An investigation of the basis for the male disadvantage in respiratory function and survival following preterm birth

A thesis submitted for the degree of Doctor of Philosophy

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Summary

Preterm birth accounts for about 8–12% of live births in most developed countries. It is widely known that male preterm infants have a greater risk of respiratory insufficiency than females, which may have long-term consequences. Male preterm infants require more initial respiratory and circulatory support than preterm females. To date, little is known about the causes and basis of this male disadvantage and further investigation is required for a better understanding. The aim of this study was to make an in-depth investigation of the possible causes for the male disadvantage in respiratory function following preterm birth.

In one study (fetal study group) we used fetal sheep at 131 days of gestation (d GA; term is 147 days) to provide lung tissue for morphometric analysis and measurement of gene expression of *surfactant proteins* and *tropoelastin* in male and female preterm fetuses. In two other groups of sheep (postnatal studies) we used an established model of moderate preterm birth for physiological studies before and after preterm birth. In the postnatal studies, preterm lambs were delivered at 133d GA as lambs born at this age demonstrate lower survival in males than females. The animals in the postnatal study group were grouped into two cohorts; one group was studied for 4 hours (h) after birth and the other group was studied for 8 h to determine whether any differences between the sexes changed with time after birth. Pregnant ewes from both cohorts underwent surgery to chronically implant fetuses with catheters at ~125d GA for the measurement of fetal physiological status at 131 and 132d GA and fluid (plasma, lung liquid and amniotic fluid) sampling and collection from 125–133d GA. A small dose of betamethasone (5.7 mg of Celestone Chronodose) was administered to the ewe at 131d GA. Unanaesthetised lambs were delivered at 133d GA, approximately 14

days before term, and then studied physiologically for 4 h or 8 h after delivery. At the end of the 4 h or 8 h study periods, the lambs were euthanised for measurement of static lung compliance and collection of bronchoalveolar lavage fluid (BALF) and lung tissue. The surfactant phospholipid composition and protein concentration of BALF and the gene expression of surfactant proteins *(SP)-A, -B, -C* and *-D* and the protein expression of SP-A and pro-SP-C in lung tissue were also measured.

We observed that, at necropsy, lungs excised from fetal sheep at 131d GA were similar in males and females in terms of lung morphology and *surfactant protein* and *tropoelastin* gene expression. Our findings indicate that these factors did not contribute to the male disadvantage. Minor differences in surfactant phospholipid composition were observed in the fetal lung liquid collected but it is unknown to what extent they affect respiratory outcome.

In the postnatal study where lambs were monitored for 4 h following preterm birth, male lambs demonstrated poorer arterial blood gas parameters (lower pH and higher $PaCO_2$,) higher arterial glucose and lactate concentrations and had a greater requirement for supplemental oxygen. Arterial cortisol concentration was lower in males than in females before birth. In males we observed altered surfactant phospholipid composition (significantly lower proportions of the molecular species PC 32:0 and higher PC 34:2 and PC 36:2 compared to females) and elevated protein concentration in the BALF, and significantly lower pro-SP-C protein expression in lung tissue; together these alterations could impair surfactant function and contribute to the lower lung volume at necropsy when the lungs were inflated at a pressure of 40 cmH_20. Overall, the observed sex differences indicate poorer postnatal adaptation in males and could account for the death of 2 males following the first hour of delivery. Consistent with the findings in the fetal sheep, the lung morphology and *surfactant protein* gene expression were similar in males and females at 4 h after birth, indicating that the male disadvantage in respiratory

function following preterm birth is unlikely to be a result of sex differences in lung architecture and *surfactant protein* gene expression.

When another group of lambs was monitored for 8 h after preterm delivery, the findings from the 4 h postnatal study were largely confirmed but there were subtle differences, some of which could be due to the different ventilation technique used in this group of lambs. As in the 4 h study, poorer gas exchange (lower pH and higher $PaCO_2$) was observed in males. Major findings were a greater inspiratory effort in male lambs than those of female lambs and that male lungs continued to be less compliant than female lungs for up to 8 h after birth. The BALF of males collected at 8 h after birth had significantly lower proportions of the molecular species PC 32:1 and PE 36:2 than in females. In lung tissue, pro-SP-C protein expression was significantly lower in male lambs than in females.

We conclude that the male disadvantage in cardio-respiratory adaptation following preterm birth is likely caused by subtle alterations in surfactant phospholipid composition. These may consequently affect lung compliance, thereby altering gas exchange and arterial blood gas parameters. This study provides evidence to support the provision of a targeted intervention in the management of male preterm babies to reduce the RDS incidence and increase the chance of survival.

Declaration

This thesis does not contain any material which has been accepted for the award of any other degree or diploma in any university or other institution. To the best of my knowledge this thesis does not contain any material previously published or written by another person, except where due reference is made in the text.

Noreen Ishak

Publications and presentations

Journal publications

Ishak N, Hanita T, Sozo F, Maritz G, Harding R, De Matteo R. Sex differences in cardiorespiratory transition and surfactant composition following preterm birth in sheep. American Journal of Physiology – Regulatory, Integrative and Comparative Physiology. 2012 Oct; 303 (7):R778-89.

Ishak N, Sozo F, Harding R, De Matteo R. Does lung development differ in male and female fetuses? Experimental Lung Research; 2014 Feb; 40 (1):30-9.

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1. **Ishak N**, Sozo F, De Matteo R, Harding R. Is there a structural basis for the male disadvantage in lung function after preterm birth? Healthy Start to Life Network, 4th Annual Scientific Conference. "Nutrition, Development and Health". Monash University, Melbourne, Australia, 2009.

2. **Ishak N**, Sozo F, De Matteo R, Stacy V, Harding R. Gender effects on lung development in preterm lambs. Australia and New Zealand Fetal and Neonatal Workshop, Darwin, Australia, 2009.

3. Sozo F, **Ishak N**, De Matteo R, Hooper S, Harding R. Structural and biochemical development of the ovine lung: Are there gender differences? Perinatal Society of Australia and New Zealand. Darwin, Australia, 2009. *J Paediatr Child Health.* 2009 Apr;45 Suppl 1

4. Sozo F, De Matteo R, **Ishak N**, Hanita T, Thompson B, Snibson K and Harding R. Gender differences in the developing lung: what is the basis for the male disadvantage? Annual Meeting of the Network in Genes and Environment in Development, Palm Cove, Australia, 2009.

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7. **Ishak N**, Hanita T, De Matteo R, Sozo F, Hooper S, Harding R. What is the basis for the male disadvantage in respiratory function following preterm birth? Perinatal Society of Australia and New Zealand, Wellington, New Zealand, 2010.

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9. Sozo F, **Ishak N**, De Matteo R, Hanita T, Weir J, Meikle P, Hooper S, Harding R. Gender differences in respiratory outcome following preterm birth: what are the mechanisms? Cool Topics in Neonatology, Royal Women's Hospital, Melbourne, Australia, 2011.

10. **Ishak N**, Hanita T, De Matteo R, Sozo F, Hooper S, Harding R. Reduced static lung compliance may account for poorer physiological respiratory function following preterm birth in males. Pediatric Academic Societies and Asian Society for Pediatric Research, Denver, Colorado, USA, 2011.

11. **Ishak N**, Hanita T, De Matteo R, Sozo F, Hooper S, Harding R. Investigating the basis of poorer respiratory function in males following

preterm delivery. Perinatal Society of Australia and New Zealand, Hobart, Tasmania, Australia, 2011.

12. **Ishak N**, Sozo F, De Matteo R, Hanita T, Weir J, Meikle P, Hooper S & Harding R. Is the male disadvantage in respiratory outcome following preterm birth a result of altered surfactant composition? Fetal and Neonatal Physiological Society, Palm Cove, Australia 2011.

13. **Ishak N**, Hanita T, De Matteo R, Sozo F, Harding R. Physiological basis of poorer respiratory outcomes for males following preterm birth. Federation of Asia and Oceania Perinatal Societies & Perinatal Society of Australia and New Zealand, Sydney, Australia, 2012.

14. Harding R, **Ishak N**, Hanita T, Sozo F, De Matteo R. Physiological basis of poorer respiratory outcomes for males following preterm birth. Experimental Biology, San Diego, USA, 2012.

15. **Ishak N**, Hanita T, De Matteo R, Sozo F, Harding R. Physiological adaptation to preterm birth differs between males and females: basis for the male disadvantage. Perinatal Society of Australia and New Zealand, Adelaide, Australia, 2013.

16. **Ishak N**, Hanita T, Sozo F, Harding R, De Matteo R. The phospholipid composition of fetal lung liquid, but not lung structure, is different between preterm male and female fetal sheep. Perinatal Society of Australia and New Zealand, Adelaide, Australia, 2013.

17. **Ishak N**, Hanita T, Sozo F, De Matteo R, Harding R. The male disadvantage following preterm birth: role of lung maturation. Developmental Origins of Health and Disease, Singapore, 2013.

Underlining indicates person who made the presentation.

Student symposia

1. **Ishak N**, Sozo F, De Matteo R, Harding R. Gender effects: are there differences in lung development in preterm fetal sheep? Higher Degree by Research Student Poster Exhibition, Monash University, Melbourne, Australia, 2009.

2. **Ishak N**, Hanita T, De Matteo R, Sozo F, Hooper S, Harding R. Investigating the mechanisms responsible for the male disadvantage in respiratory function following preterm birth. Postgraduate Student Symposium, Department of Anatomy & Developmental Biology, Monash University, 2009.

3. **Ishak N**, Hanita T, De Matteo R, Sozo F, Hooper S, Harding R. Investigating the basis for the male disadvantage in respiratory function following preterm birth. Postgraduate Student Symposium, Department of Anatomy & Developmental Biology, Monash University, 2010.

4. **Ishak N**, Hanita T, De Matteo R, Sozo F, Hooper S, Harding R. Why are males at a greater risk than females after preterm birth? Postgraduate Student Symposium, Department of Anatomy & Developmental Biology, Monash University, 2011.

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List of abbreviations and symbols

Abbreviations

I	one
II	two
III	three
4-MA	17β-N,N-diethylcarbamoyl-4-aza-4-methyl-5α-androstane-3-one
A	adenine
ACh	acetylcholine
ADMA	asymmetrical dimethlyarginine
AECs	alveolar epithelial cells
C	cytosine
cDNA	complementary deoxyribonucleic acid
CPAP	continuous positive airway pressure
Ct	cycle threshold
DAB	diaminobenzidine
dATP	deoxyribonucleic acid triphosphate adenosine
dCTP	deoxyribonucleic acid triphosphate cytosine
d GA	days of gestational age

dGTP	deoxyribonucleic acid triphosphate guanine
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPPC	dipalmitoylphosphatidylcholine
DPX	distrene plasticizer in xylene
dTTP	deoxyribonucleic acid triphosphate tyrosine
E	embryonic age (days)
EDTA	ethylenediaminetetra-acetic acid
e.g.	***exempli gratia***, for example
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EP	prostaglandin E receptor
ER	estrogen receptor
F	female
FPF	fibroblast pneumocyte factor
FRC	functional residual capacity
g	gravitational acceleration
G	guanine
G&S	Gordon and Sweet’s reticular fibre stain
h	hour(s)
HR	heart rate

HRP	horseradish peroxidase
H&E	haematoxylin and eosin
I.D.	inner diameter
i.e.	that is
IgG	immunoglobulin G
i.v.	intravenous
kDa	kilodalton
L	lecithin
L/S	lecithin/sphingomyelin
LPC	lysophosphatidylcholine
M	male
MAP	mean arterial pressure
min	minute(s)
MIS	Müllerian Inhibiting Substance
MLI	mean linear intercept
mRNA	messenger ribonucleic acid
M-MLV RT	Moloneys-murine Leukemia Virus reverse transcriptase
n	number of animals
N_2	nitrogen
NO	nitric oxide
NOS	nitric oxide synthase
PBST	phosphate buffered saline containing the detergent Tween

O.D.	outer diameter
P	probability value
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCO_2	partial pressure of carbon dioxide
PCR	polymerase chain reaction
PC/S	phosphatidylcholine/sphingomyelin
PC/PE	phosphatidylcholine/ phosphatidylethanolamine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PGE_2	prostaglandin E_2
pH	power of hydrogen ion
PI	phosphatidylinositol
PO_2	partial pressure of oxygen
PS	phosphatidylserine
P_S	probability of sex as a factor
P_T	probability of time as the repeated measure
P_{SxT}	probability of the interaction between time and sex
P-V	pressure-volume
PVDF	polyvinylidene difluoride
qPCR	quantitative polymerase chain reaction
RDS	respiratory distress syndrome

rhMIS	recombinant human Müllerian Inhibiting Substance
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RPS 29	ribosomal protein S29
S	sphingomyelin
SaO_2	saturation of oxygen in arterial blood
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
sec	second
SPC	saturated phosphatidylcholine
SPC/S	saturated phosphatidylcholine/sphingomyelin
SP	surfactant protein
SPs	surfactant proteins
SpO_2	oxygen saturation measured by pulse oximetry
T	thymine
TGF-β	transforming growth factor-β
TPE	tropoelastin
TTN	transient tachypnea of the newborn
UDG	uracil-DNA glycosylase
vs	versus
wk GA	weeks of gestational age

wk GA	weeks of gestational age

Symbols

~	approximately
=	equals/equivalent to
α	alpha
β	beta
±	plus or minus
-	minus
–	to
/	per
>	greater/more than
<	smaller/less than
x	multiplied by
X	magnification
:	ratio
#	number
↓	lower
↑	greater
↔	no change

Units of measurement

%	percent
ºC	degrees Celsius
ρ	pico (10^{-12})
µ	micro (10^{-6})
cm	centimetre
cm^3	cubic centimetre
cmH_2O	centimetres of water
Da	Dalton
g	gram
IU	international unit
kg	kilogram
L	litre
m	metre
M	molar
mcg	microgram
mg	milligram
ml	millilitre
mm	millimetre
mm^3	cubic millimetre
mM	millimolar
mmH_g	millimetres of mercury

mmol	millimoles
ms	millisecond
ng	nanogram
V	volt
v/v	volume/volume
w/v	weight/volume

Chapter 1: Literature review

1.1 Preterm birth

Adequate respiration is essential for survival following birth. This is achievable when a neonate is born with lungs that are structurally and functionally mature, as is normally observed in a baby born at term. Preterm birth, defined as birth prior to 37 completed weeks of gestation, is associated with a number of morbidities as a result of immature organ development. Immaturity of organs, especially the lung, can affect survival following preterm birth. Despite better fetal-maternal care, the incidence of preterm birth in most developed countries has continued to rise, ranging from 8–12% of annual total births. In 2009, the incidence of preterm births in Australia was 8.2% of all live births (Li et al., 2011).

1.2 Effects of sex on birth outcomes

There is now a large amount of evidence indicating that males are at a greater risk of morbidity and mortality, compared to females during infancy, childhood and throughout adulthood (Kraemer, 2000, AIHW, 2010, Balsara et al., 2013). During the perinatal period, differences in mortality, growth, and susceptibility to specific diseases are apparent between male and female infants, where males have worse outcomes. Even before birth, males are more likely to die than females (Mizuno, 2000), with the number of spontaneous abortions of male fetuses being higher than for females (Hassold et al., 1983, Engel et al., 2008). During childhood, males continue to be at greater risk of illness and death than females (Balsara et al., 2013). As adults, males continue to experience higher rates of morbidity, including a higher incidence of cancer, cardiovascular diseases, coronary heart disease, cerebrovascular disease and diabetes (AIHW, 2010).

During gestation, the vulnerability of males, relative to females, changes with development. The first trimester is the most vulnerable period for male fetuses, and it has been estimated that 4 male fetuses die for every female death during this stage of gestation (McMillen, 1979). The ratio of male to female *in utero* mortality declines as gestation progresses, reaching a value of 1:1 in late gestation (McMillen, 1979). Although the higher mortality risk in male fetuses during gestation is well documented, the causes of sex-specific mortality, and the reasons for the change in male mortality with gestational age, are largely unknown although sex hormones are likely to play a role (Ingemarsson, 2003).

Preterm birth clearly reveals sex-related differences in infant morbidity and survival. The risk of mortality and illness is known to be greater in males than in females of the same gestational age following preterm birth (Papageorgiou et al., 1981, Stevenson et al., 2000, Ingemarsson, 2003, Bhaumik et al., 2004, Elsmen et al., 2004, Mathews and MacDorman, 2011). The 'male disadvantage' has been reported in moderately preterm (30–34 weeks of gestation) birth (Altman et al., 2013) and even late preterm and term infants specifically of Caucasian ethnicity (Anadkat et al., 2012). This is largely due to lung immaturity and respiratory distress following preterm birth. Consequently, male infants are at a higher risk of neonatal mortality and are also vulnerable to neurological and motor impairments (Rosen and Bateman, 2010).

1.3 Preterm birth and respiratory distress

The Respiratory Distress Syndrome (RDS) occurs almost exclusively among infants born preterm, and is a result of lung immaturity. In preterm infants, lung immaturity can be due to structural immaturity, a deficiency in pulmonary surfactant production and also a reduced ability of the lung to clear lung liquid following birth (Seaborn et al., 2010). RDS is characterised by an increased breathing effort, acidosis and the need for supplemental oxygen. The development of RDS, commonly known as hyaline membrane disease, contributes to other

morbidities such as bronchopulmonary dysplasia, chronic lung disease and long-term neurological disabilities. The incidence of RDS is strongly influenced by gestational age at the time of birth, such that the earlier in gestation that infants are born, the greater the risk of developing RDS. Of interest to my study, the incidence of RDS and deaths from respiratory insufficiency is greater in preterm males in comparison to females of the same gestational age (Carey et al., 2007, Seaborn et al., 2010). As a result of this 'male disadvantage', preterm male infants usually need more initial respiratory and circulatory support than preterm females (Elsmen et al., 2004). However, little is known about the causes and basis of this male disadvantage and further investigation is required to gain a better understanding. In order to understand the male disadvantage in respiratory outcomes following preterm birth, it is necessary to first consider normal lung development.

1.4 Normal lung development

1.4.1 Structural lung development

As in other mammals, lung development in humans occurs in five recognisable stages. In human lung development these stages occur during gestation and continue until the infant is approximately 8 years of age (Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013). The progression of lung development is not discrete as it is a continuous process with overlapping stages (Burri, 1984, Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013). Similar stages of lung development occur in other mammalian species such as sheep, rabbits, and rats but the timing and duration of these stages vary among species because of differences in the length of gestation and maturity of the newborn animal at birth (Table 1.1).

Table 1.1: Stages of lung development in humans, sheep, rabbits and rats.

Stage of lung development	Humans (weeks)	Sheep (days)	Rabbits (days)	Rats (days)
Embryonic	~ 0–6	~0–40	~0–18	~0–13
Pseudoglandular	~ 6–16	~40–80	~18–24	~13–18
Canalicular	~ 16–26	~80–120	~24–27	~18–20
Saccular	~26–term	*	~27–term	~20–postnatal
Alveolar	~32–postnatal	~120–postnatal	Postnatal	Postnatal

Based on data in Burri (1974), Harding (1994) and Coalson (2006). *In sheep, morphological characteristics of the saccular stage are similar to those of other species but a specific gestational age range in which the stage occurs has not been defined (Alcorn et al., 1981).

1.4.1.1 Embryonic stage

Lung development begins during embryonic life. The formation of a primitive foregut endoderm, which later forms the esophagus, occurs prior to the development of future airways. The future airways first appear in the form of a ventral diverticulum from the foregut endoderm (Figure 1.1 A; (Bryden et al., 1973, Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013)). This is evident by the end of the fourth week in humans and day 17 in sheep (Bryden et al., 1973, Burri, 1984). The outgrowth separates from the future esophagus, elongates and invades the surrounding mesenchyme to form a “trachea” which continues to divide into two buds, called lung buds; these buds give rise to the left and right lungs (Figure 1.1 B). They further divide to form the lobar buds that give rise to the bronchopulmonary segments, lined with endodermal epithelium (Figure 1.1 C; (Burri, 1984, Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013)).

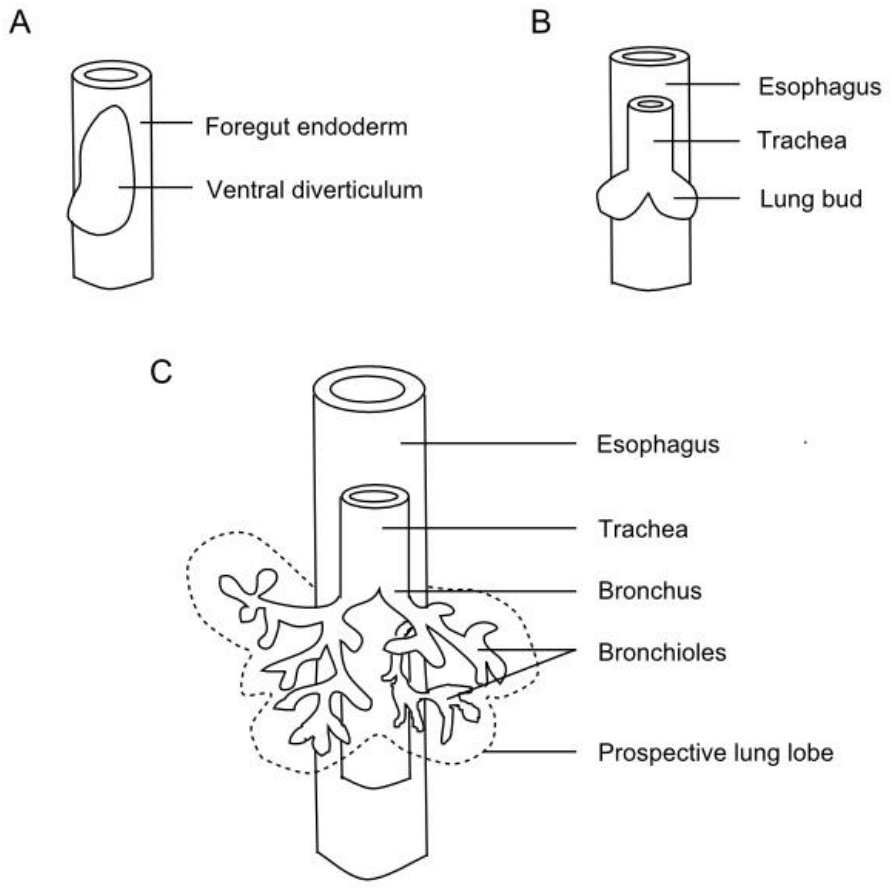

Figure 1.1 Embryonic lung development. A ventral diverticulum projects from the foregut endoderm (A) to form lung buds (B) that branch to form future airways (C). Adapted from Bryden et al. (1973).

1.4.1.2 Pseudoglandular stage

The pseudoglandular stage, named due to the lungs' glandular appearance during this stage, is stimulated by the presence of surrounding mesenchyme (Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013). A number of important outcomes such as the development of the hierarchical pattern of the pre-acinar airways and blood vessels and the formation of prospective conductive airways occur during this stage of development (Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013). The conducting airways are formed by repeated dichotomous branching that occurs in a centrifugal manner from the central region, radiating outwards towards the lung periphery (Metzger et al., 2008). These branches (epithelial-lined

pre-acinar airways) increase in size with further growth and cessation of new formation of branches occurs after the 16th week in humans (Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013). The surrounding mesenchyme eventually differentiates into cartilage, connective tissue, muscle, blood vessels and lymphatic vessels (Burri, 1984, Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013). In addition, ciliated, goblet and basal cells begin to appear in the airway epithelium (Burri, 1984, Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013).

1.4.1.3 Canalicular stage

The canalicular stage sees the development of the primitive gas exchange region where respiratory bronchioles lengthen and widen and eventually subdivide into terminal saccules (Alcorn et al., 1981, Davis and Mychaliska, 2013). Rapid expansion of peripheral respiratory units occurs to increase the volume of future airways while gradual thinning of the cuboidal epithelium and interstitial tissue takes place (Alcorn et al., 1981). This thinning of the interstitial tissue is essential for the formation of a thin blood gas barrier to allow for efficient gas exchange (Burri, 1984, Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013). During pulmonary vasculogenesis, the development of the distal pulmonary circulation begins with capillaries that penetrate into the surrounding mesenchyme and align adjacent to the epithelial cells to further reduce the thickness of the blood gas barrier (Alcorn et al., 1981, Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013). Differentiation of alveolar epithelial cells (AECs) is also initiated at this stage. Type I AECs are flattened cells that have long cytoplasmic extensions that provide a large surface area for future gas exchange, while type II AECs are more rounded or cuboidal in shape and are involved in surfactant production and secretion in the later stages of lung development (Davis and Mychaliska, 2013).

1.4.1.4 Saccular stage

During the saccular stage, the lung is characterised by terminal sacs known as saccules, which are the predecessors of alveoli (Burri, 1984, Davis and

Mychaliska, 2013, Laudy and Wladimiroff, 2000). During this stage, thinning of the interstitium continues, and tissue crests, known as secondary septa, protrude into saccules dividing them into smaller units (Figure 1.2). The projection of crests from the saccules eventually results in the formation of alveoli (Figure 1.2) (Burri, 1984, Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013). Elastin is deposited at the tips of the crests (Figure 1.2) and is important in regulating alveolar formation (Burri, 1984). During this stage, the secondary septa are about two capillaries thick and gas exchange becomes possible but is not very efficient (Burri, 1984, Davis and Mychaliska, 2013). Type I and II AECs progressively become morphologically distinct. In humans, the type II AECs become mature between 32 and 36 weeks of gestation to produce surfactant that enhances the functional maturity of the lung. Infants born at this stage of lung development may experience respiratory distress as a result of poor gas exchange due to immaturity of lung structure and surfactant deficiency, both of which reduce lung compliance; however, infants at this age are capable of surviving with medical intervention (Tucker and McGuire, 2004). In sheep, the saccular stage is not well defined; rather than being a separate stage, it overlaps with the alveolar stage (Alcorn et al., 1981).

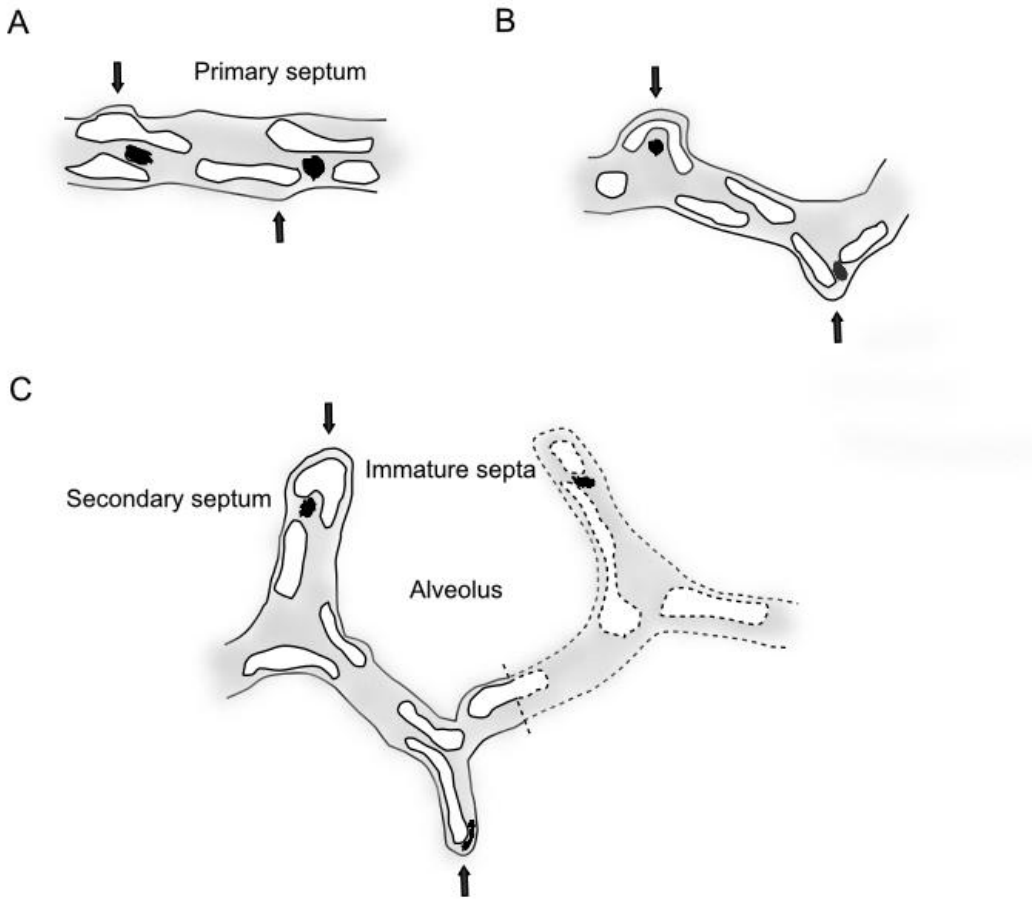

Figure 1.2 Alveolar formation.

The alveolar formation is derived from the primary septum (A), a double capillary network, of the developing lung. Arrow indicates the site of emergence of a secondary septum. (B) illustrates the folding up of one of the two capillary layers to form the future interalveolar walls, the secondary septa (C). Elastic fibers (black shading) are typically deposited at the tip of the crests. Adapted from Burri (2006).

1.4.1.5 Alveolar stage

The alveolar stage is the final stage in lung development. The septa in the saccules further extend in height and become thinner, resulting in the remodelling of the terminal saccule walls to form definitive alveoli (Langston et al., 1984, Davis and Mychaliska, 2013). These morphological changes lead to the formation of complex airspaces that promote greater surface area for gas exchange (Burri, 1984, Davis and Mychaliska, 2013). The final stage of alveolar maturation occurs

when the capillary network in the secondary septa becomes one capillary thick and the basement membrane of the capillaries fuses with the type I AECs to form a thin blood gas barrier; this reduces the distance between the air spaces and capillaries for efficient gas exchange (Burri, 1984, Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013). The alveolar stage continues postnatally both in humans and sheep but is slightly more advanced in sheep (Alcorn et al., 1981).

1.4.2 Functional lung development

1.4.2.1 Lung liquid secretion and clearance

In utero, the fetal lung is liquid-filled from early gestation until birth. The fetal lung liquid is essential for regulating fetal lung growth and development. It is now recognized that lung liquid maintains the fetal lung in a distended state creating tissue stretch that promotes lung growth (Hooper and Harding, 1995, Harding and Hooper, 1996, Davis and Mychaliska, 2013). Fetal lung liquid is a secretory product of the pulmonary epithelium (Hooper and Harding, 1995). During fetal lung development, the lung liquid leaves the lung via the trachea and pharynx at which point it is either swallowed by the fetus or exits into the amniotic sac to become a component of the amniotic fluid (Laudy and Wladimiroff, 2000, Davis and Mychaliska, 2013).

At birth, clearance of the lung liquid is necessary to prepare for air breathing. During the early stages of labour, uterine contractions are thought to change the posture of the fetus leading to flexion of the fetal trunk. This flexion increases the pressure in the fetal abdomen, subsequently elevating the diaphragm and resulting in an increase in pressure in the thoracic cavity (Davis and Mychaliska, 2013). Consequently, lung liquid leaves the lung via the trachea, reducing the amount of luminal liquid within the lung. The other mechanism that promotes lung liquid clearance is driven by the release of stress-related hormones (eg. catecholamines, arginine vasopressin) that reverses the osmotic gradient which promotes lung liquid secretion (Hooper and Harding, 2001). This eventually leads to the re-

absorption of lung liquid across the pulmonary epithelium and is most active in the second stage of labour (Hooper and Harding, 2001).

The loss of lung liquid at birth and the inhalation of air results in the generation of surface tension at the alveolar air-liquid interface (Davis and Mychaliska, 2013). This increase in surface tension within the lung increases recoil pressure, which is the tendency for the lungs to collapse; the increase in recoil pressure creates a sub-atmospheric intrapleural pressure due to the lung tending to pull away from the chest wall (Hooper and Harding, 2001). The chest wall stiffens markedly after birth and regulates lung expansion at rest by opposing lung recoil and maintaining the level of expansion (Hooper and Harding, 1995, Harding and Hooper, 1996). In term infants, the presence of surfactant at the air-liquid interface within the alveolar walls further aids in reducing surface tension at the air-liquid interface (Hooper and Harding, 1995, Harding and Hooper, 1996, Davis and Mychaliska, 2013). This enables the lung to expand easily at the next inspiration and stabilises the alveoli at the end of the respiratory cycle to prevent them from collapsing (Hooper and Harding, 1995, Harding and Hooper, 1996, Davis and Mychaliska, 2013).

1.4.2.2 Pulmonary surfactant production and secretion

Pulmonary surfactant is a lipoprotein complex comprised of 90% lipids and 10% proteins, which is synthesised and secreted by the type II AECs. Pulmonary surfactant is critical for maintaining alveolar stability by lowering the surface tension at the air-liquid interface within alveoli, thus preventing the alveoli from collapsing at the end of expiration (Batenburg, 1992, Ma and Ma, 2012, Agassandian and Mallampalli, 2013, Akella and Deshpande, 2013).

1.4.2.2.1 Surfactant lipids

Lipids make up the bulk of the pulmonary surfactant, more than 80% of which are phospholipids (Veldhuizen et al., 1998, Bersani et al., 2012, Ma and Ma, 2012, Agassandian and Mallampalli, 2013, Akella and Deshpande, 2013, Goss et al.,

2013) with the remainder being neutral lipids. Surfactant phospholipid is made up of a number of components referred to as classes. The major classes are: phosphatidylcholine (PC; 60–70%), phosphatidylglycerol (PG; 5–10%), phosphatidylethanolamine (PE; 5–10%) and phospatidylinositol (PI) and phosphatidylserine (PS) which make up 3–6%. PC in surfactant is predominantly dipalmitoylphosphatidylcholine (DPPC), a saturated PC (SPC) species, which makes up ~50% of the surfactant lipid composition (Figure 1.3). DPPC has the greatest ability to reduce surface tension at the air-liquid interface to near zero and is maximally effective in conjunction with PI and/or PG (Batenburg, 1992, Agassandian and Mallampalli, 2013). PI and PG, which are acidic phospholipids, promote adsorption and film formation. The roles of PE, a membrane lipid, and PS are unclear.

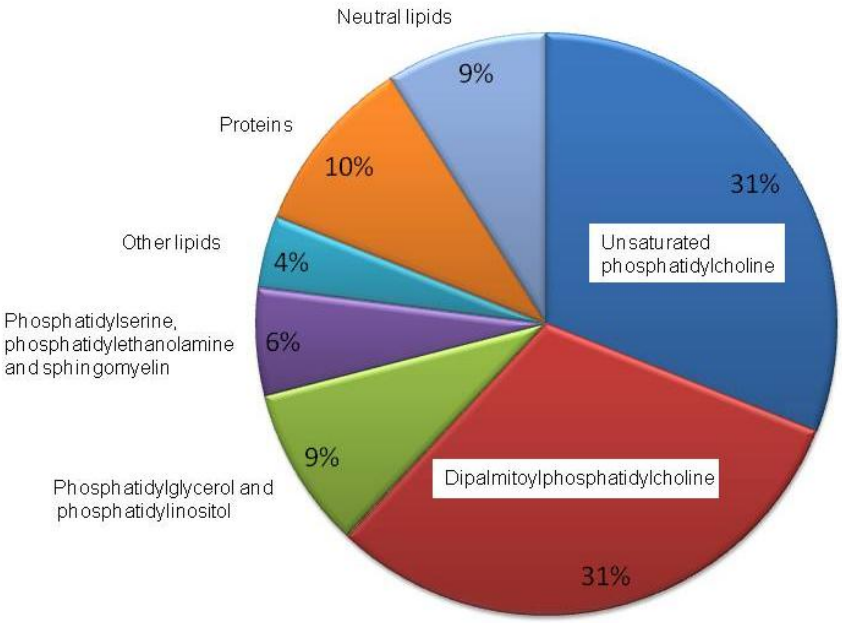

Figure 1.3: Composition of pulmonary surfactant. Adapted from Agassandian and Mallampalli (2013).

With increasing gestational age, the levels of PI and PE in ovine fetal lung liquid decrease, while PC, SPC and PG levels increase; PG appears late in gestation (~36 weeks in human) and is therefore considered to be a good indicator of lung maturity (Ikegami and Jobe, 1981, Ueda et al., 1994, Leung-Pineda and Gronowski, 2010). These maturational changes have also been observed in amniotic fluid from normal human pregnancies (Torday et al., 1981, Fleisher et al., 1985); however, similar changes were not observed in amniotic fluid of sheep (Ikegami and Jobe, 1981). Fetal rabbit lung lavage and amniotic fluid showed increasing ratios of PC and SPC as gestation progressed, with detection in the lung lavage preceding changes in the amniotic samples (Torday and Nielsen, 1981).

As lipid is the main component of pulmonary surfactant, the presence of surfactant and the extent of fetal lung maturity have been assessed clinically by a number of tests that detect indices of the pulmonary-derived lipid constituents in amniotic fluid (Leung-Pineda and Gronowski, 2010). These tests are considered to be reliable predictors of the occurrence of RDS in preterm infants. In general, a lecithin/sphingomyelin (L/S) ratio of 2 or greater, a minimum of 50% DPPC (also referred to as disaturated PC or disaturated lecithin), a low PI level and the presence of PG are useful parameters for distinguishing lung maturity. Lecithin is also referred to as PC and sphingomyelin is a type of cell membrane lipid.

1.4.2.2.2 Surfactant proteins

Surfactant proteins (SPs) make up a minor component of pulmonary surfactant. Four SPs have been identified in the lung: SP-A, -B, -C and -D. These proteins have an important role in the structure, function and metabolism of the surfactant. They can be classified as either hydrophilic (SP-A and -D) or hydrophobic (SP-B and -C). SP-A and -D are involved in host defense. Due to its location at the air-liquid interface of the lung, SP-A is the first defence barrier against toxins or inhaled pathogens (Casals, 2001, Bersani et al., 2012). SP-D, on the other hand, has a role in innate immunity by producing inflammation and promoting pathogen

clearance from the respiratory tract (Clark et al., 2002, Clark et al., 2003, Bersani et al., 2012). SP-B and -C regulate movement of surfactant lipids and also enhance their adsorption to the air-liquid interface (Possmayer et al., 2001, Bersani et al., 2012). Although SPs are a minor component of pulmonary surfactant, they are fundamentally important in preventing alveolar collapse and in pulmonary defence.

1.4.2.3 Factors that influence surfactant production

Endogenous corticosteroids are of fundamental importance in most mammalian species in achieving fetal maturation in preparation for birth. Increasing fetal cortisol concentration late in gestation stimulates maturation of the lungs both structurally and functionally (Liggins, 1994), as shown by increased synthesis and secretion of surfactant, maturation of connective tissue, alveolar epithelial cell differentiation and lung liquid re-absorption (Liggins, 1994).

Newly born preterm infants are generally unable to produce adequate amounts of mature surfactant to support respiration and consequently often develop RDS. To reduce the risk of RDS and to improve health outcomes, corticosteroid treatment is often administered prenatally to women at risk of premature labour. This has the effect of stimulating fetal lung maturation by enhancing structural development of the lung and the secretion of surfactant (Bonanno and Wapner, 2009). Additionally, synthetic or animal-derived surfactants are available for use after birth in order to reduce respiratory morbidity and mortality in babies that are incapable of supporting respiration (Engle, 2008, Akella and Deshpande, 2013, Walsh et al., 2013).

1.5 Sex differences in lung development

The greater risk of males developing RDS following preterm birth, compared to females, has been recognised for the past three decades (Seaborn et al., 2010).

This greater risk of RDS puts males at greater susceptibility to neonatal mortality and long-term morbidity. Little is known about the exact mechanisms that are responsible for the sex difference in respiratory outcome following preterm birth but these are thought to be multi-factorial.

1.5.1 Lung architecture

It is possible that structural development of the lung before birth differs between males and females. However, few studies have investigated possible differences in lung anatomy between males and females in any species during gestation or the early postnatal period. Langston et al. (1984) demonstrated no sex differences in lung morphometry in still-born and live-born infants aged from 19 weeks of gestation to 3 weeks of postnatal age; they measured excised lung volume, average distance between saccular or alveolar walls, number of alveoli per unit area and volume, total alveoli number, surface area, air-space wall thickness and volume proportions of alveolar air, duct air, airspace walls, bronchial and bronchiolar lumen, and non-parenchyma (Langston et al., 1984).

Most of the available data on sex-related differences in lung architecture are derived from postnatal humans or experimental animals. In contrast to what was observed in fetuses and newborns, sex differences in lung structure are evidently more apparent in adults. Thurlbeck (1982) reported no differences in alveolar size and alveolar number per unit area and volume in boys and girls aged from 6 weeks to 14 years. Boys however had larger lungs than girls, resulting in a greater number of alveoli and a larger alveolar surface area for any given age and stature, but these differences are eliminated when size differences are taken into account. There is some evidence that boys have more respiratory bronchioles than girls. Young males have dysanaptic growth of the large airways, which results in narrower airways as a consequence of the lag in large airway growth in relation to the lung parenchyma (Hoffstein, 1986). In animal studies, adult mice and rats show distinct sex differences in lung morphometry at maturity onset. Females have more and smaller alveoli than the males, which provide larger alveolar surface

area to body mass ratios without any difference in body mass-specific oxygen consumption between males and females (Massaro et al., 1995, Massaro et al., 1996, Massaro and Massaro, 2004).

1.5.2 Surfactant maturation

Studies investigating the ontogeny of pulmonary surfactant have found delayed development in males and this has been shown in both humans and rabbits. When the L/S ratio, and the percentage of SPC, PG and PI were assessed in human amniotic fluid between 28 and 40 weeks of gestation, there was apparently a lower degree of lung maturity in male fetuses compared to females (Torday et al., 1981, Fleisher et al., 1985). These studies led to the belief that male fetuses lag in surfactant maturation by 1.2 to 2.5 weeks of gestation compared to female fetuses (Torday et al., 1981, Fleisher et al., 1985). Similarly, lung lavage and amniotic fluid assessed in fetal rabbits from 24–30 days of gestational age (d GA; term = 31d GA) showed earlier increases in SPC/S and PC/S ratios in female fetuses than in males (Nielsen and Torday, 1981, Torday and Nielsen, 1981). Differences were not detected when rabbit fetuses were either very immature (24d GA) or close to term (30d GA) (Nielsen and Torday, 1981). However, in fetal rhesus monkeys, a lack of sex differences in the analysis of whole lung phospholipids were observed, indicating that sex difference in RDS outcome may not be due to a discordance in lung maturation (Perelman et al., 1982). However the use of whole lung tissue could mask sex differences in pulmonary phospholipids, as phospholipids are a component of cell walls.

Surfactant release in the fetal lung has been thought to be modulated by stimulation of exogenous epinephrine and other β-adrenergic agonists (Rooney, 2001). Warburton et al. (1987) reported a more rapid maturation of pulmonary β-receptors that coincides with the onset of surface active material production in female fetal sheep compared to males, and speculated that hormonal factors could be regulating the pulmonary β-receptors and surfactant production. Additionally, sex hormones are thought to have a profound influence in regulating lung

development; androgens and, estrogens and their receptors (α and β), have been shown to exert inhibitory effect in surfactant production and stimulatory effect on alveolar development respectively (Carey et al., 2007).

1.5.3 Lung liquid reabsorption

Transient tachypnea of the newborn (TTN) is caused by a delay in lung liquid reabsorption via the amiloride-sensitive sodium channels in the alveolar epithelial cells (Derbent et al., 2011). Male sex is known to be a risk factor for TTN (Tutdibi et al., 2010, Derbent et al., 2011, Altman et al., 2013). To my knowledge, no study has investigated the mechanisms for impaired clearance of the alveolar fluid in males at birth. In adult human, females are more likely to clear alveolar fluid following acute lung injury (Ware and Matthay, 2001). Lungs of adult female rats reported higher levels of alpha-epithelial sodium channel mRNA expression (Sweezey et al., 1998). If more of these channels are present in the lungs of human infants, this may enhance lung liquid clearance at birth and thus provide an explanation for the lower incidence of RDS in preterm females.

1.6 Influence of fetal hormones on pulmonary surfactant maturation and lung development

A number of studies have investigated the role of fetal hormones, including sex hormones, in regulating lung development and maturation. The major classes of hormones are discussed below.

1.6.1 Androgens

During fetal development, males are exposed to higher levels of androgens than females (Veyssiere et al., 1976, Perelman et al., 1986). Higher circulating levels of androgens in male fetuses have been considered to be a potential cause for the reported lag in lung maturation in males (Nielsen, 1992, Dammann et al., 2000).

Increased surfactant production in males to a level that is comparable to that in females has been observed in fetal rabbits treated with the anti-androgen agent, flutamide, and in a mouse model in which males have no functional androgen receptors (Nielsen, 1985). The exact mechanisms underlying the effects of androgens in lung maturation are only partially understood.

Androgen receptors are expressed in mesenchymal and epithelial cells of the rabbit lung and the receptor gene is expressed equally in both sexes with the same binding affinities (Giannopoulos and Smith, 1982); thus increased levels of circulating androgens could be the cause of retarded lung development in male fetuses, and not a difference in pulmonary androgen receptors. Potent androgens (testosterone and dihydrotestosterone) bind to the same androgen receptors at high affinities, but with no interchangeable functions; they have both been observed to delay fetal lung development by inhibiting surfactant production (Nielsen, 1992).

Nielsen (1992) investigated the effects of dihydrotestosterone (DHT) by inhibiting the conversion of testosterone into DHT by administering 17β-N,N-diethylcarbamoyl-4-aza-4-methyl-5α-androstane-3-one (4-MA), a potent inhibitor of the enzyme 5α-reductase in pregnant rabbits. This results in the absence of DHT in the serum of both male and female fetuses (Nielsen, 1992). However, lung lavage of the treated female fetuses showed higher PC/S and SPC/S ratios compared to treated male fetuses indicating a more immature lung in males compared to females, despite the absence of DHT in both sexes. This indicates that the delay in surfactant production could involve a regulator other than DHT (Nielsen, 1992). A follow-up *in vitro* study was performed to investigate the effect of testosterone and 4-MA on the production of fibroblast pneumocyte factor (FPF), which is thought to be released by pulmonary fibroblasts to induce type II AEC differentiation (Nielsen, 1989). An absence of FPF activity was observed in conditioned media treated with DHT, testosterone, or testosterone and 4-MA. However, conditioned media from untreated female fibroblasts demonstrated

significant FPF activity (Nielsen, 1992). These results demonstrate that both testosterone and DHT regulate mechanisms that could contribute to delayed fetal lung maturation in males (Nielsen, 1992).

It has been speculated that androgens play an inhibitory role in surfactant production in a variety of species by a mechanism which alters epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) signaling events that regulate fetal lung fibroblast maturation. Type II AEC differentiation is thought to be controlled by fibroblast-epithelial cell communication, which ends with the synthesis of surfactant by the type II AECs (Ballard, 1989). Exposure of the lung to exogenous chronic DHT early in murine development altered the balance of growth factor signaling (Dammann et al., 2000). It was observed that there was a down-regulation of EGF activity and an up-regulation of TGF-β activity (Dammann et al., 2000) that resulted in a reduction of SP-B and -C messenger RNA expression in both male and female fetal mice at late gestation (Dammann et al., 2000).

1.6.2 Epidermal growth factor

Epidermal growth factor (EGF) is a polypeptide hormone which regulates growth and cellular differentiation (Klein and Nielsen, 1992). EGF promotes fetal type II AEC maturation by promoting communication between fibroblasts and type II AECs (Nielsen, 1989). The onset of this communication sees a peak in EGF receptor (EGFR) activity, which occurs later in gestation in male rat fetuses compared to females (Rosenblum et al., 1998). In fetal rabbits, EGFR expression is dependent on EGF exposure and affects EGF specific binding in fetal lung, which suggests that surfactant synthesis may be controlled by the quantity of EGFR (Villanueva et al., 2000). This complements an earlier study by Klein and Nielsen (1993) where a decrease in EGF receptor density in fetal rabbit lung tissue was reported following treatment with prenatal exogenous androgen which led to decreased EGF binding. A delay in the onset of EGF activity has been suggested

to be responsible for the lag in male fetal lung maturation (Klein and Nielsen, 1992). Previous studies have reported four possible mechanisms by which EGF could enhance fetal lung differentiation. (1) Gross et al. (1986) found that EGF administration to fetal rat lung cultures led to elevated levels of PC. (2) A study by Nielsen (1989) observed accelerated fibroblast-pneumocyte factor (FPF) production, which induces type II AEC differentiation in fetal lung fibroblast cultures, following the introduction of EGF. (3) In cultures of human fetal lung tissue, EGF was found to augment SP-A synthesis (Whitsett et al., 1987). (4) *In vivo* studies of EGF-treated fetal rabbits, following either intramuscular or intraperitoneal administration, showed improvements in lung compliance, surfactant phospholipid synthesis and lung morphology (Catterton et al., 1979, Higuchi et al., 1989). A study by Klein and Nielsen (1992) on male and female rabbit lung explants at 21 and 24d GA (equivalent to pseudoglandular and early canalicular stages in humans) showed a higher level of disaturated PC (DPPC) in females than males at 24d GA when treated with EGF. At 21d GA, males were not responsive to EGF but a significant stimulation of disaturated PC by EGF was observed in females. Prenatal exposure to androgens has shown an androgen-induced lag in EGF binding during fetal lung development (Klein and Nielsen, 1993). *In vitro*, fetal rat lung fibroblasts cultured with DHT, testosterone and testosterone and 4-MA indicated no FPF activity (Nielsen, 1992).

Taken together the studies mentioned above suggest that the higher level of androgens level in male fetuses decreases both EGF and EGFR binding activity (Nielsen, 1992, Klein and Nielsen, 1993), and that this could retard lung maturation of males.

1.6.3 Transforming growth factor-β

It is possible that androgens mediate their inhibitory actions on lung maturation via TGFβ1. Unlike EGF, TGFβ1 is produced by immature fetal lung fibroblasts and inhibits fibroblast-type II cell communication, thereby down-regulating DPPC

synthesis and SP-A and SP-C synthesis (Whitsett et al., 1987, Torday and Kourembanas, 1990, Nielsen, 1992, Whitsett et al., 1992, Zhou et al., 1996). During late gestation, TGFβ receptor binding and the total number of TGFβ receptors decrease in fetal lung fibroblasts; this has the effect of reducing the inhibitory effect of TGFβ1 on lung maturation (Pereira et al., 1998). This inhibitory effect has been shown to be overcome earlier and to be more pronounced in female lung fibroblasts with advancing gestation (Pereira et al., 1998).

1.6.4 Müllerian inhibiting substance

In addition to androgens, Müllerian Inhibiting Substance (MIS) has been proposed to inhibit lung maturation in developing males. MIS is a glycoprotein hormone that is required for normal development of the male reproductive tract. It initiates regression of the Müllerian ducts in male fetuses during gestation which prevents differentiation of the Müllerian ducts into the uterus, fallopian tubes and upper one-third of the vagina. MIS is synthesised prenatally and postnatally by Sertoli cells of the testis in males. Late in gestation, MIS levels are high in males and continue to rise at birth with reports of human neonatal males being exposed to levels as high as 70 ng/ml, while levels in females are undetectable (Hudson et al., 1990); a similar observation has been made in bovine neonates (Necklaws et al., 1986). The higher MIS levels in males continue for several years after birth and decline to a basal level of 2–5 ng/ml after the first 10 years of life (Baker et al., 1990, Hudson et al., 1990, Lee and Donahoe, 1993). In contrast, MIS levels in females, produced by the granulosa cells of the ovaries, are barely detectable at gestation and at birth. In females, MIS production starts postnatally and MIS concentrations increase to basal levels similar to those observed in males after 10 years of age (Baker et al., 1990, Hudson et al., 1990). The elevated MIS level in females is thought to be involved in follicular development and in the arrest of oocyte maturation (Takahashi et al., 1986, Ueno et al., 1988, Hirobe et al., 1994).

It has been suggested that the retarding effect of MIS on lung maturation is effective from early in development; at this time the embryonic testis is anatomically close to the developing lung (Lee and Donahoe, 1993). It is unknown how MIS leads to the inhibition of lung maturation but it has been suggested that MIS could be acting on specific MIS plasma membrane binding proteins in the fetal lung (Catlin et al., 1990, Catlin et al., 1992); MIS signal transduction is also thought to be mediated by inhibition of EGF receptor autophosphorylation (Catlin et al., 1991).

Following incubation of fetal testis with nanomolar concentrations of bovine MIS or picomolar concentrations of recombinant human MIS (rhMIS), a reduction in disaturated PC was observed in female fetal rat lung at 17.5d GA (Catlin et al., 1988). In another study, explanted embryonic rat lungs incubated with rhMIS resulted in a reduced number of lung buds and reduced lung perimeter length, both of which are indicators of lung complexity or branching, which suggests an involvement in delaying airway branching (Catlin et al., 1997). In addition, MIS is associated with enhanced apoptosis as numerous apoptotic bodies were also observed in the explanted lungs (Catlin et al., 1997).

Catlin et al. (1990) performed a similar *in vivo* study in fetal rats. rhMIS (10^{-9} M, 10^{-8} M) was injected into the dorsal subcutaneous tissue of rat fetuses at 19d GA. This resulted in a depression in disaturated PC in female fetal lung compared with vehicle-injected fetuses of the same sex following 48 and 72 hours (h) of the injection (Catlin et al., 1990). The lungs of male fetuses at the same gestation were inhibited at a higher dose (10^{-8} M). Taken together, the studies cited above suggest that MIS could play a role in sex-related differences in lung development during fetal life.

1.6.5 Estrogens

Estrogens are thought to influence lung maturation, in particular, the formation of alveoli (Massaro et al., 1995, Massaro et al., 1996, Massaro and Massaro, 2004, Massaro and Massaro, 2006). In both fetal and adult mice, only estrogen receptor (ER)β but not ERα was found to be expressed in fetal tissue of both male and female mice, which is consistent with findings in other species (Takeyama et al., 2001, Knapczyk et al., 2008, Carvalho and Goncalves, 2012). Fetal expression of ERβ in the lung occurred for longer in females (E15-19) than in males (E16-18) suggesting that the longer exposure of the ERβ receptors to estrogen in female mice could be advantageous for lung development (Carvalho and Goncalves, 2012).

In the mouse, estrogens are thought to play a role in the sexual differences in alveolar formation (Massaro et al., 1995, Massaro et al., 1996, Massaro and Massaro, 2004) as well as in lung regeneration (Patrone et al., 2003). A genetic deletion of ERα reduces the septation of pre-alveolar saccules in the lung, resulting in a greater volume of an average alveolus (Massaro and Massaro, 2006) which would be expected to reduce the area available for gas exchange. Loss of ERβ in mice also reduces the septation of saccules (Massaro and Massaro, 2004). ERβ has been shown to play a role in altering the expression of platelet-derived growth factor (PDGF), a protein that has the ability to affect cell proliferation, cell migration and elastin deposition, and thus can influence alveologenesis (Patrone et al., 2003). Additionally, a deletion in both receptors decreases the number and increases the size of alveoli. These differences are however more prominent in females than males (Massaro and Massaro, 2006) which indicates a greater effect of estrogen on females than males, even though estrogen levels are similar.

Together, the above findings suggest that, in rodents, estrogens and their receptors have stimulatory effects on lung development and are crucial for alveolar

development, thereby benefiting female fetuses. However, there is little evidence on a possible role of estrogens in human lung development.

1.7 Effects of fetal sex on pulmonary sensitivity to prenatal glucocorticoid exposure

Antenatal glucocorticoid administration to pregnant women at risk of preterm labour is associated with a reduced risk of RDS and fewer neonatal deaths (Bonanno and Wapner, 2009, Mulder et al., 2009, Roberge et al., 2011). In animal studies, prenatal administration of synthetic corticosteroids (betamethasone and dexamethasone) leads to accelerated alveolar development, thinning of interstitial tissue and peripheral arterial walls, and maturation of surfactant synthesis pathways (Willet et al., 2001, Roubliova et al., 2008). Functional studies in human infants have shown higher functional residual capacity (FRC) and respiratory system compliance following administration of antenatal glucocorticoids (McEvoy et al., 2000). Betamethasone is associated with significantly fewer RDS cases in preterm male infants while dexamethasone is beneficial in significantly reducing the RDS rate in females (Roberge et al., 2011). This invites speculation that the maturational effect of the different types of glucocorticoid in fetuses might be sex-specific (Roberge et al., 2011). Willet et al. (1997) reported that prenatal administration of betamethasone in sheep fetuses at 126d GA (equivalent to ~30 weeks of gestational age (wk GA) in human) was less effective in improving lung function in males than in females when they were delivered 48 h after glucocorticoid administration. In the study by (Willet et al., 1997), preterm lambs were ventilated and monitored for 40 minutes (min) following delivery. Postnatal

lung function, such as respiratory mechanics and gas exchange, and the concentration of alveolar surfactant in bronchoalveolar lavage fluid showed considerable improvements in both male and female sheep but females displayed greater improvements in lung compliance, airway conductance, lung volume, and arterial oxygen partial pressure than males (Willet et al., 1997). A follow-up study to investigate the number and binding affinity of lung glucocorticoid receptors in preterm sheep did not show any difference between males and females (Kovar et al., 2001); thus a reduced number of glucocorticoid receptors cannot explain the lack of a beneficial effect following glucocorticoid administration in male fetuses. No sex-related differences were also observed in glucocorticoid receptors in fetal lungs of rabbits and cortisol and cortisone levels in the fetal circulation at 26d GA, which coincides with the onset of surfactant synthesis by type II AECs (Giannopoulos and Smith, 1982). These studies imply that the effect of glucocorticoids in males is inhibited by other factors that result in the slower maturation of male lungs. To my knowledge, differences in lung structure between males and females following glucocorticoid treatment have not been investigated.

Schmitz et al. (2007) has demonstrated a two-fold reduction in prostaglandin E $(EP)_2$ receptor mRNA in male fetal baboons following 48 h of betamethasone administration at 0.7 gestation. Prostaglandin E_2 (PGE_2) is produced and metabolised by the fetal lung and has been shown to induce surfactant production via EP_1 and cyclic adenosine monophosphate-coupled EP_2 and EP_4 receptors in vitro (Mendelson et al., 1991, Morsy et al., 2001). PGE_2 also has a role in modulating bronchiolar tone, where the EP_2 and EP_4 receptors cause relaxation of

airway smooth muscle, while EP_1 and EP_3 receptors have the opposite effect (Narumiya et al., 1999, Sheller et al., 2000, Fortner et al., 2001, Tilley et al., 2003, Narumiya, 2007). “Therefore, a decrease in the gene expression of EP_2 receptor following betamethasone exposure in fetal male baboons compared to females (Schmitz et al., 2007) could indicate reduced surfactant production and bronchial dilatation in males, which in turn may affect airflow and respiration after birth. However, EP_2 receptor protein expression was not different in males compared to females (Schmitz et al., 2007). Intuitively, a lack of change in EP_2 receptor protein would be unlikely to cause changes in lung function in males. However, since previous studies have shown that it takes at least 4 days for the detection of protein induction following betamethasone administration (Jobe et al., 1998, Jobe and Ikegami, 2000), EP_2 receptor protein levels could be altered at a later time point than was measured in the study by (Schmitz et al., 2007).” These studies imply that PGE_2 system acting via the EP_2 receptors has an important role in surfactant production and regulating airway tone.

1.8 Effects of fetal sex on pulmonary sensitivity to exogenous postnatal surfactant therapy

Surfactant-replacement therapy is currently used to treat infants with RDS and has the capability to substantially reduce respiratory morbidity and mortality (Willson and Notter, 2011). Engle (2008) reported that subgroup analyses of surfactant trials which studied the use of exogenous surfactant in infants below 30 weeks of gestation and/or with birth weights below 1250 g have shown that surfactant therapy is most effective in reducing the mortality rates in these subgroups of infants, with a greater effect in males (Soll et al., 1990, Liechty et al., 1991, Egberts et al., 1993, Horbar et al., 1993, Kattwinkel et al., 1993, Gaillard et al.,

2001, Engle, 2008). As this data suggests, exogenous surfactant is more effective in males than in females it is likely that male preterm infants, born prior to 30 weeks of gestation, are surfactant deficient relative to females. It is unclear whether this is true in preterm infants older than 30 weeks of gestation.

1.9 Neural control of breathing: sex differences

It is possible that sex-related differences exist in the neural control of breathing and that they could contribute to impaired respiration in male preterm infants. The development of ventilatory control in the brain is initiated early in gestation. Neural circuitry involved in respiratory control is initiated at mid-gestation and continues development throughout gestation (Zec and Kinney, 2001, Zec and Kinney, 2003). At the time of birth, the respiratory control system must be developed to a point where it is ready to allow for successful transition into air breathing at birth; however, ventilatory control mechanisms at birth are still functionally immature and unstable. Functional maturity takes place weeks or months following birth to reach mature, adult-like levels (Zec and Kinney, 2001, Zec and Kinney, 2003). Therefore, when infants are born preterm, they have an even less mature ventilatory control system, resulting in unstable breathing and periods of apnea (apnea of prematurity). However, to my knowledge there have been no studies examining sex-related differences in the neural control of breathing in infants.

1.10 Vascular development and cardiovascular changes

The vasculature of the lung is comprised of two distinct circulations: bronchial and pulmonary. The bronchial arteries (two left and one right) supply blood to structures that make up the root and supporting tissues of the lungs and the visceral pleura, while the bronchial veins drain blood supplied to the lung via the bronchial arteries (Moore, 2010). The left bronchial arteries arise from the thoracic aorta while the origin of the right bronchial artery is variable. A morphometric study of the thoracic aorta in human fetuses aged 15–34 weeks of gestation demonstrated no sex differences in the dimensions of the growth of the length,

original and terminal external diameters, and volume of the thoracic aorta across the gestational ages (Szpinda, 2007). The pulmonary arteries, on the other hand, arise from the pulmonary trunk and supply poorly oxygenated blood to the lungs while the two pulmonary veins carry oxygenated blood to the left atrium of the heart (Moore, 2010). The length, diameter and volume of the pulmonary trunk during gestation from 15–34 weeks of gestation are similar in human males and females (Szpinda, 2007).

During fetal development, the fetal circulation is characterized by low systemic resistance, at least partly due to the presence of nitric oxide (NO), a potent vasodilator. NO derived from the placenta is essential for maintaining a low vascular tone in the umbilical vein (Sladek et al., 1997). NO synthase (NOS) converts L-arginine into NO, and the released NO subsequently relaxes vascular smooth muscle (Closs et al., 1997). The endogenous NOS antagonist, asymmetrical dimethlyarginine (ADMA), which inhibits L-arginine uptake and NOS activity in endothelial cells, is present in the umbilical vein plasma (Mittermayer et al., 2006). Significantly higher concentrations of ADMA in the umbilical vein were observed in male preterm infants compared to females (Mittermayer et al., 2006); this difference could potentially affect intrauterine development by constricting the umbilical vessels, reducing intrauterine growth and resulting in pulmonary hypertension in the infant (Arrigoni et al., 2003, Mittermayer et al., 2006). ADMA concentrations in venous umbilical cord blood are inversely associated with body weight in male preterm infants (Mittermayer et al., 2006). Reduced intrauterine growth in infants has been associated with neonatal morbidity and RDS (Ott, 1995). Therefore, the presence of elevated ADMA in the umbilical vein plasma could contribute to the development of RDS in male preterm infants.

Following birth, the peripheral microvascular blood flow undergoes changes within the first few days of life. This is a period when preterm infants are most vulnerable as their circulation may be compromised (Beinder et al., 1994), increasing their risk of hypotension (Elsmen et al., 2004) due to inappropriate vasodilatation of the microvasculature. Stark et al. (2008) have demonstrated sex-specific differences in

basal microvascular blood flow in the skin circulation following preterm birth. In addition, the response of the microvasculature to vasoactive stimuli, acetylcholine (ACh) was also measured (Stark et al., 2008). Higher microvascular blood flow in the skin circulation was observed in preterm males born at 24–28 weeks of gestation during the immediate newborn period with differences not detected by 72 h of age. In response to ACh, preterm males appeared to demonstrate greater vasodilatory capacity with increasing blood flow but this was not evident in age-matched females. It appears that the vasoconstricted state of the microvasculature in females could be a protective factor that prevents further vasodilation that could otherwise lead to compromised circulation in the immediate newborn period (Stark et al., 2008).

A recent study by Polglase et al. (2012) has demonstrated that anaesthetised male and female fetal sheep have similar fetal cardiopulmonary and systemic haemodynamics (carotid arterial pressure, pulmonary arterial pressure, pulmonary blood flow and pulmonary vascular resistance) and arterial blood gas parameters (pH, glucose, lactate, haemoglobin and haematocrit). These parameters continued to show no sex differences within the initial 30 min of preterm delivery at ~128d GA (Polglase et al., 2012). The limitation of this study is that the lambs were ventilated and anaesthetised following delivery and this may mask the presence of sex differences that could have otherwise contributed to the poorer respiratory outcomes in males.

1.11 Project rationale

Important questions still remain regarding the basis for the “male disadvantage” in respiratory function following preterm birth. It is still unclear whether there are sex differences in lung anatomy, in particular, the lung parenchyma during gestation and the early postnatal period. A lack of differences in lung anatomy between fetal male and female rhesus monkeys has been reported previously (Perelman et al., 1986) but differences have not been investigated in other species and lung

architecture has not been investigated in detail. Little is also known about physiological differences between males and females during late gestation and in particular, physiological adaptation to preterm birth. Sex differences in physiological variables such as blood gas status, cardiovascular profile and static lung compliance in unanaesthetised, spontaneously breathing preterm animals have apparently not been investigated. Therefore, it is presently not known whether preterm males at birth are capable of attaining equivalent blood gas and cardiovascular status and lung function similar to that of females. Importantly, there is a lack of information on cardio-pulmonary adaptation to preterm birth and why males and females appear to adapt differently.

It is well reported that preterm males have a lower degree of lung maturity as assessed by surfactant phospholipid composition in amniotic fluid before birth and in lung lavage after birth (Torday et al., 1981, Fleisher et al., 1985). However, a detailed analysis of surfactant phospholipid composition at the time of preterm birth has not been made and surfactant phospholipid composition has not been compared to lung compliance. In understanding the male disadvantage in respiratory function following preterm birth it would be beneficial to measure these relationships.

Recently, a sheep model for exploring sex-related differences in respiratory adaptation following preterm birth has become available in our laboratory. In this model, the induction of preterm delivery in sheep born preterm at 131–133d GA (term ~147d GA; equivalent to ~32–34wk GA in humans) demonstrates a significant difference in survival outcome between males and females (De Matteo et al., 2010). The survival rate of unanaesthetised preterm lambs immediately after vaginal delivery was similar in both sexes, but a difference was observed following the 4 h time point; the difference in survival became more marked over time, with females having a 76% rate of survival at 15 days after birth compared to 46% in males (Figure 1.4). Respiratory insufficiency was considered to be the major cause of the greater mortality of male lambs, which therefore makes the sheep an ideal

experimental model to investigate the 'male disadvantage' in respiratory function following preterm birth. It is also advantageous to use sheep as they have a long gestation (term ~147d GA) and tolerate surgical manipulation. Moreover, lambs are of similar size and weight to human infants and their lung development is comparable to humans, which is of particular relevance to this study. In relation to lung development, the alveolar stage begins before term in both humans and sheep, unlike in rodents where it occurs postnatally.

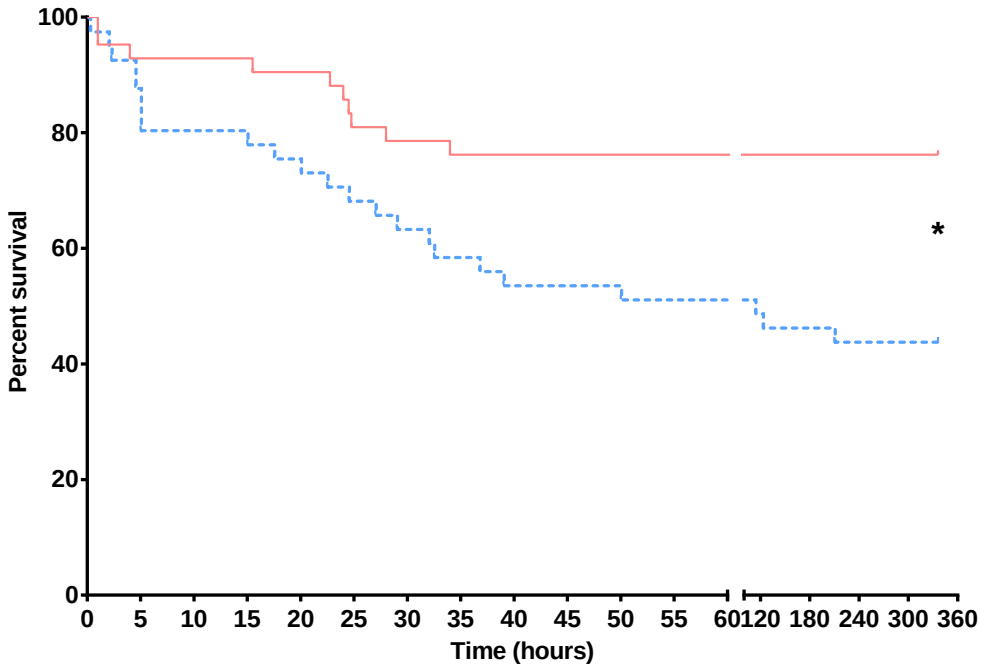

Figure 1.4: Survival rates (percentage) in females and males following preterm delivery at 131-133d GA in sheep (De Matteo et al., 2010). The survival rate in females and males following preterm delivery are represented in pink and blue lines, respectively. The asterisk shows a significant difference in survival rate between female lambs and male lambs.

1.12 Hypothesis and aims

In this study, it is hypothesised that following preterm delivery, males will have poorer cardio-respiratory adaptation than females of the same gestational age as a result of having less mature lung structure, altered pulmonary surfactant composition, and lower lung compliance.

The overall aim is to identify factors that account for previously observed differences in respiratory and survival outcomes in sheep following preterm birth. The first specific aim is to determine whether there are structural and biochemical differences in the lungs of male and female preterm fetal sheep. The second specific aim is to identify the physiological basis for the greater survival of female preterm lambs compared to males of the same gestational age, up to 4 h and 8 h after birth.

Chapter 2: General methods

2.1 Ethical approval and animal welfare

All animal procedures were approved by the Animal Ethics Committee of Monash University. The standards outlined in the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes were used in animal handling, use and care.

Date-mated pregnant crossbred ewes were obtained from an approved animal supplier and transported to the animal housing facilities (Large Animal Facility, Monash University, or Monash Medical Centre Animal Facility). The ewes were allocated to one of two study groups: a fetal study group and a postnatal study group. Details of these groups are provided below (Section 2.2). Pregnant ewes were delivered to the animal facility one week prior to necropsy (fetal study group) or surgery (postnatal study group), to allow adaptation to their new environment. After arrival at the animal facility, each pregnant ewe was placed in an individual cage, had free access to water at all times and was fed daily with ~1 kg of lucerne chaff. All ewes were kept in a controlled environment with an ambient temperature of 22°C, relative humidity of 45–55%, and a 12 hour (h) light/dark cycle (lights on at 0700 h and off at 1900 h). In the breed of sheep that was used (Merino x Border Leicester), full term is approximately 147 days after mating.

2.2 Study groups

Fetal study group: The fetal study group was used to provide lung tissue from male and female fetuses, and the results are presented in Chapter 3.

Postnatal study group: The postnatal study group was used to study lambs before and after preterm birth; these experiments are described in Chapters 4 and 5.

Fetal study group: For the fetal study, pregnant sheep carrying single, twin or triplet fetuses were euthanised at 131 days after mating, as described in Section 2.3. This age (approximately 0.9 of term) was chosen as 131–3 days of gestational age (d GA) is the limit of independent viability of lambs, without the need for assisted ventilation and intensive care; this gestational age equates to 32–34 weeks of gestation in humans (i.e. moderately preterm birth) in relation to lung development. The purpose of this group was to provide lung tissue for morphometric analysis and for measurement of gene expression of *surfactant proteins* and *tropoelastin* in male and female preterm fetuses. After euthanasia, the fetuses were removed and their lungs collected for future analysis. Some archived lung tissue that was previously collected at 131 days of gestation from the same breed of sheep was also used in this study.

Postnatal study groups: The postnatal study groups were used to assess and compare the physiological adaptation of male and female lambs delivered preterm at 133d GA via caesarean section; prior to delivery, physiological data were obtained from fetuses *in utero*. Animal experimentation on the postnatal groups entailed maternal-fetal surgery, followed by prenatal blood pressure monitoring and fluid sampling (plasma, amniotic fluid and fetal lung liquid), caesarean delivery, postnatal physiological monitoring, and assessment of lung compliance. After euthanasia, bronchoalveolar lavage fluid and selected tissues were collected for analysis. One postnatal study continued for 4 h after preterm delivery and the other for 8 h. All ewes in the postnatal study groups received analgesia, delivered via a Fentanyl transdermal patch (75 mcg/h over 72 h, Durogesic, Ortho-McNeil-Janssen Pharmaceuticals, Inc, USA) following maternal-fetal surgery (Section 2.4) and were monitored closely for signs of pain or discomfort over the next 24–48 h. A veterinary practitioner was available to assess any ewe that was of concern.

2.3 Fetal study

In the fetal study group, pregnant ewes and their fetuses were euthanised with a bolus dose of sodium pentobarbitone (130 mg/kg; Lethabarb, Virbac Pty. Ltd., Peakhurst, NSW, Australia) administered into a jugular vein of the ewe. After death of the ewe was confirmed, the fetus was removed via incisions through the abdomen and uterine wall. The body weight, body dimensions, sex and organ weights of the fetus were recorded. Soon after death, the fetal lungs were removed from the thoracic cavity. The left lung was ligated at the hilus, separated from the right lung and rapidly sectioned into small pieces (2–5 mm^3); these pieces were snap-frozen in liquid nitrogen (N_2) for storage at -80ºC for later molecular analysis. The right lung was fixed via the trachea at a distending pressure of 20 cmH_2O with 4% paraformaldehyde (in 0.1 M phosphate buffered saline, pH 7.4) and immersed in fresh 4% paraformaldehyde fixative for 24 h at 4ºC. It was then transferred into Zamboni's solution (0.1 M phosphate buffer, 0.1% formaldehyde, 0.15% saturated picric acid) overnight, followed by 70% ethanol for storage until histological processing was started. Lung volume was measured using the protocol outlined in Section 2.5. Histological assessment (Section 2.6) and measurement of mRNA levels by quantitative real-time polymerase chain reaction (qPCR; Section 2.8) were performed.

2.4 Postnatal studies

2.4.1 Surgical procedures

Surgical procedures were performed on the ewes and fetuses in the postnatal groups to prepare them for obtaining prenatal physiological measurements and studying postnatal physiological adaptation of preterm lambs.

2.4.1.1 Preparation for surgery

Pregnant ewes at approximately 124d GA (0.84 of term) underwent aseptic, recovery surgery for implantation of catheters into both ewe and fetus. Prior to

surgery, food was withheld from the ewes for 24 h but access to drinking water was not restricted. The ewe was placed upright on a sling and wool on the left side of its neck and right abdominal flank shorn to expose a jugular vein and the site of future exteriorisation of catheters, respectively. A prophylactic antibiotic (1 g ampicillin/5 ml sterile water i.v., Aspen Pharmcare Australia Pty Ltd) was then administered to the ewe followed by 1 g of sodium thiopenthone (25 ml i.v., Pentothal, Boehringer Ingelheim Pty Ltd, Australia) to induce anaesthesia. The anaesthetised ewe was then placed supine and intubated with a cuffed endotracheal tube (I.D. 8.0 mm, O.D. 10.9 mm, Portex Ltd, UK) for attachment to a mechanical ventilator (Campbell, ULCO Engineering, Australia) and anaesthetic machine (Midget 3 vaporizer; Campbell, ULCO Engineering, Australia); the ewes were ventilated by positive pressure ventilation (11 breaths per min; 800–1000 ml per breath), with anaesthesia being maintained with isoflurane (Isoflo; Abbott, Australasia) in a mixture of oxygen and nitric oxide (70:30 v/v). Some ewes were anaesthetised with halothane (0.5–2.0% in 100% oxygen, Fluothane, Zeneca, Australia) rather than isoflurane.

Once anaesthetised, the ewe was shorn over the ventral abdomen and ventral neck, which were the sites of surgical incisions. These areas were then cleansed with 4% Savlon antiseptic solution (15% w/v Cetamide, 1.5% w/v chlorhexidine gluconate; ICI Australia Operations Pty Ltd, Australia) in water, two washes with Betadine surgical scrub (7.5% w/v povidone-iodine; Faulding Pharmaceuticals, Australia), followed by administration of Betadine antiseptic solution (10% w/v povidone-iodine; Faulding Pharmaceuticals, Australia). Immediately before the commencement of surgery the incision sites were coated with 10% Hibitane solution (5% w/v Chlorhexidine Gluconate, 4% v/v isopropyl alcohol; Hibitane, Zeneca Pharmaceutical Australia Pty Ltd).

Prior to the start of surgery, surgeons thoroughly scrubbed their hands with Hibiclens antiseptic solution (4% chlorhexidine gluconate, 4% w/v isopropyl alcohol; ICI Pharmaceuticals, Australia) and wore hair nets, face masks, sterile

gowns and sterile latex gloves (Ansell International, Australia). All surgical drapes, towels, gowns and instruments used in surgery were sterilised by autoclaving.

2.4.1.2 Surgical incisions

The maternal-fetal surgery began with a midline incision on the ewe's abdominal skin, extending from the umbilicus to the upper margin of the udder, avoiding the superficial mammary veins. A diathermy was used (LCC200, Erbe, Germany) to stop bleeding from small vessels. A second incision was made through the ewe's abdominal wall via the *linea alba* to expose the uterus. The uterus was then palpated to confirm the number of fetuses and to locate the head of one fetus. With care being taken to avoid major uterine vessels and placental cotyledons, an incision was made through the uterus to expose and exteriorise the fetal head and the front legs. The uterine muscle and its membranes (amnion and chorion) were clamped to the fetal skin to minimise the loss of amniotic fluid. Fetal gestational age was estimated by measuring the head length and comparing it to a growth chart of the sheep to confirm the correct gestational age.

2.4.1.3 Fetal and maternal catheterisation

Following exteriorisation of the fetal head and neck, an incision was made to expose a carotid artery and jugular vein. These vessels were dissected free of tissue and care was taken to prevent any damage to the vagus nerves or recurrent laryngeal nerves. A fetal carotid artery and a jugular vein were catheterised with sterile 1.5 m long fetal catheters (I.D. 0.86 mm; O.D. 1.52 mm, Microtube Extrusions, Australia), each filled with heparinised saline (25 000 IU, Heparin, Pharmacia, Australia in 500 ml saline i.v. bag, 0.9% 500 ml Sodium Chloride, Baxter, USA). After recovery from surgery the fetal arterial catheter was used to collect blood samples to assess fetal well-being and to monitor arterial blood pressure and heart rate. The venous catheter was used for future drug administration.

From the same incision, the fetal trachea was located and a catheter (I.D. 0.86 mm; O.D. 1.52 mm, Microtube Extrusions, Australia) was inserted 7.5 cm into the trachea, towards the lung. This tracheal catheter allowed for sampling of fetal lung liquid, which was later used to assess lung maturity by measurement of its phospholipid content.

The fetal skin incision was closed using silk (size 3-0, Dynek Pty Ltd) and the exposed catheters were plugged to prevent leakage or entry of air.

A small balloon (approximately 2 ml capacity) was inserted into the intrapleural space of the fetus by first making a small skin incision between the right 4^{th} and 5^{th} ribs (counting from the lowermost complete rib). A second incision was made through the intercostal muscle and pleura into the thoracic cavity, taking care not to puncture the lung. The balloon was then inserted into the chest cavity and the catheter was secured to the muscle and skin. The exterior portion of the catheter was coiled and secured to the skin on the right flank with sutures, with the end of the catheter capped. After delivery, the intrapleural balloon was used to measure inspiratory efforts by the lamb.

A sterile saline-filled catheter (1.5 m polyvinyl tubing I.D. 1.5 mm; O.D. 2.5 mm) was inserted towards the heart into a jugular vein of the ewe by exposing the vein via an incision in the ventral lateral region of the neck below the level of the larynx. The procedure for this was similar to that of the fetal venous catheterisation.

2.4.1.4 Placement of umbilical cord occluder

The fetus was further removed from the uterus until the umbilical cord was exposed. A sterile inflatable vascular occluder (silicone rubber, 16 mm diameter, Model OC16HD, Invivo Metric, California, USA) was secured loosely around the umbilical cord; it was secured to the abdominal skin using silk sutures to prevent

movement and obstruction of umbilical blood flow while the fetus was still *in utero*. The occluder was used to briefly occlude the umbilical cord immediately prior to maternal anaesthesia and caesarean delivery by inflating it with 2–3 ml of sterilised water, thereby preventing anaesthesia of the fetus.

2.4.1.5 Amniotic fluid catheter

A sterile catheter (1.5 m silicone rubber tubing, I.D. 2.64 mm; O.D. 4.88 mm) with a protective casing was constructed for sampling amniotic fluid. The catheter was placed in the amniotic sac and sutured securely to the uterus. The protective casing prevented the obstruction of the lumen by membranes.

2.4.1.6 Catheter exteriorisation

At the completion of the fetal surgical procedures, all catheters, except for the amniotic fluid catheter, were secured to the skin of the fetus to prevent catheters becoming tangled or compressed as a result of fetal movement. All catheters were then exteriorised from the site of the uterine incision and the incision was sutured closed in two layers. The catheters were then passed through the ewe's right flank via a small incision. All catheters were then attached to sterile stopcocks, and the abdominal wall incision was sutured closed.

All incision sites (neck, abdomen and flank) were sprayed with iodine (50 g/L polyvinylpyrrolidone-iodine, Troy Laboratories Pty Ltd, Australia). All ewes received analgesia delivered via a Fentanyl transdermal patch (75 mcg/h over 72 h, Durogesic, Ortho-McNeil-Janssen Pharmaceuticals, Inc, USA). Once the ewe commenced spontaneous breathing, it was disconnected from the ventilator; the endotracheal tube was removed upon the return of laryngeal (swallowing) reflexes. The ewe was then returned to its pen following stabilisation of independent breathing.

2.4.2 Post-surgical care: Monitoring maternal and fetal well-being

Following surgery, the ewe was monitored closely for approximately 4 h to ensure recovery from surgery. For 3 days following surgery, incision sites were sprayed with iodine (50 g/L polyvinylpyrrolidone-iodine, Troy Laboratories Pty Ltd, Australia) and antibiotics (500 mg ampicillin, Aspen Pharmcare Australia Pty Ltd) were administered into the amniotic fluid and fetal jugular vein. Fetal blood samples were collected daily to monitor fetal well-being. All vascular catheters were flushed with heparinised saline (50 000 IU, Heparin, Pharmacia, Australia in 1000 ml saline i.v. bag (0.9% 500 ml Sodium Chloride) Baxter, USA) to prevent blood clotting.

2.4.3 Animal experimentation

2.4.3.1 Monitoring fetal physiological status

Sampling of fetal arterial blood was performed daily (125–133d GA) until the day of caesarean delivery in order to monitor fetal well-being. Samples were analysed using a Radiometer ABL blood gas analyser (ABL800, Denmark) to monitor arterial pH, PO_2, SaO_2, PCO_2, lactate and glucose. 0.5 ml blood samples were taken daily from 125–129d GA to monitor fetal well-being and 3.0 ml samples were taken from 130–133d GA to monitor fetal well-being and plasma collection. The 3 ml samples were centrifuged and fetal plasma was collected and stored at -80ºC for future analysis. Samples of fetal lung liquid (1.5 ml each) and amniotic fluid (7.0 ml each) were also collected daily from 130–133d GA and stored at -80ºC for analysis of surfactant phospholipid content. Fetal arterial blood pressure was monitored for 1 h at 131 and 132d GA by attaching the arterial and amniotic catheters to pressure transducers (“DTX Plus” transducer, Becton Dickinson, Singapore) and a computer with a data acquisition program (Chart, version 5, ADInstruments, Australia). In recording fetal arterial pressure, amniotic sac pressure was electronically subtracted to eliminate effects of maternal postural changes and changes in intra-uterine pressure. Following recording of arterial

pressure at 131d GA, betamethasone (5.7 mg of Celestone Chronodose, Schering-Plough, NSW, Australia) was administered intramuscularly to the ewe to enhance the viability of lambs after preterm birth. Betamethasone was administered to the ewe, as the majority of preterm infants are exposed to exogenous corticosteroids before birth (Polyakov et al., 2007).

2.4.3.2 Lamb delivery

Preterm lambs were delivered at 133d GA by caesarean section. Immediately prior to the intravenous administration of a short-acting anaesthetic (25 ml sodium thiopentone; 50 mg/ml i.v.; Pentothal, Boehringer Ingelheim Pty Ltd, Australia) to the ewe, the previously implanted umbilical occluder was inflated with a known volume of sterile water (~ 2 ml) to temporarily occlude the umbilical cord, thereby preventing anaesthesia of the fetus. Caesarean section was immediately performed by rapidly incising the abdominal skin, abdominal wall and then the uterus to remove the fetus. The umbilical cord was clamped and cut. The catheters that were previously implanted into the fetus and exteriorised via the ewe's flank were also clamped and cut. Following delivery of the lamb, the ewe was euthanised with an overdose of sodium pentobarbitone (0.13 g/kg; Lethabarb, Virbac Pty. Ltd., Peakhurst, NSW, Australia) via the maternal jugular vein catheter. The newly delivered lamb was weighed, dried and placed under a heat source, and its rectal temperature was monitored.

2.4.3.3 Postnatal monitoring

Following delivery, the lamb was allowed to breathe spontaneously for 4 h (short-term group; Chapter 4) or 8 h (long-term group; Chapter 5). In the event that the lamb did not start breathing spontaneously or the heart rate (evaluated via a stethoscope) did not exceed 100 beats per minute within 60 seconds (sec), assisted ventilation was provided. In the 4 h study group, intermittent mandatory ventilation using a face mask and self-inflating resuscitation bag with a reservoir, with a flow of 5 to 10 L/min of 100% oxygen, was supplied to the lamb. In the 8 h study group, a Neopuff^{TM} Infant T-piece resuscitator (Fisher & Paykel, New

Zealand) was used to resuscitate the lamb instead. Air was not heated or humidified. The CPAP level was set at 4 cmH_2O and the resuscitation techniques, including the level of CPAP, did not differ between lambs. This mandatory ventilation was continued until regular spontaneous breathing was established; respiratory assistance was only provided for a maximum of 15 min. The post-ductal percutaneous oxygen saturation (SpO_2) was continuously monitored during the entire monitoring period using a pulse oximeter (Masimo Radical-7, USA); the probe was connected to the shaved tail. Following the resuscitation period, supplemental oxygen was provided with a face mask to maintain SpO_2 of >80%, and was stopped once the lamb was able to maintain an SpO_2 of at least 80%.

Throughout the postnatal observation period, the lamb's arterial and intrapleural pressures and heart rate were recorded. Arterial pressure and heart rate were monitored from the lamb's carotid arterial catheter, which was attached to a pressure transducer. In order to assess inspiratory efforts, intrapleural pressure fluctuations were monitored from the intrapleural balloon, which was connected to a pressure transducer. The pressure transducer was connected to a computer running a data acquisition program (Chart, version 5, ADInstruments, Australia). The intrapleural balloon was emptied and then inflated with 0.5 ml of sterilised water before being connected to the transducer.

To minimise artefacts in the recorded tracings, elicited by the movement of lambs, some lambs were lightly swaddled. Blood samples were taken at 5 min after birth and every 15 min thereafter to monitor arterial pH, PO_2, SaO_2 and PCO_2, and lactate and glucose concentrations using a Radiometer ABL blood gas analyser (ABL800, Denmark). The lambs were infused with 5 ml saline via the jugular vein every 5 min for 30 min after delivery; they were also given 3 ml of 5% glucose (i.v.), within 5 min after birth and 15 min thereafter on condition that the glucose level was below 5 mmol/L. Saline and glucose were administered to maintain, respectively, blood volume and whole blood glucose levels following birth. Rectal temperature was continuously measured; we attempted to maintain rectal

temperature at 39ºC (i.e. the temperature of a healthy lamb) with the aid of an overhead warmer ("Atom" Infant Warmer Intensive Care System, Atom Medical International, Japan) and hot water bottles placed around the lamb.

During the postnatal observation period, the lambs were orally fed every few hours with ewe's colostrums milked immediately prior to caesarean section.

2.4.3.4 Measurement of static lung compliance

At the end of the 4 h or 8 h monitoring period static lung compliance was measured. Prior to measuring static lung compliance the lungs were degassed. To achieve this, lambs were anaesthetised with 1 ml sodium thiopentone (50 mg/ml i.v.; Pentothal, Boehringer Ingelheim Pty Ltd, Australia), intubated with a 4.0 mm cuffed endotracheal tube and connected to a ventilator (Drager, Babylog 8000 plus, Germany). The lambs were then mechanically ventilated using a synchronised intermittent mandatory ventilation mode with 100% oxygen at a tidal volume of 7 ml/kg and frequency of 40 breaths/min for 3 min to achieve oxygen-rich alveoli. Following that, the tracheal tube was occluded to allow oxygen uptake from the alveoli into blood. The lamb was then euthanised by intravenous administration of 150 mg sodium pentobarbitone (i.v.). The lamb's chest was opened by dividing the sternum to expose the lungs. The lungs were inflated with air via the endotracheal tube in steps of 10 cmH_2O until a pressure of 40 cmH_2O was achieved from the degassed condition. The air volume required to attain each pressure step (10, 20, 30 and 40 cmH_2O) was recorded; the air was then removed to obtain pressures of 30, 20, 10 and 0 cmH_2O, and the volume removed at each pressure step was recorded. Functional residual capacity (FRC) was not able to be measured in this study, so 0 cmH_2O is representative of FRC. Body weight was measured immediately after static lung compliance was measured.

The relative lung volume was determined by the following formula:

$$\text{Relative lung volume} = \frac{\text{Lung volume at 40 } cmH_2O}{\text{body weight (kg)}}$$

The pulmonary pressure-volume relationship was also measured (Figure 2.1).

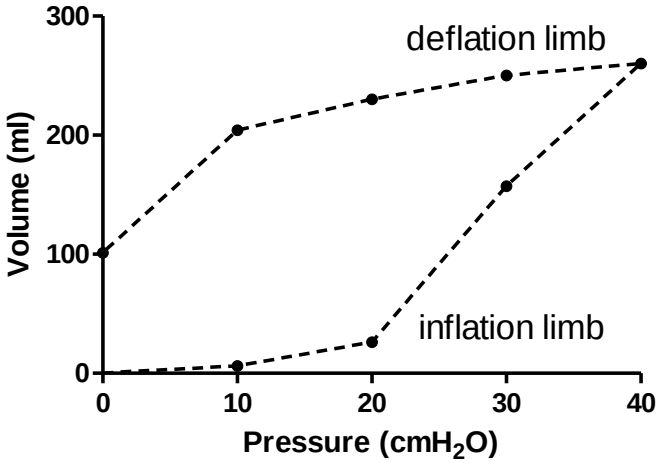

Figure 2.1 Pressure-volume curve.

Typical pressure-volume curve in a preterm lamb at 133 days of gestation.

2.4.4 Necropsy and tissue collection

After measuring static lung compliance and body weight, we measured body dimensions and major organ weights of the lambs. The lungs were removed from the thoracic cavity; the left lung was ligated at the hilus, surgically separated from the right side of the lung and cut into small pieces (2–5 mm^3). These pieces were snap-frozen in liquid N_2 for storage at -80ºC for later molecular analysis. Bronchoalveolar lavage fluid (BALF) was collected from the upper right lung lobe (see Section 2.4.4.1). The right lung was then fixed via the trachea at a distending pressure of 30 cmH_2O with 4% paraformaldehyde (in 0.1 M phosphate buffered saline, pH 7.4) and immersed in fresh 4% paraformaldehyde fixative for 24 h at 4ºC. After 24 h, the lungs were stored in Zamboni's solution (0.1 M phosphate buffer, 0.1% formaldehyde, 0.15% saturated picric acid) overnight, followed by 70% ethanol for storage until histological processing was started.

2.4.4.1 Collection of bronchoalveolar lavage fluid (BALF)

The upper lobe of the right lung was isolated from the rest of the lung by ligating the lobar bronchus just distal to the bronchus supplying the upper right lobe. The upper lobe from the right lung was lavaged three times by infusing 35–50 ml of saline via a syringe into the trachea and then withdrawing as much fluid as possible (8–26 ml) back into the syringe. The total volume of BALF collected was recorded, and the collected fluid centrifuged (1250 revolutions per minute (rpm) for 7 min at 4ºC; Heraeus Multifuge 3S-R, ThemoElectron Corporation, USA); the supernatant was removed and stored at -80ºC for later analysis of surfactant phospholipid composition.

2.5 Estimation of lung volume

The volume of the right lung was estimated using the Cavalieri method. This was achieved by positioning grids of 5 mm x 5 mm over lung tissue sections of 5 mm thickness and counting the number of points overlying the lung tissue (Michel and Cruz-Orive, 1988). The volume of the right lung was determined by multiplying the sum of points overlying the tissue by the area of each grid (25 mm^3) and the thickness of the tissue slices (5 mm).

Lung volume = sum of points x area of grid (25 mm^3) x thickness of tissue slice (5 mm)

2.6 Histological examination of the lung

The following methods were used to analyse the structure of fetal and postnatal lungs.

2.6.1 Tissue processing

Samples of lung tissue (size ~ 25 x 20 x 5 mm) from each lobe of the right lung (upper, middle and lower) were randomly selected for histological processing. These samples were post-fixed overnight in Zamboni's solution (0.1 M phosphate buffer, 0.1% formaldehyde, 0.15% saturated picric acid) and before being processed in a histokinette where they were exposed to increasing concentrations of ethanol and then xylene; tissue samples were subsequently embedded in paraffin wax and the blocks were allowed to harden for tissue sectioning.

2.6.2 Tissue sectioning

The paraffin-embedded tissue blocks were sectioned at a thickness of 5 µm using a rotary microtome (Microtec Cut4060, Germany) and mounted onto coded slides (Superfrost Plus, Menzel-Gläser, Germany). Slides were coded to mask the identification of the study group during analysis. The tissue sections were dried at room temperature initially and subsequently incubated for 2 h at 60°C for stronger adherence to the microscope slides.

2.6.3 Histological staining

Tissue sections were initially stained (see various staining procedures below) before being examined under a light microscope (Nikon Eclipse E400, Nikon, Japan).

2.6.3.1 Haematoxylin and eosin (H&E) staining

Sections stained with H&E stain were used to assess the percentage of tissue and airspace in the lung (tissue and air-space fractions), alveolar wall thickness and mean linear intercept (MLI) as an index of alveolar diameter. Paraffin sections were first dewaxed in xylene, rehydrated in absolute alcohol and rinsed in water. They were then immersed in haematoxylin for 5 min, rinsed in running water, dipped briefly in acid alcohol (0.5% hydrochloric acid in 70% ethanol) followed by a quick rinse in water, immersed in Scott's Tap Water and rinsed again. The tissues were then counterstained with eosin for 5 min, dehydrated in absolute alcohol and cleared in xylene. The tissues were mounted with Distrene plasticiser in xylene (DPX, The British Drug Houses, UK) and coverslipped.

2.6.3.2 Gordon and Sweet's reticular fibre stain

Sections stained with Gordon and Sweet's reticular fibre stain (G&S) were used to analyse the deposition of collagen types I and III in lung tissue. Tissue sections were dewaxed with xylene, rehydrated with absolute alcohol and water, immersed in acidified potassium permanganate for 5 min, rinsed, immersed in oxalic acid for 1 min, rinsed in distilled water, immersed in ferric ammonium sulphate (2%) for 5 min and impregnated with Wilder's Silver Bath for ~7 sec followed by another rinse in distilled water. The tissue was then washed in 10% neutral formalin, rinsed, counterstained with eosin for 15 min and rinsed again. The tissue was then dehydrated in absolute alcohol and cleared in xylene. Finally, the tissues were mounted with DPX and coverslipped.

2.6.3.3 Hart's elastin staining

Sections stained with Hart's resorcin fuchsin stain were used to analyse elastin deposition and measure secondary crest density in the lung interstitium. Tissue sections were dewaxed in xylene, rehydrated in absolute alcohol, rinsed in water, immersed in 0.25% potassium permanganate for 5 min, rinsed in distilled water, bleached (~30 sec) in oxalic acid and rinsed prior to incubation in Resorcin Fuchsin Solution (10 ml resorcin-fuchsin stock, 100 ml acidified 70% ethanol and 2 ml 30% hydrochloric acid) for 2 h. Tissue sections were thoroughly rinsed and then

immersed in 0.25% tartrazine in saturated picric acid for 3 min prior to dehydration in absolute alcohol and xylene. The tissues were then mounted with DPX and coverslipped.

2.6.3.4 Immunohistochemical stains

Immunohistochemistry was performed to measure cell proliferation and apoptosis using a commercially available kit (EnVision+ Dual Link System-HRP [DAB+], Dako Cytomation, Denmark). Tissue sections were dewaxed in xylene, rehydrated in decreasing graded alcohol solutions and water, washed in phosphate buffered saline (PBS), boiled in a microwave oven for 20 min in an antigen retrieval solution (0.01 M sodium citrate (pH 6.0) for Ki67 or 0.01 M citric acid (pH 6.0) for activated caspase-3) followed by incubation with dual endogenous enzyme block solution to block endogenous peroxidases. Tissue sections were then rinsed in PBS and incubated with primary antibody. Antibody incubations were performed in a humidified chamber to prevent drying of tissues. Following washes in PBS containing 0.1% Tween-20, the slides were incubated for 30 min with labelled polymer-horseradish peroxidase (HRP), a peroxidase labelled polymer conjugated to both goat anti-mouse and goat anti-rabbit immunoglobulins, and washed in PBS/0.1% Tween-20. Slides were then incubated with DAB solution (diaminobenzidine (DAB), Dako Cytomation, Denmark) for 7 min, counterstained with haematoxylin, rinsed in water, dipped in Scott's Tap Water, rinsed and dehydrated in increasing concentrations of alcohol prior to coverslipping with DPX.

2.6.4 Histological analysis of lung tissue

Sections were analysed by stereological techniques to allow quantitative three-dimensional measurements to be made from two-dimensional images. Five fields of view per tissue section, with airways or large blood vessels absent, were examined under a light microscope (Nikon Eclipse E400, Nikon, Japan); each field of view was captured using a digital camera (SPOT Insight 4Meg Fire Wire Color Mosaic 14.2, Diagnostic Instruments, USA) connected to a computer. In total, 15

fields of view (5 from each lobe) were analysed for each animal and then averaged for each animal. Image analysis was performed using the Image-Pro Plus 6.0 software (Media Cybernatics, USA).

Point counting techniques (Adkisson and Callas, 1982, Smith and Post, 1989) were used to determine the percentages of tissue and airspace, MLI, alveolar wall thickness and septal crest density, while collagen and elastin content were quantified by expressing total collagen or elastin area over the total tissue area per field of view. Cell proliferation and apoptosis were assessed by expressing the number of cells undergoing proliferation or apoptosis over the total number of cells within the examined tissue region.

2.6.4.1 Tissue space fraction

The percentages of both tissue and air space in the lung were calculated to determine tissue density as an index of lung tissue maturity, in accordance with the description by Adkisson and Callas (1982). A 21-line, 42-point grid was superimposed over images of haematoxylin and eosin-stained lung tissue taken at 100X magnification, and the number of points lying over tissue or luminal space ("airspace") was counted. The percentages of tissue and airspace were then determined by applying the following formulae:

$$\text{Percentage of tissue} = \frac{\text{Number of points overlying tissue}}{\text{Total number of points in grid}} \times 100\%$$

$$\text{Percentage of airspace} = \frac{\text{Number of points overlying airspace}}{\text{Total number of points in grid}} \times 100\%$$

2.6.4.2 Mean linear intercept (MLI)

The MLI was measured as an index of alveolar diameter. A grid with 2 straight lines of known lengths, intersecting perpendicular to each other in their centres, as described by (Weibel, 1963), was superimposed over images of H&E stained lung

tissue taken at 100X magnification. The number of septal intercepts touching the line grid was then counted to determine the approximate size of the alveoli by the following formula:

$$D_a = \frac{N \times L}{\Sigma M_i}$$

Where

D_a= alveolar diameter

N= number of lines used (2)

L= length of the line

$\sum M_i$= the sum of the number of intercepts

2.6.4.3 Alveolar wall thickness

The alveolar wall thickness (or septal thickness; i.e. the thickness of tissue between adjacent alveoli) was measured by modifying the technique described by Smith and Post (1989). The same grid used to determine the percentages of tissue and airspace was superimposed over images of haematoxylin and eosin-stained lung tissue taken at 100X magnification. The number of times tissue intercepted the 21 lines and the number of times the ends of the lines fell on tissue were counted. Alveolar (or septal) wall thickness was measured by the following formula:

$$TD = \frac{ASF \times L_r}{2 \times I_0}$$

Where

TD = mean alveolar wall thickness

ASF = volume fraction of alveolar wall (number of tissue points/ 42)

L_r= length of entire grid line (1061.9mm)

I_0 = number of intercepts

2.6.4.4 Elastin and collagen content

The content of elastin and collagen in lung tissue was determined by measuring their relative area (based on colour) in relation to the total tissue area per field of view at a magnification of 1000X by using the Image-Pro Plus software.

2.6.4.5 Density of secondary septa

The areal density of secondary septa was assessed as an indicator of the presence of definitive alveoli, as an index of lung maturity. Secondary septa were identified as outgrowths (crests) of alveolar tissue, which contained elastin (stained black with Hart's elastin stain) deposited at the tip. A grid with points 1 cm apart was superimposed on images of sections stained with Hart's elastin stain, taken at 200X magnification. The number of points of the grid that were overlying secondary septa was counted. This was expressed over the total number of points that fell on lung parenchyma.

2.7 Water content of lung tissue

The percentage of water in lung tissue was determined by drying samples at 60ºC until no change in weight was detected for 3 consecutive days. Water content was then determined by measuring the difference between the wet and dry tissue weights.

2.8 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) was used to assess the gene expression of *surfactant proteins* (*SP*)-*A*, -*B*, -*C* and -*D* in frozen lung tissue collected at necropsy from the fetal and postnatal study groups. *Tropoelastin* (*TPE*) gene expression was also measured, but only in the fetal group. Prior to using the qPCR technique, RNA was extracted from the samples and converted into cDNA.

2.8.1 RNA extraction

Frozen tissue samples were used to extract RNA using an RNeasy Mini kit for the fetal samples (Qiagen, Germany) or RNeasy Midi kit for the postnatal samples. From each animal, a maximum of 30 mg (Mini kit) or 150 mg (Midi kit) of frozen tissue was homogenised in 600 µl (Mini kit) or 4 ml (Midi kit) of buffer RLT (containing 10 µl β-mercaptoethanol per ml of buffer RLT) by using a homogeniser (IKA T10 basic, Ultra-Turrax, Germany). The resulting lysate was then centrifuged at 20238 x *g* (centrifuge 5424, Eppendorf, Germany) for 3 min (Mini kit) or 3000–5000 x *g* for 10 min (Midi kit). The supernatant was transferred into a new microcentrifuge tube and 600 µl (Mini kit) or 4 ml (Midi kit) of 70% ethanol was added to the supernatant. The solution was transferred to an RNeasy spin column inserted in a 2 ml (Mini kit) or 15 ml (Midi kit) collection tube and centrifuged at 8000 x *g* for 15 sec (Mini kit)/ 3000–5000 x *g* (Midi kit).

Purification of the RNA was performed by using DNase to digest and eliminate any trace of DNA in the samples. A volume of 350 µl (Mini kit) or 2 ml (Midi kit) of buffer RW1 (RNase-free DNase set, Qiagen, Hilden, Germany) was added to each sample and centrifuged at 8000 x *g* for 15 sec (Mini kit) or 3000–5000 x *g* for 5 min (Midi kit). The flow-through fluid was then discarded. DNase I solution (1:8 dilution in buffer RDD) was added directly onto the membrane of the spin column and incubated at room temperature for 15 min. Buffer RW1 (350 µl (Mini kit) or 2.0 ml (Midi kit)) was added to each sample, the sample was centrifuged at 8000 x *g* for 15 sec (Mini kit) or 3000–5000 x *g* for 5 min (Midi kit), and the flow-through was discarded. A volume of 500 µl (Mini kit) or 2.5 ml (Midi kit) of buffer RPE (1:4 dilution in 100% ethanol) was added to each column and the samples were centrifuged at 8000 x *g* for 15 sec (Mini kit) or 3000–5000 x *g* for 2 min (Midi kit). This step was repeated with an additional 500 µl (Mini kit)/ 2.5 ml (Midi kit) of buffer RPE and centrifuged for 2 min (Mini kit) or 5 min (Midi kit). The columns were transferred into new 2 ml (Mini kit)/ 15 ml (Midi kit) collection tubes and centrifuged at 20238 x *g* for 1 min (Mini kit) or 3000–5000 x *g* for 3 min (Midi kit). To elute the RNA, RNase-free water of volume 40 µl (Mini kit) or 250 µl (Midi kit) was added directly onto the silica-gel membrane of each column and columns

were centrifuged at 8000 x *g* for 1 min (Mini kit) or 3000–5000 x *g* for 3 min (Midi kit). The elution step was repeated by pipetting the eluate directly onto the silica-gel membrane of each column and centrifuging it at 8000 x *g* for 1 min (Mini kit) or 3000–5000 x *g* for 3 min (Midi kit). The DNase-treated RNA eluate was stored at -80°C until use.

Gel electrophoresis was performed to determine the presence of DNA contaminants or degradation of the RNA samples. An agarose gel was made by adding 3 µl Gel Red nucleic acid stain (BIOTIUM, catalogue #41004) to 50 ml 1X 0.5% Tris- 0.1% acetate- 0.04% EDTA (TAE)/1% agarose. An aliquot of the RNA samples was heated at 65ºC for 5 min and placed on ice for 5 min. A loading dye was added into each sample prior to loading into the gel. An electrical field was applied via a power supply at 80V for 25 min (Bio-Rad, PowerPac 300, USA). A gel imaging system (Bio-Rad Molecular Imager Gel Doc and ChemiDoc™ XRS+ System, USA) was then used to visualise the RNA bands.

2.8.2 cDNA synthesis

In order to synthesize cDNA, the DNase-treated RNA was incubated with an enzyme, Moloneys-Murine Leukemia Virus reverse transcriptase (M-MLV RT; RNase H minus, Point Mutant Kit, Promega, USA). This procedure involved adding 1000 ng of the DNase-treated RNA in 13.5 µl of RNase-free water (GIBCO DNase and RNase-free distilled water, Invitrogen, Australia) and incubating the RNA with 0.5 µl (250 ng) of random hexamers (Promega, USA) at 70°C for 5 min, followed by cooling on ice for 5 min. To each RNA sample, 5 µl of M-MLV RT reaction buffer, 0.125 µl each of 10 mM nucleotides (dATP, dCTP, dGTP and dTTP), 5 µl of RNase-free water and 0.5 µl M-MLV RT enzyme (100U) were added. The samples were incubated for 10 min at room temperature followed by incubation at 42°C for 1 h. The reaction was heat-inactivated at 70°C for 15 min and the cDNA was stored at -20°C until use. The concentration of cDNA was determined using Thermo Scientific NanoDrop 2000/2000c Spectrophotometers (USA).

2.8.3 Real-time polymerase chain reaction

qPCR enables the expression levels of specific mRNA transcripts to be determined. The initial step was designing the primers for the genes of interest and also the housekeeping gene, *ribosomal protein S29* (*RPS29*). The expression of *RPS29* is used to normalise the expression of the genes of interest as its expression should not normally differ between samples. The concentrations of both primers and cDNA for optimal gene amplification were then determined, the primers were combined together with the cDNA samples and the gene of interest was amplified in a PCR reaction. The addition of a fluorescent probe (SYBR green) that binds to newly synthesised double-stranded DNA enabled detection of the exponentially replicated cDNA. The exponential increase in fluorescence, and therefore DNA, relates to the mRNA levels of the particular gene of interest in the original sample.

2.8.3.1 Primer design

The genes of interest were *SP-A*, *-B*, *-C* and *-D* and *TPE* and the housekeeping gene *RPS29*. Primers for these genes were previously designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) and analysed using the NetPrimer software (www.premierbiosoft.com/netprimer/index) to ensure that no (or few) complementary sequences, such as hairpins, dimers or cross-dimers, were found within and between primer sequences. The nucleotide sequences for each primer were based on the known nucleotide sequences of each gene of interest, as outlined in Table 2.1.

The concentrations of cDNA and primers, and also annealing temperatures, for optimal amplification of each gene were previously determined and the relevant conditions are listed in Table 2.2 and Table 2.1 respectively.

2.8.3.2 PCR assay for surfactant proteins and tropoelastin

The cDNA was assayed using 96-well PCR plates (4titude, catalogue # 4ti-0750, UK) in a PCR machine (Stratagene Mx3000P, Agilent Technologies, US). For each animal, the gene of interest and *RPS29* were amplified separately. Each sample was run in triplicate so as to account for pipetting errors. Wells allocated for the samples contained 10 µl of SYBR green (Platinum SYBR® Greener® qPCR SuperMix-UDG, Invitrogen, California, USA), 7 µl of nuclease-free water, 2 µl of primers (forward and reverse primers combined) and 1 µl of cDNA. Negative controls were also run in each assay so as to ensure that there was no DNA contamination. The wells for the negative controls contained all the reagents mentioned above but with the cDNA replaced by nuclease-free water. A calibrator cDNA sample, which was taken from the same animal, was also run in triplicate for both the gene of interest and *RPS29* on each PCR plate, to enable the thresholds to be adjusted between runs.

PCR was performed using the conditions outlined in Table 2.3. A melt curve was performed to ensure that the primers used specifically amplified one product within the samples. A single peak in the melt curve (for each primer-pair) indicated that only one specific nucleotide sequence, and therefore gene, was amplified within each sample during the PCR.

Following PCR procedures, the cycle threshold (Ct) value for each sample was obtained (MxPro QPCR software, Agilent Technologies, USA) to determine the level of gene expression between groups of animals. The Ct value is the cycle at which the amplification curve crosses the threshold; the threshold is set to a value above the background fluorescence and below the plateau of the amplification plot, and is therefore located within the log-linear region of the curve, where differences in gene expression can be determined. The mean Ct value and standard deviation (from the triplicates) of each sample were calculated; triplicates with a standard deviation of greater than 0.5 indicated variability due to pipetting error and were examined for outliers by removing one of the triplicates from the

analysis in order to get a standard deviation of below 0.5. When that was not achievable, the sample was analysed again in a new PCR assay.

Table 2.1: Nucleotide sequences for each primer pair and their accession number

Gene	Accession number	Forward Primer (5'-3')	Reverse Primer (3'-5')	Melting temperature (T_m)
SP-A	NM_001009728	CAT CAA GTC CTG CAG TCA CA	GCC CAT TGG TAG AGA AGA CC	60
SP-B	AF107544	GTC CTC TGC TGG ACA AGA TG	GGA GAG GTC CTG TGT CTG AG	59
SP-C	NM_001009729	GTG AAC ATC AAA CGC CTT C	TGT GAA GAC CCA TGA GCA	58
SP-D	AJ133002	ATG ACC GAT ACC AGG AAG GA	GCC CAG TTG GAA TAG ACC AG	59
TPE	M26189	ATC TCT CAG TCA GGC ACC AG	GTT TGT TGG GAA AGA AAG CA	59
RPS29	NM_174804	CAG GGT TCT CGC TCT TGC	ACT GGC GGC ACA TAT TGA G	58-60*

* The annealing temperature of *RPS29* depends on the annealing temperature of the gene of interest.

Table 2.2: Concentrations of cDNA and primers for optimal amplification of each gene of interest

Gene	Fetal Study		Postnatal Study	
	[cDNA] (ng/µl)	[Primer] (µM)	[cDNA] (ng/µl)	Primer] (µM)
SP-A	4	10	4	10
SP-B	4	10	4	10
SP-C	4	10	4	10
SP-D	4	4	4	4
TPE	4	10	-	-
RPS29	4	10	4	10

Table 2.3: PCR stages

Step	Process	Temperature (°C)	Duration
1	UDG incubation	50.0	2 min
2	UDG inactivation and DNA polymerase activation	95.0	10 min
3	Denaturation	95.0	20 sec
4	Annealing	58.0 – 60.0 [a]	1 min
5	45 cycles	Steps 2 – 3	
6	Denaturation	95.0	1 min
7	Melt curve	55.0	30 sec
8	Melt curve	55.0 – 95.0 Temperature rises in increments of 0.6°C [b]	Time taken to reach 95.0°C
9	Melt curve	95.0	30 sec

[a] The annealing temperature of *RPS29* depends on the annealing temperature of the gene of interest.

[a] Fluorescence acquisition takes place at the end of this step for each cycle.

[b] Fluorescence acquisition occurs at every 0.6°C during the melt curve.

2.8.3.3 Normalisation of gene expression

The expression of the genes of interest was normalised to the expression of *RPS29* for each sample. This was to account for any differences in the initial cDNA concentrations among the samples and also accounts for any possible variation in RNA content and degradation, reverse-transcription efficiency and differences in handling of the samples. The normalised expression of each gene of interest was calculated using the following equations:

ΔCt (for each sample) = Ct (gene of interest) – Ct (*RPS29*)

Normalised expression (gene of interest) = $2^{-\Delta Ct}$

For each gene of interest, the mRNA levels obtained for both male and female samples were separately averaged. The mean mRNA levels for each gene of interest for males were expressed relative to the mean mRNA levels in females.

2.9 Western blot analysis

Total protein was extracted from lung tissue by homogenization in RIPA buffer [1% Igepal, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 1x PBS pH 7.4, 1x "mini" complete protease inhibitor tablet per 10 ml (Roche Diagnostics, Australia)]. The homogenate was incubated on ice for 1 h, sonicated twice for 4 sec each time and then incubated on ice for a further 1 h. Samples were then centrifuged at 16000 x *g* for 20 min at 4°C. The supernatant was retained and protein concentrations determined using the Bradford assay (Bio-Rad, USA). Total proteins (SP-A: 35 µg and pro-SP-C: 40 µg) were separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE; 12%) for SP-A and premade gradient gel (4–20% Mini-PROTEAN® TGX™ Precast Gel #456-1093, Bio-Rad, USA) for pro-SP-C under reducing conditions in 10x running buffer (3% TRIS, 14.8% Glycine, 1% SDS at 50V for 30 min and at 100V for 1.5 h. The wet electroblotting technique was used to transfer proteins onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). Prior to the transferring of proteins, the PVDF membrane was cut to size, soaked in methanol for a few minutes and soaked in transfer buffer (0.3% TRIS, 1.48% Glycine, 0.2% methanol) for 15 min. Following wet electroblotting, the membranes were removed and incubated for 1 h in blocking buffer [5% skim milk powder phosphate buffered saline Tween (PBST) filtered solution] on a rocking mixer, rinsed with PBST, transferred to a heat seal bag and incubated with primary antibodies, SP-A (Anti-Surfactant Protein A Antibody, catalogue #AB3420, Millipore, US) or pro-SP-C (Anti-Prosurfactant Protein C Antibody, #AB3786, Millipore, US), overnight on a

rotary mixer at 4°C. After washing with PBST once for 15 min and another 3 times for 5 min each, membranes were incubated for 1 h with secondary antibody (Amersham ECL HRP-linked donkey anti-rabbit IgG, 1:10000; GE Healthcare Life Sciences, UK) on a rocker. Membranes were then washed with PBST again once for 15 min and another 3 times for 5 min each. Immobilon Western Chemiluminescent HRP substrate (Kit No. WBKL S01 00, Millipore, MA, USA) was pipetted onto the membranes for 5 min at room temperature to detect immunoreactive bands.

To standardize for protein loading, membranes were also incubated with rabbit anti-actin polyclonal antibody (1:3000; A2066, Sigma-Aldrich, MO, USA). The membranes were stripped with 0.2 M NaOH on a rocker for 5 min, washed with PBST, incubated with blocking buffer [5% skim milk powder phosphate buffered saline Tween (PBST) filtered solution] for 1 h on a rocker at room temperature, and washed with PBST for 5 min. The membranes were incubated with anti-actin antibody (1:3000; A2066, Sigma-Aldrich, MO, USA, USA) for 40 min at room temperature on rocker, washed with PBST for 15 min and additional 3 washes for 5 min each. Membranes were then incubated with secondary antibody (Amersham ECL HRP-linked donkey anti-rabbit IgG, 1:10000; GE Healthcare Life Sciences, UK) on a rocker for 40 min at room temperature. Immobilon Western Chemiluminescent HRP substrate (Kit No. WBKL S01 00, Millipore, MA, USA) was pipetted onto the membranes for 5 min at room temperature to detect immunoreactive bands.

The signal was detected using x-ray film, and densitometry analysis performed using ImageQuant TL analysis software (GE Healthcare, UK). For each protein of interest, the protein levels were initially normalised by dividing by actin levels for each sample followed by taking an average obtained for both male and female samples respectively. The mean protein levels for the males were expressed relative to the mean protein levels in females for each protein of interest.

2.10 Surfactant phospholipid analysis

Determination of surfactant phospholipids was performed using liquid chromatography mass spectrometry to identify the different proportions of the lipid classes and their respective species in ovine fetal lung liquid, amniotic fluid and postnatal BALF supernatant. This technique was initiated by extracting the lipids from the relevant samples. Four standards were also required in this technique and they included a negative blank containing 10 µl purified water (Milli-Q Advantage A10 system, Millipore, Germany), a negative no-protein standard containing 10 µl water and an internal standard mix [100 pmol each of PG 17:0/17:0, PS 17:0/17:0, PC 13:0/13:0, and PE 17:0/17:0 (Avanti Polar Lipids)], a quality control sample containing the internal standard mix and 10 µl protein (20 µg protein in 10 µl Bicinchoninic acid solution), and a non-extracted standard containing internal standard mix. The first three standards underwent the same procedure as the samples whilst the last standard, in addition to 45 µl water-saturated butanol and 45 µl methanol with ammonium formate, was transferred into a 0.2 ml micro-insert in a glass vial prior to liquid chromatography mass spectrometry. Sample volumes differed depending on the fluid being analysed [amniotic fluid (10 µl), BALF (10 µl), lung liquid (50 µl), gastric aspirate (50 µl)]. Internal standards [100 pmol each of PG 17:0/17:0, PS 17:0/17:0, PC 13:0/13:0, and PE 17:0/17:0 (Avanti Polar Lipids)] were added to each sample, in addition to chloroform:methanol (2:1) at a ratio of 20:1. The samples were vortexed, sonicated for 30 min and then left to stand for 20 min at room temperature. The extracts were then centrifuged at 13000 x *g* for 10 min. The supernatant was transferred to a 96-well plate and dried under nitrogen at 40°C. Following this, the dried samples were reconstituted with 50 µl water-saturated *n*-butanol and sonicated for 10 min. Methanol containing 10 mM NH_4COOH (50 µl) was then added to each well and the plate was centrifuged at 3000 x *g* for 5 min. The supernatant was transferred into 0.2 ml micro-inserts in glass vials with Teflon insert caps for liquid chromatography mass spectrometry.

In order to analyse the phospholipids of interest, we used an electrospray

ionization-tandem mass spectrometer; this system uses a mass spectrometer (PE Sciex API 4000 Q/TRAP) with a turbo-ion spray source and Analyst 1.5 data system. Initially, liquid chromatographic separation was performed on a 1.8-µm, 50 × 2.1 mm C18 column (Zorbax) at 300 µl/min using gradient conditions as follows: starting with 100% *solvent A* (10 mM NH_4COOH, 30:20:50 tetrahydrofuran:methanol:water) then increasing to 100% *solvent B* (10 mM NH_4COOH, 75:20:5 tetrahydrofuran:methanol:water) over 8 min, continuing at that concentration for 2.5 min before returning to 0% *solvent B* over 0.5 min and finally settling at that concentration for another 3 min prior to the next injection into the column. The individual lipid species were then quantified using scheduled multiple-reaction monitoring in positive-ion mode to monitor major species, which were greater than 1% of total, that have been identified in human plasma. Multiple-reaction monitoring experiments were based on product ion of 184 mass-to-charge ratio (*m/z*) of $[phosphocholine]^+$ for PC and neutral loss of 189 Da for PG, 141 Da for PE, 185 Da for PS, and 277 Da for phosphatidylinositol (PI). Each ion pair was monitored for 10–50 ms with a resolution of 0.7 atomic mass units at half-peak height and averaged from continuous scans over the elution period. The area under the curve of each species was expressed relative to the area under the curve of the corresponding internal standard. PI species were related to the PE internal standard and the higher response of the PE standard was corrected by a factor of 0.34. The proportion of each phospholipid class (PC, PG, PE, PI and PS) was analysed by adding the concentrations of all the lipid species from each class and expressing this value as a percentage of the total phospholipid concentration measured. Within each phospholipid class, the proportions of various molecular species were also analysed by expressing each one as a percentage of their respective phospholipid class. The molecular species are denoted as A+B:x+y, where A and B are the number of carbon atoms in the fatty acid chains esterified at the respective sn-1 and sn-2 positions, while x and y are the number of double bonds in the fatty acid chains.

2.11 Total protein content of BALF

The total protein content of postnatal BALF samples was determined as there is evidence that leakage of plasma proteins into the alveolar space impairs the effectiveness of surfactant in lowering surface tension (Seeger et al., 1993, Seeger et al., 1985). Measurement of protein concentration in BALF in males and females allows us to determine if the presence of proteins within the alveolar space was related to differences in static lung compliance. In order to determine the concentration of solubilised protein in the BALF samples, a protein assay (Bio-Rad, USA) was used. Bovine serum albumin standards in 0.9% saline were diluted to concentrations of 0, 0.05, 0.1, 0.2, 0.35, 0.5, 0.75, 1 and 1.5 mg/ml. The dye reagent was prepared by diluting DyeReagent Concentrate (catalogue # 500-0006) with distilled, deionised water (1:5 dilution). The BALF samples were centrifuged at 10400 x *g* (centrifuge 5414R, Eppendorf, Germany) at 4°C for 3 min and the supernatant was used for the assay. The supernatant was diluted with dH_2O (1:4 dilution). Each standard and sample solution (10 µl) was pipetted in duplicate into a well of a 96-well plate. Diluted dye reagent (200 µl) was added to each well. The plate was incubated at room temperature for 5 min. Absorbance of the samples was then measured at 595 nm using a plate reader (FLUOstar Omega, Germany). A standard curve was created and comparison of sample data to the standard curve provided a measurement of protein concentration.

2.12 Cortisol radioimmunoassay

The concentration of cortisol in plasma was measured because cortisol is believed to regulate fetal lung maturation (Liggins, 1994). Cortisol concentrations were determined by radioimmunoassay as described by (Bocking et al., 1986). Cortisol was extracted from 100 µl of plasma (diluted 1:1 with dH_2O) using dichloromethane, vortexed briefly and left to stand until two layers formed; the upper aqueous phase and the lower organic (solvent) phase containing cortisol. Extractions were performed in duplicate. A 1 ml aliquot of the lower phase was transferred into glass tubes and dried down by evaporation at 37°C. A standard curve was constructed using a stock solution of cortisol (5 ng/ml) diluted to

concentrations of 0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25 and 50 ng/ml. A 1 ml aliquot of CH_2Cl_2 was added to 100 µl of each standard in glass tubes, vortexed and evaporated with air at 37°C. The extracted samples and standards were resuspended with cortisol antiserum (1:15000), bovine γ-globulin (0.8 mg/100 µl) and ^{3}H-cortisol (10000cpm/100µl); 100 µl each. The samples were incubated overnight at 4°C. A 1 ml aliquot of 22% polyethylene glycol was added to all samples to allow separation of antibody-bound and free ^{3}H cortisol; the samples were then vortexed and centrifuged for 15 min at 1800 x *g* at 4°C. The supernatant containing unbound ^{3}H cortisol was aspirated while the remaining pellet containing the antibody-bound ^{3}H hormone was resuspended in 200 µl of cortisol assay buffer and 100 µl was added to the recovery tubes, and vortexed. A 1 ml aliquot of Ultima Gold Scintillant (Perkin Elmer, USA) was added to all tubes and vortexed. The tubes were then analysed in a β-radiation counter (Skudtek Scientific, USA) to determine the radioactivity. The percentage recovery of cortisol was 88.8%. A standard curve was generated to determine cortisol concentration by interpolation.

2.13 Statistical analysis of data

Unpaired Student's T-test (to determine between two data at one time point): All data was tested for normality using F-test (Systat Software, Inc., SigmaPlot, Version 12.0 for Windows, California, USA). If f-test failed, non-parametric test (Mann-Whitney test) was performed. All values are presented as mean ± standard error of the mean (SEM) unless specifically mentioned and statistical significance is denoted as $P<0.05$.

One way and two ANOVA (to determine between two data at multiple time points): All data was tested for normality. No data in this study failed the F-test, so statistical tests such as one-way repeated measures ANOVA and two-way ANOVA were performed using IBM SPSS Statistics, Version 20 for Windows (IBM, Armonk, NY, USA). All values are presented as mean ± standard error of the mean (SEM) unless specifically mentioned and statistical significance is denoted as $P<0.05$.

Statistical significance tests are described in detail in each experimental chapter.

Chapter 3: Sex differences in lung morphology and surfactant composition in preterm fetal sheep

3.1 Introduction

Male preterm infants have a higher risk of respiratory morbidity and mortality than females of the same gestational age (Khoury et al., 1985, Anadkat et al., 2012). Male preterm infants are at a greater risk of developing respiratory distress syndrome (RDS) and require more doses of surfactant and longer periods of respiratory support; they also are more likely to be oxygen dependent at 36 weeks of postmenstrual age (Thomas et al., 2006). However, little is known about the underlying cause of the male disadvantage in respiratory function following preterm birth. It is likely that the causes are multi-factorial but could include differences in prenatal lung development, including lung structure and pulmonary surfactant.

Very little is currently known about differences in lung structure between the sexes, in any species, both during gestation and during the early postnatal period. Langston et al. (1984) demonstrated no sex differences in lung morphometry in both still-born and live-born infants aged from 19 weeks of gestation to 3 weeks of postnatal age; this conclusion was based on measurements of lung volume, average distance between saccular or alveolar walls, number of alveoli per unit area and volume, total alveolar number, surface area, air-space wall thickness and volume proportions of alveolar air, duct air, airspace walls, bronchial and

bronchiolar lumen, and non-parenchymal tissue (Langston et al., 1984). In contrast, sex differences in lung structure have been reported after the early postnatal period. Postnatally, from 6 weeks to 14 years, alveolar size and alveolar number per unit area and volume were found to be similar in boys and girls; however boys had larger lungs than girls (Thurlbeck, 1982). As a consequence, there were more alveoli, a larger alveolar surface area and more respiratory bronchioles in boys for any given age and stature (Thurlbeck, 1982). The growth of the large airways in young males lags behind the growth of the parenchyma, which is known as dysynaptic growth of the large airways; this results in relatively narrower airways in male lungs (Hoffstein, 1986). Women have a better correlation between tracheal cross-sectional area and expiratory air-flow than in men and this may contribute to higher airflow rate and lower airway resistance in females (Hoffstein, 1986).

In postnatal mice, rats and rhesus monkeys there are distinct sex differences in lung morphometry at the onset of maturity. In rodents, females have more alveoli and a greater alveolar surface area relative to body weight than males; this is likely because the alveoli of females are smaller than their male counterparts (Massaro and Massaro, 2004, Massaro and Massaro, 2006, Hyde et al., 2007). In contrast, female rhesus monkeys (young adults) have larger alveoli in comparison to males of the same age (Hyde et al., 2007). In adult humans, females have been shown to have fewer alveoli, probably due to their lungs being smaller than in males of the same stature (Goldman and Becklake, 1959, Thurlbeck, 1982).

Pulmonary surfactant is important in maintaining alveolar patency, thereby allowing the lungs to expand with gas after birth. During gestation, the ontogeny of pulmonary surfactant has been found to be delayed in males in both humans and rabbits in studies using amniotic fluid and/or lung lavage samples (Nielsen and Torday, 1981, Torday et al., 1981, Fleisher et al., 1985). Lung lavage and amniotic fluid assessed in fetal rabbits from 24–30 days of gestation (d GA; term is 31d GA) showed earlier increases in the ratio of SPC/S in female fetuses than in males

(Nielsen and Torday, 1981, Torday et al., 1981). In the human fetus, pulmonary surfactant release appears to occur earlier in females than males, and males are thought to lag in this aspect of lung maturation by 1.2 to 2.5 weeks (Torday et al., 1981, Fleisher et al., 1985); this is indicated by greater L/S ratio and the percentages of saturated PC and disaturated lecithin (DPPC), PG and PI in the amniotic fluid of female fetuses between 28 and 40 weeks of gestation. This apparent delay in surfactant production or maturation of surfactant composition in males may be responsible for the greater incidence of RDS seen in male preterm infants; however a detailed analysis of surfactant components in the lungs during late gestation has not been performed. Measurement of surfactant in amniotic fluid provides an indirect indication of the surfactant in the fetal lung, because fetal in amniotic fluid fetal lung liquid is diluted with other fetal fluids such as urine and oro-nasal secretions.

Since information on sex differences in fetal lung architecture and the composition of surfactant phospholipids derived from the fetal lung are largely lacking in the literature, this study was aimed to address them.

The first aim of this study was to investigate sex differences in the morphology of the preterm fetal lung of sheep. Rates of cell proliferation and apoptosis were measured as indicators of tissue growth. *Tropoelastin* gene expression was analysed because it is involved in the process of alveolarisation (Bruce, 1991, Bruce et al., 1993, Bruce and Honaker, 1998). The lungs were studied at 131d GA (0.9 of term). In terms of lung development, this is equivalent to 32–34 weeks gestation in humans. To my knowledge, this is the first study to thoroughly investigate sex differences in the architecture of lung parenchyma in an animal model of preterm birth that shows the male disadvantage with respect to respiratory function.

The second aim of this study was to investigate sex differences in *surfactant protein* gene expression and the composition of surfactant phospholipids in the lung liquid of preterm fetal sheep. Although previous studies have documented sex-related differences in surfactant composition as detected in amniotic fluid, none have examined in detail the sex differences in the composition of surfactant phospholipids collected directly from the lung lumen. Samples of fetal lung liquid are more likely to provide a more accurate representation of surfactant originating in the lung than samples of amniotic fluid because the latter is variably diluted with other fetal fluids.

3.2 Methods

Two cohorts of animals were used. In the first cohort, lung tissue was obtained from male and female ovine fetuses at ~131d GA as previously described in Section 2.2. Lung architecture was analysed using standard morphometric techniques and *surfactant protein* (*SP*) and *tropoelastin* (*TPE*) mRNA expression in lung tissue was measured.

The second cohort of sheep was to obtain fetal lung liquid. These animals were surgically prepared, as described in Section 2.4, and lung liquid collected at 131 and 133d GA from a catheter in the fetal trachea. Additional experiments were conducted on these fetuses, after 133d GA, and are discussed in Chapter 4.

3.2.1 Experimental protocol

Cohort 1: A bolus dose of sodium pentobarbitone (130 mg/kg; Lethabarb, Virbac Pty. Ltd., Peakhurst, NSW, Australia) was administered via a jugular vein to pregnant ewes of known mating date to humanely euthanise both the ewe and fetus. The fetus was removed from the uterus and the lungs were prepared for histological and gene expression analysis as described in Sections 2.6 and 2.8.

In addition, rates of cell proliferation and apoptosis were assessed by performing immunohistochemistry using a commercially available kit for each measurement. Tissue sections were dewaxed, rehydrated in decreasing graded alcohol solutions and water, washed in phosphate buffered saline (PBS), boiled in an antigen retrieval solution (0.01 M sodium citrate (pH 6.0) for Ki67 or 0.01 M citric acid (pH 6.0) for activated caspase-3) followed by incubation with dual endogenous enzyme block solution to block endogenous peroxidases. Tissue sections were then rinsed in PBS and incubated with primary antibody as detailed below. Antibody incubations were done in a humidified chamber to prevent drying of tissues. Following washes in PBS containing 0.1% Tween-20, the slides were incubated for 30 minutes (min) with labelled polymer-horseradish peroxidase (HRP), a peroxidase labelled polymer conjugated to both goat anti-mouse and goat anti-rabbit immunoglobulins, and washed in PBS/0.1% Tween-20. Slides were then incubated with DAB solution (diaminobenzidine (DAB), Dako Cytomation, Denmark) for 7 min, counterstained with haematoxylin, rinsed in water, dipped in Scott's Tap Water, rinsed and dehydrated in increasing concentrations of alcohol prior to coverslipping with DPX.

Proliferating cells were identified using the antigen, Ki67, which is expressed only in cells undergoing division (Cohen et al., 2007). For this staining, diluted mouse anti-Ki67 primary antibody (1:100 dilution in DAKO antibody diluent, DakoCytomation, North America) was applied to the tissue sections and incubated for 90 min at room temperature in a humidified incubation box. Following incubation with DAB solution, tissue sections were counterstained with haematoxylin and coverslipped.

The activated caspase-3 antigen is expressed in cells undergoing programmed cell death and is therefore used as a marker for cells undergoing apoptosis (Bem et al., 2008). As with Ki67, an immunohistochemistry kit (Dako Envision+ Dual Link System – HRP (DAB+, USA) was used for this staining. Tissue sections were incubated with diluted mouse anti-activated caspase-3 primary antibody (1:1000 in

5% normal goat serum/0.1% TritonX-100/PBS; R&D Systems, Minnneapolis, USA) for 2 h at 4°C in a humidified incubation box. Following incubation with DAB solution, tissue sections were counterstained with haematoxylin and coverslipped.

Cohort 2: Measurement of surfactant phospholipid composition in lung liquid was performed following sampling of the lung liquid (50 µl) at 131 and 133d GA, as outlined in Section 2.4.3.1. Lung liquid samples were obtained from 9 male and 9 female fetuses and were used to compare the phospholipid content between males and females. The technique has been described in Section 2.10).

3.2.2 Analytical methods

The volume of the right lung was estimated using the Cavalieri method (see Section 2.5). Tissue and air space fractions, alveolar diameter (MLI), alveolar wall thickness, collagen and elastin content and septal crest density were determined using the techniques described in Section 2.6. To quantify the percentage of lung cells undergoing proliferation or apoptosis, the number of Ki67 or activated caspase-3 labelled cells, respectively, was expressed as a proportion of the total number of cells in fields of view taken at 1000X magnification. mRNA expression of *SP-A, -B, -C* and *-D* and *TPE* was determined using the techniques described in Section 2.8. Analysis of the surfactant phospholipid composition was performed as described in Section 2.10.

3.2.3 Statistical analysis of data

Lung weights, body weights, relative lung weights, histological assessment and gene expression were analysed using the unpaired Student's t-test. These analyses were performed using SigmaPlot, Version 12.0 for Windows (Systat Software, Inc., California, USA). Surfactant phospholipid composition in lung liquid at 131 and 133d GA was analysed using one-way repeated measures ANOVA, with sex as one factor (P_S) and time (P_T) as the repeated measure, using IBM

SPSS Statistics, Version 20 for Windows (IBM, Armonk, NY). To maintain statistical power, adjustments for multiple comparisons were not performed so as to avoid the high probability of obtaining Type II errors (false negatives). All values are presented as mean ± standard error of the mean (SEM). Statistical significance was denoted as $P<0.05$.

3.3 Results

3.3.1 Fetal age and body weight

In Cohort 1 there was no difference in the gestational age of female (131 ± 0 days; n=11) and male (132 ± 0 days; n=9) fetuses. Body weights were also not significantly different between female (3.6 ± 0.3 kg) and male (3.6 ± 0.1 kg) fetuses (Figure 3.1 A).

3.3.2 Fetal lung weight

There was no difference in the absolute lung weight of female (133.2 ± 12.2 g) and male (136.4 ± 9.6 g; Figure 3.1 B) fetuses. Similarly, lung weights were not different between females (35.6 ± 1.1 g/kg) and males (36.6 ± 2.1 g/kg) when adjusted for body weight (Figure 3.1 C).

3.3.3 Fetal lung volume

Lung volume, estimated by the Cavalieri method, was not significantly different between females (80.3 ± 10.0 cm^3) and males (94.6 ± 21.7 cm^3; Figure 3.1 D).

3.3.4 Fetal lung tissue and airspace fractions

Similar values were obtained for lung tissue fraction in females (35.0 ± 1.7%) and in males (35.5 ± 1.7%; Figure 3.4 A). Likewise, the airspace fraction was

comparable in both females (65.0 ± 1.7%) and males (64.5 ± 1.7%; Figure 3.4 B). Representative images of H&E stained tissue sections from lungs of a female and male fetus are shown in Figures 3.2 A and 3.2 B respectively.

3.3.5 Mean linear intercept

The mean linear intercept, an index of alveolar size, was not significantly different between females (81.5 ± 1.3 µm) and males (81.0 ± 2.1 µm; Figure 3.4 C).

3.3.6 Alveolar wall thickness

The mean alveolar wall thickness was not significantly different between females (3.6 ± 0.2 µm) and males (3.5 ± 0.2 µm; Figure 3.4 D).

3.3.7 Septal crest density

No difference was observed between females (7.7 ± 0.9 %) and males (8.8 ± 0.7 %) in the areal density of septal crests (Figure 3.4 E). Histological representations of Hart's elastin stained tissue sections from lungs of a female and male fetus are shown in Figures 3.2 E and 3.2 F respectively.

3.3.8 Lung collagen content

There was no difference in the areal density of collagen between the lungs of females (16.6 ± 1.7 %) and males (17.0 ± 1.5 %; Figure 3.4 F). Collagen staining (Gordon and Sweet's reticular fibre stain) are shown in Figures 3.2 C and 3.2 D in lung sections from female and male fetuses respectively.

3.3.9 Lung elastin content

There was no difference in the elastin content (areal density) of the lungs of female (3.5 ± 0.3 %) and male fetuses (3.5 ± 0.3 %; Figure 3.4 G). Elastin fibres are shown in Figures 3.2 E and 3.2 F in Hart's elastin stained tissue sections of both female and male lungs. The mRNA expression of *TPE* was similar in females and males (Figure 3.5).

3.3.10 Pulmonary cell proliferation

The proportion of cells undergoing proliferation in the lungs of female (1.7 ± 0.6 %) and male (1.7 ± 0.4 %) fetuses was similar (Figure 3.4 H). Figures 3.3 A and 3.3 B show images stained for Ki67 antigen from lungs of both females and males respectively.

3.3.11 Apoptosis in lung tissue

No difference was observed in the proportion of lung cells undergoing apoptosis between female (1.1 ± 0.3 %) and male (0.8 ± 0.1 %) fetuses (Figure 3.4 I). Figures 3.3 C and 3.3 D show lung images stained for activated caspase-3 antigen taken from both female and male fetuses respectively.

3.3.12 mRNA expression in lung tissue

Male and female lambs had similar gene expression of *SP-A*, *-B*, *-C*, *-D* and *TPE* in lung tissue: *SP-A* (F: 1.0 ± 0.3 vs M: 0.9 ± 0.2), *SP-B* (F: 1.0 ± 0.2 vs M: 1.0 ± 0.1), *SP-C* (F: 1.0 ± 0.1 vs M: 1.2 ± 0.1), *SP-D* (F: 1.0 ± 0.2 vs M: 1.5 ± 0.2) and *TPE* (F: 1.0 ± 0.1 vs M: 1.0 ± 0.1) (Figure 3.5).

3.3.13 Phospholipid content of fetal lung liquid

3.3.13.1 Phospholipid classes

When the proportions of phospholipid classes, relative to total phospholipids, were analysed, no overall sex, overall time (over two different gestational ages) or interaction effects were observed in the proportions of lysophosphatidylcholine (LPC) and PC (Figures 3.6 A and 3.6 B, respectively). An overall sex effect was observed in the proportions of PE, with males having a higher proportion compared to females (Figure 3.6 C). An overall time effect was observed in the proportions of PG and PI, such that greater proportions were observed in males and females combined at 133d GA compared to 131d GA (Figure 3.6 D and E respectively). The proportions of PI and PS (Figure 3.6 F) showed an interaction effect; for PI, values in males showed a significant decrease from 131d GA to 133d GA, while values in females increased significantly. In contrast, between 131d GA to 133d GA values for PS significantly increased in males and decreased in females (Figure 3.6 F).

3.3.13.2 Phospholipid maturity ratio (PC/PE)

The ratio of phosphatidylcholine to phosphatidylethanolamine (PC/PE) is often used as an index of an index of the surface active properties of pulmonary surfactant (Banerjee and Bellare, 2001). There was no overall sex, overall time or interaction effect observed in the ratio of PC/PE (Figure 3.7).

3.3.13.3 Phospholipid species

Lysophosphatidylcholine (LPC)

There was an overall time effect for LPC 18:0 which showed a decrease in its proportion over time (131 to 133d GA). No overall sex, overall time or interaction effect were observed in the proportions of the other LPC species (14:0, 16:0, 16:1, 18:1 and 18:2; Figure 3.8 A).

Phoshatidylcholine (PC)

An overall time effect was found for PC 34:1+1; from 131d GA to 133d GA the values increased significantly in males and decreased in females. No overall sex, overall time or interaction effects were observed in the proportions of the other PC species (30:0, 32:0, 32:1+1, 34:2+1, 36:1+1, 36:3, 38:2+1, 38:3, 38:4; Figure 3.8 B).

Phosphatidylethanolamine (PE)

There was an interaction effect for PE 32:1; between 131d GA to 133d GA, the values in males significantly decreased and increased in females. No overall sex, overall time or interaction effects were observed in the proportions of the other PE species (35:1, 40:5, 16:0/18:1, 16:0/18:2, 16:0/20:4, 16:0/22:5, 16:0/22:6, 18:0/18:1, 18:0/20:4, 18:0/22:6, 18:1/18:1 and 18:1/18:2; Figure 3.8 C).

Phosphatidylglycerol (PG)

No overall sex, overall time or interaction effect was observed in the proportion of PG 16:0/18:1 (Figure 3.9 A).

Phosphatidylserine (PS)

There was an overall sex effect for PS 36:1, with females having significantly higher proportions than males. No differences were observed in the overall sex, overall time or interaction effects in the proportion of the other PS species (36:2, 40:5 and 40:6; Figure 3.9 B).

Phosphatidylinositol (PI)

An interaction effect was observed in the proportion of PI 38:5; between 131d GA to 133d GA the values significantly decreased in males while in females the values

increased. No overall sex, overall time or interaction effects were observed for the other PI species (32:1, 34:1, 36:1, 36:2, 36:3, 36:4, 38:3 and 38:4; Figure 3.9 C).

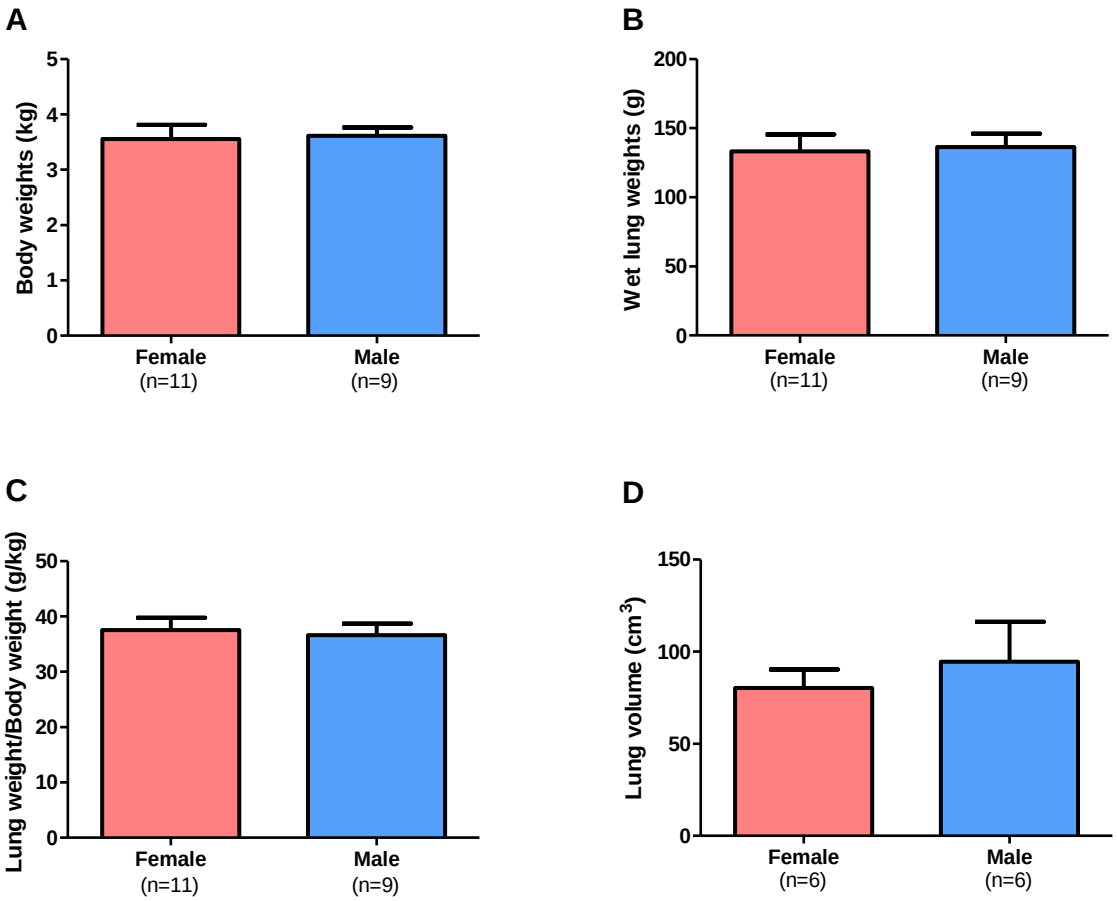

Figure 3.1 Fetal weights and lung volume.

Bar graphs show (A) body weight (kg), (B) wet lung weight (g), (C) relative wet lung weight (g/kg) and (D) lung volume (cm^3) of female fetuses (pink bars) and male fetuses (blue bars) at ~131d GA. Data are shown as mean ± SEM.

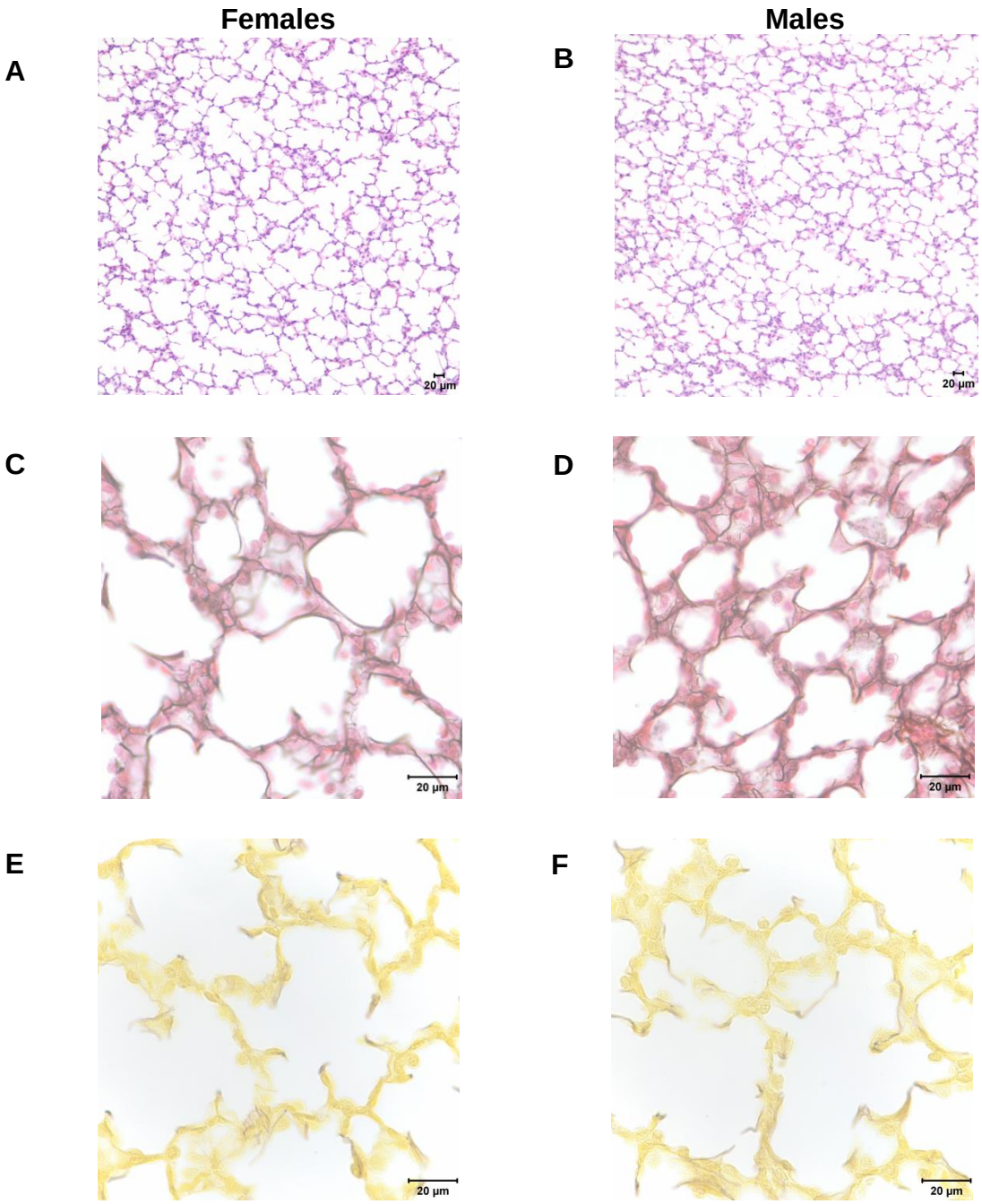

Figure 3.2 Histological images of female and male preterm sheep lungs at ~131 days of gestation.

The images are of lung tissue sections (5 µm thick) as observed under the light microscope, stained with Haematoxylin and Eosin at 200X magnification (nuclei are stained purple while cytoplasm is stained pink) in female (A) and male (B), Gordon and Sweet's reticular fibre stain at 1000X magnification (collagen fibres types I and III stained black while the interstitium is stained pink) in female (C) and male (D), and Hart's elastin stain at 1000X magnification (elastin fibres stained black while interstitium is stained yellow) in female (E) and male (F).

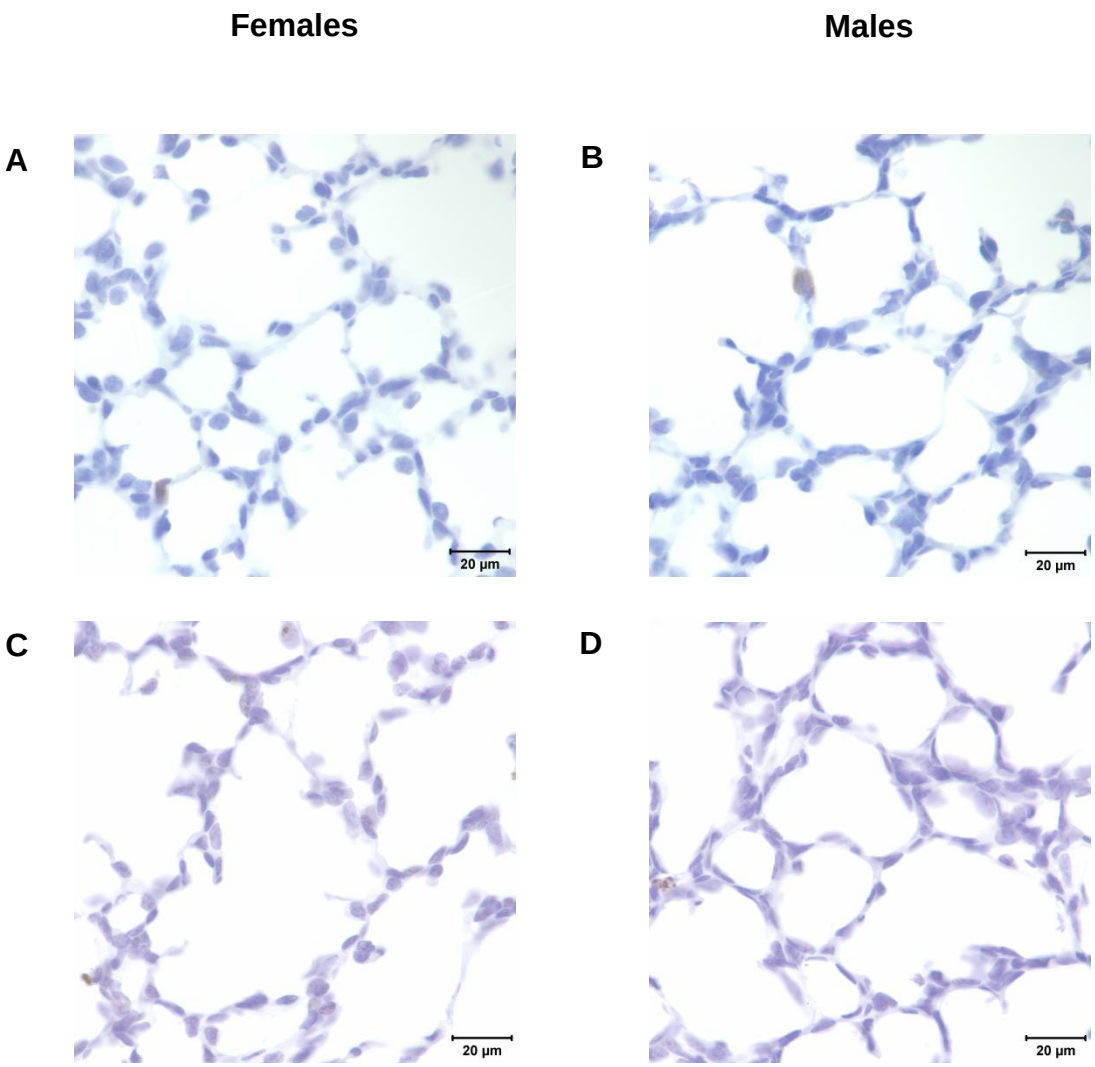

Figure 3.3 Lung tissue sections stained for Ki67 and Caspase-3 antigens.

Representative lung tissue sections of 5 µm thickness as observed under the light microscope, stained with Ki67 at 1000X magnification as a marker for proliferation in female (A) and male (B) fetuses, and Caspase-3 stain at 1000X magnification as a marker for apoptosis in female (C) and male (D) fetuses. Brown nuclei indicate cells that are undergoing proliferation or apoptosis while nuclei that are stained blue are not undergoing either process.

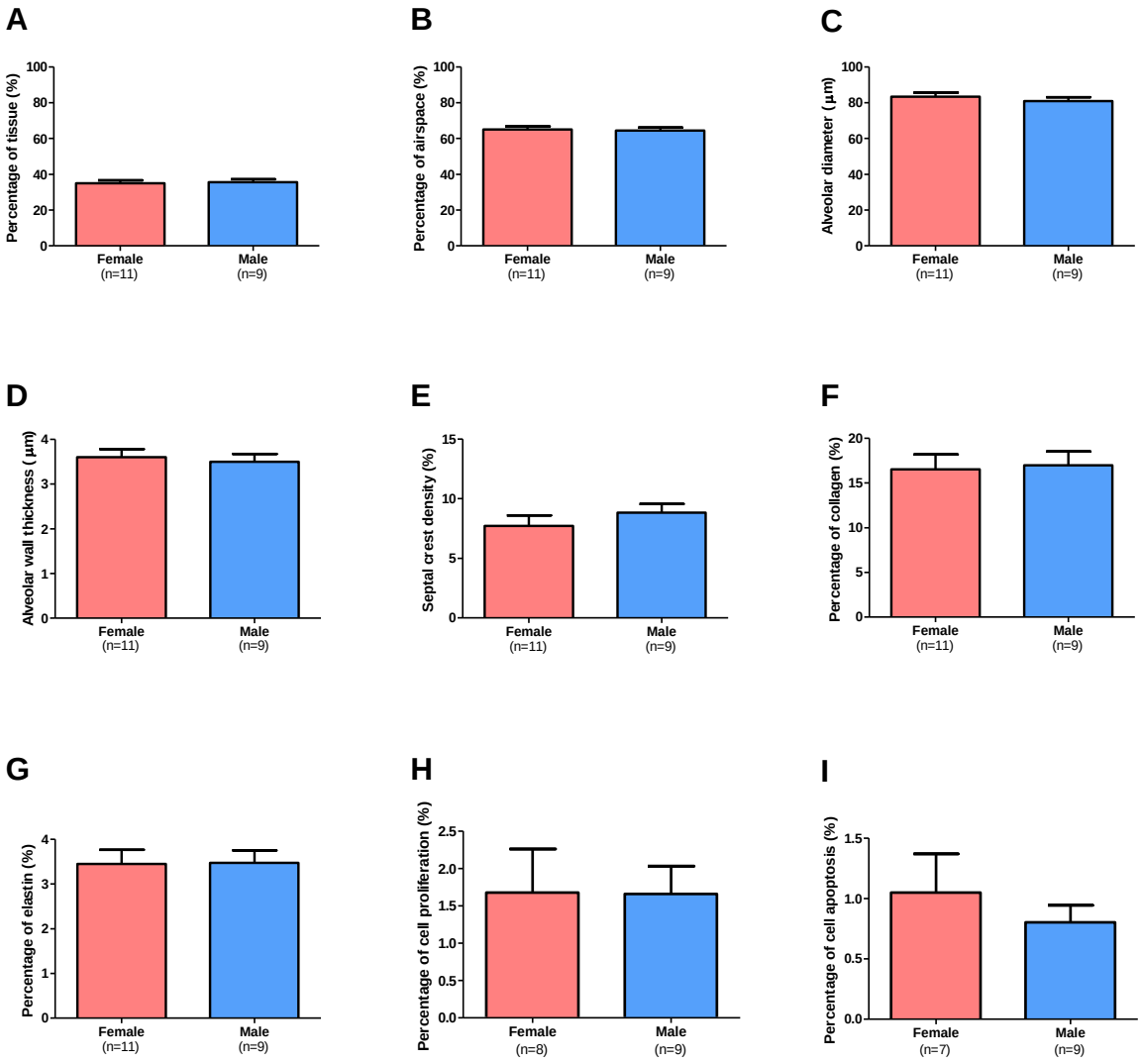

Figure 3.4: Indices of lung morphometry in fetal sheep at ~131 days of gestation.

Bar graphs show (A) lung tissue fraction, (B) air space fraction, (C) mean linear intercept, (D) alveolar wall thickness, (E) septal crest density, (F) areal density of collagen, (G) areal density of elastin, (H) percentage of cells undergoing proliferation and (I) percentage of cells undergoing apoptosis. Data are shown as mean ± SEM. Females (pink bars); males (blue bars).

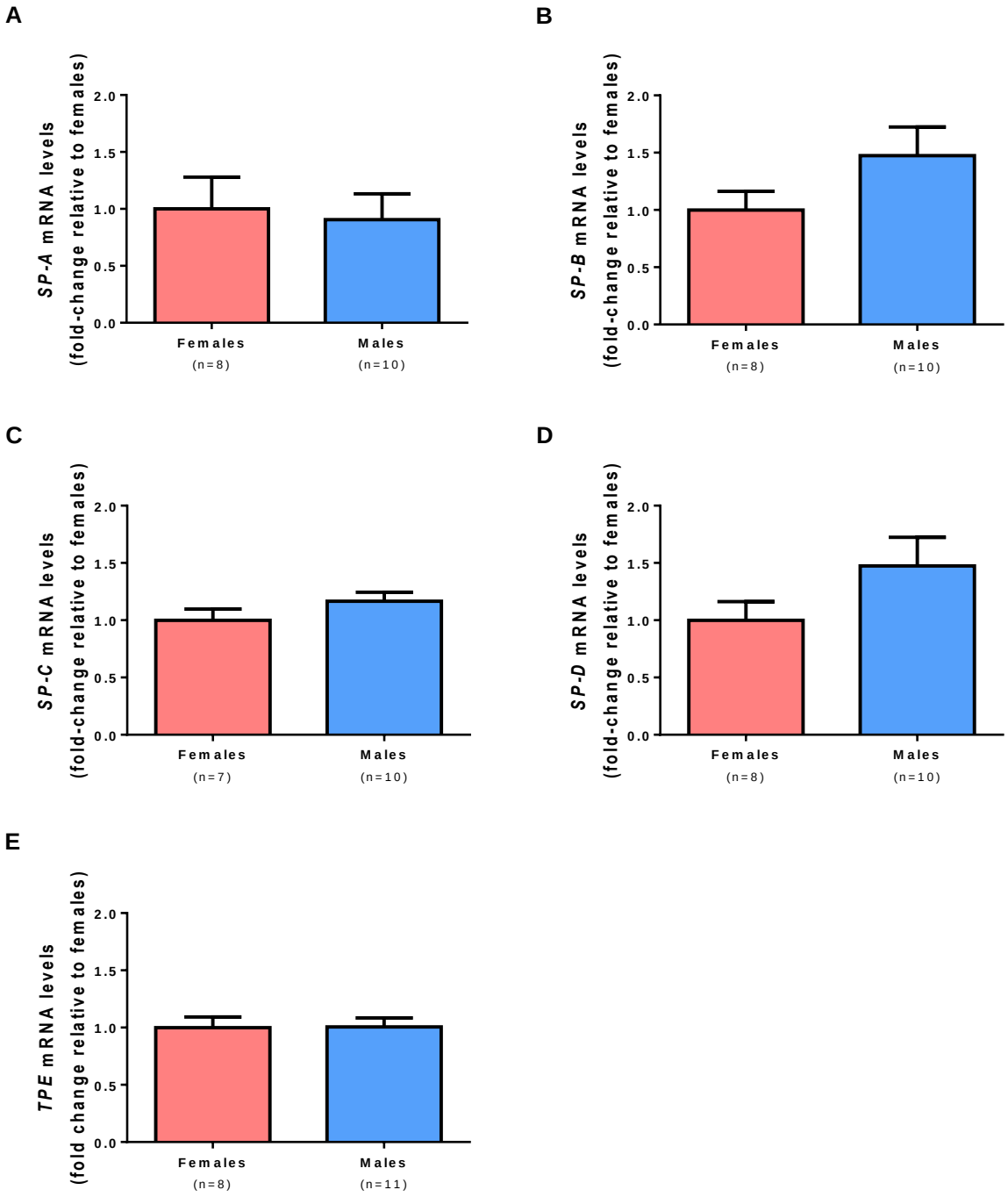

Figure 3.5 *Surfactant protein* (*SP*) and *tropoelastin* gene expression in the lungs of female and male preterm lambs.

Panels (A) to (E) show the *SP-A*, *-B*, *-C* and *-D* and *tropoelastin* gene expression, respectively, in lung tissue at 4 hours after birth in female (pink bars) and male (blue bars) lambs. Data are represented as mean ± SEM.

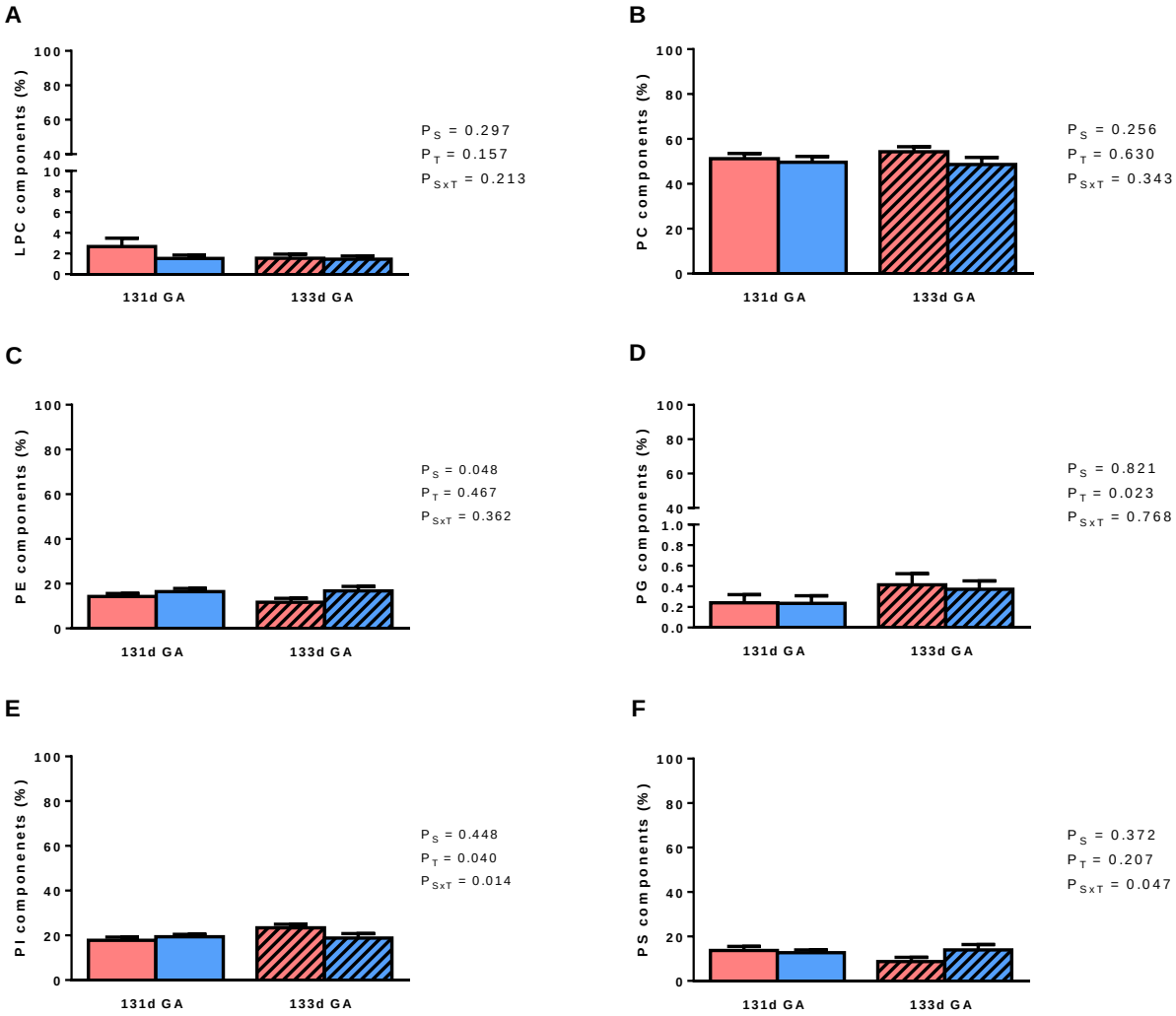

Figure 3.6. Composition of surfactant phospholipids before birth in lung liquid at 131 and 133 days of gestation.

Panels A to F show the proportions of phospholipid classes: lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS), respectively. Females (n=9) and males (n=9) are represented in pink and blue bars respectively. Unhatched bars represent samples collected at 131 days of gestation (d GA). Hatched bars represent samples collected at 133 days of gestation (d GA). Data are shown as mean ± SEM, and were analysed using one-way repeated measures ANOVA, with sex as one factor (P_S) and time (P_T) as the repeated measure. P_{SxT} is the interaction between time and sex.

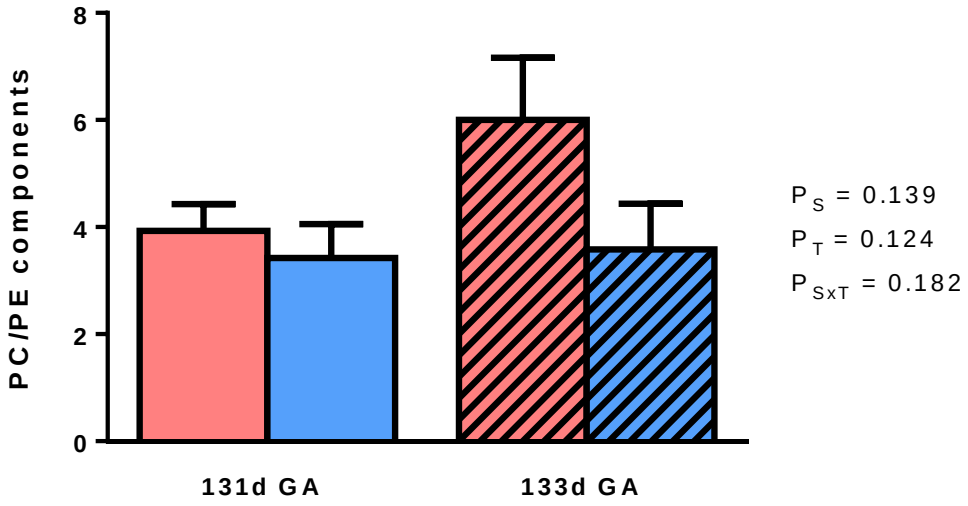

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Figure 3.7. Phospholipid maturity ratio in fetal lung liquid.

The ratio of PC/PE in fetal lung liquid at 131 and 133 days of gestation. Females (n=9) and males (n=9) are represented in pink and blue bars respectively. Unhatched bars represent samples collected at 131 days of gestation (d GA) and hatched bars represent samples collected at 133 days of gestation (d GA). Data are shown as mean ± SEM, and were analysed using one-way repeated measures ANOVA, with sex as one factor (P_S) and time (P_T) as the repeated measure. P_{SxT} is the interaction between time and sex.

Figure 3.8. Proportions of the main phospholipid molecular species of LPC (A), PC (B) and PE (C) in fetal lung liquid at 131 and 133 days of gestation (opposite page).

Females (n=9) and males (n=9) are represented in pink and blue bars respectively. Unhatched bars represent samples collected at 131 days of gestation (d GA) and hatched bars represent samples collected at 133 days of gestation (d GA). Data are shown as mean ± SEM, and were analysed using one-way repeated measures ANOVA, with sex as one factor (P_S) and time (P_T) as the repeated measure. P_{SxT} is the interaction between time and sex.

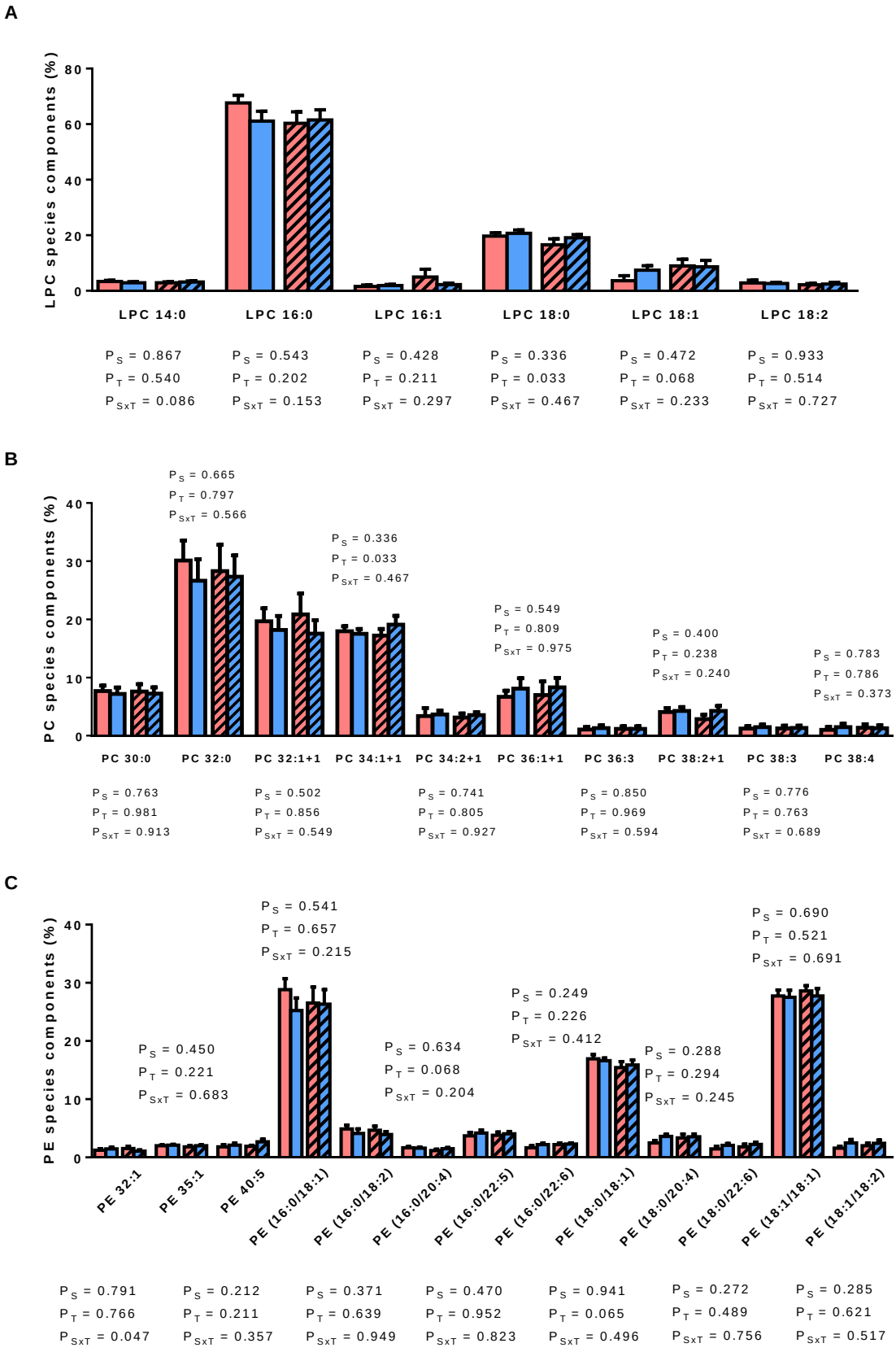
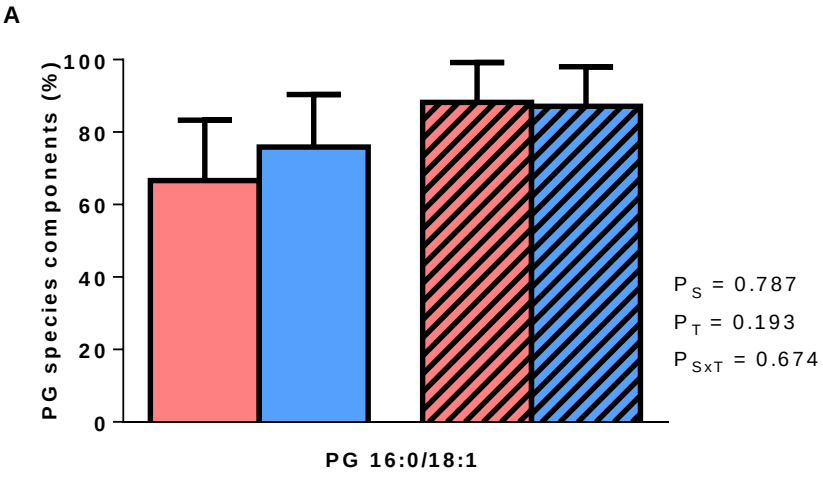
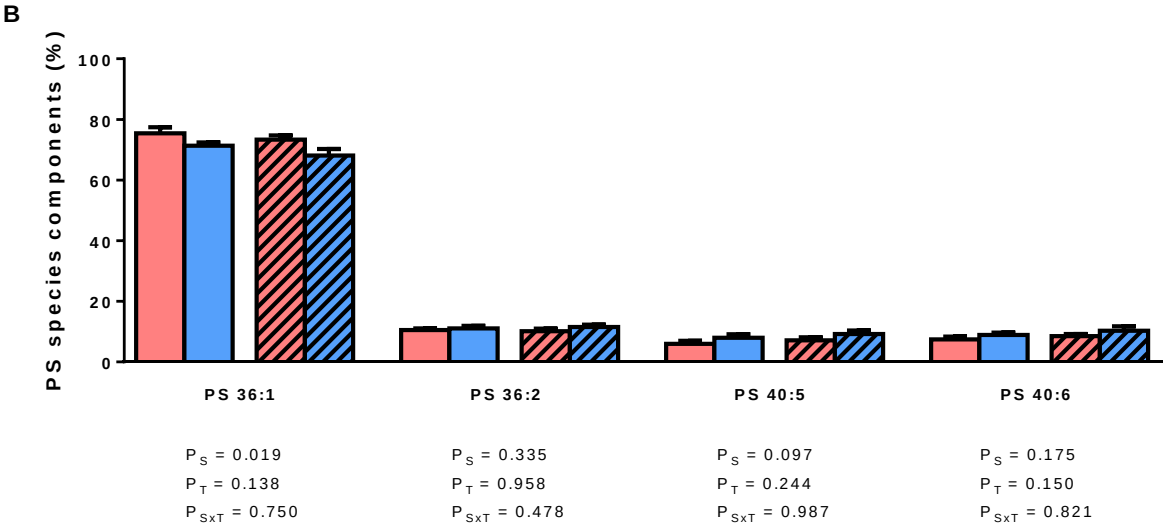
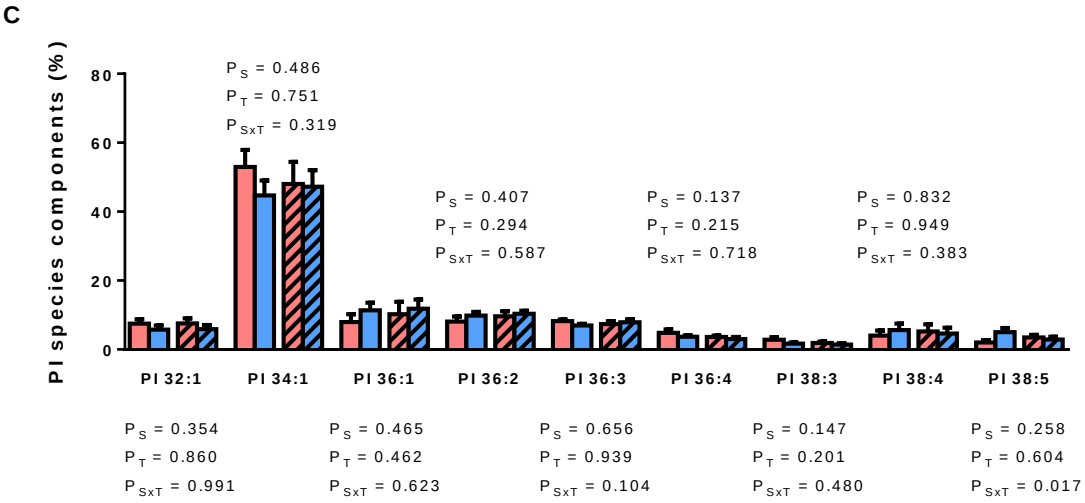

A
LPC species components (%)
LPC 14:0
LPC 16:0
LPC 16:1
LPC 18:0
LPC 18:1
LPC 18:2
P S = 0.867
P T = 0.540
P SxT = 0.086
P S = 0.543
P T = 0.202
P SxT = 0.153
P S = 0.428
P T = 0.211
P SxT = 0.297
P S = 0.336
P T = 0.033
P SxT = 0.467
P S = 0.472
P T = 0.068
P SxT = 0.233
P S = 0.933
P T = 0.514
P SxT = 0.727
B
PC species components (%)
PC 30:0
PC 32:0
PC 32:1+1
PC 34:1+1
PC 34:2+1
PC 36:1+1
PC 36:3
PC 38:2+1
PC 38:3
PC 38:4
P S = 0.665
P T = 0.797
P SxT = 0.566
P S = 0.336
P T = 0.033
P SxT = 0.467
P S = 0.549
P T = 0.809
P SxT = 0.975
P S = 0.400
P T = 0.238
P SxT = 0.240
P S = 0.783
P T = 0.786
P SxT = 0.373
P S = 0.763
P T = 0.981
P SxT = 0.913
P S = 0.502
P T = 0.856
P SxT = 0.549
P S = 0.741
P T = 0.805
P SxT = 0.927
P S = 0.850
P T = 0.969
P SxT = 0.594
P S = 0.776
P T = 0.763
P SxT = 0.689
C
PE species components (%)
PE 32:1
PE 35:1
PE 40:5
PE (16:0/18:1)
PE (16:0/18:2)
PE (16:0/20:4)
PE (16:0/22:5)
PE (16:0/22:6)
PE (18:0/18:1)
PE (18:0/20:4)
PE (18:0/22:6)
PE (18:1/18:1)
PE (18:1/18:2)
P S = 0.450
P T = 0.221
P SxT = 0.683
P S = 0.541
P T = 0.657
P SxT = 0.215
P S = 0.634
P T = 0.068
P SxT = 0.204
P S = 0.249
P T = 0.226
P SxT = 0.412
P S = 0.288
P T = 0.294
P SxT = 0.245
P S = 0.690
P T = 0.521
P SxT = 0.691
P S = 0.791
P T = 0.766
P SxT = 0.047
P S = 0.212
P T = 0.211
P SxT = 0.357
P S = 0.371
P T = 0.639
P SxT = 0.949
P S = 0.470
P T = 0.952
P SxT = 0.823
P S = 0.941
P T = 0.065
P SxT = 0.496
P S = 0.272
P T = 0.489
P SxT = 0.756
P S = 0.285
P T = 0.621
P SxT = 0.517

Figure 3.9 Proportions of the main phospholipid molecular species PG (A), PS (B) and PI (C) in fetal lung liquid at 131 and 133 days of gestation.

Data from females (n=9) and males (n=9) are represented in pink and blue bars respectively. Unhatched bars represent samples collected at 131 days of gestation (d GA) and hatched bars represent samples collected at 133 days of gestation (d GA). Data are shown as mean ± SEM, and were analysed using one-way repeated measures ANOVA, with sex as one factor (P_S) and time (P_T) as the repeated measure. P_{SxT} is the interaction between time and sex.


A
PG species components (%)
100
80
60
40
20
0
PG 16:0/18:1
P S = 0.787
P T = 0.193
P SxT = 0.674


B
PS species components (%)
100
80
60
40
20
0
PS 36:1
PS 36:2
PS 40:5
PS 40:6
P S = 0.019
P T = 0.138
P SxT = 0.750
P S = 0.335
P T = 0.958
P SxT = 0.478
P S = 0.097
P T = 0.244
P SxT = 0.987
P S = 0.175
P T = 0.150
P SxT = 0.821


C
PI species components (%)
80
60
40
20
0
P S = 0.486
P T = 0.751
P SxT = 0.319
P S = 0.407
P T = 0.294
P SxT = 0.587
P S = 0.137
P T = 0.215
P SxT = 0.718
P S = 0.832
P T = 0.949
P SxT = 0.383
PI 32:1
PI 34:1
PI 36:1
PI 36:2
PI 36:3
PI 36:4
PI 38:3
PI 38:4
PI 38:5
P S = 0.354
P T = 0.860
P SxT = 0.991
P S = 0.465
P T = 0.462
P SxT = 0.623
P S = 0.656
P T = 0.939
P SxT = 0.104
P S = 0.147
P T = 0.201
P SxT = 0.480
P S = 0.258
P T = 0.604
P SxT = 0.017

3.4 Discussion

The rationale underpinning this study was that, following preterm birth, male infants are known to have worse respiratory outcomes and survival in comparison to females born at the same gestational age (Khoury et al., 1985, Anadkat et al., 2012). However, our understanding of the basis for the sex difference in respiratory outcome following preterm birth is incomplete. Owing to the lack of knowledge on sex differences in lung structure and on pulmonary surfactant during late gestation, this study aimed to investigate lung architecture, surfactant protein gene expression and surfactant phospholipid composition in male and female fetal sheep to identify differences that could contribute to the male disadvantage following preterm birth.

In contrast to our hypothesis, we found no sex-related differences in lung architecture, surfactant protein gene expression and the majority of lung liquid surfactant phospholipids at 0.9 of term, a stage of development when newborn male lambs have poorer respiratory adaptation than female lambs (De Matteo et al., 2010). Lung liquid from male fetuses had a significantly higher proportion of PE and a lower proportion of PS 36:1 in comparison to females. In both males and females, the proportions of PG and PI increased over time (131–133d GA); over the same 2 days the proportions of species LPC 18:0 and PC 34:1+1 decreased and increased respectively. Furthermore, an interaction effect was demonstrated in the proportions of PI, PE 32:1 and PI 38:5; this was due to a significant reduction in males and a significant increase in females between 131–133d GA. Similarly, an interaction effect was observed in the proportion of PS; a significant increase in males and a significant decrease in females between 131d GA to 133d GA.

3.4.1 Sex and fetal growth

3.4.1.1 Lung and body weights

There were no differences in body or lung weights between male and female fetuses in late gestation. In contrast to the present study, Willet et al. (1997) reported an 8% lower wet lung weight in female lambs at 128d GA compared to males. In the present study, the wet lung weight was measured at necropsy when the lungs may have contained some lung liquid; in contrast the wet lung weight in the study by Willet et al. (1997) was measured following 40 min of ventilation, which could explain the differences in findings; for example the females lambs used by Willet et al. (1997) may have absorbed lung liquid more readily than the males. However, in both the present study and in the study by Willet et al. (1997), when wet lung weight was related to fetal body weight there were no differences between sexes.

In human fetuses, females, on average, weigh ~2–3% less than age-matched males throughout most of gestation (Gerards et al., 2006). This sex difference in body weight appears to be more significant before the third trimester (26 weeks of gestation) and becomes less obvious closer to term (Gerards et al., 2006). If the same is true for sheep this could explain the lack of difference in body weights that we observed in our fetal sheep as our animal model is representative of moderate preterm birth (0.9 of term); thus they may have passed the stage of gestation at which sex differences in body weight might have been more obvious. Our finding on body growth, however, is consistent with other studies in sheep which did not observe a sex difference in birth weight in lambs born in late gestation or at term (De Matteo et al., 2010, Polglase et al., 2012). In contrast, Willet et al. (1997) reported a 10% lower birth weight in female lambs at 128d GA compared to males, which could account for the sex differences in lung weight observed in their study as lung weight relative to body weight was not significantly different between sexes.

3.4.1.2 Lung Volume

In the present study, we found no sex differences in lung volume measured histologically; a similar observation was reported for lambs delivered at 128d GA and ventilated for 40 min (Willet et al., 1997). In humans, a significant sex difference was found in fetal lung volume from 18 to 34 weeks of gestation measured by three-dimensional ultrasound (Gerards et al., 2006); the mean lung volume of male fetuses was, on average, 4.3% greater than in females. However, this difference was eliminated when values were adjusted for estimated fetal body weight. Another study assessed the lungs of human fetuses by fast spin-echo T2-weighted lung magnetic resonance (MR) imaging at 21 to 38 weeks of gestation; the authors demonstrated a significant sex difference in human fetal lung volume after 35 weeks of gestation; males had a greater lung volume than females (Rypens et al., 2001). A histopathological study of human fetuses observed no sex differences in lung volumes between 19 and 40 weeks of gestation (Langston et al., 1984). A possible cause of the varying results could be the different methods used to measure lung volume. In the present study, as well as the studies by Willet et al. (1997) and Langston et al. (1984), the Cavalieri method was used to assess lung volume in histologically prepared lung tissue whereas Gerards et al. (2006) and Rypens et al. (2001) used ultrasound and MR imaging, respectively, to obtain lung volume while the fetuses were still *in utero*. Although sex differences in lung volume in fetuses and infants are not well-established they have been documented in children and adults (Rypens et al., 2001). Postnatally, boys have larger lungs than girls 6 weeks after birth to 14 years of age (Thurlbeck, 1982) which persists into adulthood (Bellemare et al., 2003). Adult male mice are also found to have larger lung volumes than females (Massaro and Massaro, 2006), probably a result of their greater body dimensions.

3.4.2 Lung morphology

3.4.2.1 Parenchyma

The lungs of male and female fetuses at 0.9 of term appeared to be structurally similar as no differences in tissue and airspace fraction, mean linear intercept (an

approximation of alveolar diameter), alveolar wall thickness, septal crest density and septal thickness were observed. Similar observations were reported in preterm lambs born at 128d GA (Willet et al., 1997); the authors of that study also found no sex differences in tissue fraction, mean linear intercept, alveolar wall thickness and surface area. However, their observations could have been confounded by fetal surgical intervention or the use of mechanical ventilation. Alveolar number and alveolar surface area were not measured in the present study; however a greater number of alveoli and larger alveolar surface area were found in human males from 6 weeks to 14 years old, resulting in a larger alveolar surface area to body mass ratio, but this difference was eliminated when body weight was taken into account (Thurlbeck, 1982).

Elastin and collagen deposition were also analysed in the present study as it has been shown that alveolarisation and probably distal airway development are controlled by the elastin-collagen network (Mercer and Crapo, 1990, Mascaretti et al., 2009). Tropoelastin is a soluble precursor of elastin. In this study, *TPE* gene expression and elastin content in the lung interstitium were similar in males and females. The lack of sex difference in elastin expression and content supports our finding of the lack of a sex difference in septal crest density because elastin appearance and septal crests are closely linked; elastic fibres are typically located at the tips of the crests (Burri, 2006). Lung collagen content, determined from the deposition of the major collagens, type I and III collagen fibres (Kaarteenaho-Wiik et al., 2004), was also not different between the sexes. In sheep, collagen and elastin volume in the lung parenchyma increases during fetal development, with significant increases between 121 and 135d GA (Willet et al., 1999). It has also been shown that infants with RDS have increased precursor proteins and mRNAs of both type I and III collagen fibres in the alveolar walls (Kaarteenaho-Wiik et al., 2004).The rate of collagen synthesis in lung development in monkeys, however, declines over gestation and is found to be lower at term than at an earlier gestation; this may be a species-specific effect (Jackson et al., 1990). At the canalicular stage of lung development in rabbits, pulmonary collagen is less mature, has fewer cross-links and is less tightly packed in comparison to collagen

in the adult lung (Bradley et al., 1974). To my knowledge, no other study has looked at sex differences in the collagen content of the developing lung.

3.4.2.2 Cell proliferation and apoptosis

Cell proliferation and apoptosis were measured to provide an insight into the progress of lung development (i.e. cell turnover) in the lungs of male and female fetuses. A lack of sex differences was observed for both cell proliferation and apoptosis, which provides support for our finding of a similar tissue and air-space fractions in the lung. Proliferation of lung cells increases throughout lung development, peaking at the canalicular stage and decreasing thereafter (Del Riccio et al., 2004). Apoptosis occurs throughout gestation in the rat and human lung (Scavo et al., 1998, Stiles et al., 2001), being more prominent during the pseudoglandular stage than in the saccular stage of lung development (Stiles et al., 2001, Wongtrakool and Roman, 2008). As the lung tissues that were quantified in the present study were at a stage of development equivalent to the late saccular/early alveolar stages, when both proliferating and apoptotic activities would have diminished, this could have prevented observation of any sex differences. As there are approximately 40 types of cells in the lung parenchyma the measurement of any particular cell type was not investigated; thus the cell type(s) that was/were still proliferating or undergoing apoptosis at the stage of gestation could not be determined.

Our inability to detect sex differences in lung architecture indicates that factors other than lung structure, as assessed by light microscopy, are likely to be responsible for the poorer respiratory outcome in males following premature birth. The structure of conducting airways and the pulmonary vasculature was not investigated in this study and it would be beneficial to examine these in future as they may play a role in contributing to the sex differences in preterm respiratory outcome. It may also be beneficial to assess lung ultrastructure by electron microscopy as this may reveal sex differences in the blood-air barrier, alveolar capillaries and alveolar type ll cells for example.

3.4.3 Surfactant composition

3.4.3.1 *Surfactant protein* mRNA expression in fetal lung tissue

Although surfactant proteins make up a minor component of pulmonary surfactant (~10%) they are important in lowering surface tension within the lung and in pulmonary innate immunity. In this study, the gene expression of *surfactant proteins A-D* was not different between fetal males and females; however a difference may have been observed at the protein level, which was not measured in the present study as frozen lung tissue was not available. It is possible that at this gestational age (0.9 of term), pulmonary surfactant protein expression is similar in males and females. Phospholipids constitute the remaining 90% of pulmonary surfactant. Being the major component of pulmonary surfactant, alterations in phospholipid composition could account for the poorer respiratory outcome in males following premature birth as suggested by the lower lung maturity index (e.g. L/S ratio) in males in animal and human studies (Nielsen and Torday, 1981, Torday et al., 1981, Fleisher et al., 1985).

3.4.3.2 Pulmonary surfactant phospholipid composition in fetal lung liquid

Previous studies have analysed the surfactant composition in the amniotic fluid and bronchoalveolar lavage fluid but there is no data from fetal lung liquid, possibly due to difficulty in obtaining this fluid. In this study, we analysed lung liquid as it is expected to provide a more direct source of surfactant than amniotic fluid, as it is less likely to be contaminated by other fetal fluids than amniotic fluid. Amniotic fluid is likely to be less reliable as an indicator of surfactant phospholipid composition as it consists of a variable mixture of mainly fetal urine and lung liquid, and also fetal oro-nasal secretions. Amniotic fluid is commonly used in humans to assess surfactant composition and maturity as it is not possible to obtain fetal lung liquid.

LPC is produced from the breakdown of PC (Agassandian and Mallampalli, 2013, Goss et al., 2013) and has toxic effects on lung function even in small amounts

(Grossmann et al., 1999). LPC, at high levels in the alveolar spaces, may destabilise alveoli at the end of expiration by fluidising the surface film and it can also increase lung vascular permeability, thus contributing to leakage of plasma protein into the alveolar spaces (Holm et al., 1991). We found no sex differences in the proportion of LPC or in the proportion of LPC species in the fetal lung liquid at either 131 or 133d GA. Therefore, LPC concentrations could not explain the male disadvantage in respiratory function in males. To my knowledge, a possible sex difference in the proportion of LPC in pulmonary surfactant has not been investigated previously.

The proportion of total PC and its species in fetal lung liquid were similar in males and females. However, percentages of disaturated lecithin (rich PC content) and saturated PC in human amniotic fluid between 30 and 41 weeks of gestational age were higher in the presence of female fetuses than with male fetuses (Torday et al., 1981, Fleisher et al., 1985). In organ cultures of fetal rabbit lungs, there were sex difference in disaturated PC and total PC, with females producing greater amount than males (Nielsen and Torday, 1981).

Surfactant PC composition has not been shown to be related directly to lung anatomy (Bernhard et al., 2001, Goss et al., 2013). This supports the lack of sex differences in lung morphometry in both stillborn human fetuses and liveborn infants aged from 19 weeks of gestation to 3 weeks of postnatal age; this finding was based on measurement of excised lung volume, average distance between saccular or alveolar walls, number of alveoli per unit area and volume, total alveoli number, surface area, air-space wall thickness and volume proportions of alveolar air, duct air, airspace walls, bronchial and bronchiolar lumen, and non-parenchyma (Langston et al., 1984). Consistent with the findings in humans, the fetal sheep lung in the present study did not show any sex differences in lung morphology.

The functional roles of PC species other than PC 32:0 (or PC 16:0/16:0) are not fully understood; however it is believed that they are required for optimal surfactant function. PC 32:0 has the greatest ability to reduce surface tension at the air-liquid interface to near zero (Bernhard et al., 2001, Agassandian and Mallampalli, 2013, Goss et al., 2013). Increasing levels of saturated PC in the developing lung correlate with increased dynamic lung compliance (Bernhard et al., 2001, Veldhuizen et al., 1998) and have also been suggested to be inversely related to the rate of ventilation (Bernhard et al., 2001). In fetal lung liquid, PC 32:0 appears to be the most abundant PC species. This finding is consistent with findings in pulmonary surfactant of human, rabbit and rat (Veldhuizen et al., 1998, Postle et al., 2001).

PE is present at higher levels in the lung liquid of males compared with females. An interaction effect was seen in PE 32:1, due to decreasing values in males with age and increasing values in females. PE is an important phospholipid that makes up cell and organelle membranes. However, a lack of sex difference in the concentrations of PE in lung was observed in fetal rhesus monkeys between 135 days gestation and term (Perelman et al., 1982). Unlike PC, PE contains little disaturated species (Batenburg, 1992) so PE may not contribute to surface tension lowering properties.

In the present study, the PC/PE ratio was measured as an index of lung maturity as a substitute for the commonly calculated L/S ratio (Banerjee and Bellare, 2001). Due to technical limitations, sphingomyelin could not be analysed in the present study. PC is also referred to as lecithin because of its large PC content. Sphingomyelin is a type of cell membrane lipid, as is PE, and therefore PE has been used as an approximation for sphingomyelin. Previous studies have shown elevated saturated PC/S and PC/S ratios in lung lavage and amniotic fluid of fetal rabbits from 24–30d GA; term is 31d GA) (Nielsen and Torday, 1981, Torday and Nielsen, 1981). Similarly, in human studies the L/S ratio is greater in the amniotic fluid of females than in males (Torday et al., 1981, Fleisher et al., 1985). In

contrast to these findings in human amniotic fluid, no difference was observed in the PC/PE ratio in the fetal lung liquid. The observed similar ratio can be explained by the lack of sex differences in the proportions of PC and PE.

An increase in PG was observed in both male and female lung liquid from 131 to 133d GA. In amniotic fluid, the appearance of PG is an indicator of fetal lung maturity. The absence or low levels of PG after birth have been suggested to increase the risk of RDS, as PG has a role in adsorption and spreading of surfactant over alveolar epithelial surface (Agassandian and Mallampalli, 2013). In the present study, we found no sex difference in the proportion of PG and its major species, PG (16:0/18:1). Similarly, a lack of sex difference in the concentrations of PG was observed in fetal rhesus monkeys between 135 days gestation and term (Perelman et al., 1982). A possible reason for the lack of sex difference observed by us could be the gestational age at which lung liquid was analysed. A sex difference may have been observed at a later gestational age as it is known that PG appears in significant amounts late in gestation. In humans, PG first appears in amniotic fluid at 35 weeks of gestation with an increased rate of increase in females than males over late gestation (Leung-Pineda and Gronowski, 2010). In the present study, however, the gestational age when samples were analysed (0.9 of term in sheep) was equivalent to ~32–34 weeks of gestation in humans, in terms of lung maturity. The gestational age chosen in the present study was selected because of the disparity in survival outcome in preterm lambs, where females had a higher chance of survival (De Matteo et al., 2010).

In our fetal sheep, the proportion of PI at 133d GA was higher than at 131d GA. This age-related increase in PI level is consistent with the increase between 30 and 35 weeks of gestation in humans, which declines thereafter (Fleisher et al., 1985). As previously mentioned, the lung liquid samples analysed in this study were collected at a stage of lung maturity equivalent to ~32–34 weeks of human gestation; this coincides with the period when PI level is increasing. An interaction effect was observed in the proportion of PI 38:5; a reduction in values was

observed in males and an increase in females. A previous study using human amniotic fluid has shown a decrease in PI level following 36 weeks of gestation and females demonstrated a significantly lower drop compared to males (Fleisher et al., 1985). It can be speculated that the differences seen in humans could be seen in the lambs at a later gestation when the proportion of PI is declining.

Little is known about the role of PS in pulmonary surfactant. In the present study, an interaction was observed between males and females, with males showing an increase and females showing a decrease. A further analysis of the PS species indicated a significantly greater proportion of PS 36:1 in females compared to males. We speculate that PS 36:1 could be a crucial component of pulmonary surfactant with an important role in lung function. As the proportion of PS 36:1 was elevated in the females, it may be beneficial in improving respiratory outcome in females.

Overall, the minor differences observed in the phospholipid composition in fetal lung liquid are unlikely to explain the male disadvantage in respiratory outcome. This could be due to the gestational ages of the samples that were analysed. A greater difference may have been observed at a later gestation. However, at 4 h following preterm birth, male lambs from which the lung liquid samples were collected were found to have lower static lung compliance than the preterm females (Chapter 4). Overall we did not find any differences in the major phospholipid classes or the PC/PE ratio, but did show minor differences in some of the phospholipid species. We consider that these minor changes in phospholipid species are unlikely to be sufficient to account for the observed respiratory insufficiency in the preterm male as reported in Chapter 4. The differences may become apparent after birth.

In our study of surfactant phospholipids, a small dose of betamethasone (5.7 mg) was administered intramuscularly via the ewe at 131d GA (Cohort 2) to increase

the chance of survival and to reduce the need for respiratory support in the lambs following preterm delivery. The time effect observed between 131 and 133d GA could therefore be a treatment effect resulting from the corticosteroid administration. Previous studies have shown that betamethasone stimulates maturation of surfactant production in infants (Ballard and Ballard, 1996, Roberge et al., 2011) and improve lung compliance in fetal sheep, particularly in females (Willet et al., 1997). However, it is not known if the small dose of betamethasone in this study is effective in inducing lung maturation or contribute to different maturation progress between males and females.

3.5 Conclusions

We conclude that there appears to be few sex differences in the fetal lung at 131d GA in terms of lung architecture, lung morphology or relative gene expression of *surfactant proteins* and *tropoelastin.* It is not known to what extent the minor differences observed in phospholipid species composition in fetal lung liquid at 131d GA and 133d GA may affect surfactant function. The difference in respiratory outcome between males and females could be due to other factors, such as sex differences in respiratory drive, clearance of lung liquid or cardiorespiratory adaptation following preterm birth. Little is known about physiological differences between males and females during gestation and in the early hours of life following preterm birth. In the next chapter, the possibility of differences in prenatal and postnatal physiology such as respiratory and cardiovascular performance will be investigated.

Chapter 4: Fetal physiological status and adaptation to preterm birth in male and female lambs: four hour postnatal study

4.1 Introduction

It is well established that males are at a greater risk of developing respiratory problems following preterm birth in comparison to their female counterparts. This consequently leads to a higher mortality rate in male preterm neonates. The exact mechanisms that are responsible for the sex difference in respiratory outcome following preterm birth are unknown but could be multi-factorial including differences in lung development (i.e. lung structure) and/or the composition of pulmonary surfactant.

A recent study has demonstrated an absence of sex differences in cardiopulmonary and systemic haemodynamics (carotid arterial pressure pulmonary arterial pressure, pulmonary blood flow and pulmonary vascular resistance) and umbilical arterial blood gas parameters (pH, glucose, lactate, haemoglobin and haematocrit) in lambs within 30 minutes (min) of preterm delivery (Polglase et al., 2012). In addition, an absence of sex differences in ventilation parameters (peak inspiratory pressure, mean airway pressure, minute ventilation, dynamic specific respiratory compliance and ventilator efficiency index) was seen in these lambs (Polglase et al., 2012). However, the use of anaesthesia and mechanical ventilation in the study could have confounded the findings.

The rationale for the study reported in this Chapter was to better understand the poorer respiratory outcomes in males following preterm birth by studying lambs born before term, in the absence of anaesthesia. Physiological analysis during prenatal life and physiological adaptation following preterm birth were investigated in this study in addition to analysing lung structure. It was hypothesised that the poorer respiratory outcome and increased mortality in preterm male lambs was due to poorer cardiorespiratory adaptation following preterm birth in comparison to female lambs. It was also hypothesised that the composition of pulmonary surfactant composition would be altered in male lambs, as previously described in other species (Nielsen and Torday, 1981, Torday et al., 1981, Fleisher et al., 1985) where sex differences in pulmonary surfactant were observed in both the amniotic fluid and BALF.

In order to determine whether sex differences in physiological status exist before preterm birth, the first aim of this study was to investigate possible sex differences in blood gas parameters, arterial pressure and circulating cortisol concentration, and in surfactant phospholipid composition in the amniotic fluid.

The second aim of this study was to identify differences in the physiological adaptation between male and female lambs in the 4 hours (h) following preterm delivery at 0.9 of term, in the absence of anaesthesia and mechanical ventilation. Blood gas parameters, arterial pressure, breathing parameters and lung compliance were also investigated.

In addition, this study was aimed at identifying sex differences in selected biochemical parameters relating to pulmonary surfactant. We assessed surfactant protein gene and protein expression in lung tissue. The composition of surfactant phospholipids and total protein content in the bronchoalveolar lavage fluid were also analysed. Morphometric analysis of the lung was also performed to determine whether sex differences were identifiable after 4 h of lung aeration.

4.2 Methods

4.2.1 Experimental protocol

This study was performed to assess and compare the physiological adaptation of male and female lambs delivered before term and studied for 4 h after birth. Surgery was performed on 21 pregnant ewes and their fetuses (Border-Leicester X Merino) at ~125 days of gestational age (d GA; term is ~147d GA) as described in Section 2.4.

Following fetal surgery, fetal physiological status was monitored daily by measuring fetal arterial pH, PaO_2, $PaCO_2$, SaO_2, lactate and glucose. Fetal arterial pressure was monitored as outlined in Section 2.4.3.1 for one hour prior to betamethasone administration on 131d GA and also on 132d GA. A small dose of betamethasone was administered intramuscularly to the ewe at 131d GA to increase the viability of the lambs following preterm delivery. Fetal blood and amniotic fluid samples were collected daily from 130–133d GA, as outlined in Section 2.4.3.1, for the analysis of cortisol concentration and surfactant phospholipid content respectively.

Following caesarean delivery at 133d GA, as described in Section 2.4.3.2, lambs were monitored for 4 h in the absence of anaesthesia and mechanical ventilation. Blood samples were collected at 5 min after birth and every 15 min thereafter to measure arterial blood gases (pH, PaO_2, $PaCO_2$, SaO_2, lactate and glucose). In addition, the mean arterial pressure (MAP), heart rate (HR), inspiratory effort and breathing frequency of the lambs were also recorded. During the 4 h of monitoring, lambs were administered supplemental oxygen (5 to 10 L/min of 100% O_2) via a face mask and self-inflating resuscitation bag when required (i.e. SaO_2 less than 80%). Saline and glucose solutions were administered to maintain blood volume and whole blood glucose concentration following birth as mentioned in Section 2.4.3.3. Rectal temperature was continuously measured and we attempted to maintain rectal temperature at 39ºC.

At the end of the 4 h of monitoring, the lambs were lightly sedated with 1 ml sodium thiopentone (50 mg/ml i.v.; Pentothal, Boehringer Ingelheim Pty Ltd, Australia), intubated and connected to a ventilator. As described in Section 2.4.3.4, the lambs were then euthanised and their thoracic cavity exposed for the measurement of static lung compliance.

Following measurement of static lung compliance, the body weight, body dimensions, and major organ weights of the lambs were measured. The lungs were removed from the thoracic cavity; the left lung was ligated at the hilus, surgically separated from the right side of the lung and sectioned into small pieces (2–5 mm^3). These pieces were snap-frozen in liquid N_2 for storage at -80ºC for later measurement of water content of lung tissue, and surfactant protein gene and protein expression of surfactant proteins. Bronchoalveolar lavage fluid (BALF) was collected from the upper right lung lobe as outlined in Section 2.4.4.1 to allow measurements of total protein and surfactant phospholipid composition. The right lung was then fixed via the trachea at a distending pressure of 30 cmH_2O with 4% paraformaldehyde (in 0.1 M phosphate buffered saline, pH 7.4) and immersed in fresh 4% paraformaldehyde fixative for 24 h at 4ºC for future histological analysis.

4.2.2 Analytical methods

Analysis of the surfactant phospholipid composition in the amniotic fluid samples was measured as outlined in Section 2.10. Blood samples that were collected were used to measure cortisol concentration in plasma, as described in Section 2.12. Investigation of sex-differences in prenatal physiological status was achieved by measuring arterial blood gas parameters (pH, PaO_2, $PaCO_2$, SaO_2, lactate and glucose), MAP and HR in fetuses while still *in utero* (see Section 2.4.3.1); sex differences in postnatal adaptation were assessed by making these measurements in the same lambs for 4 h following delivery via caesarean section (see Section 2.4.3.3). Additionally, we monitored inspiratory effort and breathing frequency in the postnatal lambs as detailed in Section 2.4.3.3. The duration in which the postnatal lambs required supplemental oxygen was also noted.

At the end of the 4 h monitoring period, the lambs were euthanised and their thoracic cavity exposed for the measurement of static lung compliance as described in Section 2.4.3.4.

Following euthanasia, the organs were weighed. Left lung tissue that was snap frozen in liquid N_2 at post-mortem was used for measuring water content in lung tissue (see Section 2.7), the expression of surfactant protein gene using the midi-kit (see Section 2.8) and protein expression of surfactant proteins was measured using Western blot analysis (see Section 2.9). For each gene and protein of interest, the mRNA levels and protein levels, respectively, obtained for both male and female samples were separately averaged. The mean mRNA and protein levels for the males were expressed relative to the mean mRNA and protein levels in females for each gene and protein of interest. The fixed right lung was used to determine tissue and luminal space volumes, mean linear intercept, alveolar wall thickness, collagen and elastin content and septal crest density using the techniques described in Section 2.6.

BALF samples were collected at post-mortem as outlined in Section 2.4.4.1 and analysed for surfactant phospholipid composition and total protein content, as described in Sections 2.10 and 2.11 respectively.

4.2.3 Statistical analysis of data

Sex differences in body weights and dimensions, organ weights, gene and protein expression, total protein content in BALF, static lung compliance, histological assessment and surfactant phospholipid composition in BALF were assessed using the unpaired Student's t-test. Surfactant phospholipid composition in amniotic fluid at 131 and 133d GA was analysed using one-way repeated measures ANOVA, with sex as one factor and time as the repeated measure. To maintain statistical power, adjustments for multiple comparisons were not performed so as to avoid the high probability of obtaining Type II errors (false

negatives). Blood gas parameters, cortisol concentration, MAP and HR during fetal life and in lambs after preterm delivery throughout the 4 h monitoring were analysed using a one-way repeated measures ANOVA. In addition, the inspiratory effort and respiratory rate in the lambs were analysed using ANOVA. Data were analysed over four different time periods; 128–133d GA, within 1 h of delivery and 2–4 h after delivery. These 3 different time periods were selected to determine if there were any sex differences in the parameters measured during gestation and following preterm delivery during the transition period (up to one hour after birth) and post-transitional period (1–3 h after birth). The data related to the requirement for supplemental oxygen were analysed by a log rank (Mantel-Cox) test.

4.3 Results

4.3.1 Weights and dimensions

At necropsy, body weights and dimensions, and organ weights and relative organ weights were not different between sexes (Tables 4.2 and 4.3). Lung weights (wet and dry) and right lung volume were also not different between sexes (Table 4.4).

4.3.2 Blood gas and cardiovascular measurements

4.3.2.1 Prenatal physiological data

Between 128–133d GA, no overall sex effect was observed in pH (Figure 4.1 A), $PaCO_2$ (Figure 4.1 B), PaO_2 (Figure 4.2 A), SaO_2 (Figure 4.2 B), glucose concentration (Figure 4.3 A), lactate concentration (Figure 4.3 B), MAP (Figure 4.4 A) and HR (Figure 4.4 B). The overall mean of each physiological parameter for each sex is shown in Table 4.1.

Table 4.1 Overall mean ± SEM of physiological parameters in fetal sheep between 128-133d GA.

	Female (n=9)	Male (n=9)	Figure
pH	7.378 ± 0.005	7.358 ± 0.010	4.1 A
$PaCO_2$ (mmHg)	49.5 ± 0.8	50.6 ± 0.8	4.1 B
PaO_2 (mmHg)	19.8 ± 1.1	20.2 ± 1.5	4.2 A
SaO_2 (%)	57.3 ± 3.1	56.2 ± 4.7	4.2 B
Glucose concentration (mmol/L)	1.00 ± 0.05	0.97 ± 0.05	4.3 A
Lactate concentration (mmol/L)	1.91 ± 0.13	1.80 ± 0.11	4.3 B
MAP (mmHg)	44.5 ± 0.5	43.9 ± 0.2	4.4 A
HR (beats/min)	162.7 ± 7.1	161.2 ± 4.1	4.4 B

Statistical analysis of prenatal data between 128-133d GA showed no significant differences between sexes and no interactions between sexes across days of gestation, thus the physiological data were pooled to obtain average values for each sex. Data are represented as mean ± SEM.

The concentration of plasma cortisol was similar in female and male fetuses at 131d GA (F: 10.8 ± 3.4 ng.ml vs M: 13.1 ± 4.4 ng/ml) and 133d GA (F: 31.6 ± 5.9 ng/ml vs 19.0 ± 6.1 ng/ml, Figure 4.6). An overall time effect and an interaction effect were seen over the two time-points; in female fetuses, plasma cortisol concentration increased more than three-fold following betamethasone administration (i.e. from 131d GA to 133d GA), while it was not significantly changed in male fetuses (Figure 4.6)."

4.3.2.2 Postnatal physiological data from 0 to 1 h

Within the first hour of delivery, males had significantly lower arterial pH (Figure 4.1 A) and significantly higher $PaCO_2$ (Figure 4.1 B), glucose concentration (Figure 4.3 A), lactate concentration (Figure 4.3 B) and MAP (Figure 4.4 A) in comparison to females. No sex difference was observed in PaO_2 (Figure 4.2 A), HR (Figure 4.4 B), breathing frequency (Figure 4.5 A) and inspiratory effort (Figure 4.5 B).

Supplemental oxygen was given to all lambs to achieve a SaO_2 value of at least 80%; which was not significantly different between females and males (Figure 4.2 B). Sex difference in oxygen dependency was assessed by comparing the amount of time male and female lambs required supplemental oxygen during the monitoring period before they were able to maintain a SaO_2 of 80% without assistance using the log rank (Mantel-Cox) test. Males were observed to be significantly more dependent on supplemental oxygen than females in order to reach SaO_2 of 80% (Figure 4.7).

4.3.2.3 Postnatal physiological data from 1 to 4 h

Following the first hour after delivery two male lambs died (at 60 and 90 min) and hence data from these animals were excluded at time points between the first hour and the time of death.

From 75 to 240 min after delivery, males had a tendency (P=0.065) to have lower pH than females (Figure 4.1 A). However, no sex differences were observed in $PaCO_2$ (Figure 4.1 B), PaO_2 (Figure 4.2 A), SaO_2 (Figure 4.2 B), arterial glucose concentration (Figure 4.3 A), lactate concentration (Figure 4.3 B), MAP (Figure 4.4 A), HR (Figure 4.4 B), breathing frequency (Figure 4.5 A) and inspiratory effort (Figure 4.5 B).

4.3.3 Static lung compliance

The lung volume at 40 cmH_2O, relative to body weight, was significantly lower in males than in females (58.3 ± 7.9 vs 90.5 ± 10.3 ml/kg; Figure 4.8).

The pulmonary pressure-volume relationship (unadjusted) is shown in Figure 4.9. Apart from values at 40 cmH_2O, no significant differences in lung volume were observed between males and females at each pressure point on both ascending and descending limbs.

4.3.4 Lung histology

Representative histological images of lung tissue from male and female lambs stained with Haematoxylin and Eosin (magnification of 200X), Gordon and Sweet's reticular fibre stain (magnification of 1000X) and Hart's elastin (magnification of 1000X) are shown in Figure 4.10.

4.3.4.1 Fetal lung tissue and airspace fractions

Similar values were obtained for lung tissue density (tissue fraction) in females (30.9 ± 0.7%) and in males (35.8 ± 2.2%; *P* = 0.06; Figure 4.11 A). Likewise, the percentage of airspace (airspace fraction) was comparable in both females (65.0 ± 1.7%) and males (64.2 ± 2.2%; Figure 4.11 B).

4.3.4.2 Mean linear intercept

The mean linear intercept was not significantly different between females (107.7 ± 2.1 µm) and males (102.6 ± 5.1 µm; Figure 4.11 C).

4.3.4.3 Alveolar wall thickness

The alveolar wall thickness was not significantly different between females (107.7 ± 2.07 µm) and males (102.6 ± 5.1 µm; Figure 4.11 D).

4.3.4.4 Septal crest density

No difference was observed between females (3.2 ± 0.3%) and males (3.0 ± 0.2%) in septal crest density (Figure 4.11 E).

4.3.4.5 Lung elastin content

There was no difference in the elastin content of the lungs of female (6.0 ± 0.5%) and male lambs (5.0 ± 0.3%; Figure 4.11 F).

4.3.4.6 Lung collagen content

There was no difference in pulmonary collagen content between females (19.1 ± 0.6%) and males (20.2 ± 1.2%; Figure 4.11 G).

4.3.5 SP gene and protein expression

Male and female lambs had similar gene expression of *SP-A*, *-B*, *-C* and *-D* in lung tissue: *SP-A* (M: 0.8 ± 0.1 vs F: 1.0 ± 0.2), *SP-B* (M: 0.8 ± 0.1 vs F: 1.0 ± 0.1), *SP-C* (M: 0.7 ± 0.1 vs F: 1.0 ± 0.1) and *SP-D* (M: 0.9 ± 0.1 vs F: 1.0 ± 0.1) (Figure 4.12).

No significant difference was observed in SP-A protein expression between male (0.7 ± 0.1) and female lambs (1.0 ± 0.4; Figure 4.13 A). When protein levels of pro-SP-C (Figure 4.13 B) were measured, values in males were significantly lower by 34%, in males (0.7 ± 0.1) than in females (1.0 ± 0.1).

4.3.6 Phospholipid content of fetal amniotic fluid and bronchoalveolar lavage fluid (BALF)

4.3.6.1 Phospholipid classes and total protein content of BALF

Amniotic fluid: When the proportions of phospholipid classes in the amniotic fluid, relative to total phospholipids, were analysed, no overall sex, overall time (over two different gestational ages) or interaction effects were observed in the proportions of PE, PI and PG; Figures 4.14 B, 4.14 C and 4.14 E, respectively). An overall time effect was observed in the proportions of PC, such that greater proportions were observed in males and females combined at 133d GA compared to 131d GA (Figure 4.14 A). An overall time effect was also observed in the proportions of PS, such that the proportions obtained in males and females combined at 131d GA were greater compared to 133d GA (Figure 4.14 D).

Bronchoalveolar lavage fluid: The proportion of PC was significantly lower in the BALF of males (68.0 ± 1.5%) than in females (72.6 ± 1.0%; Figure 4.14 A). The proportion of PE was significantly higher in the BALF of males (6.9 ± 0.6%) than in females (5.1 ± 0.4%; Figure 4.14 B). No significant sex differences were observed in the proportions of PI (M: 21.7 ± 1.3 vs F: 19.9 ± 1.1%), PS (1.9 ± 0.4 vs 1.2 ± 0.3%) and PG (1.5 ± 0.2 vs 1.3 ± 0.1%; Figure 4.14 C, D and E, respectively).

The total protein concentration in BALF collected at necropsy was significantly higher in males (3.04 ± 0.50 mg/ml) than in females (1.84 ± 0.25 mg/ml; Figure 4.17).

4.3.6.2 Phospholipid maturity ratios

In the amniotic fluid samples, there was no overall sex, overall time or interaction effect observed in the ratio of PC/PE at 131d GA (M: 3.5 ± 0.3 vs F: 3.1 ± 0.2) and 133d GA M: 3.7 ± 0.4 vs F: 3.8 ± 0.9; Figure 4.15). In the BALF collected at 4 h after delivery however, the ratio of PC/PE in males (11.0 ± 0.9) was significantly lower than in females (15.0 ± 1.1; Figure 4.15).

4.3.6.3 Phospholipid species

There was no overall sex effect in the phospholipid species within each phospholipid class (PC, PE, PI, PS and PG) of amniotic fluid. An overall time effect was observed in the PC 36:1, PC 38:2, PI 34:0, PI 36:1, PS 36:1, PE 18:0/18:1 and PE 16:0/22:6 species. An interaction effect was observed in the PC 33:1, PS 40:6 and PE 18:0/20:3 species. The mean values (± SEM) of these species for each sex at each time point are shown in Appendix (Table A1 – A5). In BALF, the relative proportions of the phospholipid classes (PE, PI, PS and PG) were not different between males and females at 4 h after preterm delivery (Figure 4.16 B, C, D and E respectively). However, males had a significantly lower proportion of PC 32:0 and higher proportions of PC 34:2 and PC 36:2 than females (Figure 4.16 A).

Table 4.2 Body weight and dimensions in female and male lambs

	Female (n=9)	Male (n=10)	P value
Dry body weight (kg)	3.15 ± 0.20	3.39 ± 0.17	0.359
Crown rump length (cm)	49.3 ± 1.4	48.7 ± 0.8	0.709
Thoracic girth (cm)	32.8 ± 1.1	34.1 ± 0.7	0.319
Hind leg length (cm)	42.4 ± 1.1	42.9 ± 0.7	0.687
Head length (cm)	12.7 ± 0.2	12.8 ± 0.1	0.552
Ponderal index (g/cm^3 x 100)	2.7 ± 0.1	2.9 ± 0.1	0.225

Data represent group mean ± SEM values.

Table 4.3 Necropsy organ weights and relative organ weights in female and male lambs

	Female (n=9)	Male (n=10)	P value
Heart (g)	23.49 ± 1.58	25.74 ± 0.95	0.225
Heart/Body weight (BW) (g/kg)	7.17 ± 0.16	7.65 ± 0.19	0.075
Liver (g)	102.30 ± 3.57	105.40 ± 6.39	0.683
Liver/BW (g/kg)	32.86 ± 1.06	31.08 ± 1.16	0.284
Kidney (g)	67.46 ± 1.78	68.25 ± 3.58	0.858
Kidney/BW (g/kg)	21.88 ± 1.01	20.22 ± 0.77	0.202
Adrenal (g)	0.23 ± 0.02	0.23 ± 0.02	0.980
Adrenal/BW (g/kg)	0.08 ± 0.01	0.07 ± 0.01	0.394
Spleen (g)	5.71 ± 0.48	5.91 ± 0.61	0.805
Spleen/BW (g/kg)	1.79 ± 0.12	1.73 ± 0.13	0.727

Data represent group mean ± SEM values.

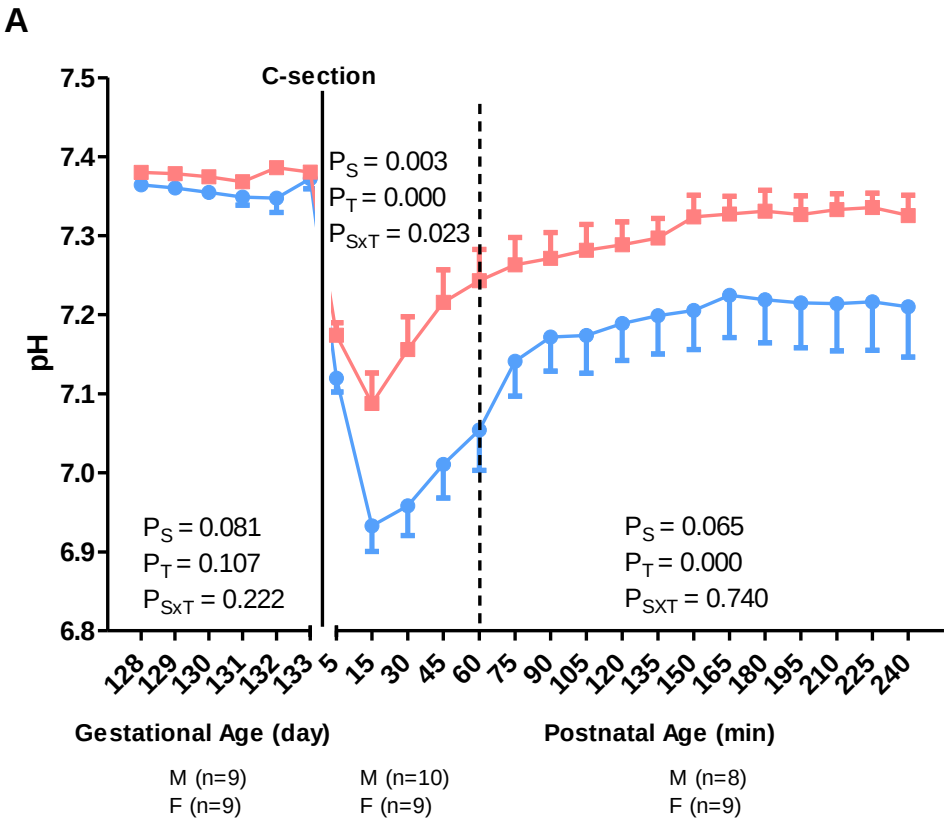
Table 4.4 Lung weights and relative weights in female and male lambs

	Female (n=9)	Male (n=10)	P value
Wet lung weight (g)	106.6 ± 11.3	116.5 ± 7.2	0.455
Wet lung weight/BW (g/kg)	31.6 ± 2.5	34.4 ± 1.6	0.331
Dry lung weight (g)	14.3 ± 1.6	15.6 ± 1.1	0.487
Dry lung weight/BW (g/kg)	4.4 ± 0.4	4.6 ± 0.2	0.471
Lung volume (cm^3)	101.9 ± 7.5	101.5 ± 5.1	0.971

Data represent group mean ± SEM values.

Figure 4.1 Arterial pH and $PaCO_2$ before and after preterm birth.

Values for arterial pH (A) and arterial partial pressure of CO_2 ($PaCO_2$) (B) on gestational days 128–133 and for up to 240 min following caesarean-section at 133 days of gestational age in female (pink) and male (blue) fetuses and postnatal preterm lambs. Separate one-way repeated ANOVAs were performed 1) before delivery, 2) between 0 and 60 min after delivery, and 3) between 75 and 240 min after delivery. P values: P_S is between factor variable (sex), P_T is repeated variable (time), and P_{SxT} is the interaction term. Data are shown as means ± SEM.


A
C-section
pH
7.5
7.4
7.3
7.2
7.1
7.0
6.9
6.8
P_S = 0.003
P_T = 0.000
P_{SxT} = 0.023
P_S = 0.081
P_T = 0.107
P_{SxT} = 0.222
P_S = 0.065
P_T = 0.000
P_{SXT} = 0.740
128 129 130 131 132 133 5 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240
Gestational Age (day)
Postnatal Age (min)
M (n=9)
F (n=9)
M (n=10)
F (n=9)
M (n=8)
F (n=9)

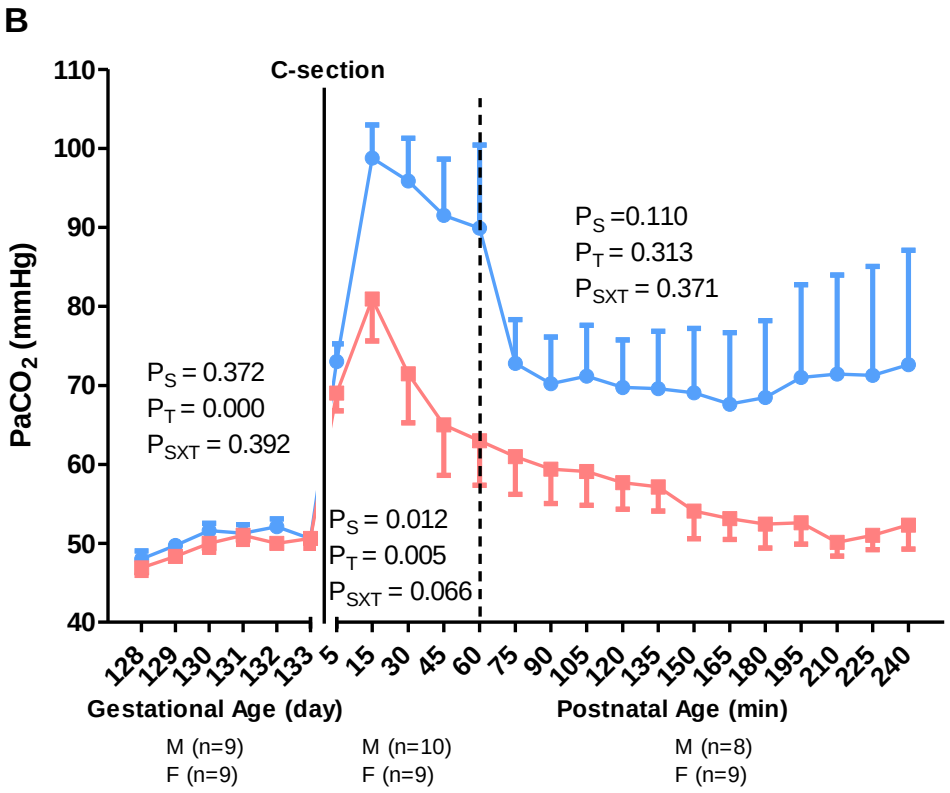
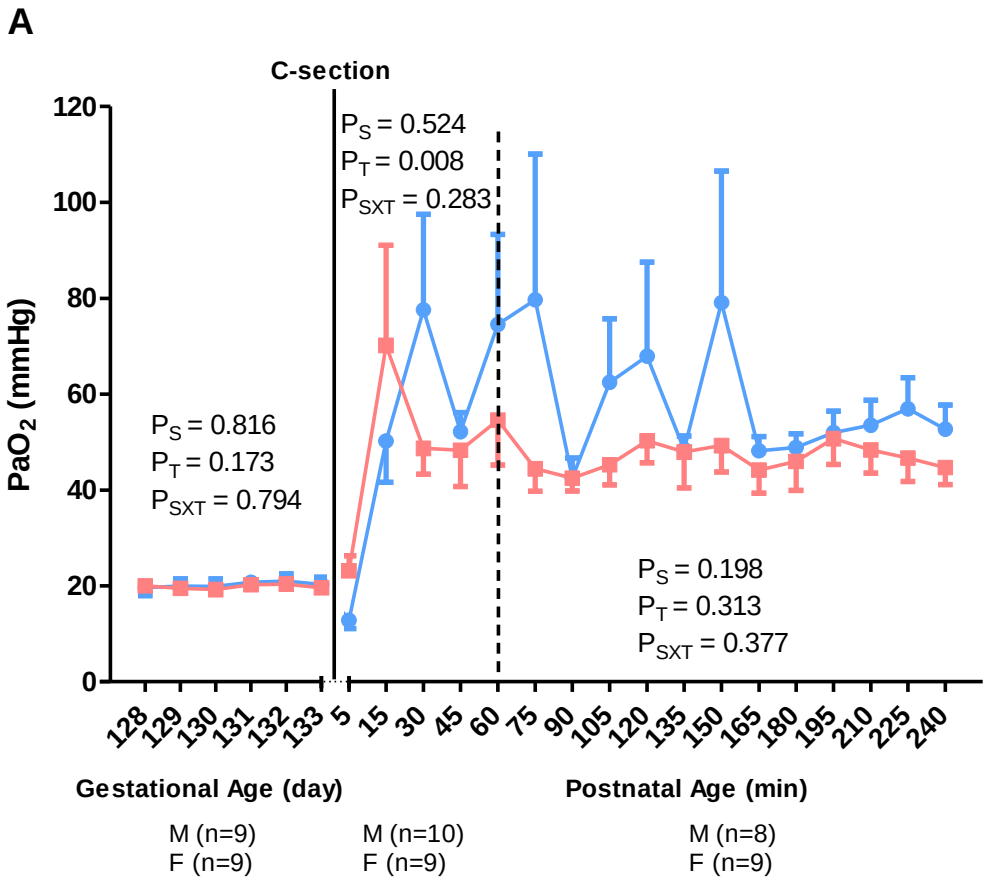

B
C-section
$PaCO_2$ (mmHg)
110
100
90
80
70
60
50
40
P_S =0.110
P_T = 0.313
P_{SXT} = 0.371
P_S = 0.372
P_T = 0.000
P_{SXT} = 0.392
P_S = 0.012
P_T = 0.005
P_{SXT} = 0.066
128 129 130 131 132 133 5 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240
Gestational Age (day)
Postnatal Age (min)
M (n=9)
F (n=9)
M (n=10)
F (n=9)
M (n=8)
F (n=9)

Figure 4.2 Arterial PaO_2 and SaO_2 before and after preterm birth.

Values for arterial partial pressure of O_2 (PaO_2) (A) and arterial saturation of O_2 (SaO_2) (B) on gestational days 128–133 and for up to 240 min following caesarean-section at 133 days of gestational age in female (pink) and male (blue) fetuses and postnatal lambs. Separate one-way repeated ANOVAs were performed 1) before delivery, 2) between 0 and 60 min after delivery, and 3) between 75 and 240 min after delivery. P values: P_S is between factor variable (sex), P_T is repeated variable (time), and P_{SxT} is the interaction term. Data are shown as means ± SEM.


A
C-section
P_S = 0.524
P_T = 0.008
P_{SXT} = 0.283
P_S = 0.816
P_T = 0.173
P_{SXT} = 0.794
P_S = 0.198
P_T = 0.313
P_{SXT} = 0.377
PaO_2 (mmHg)
0
20
40
60
80
100
120
128 129 130 131 132 133 5 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240
Gestational Age (day)
Postnatal Age (min)
M (n=9)
F (n=9)
M (n=10)
F (n=9)
M (n=8)
F (n=9)

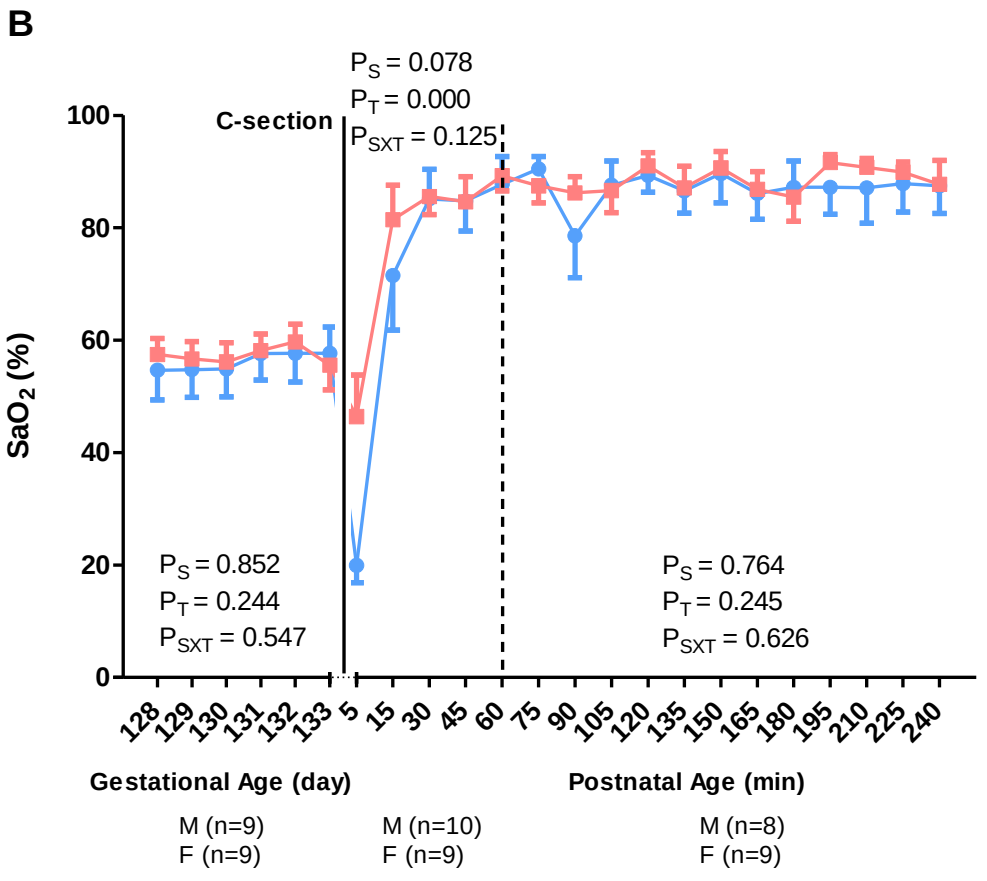
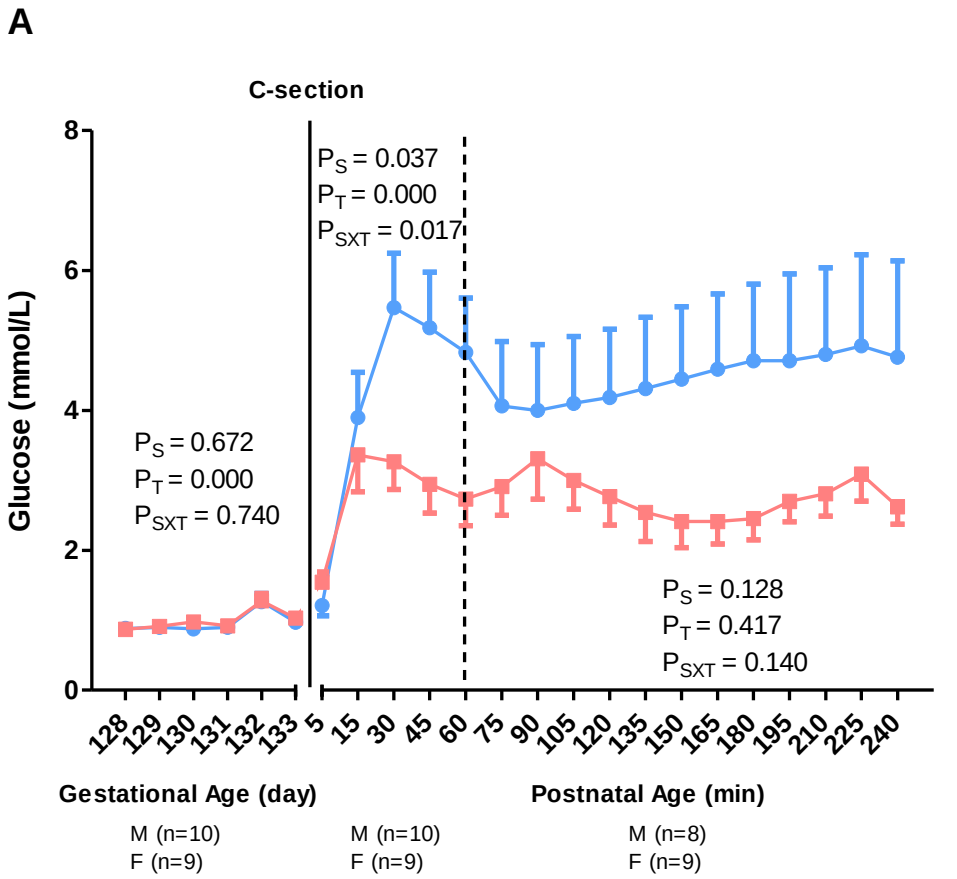

B
P_S = 0.078
P_T = 0.000
P_{SXT} = 0.125
C-section
P_S = 0.852
P_T = 0.244
P_{SXT} = 0.547
P_S = 0.764
P_T = 0.245
P_{SXT} = 0.626
SaO_2 (%)
0
20
40
60
80
100
128 129 130 131 132 133 5 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240
Gestational Age (day)
Postnatal Age (min)
M (n=9)
F (n=9)
M (n=10)
F (n=9)
M (n=8)
F (n=9)

Figure 4.3 Blood glucose and lactate concentrations before and after preterm birth.

Values for arterial concentrations of glucose (A) and lactate (B) on gestational days 128–133 and for up to 240 min following caesarean-section at 133 days of gestational age in female (pink) and male (blue) fetuses and lambs. Separate one-way repeated ANOVAs were performed 1) before delivery, 2) between 0 and 60 min after delivery, and 3) between 75 and 240 min after delivery. P values: P_S is between factor variable (sex), P_T is repeated variable (time), and P_{SxT} is the interaction term. Data are shown as means ± SEM.


A
C-section
Glucose (mmol/L)
0
2
4
6
8
P_S = 0.037
P_T = 0.000
P_{SXT} = 0.017
P_S = 0.672
P_T = 0.000
P_{SXT} = 0.740
P_S = 0.128
P_T = 0.417
P_{SXT} = 0.140
128 129 130 131 132 133 5 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240
Gestational Age (day)
Postnatal Age (min)
M (n=10)
F (n=9)
M (n=10)
F (n=9)
M (n=8)
F (n=9)

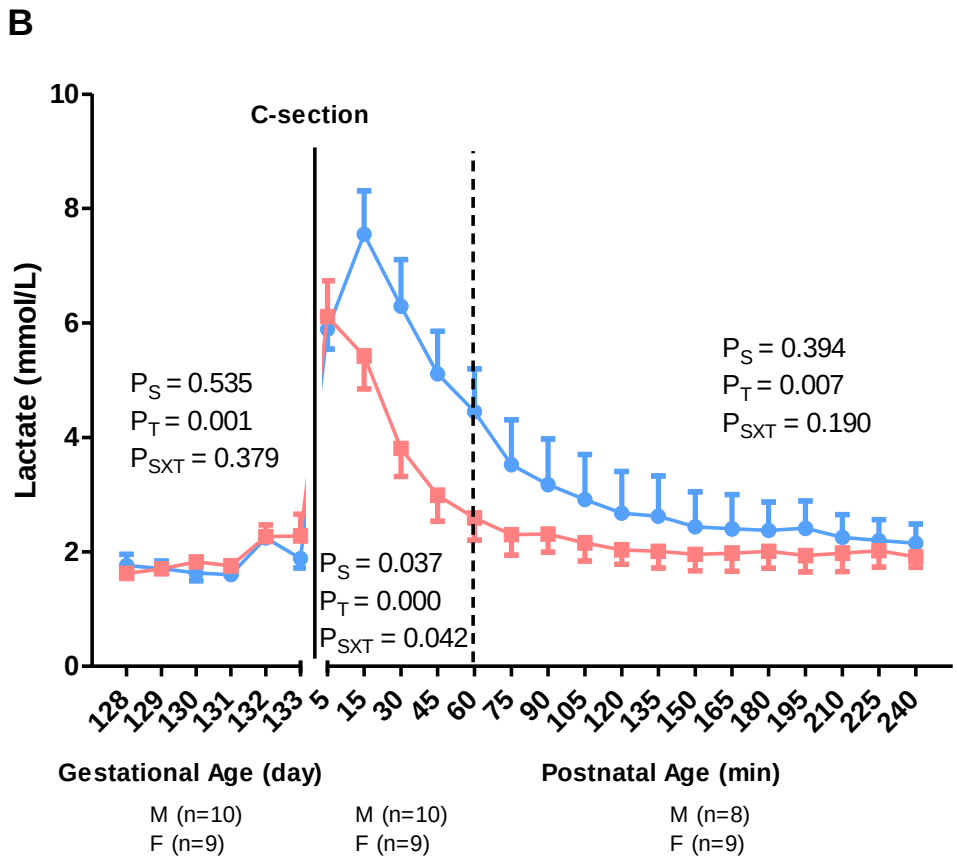
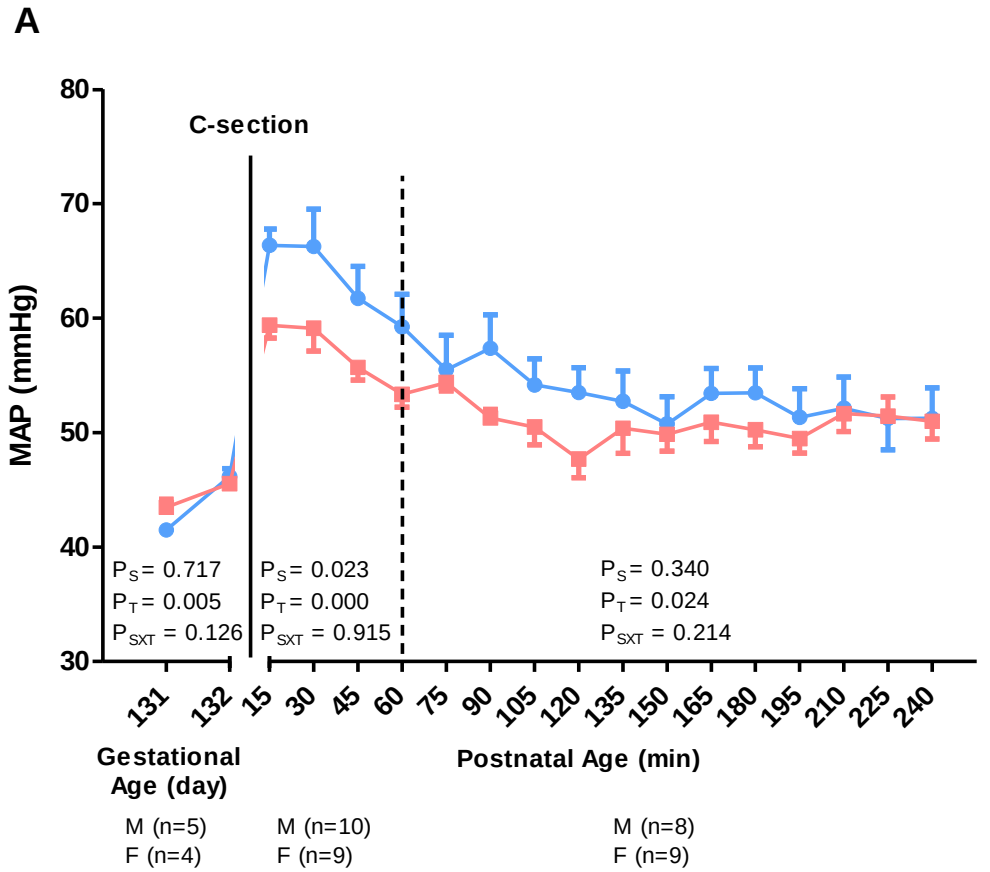

B
C-section
Lactate (mmol/L)
0
2
4
6
8
10
P_S = 0.535
P_T = 0.001
P_{SXT} = 0.379
P_S = 0.037
P_T = 0.000
P_{SXT} = 0.042
P_S = 0.394
P_T = 0.007
P_{SXT} = 0.190
128 129 130 131 132 133 5 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240
Gestational Age (day)
Postnatal Age (min)
M (n=10)
F (n=9)
M (n=10)
F (n=9)
M (n=8)
F (n=9)

Figure 4.4 Mean arterial pressure and heart rate before and after preterm birth.

Values for mean arterial pressure (A) and heart rate (B) on gestational days 131–132 and for up to 240 min following caesarean-section at 133 days of gestational age in female (pink) and male (blue) fetuses and lambs. Separate one-way repeated ANOVAs were performed 1) before delivery, 2) between 0 and 60 min after delivery, and 3) between 75 and 240 min after delivery. P values: P_S is between factor variable (sex), P_T is repeated variable (time), and P_{SxT} is the interaction term. Data are shown as means ± SEM.


A
MAP (mmHg)
80
70
60
50
40
30
C-section
P_S = 0.717
P_T = 0.005
P_{SXT} = 0.126
P_S = 0.023
P_T = 0.000
P_{SXT} = 0.915
P_S = 0.340
P_T = 0.024
P_{SXT} = 0.214
131
132
15
30
45
60
75
90
105
120
135
150
165
180
195
210
225
240
Gestational Age (day)
Postnatal Age (min)
M (n=5)
F (n=4)
M (n=10)
F (n=9)
M (n=8)
F (n=9)

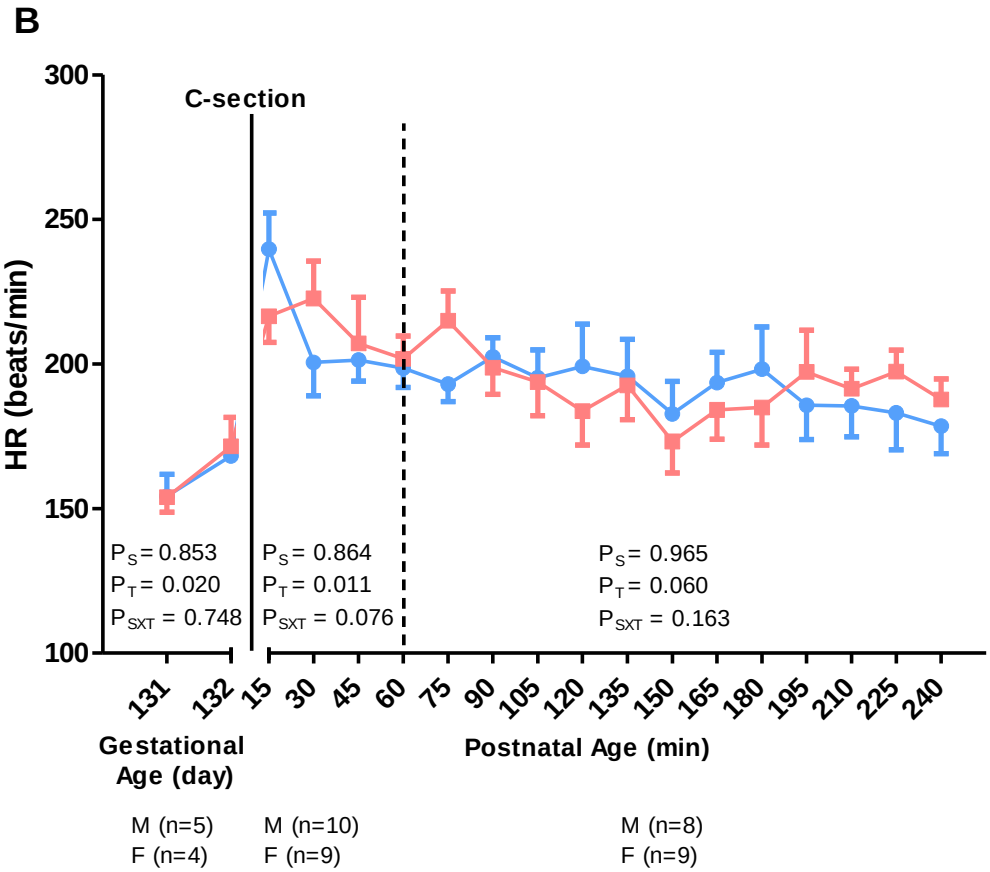
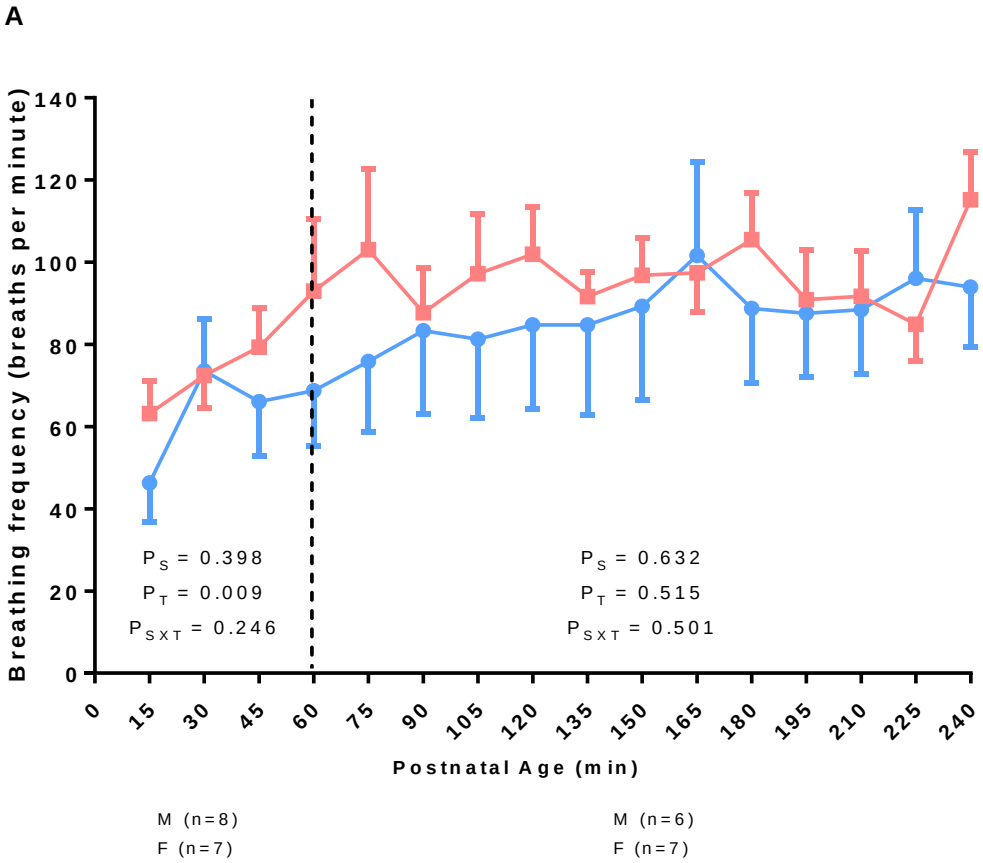
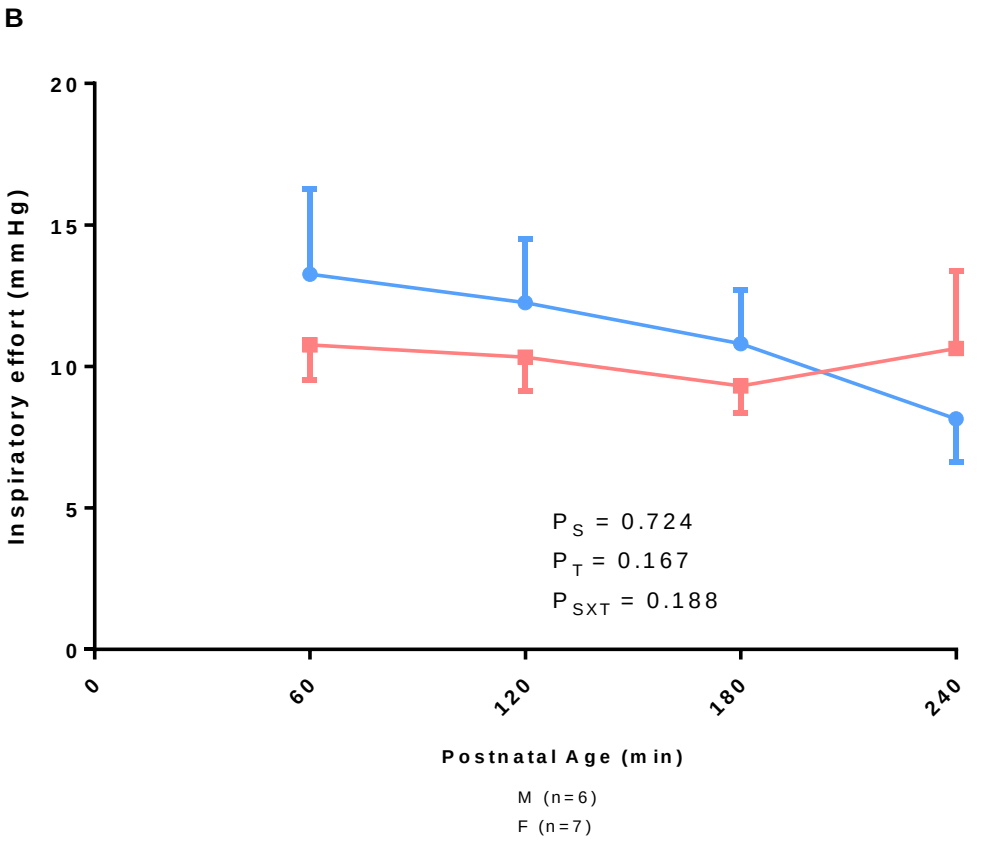

B
HR (beats/min)
300
250
200
150
100
C-section
P_S = 0.853
P_T = 0.020
P_{SXT} = 0.748
P_S = 0.864
P_T = 0.011
P_{SXT} = 0.076
P_S = 0.965
P_T = 0.060
P_{SXT} = 0.163
131
132
15
30
45
60
75
90
105
120
135
150
165
180
195
210
225
240
Gestational Age (day)
Postnatal Age (min)
M (n=5)
F (n=4)
M (n=10)
F (n=9)
M (n=8)
F (n=9)

Figure 4.5 Breathing frequency and inspiratory effort following preterm birth.

Values for breathing frequency (A) and inspiratory effort (B) following caesarean-section at 133 days of gestational age in female (pink) and male (blue) lambs. Separate one-way repeated ANOVAs were performed 1) between 0 and 60 min after delivery, and 2) between 75 and 240 min after delivery. P values: P_S is between factor variable (sex), P_T is repeated variable (time), and P_{SxT} is the interaction term. Data are shown as means ± SEM.


A
Breathing frequency (breaths per minute)
140
120
100
80
60
40
20
0
0
15
30
45
60
75
90
105
120
135
150
165
180
195
210
225
240
Postnatal Age (min)
P S = 0.398
P T = 0.009
P SXT = 0.246
P S = 0.632
P T = 0.515
P SXT = 0.501
M (n=8)
F (n=7)
M (n=6)
F (n=7)


B
Inspiratory effort (mmHg)
20
15
10
5
0
0
60
120
180
240
Postnatal Age (min)
P S = 0.724
P T = 0.167
P SXT = 0.188
M (n=6)
F (n=7)

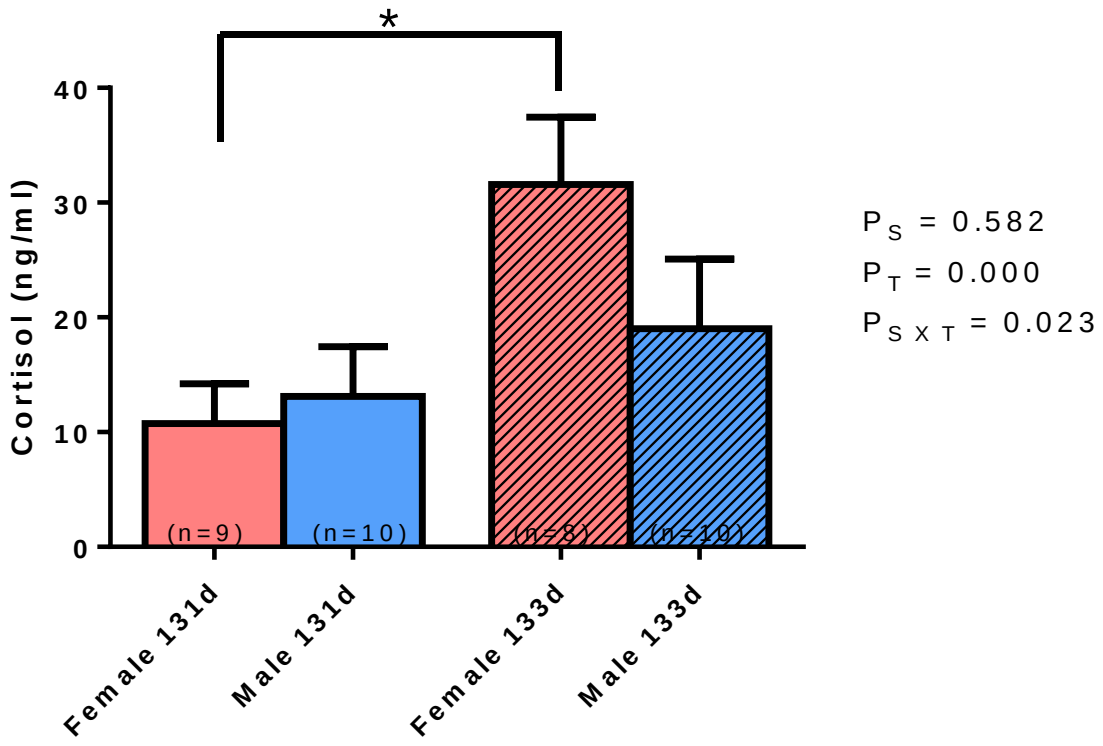


Figure 4.6 Fetal plasma cortisol concentration.

Circulating cortisol concentrations measured at 131 (open bars) and 133 (hatched bar) days of gestational age; females (pink bars) and males (blue bars). One-way repeated ANOVA was performed. P values: P_S is between factor variable (sex), P_T is repeated variable (time), and P_{SxT} is the interaction term. Data are shown as means ± SEM. The asterisk shows a significant difference between cortisol concentrations in female fetuses between 131 and 133 days of gestation.

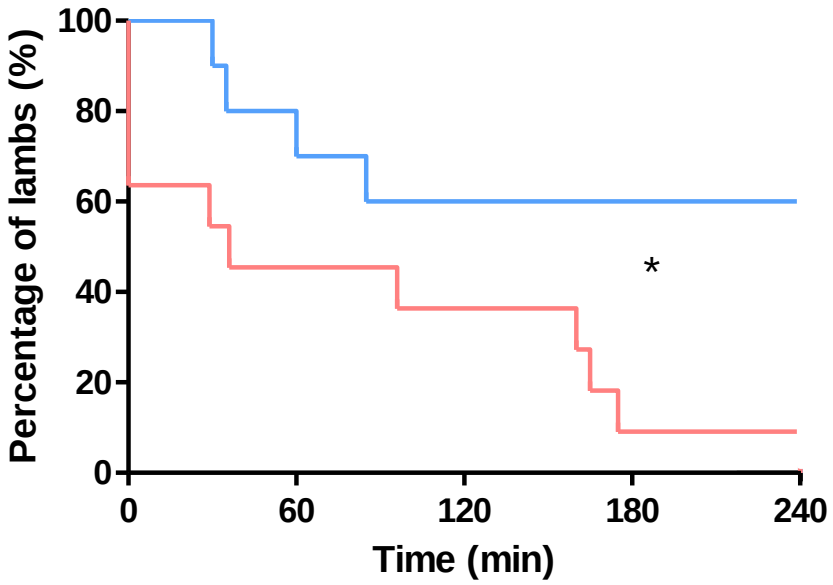


Figure 4.7 Requirement for supplemental oxygen following preterm birth.

The figure shows the percentage of female (pink; n=11) and male (blue; n=10) lambs that required supplemental oxygen at given times after delivery. The data of the duration of oxygen administration in each lamb were analysed by a log rank (Mantel-Cox) test to measure the. *P < 0.05, male vs female.

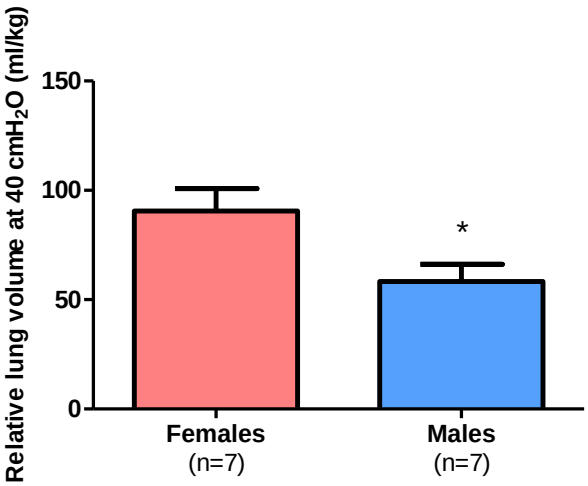


Figure 4.8 Lung volume measured at 40 cmH_2O per kg body weight.

Lung volume, relative to body weight, measured at 40 cmH_2O at 4 hours after birth in female (pink) and male (blue) lambs. Data are represented as means ± SEM. *P < 0.05, male vs female.

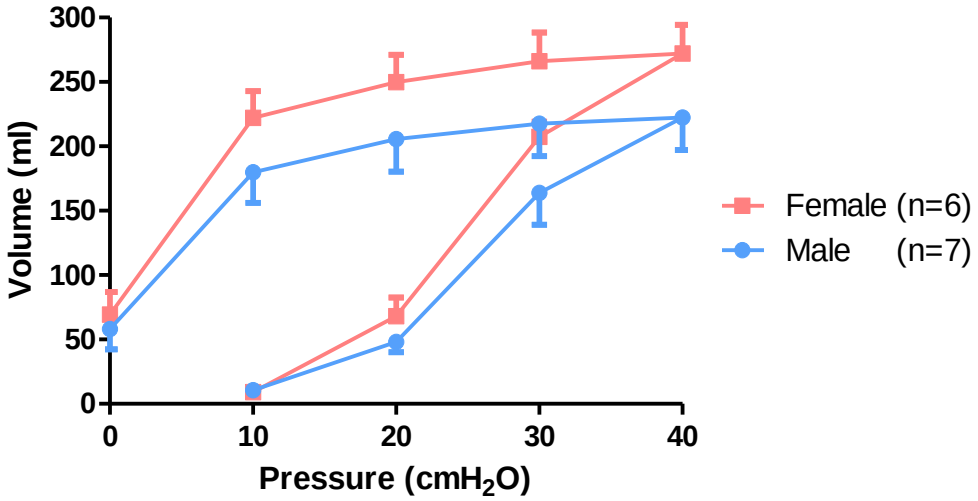


Figure 4.9 Pressure-volume (PV) relationship in lungs of male and female lambs.

Plot of pressure vs volume measured at 4 hours after birth in lungs of female (pink) and male (blue) lambs. Data are represented as means ± SEM.

Figure 4.10 Representative histological images of the lungs of female and male preterm lambs collected at 4 hours after preterm delivery at 133 days of gestation (opposite page).

The images are of lung tissue sections (5 µm thick) as observed under the light microscope, stained with Haematoxylin and Eosin at 200X magnification (nuclei are stained purple while cytoplasm is stained pink) in female (A) and male (B), Gordon and Sweet's reticular fibre stain at 1000X magnification (collagen fibres types I and III stained black while the interstitium is stained pink) in female (C) and male (D), and Hart's elastin stain at 1000X magnification (elastin fibres stained black while interstitium is stained yellow) in female (E) and male (F).

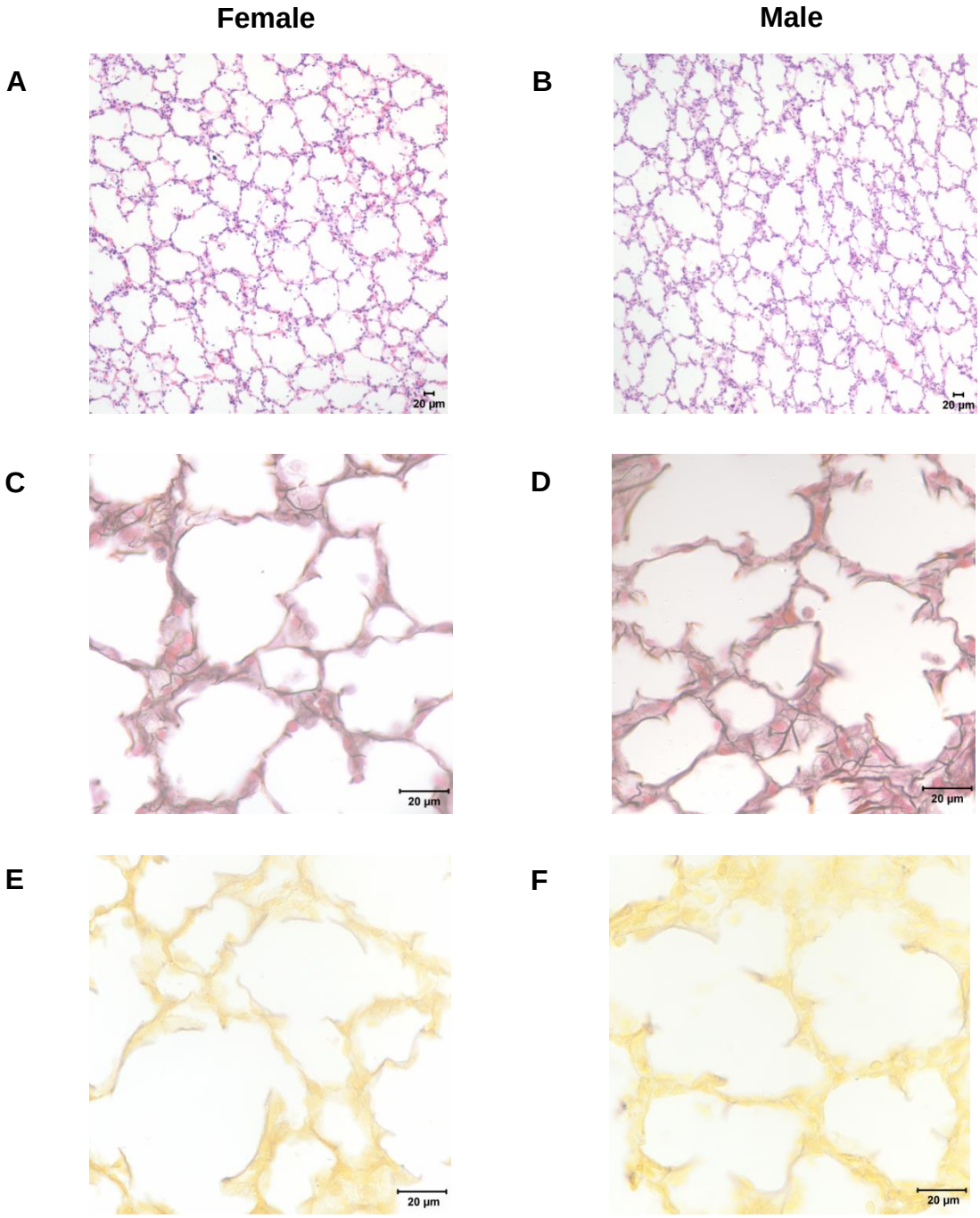
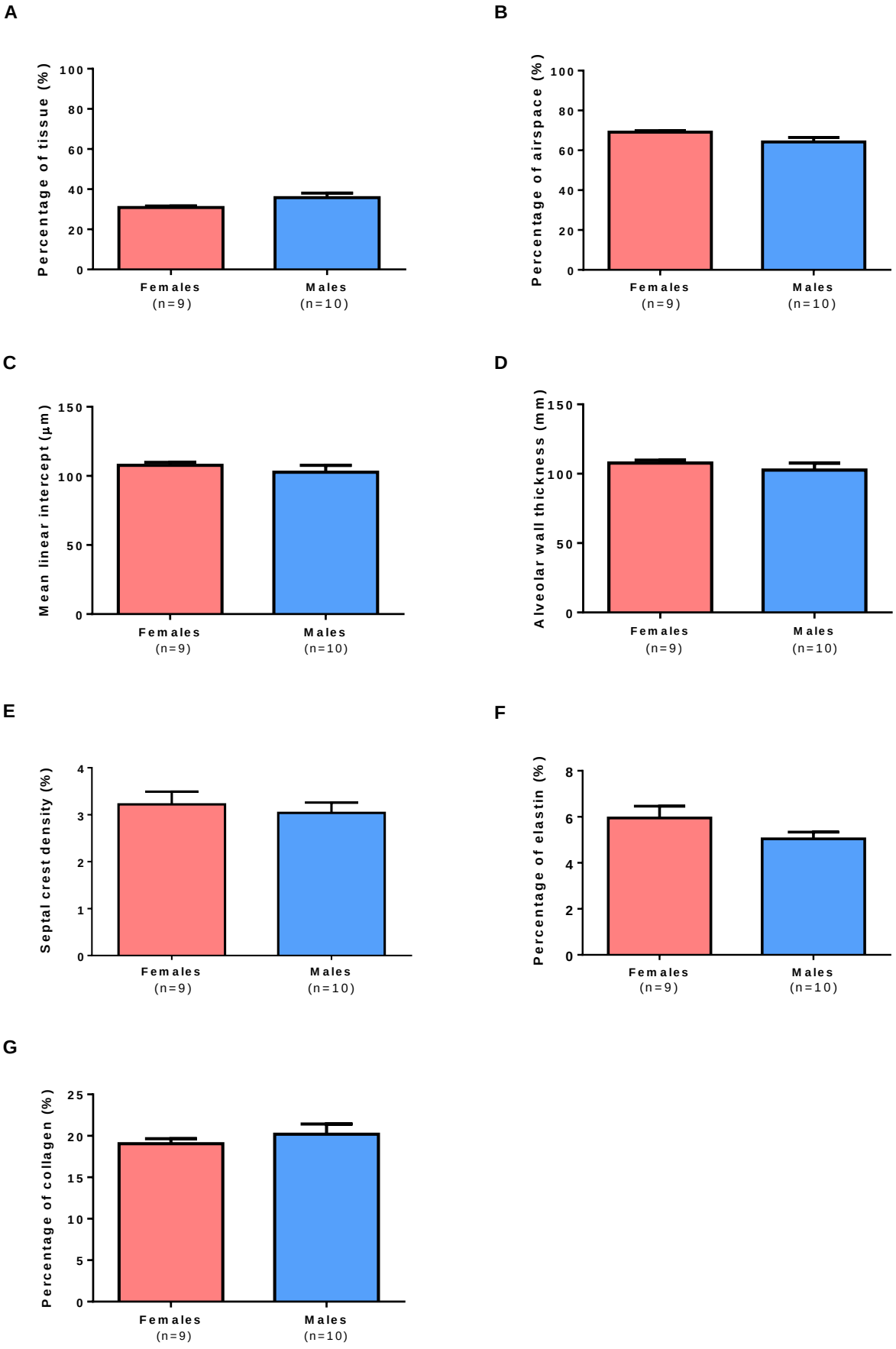

Female
Male
A
B
C
D
E
F
20 µm

Figure 4.11 Indices of lung morphometry in female and male lambs at 4 hours after preterm delivery at 133 days of gestation.

Bar graphs show (A) percentage of lung tissue (tissue fraction), (B) percentage of air space (airspace fraction), (C) mean linear intercept, (D) alveolar wall thickness, (E) septal crest density, (F) elastin content, (G) collagen content. Data are shown as mean ± SEM. Female (pink bars); male (blue bars).


A
Percentage of tissue (%)
100
80
60
40
20
0
Females
(n=9)
Males
(n=10)
B
Percentage of airspace (%)
100
80
60
40
20
0
Females
(n=9)
Males
(n=10)
C
Mean linear intercept (µm)
150
100
50
0
Females
(n=9)
Males
(n=10)
D
Alveolar wall thickness (mm)
150
100
50
0
Females
(n=9)
Males
(n=10)
E
Septal crest density (%)
4
3
2
1
0
Females
(n=9)
Males
(n=10)
F
Percentage of elastin (%)
8
6
4
2
0
Females
(n=9)
Males
(n=10)
G
Percentage of collagen (%)
25
20
15
10
5
0
Females
(n=9)
Males
(n=10)

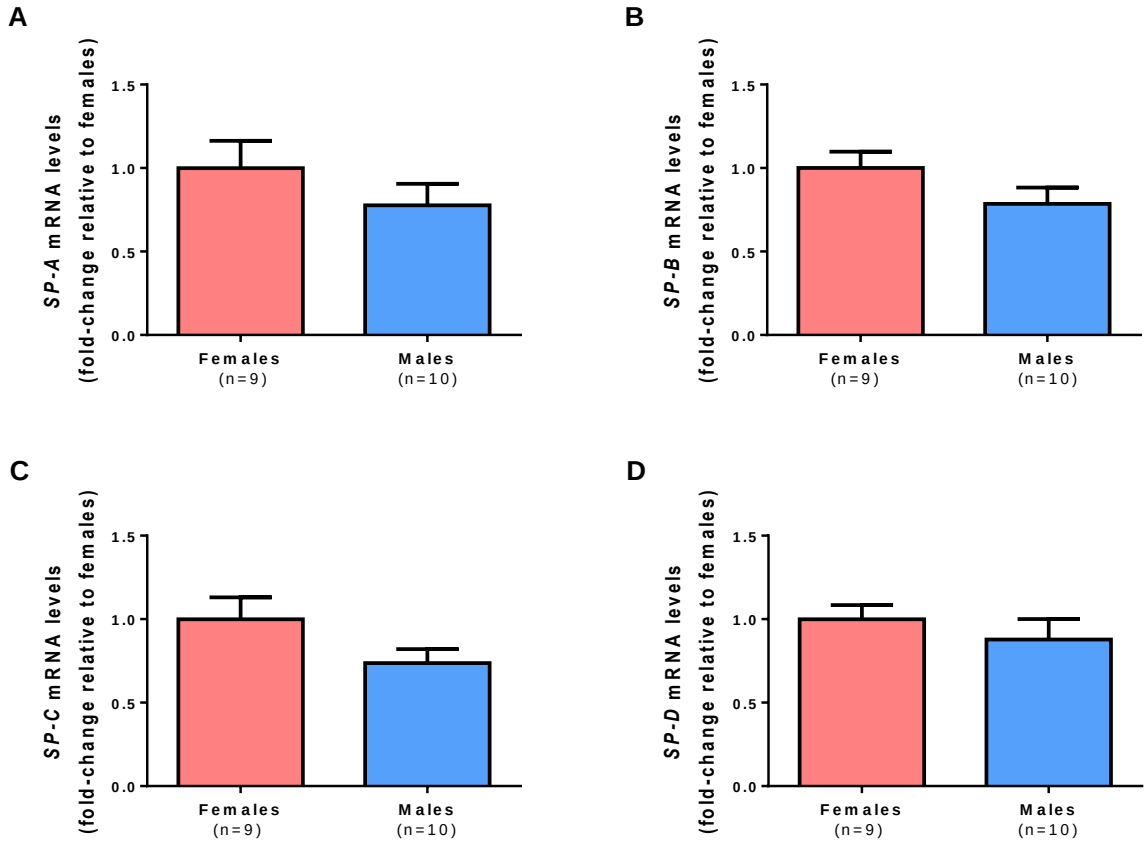


Figure 4.12 Surfactant protein (*SP*) gene expression in the lungs of female and male preterm lambs.

(A) to (D) show the *SP-A, -B, -C and -D* expression in lung tissue at 4 hours after birth in female (pink) and male (blue) lambs. Data are represented as means ± SEM.

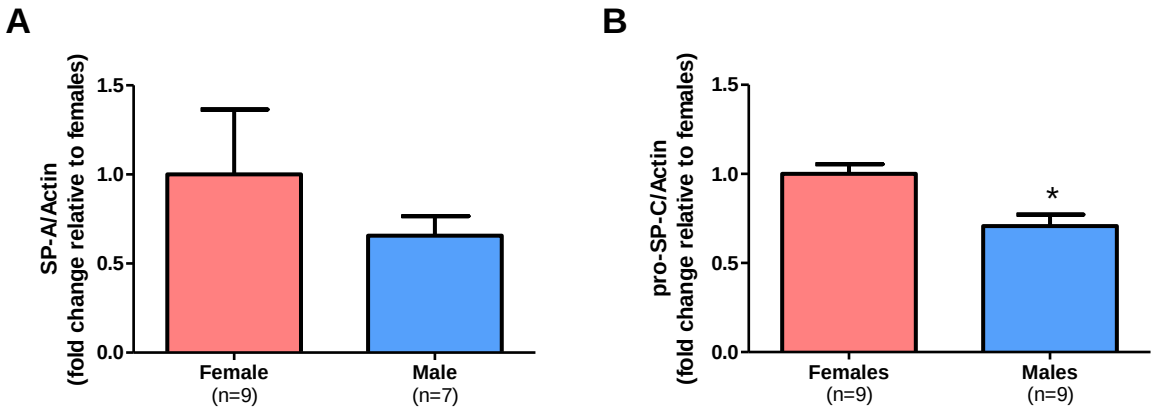


Figure 4.13 SP-A and pro-surfactant protein-C expression in the lungs of female and male preterm lambs.

(A) shows SP-A and (B) shows pro-surfactant protein-C expression in lung tissue at 4 hours after birth in female (pink) and male (blue) lambs. Data are represented as means ± SEM. *P < 0.05, female vs male.

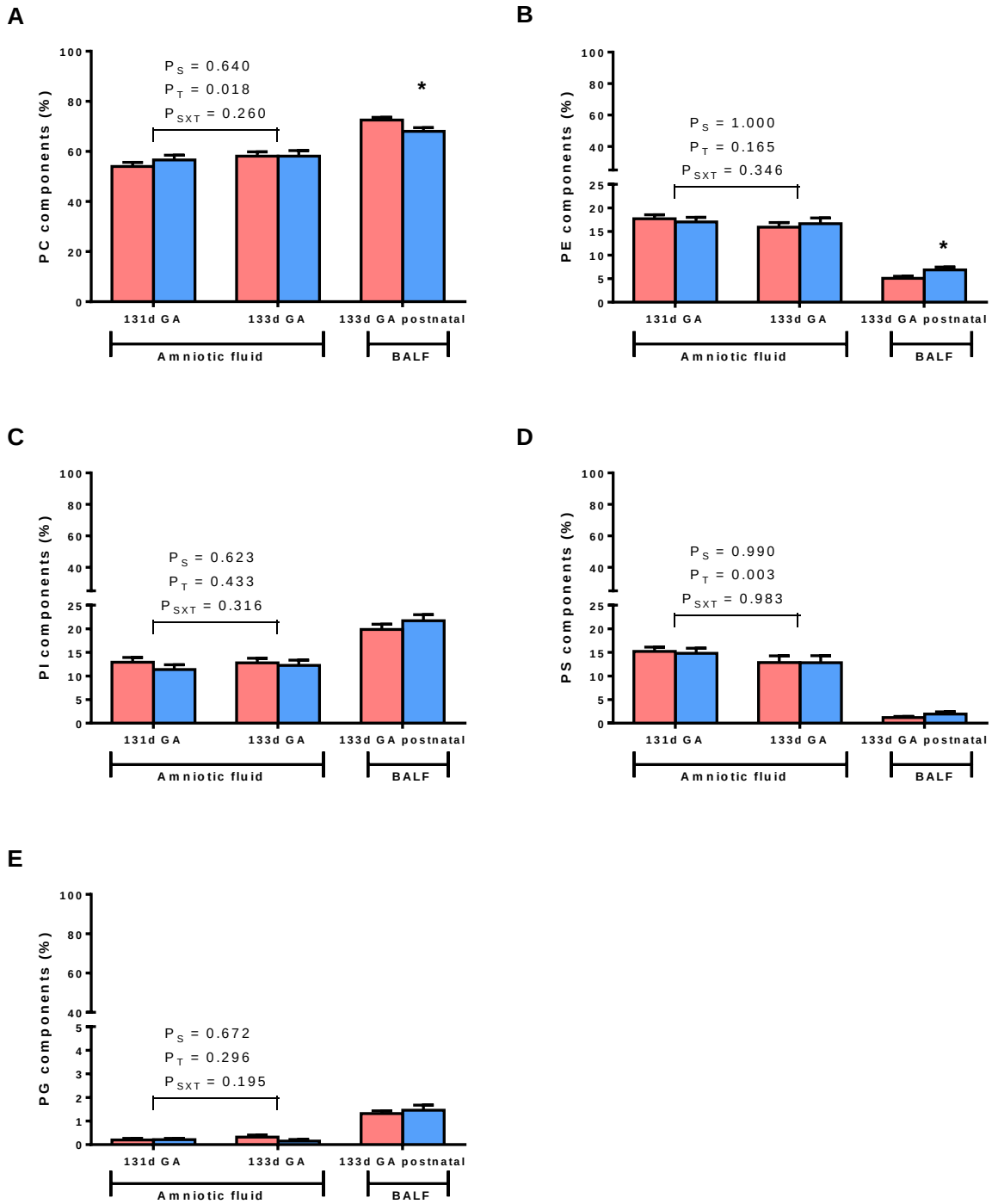


Figure 4.14 Proportion of major surfactant phospholipid classes in amniotic fluid and bronchoalveolar lavage fluid (BALF).

(A) to (E) shows the proportions of the major phospholipid classes PC, PE, PI, PS and PG compared to total phospholipid in the amniotic fluid at 131 and 133 days of gestation and in BALF collected 4 hours after preterm delivery at 133 days of gestation. Females (n=9) males (n=10) and are represented in pink and blue bars respectively. Data are shown as mean ± SEM. *$P < 0.05$, female *vs* male.

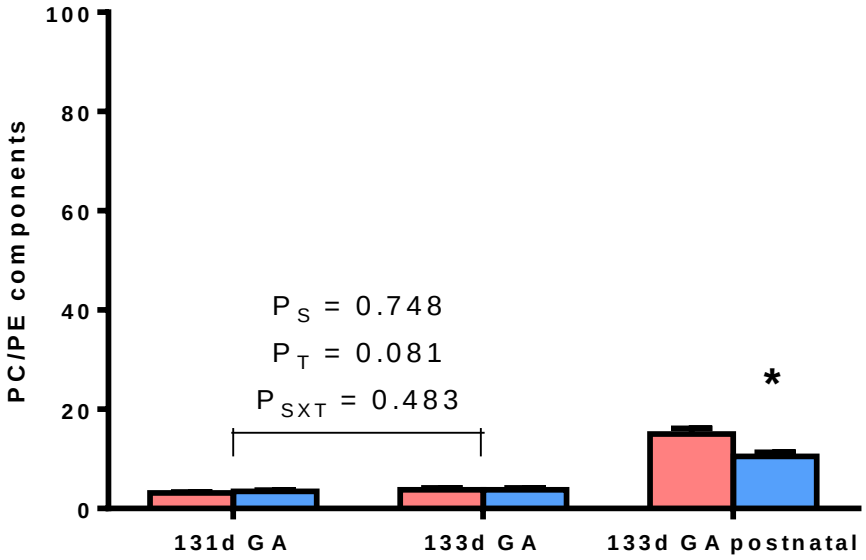


Figure 4.15 Ratio of PC/PE in amniotic fluid and bronchoalveolar lavage fluid.

The ratio of PC/PE in the amniotic fluid and BALF collected 4 hours after preterm delivery at 133 days of gestation. Females and males are represented in pink and blue bars respectively. Data are shown as mean ± SEM. *P < 0.05, female vs male.

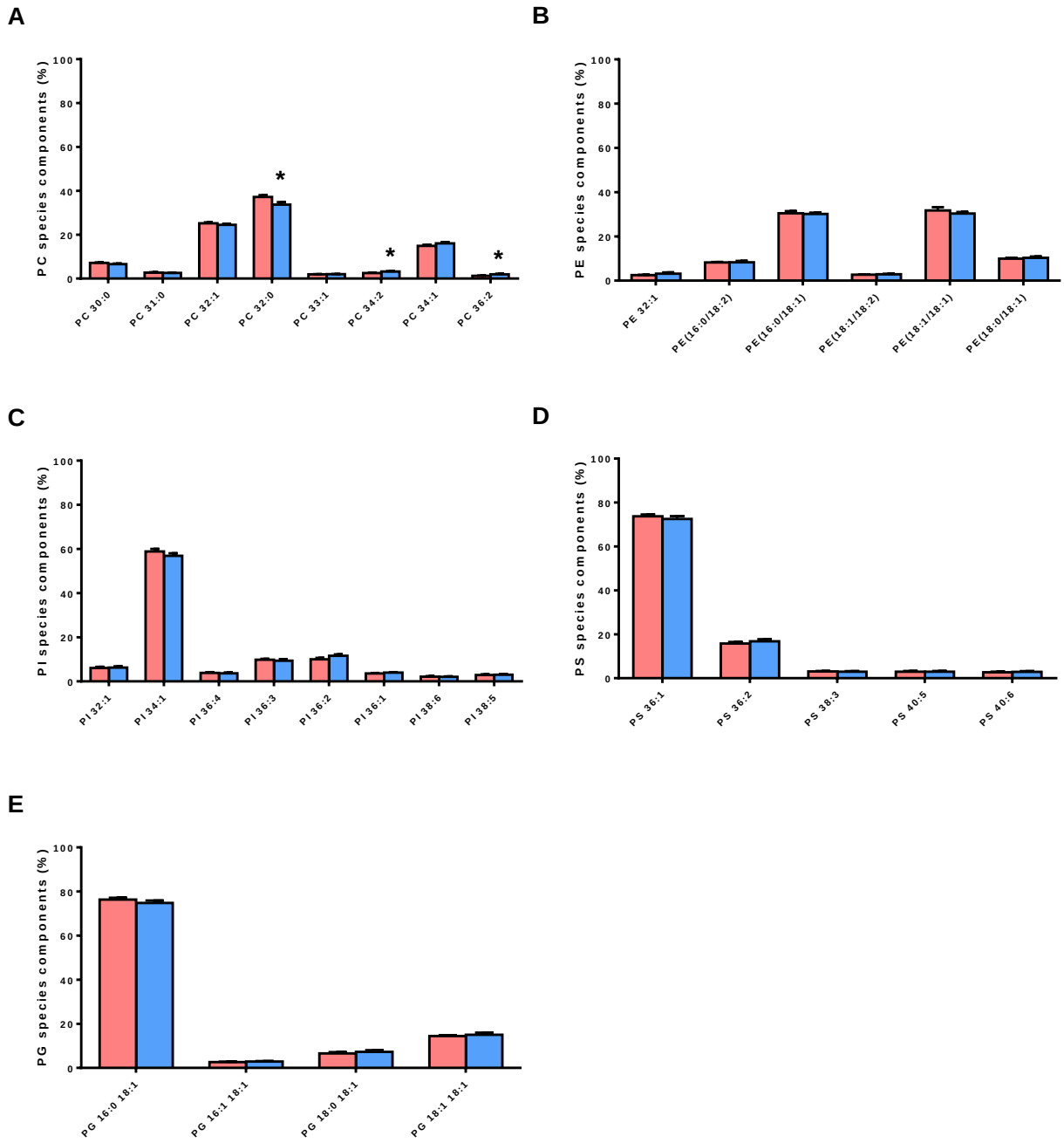


Figure 4.16 Proportion of major surfactant phospholipid species in bronchoalveolar lavage fluid collected at 4 h after preterm birth.

Figures (A) to (E) show the proportions of the phospholipid molecular species for PC, PE, PI, PS and PG compared to total phospholipid in the BALF following 4 h of preterm delivery at 133 days of gestation. Females (n=9) and males (n=9) are represented in pink and blue bars respectively. Data are shown as mean ± SEM. *$P < 0.05$, female *vs*. male.

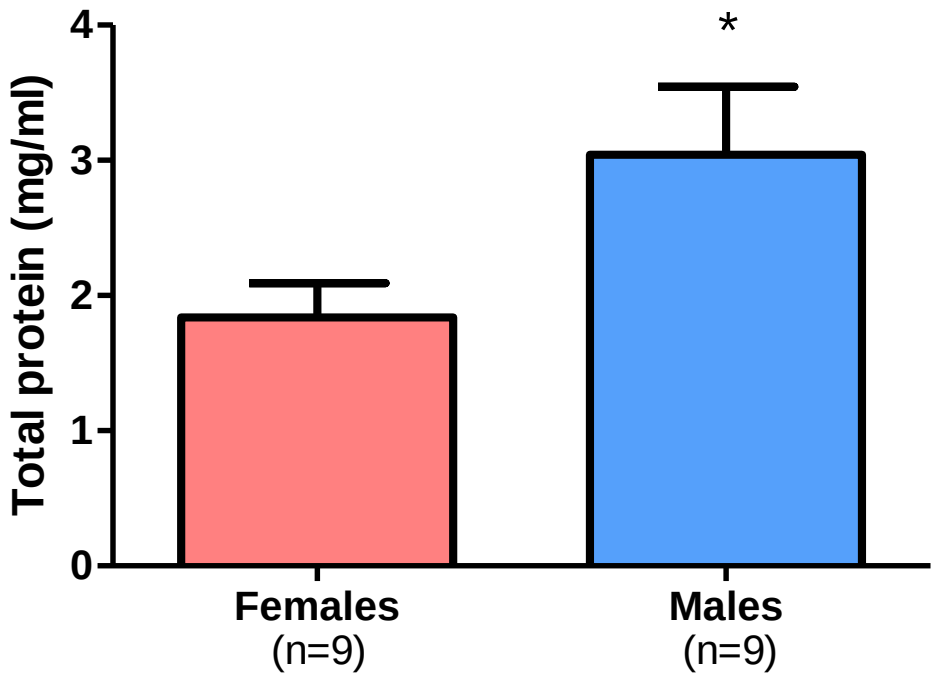


Figure 4.17 Total protein concentration in bronchoalveolar lavage fluid after preterm birth.

Total protein concentration in BALF collected at 4 hours after preterm birth in female (pink) and male (blue) lambs. Data are shown represented as means ± SEM. *P < 0.05, female *vs*. male.

4.4 Discussion

The overall aim of this study was to better understand the male disadvantage in respiratory outcome following preterm birth and also to determine if similar findings in the postnatal preterm lambs in this study were observed as in the prenatal lambs reported in Chapter 3. Similar to the observations in the fetal sheep (Chapter 3), no differences were seen in lung architecture and *surfactant protein* gene expression in lung tissue between male and female postnatal lambs at 0.9 of term, a stage of development when newborn males have poorer respiratory adaptation than females (De Matteo et al., 2010). However pro-SP-C protein expression in lung tissue was significantly lower in lung tissue of male lambs. In BALF, we found altered surfactant phospholipid composition (significantly lower proportions of the molecular species PC 32:0 and higher PC 34:2 and PC 36:2 in males) and elevated protein concentration in male lambs. Together these differences in surfactant could impair surfactant function and result in lower relative lung volume in males, thus contributing to the poorer gas exchange observed in preterm male lambs (lower pH and higher $PaCO_2$). Other major findings include higher arterial glucose and lactate in males, particularly within the first hour of preterm delivery; plasma cortisol concentration at 133d GA was significantly lower in males than in females. Overall, the sex-related differences observed in the various parameters could have contributed to the lower lung volume at 40 cmH_20 of males, which in turn was the likely cause of the loss of 2 males following the first hour of delivery and the poorer postnatal adaptation and greater requirement of supplemental oxygen of the male preterm lambs.

4.4.1 Weights and lung volume

4.4.1.1 Lung and body weights

There were no differences in body or lung weights between male and female postnatal lambs at 133d GA. In contrast to the present study, Willet et al. (1997) reported an 8% lower wet lung weight in ventilated female lambs at 128d GA compared to males. In the present study, the wet lung weight was measured at 4 h

after delivery when lung liquid would have likely been cleared; in contrast the wet lung weight in the study by Willet et al. (1997) was measured following 40 min of ventilation and may still be undergoing lung liquid clearance, which could explain the differences in findings. However, when wet lung weight was related to body weight in the fetuses in the present study and in the study by Willet et al. (1997) there were no differences between sexes. The present finding in postnatal lambs confirms our findings in fetal sheep of a similar gestational age, as reported in Chapter 3.

In human fetuses, females, on average, weigh ~2–3% less than age-matched males throughout most of gestation (Gerards et al., 2006). This sex difference in body weight appears to be more significant before the third trimester (i.e. before 26 weeks of gestation) and becomes less obvious closer to term (Gerards et al., 2006). This could explain the lack of difference in body weights that we observed in our fetal sheep as our animal model is representative of moderately preterm birth (0.9 of term) and thus may have passed the stage of gestation at which sex differences in body weight might have been more obvious. Our finding on body growth, however, is consistent with other studies in sheep which did not observe a sex difference in birth weight in lambs born in late gestation or at term (De Matteo et al., 2010, Polglase et al., 2012) and also in the fetal sheep described in Chapter 3. In contrast, Willet et al. (1997) reported a 10% lower birth weight in female lambs at 128d GA compared to males, which could account for the sex differences in lung weight observed in their study as lung weight relative to body weight was not significantly different between sexes.

4.4.1.2 Lung Volume

In the present study, we found no sex differences in lung volume measured histologically when fixed at an inflation pressure of 30 cmH_2O; a similar observation was reported for lambs delivered at 128d GA and ventilated for 40 min, fixed at the same inflation pressure (Willet et al., 1997), and also in the fetal sheep, lung fixed at an inflation pressure of 20 cmH_2O, used in the study reported

in Chapter 3. In humans, a significant sex difference was found in fetal lung volume from 18 to 34 weeks of gestation by using three-dimensional ultrasound (Gerards et al., 2006); the mean lung volume of male fetuses was, on average, 4.3% greater than in females. However, the difference in mean lung volume was eliminated when adjusted for estimated fetal weight. Another study assessed the lungs of human fetuses by fast spin-echo T2-weighted lung magnetic resonance (MR) imaging at 21 to 38 weeks of gestation; the authors demonstrated a significant sex difference in human fetal lung volume after 35 weeks of gestation; males had greater lung volume than females (Rypens et al., 2001). A histopathological study of human fetuses observed no sex differences in lung volumes between 19 and 40 weeks of gestation (Langston et al., 1984). A possible explanation for the varying results could be the different methods used to measure lung volume and different degrees of luminal inflation. In the present study, as well as the studies by Willet et al. (1997) and Langston et al. (1984), the Cavalieri method was used to assess lung volume using histologically prepared lung tissue, whereas Gerards et al. (2006) and Rypens et al. (2001) used ultrasound and MR imaging respectively to obtain lung volume while the fetuses were still *in utero*. Although sex differences in lung volume in fetuses and infants are not well-established, they have been documented in children and adults (Rypens et al., 2001). Postnatally, boys have larger lungs than girls from 6 weeks after birth to 14 years of age (Thurlbeck, 1982) which persists into adulthood (Bellemare et al., 2003). Adult male mice are also found to have larger lung volumes than females (Massaro and Massaro, 2006). However, these differences could be a result of males having larger bodies.

4.4.2 Lung morphology

The lungs of male and female postnatal lambs at 0.9 of term appeared to be structurally similar as no differences in tissue and air space fraction, alveolar diameter, alveolar wall thickness, septal crest density and septal thickness were observed. Similar observations were observed in preterm lambs born at 128d GA (Willet et al., 1997); the authors of that study found no sex differences in tissue

fraction, mean linear intercept, alveolar wall thickness and surface area. However, their observations could have been confounded by fetal surgical intervention or the use of mechanical ventilation. Alveolar number and alveolar surface area were not measured in the present study; however a greater number of alveoli and larger alveolar surface area were found in human males from 6 weeks to 14 years old, resulting in larger alveolar surface area to body mass ratios, which were eliminated when body weights were taken into account (Thurlbeck, 1982). In the present study the strong trend for male lungs to have greater tissue fraction than females (P = 0.06) could have contributed to the lower relative lung volume observed in males.

Elastin and collagen deposition were also analysed in the present study as it has been shown that alveolarisation and probably distal airway development are controlled by the elastin-collagen network (Mercer and Crapo, 1990, Mascaretti et al., 2009). In this study, elastin content in the lung interstitium was similar in males and females. The lack of sex difference in elastin expression and content supports our finding about the lack in sex difference in septal crest density because the presence of elastin and septal crests are closely linked; elastic fibers are typically located at the tips of the crests (Burri, 2006). Lung collagen content, determined from the deposition of the major collagens, type I and III collagen fibres (Kaarteenaho-Wiik et al., 2004), was also not different between the sexes. In sheep, collagen and elastin volume in the lung parenchyma increases during fetal development, with significant increases between 121 and 135d GA (Willet et al., 1999). It has also been shown that infants with RDS have increased precursor proteins and mRNAs of both type I and III collagen fibres in the alveolar walls (Kaarteenaho-Wiik et al., 2004).The rate of collagen synthesis in lung development in monkeys, however, declines over gestation and is found to be lower at term than at an earlier gestational age, probably due to species difference (Jackson et al., 1990). At the canalicular stage of lung development in rabbits, pulmonary collagen is less mature, has fewer cross-links and is less tightly packed in comparison to collagen in the adult lung (Bradley et al., 1974). To my knowledge,

no other study has assessed sex differences in the collagen content of the preterm lung.

4.4.3 Surfactant composition

4.4.3.1 Sex differences in pulmonary surfactant phospholipid composition in bronchoalveolar lavage fluid

In the postnatal lambs, differences in the surfactant phospholipid composition of BALF were observed between male and female lambs at 4 h after preterm delivery. A significant decrease was observed in the proportion of PC, which is a major component of surfactant phospholipid. A detailed analysis of the species within PC indicated a significant reduction in the proportion of PC 32:0 and increased proportions of PC 34:2 and PC 36:2. PC 32:0 is also commonly known as DPPC and it is the only surface active component which is capable of lowering the surface tension in the lung to near zero levels (Veldhuizen et al., 1998, Bernhard et al., 2001, Agassandian and Mallampalli, 2013, Goss et al., 2013). A significant 10% reduction in the proportion of PC 32:0 in the BALF of male lambs could therefore have contributed to the lower lung compliance of males which strongly suggests the impairment of surfactant function in males. PC 34:2 and PC 36:2 are plasma PCs and elevated proportions of these species in males suggest greater plasma exudation into the lung lumen as a result of increased vascular permeability in the lungs. An increase in vascular permeability within the lungs could account for the 65% increase in total protein concentration found in BALF in males compared to females. Plasma proteins are known to inhibit pulmonary surfactant function (Keough et al., 1989, Warriner et al., 2002) and could likely reduce the effectiveness of DPPC and other phospholipids in the alveolar space, thus preventing the lowering of surface tension within the lung. The proportion of PE was significantly increased in males but no differences were observed in the proportions of PE species. It is not known how PE affects the function of surfactant phospholipid in lambs.

4.4.3.2 Sex differences in pulmonary surfactant phospholipid composition in the amniotic fluid

Before birth, the proportions of PC, PE, PI, PS and PG in the amniotic fluid were similar in female and male fetuses. The proportions of PC and PS, on the other hand, indicated an increase over gestational age. Further analyses showed no differences in the proportion of their respective species between sexes. However, a few phospholipid species indicated a change over gestational age.

The increase in the proportion of PC from 131–133d GA supports findings observed in human amniotic fluid and also in fetal lamb lung where the proportion of PC increases with the progression of gestation (Fujiwara et al., 1968, Torday et al., 1981).

PE is the second most abundant phospholipid class in pulmonary surfactant, and is an important phospholipid that makes up cell and organelle membranes; it was present in similar proportions in both male and female amniotic fluid of sheep in this study. Similarly, no differences were observed between males and females in the proportion of PS in the amniotic fluid and little is known about its role. However, a decrease in the proportion of PS was observed from 131–133d GA. There have not been any previous studies that have investigated the differences in the proportion of PE and PS in amniotic fluid in any species.

It has previously been reported that the proportion of PI increases from 28 weeks and gradually decreases after 36 weeks in human pregnancy (Brown and Duck-Chong, 1982). Fleisher et al. (1985) reported a similar finding and also observed that the proportion of PI in human amniotic fluid decreased to levels below that of males after 37 weeks gestation. The decreasing trend in the proportion of PI towards late gestation is similarly seen in sheep (Fujiwara et al., 1968). In this study, however, a difference in the proportion of PI was not observed possibly due to amniotic sampling at an earlier gestation.

PG appears late in gestation in humans (Ikegami and Jobe, 1981, Ueda et al., 1994, Leung-Pineda and Gronowski, 2010), appearing at 35 weeks of gestation in significant amounts in the amniotic fluid; thus it is used as an indicator of lung maturity (Leung-Pineda and Gronowski, 2010). The low proportion of PG across the gestational ages examined in the present study could have prevented the detection of any sex differences in the proportions of PG due to the samples being collected at an earlier gestational age. In human amniotic fluid, the rate of increase in the proportion of PG was greater in females than in males, where PG was observed to initially appear at 34 weeks of gestation (Fleisher et al., 1985).

The lack of sex differences observed in the surfactant phospholipid composition in the amniotic fluid in the present study could be due to the gestational age at which the samples were obtained. It is possible that detection of any differences could not be detected due to the immaturity of the surfactant at this time point (131–133 days of the 147 day gestation). It is also possible that the placement of the amniotic catheter in the amniotic sac could have affected the data due to inaccurate sampling of amniotic fluid; that is, the catheter tip may have been close to the mouth of the fetus or near the source of fetal urine flow into the amniotic sac. This could result in the sampled amniotic fluid varying in the proportion of fetal lung liquid (the source of pulmonary surfactant) that it contains.

4.4.3.3 Phospholipid maturity ratio

In this study, the PC/PE ratio was measured as an indicator of lung maturity as a substitute to measuring the commonly calculated L/S ratio. PC is also referred to as lecithin because of its large PC content. PE is similar to sphingomyelin (S), as they are both considered to be membrane lipids that are ejected from some of the membrane of lamellar bodies during exocytosis of surfactant into the liquid lining the alveoli (Veldhuizen et al., 1998); they both remain relatively unchanged throughout gestation and are therefore used as a reference. In this study, the PC/PE ratio in BALF of male lambs (11:1) was significantly lower than the ratio of 15:1 observed in females. Our finding of a lower index of surfactant maturity in males supports the finding in the amniotic fluid of humans and rabbits and also in

the lung lavage of rabbits (Nielsen and Torday, 1981, Torday et al., 1981, Fleisher et al., 1985). Androgens are thought to inhibit the maturation of the pulmonary fibroblast and thus delay surfactant synthesis in type II alveolar epithelial cells (Dammann et al., 2000), which could explain the lower PC/PE ratio in preterm males. In contrast, no sex difference was observed in the PC/PE ratio in the amniotic fluid. This could be explained by the lack of sex differences seen in the proportions of PC and PE, which may be a result of inaccuracies in sampling of amniotic fluid.

4.4.3.4 Surfactant protein gene and protein expression in lung tissue

Although surfactant proteins make up a minor component of pulmonary surfactant (~10%), they are important in lowering surface tension, thereby preventing end-expiratory alveolar collapse, and in pulmonary defence. In this study, the gene expression of *SP-A*, *-B*, *-C* and *-D* was not different between male and female postnatal lambs; similar observations were seen in fetal lung tissue (Chapter 3). As surfactant phospholipids constitute the remaining 90% of pulmonary surfactant and have been shown to have sex differences, alterations in the phospholipid component of pulmonary surfactant rather than surfactant proteins could account for the poorer respiratory outcome in males following premature birth. However, males had significantly lower level of pro-SP-C protein than females. The mRNA and protein levels of SP-C do not correlate as protein levels can be altered in the absence of changes in gene expression. This may be contributed by the processes involved in transcription and translation. pro-SP-C is synthesised by the alveolar type II cells as a 21-kDa integral membrane propeptide, which is proteolytically processed to a 3.7-kDa secretory product (SP-C) (Beers et al., 1998, Mulugeta and Beers, 2003). pro-SP-C was measured instead of the mature SP-C protein due to optimising problems related to the small size of the mature SP-C protein being detected with the Western blot technique. SP-C facilitates the adsorption of surfactant lipids into the surface film lining the alveoli in the lung (Possmayer et al., 2001, Rodriguez-Capote et al., 2001). A lower proportion of pro-SP-C in the male lung compared to female lung is consistent with the observed

lower static lung compliance of males than females. The expression level of SP-A, which is involved in host defence (Orgeig et al., 2010), was not different between males and females. This could be due to immaturity of the immune system at this stage. In rats, low amounts of relative SP-A (in relation to total phospholipids) were detected in newborns compared to adults (Bernhard et al., 2001). As an earlier age in gestation was investigated in the present study, sex differences in the level of SP-A expression may have been absent in the lamb lung tissue we used. Since SP-B and SP-D protein expression were unable to be determined due to optimization issues related to the Western blot technique, it is unknown if their levels differ between the sexes.

4.4.4 Prenatal physiology

Prenatal arterial blood gas data (pH, $PaCO_2$, SaO_2, lactate and glucose) and MAP and HR indicated no sex differences before birth. Information on possible sex differences in blood gas status and fetal cardiovascular physiology is lacking in the literature.

Circulating plasma cortisol concentration increased in female fetuses from 131d GA to 133d GA but did not increase in males over that period. A small dose of betamethasone was administered to the ewe following sampling of fetal blood at 131d GA and could have resulted in a greater increase in fetal cortisol concentration in females than in males. Betamethasone has been shown to stimulate lung maturation (Jobe et al., 2009) and a greater corticosteroid response in females may have contributed to the better physiological transition at birth in females. In contrast to females, the circulating concentration of cortisol did not increase in male fetuses between 131 and 133d GA. This could be due to a number of reasons such as male immaturity of the hypothalamic-pituitary-adrenal (HPA) axis (Hingre et al., 1994, Ng et al., 2002), a male reduction in expression of fetal lung 11β-hydroxysteroid dehydrogenase-1, which converts cortisone to cortisol (Torday, 1992), or sex differences in the activity of placental 11β-

hydroxysteroid dehydrogenase-2, which converts cortisol to cortisone (Dammann et al., 2003).

It is not clear if sex differences in the sensitivity or activity of the HPA axis play a role in sex-related differences in the cardiorespiratory transition immediately following delivery. It would be interesting to have measured the plasma cortisol concentration in the lambs following delivery, as cortisol is known to stimulate lung maturation. In these lambs, the lung morphology was not different between males and females; however, differences were observed in surfactant composition (surfactant phospholipid and SP protein expression).

4.4.5 Postnatal physiological adaptation to preterm birth

During the transition period (the first hour) following preterm birth, male lambs had lower arterial pH and higher $PaCO_2$. This indicates that gas exchange in male lambs was less effective than in females, probably due to the relatively incompliant lungs of the males. The lower arterial pH of males is likely a result of elevated $PaCO_2$, which in turn is a likely reflection of poor alveolar ventilation and/or poor alveolar blood flow. The mean breathing frequency and inspiratory effort, however, were similar in males and females thus eliminating the involvement of sex differences in respiratory drive for the impaired gas exchange of males.

Postnatal oxygenation (PaO_2 and SaO_2) was not different between sexes because supplemental oxygen was administered to promote survival of the lambs during the 4 h postnatal study period. The high variability in PaO_2 in the males is due to their greater requirement for breathing assistance in the form of supplemental oxygen support.

Male lambs presented with higher blood lactate concentrations after preterm delivery during the transition period. This could be a result of anaerobic

metabolism in tissues of male lambs as a result of these tissues receiving insufficient oxygen for their energy needs. As males were not more hypoxemic than females, poor peripheral perfusion was likely to have contributed to the high lactate concentrations. Hypercapnic acidosis in the male lambs could also have contributed to the likelihood of generalised pulmonary vasoconstriction as it has previously been shown that the normal changes to pulmonary vascular resistance are affected by acidemia after birth (Lyrene and Philips, 1984). In addition, hypercapnic acidosis has been associated with a decrease in myocardial contractility and cerebral vasodilatation (Tuxen, 1994), which could have contributed to the higher incidence of morbidity and mortality in preterm males compared to females.

The observed greater MAP in males compared to females could be a result of the elevated $PaCO_2$, which could lead to an increase in adrenal production of catecholamine and also an increase in sympathetic drive due to hyperoxia and/or acidemia, thus contributing to vasoconstriction. The cardiac output, cerebral blood flow, or pulmonary pressures were not measured in this study.

The male lambs were hyperglycemic and this may be due to a stress response arising from the CO_2 retention and acidemia or reduced glucose metabolism. Such a stress response may increase in catecholamine release and/or HPA axis steroids. In this study, plasma catecholamine levels were not measured in the lambs.

Although SaO_2 was maintained above 80% in both male and female lambs, a survival rate of 100% was seen in female lambs but 2 out of 10 male lambs had to be euthanized following the first hour of delivery as a result of poor physiological condition, including severe, irreversible hypercapnia and acidemia. The higher mortality in male lambs in comparison to female lambs in this study is similar to a previous finding in preterm lambs (De Matteo et al., 2010).

A recent study of preterm lambs did not show sex differences in blood gas parameters and cardiopulmonary function within 30 min of preterm delivery at 128d GA (Polglase et al., 2012). However, these lambs were anaesthetized and mechanically ventilated (Polglase et al., 2012), which could have confounded the results; it is apparent that the use of anaesthesia and mechanical ventilation may mask physiological differences between males and females following preterm birth.

4.5 Conclusions

In conclusion, poorer respiratory outcome and mortality in males in this study are consistent with other studies of preterm birth. Differences in the composition or quantity of pulmonary surfactant could contribute to the lower lung compliance in the male lambs. The inactivation of surfactant function in the male lambs could also be affected by the presence of plasma proteins in the air space.

Chapter 5: Fetal physiological status and adaptation to preterm birth in male and female lambs: 8 hour postnatal study

5.1 Introduction

In the study described in Chapter 4, it seemed apparent that the male disadvantage in respiratory outcome in preterm lambs could be attributed to an impairment in surfactant function of males during the 4 hours (h) after delivery. This in turn could result in poorer cardiorespiratory adaptation in the preterm males that could consequently lead to increased mortality in males.

De Matteo et al. (2010) have demonstrated the male disadvantage following preterm birth in sheep where females have a survival rate of 76% in comparison to 44% in males over a period of 360 h after birth (Figure 1.4). The figure (Figure 1.4) shows that a greater disparity in survival outcome occurs between the sexes following 5 h of preterm delivery.

The rationale for the present study was to better understand factors contributing to the increased mortality in the male lambs in comparison to the female lambs beyond 4 h of preterm delivery, as shown in Figure 1.4. Since the figure shows that male lambs are more inclined to die after 4 h, it was hypothesised that they would have poorer blood gases, cardiorespiratory adaptation and lung compliance in comparison to the female lambs, with greater differences observed beyond 4 h

of preterm delivery. In addition, it was hypothesised that sex differences in surfactant composition would be greater between 4 and 8 h after birth.

Therefore, the aim of this study was to investigate the factors that contribute to the increased male mortality following 4 h of preterm delivery. A time point of 8 h was selected due to a marked difference observed in survival outcome between the sexes within 4 to 8 h of preterm delivery (Figure 1.4). In addition, it was also more feasible to perform this study for 8 h than for a longer period. Differences in cardiorespiratory adaptation between males and females following preterm birth were determined and these included measuring arterial blood gas parameters, blood pressure and breathing rate and effort. During the postnatal monitoring period, ultrasound imaging was also performed to measure a number of structural and functional parameters of the left ventricle, kidney dimensions, renal artery blood flow velocities, diameter and blood flow velocities within the aortic root, pulmonary arteries and the right and left common carotid arteries. The results obtained from the ultrasound imaging are not reported in this chapter but will be presented in another person's PhD thesis. Following the 8 h of monitoring, static lung compliance was measured. In addition, surfactant phospholipid composition and total protein in BALF, and surfactant protein gene and protein expression were measured to determine surfactant composition and potential function.

5.2 Methods

5.2.1 Experimental protocol

This study was performed to compare the physiological adaptation of male and female lambs for a period of 8 h after induced preterm birth. Aseptic surgery was performed on pregnant ewes and their fetuses (Border-Leicester X Merino) at ~125 days of gestational age (d GA; term is ~147d GA) for catheter implantation, as described in Section 2.4.

Following surgery, fetal physiological status was monitored daily by measuring fetal arterial pH, PaO_2, $PaCO_2$, SaO_2, lactate and glucose. Fetal arterial pressure and heart rate were monitored, as outlined in Section 2.4.3.1, for one hour prior to betamethasone administration on 131d GA and also on 132d GA. A small dose of betamethasone was administered intramuscularly to the ewe at 131d GA to increase the chance of survival in the lambs following preterm delivery.

Following caesarean delivery at 133d GA, as described in Section 2.4.3.2, lambs were monitored for 8 h. Blood samples were collected at 5 minutes (min) after birth and every 15 min thereafter to measure arterial blood gases (pH, PaO_2, $PaCO_2$, SaO_2) and blood lactate and glucose concentrations. In addition, the mean arterial pressure (MAP), heart rate (HR), inspiratory effort and breathing frequency of the lambs were recorded. During the 8 h of postnatal monitoring, lambs were given supplemental oxygen via a Neopuff^{TM} Infant T-piece resuscitator (Fisher & Paykel, New Zealand) when required. Saline and glucose were administered intravenously to maintain, respectively, blood volume and blood glucose concentration following birth as mentioned in Section 2.4.3.3. Rectal temperature was continuously measured to maintain rectal temperature at 39ºC.

At the end of the 8 h of monitoring, the lambs were lightly sedated with 1 ml sodium thiopentone (50 mg/ml i.v.; Pentothal, Boehringer Ingelheim Pty Ltd, Australia), intubated and connected to a ventilator. The lambs were then euthanised and their thoracic cavity exposed for the measurement of static lung compliance as described in Section 2.4.3.4.

Following measurement of static lung compliance, the body weight, body dimensions, and major organ weights of the lambs were collected. The lungs were removed from the thoracic cavity; the left lung was ligated at the hilus, surgically separated from the right side of the lung and sectioned into small pieces (2–5 mm^3). These pieces were snap-frozen in liquid nitrogen (N_2) for storage at -80ºC

for measuring water content in lung tissue and surfactant protein gene and protein expression. Bronchoalveolar lavage fluid (BALF) was collected from the upper right lung lobe as outlined in Section 2.4.4.1 to allow measurements of total protein and surfactant phospholipid composition. The right lung was then fixed via the trachea at a distending pressure of 30 cmH_2O with 4% paraformaldehyde (in 0.1 M phosphate buffered saline, pH 7.4) and immersed in fresh 4% paraformaldehyde fixative for 24 h at 4ºC for future histological analysis; however this was not analysed for this study.

5.2.2 Analytical methods

Investigation of sex-differences in prenatal physiological status was achieved by measuring arterial blood gas parameters (pH, PaO_2, $PaCO_2$, SaO_2, lactate and glucose), MAP and HR in fetuses while still *in utero* (see Section 2.4.3.1). Physiological variables were also measured in lambs for 8 h following preterm delivery via caesarean section (see Section 2.4.3.3). The lambs were also monitored for their inspiratory effort and breathing frequency as detailed in Section 2.4.3.3.

At the end of the 8 h of monitoring, the lambs were euthanised and their thoracic cavity exposed for the measurement of static lung compliance as described in Section 2.4.3.4.

Following the necropsy, the major organs were weighed. Left lung tissue was snap frozen in liquid N_2 and was used for measuring the water content of lung tissue (see Section 2.7), the gene expression of *surfactant proteins* using qPCR (see Section 2.8) and protein expression of surfactant proteins using Western blot analysis (see Section 2.9). For each gene and protein of interest, the mRNA levels and protein levels, respectively, obtained for both male and female samples were separately averaged. The mean mRNA and protein levels for the males were

expressed relative to the mean mRNA and protein levels in females for each gene and protein of interest.

BALF samples were collected at post-mortem as outlined in Section 2.4.4.1 and analysed for surfactant phospholipid composition and total protein content, as per Sections 2.10 and 2.11 respectively.

5.2.3 Statistical analysis of data

Sex differences in body weights and dimensions, organ weights, gene and protein expression, total protein content in BALF, static lung compliance and surfactant phospholipid composition in BALF were assessed using the unpaired Student's t-test. Blood gas parameters, MAP and HR during fetal life and in lambs after preterm delivery throughout the 8 h monitoring were analysed using a one-way repeated measures ANOVA, with sex as one factor and time as the repeated measure. In addition, the inspiratory effort and respiratory rate in the postnatal lambs were analysed using ANOVA. Data were analysed over 4 different time periods; 128–133d GA, within 1 h of delivery, 2–4 h and 4–8 h after delivery. These 4 different time periods were selected to determine if there were any sex differences in the parameters measured at gestation and following preterm delivery during the transition period and post-transitional period.

5.3 Results

5.3.1 Weights and dimensions

At necropsy, body weights and dimensions, and organ weights and relative weights were not different between sexes (Table 5.2 and 5.3). Lung weights (wet and dry) and right lung volume were also not different between sexes (Table 5.4).

5.3.2 Blood gas and cardiovascular measurements

5.3.2.1 Prenatal physiological data

Between 128–133d GA, male fetuses had significantly higher $PaCO_2$ (Figure 5.1 B) than females. No significant differences were observed in other parameters measured, including pH (Figure 5.1 A), PaO_2 (Figure 5.2 A), SaO_2 (Figure 5.2 B), glucose concentration (Figure 5.3 A), lactate concentration (Figure 5.3 B), MAP (Figure 5.4 A) and HR (Figure 5.4 B). The overall mean of the physiological parameters in fetal sheep between 128–133d GA are listed in Table 5.1

Table 5.1 Overall mean ± SEM of physiological parameters in fetal sheep between 128–133d GA.

	Female (n=9)	Male (n=8)	Figure
pH	7.376 ± 0.007	7.357 ± 0.0209	5.1 A
$PaCO_2$ (mmHg)	48.3 ± 0.5	50.3 ± 0.8	5.1 B
PaO_2 (mmHg)	22.9 ± 0.8	22.2 ± 1.1	5.2 A
SaO_2 (%)	61.6 ± 3.3	62.0 ± 2.4	5.2 B
Glucose concentration (mmol/L)	1.29 ± 0.06	1.14 ± 0.08	5.3 A
Lactate concentration (mmol/L)	1.87 ± 0.08	1.97 ± 0.11	5.3 B
MAP (mmHg)	48.0 ± 0.9	48.1 ± 2.1	5.4 A
HR (beats/min)	168.2 ± 2.3	164.3 ± 2.2	5.4 B

Statistical analysis of prenatal data between 128-133d GA showed no significant differences between sexes and no interactions between sexes across days of gestation, thus the physiological data were pooled to obtain average values for each sex. Data are represented as mean ± SEM.

5.3.2.2 Postnatal physiological data from 0 to 1 h

Between 128–133d GA, male fetuses had significantly higher $PaCO_2$ than females (Figure 5.1 B). No significant differences were observed in other parameters measured, including pH (Figure 5.1 A), PaO_2 (Figure 5.2 A), SaO_2 (Figure 5.2 B), glucose concentration (Figure 5.3 A), lactate concentration (Figure 5.3 B), MAP (Figure 5.4 A) and HR (Figure 5.4 B).

Supplemental oxygen was given to all lambs to achieve a SaO_2 of 80%; the mean SaO_2 was 78.8 ± 5.1 vs 80.9 ± 3.0% in males and females, respectively, which was not significantly different (Figure 5.2 B). Males were observed to be significantly more dependent on supplemental oxygen than females to reach SaO_2 of 80% (Figure 5.6). Oxygen dependency was assessed by the amount of time the lambs required supplemental oxygen during the monitoring period before they were able to maintain a SaO_2 of 80% on their own.

5.3.2.3 Postnatal physiological data from 1 to 4 h

One male lamb died at 106 min after delivery; therefore data from this animal were excluded at time points between the first hour and the time of death.

From 75 to 360 min after delivery, males continued to have significantly lower pH than females (Figure 5.1 A). $PaCO_2$ became significantly higher in males than in females (Figure 5.1 B). There were no significant differences in PaO_2 (Figure 5.2 A), SaO_2 (Figure 5.2 B), arterial glucose concentration (Figure 5.3 A), lactate concentration (Figure 5.3 B), MAP (Figure 5.4 A), HR (Figure 5.4 B), breathing frequency (Figure 5.5 A) and inspiratory effort (Figure 5.5 B).

5.3.2.4 Postnatal physiological data from 4 to 8 h

From 255 to 480 min after delivery, males continued to have significantly lower pH (Figure 5.1 A) and higher $PaCO_2$ (Figure 5.1 B) than females. In males, arterial

lactate concentration was significantly lower (Figure 5.3 B) and inspiratory effort was significantly higher (Figure 5.5 B) than in females during this period. No sex differences were observed in PaO_2 (Figure 5.2 A), SaO_2 (Figure 5.2 B), glucose concentration (Figure 5.3 A), MAP (Figure 5.4 A), HR (Figure 5.4 B) and breathing frequency (Figure 5.5 A).

5.3.3 Static lung compliance

The lung volume at 40 cmH_2O, relative to body weight, was significantly lower in males than in females (56.9 ± 4.0 vs 75.1 ± 3.2 ml/kg; Figure 5.7).

The pressure-volume relationship (unadjusted) is shown in Figure 5.8. Significantly lower lung volumes were observed in the descending limb of the pressure-volume curve in males than in females. In the ascending limb of male lambs, a significantly lower lung volume, compared to females, only occurred at a pressure of 30 cmH_20.

5.3.4 Surfactant protein gene and protein expression

Male and female lambs had similar gene expression of *SP-A*, *-B*, *-C* and *-D* in lung tissue: *SP-A* (Males (M): 1.3 ± 0.2 vs Females (F): 1.0 ± 0.2), *SP-B* (M: 1.1 ± 0.2 vs F: 1.0 ± 0.3), *SP-C* (M: 0.7 ± 0.1 vs F: 1.0 ± 0.2) and *SP-D* (M: 1.5 ± 0.2 vs F: 1.0 ± 0.1) (Figure 5.9).

No significant difference was observed in SP-A protein expression between male and female lambs (Figure 5.10 A). When protein levels of pro-SP-C (Figure 5.10 B) were measured, a significant reduction of 24% was observed in males (0.8 ± 0.1) compared with females (1.0 ± 0.1).

5.3.5 Surfactant phospholipid composition

In BALF, the relative proportions of the phospholipid classes (S, PC, LPC, PE, PI, PS and PG) were not different between males and females at 8 h following preterm delivery (Figure 5.11 A). Males had significantly lower proportions of PC 32:1 and PE 36:2 (Figure 5.11 C and 5.11 E respectively). However, no differences were observed in the proportion of species in S (Figure 5.11 B), LPC (Figure 5.11 D), PI (Figure 5.11 F), PS (Figure 5.11 G) and PG (Figure 5.11 H). The ratios of PC/PE and PC/S were not significantly different between males and females (Figure 5.12).

5.3.6 Protein concentration of BALF

The total protein concentration in BALF collected at necropsy was similar between males and females (M: 2.94 ± 0.33 vs F: 3.28 ± 0.43 mg/ml; Figure 5.13).

Table 5.2 Body weight and dimensions in female and male lambs

	Female (n=7)	Male (n=6)	P value
Dry body weight (kg)	4.04 ± 0.18	4.12 ± 0.21	0.768
Crown rump length (cm)	51.1 ± 2.4	53.9 ± 1.6	0.378
Thoracic girth (cm)	37.1 ± 0.6	36.4 ± 1.2	0.615
Hind leg length (cm)	47.5 ± 1.4	51.9 ± 2.8	0.167
Head length (cm)	13.5 ± 0.3	13.3 ± 0.3	0.611
Ponderal index (g/cm^3 x 100)	3.2 ± 0.5	2.7 ± 0.3	0.534

Data represent group mean ± SEM values.

Table 5.3 Necropsy organ weights and relative weights in female and male lambs

	Female (n=7)	Male (n=6)	P value
Heart (g)	32.23 ± 2.03	30.27 ± 1.13	0.439
Heart/Body weight (BW) (g/kg)	7.96 ± 0.28	7.39 ± 0.24	0.101
Liver (g)	132.55 ± 11.47	130.68 ± 14.43	0.920
Liver/BW (g/kg)	32.64 ± 1.85	31.43 ± 2.79	0.718
Kidney (g)	30.51 ± 1.64	31.79 ± 2.49	0.669
Kidney/BW (g/kg)	7.5 ± 0.2	7.7 ± 0.4	0.836
Adrenal (g)	0.6 ± 0.06	0.5 ± 0.05	0.534
Adrenal/BW (g/kg)	0.14 ± 0.01	0.12 ± 0.01	0.237
Spleen (g)	7.44 ± 0.79	6.23 ± 0.73	0.290
Spleen/BW (g/kg)	1.86 ± 0.21	1.49 ± 0.14	0.196

Data represent group mean ± SEM values.

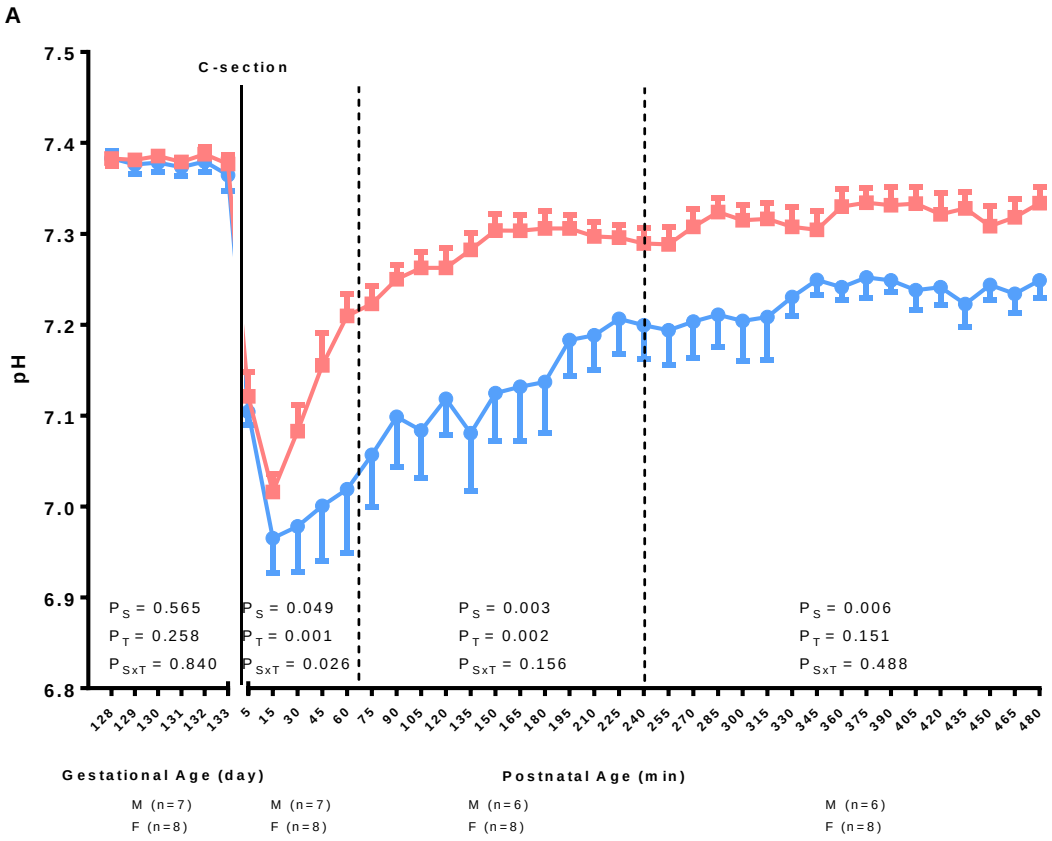
Table 5.4 Lung weights and relative weights in female and male lambs

	Female (n=7)	Male (n=6)	P value
Wet lung weight (g)	134.0 ± 4.8	152.6 ± 21.2	0.945
Wet lung weight/BW (g/kg)	33.3 ± 1.1	36.6 ± 3.8	1.000
Dry lung weight (g)	19.8 ± 1.2	21.2 ± 1.2	0.438
Dry lung weight/BW (g/kg)	5.0 ± 0.3	5.1 ± 0.1	0.798
Lung volume (cm^3)	119.0 ± 6.9	124.9 ± 14.2	0.703

Data represent group mean ± SEM values.

Figure 5.1 Arterial pH and $PaCO_2$ before and for 8 hours after preterm delivery.

Values for arterial pH and arterial partial pressure of CO_2 ($PaCO_2$) on gestational days 128–133 and for up to 480 min following caesarean-section at 133 days of gestational age in female (pink) and male (blue) fetuses and lambs. Separate one-way repeated ANOVAs were performed 1) before delivery, 2) between 0 and 60 min after delivery, and 3) between 75 and 240 min after delivery, and 4) 255 and 480 min after delivery. P values: P_S is between factor variable (sex), P_T is repeated variable (time), and P_{SxT} is the interaction term. Data are shown as means ± SEM.


A
C-section
pH
7.5
7.4
7.3
7.2
7.1
7.0
6.9
6.8
P S = 0.565
P T = 0.258
P SxT = 0.840
P S = 0.049
P T = 0.001
P SxT = 0.026
P S = 0.003
P T = 0.002
P SxT = 0.156
P S = 0.006
P T = 0.151
P SxT = 0.488
128 129 130 131 132 133
5 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375 390 405 420 435 450 465 480
Gestational Age (day)
Postnatal Age (min)
M (n=7)
F (n=8)
M (n=7)
F (n=8)
M (n=6)
F (n=8)
M (n=6)
F (n=8)

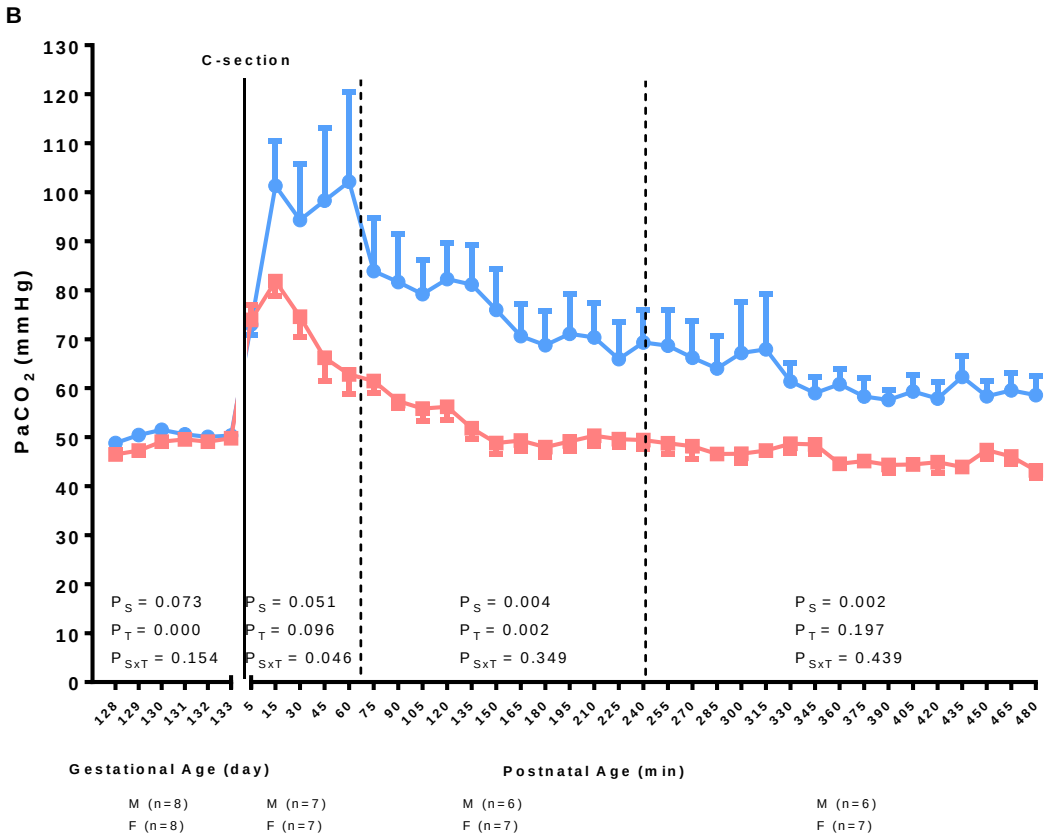
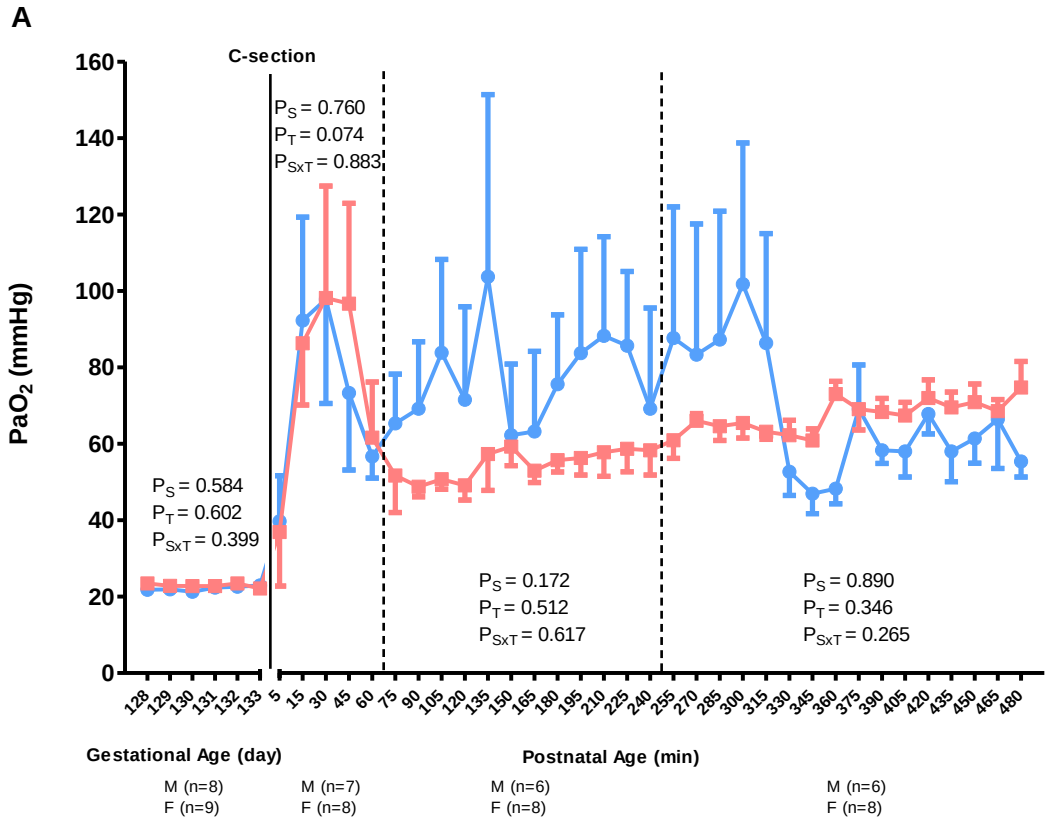

B
C-section
PaCO2 (mmHg)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
P S = 0.073
P T = 0.000
P SxT = 0.154
P S = 0.051
P T = 0.096
P SxT = 0.046
P S = 0.004
P T = 0.002
P SxT = 0.349
P S = 0.002
P T = 0.197
P SxT = 0.439
128 129 130 131 132 133
5 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375 390 405 420 435 450 465 480
Gestational Age (day)
Postnatal Age (min)
M (n=8)
F (n=8)
M (n=7)
F (n=7)
M (n=6)
F (n=7)
M (n=6)
F (n=7)

Figure 5.2 Arterial PaO_2 and SaO_2 before and for 8 hours after preterm delivery.

Values for arterial partial pressure of O_2 (PaO_2) and arterial saturation of O_2 (SaO_2) on gestational days 128–133 and for up to 480 min following caesarean-section at 133 days of gestational age in female (pink) and male (blue) fetuses and lambs. Separate one-way repeated ANOVAs were performed 1) before delivery, 2) between 0 and 60 min after delivery, and 3) between 75 and 240 min after delivery, and 4) 255 and 480 min after delivery. P values: P_S is between factor variable (sex), P_T is repeated variable (time), and P_{SxT} is the interaction term. Data are shown as means ± SEM.


A
C-section
PaO2 (mmHg)
160
140
120
100
80
60
40
20
0
PS = 0.760
PT = 0.074
PSxT = 0.883
PS = 0.584
PT = 0.602
PSxT = 0.399
PS = 0.172
PT = 0.512
PSxT = 0.617
PS = 0.890
PT = 0.346
PSxT = 0.265
128 129 130 131 132 133 5 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375 390 405 420 435 450 465 480
Gestational Age (day)
Postnatal Age (min)
M (n=8)
F (n=9)
M (n=7)
F (n=8)
M (n=6)
F (n=8)
M (n=6)
F (n=8)

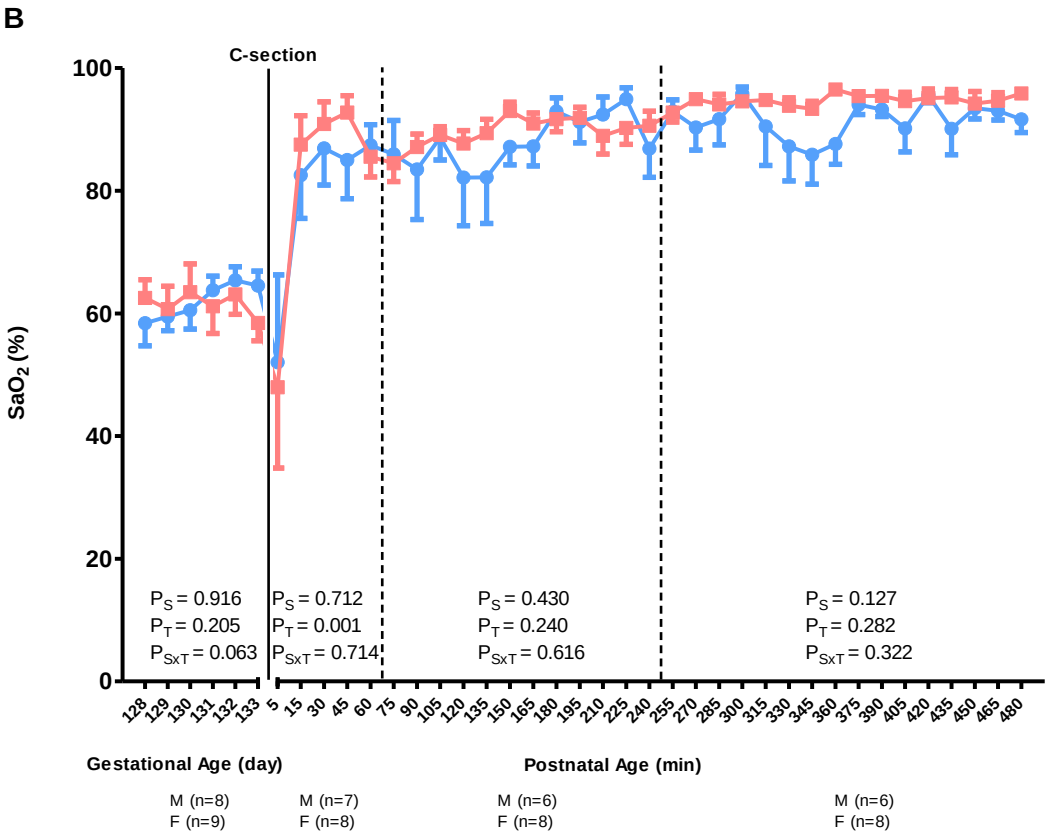
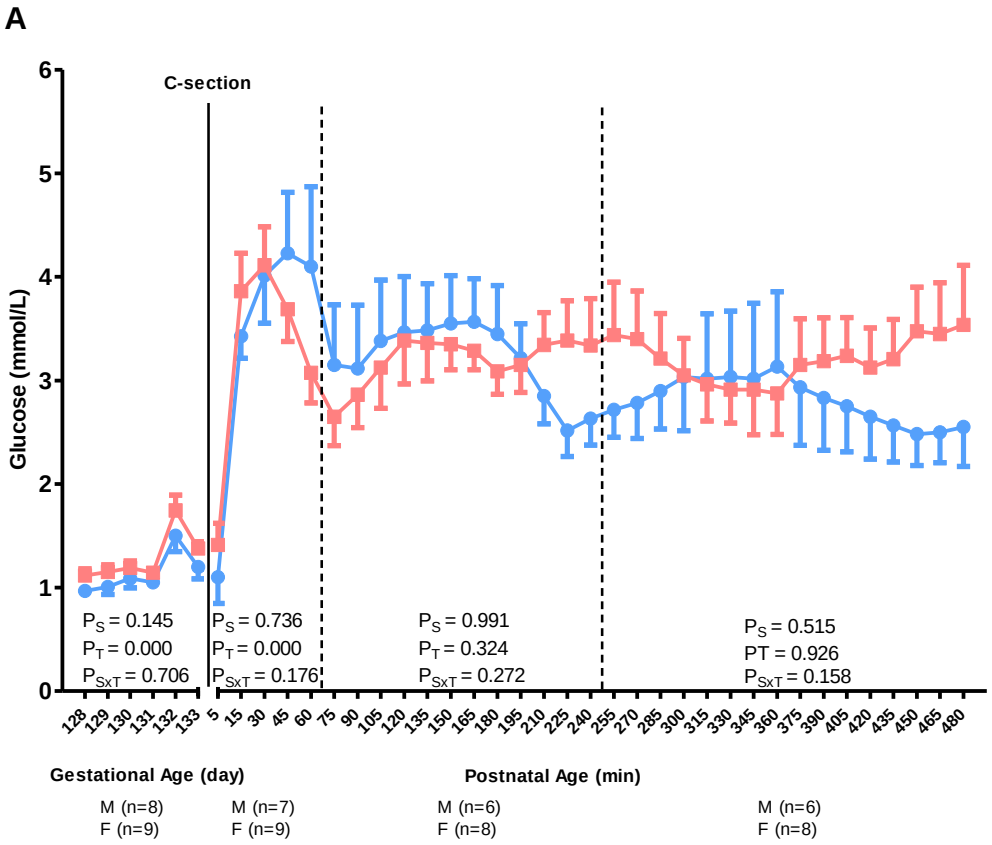

B
C-section
SaO2 (%)
100
80
60
40
20
0
PS = 0.916
PT = 0.205
PSxT = 0.063
PS = 0.712
PT = 0.001
PSxT = 0.714
PS = 0.430
PT = 0.240
PSxT = 0.616
PS = 0.127
PT = 0.282
PSxT = 0.322
128 129 130 131 132 133 5 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 315 330 345 360 375 390 405 420 435 450 465 480
Gestational Age (day)
Postnatal Age (min)
M (n=8)
F (n=9)
M (n=7)
F (n=8)
M (n=6)
F (n=8)
M (n=6)
F (n=8)

Figure 5.3 Blood glucose and lactate concentration before and for 8 hours after preterm delivery.

Values for arterial concentrations of glucose and lactate on gestational days 128–133 and for up to 480 min following caesarean-section at 133 days of gestational age in female (pink) and male (blue) fetuses and lambs. Separate one-way repeated ANOVAs were performed 1) before delivery, 2) between 0 and 60 min after delivery, and 3) between 75 and 240 min after delivery, and 4) 255 and 480 min after delivery. P values: P_S is between factor variable (sex), P_T is repeated variable (time), and P_{SxT} is the interaction term. Data are shown as means ± SEM.


A
C-section
Glucose (mmol/L)
P_S = 0.145
P_T = 0.000
P_{SxT} = 0.706
P_S = 0.736
P_T = 0.000
P_{SxT} = 0.176
P_S = 0.991
P_T = 0.324
P_{SxT} = 0.272
P_S = 0.515
PT = 0.926
P_{SxT} = 0.158
Gestational Age (day)
Postnatal Age (min)
M (n=8)
F (n=9)
M (n=7)
F (n=9)
M (n=6)
F (n=8)
M (n=6)
F (n=8)

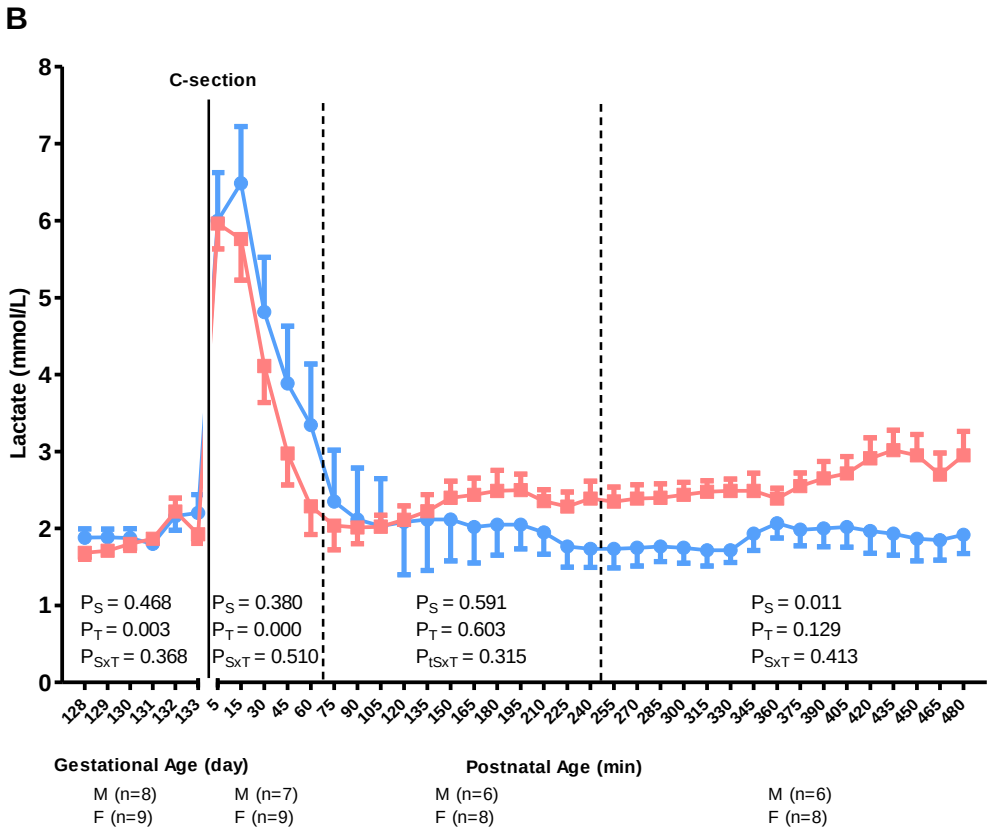
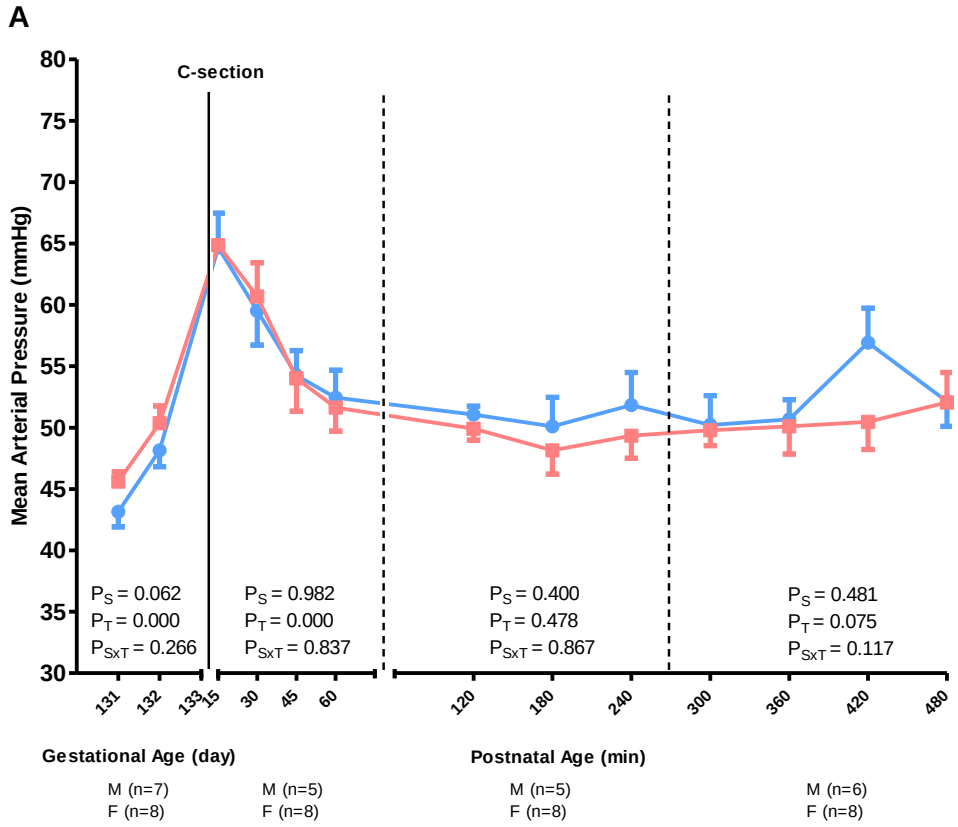

B
C-section
Lactate (mmol/L)
P_S = 0.468
P_T = 0.003
P_{SxT} = 0.368
P_S = 0.380
P_T = 0.000
P_{SxT} = 0.510
P_S = 0.591
P_T = 0.603
P_{tSxT} = 0.315
P_S = 0.011
P_T = 0.129
P_{SxT} = 0.413
Gestational Age (day)
Postnatal Age (min)
M (n=8)
F (n=9)
M (n=7)
F (n=9)
M (n=6)
F (n=8)
M (n=6)
F (n=8)

Figure 5.4 Mean arterial pressure and heart rate before and for 8 hours after preterm delivery.

Values for mean arterial pressure and heart rate on gestational days 131–132 and for up to 480 min following caesarean-section at 133 days of gestational age in female (pink) and male (blue) fetuses and lambs. Separate one-way repeated ANOVAs were performed 1) before delivery, 2) between 0 and 60 min after delivery, and 3) between 75 and 240 min after delivery, and 4) 255 and 480 min after delivery. P values: P_S is between factor variable (sex), P_T is repeated variable (time), and P_{SxT} is the interaction term. Data are shown as means ± SEM.


A
C-section
Mean Arterial Pressure (mmHg)
80
75
70
65
60
55
50
45
40
35
30
P_S = 0.062
P_T = 0.000
P_{SxT} = 0.266
P_S = 0.982
P_T = 0.000
P_{SxT} = 0.837
P_S = 0.400
P_T = 0.478
P_{SxT} = 0.867
P_S = 0.481
P_T = 0.075
P_{SxT} = 0.117
131
132
133
15
30
45
60
120
180
240
300
360
420
480
Gestational Age (day)
Postnatal Age (min)
M (n=7)
F (n=8)
M (n=5)
F (n=8)
M (n=5)
F (n=8)
M (n=6)
F (n=8)

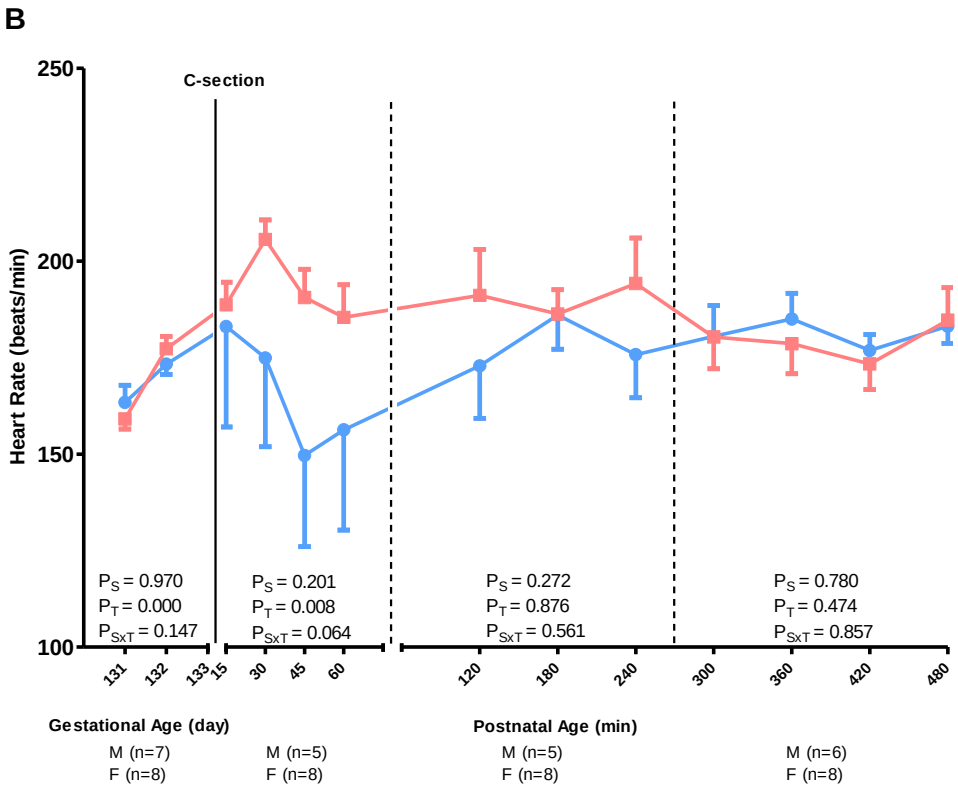
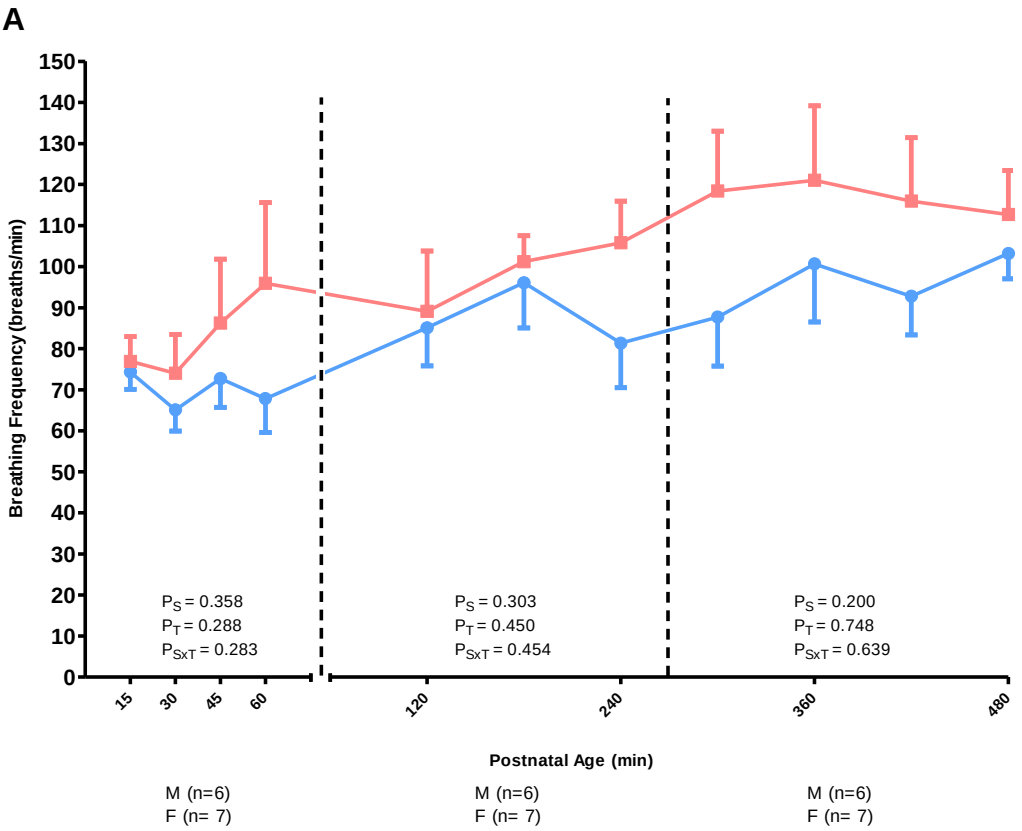
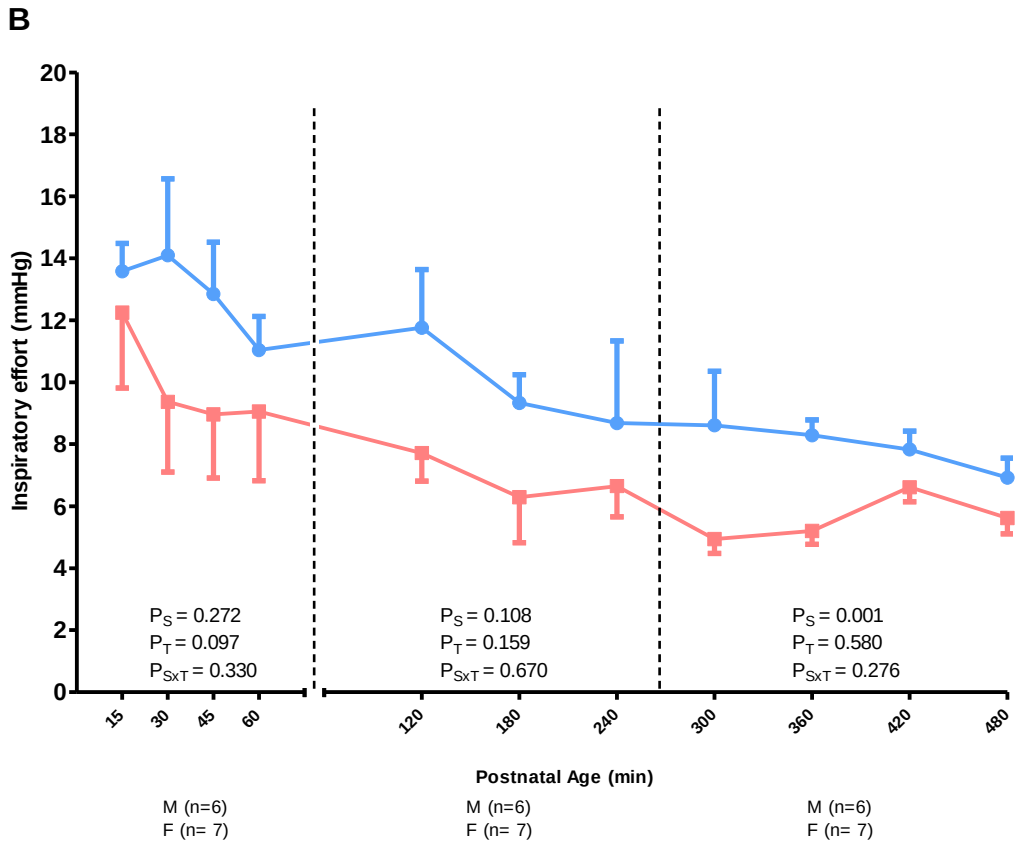

B
C-section
Heart Rate (beats/min)
250
200
150
100
P_S = 0.970
P_T = 0.000
P_{SxT} = 0.147
P_S = 0.201
P_T = 0.008
P_{SxT} = 0.064
P_S = 0.272
P_T = 0.876
P_{SxT} = 0.561
P_S = 0.780
P_T = 0.474
P_{SxT} = 0.857
131
132
133
15
30
45
60
120
180
240
300
360
420
480
Gestational Age (day)
Postnatal Age (min)
M (n=7)
F (n=8)
M (n=5)
F (n=8)
M (n=5)
F (n=8)
M (n=6)
F (n=8)

Figure 5.5 Breathing frequency and inspiratory effort for 8 hours following preterm delivery.

Values for breathing frequency and inspiratory effort following caesarean-section at 133 days of gestational age in female (pink) and male (blue) lambs. Separate one-way repeated ANOVAs were performed 1) between 0 and 60 min after delivery, and 2) between 75 and 240 min after delivery, and 3) 255 and 480 min after delivery. P values: P_S is between factor variable (sex), P_T is repeated variable (time), and P_{SxT} is the interaction term. Data are shown as means ± SEM.


A
Breathing Frequency (breaths/min)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
P_S = 0.358
P_T = 0.288
P_{SxT} = 0.283
P_S = 0.303
P_T = 0.450
P_{SxT} = 0.454
P_S = 0.200
P_T = 0.748
P_{SxT} = 0.639
15
30
45
60
120
240
360
480
Postnatal Age (min)
M (n=6)
F (n= 7)
M (n=6)
F (n= 7)
M (n=6)
F (n= 7)


B
Inspiratory effort (mmHg)
20
18
16
14
12
10
8
6
4
2
0
P_S = 0.272
P_T = 0.097
P_{SxT} = 0.330
P_S = 0.108
P_T = 0.159
P_{SxT} = 0.670
P_S = 0.001
P_T = 0.580
P_{SxT} = 0.276
15
30
45
60
120
180
240
300
360
420
480
Postnatal Age (min)
M (n=6)
F (n= 7)
M (n=6)
F (n= 7)
M (n=6)
F (n= 7)

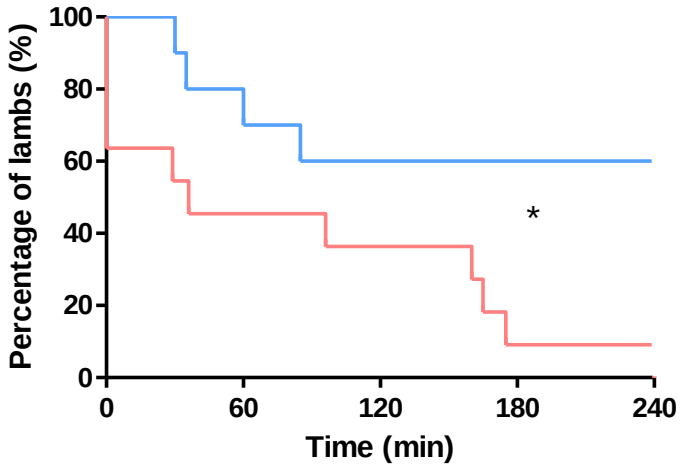


Figure 5.6 Requirement for supplemental oxygen following preterm delivery.

The figure shows the percentage of female (pink; n=8) and male (blue; n=8) lambs that required supplemental oxygen at given times after delivery. *P < 0.05, female *vs*. male.

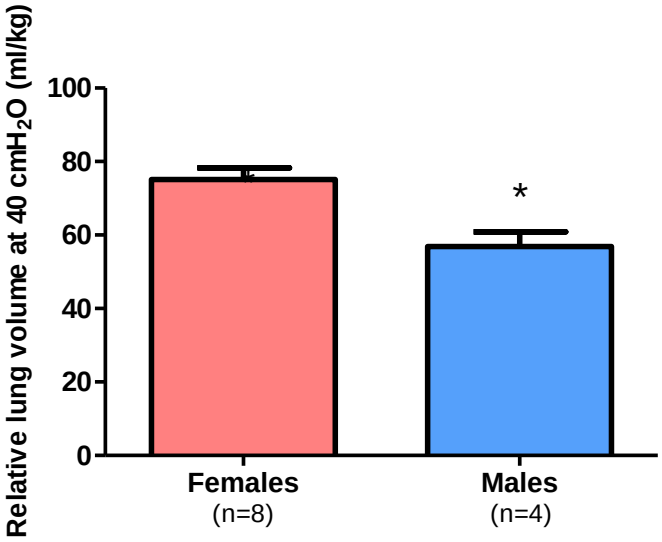


Figure 5.7 Lung volume measured at 40 cmH_2O per kg body weight.

Lung volume, relative to body weight, measured at 40 cmH_2O at 8 hours after birth in female (pink) and male (blue) lambs. Data are represented as means ± SEM. *P < 0.05, female *vs*. male.

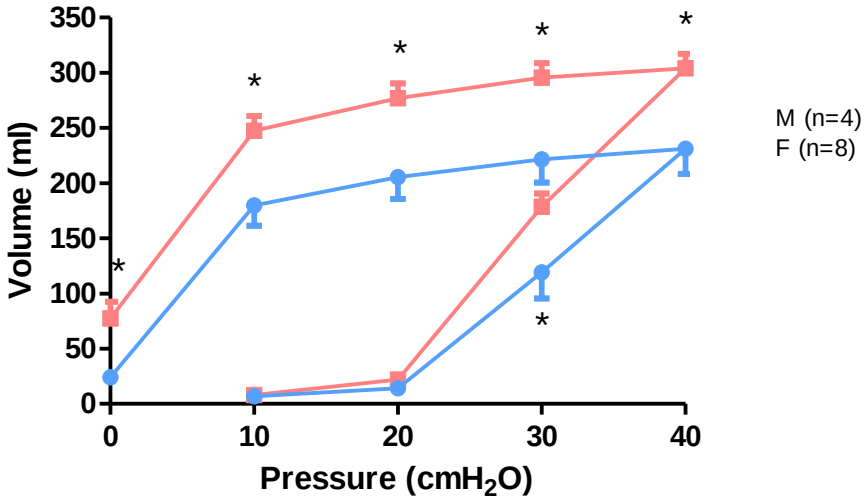


Figure 5.8 Pressure-volume (PV) relationship in lungs of male and female lambs.

Plot of pressure versus volume measured at 8 hours after birth in lungs of female (pink) and male (blue) lambs. Data are represented as means ± SEM. *P < 0.05, female *vs*. male.

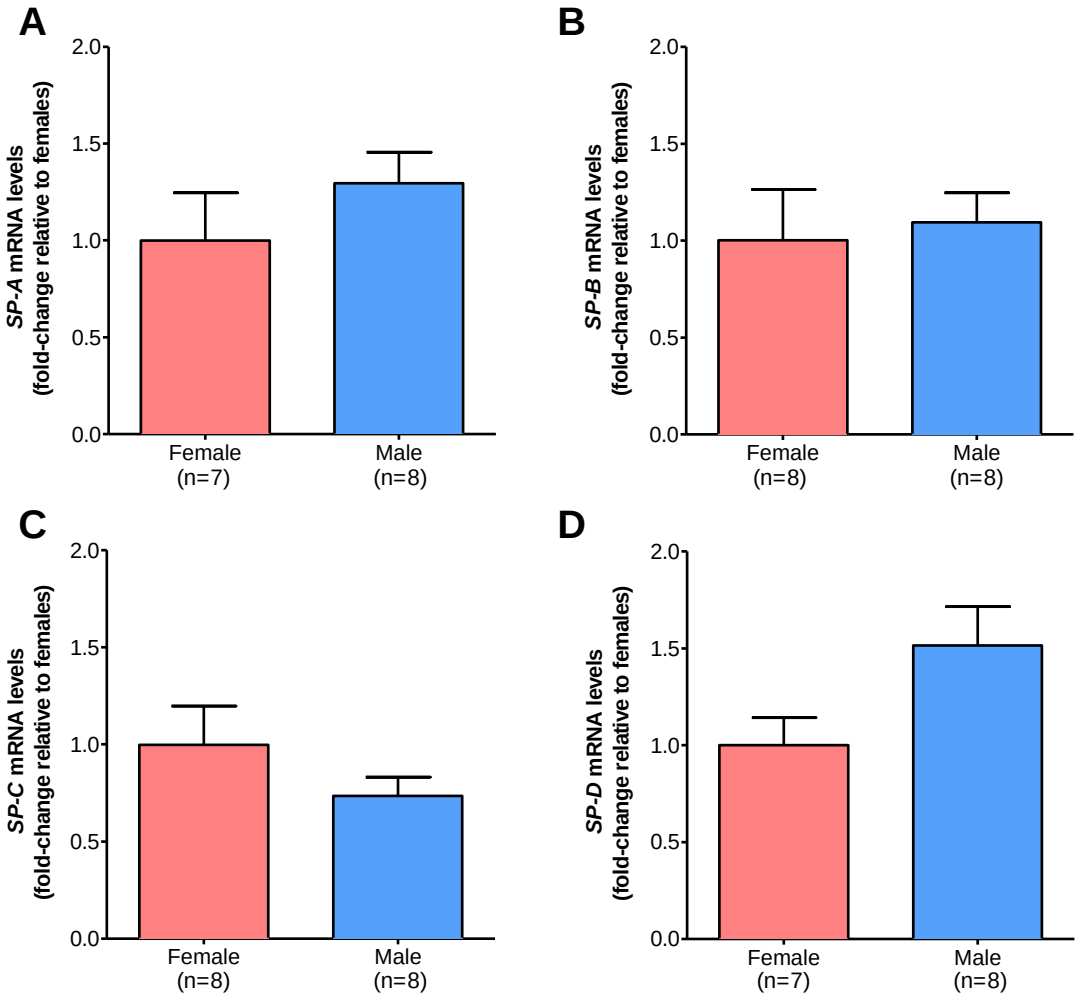


Figure 5.9 *Surfactant protein* (*SP*) gene expression in the lung.

(A) to (D) show the *SP-A*, *-B*, *-C* and *-D* expression in lung tissue at 8 hours after birth in female (pink) and male (blue) lambs. Data are represented as means ± SEM.

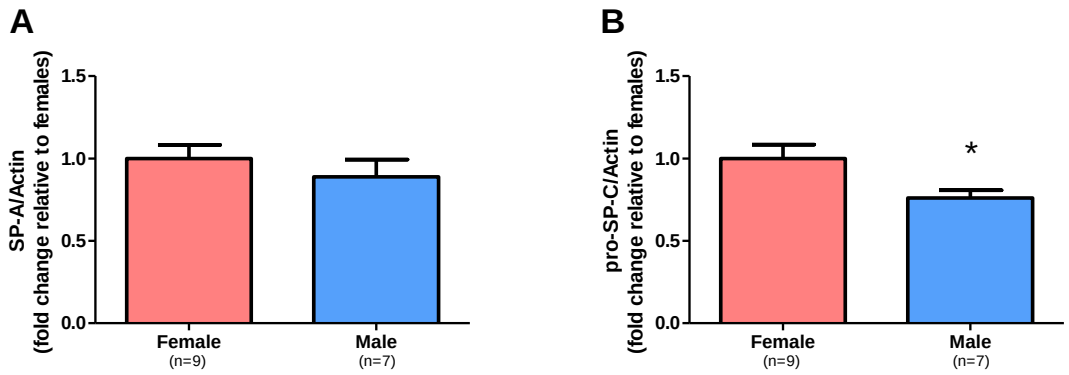
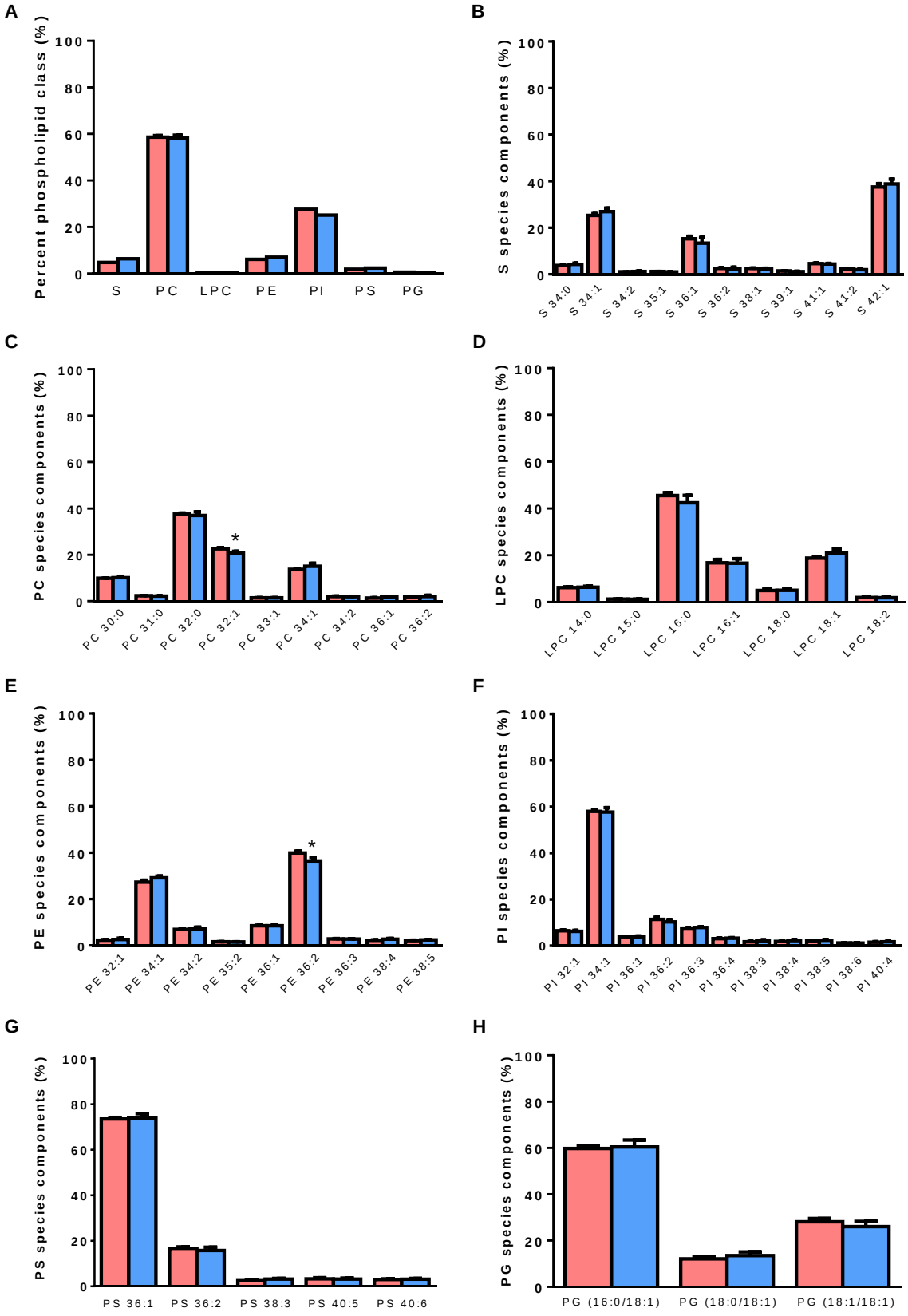


Figure 5.10 SP-A and pro-SP-C expression in the lung. (A) shows SP-A and (B) shows pro-SP-C expression in lung tissue at 8 hours after birth in female (pink) and male (blue) lambs. Data are represented as means ± SEM. *P < 0.05, female *vs*. male.

Figure 5.11 Proportions of major surfactant phospholipid classes and species in bronchoalveolar lavage fluid.

(A) shows the proportions of the phospholipid classes (S, PC, LPC, PE, PI, PS and PG) compared to total phospholipid in the BALF following 8 hours of preterm delivery at 133 days of gestation. (B) to (H) show the proportions of the phospholipid molecular species for S, PC, LPC, PE, PI, PS and PG. Females (n=9) and males (n=8) are represented in pink and blue bars respectively. Data are shown as mean ± SEM. *$P < 0.05$, female *vs*. male.


A
Percent phospholipid class (%)
S
PC
LPC
PE
PI
PS
PG
B
S species components (%)
S 34:0
S 34:1
S 34:2
S 35:1
S 36:1
S 36:2
S 38:1
S 39:1
S 41:1
S 41:2
S 42:1
C
PC species components (%)
PC 30:0
PC 31:0
PC 32:0
PC 32:1
PC 33:1
PC 34:1
PC 34:2
PC 36:1
PC 36:2
D
LPC species components (%)
LPC 14:0
LPC 15:0
LPC 16:0
LPC 16:1
LPC 18:0
LPC 18:1
LPC 18:2
E
PE species components (%)
PE 32:1
PE 34:1
PE 34:2
PE 35:2
PE 36:1
PE 36:2
PE 36:3
PE 38:4
PE 38:5
F
PI species components (%)
PI 32:1
PI 34:1
PI 36:1
PI 36:2
PI 36:3
PI 36:4
PI 38:3
PI 38:4
PI 38:5
PI 38:6
PI 40:4
G
PS species components (%)
PS 36:1
PS 36:2
PS 38:3
PS 40:5
PS 40:6
H
PG species components (%)
PG (16:0/18:1)
PG (18:0/18:1)
PG (18:1/18:1)

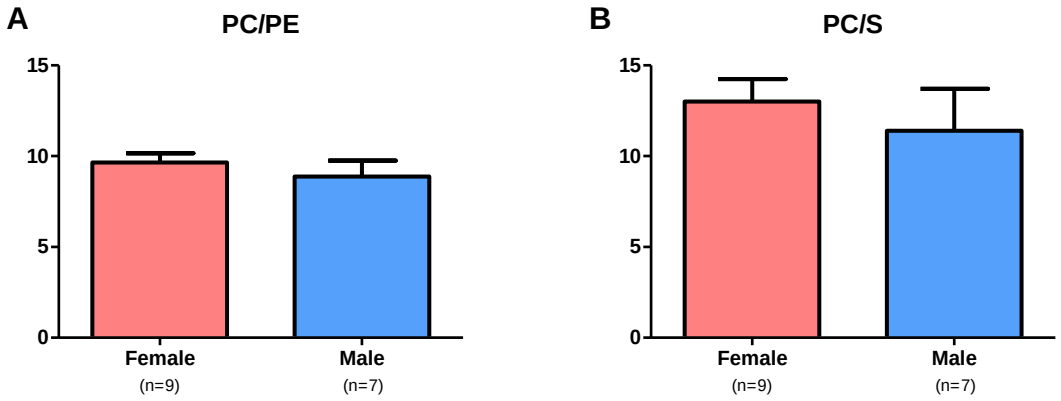


Figure 5.12 Ratio of PC/PE and PC/S in bronchoalveolar lavage fluid.

The ratios of PC/PE (A) and PC/S (B) in the BALF following 8 hours of preterm delivery at 133 days of gestation. Females and males are represented in pink and blue bars respectively. Data are shown as mean ± SEM.

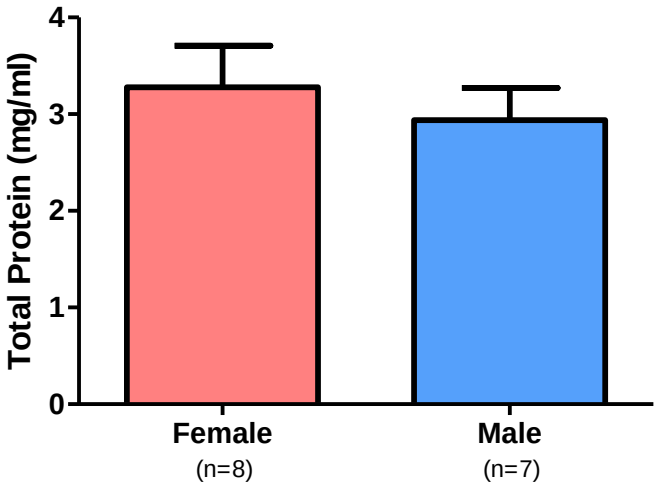


Figure 5.13 Protein concentration in bronchoalveolar lavage fluid after birth.

The figure shows the total protein concentration in BALF at 8 hours after birth in female (pink) and male (blue) lambs. Data are represented as means ± SEM.

5.4 Discussion

The purpose of this study was to further identify sex-related differences in the cardiorespiratory adaptation to preterm birth. The rationale for extending our study to 8 h was that a further reduction in male survival following 4 h of preterm delivery was observed by De Matteo et al. (2010). This study was aimed at identifying the causes that contribute to the poorer outcome in male lambs following that time point. The main findings of this study were that following 4 h of preterm delivery, inspiratory efforts in male lambs were greater than those of female lambs and male lungs continued to be less compliant than female lungs. The BALF of males collected at 8 h after birth had significantly lower proportions of the molecular species PC 32:1 and PE 36:2. In lung tissue, pro-SP-C protein expression was significantly lower in male lambs than in females. The sex differences observed in surfactant composition could have contributed to the lower lung compliance of males. It is likely that the lower lung compliance in male lambs contributed to greater inspiratory efforts and poorer gas exchange (lower pH and higher $PaCO_2$) in males. Arterial lactate was unexpectedly lower in males than females between 4 and 8 h after preterm delivery.

5.4.1 Weights and lung volume

5.4.1.1 Lung and body weights

The lack of differences in body or lung weights in this study is consistent with the findings in the preterm lambs reported in Chapter 4. Similarly, we observed no sex differences in lung or body weights in the fetal sheep reported in Chapter 3. In contrast to the present study, Willet et al. (1997) reported an 8% reduction in wet lung weight in female lambs at 128d GA compared to males. In the present study, the wet lung weight was measured at 8 h after delivery when the lungs are likely to have been cleared of lung liquid; in contrast the wet lung weight in the study by Willet et al. (1997) was measured following 40 min of mechanical ventilation where the lung is still undergoing lung liquid clearance, which could explain the differences in findings. However, when wet lung weight was related to body weight

in the fetuses in the present study and in the study by Willet et al. (1997) there were no differences between sexes.

In human fetuses, females, on average, weigh ~2–3% less than age-matched males throughout most of gestation (Gerards et al., 2006). This sex difference in body weight appears to be more significant before the third trimester (26 weeks of gestation) and becomes less obvious closer to term (Gerards et al., 2006). This could explain the lack of difference in body weights that we observed in our fetal sheep as our animal model is representative of moderate preterm birth (0.9 of term) and thus may have passed the stage of gestation at which sex differences in body weight might have been more obvious. Our finding on body growth, however, is consistent with other studies in sheep which did not observe a sex difference in birth weight in lambs born in late gestation or at term (De Matteo et al., 2010, Polglase et al., 2012) and also in the fetal sheep described in Chapter 3 and lambs described in Chapter 4. In contrast, Willet et al. (1997) reported a 10% lower birth weight in female lambs at 128d GA compared to males, which could account for the sex differences in lung weight observed in their study as lung weight relative to body weight was not significantly different between sexes.

5.4.1.2 Lung Volume

In this study, lung volume assessed histologically was not different between male and female lambs. This is consistent with our finding in the preterm lambs in Chapter 4 and fetal sheep in Chapter 3. Willet et al. (1997) also observed similar excised lung volume in both male and female lambs delivered at 128d GA following 40 min of ventilation. In humans, a significant sex difference was found in fetal lung volume from 18 to 34 weeks of gestation by using three-dimensional ultrasound (Gerards et al., 2006); the mean lung volume of male fetuses was, on average, 4.3% greater than in females. However, the difference in mean lung volume was eliminated when adjusted for estimated fetal weight. Another study assessed the lungs of human fetuses by fast spin-echo T2-weighted lung magnetic resonance (MR) imaging at 21 to 38 weeks of gestation; the authors demonstrated

a significant sex difference in human fetal lung volume after 35 weeks of gestation, with males having a greater lung volume than females (Rypens et al., 2001). A histopathological study of human fetuses observed no sex differences in lung volumes between 19 and 40 weeks of gestation (Langston et al., 1984). A possible explanation for the varying results could be the different methods used to measure lung volume. In the present study, as well as the studies by Willet et al. (1997) and Langston et al. (1984), the Cavalieri method was used to assess lung volume using histologically prepared lung tissue whereas Gerards et al. (2006) and Rypens et al. (2001) used ultrasound and MR imaging respectively to obtain lung volume while the fetuses were still *in utero*. Although sex differences in lung volume in fetuses and infants are not well-established they have been documented in children and adults (Rypens et al., 2001). Postnatally, boys have larger lungs than girls 6 weeks after birth to 14 years of age (Thurlbeck, 1982) which persists into adulthood (Bellemare et al., 2003). Adult male mice are also found to have larger lung volumes than females (Massaro and Massaro, 2006), but these differences are likely a result of males being larger.

5.4.2 Prenatal physiology

There were no sex differences in prenatal arterial blood gas data (pH, $PaCO_2$, SaO_2, lactate and glucose) and MAP and HR obtained in fetal sheep. These data were similarly seen in the fetal sheep reported in Chapter 4. There is a lack of published information on sex differences on blood gas status and fetal cardiovascular parameters.

5.4.3 Postnatal physiology

Male lambs consistently had lower arterial pH and higher $PaCO_2$ throughout the 8 h following preterm delivery. This indicates that gas exchange in male lambs was less effective than in females; the lower arterial pH of males is likely a result of elevated $PaCO_2$, which in turn is a reflection of poor alveolar ventilation, poor alveolar blood flow or both of these.

Although impaired gas exchange would be expected to result in hypoxemia, in the 8 h after preterm birth PaO_2 and SaO_2 values were similar in male and female lambs. Oxygenation (PaO_2 and SaO_2) was not different between sexes because oxygen was administered to promote survival of the lambs during the 8 h postnatal period. The high variability in PaO_2 in the males is due to their greater requirement for breathing assistance in the form of supplemental oxygen support. This observation is consistent with findings from the 4 h study group. Although SaO_2 was maintained above 80% in both male and female lambs, a survival rate of 100% was seen in female lambs but 1 out of 7 male lambs had to be euthanised following the first hour of delivery as a result of poor physiological condition. Similarly, 2 out of 10 male lambs in the 4 h study had to be euthanised, while females had a survival rate of 100%. The higher mortality of male lambs in comparison to female lambs in both the 4 h and 8 h studies is similar to a previous finding in preterm lambs (De Matteo et al., 2010).

Inspiratory effort was much greater in males compared to females in the 4–8 h after preterm delivery. This is probably a result of males having less compliant lungs which makes it more difficult to achieve the same level of alveolar ventilation. That is, to achieve the same tidal volume the males would have to make a greater inspiratory effort.

Female lambs presented with higher blood lactate concentrations between 4 and 8 h after preterm delivery. This observation was unexpected. The higher level of lactate suggests anaerobic metabolism in tissues of female lambs as a result of poor blood flow. Oxygenated tissue may still receive insufficient oxygen for their energy needs because of the poor blood flow.

In this study, we infused glucose to prevent hypoglycaemia in the lambs. No sex differences in glucose concentrations following preterm delivery were observed, indicating a similar metabolic rate in males and females.

5.4.4 Surfactant composition in BALF

5.4.4.1 Surfactant phospholipid composition of BALF

LPC is produced from the breakdown of PC (Agassandian and Mallampalli, 2013, Goss et al., 2013) and has toxic effects on lung function even in small amounts (Grossmann et al., 1999). LPC, at high levels in the alveolar spaces, may destabilise alveoli at the end of expiration by fluidising the surface film and also increasing lung permeability, thus contributing to leakage of plasma protein into the alveolar spaces (Holm et al., 1991, Holm et al., 1999). We found no sex differences in the proportion of LPC or in the proportion of LPC species in the BALF, 8 h after birth. Therefore, potential differences in LPC could not explain the male disadvantage in respiratory function in males. Fetal lung liquid that was analysed (Chapter 3) did not show any sex difference in the proportion of LPC. To my knowledge, sex difference in the proportion of LPC in pulmonary surfactant has not been investigated.

PC is the most abundant phospholipid class in the BALF samples and this is consistent with other studies in the literature (Veldhuizen et al., 1998, Akella and Deshpande, 2013, Goss et al., 2013) and from my previous findings in BALF samples collected following 4 h of preterm delivery (Chapter 4). However, no sex differences were observed in the proportion of the phospholipid class PC. PC is comprised mainly of PC 32:0, commonly known as DPPC, and this is the only surface active component which is capable of lowering the surface tension in the lung to near zero levels (Veldhuizen et al., 1998, Bernhard et al., 2001, Agassandian and Mallampalli, 2013, Goss et al., 2013). Previously, we demonstrated a significantly lower proportion of PC in relation to total phospholipids in the males compared to females following 4 h of preterm delivery. The absence of a sex difference in the proportion of PC in the BALF samples collected after 8 h of preterm delivery is not understood but it could be due to the use of a “Neopuff” with continuous positive airway pressure (CPAP) rather than via a face mask and self-inflating resuscitation bag (Chapter 4). In this study, the use of a Neopuff with CPAP could be less injurious in comparison to the use of a face

mask and self-inflating resuscitation bag where CPAP is absent. CPAP allows air to be retained in the lung by keeping the alveoli open and thus prevents the alveoli collapsing. This in turn allows for a reduced work of breathing and reduced injury due to less mechanical stress (shear stress) from the collapsing and re-expansion of alveoli.

PC 32:1 (PC 16:0/16:1) is the next major PC following PC 32:0. The specific function of PC 32:1 is unknown. It is found in abundance in lung surfactant and is not prominent in other organs. PC 16:0/16:1 was demonstrated by Bernhard et al. (2001) to be directly correlated to the respiratory rates of mammals. At term, the respiratory rate in human babies is highest, with proportions of PC16:0/16:1 the greatest in relation to PC 16:0/16:0 (Bernhard et al., 2001). In our study, we observed a higher proportion of PC16:0/16:1 in the females than in the males but this was not accompanied by any sex difference in the respiratory rate. However, the inspiratory effort in males was higher than in females during this period, characteristic of RDS. A comparison of the breathing frequency in the lambs used in the 4 and 8 h studies demonstrated a higher rate in the lambs at 8 h following preterm birth, indicating respiratory distress. However, the proportions of PC 16:0/16:1 were similar in both studies.

These findings demonstrate the complexity of pulmonary surfactant; a number of components such as the main PC 32:0, other phospholipids and proteins contribute to the dynamic properties of the surfactant. Therefore, it will be challenging to determine the factors responsible for sex differences in respiratory function.

PC 32:1 is also capable of suppressing superoxide production by phagocytic cells (Ahuja et al., 1996). Superoxide is important as a host defence mechanism for killing invading pathogens, but an excess may be detrimental as it can cause lung inflammation, injury, edema and dysfunction (Vlahos et al., 2012). An increased

secretion of PC 32:1 into the alveolar spaces of the female lung suggests a greater ability to suppress inflammatory processes in the developing lungs. SP-A also has a role in immune defence in the lung and is known to stimulate the "respiratory burst" of alveolar macrophages (Crouch, 1998). Respiratory burst is the abrupt release of reactive oxygen species from cells due to a marked increase in metabolic activity during degradation of foreign material as a host response, but this process may also result in secondary damage to host tissues (Chen and Junger, 2012). However, no difference in level of SP-A protein was observed between males and females. See Section 5.4.5.3 for detailed discussion.

5.4.4.2 Phospholipid maturity ratio in BALF

In our previous study (Chapter 4), lecithin (also referred to as PC) and sphingomyelin could not be measured; therefore, PC/PE ratio was measured as an indicator of lung maturity as a substitute to measuring the commonly calculated L/S ratio. PC is also referred to as lecithin because lecithin has a large PC content. PE is similar to S, as they are both considered to be membrane lipids that are ejected from some of the membrane of lamellar bodies during exocytosis of surfactant into the liquid lining the alveoli (Veldhuizen et al., 1998); they both remain relatively unchanged throughout gestation and are therefore used as a reference (Brown and Duck-Chong, 1982).

The lack of differences observed in the ratios of PC/PE and L/S could be accounted for by the lack of differences observed in the proportions of PC, PE and S classes. Previously (in Chapter 4), we have demonstrated that the PC/PE ratio in BALF of male lambs was significantly lower than observed in females, as the proportion of PC was reduced in males. The finding in this 8 h survival study does not support both the finding of a lower index of surfactant maturity in the BALF of males in the previous Chapter (4 h survival), and also in the amniotic fluid of humans and rabbits and in the lung lavage of rabbits (Nielsen and Torday, 1981, Torday et al., 1981, Fleisher et al., 1985).

A lower proportion of PE 36:2 was observed in BALF of male lambs compared to females but the role of this phospholipid species is not known and it is unclear how it could potentially affect surfactant function. No differences in the proportions of PE species were observed in the BALF of lambs observed for 4 h (Chapter 4).

5.4.4.3 Surfactant protein gene and protein expression in lung tissue

Surfactant proteins make up a minor component of pulmonary surfactant (~10%) but they are important in preventing alveolar collapse and in pulmonary defence. In this study, the gene expression of *surfactant proteins* was not different between males and females. Similar observations were seen in the fetal lung tissues (Chapter 3) and lamb lungs at 4 h after preterm delivery (Chapter 4). It is possible that at this gestational age (0.9 of term), pulmonary *surfactant protein* expression is similar in males and females. As surfactant phospholipids constitute the remaining 90% of pulmonary surfactant and have been shown to have sex differences, alterations in phospholipid composition could account for the poorer respiratory outcome in males following premature birth. However, males had significantly lower level of pro-SP-C at 8 h after preterm delivery. This was also previously seen in preterm lamb lungs at 4 h after birth (Chapter 4). pro-SP-C is synthesised by the alveolar type II cells as a 21-kDa integral membrane propeptide, which is proteolytically processed to a 3.7-kDa secretory product (SP-C) (Beers et al., 1998, Mulugeta and Beers, 2003). pro-SP-C was measured instead of the mature SP-C protein due to optimising difficulties with the Western blot technique for mature SP-C. SP-C facilitates the adsorption of surfactant lipids onto the surface film lining the alveoli in the lung (Possmayer et al., 2001, Rodriguez-Capote et al., 2001). A lower proportion of pro-SP-C in the male lung compared to females is consistent with lower static lung compliance of males than females.

The level of SP-A, which is involved in host defence (Orgeig et al., 2010), was not different between males and females. This was also seen in the preterm lambs

reported in Chapter 4. This lack of difference could be due to immaturity of pulmonary immune function at this stage of gestation. In rats, low amounts of relative SP-A (in relation to total phospholipids) were detected in newborns compared to adults (Bernhard et al., 2001). As an earlier age in gestation was investigated in this study, differences in SP-A could be undetectable in the lamb lung tissue and are unlikely to explain differences in respiratory outcome between the sexes.

5.4.5 Total protein in BALF

Plasma exudation in the alveoli as a result of increased vascular permeability in the lungs results in increased protein concentration in the alveolar space. Plasma proteins are known to inhibit pulmonary surfactant function (Keough et al., 1989, Warriner et al., 2002) and could likely reduce the effectiveness of DPPC and other phospholipids in the alveolar space, thus preventing the lowering of surface tension within the lung.

In this study, no difference in total protein concentration was observed in the BALF of males and females. A possible reason for the lack of sex difference at 8 h could be a lack of difference in pulmonary vascular permeability. In the 4 h postnatal lambs (Chapter 4), males had greater protein concentration in BALF than in females, which could have contributed to their poorer respiratory outcome. It is possible that differences in the ventilation technique performed between the studies could have had an effect on the degree of pulmonary vascular permeability. The use of a Neopuff^{TM} Infant T-piece resuscitator with CPAP (8 h study) could be less injurious in comparison to the use of the intermittent mandatory ventilation using a face mask and self-inflating resuscitation bag with a reservoir, with a flow of 5 to 10 L/min of 100% oxygen, where CPAP is absent (4 h study). CPAP allows air to be retained in the lung by keeping the alveoli open and thus prevents the alveoli collapsing between breaths.

Since similar proportions of LPC were observed in BALF samples from both males and females in this study, it is possible that this could contribute to the similar levels of total protein in the males and females observed. In addition, the proportions of the plasma PCs PC 34:2 and PC 36:2 in BALF, which are indicators of increased vascular permeability, were also similar in both males and females. This further supports the conclusion that there was no sex difference in vascular permeability.

Despite similar levels of total protein in both male and female BALF samples, it is possible that the amount of albumin leakage into the alveolar space could be different between the sexes. The albumin concentration in BALF could not be measured in this study as the concentrations in the samples were below the detection limit. However, the presence of plasma proteins, in particular albumin, has been shown to reduce the effectiveness of surfactant in reducing the surface tension in the lung. A higher level of albumin in the BALF of male lambs could contribute to the lower static lung compliance observed in the males.

5.5 Summary

The following tables summarize the differences observed between the 4 h and 8 h studies.

Table 5.5: Summary of major physiological findings in lambs in the 4 hour study.

		Males (compared to females)		
		Fetal	1 h	1–4 h
Blood gases	pH	↔	↓	↔
	$PaCO_2$	↔	↑	↔
Blood chemistry	Lactate	↔	↑	↔
	Glucose	↔	↑	↔
Blood pressure	MAP	↔	↑	↔
Breathing capacity	Inspiratory effort			↔

Table 5.6: Summary of major findings for surfactant in the 4 hour study.

		Males (compared to females)
Surfactant composition (BALF)	Total PC	↓
	%PC 32:0	↓
	%PC 34:2 & PC 36:2	↑
	Total protein concentration	↑
Surfactant protein (protein expression in lung)	pro-SP-C	↓

Table 5.7: Summary of major physiological findings in lambs in the 8 hour study.

		Males (compared to females)			
		Fetal	1 h	1–4 h	4–8 h
Blood gases	pH	↔	↓	↓	↓
	$PaCO_2$	↔	↑	↑	↑
Blood chemistry	Lactate	↔	↔	↔	↓
	Glucose	↔	↔	↔	↔
Blood pressure	MAP	↔	↔	↔	↔
Breathing capacity	Inspiratory effort			↔	↑

Table 5.8: Summary of major findings for surfactant in the 8 hour study.

		Males (compared to females)
Surfactant composition (BALF)	PC 32:1	↓
	PE 36:2	↓
	Total protein concentration	↔
Surfactant protein (protein expression in lung)	pro-SP-C	↓

5.6 Conclusions

The findings in this study confirm and extend the observations that were made in the 4 h survival study (Chapter 4). Both studies confirm that males have poorer respiratory adaptation than females after preterm birth. We expected to see more severe changes in the male lambs in this study compared to those in the 4 h group following an extended monitoring after preterm birth. In a previous study by De Matteo et al. (2010), a marked difference in survival outcome between male and female lambs was observed at 8 h than at 4 h following preterm delivery (Figure 1.4). In the animals used in the present study we found subtle differences that may or may not contribute to the expected poorer outcome in males. The subtle differences that we observed between the 4 h and 8 h studies could be due to the different ventilation technique used in these groups of lambs. It is also possible that major differences could be observed if the monitoring of the lambs was extended beyond 8 h. However, it was not feasible to perform such an extended study for this thesis. A more detailed discussion will be provided in the General Discussion (Chapter 6).

Chapter 6: General discussion

6.1 Overview

Preterm birth is a common medical problem that affects about 8–12% of live-births in most developed countries; in Australia, the incidence of preterm birth in 2009 was 8.2% (Li et al., 2011). It is widely known that preterm birth reveals sex-related differences in infant morbidity and survival, where males are at a disadvantage as they fare poorly when compared to females of the same gestational age (Papageorgiou et al., 1981, Stevenson et al., 2000, Ingemarsson, 2003, Bhaumik et al., 2004, Elsmen et al., 2004, Mathews and MacDorman, 2011). For example, the incidence of respiratory distress syndrome (RDS) is reported to be greater in preterm male infants than in females (Carey et al., 2007, Seaborn et al., 2010), resulting in males requiring more respiratory and circulatory support than preterm female infants (Elsmen et al., 2004). However, little is known about the causes and basis of this male disadvantage and further investigation is required for a better understanding.

The rationale for this study was to add to the limited studies on the basis for sex differences in respiratory function of preterm infants that are available in the literature. Previous studies reported limited data on sex differences in lung histology; similarly, data on pulmonary surfactant from humans and animals were limited, and were not detailed in terms of gene expression or surfactant composition. Owing to the lack of detailed information available that could explain the ‘male disadvantage’ in preterm infants, this study was aimed at providing an in-depth investigation of possible sex differences in lung histology, surfactant

phospholipid composition and surfactant protein gene and protein expression, and also the physiology of the transition at the time of preterm birth.

The present study has demonstrated that a number of factors could contribute to the male disadvantage in respiratory outcome at the time of preterm birth. This study showed that the male disadvantage in respiratory outcome could be due to sex-related differences in (1) surfactant phospholipid composition that could consequently affect lung compliance and therefore respiratory function and arterial blood gas parameters, (2) the concentration of plasma proteins within the alveolar space, which could decrease lung compliance by interfering with surfactant function at the alveolar air-liquid interface, and (3) the protein expression of SP-A and pro-SP-C in lung tissue, which could also decrease lung compliance. However, our results show that the male disadvantage in respiratory function following preterm birth does not appear to be a result of sex-related differences in lung architecture or *surfactant protein* gene expression.

This study is the first to make a detailed analysis of sex differences in lung architecture, lung compliance, surfactant phospholipid composition and physiological transition at preterm birth and therefore provides a better understanding of the sex differences in lung development and lung function following preterm birth.

6.2 Lung morphology is similar in preterm males and females

The male disadvantage in respiratory outcome is believed to be a result of a number of contributing factors, one of which could be a difference in lung architecture. Surprisingly, few studies have investigated this, and the presence of dysanaptic growth of large airways in young males is the only known difference reported in the literature (Hoffstein, 1986). In our study, both morphological

analysis in the lungs of fetal sheep and preterm postnatal lambs did not demonstrate any differences between males and females, thus indicating that possible differences in lung structure (assessed using standard histological methods) do not play a role in contributing to sex differences in preterm lung function. Further studies could be performed to investigate possible sex differences in lung structure at a higher magnification by using electron microscopy to investigate the alveolar wall, in particular the blood gas barrier; it may also be beneficial to assess the blood vessels in the lung, especially in the alveolar walls.

6.3 Sex differences in surfactant composition

In our study, surfactant phospholipid composition was analysed in three types of fluids: fetal lung liquid, amniotic fluid and bronchoalveolar lavage fluid (BALF). In fetal lung liquid, minor sex-related differences were observed in the phospholipid composition and these appear unlikely to be sufficient to account for the observed respiratory insufficiency in the preterm male; this finding was possibly due to the gestational ages at which the samples were analysed. A greater difference may have been observed at a later stage of gestation. Minor differences in surfactant phospholipid composition were also observed in the amniotic fluid. Amniotic fluid is likely to be less reliable as an indicator of surfactant phospholipid composition than samples taken from the lungs, as it mainly consists of a mixture of fetal urine and fetal lung liquid, and also fetal oro-nasal secretions. However, when the composition of surfactant phospholipid was analysed in BALF collected at 4 h after preterm delivery, major differences were observed between males and females. BALF of males showed lower proportions of a surface active component of lung surfactant, which is capable of lowering surface tension to near zero, and higher levels of plasma phosphatidylcholine species, which is indicative of greater exudation of plasma into the alveoli, most likely caused by higher vascular permeability in the lungs of males. A reduced surfactant function in males is further supported by our finding of a higher total protein concentration in the BALF of males which could consequently impair the effective functioning of surfactant.

The findings of the 8 h postnatal study confirmed and extended the observations that were seen in the 4 h postnatal study. Subtle differences were observed between the two studies but it is unclear whether these differences contribute to the expected poorer outcome in males. Differences between findings of the 4 h and 8 h postnatal studies may be a result of differences in the resuscitation technique used, as a less injurious approach was undertaken in the latter study. The sex difference in total protein concentration in BALF that was observed at 4 h after delivery was not observed at 8 h after delivery; this is possibly due to the absence of a difference in pulmonary vascular permeability between males and females in the 8 h postnatal study, as a result of the less injurious resuscitation technique.

Surfactant protein gene expression was not different between males and females in both fetal and postnatal lung tissue. However, the protein expression of SP-A and pro-SP-C in the lung tissue was lower in males at 4 h after delivery. In the 8 h study, only lower protein expression of pro-SP-C was seen in the male lung. Although surfactant proteins only constitute ~10% of surfactant, changes in these proteins may contribute to poorer respiratory function in males.

As only the protein expression of SP-A and pro-SP-C surfactant protein were measured in this study, investigating the protein expression of SP-B and SP-D would be beneficial in future studies.

6.4 Physiological transition following preterm birth

This is the first study to examine the physiological transition from fetal to postnatal life in an unanaesthetised animal model of preterm birth. This study is unique in that it presents physiological data collected from the same animals during fetal life and during the transition to early postnatal life. Before birth, there were no apparent differences in physiological data from male and female fetuses. However,

physiological data from males and females were clearly different after preterm birth, most probably due to differences in lung compliance.

Lung compliance has an important role in the ability of newborn mammals to adapt to postnatal life. Lower lung compliance leads to poorer ventilation of the lung and therefore poorer gas exchange, which consequently results in poorer blood gases, thus requiring respiratory support. It appears that lower lung compliance in males could be due to altered surfactant composition. The composition of pulmonary surfactant could have been influenced by sex hormones. However, this study did not investigate the involvement of sex hormones. Ideally, the role of sex hormones in lung development could be investigated by comparing surfactant development in prenatally castrated males with that of intact males and females. Previous studies have proposed higher concentrations of androgens and Müllerian Inhibiting Substance (MIS) in males to be potential causes for the suppression of lung maturation by inhibiting production of surfactant (Catlin et al., 1990, Nielsen, 1992). Sex hormones may also result in different fetal cortisol concentrations that consequently could affect surfactant production.

Besides affecting surfactant, sex hormones may affect respiratory function by altering the development of ventilatory control neurons of the brainstem. This may affect sensitivity to carbon dioxide and arousal.

Additionally, sex hormones could affect the endocrine control of lung liquid reabsorption which could affect lung compliance in the immediate postnatal period. Pulmonary blood flow could also be different in males and females, resulting in different respiratory outcomes, but this possibility has not been investigated in this study.

6.5 Sheep as an appropriate animal model

In our study the sheep was used as an animal model to aid in better understanding of the male disadvantage following preterm birth. The advantages of using sheep are that it is currently the only practical large animal model of preterm birth and the male disadvantage in respiratory function following preterm birth has been demonstrated in sheep (De Matteo et al., 2010). In addition, the sheep has a relatively long gestation and also its size and tolerance of surgery allow for surgical manipulation and sampling from the fetus *in utero*. However, lung maturation in relation to birth in sheep differs slightly to that in humans, in that the developing sheep lung is more advanced than human lung with respect to alveolarization at birth.

6.6 Strengths and weaknesses of this study

This study is the first to comprehensively assess the structural, molecular and functional aspects of the preterm lung and the physiological mechanisms of adaptation after preterm birth in males and females, using a valid animal model. A recent study in preterm lambs used anaesthetized and mechanically ventilated lambs and failed to show sex differences (Polglase et al., 2012); however, the use of anaesthesia and mechanical ventilation could have confounded the findings. In the present study, these confounding factors have been eliminated by using unanaesthetised lambs which were capable of breathing spontaneously throughout the duration of the monitoring period. This study also reported, for the first time, a detailed analysis of sex-related differences lung histology and surfactant composition in any species.

On the other hand, there are potential shortcomings in this study in relating the findings to humans. The sheep model may not replicate humans in all aspects. In addition, the physiological aspect of this study was only able to be examined for a relatively short period after preterm birth. Future studies could monitor postnatal lambs for longer periods.

6.7 Future directions

Further research could be done to better understand the basis of the male disadvantage following preterm birth. One parameter that can be further investigated is lung ultrastructure. The measurement of the abundance of type II alveolar epithelial cells and the abundance of lamellar bodies in the type II alveolar epithelial cells could give an insight into sex-related differences in surfactant production and secretion. Other analyses that could be performed include measuring the blood-air barrier thickness and the density of capillaries within the alveolar walls. Additionally, it may be beneficial to investigate sex differences in inflammation in the lung, as airway inflammation is reported to be associated with decreased lung function. Inflammatory markers such as immune cells in bronchoalveolar lavage fluid and abundance of macrophages in fixed lung tissue could thus give a better insight.

In terms of physiological adaptation following preterm birth, other measurements can be made to determine if there are sex differences in pulmonary blood flow, responsiveness to CO_2 and maturity of the neural control of breathing.

6.8 Conclusions

This study highlights the possible factors that contribute to the male disadvantage in cardio-respiratory adaptation following preterm birth in an animal model that is relevant to human preterm infants. Our findings demonstrate that the male disadvantage is not a result of differences in lung architecture and *surfactant protein* gene expression. However, it is more likely a result of differences in surfactant phospholipid composition that consequently affect lung compliance, thereby altering gas exchange and arterial blood gas parameters. The increased presence of plasma proteins within the alveolar space of preterm males may affect lung compliance by impairing surfactant function, potentially increasing surface tension within the alveoli. In addition, lower levels of SP-A and pro-SP-C protein expression in the lung tissue could also contribute to the male disadvantage. The

findings demonstrated in this study may be beneficial to human perinatal medicine as they may allow clinicians to provide better intervention in the management of preterm babies according to the sex of the infant. An accurate intervention particularly in male preterm infants could aid in the effort to reduce RDS incidence and also increase the chance of their survival.

Chapter 7: Appendices

Table A1: Proportions of phosphatidylcholine (PC) species relative to total PC in amniotic fluid samples of sheep collected at 131 to 133 days of gestation for female (F; n=9) and male (M; n=10) fetuses. Data are expressed as mean ± SEM. *$P < 0.05$.

Species	Sex	Days of gestation		P value
		131	**133**	
PC 30:0	F M	2.1 ± 0.5 2.7 ± 0.5	2.6 ± 0.6 3.2 ± 0.6	$P_S = 0.434$ $P_T = 0.220$ $P_{SxT} = 0.971$
PC 32:0	F M	10.4 ± 1.9 14.8 ± 2.8	13.4 ± 3.0 16.5 ± 2.9	$P_S = 0.357$ $P_T = 0.249$ $P_{SxT} = 0.853$
PC 32:1	F M	6.4 ± 1.2 8.0 ± 1.5	8.5 ± 1.8 9.6 ± 1.6	$P_S = 0.563$ $P_T = 0.124$ $P_{SxT} = 0.943$
PC 33:0	F M	1.2 ± 0.1 1.2 ± 0.1	1.1 ± 0.1 1.2 ± 0.1	$P_S = 0.612$ $P_T = 0.833$ $P_{SxT} = 0.492$
PC 33:1	F M	1.8 ± 0.1 1.5 ± 0.1	1.7 ± 0.1 1.7 ± 0.1	$P_S = 0.294$ $P_T = 0.618$ $P_{SxT} = 0.024$*
PC 34:0	F M	1.7 ± 0.1 2.1 ± 0.3	1.6 ± 0.2 1.7 ± 0.3	$P_S = 0.441$ $P_T = 0.089$ $P_{SxT} = 0.682$
PC 34:1	F M	21.3 ± 1.2 20.4 ± 1.0	19.8 ± 1.0 20.3 ± 1.3	$P_S = 0.909$ $P_T = 0.166$ $P_{SxT} = 0.212$
PC 34:2	F M	5.0 ± 0.7 4.0 ± 0.8	5.1 ± 0.6 4.1 ± 1.0	$P_S = 0.385$ $P_T = 0.807$ $P_{SxT} = 0.965$
PC 36:1	F M	15.3 ± 1.0 15.0 ± 1.4	13.6 ± 1.3 12.5 ± 1.6	$P_S = 0.746$ $P_T = 0.040$* $P_{SxT} = 0.527$
PC 36:2	F M	12.6 ± 1.0 9.7 ± 1.0	10.7 ± 1.6 9.3 ± 1.1	$P_S = 0.164$ $P_T = 0.253$ $P_{SxT} = 0.504$
PC 36:3	F	3.1 ± 0.5	3.3 ± 0.5	$P_S = 0.450$

	M	2.8 ± 0.4	2.6 ± 0.4	$P_T = 0.868$ $P_{SxT} = 0.394$
PC 36:4	F M	1.4 ± 0.4 1.4 ± 0.5	2.3 ± 0.5 2.2 ± 0.8	$P_S = 0.993$ $P_T = 0.060$ $P_{SxT} = 0.759$
PC 38:2	F M	6.3 ± 0.6 6.1 ± 0.7	4.9 ± 0.6 5.2 ± 0.7	$P_S = 0.940$ $P_T = 0.008$* $P_{SxT} = 0.356$
PC 38:4	F M	1.6 ± 0.7 1.5 ± 0.7	1.5 ± 0.6 1.8 ± 0.7	$P_S = 0.729$ $P_T = 0.536$ $P_{SxT} = 0.973$
PC 38:5	F M	2.3 ± 0.2 1.7 ± 0.3	2.1 ± 0.2 1.7 ± 0.4	$P_S = 0.245$ $P_T = 0.682$ $P_{SxT} = 0.832$

Table A2: Proportions of phosphatidylglycerol (PG) species relative to total PG in amniotic fluid samples of sheep collected at 131 to 133 days of gestation for female (F; n=9) and male (M; n=10) fetuses. Data are expressed as mean ± SEM.

Species	Sex	Days of gestation **131**	 **133**	P value
PG 16:0/18:1	F M	55.9 ± 12.3 43.1 ± 13.2	54.2 ± 15.1 26.7 ± 11.8	$P_S = 0.192$ $P_T = 0.494$ $P_{SxT} = 0.535$
PG 18:1/18:1	F M	21.9 ± 7.5 36.9 ± 12.6	45.8 ± 15.1 23.3 ± 11.0	$P_S = 0.808$ $P_T = 0.646$ $P_{SxT} = 0.152$

Table A3: Proportion of phosphatidylinositol (PI) species relative to total PI in amniotic fluid samples collected at 131 to 133 days of gestation for female (F; n=9) and male (M; n=10) fetal sheep. Data are expressed as mean ± SEM. *$P < 0.05$.

Species	Sex	Days of gestation **131**	 **133**	P value
PI 32:0	F M	1.9 ± 0.4 2.1 ± 0.4	1.8 ± 0.3 1.3 ± 0.3	$P_S = 0.868$ $P_T = 0.269$ $P_{SxT} = 0.270$
PI 32:1	F M	1.2 ± 0.3 1.4 ± 0.5	1.6 ± 0.5 2.0 ± 0.5	$P_S = 0.500$ $P_T = 0.257$

				P_{SxT} = 0.852
PI 34:0	F M	3.2 ± 0.6 2.4 ± 0.6	3.6 ± 0.3 3.1 ± 0.4	P_S = 0.425 P_T = 0.017* P_{SxT} = 0.994
PI 34:1	F M	22.6 ± 2.4 25.9 ± 3.5	26.6 ± 3.2 31.6 ± 3.3	P_S = 0.311 P_T = 0.067 P_{SxT} = 0.707
PI 36:1	F M	29.1 ± 1.7 29.5 ± 2.1	24.9 ± 2.9 24.4 ± 2.0	P_S = 0.957 P_T = 0.010* P_{SxT} = 0.644
PI 36:2	F M	15.9 ± 1.4 16.4 ± 0.8	16.0 ± 1.4 15.7 ± 0.9	P_S = 0.856 P_T = 0.889 P_{SxT} = 0.388
PI 36:3	F M	4.7 ± 0.8 5.3 ± 0.5	6.6 ± 0.5 5.3 ± 1.0	P_S = 0.640 P_T = 0.238 P_{SxT} = 0.229
PI 36:4	F M	2.3 ± 0.3 1.8 ± 0.5	2.5 ± 0.5 1.4 ± 0.6	P_S = 0.215 P_T = 0.974 P_{SxT} = 0.396
PI 38:4	F M	8.3 ± 2.5 7.8 ± 1.9	7.2 ± 2.6 8.1 ± 1.8	P_S = 0.940 P_T = 0.456 P_{SxT} = 0.324
PI 38:5	F M	3.4 ± 0.7 3.5 ± 1.3	4.5 ± 0.9 3.7 ± 1.4	P_S = 0.749 P_T = 0.583 P_{SxT} = 0.715

Table A4: Proportion of phosphatidylserine (PS) species relative to total PS in amniotic fluid samples of sheep collected at 131 to 133 days of gestation for female (F; n=9) and male (M; n=10) fetuses. Data are expressed as mean ± SEM *$P < 0.05$.

Species	Sex	Days of gestation		P value
		131	**133**	
PS 36:1	F M	75.9 ± 0.8 76.9 ± 0.8	75.1 ± 0.6 74.9 ± 1.1	$P_S = 0.662$ $P_T = 0.050$ $P_{SxT} = 0.271$
PS 36:2	F M	10.1 ± 1.2 11.7 ± 0.8	11.9 ± 1.0 10.7 ± 0.4	$P_S = 0.902$ $P_T = 0.682$ $P_{SxT} = 0.075$
PS 38:3	F M	2.2 ± 0.1 1.4 ± 0.3	2.0 ± 0.4 2.1 ± 0.4	$P_S = 0.434$ $P_T = 0.407$ $P_{SxT} = 0.132$
PS 38:4	F M	2.1 ± 0.2 1.2 ± 0.3	2.0 ± 0.1 1.6 ± 0.4	$P_S = 0.119$ $P_T = 0.437$ $P_{SxT} = 0.164$
PS 40:5	F M	4.0 ± 0.4 4.2 ± 0.5	4.2 ± 0.5 5.3 ± 0.9	$P_S = 0.452$ $P_T = 0.120$ $P_{SxT} = 0.238$
PS 40:6	F M	5.6 ± 0.6 4.5 ± 0.5	4.9 ± 0.5 5.4 ± 0.4	$P_S = 0.640$ $P_T = 0.970$ $P_{SxT} = 0.023$*

Table A5: Proportion of phosphatidylethanolamine (PE) species relative to total PE in amniotic fluid samples of sheep collected at 131 to 133 days of gestation for female (F; n=9) and male (M; n=10) lambs. Data are expressed as mean ± SEM. *$P < 0.05$.

Species	Sex	Days of gestation		P value
		131	**133**	
PE 16:0/18:1	F M	17.3 ± 1.5 18.4 ± 1.5	16.9 ± 1.7 17.6 ± 1.2	$P_S = 0.717$ $P_T = 0.399$ $P_{SxT} = 0.929$
PE 16:0/18:2	F M	3.1 ± 0.2 3.3 ± 0.3	3.1 ± 0.2 3.6 ± 0.4	$P_S = 0.288$ $P_T = 0.342$ $P_{SxT} = 0.515$
PE 35:1	F M	3.5 ± 0.1 3.7 ± 0.2	3.5 ± 0.3 3.7 ± 0.3	$P_S = 0.599$ $P_T = 0.679$ $P_{SxT} = 0.914$
PE 35:2	F M	1.5 ± 0.1 1.6 ± 0.1	1.8 ± 0.2 1.8 ± 0.2	$P_S = 0.723$ $P_T = 0.115$ $P_{SxT} = 0.613$
PE 16:0/20:4	F M	1.7 ± 0.2 1.6 ± 0.1	2.1 ± 0.2 1.6 ± 0.3	$P_S = 0.309$ $P_T = 0.409$ $P_{SxT} = 0.329$
PE 18:0/18:1	F M	22.3 ± 0.9 23.2 ± 0.7	20.5 ± 0.7 21.8 ± 0.9	$P_S = 0.243$ $P_T = 0.038$* $P_{SxT} = 0.912$
PE 18:1/18:1	F M	25.6 ± 1.7 25.2 ± 0.9	27.1 ± 1.7 25.5 ± 0.4	$P_S = 0.480$ $P_T = 0.344$ $P_{SxT} = 0.605$
PE 18:1/18:2	F M	3.3 ± 0.4 2.9 ± 0.6	3.6 ± 0.4 3.3 ± 0.5	$P_S = 0.629$ $P_T = 0.093$ $P_{SxT} = 0.901$
PE 16:0/22:5	F M	5.0 ± 0.3 4.6 ± 0.4	5.1 ± 0.3 5.3 ± 0.5	$P_S = 0.914$ $P_T = 0.183$ $P_{SxT} = 0.365$
PE 16:0/22:6	F M	2.3 ± 0.2 2.3 ± 0.1	2.6 ± 0.2 2.6 ± 0.2	$P_S = 0.961$ $P_T = 0.020$* $P_{SxT} = 0.746$
PE 18:0/20:3	F M	2.8 ± 0.2 2.6 ± 0.2	2.8 ± 0.2 2.0 ± 0.4	$P_S = 0.202$ $P_T = 0.101$ $P_{SxT} = 0.042$*
PE 18:0/20:4	F M	5.8 ± 0.4 5.4 ± 0.6	5.7 ± 0.2 5.5 ± 0.6	$P_S = 0.730$ $P_T = 0.555$ $P_{SxT} = 0.886$
PE 18:0/22:6	F M	2.2 ± 0.5 1.7 ± 0.4	1.9 ± 0.4 2.4 ± 0.3	$P_S = 0.963$ $P_T = 0.518$ $P_{SxT} = 0.292$

PE 18:1/22:6	F M	1.2 ± 0.1 1.1 ± 0.1	1.2 ± 0.1 1.1 ± 0.1	$P_S = 0.732$ $P_T = 0.986$ $P_{SxT} = 0.970$
PE 40:5	F M	1.9 ± 0.2 2.0 ± 0.3	1.6 ± 0.3 1.7 ± 0.2	$P_S = 0.633$ $P_T = 0.270$ $P_{SxT} = 0.881$

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