to species differences, techniques of assessment, the degree of prematurity and/or other confounding factors associated with studies in humans. In the MRI studies conducted by Lewandowski et al. (2013), the majority of participants were born very or extremely preterm (55% and 14%, respectively) with only 31% of the individuals born moderately preterm. In particular, the severity of the cardiac changes were inversely related to gestational age at delivery; each week of prematurity increased left ventricular mass by 1.5% and right ventricular mass by 2.7% (Lewandowski et al. 2013b). In addition to prematurity, elevated blood pressure was found to be a contributing factor to the increased LV mass found in young adults born preterm (Lewandowski et al. 2013). The effects of increased blood pressure may also contribute to the difference in findings with our study. Notably, there were a cohort of individuals studied by Lewandowski et al. (2013) that were born growth restricted, which may have contributed to the altered long-term cardiac growth. Hence, the relative lack of effects in our adult preterm sheep, may relate to the fact that they were born moderately preterm and at the time of birth they were appropriately grown for gestational age. Interestingly, in support of our findings, a recent echocardiography study conducted among 18 year old humans (n = 109) found only minor cardiac effects in participants that were born extremely preterm (Kowalski et al. 2016). Hence, the differences in findings between studies may instead relate to the methods of assessment. It is possible that MRI analyses can detect structural differences in the heart that may not be apparent using ultrasound imaging.

Evaluation of the structure and blood flow within the major arteries

With one exception, there was no evidence that moderate preterm birth resulted in any significant changes to the structure (mean internal diameter) or peak systolic blood flow velocity of the proximal ascending aorta or main, right and left pulmonary arteries compared to controls. The only detectable difference in the blood vessels examined was a significant increase in the mean internal diameter of the proximal ascending aorta when corrected for bodyweight ($p = 0.0159$) in the preterm sheep compared to term sheep. This finding is likely a consequence of lower bodyweight (albeit not statistically significant) in the preterm sheep. Certainly, in chapter 4, there was a significant decrease in body growth from weaning to 12 months of age in the preterm sheep. Notably, in the present study there was no evidence of narrowing of any of the blood vessels investigated in adulthood. These findings are in contrast to previous studies in young adulthood of individuals born preterm that report significant narrowing of the aorta (Bonamy et al. 2005; Kowalski et al. 2016). The internal diameter of the ascending aorta in a cohort (n = 109) of 18 year olds born extremely preterm (Kowalski et al. 2016) and the abdominal aorta in a cohort (n = 34) of 16 year olds born ≤ 34 (mean 29) weeks gestational age (Bonamy et al. 2005), were found to be significantly narrower when compared to age-matched individuals born at term. Transient growth arrest of the abdominal aorta, in response to the significant reduction in blood flow following early cessation of the placental circulation associated with preterm birth, can lead to narrowing of the vessel (Langille, Brownlee & Adamson 1990). The greater the prematurity at birth, the more likely that aortic structural remodelling will persist into adulthood (Bonamy et al. 2005). Therefore, the severity of prematurity (very and extremely preterm versus moderately preterm) likely accounts for the difference in findings between our investigation and other studies. Nevertheless, it is encouraging that we did not find statistically significant associations between arterial narrowing and moderate preterm birth given that the overwhelming majority of premature deliveries in humans are moderate rather than very or extremely premature.

Our results show little evidence of significant differences in the composition (internal and external vessel diameter, IMT and AT) and blood flow (resistive index) of the right and left common carotid arteries of moderately preterm sheep compared to controls. After correcting for bodyweight, the internal diameter of the right common carotid artery was significantly larger ($p = 0.0278$) in the preterm sheep compared to term sheep. The internal diameter of the left common carotid artery and the external diameters of the right and left common carotid arteries, when corrected for bodyweight, also tended to be larger in the preterm cohort but the differences were not statistically significant. Apart from the one exception, our results are in agreement with other studies involving ultrasound evaluation of young adults aged 18-27 years (Hovi et al. 2011) and children aged 7-12 years (Bonamy et al. 2008), that found that the diameter of the common carotid artery was not significantly affected by very preterm birth compared to controls. It remains unclear as to why the right common carotid artery, but not the left common carotid artery, relative to bodyweight diameter would be different to controls in our preterm sheep. A study using computerised tomography angiography (CTA) did not find any significant difference in vessel lumen between the right and left common carotid arteries at the level of the bifurcation in adults (Choudhry et al. 2016). However, it is possible that differences in the right and left common carotid artery diameter measurements could be related to the assessment method, specifically, ultrasound imaging and versus CTA. Ultrasound imaging provides less accurate morphometry of the carotid artery lumen and outer wall boundaries than CTA analyses (Shaalan et al. 2008). Further research is required, particularly studies using ultrasound imaging, to determine whether the size of the common carotid artery diameter differs significantly from the contralateral common carotid artery.

There was no significant difference in the IMT or AT of the right common carotid artery and no difference in the IMT or relative to bodyweight AT of the left common carotid artery of moderately preterm sheep compared to controls. However, the absolute AT of the left common carotid artery of moderately preterm sheep was significantly lower (p = 0.0285) relative to the term cohort. The clinical importance of a reduced AT is unknown. However, inflammation and chronic infection have been shown to lead to an increased AT which has been associated with an increased risk of hypertension in animal studies and humans (Kazmierski et al. 2009; Skilton et al. 2009). There is a significant correlation between AT and IMT (Kazmierski et al. 2009). An increased IMT is an established predictor of cardiovascular risk in asymptomatic adults (Lazdam et al. 2010) and a strong predictor of stroke and myocardial infarction in later life (O'Leary et al. 1999). The reduced AT was not accompanied by a reduced IMT of the left common carotid artery or a reduced AT of the right common carotid artery in the moderately preterm sheep. Common carotid artery wall thickness can vary from the contralateral side due to the focal nature of atherosclerotic changes (Howard et al. 1994). However, given that the IMT and relative to bodyweight AT of both common carotid arteries were not significantly different between moderately preterm and term sheep, it is likely this finding is incidental or at least not a marker of increased cardiovascular risk.

Our finding of no significant difference in the IMT of the right or left common carotid arteries of moderately preterm sheep compared to controls was in agreement with previous studies of young adults born preterm. Compared to controls, no significant difference in common carotid artery IMT was found in young adults born extremely preterm (Kowalski et al. 2016), very preterm (Finken et al. 2006) or moderately preterm (Kerkhof et al. 2012). However, there have been conflicting studies that have shown an increased carotid artery IMT relative to the lumen in young adults born moderately preterm (Hovi et al. 2011) and an increased IMT in young adults born very preterm whose mother was hypertensive during pregnancy (but not if they had a normotensive pregnancy) (Lazdam et al. 2010).

Sex-specific differences

A number of significant sex-specific differences were found in the common carotid artery parameters of 14 month old sheep. Males demonstrated greater external and internal diameter measurements of the right and left common carotid arteries compared to females. When corrected for bodyweight, the outcome was the same except for the external diameter of the left common carotid artery which was not significantly different between male and female sheep. These results are in agreement with human studies that have shown that the internal diameter of the common carotid artery is larger in men compared to women (Krejza et al. 2006; Ruan et al. 2009).

The resistive index of the common carotid artery is a measure of the downstream (cerebral) circulatory resistance to blood flow (Lima et al. 2002). The carotid artery IMT is acknowledged as being an accurate and reliable marker of cardiovascular risk (Ohta et al. 2008). The RI has been shown to be correlated with the carotid artery IMT and could therefore be used as a complementary surrogate marker of cardiovascular risk (Frauchiger et al. 2001). In our 14 month old sheep, the resistive indices of the right and left common carotid arteries were greater in the male sheep compared to females in both preterm and term cohorts. This is broadly in agreement with studies showing that the common carotid artery resistive index is either higher in healthy men compared to women (Albayrak et al. 2007), or not significantly different (Yazici, Erdogmus & Ali 2005). Having a higher common carotid artery RI compared to females does not necessarily lead to reduced brain perfusion in males. The brain also receives blood via the vertebral arteries which affects the total cerebral blood flow that has been shown not to be significantly different between men and women (Dörfler et al. 2000; Yazici, Erdogmus & Ali 2005). It is likely that the higher RI in this study is not an indicator of increased cardiovascular risk in males compared to females, given there were no sex-specific differences of the IMT of both common carotid arteries, which is a more reliable measure of cardiovascular risk (Ohta et al. 2008). Nevertheless, it is possible the significantly increased resistive indices are related to impaired cerebral perfusion due to atherosclerosis, and therefore, further investigation of sex-specific differences of the common carotid artery RI and correlation with IMT and cardiovascular risk is warranted.

Evaluation of the right kidney and blood flow within the renal artery

In regard to kidney size and function, there was only one significant difference in the size (bipolar length, width, thickness and volume) of the right kidney, or resistance to blood flow in the right renal artery, in 14 month old sheep born moderately preterm when compared to controls. When corrected for bodyweight, the bipolar length of the right kidney was significantly greater ($p = 0.0403$) in the preterm cohort compared to sheep born at term. All other measurements (absolute or relative) were not significantly different, indicating moderate preterm birth has little effect on the kidneys of sheep in early adulthood. There is a scarcity of studies in the literature on the effects of preterm birth on kidney size in adults and further research is required. However, our results are broadly in agreement with a recent study of 20 year olds, born very preterm, that reported no significant difference in the size of the right kidney compared to controls born at term (Keijzer-Veen, M et al. 2010). In contrast, a previous study of aboriginal adults found low birthweight to be associated with reduced renal size, however, the report did not state whether the subjects had IUGR which could have affected the findings (Singh, GR & Hoy 2004). The effect of preterm birth on kidney size in younger people appears to be variable. In children born very preterm, no difference in the length and volume of the kidneys compared to term controls were demonstrated at 8 years of age (Vanpée et al. 1992). Children born extremely preterm also had normal kidney length and volume at 6 to 12 (mean 8.6) years of age, but did demonstrate reduced renal function compared to controls (Rodriguez-Soriano et al. 2005). In contrast, another study of 6 to 7 year old children, also born extremely premature, found they had smaller kidney volume compared to term controls (Kwinta et al. 2011). It appears the effects of preterm birth on kidney size in children is related to the severity of prematurity and the age at which the kidneys are evaluated. Our results did not show a tendency towards reduced right kidney dimensions in moderately preterm sheep during young adulthood. In fact, when corrected for bodyweight, the right kidney width, thickness and volume of moderately preterm sheep tended to be greater than in controls, but without being statistically significant. This can be attributed to the lower bodyweight of the preterm sheep affecting the relative results, rather than any effect due to kidney size, given that the absolute kidney dimensions were not significantly different between the moderately preterm sheep and controls.

The right kidney volume of female sheep was found to be significantly lower than male sheep ($p = 0.0463$) at 14 months of age. However, this sex-specific difference was no longer apparent when the kidney volume was adjusted for bodyweight. This is in agreement with a study of 20 year olds that found very preterm females had significantly lower kidney volumes than males (Keijzer-Veen, M et al. 2010). A study of low birthweight aboriginal children and adolescents (aged 5 to 18 years) also found females had significantly smaller renal volumes (absolute and relative to body surface area) than males (Spencer, Wang & Hoy 2001). In contrast, other studies found no sex-specific differences in the kidney volume and kidney length of children and adolescents aged up to 18 years (Kim, J-H et al. 2013), or in the kidney length and width of healthy neonates, infants and children from 5 days to 16 years of age (Konuş et al. 1998). It is likely that females have smaller kidney size compared to males simply because males have greater height and bodyweight and would therefore have proportionally larger kidneys.

The renal artery resistive index is a measure of the intra-renal arterial impedance to blood flow. Any change to the renal microvasculature that causes significant haemodynamic changes to blood flow through the kidney will result in an altered renal artery RI. A raised renal artery RI is a well-established marker for renal atherosclerosis, hypertension and chronic renal failure (Petersen, L et al. 1995; Petersen, L et al. 1997; Pontremoli et al. 1999). The resistance to blood flow through the right kidney was not significantly different in our preterm sheep, compared to those born at term, indicating moderate preterm birth has little effect on the renal microvasculature in early adulthood.

Overall, the ultrasound findings at 14 months were generally similar to those reported at 12 months of age (chapter four) except for the greater diameter size and resistance to blood flow in the common carotid arteries of male sheep compared to females. The increased diameter of common carotid arteries in 14 month old male sheep cannot be attributed to the use of general anaesthesia. General anaesthesia has not been shown to significantly affect the diameter of the carotid arteries in humans and baboons (Du Boulay & Symon 1971). It is more likely that the increased diameter size is due to larger male sheep simply having bigger carotid arteries than smaller female sheep at 14 months of age. It remains unclear why the resistive indices of the common carotid arteries of male sheep would be significantly greater than females at 14 months, but not 12 months of age. General anaesthesia can alter regional vascular resistances (as in the cerebral circulation) even though blood flow may only be slightly affected (Vatner 1978). Nevertheless, potentially significant sex-specific differences in response to the administration of general anaesthesia cannot be discounted. Indeed, a study comparing men and women undergoing elective surgery revealed women were less sensitive to general anaesthetic medication, emerge faster from anaesthesia and with a poorer quality of recovery compared to men (Buchanan, Myles & Cicuttini 2011). Sex-specific differences in response to general anaesthesia appear to be influenced by female sex hormones, but the exact mechanism is uncertain (Buchanan, Myles & Cicuttini 2009). Further research is required to clarify the role of female sex hormones in the response to general anaesthesia and to determine whether any sex-specific differences are clinically important.

5.5. Conclusion.

The left ventricle, major arteries and the right kidney of sheep at 14 months of age appear to be largely unaffected by moderate preterm birth. Given the majority of preterm births are moderately preterm, this is an encouraging outcome from a clinical perspective. However, there were some significant sex-specific differences demonstrated in the volume of the right kidney and diameter size and blood flow within the common carotid arteries. Further study is required to determine if the sex-specific differences that have become apparent for the first time at 14 months of age, continue into later adulthood and are associated with an increased risk of hypertension and cardiovascular disease.

Chapter 6 Ultrasound evaluation of the cardiovascular system and kidneys of male and female moderately preterm lambs at two hours after birth.

6.1. Introduction.

Males born preterm are at greater risk of morbidity and mortality in the immediate post-natal period than females (Bennet et al. 2007; Jehan et al. 2009; Seaborn et al. 2010). The specific mechanisms between preterm birth and the 'male disadvantage' remain unclear, but appear to be multifactorial and include the effects of sex hormones, gestational age at birth, fetal nutrition and the timing of prenatal insults to the fetus (Berry, M et al. 2013; Grigore, Ojeda & Alexander 2008; Kirchengast & Hartmann 2009; Naeye et al. 1971; Schlesinger et al. 1987). Differences in short-term cardiovascular adaptations during the haemodynamic transition, from a fetal circulation to an *ex utero* circulation, between the male and the female may also be a contributing factor (De Matteo et al. 2015; Ishak et al. 2012).

We have previously evaluated the cardiovascular system and kidneys of moderately preterm lambs at two days of age (chapters 3 & 4). However, these lambs would have completed most, if not all, of the haemodynamic transition before undergoing ultrasound evaluation (Evans 2005). In this chapter, an ultrasound evaluation was conducted on a cohort of moderately preterm lambs approximately two hours after being born (Figure 2-1). The ultrasound analyses in this chapter provides important *in vivo* data to determine if there are differences in the structure and function of the heart, kidneys and major arteries of male and female moderately preterm lambs while they are still adapting to the haemodynamic transition immediately after birth.

The ultrasound evaluation in this chapter was conducted concurrently with the PhD project of Dr Noreen Ishak (a PhD student under the supervision of Professor Richard Harding, Dr Robert De Matteo and Dr Foula Sozo, Monash University) who was comparing the lung structure and function in male and female preterm lambs during the haemodynamic transition from a fetal vascular circulation to an extra-uterine circulation in the eight hours immediately after birth (De Matteo et al. 2015). The preparation, delivery and post-natal care of all the lambs evaluated in this chapter were performed and/or supervised by Dr Noreen Ishak as part of her project. The morphological and physiological analyses conducted by Dr Noreen Ishak are complementary to the ultrasound analyses. The intention is to collaborate on all findings when completed with a view to joint publication of results.

The aims of this chapter were to use ultrasound imaging to compare the:

1. structure and function of the left ventricle
2. structure and blood flow within major arteries including the proximal ascending aorta and main, right and left pulmonary arteries
3. structure of both kidneys and blood flow in the renal arteries

between male and female moderately preterm lambs during the haemodynamic transition at two hours after birth.

6.2. Methods.

6.2.1. Animal studies.

The preparation, delivery and post-natal care of all lambs in this chapter were performed or supervised by Dr Noreen Ishak as part of her PhD project as described previously in chapter two (section 2.3) and is summarised as follows:

Pressure catheters were chronically implanted in the pleura, carotid artery, jugular vein and amniotic sac of eight male and nine female fetal sheep *in utero* at 125 days gestational age. A vascular occluder was placed around the umbilical cord so that blood flow in the cord could be completely blocked briefly during caesarean delivery preventing anaesthetic agents administered to the ewe from entering the fetal circulation. The time from cord occlusion to cutting the cord at delivery was approximately 110s for the male, and 115s for the female lambs (De Matteo et al. 2015). Pressure measurements of the fetal sheep were recorded at 131 days gestational age, followed by administration of betamethasone (5.7 mg i.m., betamethasone (Celestone Chronodose, Schering-Plough, Australia) to the pregnant ewes. Two days later the moderately preterm un-anaesthetised lambs and their catheters were delivered by Caesarean section. Lambs were not mechanically ventilated but received supplemental oxygen to maintain an arterial oxygen saturation (SO_2) above 80% if required. Blood gases, arterial and intra-pleural pressures were monitored for eight hours after birth.

The un-anaesthetised preterm lambs underwent ultrasound evaluation at two hours after birth as part of this thesis.

6.2.2. Ultrasound imaging.

The ultrasound equipment and protocol for preparing the two hour old lambs for ultrasound scanning was as previously described in chapter 2 (section 2.4). Specifically, a P12-5 MHz phased array cardiac probe with a ‘pediatric cardiac/neonatal’ application setting was used for scanning the heart, proximal ascending aorta and pulmonary arteries in this cohort. A C7-4 MHz curvilinear array abdominal probe with a ‘pediatric/pediatric renal’ application setting was used for scanning the kidneys and renal arteries.

The ultrasound evaluation of the heart, proximal ascending aorta, pulmonary arteries, kidneys and renal arteries were performed as described in Chapter 2 (section 2.4). A summary of the measurements are as follows:

Cardiac measurements:

- Left ventricle anterior wall thickness (mm) in systole and diastole.
- Left ventricle posterior wall thickness (mm) in systole and diastole.
- Interventricular septum wall thickness (mm) in systole and diastole.
- Left ventricle internal short axis chamber diameter (mm) in systole and diastole.

The measurements of each of the structures were corrected for bodyweight (mm/kg). The percentage fractional shortening of the left ventricle was calculated from the acquired ultrasound measurements as previously described in section 2.4 (Singh, R et al. 2009).

Measurements of the major arteries:

Proximal ascending aorta, main, right and left pulmonary artery

- The maximum internal diameter (mm) of the proximal ascending aorta, main, right and left pulmonary arteries were measured. The measurements were corrected for bodyweight (mm/kg).
- The peak systolic velocity (cm/s) of four consecutive cardiac cycles in each of the proximal ascending aorta, main, right and left pulmonary arteries were measured and averaged.

Renal measurements:

- The maximum bipolar length (mm), width (mm) and thickness (mm) of each kidney were measured and corrected for bodyweight (mm/kg). Volume measurements were calculated based on the length, width and thickness measurements (mm^3) as previously described in section 2.4 (Weitz et al. 2013).
- The maximum systolic and end diastolic blood flow velocity (cm/s) of three consecutive cardiac cycles were measured and averaged for each renal artery and the resistive index, RI, was calculated as previously described in section 2.4 (Rabbia & Valpreda 2003).

The lambs were positioned on their back, or slightly onto one side depending on the area being investigated and held gently and securely in position on a table next to the ultrasound machine by a handler while the sonographer conducted the ultrasound examination. This restraining protocol was the same as that used for all lambs up to two weeks of age (Figures 5.2, 5.3). Devices such as an overhead heat lamp and a hot water bottle or heat pad wrapped in a towel were placed under the newborn preterm lambs to keep them warm during the ultrasound examination.

6.2.3. Statistical analysis.

Statistical analyses were performed using GraphPad Prism software (version 6.04; GraphPad software, San Diego, CA, USA). The data obtained from male and female preterm lambs were compared using an unpaired *t*-test. Data are presented as mean ± standard error of the mean (SEM). Statistical significance was accepted at $p < 0.05$.

6.3. Results.

6.3.1. Survival rates and ultrasound imaging.

Seventeen preterm lambs (8 male, 9 female) were delivered. The overall survival rate at two hours after birth was 15/17 (88%) comprising 6/8 (75%) male and 9/9 (100%) female lambs. Two male lambs were euthanased within one hour of birth due to severe hypercapnic acidosis. Three female lambs did not undergo ultrasound examination due to scheduling issues. Therefore, six male and six female preterm lambs underwent ultrasound imaging and were included in the analyses. There were no difficulties encountered in preparing the lambs or handling them during the ultrasound examination as they were small, feeble and cooperative. Images of the heart, proximal ascending aorta, main, right and left pulmonary arteries, kidneys, renal arteries and blood flow velocity data were successfully acquired for all the lambs. No gross abnormalities or pathophysiology of the left ventricular structures, kidneys or arteries were evident in any of the ultrasound images obtained. A patent ductus arteriosus was demonstrated in some male and female lambs as an incidental finding (Figure 6-1).

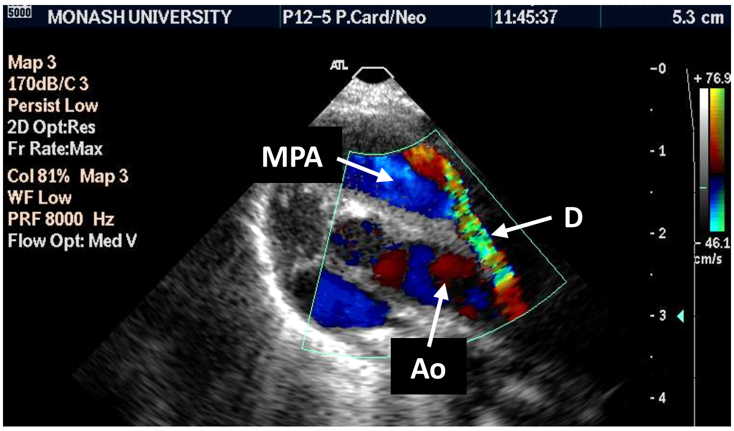

Figure 6-1: Patent ductus arteriosus of a two hour old preterm lamb.
A patent ductus arteriosus (DA) is demonstrated connecting the main pulmonary artery (MPA) and the aorta (Ao). The green colour within the ductus arteriosus indicates blood from the aorta is travelling through the ductus arteriosus and into the main pulmonary artery.

Lamb bodyweight

All the lambs were weighed immediately prior to the ultrasound examination. There was no significant difference in the average bodyweight of male and female moderately preterm sheep at two hours after birth (Figure 6-2).

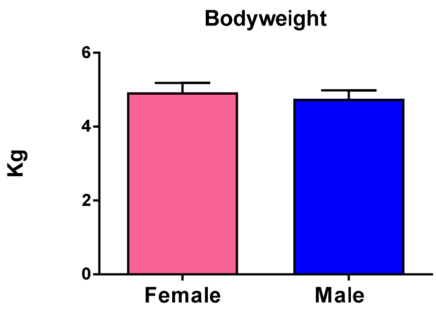

Figure 6-2: Mean bodyweight of male and female preterm lambs at two hours after birth.
Data represented as mean ± SEM. Data were analysed with an unpaired *t*-test.

6.3.2. Ultrasound measurements.

Functional and structural analysis of the left ventricle at two hours after birth

There was no significant difference in the average percentage fractional shortening of the left ventricle of male preterm lambs compared to females (Figure 6-3).

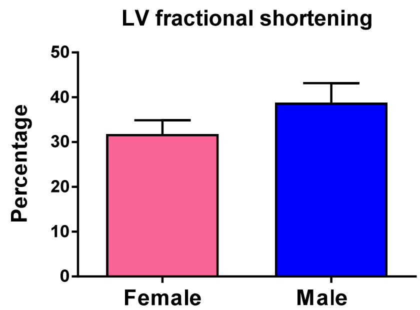

Figure 6-3: Percentage fractional shortening of the left ventricle (LV) of male and female preterm lambs at two hours after birth.
Data represented as mean ± SEM. Data were analysed with an unpaired *t*-test.

There were no significant differences in any of the structural measurements of the left ventricle between male and female preterm lambs at two hours after birth. Specifically, the mean systolic and diastolic thickness of the anterior and posterior walls of the LV, IVS and the mean systolic and diastolic internal chamber diameter of the LV in male preterm lambs were not significantly different to those of female preterm lambs. (Figures 6-4, 6-5).

There were also no significant differences in the LV parameters after being corrected for bodyweight, (Figures 6-4, 6-5).

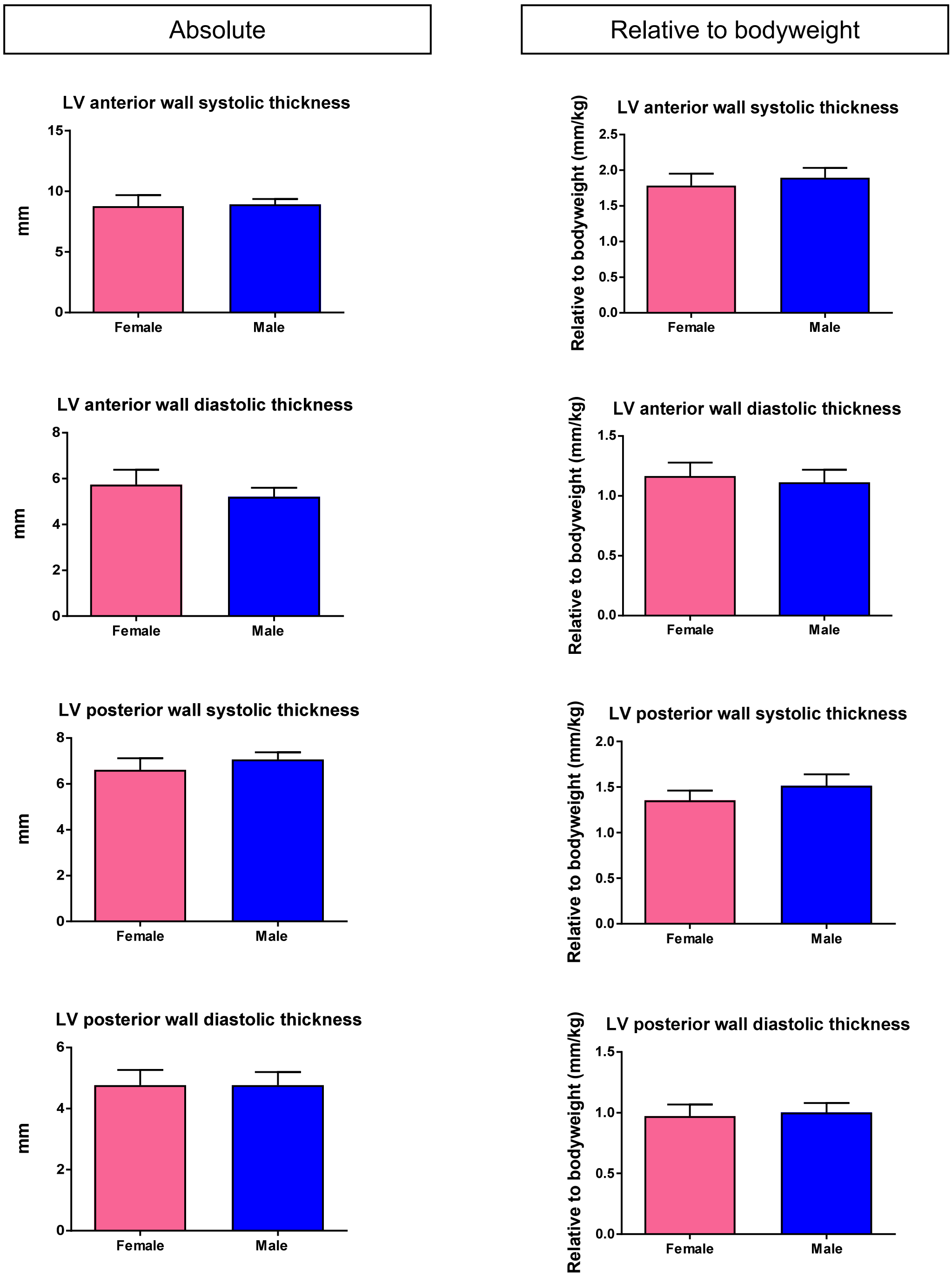

Figure 6-4: Mean systolic and diastolic left ventricle (LV) anterior and posterior wall thickness (and relative to bodyweight) of male and female preterm lambs at two hours after birth.
Data represented as mean ± SEM. Data were analysed with an unpaired *t*-test.

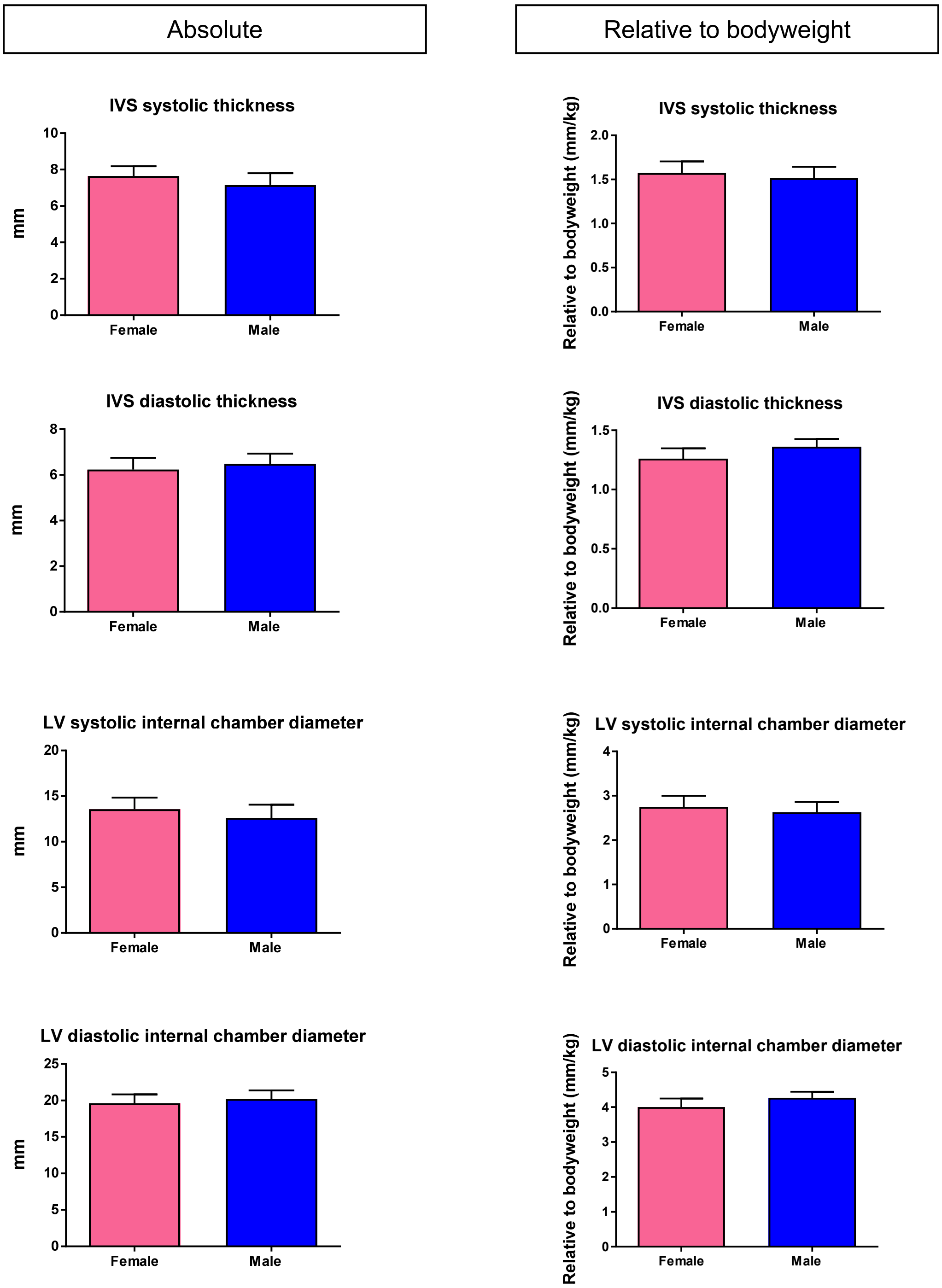

Figure 6-5: Mean systolic and diastolic interventricular septum (IVS) thickness and internal chamber diameter (and relative to bodyweight) of male and female preterm lambs at two hours after birth.
Data represented as mean ± SEM. Data were analysed with an unpaired *t*-test.

Structural and blood flow analysis of the major arteries at two hours after birth

Proximal ascending aorta, main, right and left pulmonary arteries

There were no significant differences in the mean internal diameters of the proximal ascending aorta, main, right and left pulmonary arteries of two hour old male preterm lambs compared to females (Figures 6-6).

No significant differences between male and female preterm lambs were found when these diameter measurements were corrected for bodyweight, except for the mean internal diameter of the main pulmonary artery which was significantly larger ($p = 0.0465$) in the male preterm sheep (Figure 6-6).

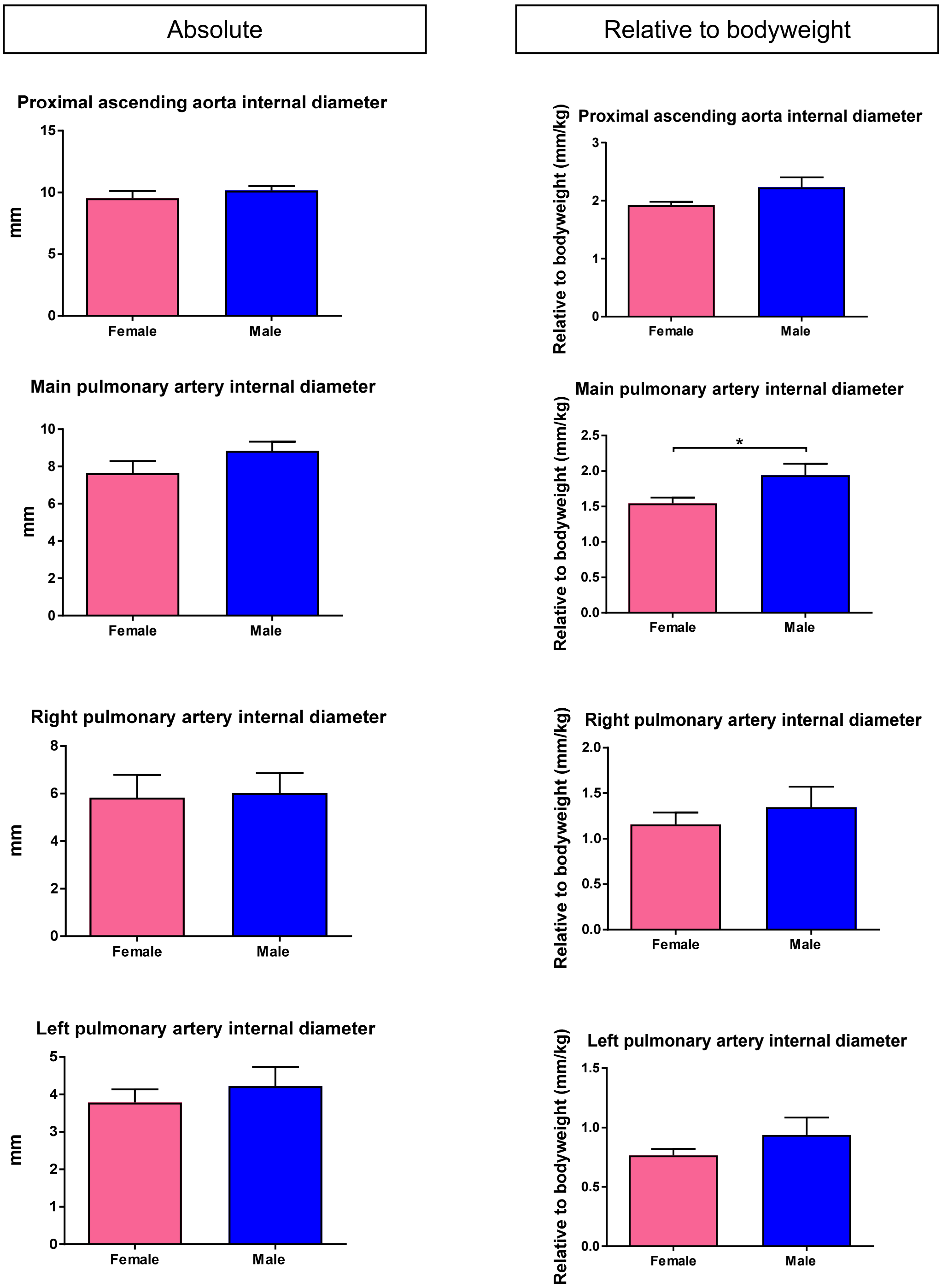

Figure 6-6: Mean internal diameter (and relative to bodyweight) of the proximal ascending aorta, main, right and left pulmonary arteries of male and female preterm lambs at two hours after birth.
Data represented as mean ± SEM. Data were analysed with an unpaired *t*-test.
*$p < 0.05$.

The average peak blood flow velocities in the proximal ascending aorta, main, right and left pulmonary arteries were not significantly different in preterm males compared to females (Figure 6-7).

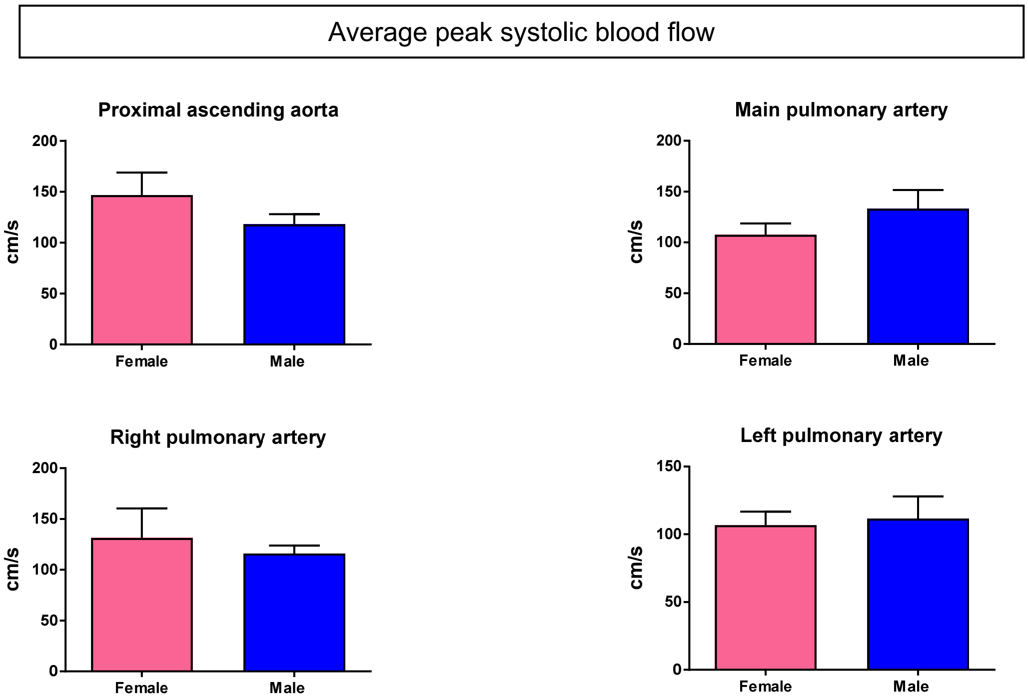

Figure 6-7: Average peak systolic blood flow velocity of the proximal ascending aorta, main, right and left pulmonary arteries of male and female preterm lambs at two hours after birth.
Data represented as mean ± SEM. Data were analysed with an unpaired *t*-test.

Analysis of renal artery blood flow and kidney dimensions at two hours after birth.

At two hours of age, the resistive indices of the right and left renal arteries in male preterm lambs were not significantly different to female lambs (Figure 6-8).

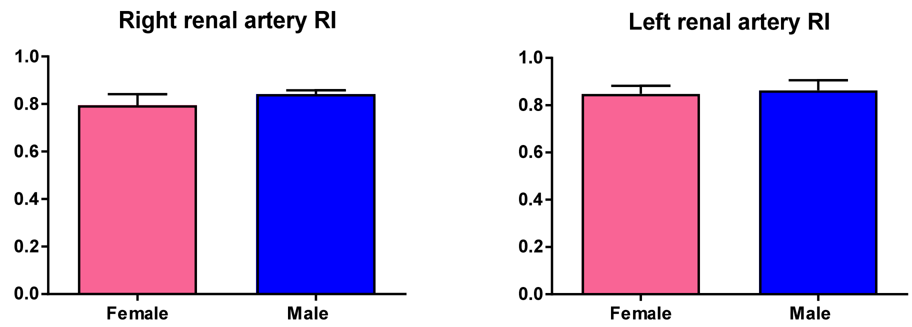

Figure 6-8: Resistive indices (RI) of the right and left renal arteries of male and female preterm lambs at two hours of age.
Data represented as mean ± SEM. Data were analysed with an unpaired *t*-test.

There were no significant differences in mean bipolar length, width, thickness or volume of the right and left kidney of preterm male lambs compared to females. No significant differences between male and female preterm lambs were found when these measurements were corrected for bodyweight (Figures 6-9, 6-10).

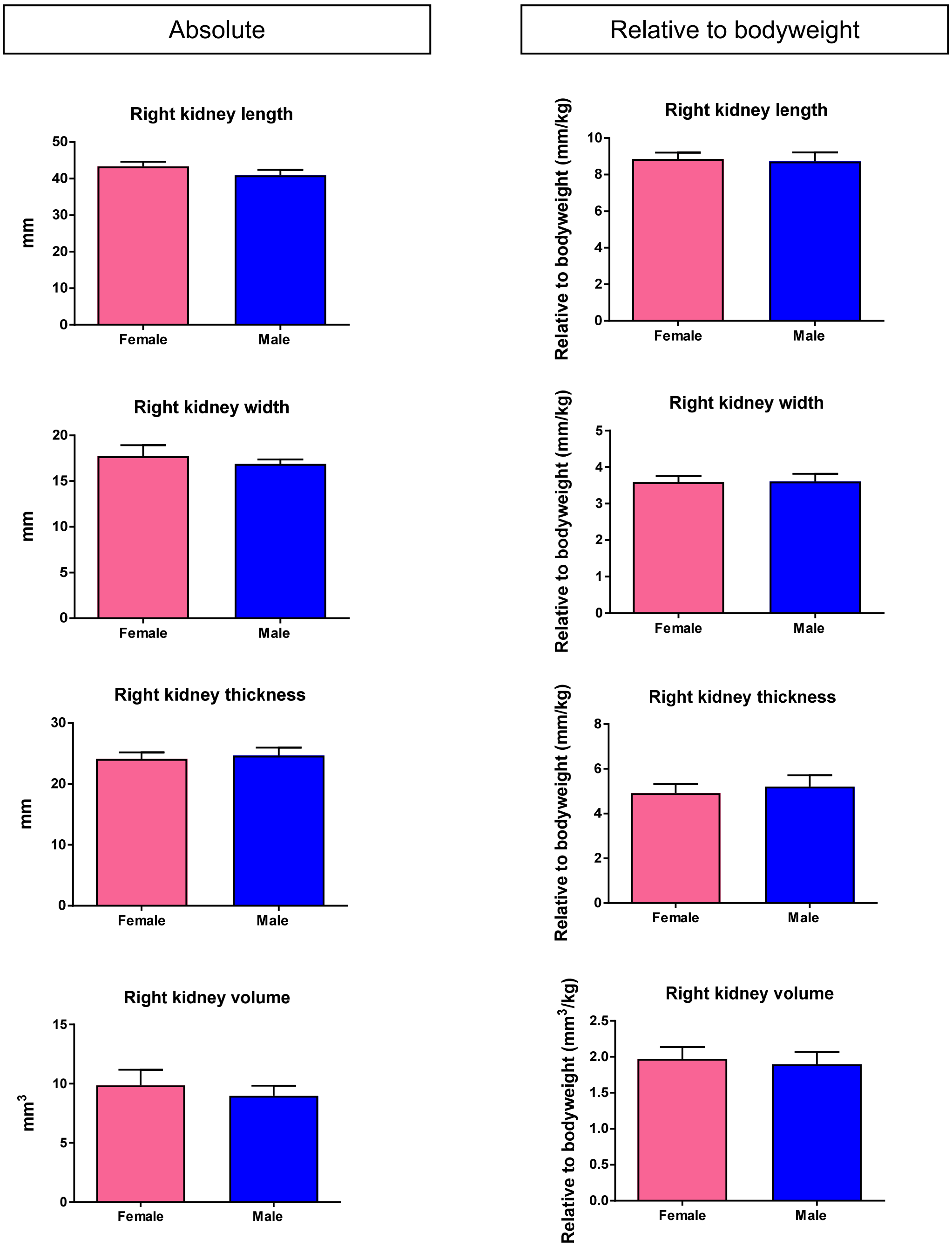

Figure 6-9: Right kidney dimensions (and relative to bodyweight) of two hour old male and female preterm lambs.
Data represented as mean ± SEM. Data were analysed with an unpaired *t*-test.

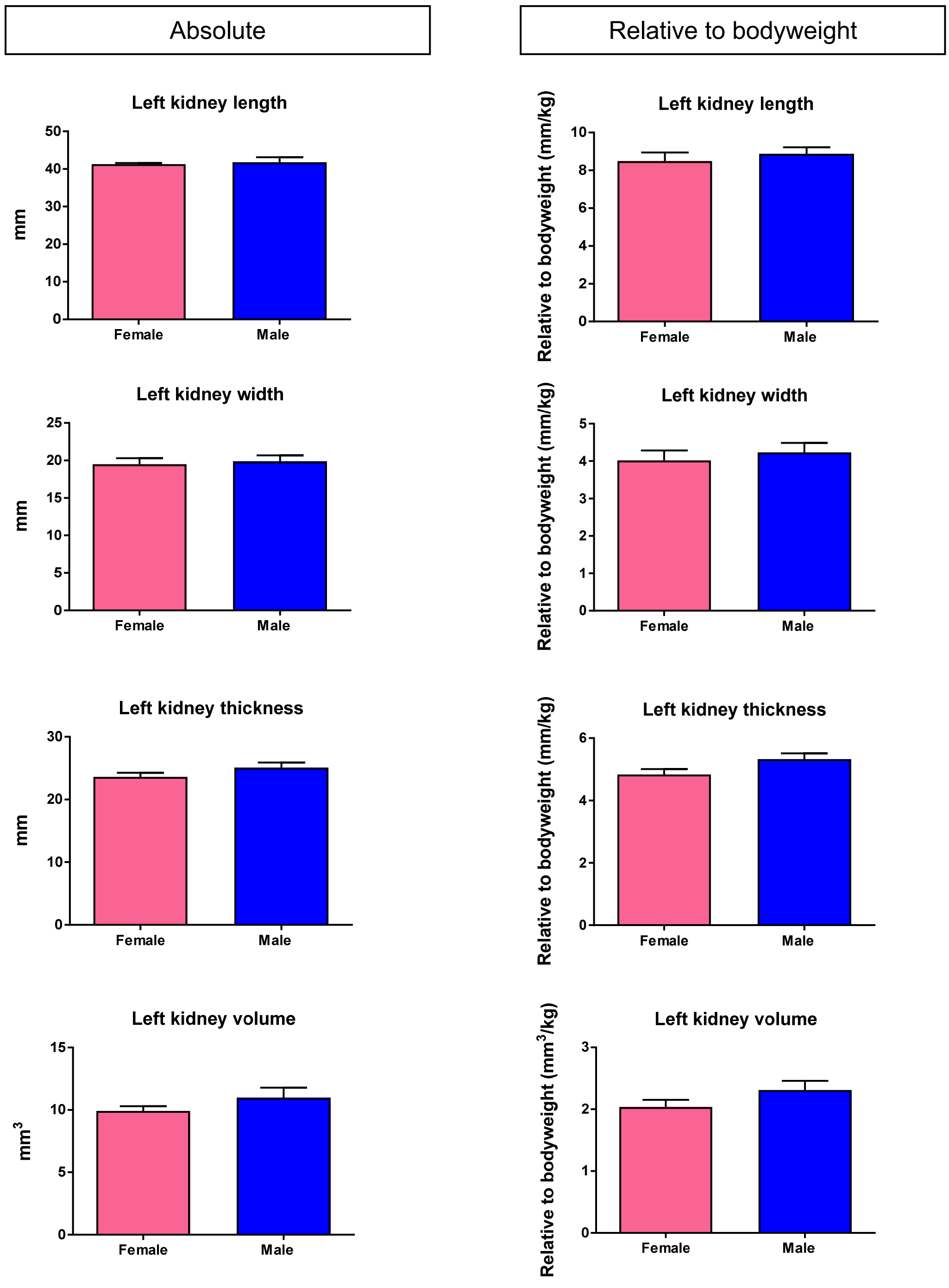

Figure 6-10: Left kidney dimensions (and relative to bodyweight) of two hour old male and female preterm lambs.
Data represented as mean ± SEM. Data were analysed with an unpaired *t*-test.

6.4. Discussion.

Imaging of the left ventricle, major arteries and kidneys of two hour old moderately preterm lambs was straightforward and easy to perform. Many lambs were comforted by sucking on a pacifier, or a gloved finger of the handler during the ultrasound examination. On most occasions, the use of a dedicated handler to hold the preterm lambs was not really necessary as they were small, weak and hardly moved once comfortably positioned on the examination table. However, to ensure lamb safety, it was still necessary for a second person to attend the examination so they could keep an eye on the lamb, allowing the sonographer to focus on data acquisition without having to watch over the lamb at the same time.

There was only one significant sex-specific difference in the structure and function of the LV, major arteries and the kidneys of male and female lambs at two hours after moderate preterm birth. When adjusted for bodyweight, the internal diameter of the main pulmonary artery was significantly larger in the male lambs. All other structural and functional measurements (absolute or relative to bodyweight) were not significantly different. The initial cardiovascular and renal responses of the male and female lambs during the haemodynamic transition after moderate preterm birth are essentially the same, and therefore, the findings do not contribute any further insight into neonatal male disadvantage.

Evaluation of the structure and function of the left ventricle

There was no significant difference in the LV wall thickness, chamber diameter or percentage fractional shortening of male and female lambs two hours after moderately preterm birth. There are no studies reported in the literature that have evaluated the male and female cardiac response during the haemodynamic transition for comparison of findings. However, our results are in agreement with other ultrasound studies that found no sex-specific differences in the LV systolic and diastolic function at two days after moderately preterm birth (Kozák-Bárány et al. 2001); or in the systolic and diastolic LV posterior wall thickness, IVS thickness or LV internal chamber diameter at three days after extremely preterm birth (Zecca et al. 2001).

Evaluation of the structure and blood flow within the major arteries

When corrected for bodyweight, there was a trend for the internal diameter of the main, right and left pulmonary arteries to be larger in the male moderately preterm lambs, compared to females, but it was only significantly greater ($p = 0.0465$) for the main pulmonary artery. There were no significant differences between male and female preterm lambs in the absolute internal diameter measurements or peak systolic blood flow within the proximal ascending aorta or main, right and left pulmonary arteries. Our findings are generally consistent with a previous study of ventilated and anaesthetised moderately preterm lambs, that found no significant difference in the fetal heart rate, pulmonary artery blood flow, pulmonary vascular resistance and left ventricular output between males and females, during the transition to an *ex utero* circulation at 30 minutes after birth (Polglase et al. 2012).

In further support of our findings, another study conducted on a cohort of human infants born moderately and extremely preterm, found no sex-specific differences in microvascular vasodilation and peripheral blood flow at 24 hours after moderate preterm birth (Stark, M., Clifton & Wright 2008). However, for up to three days following extremely preterm birth, male neonates exhibited increased peripheral microvascular vasodilation and augmented peripheral blood flow compared to females (Stark, M., Clifton & Wright 2008). The sex-specific findings appear to be related to the severity of prematurity, limited to subjects born extremely preterm and were not associated with moderately preterm birth. A transient increase in microvascular flow has the potential to contribute to circulatory vulnerability during the haemodynamic transition, including persistence of hypotension and low systemic blood flow (Stark, M., Clifton & Wright 2008). Unlike extremely preterm birth, moderately preterm birth does not appear to lead to increased microvascular vulnerability in neonates. The results support our findings that (apart from one exception) there are no significant sex-specific differences of the major arteries during the haemodynamic transition following moderate preterm birth, that would help explain neonatal male disadvantage. However, further investigation is warranted to determine if greater severity of preterm birth results in sex-specific differences of the proximal aorta and pulmonary arteries becoming evident during the circulatory transition.

It should be noted that the mother ewes of these preterm lambs were administered a much smaller amount of antenatal corticosteroid (betamethasone, 5.7 mg i.m.) than the clinically relevant dose (betamethasone, 2 times 11.4 mg i.m.) (Liggins Institute 2015) that was used in the previous chapters of this thesis. Antenatal betamethasone improves oxygenation in moderately preterm lambs by advancing lung maturity and subsequent pulmonary vasodilation in the immediate post-natal period (Konduri et al. 2013). Preterm males benefit less from the administration of antenatal corticosteroid treatment than females (Stark, M, Wright & Clifton 2009; Willet et al. 1997). In our study, all the female lambs (9/9) were alive at two hours of age but two of the eight male lambs had been euthanased shortly after birth - a similar survival rate to a previous study that utilised an identical sheep model of preterm birth, dose of antenatal betamethasone and fetal surgical intervention (Ishak et al. 2012). The two male lambs in our study were euthanased due to severe hypercapnic acidosis, suggestive of poor pulmonary gas exchange due to lung immaturity. The lower survival rate in males compared to females could be due to innate male disadvantage relating to fetal acid-base balance, a poorer response to antenatal corticosteroid treatment and/or less resilience to the surgical interventions that were conducted as part of Dr Noreen Ishak's PhD experiments (De Matteo et al. 2015). Our ultrasound findings did not show evidence of a compromised cardiovascular response during the haemodynamic transition in the male lambs that underwent ultrasound examination. However, changes arising subsequent to the haemodynamic transition cannot be excluded.

Adaptations of a structurally immature cardiovascular system to the haemodynamic transition after preterm birth may be further complicated by the persistence of fetal shunts (Kluckow, Martin & Evans 2001; Kupinski 2013; Polglase et al. 2009). For example, a patent ductus arteriosus has been associated with high pulmonary blood flow, increased risk of pulmonary haemorrhage, cardiac failure and impairment of renal perfusion (Finnemore & Groves 2015; Kluckow, Martin & Evans 2000; Koskinen et al. 2009; Touboul et al. 2007). A patent ductus arteriosus was sometimes visualised on the ultrasound images of both male and female preterm lambs, as an incidental finding, while the data was being collected for this chapter. There was no attempt to routinely demonstrate the ductus arteriosus, as it was not part of this study, with the expectation that it would be functionally open in all lambs at two hours after moderately preterm birth (Clyman & Roman 2007; Dawes, Mott & Widdicombe 1955; Popat & Kluckow 2012). Left-to-right shunting of blood flow was present in all instances where the ductus arteriosus was visualised. This finding was consistent with a previous study that demonstrated left-to-right shunting through the ductus arteriosus, with no significant difference in flow direction between anaesthetised male and female lambs at 30 minutes after moderately preterm Caesarean delivery (Polglase et al. 2012).

Evaluation of the kidneys and blood flow within the renal arteries

There were no significant sex-specific differences in the renal dimensions of the right and left kidneys, or resistive indices of the renal arteries, demonstrated in the moderately preterm lambs at two hours after birth. A paucity of studies reported in the literature investigating sex-specific differences in kidney size and renal artery blood flow, during the haemodynamic transition, makes direct comparison to other findings problematic.

Nevertheless, our findings are in general agreement with ultrasound studies involving preterm neonates, born 26 to 37 weeks gestation, that found no significant sex-specific difference in the kidney length (Chiara et al. 1989) or kidney length and volume (Gupta, Anand & Lamba 1993) at two days of age. Further studies showed no significant difference in the kidney length between male and female neonates born less than 37 weeks gestation at three days of age (Schlesinger et al. 1987), or in the kidney volume of infants born less than 34 weeks gestation at 14 to 96 days of age (Huang et al. 2007). However, in a contradictory study of neonates born less than 37 weeks gestation, males had a combined right and left kidney volume that was significantly greater than in females when assessed at five days of age (Schmidt et al. 2005). Supporting our renal artery RI findings, a study of neonates born extremely preterm did not find any significant sex-specific differences in the resistance to blood flow in the renal arteries at six days of age (Armstrong et al. 2001). Although the outcomes of the other studies involve subjects that are at least two days old, and therefore, have already transitioned through most of the haemodynamic changes associated with an *ex-utero* circulation, their results are broadly in support of our findings.

Our results did not show any evidence of structurally or functionally compromised kidneys or an altered reno-vascular response during the haemodynamic transition, in male lambs compared to females that might contribute to neonatal male disadvantage. However, microvascular and structural changes that are too small to be detected using ultrasound analyses cannot be discounted. In addition, significant remodelling of the kidney structure and alterations to the renal blood flow in response to the haemodynamic changes may not be possible within two hours of moderate preterm birth. More time may be required for substantial adaptations in renal structure and blood flow to arise and subsequently become evident in response to the major haemodynamic changes following birth. Further research involving microvascular and histological analyses of the kidneys and renal blood flow immediately after moderate preterm birth is recommended to complement the ultrasound findings.

6.5. Conclusion.

With one exception, there are no significant sex-specific differences in the initial short-term cardiovascular and renal responses to the haemodynamic transition after moderate preterm birth that help explain neonatal male disadvantage. Our findings lend further support to the view that male morbidity and mortality immediately after moderate preterm birth is most likely due to poor respiratory function rather than mal-adaptation to the rapid haemodynamic transition to a post-natal circulation (De Matteo et al. 2015; Ishak et al. 2012; Seaborn et al. 2010; Torday & Nielsen 1987). It is possible that structural and functional changes following moderate preterm birth are present, but not great enough to be demonstrated using ultrasound imaging. Additional complementary studies are recommended involving greater severity of preterm birth and histological analyses to detect microscopic changes that cannot be detected using ultrasound analyses.

Chapter 7 General discussion and conclusion.

7.1. Summary discussion.

In this thesis I present the findings of four ultrasound studies that assessed the effects of moderate preterm birth on the structure and function of the left ventricle, major arteries and kidneys of sheep immediately after birth and serially to early adulthood. Ultrasound analyses were also conducted to ascertain if there were any significant sex-specific differences, in particular male disadvantage, demonstrated in response to moderately preterm birth. A well-established sheep model of moderate preterm birth was slightly modified for use in my ultrasound studies in order to obtain clinically relevant findings. I developed imaging protocols for the ultrasound examinations so they could be performed on non-sedated animals ranging in size from small newborn lambs to large adult sheep. In addition, because my imaging protocols were created using standard ultrasound equipment and simple manual handling techniques, they can be readily employed by other investigators to conduct future *in vivo*, non-invasive ultrasound evaluations of the cardiovascular structures and kidneys.

Initially after birth, my ultrasound findings demonstrate that moderately preterm sheep have significantly greater left ventricular wall thickness, diameter of the major arteries and dimensions of the kidneys compared to sheep born at term, but only after correction for bodyweight. The relative to bodyweight differences are essentially no longer evident by three months of age, subsequent to a substantial increase in bodyweight of the preterm sheep. My findings demonstrate that moderately preterm birth has little effect on the structure and function of the left ventricle, major arteries and kidneys of sheep after three months of age and into early adulthood. In addition, there is no evidence in any of my ultrasound analyses of a male disadvantage in lambs born moderately preterm using our sheep model.

Moderately preterm birth and the structure and function of the left ventricle

In a study using a sheep model of moderate preterm birth similar to that used in our long term evaluations (chapters 4 and 5), significantly increased collagen deposits in the myocardium, altered cardiomyocyte maturation and cardiomyocyte hypertrophy was demonstrated in 11 week old lambs born moderately preterm, compared to term controls (Bensley, J et al. 2010). The moderately preterm lambs had no significant difference in the number of cardiomyocytes compared to lambs born at term, but had fewer binucleated and more mononucleated (with increased ploidy) cardiomyocytes, indicating structural immaturity and remodelling of the heart wall in the neonatal period that could lead to long term cardiac vulnerability (Bensley, J et al. 2010). A low, non-clinical dose of betamethasone (3.7-5.0 mg i.m.) was administered maternally to the preterm lambs in the study and there were no maternal or fetal co-morbidities to potentially confound the results. Increased collagen deposition in the myocardium can result in stiffer ventricular walls and decreased contractility (Brower et al. 2006; Weber 1989). However, Bensley *et al* (2010) found no evidence of cardiac dysfunction and no significant difference in the mean, systolic and diastolic arterial pressures between the moderately preterm lambs and controls in the neonatal period.

In agreement with the Bensley *et al* (2010) findings, the results of Dr Nguyen's concurrent PhD morphometric and physiological investigation of our short term (two days of age) cohort of lambs (chapter 3) found no significant difference in the number of cardiomyocytes in the left ventricle and IVS, a greater proportion of mononucleated cardiomyocytes and a lower proportion of binucleated cardiomyocytes and no significant differences in the mean, systolic and diastolic arterial blood pressures and heart rate between moderately preterm and term lambs at two days of age (Nguyen, V. 2016). When corrected for bodyweight, the relative thickness of the left ventricle and IVS were significantly greater in the preterm lambs (in accordance with our ultrasound findings), however, there was no significant difference in the amount of collagen deposited in the heart walls compared to term controls (Nguyen, V. 2016).

In moderately preterm infants during the first four weeks after birth, echocardiographic evaluation has demonstrated mild hypertrophy of the IVS and altered ventricular shape (Kozák-Bárány et al. 2001). When corrected for body surface area, the LV wall mass was not significantly different to term infants at the same post-natal age, however, the LV mass increased more rapidly in the preterm than term infants, suggesting marked myocardial adaptation to the haemodynamic transition following moderately premature birth (Kozák-Bárány et al. 2001). In another echocardiographic study, very preterm infants were found to have LV diastolic dysfunction compared to term controls at six months of age (Schmitz et al. 2004).

In recent studies of young adult men and women using magnetic resonance imaging (MRI), preterm birth was associated with an increased wall mass, decreased internal chamber diameter, altered wall geometry and reduced systolic and diastolic function of the right and left ventricle compared to term controls (Lewandowski et al. 2013; Lewandowski et al. 2013b). The preterm participants in these studies were mostly born very premature (55%), with the rest comprising moderately premature (31%) and extremely premature (14%) individuals. The severity of the cardiac changes were inversely related to gestational age at delivery; with each week of prematurity increasing left ventricular mass by 1.5% and right ventricular mass by 2.7% (Lewandowski et al. 2013b). An increased LV mass is an independent predictor of cardiovascular disease in adulthood (de Simone et al. 2002; Levy et al. 1990). Elevated blood pressure was found to be a contributing factor to the increased LV mass and altered long-term cardiac growth found in the young adults born preterm, some of which were born growth restricted in addition to being premature (Lewandowski et al. 2013). Being small for gestational age has been shown to increase the risk of hypertension in young adults born moderately preterm (Johansson et al. 2005), however, other conflicting studies have shown the risk is independent of birthweight (Kistner et al. 2005; Rotteveel et al. 2008). Although Lewandowski *et al* found that the severity of the prematurity was associated with a greater risk of cardiovascular disease, it remains unclear whether individuals born moderately preterm are at significant risk of cardiac events beyond early adulthood and follow up studies of cardiac structure and function in later life has been recommended (Lewandowski et al. 2013; Norman 2013).

Based on previous studies in animals and humans, it was expected that our sheep would exhibit some evidence of structural or functional mal-adaptation of the heart in response to moderate preterm birth. My ultrasound findings demonstrate that the fetal growth of the LV is mostly complete by late gestation and that post-natal LV growth in the moderately preterm sheep is similar to that of sheep born at term. Compared to controls, increased LV wall thickness and LV internal chamber diameter, relative to bodyweight, were the only significant differences in cardiac structure and function that were demonstrated in the preterm sheep in the immediate post-natal period. None of the LV differences in the preterm sheep were still evident by three months of age, most likely due to the substantial post-natal increase in bodyweight rather than remodelling of the myocardium. The absolute measurements of the LV structures were not significantly different in the moderately preterm sheep compared to controls at any age, further supporting the view that initial differences in the moderately preterm sheep are primarily due to lower bodyweight, rather than significant remodelling of the LV wall. The histological analyses on our preterm sheep performed by Dr Nguyen as part of her concurrent PhD study clearly demonstrate that microscopic changes to the structure of the heart wall were present, however, the structural changes were not large enough in early adulthood to be demonstrated on ultrasound imaging. It is possible that moderate preterm birth does not adversely affect LV structure and function or arterial blood pressure until after early adulthood, therefore, further ultrasound and complementary morphometric and physiological analyses in mid and later life are warranted.

Moderately preterm birth and the structure of the major arteries and blood flow

Preterm birth is associated with altered vascular development that can lead to reduced arterial dimensions, arterial elasticity and impaired endothelial function (Ligi et al. 2010). Large vessels such as the aorta, pulmonary and common carotid arteries, require an adequate amount of elastin in their walls in order to successfully adapt to the increased arterial pressure during the haemodynamic transition that occurs after birth (Abitbol & Rodriguez 2012). Premature birth interrupts the deposition of elastin in the arterial wall, which occurs mostly during late gestation, and further elastin accumulation in the arteries is limited after birth (Martyn & Greenwald 1997). Reduced levels of elastin has been associated with a diminished vascular compliance of the large arteries and is a marker for hypertension and cardiovascular disease in adults (Bonamy et al. 2005; Ligi et al. 2010; Martyn & Greenwald 1997).

In a sheep model of moderate preterm birth similar to the one used in our serial long term evaluation, 11 week old preterm lambs had significantly thicker aortic walls and a smaller lumen, compared to term lambs at the same age (Bensley, J et al. 2012). Notably, in the wall of the aorta in four out of the seven preterm lambs, there were focal areas of mostly collagen deposits, most likely representing injury as a consequence of a structurally immature aorta being exposed to high arterial pressure after moderate preterm birth (Bensley, J et al. 2012). In our serial ultrasound evaluation, the internal diameter of the proximal ascending aorta was also significantly decreased in all the preterm sheep, compared to term controls, as were the internal diameters of the main pulmonary artery of the female preterm sheep and the right and left pulmonary artery diameters of the male preterm sheep. These results are also in agreement with the findings in previous studies of children (Jiang et al. 2006) and adolescent girls born prematurely (Bonamy et al. 2005) that had significantly decreased arterial diameters. The results of Dr Nguyen’s concurrent PhD histological investigation of our short term (two days of age) cohort of lambs found no significant differences in the composition of the walls (elastin, collagen layers) of the ascending aorta or left common carotid artery of preterm lambs compared to controls (Nguyen, V. 2016). However, by early adulthood (14 months of age), there was significantly reduced elastin content and increased muscle content in the left common carotid artery of preterm sheep compared to term controls.

Preterm birth has been associated with an increased IMT of the aorta in preschool children (Shimizu et al. 2014), thickened intima-media of the carotid artery in children born very preterm (Lee et al. 2014) and in young adults born very preterm whose mother was hypertensive during pregnancy (Lazdam et al. 2010). However, there are inconsistencies reported in the literature, with studies of newborns (Schubert et al. 2013) and adolescents (Hovi et al. 2011) finding no significant difference in the common carotid artery IMT values between individuals born preterm and term. Our findings of no significant difference in the IMT of the right and left common carotid arteries of moderately preterm sheep in young adulthood, compared to controls, were in agreement with other studies that found no significant difference in common carotid artery IMT of young adults born extremely preterm (Kowalski et al. 2016), very preterm (Finken et al. 2006) or moderately preterm (Kerkhof et al. 2012). Further research is required to clarify the association between preterm birth and IMT, particularly given increased IMT is a marker for subclinical atherosclerosis, impaired arterial endothelial function in early adulthood (Skilton et al. 2011), as well as hypertension, myocardial infarction and stroke in later life (O'Leary et al. 1999; Raitakari et al. 2003).

There is emerging evidence of a direct association between preterm birth and the adult onset of hypertension (Bayman, Drake & Piyasena 2014; Bonamy et al. 2005; Kerkhof et al. 2012; Lee et al. 2014). The risk of developing hypertension in adulthood increases with decreasing gestational age at birth (Keijzer-veen, MG et al. 2010; Kerkhof et al. 2012). In a Swedish study of young adult men, those born extremely preterm were reported to have almost two times the risk of developing high systolic blood pressure compared to men born at term (Johansson et al. 2005). A systematic review and meta-analysis reported that the increase in arterial blood pressure attributed to preterm birth in young adults is in the order of 3 to 4 mm Hg (Parkinson et al. 2013). Although this increase in arterial blood pressure appears small, a reduction in arterial blood pressure of only 2 mm Hg can significantly reduce the risk of coronary heart disease and stroke (Cook et al. 1995). Therefore, ongoing research to clarify the association between prematurity and hypertension is warranted to help inform monitoring of the expanding ex-preterm population in order to reduce the risk of cardiovascular disease (Bayman, Drake & Piyasena 2014). Physiological analyses of our long term sheep cohort (chapters 4 and 5) performed from birth to 14 months of age by Dr Nguyen as part of her concurrent PhD study, did not demonstrate any evidence of hypertension in the preterm sheep (Nguyen, V. 2016). When assessed at two days after birth and then again at 14 months of age, there were no significant differences in arterial pressure or heart rate between preterm sheep and those born at term (Nguyen, V. 2016). However, our preterm sheep exhibited significantly decreased systolic, diastolic and mean arterial blood pressure compared to term sheep when evaluated over the 14 month study period from birth to early adulthood (Nguyen, V. 2016). This finding is in contrast to studies that have reported elevated arterial pressures in children (Cheung et al. 2004; Gunay et al. 2014), adolescents (Bonamy et al. 2005; Kowalski et al. 2016) and young adults (Keijzer-veen, MG et al. 2010; Kerkhof et al. 2012) born prematurely. The discrepancy in findings may relate to the greater severity of prematurity in these studies (very and extremely preterm compared to our moderately preterm), or that hypertension as a consequence of moderately preterm birth may not become evident until mid or later adulthood. However, in agreement with the finding of hypotension in the period from birth to early adulthood in our sheep, there are other studies that have reported significantly reduced arterial pressure in premature infants during the neonatal period as they adapt to the *ex-utero* circulation following birth (Shead 2015; Wu, Azhibekov & Seri 2016).

Moderately preterm birth and the structure of the kidneys and blood flow in the renal arteries

Nephrogenesis, which is normally completed by 34 to 36 weeks gestational age in humans, is interrupted by preterm birth resulting in a reduced nephron endowment (Hinchliffe et al. 1991; Rodríguez et al. 2004; Rudolph 2000). A reduced nephron number is associated with an increased risk of chronic kidney disease and systemic hypertension in later life (Franke et al. 2010; Hoy et al. 2006; Keller et al. 2003). Nephrogenesis has been shown to continue after birth in preterm infants, but the nephrons formed *ex utero* have an elevated number of structural abnormalities, most likely in response to the marked increase in systemic blood pressure, renal blood flow and glomerular filtration that occurs following birth (Arant 1987; Black et al. 2013; Gubhaju et al. 2009; Sutherland et al. 2011). Any adverse occurrence that disturbs the normal completion of nephrogenesis, such as premature birth, is likely to compromise normal renal growth and subsequent kidney function (Gubhaju et al. 2014; Kandasamy, Smith & Wright 2012; Saint-Faust, Boubred & Simeoni 2014).

The effect of preterm birth on kidney size in younger people appears to be variable depending on the severity of prematurity and the age at which the kidneys are evaluated. In children born very preterm, no difference in the length and volume of the kidneys compared to term controls were demonstrated at 8 years of age (Vanpée et al. 1992). Children born extremely preterm also had normal kidney length and volume at 6 to 12 (mean 8.6) years of age, but demonstrated reduced renal function compared to controls (Rodriguez-Soriano et al. 2005). In contrast, another study of 6 to 7 year old children, also born extremely premature, found they had smaller kidney volume compared to term controls (Kwinta et al. 2011). An ultrasound study of 20 year old male and females, born very preterm (including appropriate for gestational age, AGA, and small for gestational age, SGA, individuals), reported no significant difference in the size of the right kidney compared to controls born at term (Keijzer-Veen, M et al. 2010). However, the female individuals had significantly smaller left renal length and volume compared with age-matched controls. IUGR had no significant effect on renal size (Keijzer-Veen, M et al. 2010). By contrast, a previous study of aboriginal adults found low birthweight to be associated with reduced renal size, however, the report did not state whether the subjects had IUGR which could have affected the findings (Singh, GR & Hoy 2004).

The period of nephrogenesis in sheep is similar to that of humans (from 0.2 - 0.9 of term gestation) (Singh, RR, Cuffe & Moritz 2012). Low nephron endowment in sheep has also been associated with increased cardiovascular and renal risk in later life (Gilbert et al. 2005; Moritz et al. 2005; Singh, R et al. 2009; Singh, R et al. 2012). In our long term serial sheep study, the only significant difference found was a greater bipolar length of the right kidney, when corrected for bodyweight, in the preterm cohort compared to sheep born at term. All other measurements (absolute or relative) were not significantly different, indicating moderate preterm birth had little effect on the size of the kidneys of preterm sheep in early adulthood. The renal artery resistive index is a measure of the intra-renal arterial impedance to blood flow. Changes to the renal microvasculature that produce significant haemodynamic changes to blood flow through the kidney will result in an altered renal artery RI. A raised renal artery resistive index is a well-established marker for renal atherosclerosis, hypertension and chronic renal failure (Petersen, L et al. 1995; Petersen, L et al. 1997; Pontremoli et al. 1999). The resistance to blood flow through the right kidney was not significantly different in our preterm sheep, compared to those born at term, indicating moderate preterm birth had little effect on the renal microvasculature in early adulthood. However, adverse renal adaptations in response to moderate preterm birth may not become apparent until after early adulthood. Therefore, further serial ultrasound studies with complementary physiological and histological analyses are recommended to determine whether renal changes in mid and later life result in increased cardiovascular risk.

Based on previous studies in sheep and humans, it was expected that moderate preterm birth would result in significant structural and functional mal-adaptations of the heart, major arteries and kidneys and that these changes would be more severe in males (Bayman, Drake & Piyasena 2014; Bensley, J et al. 2012; Black et al. 2013; Elsmen, Steen & Hellstrom-Westas 2004; Lewandowski et al. 2013). My results are therefore a little surprising, but there are a number of possible explanations as to why the effects of moderately preterm birth revealed in my findings are relatively minor. The moderately preterm sheep model used in my studies is free of the potential confounders that are commonly present in other studies, such as pregnancy related high blood pressure, low birth weight and intra-uterine growth restriction (IUGR)(Lazdam et al. 2010; Singh, GR & Hoy 2004; Skilton et al. 2011). In our model, moderately preterm birth was induced in healthy ewes with normal fetuses that would have been delivered naturally at term gestation, if birth had not been induced. Therefore, the effects of moderate preterm birth that are revealed using our model are independent of the potentially confounding effects of maternal and fetal co-morbidities (such as pre-eclampsia, chorioamnionitis and IUGR). Some studies have shown the contribution of co-morbidities, such as IUGR, can be more significant than the effects of moderate preterm birth *per se* in increasing cardiovascular risk (Cheung et al. 2004; Johansson et al. 2005; Skilton et al. 2011) However, there are also contradictory findings that have demonstrated that gestational age has a greater impact on adult blood pressure than birth weight in preterm subjects (Siewert-Delle & Ljungman 1998). Nevertheless, my findings that moderate preterm birth *per se* has few significant effects up until early adulthood in a clinically relevant large animal model are encouraging, and suggests a shift in focus to the contribution of co-morbidities to cardiovascular risk in future ultrasound serial studies of moderately preterm birth is warranted.

Another reason my findings may differ from other studies is that ultrasound imaging is unable to display microscopic structure. Therefore, any structural adaptations at the cellular level in response to moderate preterm birth cannot be demonstrated in my ultrasound findings unless the changes are so great that they produce an altered macroscopic appearance or function. In addition, the sheep used in my studies were still in early adulthood when they underwent their final ultrasound assessment at 14 months of age. Preterm birth is associated with an increased risk of chronic conditions emerging in mid- and later life including cardiovascular and renal disease (Bayman, Drake & Piyasena 2014; Cooper, Atherton & Power 2009; Grobman 2012; Gubhaju, Sutherland & Black 2011; Keijzer-Veen, M et al. 2010; Kerkhof et al. 2012; Saigal & Doyle 2008). Early adulthood may be too soon for the longer term effects of moderate preterm birth to become evident. Additional ultrasound studies of the cardiovascular system and kidneys are recommended, including complementary histological analyses to demonstrate changes at a cellular level, to determine if effects of moderately preterm birth emerge after early adulthood.

7.2. Moderate preterm birth and male disadvantage.

My ultrasound findings did not reveal any evidence in our sheep model that moderate preterm birth leads to male disadvantage. Essentially, there were no significant sex-specific effects of moderately preterm birth demonstrated, until a number of differences of the common carotid artery emerged for the first time when the sheep were evaluated at 14 months of age (early adulthood). Males had larger internal and external diameters of the right and left common carotid arteries compared to females. When corrected for bodyweight, the outcome was the same except for the external diameter of the left common carotid artery which was not significantly different between male and female sheep. These results are in agreement with human studies that found that the internal diameter of the common carotid artery is larger in men compared to women in early adulthood (mean 36 years) (Ruan et al. 2009) and during middle age (mean 52 years) (Krejza et al. 2006). In addition, the resistive indices of the right and left common carotid arteries were significantly greater in the male sheep compared to females at 14 months of age. This finding is broadly in agreement with studies showing that the resistive indices of the common carotid arteries can be higher in middle aged men (mean 50 years) compared to women (mean 50 years) (Albayrak et al. 2007; Yazici, Erdogmus & Ali 2005) and is not necessarily an indicator of increased cardiovascular risk. Notably, there were no sex-specific differences of the IMT of the right and left common carotid arteries, which is a more reliable measure of cardiovascular risk than elevated resistive indices (Ohta et al. 2008). Nevertheless, it is possible the significantly increased resistive indices of both common carotid arteries is related to impaired cerebral perfusion associated with atherosclerosis (Frauchiger et al. 2001) and represents the early signs of increased cardiovascular risk in the male sheep compared to females. Additional ultrasound evaluation of the common carotid artery diameter and resistive indices are warranted in older sheep born moderately premature, to determine whether these sex-specific differences continue beyond early adulthood and are ultimately revealed as the first signs of male disadvantage in later life.

My findings demonstrate that moderate preterm birth, *per se*, is not associated with male disadvantage from birth to early adulthood. It should be noted that our preterm sheep model is independent of maternal and fetal co-morbidities that are commonly present in other studies, particularly human studies. It is likely that morbidities that either result in preterm birth (such as PPROM), or are associated with preterm birth (such as IUGR) are more likely to contribute to male disadvantage than moderate preterm birth *per se* (Bennet et al. 2007; Moutquin 2003).

7.3. Moderately preterm birth and antenatal administration of betamethasone.

The model of preterm birth used in the short term (2 days) evaluation (chapter 3) and long term studies (chapters 4 to 5), exposed the fetal sheep to a single course of antenatal betamethasone that was administered to the maternal ewes a few hours prior to the induction of delivery. The clinically relevant dose that was administered (2 x 11.4 mg i.m. 24 hrs apart) is identical to that recommended for women at risk of preterm delivery (Berry, M et al. 2013; Liggins Institute 2015). Glucocorticoids such as betamethasone enhance survival after preterm delivery by accelerating fetal lung maturation (Berry, L et al. 1997; Roberts & Dalziel 2013). Betamethasone is an essential element of the moderately preterm sheep model because it significantly decreases mortality caused by respiratory insufficiency and reduces the need for further post-natal intervention such as the use of surfactants and mechanical ventilation (De Matteo et al. 2010). The clinical dose of betamethasone administered to our long term sheep cohort appears to have increased male preterm lamb survival at 14 days after birth to 12/16 (75%), when compared to the 18/44 (44%) survival that was achieved in a previous study that utilised a similar sheep model that administered non-clinical doses of 3 mg or 5.7 mg of betamethasone (De Matteo et al. 2010).

The model of preterm birth used in the short term (two hours) evaluation (chapter 6), comparing male and female preterm lambs, utilised very different protocols to those used in the other studies of this thesis (chapters 3 to 5). As part of Dr Ishak's PhD experiments that were conducted concurrently, the fetal sheep underwent surgical intervention *in utero* at 125 days gestational age to chronically implant pressure catheters into a carotid artery and the amniotic sac. Fetal arterial and amniotic pressure measurements were recorded at 131 days gestational age, immediately followed by administration of betamethasone (5.7 mg i.m., betamethasone (Celestone Chronodose, Schering-Plough, Australia) to the pregnant ewes. The antenatal dose of betamethasone administered to the short term (two hours) cohort of lambs was significantly lower than the recommended clinical dose administered to the other sheep cohorts that were investigated in this thesis (chapters 3 to 5). The lower survival rate of the male preterm lambs, 6/8 (75%) compared to the females, 9/9 (100%) at two hours after birth, could have been due to several factors. Exposure to antenatal betamethasone has previously been shown to provide a lesser prophylactic effect in male infants compared to females (Stark, M, Wright & Clifton 2009). The non-clinical dose of betamethasone administered to the short term (two hours) lamb cohort further reduces the already lower benefit to the male lambs compared to females. Male preterm lambs have an inherently higher risk of developing pulmonary insufficiency and breathing complications (Elsmen, Steen & Hellstrom-Westas 2004) and are, therefore, less likely to survive than female lambs. It has also been suggested that male lambs may be simply less resilient to fetal surgical intervention compared to female lambs (De Matteo et al. 2015).

It should be noted that the use of betamethasone to increase the viability of newborn preterm lambs potentially introduces a confounding variable. My findings may be the result of moderate preterm birth and/or the effects of betamethasone. It is difficult to differentiate the effects of glucocorticoids *per se* from the effects of prematurity in human studies because most individuals born preterm are routinely exposed to antenatal glucocorticoids (Bayman, Drake & Piyasena 2014). In addition, lamb survival in the sheep model of preterm birth necessitates the administration of antenatal betamethasone (De Matteo et al. 2010). The benefits of glucocorticoid treatment in preterm delivery are well documented (Liggins & Howie 1972; Roberts & Dalziel 2013), but it has been shown to adversely affect the development of various organs including the heart; enhanced cardiomyocyte proliferation and maturation (Giraud et al. 2006; Kim, M et al. 2014), blood vessels; alterations in wall structure and endothelial dysfunction (Pulgar & Figueroa 2006) and kidneys; decreased number of glomeruli and reduced renal weight (Figueroa et al. 2005; Li, S et al. 2013). The effects of glucocorticoid treatment depend on the type of glucocorticoid administered, dose and timing of delivery (Molnar et al. 2002; Moss et al. 2005). Our study is not able to separate the effects of antenatal betamethasone from the effects of moderately reduced gestation, however, our results are clinically relevant given antenatal betamethasone treatment is currently recommended for all women at risk of preterm delivery in Australia and New Zealand (Berry, M et al. 2013; Liggins Institute 2015). It appears the benefits of significantly decreasing newborn mortality, morbidity and the likelihood of medical intervention and mechanical ventilation (which would also potentially introduce confounding variables) outweigh the potential risks of using betamethasone in our moderately preterm sheep model. Nevertheless, further research is warranted to verify that the benefits do indeed offset the possible short and longer term risks of betamethasone administration.

7.4. Ultrasound imaging of sheep from moderately preterm birth to adulthood.

One of the main outcomes of my thesis was the establishment of scanning protocols for the serial ultrasound evaluation of sheep from shortly after moderately preterm birth to adulthood. I developed scanning protocols so that images of the left ventricle, major arteries and kidneys could be obtained using readily available clinical ultrasound equipment, without the need for general anaesthesia or sedation of the animals. Standard B-mode, M-mode, and Doppler modalities were employed using conventional ultrasound probes and system settings, together with size appropriate sheep handling techniques, to successfully acquire the relevant structural and functional data. I developed specific sheep handling protocols to accommodate the size of the animals as they grew larger from birth through to adulthood. A specialised sheep handling device called a V-drive was used to assist in the positioning and restraining of sheep from six months of age. However, the use of specialised sheep handling equipment is not essential and any raised platform that can comfortably support the sheep and be located next to the ultrasound system is sufficient. Of course, it is preferable to use specialised sheep handling equipment like a V-drive, if available, to reduce the need to manually lift unsupported older sheep that are large and heavy.

The use of animal handlers in my scanning protocols removed the need to use sedation or general anaesthesia to control the sheep during the ultrasound examination. This eliminated the requirement to medicate the sheep and introduce a potentially confounding variable for subsequent findings. Sheep handlers are essential because the sonographer cannot safely and securely restrain an animal being scanned (even a newborn lamb) and perform the ultrasound examination at the same time. Only one animal handler is required to adequately restrain a lamb of up to two weeks of age. My protocols involve the use of two handlers to restrain the sheep from three months of age, as the animals are too large and strong to be held safely by one average sized adult. Surprisingly, the animal handlers found it tiring to gently and securely restrain the animals for the usual 30 to 40 minutes examination time. A typical ultrasound scanning session, involving the examination of several sheep in a row, can amount to many hours of continuous sheep handling. Therefore, my scanning protocols also include taking a short break in-between each ultrasound examination during long sessions to give the handlers a rest and to keep them alert. Given the scanning protocols created for my studies were predicated on the use of easily accessible clinical ultrasound equipment and the simple manual handling of sheep, they can be readily utilised by other investigators in future *in vivo* non-invasive ultrasound evaluations of the cardiovascular structures and kidneys of sheep.

7.5. Limitations of this study and recommendations for future research.

Unless changes to the cellular structure of organs in response to moderate preterm birth results in an altered macroscopic appearance, they cannot be detected using ultrasound imaging. Previous histological analyses have shown cellular changes to the wall of the heart (cardiomyocyte hypertrophy and increased collagen deposits) and the wall of the aorta and pulmonary arteries (increased elastin deposits and reduction in smooth muscle content) in sheep after moderate preterm birth (Bensley, J et al. 2012; Bensley, J et al. 2010). In addition, the histological analysis conducted on our short term (two day) cohort of lambs by Dr Nguyen as part of her PhD studies found a significantly altered cardiomyocyte structure (increased number and altered nuclearity) in the preterm lambs consistent with a structurally immature cardiac wall (Nguyen, V. 2016). Further histological analysis by Dr Nguyen on our long term sheep cohort (at 14 months of age) found significantly altered elastin and muscle content of the left CCA wall compared to term controls (Nguyen, V. 2016). None of the histological changes in our sheep were significant enough to change the macroscopic structure of the heart or vessel walls and be demonstrated in our ultrasound findings. However, one advantage of ultrasound imaging over histological analysis is that individual sheep can be serially evaluated in a non-invasive manner over a long time period, to discover when substantial changes associated with preterm birth first arise and then be able to track those changes to determine when they become significantly different to control animals.

Ultrasound imaging is restricted by the inability of the ultrasound beam to penetrate gas or dense bone. This limitation was generally overcome in my studies by utilising the routine scanning strategies of changing the positioning of the sheep and/or using different ‘lines of sight’ to vary the angle of the ultrasound beam so it did not intersect any air or bone on its way to the organ of interest (Coombs 2004). However, this was not always possible. For example, the bowel gas that was present in the rumen of sheep from three months of age could not be avoided by modifying the scanning angle or repositioning the animals during the scans, resulting in non-visualisation of the left kidney. An attempt was made to scan the sheep in the early morning before their first feed of the day in an effort to reduce the amount of bowel gas, but that did not help. Therefore, the renal analyses was limited to the right kidney and right renal artery in the cohort of sheep undergoing serial evaluation to twelve months of age (chapter 4) and again at the fourteen month evaluation (chapter 5). The non-visualisation of the left kidney in the older animals is unlikely to significantly impact my findings. There is no evidence in my results, or reported in the literature, to suggest that the left kidney is disadvantaged or responds differently to preterm birth or other physiological challenges compared to the right kidney. Any future sheep imaging studies involving evaluation of the left kidney beyond three months of age will require an alternative imaging modality that is not restricted by bowel gas, such as CT or MRI. However, the use of such imaging modalities would create new challenges for sheep handling, require general anaesthesia, limit accessibility and increase financial costs compared to ultrasound imaging.

At twelve months of age, sheep are sexually mature but have only reached early adulthood (De Matteo et al. 2010). Although the effects of preterm birth have been demonstrated in early adulthood (Finken et al. 2006; Skilton et al. 2011), the long term effects of moderate preterm birth may only become evident in mid-life or later (Burke et al. 1995; Cooper, Atherton & Power 2009; Kerkhof et al. 2012). My serial ultrasound evaluation, from two days to twelve months of age, demonstrated few significant differences in the structure and function of the left ventricle, major arteries and kidneys of moderately preterm sheep compared to controls. Notably, in the same cohort of sheep the ultrasound study at fourteen months of age demonstrated for the first time the emergence of sex-specific differences in the diameter and blood flow within both common carotid arteries. Findings in human studies suggest these results reflect the normal variation between males and females in adulthood (Albayrak et al. 2007; Krejza et al. 2006; Ruan et al. 2009). However, additional long term ultrasound studies conducted in older sheep are required to determine if these sex-specific differences are the first signs of increased cardiovascular risk leading to male disadvantage in mid or later life.

The incidence of morbidity and other complications following preterm birth are inversely related to gestational age (Dani et al. 2009). Future ultrasound investigations evaluating the effects of very and extremely premature birth should be conducted as they are more likely to demonstrate cardiovascular and renal changes than in moderately preterm birth (Kerkhof et al. 2012; Siewert-Delle & Ljungman 1998). However, the evaluation of very and extremely preterm birth will necessitate the use of neonatal intensive care and other medical support in order to maximise the survival of these subjects. Further investigation of the effects of severe prematurity is worthwhile not only to help determine the general mechanisms leading to increased cardiovascular risk and male disadvantage, but to provide a greater understanding of the specific effects on individuals born very and extremely premature who are increasingly likely to survive due to improved neonatal intensive care (Saigal & Doyle 2008; Vohr & Allen 2005).

The number of sheep in each cohort in my studies are typical of large animal studies (Bubb et al. 2007; Cock, M et al. 2005; Tare et al. 2014). Financial limitations and ethical considerations strictly limit the number of animals to the minimum required to conduct the investigation and still obtain meaningful results. The number of male versus female and preterm versus term sheep in each cohort of this thesis were balanced, except for the short term cohort that was scanned at two days of age (chapter 3). That cohort had 5 male term lambs compared to 10 female term lambs, 10 male preterm lambs and 11 female preterm lambs. The relatively lower number of male term lambs compared to the other categories of sheep in the cohort could introduce a potential bias in the results.

A limitation of the investigations conducted in this thesis is that only the animals that survive to the end of the experimental period undergo ultrasound evaluation. As a consequence, it is the more robust offspring that are assessed in the studies. In the short term (two day) cohort of sheep (chapter 3), 4/10 (40%) of the male and 4/11 (36%) of the female preterm lambs did not survive to two days of age and were not included in the analyses. In the long term cohort of sheep (chapters 4 & 5), 7/16 (44%) of the male and 5/12 (42%) of the female preterm sheep did not survive to 14 months of age and were excluded from the analyses. In the short term (two hour) cohort of lambs (chapter 6), where male and female preterm lambs were compared, all the females (9/9) survived, but 2/8 (25%) of the males died and were not included in the ultrasound evaluation. When subject numbers are small, not being able to include the data from one or two of the weaker animals that do not survive may introduce a selection bias that could potentially affect the overall findings. The results of our comparison of male and female lambs at two hours after birth could potentially be different if it were possible to include the two weaker males that did not survive, particularly when looking for evidence of male disadvantage.

Another limitation is the lack of blinding in the study which could potentially introduce bias. It is problematic for the investigator conducting the ultrasound examinations to be blinded to whether the animals were born preterm/term or male/female as the size discrepancy and sex is readily apparent. The ultrasound examination protocols for all the sheep were identical and this would help reduce the potential for bias. The investigator was blinded to the sex and gestational age at birth of the animals when performing the off-line measurements on the collected data.

7.6. Conclusion.

Serial ultrasound imaging has been successfully employed to evaluate the cardiovascular system and kidneys of moderately preterm sheep from birth to early adulthood. Ultrasound analyses have demonstrated that overall, in the absence of maternal and fetal co-morbidities, moderately premature birth *per se* does not result in significant structural and functional mal-adaptations of the heart, major arteries and kidneys or lead to significant sex-specific differences that indicate male disadvantage in sheep. These findings are very encouraging given the vast majority of premature deliveries are moderately preterm. Future serial ultrasound studies directed at the contribution of co-morbidities to increased cardiovascular risk associated with moderate preterm birth are recommended. The results of my studies will help inform the management of individuals born moderately preterm and aid in the development of future serial non-invasive *in vivo* surveillance strategies for cardiovascular disease, with the objective of identifying risk in the growing population of those born prematurely.

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