Antenatal and postnatal influences on preterm diaphragm function

By

Christine Astell, BSc (Hon)

This thesis is presented for the degree of Doctor of Philosophy

The University of Western Australia

School of Human Sciences

Physiology

Submitted: 1st May 2018

Declaration

I, Christine Astell, certify that:

This thesis has been accomplished during enrolment in the degree.

This thesis does not contain material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

No part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia and where applicable, any partner institution responsible for the joint-award of this degree.

This thesis does not contain any material previously published or written by another person, except where due reference has been made in the text.

The work(s) are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

The research involving animal data reported in this thesis was assessed and approved by the the Animal Ethics Committee of the University of Western Australia (3/100/1301) or the Telethon Kids Institute Animal Ethics Committee (AEC #304). All experiments were conducted in accordance with the guidelines of the National Health and Medical Research Council (NHMRC) Australian code for the care and use of animals for scientific purposes (2013).

The work described in this thesis was funded by a National Health and Medical Research Council (NHMRC) Centre of Research Excellence (GRT1057514), NHMRC Project Grant (GRT1057759), and Telethon New Children's Hospital Medical Research Fund. Financial assistance was provided by an Australian Government Research Training Program (RTP) Scholarship, Australian Postgraduate Award, UWA safety net to-up scholarship, and NHMRC Preterm Infants Centre of Research Excellence top-up scholarship.

This thesis contains published work and/or work prepared for publication, which has been co-authored. Author contribution of this work is specified in the relevant appendices.

Christine Astell

Gavin Pinniger

Coordinating Supervisor

Abstract

Respiratory disease is the leading cause of perinatal morbidity and mortality in the preterm infant. Until recently, respiratory disease in preterm infants was considered primarily a disease of lung immaturity. The contribution of respiratory musculature to the development of respiratory complications after preterm birth has been infrequently considered, despite the vital role of respiratory musculature in spontaneous breathing. The diaphragm is the primary respiratory muscle, and appropriate *in utero* development of the diaphragm is essential for establishing and sustaining spontaneous breathing from birth. The structural and functional immaturity of the preterm diaphragm, combined with the increased work of breathing of neonates, may contribute to the development of respiratory disease in preterm infants. Diaphragm function may be further impaired by external influences during preterm birth and postnatal development. The overall aim of this thesis was, therefore, to investigate the impact of three clinically common perinatal exposures, inflammation, glucocorticoid therapy, and mechanical ventilation, on preterm diaphragm function.

In utero inflammation leads to fetal diaphragm weakness. However, it is unknown whether inflammation-induced diaphragm weakness persists into postnatal life. The first study of this thesis investigated the combined effects of preterm birth and *in utero* inflammation on postnatal diaphragm structure and function, using an established sheep model of chorioamnionitis. In this study, lambs received intra-amniotic injections of saline or lipopolysaccharide (LPS; 4 mg) 48 hours (h) prior to premature delivery at 129 days (d) gestational age (GA; term = 145 – 150 d GA). Saline and LPS exposed lambs were raised in an intensive care environment and killed at 7 d postnatal age (PNA; 136 d GA equivalent). Fetal control lambs, representing a pure maturational control, were delivered at 136 d GA, without prior intervention, and were killed immediately. The diaphragm was excised immediately for *in vitro* assessment of contractile function and myosin heavy chain (MHC) fibre characteristics. *In utero* LPS exposure did not affect diaphragm structure or function at 7 d PNA. Instead, the preterm diaphragm underwent rapid functional adaptation in the first week of life, indicated by an increase in specific force and shorter twitch contraction times in the saline and LPS groups. Furthermore, postnatal management of

preterm lambs with mechanical ventilation, and the nutritional protein intake of preterm lambs, significantly predicted diaphragm function and MHC characteristics at 7 d PNA. Interestingly, LPS exposure increased dependency of the preterm lamb on mechanical ventilation. While postnatal events in managing the preterm lambs are complex, this study emphasises the impact of postnatal management on minimising the influence of *in utero* inflammation on diaphragm function in the preterm infant.

Dexamethasone treatment can accelerate lung development and weaning of preterm infants from mechanical ventilation. However, high-dose dexamethasone treatment has been associated with serious long-term side effects, including protein catabolism. The effects of low-dose dexamethasone, more commonly used in clinical practice today, on preterm diaphragm function are unknown. The second experimental chapter of this thesis investigated the short-term and long-term effects of postnatal dexamethasone exposure on diaphragm structure and function. Specifically, the short-term study investigated the dose-dependent effects of postnatal dexamethasone treatment on diaphragm structure and function. Lambs assigned to the short-term study were delivered prematurely at 129 d GA, received either saline, low-dose dexamethasone, or high-dose dexamethasone, were managed in an intensive care environment, and were killed at 7 d PNA. Low-dose dexamethasone had no significant effect on diaphragm function or structure. High-dose dexamethasone increased twitch contraction time; however, the longer contraction time did not influence the force-frequency relationship or the maximum force generating capacity of diaphragm, and was not associated with changes in MHC fibre characteristics. Instead, the duration of mechanical ventilation required by preterm lambs was the most significant predictor of diaphragm function at 7 d PNA, with longer durations of mechanical ventilation associated with reduced diaphragm strength.

The second dexamethasone study investigated the long-term interactive effects of postnatal dexamethasone treatment and mechanical ventilation, and the long-term effects of preterm birth, on diaphragm function and structure. Lambs assigned to the long-term study were delivered at 129 d GA and assigned to one of four groups: they received saline with or without mechanical ventilation, or received dexamethasone with or without mechanical ventilation, and were

killed at 2 m term-equivalent. Lambs from a comparative term control group were delivered naturally and received no intervention, before they were killed at 2 months (m) PNA. Postnatal dexamethasone increased the twitch half relaxation time; however, changes in half relaxation time did not influence the force-generating capacity of the diaphragm. Postnatal dexamethasone had no significant long-term effect on myofibre size. Mechanical ventilation did not have a significant main effect, or interactive effect with dexamethasone, on diaphragm function or structure. Lastly, preterm birth had no significant effect on diaphragm function or structure by 2 m post-term. These data suggest that the potential benefits of dexamethasone treatment on preterm lung function are not compromised by impaired diaphragm function. Postnatal administration of dexamethasone to accelerate weaning of preterm infants from mechanical ventilation may benefit preterm diaphragm function, given the negative correlation between duration of mechanical ventilation and diaphragm strength. Furthermore, these data suggest that the diaphragm dysfunction resulting from preterm birth and mechanical ventilation, which is evident in the acute postnatal period, may be overcome during subsequent postnatal development.

Mechanical ventilation induces diaphragm dysfunction in the adult, a phenomenon referred to as ventilation-induced diaphragm dysfunction (VIDD). The susceptibility of the developing diaphragm to VIDD is unknown. The previous two studies suggested that the duration of mechanical ventilation was the most significant predictor of diaphragm function in the preterm lamb at 7 d PNA. However, a causal relationship between mechanical ventilation and diaphragm function could not be determined, as preterm lambs were unable to breathe independently after birth and were susceptible to postnatal complications. Therefore, the aim of the final study was to determine the susceptibility of the developing diaphragm to mechanical ventilation-induced dysfunction, using a newborn rat model. Postnatal respiratory development of the rat is similar to prenatal respiratory development in the human fetus, disrupted by preterm birth. Results from this study showed that mechanical ventilation for 4-12 h reduced maximum specific force by ~ 18 % in immature and adult rat diaphragm. However, the force-frequency results suggest that the developing diaphragm may be less susceptible to ventilator-induced dysfunction, than the developed adult diaphragm.

The studies in this thesis demonstrate that LPS-induced diaphragm weakness at birth does not persist into postnatal life. Preterm diaphragm function rapidly changes during postnatal life, and the functional change is likely influenced by postnatal management in the intensive care unit. Mechanical ventilation is a major predictor of diaphragm function in the preterm lamb at 1 w PNA, and significantly impairs diaphragm function in the newborn rat. Interestingly, LPS exposure increased dependency of the preterm lamb on mechanical ventilation. Therefore, it is important to consider the combined effects of *in utero* inflammation and mechanical ventilation, given the impact of mechanical ventilation on preterm diaphragm function. Our results suggest that dexamethasone can be used to accelerate weaning from mechanical ventilation without further impairing diaphragm function. Accelerated weaning from mechanical ventilation is likely important to maintain preterm diaphragm function. The immature diaphragm is vulnerable to ventilation-induced diaphragm dysfunction, although to a lesser extent then the adult diaphragm. Nevertheless, even a small reduction in strength may severely compromise the ability of the preterm diaphragm to support independent ventilation once weaned from mechanical ventilation, given the diaphragm of the preterm infant is already weak due to immaturity.

Table of contents

List of figures

List of tables

Acknowledgement

I would like to thank my supervisors, Dr Gavin Pinniger, Assoc/Prof Jane Pillow, Dr Rob White, and Dr Tony Bakker, for their continued support and advice throughout my candidature. In particular, I thank Gavin for teaching me the dissection techniques used during this project, and for his tireless help during the drafting of this thesis; Jane, for establishing PICRU, which allowed me to use a preclinical lamb model for my experiments; Rob, for assisting me with western blot and immunohistochemistry experiments; and Tony, for his ideas and suggestions when I struggled with the interpretation of data.

I would also like to thank my friends for being so supportive of my PhD studies. I thank Sophie, for proofreading my writing on many occasions; Tom, who started postgraduate studies on the same day as I did, and who has been there through all the ups-and-downs of my project; and Phoebe and Chiara, whose friendship has made my PhD journey even more enjoyable.

A special thank you to Alex and my family. There is no way I could have done this without all of the love and encouragement you have given me.

This research was supported by an Australian Government Research Training Program (RTP) Scholarship, Australian Postgraduate Award, UWA safety net to-up scholarship, and NHMRC Preterm Infants Centre of Research Excellence top-up scholarship

Abbreviations

Akt1	Protein kinase B
BPD	Bronchopulmonary dysplasia
Ca^{2+}	Calcium ions
$CaCl_2$	Calcium chloride
CLD	Chronic lung disease
CPAP	Continuous positive airway pressure
CSA	Cross-sectional area
d	Day(s)
DEX	Dexamethasone
DHPR	Dihydropyridine receptor
FI	Fatigue index
FiO_2	Fraction of inspired oxygen
GA	Gestational age
h	Hour(s)
H&E	Haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IA	Intra-amniotic
Ig	Immunoglobulin
IGF-1	Insulin like growth factor 1
IM	Intra-muscular
IV	Intra-venous
KCl	Potassium chloride
L_o	Optimal muscle length
LPS	Lipopolysaccharide
m	Month(s)
$MgSO_4(H_2O)_7$	Magnesium sulfate heptahydrate
MHC	Myosin heavy chain
MV	Mechanical ventilation
NaCl	Sodium chloride
$NaHCO_3$	Sodium Bicarbonate
NP-40	Nonidet P-40
Pa_{CO2}	Partial pressure of CO_2
PCA	Post-conceptional age

Pdi	Transdiaphragmatic pressure
PM	Post-mortem
PNA	Postnatal age
P_o	Maximum specific force
P_t	Twitch force
RDS	Respiratory distress syndrome
RYR	Ryanodine receptor
SDS	Sodium dodecyl sulfate
SERCA	Sarcoplasmic reticulum Ca^{2+} ATPase pump
SpO_2	Peripheral capillary oxyhaemoglobin saturation
SR	Sarcoplasmic reticulum
TBST	Tris-buffered saline with tween
TRPC1	Transient receptor potential channel 1
TTP	Time to peak twitch force
VIDD	Ventilator-induced diaphragm dysfunction
y	Year(s)
½ RT	Twitch half relaxation time
4EBP1	Eukaryotic translation initiation factor 4E -binding protein 1

Publications and abstracts arising from this thesis

1. Astell CJ, Wang KC, Larcombe AL, James AL, Le Cras TD, Pinniger GJ, Noble PB. Transforming growth factor alpha overexpression impairs diaphragm function in transgenic mice. 2015. Science on the Swan. Perth, Australia. Poster.
2. Astell CJ, Bakker AT, Ahmadi-Noorbakhsh S, Noble PB, Pillow JJ, Pinniger GJ. Antenatal and postnatal influences on diaphragm function in preterm lambs. 2016. Fetal and Neonatal Workshop of Australia and New Zealand. Magnetic Island, Australia. Oral.
3. Astell CJ, Bakker AT, Ahmadi-Noorbakhsh S, Noble PB, Pillow JJ, Pinniger GJ. Antenatal and postnatal influences on diaphragm function in preterm lambs. 2016. Australian Society of Medical Research. Perth, Australia. Oral.
4. Pinniger GJ, Astell CJ, Bakker AT, Ahmadi-Noorbakhsh S, Noble PB, Pillow JJ. The effect of *in utero* inflammation on postnatal diaphragm function in preterm lambs. 2016. European Muscle Conference. Montpellier, France. Poster
5. Wang KCW, Astell CJ, Wijesignhe P, Larcombe AN, Pinniger GJ, Zosky GR, Kennedy BF, Berry LJ, Sampson DD, James AL, Le Cras TD, Noble RB. 2017. 'Optical coherence tomography-based contact indentation for diaphragm mechanics in a mouse model of transforming growth factor alpha induced lung disease', *Scientific Reports*, vol.7, no.1, pp.1517
6. Astell CJ, Bakker AT, Ahmadi-Noorbakhsh S, Noble PB, Pillow JJ, Pinniger G. Perinatal influences on preterm diaphragm function. Endocrine and Reproductive Sciences Meeting. 2018. Perth, Australia. Abstract.

1 General introduction

1.1 Epidemiology of preterm birth

Approximately 15 million infants worldwide are born prematurely each year (Blencowe et al. 2012). This figure equates to approximately 1 in 10 births. Preterm birth is defined as birth occurring before 37 completed weeks (w) gestational age (GA) (Goldenberg et al. 2008), and is classified further as: late preterm (34 – 36 w), moderate preterm (32 – 33 w), very preterm (28 – 31 w) and extremely preterm (<28 w) (Blencowe et al. 2012). Very preterm and extremely preterm births comprise ~10 % and ~5 % of preterm births, respectively (Blencowe et al. 2012). The rate of preterm birth in developed economies increased by ~ 19 % from 1990 to 2010 (Blencowe et al. 2012), largely due to an increase in the number of medically indicated preterm births (Goldenberg et al. 2008). The increasing rate of preterm birth is a major public health concern, with preterm infants being the group most susceptible to perinatal morbidity and mortality. The annual socioeconomic costs to prevent and treat problems associated with preterm birth equates to over 26 billion dollars in the USA alone (Behrman & Butler 2007).

The aetiology of preterm birth is complex, and as a result, successfully preventing preterm birth is challenging (Newnham et al. 2014). Preterm birth can occur spontaneously, after premature rupture of placental membranes, or can be medically indicated, e.g. from preeclampsia or intrauterine growth restriction. Intrauterine inflammation is a major trigger of premature rupturing of membranes and is associated with 70 % of preterm births before 30 w GA (Goldenberg, Hauth & Andrews 2000). This inflammation is commonly a consequence of an ascending vaginal or cervical infection. The extension of inflammation to the fetal compartment often results in a fetal inflammatory response syndrome and multi-organ injury (Gantert et al. 2010). While the inflammatory cascade caused by intrauterine infection and resulting in preterm birth is well described (Kota et al. 2013), there is currently no widely accepted treatment strategy for intrauterine infection (Newnham et al. 2014).

Medical and technological advances have improved survival rates of preterm infants, particularly of very and extremely preterm infants (Costeloe et al. 2012; Horbar et al. 2002; Thompson et al. 2015), however, serious morbidity after premature birth remains a major problem; in England, survival rates in extremely premature infants (born at 23 - 25 w GA) increased by 10-16 % between 1995 and 2006, but the incidence of major morbidities, bronchopulmonary dysplasia (BPD) and neurological impairment remained unchanged (Costeloe et al. 2012). Furthermore, the increase in both the delivery rate and survival rate of preterm infants means an increasing number of preterm infants face perinatal morbidities. Extremely preterm and very preterm infants are at greatest risk of impaired cardiorespiratory, neurological, immunologic and gastrointestinal function and development (Behrman & Butler 2007). However, even moderate to late preterm birth significantly increases the incidence of morbidity during the perinatal period and in later childhood (Shapiro-Mendoza & Lackritz 2012).

1.2 Respiratory complications in preterm infants

Respiratory disease is the single greatest cause of morbidity and mortality in the preterm infant. Approximately 75 % of infants born at 28 – 29 w GA (very preterm) suffer from immediate respiratory distress syndrome (RDS) (Sweet et al. 2010). RDS is characterised by alveolar surfactant deficiency and structural immaturity of the lungs and chest wall, which in combination results in reduced functional residual capacity and alveolar collapse during expiration (Garvey 1975; Verma 1995). Additionally, extremely preterm infants who do not show signs of RDS at birth can still develop chronic lung disease (CLD) such as BPD within the first weeks of life, necessitating mechanical ventilation (Bland 2005; Eber & Zach 2001). BPD is characterised by abnormal distal lung development, predominantly failure of alveolarisation (Bland 2005; Eber & Zach 2001).

1.3 Normal development of the respiratory system and disruption with preterm birth

The respiratory system consists of two components: 1) the lungs, the gas-exchange organ; and 2) the respiratory pump, which ventilates the lungs, and includes the neural respiratory center and nerves, chest wall and respiratory

muscles. Air enters the lungs through the nose/mouth during inhalation. Air is warmed and humidified in the upper airways, and then distributed to the deeper parts of the lungs where gas exchange occurs in the alveoli by interaction with the pulmonary circulation.

Neural activation of the respiratory pump facilitates inspiration by reducing intrathoracic pressure and inflating the lungs. The diaphragm is the primary respiratory muscle and contributes approximately 70 – 80 % of the work of breathing (Reid & Dechman 1995). Diaphragm contraction is responsible for expanding the chest wall and enlarging the thoracic cavity, thereby reducing thoracic pressure, during tidal breathing. The intercostal muscles stabilize the chest wall during tidal breathing. Increased neural activation of the diaphragm and external intercostal muscles further expands the chest wall and further enlarges the thoracic cavity during forced inspiration. The effectiveness of the lungs and respiratory pump in facilitating spontaneous ventilation increases over the course of development, meaning that the more premature the infant, the greater the compromise in lung and respiratory pump function.

1.3.1 Lungs

The lungs are among the last organs to fully develop in the infant. Normal lung development begins proximally, at 7 w GA, and progresses distally in 6 overlapping sequential stages: embryonic, pseudoglandular, canalicular, saccular, alveolar and microvascular maturation (Table 1-1). Lung development continues into adolescence, although the rate of development slows after 2 – 3 years (y) postnatal age (PNA) (Herring et al. 2014; Schittny 2017).

Preterm birth disrupts normal lung development resulting in lungs which are structurally and functionally immature at birth. Extremely preterm babies are born during the canalicular phase of lung development. Differentiation of epithelial cells into type I (gas exchange cells) and type II (surfactant-producing cells), vascularisation of the mesenchyme, and formation of the alveolar capillary barrier are only just beginning in extremely preterm lungs (Joshi & Kotecha 2007; Moss 2006). Very preterm babies are born during the early saccular phase of lung development; a time when type I and type II epithelial cells are immature, type II cells produce an inadequate quantity and quality of

surfactant, the alveolar-capillary barrier is only beginning to thin and vascularisation is not fully developed (Joshi & Kotecha 2007; Moss 2006). Consequently, extremely and very preterm infants have insufficient lung maturity to support independent ventilation.

Table 1-1. Stages of lung growth. Adapted from Joshi & Kotecha (2007) and Schittny (2017).

Stage	Time	Events
Embryonic	0 – 7 weeks	Formation of trachea, right and left main bronchi, segmental bronchi, and vasculogenesis around airway buds
Pseudoglandular	7 – 17 weeks	Differentiation of epithelial cells, formation of conduction airway and terminal bronchioles, formation of pulmonary arteries and veins
Canalicular	17 – 27 weeks	Formation of respiratory bronchioles, alveolar ducts and primitive alveoli, differentiation of type I and type II pneumocytes and formation of alveolar capillary barrier
Saccular	28 – 36 weeks	Increment in gas exchange areas, further differentiation of type I and type II cells
Alveolar	36 weeks – 2-3 years	First phase alveolarisation: Septation and multiplication of alveoli
	2-3 years – adolescence	Second phase alveolarisation: Septation and multiplication of alveoli
	Until 18 – 22 years	Enlargement of terminal bronchioles and alveoli
Microvascular maturation	Birth to 3 – 21 years (time uncertain)	Fusion of double alveolar capillary network into a single layer

1.3.2 Respiratory pump

Diaphragm development and function is vital for normal lung growth and lung maturation. Fetal breathing movements primarily result from *in utero* diaphragm contractions, and act to intermittently reduce intrathoracic pressure, stretch the lungs and increase lung fluid volume (Harding 1997). Fetal breathing movements first occur at 11 w GA in the human fetus and become deeper and

increasingly frequent; fetal breathing movements occur 30 % of the time by mid-saccular stage (Joshi & Kotecha 2007). Similarly, fetal breathing movements increase in frequency and depth from 40 days (d) GA (term = 145 – 150 d) in sheep (Dawes et al. 1972), and lung volume increases exponentially over the same period (Harding & Hooper 1996). Failure of the diaphragm muscle to develop normally in patients with congenital diaphragmatic hernia can halt lung development in the canalicular and saccular phase (Clugston & Greer 2007). Additionally, elimination of fetal breathing movements (via experimental spinal sectioning above the phrenic nerve in animal models) causes lung hypoplasia and delayed lung development, as evident by poor alveolar development and reduced lung compliance (Liggins et al. 1981; Wigglesworth & Desai 1979). Babies born prematurely have had less exposure to fetal breathing movements and are born with much lower lung volumes: the total lung capacity very preterm infants born at 30 w GA is only 46 % of the total lung capacity of term infants born at 40 w GA, when total lung capacity is normalised to body size (Langston et al. 1984).

Like the lung, the diaphragm is also structurally and functionally immature in preterm infants. The diaphragm undergoes rapid neuromuscular development during the second half of pregnancy (Lavin et al. 2013) which is essential for appropriate diaphragm function at birth. Premature birth interrupts normal diaphragm development and significantly reduces diaphragm strength (Dimitriou et al. 2003, 2001; Keens et al. 1978). A detailed review of normal diaphragm development and the functional characteristics of the preterm diaphragm are presented in Chapter 2.

The preterm diaphragm faces an increased workload during breathing, in addition to the reduced functional capacity brought about by premature birth. As a consequence of immature lung development, the preterm diaphragm is required to ventilate stiff lungs characterised by inadequate surfactant production and immature, non-compliant gas-exchange regions. Furthermore, the chest wall of the preterm infant is more compliant than the chest wall of term infants (Gerhardt & Bancalari 1980) and thus, susceptible to distortion by negative pleural pressures during inspiration (Heldt & McIlroy 1987). Chest wall

distortion increases the volume displacement of the diaphragm during inspiration (Heldt & McIlroy 1987). The preterm diaphragm needs to generate sufficient force to overcome the mechanical disadvantages imposed by the immature lungs and highly compliant chest wall. The high load-to-capacity ratio of the preterm diaphragm, due to the increased workload and reduced functional capacity, may predispose the preterm infant to respiratory distress. Additionally, perinatal exposures can also adversely affect diaphragm function (Mahzabin et al. 2017; Song et al. 2013), potentially contributing further to the development of respiratory distress and failure.

1.4 Current prevention and management of preterm respiratory diseases

Preterm respiratory diseases are managed primarily as diseases of lung immaturity, despite the increasing evidence that preterm respiratory disease has a complex aetiology, including pulmonary, respiratory musculature and neurological contributions (American Thoracic Society 2003). Clinical management of respiratory disease in preterm infants aims to stimulate lung maturation in addition to supporting ventilation and oxygenation. Preterm respiratory diseases are managed primarily with mechanical ventilation, non-invasive ventilation, oxygen supplementation, administration of surfactant and administration of antenatal corticosteroids (Roberts & Dalziel 2006; Sweet et al. 2013). Specifically, antenatal corticosteroids stimulate surfactant production and maturation of the lung antioxidant system (Bolt et al. 2001). Antenatal corticosteroids, as well as exogenous surfactant and ventilatory support, reduce the impact and incidence of RDS (Davis & Barrington 2005; Roberts & Dalziel 2006), but are not necessarily linked with a decreased incidence of BPD (Roberts & Dalziel 2006; Ward & Beachy 2003). Many preterm infants still require extended respiratory support (Chow et al. 2017). The contribution of other components of the respiratory system that are vital for spontaneous ventilation, such as the diaphragm, should be considered when managing/treating preterm respiratory disease. Notably, little is known on how preterm diaphragm development and function is affected by current clinical exposures and treatments. The limitations that exist in the current literature on the effects of antenatal and postnatal exposures on preterm diaphragm function and structure are detailed in Chapter 2.

1.5 Diaphragm structure and function

The diaphragm is a muscular dome-shaped barrier separating the thoracic and abdominal cavities (Bordoni & Zanier 2013); it is convex on the superior thoracic surface and concaved on the inferior abdominal surface, forming an area of apposition with the lower rib cage (Figure 1-1A). The left and right phrenic nerves that innervate the diaphragm are formed by motoneurons originating from the ventral horn of the C3-C5 segment of the spinal cord. Phrenic nerve activation and subsequent muscle contraction flattens the diaphragm and expands the thoracic cavity (Rochester, Arora & Braun 1982). Simultaneously, diaphragm contraction acts through the zone of apposition to elevate the lower ribs and expand the lower rib cage (Rochester, Arora & Braun 1982). Overall, pleural pressure decreases and abdominal pressure increases in response to contraction of the diaphragm (Figure 1-1B). The difference in pleural and abdominal pressure during inspiration is referred to as transdiaphragmatic pressure (Pdi) and can be measured as an index of diaphragm force output.

The diaphragm is composed of two muscles, the costal and crural diaphragm, which radiate from a large central tendon (Reid & Dechman 1995). Costal diaphragm fibres form sternal attachments to the xiphoid process, and costal attachments to the inner surface of 7^{th} – 12^{th} ribs, whilst the crural diaphragm attaches to the first three lumbar vertebrae. Three main hiatuses within the diaphragm allow passage of the oesophagus, aorta and vena cava (Figure 1-1C).

The diaphragm has a diverse range of functions. Increasing neural respiratory drive, leading to increasing motoneuron recruitment and Pdi production, occurs during increased respiratory demand, e.g. during exercise (Aliverti et al. 1997). Maximum motoneuron recruitment and maximum Pdi occurs during non-ventilatory behaviours such as the gag reflex and sneezing (Sieck & Fournier 1989). During diaphragm activation, crural fibres constrict the oesophageal hiatus, acting as a sphincter to prevent gastrointestinal reflux when abdominal pressure rises (Collis, Kelly & Wiley 1954; Downey 2011; Nason et al. 2012). Furthermore, contraction of costal and crural fibres widens the vena cava

hiatus, facilitating venous return (Moore, Dalley & Agur 2013; Nason et al. 2012).

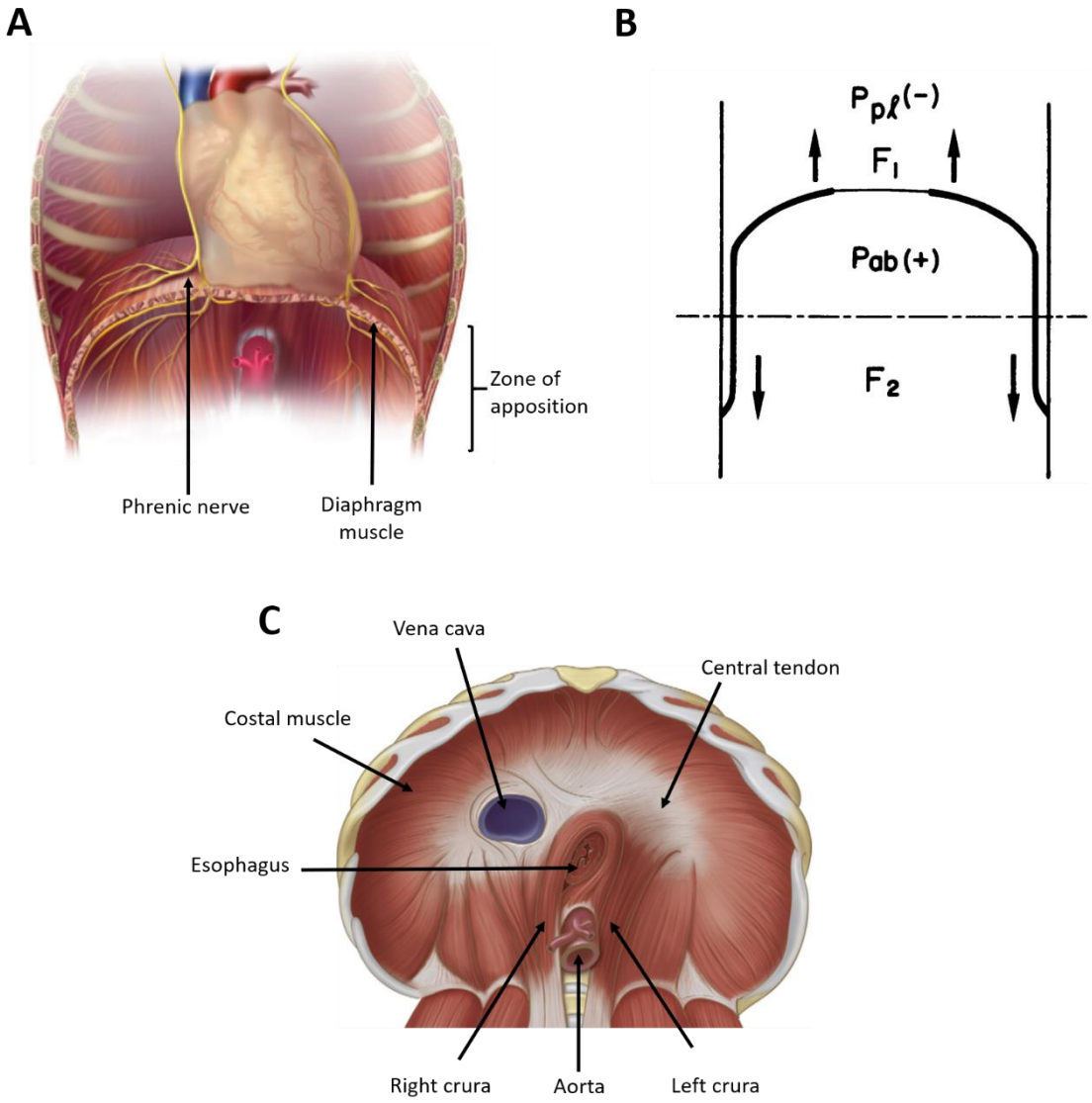


Figure 1-1. Diaphragm structure and function

(A) The dome-shaped arrangement of the diaphragm forming the zone of apposition *in situ.* Phrenic nerve innervation is depicted. Adapted from Nason et al (2012); (B) Diaphragm contraction results in an upward (F_1) and downward (F_2) force, which expands the thoracic cavity, decreasing pleural pressure (P_{pl}), and reduces the size of the abdominal cavity, increases abdominal pressure (P_{ab}). Adapted from Rochester, Arora & Braun (1982); (C) Abdominal view of diaphragm costal muscle, crural muscle, central tendon, and diaphragmatic apertures. Adapted from Downey (2011).

Costal and crural diaphragm fibres, like all skeletal muscle fibres, have a highly organised intracellular arrangement. They are primarily composed of myofibrils,

which contain bundles of myofilaments arranged into small contractile units known as sarcomeres (for review: (Au 2004)). Contraction occurs by cross-bridge cycling between the contractile proteins of the thick and thin sarcomeric filaments, myosin and actin, respectively. Thin filament regulatory proteins, tropomyosin and troponin, form a complex which acts as an 'on-off' switch, regulating cross-bridge cycling (Farah & Reinach 1995; Galińska-Rakoczy et al. 2008). In relaxed muscle, the troponin-tropomyosin complex blocks actin and myosin interactions and inhibits cross-bridge cycling. The inhibitory function of troponin-tropomyosin complex is removed in the presence of Ca^{2+}, allowing contraction to occur (Farah & Reinach 1995).

Ca^{2+} release is regulated by the excitation contraction-coupling pathway (for review: (Lamb 2000; Sandow 1965)); a chain of cellular events that links action potential generation to muscle contraction. Firstly, sarcolemmal depolarisation activates voltage-sensitive dihydropyridine receptors (DHPRs), located in invaginations of the sarcolemmal membrane (T-tubule membrane). Upon activation, the DHPRs interact with, and open adjacent ryanodine receptors (RYRs), on the sarcoplasmic reticulum (SR). Ca^{2+} released into the sarcoplasm via RYR binds to troponin C, removing the inhibitory function of the troponin-tropomyosin complex and enabling cross-bridge cycling. When action potential generation and Ca^{2+} release has ceased, sarcoplasmic Ca^{2+} concentrations decrease as Ca^{2+} is removed from the cytoplasm through the action of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps. The activity of the SERCA pumps lowers the cytosolic Ca^{2+}, which leads to the release of Ca^{2+} from troponin C, and results in muscle relaxation. Alteration to any component of the excitation-contraction coupling pathway will affect the contractile function of the diaphragm.

1.6 Animal models of respiratory development and disease

Respiratory development, function, and disease have been studied in a range of small (rodent) and large (pigs, sheep) animal models. Prenatal developmental processes in humans have similarities to postnatal respiratory development in altricial rodents, including postnatal diaphragm development. For instance, the expression of developmental myosin heavy chain (MHC) isoforms decreases

from 80 % to 15 % between 16 - 24 w GA and term gestation in the human diaphragm (Watchko, Daood & Sieck 1998). In the rat, all diaphragm fibres express developmental myosin heavy chain isoforms at birth (LaFramboise et al. 1991) and developmental myosin heavy chain isoforms disappear by 28 d PNA (Johnson et al. 1994). Alterations of the newborn rodent diaphragm by postnatal exposures can provide insight into the effects of such postnatal exposures on the preterm human diaphragm. However, differences in gestational and developmental trajectories limit the interpretation of alterations in respiratory function by antenatal exposures in rodent models.

1.6.1 The ovine model of respiratory development

The preterm ovine provides a better model for preterm respiratory disease than rodent models. Newborn sheep have similar physical features (weight and size) and precocial developmental trajectories (lung, skeletal muscle, and gastrointestinal development times) to humans. Additionally, sheep have a long gestation (145 – 150 d), making it easier to investigate antenatal exposures and treatments. The ovine model is used extensively to investigate the systemic effects of antenatal corticosteroids (Jobe et al. 1998; Moss et al. 2005) and chorioamnionitis, including effects of chorioamnionitis on the preterm diaphragm (Karisnan et al. 2015a, 2015b, 2017; Song et al. 2013). Respiratory and neurological outcomes are similar in the offspring of sheep and human after exposure to maternal antenatal glucocorticoid treatment (French et al. 1999; Jobe et al. 1998). Similarly, respiratory, neurological, immunological and gastrointestinal outcomes after lipopolysaccharide (LPS)-induced inflammation in the lamb are comparable to those associated with chorioamnionitis in the human infant (Kuypers et al. 2012b). A further advantage of the ovine model is that sheep frequently have singleton pregnancies and hence accurate delivery of LPS to a specific fetus is more practical than in multiple pregnancies of the pig. Lastly, given the similar size of newborn lambs and human infants, lamb respiratory function can be monitored and mechanical ventilation managed using contemporary neonatal equipment and neonatal medicine. Therefore, the ovine model provides an appropriate model of antenatal and postnatal human respiratory development.

2 Neonatal development of diaphragm structure and function – the influence of antenatal and postnatal exposures associated with preterm birth

The diaphragm is the primary inspiratory muscle, responsible for establishing spontaneous unsupported breathing after birth. The functional capacity of the diaphragm is highly dependent on its development during the late antenatal period. Premature birth before, or during, this period interrupts normal diaphragm development and significantly impairs the ability of the diaphragm to support spontaneous breathing. The diaphragm of the preterm infant comprises predominantly small, undeveloped fibres (Maxwell et al. 1983; Watchko, Daood & Sieck 1998), and produces lower Pdi than that of the term infant and adult diaphragm (Dimitriou et al. 2003, 2001; Keens et al. 1978).

The functional capacity of the immature diaphragm may be further compromised by the antenatal and postnatal exposures that are typically faced by preterm infants, such as undernutrition (Brozanski et al. 1993; Prakash, Fournier & Sieck 1993), inflammation (Song et al. 2013), mechanical ventilation (Knisely, Leal & Singer 1988), and glucocorticoids (Song et al. 2014) (Figure 2-1). These clinically common exposures impact on the development and severity of respiratory insufficiency. The effects of these exposures on preterm diaphragm function are under-investigated but are an essential consideration when evaluating the respiratory function of the preterm infant. Diaphragmatic dysfunction induced by antenatal and postnatal insults may compromise further diaphragm function consequent to preterm delivery and likely accelerate the development of respiratory failure in early postnatal life.

The purpose of this chapter is to: 1) describe developmental changes in diaphragm structure and function; and 2) describe the effects of antenatal and postnatal insults, commonly associated with premature birth, on the diaphragm. Overall, this review will highlight the potential contribution of impaired diaphragm function, resulting from immature respiratory development and adverse exposures, to respiratory failure in preterm infants.

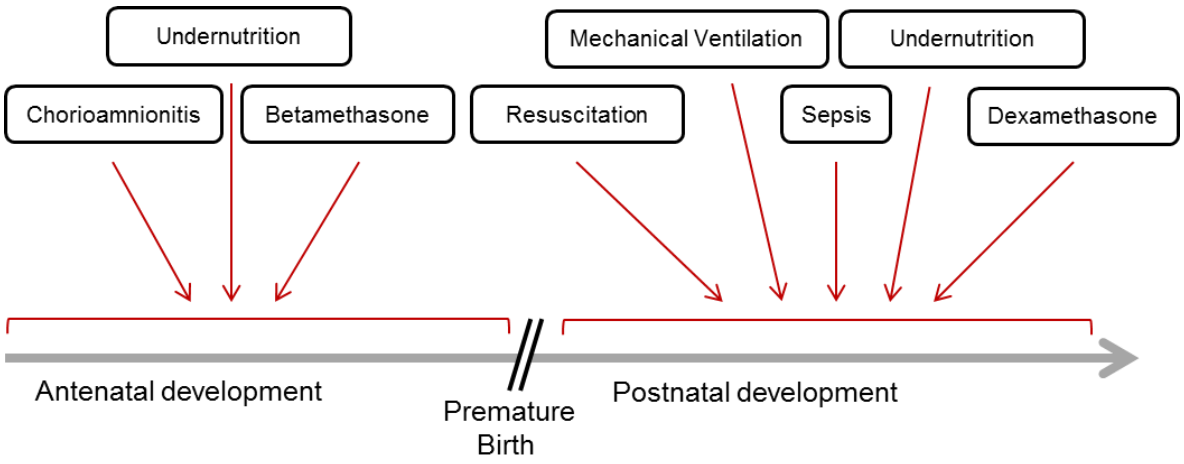


Figure 2-1. Antenatal and postnatal exposures commonly associated with preterm birth. Adapted from Gantert et al (2010).

2.1 Normal diaphragm development

To fully understand the effects of preterm birth on diaphragm function, it is important first to understand normal structural and functional development of the diaphragm muscle and phrenic nerves. Postnatal diaphragm development in altricial species (e.g. rodents and cats) and prenatal diaphragm development in precocial species (e.g. sheep and primates) have many similarities to prenatal developmental processes in humans (Lavin et al. 2013; Maxwell et al. 1983; Polla et al. 2004; Watchko, Daood & Sieck 1998).

2.1.1 Embryology

Human diaphragm embryogenesis occurs during the 4^{th} – 12^{th} weeks of gestation, and is facilitated by embryonic folding. The embryological origins of the diaphragm are not completely understood. 'Classical' textbook descriptions based on anatomical dissections of embryological tissue, state that diaphragm muscle develops from four structures: the septum transversum, the pleuroperitoneal folds, the oesophageal mesentery and the muscular body wall. Classically, the costal and crural diaphragm are considered likely to have separate embryological origins; the costal diaphragm developing from musculature in the septum transversum and muscular body wall, and the crural diaphragm developing from musculature of the oesophageal mesentery. The use of immunohistochemical markers and rat gene knockout models has

changed our understanding of diaphragm embryogenesis from that of the 'classical' description. In the rat model, the costal and crural diaphragm muscles arise from muscle precursor cells in the pleuroperitoneal folds, with no contribution from the septum transversum, oesophageal mesentery or muscular body wall (Babiuk et al. 2003). Muscle precursor cells and phrenic axons target the pleuroperitoneal folds at the level of the cervical spinal cord, under the guidance of a number of signaling molecules and growth factors (Molkentin & Olson 1996), after which, the folds descend to the level of the thoracic spinal cord. Muscle precursor cells and phrenic axons migrate along three axes, giving rise to the dorsolateral costal, sternal costal and crural diaphragm musculature (Allan & Greer 1997; Babiuk et al. 2003). However, the origin of the non-muscular component of the diaphragm remains unknown.

2.1.2 Myogenesis

Myogenesis occurs during and after embryogenesis, and is the sequential process where myogenic precursor cells develop into myoblasts, proliferate and differentiate into myotubes, and then fuse into myofibres. Myogenesis occurs in three distinct 'waves'; a small proportion of total fibres are formed during primary myogenesis at 8 – 10 w human gestation (Barbet, Thornell & Butler-Browne 1991), which provides the scaffolding for the development of secondary myofibres at 10 -19 w human gestation, and then tertiary myofibres, which are first visible at 16 - 17 w human gestation (Barbet, Thornell & Butler-Browne 1991; Draeger, Weeds & Fitzsimons 1987). The total number of muscle fibres is fixed after tertiary myogenesis (Wigmore & Stickland 1983) and subsequent muscle growth is characterised by hypertrophy through the proliferation and fusion of satellite cells with established myofibres (Pallafacchina, Blaauw & Schiaffino 2013; White et al. 2010).

2.1.3 Myofibre development

Skeletal muscle fibres are normally classified based on the MHC isoform expression. Embryonic fibres express the MHC_{emb} isoforms, neonatal fibres express MHC_{neo} isoforms; while adult slow-twitch Type I fibres express the MHC_{I} isoforms, and adult fast-twitch Type II fibres express either MHC_{IIa}, MHC_{IIx} or MHC_{IIb} isoforms. Fibres may also be "hybrids" which express multiple MHC

isoforms. For example, adult fibres may co-express MHC_{I} and MHC_{IIa}; MHC_{IIa} and MHC_{IIx}; or MHC_{IIx} and MHC_{IIb} (Schiaffino & Reggiani 1994).

Skeletal muscle undergoes notable structural changes during development in the second half of gestation; muscle fibre cross-sectional area (CSA) and fibre length increase, and there is a shift in fibre type proportions. Fetal muscle predominantly comprises fibres expressing the MHC_{emb} and MHC_{neo} isoforms and a small proportion of fibres expressing the MHC_{I} isoform. The human diaphragm consists of 80 % MHC_{emb}/MHC_{neo} and 20 % MHC_{I} isoforms at 16-24 w GA (Watchko, Daood & Sieck 1998). With development, the MHC_{emb} and subsequently MHC_{neo} isoforms gradually disappear, and adult MHC_{I}, MHC_{IIa}, MHC_{IIx} and MHC_{IIb} proportions progressively increase. Specifically, the development of primary myofibres is associated with the disappearance of developing MHC isoforms and the appearance of the adult MHC_{I} isoform (Schiaffino & Reggiani 1994). The development of secondary and tertiary myofibres is associated with the disappearance of developing MHC isoforms and the expression of MHC_{IIa}, MHC_{IIx}, MHC_{IIb} and/or MHC_{I} (Schiaffino & Reggiani 1994). By full term gestation, the proportion of MHC_{emb} and MHC_{neo} decreases to 15 %, MHC_{I} increases to 30 %, and the proportions of MHC_{IIa} and MHC_{IIx} are 45 % and 10 %, respectively (Watchko, Daood & Sieck 1998). A high proportion of hybrid fibres are observed during the developmental transition to adult fibre type proportions in the rat diaphragm, with some myofibres co-expressing MHC_{emb}, MHC_{neo}, MHC_{IIa} and MHC_{IIx} isoforms (LaFramboise et al. 1991). Overall, the developmental change in diaphragm fibre type proportions is likely to increase diaphragm strength and facilitate the range of functions performed by the diaphragm, including the non-ventilatory behaviors of coughing and sneezing.

Muscle fibres of the immature diaphragm are small and intrinsically weak (Johnson et al. 1994; Sieck, Fournier & Blanco 1991), with fibres expressing MHC_{neo} and MHC_{I} producing less force per cross-bridge cycle than fibres expressing MHC_{IIa} and MHC_{IIx} isoforms (Geiger et al. 2001). Maturation of the diaphragm is associated with a shift to adult fibre types, an increase in fibre size, and myofibril development including increased myosin density, new

sarcomere formation and alignment (Geiger et al. 2001; Maxwell et al. 1983; Orliaguet et al. 2002; West et al. 1999; Williams & Goldspink 1971). Myofibril developmental changes increase the capacity of the diaphragm to generate force, and likely increase the transmission of force into mechanical work. The maximum specific force (maximum force normalised to CSA) of the diaphragm increases with maturation (rat: (Johnson et al. 1994), baboon: (Maxwell et al. 1983), and lamb: (Lavin et al. 2013)). Additionally, non-contractile scaffolding proteins, titin and nebulin, facilitate sarcomere formation and arrangement (Isaacs et al. 1992; Wang 1996), and stabilise skeletal muscle during contraction and stretch (Kontrogianni-Konstantopoulos et al. 2009). The association of titin with sarcomere formation and alignment in the developing diaphragm likely explains the reduction in susceptibility to stretch-induced damage with increased gestational age in the lamb (Lavin et al. 2013). The preterm diaphragm is likely more susceptible to injury from increased resistive loading by the high ratio of chest wall to lung compliance, and from eccentric activation during expiration for the maintenance of end-expiratory lung volume (Kosh & Stark 1984). Overall, these results explain the lower Pdi produced in response to phrenic nerve stimulation and the lower maximal inspiratory pressures that occur during crying in preterm infants compared to term infants (Dimitriou et al. 2001, 2003).

Contractile function is also dependent on SR Ca^{2+} handling. Ca^{2+} is the trigger for skeletal muscle contraction. However, the capacity of the SR to release and re-sequester Ca^{2+} is likely reduced, due to the immaturity of the SR Ca^{2+} release system, in the preterm diaphragm (Arai et al. 1992; Brandl et al. 1987; Maxwell et al. 1983; Schiaffino & Margreth 1969; Zubrzycka-Gaarn & Sarzala 1980). A reduction in the SR Ca^{2+} release will reduce cross-bridge interaction and force production. The SR is underdeveloped in the preterm baboon diaphragm compared to the term baboon (Maxwell et al. 1983). Development of the skeletal muscle Ca^{2+} release system occurs during the first two postnatal weeks in the rat, and is characterised by increased depth of t-tubule invaginations, increased size of the SR, and an increase in junctional cisternae between the t-tubules and the SR (Schiaffino & Margreth 1969). Messenger RNA expression of the RYR, the channel responsible for the release of Ca^{2+}

from the SR, and calsequestrin, the Ca^{2+} binding protein within the SR, increases with muscle development in the rabbit (Zubrzycka-Gaarn & Sarzala 1980). Protein density and ATPase activity of the SERCA, responsible for the reuptake of Ca^{2+}, also increases with development in the rabbit (Zubrzycka-Gaarn & Sarzala 1980). Isoforms of SERCA and calsequestrin switch from neonatal and slow isoforms to fast isoforms in Type II fibres with development (Arai et al. 1992; Brandl et al. 1987). Consequently, the contraction and relaxation times in the immature diaphragm are significantly longer than in the mature diaphragm (Lavin et al. 2013; Maxwell et al. 1983; Sieck, Fournier & Blanco 1991). This presents an interesting paradox, in that longer contractile times result in enhanced force summation and an increased relative force output at lower frequencies of stimulation.

Lastly, *in vitro* studies report a high level of fatigue resistance in the immature diaphragm. Diaphragm fatigue resistance is high at birth and decreases thereafter in altricial species, including the rat (Watchko & Sieck 1993; Zhan et al. 1998) and the cat (Sieck, Fournier & Blanco 1991). Similarly, diaphragm fatigue resistance is high in the preterm and decreases during late prenatal development in precocial species, such as the baboon (Maxwell et al. 1983) and sheep (Lavin et al. 2013). Fatigue resistance is largely determined by the energy requirements and oxidative capacity of the muscle, and is typically classified based on the oxidative capacity of the muscle (Burke 1981). Adult slow-twitch fibres (Type I) and fast-twitch fibres with high oxidative capacity (i.e. Type IIa) are more resistant to fatigue than adult fast-twitch fibres with low oxidative capacity (i.e. Type IIb fibres). Muscle fibres of the immature diaphragm have low oxidative capacity, measured by the activity of the oxidative enzyme succinate dehydrogenase (Fratacci et al. 1996; Sieck & Blanco 1991; Sieck, Cheung & Blanco 1991; Smith et al. 1988) and a low proportion of slow-twitch fibres (Watchko, Daood & Sieck 1998). However, the energy requirements of immature muscle are also low; characterised by low myosin density, low actomyosin ATPase activity and slow, weak contractions. There is a strong inverse correlation between the developmental increase in MHC ATPase activity and fatigue resistance in the rat diaphragm (Watchko, Daood & Sieck 1998). It may be, therefore, that the low energy requirement of

the immature muscle outweighs the reduced oxidative capacity and overall improves fatigue resistance.

The high fatigue resistance of the immature diaphragm reported from *in vitro* studies may not be comparable to *in vivo* measures of fatigue resistance; e.g. the newborn rabbit diaphragm is reportedly less fatigue resistant than the adult rabbit diaphragm based on *in situ* measures in which fatigue resistance was evaluated as the ability to maintain mouth occlusion pressure during repetitive stimulations (Le Souef et al. 1988). Respiratory fatigue can result from central fatigue or peripheral muscle fatigue during respiratory loading (ATS 2002). The *in situ* study may indicate that the newborn rabbit is more susceptible to central respiratory fatigue, rather than overt diaphragm fatigue, when compared to the adult rabbit. Based on the contrasting observations, extrapolation of *in vitro* measures of diaphragm fatigability to preterm infants should be made with caution. It should also be noted that there is a lack of data showing diaphragm fatigue in preterm infants, likely due to the difficulty in obtaining accurate measures of diaphragm fatigue in infants (ATS 2002).

Adult fibre-type characteristics develop as the diaphragm matures during the perinatal period. Size and oxidative capacity increase in all fibre types but there is a preferential increase in the size of Type II fibres (Fratacci et al. 1996; Maxwell et al. 1983; Sieck, Fournier & Blanco 1991) and the oxidative capacity of Type I fibres (Fratacci et al. 1996; Sieck & Blanco 1991; Sieck, Cheung & Blanco 1991; Smith et al. 1988). Variations in MHC isoforms, SERCA isoforms and SR size, typical of adult fibre types, develop during the perinatal period. These developmental variations between fibre-types facilitate the range of functions of the adult diaphragm.

Overall, the immature diaphragm muscle is weaker than the mature diaphragm and also faces an increased work of breathing, due to the decreased lung and increased chest wall compliance of the preterm infant. The weak immature diaphragm is required to operate at a higher proportion of its maximal capacity to sustain normal tidal breathing and therefore has less reserve capacity to cope with the increased work of breathing, further increasing the likelihood of respiratory distress of the preterm infant.

2.1.4 Phrenic motoneuron development

Diaphragm function is dependent not only on contractile function of the muscle fibres, but also on the activity of the innervating phrenic motoneurons. Phrenic motoneuron development, primarily studied in rats and cats (Cameron et al. 1991a; ed. Haddad & ed. Farber 1991; Mantilla & Sieck 2008; Prakash et al. 2000), is associated with significant changes in the size, electrophysiological properties, and motor unit innervation ratio. The developmental changes of the phrenic motoneuron match the developmental changes of diaphragm muscle fibres, and the overall respiratory demands.

In the adult, motoneurons innervating Type I muscle fibres are typically smaller than motoneurons innervating Type II fibres: Type I motoneurons have the smallest diameter, followed by Type IIa, with Type IIb and IIx being the largest (Zajac & Faden 1985). Small motoneurons have a higher input resistance, lower rheobase and slower axonal conduction velocity relative to large motoneurons (Burke 1981). Small motoneurons, innervating fatigue-resistant Type I fibres, are recruited before larger motoneurons, innervating fatigable Type II fibres (Burke 1981; Dick, Kong & Berger 1987; Henneman 1957; Mendell 2005; Zajac & Faden 1985). Consequently, motoneuron recruitment order matches the contractile properties of the muscle fibre that it innervates.

The functional recruitment of motoneurons and regulation of force production differs between the adult and developing diaphragm. In the adult cat, tidal breathing requires only 24 % of the maximum Pdi produced during airway occlusion, and is achievable by activation of Type I fibres alone (Sieck & Fournier 1989). Motoneuron recruitment and Pdi increases in response to increased respiratory demands reaching ~30 % of its maximum force generating capacity during hypoxia or hypercapnia (Sieck & Fournier 1989); where maximum force generating capacity is the maximum force produced during total airway occlusion. Increased Pdi is facilitated by a progressive recruitment of Type I, Type IIa, Type IIx/IIb fibres. Hence, during normal tidal breathing the adult diaphragm has a ‘reserve capacity’, meaning that motor unit recruitment, and thus force production, can be increased during times of increased respiratory demand. In comparison to the adult diaphragm, the

developing diaphragm works close to maximal capacity during tidal breathing; with ~94 % of phrenic motoneurons activated during tidal breathing in the two week-old cat (Cameron et al. 1991b). The electrophysiological properties of immature phrenic motoneurons facilitate increased activation, as they are small in size, with high input resistance and low rheobase (Cameron et al. 1991a, 1991b; Carrascal et al. 2005; Fulton & Walton 1986; Prakash et al. 2000). The high recruitment of developing phrenic motoneurons is likely vital for the generation of adequate Pdi during tidal breathing, but reduces the capacity for increased motor unit recruitment in response to increased respiratory demands.

Greater force production in response to increasing respiratory demand is also achieved by increasing motor unit firing frequency. Increased firing frequency is particularly important for the progressive increase in Pdi during the second half of inspiration (Iscoe et al. 1976), particularly in adult muscle. However, developing motoneurons have a prolonged action potential after-hyperpolarisation (Fulton & Walton 1986), which may limit their capacity to increase motor unit firing frequency (Navarrete & Vrbov 1983). Given that the developing diaphragm requires activation of a high proportion of motor units for the generation of tidal breathing, limitations to the firing frequency of developing phrenic motoneurons could further compromise the ability of the developing diaphragm to cope with increased respiratory demands. Alternatively, a reduced firing frequency of developing phrenic motoneurons may go hand-in-hand with the long contractile times of the developing diaphragm muscle. The long contractile times result in enhanced force summation and maximum force production at lower stimulation frequencies. As such, stimulation at higher frequencies may not necessarily result in additional gains in force production.

Lastly, immature motor units are also characterised by high innervation ratios and polyneuronal innervation of immature muscle fibres (Bagust, Lewis & Westernman 1973; Brown, Jansen & Van Essen 1976; Burke 1981; O'Brien, Ostberg & Vrbová 1978). Consequently, activation of individual motoneurons results in the recruitment of many muscle fibres and high force production. Increased fibre recruitment is likely important in the generation of adequate Pdi during tidal breathing in the preterm infant, given the intrinsic weakness of

immature diaphragm muscle fibres. However, polyneuronal innervation reduces the capacity of the developing diaphragm to produce graded changes in force. Synapse elimination of polyneuronally innervated muscle fibres underlies the transition to mature mononeuronal fibre innervation and smaller motor units during phrenic motoneuron development (Bagust, Lewis & Westernman 1973; Brown, Jansen & Van Essen 1976; Burke 1981; O'Brien, Ostberg & Vrbová 1978). These changes in motor unit innervation coincide with reduced motoneuron excitability and match the developmental increase in diaphragm muscle strength and stabilisation of the chest wall.

Overall, the preterm diaphragm works at a high proportion of maximum capacity during tidal breathing, due to the intrinsic weakness of the diaphragm fibres and the immature motor unit properties. High motor unit recruitment of the diaphragm is required to match the high respiratory demands of the preterm infant; however, increased demands on the respiratory muscle pump may contribute to the development of respiratory failure in the preterm infant.

2.2 External influences on preterm diaphragm structure and function

The functional capacity of the immature preterm diaphragm may be compromised further by external influences during preterm birth and postnatal development. Antenatal exposure to undernutrition, inflammation and glucocorticoids are commonly associated with preterm birth, complicated by postnatal undernutrition, mechanical ventilation and postnatal glucocorticoid therapy. The effects of these external insults on the diaphragm are an essential consideration when evaluating the respiratory function of the preterm infant. Given diaphragm function is already compromised by preterm birth, further dysfunction induced by these external insults will likely contribute to respiratory failure in early postnatal life.

The effects of common antenatal and postnatal insults on the structure and function of the preterm diaphragm is the focus of the remainder of this chapter. Inferences about the response of preterm diaphragm to undernutrition, inflammation, mechanical ventilation and glucocorticoids are drawn from extended adult skeletal muscle literature, when such information is missing from the preterm literature.

2.2.1 Nutrition

Antenatal and postnatal undernutrition are commonly associated with premature birth and likely influence diaphragm development. Maternal undernutrition and inadequate nutritional transfer via placental insufficiency can induce intrauterine growth restriction (Brown & Hay 2016; Godfrey & Barker 1995), and babies born at 26 - 30 w gestation are up to 10 times more likely to have intrauterine growth restriction than babies born at 40 w gestation (Gilbert & Danielsen 2003). Additionally, postnatal growth restriction resulting from low energy reserves (Frank & Sosenko 1988) and inadequate nutritional intake during early postnatal life is referred to as a 'universal problem' in preterm infants (Cooke, Ainsworth & Fenton 2004); postnatal growth restriction affected ~50 % of infants born at 501 - 1500 g in North American hospitals in 2013 (Horbar et al. 2015). Thus, preterm infants are at an increased risk of both intrauterine and postnatal growth restriction resulting from undernutrition. Intrauterine growth restriction is associated with an increased risk of RDS development in late preterm babies (Gilbert & Danielsen 2003): both intrauterine and postnatal growth restriction are implicated in the pathogenesis of BPD (Korhonen et al. 1999; Lal et al. 2003; Wilson et al. 1991). In adults, nutritional deprivation is associated with respiratory muscle weakness and likely compromises respiratory function (Arora & Rochester 1982). Experimental studies indicate undernutrition delays diaphragm development and weakens the developing diaphragm (Brozanski et al. 1991, 1993; Prakash, Fournier & Sieck 1993). Therefore, antenatal and/or postnatal undernutrition is likely to further compromise the contractile function of the immature diaphragm, thereby contributing to the increased risk of respiratory insufficiencies in the preterm infant.

Effects of undernutrition on skeletal muscle

The effects of undernutrition on diaphragm development have not been studied extensively. Nonetheless, current evidence is highly consistent with the well-known effects of undernutrition on the development of peripheral skeletal muscles. Undernutrition negatively affects growth rate, resulting in significantly lower body weights in undernourished babies. Nutritional status also impacts muscle development; undernutrition is associated with significantly fewer and smaller muscle fibres (Brozanski et al. 1993; Fahey et al. 2005; Prakash,

Fournier & Sieck 1993; Wilson, Ross & Harris 1988), resulting in significantly lower muscle weights (Brozanski et al. 1993; Fahey et al. 2005; Lefaucheur et al. 2003; Prakash, Fournier & Sieck 1993; Ruiz-Rosado et al. 2013). The effect of the nutritional insult on the fetal and postnatal diaphragm muscle varies depending on developmental stage, and the type of undernutrition, as described below.

Comparative studies in sheep, rats, and guinea-pigs indicate undernutrition immediately before or during secondary myogenesis reduces the number of secondary myofibres formed; impaired myofibre formation affects fast Type II fibres preferentially (Brozanski et al. 1993; Dwyer et al. 1995; Prakash, Fournier & Sieck 1993; Zhu et al. 2004). The specific reduction in fast Type II fibres with maternal undernutrition is associated with changes to the contractile properties of the rat diaphragm. For example, maternal undernutrition in the rat causes intrinsic diaphragm weakness and an increase in fatigue resistance *in vitro* (Brozanski et al. 1993; Prakash, Fournier & Sieck 1993). Thus, the reduction in the number of fibres and intrinsic strength of the diaphragm by undernutrition likely exacerbates diaphragm weakness associated with preterm birth, assuming undernutrition-induced changes to diaphragm function, identified in sheep, rats, and guinea-pigs, are evident in humans.

Hypoplasia induced by maternal undernutrition before/during secondary myogenesis is widely regarded as irreversible, given the total number of muscle fibres is fixed after tertiary myogenesis, at least in pigs (Wigmore & Stickland 1983) and rodents (White et al., 2010). Alterations to diaphragm development in the rat by trans-gestational maternal undernutrition, including hypotrophy of Type II fibres, intrinsic diaphragm weakness and increased fatigue resistance, persist into adulthood despite postnatal nutritional rehabilitation restoring body weight (Brozanski et al. 1993; Prakash, Fournier & Sieck 1993). Similarly, hypotrophy induced by a 50 % reduction in nutrition at 28 – 78 d gestation in fetal lambs, before/during secondary myogenesis, is still evident after 8 months (m) of postnatal life (Zhu et al. 2006). Although hypoplasia was irreversible, an increase in fast Type II (glycolytic) fibres was noted with 8 m of adequate postnatal nutrition (Zhu et al. 2006). It is possible that fibre type shifts reverse

the fibre type specific effects of undernutrition during myogenesis. However, it should be noted that while hypoplasia induced by undernutrition before/during secondary myogenesis is widely regarded as irreversible, Daniel et al. (2007), using a similar dietary profile as Zu et al. (50 % restriction in nutrition at 30 – 70 d gestation in fetal lambs) reported the recovery of myofibre numbers to normal levels following 24 weeks of adequate postnatal nutrition. The mechanisms underlying the recovery in fibre number with adequate postnatal nutrition are unclear but potentially clinically relevant. Overall, maternal undernutrition occurring before or during secondary myogenesis in the human fetus will likely induce diaphragmatic hypoplasia and weakness, but whether these changes are reversible remains controversial. Irreversible hypoplasia and weakness of the preterm diaphragm could contribute to respiratory insufficiencies in the preterm infant particularly if the effects are prolonged during postnatal development.

Undernutrition subsequent to the completion of myogenesis likely compounds the effects of maternal undernutrition before/during secondary myogenesis, further impairing diaphragm development and function. Undernutrition during postnatal life in mice, guinea-pigs and pigs is associated with reduced body and skeletal muscle weight gain (Goldspink & Ward 1979; Lefaucheur et al. 2003; Ward & Stickland 1993), similar to the effects of maternal undernutrition before/during secondary myogenesis. However, the reduction in muscle weight with postnatal undernutrition in these animal models results from a reduction in fibre CSA, preferentially affecting fast Type IIb fibres (Goldspink & Ward 1979; Lefaucheur et al. 2003; Ward & Stickland 1993). The reduction in fibre size is likely due, at least in part, to a reduction in the population of satellite cells during undernutrition (Woo et al. 2011). Furthermore, in rats, guinea pigs and pigs, muscle development is delayed when undernutrition extends beyond secondary myogenesis into postnatal life; the delayed muscle development is indicated by a delayed disappearance of developmental fibre types and a reduction in muscle protein concentration (Brozanski et al. 1991; Lefaucheur et al. 2003; Ward & Stickland 1993). Glycolytic activity is also reduced as a consequence of postnatal undernutrition in pigs, consistent with a higher proportion of developmental fibres and lower proportions of adult fast Type IIb fibres

(Lefaucheur et al. 2003). Overall, diaphragm atrophy and delayed development induced by antenatal and postnatal undernutrition will likely further reduce preterm diaphragm strength and delay the developmental increase in the functional capacity of the diaphragm.

Effects of vitamin deficits on skeletal muscle

The effects of undernutrition on skeletal muscle structure and function are dependent on the type of nutrient deprivation as well as the timing of undernutrition during development. The aforementioned animal studies have induced undernutrition by reducing total calorie intake. However, certain vitamins are essential for normal diaphragm development. For instance, dietary intake of vitamin A is important in retinoid signalling, and a maternal diet deficient in vitamin A during early pregnancy in rodents is associated with the development of congenital diaphragmatic hernia, a failure of the pleuroperitoneal folds to close during embryogenesis (Clugston & Greer 2007; Montedonico, Nakazawa & Puri 2008; Wilson, Roth & Warkany 1953). Clinical trials in newborn infants with congenital diaphragmatic hernia also suggest a possible deterioration of retinol transport across the placenta is involved in the pathogenesis of congenital diaphragmatic hernia (Beurskens et al. 2010), indicating that adequate retinoic acid signalling is required for normal diaphragm development.

More recently, vitamin D has been implicated in normal skeletal muscle development, which is particularly relevant as reports of the prevalence of vitamin D deficiency at birth in preterm infants range from 13 – 99 % (Monangi et al. 2014; Park et al. 2015; Tergestina et al. 2014). Vitamin D receptors are more prevalent in skeletal muscle of young rats compared to older rats (Ray et al. 2016) and both vitamin D receptors and CYP27B1, the enzyme responsible conversion of precursor (25-hydroxyvitaminD) to its active vitamin D3 (1,25-dihydroxyvitamin D), are present in myoblasts and myotubes (Srikuea et al. 2012). Both active and inactive forms of vitamin D3 regulate myoblast proliferation, differentiation and myotube size (Girgis et al. 2014; Srikuea et al. 2012), further emphasising the importance of vitamin D for skeletal muscle growth and differentiation. Vitamin D receptor knockout rats have reduced fibre

size and delayed disappearance of developmental fibre types compared to wild type rats (Endo et al. 2003), and maternal diets low in vitamin D reduce skeletal muscle CSA, increase inter-myofibrillar spaces and alter the expression of genes involved in myogenesis, protein catabolism and cytoskeletal organisation in muscles from rat offspring (Max et al. 2014). Furthermore, a postnatal diet deficient in vitamin D induces a selective atrophy and weakness of the diaphragm, while sparing the fast-twitch peripheral EDL muscle (Ray et al. 2016), indicating a possible fibre or muscle type-specific effect. These results indicate vitamin D has an important and complex role in regulating molecular pathways which drive muscle growth and development. The same vitamin D deficient diet does not have an effect on the skeletal muscle structure and function in older rats (Ray et al. 2016), which is consistent with a decrease in vitamin D receptor expression with development and emphasises the importance of vitamin D in skeletal muscle development. Therefore, the developing diaphragm may be particularly vulnerable to vitamin D deficiency.

Overview and clinical relevance

Overall, based on the evidence available from other muscles, it is highly likely that undernutrition will impair diaphragm force production, exacerbating preterm diaphragm weakness. Undernutrition impairs lung maturation and function (Pike, Pillow & Lucas 2012) and reduces chest wall muscle strength (Dias et al. 2004), which further increases the load-to-capacity ratio of the preterm diaphragm. Thus, the nutritionally deprived, weak, preterm diaphragm may not have the functional capacity to support independent ventilation. It is possible that diaphragm weakness contributes to the increased incidence of BPD in preterm infants with low birth weight, who are nutritionally deprived and have increased energy demands (Kurzer et al. 1988; Lal et al. 2003; Wilson et al. 1991; Yeh et al. 1989).

Early nutritional intervention to increase growth rate and reduce co-morbidities has gained considerable attention amongst neonatal researchers. Vitamin A supplementation during gestation significantly reduces the incidence of congenital diaphragmatic hernia and improves lung maturation in rats (Babiuk, Thébaud & Greer 2004). Postnatal vitamin A supplementation is protective

against ventilator-induced oxidative and catabolic injury of the rat diaphragm (unpublished data, Song et al), and in the clinical setting, reduces the severity of BPD in very low weight infants dependent on mechanical ventilation (Shenai et al. 1987). On the other hand, nutritional intervention with earlier and/or larger amounts of amino acids, earlier and/or more lipids and increased total energy intake in very low weight infants with respiratory distress improves growth rate but does not reduce the incidence of BPD (Wilson et al. 1997); emphasising the importance of adequate nutrition *in utero*. Protracted diaphragm hypoplasia induced by undernutrition before/during secondary myogenesis may contribute to the persistence of BPD with postnatal nutritional interventions. Antenatal nutritional interventions to increase total energy intake before/during secondary myogenesis may be beneficial in preventing diaphragm hypoplasia and diaphragm contribution to respiratory insufficiencies.

2.2.2 Inflammation

Intrauterine inflammation and postnatal sepsis are commonly associated with preterm birth and influence the respiratory function of preterm infants. Chorioamnionitis, inflammation of the feto-maternal placental membranes, is a risk factor for preterm birth (Goldenberg, Hauth & Andrews 2000). Typically, chorioamnionitis remains undetected throughout gestation and is identified as a histologic finding on placental examination after birth. About 70 % of preterm births before 30 w of gestation are associated with histologic chorioamnionitis (Goldenberg, Hauth & Andrews 2000). The effects of chorioamnionitis on respiratory function in the preterm infant is complex, being associated with an initial decreased incidence of RDS but later increase in BPD (Been et al. 2011; Gantert et al. 2010; Kramer et al. 2009). Inflammation-induced lung maturation and increased susceptibility of the lung to secondary insults underlie the decrease in RDS and increase in BPD, respectively. Preterm infants are also at a high risk of morbidities and mortality caused by postnatal sepsis (Kaufman & Fairchild 2004) and postnatal sepsis is a risk factor for the development of BPD (Van Marter et al. 2002).

The involvement of the premature diaphragm in respiratory complications after exposure to both chorioamnionitis and postnatal sepsis is unknown. Diaphragm

dysfunction is the main contributor to respiratory failure in experimental models of adult sepsis (Hussain, Simkus & Roussos 1985). Chorioamnionitis and sepsis-induced diaphragm dysfunction will likely contribute to and accelerate respiratory failure in the preterm infant, given results of sepsis-associated diaphragm dysfunction in adults, and the increased susceptibility of the preterm infant to respiratory distress.

Post-developmental alterations of the diaphragm by inflammation

Sepsis-induced diaphragm dysfunction in the adult is attributed to both impairment of the contractile filaments and atrophy of muscle fibres. Contractile dysfunction is mediated by increased levels of pro-inflammatory cytokines and oxidative stress (Lanone et al. 2005), which are associated with damage or alterations in multiple key components of the excitation-contraction coupling pathway. Damage to the sarcolemma and hyperpolarisation of the resting membrane potential is linked to reduced diaphragm electromyography activity, indicating failed action potential generation and propagation (Leon et al. 1992; Lin et al. 1998). Alterations in Ca^{2+} handling (Stamm et al. 2001) and reduced myofilament Ca^{2+} sensitivity likely reduce the force producing capacity (Andrade et al. 1998). Reduced mitochondrial oxygen utilisation and reduced ATP production diminish energy available for force production (Callahan & Supinski 2005). The resulting marked diaphragm dysfunction is the main contributor to respiratory failure in experimental models of adult sepsis (Hussain, Simkus & Roussos 1985).

Evidence for alterations of the developing diaphragm by inflammation

In contrast to the adult diaphragm, the effects of inflammation on the preterm diaphragm are largely unknown. The preterm diaphragm is likely more susceptible to oxidative stress than the term or adult diaphragm because it has immature antioxidant defences (Song & Pillow 2012) and is highly oxidative; having larger, more abundant mitochondria (Maxwell et al. 1983). A brief exposure to LPS impairs contractile function of the preterm lamb diaphragm, reducing maximum specific force by ~30 % at birth (Song et al. 2013). Conversely, the same LPS exposure under the same experimental conditions, impairs contractile function of the term lamb diaphragm by only ~20 %

(Karisnan et al. 2017). These results indicate that the preterm diaphragm is more vulnerable to inflammation-induced dysfunction than its term counterpart. Furthermore, LPS-induced weakness of the preterm lamb diaphragm at birth persists for up to 21 d after exposure (Karisnan et al. 2015a). Long-lasting diaphragm dysfunction indicates that *in utero* exposure to LPS may interfere with critical developmental programming of the diaphragm. A 30 % reduction in diaphragm strength, in an already compromised preterm diaphragm, could have major implications for the establishment of spontaneous breathing at birth and the development of respiratory insufficiencies in early postnatal life.

Overview and clinical relevance

Inflammation induces major diaphragm dysfunction in animal models of adult sepsis, which results in respiratory failure and mortality (Hussain, Simkus & Roussos 1985). Exposure to inflammation *in utero* impairs preterm diaphragm function at birth and the preterm diaphragm is more susceptible to *in utero* inflammation than the term diaphragm (Karisnan et al. 2017). Based on the results of diaphragm weakness following prolonged *in utero* exposure to inflammation (Karisnan et al. 2015a), diaphragm dysfunction present at birth may persist into postnatal life and compromise respiratory function of the preterm infant; however, the long-lasting effects of chorioamnionitis on postnatal diaphragm function remain to be studied. Furthermore, the effects of postnatal sepsis on preterm diaphragm function are unknown and require investigation. Extrapolation of the known effects of sepsis on the adult diaphragm to the preterm diaphragm should be made with caution, as the preterm diaphragm is likely more vulnerable to oxidative stress. Given the increased susceptibility of the preterm diaphragm to antenatal and postnatal inflammation, and the already compromised nature of the preterm diaphragm, inflammation is likely a major complication for postnatal diaphragm and respiratory function of the preterm infant.

2.2.3 Mechanical ventilation

Mechanical ventilation is commonly used to facilitate gas exchange in preterm infants who are unable to breathe independently. According to the 2015 report of the Australian and New Zealand neonatal network, over 87 % of extremely

preterm infants require invasive respiratory support (Chow et al. 2017). Despite the essential requirement of respiratory support to sustain life, mechanical ventilation induces an inflammatory response that arrests alveolarisation and depresses surfactant production within the lung (Attar & Donn 2002). Mechanical ventilation also impairs diaphragm funtion in adults; referred to as VIDD (Vassilakopoulos & Petrof 2004). VIDD is characterised by diaphragm wasting and weakness that has been identified from *in vivo* measures, including sonography in humans, showing reduced diaphragm thickness and transdiaphragmatic pressures (Grosu et al. 2012; Hermans et al. 2010). The combination of ventilator-induced lung injury and diaphragm dysfunction impedes weaning of adult patients from mechanical ventilation to spontaneous breathing: 40 % of the time patients spend on mechanical ventilation is devoted to weaning (Esteban et al. 1994), with extended time on mechanical ventilation due, at least in part, to VIDD. VIDD in the preterm infant, although not well characterised, is likely to effect the ability of the preterm infant to sustain spontaneous breathing. Measurements of diaphragm function may be useful tools to predict weaning success in infants, children and adult patients (Barwing et al. 2013; Currie et al. 2011; Dres et al. 2012; Harikumar et al. 2009; Kraaijenga et al. 2017); previous studies have predicted extubation failure in ventilated infants and children with 100 % accuracy using the the tension-time index (representing the load-to-capacity ratio of the diaphragm) (Currie et al. 2011; Harikumar et al. 2009); demonstrating the important balance between the functional capacity of the diaphragm and the load placed on it by the lungs and chest wall in the esablishment of adequate spontaneous ventilation.

Much of what we understand about the characteristics and mechanisms of VIDD is based on adult animal studies, or adult human studies on intensive care unit patients. In contrast, little is known about diaphragm function following mechanical ventilation in preterm infants. Consequently, the literature regarding VIDD in adults is reviewed briefly to identify the important questions regarding VIDD in the preterm infant.

Ventilator-induced dysfunction of the developed diaphragm: Implications for the developing diaphragm

In animal models of adult VIDD, diaphragm contractile function declines with increasing duration of mechanical ventilation. The relationship between mechanical ventilation and *in vitro* diaphragm specific force is sigmoidal: 12 h of mechanical ventilation induces rapid onset and large reductions in diaphragm specific force, which doubles over the next 12 h of mechanical ventilation (Figure 2-2). Intrinsic strength of the diaphragm is nearly halved within just 24 h of mechanical ventilation in rats (Powers et al. 2002), indicating that even short durations of mechanical ventilation induce severe diaphragm weakness. Mechanical ventilation induces major structural alterations to the diaphragm, including a decrease in fibre size, myofibrillar disarray, an increased number of lipid vacuoles in the sarcoplasm, and damage to mitochondria, which likely contributes to the diaphragm weakness (Bernard et al. 2003). The proportion of abnormal myofibrils following mechanical ventilation correlates with, and likely contributes to, the reduced force capacity of the diaphragm after mechanical ventilation (Sassoon et al. 2002; Vassilakopoulos & Petrof 2004). Furthermore, atrophy of the diaphragm during mechanical ventilation amplifies diaphragm weakness. For example, mechanical ventilation in rats for 9 – 14 d reduces the CSA of individual muscle fibres by 50 % and the maximum specific force production of individual firbes is reduced by 70 %; these changes correspond to a loss in force generating capacity of the whole inteact diaphragm by 85 % (Corpeno et al. 2014). Thus, exacerbating the existing functional deficits of the preterm diaphragm by VIDD is likely to critically impair the capacity to sustain independent ventilation.

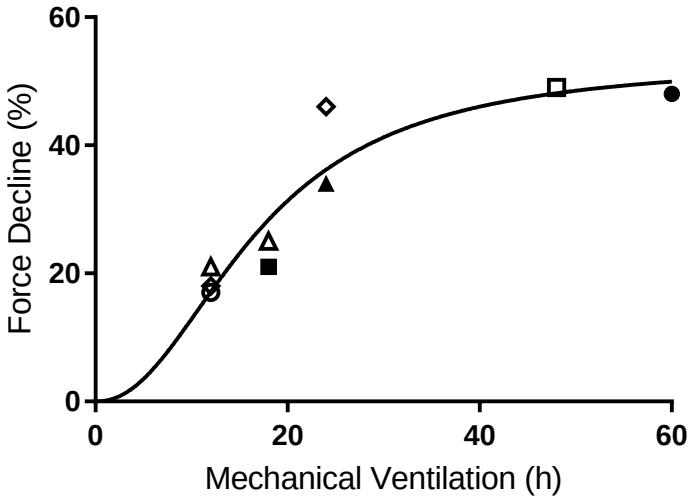


Figure 2-2. Mechanical ventilation reduces specific force of adult rat diaphragm in a sigmoidal fashion (R^2 = 0.87).

Data are drawn from Betters et al (2004; ○), Gayan-Ramirez et al (2003; ▲), Le Bourdelles et al (1994; □), Powers et al (2002; ◇), Shanely et al (2003; ■), Whidden et al (2009; △)and Yang et al (2002; ●).

Interestingly, it appears that the severity of VIDD is strongly related to respiratory rate. VIDD is more rapid in onset/progression in small animals with high respiratory rates compared with large animals with low respiratory rates: In rabbits, mechanical ventilation with a respiratory rate of 40 – 50 breaths per minute for 3 d reduced diaphragm strength by 50 % (Sassoon et al. 2002); in young adult pigs (20 – 30 kg) mechanical ventilation with a respiratory rate of 16 – 19 breaths per minute for 5 d reduced diaphragm strength by 30 % (Radell et al. 2002); while in baboons, mechanical ventilation at a respiratory rate of 12 breaths per minute for 11 d only reduced maximum transdiaphragmatic pressure generation by 25 % (Anzueto et al. 1997). The mechanisms underlying the increased severity of VIDD in small animals is unclear, but could relate to either: i) species differences in susceptibility to VIDD; ii) animals that have a high respiratory rate are more susceptible to the diaphragm unloading and disuse atrophy associated with mechanical ventilation; and/or ii) the higher rate of ventilation increases exposure of the vulnerable diaphragm to potentially harmful passive stretching during the ventilation cycle. The relationship between respiratory rate and VIDD may have important implications for the preterm

infant, as respiratory rate decreases with development; the respiratory rate of healthy term infants is ~44 breaths per minute and decreases to ~26 breaths per minute by 2 years of age (Fleming et al. 2011). The preterm infant with a high respiratory rate may therefore be more susceptible to VIDD.

Overview and clinical relevance

Mechanical ventilation is associated with reduced diaphragm strength and diaphragm atrophy in the neonate and infant (Knisely, Leal & Singer 1988). Nonetheless, the effects of mechanical ventilation on the preterm diaphragm remain relatively unexplored. The preterm diaphragm is likely more susceptible to VIDD than the adult diaphragm due to the high respiratory rates of infants (Fleming et al. 2011) and the increased susceptibility of the preterm diaphragm to oxidative stress (Maxwell et al. 1983; Song & Pillow 2012). Diaphragm dysfunction likely plays a fundamental role in the duration of mechanical ventilation infants require; considering that the time-tension index of the diaphragm predicts extubation failure of ventilated infants and children with with 100 % accuracy (Currie et al. 2011; Harikumar et al. 2009). Furthermore, the duration of mechnical ventilation is a strong positive predictor for BPD (Morley et al. 2008). It is critical that the effects of mechanical ventilation on the preterm diaphragm are investigated and the contribution of the diaphragm to respiratory complications is considered when managing preterm respiratory disease.

2.2.4 Glucocorticoids

Antenatal and postnatal glucocorticoids are another common form of prevention and treatment of respiratory diseases in the preterm infant. Antenatal betamethasone is administered to mothers expected to deliver prematurely to facilitate lung maturation and surfactant production (Bolt et al. 2001). Similarly, postnatal dexamethasone has been used since the 1980's to facilitate weaning of infants with severe CLD off mechanical ventilation. The potent anti-inflammatory properties of glucocorticoids make them an effective treatment to reduce the lung inflammation induced by chorioamnionitis, postnatal sepsis and mechanical ventilation that contribute to development of CLD (Gupta et al. 2012). However, there is minimal research into the effects of glucocorticoids on

the preterm diaphragm despite their wide application in the preterm infant for the prevention and treatment of respiratory disorders.

Alterations of the developed diaphragm by exogenous glucocorticoids: Implications for the developing diaphragm

In adults, glucocorticoid administration is associated with serious adverse effects on skeletal muscle, including the diaphragm. Prolonged exposure to high-dose glucocorticoids is associated with reduced inspiratory muscle strength in patients treated for asthma and chronic obstructive pulmonary disease (Decramer & Stas 1992), and reduced inspiratory muscle strength and endurance in patients without prior respiratory conditions (Weiner, Azgad & Weiner 1993). Diaphragm weakness results from glucocorticoid-induced atrophy, selective to Type IIb/x fibres (Van Balkom et al. 1997; Dekhuijzen et al. 1995; Lewis, Monn & Sieck 1992; Nava et al. 1996; Prezant et al. 1998). The direct effects of excessive glucocorticoids on skeletal muscle are further compounded by glucocorticoid-induced myopathy due to appetite suppression and consequent undernutrition and weight loss (*see nutrition section 2.2.1*) (Lewis, Monn & Sieck 1992; Nava et al. 1996).

The effects of glucocorticoid-administration on diaphragm contractile function are variable in *in vivo* animal models of adult glucocorticoid-induced myopathy with reports that glucocorticoid use either reduces maximum Pdi (Viires et al. 1990) or reduces diaphragm endurance without altering maximum Pdi (Ferguson, Irvin & Cherniack 1990). Similar contrasting effects of glucocorticoids are found *in vitro*: glucocorticoids have been reported to have no effect on maximum specific force of the diaphragm (Dekhuijzen et al. 1995; Lewis, Monn & Sieck 1992; Moore et al. 1989; Prezant et al. 1998) or to reduce the specific force of the diaphragm (Van Balkom et al. 1997; Sasson et al. 1991; Sassoon et al. 2008). When present, reduced diaphragm maximum specific force is correlated with a proportional increase in myofibrillar damage (Sassoon et al. 2008). Likewise, diaphragm fatigue may increase (Lewis, Monn & Sieck 1992), decrease (Van Balkom et al. 1997; Prezant et al. 1998) or be unchanged (Dekhuijzen et al. 1995; Nava et al. 1996; Sasson et al. 1991) by glucocorticoid exposure. The different outcomes between studies are likely to be due to

differences in dose and duration of steroid administration, and the use of different animal models and fatigue protocols (Dekhuijzen & Decramer 1992). Glucocorticoid-induced contractile dysfunction and atrophy are dose- and duration-dependent (Prezant et al. 1998; Sasson et al. 1991; Sassoon et al. 2008). For example, specific force and muscle fibre CSA of the rabbit diaphragm was unaffected by a 3 d, 10 mg/kg/d, course of methylprednisolone (Sassoon et al. 2008). However, at a higher methylprednisolone dose of 80 mg/kg/d, the force response of the rabbit diaphragm to high stimulation frequencies was reduced after just one day, and after 3 d both the force response to all stimulation frequencies and Type IIb fibre CSA were significantly reduced (Sassoon et al. 2008). Similarly, the decrease in *MHC IIb* mRNA and Type II fibre CSA of the rat diaphragm are greater after a 10 w administration of dexamethasone than after a 2.5 w administration (Prezant et al. 1998).

Evidence of alterations of the developing diaphragm by exogenous glucocorticoids

The effects of glucocorticoids on the adult diaphragm should be extrapolated to the immature diaphragm with caution due to the following reasons: 1) glucocorticoids inhibit IGF-1 signalling, an important regulator of myogenesis and hyperplasia (Engert, Berglund & Rosenthal 1996; Schiaffino & Mammucari 2011), therefore, the time of glucocorticoid exposure relative to diaphragm development likely influences its effects; 2) glucocorticoids induce atrophy by both decreasing the activity of anabolic pathways and increasing activity of catabolic pathways. Developmental changes in the activity of these anabolic and catabolic pathways of the diaphragm, such as IGF-1 expression, may influence the effects of glucocorticoids (Song & Pillow 2013); and 3) glucocorticoid exposure during *in utero* organ development may also alter genetic programming and long-term function (Fowden, Giussani & Forhead 2006).

Studies examining glucocorticoid exposure during development indicate that administration of glucocorticoids in preterm infants and animal models is associated with reduced fetal weight and fetal growth (Frank & Roberts 1979; Halliday, Ehrenkranz & Doyle 2003; Kumar & Seshadri 2005; Pratt et al. 1999).

Therefore, glucocorticoid administration may induce diaphragm dysfunction indirectly, through growth restriction. Additionally, two experimental studies examining the direct effects of antenatal betamethasone and postnatal dexamethasone on the rat diaphragm noted that: 1) two doses of antenatal betamethasone, one prior to and one during secondary myogenesis (Harris et al. 1989) induced long-term diaphragm weakness, long-term reduction in fast fibre gene and protein expression, and long-term reduction of Akt and 4EBP1 anabolic signalling, consistent with a genetic reprogramming (Song et al. 2014); and 2) postnatal dexamethasone effects in rats are dependent on the age at the time of exposure with dexamethasone reducing the intrinsic force generating capacity and atrophy in newborn rats, but only inducing atrophy in adolescent and adult rats (Trang, Viires & Aubier 1992). While the rodent model is a valid model for studying the effects of steroids on developed skeletal muscle, differences in gestational and developmental trajectories between rats and humans limits translation of the above-mentioned studies. Additionally, both studies used high doses of glucocorticoids, well above the levels used in clinical practice. Given the dose-dependent effects of glucocorticoids in adult skeletal muscle, experimental outcomes of such high-dose glucocorticoid studies may have little clinical relevance. Therefore, despite the wide use of glucocorticoids to reduce respiratory morbidity in the neonate, the effects of glucocorticoids on the neonatal diaphragm remain largely unknown.

Overview and clinical relevance

Early dexamethasone use as a prevention/treatment of CLD has diminished (Blackmon et al. 2002) as the use of high-dose postnatal glucocorticoids in neonates has been associated with skeletal muscle catabolism (Tsai et al. 1996), gastrointestinal perforation and long-term neurological deficits (Stark et al. 2001; Yeh et al. 1997). However, delayed postnatal glucocorticoid use is associated with prolonged mechanical ventilation and an increased incidence of CLD, which is also a risk factor for neurological impairment (Cheong et al. 2013; Walsh et al. 2005). Whether there is a safe and effective dexamethasone dose which will improve pulmonary function with minimal side effects is unknown. Investigating the effects of glucocorticoids on the immature diaphragm would inform best practice for postnatal glucocorticoid use in the preterm infant.

2.3 Interactive effects of undernutrition, inflammation, mechanical ventilation and glucocorticoids on diaphragm muscle function

An added complexity to the effects of undernutrition, inflammation, mechanical ventilation and glucocorticoids on the diaphragm is that preterm infants are often subject to multiple adverse exposures in series or in parallel (Figure 2-1). The effects of these antenatal and postnatal exposures on the diaphragm may be interactive.

Exposure of preterm infants to undernutrition and glucocorticoids likely occurs simultaneously. Repeated maternal glucocorticoid administration reduces fetal birth weight (French et al. 1999) and, in the rat, reduces nutritional transfer to the fetus by altering placental size and structure (Hewitt, Mark & Waddell 2006). Maternal undernutrition also increases fetal exposure to endogenous glucocorticoids in the rat (Lesage et al. 2001). Therefore, undernutrition and glucocorticoid administration may induce myopathy both directly and indirectly. Furthermore, chorioamnionitis is associated with *in utero* fetal growth restriction (Williams et al. 2000), and the effects of chorioamnionitis and growth restriction on the diaphragm may be additive.

The pro-inflammatory effects of chorioamnionitis, postnatal sepsis and mechanical ventilation and the anti-inflammatory effects of glucocorticoids may be influenced by previous exposures. For example, chorioamnionitis alters the innate immune response of the preterm lamb (Kramer et al. 2007); and while a single exposure to LPS-induced chorioamnionitis causes diaphragm dysfunction at birth, repeated subsequent exposures do not increase the extent of diaphragm dysfunction further, despite increasing white blood cell counts (Karisnan et al. 2015a). Furthermore, the anti-inflammatory action of antenatal glucocorticoids is dependent on glucocorticoid administration timing relative to the onset of chorioamnionitis (Wolfe et al. 2013). Interactive effects between mechanical ventilation and sepsis as well as mechanical ventilation and glucocorticoids on the diaphragm are also reported from adult animal models. Mechanical ventilation protects the diaphragm from concurrent sepsis-induced dysfunction in the adult rat by reducing sarcolemmal damage from oxidative stress (Ebihara et al. 2002); indicating mechanical ventilation may benefit

diaphragm function in septic preterm infants in some circumstances. However, the timing of mechanical ventilation in relation to the onset of sepsis is likely crucial in determining diaphragm outcome, as mechanical ventilation initiated after sepsis exacerbates sepsis-induced diaphragm dysfunction (Maes et al. 2014). Lastly, glucocorticoids alter VIDD in a dose-dependent manner: low-dose steroids exacerbate VIDD, while high-dose steroids may actually prevent VIDD by inhibiting calpain activity associated with mechanical ventilation in adult rats (Maes et al. 2010; Sassoon et al. 2011). Consequently, the dose, timing and interaction between antenatal and postnatal exposures/treatments require careful consideration when evaluating their effects on the neonatal diaphragm and respiratory function.

2.4 Conclusions and future perspectives

The preterm diaphragm has a reduced functional capacity and a high mechanical load, due to immaturity of the respiratory system with premature birth. Common exposure of the preterm infant to undernutrition, inflammation, mechanical ventilation and exogenous glucocorticoids may further compromise the functional capacity of the preterm diaphragm. The susceptibility of the diaphragm to injury and dysfunction caused by external insults is likely linked intrinsically to the timing of the external insult in relation to diaphragm development, and influenced by previous exposures. Diaphragm weakness may contribute to respiratory distress and chronic respiratory diseases seen in the infant. The diaphragm needs to be considered when reviewing and treating respiratory insufficiencies in the preterm infant. To date, the influence of clinical exposures on the preterm diaphragm has garnered little attention and there are major outstanding questions that need to be addressed. The individual and combined effects of common clinical antenatal and postnatal exposures on the developing diaphragm warrant further investigation.

2.5 Statement of aims

The aims of this project are to determine the effect of clinically relevant antenatal and postnatal exposures on the structure and function of the preterm diaphragm.

The specific aims of the PhD project are:

Aim 1: To determine the effect of *in utero* inflammation on postnatal diaphragm function, in the preterm lamb.

Objective: To assess the combined effects of preterm birth and a 2 d IA LPS exposure on diaphragm function and structure in lambs delivered prematurely (at 129 d GA) and raised to 7 d PNA.

Hypothesis: *In utero* LPS exposure, preceding preterm birth, will promote structural and physiological changes which would exacerbate diaphragm dysfunction at 7 d PNA.

Aim 2: To determine the short-term and long-term effects of clinically relevant, postnatal dexamethasone administration to preterm lambs on the structure and function of the diaphragm.

Objectives: To assess: i) the acute effects of high and low-dose postnatal dexamethasone exposure on the functional development of the preterm lamb diaphragm at 7 d PNA; ii) the long-term combined effects of postnatal dexamethasone administration and mechanical ventilation on the functional development of the preterm lamb diaphragm at 2 m post-term; and iii) the long-term effects of preterm birth on diaphragm function at 2 m post-term.

Hypotheses: i) The dose of dexamethasone administration will determine the extent of the functional impairment of the preterm lamb diaphragm; ii) dexamethasone-induced diaphragm dysfunction, after preterm birth, will be long-lasting and influenced by concurrent mechanical ventilation; and iii) diaphragm dysfunction induced by preterm birth would be long-lasting.

Aim 3: To determine the susceptibility of the developing diaphragm to ventilator-induced dysfunction, in the newborn rat.

Objective: To determine the susceptibility to ventilator-induced diaphragm dysfunction in the developing rat relative to the adult rat.

Hypothesis: The severity of VIDD is inversely related to diaphragm development.

Chapter 3

Functional adaptations of the preterm lamb diaphragm in the first week of life are not influenced by *in utero* lipopolysaccharide exposure

Preface

This study examines the combined effects of preterm birth and a 2 d IA LPS exposure on diaphragm function and structure in preterm lambs during early postnatal life. At the time of thesis submission, this chapter was part of a manuscript under preparation for submission. The manuscript in preparation is presented in Appendix IV.

3 Functional adaptations of the preterm lamb diaphragm in the first week of life are not influenced by *in utero* lipopolysaccharide exposure

3.1 Abstract

In utero inflammation exacerbates preterm diaphragm weakness at birth. Persistent diaphragm dysfunction, resulting from immaturity and *in utero* inflammation, may predispose preterm infants to postnatal respiratory failure. We determined the combined effects of preterm birth and *in utero* inflammation on postnatal diaphragm function in lambs. Lambs were exposed to intra-amniotic (IA) saline (n = 8) or 4 mg LPS (n = 8) 48 h before caesarean delivery at 129 d gestational age (GA, term = 145 - 150 d). Lambs were managed in an intensive care environment in accordance with routine clinical practice then killed at 7 d PNA (136 d GA). Naïve fetal control lambs were delivered at 136 d GA and killed immediately (n = 7). There were no significant differences in diaphragm contractile function *in vitro* or MHC fibre characteristics between saline and LPS groups. However, the contractile properties of the saline and LPS groups were significantly different to the fetal control group, indicative of postnatal adaptations to extra-uterine life. Duration of mechanical ventilation and nutritional intake were important predictors of diaphragm function at 7 d PNA. Importantly, LPS exposure correlated positively with the duration of mechanical ventilation required by lambs in the intensive care unit. These findings suggest that LPS-induced diaphragm weakness at birth does not persist at 7 d PNA. However, the rapid adaptation in diaphragm function after birth is heavily influenced by the combined effects of common clinical antenatal and postnatal exposures on the developing respiratory system.

3.2 Introduction

A functional respiratory system is essential for successful transition to independent gas exchange after birth. The underdeveloped and surfactant deficient respiratory system of the extremely preterm infant is inadequately prepared for this transition, often resulting in development of respiratory distress syndrome soon after birth. Additionally, preterm infants without significant respiratory disease at birth may develop progressive respiratory failure over the first week of life (Bland 2005; Eber & Zach 2001). Accordingly, many preterm infants require mechanical respiratory support: recent data suggest at least 50 % of infants born earlier than 30 weeks' gestation require intubation and mechanical ventilation (Sweet et al. 2017).

Until recently, respiratory disease in preterm infants was considered primarily a disease of lung immaturity. Consequently, the contribution of the diaphragm to respiratory disease in preterm infants has garnered little attention, despite the pivotal role of the diaphragm in spontaneous breathing. Like the lungs, the preterm diaphragm is structurally and functionally immature at birth (Dimitriou et al. 2003, 2001; Keens et al. 1978). Appropriate development of the diaphragm *in utero* is vital for establishing and sustaining spontaneous breathing from the time of birth (Mantilla & Sieck 2008). The newborn diaphragm needs to generate sufficient transpulmonary pressure to overcome the high surface tension of the newborn lungs, thereby facilitating resorption of fetal lung fluid and establishment of a functional residual capacity (te Pas et al. 2008). Thereafter the diaphragm must continuously produce adequate trans-pulmonary pressures to sustain spontaneous breathing throughout postnatal life (te Pas et al. 2008).

Preterm birth disrupts normal diaphragm development: the immature diaphragm of the preterm infant is characterised by small muscle fibres (Fratacci et al. 1996; Sieck, Cheung & Blanco 1991; Sieck, Fournier & Blanco 1991), which have underdeveloped SR (Maxwell et al. 1983; Schiaffino & Margreth 1969), underdeveloped myofibrils (Geiger et al. 2001; Maxwell et al. 1983; Orliaguet et al. 2002; West et al. 1999; Williams & Goldspink 1971) and a reduced antioxidant capacity (Song & Pillow 2012). Consequently, the force-generating

capabilities of the preterm diaphragm are reduced (Dimitriou et al. 2001, 2003) and the diaphragm must operate at a greater proportion of its maximum capacity to achieve adequate ventilation. The impact of preterm birth on the capacity of the diaphragm to initiate and sustain independent ventilation after birth is unclear.

The preterm diaphragm is also vulnerable to environmental insults associated with preterm birth. *In utero* inflammation is a common exposure among preterm infants and further compromises diaphragm integrity at birth (Karisnan et al. 2015a, 2015b, 2017; Song et al. 2013). Chorioamnionitis, inflammation of the fetal membranes, is a histological finding in 70 % of preterm births before 30 w GA (Goldenberg, Hauth & Andrews 2000). A short duration exposure to IA LPS at 2 d or 7 d before premature delivery at 129 d GA (term = 145 – 150 d GA) significantly impairs diaphragm development and function in an ovine model of chorioamnionitis; reducing fetal diaphragm contractile strength by ~30 % at birth (Song et al. 2013). Importantly, *in utero* inflammation induced by IA LPS exposure 21 d before premature birth produces similar levels of diaphragm weakness at birth, despite the absence of any overt signs of inflammation (Karisnan et al. 2015a). These findings indicate *in utero* inflammation severely compromises diaphragm development and may have long lasting effects on diaphragm function. However, the impact of *in utero* LPS exposure on postnatal diaphragm function is still unknown.

Using an established ovine model of chorioamnionitis (Berry et al. 2011; Kuypers et al. 2012b), we investigated the effects of a 2 d IA LPS exposure on diaphragm function and structure in lambs delivered preterm and raised to 7 d PNA. We hypothesised that diaphragm dysfunction at 7 d PNA will be exacerbated when *in utero* LPS exposure precedes preterm birth. We expected that the additional dysfunction of the preterm diaphragm, by *in utero* inflammation, would reduce the capacity of the preterm diaphragm to cope with the demands of spontaneous breathing after birth.

3.3 Methods

All experiments were approved by the Animal Ethics Committee of the University of Western Australia (3/100/1301) and were conducted in accordance

with the guidelines of the National Health and Medical Research Council (NHMRC) Australian code for the care and use of animals for scientific purposes (2013).

3.3.1 Experimental outline

Date-mated merino ewes (4 – 7 years of age) were randomly assigned to one of three groups: i) LPS group (n = 8) receiving an ultrasound guided IA injection of LPS (4 mg [2 ml], *E. coli* 055:B5; Sigma Aldrich) 48 h before premature delivery at 129 d GA (term = 145 – 150 d GA); ii) saline group (n = 8) receiving an equivalent volume IA injection of saline 48 h before premature delivery at 129 d GA; and iii) end-point fetal control group (n = 7), representing a pure maturational control and, which received no intervention before premature delivery at 136 d GA. LPS and saline lambs were then managed postnatally in an intensive care environment and killed at 7 d PNA (136 d post-conceptional age; PCA). Fetal controls were killed immediately prior to delivery, before the initiation of spontaneous ventilation. The experimental outline is depicted in Figure 3-1.

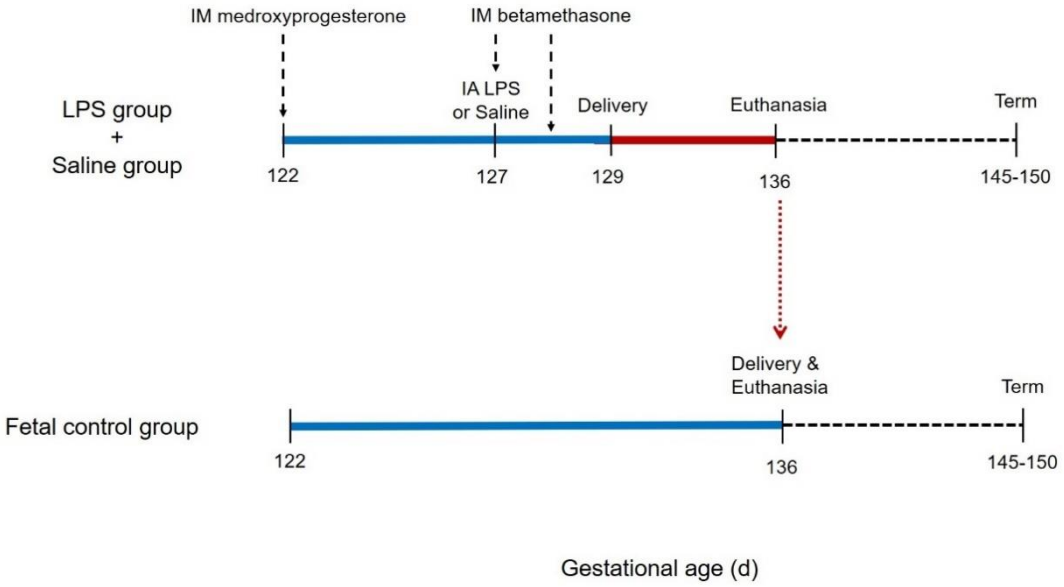


Figure 3-1. Diagram of the experimental outline for fetal control, saline and LPS groups.

Ewes assigned to the LPS and saline groups received IM medroxyprogesterone at 122 d GA, 2 doses of IM betamethasone at 127 d and 128 d GA, and either IA LPS or saline injections at 127 d GA, before premature delivery at 129 d GA. Preterm lambs were managed in an intensive care environment for 7 d, after which they were killed at 136 d PCA. Fetal control lambs were killed on delivery at 136 d GA. Prenatal development is represented by the blue line, postnatal development in an intensive care unit is represented by the red line. LPS, Lipopolysaccharide; IM, intra-muscular; IA, intra-amniotic; GA, gestational age; PCA, post-conceptual age.

3.3.2 Animal management

Antenatal treatment and delivery

Ewes randomly assigned to the LPS and saline groups received intra-muscular (IM) medroxyprogesterone (150 mg; Pfizer) 7 d prior to planned caesarean section (at 122 d PCA), to prevent onset of spontaneous labour associated with maternal betamethasone administration. Maternal betamethasone was administered to LPS and saline groups to accelerate lung maturation and surfactant production, in accordance with routine clinical practice. Ewes assigned to the LPS and saline groups received 2 doses of IM betamethasone (Celestone®, 5.7 mg; Merck Sharp and Dohme) at 24 h intervals in the 2 d prior to surgical hysterotomy, commencing 6 h after the IA LPS/IA saline injection.

Ewes were pre-medicated with IM Buprenorphine (0.01 mg/kg; Indivior Pty Ltd) and IM Acepromazine (0.05 mg/kg; Ceva Animal Health Pty Ltd) one hour prior to anaesthetic induction with Thiopentone (15 mg/kg; Troy Laboratories), and maintenance anaesthetic with isofluorane (1.5 – 2.5 %). The fetal head and neck of saline and LPS lambs were exteriorised for insertion of carotid arterial and jugular venous catheters, oral intubation, removal of lung fluid and administration of intra-tracheal surfactant (Curosurf®, 100 mg/kg; Chiesi Farmaceutici S.p.A.). Lambs were then delivered. Fetal control lambs were killed at delivery without intervention (pentobarbitone, 150 mg/kg, intravenously, IV; Pitman-Moore). Ewes were killed immediately after lamb delivery (pentobarbitone, 150 mg/kg, IV; Pitman-Moore). Successful IA LPS injection was confirmed by performing a limulus amebocyte lysate assay on amniotic fluid samples, according to the manufacturers' protocol (limulus amebocyte lysate QCL-1000; Lonza).

Postnatal treatment

Lambs were resuscitated immediately after delivery and managed in accordance with routine clinical practice. Assist/control volume-guaranteed ventilation (5 – 7 mL/kg) was commenced immediately following delivery and changed to mandatory minute ventilation after commencement of spontaneous respiratory efforts. Ventilatory settings (volume guarantee, positive end-expiratory pressure, fractional inspired oxygen, inspiratory time and respiratory rate) were adjusted to target mild permissive hypercapnia (partial pressure of carbon dioxide in arterial blood ($PaCO_2$) of 45 – 55 mmHg) and peripheral capillary oxyhaemoglobin saturation (SpO_2) of 90 – 95 %. Maximum peak inspiratory pressure was 30 cmH_2O. Lambs were weaned from mechanical ventilation if they were breathing spontaneously and maintaining SpO_2 of 90 – 95 % at a peak inspiratory pressure < 15 mmHg and inspired oxygen fraction (FiO_2) < 0.25. Lambs were reintubated if they displayed any of the following: persistent SpO_2 < 80 % despite FiO_2 > 0.8 and increasing mean airway pressure to a maximum of 12 cmH_2O; ≥ 4 apnoea events requiring resuscitation within an hour; ventilatory failure ($PaCO_2$ > 80 mmHg on two consecutive arterial gases); or severe metabolic acidosis (base excess < -10 mmol).

Arterial blood pressure was monitored continuously and arterial blood was sampled intermittently for blood-gas analysis to help in the assessment of respiratory status. Lambs received intravenous fluid support (5 % dextrose) and commenced enteral feeds at 2 h (20 mL/kg/d) with fresh maternal colostrum, incrementing enteral feeds by 20 mL/kg/d and transferring to formula feeds at 24 h (MaxCare® Lamb & Kid; MaxumAnimal). Supplemental heating was adjusted to target normothermia (38.5-39.5 °C) throughout postnatal life. Saline and LPS lambs were killed at 7 d (pentobarbitone, 150 mg/kg IV; Pitman-Moore).

3.3.3 Tissue collection

The diaphragm was excised immediately after lambs were killed. The mid-costal portion of the right hemi-diaphragm, including the attached ribs and central tendon, was maintained in mammalian Ringer's solution (in mM; NaCl, 121; KCl, 5.4; $MgSO_4(H_2O)_7$, 1.2; $NaHCO_3$, 25; HEPES, 5; glucose, 11.5 and $CaCl_2$, 2.5: pH 7.3) bubbled with carbogen (5 % CO_2 in O_2) throughout careful dissection of muscle strips and ensuing measurements of contractile function. Sections of the mid-costal portion of the left hemi-diaphragm were imbedded in tragacanth gum and frozen in isopentane cooled in liquid nitrogen for histochemical analysis. Frozen samples were stored at -80 ºC until further analysis

3.3.4 Diaphragm contractile properties

A longitudinal strip (3-5 mm wide) of intact muscle fibres was mounted in an *in vitro* muscle test system (1200A, Aurora Scientific). Diaphragm contractile properties were measured at optimal muscle length (L_o) in response to 0.2 ms supramaximal square wave pulses (biphasic stimulator, 701B, Aurora Scientific). Measured contractile properties included maximum specific force (P_o), force-frequency relationship, twitch force (P_t), time to peak (TTP), half-relaxation time (1/2 RT), and fatigue index (FI). FI was determined from the ratio of the force produced during the 150^{th} contraction relative to the 1^{st} contraction of a fatigue protocol, in which a higher number indicates a greater fatigue resistance. Force (g) was normalised to CSA calculated from muscle fibre length, muscle mass and density (1.056 $g \cdot cm^{-3}$) (Mendez & Keys 1960) and

presented as specific force ($N \cdot cm^{-2}$). The detailed protocol is described in Appendix I.

3.3.5 Histochemistry

Myosin heavy chain (MHC) fibre typing

Transverse sections (8 µm) of frozen muscle were stained with anti-myosin heavy chain (MHC) specific antibodies: MHC_{IIa} (mouse monoclonal immunoglobulin (Ig) G1; SC-71; DSHB, University of Iowa; dilution 1:25), MHC_{I} (mouse monoclonal IgG2b; BA-D5; DSHB, University of Iowa; dilution 1:25), MHC embryonic (MHC_{emb}) (rabbit polyclonal IgG; 2400104; Mimotopes; dilution 1:25) and MHC fast (MHC_{IIabx}) (rabbit polyclonal IgG; 2400107; Mimotopes; dilution 1:25). Primary MHC_{IIa} was detected by goat anti-mouse IgG1 Alexa Fluor 488 (Jackson ImmunoResearch; dilution 1:500), MHC_{I} was detected by goat anti-mouse IgG2b dylight 405 (Jackson ImmunoResearch; dilution 1:500), and MHC_{emb} and MHC_{IIabx} were detected by anti-rabbit IgG Alexa Fluor 594 (Invitrogen; dilution 1:500). Tiled images were captured at 20x using a Nikon C2+ Confocal microscope (Nikon Corporation). The detailed protocol is described in Appendix I. MHC_{emb} and MHC_{IIabx} were designed by our laboratory and validated before use, as described in Appendix II.

Myofibre cross-sectional area (CSA)

Transverse sections (8 µm) of frozen muscle were stained with haematoxylin and eosin (H&E) to quantify myofibre CSA. Tiled images were captured at 20x using a Nikon Eclipse Ti inverter microscope (Nikon Corporation).

Image analysis

The CSA of each MHC fibre type was determined by matching the H&E images with images of serial sections stained for MHC expression. Fibre type proportions and MHC fibre CSA were determined manually using ImageJ (v1.51j8) software and ImageJ cell counter plugin (Abramoff, Magalhaes & Ram 2004).

3.3.6 Statistical analysis

A sample size of 8 lambs/group has an 80 % power to detect a 2 SD mean difference with significance at the 5 % level. Statistical differences among

treatment groups were assessed with one-way analysis of variance (ANOVA) with *a priori* Tukey multiple comparisons or Kruskal-Wallis H Test with *a priori* Mann-Whitney U tests, as appropriate. Mann-Whitney U tests were Bonferroni adjusted, with significance at the 0.017 level, to account for multiple comparisons. The force frequency relationships were analysed using 2-way ANOVA. Independent one-way ANOVA with Tukey multiple comparisons or nonparametric Kruskal-Wallis with Mann-Whitney U tests were performed, as appropriate, where an interaction between both factors was detected with 2-way ANOVA Greenhouse-Geisser.

The relationship between diaphragm functional and structural properties at 7 d PNA and several potential confounding experimental variables were also evaluated. The potential confounding variables evaluated were average protein intake and duration of mechanical ventilation, as both nutrition and mechanical ventilation are known to influence diaphragm function and structure (*see section 2.2.1 on nutrition and 2.2.3 on mechanical ventilation*). The duration of mechanical ventilation was normalised to absolute PNA, to account for variability in PNA, by dividing total time on mechanical ventilation by absolute PNA and multiplying by 7 d. Collinearities between the potential confounding variables were analysed initially with Pearson's bivariate correlation. The relationships between potential confounding variables and outcome variables were analysed using Pearson's bivariate correlation and Spearman's bivariate correlation analyses, as appropriate. Outcome variables that correlated significantly with the confounding experimental variables were analysed with linear regression analyses, as all significant correlations were with normally distributed outcome variables. Lastly, outcome variables that correlated significantly with the independent variables were re-analysed while controlling for the independent experimental variables using one-way analysis of variance (ANCOVA) and two-way ANCOVA, as appropriate.

Data are presented as mean ± standard deviation (SD) or as median (range). Significance was at the 0.05 level, unless otherwise stated. Analyses were performed on IBM SPSS Statistics software (Version 19, IBM Company 2010).

3.4 Results

3.4.1 Lamb descriptive data at post-mortem

Lamb post-conceptual age at post-mortem, body weight at post-mortem, sex and optimal muscle length for all groups are presented in Table 3-1, along with body weight at birth for saline and LPS groups. Body weight at birth of the saline and LPS lambs and body weight at post-mortem of the fetal control lambs was corrected for amniotic fluid in the wool (dry body weight ≈ 0.9 wet body weight). Body weight was significantly lower in the saline ($p = 0.001$) and LPS ($p = 0.009$) groups compared with the fetal control group at post-mortem.

3.4.2 Diaphragm contractile properties

Diaphragm contractile function was not significantly different between LPS and saline groups ($p > 0.05$, $n = 7$, Figure 3-2, Figure 3-3). However, diaphragm contractile properties of the LPS and saline groups were significantly different to that of the fetal control group, as detailed below.

Maximum specific force and twitch characteristics

Maximum specific force and twitch characteristics for fetal control, saline and LPS groups are presented in Figure 3-2. Maximum specific force was significantly higher in the saline group compared to the fetal control group ($p = 0.007$). Peak twitch force was significantly lower in the saline group ($p = 0.003$) and LPS group ($p < 0.001$) compared with the fetal control group. Time to peak twitch force was significantly shorter in the saline and LPS groups compared with the fetal control group ($p < 0.001$). Similarly, half relaxation time was significantly shorter in the saline group ($p = 0.01$) and LPS group ($p < 0.001$) compared with the fetal control group. Peak twitch force as a ratio of maximum specific force was significantly lower in the saline and LPS groups compared with the fetal control group ($p < 0.001$).

Force-frequency relationships

The relationship between stimulation frequency and force production was investigated from isometric contractions at 5 – 80 Hz, as displayed in Figure 3-3A. There was a significant interaction between treatment group and stimulation frequency ($p < 0.001$). The saline and LPS groups produced

significantly lower specific force at 5 Hz stimulation, and the LPS group also produced significantly lower specific force at 10 Hz stimulation, compared to the fetal control group ($p < 0.05$). Additionally, the saline group produced significantly higher specific force at stimulation frequencies 30 Hz and above compared to the fetal control group ($p < 0.05$). There was a significant interaction between treatment group and stimulation frequency when the force-frequency relationships were normalised to maximum force produced within each group ($p < 0.001$; Figure 3-3B). The normalised force-frequency relationship was shifted to the right in the saline and LPS groups: saline and LPS groups produced significantly lower relative force at low stimulation frequencies 5 – 40 Hz, compared with the fetal control group ($p < 0.05$).

Susceptibility to fatigue

The fatigue protocol successfully reduced force to a 40 Hz stimulation by approximately 50 – 55 % in all treatment groups. The FI did not differ significantly between groups ($p = 0.393$; Figure 3-2).

Table 3-1. Lamb descriptive data of the fetal control group, saline, and LPS groups, at 7 d PNA.

	Fetal control (n = 7)	Saline (n = 8)	LPS (n = 8)
PCA at PM (d)	136.1 ± 0.9	136.8 ± 1.5	135.8 ± 1.0
Sex ratio (M:F)	4:3	3:5	4:4
Body weight at birth (kg)	N/A	2.94 ± 0.41 (wet) 2.64 ± 0.37 (dry equiv)	3.30 ± 0.42 (wet) 2.97 ± 0.38 (dry equiv)
Body weight at PM (kg)	4.04 ± 0.44 (wet) 3.64 ± 0.40 (dry equiv)	2.84 ± 0.37**	3.02 ± 0.31**
Normalised MV (h)	N/A	25.94 ± 20.90	77.61 ± 62.19
Normalised CPAP (h)	N/A	5.12 (0 – 22.08)	21.08 (0 – 82.33)
Average protein intake (mg/kg/d)	N/A	38.07 ± 12.44	28.82 ± 9.26
L_o (mm)	39.8 ± 6.1	36.6 ± 4.3 (n = 7)	38.7 ± 4.0 (n = 7)

Data are presented as mean ± SD or median (min – max), except for sex ratio. PCA, postconceptional age; PM, post-mortem; MV, mechanical ventilation; L_o, optimal muscle length; ** significantly different from the dry body weight at PM of the fetal control group at $p < 0.01$

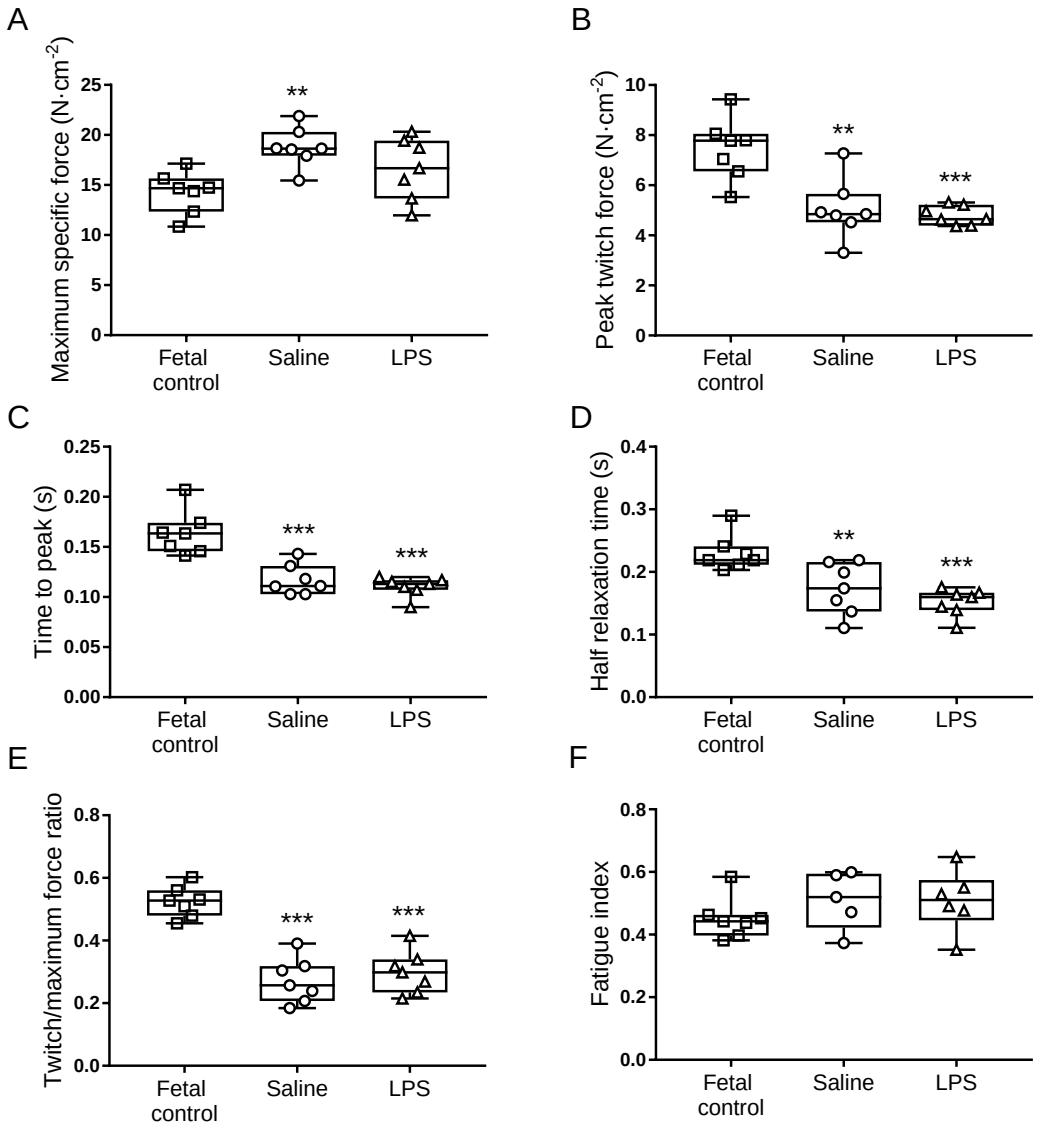


Figure 3-2. Diaphragm contractile properties of fetal control, saline and LPS groups.

(A) maximum specific force (P_o); (B) peak twitch force (P_t); (C) time to peak (TTP); (D) half relaxation time (1/2 RT); (E) twitch force/maximum force ratio (P_t:P_o) and (F) fatigue index (FI). Box and whisker plots represent median, minimum and maximum values, and all data points are visible (n = 7). ** and *** significantly different to the fetal control group at $p < 0.01$ and $p < 0.001$, respectively.

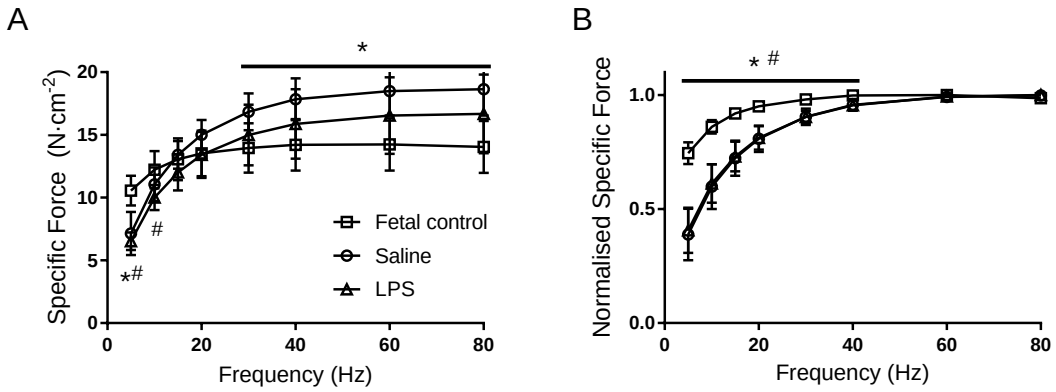


Figure 3-3. Force-frequency relationship of fetal control, saline and LPS groups.

(A) Force is displayed as specific force ($N \cdot cm^{-2}$) and (B) normalised to maximum specific force. Values represent mean ± SD (n = 7). * significant difference between the fetal control and saline groups ($p < 0.05$). # significant difference between the fetal control and LPS groups ($p < 0.05$).

3.4.3 MHC fibre characteristics

Transverse sections of frozen diaphragm were stained for expression of MHC_{emb}, MHC_{I}, MHC_{IIa} and MHC_{IIabx}. In all treatment groups, all myofibres that stained positively for MHC_{IIabx} also stained positively for MHC_{IIa}; indicating that $MHC_{IIb/x}$ isoforms are always co-expressed with MHC_{IIa} or that MHC_{IIa} is the only fast adult isoform expressed at 136 d PCA. Expression and/or co-expression of MHC isoforms in fibres included four combinations: i) MHC_{I} only; ii) MHC_{emb} and MHC_{I}; iii) MHC_{emb} and $MHC_{IIabx/IIa;}$ and iv) MHC_{emb}, MHC_{I} and $MHC_{IIabx/IIa}$. No fibres expressed only MHC_{emb} or only $MHCI_{IIabx/IIa}$. The percentage distribution of MHC-type myofibres was not significantly different between treatment groups ($p > 0.05$; Figure 3-4B).

The CSA of individual myofibre types were determined from transverse sections stained with H&E and matched to serial transverse sections stained for MHC expression. In all treatment groups, MHC_{I} only fibres were significantly larger than any other fibre type ($p < 0.01$; Figure 3-4C). In the LPS group, $MHC_{emb + I}$ fibres were significantly larger than $MHC_{emb + I + IIabx/IIa}$ fibres ($p < 0.05$; Figure 3-4C). The CSA of individual myofibre types were not significantly different between treatment groups ($p > 0.05$; Figure 3-4C).

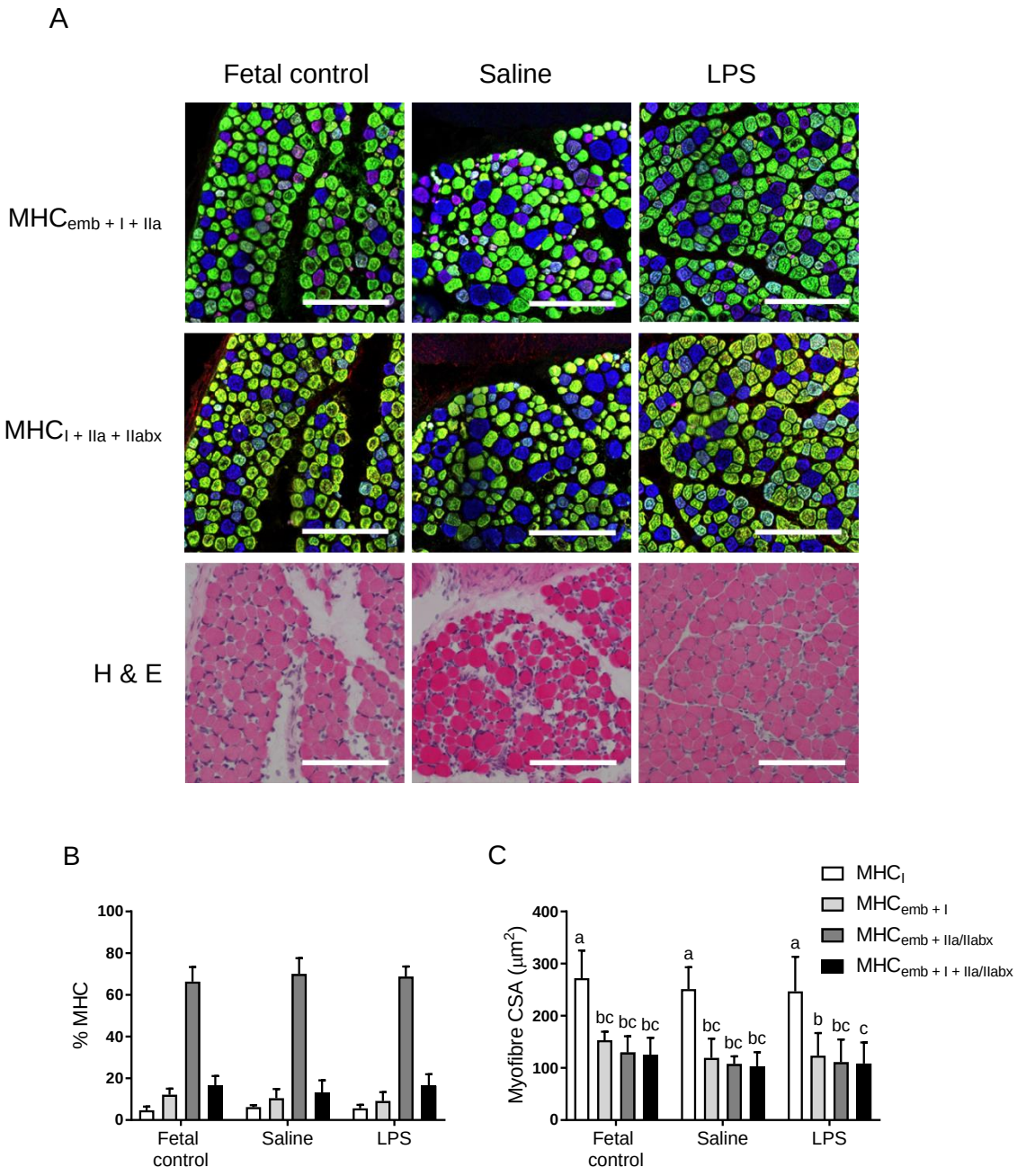


Figure 3-4. Diaphragm muscle fibre types and cross-sectional area (CSA) from fetal control, saline and LPS groups.

(A) Muscle fibre sections were stained with a combination of MHC_{emb} (red), MHC_{I} (blue), MHC_{IIa} (green) antibodies, a combination of MHC_{I} (blue), MHC_{IIa} (green), and MHC_{IIabx} (red) antibodies, or H&E. Myofibre size (measured by CSA) was quantified from transverse sections stained with H&E. Graphs show (B) the proportion of myofibre types and (C) myofibre CSA. Scale bar represents 100 μm. Data are presented as mean ± SD (n = 6), and statistical differences in myofibre CSA are indicated by different letters ($p < 0.05$). MHC, myosin heavy chain.

3.4.4 Independent predictors of diaphragm structural and functional properties at 7 d PNA

Differences between saline and LPS groups may have been influenced by confounding postnatal management that included mechanical ventilation and altered nutritional intake. Normalised duration of MV and average protein intake for LPS and saline groups are presented in Table 3-1. The relationship between diaphragm outcome variables of combined LPS and saline groups at 7 d PNA, LPS exposure, and potential confounding experimental variables (average protein intake and normalised duration of mechanical ventilation), were evaluated using correlation and linear regression (n = 14 for diaphragm contractile function; n = 12 for MHC fibre characteristics).

Correlation coefficients between the independent variables are presented in Table 3-2. Correlation coefficients between independent variables and outcome measures are presented in Table 3-3. Linear regression analyses are presented in Table 3-4.

LPS exposure

The normalised duration of mechanical ventilation required by LPS and saline lambs was not significantly different ($p = 0.054$). However, it should be noted, that the statistical comparison between the normalised duration of mechanical ventilation required by LPS and saline lambs just failed significance. The normalised duration of mechanical ventilation ranged from 3-54 h among saline lambs, and all lambs were successfully extubated before study completion. In comparison, normalised duration of mechanical ventilation among LPS lambs ranged from 4-143 h, with 4/8 lambs requiring respiratory support up until study completion. LPS exposure correlated positively with the normalised duration of mechanical ventilation required by postnatal lambs, after LPS and saline groups were combined into a single group and analysed with Pearson's bivariate correlation ($R^2 = 0.270$, $p = 0.032$).

Mechanical ventilation

As collinearities were identified between the normalised duration of mechanical ventilation and LPS exposure, the unstandardised residual duration of mechanical ventilation, that is the component of mechanical ventilation not due

to LPS exposure, was entered into the correlational model. The unstandardised residual mechanical ventilation significantly predicted several outcome measures. Duration of mechanical ventilation (residual) exhibited a moderate negative correlation with specific force produced at 15 - 80 Hz (R^2 ranged between 0.456 to 0.528) and a moderate negative correlation with the proportion of fibres expressing $MHC_{emb + I}$ (R^2 = 0.363). Specifically, duration of mechanical ventilation (residual) explained ~51 % of the variation observed in diaphragm maximum specific force at 7 d PNA. The negative correlation between mechanical ventilation (residual) and specific force suggests that as duration of ventilation increases, specific force decreases.

Protein intake

Average protein intake of LPS and saline lambs did not differ significantly (p = 0.114). However, average protein intake correlated negatively with the duration of mechanical ventilation (residual) required by lambs. Therefore, the unstandardised residual protein intake, that is the component of protein intake not due to mechanical ventilation, was entered into correlation and linear regression analyses.

Average protein intake exhibited a strong positive correlation with the $MHC_{emb + IIabx/IIa}$ proportions (R^2 = 0.631) and a strong negative correlation with the $MHC_{emb + I + IIabx/IIa}$ proportions (R^2 = 0.634).

3.4.5 Diaphragm contractile function and MHC fibre characteristics of saline and LPS lambs after correcting for respiratory support

LPS exposure had no significant effect on P_o (p = 0.164; data not shown), force-frequency relationship (p = 0.577; data not shown), or MHC fibre characteristics (p > 0.05; data not shown) after correcting for the unstandardised residual duration of mechanical ventilation or the unstandardised residual average protein intake, as appropriate.

Table 3-2. Collinearities between potential confounding postnatal variables for the saline and LPS groups.

	LPS exposure	Normalised MV (h)	Average protein intake (g/kg/d)	Normalised MV (residual) (h)	Average protein intake (residual) (g/kg/d)
LPS exposure	N/A	0.511 0.043	-0.411 0.114	0.000 1.000	-0.481 0.059
Normalised MV (h)	0.511 0.043	N/A	-0.657 0.006	0.859 0.000	-0.246 0.358
Average protein intake (g/kg/d)	-0.411 0.114	-0.657 0.006	N/A	-0.520 0.039	0.854 0.000
Normalised MV (residual) (h)	0.000 1.000	0.859 0.000	-0.520 0.039	N/A	0.000 1.000
Average protein intake (residual) (g/kg/d)	-0.481 0.059	-0.246 0.358	0.854 0.000	0.000 1.000	N/A

Top values represent the correlation coefficient, bottom values represent the p-value. MV, mechanical ventilation.

Table 3-3. Bivariate correlation between potential confounding postnatal variables (protein intake and duration of MV) and diaphragm structural and functional properties of saline and LPS groups at 7d PNA.

	Normalised MV (residual) (h)		Average protein intake (residual) (g/kg/d)	
	R	p	R	p
P_o (N·cm^{-2})	-0.716	0.004	0.351	0.218
P_t (N·cm^{-2})	0.055	0.853	-0.198	0.498
TTP (s)	-0.127	0.665	-0.457	0.101
½ RT (s)	-0.142	0.627	-0.178	0.543
5 Hz (N·cm^{-2})	-0.049	0.869	-0.325	0.257
10 Hz (N·cm^{-2})	-0.476	0.086	-0.147	0.616
15 Hz (N·cm^{-2})	-0.676	0.008	0.052	0.859
20 Hz (N·cm^{-2})	-0.721	0.004	0.187	0.522
30 Hz (N·cm^{-2})	-0.726	0.003	0.280	0.332
40 Hz (N·cm^{-2})	-0.723	0.004	0.303	0.292
60 Hz (N·cm^{-2})	-0.717	0.004	0.332	0.246
80 Hz (N·cm^{-2})	-0.715	0.004	0.351	0.218
FI	0.052	0.880	0.346	0.298
MHC_I (%)	0.232	0.469	0.504	0.095
$MHC_{emb + I}$ (%)	-0.602	0.038	-0.243	0.447
$MHC_{emb + IIabx/IIa}$ (%)	0.178	0.580	0.795	0.002
$MHC_{emb + I + IIabxIIa}$ (%)	0.215	0.501	-0.796	0.002
MHC_I CSA (µm^2)	0.017	0.958	-0.060	0.854
$MHC_{emb + I}$ CSA (µm^2)	-0.112	0.729	-0.038	0.906
$MHC_{emb + IIabx/IIa}$ CSA (µm^2)	-0.204	0.525	-0.284	0.371
$MHC_{emb + I + IIabx/IIa}$ CSA (µm^2)	0.019	0.954	-0.179	0.578

Significant correlations are shaded. n = 14 for all contractile properties; n = 12 for all MHC fibre characteristics. MV, mechanical ventilation; P_o, maximum specific force; P_t, twitch force; TTP, time to peak twitch force; ½ RT, half relaxation time; FI, fatigue index; CSA, cross-sectional area

Table 3-4. Linear regression analysis between the significantly correlated postnatal variables (protein intake and duration of MV) and diaphragm structural and functional properties of saline and LPS groups at 7 d PNA.

	Normalised MV (residual) (h)			Average protein intake (residual) (g/kg/d)		
	R^2	SEM	p	R^2	SEM	p
P_o ($N \cdot cm^{-2}$)	0.513	0.013	0.004			
15 Hz ($N \cdot cm^{-2}$)	0.456	0.007	0.008			
20 Hz ($N \cdot cm^{-2}$)	0.520	0.008	0.004			
30 Hz ($N \cdot cm^{-2}$)	0.528	0.010	0.003			
40 Hz ($N \cdot cm^{-2}$)	0.522	0.012	0.004			
60 Hz ($N \cdot cm^{-2}$)	0.514	0.013	0.004			
80 Hz ($N \cdot cm^{-2}$)	0.511	0.014	0.004			
$MHC_{emb + I}$ (%)	0.363	0.022	0.038			
$MHC_{emb + IIabx/IIa}$ (%)				0.631	0.122	0.002
$MHC_{emb + I + IIabxIIa}$ (%)				0.634	0.112	0.002

Significant correlations are shaded. n = 14 for all contractile properties; n = 12 for all MHC fibre characteristics. MV, mechanical ventilation; P_o, maximum specific force.

3.5 Discussion

It is well established that *in utero* inflammation significantly impairs preterm diaphragm contractile function and alters diaphragm structure at birth (Karisnan et al. 2015a, 2015b, 2017; Song et al. 2013). This study sought to determine if this inflammation-induced diaphragm weakness persists after birth, specifically in the acute (7 d) postnatal period. Identifying such associations may aid in better understanding of the respiratory insufficiencies in the early postnatal life of the preterm infant, following exposure to chorioamnionitis.

In vitro diaphragm contractile function and diaphragm MHC fibre characteristics were not significantly different between the saline and LPS groups. However, exposure to *in utero* LPS correlated positively with the duration of mechanical ventilation required by lambs in the intensive care unit. Furthermore, the duration of mechanical ventilation required by lambs, and the average protein intake during the first week of life, independently predicted diaphragm contractile function and MHC fibre characteristics at 7 d PNA. Our results emphasise how complex interaction between common clinical exposures in the perinatal period influence the functional and structural development of the diaphragm after birth.

Interestingly, *in vitro* diaphragm contractile properties of the saline and LPS groups, who developed postnatally for 7 d, were significantly different to the age-matched fetal control group that were killed at delivery. The diaphragm function of the saline and LPS lambs are typical of a more mature fetal lamb diaphragm, compared to the fetal control lambs, as described below. These results suggest that the immature diaphragm adapts rapidly to the marked increase in workload associated with the transition from fetal to postnatal life in the first 7 d after preterm birth.

3.5.1 Diaphragm contractile properties

The contractile properties of the fetal control diaphragm are typical of an immature diaphragm. The fetal control diaphragm was weak, relative to the saline diaphragm, and slower to contract and relax, relative to both the saline and LPS diaphragm. Incomplete myofibrillar development reduces the force-generating capacity of the immature diaphragm (Johnson et al. 1994; Lavin et

al. 2013; Maxwell et al. 1983); while incomplete development of the SR (and Ca^{2+} storage and release mechanisms) will likely reduce the capacity of the immature muscle to release and re-sequester Ca^{2+}. Impaired Ca^{2+} handling may impair force-generating capacity of the diaphragm and may account for the longer contraction and relaxation times of immature diaphragm (Lavin et al. 2013; Maxwell et al. 1983; Sieck et al. 1991). The fetal control diaphragm also produced higher specific force at low stimulation frequencies compared to the saline and LPS diaphragm. The force increase at low stimulation frequencies in the fetal control lambs may be explained by the slower contraction times resulting in enhanced force summation. Together, the reduction in the maximal force-generating capacity and the increase in force production at low stimulation frequencies is reflected in a higher P_t:P_o ratio and leftward shift of the force-frequency relationship of the fetal control diaphragm, relative to the saline and LPS diaphragm. A previous study has reported that the preterm fetal diaphragm has a higher P_t:P_o ratio and leftward shift of the force-frequency relationship compared with the near-term fetal diaphragm (Lavin et al. 2013).

In contrast to the fetal control group, the contractile properties of the saline and LPS diaphragm are typical of more mature fetal lambs. The greater specific force and faster contraction and relaxation times likely reflect an accelerated development of the structural and functional properties of the diaphragm: accelerated development may include altered myofilament arrangement (Geiger et al. 2001; Maxwell et al. 1983; Orliaguet et al. 2002; Racca et al. 2013; West et al. 1999; Williams & Goldspink 1971), altered Ca^{2+} handling (Schiaffino & Margreth 1969; Zubrzycka-Gaarn & Sarzala 1980), and/or the transition from immature to adult protein isoforms (Arai et al. 1992; Brandl et al. 1987; Watchko, Daood & Sieck 1998). We hypothesise that mechanical stresses at birth and in postnatal life accelerate development of the saline and LPS diaphragm.

The diaphragm undergoes a dramatic change in mechanical stimuli, including stretch, immediately after birth that may, in part, contribute to the rapid development of diaphragm function (Cannata et al. 2011). Lung luminal volume decreases by ~ 40 % when lung fluid is removed and functional residual

capacity is established in the first few hours of life in the lamb (Harding & Hooper 1996). Consequently, the increased negative intrathoracic pressure pulls the diaphragm to a longer resting muscle length after birth (Damm & Egli 2014; Louis et al. 2008). Passive stretch increases the Ca^{2+} sensitivity and the force-generating capacity of isolated single fibres from preterm and term lamb diaphragm (Cannata et al. 2011). Additionally, stretch-induced Ca^{2+} influx, via stretch-activated Ca^{2+} channel (e.g. TRPC1), can promote myofibre development, myofibre growth and myofibre survival (Damm & Egli 2014; Louis et al. 2008). TRPC1 expression is 7 – 8 times higher in the diaphragm than in limb muscles (Matsumura et al. 2011), and the diaphragm is therefore likely to be more susceptible to Ca^{2+} influx via stretch-induced TRPC1 activation.

The diaphragm is activated continuously to establish and maintain independent breathing after birth. Lungs with poor alveolar development and surfactant deficiency (Moss 2006), combined with high chest wall compliance (Heldt & McIlroy 1987), markedly increase the workload of the newborn preterm diaphragm. Imposed workload is important for diaphragm function, as evident by the rapid and severe weakness and atrophy of the adult diaphragm when unloaded during mechanical ventilation (Vassilakopoulos & Petrof 2004). It is likely that the increased mechanical loading of the diaphragm after birth lead to the rapid development of diaphragm function in the postnatal lambs that was not evident in the fetal control lambs.

In contrast to our initial hypothesis, *in utero* LPS exposure did not impair diaphragm structure or function at 7 d PNA. Instead, our results suggest that the dynamic combination of cyclical stretches and high workload during postnatal breathing may accelerate preterm diaphragm development in the lamb. Furthermore, we speculate that the improvement in diaphragm contractile function after 7 d of postnatal life may have been greater in the LPS group than the saline group. Saline and LPS groups did not significantly differ at 7 d PNA. If the diaphragms of the LPS group were 30 % weaker than the control group at birth, as previous studies have shown (Song et al. 2013), our results may reflect a greater improvement in diaphragm contractile function of the LPS group. Chorioamnionitis alters the innate immune response of the preterm lamb

(Kramer et al. 2007); and while a single exposure to LPS-induced chorioamnionitis causes diaphragm dysfunction at birth, repeated subsequent exposures do not increase the extent of diaphragm dysfunction further, despite increasing white blood cell levels (Karisnan et al. 2015a). Similarly, chorioamnionitis may have protected the diaphragm against subsequent oxidative damage induced by mechanical ventilation and other postnatal inflammatory stimuli in the LPS group.

3.5.2 MHC fibre characteristics

MHC isoforms were expressed in 4 combinations in the lamb diaphragm at 136 d PCA. Fibres which only expressed MHC_{I} were significantly larger than the 3 other fibre types, which co-express MHC_{emb}. The larger size of MHC_{I} fibres compared with other fibre types is a distinguishing feature of an immature muscle (Fratacci et al. 1996; Maxwell et al. 1983; Sieck, Fournier & Blanco 1991). With development, the MHC_{emb} isoform disappears (Strbenc et al. 2006; Watchko, Daood & Sieck 1998) and there is preferential growth of secondary myofibres expressing MHC_{IIabx} (Fratacci et al. 1996; Maxwell et al. 1983; Sieck, Fournier & Blanco 1991). However, MHC fibre characteristics (MHC fibre type and size) did not significantly differ between fetal control, saline and LPS groups. Stretch-induced changes to Ca^{2+} handling and/or stretch-induced post-translational modifications of contractile and non-contractile proteins (Cannata et al. 2011; Chambers et al. 2009; Monasky, Biesiadecki & Janssen 2011; Tavi, Han & Weckstrom 1998) may account for differences in the contractile properties of the saline and LPS diaphragm, compared with the fetal control diaphragm, despite the lack of difference in MHC fibre characteristics.

3.5.3 Independent postnatal predictors of diaphragm contractile properties and MHC fibre characteristics

Lambs have similar developmental trajectories (lung, skeletal muscle, and gastrointestinal development times) to humans. Consequently, the premature birth of saline and LPS lambs was associated with postnatal complications, similar to the postnatal complications experienced by preterm newborn infants in the neonatal intensive care unit. Lambs were managed with a graded de-escalation of respiratory support to match, as closely as possible, the clinical

management of preterm infants. Additionally, the immature intestine of the preterm lamb, like that of the preterm infant, does not tolerate large protein or osmotic loads (Book et al. 1975). Hence, slow progressive increases in feeding resulted in suboptimal enteral nutritional intakes. Feed was also withheld when lambs were not tolerating enteral nutrition. This management strategy, whilst similar to the clinical setting, may have masked differences between the saline and LPS lambs by introducing an inherent variability.

Bivariate correlation and subsequent linear regression analyses were conducted to identify the variables that significantly predicted measures of diaphragm contractile properties and MHC characteristics at 7d PNA. The contractile properties and MHC fibre characteristics of the saline and LPS lambs, while controlling for confounding experimental variables, were analysed with ANCOVA.

Collinearities between LPS exposure and mechanical ventilation

The duration of mechanical ventilation varied depending on the clinical requirements for each lamb. LPS exposure was collinear with the duration of mechanical ventilation, with prior LPS exposure associated with an increased requirement for mechanical ventilation at 7 d PNA; despite the P value for the statistical comparison between the average normalised duration of mechanical ventilation required by LPS and saline lambs falling just below the 0.05 level ($p = 0.054$).

LPS lambs typically had compliant lungs but appeared neurologically inert upon observation. It is possible that LPS negatively impacted the respiratory control system and modulated the respiratory rhythm generation and diaphragm activity of LPS lambs, as found previously (Huxtable et al. 2011).

The absence of significant differences in diaphragm function or structure between saline and LPS groups is interesting, particularly given that LPS exposure was collinear with duration of mechanical ventilation required by lambs. As previously stated, it is possible that LPS protected the diaphragm from damage by pro-inflammatory stimuli during early postnatal life, similar to the protection afforded to the diaphragm by LPS-induced chorioamnionitis

against subsequent repeated LPS exposure (Karisnan et al. 2015a). This result emphasises the importance of studying not just the individual effects but the combined effects of common clinical antenatal and postnatal exposures on the developing diaphragm.

Mechanical ventilation

The unstandardised residual duration of mechanical ventilation (the duration of mechanical ventilation independent of LPS exposure) significantly predicted diaphragm contractile function and MHC fibre characteristics at 7 d PNA. Increased durations of mechanical ventilation (residual) were associated with reduced specific force across a range of maximal and submaximal stimulation frequencies (15 – 80 Hz). These results are consistent with previous studies, which show diaphragm weakness increases the need for mechanical ventilation in patients with chronic obstructive pulmonary disease (Budweiser, Jörres & Pfeifer 2008), and that mechanical ventilation reduces diaphragm strength in adult humans (Jaber et al. 2011; Levine et al. 2008) and adult animals (Anzueto et al. 1997; Betters et al. 2004; Corpeno et al. 2014; Radell et al. 2002; Sassoon et al. 2002).

The duration of ventilation (residual) correlated negatively with the proportion of fibres expressing MHC_{emb+I}. Although the functional implications of this relationship remain unclear, as the proportion of fibres expressing MHC_{emb+I} was low relative to other fibre types (Figure 3-4B), this finding does indicate an influence of mechanical ventilation on the structural development of the immature diaphragm.

Postnatal nutrition

Postnatal growth restriction resulting from inadequate nutrition intake during early postnatal life is a universal problem among preterm infants (Cooke, Ainsworth & Fenton 2004; Horbar et al. 2015). In this study, suboptimal enteral nutritional intake was associated with impaired postnatal growth of both postnatal lamb groups, compared with *in utero* growth of the fetal control lambs as defined by dry body weight at postmortem. The saline and LPS lambs did not gain appreciable weight over the 7 postnatal days. Consequently, the saline and LPS lambs weighed significantly less than the fetal control lambs at post-

mortem. Importantly, diaphragm function of the postnatal lambs is typical of the more mature fetal lambs, despite postnatal lambs having a lower body weight.

Previous studies indicate that undernutrition results in respiratory muscle weakness, and that developing skeletal muscle is more susceptible to changes induced by undernutrition than adult skeletal muscle (Wilson, Mcclure & Dodge 1992). Undernutrition during development induces diaphragm weakness primarily by reducing fibre numbers and size (Brozanski et al. 1993; Fahey et al. 2005; Prakash, Fournier & Sieck 1993; Wilson, Ross & Harris 1988). Undernutrition results in the preferential atrophy of fibres expressing MHC_{IIabx}, which are primarily formed during secondary and tertiary myogenesis (Goldspink & Ward 1979; Lefaucheur et al. 2003; Ward & Stickland 1993). Furthermore, studies in which undernutrition extended beyond secondary myogenesis report the delayed disappearance of developmental MHC isoforms, indicative of delayed diaphragm development (Brozanski et al. 1991).

In this study, postnatal protein intake was monitored between 129 – 136 d PCA, which is after secondary myogenesis (Fahey et al. 2005). We found no correlation between average protein intake (residual) and the myofibre CSA or the proportion of fibres which co-expressed MHC_{emb}. However, protein intake (residual) significantly predicted $MHC_{emb + IIabx/IIa}$ proportions and $MHC_{emb+ I + IIabx/IIa}$ proportions at 7 d PNA. Average protein intake (residual) correlated positively with $MHC_{emb + IIabx/IIa}$ and negatively with $MHC_{emb + I + IIabx/IIa}$; these correlations suggest a shift in fibre type proportions from $MHC_{emb + I + IIabx/IIa}$ to $MHC_{emb + IIabx/IIa}$ in lambs with higher protein intake. These results are consistent with previous studies, which found that undernutrition during the postnatal period increased MHC_I expression at the mRNA and protein level (Harrison, Rowlerson & Dauncey 1996; White, Cattaneo & Dauncey 2000).

3.5.4 Study limitations

This study was designed to investigate the effects of a 2 d IA LPS exposure on diaphragm structure and function in lambs delivered preterm and raised to 7 d PNA. The ovine model is an appropriate model of antenatal and postnatal human respiratory development. We calculated that a sample size of 8 lambs/group would ensure a study power of 80 % to detect differences of 2 SD

between treatment groups using one-way or two-way ANOVA, as appropriate. However, common postnatal clinical events associated with preterm birth impacted on results from the saline and LPS groups, leading to higher than anticipated variance in our outcome measures. We controlled for mechanical ventilation and postnatal protein intake by incorporating each as covariates in our analyses. However, an unexpectedly high incidence of interrupted feeding and suspected postnatal sepsis may have impacted outcomes further.

The preterm lamb, like the preterm infant, is highly susceptible to postnatal sepsis given the immaturity of its immune system (Kallapur et al. 2007; Kramer et al. 2007). Several preterm lambs displayed symptoms consistent with postnatal infection, including: hypothermic and hyperthermia, vomiting, hypotension and either neutropenia or neutrophilia. However, postnatal sepsis could not be diagnosed decisively as routine blood cell cultures were not obtained prospectively, lambs were on continuous intravenous antibiotics (confounding microbiological detection), and white blood cell counts are dependent on the time blood samples were taken in relation to the onset of postnatal sepsis.

Saline and LPS lambs were treated postnatally in accordance with routine clinical practice; which included caffeine administration during de-escalation of respiratory support as required. Previous studies have shown *in vivo* diaphragm strength increases for up to 6 h after caffeine administration in preterm infants (Kassim, Greenough & Rafferty 2009; Kraaijenga et al. 2015). Therefore, caffeine treatment may have influenced diaphragm function of the saline and LPS groups, and contributed to the difference in diaphragm contractile properties of the saline and LPS groups, compared with the fetal control group.

It should also be noted that saline and LPS groups were exposed to maternal intramuscular medroxyprogesterone 5 d prior to intra-amniotic injections and maternal intramuscular betamethasone, which were given 2 d prior to planned delivery at 129 d gestation: medroxyprogesterone reduces the risk of spontaneous abortion associated with betamethasone administration to the pregnant ewe. Betamethasone facilitates lung maturation and surfactant production. However, in contrast, the fetal control group received neither

medroxyprogesterone nor betamethasone, and hence represents the naïve condition, representing normal *in utero* development. The ideal study design would include a second fetal control group delivered at 136 d but exposed to the same maternal interventions at the same gestational age as the postnatal control (saline) lambs. However, this was considered impractical as a 9 d betamethasone exposure often precipitates a spontaneous abortion, even with prior medroxyprogesterone administration, in the sheep, and the risk of fetal loss was considered too high.

Antenatal medroxyprogesterone and betamethasone may influence diaphragm contractile function at 7 d PNA. Progesterone receptors are present in skeletal muscle myoblasts and myocytes, and progestins reduce the proliferation of bovine satellite cells *in vitro* (Sissom, Reinhardt & Johnson 2006). However, the influence of progesterone on skeletal muscle function is largely unknown (Kim et al. 2016), and it is unclear whether medroxyprogesterone exposure during late gestation effects diaphragm function and structure in the lamb. Furthermore, while it is possible that betamethasone may influence diaphragm function at 7 d PNA, it is considered unlikely given the same 2 d betamethasone exposure has no effect on diaphragm function in the preterm lamb at birth (Mahzabin et al. 2017).

3.5.5 Conclusions

Contrary to our hypothesis, diaphragm structure and function at 7 d PNA did not differ between saline and LPS groups. Furthermore, the difference in diaphragm function of the saline and LPS groups compared with that of the fetal control group suggests that the diaphragm adapts rapidly to the marked increase in workload associated with the transition from fetal to postnatal life in the first 7 d after preterm birth. Interestingly, exposure to *in utero* LPS correlated positively with duration of mechanical ventilation lambs required in the intensive care unit. While postnatal events in managing the preterm lambs are complex, this study emphasises the importance of investigating the combined effects of common clinical antenatal and postnatal exposures on respiratory development in an animal model with similar developmental trajectories to humans.

Chapter 4

Postnatal dexamethasone treatment does not compromise diaphragm function of the preterm lamb.

Preface

This study examines the effect of high and low-dose postnatal dexamethasone exposure on the functional development of the preterm lamb diaphragm at 7 d PNA. Furthermore, this study examines the combined effects of postnatal dexamethasone administration and mechanical ventilation on the functional development of the preterm lamb diaphragm at 2 m post-term. Lastly, this study assesses the long-term effects of preterm birth on diaphragm function at 2 m post-term.

4 Postnatal dexamethasone treatment does not compromise diaphragm function of the preterm lamb

4.1 Abstract

Many preterm infants require extended ventilator support due to the immaturity of their respiratory system. Dexamethasone treatment can accelerate lung development and weaning of preterm infants from mechanical ventilation. However, dexamethasone use has been associated with serious long-term side effects, including protein catabolism. The effects of dexamethasone on the developing diaphragm are unclear. We aimed to determine the short-term and long-term effects of postnatal dexamethasone treatment, and the long-term effects of preterm birth, on diaphragm structure and function in preterm lambs. Specifically, we aimed to determine the short-term dose-dependent effects of dexamethasone, and the long-term interactive effects between dexamethasone and mechanical ventilation, on diaphragm structure and function.

Lambs were delivered prematurely at 129 d and assigned to a short-term or long-term study. Lambs assigned to the short-term study received saline (n = 8), low-dose dexamethasone (0.45 mg/kg total; n = 8) or high-dose dexamethasone (2.67 mg/kg total; n = 7) from birth, for the first week of life, before they were killed at 7 d PNA. Preterm lambs in the long-term study were assigned to one of four groups: they received saline with continuous positive airway pressure (n = 5), saline with mechanical ventilation (n = 7), dexamethasone (0.89 mg/kg total) with continuous positive airway pressure, or dexamethasone with mechanical ventilation (n = 5). Saline and dexamethasone treatments were commenced at 72 h. Preterm lambs assigned to the long-term study were killed at 2 m post-term. A full-term control group (n = 5), used for comparison to the 2 m preterm groups, was delivered naturally and received no intervention, before they were killed at 2 m PNA. Diaphragm contractile properties were measured *in vitro*. Diaphragm myofibre type and CSA were measured using immunofluorescence staining and H&E staining, respectively.

Low-dose dexamethasone exposure had no effect on diaphragm structure or function at 7 d PNA. High-dose dexamethasone increased time to peak twitch

force at 7 d, and dexamethasone treatment increased half relaxation time at 2 m post-term. However, changes in the contractile times did not influence the force-frequency relationship or maximum force generating capacity of the diaphragm, at 7 d or 2 m post-term. The duration of mechanical ventilation was the most significant predictor of diaphragm function at 7 d PNA, however duration of mechanical ventilation had no significant effect on diaphragm function at 2 m post-term. Lastly, preterm birth had no significant effect on diaphragm function or myofibre CSA at 2 m post-term.

Overall, postnatal dexamethasone exposure had no significant detrimental short-term or long-term effects on diaphragm structure or function in prematurely born lambs. Our results suggest that dexamethasone treatment does not compromise diaphragm structure or function. Furthermore, our data suggest that the diaphragm dysfunction associated with mechanical ventilation during the acute postnatal period may be overcome with subsequent postnatal development; and that preterm birth has no long-lasting effect on diaphragm structure or function.

4.2 Introduction

Respiratory disease is a major consequence of the incomplete development of the respiratory system associated with preterm birth. Poor alveolar development, combined with surfactant deficiency and high chest wall compliance, increases the work of breathing in preterm infants (Heldt & McIlroy 1987; Joshi & Kotecha 2007; Moss 2006). The preterm diaphragm is weak and structurally underdeveloped (Dimitriou et al. 2001, 2003; Keens et al. 1978; Lavin et al. 2013; Maxwell et al. 1983), which compromises its capacity to cope with the high workload of breathing. Antenatal and postnatal insults may further compromise preterm respiratory function, resulting in respiratory distress, and the need for respiratory support (Gantert et al. 2010; Kramer et al. 2009; Lal et al. 2003; Van Marter et al. 2002; Wilson et al. 1991). Mechanical ventilation, while vital in supporting gas-exchange in preterm infants who are unable to breath independently, is associated with lung injury (Morley et al. 2008) and diaphragm weakness and wasting (Vassilakopoulos & Petrof 2004). For these reasons, infants should be weaned from mechanical ventilation as soon as possible. Dexamethasone is the most widely use glucocorticoid treatment to accelerated lung maturation and weaning from mechanical ventilation.

Dexamethasone was first adopted in the 1980s pre-surfactant era, based on observational studies that concluded high-dose dexamethasone improves short-term respiratory function (Avery et al. 1985; Cummings, D'Eugenio & Gross 1989; Mammel et al. 1983; Rastogi et al. 1996). Glucocorticoid administration accelerates lung maturation and surfactant production (Bolt et al. 2001), and the potent anti-inflammatory properties of glucocorticoids make them an effective treatment to reduce the lung inflammation that precedes BPD (Gupta et al. 2012). However, early dexamethasone studies used extremely high-doses (0.5 – 1.0 mg/kg/d), over 20 times higher than basal cortisol levels. High-dose dexamethasone is associated with long-term adverse outcomes, including increased protein catabolism, gastrointestinal proliferations and adverse neurological outcomes (Stark et al. 2001; Tsai et al. 1996; Yeh et al. 1997). Although low-dose dexamethasone treatments are less likely to result in adverse long-term outcomes, they may not necessarily produce the same improvements in preterm respiratory function (Cummings, D'Eugenio & Gross

1989). The effects of high and low-dose dexamethasone treatment on other components of the respiratory system, such as the diaphragm, are unknown in the preterm infant.

Glucocorticoid treatment reduces muscle mass and impairs function of the adult diaphragm (Van Balkom et al. 1997; Prezant et al. 1998; Sasson et al. 1991; Sassoon et al. 2008), which is explained in detail in Chapter 2. Glucocorticoid-induced diaphragm weakness and atrophy are associated with the inhibition of protein synthesis, upregulation of proteolytic pathways, vacuolisation of muscle fibres, structural damage to the contractile apparatus, and mitochondrial dysfunction (Ferguson, Irvin & Cherniack 1990; Menconi et al. 2007; Sassoon et al. 2008; Schakman et al. 2009). If dexamethasone has similar effects on the preterm diaphragm, then dexamethasone treatment may induce preterm diaphragm weakness and atrophy via multiple mechanisms.

Glucocorticoid-induced weakness and atrophy of the adult diaphragm are dose and duration-dependent (Prezant et al. 1998; Sasson et al. 1991; Sassoon et al. 2008). For example, diaphragm specific force and muscle fibre CSA of rabbits are unaffected by a 3 d 10 mg/kg/d course of methylprednisolone (Sassoon et al. 2008). However, diaphragmatic force response is reduced after just one day of a higher methylprednisolone dose (80 mg/kg/d) treatment. The force response is further reduced and after 3 d of high-dose methylprednisolone, accompanied by preferential atrophy of Type IIb fibres (Sassoon et al. 2008). Furthermore, glucocorticoid use and mechanical ventilation have interactive effects on adult diaphragm structure and function that are also dose and duration dependent. Low-dose glucocorticoids can exacerbate diaphragm dysfunction caused by mechanical ventilation (Maes et al. 2010; Sassoon et al. 2011). High-dose steroids can, however, prevent ventilation induced diaphragm dysfunction in rats, by inhibiting calpain activity (Maes et al. 2010; Sassoon et al. 2011). The dose and duration of dexamethasone treatment, and potential interactive effects of dexamethasone treatment and mechanical ventilation, may have important implications for the function of the preterm diaphragm.

Overall, the long-term implications of preterm birth and postnatal exposure to glucocorticoids and mechanical ventilation on diaphragm function are unknown. The current study aimed to:

1. Determine the short-term and long-term effects of clinically relevant, postnatal dexamethasone administration to preterm lambs on the structure and function of the diaphragm. Specifically:

    i. The study aimed to determine the effects of low and high-dose dexamethasone on the function and structure of the preterm lamb diaphragm at 7 d PNA, as the effects of glucocorticoid treatment on the adult diaphragm are dose-dependent.
    ii. The study aimed to investigate the independent and combined effects of postnatal dexamethasone administration and mechanical ventilation on diaphragm function at 2 m post-term, as dexamethasone is used clinically to accelerate weaning of preterm infants off mechanical ventilation.

2. Determine the long-term effects of preterm birth on the function of the diaphragm.

We hypothesised that diaphragm dysfunction induced by dexamethasone would be dose-dependent, long-lasting and influenced by concurrent mechanical ventilation. Furthermore, we hypothesised that diaphragm dysfunction induced by preterm birth would be long-lasting.

4.3 Methods

All experiments were approved by the Animal Ethics Committee of the University of Western Australia (3/100/1301) and were conducted in accordance with the guidelines of the National Health and Medical Research Council (NHMRC) Australian code for the care and use of animals for scientific purposes (2013).

4.3.1 Experimental outline

Merino lambs were assigned randomly to a short-term (7 d) study or to a long-term (2 m) study; examining the short and long-term effects of dexamethasone exposure on preterm diaphragm function, respectively.

Experiment 1: Short-term (7 d) study

To determine the short-term impact of postnatal dexamethasone exposure on preterm diaphragm function, lambs, delivered at 129 d GA (term = 145 - 150 d GA) were randomly assigned to one of three groups:

i. A saline group (n = 8) receiving no dexamethasone treatment.
ii. A low-dose dexamethasone (Low DEX) treated group (n = 8) receiving dexamethasone intravenously for 7 d via a modified Dart trial protocol (Doyle et al. 2007): 0.15 mg/kg/d for 3 d, then 0.10 mg/kg/d for 2 d, then 0.05 mg/kg/d for 2 d; 0.75 mg/kg total. A dexamethasone dosing regimen which starts at < 0.2 mg/kg/d is considered to be a low dose.
iii. High-dose dexamethasone (High DEX) treated group (n = 7), receiving high dexamethasone intravenously for 7 d in accordance with Cummings (1989): commencing dose 0.5 mg/kg/d for 3 d, then 0.3 mg/kg/d for 3 d, then 0.27 mg/kg for 1 d; 2.67 mg/kg total. A dexamethasone dosing regimen which starts at ≥ 0.5 mg/kg/d is considered to be a high dose.

Preterm lambs were managed in an intensive care environment and killed (pentobarbitone, 150 mg/kg IV; Pitman-Moore, Australia) at 7 d PNA (136 d PCA).

Experiment 2: Long-term (2 month) study

To determine the long-term impact of low-dose postnatal dexamethasone exposure on diaphragm function, preterm lambs, delivered at 129 d GA, were assigned randomly to one of four groups:

i. Saline group that received non-invasive respiratory support with continuous positive airway pressure (SAL + CPAP; n = 5).

ii. Saline group that received mechanical ventilation (SAL + MV; n = 7).
iii. Dexamethasone group that received non-invasive respiratory support with continuous positive airway pressure (DEX+ CPAP; n = 5).
iv. Dexamethasone group that received mechanical ventilation (DEX + MV; n = 5).

A term control group (n = 5), that was delivered naturally at 145 – 150 d and received no intervention, was included for comparison with preterm lambs to enable evaluation of the long-term impact of preterm birth on diaphragm function at 2 m corrected PNA.

Dexamethasone (100 µg/mL) or an equivalent volume of saline was administered to preterm lambs from 72 h of life (~equivalent to 1 w human PNA), in the 2 m study. Dexamethasone was administered intravenously in two divided doses at 12 h intervals in accordance with the protocol described in the DART trial: commencing dose 0.15 mg/kg/d for 3 d, then 0.10 for 3 d, then 0.05 for 2 d, then 0.02 for 2 d; Cumulative dexamethasone dose was 0.89 mg/kg (Doyle et al. 2007). Preterm lambs were managed in an intensive care environment until post-term equivalent (150 d PCA), during which they received varying durations of mechanical ventilation and non-invasive ventilation, as described below. Preterm lambs were then moved to a nursey pen and killed at 2 m post-term (~210 d PCA). Term lambs were penned with their mother for 7 d, after which they were moved to the nursery pen and killed at 2 m PNA.

4.3.2 Animal management

Prenatal treatment and delivery of preterm lambs

Date-mated merino ewes (4 – 7 years of age) assigned to a preterm group received IM medroxyprogesterone (150 mg; Pfizer) at 122 d PCA, and 2 doses of IM betamethasone (Celestone®, 0.15mg/kg/dose; Merck Sharp and Dohme) at 24 h intervals in the 2 d prior to delivery via surgical hysterotomy. Ewes were pre-medicated with IM Buprenorphine (0.01 mg/kg; Indivior Pty Ltd) and IM Acepromazine (0.05 mg/kg; Ceva Animal Health Pty Ltd) one hour prior to anaesthetic induction with Thiopentone (15 mg/kg; Troy Laboratories), and

maintenance anaesthetic with isofluorane (1.5 – 2.5 %). Arterial and venous catheters were inserted into the carotid artery and jugular vein of fetuses assigned to the short-term 7 d study, and into the umbilical artery and vein of fetuses assigned to the long-term 2 m study. All fetuses were intubated orally, lung fluid was suctioned, and intra-tracheal surfactant was administered (Curosurf®, 100 mg/kg; Chiesi Farmaceutici S.p.A) before delivery. Ewes were killed immediately after lamb delivery (pentobarbitone, 150mg/kg, IV; Pitman-Moore).

Postnatal treatment

Preterm lambs were weighed, resuscitated and commenced on assist/control volume-guaranteed ventilation (5 – 7 mL/kg) immediately following delivery, in accordance with routine clinical practice. Ventilatory settings (volume guarantee, positive end-expiratory pressure, fractional inspired oxygen, inspiratory time and respiratory rate) were adjusted to target mild permissive hypercapnia ($PaCO_2$ of 45 – 55 mmHg) and SpO_2 of 90 – 95 %. Maximum peak inspiratory pressure was 30 cmH_2O. Three lambs in the short-term high-dose dexamethasone group were initially assigned to another study and received 96 h of mandatory ventilation before weaning to less invasive ventilatory modes if they were spontaneously breathing and maintaining SpO_2 of 90 – 95 % at a peak inspiratory pressure < 15 mmHg and FiO_2 < 0.25. Preterm lambs assigned to a mechanical ventilation group in the 2 m study received a mandatory 96 h of mechanical ventilation after delivery, after which they were weaned according to the aforementioned criteria. All other preterm lambs assigned to the 7 d study and 2 m study were mechanically ventilated as required after birth and weaned as soon as possible, based on the aforementioned criteria, usually within 3-6 hours after delivery. All preterm lambs were re-intubated if they displayed any of the following: persistent SpO_2 < 80 % despite FiO_2 > 0.8 and increasing mean airway pressure to a maximum of 12 cmH_2O; ≥ 4 apnoea events requiring resuscitation within an hour; ventilatory failure ($PaCO_2$ > 80 mmHg on two consecutive arterial gases); or severe metabolic acidosis (Base excess < -10 mmol).

Arterial blood pressure was monitored continuously and arterial blood was sampled intermittently for blood-gas analysis to aid in the assessment of respiratory status. Preterm lambs received intravenous fluid support (5 % dextrose) and commenced enteral feeds at 2 h (20 mL/kg/d) with fresh maternal colostrum, incrementing enteral feeds by 20 mL/kg/d and transferring to formula feeds at 24 h (MaxCare® Lamb & Kid; MaxumAnimal). Supplemental heating was adjusted to target normothermia (38.5-39.5 °C) throughout early postnatal life (primarily first week). Lambs assigned to the short-term study were killed at 7 d (pentobarbitone, 150 mg/kg IV; Pitman-Moore).

Preterm lambs assigned to the long-term study were transferred from the intensive care unit into in a nursery pen from 2 w PNA (~term equivalent) and were housed in the nursery pen until they were killed at 2 m post-term (~210 d PCA) (pentobarbitone, 150 mg/kg, IV; Pitman-Moore). Lambs received ad-lib access to formula milk (approx. 1500-2000 mL/d) and solid feed (finely ground modified Maccos 707 pellet feed was added initially as a small pinch per milk feed and increased to 100 g/d, plus oaten chaff) for 5 weeks, after which milk feeds were decreased to 1200 mL/d for 1 week, then 1000 mL/d for 2 weeks, while in the nursey pen. Lamb vital signs and physical appearance were monitored twice a day.

Term control lambs assigned to the 2 m study, were penned with their mother for 7 d, after which they were moved to the nursery pen, where they were housed, fed and monitored, as preterm lambs were. Term control lambs were killed at 2 m PNA.

4.3.3 Tissue collection

The diaphragm was excised immediately after lambs were killed. The mid-costal portion of the right hemi-diaphragm, including the attached ribs and central tendon, was maintained in mammalian Ringer's solution (in mM; NaCl, 121; KCl, 5.4; $MgSO_4(H_2O)_7$, 1.2; $NaHCO_3$, 25; HEPES, 5; glucose, 11.5 and $CaCl_2$, 2.5: pH 7.3) bubbled with carbogen (5 % CO_2 in O_2) throughout careful dissection of muscle strips and ensuing measurements of contractile function. Sections of the mid-costal portion of the left hemi-diaphragm were imbedded in tragacanth gum and frozen in isopentane cooled in liquid nitrogen for

histochemical analysis. Frozen samples were stored at -80 ºC until further analysis.

4.3.4 Diaphragm contractile properties

A longitudinal strip (3-5 mm wide) of intact muscle fibres was mounted in an *in vitro* muscle test system (1200A, Aurora Scientific). Diaphragm contractile properties were measured at optimal muscle length (L_o) in response to 0.2 ms supramaximal square wave pulses (biphasic stimulator, 701B, Aurora Scientific). Measured contractile properties included maximum specific force (P_o), force-frequency relationship, twitch force (P_t), time to peak (TTP), half-relaxation time (1/2 RT), and fatigue index (FI). FI was determined from the ratio of the force produced during the 150th contraction relative to the 1st contraction of a fatigue protocol, in which a higher number indicates a greater fatigue resistance. Force (g) was normalised to CSA calculated from muscle fibre length, muscle mass and density (1.056 g·cm^{-3}) (Mendez & Keys 1960) and presented as specific force (N·cm^{-2}). The detailed protocol is described in Appendix I.

4.3.5 Histochemistry

Myosin heavy chain (MHC) fibre typing

MHC fibre typing was conducted for the short-term study only.

Transverse sections (8 µm) of frozen muscle were stained with anti-myosin heavy chain (MHC) specific antibodies: MHC_{IIa} (mouse monoclonal IgG1; SC-71; DSHB, University of Iowa; dilution 1:25), MHC_I (mouse monoclonal IgG2b; BA-D5; DSHB, University of Iowa; dilution 1:25), MHC embryonic (MHC_{emb}) (rabbit polyclonal IgG; 2400104; Mimotopes; dilution 1:25) and MHC_{IIabx} (rabbit polyclonal IgG; 2400107; Mimotopes; dilution 1:25). Primary MHC_{IIa} was detected by goat anti-mouse IgG1 Alexa Fluor 488 (Jackson ImmunoResearch; dilution 1:500), MHC_I was detected by goat anti-mouse IgG2b dylight 405 (Jackson ImmunoResearch; dilution 1:500), and MHC_{emb} and MHC_{IIabx} were detected by anti-rabbit IgG Alexa Fluor 594 (Invitrogen; dilution 1:500). Tiled images were captured at 20x using a Nikon C2+ Confocal microscope (Nikon Corporation). The detailed protocol is described in Appendix I. MHC_{emb} and

MHC_{IIabx} were designed by our laboratory and validated before use, as described in Appendix II.

Myofibre cross-sectional area (CSA)

Transverse sections (8 µm) of frozen muscle were stained with H&E to quantify myofibre CSA. Tiled images were captured at 20x using a Nikon Eclipse Ti inverter microscope (Nikon Corporation).

Image analysis

Fibre type proportions and MHC fibre CSA were determined manually using ImageJ (v1.51j8) software and ImageJ cell counter plugin (Abramoff, Magalhaes & Ram 2004). The CSA of each MHC fibre type was determined by matching the H&E images with images of serial sections stained for MHC expression, for the short-term study.

4.3.6 Statistical analysis

Data are presented as mean ± SD or as median (range). Significance was at the 0.05 level, unless otherwise stated. Analyses were performed on IBM SPSS Statistics software (Version 19, IBM Company 2010), with the statistical analysis methods described below.

Statistical analysis of the short-term (7 d) study

A sample size of 8 lambs/group has an 80 % power to detect differences of 2 SD between treatment groups at the 5 % level. Differences in the lamb descriptive data of 7 d treatment groups were analysed with one-way ANOVA. Differences in diaphragm contractile properties and MHC fibre characteristics among 7 d treatment groups were assessed with one-way ANCOVA and pairwise comparisons, expect for the force frequency relationships, which were analysed using two-way ANCOVA. Sex was controlled for during all statistical analyses, as dexamethasone treatment is known to have sex specific effects on skeletal muscle (Prezant et al. 1997).

The relationship between diaphragm functional and structural properties at 7 d PNA and several potential confounding experimental variables were also evaluated. The potential confounding variables evaluated were average protein intake and duration of mechanical ventilation (normalised to absolute PNA), as

both nutritional and mechanical ventilation are known to influence diaphragm function (*see section 2.2.1 on nutrition and 2.2.3 on mechanical ventilation*). Collinearities between the potential confounding variables was first analysed with Pearson's bivariate correlation. The relationship between potential confounding variables and outcome variables at 7 d PNA were analysed using Pearson's bivariate correlation and Spearman's bivariate correlation analyses, as appropriate. Outcome variables that correlated significantly with the confounding experimental variables were analysed with linear regression analyses, as all significant correlations were with normally distributed outcome variables. Lastly, outcome variables that significantly correlated with the independent variables were re-analysed while controlling for the independent experimental variables, and sex, using one-way ANCOVA and two-way ANCOVA, as appropriate.

Statistical analysis of the long-term (2 m) study

A sample size of 8 lambs/group has an 80 % power to detect a 2 SD mean difference with significance at the 5 % level. Differences in the lamb descriptive data of 2 m treatment groups were analysed with one-way ANOVA. The combined and independent effects of dexamethasone and mechanical ventilation on diaphragm function and structure at 2 m post-term was analysed with two-way ANCOVA controlling for sex, except for the force-frequency relationship, which was analysed with 3–way ANCOVA controlling for sex. The relationship between diaphragm functional and structural properties at 2 m post-term and the duration of mechanical ventilation were analysed with Pearson's bivariate and Spearman's bivariate correlation analyses, as appropriate. Lastly, differences among term control and the combined saline group (SAL+CPAP and SAL+MV), were analysed with one-way ANCOVA while controlling for sex.

4.4 Results

4.4.1 Experiment 1: Short-term effects of dexamethasone on postnatal diaphragm function and structure

Lamb descriptive data at post-mortem

Lamb characteristics of each group are presented in Table 4-1. Lamb PCA at post-mortem, body weight at post-mortem, and optimal muscle length did not significantly differ between treatment groups ($p > 0.05$).

The short-term effects of dexamethasone on diaphragm contractile properties

Statistical analysis identified no significant differences in the diaphragm contractile function of the three high-dose dexamethasone lambs whom received 96 h mandatory mechanical ventilation before weaning and the four high-dose dexamethasone lambs whom were weaned as soon as possible (Appendix III). Therefore, all high-dose dexamethasone lambs were grouped together as a single high-dose dexamethasone group for subsequent analyses.

Both low-dose and high-dose dexamethasone exposure had no effect on the force-generating capacity of the preterm diaphragm at 7 d PNA (Figure 4-1). Maximum specific force was not significantly different between the diaphragm of the saline group, low-dose dexamethasone group and high-dose dexamethasone group ($p = 0.494$). There was no significant interaction between treatment group and the force-frequency relationship when force was measured as specific force ($p = 0.102$) or when force was normalised to the maximum force produced within each group ($p = 0.109$). There was no significant difference in peak twitch force ($p = 0.914$) twitch half relaxation time ($p = 0.167$), or the peak twitch force as a ratio of maximum specific force ($p = 0.722$), between treatment groups. However, time to peak twitch force significantly differed between treatment groups ($p = 0.002$). The time to peak twitch force was significantly longer in the diaphragm from the high-dose dexamethasone group compared to the saline ($p = 0.004$) and low-dose dexamethasone groups ($p = 0.001$).

The fatigue protocol successfully reduced force elicited by a 40 Hz stimulation by approximately 40 – 50 % in all treatment groups. There was no significant difference in the fatigue index between groups (p = 0.090; Figure 4-1F).

Table 4-1. Lamb descriptive data of saline, low DEX and high DEX groups at 7 d PNA.

	Saline (n = 8)	**Low DEX (n = 8)**	**High DEX (n = 7)**
PCA (d)	136.5 (135 – 139)	136.0 (135 – 137)	136.0 (135 – 137)
Sex ratio (M:F)	3:5	6:2	4:3
Body wt (kg)	2.84 ± 0.37	2.74 ± 0.30	2.67 ± 0.47
Normalised MV (h)	25.94 ± 20.90	22.19 ± 23.66	49.32 ± 52.13
Normalised CPAP (h)	7.99 ± 7.97	26.84 ± 27.79	17.63 ± 10.44
Average protein intake (mg/kg/d)	38.07 ± 12.44	28.92 ± 11.19	25.67 ± 4.71
L_o (mm)	36.6 ± 4.3 (n = 7)	38.4 ± 4.4	38.5 ± 5.3

Data presented as mean ± SD or median (min - max), except for sex ratio. PCA, postconceptional age; MV, mechanical ventilation; CPAP, continuous positive airway pressure; PM, post-mortem; L_o, optimal muscle length.

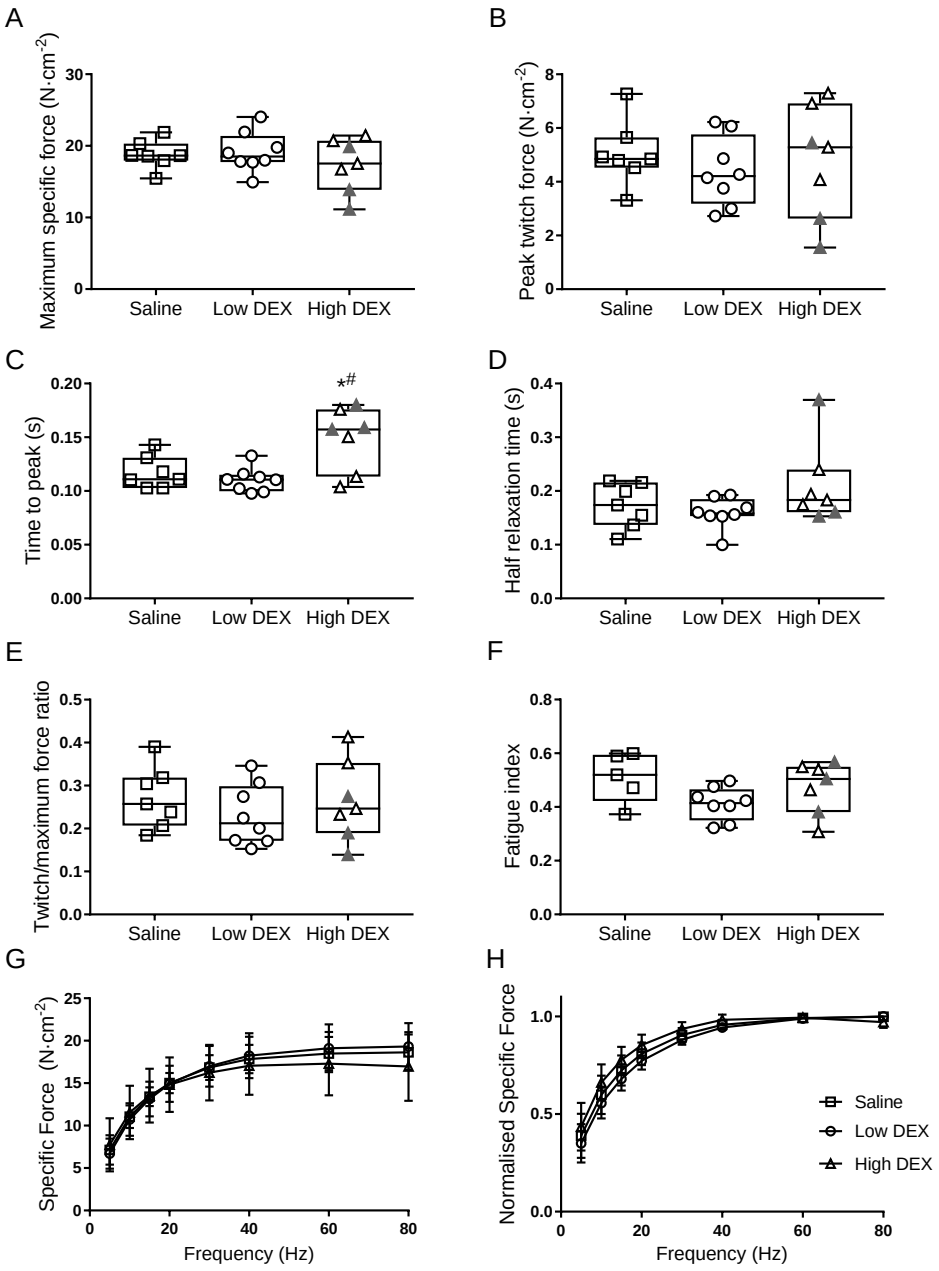


Figure 4-1. Diaphragm contractile properties of saline, low DEX and high DEX groups at 7 d PNA.

(A) maximum specific force (P_o); (B) peak twitch force (P_t); (C) time to peak (TTP); (D) half relaxation time (1/2 RT); (E) peak twitch force as a ratio of maximum force (P_t:P_o); (F) fatigue index (FI); (G) force-frequency relationship displayed as specific force (N·cm^{-2}); and (H) force-frequency relationship normalised to maximum specific. Box and whisker plots represent median, minimum and maximum values, and all data points are visible. Data points from high dexamethasone lambs whom received 96 h mandatory MV are shaded. Force-frequency data are presented as mean ± SD. n = 7 for saline and high dexamethasone groups, n = 8 for low dexamethasone group. * and # significantly different to the saline and low dexamethasone group at $p < 0.05$, respectively.

The short-term effects of dexamethasone on diaphragm MHC fibre characteristics

As in Chapter 3, all myofibres that stained positively for MHC_{IIabx} also stained positively for MHC_{IIa}, and MHC isoforms were expressed in the following four combinations: i) 6-8 % of fibres expressed MHC_{I} only; ii) 9-11 % fibres co-expressed MHC_{I} and MHC_{emb}; iii) 65-71 % of fibres co-expressed $MHC_{IIabx/IIa}$ and MHC_{emb}; iv) 12-16 % of fibres co-expressed MHC_{I}, $MHC_{IIabx/IIa}$ and MHC_{emb}. The percentage distribution of the four MHC-type myofibres were not significantly different between treatment groups ($p > 0.05$; Figure 4-2B). Fibres that only expressed MHC_{I} were significantly larger than all other fibre types ($p < 0.01$; Figure 4-2C), however the CSA of individual myofibre types were not significantly affected by dexamethasone exposure ($p > 0.05$; Figure 4-2C).

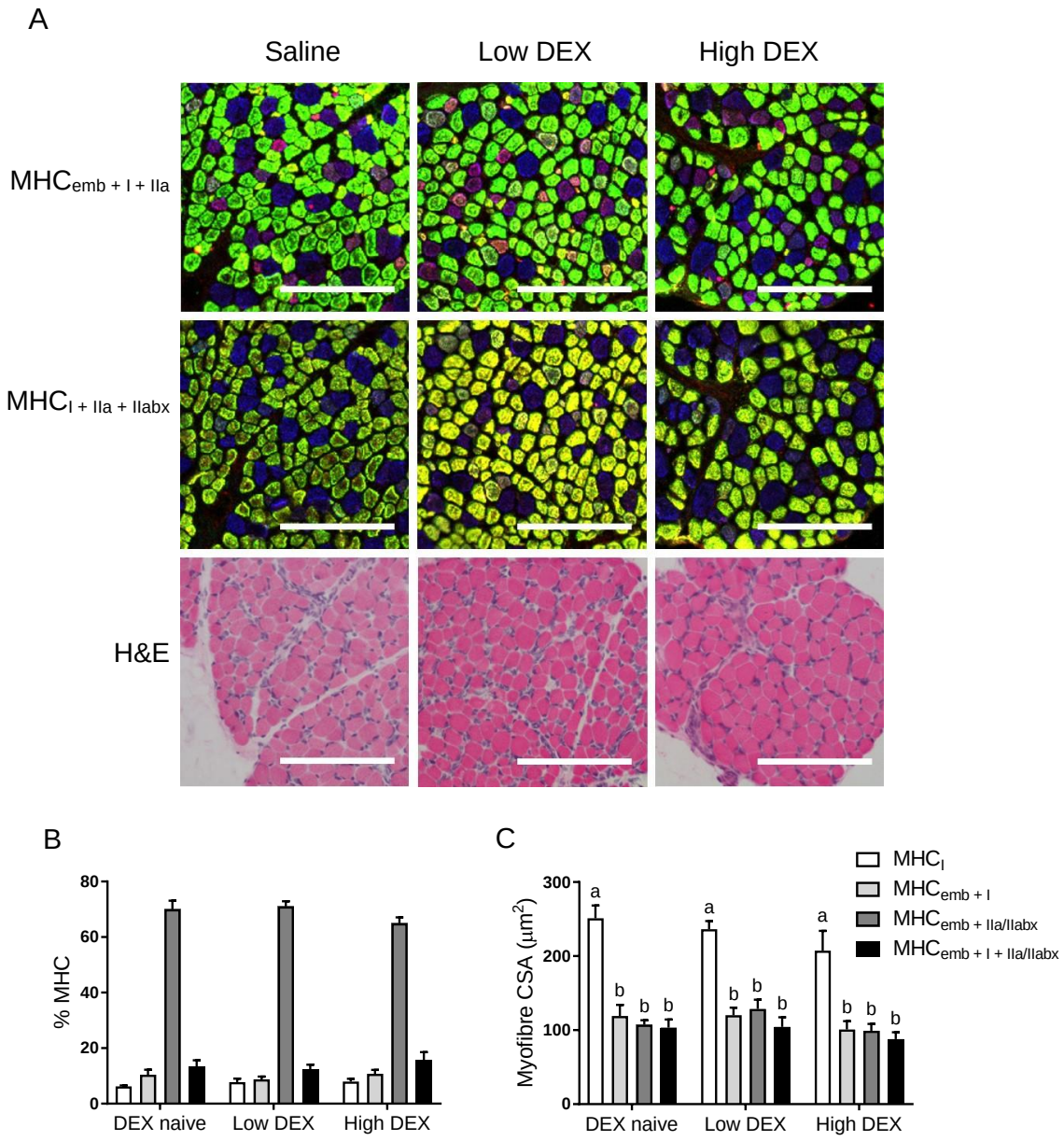


Figure 4-2. Diaphragm myofibre types (%) and cross-sectional area (CSA) from saline, low DEX and high DEX groups at 7 d PNA.

(A) Muscle fibre sections were stained with a combination of MHC_{emb} (red), MHC_{I} (blue), MHC_{IIa} (green) antibodies, a combination of MHC_{I} (blue), MHC_{IIa} (green), and MHC_{IIabx} (red) antibodies, or H&E. Myofibre size (measured by CSA) was quantified from transverse sections stained with H&E. Graphs show (B) the proportion of myofibre types and (C) myofibre CSA. Sale bars represent 100 μm. Data are presented as mean ± SD (n = 6), and statistical differences in the myofibre CSA are indicated by different letters ($p < 0.05$). MHC, myosin heavy chain.

Independent predictors of diaphragm structural and functional properties at 7 d PNA

Differences between saline, low-dose dexamethasone and high-dose dexamethasone groups may have been influenced by confounding postnatal management that included altered nutritional intake and duration of mechanical ventilation. Average protein intake and normalised duration of mechanical ventilation for saline, low-dose dexamethasone and high-dose dexamethasone groups are presented in Table 4-1. The relationships between diaphragm outcome variables of combined saline, low-dose dexamethasone and high-dose dexamethasone groups at 7 d PNA, dexamethasone treatment and potential confounding experimental variables (average protein intake and normalised duration of mechanical ventilation) were evaluated using correlation and linear regression (n = 23 for diaphragm contractile function; n = 18 for MHC fibre characteristics). Stepwise multivariate regression was used in cases where both the average protein intake and duration of mechanical ventilation significantly correlated with diaphragm measurements.

Correlation coefficients between the independent variables are presented in Table 4-2. Correlation coefficients between independent variables and outcome measures are presented in Table 4-3. Linear regression analyses are presented in Table 4-4.

Dexamethasone treatment

Dexamethasone treatment (0 = saline, 1 = low dexamethasone, 2 = high dexamethasone) correlated negatively with the protein intake by lambs in the 7 d of postnatal life ($R^2 = 0.215$, $p = 0.026$); despite average protein intake by saline, low-dose dexamethasone, and high-dose dexamethasone lambs, not differing significantly ($p = 0.071$) when analysed with one-way ANOVA.

Protein intake

As collinearities were identified between normalised duration of average protein intake and dexamethasone treatment, the unstandardised residual protein intake, that is the component of protein intake not due to dexamethasone treatment, was entered into the correlational model. Average protein intake (residual) did not remain significant when added to any stepwise multivariate

regression analyses comprising of duration of mechanical ventilation; hence, average protein intake did not independently predict diaphragm MHC characteristics at 7 d PNA.

Mechanical ventilation

Normalised duration of mechanical ventilation required by saline, low-dose dexamethasone, and high-dose dexamethasone lambs, did not differ significantly ($p = 0.280$). However, as in Chapter 3, duration of mechanical ventilation independently predicted diaphragm contractile properties and MHC characteristics. Duration of mechanical ventilation exhibited a moderate negative correlation with specific force produced at 20-80 Hz (R^2 ranged between 0.233 to 0.378), and a weak negative correlation with peak twitch force ($R^2 = 0.190$). Specifically, duration of mechanical ventilation (residual) explained ~35 % of the variation observed in diaphragm maximum specific force at 7 d PNA. The negative correlation between mechanical ventilation (residual) and specific force suggests that specific force decreases with increasing duration of ventilation.

Duration of mechanical ventilation also exhibited a moderate negative correlation with the proportion of fibres expressing MHC_I ($R^2 = 0.265$) and a moderate positive correlation with the proportion of fibres expressing $MHC_{emb + I + IIabx/IIa}$ ($R^2 = 0.406$).

Diaphragm contractile properties and MHC fibre characteristics after correcting for respiratory support

After controlling for the duration of mechanical ventilation, low-dose and high-dose dexamethasone treatment had no significant effect on the maximum specific force ($p = 0.926$; data not shown), force-frequency relationship ($p = 0.094$; data not shown), twitch force ($p = 0.498$; data not shown), or MHC fibre characteristics ($p > 0.05$; data not shown).

Table 4-2. Collinearities between potential confounding postnatal variables for saline, low DEX and high DEX groups at 7 d PNA.

	Dexamethasone	Sex	Normalised MV (h)	Average protein intake (g/kg/d)	Average protein intake (residual) (g/kg/d)
Dexamethasone	N/A	0.288 0.182	0.269 0.214	-0.464 0.026	0.000 1.000
Sex	0.288 0.182	N/A	0.099 0.653	-0.064 0.770	0.078 0.722
Normalised MV (h)	0.269 0.214	0.099 0.653	N/A	-0.430 0.041	-0.344 0.108
Average protein intake (g/kg/d)	-0.464 0.026	-0.064 0.770	-0.430 0.041	N/A	0.886 0.000
Average protein intake (residual) (g/kg/d)	0.000 1.000	0.078 0.722	-0.344 0.108	0.886 0.000	N/A

Top values represent the correlation coefficient, bottom values represent the p-value. Significant correlations are shaded. MV, mechanical ventilation

Table 4-3. Bivariate correlation between potential confounding postnatal variables and diaphragm structural and functional properties of saline, low DEX and high DEX groups at 7d PNA.

	Normalised MV (h)		Average protein intake(residual) (g/kg/d)	
	R	p	R	p
P_o (N·cm^{-2})	-0.590	0.004	0.100	0.659
P_t (N·cm^{-2})	-0.436	0.043	-0.032	0.887
TTP (s)	0.268	0.228	-0.042	0.851
½ RT (s)	-0.155	0.490	-0.030	0.895
5 Hz (N·cm^{-2})	-0.227	0.309	-0.095	0.674
10 Hz (N·cm^{-2})	-0.308	0.163	-0.107	0.635
15 Hz (N·cm^{-2})	-0.416	0.054	-0.052	0.817
20 Hz (N·cm^{-2})	-0.483	0.023	0.011	0.962
30 Hz (N·cm^{-2})	-0.536	0.010	0.067	0.767
40 Hz (N·cm^{-2})	-0.547	0.008	0.079	0.726
60 Hz (N·cm^{-2})	-0.583	0.004	0.088	0.696
80 Hz (N·cm^{-2})	-0.615	0.002	0.102	0.653
FI	-0.005	0.983	0.293	0.210
MHC_I (%)	-0.515	0.029	0.486	0.041
$MHC_{emb + I}$ (%)	-0.126	0.617	-0.076	0.766
$MHC_{emb + IIabx/IIa}$ (%)	-0.265	0.289	0.261	0.296
$MHC_{emb + I + IIabx/IIa}$ (%)	0.637	0.004	-0.482	0.043
MHC_I CSA (µm^2)	0.347	0.158	-0.161	0.522
$MHC_{emb + I}$ CSA (µm^2)	0.171	0.497	0.136	0.590
$MHC_{emb + IIabx/IIa}$ CSA (µm^2)	-0.243	0.332	0.319	0.197
$MHC_{emb + I + IIabx/IIa}$ CSA (µm^2)	-0.044	0.862	0.252	0.313

Significant correlations are shaded. n = 23 for all contractile properties; n = 18 for all MHC fibre characteristics. MV, mechanical ventilation; P_o, maximum specific force; P_t, twitch force; TTP, time to peak twitch force; ½ RT, half relaxation time; FI, fatigue index; CSA, cross-sectional area

Table 4-4. Linear regression analysis between the significantly correlated postnatal variables (duration of MV and protein intake) and diaphragm structural and functional properties of saline, low DEX and high DEX groups at 7 d PNA.

	Normalised MV (h)			Average protein intake(residual) (g/kg/d)		
	R^2	SEM	p	R^2	SEM	p
P_o ($N \cdot cm^{-2}$)	0.348	0.015	0.004			
P_t ($N \cdot cm^{-2}$)	0.190	0.009	0.043			
20 Hz ($N \cdot cm^{-2}$)	0.233	0.012	0.023			
30 Hz ($N \cdot cm^{-2}$)	0.287	0.013	0.010			
40 Hz ($N \cdot cm^{-2}$)	0.299	0.014	0.008			
60 Hz ($N \cdot cm^{-2}$)	0.340	0.015	0.004			
80 Hz ($N \cdot cm^{-2}$)	0.378	0.015	0.002			
MHC_I (%)	0.265	0.018	0.029	#	0.067	0.278
$MHC_{emb + I + IIabxIIa}$ (%)	0.406	0.038	0.004	#	0.146	0.452

Predictor variables are shaded, and # indicates variables were not selected as predictor variables in the multiple linear regression model. n = 23 for all contractile properties; n = 18 for all MHC fibre characteristics. MV, mechanical ventilation; P_o, maximum specific force; P_t, twitch specific force.

4.4.2 Experiment 2: Long-term effects of dexamethasone on postnatal diaphragm function

Although dexamethasone exposure had minimal short-term effects on preterm diaphragm function, high-dose dexamethasone exposure has long-term effects on organ development (Watterberg 2012). Therefore, it was important to investigate potential long-term adverse outcomes of postnatal dexamethasone exposure on diaphragm function.

Lamb descriptive data at post-mortem

Lamb post-conceptual age at post-mortem, body weight at post-mortem and optimal muscle length did not significantly differ between the term control group, SAL + CPAP, SAL + MV, DEX + CPAP and DEX + MV groups ($p > 0.05$, Table 4-5). As expected, lambs assigned to a mechanical ventilation group (SAL + MV or DEX + MV) experienced longer durations of mechanical ventilation, compared to lambs assigned to a continuous positive airway pressure group (SAL + CPAP or DEX + CPAP; $p < 0.05$).

Table 4-5. Lamb group descriptive data at 2 months post-term.

	Term control (n = 6)	**SAL + CPAP (n = 6)**	**SAL + MV (n = 7)**	**DEX + CPAP (n = 5)**	**DEX + MV (n = 5)**
PCA (d)	211 ± 5	210 ± 5	204 ± 4	208 ± 8	207 ± 3
Sex ratio (M:F)	2:4	1:5	2:5	2:3	2:3
Body wt (kg)	16.8 ± 3.2	18.4 ± 2.8	19.5 ± 2.1	20.6 ± 1.8	17.0 ± 4.0
MV (h)	N/A	39.8 ± 41.9[a]	100.1 ± 6.5[b]	25.6 ± 46.3[a]	111.2 ± 18.3[b]
L_o (mm)	62 (48 – 75)*	62 (53 – 68)*	68 (54 – 78)	66 (60 – 74)	68 (63 – 72)

PCA, postconceptional age; MV, mechanical ventilation; L_o, optimal muscle length. Values represent mean ± SD or median (min – max), except for sex ratio. *n = 5. Statistical differences in the duration of mechanical ventilation are indicated by different letters ($p < 0.05$).

The long-term effects of dexamethasone on diaphragm contractile properties

Diaphragm contractile properties of SAL+ CPAP, SAL + MV, DEX + CPAP and DEX + MV groups are presented in Figure 4-3. Dexamethasone had a significant main effect on diaphragm contractile properties. Dexamethasone significantly increased half relaxation time ($p = 0.049$) and significantly reduced the peak twitch force to maximum force ratio ($p = 0.020$). The assigned ventilation strategy (CPAP or MV) had no main effect on any measure of diaphragm contractile function ($p > 0.05$). Furthermore, there was no significant interaction effect between dexamethasone and assigned ventilation strategy on any measure of diaphragm contractile function ($p > 0.05$).

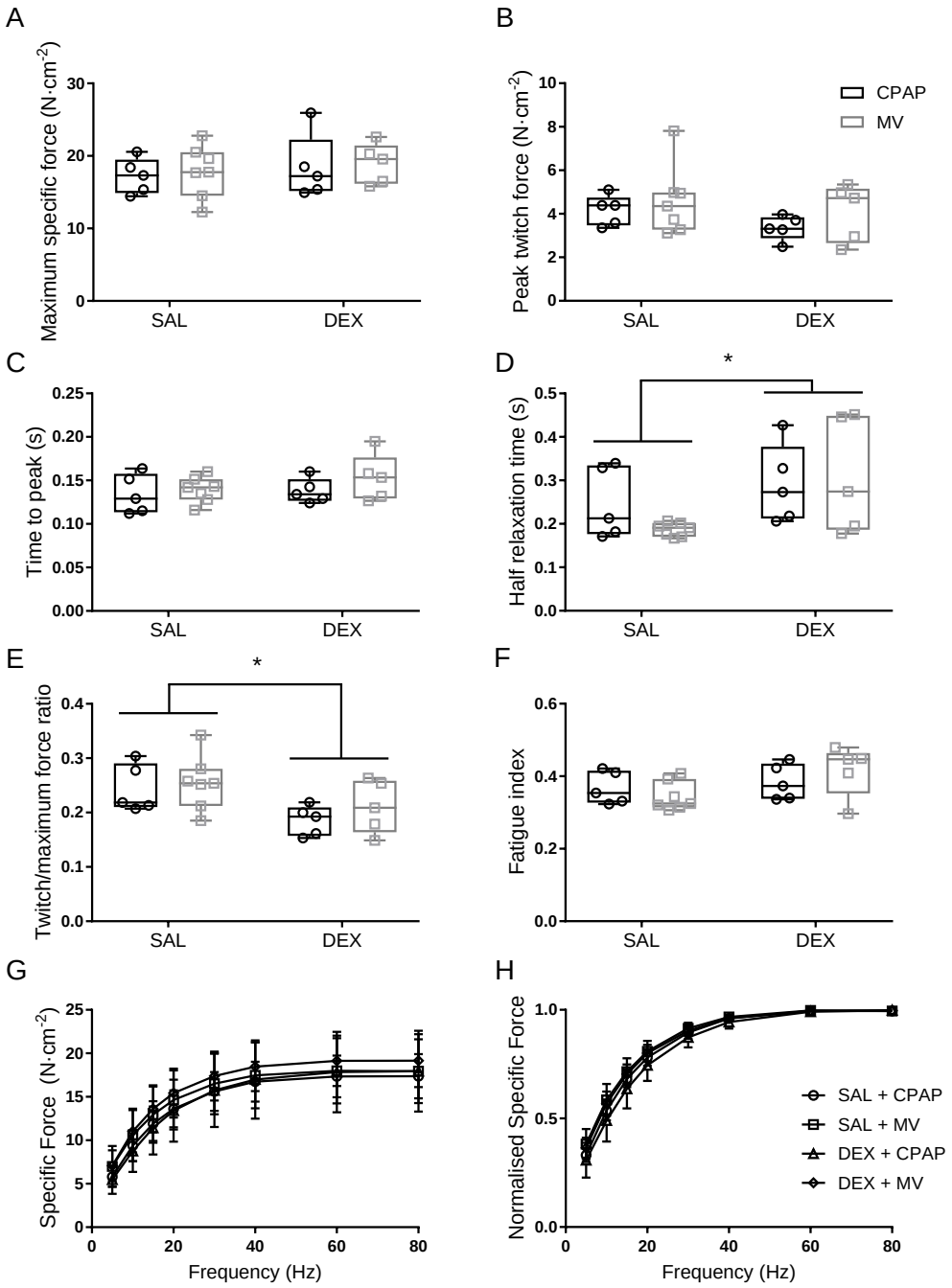


Figure 4-3. Diaphragm contractile properties of SAL + CPAP, SAL + MV, DEX + CPAP, and DEX + MV groups at 2 m post-term.

(A) maximum specific force (P_o); (B) peak twitch force (P_t); (C) time to peak (TTP); (D) half relaxation time (1/2 RT); (E) peak twitch force as a ratio of maximum force (P_t:P_o); (F) fatigue index (FI); (G) force-frequency relationship displayed as specific force ($N \cdot cm^{-2}$); and (H) force-frequency relationship normalised to maximum specific. Box and whisker plots represent median, minimum and maximum values, and all data points are visible. Force-frequency data are presented as mean ± SD. n = 5 for SAL + CPAP, DEX + CPAP, and DEX + MV groups, n = 7 for the SAL + MV group * significantly different to saline groups at $p < 0.05$.

The long-term effects of dexamethasone on myofibre CSA

Dexamethasone (p = 0.079) and assigned ventilation strategy (p = 0.349) had no main effect or interaction effect (p = 0.559) on myofibre CSA (Figure 4-4B).

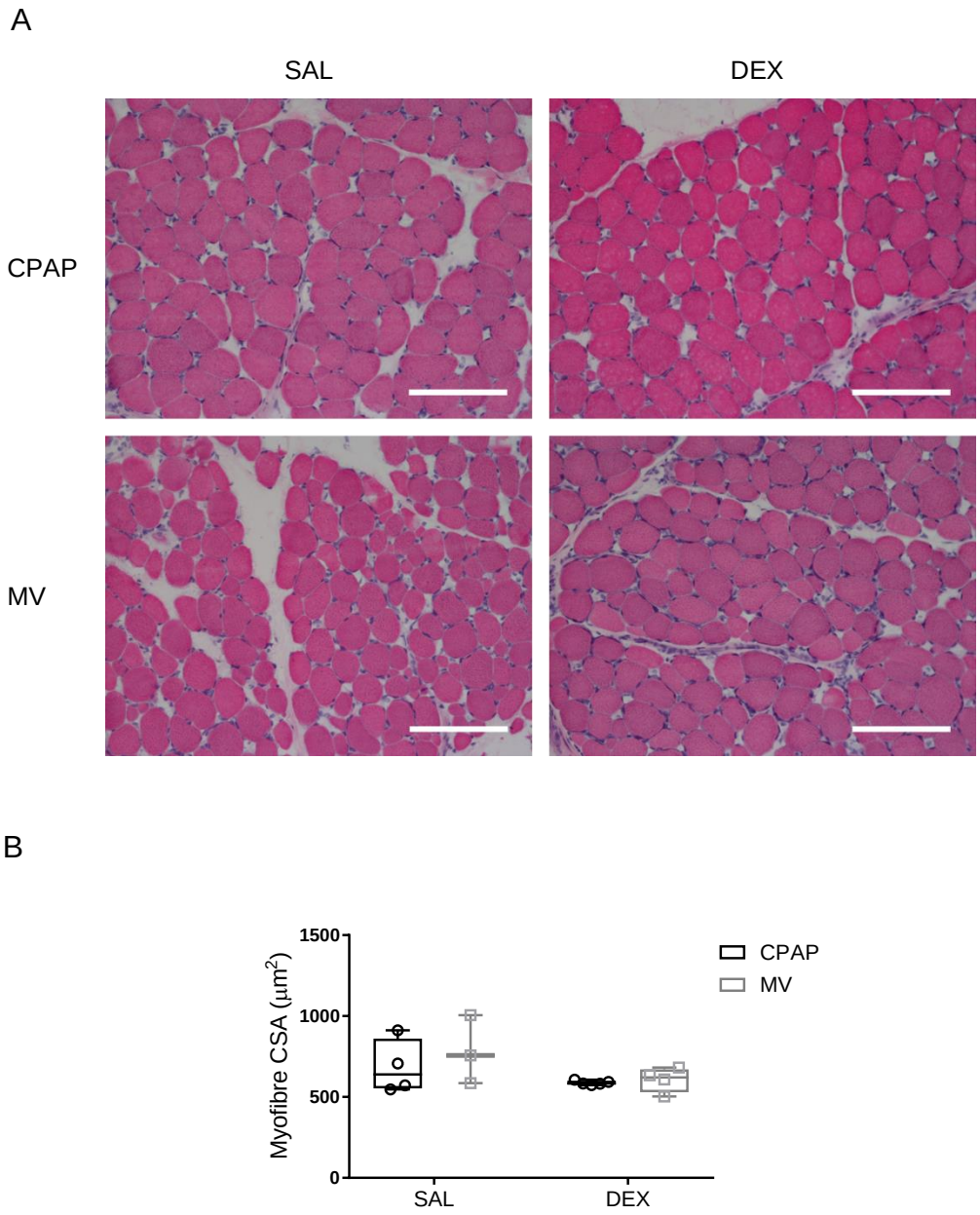


Figure 4-4. Diaphragm muscle fibre cross-sectional area (CSA) of SAL + CPAP, SAL + MV, DEX + CPAP and DEX + MV groups at 2 m post-term.

(A) Muscle fibre sections were stained with H&E. Scale bar represents 100 µm. (B) Myofibre size (measured by CSA) was quantified from H&E stained transverse sections of muscle. Box and whisker plots represent median, minimum and maximum values, and all data points are visible. n = 4 for SAL + CPAP and DEX + MV groups, n = 3 for the SAL + MV group, and n = 5 for the DEX + CPAP group.

Correlation between duration of MV and diaphragm contractile properties and myofibre CSA at 2 m post-term

The assigned ventilation strategy did not significantly affect diaphragm function or myofibre CSA at 2 m post-term, however, a number of lambs assigned to a CPAP group required extended ventilation, in accordance with the criteria outlined in 2.2.2. The duration of mechanical ventilation required by SAL + CPAP lambs ranged from 1 – 106 h, and the duration of mechanical ventilation required by DEX + CPAP lambs ranged from 2 – 108 h. Bivariate correlational analyses were performed to determine the relationship between outcome measures and the duration of mechanical ventilation, and found the duration of mechanical ventilation did not significantly correlate with diaphragm function or myofibre CSA at 2 m post-term (Table 4-6).

Table 4-6. Bivariate correlation between duration of MV and diaphragm function and myofibre CSA of the 2 m preterm groups.

	MV (h)	
	R	**p**
P_o ($N \cdot cm^{-2}$)	0.321	0.146
P_t ($N \cdot cm^{-2}$)	0.216	0.334
TTP (s)	0.172	0.443
½ RT (s)	-0.338	0.124
5 Hz ($N \cdot cm^{-2}$)	0.315	0.153
10 Hz ($N \cdot cm^{-2}$)	0.342	0.120
15 Hz ($N \cdot cm^{-2}$)	0.346	0.115
20 Hz ($N \cdot cm^{-2}$)	0.361	0.099
30 Hz ($N \cdot cm^{-2}$)	0.359	0.101
40 Hz ($N \cdot cm^{-2}$)	0.341	0.120
60 Hz ($N \cdot cm^{-2}$)	0.334	0.128
80 Hz ($N \cdot cm^{-2}$)	0.322	0.144
FI	-0.072	0.751
CSA (μm^2)	0.236	0.379

N = 22 for all contractile properties; N = 16 for myofibre CSA. MV, mechanical ventilation; P_o, maximum specific force; P_t, twitch force; TTP, time to peak twitch force; ½ RT, half relaxation time; FI, fatigue index; CSA, cross-sectional area.

The long-term effects of preterm birth on diaphragm function and myofibre CSA

The SAL + CPAP and SAL + MV groups were combined into a single saline group (n = 12) for comparison with the term control group, given mechanical ventilation had no significant effect on diaphragm function or structure at 2 m post-term. Diaphragm contractile properties did not significantly differ between term control and combined saline groups (Figure 4-5). Maximum specific force ($p = 0.526$), twitch force characteristics ($p > 0.05$), twitch:max force ratio ($p = 0.149$), raw force-frequency relationship ($p = 0.833$), normalised force-frequency relationship ($p = 0.626$), and fatigue index ($p = 0.437$) were not significantly different between the term control group and combined saline group. Furthermore, there was no significant difference in myofibre CSA between term control and saline groups ($p = 0.061$; Figure 4-6B).

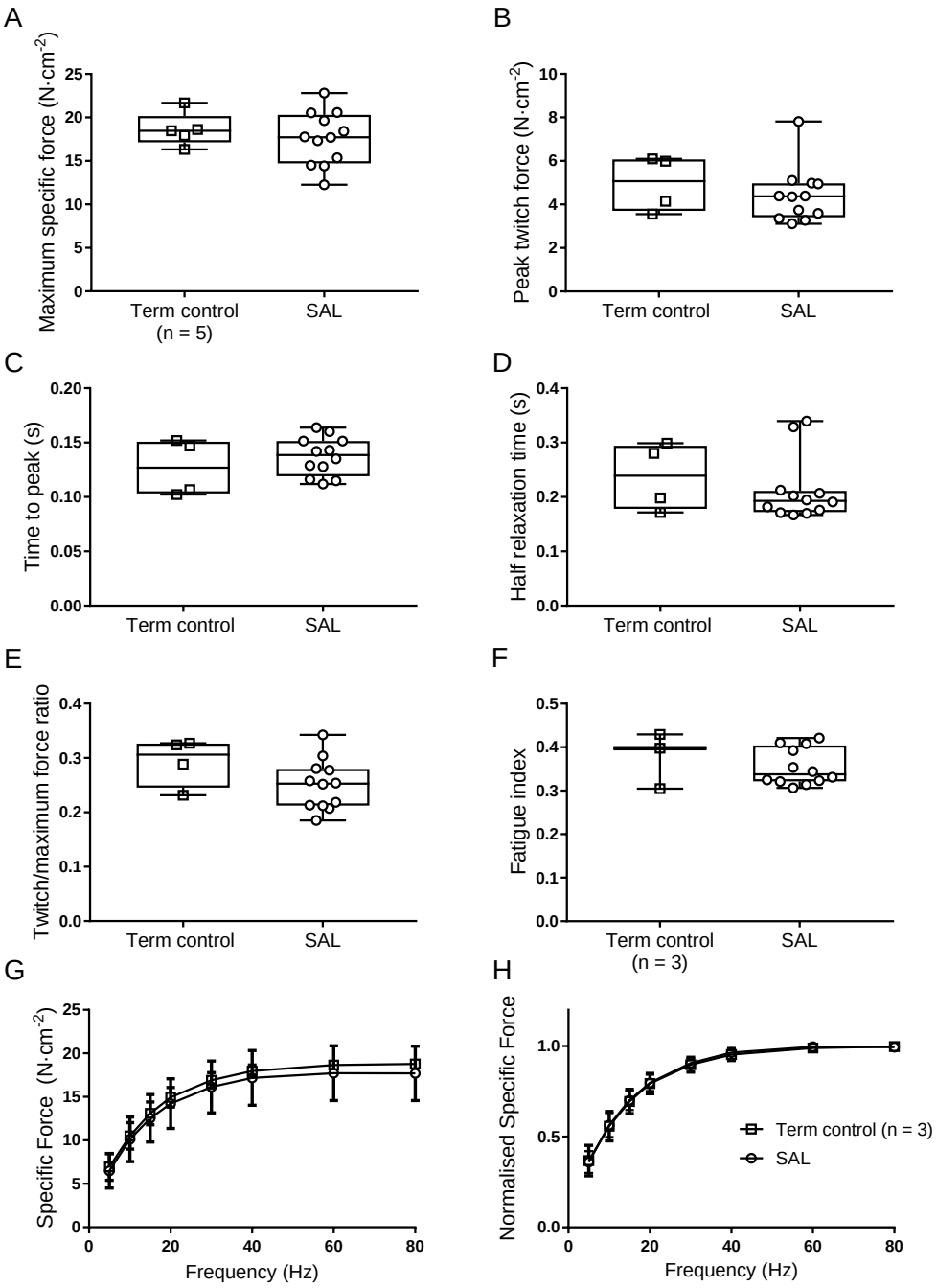
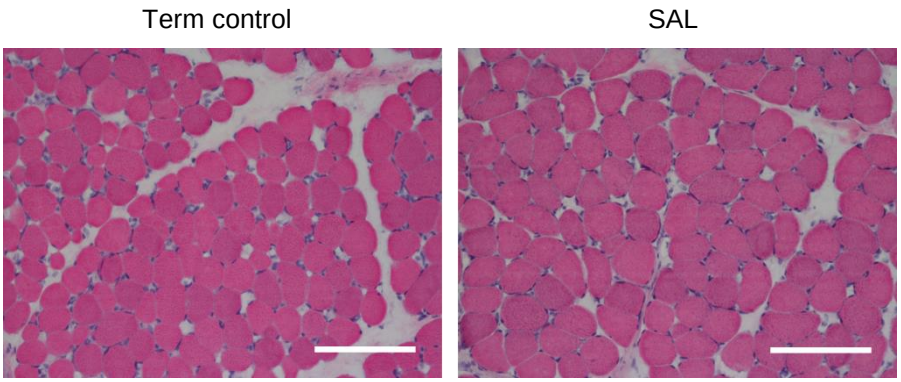


Figure 4-5. Diaphragm contractile properties of the term control group and combined saline group at 2 m post-term.

(A) maximum specific force (P_o); (B) peak twitch force (P_t); (C) time to peak (TTP); (D) half relaxation time (1/2 RT); (E) peak twitch force as a ratio of maximum force (P_t:P_o); (F) fatigue index (FI); (G) force-frequency relationship displayed as specific force ($N{\cdot}cm^{-2}$); and (H) force-frequency relationship normalised to maximum specific. Box and whisker plots represent median, minimum and maximum values, and all data points are visible. Force-frequency data are presented as mean ± SD. n = 4 for the term control group (unless otherwise stated), and n = 12 for the combined saline group.

A



B

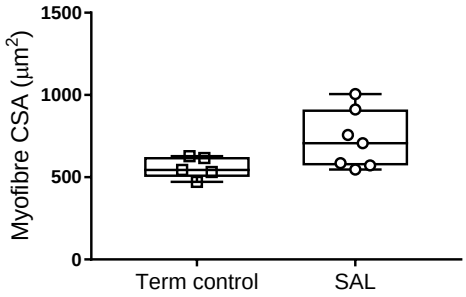


Figure 4-6. Diaphragm muscle fibre cross-sectional area (CSA) of term control and combined saline group at 2 m post-term.

(A) Muscle fibre sections were stained with H&E. Scale bar represents 100 µm. (B) Myofibre size (measured by CSA) was quantified from H & E stained transverse sections of muscle. Box and whisker plots represent median, minimum and maximum values, and all data points are visible. n = 5 for the term control group, n = 7 for the combined saline group.

4.5 Discussion

In this study, we investigated the short-term and long-term effects of postnatal dexamethasone exposure, and the long-term effects of preterm birth, on diaphragm structure and function in preterm lambs. Specifically, we investigated the effect of high and low-dose dexamethasone on preterm diaphragm structure and function at 7 d PNA; and we investigated the independent and combined effects of dexamethasone treatment and mechanical ventilation, on diaphragm structure and function at 2 m post-term. Contrary to our hypothesis, postnatal dexamethasone did not impair diaphragm structure or function in the short-term or long-term, and did not have long-term interactive effects with mechanical ventilation on diaphragm structure or function. The duration of mechanical ventilation was the most significant predictor of diaphragm function at 7 d PNA, however duration of mechanical ventilation had no significant effect on diaphragm function at 2 m post-term. Lastly, preterm birth had no significant effect on diaphragm function or myofibre CSA by 2 m post-term. Our data suggest that dexamethasone treatment does not impair diaphragm structure or function. Furthermore, our data suggest that the diaphragm dysfunction associated with mechanical ventilation during the acute postnatal period may be overcome with subsequent postnatal development, and that preterm birth has no long-lasting effect on diaphragm structure or function.

4.5.1 Short-term effects of dexamethasone on postnatal diaphragm function

Low-dose dexamethasone treatment had no effect on diaphragm function or MHC characteristics in the preterm lamb at 7 d PNA. Time to peak twitch force was significantly longer in the high-dose dexamethasone group, compared with the saline and low dexamethasone groups, however no other measures of diaphragm contractile function or MHC characteristics differed between the three treatment groups as 7d PNA. Our results do not support our hypothesis, that postnatal dexamethasone exposure would induce preterm diaphragm dysfunction in a dose-dependent manner.

The prolonged time to peak twitch force in the high dexamethasone group is consistent with findings in the adult rat diaphragm (Nava et al. 1996). Glucocorticoids have been reported to preferentially decrease the CSA of Type

II fibres and decrease the proportion of fibres expressing MHC_{IIb}, in a dose-dependent manner, in the diaphragm of adult rats and rabbits (Van Balkom et al. 1997; Lewis, Monn & Sieck 1992; Prezant et al. 1998; Sassoon et al. 2008). A reduction in the relative expression of MHC_{II}, and hence an increase in the relative expression of MHC_{I}, would explain the longer time to peak twitch force. However, we saw no significant difference in the distribution of fibre types or fibre CSA between dexamethasone treated and saline groups. In the absence of any notable changes in fibre type proportions, the mechanisms underlying the change in time to peak are unclear, but may result from alterations in SR Ca^{2+} handing (Shoji et al. 1976) and/or reduced myofibrillar Ca^{2+} sensitivity (Laszewski & Ruff 1985), consistent with previous reports. A prolonged time to peak can be associated with greater summation of forces at low stimulation frequencies. However, this was not reflected in our study, as the force-frequency relationship was not significantly different between high dexamethasone and saline diaphragm. Overall, the longer time to peak is unlikely to have any major clinical implications to preterm diaphragm function.

High-dose glucocorticoid treatment is consistently associated with diaphragm atrophy (Van Balkom et al. 1997; Dekhuijzen et al. 1995; Lewis, Monn & Sieck 1992; Nava et al. 1996; Prezant et al. 1998), and often associated with diaphragm weakness (Van Balkom et al. 1997; Sasson et al. 1991; Sassoon et al. 2008), in adult animal models. A previous study suggests that glucocorticoid treatment induces greater dysfunction of the developing diaphragm, compared to adult diaphragm of rats (Trang, Viires & Aubier 1992). It is unclear why our findings differ from the earlier study. However, it may result from differences in species and/or animal management. Previous studies used controlled experimental designs, while in this study, preterm lambs were managed in accordance with routine clinical practice. Respiratory support and altered nutritional intake may have masked differences between the saline, low-dose dexamethasone and high-dose dexamethasone groups by introducing an inherent variability. Additionally, it is possible that there were interactive effects between dexamethasone treatment and mechanical ventilation (Maes et al. 2010; Sassoon et al. 2011) on diaphragm function, as previously found in adult animal models.

Independent postnatal predictors of diaphragm contractile properties and MHC fibre characteristics

To compare the contractile properties and MHC fibre characteristics of the saline, low-dose dexamethasone, and high-dose dexamethasone lambs, while controlling for confounding experimental variables, bivariate correlation and regression analyses were conducted to identify the variables that significantly predicted measures of diaphragm contractile properties and MHC characteristics at 7 d PNA.

Collinearities between dexamethasone treatment and average protein intake

Dexamethasone treatment was collinear with average protein intake, with dexamethasone treatment associated with a reduced intake of protein; despite statistical comparison between the average protein intake by saline, low-dose dexamethasone, and high-dose dexamethasone lambs failing significance ($p = 0.071$). The association between dexamethasone treatment and protein intake results from the withholding of food from three lambs, two high dexamethasone and one low dexamethasone lamb, for extended periods of time. Feed was withheld when lambs were not tolerating enteral nutrition and presented with abdominal bloating.

Average protein intake

The unstandardised residual protein intake (protein intake independent of dexamethasone treatment) positively correlated with MHC_{I} and negatively correlated with $MHC_{emb + I + IIabx/IIa}$. However, the unstandardised residual protein intake did not independently predict the proportion of fibres expressing MHC_{I} and $MHC_{emb + I + IIabx/IIa}$. The P values for the correlation between protein intake and proportion of fibres expressing MHC_{I} and $MHC_{emb + I + IIa}$ fell just below the 0.05 level, and given the small sample size, it is not surprising that average protein intake fell below the F-to-remove threshold of the multivariate regression analyses. Sample size was originally planned for 8 lambs/group at study endpoint. However, the higher than expected mortality due to postnatal sepsis and gastrointestinal disease resulted in lower study group sizes. As discussed in Chapter 3, further supplementing our sample size isn't practical given limited

funding and the high costs of caring for each preterm lamb in a preclinical intensive care unit for 7 days.

Duration of mechanical ventilation

The duration of mechanical ventilation depended on the clinical requirements for each lamb of the saline and low-dose dexamethasone groups. As previously stated, four high-dose dexamethasone lambs were treated with the same graded de-escalation of respiratory support, while three high-dose dexamethasone lambs received 96 h of mandatory mechanical ventilation before weaning. Overall, the duration of mechanical ventilation which lambs received was highly variable and did not differ significantly between groups.

The duration of mechanical ventilation independently predicted diaphragm contractile strength and MHC characteristics at 7 d PNA. Increased duration of mechanical ventilation was associated with reduced specific force at stimulation frequencies between 20 – 80 Hz and with reduced twitch force. These results are consistent with the correlational results between duration of mechanical ventilation (residual) and diaphragm strength in Chapter 3, and are consistent with the effects of mechanical ventilation on contractile function in the developed diaphragm (Betters et al. 2004; Le Bourdelles et al. 1994; Corpeno et al. 2014; Powers et al. 2002; Whidden et al. 2009; Yang et al. 2002).

The duration of mechanical ventilation negatively correlated with the proportion of fibres expressing MHC_{I} and positively correlated with the proportion of fibres expressing $MHC_{emb + I + IIabx/IIa}$. This may indicate a delayed disappearance of developing MHC_{emb} isoform and hence delayed myofibre development with increased durations of mechanical ventilation, and impaired diaphragm excursions due to positive pressure ventilation.

4.5.2 Long-term effects of dexamethasone on postnatal diaphragm function

Dexamethasone treatment was associated with minor changes in diaphragm function at 2 m post-term. Dexamethasone increased the twitch half relaxation time and reduced the twitch to maximum force ratio, however there was no effect of dexamethasone on the overall strength of the 2 m diaphragm. Mechanical ventilation had no significant main effect on diaphragm function or

myofibre size, and had no significant interaction effect with dexamethasone on diaphragm function or myofibre size. These findings suggest that if any ventilation-induced diaphragm weakness was evident early in life, as was seen in the 7 d PNA group, it does not persist at 2 m post-term. Furthermore, diaphragm contractile function and myofibre size did not differ between term control and saline lambs, indicating that preterm birth does not have long-lasting effects on diaphragm function and structure. Our results do not support our hypothesis that preterm birth and postnatal dexamethasone exposure would induce long-lasting diaphragm dysfunction, nor do they support our hypothesis that dexamethasone-induced diaphragm dysfunction would be influenced by concurrent mechanical ventilation.

Dexamethasone increased half relaxation time of the 2 m post-term diaphragm, despite having no effect on half relaxation time at 7 d PNA. The longer half relaxation time in the sheep diaphragm at 2 m post-term is consistent with glucocorticoid use increasing half relaxation time in diaphragm of adult rats (Nava et al. 1996; Prezant et al. 1998). A longer half relaxation time may result from slower Ca^{2+} uptake by SERCA (Shoji et al. 1976) or a reduction of Type II fibre composition of the diaphragm (Van Balkom et al. 1997; Lewis, Monn & Sieck 1992; Prezant et al. 1998), as previously reported in adult animal models. While the longer half relaxation time and reduced twitch to maximum force ratio indicate that dexamethasone has long-term effects on the diaphragm, the changes are minimal and don’t affect overall diaphragm strength or the diaphragm force-frequency relationship.

The statistical comparison of myofibre CSA between the dexamethasone treated groups and the saline groups did not reach statistical significance ($p = 0.079$). However, there appeared to be a trend with a decrease in CSA with dexamethasone treatment, which could be consistent with glucocorticoid-induced atrophy reported in the adult diaphragm (Van Balkom et al. 1997; Dekhuijzen et al. 1995; Lewis, Monn & Sieck 1992; Nava et al. 1996; Prezant et al. 1998). Statistical comparison of myofibre CSA between the term control and the combined saline group also did not reach significance ($p = 0.061$), but indicates a trend increase in myofibre CSA of the preterm saline group,

compared to the term control group. Our statistical analyses may be underpowered by a low sample size (n = 3 – 7; Figure 4-4, Figure 4-6). and greater than anticipated variability in myofibre size at 2 m post-term. Further research is required to determine whether dexamethasone exposure and/or preterm birth have significant long-term effects on diaphragm myofibre size.

Overall, our data indicate that preterm birth and dexamethasone exposure have no major adverse long-term functional effects on the diaphragm. There was a trend of increased myofibre CSA with preterm birth and decrease myofibre CSA with dexamethasone treatment. However, given myofibre CSA of dexamethasone group was not significantly different to that of the term group, postnatal dexamethasone treatment is unlikely to impair diaphragm strength of preterm infants, compared to diaphragm function of term infants, in the long-term.

4.5.3 Study limitations

The limitations of this study are similar to the limitations of the study outlined in Chapter 3. We were unable to control for several postnatal exposures during our experiments, due to postnatal complications in lambs after preterm birth. The relationship between outcome variables and the postnatal exposures, average protein intake and mechanical ventilation, were investigated using correlational and regression analyses. We controlled for duration of mechanical ventilation during our analyses of the short-term study, as duration of mechanical ventilation independently predicted diaphragm structural and functional measures at 7 d PNA. However, we were unable to diagnose, and therefore control for, sepsis. As in Chapter 3, several lambs displayed symptoms consistent with postnatal infection (fever, vomiting, hypotension, neutropenia or neutrophilia), but could not be decisively diagnosed as routine blood cell cultures were not obtained. This is a limitation to this study, as the anti-inflammatory effects of postnatal dexamethasone are likely influenced by the inflammatory status of the lamb (Wolfe et al. 2013).

Another limitation to this study is that three lambs in the 7 d high dexamethasone group received 96 h of mandatory mechanical ventilation before de-escalation of respiratory support, while all other lambs were weaned

as soon as possible. Lambs which received 96 h of mandatory mechanical ventilation were initially part of a long-term study, but were switched to this short-term study as they were sick and unlikely to survive until 2 months post-term. Statistical analyses revealed no significant difference in the diaphragm contractile properties of the three lambs receiving mandatory ventilation and the four lambs whom were weaned from mechanical ventilation as soon as possible Appendix III), and therefore, these lambs were combined into a single high dexamethasone group. However, our statistical comparison was likely underpowered given our low sample size (n = 4 and n = 3). For instance, statistical differences in the maximum specific force of lambs receiving high dexamethasone and 96 h of mandatory ventilation (14.96 ± 2.57 $N \cdot cm^{-2}$) and those weaned from mechanical ventilation as soon as possible (19.10 ± 1.16 $N \cdot cm^{-2}$) may become apparent with a higher sample size. The high dexamethasone group was included in the analyses because of the following reasons: 1) adverse effects of dexamethasone treatment in the preterm infant, including protein catabolism, were reported in studies after the adoption of dexamethasone in the 1980's at high doses (0.5 – 1 mg/kg/d tapered over 6 weeks) (Tsai et al. 1996); and 2) many studies which investigated the effects of glucocorticoid treatment on the adult diaphragm, and the few studies which have investigated the effects of glucocorticoid treatment on the developing rodent diaphragm, used high doses of glucocorticoids, well above levels used in contemporary clinical practice (Song et al. 2014; Trang, Viires & Aubier 1992). Therefore, it was important that we include a high-dose dexamethasone group as an initial comparison to previous studies (Song et al. 2014; Trang, Viires & Aubier 1992), before investigating the effects of low-dose dexamethasone (0.75 mg/kg cumulative dose), similar to contemporary dosing protocols.

4.5.4 Conclusions

Contrary to our hypothesis, our data suggest that postnatal dexamethasone treatment does not adversely affect the functional or structural integrity of the preterm diaphragm. Postnatal dexamethasone, even at high doses, had no detrimental effect on preterm diaphragm function or structure at 7 d PNA. Postnatal dexamethasone had no adverse long-term effects on diaphragm function or structure at 2 m post-term, and did not have long-term interactive

effects with mechanical ventilation on diaphragm function. Instead, duration of mechanical ventilation was the most significant predictor of diaphragm strength in the preterm lamb at 7 d PNA. As dexamethasone is used to wean infants off mechanical ventilation, our study suggests that dexamethasone treatment could benefit preterm diaphragm function, given the duration of mechanical ventilation is negatively associated with diaphragm strength, and given dexamethasone treatment does not negatively impact diaphragm function or structure. Furthermore, our data suggests that that the diaphragm dysfunction associated with mechanical ventilation during the acute postnatal period may be overcome with subsequent postnatal development, and that preterm birth has no long-lasting effect on diaphragm structure or function.

Chapter 5

A rat model of early-life ventilation-induced diaphragm dysfunction

Preface

This study assesses the susceptibility of the developing diaphragm to contractile dysfunction from mechanical ventilation, using a newborn rat model.

5 A rat model of early-life ventilator-induced diaphragm dysfunction

5.1 Abstract

Mechanical ventilation is commonly used to manage preterm respiratory disease. Mechanical ventilation impairs diaphragm function in adults, a process referred to as ventilator-induced diaphragm dysfunction. However, the susceptibility of the preterm diaphragm to ventilator-induced dysfunction is unknown. Ventilator-induced diaphragm dysfunction may contribute to weaning difficulties of the preterm infant. We aimed to determine the susceptibility of the developing diaphragm to ventilator-induced dysfunction, using a newborn rat model. We hypothesised that the developing diaphragm would be more susceptible to ventilator-induced dysfunction, as a result of immature antioxidant defences, compared to the adult diaphragm.

Neonatal rats (10 – 14 d PNA; n = 9) and adult rats (n = 6) were mechanically ventilated with a high tidal volume, low peak end expiratory pressure, ventilation strategy for 4 – 12 h. Rats were killed and diaphragm contractile properties were measured *in vitro*, immediately after mechanical ventilation. Diaphragm contractile properties of the mechanically ventilated groups were compared to neonatal control rats (n = 11) and adult control rats (n = 8), who received no intervention before they were killed.

Mechanical ventilation significantly reduced maximum specific force and twitch force of the neonatal and adult rat diaphragm, and there was no interaction effect between mechanical ventilation and age. Mechanical ventilation also significantly reduced force produced by the adult diaphragm at all stimulation frequencies. Furthermore, the duration of mechanical ventilation negatively correlated with force produced by the adult diaphragm at all stimulation frequencies. However, the force-frequency relationship did not significantly differ between neonatal control and mechanical ventilation groups. The duration of mechanical ventilation negatively correlated with force produced by the neonatal diaphragm at high stimulation frequencies, but not low stimulation

frequencies, when controlling for age-dependent variability in diaphragm function between 10 – 14 d.

The force-frequency results indicate that the onset of ventilation-induced diaphragm dysfunction is not as severe in the neonatal rat compared to the adult rat. These results suggest that the developing diaphragm of the preterm infant may be less susceptible to ventilator-induced dysfunction, than the developed adult diaphragm. However, even a small reduction in strength may severely compromise the ability of the preterm diaphragm to support independent ventilation, given the diaphragm of the preterm infant is already weak due to immaturity.

5.2 Introduction

Mechanical ventilation is commonly used to facilitate respiration in preterm infants who are unable to breathe independently. It is becoming increasingly evident that antenatal and postnatal exposure to inflammation, glucocorticoids and undernutrition act to influence the respiratory capacity and requirement for mechanical ventilation of the preterm infant (Cummings, D'Eugenio & Gross 1989; Gantert et al. 2010; Lal et al. 2003; Van Marter et al. 2002; Thomas & Speer 2014; Wilson et al. 1991). In Chapter 3, fetal exposure to inflammation *in utero* was associated with an increased dependency of the preterm lamb on mechanical ventilation; this finding is consistent with the increased incidence of BPD in preterm infants after exposure to chorioamnionitis (Gantert et al. 2010; Thomas & Speer 2014). Approximately 87 % of extremely preterm infants require invasive respiratory support (Chow et al. 2017), despite the recent wider use of non-invasive modes of respiratory support. Clinicians attempt to limit the time preterm infants spend on mechanical ventilation as mechanical ventilation causes lung injury, which is a major risk factor for BPD (Morley et al. 2008). Furthermore, mechanical ventilation causes diaphragm weakness and wasting in adults, termed ventilator-induced diaphragm dysfunction (VIDD) (Vassilakopoulos & Petrof 2004). The immaturity of the preterm respiratory system, and subsequent lung injury induced by mechanical ventilation, makes weaning of the preterm infant from mechanical ventilation difficult. Diaphragm injury induced by mechanical ventilation, may also contribute to weaning difficulties in the preterm infant. In extreme cases, preterm infants can require respiratory support for up to several months (Chow et al. 2017).

Published studies on VIDD refer almost exclusively to the adult rat diaphragm and these findings were described in detail in Chapter 2. VIDD is rapid in onset, with just 6 h of mechanical ventilation reducing maximum diaphragm specific force and increasing proteolytic markers in the adult rat diaphragm (Le Bourdelles et al. 1994; Corpeno et al. 2014). Prolonged mechanical ventilation causes further deficits in force: maximum diaphragm specific force is reduced by 50 % after 48 h of mechanical ventilation in the rat (Le Bourdelles et al. 1994). Atrophy of the diaphragm during mechanical ventilation amplifies the diaphragm weakness caused by contractile dysfunction (Corpeno et al. 2014).

The combination of atrophy and intrinsic weakness reduces overall diaphragm function by 85 % after 9 -14 d of mechanical ventilation in the rat (Corpeno et al. 2014). Subsequently, impaired diaphragm function impedes weaning from mechanical ventilation (Barwing et al. 2013; Currie et al. 2011; Dres et al. 2012; Harikumar et al. 2009). Oxidative damage and oxidative stress-induced upregulation of proteolytic pathways after instituting mechanical ventilation have been proposed as key causes of contractile dysfunction and atrophy of the diaphragm, respectively (Kavazis et al. 2009; McClung et al. 2008; Powers, Kavazis & DeRuisseau 2005; Powers, Kavazis & McClung 2007).

Data on VIDD among preterm infants are scarce, despite preterm infants often requiring mechanical ventilation. The preterm diaphragm has an inadequate antioxidant protection and an increased susceptibility to oxidative stress (Maxwell et al. 1983; Song & Pillow 2012), which may increase the likelihood of VIDD and weaning failure. Extremely preterm infants are the group most prone to weaning failure, with up to 40 % failing extubation (Stefanescu et al. 2003). Additionally, a high diaphragm work-to-capacity ratio has been linked to extubation failure in infants and children (Currie et al. 2011; Harikumar et al. 2009). In the studies outlined in Chapter 3 and 4, duration of mechanical ventilation was the most significant predictor of diaphragm strength in the preterm lamb at 7 d PNA. Duration of mechanical ventilation accounted for 42 % of the explained variability in maximum specific force of the preterm lamb diaphragm, when all 7 d postnatal treatment groups from Chapter 3 and 4 are analysed together (Figure 5-1). The association between the duration of mechanical ventilation and diaphragm function is therefore of important consideration in the study of preterm respiratory failure.

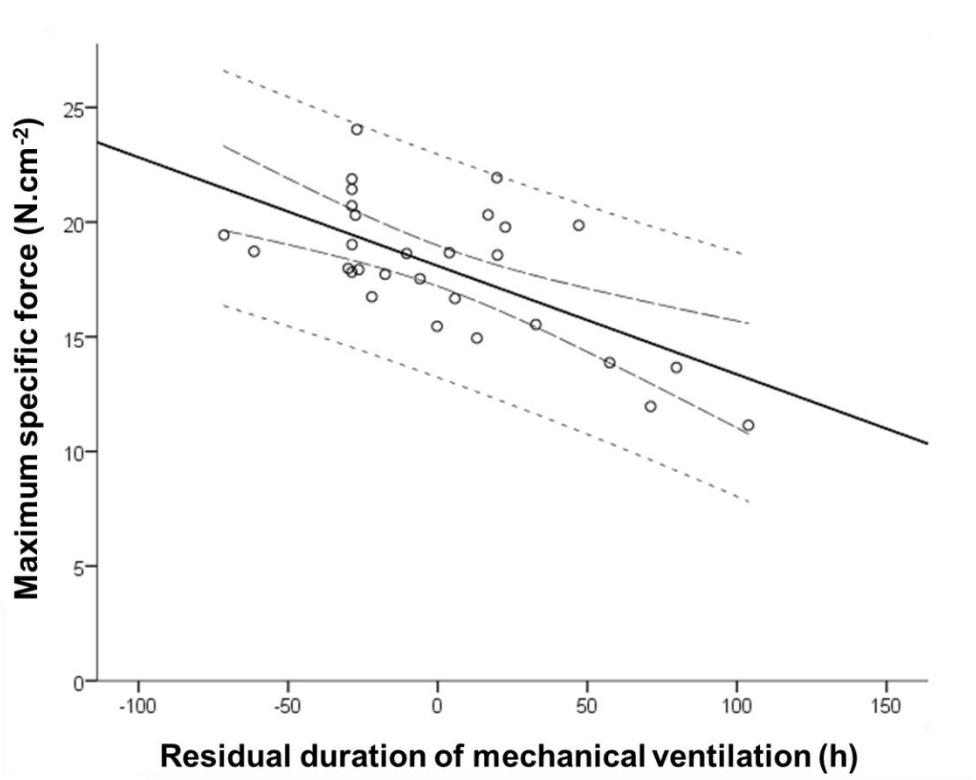


Figure 5-1. The relationship between maximum diaphragm specific force and duration of mechanical ventilation, in preterm lambs.

Duration of mechanical ventilation (residual) independently predicted diaphragm specific force ($F(1,27) = 19.591$, $p < 0.001$). Data are taken from Chapter 3 and 4 and includes saline, LPS, low DEX and high DEX groups ($n = 31$). Mechanical ventilation is presented as the unstandardised residual duration of mechanical ventilation, the component of mechanical ventilation not due to LPS exposure. The solid line represents the linear regression ($R^2 = 0.420$). The region between the two dashed lines and the region between the two dotted lines represent the 95% confidence interval of the mean and 95 % predictive interval, respectively.

The preterm lamb is used widely to model preterm respiratory disease, and has been specifically used to investigate the effects of *in utero* inflammation and antenatal glucocorticoids (Jobe et al. 1998; French et al. 1999; Kuypers et al. 2012a, 2012b; Song et al. 2013; Mahzabin et al. 2017). However, it is difficult to conduct a controlled experiment which tests medical interventions associated with mechanical ventilation in preterm lambs, given their susceptibility to adverse postnatal events, including feeding intolerance and infection. This study is designed to evaluate a rat model of early-life VIDD. The development of the respiratory system in the first few weeks of life in the rat is similar to respiratory

system development during the last gestational trimester in humans (Burri 1984; Gaultier, Matrot & Gallego 2006; Johnson et al. 1994; Powell & Whitney 1980), when preterm birth occurs and infants require respiratory support. Specifically, the increase in diaphragm strength and speed of contraction, the increase in diaphragm fibre size, and the development of the myofilaments and Ca^{2+} release system, which occurs postnatally in the rat is similar to that which occurs prenatally in humans and precocious animals (Johnson et al. 1994; LaFramboise et al. 1991; Lavin et al. 2013; Schiaffino & Reggiani 1994; Watchko, Daood & Sieck 1998). Loss of contractile function during mechanical ventilation precedes diaphragm atrophy, and is one of the first indications of VIDD, in adult rats (Corpeno et al. 2014). Hence, the aim of this study is to assess the susceptibility of the developing diaphragm to contractile dysfunction from mechanical ventilation, using a newborn rat model. As the developing diaphragm has reduced antioxidant defences (Maxwell et al. 1983; Song & Pillow 2012), we hypothesise the developing diaphragm will be more susceptible to VIDD than the adult diaphragm.

5.3 Methods

All experiments were approved by the Telethon Kids Institute Animal Ethics Committee (AEC #304) and notification provided to the UWA Animal Ethics Committee. Experiments were conducted in accordance with the guidelines of the National Health and Medical Research Council (NHMRC) Australian code for the care and use of animals for scientific purposes (2013).

5.3.1 Experimental outline

Neonatal female Piebald-Virol-Glaxo (PVG/c) rats (9 – 14 d old) were assigned randomly to: i) an unventilated control group (neonatal control; n = 11); or, ii) a mechanical ventilation group (neonatal MV; n = 9). Two groups of adult female PVG/c rats (8 - 12 w old) were used for comparison with the neonatal rats. Adult rats were assigned randomly to: i) an unventilated control group (adult control; n = 8); or ii) a mechanical ventilation group (adult MV; n = 6) in which adult rats received the same ventilation strategy as the neonatal mechanical ventilation group.

The neonatal and adult rats assigned to the control groups were killed without intervention with an overdose of ketamine/xylazine (IP injection). All mechanically ventilated rats were managed as described below.

5.3.2 Management of mechanically ventilated rats

Anaesthetised rats (Ketamine, 80 ug/g, and xylazine 13 ug/g; intraperitoneal injection) were tracheostomised, and ventilated using a computer-controlled closed-circuit ventilatory system (flexiVent®; Scireq). Neonatal and adult rats were mechanically ventilated with the following settings: respiratory rate 80 breaths/min, tidal volume of 20 mL/kg, peak expiratory end pressure of 2 cmH_2O and FiO_2 of 0.5.

Rat vital signs, including temperature (Digitron 2006T digital thermocouple inserted into the rectum), heart rate, SpO_2 (MouseOx pulse oximeter; STARR Life Sciences Corporation), colour and hydration, were monitored every 15 mins, and blood glucose and ketone levels (1-2 mm tail-tip amputation; Accu-Chek Performa; Roche Diabetes Care) were measured every 2-3 h to ensure rats were nutritionally stable throughout mechanical ventilation. Lung function was tested every 15 mins using the forced oscillation technique (Zosky et al. 2008).

Standard care throughout mechanical ventilation included expressing the bladder, passively moving the limbs, and keeping the site of tracheotomy lubricated. Supplemental heating was adjusted to target normothermia (37 ºC; Far infrared warming pads, CDT-20, Kent Scientific), and supplementary saline and glucose were administered to maintain hydration and plasma glucose concentration, throughout mechanical ventilation. Hydration was maintained in rats with 5 mL/h/kg saline IP, in accordance with Chiumello et al (1999). Anaesthesia was monitored, via heart rate and reflex withdrawal, and maintained with top-up doses of ketamine/xylazine, as required. To minimise the number of IP injections, top-up doses of anaesthetic were administered in combination with saline or in combination with saline and glucose, as appropriate.

A minor change in the experimental procedure to administer antibiotics to rats was made approximately half way through the study. Long-lasting antibiotics (penicillin G procaine, 300,000 U, IM) were administered to anaesthetised rats prior to the onset of mechanical ventilation to reduce the likelihood of rats developing infection during the experimental procedure (Sassoon et al. 2002).

Experiments were terminated with an overdose of ketamine/xylazine after varying study durations (between 4 – 12 h). The experimental set up is depicted in Figure 5-2.

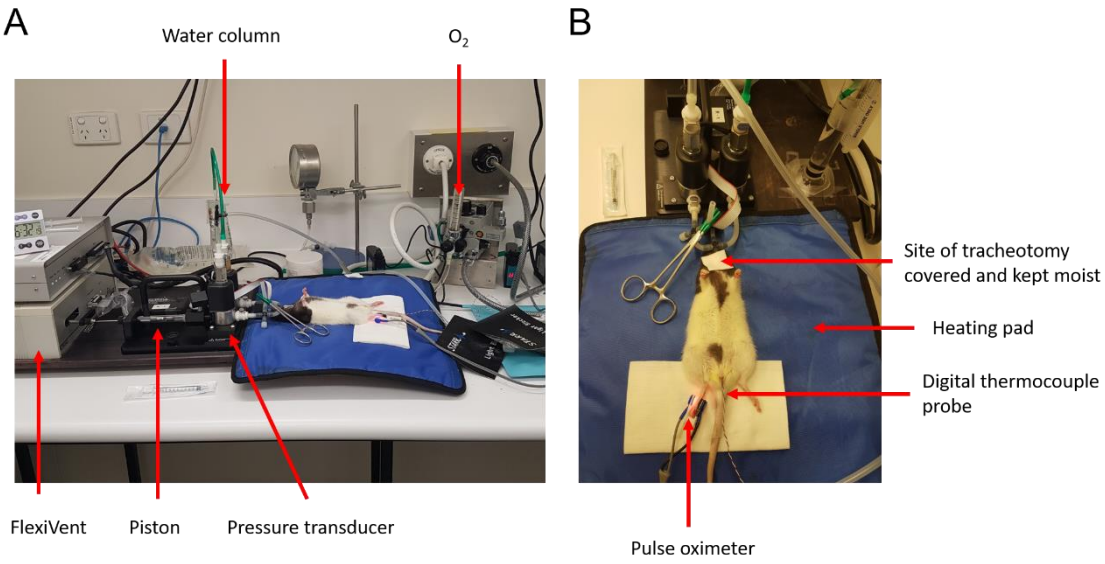


Figure 5-2. Experimental set up.

(A) Rats were anaesthetised, tracheostomised, and attached to a computer-controlled flexiVent ventilator system. (B) Rat SpO_2 and temperature was monitored via pulse oximeter and digital thermocouple probe. Basic care throughout ventilation included keeping the site of tracheotomy covered and moist at all times, and maintaining normothermia with a heating pad.

5.3.3 Diaphragm contractile properties

The diaphragm was excised immediately after the rats were killed. A longitudinal strip (2-3 mm wide) of intact muscle fibres were dissected from the right hemidiaphragm and mounted in an *in vitro* muscle test system (1200A, Aurora Scientific) containing mammalian Ringer's solution (pH 7.3) bubbled with carbogen at 25 ºC (Segal & Faulkner 1985). Diaphragm contractile properties were measured at optimal muscle length (L_o) in response to 0.2 ms

supramaximal square wave pulses (biphasic stimulator, 701B, Aurora Scientific). Measured contractile properties included maximum tetanic force (P_o), force-frequency relationship, twitch force (P_t), time to peak (TTP), half-relaxation time (1/2 RT), and fatigue index (FI). The force-frequency relationship was evaluated between 4 – 60 Hz in the neonatal diaphragm and between 5 – 100 Hz in the adult diaphragm. The susceptibility to fatigue was evaluated from a series of 900 tetanic contractions of 500 ms durations repeated once every 2 sec. The neonatal diaphragm was fatigued at 30 Hz and the adult diaphragm was fatigued at 60 Hz; the neonatal and adult diaphragm produced 95 – 100 % of maximum force at 30 Hz and 60 Hz, respectively. The fatigue index (FI) was determined from the ratio of the force produced during the 900^{th} contraction relative to the 1^{st} contraction of the fatigue protocol, in which a higher number indicates a greater fatigue resistance.

Force (g) was normalised to CSA calculated from muscle fibre length, muscle mass and density (1.056 $g \cdot cm^{-3}$) (Mendez & Keys 1960) and presented as specific force ($N \cdot cm^{-2}$). The detailed protocol is described in Appendix I.

5.3.4 Statistical analysis

Two-way interactions between age and mechanical ventilation on diaphragm contractile properties were analysed using two-way ANOVA with pairwise comparisons. Data with non-normal distribution were first log-transformed. However, it should be noted that the P_t:P_o ratio remained non-normally distributed after log transformation. The effect of mechanical ventilation on the force-frequency relationships of adult and neonatal diaphragm were analysed using two-way ANOVA. Independent sample t-tests were performed, as appropriate, where an interaction between both factors was detected with two-way ANOVA Greenhouse-Geisser.

The relationship between neonatal diaphragm functional properties and the experimental variables, duration of mechanical ventilation and age, were evaluated using Pearson's bivariate correlation. Partial correlations were also conducted between neonatal diaphragm contractile function and duration of mechanical ventilation, while controlling for age. Furthermore, the relationship between adult diaphragm functional properties and the duration of mechanical

ventilation were evaluated using Pearson's bivariate correlation. Diaphragm contractile properties that correlated significantly with the experimental variables were analysed with linear regression. Data are presented as mean ± SD or median, minimum and maximum values, as appropriate. Statistical significance was accepted at $p < 0.05$.

5.4 Results

5.4.1 Rat descriptive characteristics

Rat postnatal age at post-mortem, body weight at post-mortem and optimal muscle length for the neonatal control, neonatal mechanical ventilation, adult control and adult mechanical ventilation groups are also presented in Table 5-1. Age, body weight and optimal muscle length were significantly greater in the adult groups, compared to the neonatal groups ($p < 0.001$). There was no significant difference in age, body weight or optimal muscle length between the two neonatal groups or between the two adult groups ($p > 0.05$).

The duration of mechanical ventilation, blood glucose, and blood ketone levels for the neonatal mechanical ventilation and adult mechanical ventilation groups are presented in Table 5-1. There was no significant difference in the duration of ventilation ($p = 0.591$) or blood glucose levels ($p = 0.707$) between neonatal mechanical ventilation and adult mechanical ventilation groups.

Lung tissue mechanics were significantly altered by mechanical ventilation. Airway damping and airway elastance were significantly higher at the end of mechanical ventilation, compared to the start of mechanical ventilation ($p < 0.05$; data not shown). Airway damping increased significantly more during mechanical ventilation in the adult mechanical ventilation group compared to the neonatal mechanical ventilation group ($p = 0.019$; Table 5-1).

5.4.2 Diaphragm function

Maximum specific force and twitch characteristics

Maximum specific force and twitch characteristics for neonatal control, neonatal mechanical ventilation, adult control, and adult mechanical ventilation groups are presented in Figure 5-3. Age and mechanical ventilation had significant

main effects on diaphragm maximum specific force and twitch characteristics, as detailed below.

The adult diaphragm produced significantly higher P_o, compared to neonatal diaphragm ($p < 0.001$). P_t did not differ significantly between adult and neonatal diaphragm ($p = 0.758$). However, TTP ($p < 0.001$) and ½RT ($p = 0.004$) were significantly shorter in the adult diaphragm, compared to neonatal diaphragm. Furthermore, P_t:P_o was significantly lower in the adult diaphragm, compared to neonatal diaphragm ($p = 0.001$).

Diaphragm from mechanically ventilated rats produced significantly lower P_o ($p < 0.001$) and P_t ($p = 0.027$), compared to diaphragm from control rats. There was no significant difference in the speed of contraction (TTP and ½RT; $p > 0.05$) or P_t:P_o ($p = 0.721$) between mechanically ventilated and control diaphragm.

There was no statistically significant interaction effect between age and mechanical ventilation on maximum specific force or twitch characteristics ($p > 0.05$)

Table 5-1. Descriptive data of the neonatal control group, neonatal MV group, adult control group, and adult MV group at post-mortem.

	Neonatal control (n = 11)	Neonatal MV (n = 9)	Adult Control (n = 8)	Adult MV (n = 6)
Age (d)	11.6 (9 – 14)	11.6 (10 – 14)	82.3 (69 – 104)*#	80.2 (69 – 104)*#
Body wt (g)	23.0 (18 – 30)	23.1 (18 – 30)	152.5 (135 – 165)*#	154.3 (142 – 160)*#
MV (h)	–	7.7 ± 2.9	–	8.5 ± 2.4
R_{aw} (% of initial)	–	108.3 ± 17.0[a]	–	84.7 ± 56.8
G (% of initial)	–	113.0 ± 20.5[a]	–	147.0 ± 26.6#
H (% of initial)	–	145.6 ± 29.5[a]	–	121.7 ± 19.4
Glucose (mmol/L)	–	3.5 ± 1.8 (n = 7)	–	3.9 ± 2.4
Ketones (mmol/L)	–	12.1 ± 1.1[b]	–	1.4 ± 0.2[c]
L_o (mm)	6.4 (4.6 – 7.2)	6.2 (5.2 – 7.4)	16.9 (15 – 18)*#	16.9 (16 – 18)*#

MV, mechanical ventilation; R_{aw}, airway resistance; G, airway damping; H, airway elastance; L_o, optimal muscle length. Airway mechanics (R_{aw}, G, and H) are presented as the values at the end of MV as a percentage of values at the start of MV. Values represent mean ± SD or median (min – max). * and # statistically different from neonatal control and neonatal MV groups at $p < 0.05$, respectively. [a]n = 8; [b]n = 6; [c]n = 5.

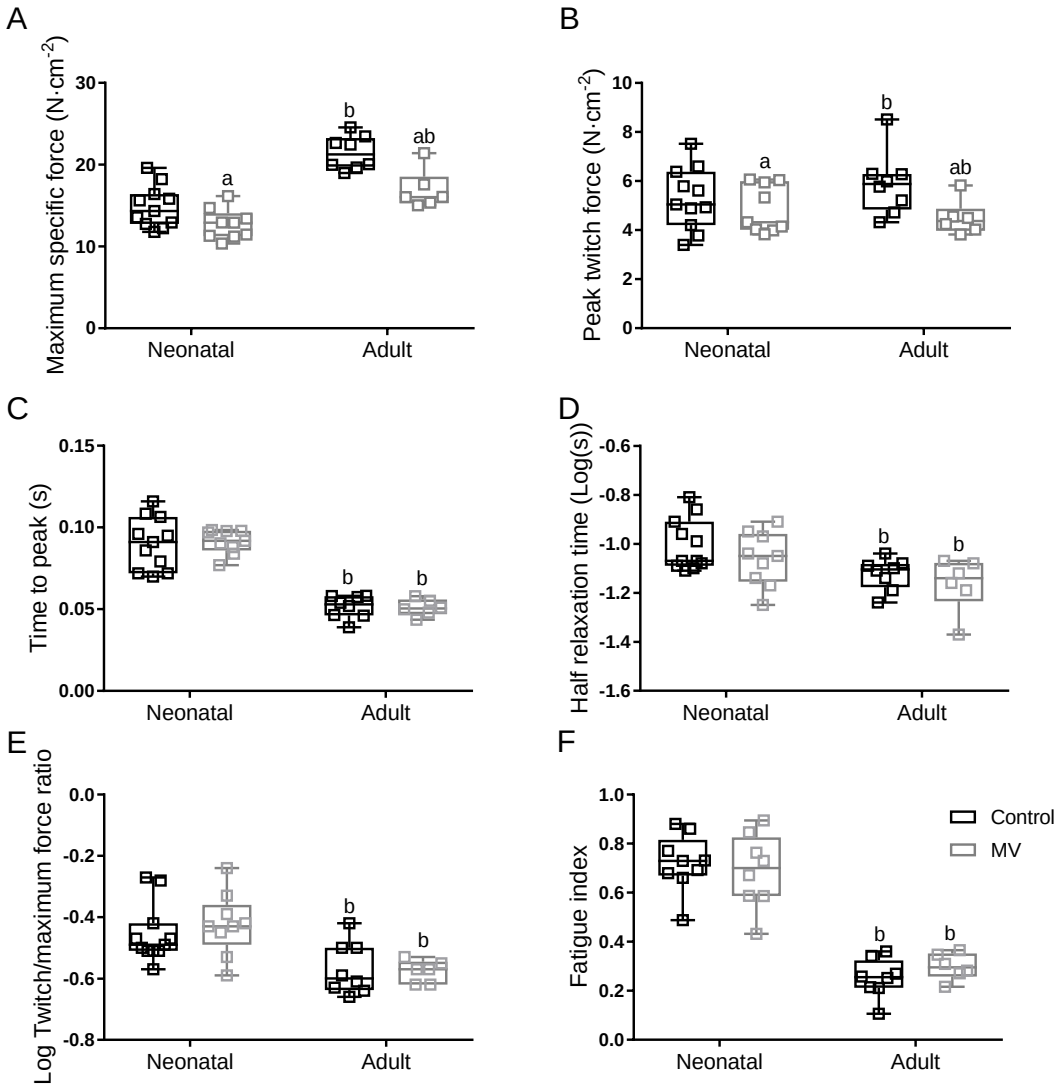


Figure 5-3. Diaphragm contractile properties of neonatal control, neonatal MV, adult control, and adult MV groups.

(A) maximum specific force (P_o); (B) peak twitch force (P_t); (C) time to peak (TTP); (D) half relaxation time (1/2 RT); (E) twitch force/maximum force ratio (P_t:P_o) and (F) fatigue index (FI). Box and whisker plots represent median, minimum and maximum values, and all data points are visible. n = 11 for the neonatal control group, n = 9 for the neonatal MV group, n = 8 for the adult control group, and n = 6 for the adult MV group. [a] indicates significantly different to control, [b] indicates significantly different to the neonate ($p < 0.05$).

Force-frequency relationships

The force-frequency relationships of the neonatal control and neonatal mechanical ventilation groups were investigated between 4 – 60 Hz (Figure 5-4A), and the force-frequency relationships of the adult control and adult mechanical ventilation groups were investigated between 5 – 100 Hz (Figure 5-4B). Mechanical ventilation had neither a significant main effect ($p = 0.258$) or interactive effect ($p = 0.152$) on the force-frequency relationship of the neonatal diaphragm. However, mechanical ventilation significantly reduced specific force produced by the adult diaphragm at all stimulation frequencies ($p < 0.05$).

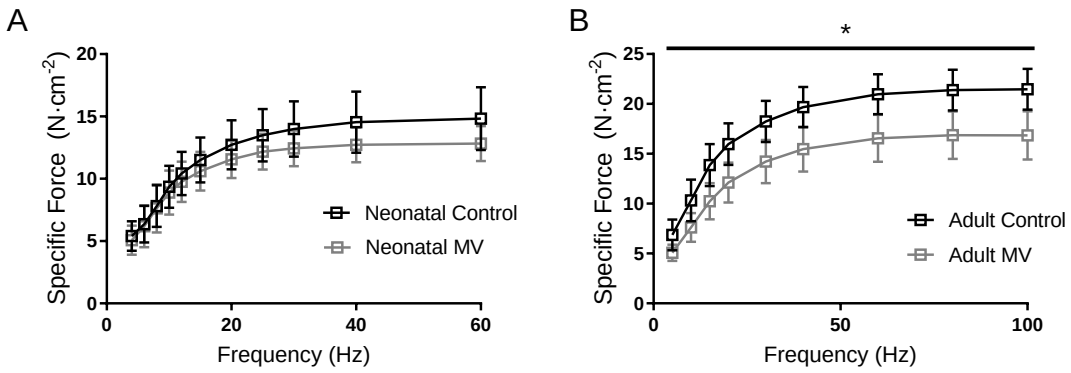


Figure 5-4. Force-frequency relationships of (A) neonatal control and neonatal MV groups, and (B) adult control and adult MV groups.

Values represent mean ± SD. n = 8 for the neonatal control group, n = 7 for the neonatal MV group, n = 8 for the adult control group, and n = 6 for the adult MV group. * significantly different between the adult control and adult MV groups ($p < 0.05$).

Susceptibility to fatigue

The fatigue index was significantly lower in adult diaphragm, compared to neonatal diaphragm ($p < 0.001$; Figure 5-3F), indicating increased fatigability. There was no statistically significant main effect of mechanical ventilation ($p = 0.566$) or interaction effect between age and mechanical ventilation ($p = 0.672$) on the fatigue index (Figure 5-3F).

Correlational analyses

Bivariate correlation and linear regression analyses were performed to determine the relationship between the duration of mechanical ventilation and

diaphragm contractile properties for neonatal and adult groups; as the duration of mechanical ventilation varied between 4 – 12 hours. Analyses were performed on neonatal and adult groups separately, given diaphragm function differed significantly between neonatal and adult groups (Figure 5-3). Furthermore, bivariate correlation and linear regression analyses were performed to determine the relationship between diaphragm contractile properties and the age of neonatal rats, as the diaphragm develops rapidly during the first two weeks of life in the rat (Johnson et al. 1994), and the age of neonatal rats varied between 10 – 14 d.

The duration of mechanical ventilation did not correlate significantly with neonatal diaphragm contractile function (Table 5-2), despite P_o and P_t being significantly lower in mechanically ventilated diaphragm compared to control diaphragm (Figure 5-3). However, age significantly predicted diaphragm strength (Table 5-2; Table 5-3). Age positively correlated with maximum specific force (r^2 = 0.352) and specific force produced at 10 – 40 Hz (r^2 ranged between 0.252 to 0.377) of neonatal rats. Partial correlational analyses between the duration of mechanical ventilation and diaphragm contractile properties were conducted with age as a covariate, given the correlation between diaphragm strength and age between 10 – 14 d PNA. The duration of mechanical ventilation correlated negatively with maximum specific force (r^2 = 0.242) and force produced at 60 Hz (r^2 = 0.274) in neonatal rats, when controlling for age (Table 5-2).

The duration of mechanical ventilation significantly predicted diaphragm strength in the adult rat (Table 5-4; Table 5-5). The duration of mechanical ventilation correlated negatively with force produced by the adult rat diaphragm at all stimulation frequencies: maximum specific force (r^2 = 0.648), twitch force (r^2 = 0.319), and force produced at 10 – 60 Hz (r^2 ranged from 0.384 to 0.635). Specifically, the duration of mechanical ventilation explained 64.8 % of the variability in maximum specific force of the adult diaphragm.

Table 5-2. Bivariate correlation between experimental variables (duration of MV and age) and diaphragm structural and functional properties of neonatal control and neonatal MV groups.

	MV (h)			Age (d)			MV (h) (controlling for age)		
	R	**p**	**n**	**R**	**p**	**n**	**R**	**p**	**n**
P_o ($N \cdot cm^{-2}$)	-0.287	0.221	20	0.593	0.006	20	-0.492	0.032	17
P_t ($N \cdot cm^{-2}$)	-0.175	0.460	20	0.419	0.066	20	-0.278	0.249	17
TTP (s)	0.060	0.801	20	-0.359	0.120	20	0.134	0.585	17
½ RT (s)	-0.146	0.540	20	-0.079	0.742	20	-0.134	0.584	17
10 Hz ($N \cdot cm^{-2}$)	-0.071	0.785	17	0.565	0.018	17	-0.210	0.436	14
15 Hz ($N \cdot cm^{-2}$)	-0.123	0.650	16	0.614	0.011	16	-0.296	0.284	13
20 Hz ($N \cdot cm^{-2}$)	-0.202	0.437	17	0.580	0.015	17	-0.378	0.149	14
30 Hz ($N \cdot cm^{-2}$)	-0.239	0.356	17	0.529	0.029	17	-0.396	0.128	14
40 Hz ($N \cdot cm^{-2}$)	-0.268	0.299	17	0.502	0.040	17	-0.417	0.108	14
60 Hz ($N \cdot cm^{-2}$)	-0.372	0.156	16	0.472	0.085	16	-0.523	0.045	13
FI	-0.097	0.703	18	-0.413	0.089	18	-0.027	0.917	15

Significant correlations are shaded. MV, mechanical ventilation; P_o, maximum specific force; P_t, twitch force; TTP, time to peak twitch force; ½ RT, half relaxation time; FI, fatigue index.

Table 5-3. Linear regression analysis between age and diaphragm structural and functional properties of neonatal control and neonatal MV groups.

	Age (d)			
	R^2	**SEM**	**p**	**n**
P_o ($N \cdot cm^{-2}$)	0.352	0.279	0.006	20
10 Hz ($N \cdot cm^{-2}$)	0.320	0.206	0.018	17
15 Hz ($N \cdot cm^{-2}$)	0.376	0.204	0.011	16
20 Hz ($N \cdot cm^{-2}$)	0.336	0.213	0.015	17
30 Hz ($N \cdot cm^{-2}$)	0.280	0.277	0.029	17
40 Hz ($N \cdot cm^{-2}$)	0.5252	0.308	0.040	17

Predictor variables are shaded. MV, mechanical ventilation; P_o, maximum specific force.

Table 5-4. Bivariate correlation between duration of MV and diaphragm structural and functional properties of adult control and adult MV groups.

	MV (h)		
	R	**p**	**n**
P_o ($N \cdot cm^{-2}$)	-0.805	0.001	14
P_t ($N \cdot cm^{-2}$)	-0.565	0.035	14
TTP (s)	0.028	0.924	14
½ RT (s)	-0.124	0.672	14
10 Hz ($N \cdot cm^{-2}$)	-0.620	0.018	14
15 Hz ($N \cdot cm^{-2}$)	-0.714	0.004	14
20 Hz ($N \cdot cm^{-2}$)	-0.739	0.003	14
30 Hz ($N \cdot cm^{-2}$)	-0.761	0.002	14
40 Hz ($N \cdot cm^{-2}$)	-0.780	0.001	14
60 Hz ($N \cdot cm^{-2}$)	-0.797	0.001	14
FI	0.393	0.164	14

Significant correlations are shaded. MV, mechanical ventilation; P_o, maximum specific force; P_t, twitch force; TTP, time to peak twitch force; ½ RT, half relaxation time; FI, fatigue index.

Table 5-5. Linear regression analysis between duration of MV and diaphragm structural and functional properties of adult control and adult MV groups.

	MV (h)		
	R^2	**SEM**	**p**
P_o ($N \cdot cm^{-2}$)	0.648	0.117	0.001
P_t ($N \cdot cm^{-2}$)	0.319	0.065	0.035
10 Hz ($N \cdot cm^{-2}$)	0.384	0.111	0.018
15 Hz ($N \cdot cm^{-2}$)	0.510	0.117	0.004
20 Hz ($N \cdot cm^{-2}$)	0.547	0.118	0.003
30 Hz ($N \cdot cm^{-2}$)	0.579	0.118	0.002
40 Hz ($N \cdot cm^{-2}$)	0.609	0.117	0.001
60 Hz ($N \cdot cm^{-2}$)	0.636	0.117	0.001

Predictor variables are shaded, and n = 14. MV, mechanical ventilation; P_o, maximum specific force.

5.5 Discussion

Ventilator-induced diaphragm dysfunction has been characterised extensively in the adult (Bernard et al. 2003; Betters et al. 2004; Corpeno et al. 2014; Kavazis et al. 2009; Petrof, Jaber & Matecki 2010; Powers, Kavazis & Levine 2009; Vassilakopoulos & Petrof 2004), but not the neonate. This study aimed to determine the susceptibility of the developing diaphragm to VIDD, using a newborn rat model, to aid in better understanding of ventilator dependency and weaning failure among preterm infants. We hypothesised that the neonatal diaphragm would be more susceptible to VIDD than the adult counterpart, given the neonatal diaphragm has immature antioxidant defences (Maxwell et al. 1983; Song & Pillow 2012). Contrary to our hypotheses, mechanical ventilation induced diaphragm dysfunction to a lesser extent in the neonatal rat, compared to the adult rat. Our data indicate that the onset of VIDD may not be as severe in the developing diaphragm of the preterm infant, compared to the developed adult diaphragm. However, a small reduction in strength may severely compromise the ability of the preterm diaphragm to support independent ventilation, given the diaphragm of the preterm infant is already weak due to immaturity.

Diaphragm contractile properties change significantly with development in the rat. The adult diaphragm produced significantly higher maximum specific force, contracted and relaxed faster, and was more susceptible to fatigue, than the neonatal diaphragm. Furthermore, development of the neonatal rat between 10 – 14 d correlated positively with diaphragm force production. The developmental increase in diaphragm strength, contractile speeds, and susceptibility to fatigue, are well characterised in the literature (Johnson et al. 1994; Lavin et al. 2013; Maxwell et al. 1983; Sieck, Fournier & Blanco 1991). The positive correlation between neonatal rat age and diaphragm strength is consistent with the rapid developmental changes that occur in the rat diaphragm during the first two weeks of life (Johnson et al. 1994; Schiaffino & Margreth 1969).

Mechanical ventilation induced diaphragm dysfunction of both adult and neonatal rats. Overall, mechanical ventilation reduced maximum specific force

by ~18 %. There was no interaction between mechanical ventilation and age on maximum specific force or twitch characteristics. However, mechanical ventilation did not significantly affect the force-frequency relationship of the neonatal diaphragm, despite reducing specific force produced by the adult diaphragm at all stimulation frequencies. The force-frequency results suggest that the neonatal diaphragm is less susceptible to ventilation-induced dysfunction, compared to the adult diaphragm.

The neonatal diaphragm may be less susceptible to VIDD due to a reduced susceptibility to eccentric contraction -induced injury during the ventilatory cycle. Although VIDD has primarily been attributed to diaphragm unloading, eccentric diaphragm contraction due to patient-ventilator desynchrony can occur during mechanical ventilation, and likely contributes to VIDD (Goligher 2016). Eccentric contractions can cause diaphragm injury, in the rat and dog (Gea et al. 2009; Watchko et al. 1994). However, *in vitro* experiments on isolated diaphragm fibres indicate that the developing muscle fibres are less susceptible to injury from eccentric contraction, than the adult rat diaphragm (Watchko et al. 1994), as evident by a less severe reduction in diaphragm strength and less ultrastructure damage (e.g. z-disk streaming) (Watchko et al. 1994). The mechanisms which underlie the reduced susceptibility of the developing rat diaphragm to stretch-induced damage from eccentric contractions are unclear (Watchko et al. 1994), but may result from immature formation of sarcomeres reducing the transmission of force in the developing muscle (Kontrogianni-Konstantopoulos et al. 2009).

The severity of VIDD may have been influenced by the extent of lung damage induced by mechanical ventilation (Bruells et al. 2013). Mechanical ventilation increased airway damping and airway elastance, indicative of lung damage in neonatal and adult rats (Al-Jamal & Ludwig 2001). The increase in airway damping was significantly greater in adult rats, compared to the neonatal rats. In comparison, the percentage increase in airway elastance tended to be higher in the neonatal rats (145 % increase in airway elastance), compared to the adult rats (121 % increase in airway elastance); although statistical analyses comparing the percentage increase in airway elastance in neonatal and adult

rats did not reach significance (p = 0.111). These results may indicate that the neonatal and adult lungs respond differently to the same ventilation strategy. As mechanical ventilation-induced lung injury is another mechanism that may contribute to VIDD, these findings may contribute to the increased severity of VIDD in adult rats.

Age-dependent variability in diaphragm contractile properties of the neonatal group may have influenced our statistical analyses. There was no significant direct correlation between duration of mechanical ventilation and diaphragm strength. However, there was a significant negative correlation between the duration of mechanical ventilation and specific force produced to high stimulation frequencies, when controlling for age in the neonatal group. Consequently, the differential effects of mechanical ventilation on the force-frequency relationship of the adult and neonatal diaphragm may result, in part, from the age-dependent variability of the neonatal diaphragm contractile properties. However, even when we control for postnatal age, mechanical ventilation has no significant effect on force produced by the neonatal diaphragm to submaximal stimulation frequencies. This is an important observation, given that the diaphragm usually contracts at submaximal frequencies during tidal breathing (De Troyer et al. 1997).Overall, our statistical analyses indicate that VIDD is less severe in the neonatal diaphragm, compared to the adult diaphragm.

5.5.1 Study limitations

We measured diaphragm contractile function to assess the susceptibility of the neonatal diaphragm to VIDD, as loss of contractile function during mechanical ventilation is one of the first indications of VIDD in adult rats (Corpeno et al. 2014). However, diaphragm atrophy will also contribute to diaphragm dysfunction during mechanical ventilation. Although our contractile data indicate that VIDD is less severe in the neonatal diaphragm, compared to the adult diaphragm, ventilation-induced atrophy of the developing diaphragm needs to be evaluated.

We aimed to induce diaphragm dysfunction by 12 h mechanical ventilation. However, experiments were stopped between 4 – 12 h, when rats became

clinically unstable. Experiments were stopped for the following reasons: 1) inability to obtain a SpO_2 measurement, indicating a drop in blood pressure; 2) hypoxemia; or 3) a significant change in lung mechanics, indicating poor lung function.

Blood glucose levels dropped, blood ketones rose, and occasionally rats appeared dehydrated, towards the end of mechanical ventilation, despite regular IP injections of glucose and saline. It is likely that glucose and saline were not absorbed when rats were hypotensive. Subsequent analyses confirmed that blood glucose and ketone levels did not correlate with diaphragm contractile function (data not shown). Venous cannulation would allow us to administer glucose and saline IV, and arterial cannulation would also allow us to monitor blood pressure. However, arterial cannulation is particularly challenging in the neonatal rats given their small size.

5.5.2 Conclusions

There are practical limitations that need to be overcome before using the newborn rat as a model of early-life VIDD. However, our results emphasise the importance of researching respiratory diseases using age-specific animal models. Mechanical ventilation induced dysfunction of the developing rat diaphragm to a lesser extent than the developed rat diaphragm. Despite the reduced susceptibility of the developing diaphragm to VIDD, small reductions in diaphragm strength induced by mechanical ventilation may have serious implications for the respiratory function of the preterm infant.

6 General Discussion

This research addresses the impact of three clinically common antenatal and postnatal exposures on preterm diaphragm function during early postnatal life. The role of the diaphragm in preterm respiratory disease is seldom considered, despite the major contribution of the diaphragm to sustaining ventilation. The diaphragm normally contributes ~70 – 80 % of the work of breathing (Reid & Dechman 1995). However, the low lung and high chest wall compliance of the preterm infant increases the workload placed on the preterm diaphragm. Furthermore, the preterm diaphragm has a reduced functional capacity, as a result of immature development. Importantly, the functional capacity of the immature preterm diaphragm may be compromised further by external influences during preterm birth and postnatal development. Given the preterm diaphragm must operate at high relative workload, and preterm diaphragm function is already compromised at birth, further dysfunction induced by these external insults will likely contribute to respiratory failure in early postnatal life. This thesis investigated the impact of *in utero* inflammation (Chapter 3), postnatal dexamethasone (Chapter 4) and mechanical ventilation (Chapter 5), on immature diaphragm function and structure in early postnatal life. The novel data generated from these studies are discussed below, and emphasise the importance of considering the preterm diaphragm when treating preterm respiratory diseases.

6.1 Study importance and novel findings

6.1.1 Chapter 3: Functional adaptations of the preterm lamb diaphragm during the first week of life are not influenced by in utero lipopolysaccharide exposure

The purpose of this study was to investigate the effects of *in utero* inflammation on postnatal diaphragm function and structure, using a preterm lamb model. This study shows, for the first time, that preterm diaphragm function changes markedly after 1 w of postnatal life, in the lamb. The diaphragm of preterm lambs that developed postnatally for 1 w, were significantly stronger and contracted faster than the diaphragm of lambs who developed antenatally to the

same PCA. The diaphragm contractile properties of preterm lambs, whom developed postnatally were typical of the diaphragm contractile properties reported in lambs of older PCA (Lavin et al. 2013). These results suggest that the preterm diaphragm undergoes accelerated functional development during early postnatal life. Accelerated functional development likely increases the capacity of the preterm diaphragm to cope with the high workload placed on it by the stiff lungs and compliant chest wall of the preterm infant. However, accelerated development of diaphragm function in our study, was not accompanied by an accelerated disappearance of developmental MHC expression or an increase in myofibre size, as one might expect. Further research is necessary to determine the mechanisms which underlie the alteration of preterm diaphragm function in early postnatal life. Given the importance of stretch and workload on muscle development and function (Cannata et al. 2011; Damm & Egli 2014; Louis et al. 2008; Vassilakopoulos & Petrof 2004), we hypothesise that the cyclic contraction and relaxation of the diaphragm, combined with high workload, are, at least in part, responsible for the rapid acceleration in preterm diaphragm development after birth.

The functional adaptations of the preterm lamb diaphragm during the first week of life were not influenced by prior exposure to *in utero* inflammation. LPS had no significant effect on diaphragm contractile function or MHC fibre characteristics at 7 d PNA, in the lamb. However, LPS treated lambs appeared neurologically inert and had an increased dependency on mechanical ventilation, which may indicate that *in utero* inflammation impacts negatively on the respiratory control system (Huxtable et al. 2011).

The duration of mechanical ventilation required by lambs was the most significant predictor of diaphragm strength at 7 d PNA. Lambs that required extended mechanical ventilation had weaker diaphragms. This relationship is consistent with prior findings that poor diaphragm function necessitates mechanical ventilation in adult respiratory disease (Budweiser, Jörres & Pfeifer 2008), and that ventilation induces diaphragm dysfunction in adults (Jaber et al. 2011; Levine et al. 2008). However, this is the first study to demonstrate the

negative relationship between mechanical ventilation and diaphragm function in the developing diaphragm.

The evidence that LPS exposure increased dependency of the lamb on mechanical ventilation, and that duration of mechanical ventilation was the most significant predictor of lamb diaphragm function, are particularly important. Histologic chorioamnionitis is associated with approximately 70 % of preterm births before 30 w (Allen et al. 2000), and over 87 % of infants born extremely prematurely require invasive respiratory support (Chow et al. 2017).

It is interesting that there were no significant differences in diaphragm function or structure between saline and LPS groups, despite LPS lambs requiring longer durations of mechanical ventilation. This finding suggests a complex interaction between *in utero* inflammation, preterm birth and mechanical ventilation. The combine effects of these complications have been investigated previously. Ebihara et al. (2002) demonstrated a protective effect of mechanical ventilation on rat diaphragm function in the presence of LPS-induced sepsis. The protective effect occurred despite an increase in markers of oxidative stress, suggesting a complex interaction between mechanical and oxidative stresses in the presence of sepsis. Using an *in vitro* cell culture model, the authors demonstrated a synergistic interaction in which mechanical stress applied in the presence of oxidative stress enhanced sarcolemmal injury (Ebihara et al. 2002). Thus, they propose that mechanical ventilation could prevent sarcolemmal injury by reducing the diaphragm myofiber mechanical stresses inherently associated with spontaneous breathing efforts. The potential protective effects of mechanical ventilation must be weighed against the likely development of ventilator-induced diaphragm dysfunction and the increased risk of CLD, including BPD, that are well established complications associated with prolonged mechanical ventilation.

6.1.2 Chapter 4: Postnatal dexamethasone treatment does not compromise diaphragm function of the preterm lamb

Mechanical ventilation damages the preterm lung (Attar & Donn 2002), and, as found in Chapter 3, is associated with a reduced strength of the preterm diaphragm. It is therefore important that preterm infants are weaned from mechanical ventilation as soon as possible. Dexamethasone accelerates lung maturation and facilitates weaning of the preterm infant dependent on mechanical ventilation. However, dexamethasone use remains a highly contentious practice since the identification of long-term adverse outcomes with high-dose dexamethasone treatment (Stark et al. 2001; Tsai et al. 1996; Yeh et al. 1997). This study aimed to identify short-term and long-term effects of dexamethasone on diaphragm function and structure. Given the short-term effects of preterm birth and mechanical ventilation on diaphragm function and structure reported in Chapter 3, this study also aimed to determine the long-term effects of preterm birth and mechanical ventilation on diaphragm function and structure.

Postnatal dexamethasone did not compromise preterm diaphragm function or structure in the short-term or long-term. The short-term effects of both low and high-doses of dexamethasone were investigated, and neither dose had any significant detrimental effect on preterm diaphragm function or MHC fibre characteristics. Our results suggest that the potential benefits of dexamethasone treatment on preterm lung function are not compromised by impaired diaphragm function. However, our results are inconsistent with a previous study which reported that high-dose dexamethasone induced dysfunction of the newborn rat diaphragm to a greater extent than the adult diaphragm (Trang, Viires & Aubier 1992). The cause of the discrepancy between these studies is unclear, but may result from differing experimental designs. The previous newborn rat study had a controlled experimental design, while in this study, preterm lambs were managed in accordance with routine clinical practice. Preterm lambs required varying durations of mechanical ventilation. Interactive effects between dexamethasone treatment and mechanical ventilation (Maes et al. 2010; Sassoon et al. 2011) on diaphragm function have been demonstrated in adult animal models, and may have

influenced the results of our study. High-dose dexamethasone administration protected against VIDD in the adult rabbit (Maes et al. 2010). High-dose dexamethasone administration protecting against VIDD in the lamb may explain why we saw no significant difference in the diaphragm function of saline control and high-dose dexamethasone groups.

Importantly, our data suggests that accelerated weaning from mechanical ventilation with dexamethasone will benefit diaphragm function in the preterm infant. As in Chapter 3, mechanical ventilation was the most significant predictor of preterm diaphragm function at 7 d PNA. Lambs that required extended mechanical ventilation had weaker diaphragms. The duration of mechanical ventilation explained 42 % of variability in maximum specific force of the preterm diaphragm at 1 w PNA, when all data from Chapter 3 and Chapter 4 are analysed together. Collectively, the negative correlation between mechanical ventilation and diaphragm strength, the fact that dexamethasone has no detrimental effects on diaphragm function, supports the use of dexamethasone to accelerate weaning of the preterm infants from mechanical ventilation.

This study also found that diaphragm dysfunction induced by preterm birth does not persist at 2 m post-term in the lamb. Furthermore, the negative correlation between duration of mechanical ventilation and diaphragm strength at 7 d PNA, are not evident at 2 m post-term in the lamb. These results indicate that diaphragm dysfunction induced by preterm birth, and associated with mechanical ventilation, during early postnatal life is reversible with subsequent development. Diaphragm dysfunction may contribute to respiratory disease during early postnatal life, but is unlikely to contribute to chronic respiratory diseases in childhood and adulthood, after preterm birth.

6.1.3 Chapter 5: A rat model of early-life ventilator-induced diaphragm dysfunction

The duration of mechanical ventilation was the most significant predictor of diaphragm function at 1 w PNA, in Chapter 3 and Chapter 4. However, a causal relationship between mechanical ventilation and diaphragm function could not be determined in the preterm lamb, because the lamb is unable to breathe

independently after preterm birth and is susceptible to postnatal complications. The purpose of Chapter 5 was to determine the susceptibility of the developing diaphragm to VIDD using a newborn rat model.

This rat study shows, for the first time, that VIDD appears to be is less severe in the neonatal rat compared to the adult rat. Mechanical ventilation, for 4 – 12 h, reduced diaphragm specific force of the adult diaphragm at all stimulation frequencies, but only reduced maximum specific force produced by the neonatal diaphragm. It is unclear as to why the neonatal diaphragm is less susceptible to VIDD, but we hypothesise the neonatal diaphragm is less susceptible to eccentric contraction-induced injury during mechanical ventilation, due to reduced force transmission of the immature sarcomere.

Our results emphasise the importance of researching respiratory diseases using age-specific animal models. The known effects of mechanical ventilation on the adult diaphragm cannot be extrapolated to the preterm diaphragm.

6.2 Challenges and limitations

This study highlights the challenges faced when using animal model of preterm birth that make it difficult to distinguish between multiple factors that may impact on respiratory muscle function. Lambs are a suitable model for studying preterm respiratory development, because lambs have long gestations and similar developmental trajectories to humans. The long gestation and the developmental trajectories of the preterm lamb were particularly important when studying the effects of *in utero* inflammation on diaphragm function, as the effects of *in utero* inflammation on diaphragm function are dependent on the time of exposure in relation to development (Karisnan et al. 2017). However, the preterm lamb, like the preterm infant, experiences postnatal complications. Lambs were subject to mechanical ventilation, feeding intolerance, and infection, which complicated the interpretation of our results and increased the variability in our data.

The variability in the data presented in Chapter 3 and Chapter 4 was higher than expected and likely limits our statistical power to detect significant differences and increases the risk of a type II error. Protein intake correlated

with, but did not predict, diaphragm function and MHC fibre characteristics in Chapter 3 and Chapter 4. It is possible that we would find protein intake to independently predict diaphragm function and/or MHC fibre characteristics in the preterm lamb if we had a larger final sample size. Final sample size was compromised by unexpected high initial mortality, and increasing the sample size further wasn't practical given costs associated with using a large animal model. We were also unable to identify sex specific effects of dexamethasone in Chapter 4, due to the small sample size. For instance, sex comparisons could not be made in the low-dose dexamethasone group which only had 2 females. Instead, sex was controlled for during ANCOVA. This is a limitation of the study outlined in Chapter 4, as dexamethasone induces greater diaphragm atrophy in the adult male rat, compared with the adult female rat (Prezant et al. 1997).

We used a newborn rat to model early-life VIDD in Chapter 5, because of the aforementioned complications associated with using the preterm lamb model. An additional benefit of using a rat model of early-life VIDD, is that VIDD is quicker in onset/progression in small animals with higher respiratory rates, compared with large animals with low respiratory rates (Anzueto et al. 1997; Radell et al. 2002; Sassoon et al. 2002). However, it was difficult to cannulate and deliver adequate saline and nutrients to newborn rat given their small size. An animal model needs to be optimised before further investigation into the underlying causes and the prevention early-life ventilator-induced diaphragm dysfunction can be conducted.

6.3 Conclusion and future directions

The findings presented in this thesis provide novel evidence on the effects of three clinically common exposures, *in utero* inflammation, mechanical ventilation and postnatal dexamethasone, on preterm diaphragm function during postnatal life, using preterm lamb and newborn rat models. These studies demonstrate the challenges when researching perinatal physiology in animal models with similar immature physiology to preterm infants.

In summary, we find diaphragm weakness induced by *in utero* inflammation at birth does not persist into early postnatal life, in the preterm lamb. Instead, major changes in diaphragm function after preterm birth indicate an accelerated

diaphragm development during early postnatal life. Mechanical ventilation likely influences postnatal diaphragm development, as it was the most significant predictor of diaphragm function in the preterm lamb at 1 w PNA, and significantly impaired diaphragm function in the newborn rat. Given that dexamethasone had no short-term or long-term detrimental effects on the preterm lamb diaphragm, our data indicate that accelerated weaning from mechanical ventilation with dexamethasone will benefit preterm diaphragm function. Furthermore, *in utero* LPS exposure was associated with an increased dependency of preterm lambs on mechanical ventilation in the intensive care unit, which emphasises the importance of investigating the combined effects of common clinical antenatal and postnatal exposures on respiratory development.

The influence of clinically relevant ventilation strategies on the preterm diaphragm needs to be investigated, given the vital role of the diaphragm in maintaining independent ventilation and our results showing mechanical ventilation was the most significant predictor of diaphragm function in the lamb at 7 d PNA. Ventilation parameters, including tidal volume and positive end expiratory pressure, may impact the onset/progression of ventilation-induced dysfunction of the developing diaphragm. Ventilation-induced diaphragm dysfunction likely contributes to ventilator-dependence and weaning difficulties among preterm infants. Thus, it is vital that management and treatment plans for respiratory failure among preterm infants aim to optimise, not only lung function, but diaphragm function as well.

7 References

Abramoff, MD, Magalhaes, PJ & Ram, SJ 2004, 'Image processing with ImageJ', *Biophotonics International*, vol. 11, no. 7, pp.36–42.

Al-Jamal, R & Ludwig, MS 2001, 'Changes in proteoglycans and lung tissue mechanics during excessive mechanical ventilation in rats', *American Journal of Physiology - Lung Cellular and Molecular Physiology.*, vol. 281, pp.L1078–L1087.

Aliverti, A, Cala, SJ, Duranti, R, Ferrigno, G, Kenyon, CM, Pedotti, A, Scano, G, Sliwinski, P, Macklem, PT & Yan, S 1997, 'Human respiratory muscle actions and control during exercise', *Journal of Applied Physiology*, vol. 83, no. 4, pp.1256–1269.

Allan, DW & Greer, JJ 1997, 'Embryogenesis of the phrenic nerve and diaphragm in the fetal rat', *Journal of Comparative Neurology*, vol. 382, no. 4, pp.459–468.

Allen, MC, Alexander, GR, Tompkins, ME & Hulsey, TC 2000, 'Racial differences in temporal changes in newborn viability and survival by gestational age', *Paediatric and Perinatal Epidemiology*, vol. 14, no. 2, pp.152–158.

American Thoracic Society 2003, 'Statement on the care of the child with chronic lung disease of infancy and childhood', *American Journal of Respiratory and Critical Care Medicine*, vol. 168, no. 3, pp.356–396.

Andrade, FH, Reid, MB, Allen, DG & Westerblad, H 1998, 'Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse', *Journal of Physiology*, vol. 509, no. 2, pp.565–575.

Anzueto, A, Peters, JI, Tobin, MJ, De Los Santos, R, Seidenfeld, JJ, Moore, G, Cox, WJ & Coalon, JJ 1997, 'Effects of prolonged controlled mechanical ventilation on diaphragmatic function in healthy adult baboons', *Critical Care Medicine*, vol. 25, no. 7, pp.1187–1190.

Arai, M, Otsu, K, MacLennan, DH & Periasamy, M 1992, 'Regulation of

sarcoplasmic reticulum gene expression during cardiac and skeletal muscle development', *American Journal of Physiology*, vol. 262, no. 3 Pt 1, pp.C614–C620.

Arora, NS & Rochester, DF 1982, 'Respiratory muscle strength and maximal voluntary ventilation in undernourished patients', *American Review of Respiratory Disease*, vol. 126, no. 1, pp.5–8.

ATS 2002, 'ATS/ERS Statement on Respiratory Muscle Testing.', *American Journal of Respiratory and Critical Care Medicine*, vol. 166, no. 4, pp.518–624. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12186831. [26 May 2014].

Attar, MA & Donn, SM 2002, 'Mechanisms of ventilator-induced lung injury in premature infants', *Seminars in Neonatology*, vol. 7, no. 5, pp.353–360.

Au, Y 2004, 'The muscle ultrastructure: a structural perspective of the sarcomere', *Cellular and Molecular Life Sciences*, vol. 61, no. 24, pp.3016–3033.

Avery, GB, Fletcher, AB, Kaplan, M & Brudno, DS 1985, 'Controlled trial of dexamethasone in respirator-dependent infants with bronchopulmonary dysplasia', *Pediatrics*, vol. 75, no. 1, pp.106–111.

Babiuk, RP, Thébaud, B & Greer, JJ 2004, 'Reductions in the incidence of nitrofen-induced diaphragmatic hernia by vitamin A and retinoic acid', *American Journal of Physiology. Lung Cellular and Molecular Physiology*, vol. 286, no. 5, pp.L970–L973.

Babiuk, RP, Zhang, W, Clugston, R, Allan, DW & Greer, JJ 2003, 'Embryological origins and development of the rat diaphragm', *Journal of Comparative Neurology*, vol. 455, no. 4, pp.477–487.

Bagust, J, Lewis, DM & Westernman, RA 1973, 'Polyneuronal innervation of kitten skeletal muscle', *Journal of Physiology*, vol. 229, no. 1, pp.241–255.

Van Balkom, RH, Zhan, WZ, Prakash, YS, Dekhuijzen, PN & Sieck, GC 1997, 'Corticosteroid effects on isotonic contractile properties of rat diaphragm

muscle', *Journal of Applied Physiology*, vol. 83, no. 4, pp.1062–1067.

Barbet, JP, Thornell, LE & Butler-Browne, GS 1991, 'Immunocytochemical characterisation of two generations of fibers during the development of the human quadriceps muscle', *Mechanisms of Development*, vol. 35, no. 1, pp.3–11.

Barwing, J, Pedroni, C, Olgemöller, U, Quintel, M & Moerer, O 2013, 'Electrical activity of the diaphragm (EAdi) as a monitoring parameter in difficult weaning from respirator: a pilot study', *Critical Care*, vol. 17, no. 4, p.R182.

Been, J V, Degraeuwe, PL, Kramer, BW & Zimmermann, LJI 2011, 'Antenatal steroids and neonatal outcome after chorioamnionitis: a meta-analysis', *BJOG : An International Journal of Obstetrics and Gynaecology*, vol. 118, no. 2, pp.113–122.

Behrman, RE & Butler, AS 2007, *Preterm Birth: Causes, Consequences and Prevention* , National Academies Press, Washington.

Bernard, N, Matecki, S, Py, G, Lopez, S, Mercier, J & Capdevila, X 2003, 'Effects of prolonged mechanical ventilation on respiratory muscle ultrastructure and mitochondrial respiration in rabbits', *Intensive Care Medicine*, vol. 29, no. 1, pp.111–118.

Berry, CA, Nitsos, I, Hillman, NH, Pillow, JJ, Polglase, GR, Kramer, BW, Kemp, MW, Newnham, JP, Jobe, AH & Kallapur, SG 2011, 'Interleukin-1 in lipopolysaccharide induced chorioamnionitis in the fetal sheep', *Reproductive Sciences*, vol. 18, no. 11, pp.1092–1102.

Betters, JL, Criswell, DS, Shanely, RA, Van Gammeren, D, Falk, D, DeRuisseau, KC, Deering, M, Yimlamai, T & Powers, SK 2004, 'Trolox attenuates mechanical ventilation-induced diaphragmatic dysfunction and proteolysis', *American Journal of Respiratory and Critical Care Medicine*, vol. 170, no. 11, pp.1179–1184.

Beurskens, LW, Tibboel, D, Lindemans, J, Duvekot, JJ, Cohen-Overbeek, TE, Veenma, DC, de Klein, A, Greer, JJ & Steegers-Theunissen, RP 2010,

‘Retinol status of newborn infants is associated with congenital diaphragmatic hernia’, *Pediatrics*, vol. 126, no. 4, pp.712–720.

Blackmon, LR, Bell, EF, Engle, WA, Kanto, WP, Martin, GI, Miller, CA, Rosenfeld, W, Speer, ME & Stark, AR 2002, ‘Postnatal corticosteroids to treat or prevent chronic lung disease in preterm infants’, *Pediatrics*, vol. 109, no. 2, pp.330–338.

Bland, RD 2005, ‘Neonatal chronic lung disease in the post-surfactant era’, *Biology of the Neonate*, vol. 88, no. 3, pp.181–191.

Blencowe, H, Cousens, S, Oestergaard, MZ, Chou, D, Moller, AB, Narwal, R, Adler, A, Garcia, C V, Rohde, S, Say, L & Lawn, JE 2012, ‘National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications’, *Lancet*, vol. 379, no. 9832, pp.2162–2172.

Bolt, RJ, van Weissenbruch, MM, Lafeber, HN & Delemarre-van de Waal, HA 2001, ‘Glucocorticoids and lung development in the fetus and preterm infant’, *Pediatric Pulmonology*, vol. 32, no. 1, pp.76–91.

Book, L, Herbst, J, Atherton, S & Jung, A 1975, ‘Necrotizing enterocolitis in low-birth-weight infants fed an elemental formula’, *Journal of Pediatrics*, vol. 87, no. 4, pp.602–605.

Bordoni, B & Zanier, E 2013, ‘Anatomic connections of the diaphragm: influences of respiration on the body system’, *Journal of Multidisciplinary Healthcare*, vol. 6, pp.281–291.

Le Bourdelles, G, Viires, N, Boczkowski, J, Seta, N, Pavlovic, D & Aubier, M 1994, ‘Effects of mechanical ventilation on diaphragmatic contractile properties in rats’, *American Journal of Respiratory and Critical Care Medicine*, vol. 149, no. 6, pp.1539–1544.

Brandl, CJ, DeLeon, S, Martin, DR & MacLennan, DH 1987, ‘Adult forms of the Ca2+-ATPase of sarcoplasmic reticulum’, *Journal of Biological Chemistry*, vol. 262, no. 8, pp.3768–3774.

Brown, LD & Hay, WW 2016, ‘Impact of placental insufficiency on fetal skeletal muscle growth’, *Molecular and Cellular Endocrinology*, vol. 435, pp.69–77.

Brown, MC, Jansen, JK & Van Essen, D 1976, ‘Polyneuronal innervation of skeletal muscle in new-born rats and its elimination during maturation’, *Journal of Physiology*, vol. 261, no. 2, pp.387–422.

Brozanski, BS, Daood, MJ, LaFramboise, WA, Watchko, JF, Foley, TP, Butler-Browne, GS, Whalen, RG, Guthrie, RD & Ontell, M 1991, ‘Effects of perinatal undernutrition on elimination of immature myosin isoforms in the rat diaphragm’, *American Journal of Physiology*, vol. 261, no. 2, pp.L49–L54.

Brozanski, BS, Watchko, JF, O’Day, TL & Guthrie, RD 1993, ‘Effect of undernutrition on contractile and fatigue properties of rat diaphragm during development’, *Journal of Applied Physiology*, vol. 74, no. 5, pp.2121–2126.

Bruells, CS, Smuder, AJ, Reiss, LK, Hudson, MB & Nelson, WB 2013, ‘Negative pressure ventilation and positive pressure ventilation promote comparable levels of ventilator-induced diaphragm dysfunction in rats’, *Anesthesiology*, vol. 119, pp.652–662.

Budweiser, S, Jörres, R & Pfeifer, M 2008, ‘Treatment of respiratory failure in COPD’, *International Journal of Chronic Obstructive Pulmonary Disease*, vol. 3, no. 4, pp.605–618.

Burke, RE 1981, ‘‘Motor units: anatomy, physiology, and functional organization’’ in *Handbook of Physiology. The Nervous System. Motor Control. Vol II* , pp.345–422.

Burke, RE, Levine, NE, Tsairis, P & Zajac, FE 1973, ‘Physiological types and histochemical profiles in motor units of the cat gastrocnemius’, *Journal of Physiology*, vol. 234, no. 3, pp.732–748.

Burri, PH 1984, ‘Fetal and postnatal development of the lung’, *Annual Review of Physiology*, vol. 46, pp.617–628.

Callahan, LA & Supinski, GS 2005, ‘Sepsis induces diaphragm electron

transport chain dysfunction and protein depletion', *American Journal of Respiratory and Critical Care Medicine*, vol. 172, no. 7, pp.861–868.

Cameron, WE, He, F, Kalipatnapu, P, Jodkowski, JS & Guthrie, RD 1991a, 'Morphometric analysis of phrenic motoneurons in the cat during postnatal development', *Journal of Comparative Neurology*, vol. 314, no. 4, pp.763–776.

Cameron, WE, Jodkowski, JS, Fang, H & Guthrie, RD 1991b, 'Electrophysiological properties of developing phrenic motoneurons in the cat', *Journal of Neurophysiology*, vol. 65, no. 3, pp.671–679.

Cannata, DJ, Crossley, KJ, Barclay, CJ, Walker, DW & West, JM 2011, 'Contribution of stretch to the change of activation properties of muscle fibers in the diaphragm at the transition from fetal to neonatal Life', *Frontiers in Physiology*, vol. 2, no. 109, pp.1–13.

Carrascal, L, Nieto-Gonzalez, JL, Cameron, WE, Torres, B & Nunez-Abades, P 2005, 'Changes during the postnatal development in physiological and anatomical characteristics of rat motoneurons studied in vitro', *Brain Research Reviews*, vol. 49, no. 2, pp.377–387.

Chambers, MA, Moylan, JS, Smith, JD, Goodyear, LJ & Reid, MB 2009, 'Stretch-stimulated glucose uptake in skeletal muscle is mediated by reactive oxygen species and p38 MAP-kinase', *Journal of Physiology*, vol. 587, no. 13, pp.3363–3373.

Cheong, JL, Anderson, P, Roberts, G, Duff, J & Doyle, LW 2013, 'Postnatal corticosteroids and neurodevelopmental outcomes in extremely low birthweight or extremely preterm infants: 15-year experience in Victoria, Australia', *Archives of Disease in Childhood. Fetal and Neonatal Edition*, vol. 98, no. 1, pp.F32–F36.

Chow, SSW, Le Marsney, R, Creighton, P, Kander, V, Haslam, R & Lui, K 2017, *Report of the Australian and New Zealand neonatal network (2015)*.

Clugston, RD & Greer, JJ 2007, 'Diaphragm development and congenital

diaphragmatic hernia', *Seminars in Pediatric Surgery*, vol. 16, no. 2, pp.94–100.

Collis, JL, Kelly, TD & Wiley, AM 1954, 'Anatomy of the crura of the diaphragm and the surgery of hiatus hernia', *Thorax*, vol. 9, no. 3, pp.175–189.

Cooke, RJ, Ainsworth, SB & Fenton, AC 2004, 'Postnatal growth retardation: a universal problem in preterm infants', *Archives of Disease in Childhood. Fetal and Neonatal Edition*, vol. 89, no. 5, pp.F428–F430.

Corpeno, R, Dworkin, B, Cacciani, N, Salah, H, Bergman, H-M, Ravara, B, Vitadello, M, Gorza, L, Gustafson, A-M, Hedström, Y, Petersson, J, Feng, H-Z, Jin, J-P, Iwamoto, H, Yagi, N, Artemenko, K, Bergquist, J & Larsson, L 2014, 'Time course analysis of mechanical ventilation-induced diaphragm contractile muscle dysfunction in the rat.', *Journal of Physiology*, vol. 592, pp.3859–3880.

Costeloe, KL, Hennessy, EM, Haider, S, Stacey, F, Marlow, N & Draper, ES 2012, 'Short term outcomes after extreme preterm birth in England : comparison of two birth cohorts in 1995 and 2006 (the EPICure studies)', *British Medical Journal*, vol. 345, pp.e7976–e7989.

Cummings, JJ, D'Eugenio, DB & Gross, SJ 1989, 'A controlled trial of dexamethasone in preterm infants at high risk for bronchopulmonary dysplasia', *New England Journal of Medicine*, vol. 320, no. 23, pp.1505–1510.

Currie, A, Patel, D-S, Rafferty, GF & Greenough, A 2011, 'Prediction of extubation outcome in infants using the tension time index', *Archives of Disease in Childhood. Fetal and Neonatal Edition*, vol. 96, no. 4, pp.F265–F269.

Damm, TB & Egli, M 2014, 'Calcium's role in mechanotransduction during muscle development', *Cellular Physiology and Biochemistry*, vol. 33, no. 2, pp.249–272.

Davis, DJ & Barrington, KJ 2005, 'Recommendations for neonatal surfactant

therapy', *Paediatrics and Child Health*, vol. 10, no. 2, pp.109–116.

Dawes, GS, Fox, HE, Leduc, BM, Liggins, GC & Richards, RT 1972, 'Respiratory movements and rapid eye movement sleep in the foetal lamb', *Journal of Physiology*, vol. 220, no. 1, pp.119–143.

Decramer, M & Stas, KJ 1992, 'Corticosteroid-induced myopathy involving respiratory muscles in patients with chronic obstructive pulmonary disease or asthma', *American Review of Respiratory Disease*, vol. 146, no. 3, pp.800–802.

Dekhuijzen, PNR & Decramer, M 1992, 'Steroid-induced myopathy and its significance to respiratory disease: a known disease rediscovered', *European Respiratory Journal*, vol. 5, no. 8, pp.997–1003.

Dekhuijzen, PNR, Gayan-Ramirez, G, Bisschop, A, De Bock, V, Dom, R & Decramer, M 1995, 'Corticosteroid treatment and nutritional deprivation cause a different pattern of atrophy in rat diaphragm', *Journal of Applied Physiology*, vol. 78, no. 2, pp.629–637.

Dias, CM, Pássaro, CP, Cagido, VR, Einicker-Lamas, M, Lowe, J, Negri, EM, Capelozzi, VL, Zin, WA & Rocco, PRM 2004, 'Effects of undernutrition on respiratory mechanics and lung parenchyma remodeling', *Journal of Applied Physiology*, vol. 97, no. 5, pp.1888–1896.

Dick, TE, Kong, FJ & Berger, AJ 1987, 'Correlation of recruitment order with axonal conduction velocity for supraspinally driven diaphragmatic motor units', *Journal of Neurophysiology*, vol. 57, no. 1, pp.245–259.

Dimitriou, G, Greenough, A, Moxham, J & Rafferty, GF 2003, 'Influence of maturation on infant diaphragm function assessed by magnetic stimulation of phrenic nerves', *Pediatric Pulmonology*, vol. 35, no. 1, pp.17–22.

Dimitriou, G, Greenough, A, Rafferty, GF & Moxham, J 2001, 'Effect of maturity on maximal transdiaphragmatic pressure in infants during crying', *American Journal of Respiratory and Critical Care Medicine*, vol. 164, no. 3, pp.433–436.

Downey, R 2011, ‘Anatomy of the normal diaphragm’, *Thoracic Surgery Clinics*, vol. 21, no. 2, pp.273–279.

Doyle, LW, Davis, PG, Morley, CJ, McPhee, A & Carlin, JB 2007, ‘Outcome at 2 years of age of infants from the DART study: a multicenter, international, randomized, controlled trial of low-dose dexamethasone’, *Pediatrics*, vol. 119, no. 4, pp.716–721.

Draeger, A, Weeds, AG & Fitzsimons, RB 1987, ‘Primary, secondary and tertiary myotubes in developing skeletal muscle: a new approach to the analysis of human myogenesis’, *Journal of the Neurological Sciences*, vol. 81, no. 1, pp.19–43.

Dres, M, Schmidt, M, Ferre, A, Mayaux, J, Similowski, T & Demoule, A 2012, ‘Diaphragm electromyographic activity as a predictor of weaning failure’, *Intensive Care Medicine*, vol. 38, no. 12, pp.2017–2025.

Dwyer, CM, Madgwick, AJA, Ward, SS & Stickland, NC 1995, ‘Effect of maternal undernutrition in early gestation on the development of fetal myofibres in the guinea-pig’, *Reproduction, Fertility and Development*, vol. 7, no. 5, pp.1285–1292.

Eber, E & Zach, MS 2001, ‘Long term sequelae of bronchopulmonary dysplasia (chronic lung disease of infancy)’, *Thorax*, vol. 56, no. 4, pp.317–323.

Ebihara, S, Hussain, SNA, Danialou, G, Cho, WK, Gottfried, SB & Petrof, BJ 2002, ‘Mechanical ventilation protects against diaphragm injury in sepsis: interaction of oxidative and mechanical stresses’, *American Journal of Respiratory and Critical Care Medicine*, vol. 165, no. 2, pp.221–228.

Endo, I, Inoue, D, Mitsui, T, Umaki, Y, Akaike, M, Yoshizawa, T, Kato, S & Matsumoto, T 2003, ‘Deletion of vitamin D receptor gene in mice results in abnormal skeletal muscle development with deregulated expression of myoregulatory transcription factors’, *Endocrinology*, vol. 144, no. 12, pp.5138–5144.

Engert, JC, Berglund, EB & Rosenthal, N 1996, ‘Proliferation precedes

differentiation in IGF-I-stimulated myogenesis', *Journal of Cell Biology*, vol. 135, no. 2, pp.431–440.

Esteban, A, Alia, I, Ibañez, J, Benito, S & Tobin, MJ 1994, 'Modes of mechanical ventilation and weaning: a national survey of spanish hospitals', *Chest*, vol. 106, no. 4, pp.1188–1193.

Fahey, AJ, Brameld, JM, Parr, T & Buttery, PJ 2005, 'The effect of maternal undernutrition before muscle differentiation on the muscle fiber development of the newborn lamb', *Journal of Animal Science*, vol. 83, no. 11, pp.2564–2571.

Farah, CS & Reinach, FC 1995, 'The troponin complex and regulation of muscle contraction', *FASEB Journal*, vol. 9, pp.755–767.

Ferguson, GT, Irvin, CG & Cherniack, RM 1990, 'Effect of corticosteroids on diaphragm function and biochemistry in the rabbit', *American Review of Respiratory Disease*, vol. 141, no. 1, pp.156–163.

Fleming, S, Thompson, M, Stevens, R, Heneghan, C, Plüddemann, A, Maconochie, I, Tarassenko, L & Mant, D 2011, 'Normal ranges of heart rate and respiratory rate in children from birth to 18 years of age: a systematic review of observational studies', *Lancet*, vol. 377, no. 9770, pp.1011–1018.

Fowden, AL, Giussani, DA & Forhead, AJ 2006, 'Intrauterine programming of physiological systems: causes and consequences', *Physiology*, vol. 21, no. 1, pp.29–37.

Frank, L & Roberts, RJ 1979, 'Effects of low-dose prenatal corticosteroid administration on the premature rat', *Biology of the Neonate*, vol. 36, no. 1–2, pp.1–9.

Frank, L & Sosenko, IR 1988, 'Undernutrition as a major contributing factor in the pathogenesis of bronchopulmonary dysplasia', *American Review of Respiratory Disease*, vol. 138, no. 3, pp.725–729.

Fratacci, MD, Levame, M, Rauss, A, Bousbaa, H & Atlan, G 1996, 'Rat diaphragm during postnatal development. I. Changes in distribution of

muscle fibre type and in oxidative potential', *Reproduction, Fertility and Development*, vol. 8, no. 3, pp.391–398.

French, NP, Hagan, R, Evans, SF, Godfrey, M & Newnham, JP 1999, 'Repeated antenatal corticosteroids: size at birth and subsequent development', *American Journal of Obstetrics and Gynecology*, vol. 180, no. 1, pp.114–121.

Fulton, BP & Walton, K 1986, 'Electrophysiological properties of neonatal rat motoneurones studied in vitro', *Journal of Physiology*, vol. 370, no. 1, pp.651–678.

Galińska-Rakoczy, A, Engel, P, Xu, C, Jung, H, Craig, R, Tobacman, LS & Lehman, W 2008, 'Structural basis for the regulation of muscle contraction by troponin and tropomyosin', *Journal of Molecular Biology*, vol. 379, no. 5, pp.929–935.

Gantert, M, Been, J V, Gavilanes, AWD, Garnier, Y, Zimmermann, LJI & Kramer, BW 2010, 'Chorioamnionitis: a multiorgan disease of the fetus?', *Journal of Perinatology*, vol. 30, pp.S21–S30.

Gaultier, C, Matrot, B & Gallego, J 2006, 'Transgenic models to study disorders of respiratory control in newborn mice', *Institute for Laboratory Animal Research*, vol. 47, no. 1, pp.15–21.

Gea, J, Zhu, E, Gáldiz, JB, Comtois, N, Salazkin, I, Fiz, JA & Grassino, A 2009, '[Functional consequences of eccentric contractions of the diaphragm]', *Archivos de Bronconeumologia*, vol. 45, no. 2, pp.68–74.

Geiger, PC, Cody, MJ, Macken, RL, Bayrd, ME, Fang, YH & Sieck, GC 2001, 'Plasticity in skeletal, cardiac and smooth muscle selected contributions: mechanisms underlying increased force generation by rat diaphragm muscle fibers during development', *Journal of Applied Physiology*, vol. 90, no. 1, pp.380–388.

Gerhardt, T & Bancalari, E 1980, 'Chestwall compliance in full-term and premature infants', *Acta Paediatrica Scandinavica*, vol. 69, no. 3, pp.359–

364.

Gilbert, WM & Danielsen, B 2003, ‘Pregnancy outcomes associated with intrauterine growth restriction’, *American Journal of Obstetrics and Gynecology*, vol. 188, no. 6, pp.1596–1601.

Girgis, CM, Clifton-Bligh, RJ, Mokbel, N, Cheng, K & Gunton, JE 2014, ‘Vitamin D signaling regulates proliferation, differentiation, and myotube size in C2C12 skeletal muscle cells’, *Endocrinology*, vol. 155, no. 2, pp.347–357.

Godfrey, KM & Barker, DJP 1995, ‘Maternal nutrition in relation to fetal and placental growth’, *European Journal of Obstetrics and Gynecology and Reproductive Biology*, vol. 61, no. 1, pp.15–22.

Goldenberg, RL, Culhane, JE, Iams, JD & Romero, R 2008, ‘Epidemiology and causes of preterm birth’, *Lancet*, vol. 371, no. 9606, pp.75–84.

Goldenberg, RL, Hauth, JC & Andrews, WW 2000, ‘Intrauterine infection and preterm delivery’, *New England Journal of Medicine*, vol. 342, no. 20, pp.1500–1507.

Goldspink, G & Ward, PS 1979, ‘Changes in rodent muscle fibre types during post-natal growth undernutrition and exercise’, *Journal of Physiology*, vol. 296, no. 1, pp.453–469.

Goligher, E 2016, *Diaphragm activity and function during mechanical ventilation*.

Grosu, HB, Lee, YI, Lee, J, Eden, E, Eikermann, M & Rose, KM 2012, ‘Diaphragm muscle thinning in patients who are mechanically ventilated’, *Chest*, vol. 142, no. 6, pp.1455–1460.

Gupta, S, Prasanth, K, Chen, CM & Yeh, TF 2012, ‘Postnatal corticosteroids for prevention and treatment of chronic lung disease in the preterm newborn’, *International Journal of Pediatrics*, vol. 315642.

Haddad, GG & Farber, JP (eds) 1991, *Developmental Neurobiology of Breathing* , CRC Press.

Halliday, HL, Ehrenkranz, RA & Doyle, LW 2003, 'Early postnatal (<96 hours) corticosteroids for preventing chronic lung disease in preterm infants', *Cochrane Database of Systematic Reviews*, vol. 1, no. CD001146.

Harding, R 1997, 'Fetal pulmonary development: the role of respiratory movements', *Equine Veterinary Journal*, vol. 29, no. S24, pp.32–39.

Harding, R & Hooper, SB 1996, 'Regulation of lung expansion and lung growth before birth', *Journal of Applied Physiology*, vol. 81, no. 1, pp.209–224.

Harikumar, G, Egberongbe, Y, Nadel, S, Wheatley, E, Moxham, J, Greenough, A & Rafferty, GF 2009, 'Tension-time index as a predictor of extubation outcome in ventilated children', *American Journal of Respiratory and Critical Care Medicine*, vol. 180, no. 10, pp.982–988.

Harris, AJ, Duxson, MJ, Fitzsimons, RB & Rieger, F 1989, 'Myonuclear birthdates distinguish the origins of primary and secondary myotubes in embryonic mammalian skeletal muscles', *Development*, vol. 107, no. 4, pp.771–784.

Harrison, AP, Rowlerson, AM & Dauncey, MJ 1996, 'Selective regulation of myofiber differentiation by energy status during postnatal development', *American Journal of Physiology*, vol. 270, no. 3, pp.R667–R674.

Heldt, GP & McIlroy, MB 1987, 'Distortion of chest wall and work of diaphragm in preterm infants', *Journal of Applied Physiology*, vol. 62, no. 1, pp.164–169.

Henneman, E 1957, 'Relation between size of neurons and their susceptibility to discharge', *Science*, vol. 126, no. 3287, pp.1345–1347.

Hermans, G, Agten, A, Testelmans, D, Decramer, M & Gayan-Ramirez, G 2010, 'Increased duration of mechanical ventilation is associated with decreased diaphragmatic force: a prospective observational study', *Critical Care*, vol. 14, no. 4, p.R127.

Herring, MJ, Putney, LF, Wyatt, G, Finkbeiner, WE & Hyde, DM 2014, 'Growth of alveoli during postnatal development in humans based on stereological

estimation', *American Journal of Physiology: Lung Cellular and Molecular Physiology*, vol. 307, no. 4, pp.L338–L344.

Hewitt, DP, Mark, PJ & Waddell, BJ 2006, 'Glucocorticoids prevent the normal increase in placental vascular endothelial growth factor expression and placental vascularity during late pregnancy in the rat', *Endocrinology*, vol. 147, no. 12, pp.5568–5574.

Horbar, JD, Badger, GJ, Carpenter, JH, Fanaroff, AA, Kilpatrick, S, LaCorte, M, Phibbs, R & Soll, RF 2002, 'Trends in mortality and morbidity for very low birth weight infants, 1991-1999', *Pediatrics*, vol. 110, no. 1, pp.143–151.

Horbar, JD, Ehrenkranz, RA, Badger, GJ, Edwards, EM, Morrow, KA, Soll, RF, Buzas, JS, Bertino, E, Gagliardi, L & Bellù, R 2015, 'Weight growth velocity and postnatal growth failure in infants 501 to 1500 grams: 2000 - 2013', *Pediatrics*, vol. 136, no. 1, pp.e84-92.

Hussain, SN, Simkus, G & Roussos, C 1985, 'Respiratory muscle fatigue: a cause of ventilatory failure in septic shock', *Journal of Applied Physiology*, vol. 58, no. 6, pp.2033–2040.

Huxtable, AG, Vinit, S, Windelborn, JA, Crader, SM, Guenther, CH, Watters, JJ & Mitchell, GS 2011, 'Systemic inflammation impairs respiratory chemoreflexes and plasticity', *Respiratory Physiology & Neurobiology*, vol. 178, no. 3, pp.482–489.

Isaacs, WB, Kim, IS, Struve, A & Fulton, AB 1992, 'Association of titin and myosin heavy chain in developing skeletal muscle', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 16, pp.7496–7500.

Iscoe, S, Dankoff, J, Migicovsky, R & Polosa, C 1976, 'Recruitment and discharge frequency of phrenic motoneurones during inspiration', *Respiration Physiology*, vol. 26, no. 1, pp.113–128.

Jaber, S, Petrof, BJ, Jung, B, Chanques, G, Berthet, JP, Rabuel, C, Bouyabrine, H, Courouble, P, Koechlin-Ramonatxo, C, Sebbane, M,

Similowski, T, Scheuermann, V, Mebazaa, A, Capdevila, X, Mornet, D, Mercier, J, Lacampagne, A, Philips, A & Matecki, S 2011, 'Rapidly progressive diaphragmatic weakness and injury during mechanical ventilation in humans', *American Journal of Respiratory and Critical Care Medicine*, vol. 183, no. 3, pp.364–371.

Jobe, AH, Wada, N, Berry, LM, Ikegami, M & Ervin, MG 1998, 'Single and repetitive maternal glucocorticoid exposures reduce fetal growth in sheep', *American Journal of Obstetrics and Gynecology*, vol. 178, no. 5, pp.880–885.

Johnson, BD, Wilson, LE, Zhan, WZ, Watchko, JF, Daood, MJ & Sieck, GC 1994, 'Contractile properties of the developing diaphragm correlate with myosin heavy chain phenotype', *Journal of Applied Physiology*, vol. 77, no. 1, pp.481–487.

Joshi, S & Kotecha, S 2007, 'Lung growth and development', *Early Human Development*, vol. 83, no. 12, pp.789–794.

Kallapur, SG, Jobe, AH, Ball, MK, Nitsos, I, Moss, TJM, Hillman, NH, Newnham, JP & Kramer, BW 2007, 'Pulmonary and systemic endotoxin tolerance in preterm fetal sheep exposed to chorioamnionitis', *Journal of Immunology*, vol. 179, no. 12, pp.8491–8499.

Karisnan, K, Bakker, AJ, Song, Y, Noble, PB, Pillow, JJ & Pinniger, GJ 2015a, 'Gestational age at initial exposure to in utero inflammation influences the extent of diaphragm dysfunction in preterm lambs', *Respirology*, vol. 20, no. 8, pp.1255–1262.

Karisnan, K, Bakker, AJ, Song, Y, Noble, PB, Pillow, JJ & Pinniger, GJ 2015b, 'Interleukin-1 receptor antagonist protects against lipopolysaccharide induced diaphragm weakness in preterm lambs', *PLoS ONE*, vol. 10, no. 4, p.e0124390.

Karisnan, K, Mahzabin, T, Bakker, AJ, Song, Y, Noble, PB, Pillow, JJ & Pinniger, GJ 2017, 'Gestational age at time of in utero lipopolysaccharide exposure influences the severity of inflammation-induced diaphragm

weakness in lambs', *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology.*

Kassim, Z, Greenough, A & Rafferty, GF 2009, 'Effect of caffeine on respiratory muscle strength and lung function in prematurely born, ventilated infants', *European Journal of Pediatrics*, vol. 168, no. 12, pp.1491–1495.

Kaufman, D & Fairchild, KD 2004, 'Clinical microbiology of bacterial and fungal sepsis in very-low-birth-weight infants', *Clinical Microbiology Reviews*, vol. 17, no. 3, pp.638–680.

Kavazis, AN, Talbert, EE, Smuder, AJ, Hudson, MB, Nelson, B & Powers, SK 2009, 'Mechanical ventilation induces diaphragmatic mitochondrial dysfunction and increased oxidant production', *Free Radical Biology & Medicine*, vol. 46, no. 6, pp.842–850.

Keens, TG, Brydan, AC, Levison, H & Ianuzzo, CD 1978, 'Developmental pattern of muscle fibre types in human ventilatory muscles', *Journal of Applied Physiology*, vol. 44, no. 6, pp.909–913.

Kim, YJ, Tamadon, A, Park, HT, Kim, H & Ku, S 2016, 'The role of sex steroid hormones in the pathophysiology and treatment of sarcopenia', *Osteoporosis and Sarcopenia*, vol. 2, no. 3, pp.140–155.

Knisely, AS, Leal, SM & Singer, DB 1988, 'Abnormalities of diaphragmatic muscle in neonates with ventilated lungs', *Journal of Pediatrics*, vol. 113, no. 6, pp.1074–1077.

Kontrogianni-Konstantopoulos, A, Ackermann, MA, Bowman, AL, Yap, S V & Bloch, RJ 2009, 'Muscle giants: molecular scaffolds in sarcomerogenesis', *Physiological Reviews*, vol. 89, no. 4, pp.1217–1267.

Korhonen, P, Tammela, O, Koivisto, A-M, Laippala, P & Ikonen, S 1999, 'Frequency and risk factors in bronchopulmonary dysplasia in a cohort of very low birth weight infants', *Early Human Development*, vol. 54, no. 3, pp.245–258.

Kosh, PC & Stark, AR 1984, 'Dynamic maintenance of end-expiratory lung

volume in full-term infants', *Applied Journal of Physiology: Respiratory, Environmental and Exercise Physiology*, vol. 57, no. 4, pp.1126–1133.

Kota, SK, Gayatri, K, Jammula, S, Kota, SK, Krishna, SVS, Meher, LK & Modi, KD 2013, 'Endocrinology of parturition', *Indian Journal of Endocrinology and Metabolism*, vol. 17, no. 1, pp.50–59.

Kraaijenga, J V., Hutten, GJ, de Jongh, FH & van Kaam, AH 2015, 'The effect of caffeine on diaphragmatic activity and tidal volume in preterm infants', *Journal of Pediatrics*, vol. 167, no. 1, pp.70–75.

Kraaijenga, J V, de Waal, CG, Hutten, GJ, de Jongh, FH & van Kaam, AH 2017, 'Diaphragmatic activity during weaning from respiratory support in preterm infants', *Archives of Disease in Childhood. Fetal and Neonatal Edition*, vol. 102, no. 4, pp.F307–F311.

Kramer, BW, Joshi, SN, Moss, TJM, Newnham, JP, Sindelar, R, Jobe, AH & Kallapur, SG 2007, 'Endotoxin-induced maturation of monocytes in preterm fetal sheep lung', *American Journal of Physiology. Lung Cellular and Molecular Physiology*, vol. 293, no. 2, pp.L345–L353.

Kramer, BW, Kallapur, S, Newnham, J & Jobe, AH 2009, 'Prenatal inflammation and lung development', *Seminars in Fetal and Neonatal Medicine*, vol. 14, no. 1, pp.2–7.

Kumar, P & Seshadri, R 2005, 'Neonatal morbidity and growth in very low birth-weight infants after multiple courses of antenatal steroids', *Journal of Perinatology*, vol. 25, no. 11, pp.698–702.

Kurzer, SI, Garg, M, Bautista, DB, Bader, D, Merrit, RJ, Warburton, D & Keens, TG 1988, 'Growth failure in infants with bronchopulmonary dysplasia: nutrition and elevated resting metabolic expenditure', *Pediatrics*, vol. 81, no. 3, pp.379–384.

Kuypers, E, Collins, JJP, Kramer, BW, Ofman, G, Nitsos, I, Pillow, JJ, Polglase, GR, Kemp, MW, Newnham, JP, Gavilanes, AWD, Nowacki, R, Ikegami, M, Jobe, AH & Kallapur, SG 2012a, 'Intra-amniotic LPS and antenatal

betamethasone: inflammation and maturation in preterm lamb lungs', *American Journal of Physiology: Lung Cellular and Molecular Physiology*, vol. 302, no. 4, pp.L380–L389.

Kuypers, E, Ophelders, D, Jellema, RK, Kunzmann, S, Gavilanes, AW & Kramer, BW 2012b, 'White matter injury following fetal inflammatory response syndrome induced by chorioamnionitis and fetal sepsis: lessons from experimental ovine models', *Early Human Development*, vol. 88, no. 12, pp.931–936.

LaFramboise, WA, Daood, MJ, Guthrie, RD, Schiaffino, S, Moretti, P, Brozanski, B, Ontell, MP, Butler-Browne, GS, Whalen, RG & Ontell, M 1991, 'Emergence of the mature myosin phenotype in the rat diaphragm muscle', *Developmental Biology*, vol. 144, no. 1, pp.1–15.

Lal, MK, Manktelow, BN, Draper, ES & Field, DJ 2003, 'Chronic lung disease of prematurity and intrauterine growth retardation: a population-based study', *Pediatrics*, vol. 111, no. 3, pp.483–487.

Lamb, GD 2000, 'Excitation-contraction coupling in skeletal muscle: comparisons with cardiac muscle', *Clinical and Experimental Pharmacology & Physiology*, vol. 27, no. 3, pp.216–224.

Langston, C, Kida, K, Reed, M & Thurlbeck, WM 1984, 'Human lung growth in late gestation and in the neonate', *American Revue of Respiratory Disease*, vol. 4, no. 129, pp.607–613.

Lanone, S, Taillè, C, Boczkowski, J & Aubier, M 2005, 'Diaphragmatic fatigue during sepsis and septic shock', *Intensive Care Medicine*, vol. 31, no. 12, pp.1611–1617.

Laszewski, B & Ruff, RL 1985, 'Effects of glucocorticoid treatment on excitation-contraction coupling', *American Journal of Physiology. Endocrinology and Metabolism*, vol. 248, no. 3, pp.E363–E369.

Lavin, T, Song, Y, Bakker, AJ, McLean, CJ, Macdonald, WA, Noble, PB, Berry, CA, Pillow, JJ & Pinniger, GJ 2013, 'Developmental changes in diaphragm

muscle function in the preterm and postnatal lamb', *Pediatric Pulmonology*, vol. 48, no. 7, pp.640–648.

Lefaucheur, L, Ecolan, P, Barzic, Y-M, Marion, J & Le Dividich, J 2003, 'Early postnatal food intake alters myofiber maturation in pig skeletal muscle', *Journal of Nutrition*, vol. 133, no. 1, pp.140–147.

Leon, A, Boczkowski, J, Dureuil, B, Desmonts, J-M & Aubier, M 1992, 'Effects of endotoxic shock on diaphragmatic function in mechanically ventilated rats', *Journal of Applied Physiology*, vol. 72, no. 4, pp.1466–72.

Lesage, J, Blondeau, B, Grino, M, Bréant, B & Dupouy, JP 2001, 'Maternal undernutrition during late gestation induces overexposure to glucocorticoids and intrauterine growth retardation, and disturbs the hypothalamo-pituaitary adrenal axis in the newborn rat', *Endocrinolgy*, vol. 142, no. 5, pp.1692–1702.

Levine, S, Nguyen, T, Taylor, N, Friscia, ME, Budak, MT, Rothenberg, P, Zhu, J, Sachdera, R, Sonnad, S, Kaiser, LR, Rubinstein, NA, Powers, SK & Shrager, JB 2008, 'Rapid disuse atrophy of diaphragm fibres in mechanically ventilated humans', *The New England journal of medicine*, vol. 358, no. 13, pp.1327–1335.

Lewis, MI, Monn, SA & Sieck, GC 1992, 'Effect of corticosteroids on diaphragm fatigue, SDH activity, and muscle fiber size', *Journal of Applied Physiology*, vol. 72, no. 1, pp.293–301.

Liggins, GC, Vilos, GA, Campos, GA, Kitterman, JA & Lee, CH 1981, 'The effect of spinal cord transection on lung development in fetal sheep', *Journal of Developmental Physiology*, vol. 3, no. 4, pp.267–274.

Lin, MC, Ebihara, S, El Dwairi, Q, Hussain, SNA, Yang, L, Gottfried, SB, Comtois, A & Petrof, BJ 1998, 'Diaphragm sarcolemmal injury is induced by sepsis and alleviated by nitric oxide synthase inhibition', *American Journal of Respiratory and Critical Care Medicine*, vol. 158, no. 5, pp.1656–1663.

Louis, M, Zanou, N, Van Schoor, M & Gailly, P 2008, 'TRPC1 regulates skeletal

myoblast migration and differentiation', *Journal of Cell Science*, vol. 121, no. 23, pp.3951–3959.

Maes, K, Agten, A, Smuder, A, Powers, SK, Decramer, M & Gayan-Ramirez, G 2010, 'Corticosteroid effects on ventilator-induced diaphragm dysfunction in anesthetized rats depend on the dose administered', *Respiratory Research*, vol. 11, p.178.

Maes, K, Stamiris, A, Thomas, D, Cielen, N, Smuder, A, Powers, SK, Leite, FS, Hermans, G, Decramer, M, Hussain, SN & Gayan-Ramirez, G 2014, 'Effects of controlled mechanical ventilation on sepsis-induced diaphragm dysfunction in rats', *Critical Care Medicine*, vol. 42, no. 12, pp.e772-782.

Mahzabin, T, Pillow, JJ, Pinniger, GJ, Bakker, AJ, Noble, PM, White, RB, Karisnan, K & Song, Y 2017, 'Influence of antenatal glucocorticoid on preterm lamb diaphragm', *Pediatric Research*, vol. 82, no. 3, pp.509–517.

Mammel, MC, Johnson, DE, Green, TP & Thompson, TR 1983, 'Controlled trial of dexamethasone therapy in infants with bronchopulmonary dysplasia', *Lancet*, vol. 1, pp.1356–1358.

Mantilla, CB & Sieck, GC 2008, 'Key aspects of phrenic motoneuron and diaphragm muscle development during the perinatal period', *Journal of Applied Physiology*, vol. 104, no. 6, pp.1818–1827.

Van Marter, LJ, Dammann, O, Allred, EN, Leviton, A, Pagano, M, Moore, M & Martin, C 2002, 'Chorioamnionitis, Mechanical Ventilation, and Postnatal Sepsis as Modulators of Chronic Lung Disease in Preterm Pnfants', *Journal of Pediatrics*, vol. 140, no. 2, pp.171–176.

Matsumura, CY, Taniguti, APT, Pertille, A, Neto, HS & Marques, MJ 2011, 'Stretch-activated calcium channel protein TRPC1 is correlated with the different degrees of the dystrophic phenotype in mdx mice', *American Journal of Physiology. Cell Physiology*, vol. 301, no. 6, pp.C1344–C1350.

Max, D, Brandsch, C, Schumann, S, Kuhne, H, Frommhagen, M, Schutkowski, A, Hirche, F, Staege, MS & Stangl, GI 2014, 'Maternal vitamin D deficiency

causes smaller muscle fibers and altered transcript levels of genes involved in protein degradation, myogenesis, and cytoskeleton organization in the newborn rat', *Molecular Nutrition and Food Research*, vol. 58, no. 2, pp.343–352.

Maxwell, LC, McCarter, RJM, Kuehl, TJ & Robotham, JL 1983, 'Development of histochemical and functional properties of baboon respiratory muscles', *Journal of Applied Physiology*, vol. 54, no. 2, pp.551–561.

McClung, JM, Whidden, MA, Kavazis, AN, Falk, DJ, Deruisseau, KC & Powers, SK 2008, 'Redox regulation of diaphragm proteolysis during mechanical ventilation', *American journal of physiology. Regulatory, Integrative and Comparative Physiology*, vol. 294, no. 5, pp.R1608–R1617.

Menconi, M, Fareed, M, O'Neal, P, Poylin, V, Wei, W & Hasselgren, PO 2007, 'Role of glucocorticoids in the molecular regulation of muscle wasting', *Critical Care Medicine*, vol. 35, no. 9, pp.S602–S608.

Mendell, LM 2005, 'The size principle: a rule describing the recruitment of motoneurons', *Journal of Neurophysiology*, vol. 93, no. 6, pp.3024–3026.

Mendez, J & Keys, A 1960, 'Density and composition of mammalian muscle', *Metabolism*, vol. 9, pp.184–188.

Molkentin, JD & Olson, EN 1996, 'Defining the regulatory networks for muscle development', *Current Opinion in Genetics & Development*, vol. 6, no. 4, pp.445–453.

Monangi, N, Slaughter, JL, Dawodu, A, Smith, C & Akinbi, HT 2014, 'Vitamin D status of early preterm infants and the effects of vitamin D intake during hospital stay', *Archives of Disease in Childhood. Fetal and Neonatal Edition*, vol. 99, no. 2, pp.F166–F168.

Monasky, MM, Biesiadecki, BJ & Janssen, PML 2011, 'Increased phosphorylation of tropomyosin, troponin I, and myosin light chain-2 after stretch in rabbit ventricular myocardium under physiological conditions', *Journal of Molecular and Cellular Cardiology*, vol. 48, no. 5, pp.1023–1028.

Montedonico, S, Nakazawa, N & Puri, P 2008, 'Congenital diaphragmatic hernia and retinoids: searching for an etiology', *Pediatric Surgery International*, vol. 24, no. 7, pp.755–761.

Moore, BJ, Miller, MJ, Feldman, HA & Reid, MB 1989, 'Diaphragm atrophy and weakness in cortisone-treated rats', *Journal of Applied Physiology*, vol. 67, no. 6, pp.2420–2426.

Moore, KL, Dalley, AF & Agur, AMR 2013, *Clinically oriented anatomy* , 7th edn , Lippincott Williams & Wilkins, Baltimore.

Morley, CJ, Davis, PG, Doyle, LW, Brion, LP, Hascoet, JM & Carlin, JB 2008, 'Nasal CPAP or intubation at birth for very preterm infants', *New England Journal of Medicine*, vol. 358, no. 7, pp.700–708.

Moss, TJM 2006, 'Respiratory consequences of preterm birth', *Clinical and Experimental Pharmacology and Physiology*, vol. 33, no. 3, pp.280–284.

Moss, TJM, Doherty, DA, Nitsos, I, Sloboda, DM, Harding, R & Newnham, JP 2005, 'Effects into adulthood of single or repeated antenatal corticosteroids in sheep', *American Journal of Obstetrics and Gynecology*, vol. 192, no. 1, pp.146–152.

Nason, LK, Walker, CM, McNeeley, MF, Burivong, W, Fligner, CL & Godwin, FD 2012, 'Imaging of the diaphragm: anatomy and function', *Radiographics*, vol. 32, no. 2, pp.51–71.

Nava, S, Gayan-Ramirez, G, Rollier, H, Bisschop, A, Dom, R, de Bock, V & Decramer, M 1996, 'Effects of acute steroid administration on ventilatory and peripheral muscles in rats', *American Journal of Respiratory and Critical Care Medicine*, vol. 153, no. 6, pp.1888–1896.

Navarrete, R & Vrbov, G 1983, 'Changes of activity patterns in slow and fast muscles during postnatal development', *Developmental Brain Research*, vol. 8, no. 1, pp.11–19.

Newnham, JP, Dickinson, JE, Hart, RJ, Pennell, CE, Arrese, CA & Keelan, JA 2014, 'Strategies to prevent preterm birth', *Frontiers in Immunology*, vol. 5,

p.584.

O'Brien, RAD, Ostberg, JC & Vrbová, G 1978, 'Observations on the elimination of polyneuronal innervation in developing mammalian skeletal muscle', *Journal of Physiology*, vol. 282, no. 1, pp.571–582.

Orliaguet, G, Langeron, O, Bouhemad, B, Coriat, P, LeCarpentier, Y & Riou, B 2002, 'Effects of postnatal maturation on energetics and cross-bridge properties in rat diaphragm', *Journal of Applied Physiology*, vol. 92, no. 3, pp.1074–1082.

Pallafacchina, G, Blaauw, B & Schiaffino, S 2013, 'Role of satellite cells in muscle growth and maintenance of muscle mass', *Nutrition, Metabolism & Cardiovascular Diseases*, vol. 23, no. Suppl 1, pp.S12–S18.

Park, S-H, Lee, G-M, Moon, J-E & Kim, H-M 2015, 'Severe vitamin D deficiency in preterm infants: maternal and neonatal clinical features', *Korean Journal of Pediatrics*, vol. 58, no. 11, pp.427–33.

te Pas, AB, Davis, PG, Hooper, SB & Morley, CJ 2008, 'From liquid to air: Breathing after birth', *Journal of Pediatrics*, vol. 152, no. 5, pp.607–611.

Petrof, BJ, Jaber, S & Matecki, S 2010, 'Ventilator-induced diaphragmatic dysfunction', *Current Opinion in Critical Care*, vol. 16, no. 1, pp.19–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19935062. [29 March 2014].

Pike, K, Pillow, JJ & Lucas, JS 2012, 'Long term respiratory consequences of intrauterine growth restriction', *Seminars in Fetal & Neonatal Medicine*, vol. 17, no. 2, pp.92–98.

Polla, B, D'Antona, G, Bottinelli, R & Reggiani, C 2004, 'Respiratory muscle fibres: specialisation and plasticity', *Thorax*, vol. 59, no. 9, pp.808–817.

Powell, JT & Whitney, PL 1980, 'Postnatal development of rat lung. Changes in lung lectin, elastin, acetylcholinesterase and other enzymes', *Biochemical Journal*, vol. 188, no. 1, pp.1–8.

Powers, SK, Kavazis, AN & DeRuisseau, KC 2005, ‘Mechanisms of disuse muscle atrophy: role of oxidative stress’, *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, vol. 288, no. 2, pp.R337–R344.

Powers, SK, Kavazis, AN & Levine, S 2009, ‘Prolonged mechanical ventilation alters diaphragmatic structure and function’, *Critical Care Medicine*, vol. 37, no. 10 Suppl, pp.S347–S353.

Powers, SK, Kavazis, AN & McClung, JM 2007, ‘Oxidative stress and disuse muscle atrophy’, *Journal of Applied Physiology*, vol. 102, no. 6, pp.2389–2397.

Powers, SK, Shanely, RA, Coombes, JS, Koesterer, TJ, McKenzie, M, Van Gammeren, D, Cicale, M & Dodd, SL 2002, ‘Mechanical ventilation results in progressive contractile dysfunction in the diaphragm’, *Journal of Applied Physiology*, vol. 92, no. 5, pp.1851–1858.

Prakash, YS, Fournier, M & Sieck, GC 1993, ‘Effects of prenatal undernutrition on developing rat diaphragm’, *Journal of Applied Physiology*, vol. 75, no. 3, pp.1044–1052.

Prakash, YS, Mantilla, CB, Zhan, WZ, Smithson, KG & Sieck, GC 2000, ‘Phrenic motoneuron morphology during rapid diaphragm muscle growth’, *Journal of Applied Physiology*, vol. 89, no. 2, pp.563–572.

Pratt, L, Magness, RR, Phernetton, T, Hendricks, SK, Abbott, DH & Bird, IM 1999, ‘Repeated use of betamethasone in rabbits: effects of treatment variation on adrenal suppression, pulmonary maturation, and pregnancy outcome’, *American Journal of Obstetrics and Gynecology*, vol. 180, no. 4, pp.995–1005.

Prezant, DJ, Karwa, ML, Richner, B, Maggiore, D, Gentry, EI & Cahill, J 1997, ‘Gender-specific effects of dexamethasone treatment on rat diaphragm structure and function’, *Journal of Applied Physiology*, vol. 82, no. 1, pp.125–133.

Prezant, DJ, Karwa, ML, Richner, B, Maggiore, D, Gentry, EI, Chung, V & Cahill, J 1998, 'Short-term vs long-term dexamethasone treatment: effects on rat diaphragm structure and function', *Lung*, vol. 176, no. 4, pp.267–280.

Racca, A, Beck, AE, Rao, VS, Flint, G V, Lundy, SD, Born, DE, Bamshad, MJ & Regnier, M 2013, 'Contractility and kinetics of human fetal and human adult skeletal muscle', *The Journal of Physiology*, vol. 591, no. 12, pp.3049–3061.

Radell, PJ, Remahl, S, Nichols, DG & Eriksson, LI 2002, 'Effects of prolonged mechanical ventilation and inactivity on piglet diaphragm function', *Intensive Care Medicine*, vol. 28, no. 3, pp.358–364.

Rastogi, A, Akintorin, S, Bez, M, Morales, P & Pildes, R 1996, 'A controlled trial of dexamethasone to prevent bronchopulmonary dysplasia in surfactant-treated infants', *Pediatrics*, vol. 98, no. 2, pp.204–210.

Ray, AD, Personius, KE, Williamson, DL, Dungan, CM, Dhillon, SS & Hershberger, PA 2016, 'Vitamin D3 intake modulates diaphragm but not peripheral muscle force in young mice', *Journal of Applied Physiology*, vol. 120, no. 10, pp.1124–1131.

Roberts, D & Dalziel, SR 2006, 'Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth', *Cochrane Database of Systematic Reviews*, vol. 3, p.CD004454.

Rochester, DF, Arora, NS & Braun, NM 1982, 'Maximum contractile force of human diaphragm muscle, determined in vivo', *Transactions of the American Clinical and Climatological Association*, vol. 93, no. 7, pp.200–208.

Ruiz-Rosado, A, Fernández-Valverde, F, Mariscal-Tovar, S, Hinojosa-Rodriguez, CX, Hernández-Valencia, JA, Anzueto-Rios, A, Guadarrama-Olmos, JC, Segura-Alegría, B & Jiménez-Estrada, I 2013, 'Histoenzymatic and morphometric analysis of muscle fiber type transformation during the postnatal development of the chronically food-deprived rat', *Journal of Histochemistry and Cytochemistry*, vol. 61, no. 5, pp.372–381.

Sandow, A 1965, ‘Excitation-contraction coupling in skeletal muscle’, *Pharmacological Reviews*, vol. 17, no. 3, pp.265–320.

Sasson, L, Tarasiuk, A, Heimer, D & Bark, H 1991, ‘Effect of dexamethasone on diaphragmatic and soleus muscle morphology and fatigability’, *Respiration Physiology*, vol. 85, no. 1, pp.15–28.

Sassoon, CSH, Caiozzo, VJ, Manka, A & Sieck, GC 2002, ‘Altered diaphragm contractile properties with controlled mechanical ventilation’, *Journal of Applied Physiology*, vol. 92, no. 6, pp.2585–2595.

Sassoon, CSH, Zhu, E, Fang, L, Ramar, K, Jiao, G-Y & Caiozzo, VJ 2011, ‘Interactive effects of corticosteroid and mechanical ventilation on diaphragm muscle function’, *Muscle & Nerve*, vol. 43, no. 1, pp.103–111.

Sassoon, CSH, Zhu, E, Pham, HT, Nelson, RS, Fang, L, Baker, MJ & Caiozzo, VJ 2008, ‘Acute effects of high-dose methylprednisolone on diaphragm muscle function’, *Muscle & Nerve*, vol. 38, no. 3, pp.1161–1172.

Schakman, O, Gilson, H, Kalista, S & Thissen, JP 2009, ‘Mechanisms of muscle atrophy induced by glucocorticoids’, *Hormone Research*, vol. 72, no. suppl 1, pp.36–41.

Schiaffino, S & Mammucari, C 2011, ‘Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models’, *Skeletal Muscle*, vol. 1, p.4.

Schiaffino, S & Margreth, A 1969, ‘Coordinated development of the sarcoplasmic reticulum and T system during postnatal differentiation of rat skeletal muscle’, *Journal of Cell Biology*, vol. 41, no. 3, pp.855–875.

Schiaffino, S & Reggiani, C 1994, ‘Myosin isoforms in mammalian skeletal muscle’, *Journal of Applied Physiology*, vol. 77, no. 2, pp.493–501.

Schittny, JC 2017, ‘Development of the lung’, *Cell and Tissue Research*, vol. 367, no. 3, pp.427–444.

Segal, SS & Faulkner, JA 1985, ‘Temperature-dependent physiological stability

of rat skeletal muscle in vitro', *American Journal of Physiology. Cell Physiology*, vol. 248, no. 3, pp.C265–C270.

Shapiro-Mendoza, CK & Lackritz, EM 2012, 'Epidemiology of late and moderate preterm birth', *Seminars in Fetal and Neonatal Medicine*, vol. 17, no. 3, pp.120–125.

Shenai, JP, Kennedy, KA, Chytil, F & Stahlman, MT 1987, 'Clinical trial of vitamin A supplementation in infants susceptible to bronchopulmonary dysplasia', *Journal of Pediatrics*, vol. 111, no. 2, pp.269–277.

Shoji, S, Takagi, A, Sugita, H & Toyokura, Y 1976, 'Dysfunction of sarcoplasmic reticulum in rabbit and human steroid myopathy', *Experimental Neurology*, vol. 51, no. 2, pp.304–309.

Sieck, GC & Blanco, CE 1991, 'Postnatal changes in the distribution of succinate dehydrogenase activities among diaphragm muscle fibers', *Pediatric Research*, vol. 29, no. 6, pp.586–593.

Sieck, GC, Cheung, TS & Blanco, CE 1991, 'Diaphragm capillarity and oxidative capacity during postnatal development', *Journal of Applied Physiology*, vol. 70, no. 1, pp.103–111.

Sieck, GC & Fournier, M 1989, 'Diaphragm Motor unit recruitment during ventilatory and nonventilatory behaviors', *Journal of Applied Physiology*, vol. 66, no. 6, pp.2539–2545.

Sieck, GC, Fournier, M & Blanco, CE 1991, 'Diaphragm muscle fatigue resistance during postnatal development', *Journal of Applied Physiology*, vol. 71, no. 2, pp.458–464.

Sissom, EK, Reinhardt, CD & Johnson, BJ 2006, 'Melengestrol acetate alters muscle cell proliferation in heifers and steers', *Journal of Animal Science*, vol. 84, no. 11, pp.2950–2958.

Smith, D, Green, H, Thomson, J & Sharratt, M 1988, 'Oxidative potential in developing rat diaphragm, EDL, and soleus muscle fibers', *American Journal of Physiology*, vol. 254, no. 22, pp.C661–C668.

Song, Y, Demmer, DL, Pinniger, GJ, Lavin, T, MacMillan, M V., Pillow, JJ & Bakker, AJ 2014, 'Effect of maternal steroid on developing diaphragm integrity', *Plos One*, vol. 9, no. 3, p.e93224.

Song, Y, Karisnan, K, Noble, PB, Berry, CA, Lavin, T, Moss, TJM, Bakker, AJ, Pinniger, GJ & Pillow, JJ 2013, 'In utero LPS exposure impairs preterm diaphragm contractility', *American Journal of Respiratory Cell and Molecular Biology*, vol. 49, no. 5, pp.866–874.

Song, Y & Pillow, JJ 2012, 'Ontogeny of proteolytic signaling and anti-oxidant capacity in fetal and neonatal diaphragm', *Anatomical Record*, vol. 295, no. 5, pp.864–871.

Song, Y & Pillow, JJ 2013, 'Developmental regulation of molecular signalling in fetal and neonatal diaphragm protein metabolism', *Experimental Biology and Medicine*, vol. 238, no. 8, pp.913–922.

Le Souef, PN, England, SJ, Stogryn, HAF & Bryan, AC 1988, 'Comparison of Diaphragmatic Fatigue in Newborn and Older Rabbits', *Journal of Applied Physiology*, vol. 65, no. 3, pp.1040–1044.

Srikuea, R, Zhang, X, Park-Sarge, O-K & Esser, KA 2012, 'VDR and CYP27B1 are expressed in C2C12 Cells and regenerating skeletal muscle: potential role in suppression of myoblast proliferation.', *American Journal of Physiology. Cell Physiology*, vol. 303, no. 4, pp.C396–C405.

Stamm, C, Cowan, DB, Friehs, I, Noria, S, del Nido, PJ & McGowan, FX 2001, 'Rapid endotoxin-induced alterations in myocardial calcium handling: obligatory role of cardiac TNF-α', *Anesthesiology*, vol. 95, no. 6, pp.1396–1405.

Stark, AR, Carlo, WA, Tyson, JE, Papile, LA, Wright, LL, Shankaran, S, Donovan, EF, Oh, W, Bauer, CR, Saha, S, Poole, K & Stoll, BJ 2001, 'Adverse effects of early dexamethasone treatment in extremely-low-birth-weight infants', *New England Journal of Medicine*, vol. 344, no. 2, pp.95–101.

Stefanescu, BM, Murphy, WP, Hansell, BJ, Fuloria, M, Morgan, TM & Aschner, JL 2003, 'A randomised, controlled trial comparing two different continuous positive airway pressure systems for the successful extubation of extremely low birth weight infants', *Pediatrics*, vol. 112, no. 5, pp.1031–1038.

Strbenc, M, Smerdu, V, Pogacnik, A & Fazarinc, G 2006, 'Myosin heavy chain isoform transitions in canine skeletal muscles during postnatal growth', *Journal of Anatomy*, vol. 209, no. 2, pp.149–163.

Sweet, DG, Carnielli, V, Greisen, G, Hallman, M, Ozek, E, Plavka, R, Saugstad, OD, Simeoni, U, Speer, CP & Halliday, HL 2010, 'European consensus guidelines on the management of neonatal respiratory distress syndrome in preterm infants - 2010 update', *Neonatology*, vol. 97, no. 4, pp.402–417.

Sweet, DG, Carnielli, V, Greisen, G, Hallman, M, Ozek, E, Plavka, R, Saugstad, OD, Simeoni, U, Speer, CP, Vento, M & Halliday, HL 2013, 'European consensus guidelines on the management of neonatal respiratory distress syndrome in preterm infants - 2013 update', *Neonatology*, vol. 103, no. 4, pp.353–368.

Sweet, DG, Carnielli, V, Greisen, G, Hallman, M, Ozek, E, Plavka, R, Saugstad, OD, Simeoni, U, Speer, CP, Vento, M, Visser, GH & Halliday, HL 2017, 'European consensus guidelines on the management of respiratory distress syndrome - 2016 update', *Neonatology*, vol. 111, no. 2, pp.107–125.

Tavi, P, Han, C & Weckstrom, M 1998, 'Mechanisms of stretch-induced changes in [Ca2+]i in rat atrial myoctes: role of increased troponin C affinity and stretch-activated ion channels', *Circulation Research*, vol. 83, pp.1165–1177.

Tergestina, M, Jose, A, Sridhar, S, Job, V, Rebekah, G, Kuruvilla, KA & Thomas, N 2014, 'Vitamin D status and adequacy of standard supplementation in preterm neonates from South India', *Journal of Pediatric Gastroenterology and Nutrition*, vol. 58, no. 5, pp.661–665.

Thomas, W & Speer, CP 2014, 'Chorioamnionitis is essential in the evolution of bronchopulmonary dysplasia – the case in favour', *Paediatric Respiratory*

Reviews, vol. 15, no. 1, pp.49–52.

Thompson, K, Conlon, J, Magowan, BA & Bain, M 2015, 'Neonatal mortality amongst Scottish preterm singleton births (2001-2010): record linkage of maternity data and neonatal mortality data', *Public Health*, vol. 129, no. 12, pp.1598–1601.

Trang, TT, Viires, N & Aubier, M 1992, 'Effect of steroids on diaphragm of newborn, weaning adolescent, and adult rats', *American Review of Respiratory Disease*, vol. 146, no. 1, pp.26–31.

De Troyer, A, Leeper, J, McKenzie, D & Gandevia, S 1997, 'Neural drive to the diaphragm in patients with severe COPD', *American Journal of Respiratory and Critical Care Medicine*, vol. 155, no. 4, pp.1335–1340.

Tsai, FJ, Tsai, CH, Wu, SF, Liu, YH & Yeh, TF 1996, 'Catabolic effect in premature infants with early dexamethasone treatment', *Acta Paediatrica*, vol. 85, no. 12, pp.1487–90.

Vassilakopoulos, T & Petrof, BJ 2004, 'Ventilator-induced diaphragmatic dysfunction', *American journal of Respiratory and Critical Care Medicine*, vol. 169, no. 3, pp.336–341.

Viires, N, Pavlovic, D, Pariente, R & Aubier, M 1990, 'Effects of steroids on diaphragmatic function in rats', *American Review of Respiratory Disease*, vol. 142, no. 1, pp.34–38.

Walsh, MC, Morris, BH, Wrage, LA, Vohr, BR, Poole, WK, Tyson, JE, Wright, LL, Ehrenkranz, RA, Stoll, BJ & Fanaroff, AA 2005, 'Extremely low birthweight neonates with protracted ventilation: mortality and 18-month neurodevelopmental outcomes', *Journal of Pediatrics*, vol. 146, no. 6, pp.798–804.

Wang, K 1996, 'Titin/connectin and nebulin: giant protein rulers of muscle structure and function', *Advances in Biophysics*, vol. 33, pp.123–134.

Ward, RM & Beachy, JC 2003, 'Neonatal complications following preterm birth', *BJOG: An International Journal of Obstetrics and Gynaecology*, vol. 110,

no. suppl 20, pp.8–16.

Ward, SS & Stickland, NC 1993, ‘The effect of undernutrition in the early postnatal period on skeletal muscle tissue’, *British Journal of Nutrition*, vol. 69, no. 1, pp.141–150.

Watchko, JF, Daood, MJ & Sieck, GC 1998, ‘Myosin heavy chain transitions during development: functional implications for the respiratory musculature’, *Comparative Biochemistry and Physiology B. Biochemistry and Molecular Biology*, vol. 119, no. 3, pp.459–470.

Watchko, JF, Johnson, BD, Gosselin, LE, Prakash, YS & Sieck, GC 1994, ‘Age-related differences in diaphragm muscle injury after lengthening activations.’, *Journal of Applied Physiology*, vol. 77, no. 5, pp.2125–2133.

Watchko, JF & Sieck, GC 1993, ‘Respiratory Muscle Fatigue Resistance Relates to Myosin Phenotype and SDH Activity during Development.’, *Journal of Applied Physiology*, vol. 75, no. 3, pp.1341–1347.

Watterberg, K 2012, ‘Evidence-based neonatal pharmacotherapy: postnatal corticosteroids’, *Clinics in Perinatology*, vol. 39, no. 1, pp.47–59.

Weiner, F, Azgad, Y & Weiner, M 1993, ‘The effect of corticosteroids on inspiratory muscle performance in humans’, *Chest*, vol. 104, no. 6, pp.1788–1791.

West, JM, Barclay, CJ, Luff, AR & Walker, DW 1999, ‘Developmental changes in the activation properties and ultrastructure of fast- and slow-twitch muscles from fetal sheep’, *Journal of Muscle Research and Cell Motility*, vol. 20, no. 3, pp.249–264.

Whidden, MA, McClung, JM, Falk, DJ, Hudson, MB, Smuder, AJ, Nelson, WB & Powers, SK 2009, ‘Xanthine oxidase contributes to mechanical ventilation-induced diaphragmatic oxidative stress and contractile dysfunction’, *Journal of Applied Physiology*, vol. 106, no. 2, pp.385–394.

White, P, Cattaneo, D & Dauncey, MJ 2000, ‘Postnatal regulation of myosin heavy chain isoform expression and metabolic enzyme activity by nutrition’,

The British Journal of Nutrition, vol. 84, no. 2, pp.185–194.

White, RB, Biérinx, A-S, Gnocchi, VF & Zammit, PS 2010, 'Dynamics of muscle fibre growth during postnatal mouse development', *BMC Developmental Biology*, vol. 10, p.21.

Wigglesworth, JS & Desai, R 1979, 'Effect of lung growth of cervical cord section in the rabbit fetus', *Early Human Development*, vol. 3, no. 1, pp.51–65.

Wigmore, PMC & Stickland, NC 1983, 'Muscle development in large and small pig fetuses', *Journal of Anatomy*, vol. 137, no. 2, pp.235–245.

Williams, MC, O'Brien, WF, Nelson, RN & Spellacy, WN 2000, 'Histologic chorioamnionitis is associated with fetal growth restriction in term and preterm infants', *American Journal of Obstetrics and Gynecology*, vol. 183, no. 5, pp.1094–1099.

Williams, PE & Goldspink, G 1971, 'Longitudinal growth of striated muscle fibres', *Journal of Cell Science*, vol. 9, pp.751–767.

Wilson, DC, Cairns, P, Halliday, HL, Reid, M, McClure, G & Dodge, JA 1997, 'Randomised controlled trial of an aggressive nutritional regimen in sick very low birthweight infants', *Archives of Disease in Childhood. Fetal and Neonatal Edition*, vol. 77, no. 1, pp.F4–F11.

Wilson, DC, Mcclure, G & Dodge, JA 1992, 'The influence of nutrition on neonatal respiratory muscle function', *Intensive Care Medicine*, vol. 18, no. 2, pp.105–108.

Wilson, DC, McClure, G, Halliday, HL, Reid, MM & Dodge, JA 1991, 'Nutrition and bronchopulmonary dysplasia', *Archives of Disease in Childhood*, vol. 66, no. 1, pp.37–38.

Wilson, JG, Roth, CB & Warkany, J 1953, 'An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation', *American Journal of Anatomy*, vol. 92, no. 2, pp.189–217.

Wilson, SJ, Ross, JJ & Harris, AJ 1988, ‘A critical period for formation of secondary myotubes defined by prenatal undernourishment in rats’, *Development*, vol. 102, no. 4, pp.815–821.

Wolfe, KB, Snyder, CC, Gisslen, T, Kemp, MW, Newnham, JP, Kramer, BW, Jobe, AH & Kallapur, S 2013, ‘Modulation of lipopolysaccharide-induced chorioamnionitis in fetal sheep by maternal betamethasone’, *Reproductive Sciences*, vol. 20, no. 12, pp.1447–1454.

Woo, M, Isganaitis, E, Cerletti, M, Fitzpatrick, C, Wagers, AJ, Jimenez-Chillaron, J & Patti, ME 2011, ‘Early life nutrition modulates muscle stem cell number: implications for muscle mass and repair’, *Stem Cells and Development*, vol. 20, no. 10, pp.1763–1769.

Yang, L, Luo, J, Bourdon, J, Lin, MC, Gottfried, SB & Petrof, BJ 2002, ‘Controlled mechanical ventilation leads to remodeling of the rat diaphragm’, *American Journal of Respiratory and Critical Care Medicine*, vol. 166, no. 8, pp.1135–1140.

Yeh, TF, Lin, YJ, Hsieh, WS, Lin, HC, Lin, CH, Chen, JY, Kao, HA & Chien, CH 1997, ‘Early postnatal dexamethasone therapy for the prevention of chronic lung disease in preterm infants with respiratory distress syndrome: a multicenter clinical trial’, *Pediatrics*, vol. 100, no. 4.

Yeh, TF, McClenan, DA, Ajayi, OA & Pildes, RS 1989, ‘Metabolic Rate and Energy Balance in Infants with Bronchopulmonary Dysplasia’, *Journal of Pediatrics*, vol. 114, no. 3, pp.448–451.

Zajac, FE & Faden, JS 1985, ‘Relationship among recruitment order, axonal conduction velocity, and muscle-unit properties of type-identified motor units in cat plantaris muscle’, *Journal of Neurophysiology*, vol. 53, no. 5, pp.1303–1322.

Zhan, WZ, Watchko, JF, Prakash, YS & Sieck, GC 1998, ‘Isotonic contractile and fatigue properties of developing rat diaphragm muscle’, *Journal of Applied Physiology*, vol. 84, no. 4, pp.1260–1268.

Zhu, MJ, Ford, SP, Means, WJ, Hess, BW, Nathanielsz, PW & Du, M 2006, 'Maternal nutrient restriction affects properties of skeletal muscle in offspring', *Journal of Physiology*, vol. 575, no. 1, pp.241–250.

Zhu, MS, Ford, SP, Nathanielsz, PW & Du, M 2004, 'Effect of maternal nutrient restriction in sheep on the development of fetal skeletal muscle', *Biology of Reproduction*, vol. 71, no. 6, pp.1968–1973.

Zosky, GR, Larcombe, AN, White, OJ, Burchell, JT, Janosi, TZ, Hantos, Z, Holt, PG, Sly, PD & Turner, DJ 2008, 'Ovalbumin-sensitized mice are good models for airway hyperresponsiveness but not acute physiological responses to allergen inhalation', *Clinical and Experimental Allergy*, vol. 38, no. 5, pp.829–838.

Zubrzycka-Gaarn, E & Sarzala, MG 1980, ''Sarcoplasmic reticulum and sarcolemma during development'' in *Plasticity of Muscle* , ed.D Pette, Walter de Gruyter, Walter de Gruyter, Berlin, pp.209–223.

8 Appendices

8.1 Appendix I: Detailed experimental protocols

Detailed methods for ewe and lamb management, *in vitro* diaphragm contractile measurements, and histochemical techniques, are described below. Ewe and lamb management specific to a treatment group is explained in the relevant experimental chapters (Chapter 3 and chapter 4). The management of rats used for the experiments outlined in chapter 5 are specific to that chapter and explained in chapter 5.

8.1.1 Ewe management

Breeding and Transport

Breeding was undertaken at the UWA Ridgefield Farm (Pingelly, WA). Ewes with a condition score of at least 2.5 and weighing at least 50 kg were deemed eligible for mating. Synchronised induction of anoestrus was achieved via insertion of a progesterone sponge (30 mg, Eazi-Breed^{TM} CIDR®, Zoetis, Sydney, Aust) to a depth of 7-10 cm into the ewe's vagina. Sponges were removed after 14 d, and ewes were joined with a ram 1 day later (D0). Parity was determined by ultrasound scan at 50 - 80 d gestation. All ewes underwent veterinary inspection approximately 1 week prior to transport from Ridgefield Farm to the Large Animal Facility at UWA. Ewes approved for inclusion were commenced on pellet feed (Macco Feeds 707: 16% protein in dry matter, 12 MJ/k dry matter; 200 gm/d) 1 week prior to transport from Ridgefield Farm to the Large Animal Facility at UWA. Pellet feed was gradually increased according to gestation (to a total of 600 gm/ d preterm ewes or 875 gm/d for term ewes to meet the needs of a 60 kg pregnant ewe). Mineral supplements (20 mg/d) and oaten chaff (750 g/d) were provided daily. Feeds were divided into morning and afternoon feeds. Small amounts of oaten hay or lucerne were provided in the afternoon feed. Ewes were vaccinated against Cheesy Gland and 5 clostridial diseases (Cydectin Eweguard SE B12 6 in 1 Vaccine and Wormer, Virbac Australia Pty. Ltd., Milperra, Australia) on arrival at UWA Large Animal Facility.

Antenatal Treatment

Preterm ewes were injected with IM medroxyprogesterone acetate (150 mg, Pfizer, Australia) at 122 d gestation to prevent premature labour. Antenatal betamethasone (5.7 mg IM, Merck Sharp & Dome, Australia) was commenced 48 hours prior to delivery and repeated 24 hours later to promote fetal lung maturation. Term ewes received no antenatal exposure to medroxyprogesterone or betamethasone prior to delivery.

Delivery and euthanasia

Preterm ewes were fasted for 24 h prior to delivery to prevent aspiration of vomited food, but had ad-lib access to water at all times. Preterm ewes were premedicated with buprenorphine (0.01 mg/kg; Indivior Pty Ltd) and acepromazine (0.05 mg/kg; Ceva Animal Health Pty Ltd) one hour prior to induction of anaesthesia. Colostrum was collected and refrigerated, prior to IV anaesthetic induction with thiopentone (15 mg/kg; Troy Laboratories). Ewes were intubated with a 9.0 mm tracheal tube, commenced on inhalational anaesthetic (Isofluorane 1.5 - 2.5 %) and transferred to a surgical table. Ewe anaesthetic monitoring included ECG, end-tidal CO_2, and SpO_2. Preterm lambs were delivered via hysterotomy after instrumentation, using standard sterile procedures. Additional colostrum was collected at completion of delivery with assistance of 0.5 mL oxytocin (10 IU/ml; Provet), after which ewes were killed humanely (pentobarbitone, 150mg/kg, IV; Pitman-Moore)

Term ewes delivered naturally (vaginally) in their pens, and were allowed to stay with their lambs for 1 week prior to euthanasia (pentobarbitone 150 mg/kg).

8.1.2 Lamb management

Fetal instrumentation

Lambs were exteriorised via hysterotomy, and intubated with a 3.5 mm tracheal tube. Fetal lung fluid (~ 60 mL) was suctioned from the tracheal tube with gentle negative pressure, exogenous surfactant (100 mg/kg, Chiesi Farmaceutici, Palma, Italy) was instilled into the distal trachea with a feeding tube, and the tracheal tube was occluded until delivery. Vascular catheters were inserted and suture secured into the carotid arterial and jugular vein, or the umbilical vein

and umbilical artery, for delivery of IV fluids, antibiotics, inotropes, and monitoring of arterial blood gases. Cord blood was collected from the placenta for baseline blood gas, plasma and full blood counts after lambs were delivered and prior to ewe euthanasia (150 mg/kg pentobarbitone). Lambs were dried and the umbilical cord clamped and cut.

Resuscitation

Newborn lambs were weighed and positioned prone on the neonatal warmer. Lamb tracheal tubes were connected to an infant resuscitating device (Neopuff^{TM}, Fisher Pike healthcare, New Zealand) and a sustained inflation delivered (30 cmH_2O, 30 s, flow 8 L/min) using a F_IO_2 of 0.30.

Respiratory management

Lambs were commenced on assist/control ventilation (VN500, Drager Medical, Lubeck, Germany) with volume guarantee of 5 - 7 mL/kg, using a maximum peak inspiratory pressure (PIP) of 30 cmH_2O and an initial positive end expiratory pressure (PEEP) of 9 cmH_2O. Ventilatory settings (VG, PEEP, FiO_2, inspiratory time and rate) were adjusted subsequently to target mild permissive hypercapnia ($PaCO_2$ 45-55 mmHg) and SpO_2 90-95% whilst using the lowest achievable FiO_2. Maximum PIP was subsequently maintained at 4 - 5 cmH_2O above average PIP. PEEP was weaned to 7 cmH_2O within 15 min after delivery.

The ventilatory mode changed to mandatory minute ventilation using a volume guarantee of 7 mL/kg, once lambs commenced breathing spontaneously. Pressure support was adjusted as required to target permissive hypercapnia using triggered breath volumes of ~6 mL/kg, usually achieved with a physiological triggered breathing frequency of 50-60 breaths/min.

Graded de-escalation of respiratory support commenced once lambs could sustain 100 % spontaneous ventilation on mandatory minute ventilation with an $FiO_2 < 0.3$ and a PIP of ≤ 20 cmH_2O to achieve a $PaCO_2 < 55$ mmHg with a volume guarantee set at < 7 mL/kg. Lambs were loaded with caffeine (15 mg/kg IV over 20 min) and commenced on bubble CPAP (FisherPaykel Healthcare) via the tracheal tube. Caffeine was continued (5 mg/kg/d) as required over the

first week of life. Glycopyrrolate (Robinul, 0.1 mg/kg IM) was administered for excessive secretions in the upper respiratory tract, as required.

Lambs sustaining tracheal tube CPAP for 12 hours had further de-escalation of respiratory support via extubation to humidified high flow (8 L/min), and subsequent low flow oxygen if required prior to removal of respiratory support. Respiratory support was ceased when lambs were able to sustain a SpO_2 of > 90 % without apnoea.

Reintubation was mandated by any one of the following criteria: 1) ventilatory failure (*$PaCO_2$* > 80 mmHg on 2 consecutive blood gases > 30 min apart unresponsive to altered nasal HFOV amplitude +/- frequency); 2) severe metabolic acidosis associated with sepsis; 3) ≥ 4 apnoea within an hour requiring bag and mask ventilation, or 4) persistent SpO_2 < 90 % despite FiO_2 > 0.5 and increase in mean airway pressure to 12 cmH_2O.

General care, Fluids, Nutrition and Growth

Preterm Lambs

Continuous cardiorespiratory monitoring was maintained until lambs were weaned from all respiratory support and supplemental oxygen. Preterm lambs were actively warmed (Fisher & Paykel open bed warmers) as required to target a core temperature of 38.5-39.5 °C (normothermic for lamb). Sedation was administered intermittently if the lamb became very active or for analgesia if required (0.01 mg/kg IM buprenorphine). Most lambs required minimal or no sedation.

Preterm lambs were commenced on total daily fluids of 100 mL/kg (5% glucose). Fluid intake was incremented by 20 mL/kg/d until they reached a total intake of 200 mL/kg/d. Oral feeds were commenced at two hours of life (20 mL/kg/d) with fresh ewe colostrum, and transitioned to lamb replacement formula (MaxCare® Lamb & Kid; MaxumAnimal) after 24 hours. Oral feeds were increased by 15 - 20 mL/kg/d as tolerated, and intravenous fluids were decreased accordingly. Feeds were suspended and full intravenous fluids recommenced if girth measurement increased by more than 3 cm within 24 hours, or gastric aspirate exceeded 5 mL.

Lambs assigned to a 2 m treatment group were graduated from the intensive care unit to the nursery at 2 w PNA (~ term equivalent). Solid creep feed was commenced at 2 w PNA (finely ground modified Maccos 707 pellet feed was added initially as a small pinch per milk feed and increased to 100 g/d, plus oaten chaff). Lambs received ad-lib access to formula milk and solid feed from 2 – 7 w PNA, after which after which milk feeds were decreased to 1200 mL/d for 1 week, then 1000 mL/d for 2 weeks.

Term Lambs

Term lambs were kept with their respective ewes for 7 day then separated and placed in the nursery pen with the same ad-lib exposure to formula milk, creep and chaff as the preterm lambs after graduation from the intensive care unit to the nursery.

Prophylactic Antibiotics and Infection

Lambs receive prophylactic IV antibiotic cover (100 mg/kg piperacillin/tazobactam every 12 hours and 6 mg/kg gentamicin daily). Prophylactic antibiotics were continued until removal of intravascular catheters (usually 5 - 7 d). Gentamicin was changed to amikacin (10 mg/kg) in the event of suspected sepsis (hypothermia, hyperthermia, decreased interest in feeds, lethargy, neutropenia < 1000 x 10^6 cells/mL or neutrophilia > 5000 x 10^6 cells/mL).

8.1.3 Diaphragm contractile properties

The diaphragm and attached ribs were excised immediately following euthanasia, and maintained in a petri dish containing mammalian Ringer's solution (in mM; NaCl, 121; KCl, 5.4; $MgSO_4(H_2O)_7$, 1.2; $NaHCO_3$, 25; HEPES, 5; glucose, 11.5 and $CaCl_2$, 2.5: pH 7.3) and bubbled with carbogen (5 % CO_2 in O_2). A longitudinal strip (3-5 mm wide) of intact muscle fibres, central tendon and attached ribs were dissected from the mid-costal portion of the right hemidiaphragm. During the dissection the mammalian Ringer's solution was replaced ~15-20 min to avoid damage to the intact muscle strip from proteases released by fibres which had been cut. The diaphragm strip was mounted in an *in vitro* muscle test system (1200A; Aurora Scientific) containing mammalian Ringer's solution and bubbled with carbogen (5 % CO_2 in O_2) at 25 °C. Rat

diaphragm function was measured in an 800A *in vitro* muscle apparatus with an operating range of 0 – 0.5 N and a resolution of 0.3 mN. Lamb diaphragm function was measured in an 805A *in vitro* muscle apparatus with an operating range of 0 – 5 N and a resolution of 1 mN. The tissue bath of the *in vitro* muscle test system was adapted for larger diaphragm strips from 2 m old lambs. A glass tissue bath was designed to facilitate an 85 mm maximum muscle length, compared to the 55 mm maximum muscle length of the standard 805A *in vitro* muscle bath (Aurora Scientific). The glass tissue bath was water-jacketed for stable temperature control. Bath dimension: 55 mm ID X 80 mm OD X 120 mm depth (145 mm overall height). Equipment was routinely manually calibrated with weights spanning the operating range.

All measurements of contractile function were performed at L_o, the length at which maximum twitch force was recorded. L_o was determined by incrementally increasing muscle length, and recording twitch force at each length, until an increase in muscle length did not produce an increase in twitch force, i.e. maximum twitch force was recorded. The diaphragm strip was stimulated by 0.2 ms supramaximal square wave pulses supplied by a constant current, biphasic stimulator (701B, Aurora Scientific). Twitch force characteristics were examined, including P_t, TTP, and ½ RT. The force response to a range of different stimulation frequencies were measured, because the diaphragm activates sub-maximally and maximally *in vivo*. The force-frequency relationship was determined from 700 ms -1 s stimulations elicited at 4-100 Hz. Stimulation durations and frequencies differed between the 7 d old lamb experiments and 2 m old lamb experiments and differed between the neonatal rat experiments and adult rat experiments to ensure maximum tetanic force responses plateaued and to ensure there were sufficient data points along the steep incline of the force-frequency relationship, respectively. Peak twitch force as a ratio of maximum specific force was calculated manually. The fatigue index of the sheep diaphragm was determined from the ratio of force produced before and after a Burke fatigue test: 330 ms duration stimulations at 40 Hz every second for 2 minutes (Burke et al. 1973). The fatigue index of the rat diaphragm was determined from the ratio of force produced before and after a series of 900 tetanic contractions of 500 ms durations repeated once every 2 seconds. The

neonatal rat diaphragm was fatigued at 30 Hz and the adult rat diaphragm was fatigued at 60 Hz; the neonatal and adult diaphragm produced 95 – 100 % of maximum force at 30 Hz and 60 Hz, respectively. All contractile properties were examined using ASI Dynamic Control Data Analysis Program (Aurora Scientific). Typical traces of a twitch contraction, sub-maximal contraction, and maximal contraction are presented in Figure 8-1.

Specific force, force (g) normalised to CSA ($N \cdot cm^{-2}$), was calculated to account for size variation of the diaphragm strips. CSA (mm^2) was calculated from L_o (mm), muscle mass (g) and muscle density (1.056 $g \cdot cm^{-3}$) (Mendez & Keys 1960). L_o was measured using digital calipers and the diaphragm strip was weighed after ribs, central tendon and ligament was removed.

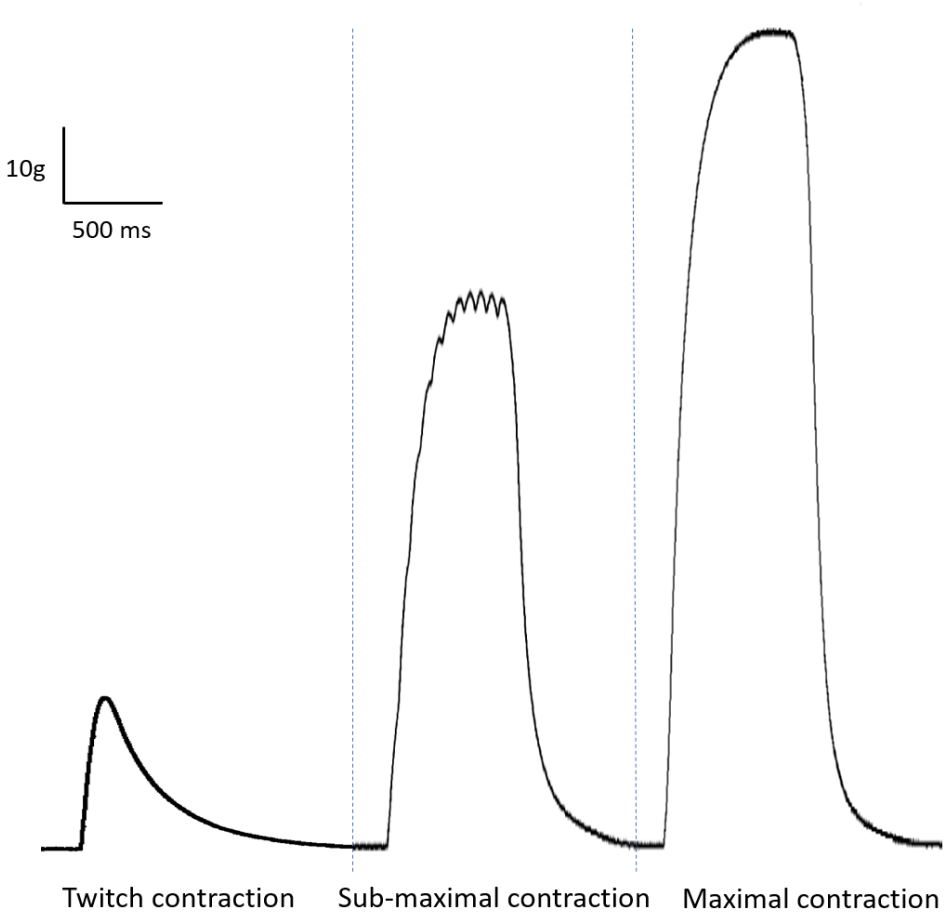


Figure 8-1. Representative traces of a twitch contraction, sub-maximal contraction (20 Hz), and maximal contraction (80 Hz) of a diaphragm strip from a saline control preterm lamb.

8.1.4 Histochemistry

Myosin heavy chain (MHC) fibre typing

Transverse (8 µm) sections were cut from tragacanth imbedded, frozen diaphragm samples using a cryostat microtome (Leica Biosystems CM3050S) and adhered to Superfrost® Plus slides (Menzel-Gläser). Sections were stained for MHC_{emb}, MHC_{IIabx}, MHC_{IIa}, and MHC_{I} expression. Anti-myosin antibodies specific to MHC_{I} (mouse monoclonal IgG2b, BA-D5, supernatant) and MHC_{IIa} (mouse monoclonal IgG1, SC-71, supernatant) were purchased from Developmental Studies Hybridoma Bank (DSHB, University of Iowa). Anti-myosin antibodies specific to MHC_{emb} (rabbit polyclonal IgG) MHC_{IIabx} (rabbit polyclonal IgG) were developed by our laboratory and produced by Mimotopes. The development and validation of MHC_{emb} and MHC_{IIabx} antibodies are outlined in Appendix II. MHC neonatal expression was not evaluated due to the unavailability of a suitable antibody.

Two sections cut from each muscle were incubated with blocking solution (phosphate-buffered saline; normal goat serum, 10 %; bovine serum albumin, 0.5 %) for 1 h at room temperature, then briefly washed twice with phosphate-buffered saline for 2 min. The first section from each muscle was incubated with a solution of primary antibodies (dilution 1:25) to MHC_{emb}, MHC_{I} and MHC_{IIa} in phosphate-buffered saline containing 0.5 % bovine serum albumin for 2 h at room temperature. The second section from each muscle was incubated with a solution of primary antibodies (dilution 1:25) to MHC_{I}, MHC_{IIa} and MHC_{IIabx} in phosphate-buffered saline containing 0.5 % bovine serum albumin for 2 h at room temperature. After 3 washes (5 min each) with phosphate-buffered saline, all sections were incubated with a cocktail of anti-mouse IgG2b dylight 405 (Jackson ImmunoResearch; dilution 1:500) specific for the MHC_{I} primary antibody, anti-mouse IgG1 Alexa Fluor 488 (Jackson ImmunoResearch; dilution 1:500) specific for the MHC_{IIa} primary antibody; and anti-rabbit IgG Alexa Flour 594 (Invitrogen; dilution 1:500) specific for the MHC_{emb} and MHC_{IIabx} primary antibodies, in blocking solution for 1 h at room temperature. After 3 washes (5 min each) with miliQ water, sections were mounted with hydromount™ (Electron Microscopy Sciences).

As antibody staining was performed in multiple rounds, one muscle (control) was repeated in each round to confirm staining consistency. Two sections of the control muscle were stained in each round, the first section was incubated with primary antibodies to MHC_{emb}, MHC_{I} and MHC_{IIa}, and the second section was incubated with primary antibodies to MHC_{I}, MHC_{IIa} and MHC_{IIabx}. A control incubation with no primary antibodies was also performed in each round.

Tiled images were captured at 20x using a C2+ Confocal microscope system (Nikon Instruments Inc.) and NIS-Elements BR 4.1 software.

Myofibre cross-sectional area (CSA)

Transverse (8 µm) sections were cut from tragacanth imbedded, frozen diaphragm samples using a cryostat microtome (Leica) and adhered to Superfrost® Plus slides (Menzel-Gläser). Sections were stained with H&E to quantify myofibre CSA. Tiled images were captured at 20x using a Nikon Eclipse Ti inverter microscope (Nikon Corporation).

Image analysis

Fibre type proportions were determined by counting the fibres which stained positively for each antibody and dividing by the total number of fibres counted. The CSA of each MHC fibre type was determined by matching the H&E images with images of serial sections stained for MHC expression. Fibre proportions and CSA were analysed manually using ImageJ (v1.51j8) software and ImageJ cell counter plugin (Abramoff, Magalhaes & Ram 2004). Over 900 fibres were counted and over 500 fibres were measured for CSA, per animal. Areas affected by freeze fracturing were excluded from analyses.

8.2 Appendix II: Antibody Validation

Polyclonal antibodies (IgG) specific to sheep myosin heavy chain embryonic (MHC_{emb}) and sheep myosin heavy chain fast (MHC_{IIabx}) were designed by our laboratory and produced by Mimotopes, as they were not commercially available at the time of the study. MHC_{emb} epitope was produced from a 14-aa peptide (sequence: DFTS SRMVV HESEE) corresponding to amino acids 1927 – 1940 (Genbank sequence ID. XP_011977815.1). MHC_{IIabx} epitope was produced from a 10-aa peptide (sequence: EVHT KIISE E) corresponding to amino acids 1937 - 1947 (Genbank sequence ID. XP_011977812.1). MHC protein sequences were retrieved using Genbank and compared using Clusterw. An N-terminal cysteine was added and conjugated with keyhole limpet haemocyanin and 6-maleimidocaproyl acid N-hydroxysuccinimide ester to each epitope. Two rabbits were then immunised using a combination of complete Freund's adjuvant and incomplete Freund's adjuvant for each conjugate. Polyclonal antibodies against MHC_{emb} and MHC_{IIabx} were affinity purified, and subsequently analysed for purity (high performance liquid chromatography), identity (mass spectrometry) and epitope binding capability (enzyme-linked immunosorbent assay).

Antibody specificity to the MHC of interest was determined using western blot analysis (Figure 8-2). Antibody specificity to MHC_{emb} was demonstrated by the presence of a single band at the expected molecular weight of MHC (223 kDa) in diaphragm samples from a 7 d old lamb and a 2 m old lamb after premature birth at 129 d GA (term = 145 - 150 d GA). The MHC_{emb} antibody stained more protein in the 7 d old lamb sample compared with the 2 m old lamb sample. The antibody to MHC_{IIabx} was deemed specific based on the presence of a dominant band at the expected molecular weight of MHC (223 kDa) in diaphragm samples from a 7 d old lamb and a 2 m old lamb.

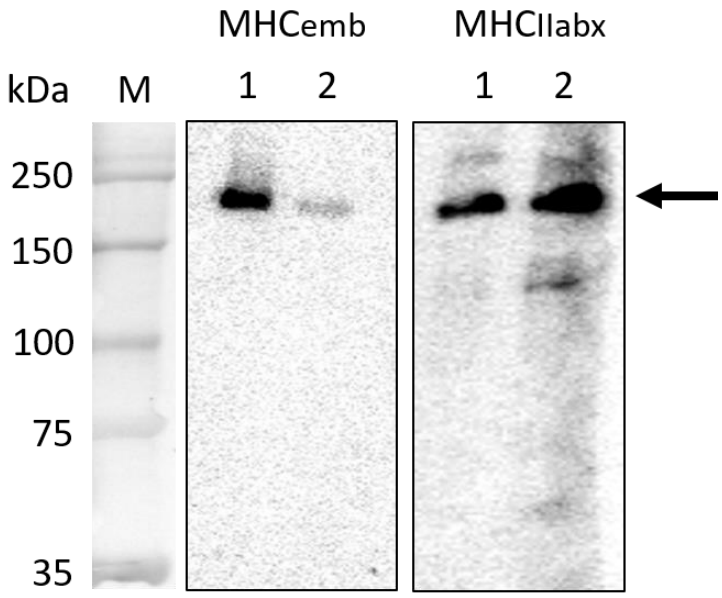


Figure 8-2. Representative blots of (1) 7 d old lamb diaphragm muscle and (2) 2 m old lamb diaphragm muscle with antibody staining against MHC_{emb} and MHC_{IIabx}. Lambs were delivered at 129 d GA (term = 145 - 150 d GA).

8.2.1 Methods: Muscle protein extraction and Western blot

Left hemidiaphragm muscle from a 7 d old lamb and 2 m old lamb, each delivered at 129 d GA (term = 145 – 150 d GA), were snap frozen in liquid nitrogen for analysis of antibody validity. Frozen muscles were crushed using a mortar and pestle under liquid nitrogen and homogenised in ice-cold lysis buffer containing 50 mM Tris, 150 mM NaCl, 1 % NP-40, 2 % SDS, and protease inhibitor cocktail tablet (Roche). Samples were centrifuged and the total protein content of suspensions were measured by DC protein assay, using the manufacturer's (Bio-Rad) instructions. Equal amounts of total protein (2µg for MHC_{emb} and 5µg for MHC_{IIabx} antibodies) were separated by a 4 -15 % SDS-PAGE TGX gel (Bio-Rad) and transferred to a nitrocellulose membrane (Bio-Rad) using a Trans Turbo Blot system (Bio-Rad). After blocking for 1.5 h with Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) with 0.1 % Tween-20 (TBST), containing 5 % non-fat dry milk, the membrane was incubated overnight with primary antibodies against MHC_{emb} or MHC_{IIabx} at 4ºC. MHC_{emb} and MHC_{IIabx} antibodies were used at 1:200 of 1 mg/mL stock (Mimotopes),

diluted in TBST. Bound antibodies were detected with HRP-conjugated anti-rabbit IgG (Invitrogen) (1:2000 in TBST, incubated at 25 °C for 1 h). The blots were developed with Luminata Classico Western horseradish peroxidase substrate (Millipore) and scanned using the ChemiDoc MP Imaging System (Bio-Rad).

8.3 Appendix III: Comparison of the diaphragm contractile properties of high dexamethasone lambs with 96 h mandatory MV and high dexamethasone lambs with CPAP at 7 d PNA

Diaphragm contractile measurements including maximum specific force, force-frequency relationship, twitch characteristics, peak twitch to maximum specific force ratio and the fatigue index were not significantly different between the three high dexamethasone lambs whom received 96 h mandatory mechanical ventilation before weaning and the four high dexamethasone lambs whom were weaned as soon as possible (Table 8-1; Figure 8-3).

Table 8-1. Diaphragm contractile properties of lambs assigned to high dexamethasone with mechanical ventilation (High DEX + MV) and high dexamethasone with non-invasive mechanical ventilation (High DEX + CPAP).

	High DEX + MV (n = 3)	**High DEX + CPAP (n = 4)**
PCA (d)	136.7 ± 0.6	136.0 ± 0.8
Sex ratio (M:F)	2:1	2:2
Body wt (kg)	3.08 ± 0.46	2.36 ± 0.12
P_o ($N \cdot cm^{-2}$)	14.96 ± 4.46	19.10 ± 2.31
P_t ($N \cdot cm^{-2}$)	3.21 ± 2.01	5.89 ± 1.49
TTP (ms)	165 ± 13	136 ± 33
½ RT (ms)	228 ± 123	198 ± 29
FI	0.48 ± 0.09	0.47 ± 0.11

P_o, maximum specific force; P_t, peak twitch force; TTP, time to peak twitch force; ½ RT, half relaxation time; FI, fatigue index. Values represent mean ± SD.

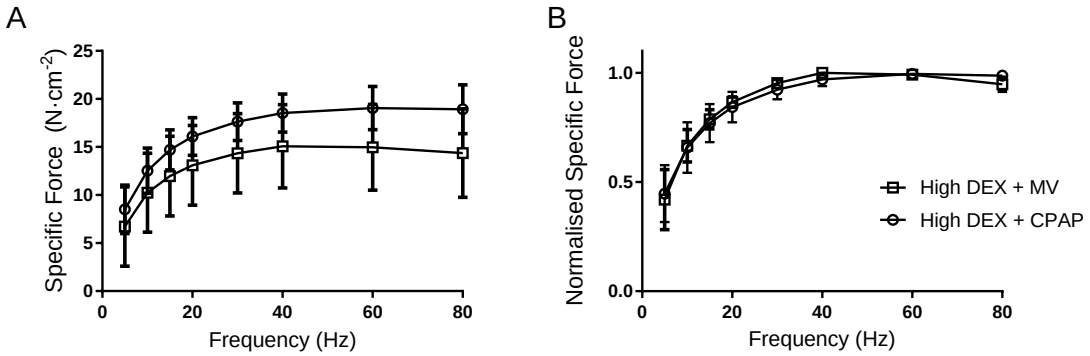


Figure 8-3. Force-frequency relationships of lambs assigned to high dexamethasone with mechanical ventilation (High DEX + MV) and high dexamethasone with non-invasive mechanical ventilation (High DEX + CPAP).

(A) Force is displayed as specific force ($N \cdot cm^{-2}$) and (B) normalised to maximum specific force. Values represent mean ± SD; n = 3 for the high DEX + MV group, n = 4 for the high DEX + CPAP group.

8.4 Appendix IV: Manuscripts in preparation and published

Manuscript in preparation

Manuscript title: Functional adaptations of the preterm lamb diaphragm in the first week of life are not influenced by *in utero* lipopolysaccharide exposure.

Student contribution to work: The candidate is the lead first author of this experimental paper. The candidate conducted the *in vitro* whole muscle contractile experiments and immunohistochemistry experiments. The candidate took the lead role in data analysis, data interpretation, writing and editing of the manuscript.
Location of student contribution in thesis: Chapter 3

Published manuscript

Manuscript title: Optical coherence tomography-based indentation of diaphragm mechanics in a murine model of transforming growth factor alpha induced lung disease.
Student contribution to work: The candidate is second author of the experimental paper. The candidate conducted the *in vitro* whole muscle contractile experiments and histochemistry experiments, and contributed to manuscript revision.

Manuscript in preparation

Functional adaptation of the preterm lamb diaphragm in the first week of life is not influenced by *in utero* lipopolysaccharide exposure

Chrissie J. Astell[a1], Tanzila Mahzabin[a1], Jenny Lam[1], Robert B. White[1], Anthony J. Bakker[1], Siavash Ahmadi-Noorbakhsh[1], Peter B. Noble[1], J. Jane Pillow[b1,2] and Gavin J. Pinniger[b1]

[1]School of Human Sciences and [2]Centre for Neonatal Research and Education, Division of Paediatrics and Child Health, Medical School, The University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia.

[a] joint first authors

[b] joint senior authors

Running title: Antenatal and postnatal impacts on preterm diaphragm function

Keywords: inflammation, respiratory function, diaphragm, preterm infant, respiratory distress syndrome

Correspondence: Gavin J. Pinniger, School of Human Sciences, The University of Western Australia, 35 Stirling Highway, M309, Crawley 6009, Western Australia. Email: gavin.pinniger@uwa.edu.au . Phone: +61 8 6488 3380. Fax: +61 8 6488 1025

Abstract

In utero inflammation exacerbates preterm diaphragm weakness at birth. Persistent diaphragm dysfunction, resulting from immaturity and *in utero* inflammation, may predispose preterm infants to postnatal respiratory failure. We determined the combined effects of preterm birth and *in utero* inflammation on postnatal diaphragm function in lambs. Lambs were exposed to intra-amniotic saline (N = 8) or 4 mg lipopolysaccharide (LPS; N = 9) 48 h before caesarean delivery at 129 d gestational age (GA, term = 150 d). Lambs were managed in an intensive care environment in accordance with routine clinical practice then killed at 7 d postnatal age (PNA; 136 d GA). Fetal control lambs were delivered at 136 d GA and killed immediately (N = 7). There were no significant differences in diaphragm contractile function, myosin heavy chain fibre characteristics, molecular markers of inflammation, protein metabolism or oxidative status, between saline and LPS groups. However, the contractile properties of the saline and LPS groups were significantly different to the fetal control group, indicating postnatal adaptations to extra-uterine life. Duration of mechanical ventilation and nutritional intake were important predictors of diaphragm function and structure at 7 d PNA. Importantly, LPS lambs had a greater reliance on mechanical ventilation than saline lambs. Although LPS-induced diaphragm weakness at birth did not persist at 7 d PNA, the increased reliance on mechanical ventilation after *in utero* inflammation highlights potential interactions between clinical complications associated with preterm birth that may affect diaphragm function and contribute to the development of respiratory failure during early postnatal life.

Introduction

A functional respiratory system is essential for successful transition to independent gas exchange after birth. The underdeveloped and surfactant deficient respiratory system of the extremely preterm infant is inadequately prepared for this transition, resulting in development of respiratory distress syndrome soon after birth. Additionally, preterm infants without significant respiratory disease at birth may develop progressive respiratory failure over the first week of life (Eber & Zach, 2001; Bland, 2005). Accordingly, many preterm infants require mechanical respiratory support: recent data suggest at least 50 % of infants born earlier than 30 weeks' gestation require intubation and mechanical ventilation (Sweet et al., 2017).

Until recently, respiratory disease in preterm infants was considered primarily a disease of lung immaturity. Consequently, the contribution of the diaphragm to respiratory disease in preterm infants has garnered little attention, despite the pivotal role of the diaphragm in spontaneous breathing. Like the lungs, the preterm diaphragm is structurally and functionally immature at birth (Keens et al., 1978; Dimitriou et al., 2001, 2003). Appropriate development of the diaphragm *in utero* is vital for establishing and sustaining spontaneous breathing from the time of birth (Mantilla & Sieck, 2008). The newborn diaphragm needs to generate sufficient trans-pulmonary pressure to overcome the high surface tension of the newborn lungs, thereby facilitating resorption of fetal lung fluid and establishment of a functional residual capacity (te Pas et al., 2008). Thereafter the diaphragm must continuously produce adequate trans-pulmonary pressures to sustain spontaneous breathing throughout postnatal life (te Pas et al., 2008).

Preterm birth disrupts normal diaphragm development: the immature diaphragm of the preterm infant is characterised by small muscle fibres (Sieck et al., 1991a, 1991b; Fratacci et al., 1996), which have underdeveloped sarcoplasmic reticulum (Schiaffino & Margreth, 1969; Maxwell et al., 1983), underdeveloped myofibrils (Williams & Goldspink, 1971; Maxwell et al., 1983; West et al., 1999; Geiger et al., 2001; Orliaguet et al., 2002) and a reduced antioxidant capacity (Song & Pillow, 2012). Consequently, the force-generating capabilities of the

preterm diaphragm are reduced (Dimitriou et al., 2001, 2003) and the diaphragm must operate at a greater proportion of its maximum capacity to achieve adequate ventilation. The impact of preterm birth on the capacity of the diaphragm to initiate and sustain independent ventilation after birth is unclear.

Adverse antenatal and postnatal complications can further exacerbate respiratory dysfunction of the preterm infant. For example, chorioamnionitis, an inflammation of the fetal membranes, is a histological finding in 70 % of preterm births before 30 w gestational age (GA) (Goldenberg et al., 2000); the group most likely to develop early respiratory failure and chronic respiratory illness. Antenatal exposure of the fetus to inflammation alters postnatal organ development (Galinsky et al., 2013). The effects of antenatal inflammation on the fetal respiratory system are complex with initial acceleration of lung development contributing to decreased incidence of respiratory distress syndrome (Watterberg et al., 1996), but increasing the risk of long-term respiratory disorders such as bronchopulmonary dysplasia (Bose et al., 2011). The impact of *in utero* inflammation on diaphragm function in preterm infants is largely unknown. However, using an ovine model of chorioamnionitis, we have shown that *in utero* LPS exposure further compromises the integrity of the already weakened preterm diaphragm (Song et al., 2013a; Karisnan et al., 2015a, 2015b, 2017).

A short duration exposure to intra-amniotic (IA) lipopolysaccharide (LPS) at 2 d or 7 d before premature delivery at 129 d GA (term = 145 – 150 d GA) significantly impairs diaphragm development and function in the sheep, reducing fetal diaphragm contractile strength by ~30 % at birth (Song et al. 2013). The LPS-induced diaphragm weakness at birth results from a transient increase in local and systemic pro-inflammatory cytokines (interleukin-1 (IL-1) and interleukin-6 (IL-6)) and is associated with an upregulation of proteolytic pathways mediated by NF-kB, followed by a secondary reduction in protein synthesis (Song et al. 2013). Importantly, when IA LPS injections were administered 21 d before premature birth the level of diaphragm weakness was similar to that when the LPS exposure occurred 7 d before birth, despite the absence of any overt differences in the level of inflammation (Karisnan et al.,

2015a). These findings indicate *in utero* inflammation severely compromises diaphragm development. The combined effects of preterm birth and persistent diaphragm weakness resulting from *in utero* exposure to inflammation may contribute to the development of respiratory insufficiency over the first minutes, hours or days of life of the preterm infant. However, the impact of *in utero* LPS exposure on postnatal diaphragm function is still unknown.

Using an established ovine model of chorioamnionitis (Berry et al., 2011; Kuypers et al., 2012), we investigated the combined effects of preterm birth and a 2 d IA LPS exposure on diaphragm function and structure in lambs delivered at 129 d GA and raised to 7 d postnatal age (PNA). Furthermore, we aimed to identify the mechanisms underlying any potential changes in postnatal diaphragm structure and function. We hypothesised that *in utero* LPS exposure would promote structural and physiological changes which would exacerbate diaphragm dysfunction at 7 d PNA after preterm birth. We predicted that the additional dysfunction of the preterm diaphragm imposed by *in utero* exposure to a pro-inflammatory stimulus would reduce the capacity of the preterm diaphragm to cope with the strenuous demands of spontaneous breathing after birth.

Methods

Ethical Approval

Ewes were bred at the University of Western Australia (UWA) Ridgefield Farm. All experiments were performed at the University of Western Australia (UWA), with approval by the UWA Animal Ethics Committee (3/100/1301). All experiments conform to the guidelines of the National Health and Medical Research Council (NHMRC) Australian code for the care and use of animals for scientific purposes (2013) and the principles and regulations as described in the Editorial by Grundy (Grundy, 2015).

Experimental outline and animal management

Antenatal management

Date-mated ewes, pretreated with intra-muscular (IM) medroxyprogesterone (150 mg; Pfizer) at 122 d gestation, were assigned randomly to an ultrasound guided IA injection of LPS in saline (LPS group; 4 mg, 2 mg/mL; E. coli 055:B5; Sigma Aldrich, USA; n = 9) or an equivalent volume IA saline injection (saline group; n = 8) at 127 d gestation. Ewes also received two doses of IM betamethasone (5.7 mg, Merck Sharp & Dome, USA) given at 24 h intervals, commencing 6 h after the IA injections, prior to caesarean section delivery at 129 d gestation of preterm lambs destined for 7 d postnatal care. Ewes were pre-medicated with IM Acepromazine (0.05 mg/kg, Ceva Animal Health Pty Ltd, Australia) and IM Buprenorphine (0.01 mg/kg, Indivior Pty Ltd, Australia) prior to anaesthetic induction with Thiopentone (15 mg/kg, Troy Laboratories, Australia), and maintenance anaesthesia with isofluorane (1.5-2.5 %). The fetal head and neck of saline and LPS lambs were exteriorised for insertion of carotid arterial and jugular venous catheters, oral intubation, and removal of lung fluid and administration of intratracheal surfactant (Curosurf®, 100 mg/kg; Chiesi Farmaceutici S.p.A., Italy). Lambs were then delivered. Fetal control lambs (N = 7; no antenatal maternal interventions) were delivered at the study endpoint (136 d gestation) and killed at delivery without intervention (pentobarbitone, 150 mg/kg IV; Pitman-Moore, Australia). IA LPS injection was confirmed by a Limulus amebocyte lysate (LAL) assay on amniotic fluid samples obtained at delivery, in accordance with the manufacturers protocol (LAL QCL-1000; Lonza).

Postnatal management

Lambs were resuscitated immediately after delivery and were managed in accordance with routine clinical practice. Assist/control volume-guaranteed ventilation (5 – 7 mL/kg) was commenced immediately following delivery and changed to mandatory minute ventilation after commencement of spontaneous respiratory efforts. Ventilatory settings (volume guarantee, positive end-expiratory pressure, fractional inspired oxygen, inspiratory time and respiratory

rate) were adjusted to target mild permissive hypercapnia (partial pressure of carbon dioxide in arterial blood (PaCO2) of 45 – 55 mmHg) and peripheral capillary oxyhaemoglobin saturation (SpO2) of 90 – 95 %. Maximum peak inspiratory pressure was 30 cmH2O. Lambs were weaned from mechanical ventilation if they were breathing spontaneously and maintaining SpO2 of 90 – 95 % at a peak inspiratory pressure < 15 mmHg and FiO2 < 0.25. Lambs were reintubated if they displayed any of the following: persistent SpO2 < 90 % despite FiO2 > 0.5 and mean airway pressure > 12 cmH2O; ≥ 4 apnoea events requiring resuscitation within an hour; ventilatory failure (PaCO2 > 80 mmHg on two consecutive arterial gases); or severe metabolic acidosis (base excess (BE) < -10 mmol/L). Arterial blood pressure was monitored continuously and ionotropic support (dopamine 10 µg/kg/min) commenced for sustained mean pressure < 45 mmHg.

Lambs received intravenous fluid support (5 % dextrose) and commenced enteral feeds at 2 h (20 mL/kg/d) with fresh maternal colostrum, incrementing enteral feeds by 20 mL/kg/d and transferring to formula feeds at 24 h (MaxCare® Lamb & Kid, MaxumAnimal, Australia). Supplemental heating was adjusted to target normothermia (38.5-39.5 □C) throughout postnatal life. Saline and LPS lambs were killed at 7 d (pentobarbitone, 150 mg/kg IV; Pitman-Moore, Australia).

Tissue collection

The diaphragm was excised immediately and the mid-costal portion of the right hemi-diaphragm, including the attached ribs and central tendon, was maintained in mammalian Ringer's solution (in mM; NaCl, 121; KCl, 5.4; MgSO47H2O, 1.2; NaHCO3, 25; HEPES, 5; glucose, 11.5 and CaCl2, 2.5: pH 7.3) bubbled with carbogen (5 % CO2 in O2) throughout careful dissection of muscle fibres for use in measurements of contractile function. Sections of the mid-costal portion of the left hemi-diaphragm were imbedded in tragacanth gum and frozen in isopentane cooled in liquid nitrogen for histochemical analysis, and snap-frozen in liquid nitrogen for molecular analysis. Frozen samples were stored at -80 ºC until further analysis.

Whole muscle diaphragm contractile properties

Diaphragm contractile measurements were performed according to Lavin et al (2013) with modifications. Briefly, a longitudinal strip of intact muscle fibres were mounted in an *in vitro* muscle test system (1200A, Aurora Scientific, Aurora, Canada) containing mammalian Ringer's solution (pH 7.3) bubbled with carbogen at 25 ºC (Segal & Faulkner, 1985). Diaphragm contractile properties were measured at optimal muscle length (Lo) and included maximum tetanic force (Po), force-frequency relationship, peak twitch force (Pt), time to peak twitch force (TTP), twitch half-relaxation time (1/2 RT). Susceptibility to fatigue was evaluated from a series of 150 tetanic contractions of 330 ms duration repeated once every second. The fatigue index (FI) was determined from the ratio of the force produced during the 150th contraction relative to the 1st contraction (Javen et al., 1996), in which a higher number indicates a greater fatigue resistance. Force (g) was normalised to cross-sectional area (CSA) calculated from muscle fibre length, muscle mass and density (1.056 g·cm-3) (Mendez & Keys, 1960) and presented as specific force (N·cm-2).

Skinned fibre contractile function

Individual fibres were dissected from the diaphragm, mounted on a force transducer (model BAM4C, SI Heidelberg, Germany) at 120 % resting length and chemically skinned by exposure to Triton X-100 for 12 min. All experiments were undertaken at room temperature (22-24 °C). The force-pCa relationship was determined by exposing skinned fibres to a sequence of highly buffered Ca^{2+}-EGTA solutions of different known free Ca^{2+} concentrations. The Ca^{2+} buffered solutions were prepared by mixing different proportions of solution A and B (Table 1) to produce solutions with pCa in the range 7.00-4.70. Broad fibre type classifications were determined by exposing fibres to a sequence of Sr^{2+}-EGTA buffered solutions of different known Sr^{2+} concentrations with pSr in the range 6.37-3.70. Maximum Ca^{2+}-activated force was normalised to fibre cross-sectional area (estimated from fibre diameter measurements), and presented as specific force (mN·cm-2).

The Ca^{2+}- and Sr^{2+}-activated force responses were expressed as a percentage of their respective maximum Ca^{2+}- or Sr^{2+}-activated force and plotted as a function of pCa or pSr. The analysis of force-pCa and force-pSr curves have been described previously (Bortolotto et al., 2000; Cannata et al., 2010, 2011). The data collected from pCa/pSr experiments were fitted with sigmoidal curves using the software package GraphPad Prism (GraphPad Software, Inc., San Diego). Force-pCa curves were always described by a single sigmoid function and the parameters derived were the Hill coefficient (hCa) (maximum slope of the force-pCa curve) pCa10 and pCa50 (the amount of Ca^{2+} needed to produce 10 % and 50 % of maximum force, respectively).

Fibres in which the force-pSr relationship could be described by a single sigmoid function were classified as 'fast' or 'slow' based on the difference in their relative sensitivity to Ca^{2+} and Sr^{2+} (West et al., 1999; Cannata et al., 2011). Fibres with pCa50 – pSr50 > 1.0 unit were classified as fast, and fibres with pCa50 – pSr50 < 1.0 unit were classified as slow. Fibres that could not be described by a single sigmoid function showed discontinuity in the central region and were better described as the sum of two sigmoid curves (F1 and F2). When pSr curves were best described by a double sigmoid, separate parameters of pSr50 and hsr were derived for the first and second sigmoid curves (pSr501, pSr502 and hSr1, hSr2) along with F1% (the proportion of the entire force-pSr curve described by the first sigmoid curve). These fibres were categorised as intermediate (pSr501 < 5.90, hSr1 < 2.0) or hybrid (pSr501 > 5.90, hSr1 > 2.0), based on classifications of diaphragm muscle fibres described by Cannata et al. (2011).

Immunohistochemistry: Myosin heavy chain (MHC) fibre typing and cross-sectional area (CSA)

Transverse sections (8 µm) of frozen muscle were stained with anti-myosin heavy chain (MHC) specific antibodies: MHCIIa (mouse monoclonal IgG1; SC-71; DSHB, University of Iowa; dilution 1:25), MHCI (mouse monoclonal IgG2b; BA-D5; DSHB, University of Iowa; dilution 1:25), MHC embryonic (MHCemb) (rabbit polyclonal IgG; 2400104; Mimotopes; dilution 1:25) and MHC fast (MHCIIabx) (rabbit polyclonal IgG; 2400107; Mimotopes; dilution 1:25). Primary

MHCIIa was detected by goat anti-mouse IgG1 Alexa Fluor 488 (Jackson ImmunoResearch; dilution 1:500), MHCI was detected by goat anti-mouse IgG2b dylight 405 (Jackson ImmunoResearch; dilution 1:500), and MHCemb and MHCIIabx were detected by anti-rabbit IgG Alexa Fluor 594 (Invitrogen; dilution 1:500). Tiled images were captured at 20x magnification using a Nikon C2+ Confocal microscope (Nikon Corporation). Myofibre CSA was determined from haematoxylin and eosin (H&E) stained sections with tiled images captured at 20x using a Nikon Eclipse Ti inverter microscope (Nikon Corporation) and image analysis performed using ImageJ (v1.51j8) software and ImageJ cell counter plugin https://imagej.nih.gov/ij/plugins/cell-counter.html).

Molecular analyses

RNA Isolation, Reverse Transcription and Quantitative PCR

RNA purification, reverse transcription and quantitative PCR conditions were performed according to Mahzabin et al (2017). Diaphragm gene expression was assessed for atrophy-related genes (muscle ring-finger protein 1 (MuRF1) and muscle atrophy F-box (Atrogin-1/MAFbx), cytokine genes (interleukin-10 (IL-10), interleukin-1β (IL-1 β), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α)), antioxidant genes (glutathione peroxidase 1 (GPX1), and superoxide dismutase 1 (SOD1)), myogenic regulatory factors (MRF) genes (paired box (PAX3, PAX7), myogenic factor 5 (MYF5), myogenic differentiation (MYOD and MYOG) and MHC genes (MHCemb, MHCI and MHCIIa). Novel primers are listed in Table 2; all other primers were described previously (Smeed et al., 2007; Zhu et al., 2010; Song & Pillow, 2012; Mahzabin et al., 2017). Each primer set (total 19) was validated by generating standard curves from serial, 10-fold, dilutions of purified cDNA using Wizard SV gel and PCR clean up system (Promega, Madison, WI, USA). The fluorescent signal of samples, amplified from qPCR reaction, are presented as arbitrary concentrations normalised against housekeeping genes (18S rRNA, GAPDH and β-actin) using GeNorm algorithm (Vandesompele et al., 2002).

Total Protein Extraction

Total cellular protein was extracted from 30 mg frozen diaphragm tissue according to Song & Pillow (2012). Protein concentration was measured in whole cell lysates using the Bradford method (Bradford, 1976). Whole cell lysates were used for Western Blot and biochemical assays.

Western Blot

Total protein lysates (50 mg) were separated by electrophoresis using pre-cast 4-15 % TGX stain free gels (Bio-Rad, Gladesville, NSW, Australia) and transferred to nitrocellulose membranes using a Trans Turbo Blot system (Bio-Rad). Ponceau S staining (P-7170-1L, Sigma) was used to check the quality of protein transfer as well as a loading control. After blocking with TBST (20 mM Tris, 150 mM NaCl, 0.1% v/v Tween-20, pH 7.5) containing 5% w/v bovine serum albumin (BSA), the membranes were incubated overnight with primary antibodies (Cell Signaling Technology, Carlsbad, CA, USA) against protein kinase B, Akt {p-Akt (Ser473) (#9271); t- Akt (#9272)}, mammalian target of rapamycin, mTOR{ p-mTOR (Ser 2448) (#2971); t-mTOR (#2983)}; translation initiation factors 4e-binding protein 1, 4EBP1{ p-4E-BP1 (Thr70) (#9455), t- 4E-BP1 (#9452)} at 4 ºC. Antibodies were used at a dilution of 1:1000. Bound antibodies were detected with anti-rabbit immunoglobulin conjugated with horseradish peroxidase at 1:3000 dilution (Cell Signaling Technology) for 1 h at room temperature (22 - 24 °C). The blots were developed by adding Perkin Elmer Western Lightning ultra (NEL111001EA) and protein signals were quantified using ChemiDoc MP Imaging system (Bio-Rad). The membranes blotted with phosphorylated proteins (P) were stripped with Restore Western Stripping Buffer (Life Technologies, VIC, Australia, #2105) for 10 min at room temperature, and re-blotted with corresponding total (T) proteins. The protein expression data are presented as normalised values for phosphorylated protein against total protein content of the same blot.

Biochemical assays of proteolysis and oxidative stress

The chymotrypsin like peptidase activity of the 20S proteasome (a major component of ubiquitin proteasome pathway, UPP) in the diaphragm was

measured fluorometrically in crude extracts following the release of free 7-amido-4-methylcoumarin (AMC) from synthetic peptide (Suc-LLVYAMC) substrates (BML-AK 740 assay kit, Enzo Life sciences, Farmingdale, NY).

The protein carbonyl content (an indicator of cellular oxidation status) was measured using a commercially available colorimetric kit (Protein Carbonyl Colorimetric assay kit, Cayman, Ann Arbor, MI).

Statistical analysis

Differences among treatment groups were assessed with one-way analysis of variance (ANOVA) with a priori Tukey multiple comparisons or Kruskal-Wallis H Test with a priori Mann-Whitney U tests, as appropriate. Mann-Whitney U tests were Bonferroni adjusted, with statistical significance accepted at $p \leq 0.017$. The force frequency relationships were analysed using 2-way ANOVA. Independent one-way ANOVA with Tukey multiple comparisons or nonparametric Kruskal-Wallis with Mann-Whitney U tests were performed, as appropriate, where an interaction between both factors was detected with 2-way ANOVA Wilks Lambda. Myofilament function in hybrid skinned muscle fibres were analysed by unpaired Students t-test.

The outcome measures of diaphragm function may be affected by potential confounding variables associated with the postnatal management of preterm lambs including exposure to mechanical ventilation and nutritional status. The relationship between potential confounding variables and outcome variables was analysed using Pearson's and Spearman's bivariate correlation analyses, as appropriate. Outcome variables that correlated significantly with the confounding experimental variables were analysed via linear regression and ANCOVA analyses, as appropriate.

Data are presented as mean ± standard deviation (SD) or as median (range). N denotes number of animals, n denotes number of diaphragm fibres. Statistical significance was accepted at $p \leq 0.05$, unless otherwise stated. Analyses were performed on IBM SPSS Statistics software (Version 19, IBM Company 2010).

Results

Lamb descriptive data

Lamb post-conceptual age at post-mortem (PM), body weight at PM, sex, pregnancy ratio and optimal muscle length for all groups are presented in Table 3. Body weight at birth is presented for saline and LPS groups in Table 3. Body weights at birth were corrected for amniotic fluid in the wool (dry body weight = 0.9 wet body weight). Body weight at PM was significantly lower in the saline ($p = 0.004$) and LPS ($p < 0.001$) groups compared with the fetal control group (dry weight equivalent).

In vitro whole muscle contractile properties

There were significant differences in diaphragm whole muscle contractile function between the fetal control group compared with the saline and LPS groups, but not between the saline and LPS groups (Figure 1).

Maximum specific force (Po) was significantly higher in the saline group compared to the fetal control group ($p = 0.007$). Peak twitch force (Pt) was significantly lower in the saline group ($p = 0.003$) and LPS group ($p < 0.001$) compared with the fetal control group. TTP was significantly shorter in the saline and LPS groups compared with the fetal control group ($p < 0.001$). Similarly, ½ RT was significantly shorter in the saline group ($p = 0.01$) and LPS group ($p < 0.001$) compared with the fetal control group. Peak twitch force as a ratio of maximum tetanic force (Pt:Po) was significantly lower in the saline and LPS groups compared with the fetal control group ($p < 0.001$). The fatigue protocol reduced the force response to 40 Hz stimulation by approximately 50 – 55 %. There was no significant difference in the fatigue index between groups ($p = 0.393$).

The relationship between stimulation frequency and force production was investigated from isometric contractions at 5-80 Hz, as displayed in Figure 1G. There was a significant interaction between treatment group and stimulation frequency ($p = 0.014$). The saline and LPS groups produced significantly lower specific force at 5 Hz stimulation, and the LPS group also produced significantly

lower specific force at 10 Hz stimulation, compared to the fetal control group ($p < 0.05$). Additionally, the saline group produced significantly higher specific force at stimulation frequencies 30 Hz and above compared to the fetal control group ($p < 0.05$).

There was a significant interaction between treatment group and stimulation frequency when the force-frequency relationships were normalised to maximum force produced within each group ($p < 0.001$; Figure 1H). The normalised force-frequency relationship was shifted to the right in the saline and LPS groups, producing significantly lower relative force at stimulation frequencies of 5-40 Hz, compared to the fetal control group ($p < 0.05$).

Skinned fibre contractile properties

The Ca^{2+}- and Sr^{2+}- activation parameters for single muscle fibres are presented in Table 4. All force-pCa curves were described as single sigmoid curves (Figure 2). The maximum Ca^{2+}-activated force was not significantly different between groups for fast, intermediate or slow fibres, but was significantly lower in hybrid fibres from the saline group compared to the fetal control group (unpaired Students t-test; $p = 0.004$; Table 4). There were no significant differences in the pCa10, pCa50 or hCa in fibres between the three groups (Table 4). The force-pSr curves were classified as normal, intermediate or hybrid based on Sr^{2+}-activation parameters that govern the shape of the force-pSr curve (Figure 1B-D. A total of 82 fibres were studied, of which 62 (75 %) had force-pSr curves that were described by a single sigmoid curve. Of these 62 fibres, 59 fibres (95 %) were classified as fast fibres (pCa50 – pSr50 > 1.0 unit). The fetal control group and saline group had one and two fibre/s respectively, that were classified as slow (pCa50 – pSr50 < 1.0 unit). The proportion of fast, slow, hybrid and intermediate fibres for each treatment group are presented in Table 4.

MHC gene expression and fibre characteristics

MHC expression was investigated at both the mRNA and protein level. MHCemb, MHCI, and MHCIIa mRNA expression was not significantly different between treatment groups ($p > 0.05$; data not presented). Furthermore, the

percentage distribution of myofibres, stained for the expression of MHCemb, MHCI, MHCIIa, and MHCIIabx, was not significantly different between treatment groups ($p > 0.05$; Figure 3). In all treatment groups, all myofibres that stained positively for MHCIIabx also stained positively for MHCIIa; indicating that MHCIIb/x isoforms are always co-expressed with MHCIIa or that MHCIIa is the only fast adult isoform expressed at 136 d PCA. MHC isoforms were expressed in four combinations: i) fibres expressed MHCI only; ii) fibres co-expressed MHCemb and MHCI; iii) fibres co-expressed MHCemb and MHCIIabx/IIa; and iv) fibres co-expressed MHCemb, MHCI and MHCIIabx/IIa. No fibres expressed MHCemb only or MHCIIIabx/IIa only. The CSA of individual myofibre types were not significantly different between treatment groups ($p > 0.05$; Figure 3).

Molecular analyses

Cytokine levels

There was no significant difference in the local mRNA expression levels of pro-inflammatory genes, IL-1β ($p = 0.412$), IL-6 ($p = 0.974$), or TNF-α ($p = 0.618$), or anti-inflammatory gene, IL-10 ($p = 0.875$) between treatment groups (Figure 4).

Anabolic and proteolytic markers

Protein expression (phosphorylated over total, P/T) of three intracellular signalling components (Akt, mTOR, 4EBP 1) were measured to evaluate anabolic signalling transduction cascades (Figure 5A - C). There was no significant difference in P/T-Akt ($p = 0.572$) or P/T-4EBP1 ($p = 0.786$) protein activity between treatment groups. There was a significant main effect of treatment on P/T-mTOR ($p = 0.047$; Kruskal-Wallis). However, a priori analyses did not reveal any significant difference in P/T-mTOR activity between treatment groups.

MuRF1 gene expression, MAFbx gene expression, and 20 S proteasome activity were measured to evaluate the proteolytic response of diaphragm (Figure 5D - F). There was no significant difference in MuRF1 mRNA expression ($p = 0.152$), MAFbx mRNA expression ($p = 0.431$), or 20 S proteasome activity ($p = 0.426$) between treatment groups.

Oxidative status

Expression (mRNA) of the antioxidant gene, SOD 1 was not significantly different between treatment groups (p = 0.092; Figure 6A). However, mRNA expression of the antioxidant gene GPX 1, was significantly higher in the saline group compared to the fetal control group (p = 0.022; Figure 6B). Protein carbonyl activity was significantly lower in the saline group compared to the fetal control group (p = 0.006; Figure 6C).

Myogenic regulatory factors (MRF) gene expression

There was no significant difference in the gene expression for the myogenic regulatory factors, PAX3 (p = 0.610), PAX7 (p = 0.127), MYF5 (p = 0.465), MYOD (p = 0.161), and MYOG (p = 0.371), between treatment groups (data not presented).

Independent predictors of diaphragm functional properties, structural properties and molecular properties at 7 d PNA

Differences between saline and LPS groups may have been influenced by confounding postnatal management that included mechanical ventilation and altered nutritional intake. Thus, for these two groups the relationship between diaphragm outcome variables at 7 d PNA, LPS exposure and potential confounding experimental variables (average protein intake and normalised duration of mechanical ventilation) was evaluated using correlation and linear regression. Availability of tissues varied for individual regression analyses (N = 14 for diaphragm contractile function; N = 16 for skinned fibre measurements; N = 12 for MHC fibre characteristics; N = 16-17 for all molecular analyses).

LPS exposure

LPS exposure correlated positively with the normalised duration of mechanical ventilation required by postnatal lambs (R2 = 0.270, p = 0.032). The normalised duration of mechanical ventilation ranged from 3-54 h among saline lambs, and all lambs were successfully extubated before study completion. In comparison, normalised duration of mechanical ventilation among LPS lambs ranged from 4-143 h, with 4/8 lambs requiring respiratory support up until study completion.

Mechanical ventilation

The unstandardised residual mechanical ventilation (component of mechanical ventilation not due to LPS exposure) significantly predicted several outcome measures. Duration of mechanical ventilation (residual) exhibited a moderate negative correlation with whole muscle specific force produced at stimulation frequencies ranging from 15-80 Hz (R2 ranged between 0.456 to 0.528; $p < 0.01$). Duration of mechanical ventilation explained ~51 % of the variation observed in diaphragm maximum specific force at 7 d PNA, independent of LPS exposure.

Duration of mechanical ventilation (residual) exhibited a moderate negative correlation with the proportion of fibres expressing MHCemb + I (R2 = 0.363, p = 0.038), and a moderate positive correlation with IL-6 mRNA expression (R2 = 0.326, p = 0.021).

Protein intake

Average protein intake exhibited a moderate positive correlation with the MHCemb + IIa/IIabx proportions (R2 = 0.482, p = 0.012) and a strong negative correlation with the MHCemb + I + IIa/IIabx proportions (R2 = 0.731, $p < 0.001$).

Diaphragm contractile properties and MHC fibre characteristics after correcting for respiratory support.

Diaphragm contractile function and MHC fibre characteristics of saline and LPS lambs were compared after correcting for significant independent experimental variables. LPS exposure had no significant effect on the maximum specific force (p = 0.164; data not presented), force-frequency relationship (p = 0.577; data not presented), MHC fibre characteristics ($p > 0.05$; data not presented), or IL-6 mRNA expression (p = 0.797; data not shown) after correcting for the unstandardised residual duration of mechanical ventilation or average protein intake, as appropriate.

Discussion

Diaphragm weakness associated with preterm birth is exacerbated by *in utero* exposure to inflammation (Song et al., 2013a; Karisnan et al., 2015a, 2015b, 2017). We hypothesised that the combined effects of preterm birth *and in utero* inflammation would severely compromise the capacity of the diaphragm to cope with the increased work of breathing in the acute (7 d) postnatal period. Contrary to our hypothesis, diaphragm function and structure at 7 d PNA was not significantly affected by *in utero* exposure to LPS. There was no significant difference in the contractile measurements from intact diaphragm fibres, Ca^{2+}-activation properties of single skinned fibres, MHC fibre characteristics, or molecular markers of diaphragm development, growth and metabolism between saline and LPS lambs. Interestingly, the functional properties of diaphragms from the saline and LPS groups were significantly different to the age-matched fetal control lambs that were killed at delivery. The contractile properties of the diaphragms from saline and LPS lambs were typical of more mature animals, compared to the fetal control lambs, as described below. A clinically important observation from this study, however, was that lambs exposed to *in utero* inflammation were more dependent on mechanical ventilation in the intensive care unit. Furthermore, the duration of mechanical ventilation required by lambs, and the average protein intake during the first week of life, independently predicted diaphragm contractile function and MHC fibre characteristics at 7 d PNA. Our results emphasise how complex interactions between common clinical complications in the perinatal period influence the functional, structural and molecular development of the diaphragm after birth.

Postnatal development of contractile properties in the immature diaphragm

Despite having the same postconceptional age at death, the contractile properties of the preterm fetal control diaphragm differed markedly from the saline and LPS diaphragm. Contractile properties of the fetal control diaphragm were typical of immature skeletal muscle, displaying lower maximum specific force, higher peak twitch force, slower contraction times and a leftward shift of the force-frequency curve, compared to the saline and LPS groups. Slower

twitch contraction and relaxation times would facilitate force summation thereby accounting for the higher specific force produced at low stimulation frequencies in the fetal control group. The higher twitch force to tetanic force ratio (Tw:Te) and leftward shift of the force-frequency relationship displayed by the fetal control group are typical contractile features of the immature diaphragm, as reported previously for sheep and cats (Lavin et al. 2013; Sieck et al. 1991). In contrast to the fetal control group, the contractile properties of the saline and LPS diaphragm were typical of more mature fetal lambs (Lavin et al. 2013). The greater specific force and faster contraction and relaxation times are likely to reflect an accelerated development of the structural and functional properties of the diaphragm that may include the transition from immature to adult protein isoforms (Brandl et al., 1987; Arai et al., 1992; Watchko et al., 1998), improved myofilament function (Williams & Goldspink, 1971; Maxwell et al., 1983; West et al., 1999; Geiger et al., 2001; Orliaguet et al., 2002; Racca et al., 2013), and/or improved Ca^{2+} handling (Schiaffino & Margreth, 1969; Zubrzycka-Gaarn & Sarzala, 1980; Froemming & Ohlendieck, 1998).

The longer contraction and relaxation times observed in the fetal control diaphragms could be accounted for by an increase in the proportion of slow, fatigue resistant muscle fibres. However, MHC mRNA and protein expression was not significantly different between fetal control, saline and LPS groups, nor was there any significant difference in the susceptibility to fatigue between treatment groups. Immunohistochemical analysis showed that a small proportion of fibres expressed only MHCI isoforms; all other fibres co-expressed various combinations of MHC isoforms with MHCemb+IIa/IIabx the most abundant (~70%). Skeletal muscle development is characterised by the disappearance of MHCemb isoforms (Watchko et al., 1998; Strbenc et al., 2006) and the preferential growth of secondary myofibres expressing MHCIIabx (Maxwell et al., 1983; Sieck et al., 1991b; Fratacci et al., 1996). It was surprising that we did not observe any changes in MHC expression, considering differences in whole muscle contractile properties between the saline and LPS diaphragm, compared with the fetal control diaphragm. However, the relationship between MHC expression and contractile function is reportedly less

robust in fetal muscle compared to adult muscle (West et al., 1999; Lavin et al., 2013).

The activation profiles of single muscle fibres were characterised based on differential sensitivity to Ca^{2+} and Sr^{2+}, independently of MHC expression (West et al., 1999; Cannata et al., 2011). The proportion of fibres classified as pure fast-twitch fibres was 41 % in the fetal control group, 65 % in the saline group and 75 % in the LPS group. These proportions, are broadly consistent with the predominant fast-twitch phenotype of diaphragm fibres from fetal lambs of similar gestational age (Cannata et al., 2011), but do suggest a difference between the fetal control and postnatal saline and LPS groups that was not evident based on MHC expression. Slow-twitch muscle characteristics are thought to emerge later in gestation or after birth, with fibres from adult sheep diaphragm shown to exhibit a hybrid phenotype, where both fast- and slow-twitch muscle characteristics are present within each fibre (Cannata et al., 2011). In this study, transition from fast to slow fibres was seen in some fibres that exhibited either an intermediate or hybrid contractile phenotype. The combined proportions of slow, intermediate and hybrid fibres were higher in the fetal control group (~59 %) compared to the postnatal saline group (~38 %). Interestingly only 25 % of fibres in the LPS group were classified as intermediate, and there were no slow or hybrid fibres present. These differences in activation characteristics suggest possible antenatal and postnatal influences on the transition to a typical hybrid phenotype of adult diaphragm. Importantly, these results indicate that the activation characteristics can be affected independently of changes in MHC expression, and may reflect the increased mechanical load placed on the diaphragm during the transition from fetal to postnatal life, as suggested previously by Cannata et al (2011).

The effects of preterm birth and *in utero* LPS exposure on myofilament function were also evaluated in single skinned fibres. The low and variable proportions of intermediate, hybrid and slow fibres expressed between the three groups made interpretation of the statistical analyses difficult. However, when considering the ‘pure fast’ fibres only, there were no significant differences in maximum Ca^{2+}-activated force, Ca^{2+} activation threshold (pCa10) or Ca^{2+}

sensitivity (pCa50) between all groups. Overall, our results suggest that the differences in whole muscle contractile function of the postnatal groups, compared to the fetal control group, are not due to differences in myofilament function.

In the absence of any significant difference in MHC expression or myofilament function, the differences in contractile properties between the fetal control and postnatal lambs are likely related to developmental differences in Ca^{2+} handling. In rabbit skeletal muscle, expression of SERCA and the sarcoplasmic reticulum Ca^{2+} binding protein, calsequestrin, increase markedly in the first week after birth, while the expression of Ca^{2+} release channels remains constant (Froemming and Ohlendieck, 1998). A similar postnatal transition of Ca^{2+} handling proteins in the preterm lamb could explain the differences in twitch contraction times and maximum force production between fetal control and postnatal lambs observed in this study. Decreased SERCA activity in the fetal control diaphragm would increase the Ca^{2+} available to bind to troponin C, which would increase peak twitch force, while decreased calsequestrin levels would limit the total SR Ca^{2+} available for release, decreasing maximal force production.

We demonstrated previously that protein synthesis in the diaphragm of preterm fetal lambs is suppressed by *in utero* LPS 7 d before birth (Song et al. 2013). Contrary to these findings, LPS exposure in the present study had only minimal effects on the anabolic or proteolytic signalling pathways of the diaphragm at 7 d PNA. Although our previous study used a higher dose and earlier GA at time of LPS administration, it appears that the inflammation induced decrease in protein synthesis that is present at birth (Song et al. 2013), does not persist after 7 d PNA. Thus the adaptation of the protein synthesis pathways to early postnatal life appears consistent with the development of diaphragm function over the same period, which is not altogether surprising considering the regulation of anabolic signalling differs markedly between antenatal and postnatal development (Suzuki et al., 1998). We did observe a main effect of treatment group on mTOR phosphorylation levels, but post-hoc analyses did not reveal any specific group differences. These findings indicate that the anabolic

pathway may be differentially regulated in the preterm diaphragm but we cannot identify whether this may be mediated via *in utero* LPS exposure or postnatal development.

In support of the molecular analysis of protein synthesis and degradation pathways, the CSA of the 4 fibre types did not differ significantly between treatment groups. Fibres which only expressed MHCI were significantly larger than the 3 other fibre types, which co-express MHCemb. The larger size of MHCI fibres compared with other fibre types is a distinguishing feature of an immature muscle (Maxwell et al., 1983; Sieck et al., 1991b; Fratacci et al., 1996). The absence of any treatment effects of fibre CSA or protein synthesis/degradation pathways indicate that the functional capacity of the intact diaphragm was not influenced by myofibre atrophy or hypertrophy.

Exposure to IA LPS had no significant effect on the pro-inflammatory or anti-inflammatory cytokine profiles of the preterm diaphragm at 7 d PNA. We showed previously that *in utero* LPS exposure induces a transient pro-inflammatory response that is resolved within 7 days (Song et al. 2013). Therefore, it is unsurprising that we observed no significant change in the cytokine profile after 7 d PNA.

Despite the similarity in inflammatory profiles between the three groups, the molecular markers of oxidative stress were significantly different between the fetal control and saline groups. The saline lambs had higher antioxidant GPX 1 expression and lower protein carbonyl content compared to the fetal control lambs; which is consistent with the developmental changes in antioxidant capacity of the lamb diaphragm (Song & Pillow, 2012). Interestingly these changes in oxidative state were not evident in the LPS lambs, suggesting that the *in utero* LPS exposure may compromise the postnatal development of antioxidant defences. We showed previously that IA LPS exposure further impairs the already compromised anti-oxidant defences in preterm lambs and this effect is mediated by deficiency in erythroid 2-related factor 2 (Nrf2) signalling (Song et al., 2013b). However, it is unclear whether these changes in redox state would persist in the early postnatal period.

In summary, the preterm diaphragm in an environment of standard postnatal care exhibits a clear functional advantage over the age-matched fetal control. Accelerated functional adaptation in the preterm diaphragm subject to postnatal care appears to be mediated by changes in Ca^{2+} handling proteins and activation status. The diaphragm also appears surprisingly resilient to prenatal LPS exposure, although reduced antioxidant capacity may infer a susceptibility to pathological stimuli in the long term.

Independent postnatal predictors of diaphragm contractile properties and MHC fibre characteristics

The premature birth of saline and LPS lambs was associated with postnatal complications, similar to the postnatal complications experienced by preterm newborn infants in the neonatal intensive care unit. Lambs were managed with a graded de-escalation of respiratory support to match, as closely as possible, the clinical management of preterm infants. Additionally, the immature intestine of the preterm lamb, like that of the preterm infant, does not tolerate large protein or osmotic loads (Book et al., 1975). Hence, slow progressive increases in feeding resulted in suboptimal enteral nutritional intake. Feed was also withheld when lambs were not tolerating enteral nutrition. This management strategy, whilst similar to the clinical setting, may have masked differences between the saline and LPS lambs by introducing an inherent variability. In the absence of any effect of *in utero* LPS exposure on our outcome measures of diaphragm structure and function, the saline and LPS groups were combined to investigate the independent effects of mechanical ventilation and postnatal protein intake on diaphragm function at 7 d PNA.

Mechanical ventilation

The duration of mechanical ventilation varied depending on the clinical requirements for each lamb, but significantly predicted diaphragm contractile function, MHC fibre characteristics and inflammatory status at 7 d PNA. Increased duration of mechanical ventilation was associated with i) reduced specific force across a range of submaximal and maximal stimulation frequencies (15 – 80 Hz); and ii) and a decreased proportion of fibres

expressing MHCemb + I. These results are consistent with previous studies, which show diaphragm weakness increases the need for mechanical ventilation in COPD patients (Budweiser et al., 2008), and that mechanical ventilation weakens the diaphragm in adult humans (Levine et al., 2008; Jaber et al., 2011) and adult animal models (Anzueto et al., 1997; Radell et al., 2002; Sassoon et al., 2002; Betters et al., 2004; Corpeno et al., 2014). It is important to acknowledge that prior LPS exposure was associated with an increased requirement for mechanical ventilation during the 7 days of postnatal care. It is interesting then that there were no significant differences in diaphragm function or structure between saline and LPS groups, despite LPS lambs requiring longer durations of mechanical ventilation. This observation highlights the complex interaction between antenatal and postnatal complications associated with preterm birth that will be discussed in more detail below.

Surprisingly, duration of mechanical ventilation was not associated with any changes in fatigue resistance, or markers of muscle atrophy or oxidative stress, which are typical hallmarks of ventilator-induced diaphragm dysfunction in adults (Anzueto et al., 1997; Bernard et al., 2003; Kavazis et al., 2009; Corpeno et al., 2014). The differential effect of mechanical ventilation between adult and neonatal subjects may be influenced by the postnatal development of the diaphragm in preterm lambs. While the effects of mechanical ventilation on the preterm diaphragm remain relatively unexplored, mechanical ventilation is associated with smaller diaphragm fibres in the neonate and infant (Knisely et al., 1988). Although the duration of ventilation correlated negatively with the proportion of fibres expressing MHCemb + I, there was no relationship between mechanical ventilation and diaphragm fibre cross-sectional area in this study. The functional implications of this relationship remain unclear, as the proportion of fibres expressing MHCemb + I was low relative to other fibre types. This finding does however indicate an influence of mechanical ventilation on the structural development of the immature diaphragm.

Postnatal nutrition

Postnatal growth restriction resulting from inadequate nutritional intake during early postnatal life is a universal problem among preterm infants (Cooke et al.,

2004; Horbar et al., 2015). In this study, sub-optimal enteral protein intake was associated with impaired postnatal growth of both saline and LPS lambs, compared with *in utero* growth of the fetal control lambs. The saline and LPS lambs did not gain weight over the 7 postnatal days and consequently, weighed significantly less than the fetal control lambs at postmortem. In contrast, De Matteo et al. (2010) reported a ~30 % increase in relative body weight during the first week of life in preterm lambs of a similar GA to that used in our study. The absence of postnatal weight gain in our study is likely to reflect the added effects of *in utero* LPS exposure and mechanical ventilation on the already impaired gastrointestinal function of the preterm lamb. Importantly, diaphragm function of the postnatal lambs is typical of a more mature fetal lamb, despite postnatal lambs having a lower body weight.

Previous studies indicate that undernutrition results in respiratory muscle weakness, and that developing skeletal muscle is more susceptible to nutritional regulation than adult skeletal muscle (Wilson et al., 1992). Undernutrition during development induces diaphragm weakness primarily by reducing the number and size of muscle fibres (Wilson et al., 1988; Brozanski et al., 1993; Prakash et al., 1993; Fahey et al., 2005). Undernutrition causes preferential atrophy of fibres expressing MHCIIabx (Goldspink & Ward, 1979; Ward & Stickland, 1993; Lefaucheur et al., 2003) and delays the disappearance of developmental MHC isoforms, indicative of delayed diaphragm development (Brozanski et al., 1991).

In the current study, postnatal protein intake was monitored between 129-136 d PCA, which is after secondary myogenesis (Fahey et al., 2005). Average protein intake correlated with, but did not independently predict, diaphragm specific force produced at 30-80 Hz at 7 d PNA. However, we found no correlation between average protein intake and the myofibre CSA or the proportion of fibres which co-expressed MHCemb. Protein intake did significantly predict the proportions of MHCemb + IIabx/IIa and MHCemb+ I + IIabx/IIa fibres at 7 d PNA. The positive correlation between average protein intake and MHCemb + IIabx/IIa and negative correlation with MHCemb + I + IIabx/IIa may indicate a shift towards a ‘fast fibre’ phenotype in lambs with higher protein intake. These results are consistent with previous studies, which

found that undernutrition during the postnatal period increased MHCI expression at the mRNA and protein level (Harrison et al., 1996; White et al., 2000).

Challenges and translational advantages of large animal preclinical perinatal research

We used a well-established ovine model of chorioamnionitis to determine the impact of *in utero* inflammation and preterm birth on postnatal diaphragm function. The preterm lamb model provides unique insight into the complex pathophysiological processes in the perinatal period that contribute to respiratory dysfunction in early postnatal life. In lambs, the developmental trajectories of major organs including the lungs, skeletal muscles, and gastrointestinal systems are similar to humans. The brain, however, is more developed in the preterm sheep compared to preterm humans (Bernhard et al., 1967). The stage of respiratory development at birth in our preterm lambs (129 d GA) is similar to that of human infants at 32 to 34 weeks of gestation (De Matteo et al., 2010) and our preterm lambs were managed in an extended postnatal care model using contemporary neonatal equipment and treatment protocols. Nonetheless, common postnatal clinical complications associated with chorioamnionitis and preterm birth impacted on the management of lambs in the saline and LPS groups, leading to higher than anticipated variance in our outcome measures. This study highlights the challenges faced in a large pre-clinical animal model of preterm birth that make it difficult to distinguish between multiple factors that may impact on respiratory muscle function.

Along with several other groups (Kallapur et al., 2001; Berry et al., 2011; Kuypers et al., 2012) we have routinely used IA LPS administration in sheep as a model for chorioamnionitis. LPS is a component of Gram-negative bacteria that initiates an inflammatory response activated via CD14/TLR-4 receptors (Poltorak et al., 1998) and characterised by increased expression of IL-1, and IL-6, which is the dominant feature of clinical and subclinical chorioamnionitis, in the absence of bacterial infection (Dziegielewska et al., 1998; Garnier et al., 2001). Whilst severe forms of clinical chorioamnionitis can be induced by intra-venous and intra-peritoneal exposure to LPS, these models cause high-grade

fetal inflammatory responses and are associated with high rates (>50%) of fetal loss (Galinsky et al., 2013). IA LPS exposures, as used in our study, reflect subclinical chorioamnionitis resulting in a low grade fetal inflammatory response and reduced fetal loss.

We showed previously, that our ovine model of chorioamnionitis promotes accelerated lung development and enhanced surfactant production, but also disrupts normal alveolar development (Pillow et al., 2004) and significantly impairs diaphragm function at birth in preterm lambs (Song et al., 2013a; Karisnan et al., 2015a, 2015b, 2017). Furthermore, chorioamnionitis impairs development of the innate immune system and enhances the already increased risk of postnatal sepsis in preterm infants (Kallapur et al., 2007; Kramer et al., 2007; Wolfs et al., 2012; Melville & Moss, 2013). LPS induced lung maturation is consistent with the reduced initial severity of postnatal respiratory distress in preterm infants born preterm in the presence of chorioamnionitis (Watterberg et al, 1996). However, it is not uncommon for such infants to develop respiratory distress several days after delivery, progressing onto chronic respiratory illness. Thus, the acute postnatal care of preterm infants that have been exposed to chorioamnionitis often necessitates mechanical ventilation in the presence of inflammation and/or sepsis.

These well-established effects of *in utero* inflammation are consistent with the observation that our LPS exposed preterm lambs had an increased requirement for mechanical ventilation compared to the saline group. Several preterm lambs displayed symptoms consistent with postnatal infection, including: hypothermia or fever, vomiting, hypotension and either neutropenia or neutrophilia. However, postnatal sepsis was not determined decisively as routine blood cultures were not obtained, lambs were on continuous intravenous antibiotics, and white blood cell counts are dependent on the time blood samples were taken in relation to the onset of postnatal sepsis. It is also possible that LPS negatively impacted the respiratory control system and modulated the respiratory rhythm generation and diaphragm activity of LPS lambs, as found previously (Huxtable et al., 2011), thus contributing to the increased requirement on mechanical ventilation.

It is interesting that there were no significant differences in diaphragm function or structure between saline and LPS groups, despite LPS lambs requiring longer durations of mechanical ventilation. This finding suggests a complex interaction between *in utero* inflammation, preterm birth and mechanical ventilation. The combine effects of these complications have been investigated by several previous studies. Ebihara et al. (2002) demonstrated a protective effect of mechanical ventilation on rat diaphragm function in the presence of LPS-induced sepsis. The protective effect occurred despite an increase in markers of oxidative stress, suggesting a complex interaction between mechanical and oxidative stresses in the presence of sepsis. Using an *in vitro* cell culture model, the authors demonstrated a synergistic interaction in which mechanical stress applied in the presence of oxidative stress enhanced sarcolemmal injury (Ebihara et al., 2002). Thus they propose that mechanical ventilation could prevent sarcolemmal injury by reducing the diaphragm myofibre mechanical stresses inherently associated with spontaneous breathing efforts. The potential protective effects of mechanical ventilation must be weighed against the likely development of ventilator-induced diaphragm dysfunction and the increased risk of chronic lung diseases including bronchopulmonary dysplasia that are well established complications associated with prolonged mechanical ventilation. Van Marter et al. (2002) investigated the combined effects of chorioamnionitis, mechanical ventilation and postnatal sepsis on the chronic lung disease outcomes of preterm infants. They found that although isolated chorioamnionitis is protective for chronic lung disease, prolonged mechanical ventilation and chorioamnionitis together appear to be more harmful than mechanical ventilation alone (Van Marter et al., 2002). Collectively, these findings emphasise the importance of studying not just the individual effects but the combined effects of common clinical antenatal and postnatal exposures on the developing diaphragm and the potential development of chronic lung disease.

It should also be noted that saline and LPS treated groups were exposed to medroxyprogesterone in the 7 d prior to premature delivery, and to betamethasone in the 2 d prior to premature delivery. Medroxyprogesterone was administered to ewes to prevent spontaneous abortion associated with

later betamethasone use. Betamethasone was administered to facilitate lung maturation and surfactant production. The fetal control group received neither medroxyprogesterone nor betamethasone as it is a complete naïve group, representing normal *in utero* development. It was not considered practical to include a second fetal control group receiving both antenatal medroxyprogesterone and betamethasone due to the high risk of preterm delivery in ewes after more than 48 hours of betamethasone exposure in late gestation. We previously showed that betamethasone exposure 2 days before preterm delivery has no effect on diaphragm function at birth (Mahzabin et al., 2017) so it is unlikely that it would affect diaphragm function at 7 d PNA. We cannot exclude the possibility that antenatal medroxyprogesterone influenced diaphragm contractile function in our preterm lambs; progesterone receptors are present in skeletal muscle myoblasts and myocytes, and progestins reduce the proliferation of bovine satellite cells *in vitro* (Sissom et al., 2006). However, the influence of progesterone on skeletal muscle function is largely unknown (Gras et al., 2007; Smith et al., 2014) .

Conclusions

Contrary to our hypothesis, diaphragm structure and function at 7 d PNA did not differ between saline and LPS groups. Furthermore, the difference in diaphragm function of the saline and LPS groups compared with that of the fetal control group suggests that the diaphragm adapts rapidly to the marked increase in workload associated with the transition from fetal to postnatal life in the first 7 d after preterm birth. However, *in utero* LPS exposure was associated with an increased dependency on mechanical ventilation in the intensive care unit. While postnatal events in managing the preterm lambs are complex, this study emphasises the importance of investigating the combined effects of common clinical antenatal and postnatal complications on respiratory development in an animal model with similar developmental trajectories to humans. The multitude of systems affected by preterm birth and in utero inflammation pose specific challenges for the isolation and investigation of *in vivo* mechanisms of muscle dysfunction and other clinical problems associated with the clinical care of preterm infants.

Conflict of interest statement

All authors have no financial or personal conflict with other people or organisations that could inappropriately influence our work.

Acknowledgements

We would like to thank all lamb carers who worked at the Pre-Clinical Intensive Care Unit (PICRU).

Funding

This research was made possible by a National Health and Medical Research Council (NHMRC) Centre of Research Excellence (GRT1057514), NHMRC Project Grant (GRT1057759), and Telethon New Children's Hospital Research Fund. JJP was supported by NHMRC Senior Research Fellowship (RF1077691) and PBN was supported by NHMRC Career Development Fellowship (RF1045824). Equipment and consumable support were generously provided by Chiesi Farmaceutici S.p.A. (poractant alfa), Fisher & Paykel Healthcare (ventilation circuits), Draeger Medical (ventilators), and ICU Medical (arterial circuits).

Author contributions

Experiments were performed at The University of Western Australia. JJP, PBN, GJP and AJB contributed to the conception and/or design of the work. PBN and SAN performed the surgical preparation and caesarean delivery. CJA performed the *in vitro* whole muscle diaphragm experiments and histology; JL performed the *in vitro* skinned fibre experiments; TM performed the molecular experiments. CJA and TM drafted the article. All authors revised the article critically for important intellectual content, have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship.

References

Anzueto A, Peters J, Tobin MJ, De Los Santos R, Seidenfeld J, Moore G, Cox W & Coalon J (1997). Effects of prolonged controlled mechanical ventilation on diaphragmatic function in healthy adult baboons. Crit Care Med 25, 1187–1190.

Arai M, Otsu K, MacLennan DH & Periasamy M (1992). Regulation of sarcoplasmic reticulum gene expression during cardiac and skeletal muscle development. Am J Physiol 262, C614–C620.

Bernard N, Matecki S, Py G, Lopez S, Mercier J & Capdevila X (2003). Effects of prolonged mechanical ventilation on respiratory muscle ultrastructure and mitochondrial respiration in rabbits. Intensive Care Med 29, 111–118.

Bernhard C, Kolmodin G & Meyerson B (1967). On the prenatal development of function and structure in the somesthetic cortex of the sheep. Prog Brain Res 26, 60–77.

Berry CA, Nitsos I, Hillman NH, Pillow JJ, Polglase GR, Kramer BW, Kemp MW, Newnham JP, Jobe AH & Kallapur SG (2011). Interleukin-1 in lipopolysaccharide induced chorioamnionitis in the fetal sheep. Reprod Sci 18, 1092–1102.

Betters JL, Criswell DS, Shanely RA, Van Gammeren D, Falk D, DeRuisseau KC, Deering M, Yimlamai T & Powers SK (2004). Trolox attenuates mechanical ventilation-induced diaphragmatic dysfunction and proteolysis. Am J Respir Crit Care Med 170, 1179–1184.

Bland RD (2005). Neonatal chronic lung disease in the post-surfactant era. Biol Neonate 88, 181–191.

Book L, Herbst J, Atherton S & Jung A (1975). Necrotizing enterocolitis in low-birth-weight infants fed an elemental formula. J Pediatr 87, 602–605.

Bortolotto S, Cellini M, Stephenson D & Stephenson G (2000). MHC isoform composition and Ca(2+)- or Sr(2+)-activation properties of rat skeletal muscle fibres. Am J Physiol Cell Physiol 279, C1564-77.

Bose C, Laughon M, Allred E, Van Marter L, O'Shea T, Ehrenkranz R, Fichorova R & Leviton A (2011). Blood protein concentrations in the first two postnatal weeks that predict bronchopulmonary dysplasia among infants born before the 28th week of gestation. Pediatr Res 69, 347–353.

Bradford M (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248–254.

Brandl CJ, DeLeon S, Martin DR & MacLennan DH (1987). Adult forms of the Ca2+-ATPase of sarcoplasmic reticulum. J Biol Chem 262, 3768–3774.

Brozanski B, Daood M, LaFramboise W, Watchko J, Foley T, Butler-Browne G, Whalen R, Guthrie R & Ontell M (1991). Effects of perinatal undernutrition on elimination of immature myosin isoforms in the rat diaphragm. Am J Physiol 261, L49–L54.

Brozanski B, Watchko J, O'Day T & Guthrie R (1993). Effect of undernutrition on contractile and fatigue properties of rat diaphragm during development. J Appl Physiol 74, 2121–2126.

Budweiser S, Jörres R & Pfeifer M (2008). Treatment of respiratory failure in COPD. Int J Chron Obstruct Pulmon Dis 3, 605–618.

Cannata D, Ireland Z, Dickinson H, Snow R, Russell A, West J & Walker D (2010). Maternal creatine supplementation from mid-pregnancy protects the diaphragm of the newborn spiny mouse from intrapartum hypoxia-induced damage. Pediatr Res 68, 393–398.

Cannata DJ, Crossley KJ, Barclay CJ, Walker DW & West JM (2011). Contribution of stretch to the change of activation properties of muscle fibers in the diaphragm at the transition from fetal to neonatal Life. Front Physiol 2, 1–13.

Cooke RJ, Ainsworth SB & Fenton AC (2004). Postnatal growth retardation: a universal problem in preterm infants. Arch Dis Child Fetal Neonatal Ed 89, F428–F430.

Corpeno R, Dworkin B, Cacciani N, Salah H, Bergman H-M, Ravara B, Vitadello M, Gorza L, Gustafson A-M, Hedström Y, Petersson J, Feng H-Z, Jin J-P, Iwamoto H, Yagi N, Artemenko K, Bergquist J & Larsson L (2014). Time course analysis of mechanical ventilation-induced diaphragm contractile muscle dysfunction in the rat. J Physiol 592, 3859–3880.

Dimitriou G, Greenough A, Moxham J & Rafferty GF (2003). Influence of maturation on infant diaphragm function assessed by magnetic stimulation of phrenic nerves. Pediatr Pulmonol 35, 17–22.

Dimitriou G, Greenough A, Rafferty GF & Moxham J (2001). Effect of maturity on maximal transdiaphragmatic pressure in infants during crying. Am J Respir Crit Care Med 164, 433–436.

Dziegielewska K, Andersen N & Saunders N (1998). Modification of macrophage response to lipopolysaccharide by fetuin. Immunol Lett 60, 31–35.

Eber E & Zach MS (2001). Long term sequelae of bronchopulmonary dysplasia (chronic lung disease of infancy). Thorax 56, 317–323.

Ebihara S, Hussain SNA, Danialou G, Cho WK, Gottfried SB & Petrof BJ (2002). Mechanical ventilation protects against diaphragm injury in sepsis: interaction of oxidative and mechanical stresses. Am J Respir Crit Care Med 165, 221–228.

Fahey AJ, Brameld JM, Parr T & Buttery PJ (2005). The effect of maternal undernutrition before muscle differentiation on the muscle fiber development of the newborn lamb. J Anim Sci 83, 2564–2571.

Fratacci MD, Levame M, Rauss A, Bousbaa H & Atlan G (1996). Rat diaphragm during postnatal development. I. Changes in distribution of muscle fibre type and in oxidative potential. Reprod Fertil Dev 8, 391–398.

Froemming G & Ohlendieck K (1998). Oligomerisation of Ca2+-regulatory membrane components involved in the excitation-contraction-relaxation cycle during postnatal development of rabbit skeletal muscle. Biochim Biophys Acta 1387, 226–238.

Galinsky R, Polglase GR, Hooper SB, Black MJ & Moss TJM (2013). The Consequences of Chorioamnionitis: Preterm Birth and Effects on Development. J Pregnancy 2013, Article ID 412831.

Garnier Y, Coumans A, Berger R, Jensen A & Hasaart T (2001). Endotoxemia severely affects circulation during normoxia and asphyxia in immature fetal sheep. J Soc Gynecol Investig 8, 134–142.

Geiger PC, Cody MJ, Macken RL, Bayrd ME, Fang YH & Sieck GC (2001). Plasticity in skeletal, cardiac and smooth muscle selected contributions: mechanisms underlying increased force generation by rat diaphragm muscle fibers during development. J Appl Physiol 90, 380–388.

Goldenberg R, Hauth J & Andrews W (2000). Intrauterine infection and preterm delivery. N Engl J Med 342, 1500–1507.

Goldspink G & Ward PS (1979). Changes in rodent muscle fibre types during post-natal growth undernutrition and exercise. J Physiol 296, 453–469.

Gras F, Brunmair B, Quarré L, Szöcs Z, Waldhäusl W & Fürnsinn C (2007). Progesterone impairs cell respiration and suppresses a compensatory increase in glucose transport in isolated rat skeletal muscle: a non-genomic mechanism contributing to metabolic adaptation to late pregnancy? Diabetologia 50, 2544–2552.

Grundy D (2015). Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology. J Physiol 593, 2547–2549.

Harrison AP, Rowlerson AM & Dauncey MJ (1996). Selective regulation of myofiber differentiation by energy status during postnatal development. Am J Physiol 270, R667–R674.

Horbar JD, Ehrenkranz RA, Badger GJ, Edwards EM, Morrow KA, Soll RF, Buzas JS, Bertino E, Gagliardi L & Bellù R (2015). Weight growth velocity and postnatal growth failure in infants 501 to 1500 grams: 2000 - 2013. Pediatrics 136, e84-92.

Huxtable AG, Vinit S, Windelborn JA, Crader SM, Guenther CH, Watters JJ & Mitchell GS (2011). Systemic inflammation impairs respiratory chemoreflexes and plasticity. Respir Physiol Neurobiol 178, 482–489.

Jaber S, Petrof BJ, Jung B, Chanques G, Berthet JP, Rabuel C, Bouyabrine H, Courouble P, Koechlin-Ramonatxo C, Sebbane M, Similowski T, Scheuermann V, Mebazaa A, Capdevila X, Mornet D, Mercier J, Lacampagne A, Philips A & Matecki S (2011). Rapidly progressive diaphragmatic weakness and injury during mechanical ventilation in humans. Am J Respir Crit Care Med 183, 364–371.

Javen I, Williams N, Young R, Luff A & Walker D (1996). Growth and differentiation of fast and slow muscles in fetal sheep, and the effects of hypophysectomy. J Physiol 494, 839–849.

Johnson BD, Wilson LE, Zhan WZ, Watchko JF, Daood MJ & Sieck GC (1994). Contractile properties of the developing diaphragm correlate with myosin heavy chain phenotype. J Appl Physiol 77, 481–487.

Kallapur S, Willet K, Jobe A, Ikegami M & Bachurski C (2001). Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. Am J Physiol Lung Cell Mol Physiol 280, L527–L536.

Kallapur SG, Jobe AH, Ball MK, Nitsos I, Moss TJM, Hillman NH, Newnham JP & Kramer BW (2007). Pulmonary and systemic endotoxin tolerance in preterm fetal sheep exposed to chorioamnionitis. J Immunol 179, 8491–8499.

Karisnan K, Bakker A, Song Y, Noble P, Pillow J & Pinniger G (2015a). Gestational age at initial exposure to in utero inflammation influences the extent of diaphragm dysfunction in preterm lambs. Respirology 20, 1255–1262.

Karisnan K, Bakker AJ, Song Y, Noble PB, Pillow JJ & Pinniger GJ (2015b). Interleukin-1 receptor antagonist protects against lipopolysaccharide induced diaphragm weakness in preterm lambs. PLoS One 10, e0124390.

Karisnan K, Mahzabin T, Bakker AJ, Song Y, Noble PB, Pillow JJ & Pinniger GJ (2017). Gestational age at time of in utero lipopolysaccharide exposure

influences the severity of inflammation-induced diaphragm weakness in lambs. Am J Physiol Regul Integr Comp Physiol; DOI: 10.1152/ajpregu.00150.2017.

Kavazis AN, Talbert EE, Smuder AJ, Hudson MB, Nelson B & Powers SK (2009). Mechanical ventilation induces diaphragmatic mitochondrial dysfunction and increased oxidant production. Free Radic Biol Med 46, 842–850.

Keens TG, Brydan AC, Levison H & Ianuzzo CD (1978). Developmental pattern of muscle fibre types in human ventilatory muscles. J Appl Physiol 44, 909–913.

Knisely AS, Leal SM & Singer DB (1988). Abnormalities of diaphragmatic muscle in neonates with ventilated lungs. J Pediatr 113, 1074–1077.

Kramer BW, Joshi SN, Moss TJM, Newnham JP, Sindelar R, Jobe AH & Kallapur SG (2007). Endotoxin-induced maturation of monocytes in preterm fetal sheep lung. Am J Physiol Lung Cell Mol Physiol 293, L345–L353.

Kuypers E, Ophelders D, Jellema RK, Kunzmann S, Gavilanes AW & Kramer BW (2012). White matter injury following fetal inflammatory response syndrome induced by chorioamnionitis and fetal sepsis: lessons from experimental ovine models. Early Hum Dev 88, 931–936.

Lavin T, Song Y, Bakker AJ, McLean CJ, Macdonald WA, Noble PB, Berry CA, Pillow JJ & Pinniger GJ (2013). Developmental changes in diaphragm muscle function in the preterm and postnatal lamb. Pediatr Pulmonol 48, 640–648.

Lefaucheur L, Ecolan P, Barzic Y-M, Marion J & Le Dividich J (2003). Early postnatal food intake alters myofiber maturation in pig skeletal muscle. J Nutr 133, 140–147.

Levine S, Nguyen T, Taylor N, Friscia ME, Budak MT, Rothenberg P, Zhu J, Sachdera R, Sonnad S, Kaiser LR, Rubinstein NA, Powers SK & Shrager JB (2008). Rapid disuse atrophy of diaphragm fibres in mechanically ventilated humans. N Engl J Med 358, 1327–1335.

Mahzabin T, Pillow JJ, Pinniger GJ, Bakker AJ, Noble PB, White RB, Karisnan K & Song Y (2017). Influence of antenatal glucocorticoid on preterm lamb diaphragm. Pediatr Res 82, 509–517.

Mantilla CB & Sieck GC (2008). Key aspects of phrenic motoneuron and diaphragm muscle development during the perinatal period. J Appl Physiol 104, 1818–1827.

Van Marter LJ, Dammann O, Allred EN, Leviton A, Pagano M, Moore M & Martin C (2002). Chorioamnionitis, Mechanical Ventilation, and Postnatal Sepsis as Modulators of Chronic Lung Disease in Preterm Pnfants. J Pediatr 140, 171–176.

De Matteo R, Blasch N, Stokes V, Davis P & Harding R (2010). Induced preterm birth in sheep: a suitable model for studying the developmental effects of moderately preterm birth. Reprod Sci 17, 724–733.

Maxwell LC, McCarter RJM, Kuehl TJ & Robotham JL (1983). Development of histochemical and functional properties of baboon respiratory muscles. J Appl Physiol 54, 551–561.

Melville J & Moss T (2013). The immune consequences of preterm birth. Front Neurosci.

Mendez J & Keys A (1960). Density and composition of mammalian muscle. Metabolism 9, 184–188.

Orliaguet G, Langeron O, Bouhemad B, Coriat P, LeCarpentier Y & Riou B (2002). Effects of postnatal maturation on energetics and cross-bridge properties in rat diaphragm. J Appl Physiol 92, 1074–1082.

te Pas AB, Davis PG, Hooper SB & Morley CJ (2008). From liquid to air: Breathing after birth. J Pediatr 152, 607–611.

Pillow J, Jobe A, Collins R, Hantos Z, Ikegami M, Moss T, Newnham J, Willet K & Sly P (2004). Variability in preterm lamb lung mechanics after intra-amniotic endotoxin is associated with changes in surfactant pool size and morphometry. Am J Physiol Lung Cell Mol Physiol 287, L992–L998.

Poltorak A, He X, Smirnova I, Liu M, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B & Beutler

B (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science (80-) 282, 2085–2088.

Prakash YS, Fournier M & Sieck GC (1993). Effects of prenatal undernutrition on developing rat diaphragm. J Appl Physiol 75, 1044–1052.

Racca A, Beck AE, Rao VS, Flint G V, Lundy SD, Born DE, Bamshad MJ & Regnier M (2013). Contractility and kinetics of human fetal and human adult skeletal muscle. J Physiol 591, 3049–3061.

Radell PJ, Remahl S, Nichols DG & Eriksson LI (2002). Effects of prolonged mechanical ventilation and inactivity on piglet diaphragm function. Intensive Care Med 28, 358–364.

Sassoon CSH, Caiozzo VJ, Manka A & Sieck GC (2002). Altered diaphragm contractile properties with controlled mechanical ventilation. J Appl Physiol 92, 2585–2595.

Schiaffino S & Margreth A (1969). Coordinated development of the sarcoplasmic reticulum and T system during postnatal differentiation of rat skeletal muscle. J Cell Biol 41, 855–875.

Segal SS & Faulkner JA (1985). Temperature-dependent physiological stability of rat skeletal muscle in vitro. Am J Physiol Cell Physiol 248, C265–C270.

Sieck GC, Cheung TS & Blanco CE (1991a). Diaphragm capillarity and oxidative capacity during postnatal development. J Appl Physiol 70, 103–111.

Sieck GC, Fournier M & Blanco CE (1991b). Diaphragm muscle fatigue resistance during postnatal development. J Appl Physiol 71, 458–464.

Sissom EK, Reinhardt CD & Johnson BJ (2006). Melengestrol acetate alters muscle cell proliferation in heifers and steers. J Anim Sci 84, 2950–2958.

Smeed JA, Watkins CA, Rhind SM & Hopkins J (2007). Differential cytokine gene expression profiles in the three pathological forms of sheep paratuberculosis. BMC Vet Res 3, 18.

Smith G, Yoshino J, Reeds D, Bradley D, Burrows R, Heisey H, Moseley A & Mittendorfer B (2014). Testosterone and progesterone, but not estradiol, stimulate muscle protein synthesis in postmenopausal women. J Clin Endocrinol Metab 99, 256–265.

Song Y, Karisnan K, Noble PB, Berry C a, Lavin T, Moss TJM, Bakker AJ, Pinniger GJ & Pillow JJ (2013a). In utero LPS exposure impairs preterm diaphragm contractility. Am J Respir Cell Mol Biol 49, 866–874.

Song Y & Pillow J (2012). Ontogeny of proteolytic signaling and anti-oxidant capacity in fetal and neonatal diaphragm. Anat Rec 295, 864–871.

Song Y, Pinniger GJ, Bakker AJ, Moss TJM, Noble PB, Berry C a & Pillow JJ (2013b). Lipopolysaccharide-induced weakness in the preterm diaphragm is associated with mitochondrial electron transport chain dysfunction and oxidative stress. PLoS One 8, e73457.

Strbenc M, Smerdu V, Pogacnik A & Fazarinc G (2006). Myosin heavy chain isoform transitions in canine skeletal muscles during postnatal growth. J Anat 209, 149–163.

Suzuki Y, Shen T, Poyard M, Best-Belpomme M, Hanoune J & Defer N (1998). Expression of adenylyl cyclase mRNAs in the denervated and in the developing mouse skeletal muscle. Am J Physiol 274, C1674-85.

Sweet D, Carnielli V, Greisen G, Hallman M, Ozek E, Plavka R, Saugstad O, Simeoni U, Speer C, Vento M, Visser G & Halliday H (2017). European consensus guidelines on the management of respiratory distress syndrome - 2016 update. Neonatology 111, 107–125.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3, 34–1.

Ward SS & Stickland NC (1993). The effect of undernutrition in the early postnatal period on skeletal muscle tissue. Br J Nutr 69, 141–150.

Watchko JF, Daood MJ & Sieck GC (1998). Myosin heavy chain transitions during development: functional implications for the respiratory musculature. Comp Biochem Physiol B Biochem Mol Biol 119, 459–470.

Watterberg K, Demers L, Scott S & Murphy S (1996). Chorioamnionitis and Early Lung Inflammation in Infants in Whom Bronchopulmonary Dysplasia Develops. Pediatrics 97, 210–215.

West JM, Barclay CJ, Luff AR & Walker DW (1999). Developmental changes in the activation properties and ultrastructure of fast- and slow-twitch muscles from fetal sheep. J Muscle Res Cell Motil 20, 249–264.

White P, Cattaneo D & Dauncey MJ (2000). Postnatal regulation of myosin heavy chain isoform expression and metabolic enzyme activity by nutrition. Br J Nutr 84, 185–194.

Williams PE & Goldspink G (1971). Longitudinal growth of striated muscle fibres. J Cell Sci 9, 751–767.

Wilson DC, Mcclure G & Dodge JA (1992). The influence of nutrition on neonatal respiratory muscle function. Intensive Care Med 18, 105–108.

Wilson SJ, Ross JJ & Harris AJ (1988). A critical period for formation of secondary myotubes defined by prenatal undernourishment in rats. Development 102, 815–821.

Wolfs T, Jellema R, Turrisi G, Becucci E, Buonocore G & Kramer B (2012). Inflammation-induced immune suppression of the fetus: a potential link between chorioamnionitis and postnatal early onset sepsis. J Matern Neonatal Med 25, 8–11.

Zhu MJ, Du M, Nathanielsz PW & Ford SP (2010). Maternal obesity up-regulates inflammatory signaling pathways and enhances cytokine expression in the mid-gestation sheep placenta. Placenta 31, 387–391.

Zubrzycka-Gaarn E & Sarzala MG (1980). Sarcoplasmic reticulum and sarcolemma during development. In Plasticity of Muscle, ed. Pette D, pp. 209–223. Walter de Gruyter, Berlin.

Tables

Table 1. Composition (mM) of solutions used in skinned fibre experiments.

Solution	[EGTA]	$[Mg^{2+}]_{total}$	$[Ca^{2+}]_{total}$	$[Sr^{2+}]_{total}$
A	50	10.3	-	-
B	50	8.5	47.5	-
S	50	8.5	-	40

All solutions contained (mM): K^+ (126); Na^+ (37); HEPES (90); total ATP (8); creatine phosphate (10) and sodium azide (1). pH was 7.10 ± 0.01 at 23-25 °C. EGTA: ethylene Glycol-bis(β-aminoethyl Ether) N,N,N',N'-Tetraacetic acid.

Table 2. Novel primer sequences designed on ovine specific mRNA sequences for real time PCR

Gene	Primer Name	Primer sequence (5'-3')	Accession No.	Tm (°c)	Product Size (bp)
PAX3	FP	AGGCCACTTTACCACGGG	XM_015093551.1	60	256
	RP	TGCCGGGGTTCTCTCTTTTG			
PAX7	FP	CTACCGGCATCTGTCATCCC	XM_015092880.1	60	262
	RP	TGCTCAGGATGCTCATCACC			
MYF5	FP	TGCCATCCGCTACATTGAGA	XM_015094556.1	55	287
	RP	ATCCAGGTTGCTCTGAGTTGG			
MYOD	FP	CCCTGGTGACTTCAGCTGTT	NM_001009390.1	60	220
	RP	TACAAAGTCCCTGTCGCACC			
MYOG	FP	TGTAAGGTGTGCAAGCGGAA	NM_001174109.1	60	191
	RP	TGCAGGCGCTCTATGTACTG			

FP, forward primer; RP, reverse primer; *PAX3*, paired box 3; *PAX7*, paired box 7; *MYF5*, myogenic factor 5; *MYOD*, myogenic differentiation; *MYOG*, myogenin.

Table 3. Lamb descriptive data.

	Fetal control (N = 7)	**Saline (N = 8)**	**LPS (N = 9)**
PCA at PM (d)	136.1 ± 0.9	136.8 ± 1.5	135.9 ± 1.1
Sex ratio (M: F)	4:3	3:5	4:5
Pregnancy ratio (singleton: twin)	2:5	5:3	7:2
Body weight at birth (kg)	N/A	2.94 ± 0.41 (wet) 2.65 ± 0.37 (dry equiv)	3.26 ± 0.42 (wet) 2.93 ± 0.38 (dry equiv)
Body weight at PM (kg)	4.04 ± 0.44 (wet) 3.64 ± 0.4 (dry equiv)	2.84 ± 0.37** (dry)	3.00 ± 0.31*** (dry)
L_o (mm)	39.8 ± 6.1 (n = 7)	36.6 ± 4.3 (n = 7)	38.7 ± 4.0 (n = 7)

Data are presented as mean ± SD, except for sex ratio and pregnancy ratio. PCA, postconceptional age; PM, post mortem; L_o, optimal muscle length; ** and *** significantly different from the dry body weight at PM of the fetal control group at $p < 0.01$ and $p < 0.001$, respectively.

Table 4. Ca^{2+} activation parameters of diaphragm fibres from fetal control, postnatal control and LPS treated preterm lambs.

		Fetal control (n = 22, N = 5)	**Saline (n = 37, N = 6)**	**LPS (n = 19, N = 4)**
Fibre type proportion (number)	Fast	41 % (9)	65 % (24)	75 % (15)
	Intermediate	36.5 % (8)	11 % (4)	25 % (4)
	Hybrid	18 % (4)	19 % (7)	0
	Slow	4.5 % (1)	5 % (2)	0
Max Ca^{2+}-activated force (mN/mm^2)	Fast	126.9 ± 48.9	106.3 ± 38.7	103.2 ± 23.5
	Intermediate	137.0 ± 24.5	111.2 ± 47.5	90.0 ± 35.4
	Hybrid	154.6 ± 23.7	87.4 ± 29.7*	N/A
	Slow	53.9	114.6 ± 8.0	N/A
pCa_{10}	Fast	6.58 ± 0.04	6.61 ± 0.07	6.62 ± 0.09
	Intermediate	6.61 ± 0.05	6.65 ± 0.05	6.56 ± 0.07
	Hybrid	6.65 ± 0.09	6.58 ± 0.05	N/A
pCa_{50}	Fast	6.34 ± 0.06	6.34 ± 0.08	6.34 ± 0.08
	Intermediate	6.36 ± 0.06	6.39 ± 0.05	6.27 ± 0.11
	Hybrid	6.38 ± 0.07	6.34 ± 0.03	N/A
h_{Ca}	Fast	4.11 ± 0.57	3.56 ± 0.46	3.69 ± 0.74
	Intermediate	3.91 ± 0.83	3.85 ± 1.18	3.46 ± 0.67
	Hybrid	3.66 ± 0.89	3.99 ± 0.73	N/A

Values are mean ± SD. n denotes number of fibres and N denotes number of animals. * significantly different to Fetal control ($P < 0.05$). pCa_x = indicates the amount of Ca^{2+} needed to produce "x" amount of force; h_{Ca} = the maximum slope of the force-pCa curve.

Figure Legends

Figure 1. Diaphragm contractile properties: (A) maximum specific force (Po); (B) peak twitch force (Pt); (C) time to peak (TTP); (D) half relaxation time (1/2 RT); (E) peak twitch force as a ratio of maximum force (Pt:Po); (F) fatigue index (FI); (G) force-frequency relationship displayed as specific force (N·cm-2); and (H) force-frequency relationship normalised to maximum specific for the fetal control, saline and LPS groups (N = 7). Box and whisker plots represent median, minimum and maximum values, and all data points are visible. Force-frequency data are presented as mean ± SD. *, ** and *** significant difference between the saline group and fetal control group at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. # and ### significant difference between the LPS group and fetal control group at $p < 0.05$ and $p < 0.001$, respectively.

Figure 2. (A) Force-pCa curve of 'fast' diaphragm fibres from fetal control (n = 13), saline (n = 24) and LPS (n = 15) preterm lambs, expressed as a percentage of maximum Ca^{2+}-activated force at pCa (-log [Ca^{2+}]) 4.7. Data are presented as mean ± SD, and n = number of fibres. (B-D) Fibres were classified as normal (single sigmoid curve), intermediate (double sigmoid curve, $pSr_{50}1 < 5.90$, $h_{Sr}1 < 2.0$) or hybrid (double sigmoid curve, $pSr_{50}1 > 5.90$, $h_{Sr}1 > 2.0$) based on Sr^{2+} activation parameters.

Figure 3. Diaphragm muscle fibre types and cross-sectional area (CSA) from fetal control, saline and LPS groups (n = 6). (A) Muscle fibre sections were stained with a combination of MHC_{emb} (red), MHC_{I} (blue), MHC_{IIa} (green) antibodies, a combination of MHC_{I} (blue), MHC_{IIa} (green), and MHC_{IIabx} (red) antibodies, or H & E. Myofibre size (measured by CSA) was quantified from transverse sections stained with H & E. Graphs show (B) the proportion of myofibre types and (C) myofibre CSA. Data are presented as mean ± SD. MHC, myosin heavy chain.

Figure 4. Relative mRNA expression for (A) *IL-1β*, (B) *IL-6*, (C) *IL-10*, and (D) *TNF-α* in the fetal control group (N = 7), saline group (N = 8) and LPS group (N = 8). Values are presented as arbitrary concentrations normalised against a

geometric mean of three reference genes. Box and whisker plots represent median, minimum and maximum values, and all data points are visible.

Figure 5. Anabolic and proteolytic signalling in diaphragm from fetal control (N = 7), saline (N = 8) and LPS groups (N = 8, unless otherwise stated). (A) P/T-Akt protein content; (B) P/T-mTOR protein content; (C) P/T-4EBP1 protein content; (D) *MuRF1* mRNA; (E) *MAFbx* mRNA; and (F) 20 S proteasome activity (N = 9 for LPS group). Protein content is presented as phosphorylated (P) protein content normalised to total (T) protein content. *MuRF1* and *MAFbx* are presented as arbitrary concentrations normalised against a geometric mean of three reference genes. 20 S proteasome activity is normalised against total protein concentration. Box and whisker plots represent median, minimum and maximum values, and all data points are visible. Inserts show representative Western blots of phosphorylated and total protein for AKT, mTOR and 4EBP1. * significant main effect between treatment groups at $p < 0.05$.

Figure 6. Expression (mRNA) of the antioxidant genes, (A) *SOD 1* , (B) *GPX 1* , and (C) protein carbonyl content in the fetal control (N = 7), saline (N = 8) and LPS groups (N = 8 for *GPX1* and *SOD 1* mRNA, N = 9 for protein carbonyl). Protein carbonyl content is normalised against total protein concentration. Box and whisker plots represent median, minimum and maximum values, and all data points are visible. * significant difference between the saline group and fetal control group at $p < 0.05$.

Figure 1

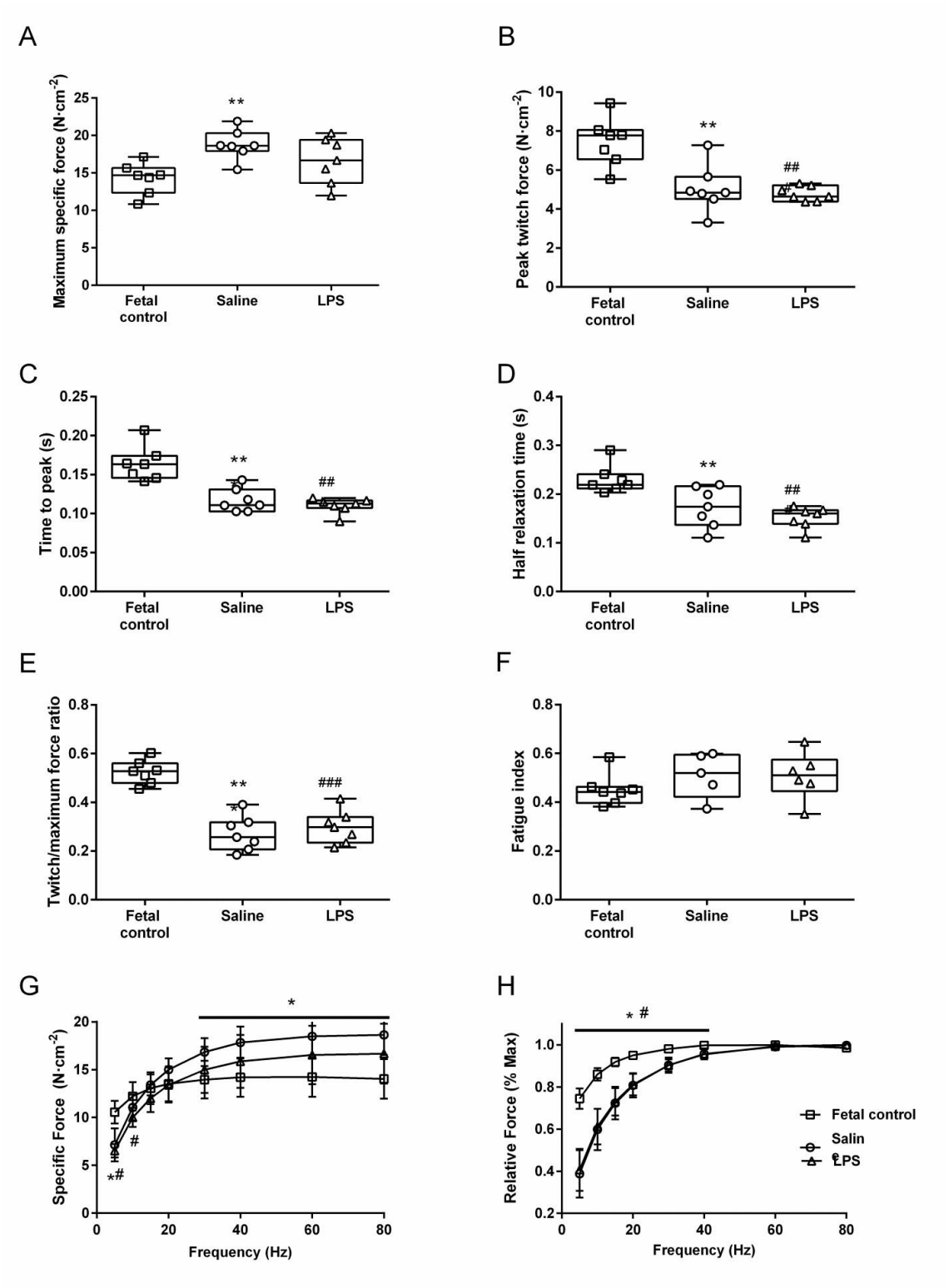

A
Maximum specific force (N·cm-2)
25
20
15
10
5
0
**
Fetal control
Saline
LPS
B
Peak twitch force (N·cm-2)
10
8
6
4
2
0
**
##
Fetal control
Saline
LPS
C
Time to peak (s)
0.25
0.20
0.15
0.10
0.05
0.00
**
##
Fetal control
Saline
LPS
D
Half relaxation time (s)
0.4
0.3
0.2
0.1
0.0
**
##
Fetal control
Saline
LPS
E
Twitch/maximum force ratio
0.8
0.6
0.4
0.2
0.0
**
###
Fetal control
Saline
LPS
F
Fatigue index
0.8
0.6
0.4
0.2
0.0
Fetal control
Saline
LPS
G
Specific Force (N·cm-2)
20
15
10
5
0
*
#
*#
0
20
40
60
80
Frequency (Hz)
H
Relative Force (% Max)
1.0
0.8
0.6
0.4
0.2
* #
Fetal control
Salin
LPS
0
20
40
60
80
Frequency (Hz)

Figure 2

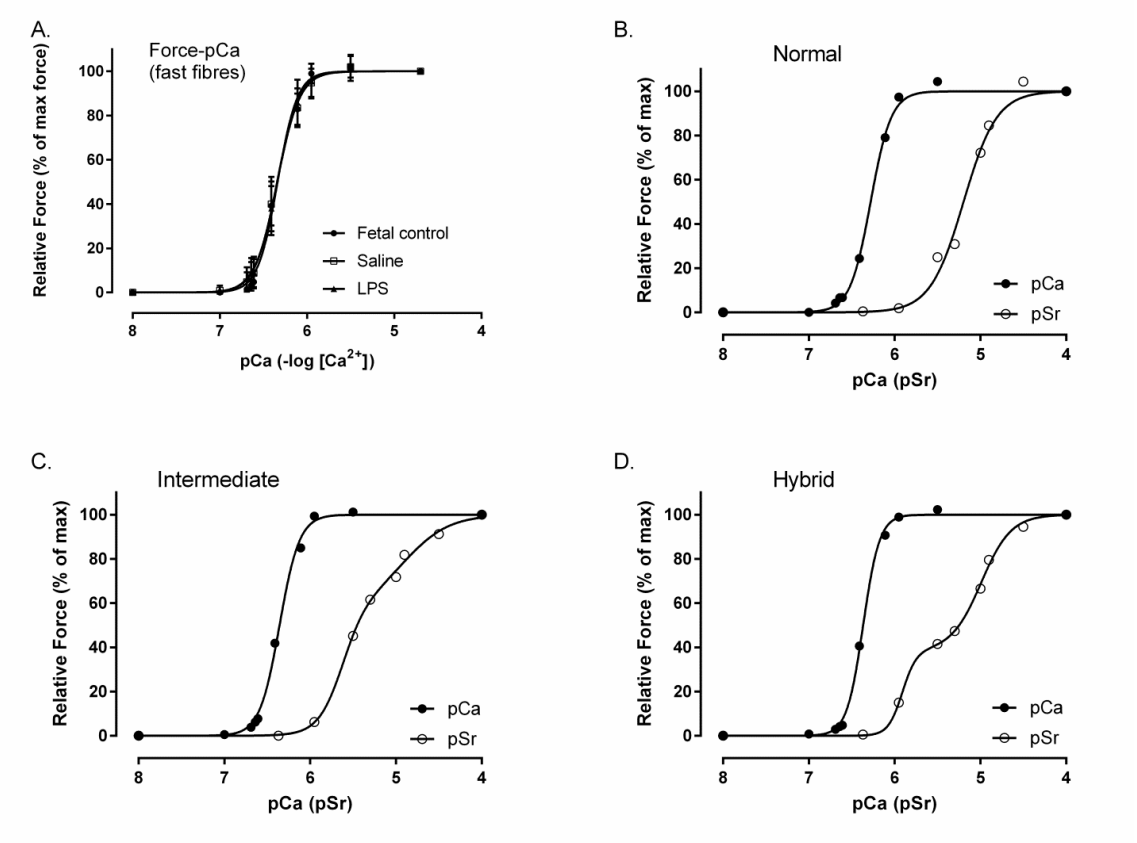

A.
Force-pCa
(fast fibres)
Relative Force (% of max force)
pCa (-log [Ca2+])
Fetal control
Saline
LPS
B.
Normal
Relative Force (% of max)
pCa (pSr)
pCa
pSr
C.
Intermediate
Relative Force (% of max)
pCa (pSr)
pCa
pSr
D.
Hybrid
Relative Force (% of max)
pCa (pSr)
pCa
pSr

Figure 3

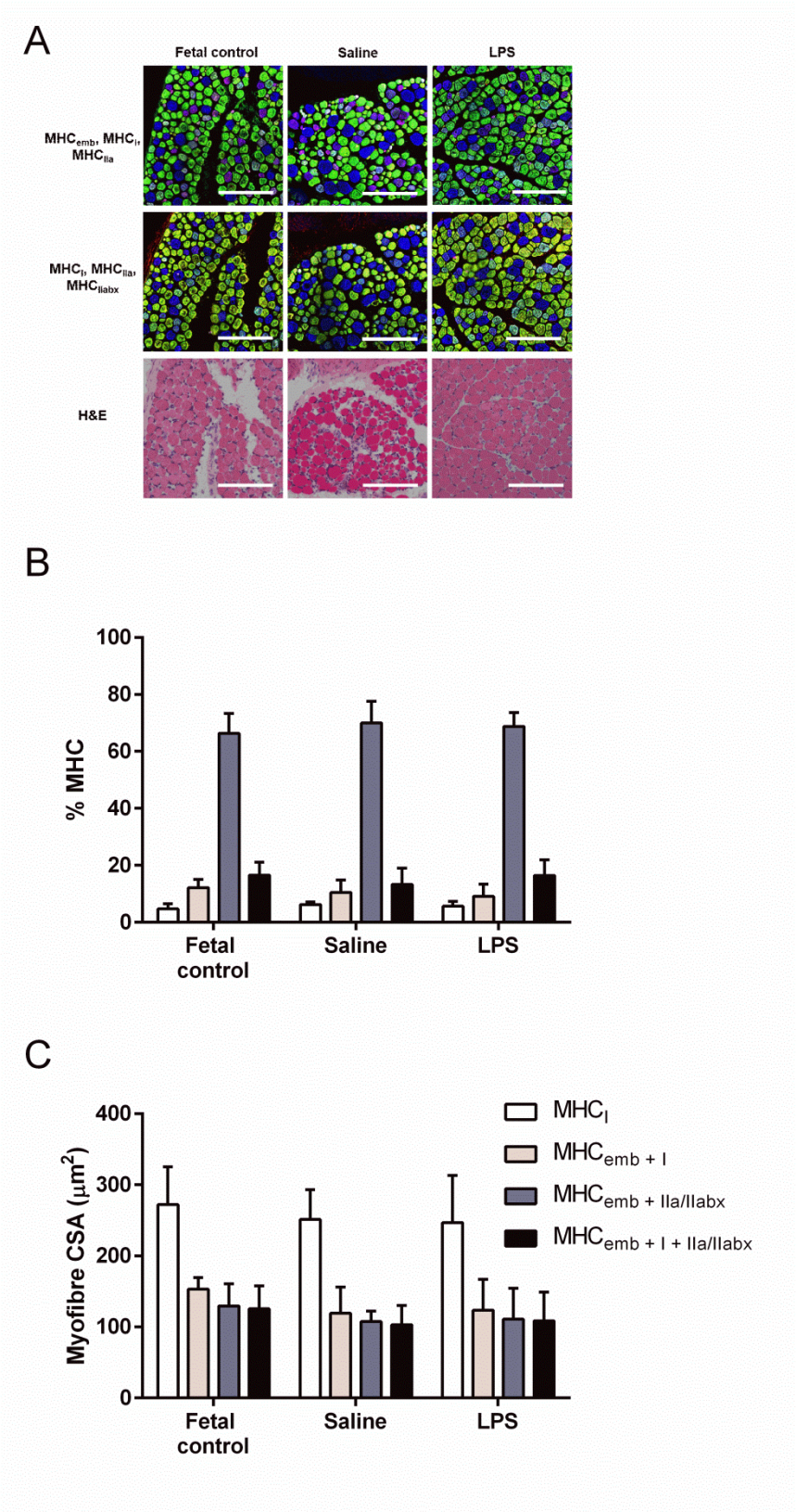

A
Fetal control
Saline
LPS
MHCemb, MHCI, MHCIIa
MHCI, MHCIIa, MHCIIabx
H&E
B
% MHC
100
80
60
40
20
0
Fetal control
Saline
LPS
C
Myofibre CSA (μm2)
400
300
200
100
0
Fetal control
Saline
LPS
MHCI
MHCemb + I
MHCemb + IIa/IIabx
MHCemb + I + IIa/IIabx

Figure 4

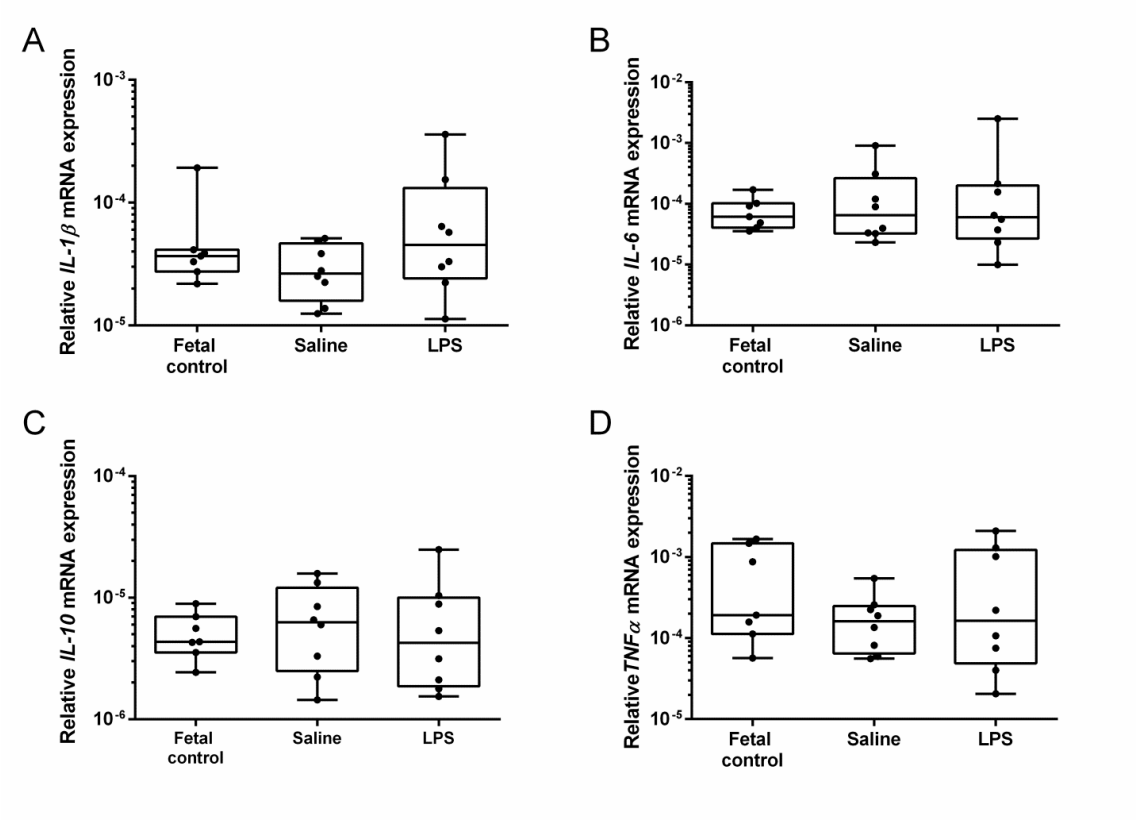

A
Relative IL-1β mRNA expression
10-3
10-4
10-5
Fetal control
Saline
LPS
B
Relative IL-6 mRNA expression
10-2
10-3
10-4
10-5
10-6
Fetal control
Saline
LPS
C
Relative IL-10 mRNA expression
10-4
10-5
10-6
Fetal control
Saline
LPS
D
Relative TNFα mRNA expression
10-2
10-3
10-4
10-5
Fetal control
Saline
LPS

Figure 5

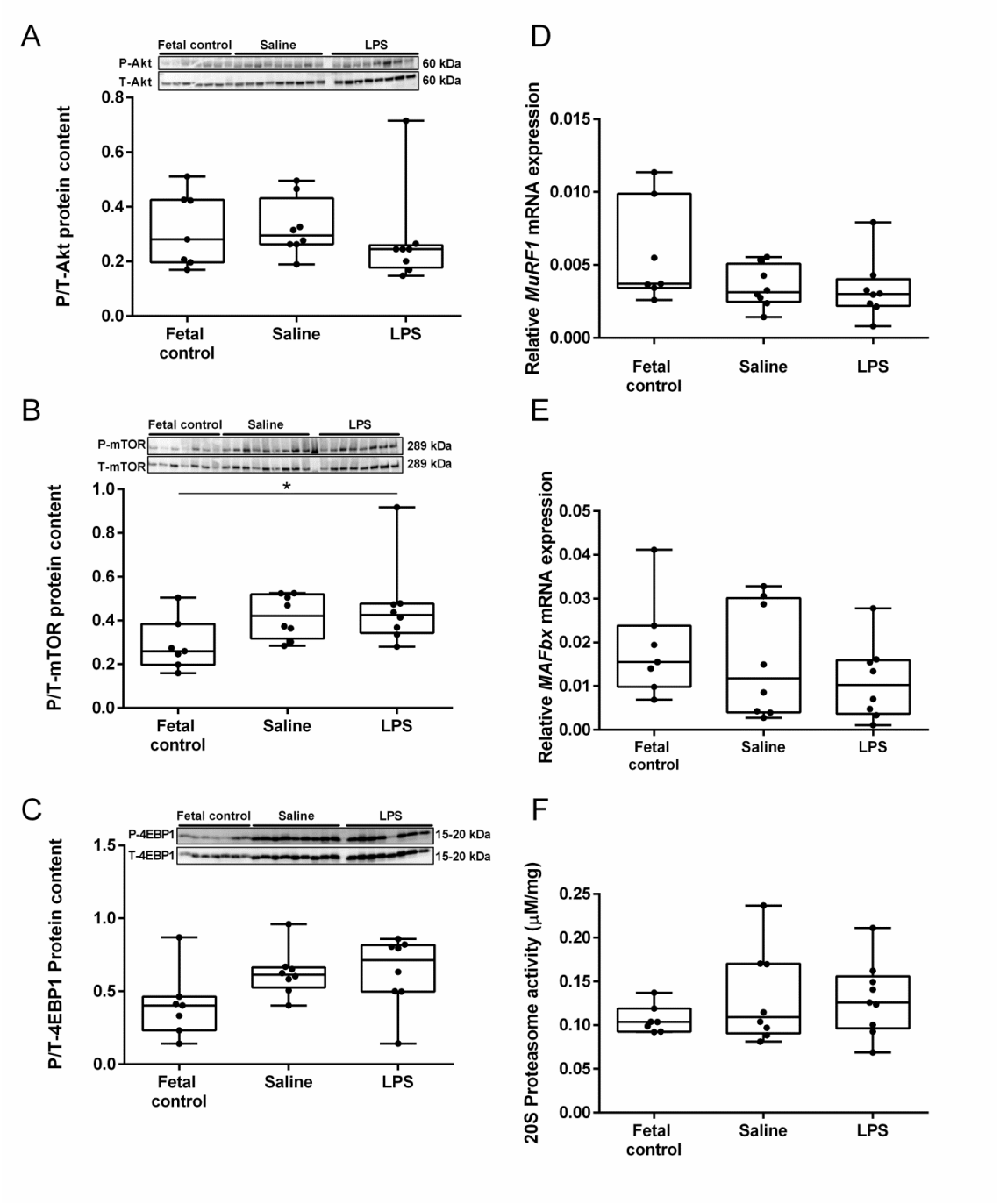
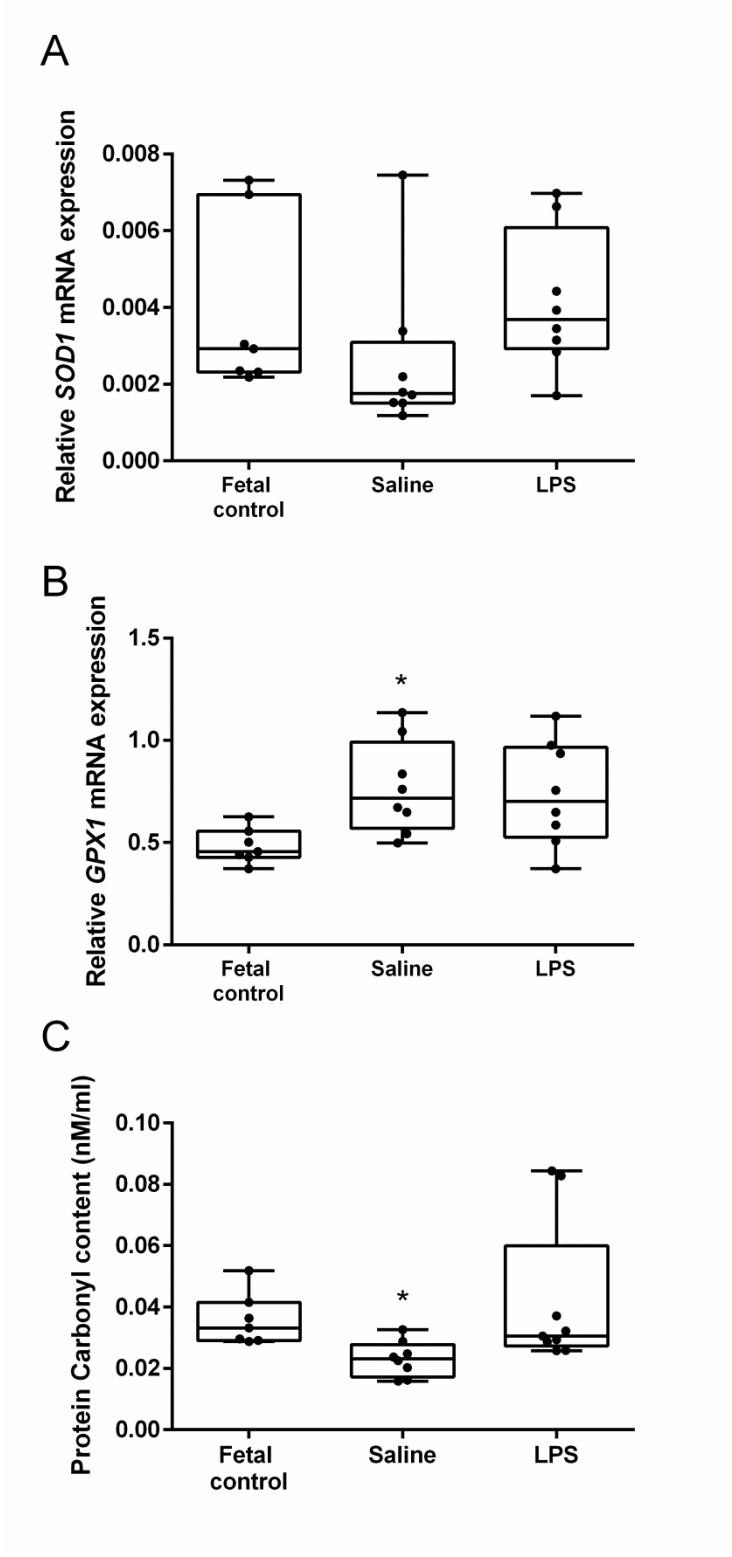

A
Fetal control
Saline
LPS
P-Akt
T-Akt
60 kDa
60 kDa
P/T-Akt protein content
0.8
0.6
0.4
0.2
0.0
Fetal control
Saline
LPS
B
Fetal control
Saline
LPS
P-mTOR
T-mTOR
289 kDa
289 kDa
*
P/T-mTOR protein content
1.0
0.8
0.6
0.4
0.2
0.0
Fetal control
Saline
LPS
C
Fetal control
Saline
LPS
P-4EBP1
T-4EBP1
15-20 kDa
15-20 kDa
P/T-4EBP1 Protein content
1.5
1.0
0.5
0.0
Fetal control
Saline
LPS
D
Relative MuRF1 mRNA expression
0.015
0.010
0.005
0.000
Fetal control
Saline
LPS
E
Relative MAFbx mRNA expression
0.05
0.04
0.03
0.02
0.01
0.00
Fetal control
Saline
LPS
F
20S Proteasome activity (µM/mg)
0.25
0.20
0.15
0.10
0.05
0.00
Fetal control
Saline
LPS

Figure 6


A
Relative SOD1 mRNA expression
0.008
0.006
0.004
0.002
0.000
Fetal control
Saline
LPS
B
Relative GPX1 mRNA expression
1.5
1.0
0.5
0.0
*
Fetal control
Saline
LPS
C
Protein Carbonyl content (nM/ml)
0.10
0.08
0.06
0.04
0.02
0.00
*
Fetal control
Saline
LPS

SCIENTIFIC REPORTS

OPEN

Optical coherence tomography-based contact indentation for diaphragm mechanics in a mouse model of transforming growth factor alpha induced lung disease

Received: 17 November 2016

Accepted: 30 March 2017

Published: xx xx
Published online: 04 May 2017

Kimberley C. W. Wang[1], Chrissie J. Astell[2], Philip Wijesinghe[3,4], Alexander N. Larcombe[1,5], Gavin J. Pinniger[2], Graeme R. Zosky[1,6], Brendan F. Kennedy[4,7], Luke J. Berry[1], David D. Sampson[3,8], Alan L. James[9], Timothy D. Le Cras[10] & Peter B. Noble[1,2,11]

This study tested the utility of optical coherence tomography (OCT)-based indentation to assess mechanical properties of respiratory tissues in disease. Using OCT-based indentation, the elastic modulus of mouse diaphragm was measured from changes in diaphragm thickness in response to an applied force provided by an indenter. We used a transgenic mouse model of chronic lung disease induced by the overexpression of transforming growth factor-alpha (TGF-α), established by the presence of pleural and peribronchial fibrosis and impaired lung mechanics determined by the forced oscillation technique and plethysmography. Diaphragm elastic modulus assessed by OCT-based indentation was reduced by TGF-α at both left and right lateral locations ($p < 0.05$). Diaphragm elastic modulus at left and right lateral locations were correlated within mice ($r = 0.67$, $p < 0.01$) suggesting that measurements were representative of tissue beyond the indenter field. Co-localised images of diaphragm after TGF-α overexpression revealed a layered fibrotic appearance. Maximum diaphragm force in conventional organ bath studies was also reduced by TGF-α overexpression ($p < 0.01$). Results show that OCT-based indentation provided clear delineation of diseased diaphragm, and together with organ bath assessment, provides new evidence suggesting that TGF-α overexpression produces impairment in diaphragm function and, therefore, an increase in the work of breathing in chronic lung disease.

Optical coherence tomography (OCT) is an interferometric imaging technique that forms volumetric images of tissue structure based on contrast generated by the back-scattering of light waves[1]. In the respiratory field, an increasing number of new applications for OCT are emerging[2]. Various endoscopic, needle and bench top OCT systems have been used to measure lumen dimensions in small[3], large[4, 5] and upper airways[6], to assess wall morphology and thickness[4, 7], and to visualise individual alveoli[8]. However, one feature of OCT in the respiratory

[1]Telethon Kids Institute, The University of Western Australia, Subiaco, Western Australia, Australia. [2]School of Anatomy, Physiology and Human Biology, The University of Western Australia, Perth, Western Australia, Australia. [3]Optical+Biomedical Engineering Laboratory, School of Electrical, Electronic & Computer Engineering, The University of Western Australia, Perth, Western Australia, Australia. [4]BRITElab, Harry Perkins Institute of Medical Research QEII Medical Centre, Crawley, Western Australia, Australia. [5]School of Public Health, Curtin University, Perth, Western Australia, Australia. [6]University of Tasmania, Hobart, Tasmania, Australia. [7]School of Electrical, Electronic & Computer Engineering, The University of Western Australia, Perth, Western Australia, Australia. [8]Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, Perth, Western Australia, Australia. [9]Sir Charles Gairdner Hospital, Nedlands, Western Australia, Australia. [10]Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA. [11]Centre for Neonatal Research and Education, School of Paediatrics and Child Health, The University of Western Australia, Perth, Western Australia, Australia. Correspondence and requests for materials should be addressed to K.C.W.W. (email: kimberley.wang@telethonkids.org.au)

system that could provide significant clinical application has yet to be fully exploited – assessment of respiratory tissue mechanics.

While the primary application of imaging techniques such as OCT is to provide information on tissue structure, the approach may be extended to the assessment of tissue mechanics (e.g., elastic modulus) by measuring deformation of structure in response to an applied force[9,10]. Indentation assessed by OCT, hereafter referred to as 'OCT-based indentation'[11–14] measures tissue deformation and structure, providing complementary information pertaining to organ function. This promising technology provides an opportunity to better understand the functional consequences of disease-related changes in tissue structure and mechanics, not possible using more standard techniques in respiratory mechanics[15].

We used OCT-based indentation to characterise diaphragm dysfunction in a transgenic mouse model of transforming growth factor-alpha (TGF-α)-induced lung disease. Overexpression of TGF-α in mice has been shown to produce lung and pleural fibrosis and impair respiratory mechanics[16,17]. We further questioned whether the pathology would extend to the diaphragm and alter its function. Chronic lung disease produced by TGF-α was initially established by histology and changes in lung mechanics assessed by the forced oscillation technique (FOT) and plethysmography, and the proposed changes in diaphragm function were demonstrated by the assessment of contractile force in organ bath chambers. The aim of the study was to determine whether OCT-based indentation could delineate changes in diaphragm mechanics in a mouse model of chronic lung disease.

Results

As fully described in the Methods, lung disease was induced by TGF-α overexpression in transgenic mice, triggered by doxycycline (Dox) in the diet. Levels of TGF-α in lung homogenates were increased by Dox (Fig. 1A). Animal characteristics are provided in Table 1. In the FOT study, mice treated with Dox were marginally smaller than the Control group which contained a greater proportion of males. There were no differences between Dox and Control groups in the other studies.

Establishment of lung disease. Changes in lung function and structure after Dox were assessed to confirm the presence of lung disease. Mechanical measurements were performed on two different mouse genotypes: wild type (++) or heterozygote (+−); for the early growth response one (Egr-1) gene, which potentially effects the severity of the response to Dox (see Methods). Lung elastance (Fig. 1B), damping (Fig. 1C) and airway resistance (Fig. 1D) were increased in the Dox group compared with the Control group. Thoracic gas volume was also increased in the Dox group compared with Control: Control/Egr-1++, 0.29 ± 0.05 mL, n = 7; Control/Egr-1+−, 0.27+− 0.02 mL, n = 8; Dox/Egr-1++, 0.38 ± 0.04 mL, n = 7; Dox/Egr-1+−, 0.35 ± 0.05 mL, n = 8; $p < 0.05$. There was no effect of genotype (Egr-1++ vs. +−) on the response to Dox. A single genotype (Egr-1+−) was therefore used for all subsequent analyses.

Consistent with previous studies[16,17], Dox produced pleural thickening and peribronchial fibrosis (Fig. 2) with no histological evidence of lung inflammation. Semi-quantitative fibrosis score (median and range) was 1 [0–3] in the Dox group (n = 10) which was greater than the Control group (0 [0-0], n = 9, $p < 0.001$).

OCT-based indentation of diaphragm tissue. Figure 3 shows transverse images of diaphragm tis- sue acquired by OCT, and includes a thickness map that was used to identify the regions for indentation (see Methods). Normal fibre orientation was apparent in diaphragms from the Control group (Fig. 3C). In comparison, a fibrotic layered appearance of the diaphragm from the Dox group (Fig. 3D) was common (6/10) compared with the Control group (0/8; chi-squared = 7.2, $p < 0.01$). Diaphragms in the Control group tended to be associated with adipose tissue (porous spongy appearance) at anterior locations (Fig. 3E). Diaphragms were less likely to be associated with adipose tissue in the Dox group (0/10) compared with the Control group (3/8, chi-squared = 4.5, $p < 0.05$).

Due to the potential confounding effects of soft adipose tissue in the determination of diaphragm elastic modulus, OCT-based indentation data are only reported for the left and right lateral locations. There was a correlation between the elastic modulus of the left and right diaphragm (Fig. 4A). The elastic modulus of the diaphragm from the Dox group was reduced compared with Control indicating that the diseased diaphragm was softer (Fig. 4B). There was no significant difference in the thicknesses of the diaphragm between groups: 0.68 ± 0.02 mm and 0.67 ± 0.02 mm at right and left locations respectively, in the Control group, and 0.67 ± 0.03 and 0.63 ± 0.03 mm in the Dox group.

Organ bath assessment of diaphragmatic force. Cross sectional histology of diaphragm tissue from Control and Dox groups is shown in Fig. 5(A and B). Thickening of the diaphragm ligament and collagen deposition was evident in the Dox group. The percentage of collagen within the diaphragm was positively correlated with lung fibrosis score from the same group of mice (n = 16, r = 0.55, $p < 0.05$). Maximum specific force in the diaphragm was reduced in Dox-treated animals with greater fibrosis score (Fig. 5C). Whilst force was reduced as the percentage of collagen within the muscle increased, the reduction in force was far greater than that predicted from a simple loss of muscle mass (Fig. 5D). There was no difference in passive diaphragmatic force or cross sectional area between groups.

Discussion

The purpose of the study was to investigate the assessment of respiratory mechanics, specifically elastic properties of diaphragm tissue, using a new (for the respiratory system) method; OCT-based indentation. We demonstrated that OCT-based indentation clearly delineated changes in diaphragm mechanics and altered structure in a mouse model of TGF-α-induced lung disease. Below, we discuss important methodological considerations, the utility of OCT-based assessment of respiratory mechanics, and new findings on TGF-α induced respiratory disease.

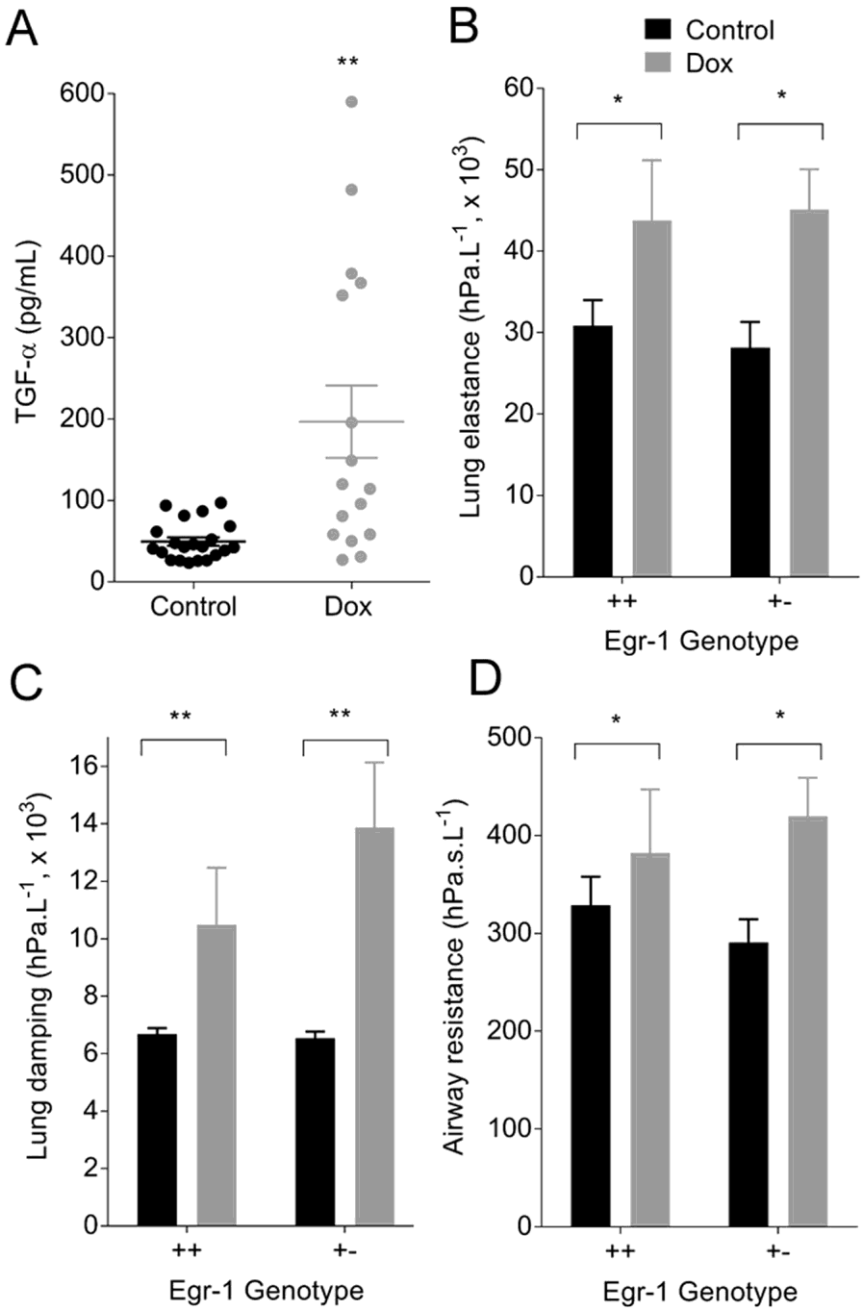


Figure 1. Dox induced TGF-α overexpression (**A**, Control n = 21; Dox n = 16; $^{**}p < 0.01$) and increased lung elastance (**B**, $^{*}p < 0.05$), lung damping (**C**, $^{***}p < 0.01$) and airway resistance (**D**, $^{*}p < 0.05$) compared with the Control group. There was no effect of genotype. Data are mean ± SE; Egr-1++ Control n = 7, Dox n = 7; Egr-1+− Control n = 7, Dox n = 12.

	Control		Doxycycline	
	Sex (M:F)	Mass (g)	Sex (M:F)	Mass (g)
FOT	12:6	21.9 ± 0.8	9:11	19.5 ± 0.7*
OCT	2:6	20.8 ± 1.0	5:5	22.2 ± 0.8
Organ bath	4:5	21.8 ± 1.1	6:4	21.5 ± 1.1

Table 1. Group characteristics. Mean ± SE, $^{*}p < 0.05$. FOT, forced oscillation technique; OCT, optical coherence tomography.

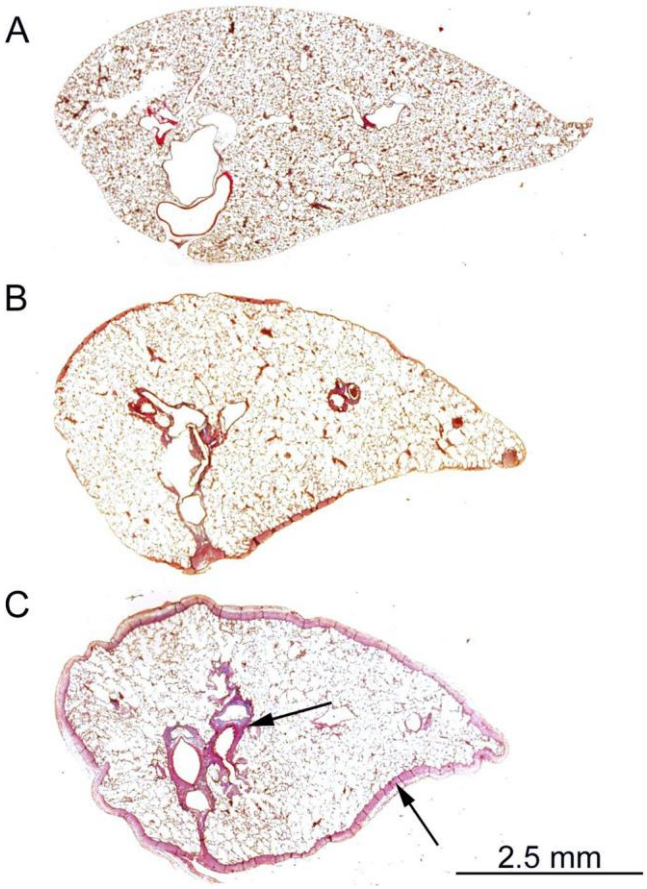


Figure 2. Dox produced pleural fibrosis and peribronchial fibrosis (indicated by arrow): (**A**), Control; (**B** and **C**) moderate and severe pleural fibrosis in the presence of Dox respectively.

The effects of TGF-α occur through activation of the epidermal growth factor receptor, which is implicated in numerous chronic respiratory diseases, including lung fibrosis, cancer, chronic obstructive pulmonary disease, asthma and cystic fibrosis[18]. In our transgenic mouse model of lung disease, TGF-α levels were increased in response to Dox. Importantly, in this model, TGF-α levels are increased in the absence of lung inflammation[16,17]; thereby, allowing the direct effects of TGF-α to be examined. In alignment with an increasing world-wide focus on non-inflammatory mechanisms of lung disease[19], such transgenic mouse models provide opportunities for new data on the physiological consequences of growth factors liberated as part of the disease cascade.

We initially confirmed the presence of TGF-α induced lung disease, through changes in lung mechanics (discussed further below) and the presence of pleural and peribronchial fibrosis. TGF-α effects are potentially regulated by the Egr-1 gene which is dependent upon the duration of exposure. Following extended periods of TGF-α exposure (~8 weeks on Dox), lung fibrosis is substantially enhanced in mice deficient or heterozygote for the Egr-1 gene, compared with wildtype[17]. However, we saw no differences in lung mechanics between Egr-1 genotypes (+/+ versus +/−) following a shorter 3-week exposure. For this reason, a single genotype was chosen for subsequent protocols.

Since fibrosis develops at the serosal pleura after TGF-α exposure (present study)[16,17], we queried whether this would also extend to parietal surfaces and infiltrate the diaphragm, thereby altering muscle mechanics. OCT-based indentation revealed a softening of the diaphragm tissue, which was accompanied by an abnormal layered appearance, consistent with fibrotic disease. While normal fibre orientation was apparent in diaphragms from control mice, this was disrupted in the Dox group. Histological sectioning revealed thickening of the diaphragm ligament in the Dox group, and increased collagen between fibres that was inversely associated with muscle force in organ bath studies. It is notable that the organ bath approach was only sufficient to detect more severe disease (Fibrosis score 1–2), in comparison with OCT-based indentation which was able to clearly delineate Control and Dox groups.

A strength of imaging-based assessment of tissue mechanics is that functional measurements are co-localised with images of structure. In the present study, OCT images revealed regional diversity between Control and Dox groups, characterised by a greater deposition of fat at anterior diaphragm locations. The presence of 'spongy' adipose tissue significantly reduced diaphragm elastic modulus in control animals (data not shown) and, therefore, masked real differences between Control and Dox groups. Images acquired by OCT were, therefore, used to identify regions free of adipose tissue (left and right lateral locations) so that measurements better reflected the intrinsic elastic properties of the diaphragm. Elastic modulus of diaphragm tissue at left and right locations (free

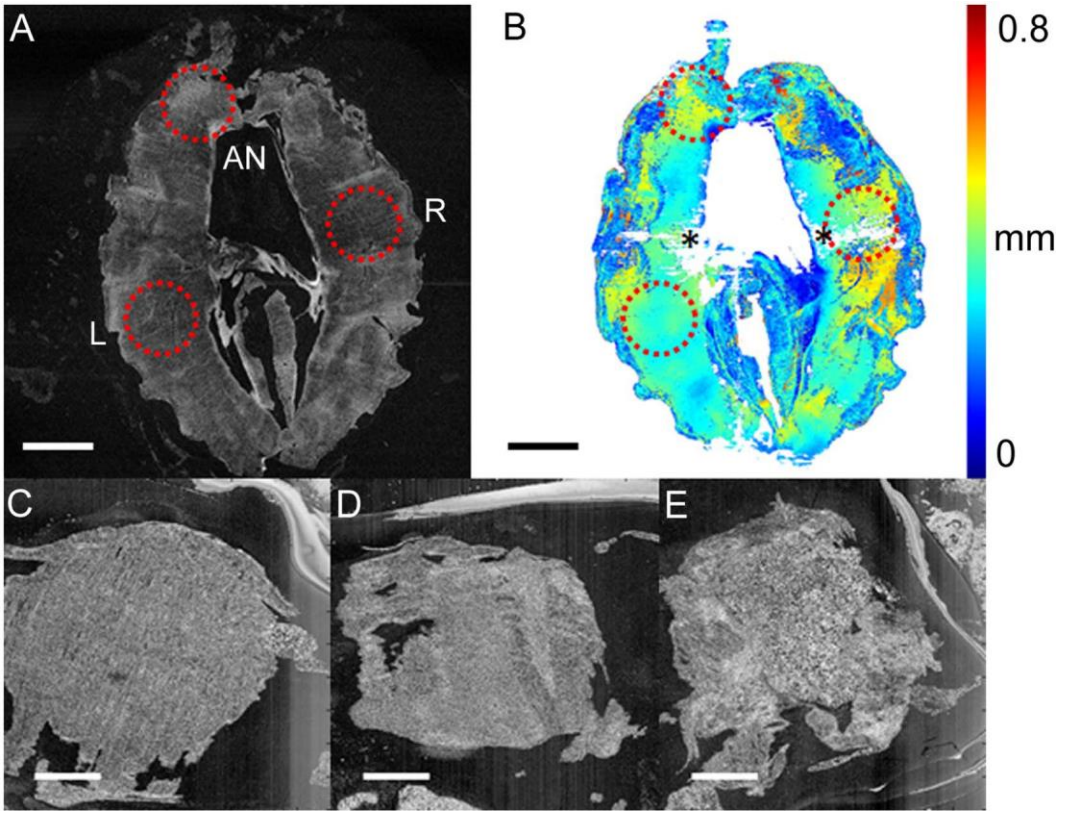


Figure 3. Transverse (*en face*) images of diaphragm recorded by OCT (**A**). Circles indicate the locations (left and right lateral, L and R) and anterior (AN) at which indentation was performed. Thickness map of the diaphragm (**B**); asterisks denote artefacts in thickness detection. At left and right lateral locations, normal diaphragm fibre orientation is evident in the Control group (**C**), compared with the Dox group (**D**), where connective tissue (speckled white appearance) occludes muscle. At anterior locations, diaphragm in the Control group was associated with adipose tissue, exhibiting a porous or spongy appearance (**E**). Scale bar is equal to 3 mm in (**A**) and (**B**), and 1 mm in (**C–E**).

of adipose tissue) was correlated ($r = 0.67$), suggesting that measurements were representative of tissue beyond the indenter field. To further minimise measurement error by ensuring complete and uniform contact of the indenter with the tissue, specific regions of assessment were those with the least surface heterogeneity, as determined from the thickness map.

In vivo translation of OCT-based indentation is a technical challenge that is dependent upon the anatomical location of the tissue of interest. With respect to the diaphragm, measurements are likely to be invasive but are feasible. In a recent study, the safety of diaphragm biopsy by laparoscopy was demonstrated without evidence of pneumothorax[20]. It is possible that OCT-based indentation could be performed under direct visualisation during laparoscopy and provide additional information than biopsy alone. Other than the diaphragm, one immediate application of OCT-based indentation is the measurement of airway elastance that may be altered in obstructive disease[21]. Elastic properties of airway passages are relatively difficult to assess in comparison with lung tissue, which dominates volume and pressure signals recorded at the mouth. Bronchoscopic delivery of OCT probes to the airway surface has been used to quantify lumen area and examine wall morphology[3,5]. However, for assessment of airway elastance, it is necessary to control and measure the applied stress, easily achieved *in vitro* through application of a known force. *In vivo*, one solution is to integrate imaging probes with an elastic membrane of known elasticity and to subsequently calculate the applied stress from the change in membrane thickness[22].

In an extension to OCT-based indentation, local measurements of tissue strain can be performed, referred to as compression optical coherence elastography[9,10,23]. By this approach, local strain is mapped across the tissue volume undergoing compression, forming mechanical contrast images (elastograms) to detect micro-mechanical features not identified by structural imaging. In the present study, compression elastography was performed alongside OCT-based indentation. An important distinction to OCT-based indentation is that compression elastography, in its simplest form, only provides measurement of strain, which in the absence of known local stress, is a relative measure of the mechanical properties. Strain has been used to identify tissue pathology via textural features[24]; however, in this study, there was no significant change in mechanical texture between animal groups, suggesting that the scale at which the mechanical properties vary in the disease model is lower than the elastogram resolution (~10–100 μm). Assessment based solely on local strain is somewhat limited and would be greatly advanced if local stress could also be determined. Recently, aided by the aforementioned elastic membrane to

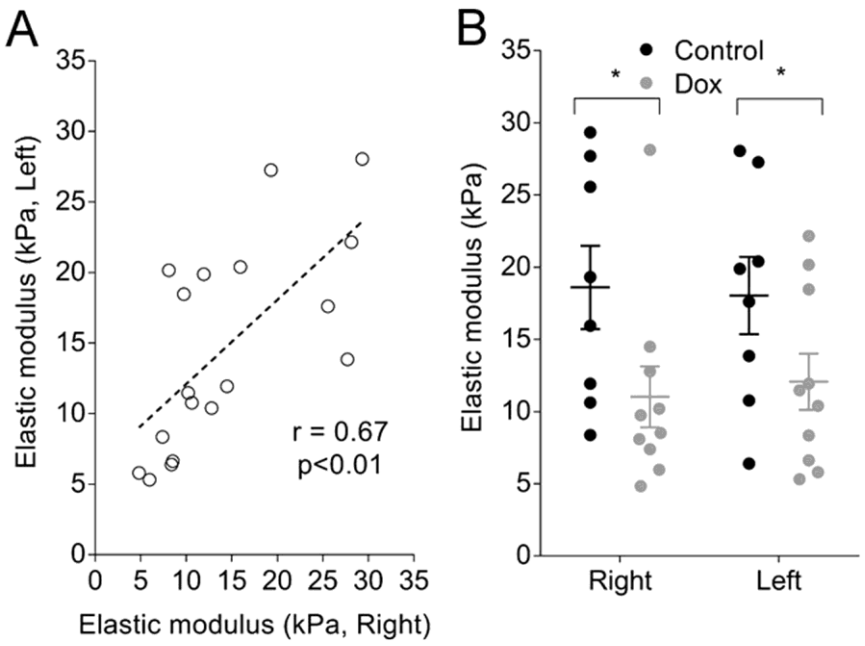


Figure 4. Elastic modulus of mouse diaphragm measured by OCT-based indentation. Right and left lateral measurements (data are from Control and Dox groups combined) were positively correlated (**A**, n = 18). Elastic modulus of diaphragm from the Dox group was reduced compared with the Control group (**B**). There was no effect of location. Data are mean ± SE. *$p < 0.05$.

measure local stress, images of absolute local elastance (local stress/local strain) were achieved with elastography in breast tissue[25]. A combination of indentation loading with local quantification of elastance via OCT elastography may prove to be the most useful approach to assess mechanical properties in respiratory science.

Due to technical constraints, it was not possible to directly correlate data generated by OCT-based indentation with organ bath assessment; instead, the organ bath approach was employed to provide an initial indication that the diaphragm was affected in the mouse model of lung disease, before testing a new methodology. Moreover, data generated from OCT-based indentation provides different and complementary information. OCT-based indentation reflects the mechanical properties of the tissue in the orientation perpendicular to the muscle fibres (as would be the case *in vivo*, analogous to a biopsy procedure); whereas, the organ bath approach assesses muscle mechanics in parallel to the contractile fibres. Together, these data provide some insight as to how diaphragm function may be altered in TGF-α induced lung disease. We propose that softening of the diaphragm tissue observed by OCT-based indentation occurs due to decoupling of skeletal muscle fibres by fibrotic infiltration, resulting in an energy-inefficient contractile process that attenuates the production and transmission of force throughout the diaphragm tissue.

There are several other less likely mechanisms that may lead to a reduction in diaphragmatic force after TGF-α exposure. There is evidence that TGF-α promotes muscle wastage of distal hind limbs in mice[26]. A decrease in muscle bulk cannot explain our findings since diaphragmatic force was normalised to muscle cross-sectional area. Changes in diaphragmatic force may be a direct consequence of collagen deposition. Intra-diaphragmatic collagen has been documented in patients with chronic obstructive pulmonary disease[27], and may increase the work of breathing. An increased proportion of collagen within the muscle layer reduces the number of functional contractile units in a given cross sectional area of muscle. However, decreases in diaphragm force were far greater than expected from a simple change in muscle composition (Fig. 5D).

Additional studies are required to determine if there is a consistent decrease in diaphragmatic adipose tissue in the current mouse model of TGF-α lung disease. Other studies have reported decreases in body weight[16,17] and absolute fat content following TGF-α exposure[26]. There is also evidence that mesenchymal progenitor cells are able to differentiate into both collagen type-I producing cells and adipocytes[28]. We can only speculate that a TGF-α driven fibrotic process may be associated with a down regulation of differentiation of adipocyte cells.

The present study provides further mechanistic insight into changes in lung mechanics that occur in TGF-α induced lung disease. Previous studies reported changes in total respiratory compliance and resistance[16,17], whereas, our study isolated these changes to lung elastance and damping, airway (Newtonian) resistance and lung volume. In the absence of pulmonary fibrosis (fibrosis was not present in alveolar walls), increased lung elastance can be attributed to pleural thickening and greater elastic load. Tissue damping on the other hand reflects viscous properties of the lung which will be affected by fibrosis in the peribronchial space, as well as pleural fibrosis. Peribronchial fibrosis may also account for increased airway resistance, likely as a result of airway-parenchymal decoupling[29], promoting airway collapse and increasing gas volume determined by plethysmography at atmospheric elastic-equilibrium lung volume.

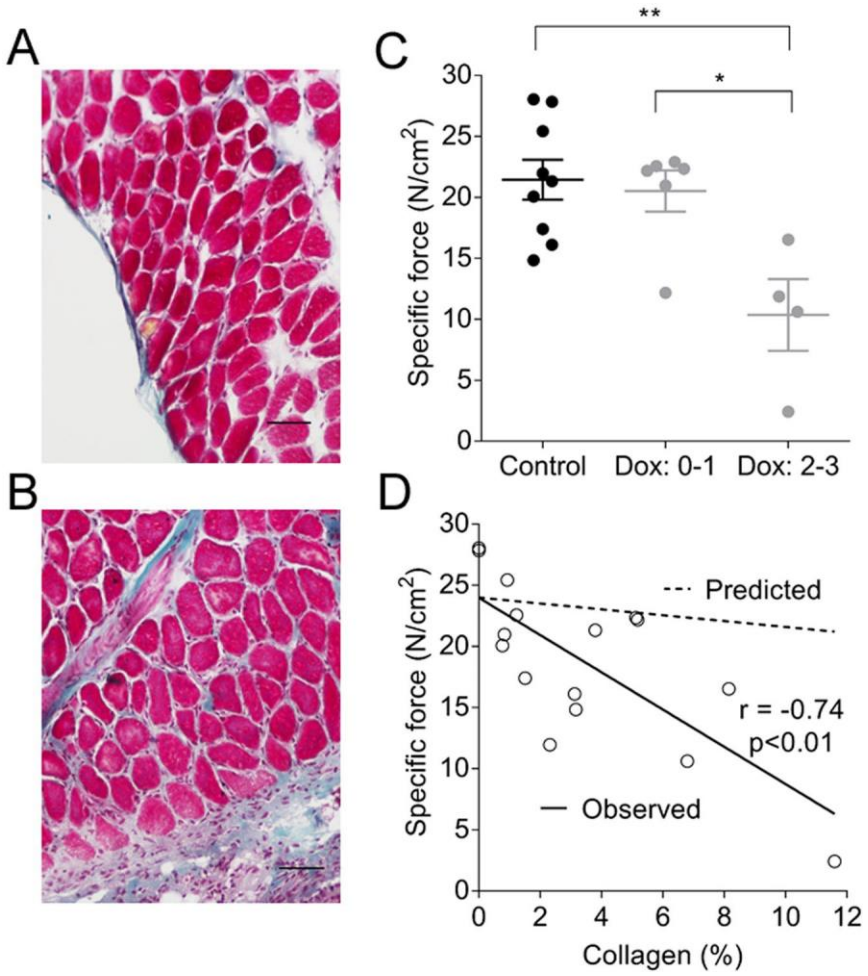


Figure 5. Cross sectional (scale bar = 0.1 mm) histological images of diaphragm from Control (**A**) and Dox groups (**B**). Blue collagen infiltration between muscle fibres and ligament thickening are apparent in the Dox group. Diaphragm maximum specific force (**C**) was reduced with more severe lung disease (fibrotic scores 2–3, $^{**}p < 0.01$, $^{*}p < 0.01$). There was an inverse correlation between diaphragm collagen (%) and force (**D**, n = 16). The predicted line shows the change in force that would be expected if muscle mass was replaced by collagen. Predicted force was calculated from the *y*-intercept of the observed line (24 N/cm^2) multiplied by the fraction of muscle (1-%collagen/100). Data are mean ± SE.

Finally, taken together, findings on lung tissue and diaphragm have clinical significance. In human disease, a variety of methods are used to assess chest wall disease in those with varying degrees of breathlessness, often in relation to exposures to agents such as asbestos. Often the degree of symptoms, such as breathlessness is out of proportion with functional and radiological changes. It has been suggested that pleural thickening involving the diaphragm has a greater effect on breathlessness than thickening elsewhere on the chest wall[30]. The present study suggests that changes within the diaphragm not observable on routine imaging may still alter diaphragm function and contribute to symptoms.

In conclusion, the present study used OCT-based indentation to demonstrate impaired diaphragm mechanics following TGF-α exposure, which may contribute to morbidity and mortality in chronic lung disease. We propose that assessment of tissue mechanics by OCT has diagnostic potential across a diverse range of chronic lung diseases.

Methods

Our experimental approach was to establish TGF-α-induced lung disease in transgenic mice and to quantitatively measure these changes using established functional and histological methodologies, notably the FOT and plethysmography, and *in vitro* organ bath experiments on excised diaphragm tissue. The utility of OCT-based indentation was then investigated through the assessment of diaphragm tissue structure and mechanics from mice with and without TGF-α-induced lung disease.

Animal handling. The mouse model used is described fully in a previous publication[17], which in this study was re-derived on a BALB/c background (The Jackson Laboratory). Mice bi-transgenic for Clara cell secretory protein-rtTA(+/−)/[tetO](7)-TGF-α(+/−) and wild type (++) or heterozygote (+−) for the Egr-1 gene,

conditionally expressing TGF-α in the lungs in response to Dox were used in this study. Young mice (4–5 weeks) were fed Dox or a control diet (Control) for three weeks before being randomly allocated to the *in vivo* or *in vitro* protocol. Mice were anaesthetised by intraperitoneal injection of xylazine (2 mg/mL) and ketamine (40 mg/mL) at a dose 0.1 mL/10 g body weight for *in vivo* lung function, or overdosed with the same solution (0.2 mL/10 g). All experiments were approved by the Animal Ethics Committee at Telethon Kids Institute. All methods were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, Australia, 7th Edition).

Forced oscillation technique and plethysmography. Lung mechanics were assessed in anaesthe- tised and mechanically ventilated mice by low frequency FOT[31]. Outcome variables included airway resist- ance, lung elastance and damping. Plethysmography was used to measure thoracic gas volume at atmospheric elastic-equilibrium lung volume[32]. The trachea was occluded at end expiration and the intercostal muscles were stimulated with intramuscular electrodes to induce inspiratory efforts. Associated changes in tracheal pressure and plethysmograph box pressure were used to calculate volume according to Boyle's law.

Organ bath studies. Longitudinal strips (2–3 mm wide) of intact muscle fibres were dissected from the right hemi-diaphragm and mounted in an *in vitro* muscle test system (1200A, Aurora Scientific, Canada) containing mammalian Ringer solution (pH 7.3) bubbled with carbogen at 25 °C[33]. Diaphragm strips were stimulated at optimal muscle length (L_o) with 0.2 ms supramaximal square wave pulses at 80 Hz to determine maximum tetanic force (g). Passive force development was determined by passive lengthening from 0.8 L_o–1.1 L_o at 0.05 L_o/s. Force (g) was normalised to cross-sectional area (N/cm^2) calculated from muscle fibre length, muscle mass and density ($1.056\ g/cm^3$).

OCT-based indentation. Whole diaphragms were imaged (Fig. 3A) using a benchtop spectral-domain OCT system[34]. OCT captured volumetric images of tissue microstructure, at an axial and lateral resolution of 8 μm and 11 μm, respectively[34]. Indentation was performed at left and right lateral, and anterior locations (Fig. 3A). The diaphragm muscle was compressed by a 3-mm diameter, flat, cylindrical indenter (a weighted rod), which applied a physiologically relevant stress onto the sample, i.e., 0.92 kPa or ~10 cmH_2O. The imaging beam was incident on the sample (typically 500 mm thick) from the opposite side with respect to the indenter. The stress at the tissue surface was determined from the known weight placed on the indenter and the OCT-measured surface area of tissue in contact with the indenter. The strain of the diaphragm was captured using OCT by measuring its thickness in the uncompressed and compressed states. To reduce artefacts associated with tissue inhomogeneity, flat regions of the diaphragm were chosen for assessment, qualitatively, aided by a thickness map (Fig. 3B), generated from OCT volumes using a Canny edge detector. OCT-based indentation was performed on the same regions (to within 1 mm) in all diaphragms; these regions were chosen prior to the measurements presented in this paper.

At each location, the elastic (Young's) modulus of the muscle was calculated as the ratio of the stress applied to the tissue over the strain (Δthickness/initial thickness), i.e., Hooke's Law. This model was employed, in contrast to common indentation models, as the diaphragm thickness (~500 μm) was significantly smaller than the indenter diameter (3 mm). A single value of elastic modulus was obtained by determining the average strain in the region under test and relating this to the measured stress. The error in the measured elastic modulus (derivation in Supplement 1), in an ideal case, can be calculated by propagating the error associated with the measured parameters, *i.e.*, the mass, the diameter and the displacement of the indenter, and the thickness of the sample. The error, given that the elastic modulus of the tissue is 14 kPa (combined average of Control and Dox groups), was measured as 0.83 kPa (standard deviation). Furthermore, biological tissue is typically non-linear elastic, exhibiting a higher effective elastic modulus with increased applied stress. The non-linearity was measured by applying a higher stress of 2.4 kPa to all diaphragms; at 2.4 kPa the average elastic modulus was 6.5% greater than at 0.92 kPa. The non-linearity should be taken into account when comparing diaphragm elastic modulus measured under different stresses.

Tissue analyses and histology. After the removal of the diaphragm for *in vitro* studies, lungs were fixed by formalin immersion and embedded longitudinally to acquire cross sections stained by Masson's Trichrome. Fibrosis was assessed semi-quantitatively by a blinded observer (L.B): 0 (none); 1 (mild); 2 (moderate) and 3 (severe). Diaphragm strips (16/19) were embedded in tragacanth and frozen in isopentane cooled in liquid nitrogen. Cross sections were also stained with Masson's Trichrome to measure collagen as a percentage of cross sectional area. In some animals (Egr-1+−), concentration of TGF-α in lung homogenates was assessed by enzyme-linked immunosorbent assay (Elabscience, E-EL-H1586).

Analysis and statistics. Data were transformed where necessary to ensure the assumptions of normality and homoscedasticity of variances for the parametric tests were satisfied. Where this was not possible equivalent non-parametric tests were used. Linear associations were assessed by correlation analysis (Pearson or Spearman based on normality). All statistics were performed by GraphPad Prism (GraphPad Software, version 7.02).

References

1. Drexler, W. & Fujimoto, J. G. *Optical coherence tomography : technology and applications*. 2nd edn (Springer, 2015).
2. McLaughlin, R. A., Noble, P. B. & Sampson, D. D. Optical coherence tomography in respiratory science and medicine: from airways to alveoli. *Physiology (Bethesda)* **29**, 369–380, doi:10.1152/physiol.00002.2014 (2014).
3. Ding, M. *et al*. Measuring airway remodeling in patients with different COPD staging using endobronchial optical coherence tomography. *Chest* **150**, 1281–1290, doi:10.1016/j.chest.2016.07.033 (2016).

4. Noble, P.B. *et al*. Airway narrowing assessed by anatomical optical coherence tomography *in vitro*: dynamic airway wall morphology and function. *J Appl Physiol (1985)* **108**, 401–411, doi:10.1152/japplphysiol.00511.2009 (2010).
5. Williamson, J. P. *et al*. Elastic properties of the central airways in obstructive lung diseases measured using anatomical optical coherence tomography. *Am J Respir Crit Care Med* **183**, 612–619, doi:10.1164/rccm.201002-0178OC (2011).
6. Armstrong, J. J. *et al*. Quantitative upper airway imaging with anatomic optical coherence tomography. *Am J Respir Crit Care Med* **173**, 226–233, doi:10.1164/rccm.200507-1148OC (2006).
7. Lee, A. M. *et al*. Validation of airway wall measurements by optical coherence tomography in porcine airways. *PLoS One* **9**, e100145, doi:10.1371/journal.pone.0100145 (2014).
8. Quirk, B. C. *et al*. Optofluidic needle probe integrating targeted delivery of fluid with optical coherence tomography imaging. *Opt Lett* **39**, 2888–2891, doi:10.1364/OL.39.002888 (2014).
9. Kennedy, B. F., Kennedy, K. M. & Sampson, D. D. A review of optical coherence elastography: Fundamentals, emerging methods and prospects. *IEEE J. Selected Topics in Quantum Electronics* **20**, 7101217–288, doi:10.1109/JSTQE.2013.2291445 (2014).
10. Larin, K. & Sampson, D. D. Optical coherence elastography–OCT at work in tissue biomechanics. *Biomedical Optics Express* **8**, 1172–1202, doi:10.1364/BOE.8.001172 (2017).
11. Lee, S. J. *et al*. Optically based-indentation technique for acute rat brain tissue slices and thin biomaterials. *J Biomed Mater Res B Appl Biomater* **97**, 84–95, doi:10.1002/jbm.b.31789 (2011).
12. Yang, Y., Bagnaninchi, P. O., Ahearne, M., Wang, R. K. & Liu, K. K. A novel optical coherence tomography-based micro-indentation technique for mechanical characterization of hydrogels. *J R Soc Interface* **4**, 1169–1173, doi:10.1098/rsif.2007.1044 (2007).
13. Chao, C. Y., Zheng, Y. P. & Cheing, G. L. A novel noncontact method to assess the biomechanical properties of wound tissue. *Wound Repair Regen* **19**, 324–329, doi:10.1111/j.1524-475X.2011.00694.x (2011).
14. Alonso-Caneiro, D., Karnowski, K., Kaluzny, B. J., Kowalczyk, A. & Wojtkowski, M. Assessment of corneal dynamics with high-speed swept source optical coherence tomography combined with an air puff system. *Opt Express* **19**, 14188–14199, doi:10.1364/OE.19.014188 (2011).
15. Oostveen, E. *et al*. The forced oscillation technique in clinical practice: methodology, recommendations and future developments. *Eur Respir J* **22**, 1026–1041, doi:10.1183/09031936.03.00089403 (2003).
16. Hardie, W. D. *et al*. Conditional expression of transforming growth factor-alpha in adult mouse lung causes pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* **286**, L741–749, doi:10.1152/ajplung.00208.2003 (2004).
17. Kramer, E. L. *et al*. Early growth response-1 suppresses epidermal growth factor receptor-mediated airway hyperresponsiveness and lung remodeling in mice. *Am J Respir Cell Mol Biol* **41**, 415–425, doi:10.1165/rcmb.2008-0470OC (2009).
18. Vallath, S., Hynds, R. E., Succony, L., Janes, S. M. & Giangreco, A. Targeting EGFR signalling in chronic lung disease: therapeutic challenges and opportunities. *Eur Respir J* **44**, 513–522, doi:10.1183/09031936.00146413 (2014).
19. Noble, P. B. *et al*. Airway smooth muscle in asthma: linking contraction and mechanotransduction to disease pathogenesis and remodelling. *Pulm Pharmacol Ther* **29**, 96–107, doi:10.1016/j.pupt.2014.07.005 (2014).
20. Noullet, S. *et al*. A novel technique for diaphragm biopsies in human patients. *J Surg Res* **196**, 395–398, doi:10.1016/j.jss.2015.02.057 (2015).
21. Brown, N. J., Salome, C. M., Berend, N., Thorpe, C. W. & King, G. G. Airway distensibility in adults with asthma and healthy adults, measured by forced oscillation technique. *Am J Respir Crit Care Med* **176**, 129–137, doi:10.1164/rccm.200609-1317OC (2007).
22. Kennedy, K. M. *et al*. Optical palpation: optical coherence tomography-based tactile imaging using a compliant sensor. *Opt Lett* **39**, 3014–3017, doi:10.1364/OL.39.003014 (2014).
23. Chin, L. *et al*. Three-dimensional optical coherence micro-elastography of skeletal muscle tissue. *Biomed Opt Express* **5**, 3090–3102, doi:10.1364/BOE.5.003090 (2014).
24. Kennedy, B. F. *et al*. Investigation of optical coherence microelastography as a method to visualize cancers in human breast tissue. *Cancer Res* **75**, 3236–3245, doi:10.1158/0008-5472.CAN-14-3694 (2015).
25. Kennedy, K. M. *et al*. Quantitative micro-elastography: imaging of tissue elasticity using compression optical coherence elastography. *Sci Rep* **5**, 15538, doi:10.1038/srep15538 (2015).
26. Luetteke, N. C., Lee, D. C., Palmiter, R. D., Brinster, R. L. & Sandgren, E. P. Regulation of fat and muscle development by transforming growth factor alpha in transgenic mice and in cultured cells. *Cell Growth Differ* **4**, 203–213 (1993).
27. Scott, A., Wang, X., Road, J. D. & Reid, W. D. Increased injury and intramuscular collagen of the diaphragm in COPD: autopsy observations. *Eur Respir J* **27**, 51–59, doi:10.1183/09031936.06.00143004 (2006).
28. Uezumi, A. *et al*. Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle. *J Cell Sci* **124**, 3654–3664, doi:10.1242/jcs.086629 (2011).
29. Macklem, P. T. Factors determining bronchial smooth muscle shortening. *Am Rev Respir Dis* **143**, S47–48, doi:10.1164/ajrccm/143.3_Pt_2.S47 (1991).
30. Copley, S. J. *et al*. Functional consequences of pleural disease evaluated with chest radiography and CT. *Radiology* **220**, 237–243, doi:10.1148/radiology.220.1.r01jl27237 (2001).
31. Zosky, G. R. *et al*. Ovalbumin-sensitized mice are good models for airway hyperresponsiveness but not acute physiological responses to allergen inhalation. *Clin Exp Allergy* **38**, 829–838, doi:10.1111/j.1365-2222.2007.02884.x (2008).
32. Janosi, T. Z. *et al*. Plethysmographic estimation of thoracic gas volume in apneic mice. *J Appl Physiol (1985)* **101**, 454–459, doi:10.1152/japplphysiol.00011.2006 (2006).
33. Song, Y. *et al*. Effect of maternal steroid on developing diaphragm integrity. *PLoS One* **9**, e93224, doi:10.1371/journal.pone.0093224 (2014).
34. Kennedy, B. F. *et al*. Optical coherence micro-elastography: mechanical-contrast imaging of tissue microstructure. *Biomed Opt Express* **5**, 2113–2124, doi:10.1364/BOE.5.002113 (2014).

Acknowledgements

We thank Mr John Elliot for expert histological assessment. Funding provided by the National Health and Medical Research Council (NHMRC) of Australia (1027218). P.N. and K.W. are supported by NHMRC Fellowships (1045824, 1090888). P.W. was supported by the William and Marlene Schrader Postgraduate Scholarship, The University of Western Australia, and C.A. by an NHMRC Preterm Infants CRE top-up scholarship.

Author Contributions

K.W. performed the mouse breeding, genotyping and lung assessment; C.A. and G.P. performed the *in vitro* diaphragm experiments; P.W., B.K. and D.S. developed and performed the optical coherence tomography-based indentation; C.A., L.B. performed the histology; K.W., A.L., G.Z., A.J., T.L. and P.N. designed the study; K.W. and P.N. drafted the manuscript. All authors contributed to manuscript revision.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-01431-x

Competing Interests: The authors declare that they have no competing interests.