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Developmental changes in diaphragm muscle function in the preterm and postnatal lamb

T. Lavin BSc^{1,2,†}, Y. Song PhD^{1,2,†}, A.J. Bakker PhD¹, C.J. McLean BSc², W.A. Macdonald PhD², P.B. Noble PhD^{1,2}, C.A. Berry PhD², J.J. Pillow PhD^{2,3} & G.J. Pinniger PhD^{1*}

- ^{1.} School of Anatomy, Physiology and Human Biology, The University of Western Australia, Crawley, Western Australia, Australia, 6009.
- ^{2.} Centre for Neonatal Research and Education, School of Women's and Infants' Health, The University of Western Australia, Crawley, Western Australia, Australia, 6009.
- ^{3.} King Edward Memorial Hospital, Women and Newborn Health Service, Western Australia, Australia, 6008

[†] Both authors contributed equally.

* Corresponding Author

Gavin J Pinniger
M309, School of Anatomy, Physiology and Human Biology
University of Western Australia
35 Stirling Highway
Crawley, Western Australia
Australia, 6009

Ph: +61 8 6488 3380
Fax: +61 8 6488 1025
Email: gavin.pinniger@uwa.edu.au

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Abbreviated Title: Functional development of the preterm diaphragm

SUMMARY

Rationale: The preterm diaphragm is structurally and functionally immature, potentially contributing to an increased risk of respiratory distress and failure. We investigated developmental changes in contractile function and susceptibility to fatigue of the costal diaphragm in the fetal lamb to understand factors contributing to the risk of developing diaphragm dysfunction and respiratory disorders. We hypothesized that the functional capacity of the diaphragm will vary with maturational stage as will its susceptibility to fatigue.

Methods: Lambs were studied at 75, 100, 125, 145, 154, 168 and 200 d postconceptional age (term=147 d). Lambs were euthanized (sodium pentobarbitone, 100 mg/kg) either at delivery or immediately prior to post-mortem for postnatal lambs. Contractile function was assessed on longitudinal strips of intact muscle fibres and the remaining tissue frozen in liquid nitrogen for analysis of myosin heavy chain (MHC) mRNA expression and protein content.

Results: Fetal development of diaphragm function was characterized by a significant increase in maximum specific force, increased susceptibility to fatigue, reduced twitch contraction times, and a progressive increase in MHCI and MHCII protein content. Postnatally, there was a progressive decrease in the susceptibility to fatigue that coincided with an increase in the MHC I:II protein ratio.

Conclusion: These data indicate that the functional capacity of the diaphragm varies with maturational age and may be an important determinant of the susceptibility to preterm respiratory failure.

KEYWORDS

Respiratory Muscle, Ventilation, Skeletal Muscle, Fatigue, Ontogeny

INTRODUCTION

A functional respiratory system is vital for the successful establishment of unsupported spontaneous breathing. Due to developmental immaturity of the gas exchanging regions of the lung, preterm infants are prone to respiratory distress and failure during transition to postnatal life. Generation of negative inspiratory pressures and efficient independent ventilation are also critically dependent on a functioning diaphragm and intercostal muscles. However, little is known about the integrity of the respiratory muscle pump in the premature infant.

Like the lung, the diaphragm is also structurally and functionally immature at birth in the preterm infant. Preterm infants who breathe spontaneously at birth may develop insidious respiratory insufficiency over the first minutes, hours or days of life, often necessitating mechanical ventilation. These infants have lower measured twitch transdiaphragmatic pressures compared to term infants (1), indicating that functional immaturity of the diaphragm may contribute to preterm respiratory failure. Diaphragmatic integrity may be compromised further by adverse environmental exposures *in utero* (antenatal steroids, infection, under nutrition) or by the postnatal disuse atrophy associated with the use of controlled mechanical ventilation and the use of paralysing agents.

There are notable differences between the immature and adult diaphragm in myosin heavy chain (MHC) content, metabolism, contractile function and oxidative capacity. Diaphragm fibre type analysis in a range of species indicate a low content of slow Type I (fatigue resistant) muscle fibres in the immature diaphragm (<10% at birth) versus a higher content in the mature diaphragm (~50-60%) (2-4). In humans, adult proportions of Type I fibres are not attained until 1 year (3). Additionally, embryonic

and neonatal MHC isoforms are evident in fetal and early life, but progressively disappear with advancing maturation.

The functional consequences of developmental fibre type changes are poorly understood. For example, Type II MHC preponderance in the fetal respiratory muscles might indicate predilections to respiratory failure and fatigue. Although increased fatigability of the diaphragm was reported after direct phrenic nerve stimulation in full-term newborn rabbit pups compared to adult rabbits (5), several others report the opposite finding of a high fatigue resistance in the diaphragm of newborn rats (6), cats (7) and baboons (8). Subsequent increased fatigability during postnatal development is also contrary to the increased proportion of fatigue resistant Type I fibres reported in the adult compared to the newborn diaphragm (3).

These data suggest that the relationship between MHC expression, contractile function and fatigability is less robust in fetal muscle compared to adult muscle, likely due to the influence of other cellular factors involved in contractile function and endurance. In addition to changes in MHC fibre content, pre-natal development is also characterized by changes in intracellular Ca^{2+} handling (9), oxidative capacity (10,11), and myofilament structure (9) which will impact on force generation and susceptibility to fatigue and damage. These developmental changes occur at different rates and may impact on the resilience of the immature newborn infant to increased respiratory mechanical loads and the inflammatory and metabolic challenges that occur after birth. Therefore, the level of functional development of the diaphragm will vary with gestational age as will its contribution to respiratory failure due to diaphragmatic fatigue, damage and/or contractile dysfunction in preterm babies.

Developmental changes in fibre type composition and contractile properties of the diaphragm have been examined primarily in small laboratory animals. However, the

relevance of these observations to human diaphragm development is limited as most developmental changes in rodents occur *ex utero* while the majority of development in human neonates occurs *in utero* (12). Furthermore, small laboratory animals have a higher proportion of Type II muscle fibres and a higher respiratory rate compared to humans and larger mammals which may impact on the contractile properties of the diaphragm and therefore affect the susceptibility to muscle damage and fatigue (13).

A more representative model of the *in utero* development of the diaphragm in human neonates may be gained from a comparable ovine animal model. The ovine model is also a convenient model to study the effect of chronic external influences such as nutritional imbalance, infection and steroid exposures on the structural and functional integrity of the immature diaphragm. Although the ontogeny of skeletal limb muscle in the lamb has been documented (2), there are no physiological data on the ovine fetal diaphragm. Therefore, we investigated developmental changes in contractile function of the lamb costal diaphragm to understand factors contributing to the risk of developing respiratory disorders and diaphragm dysfunction at different fetal and postnatal ages. We hypothesized that the functional capacity of the diaphragm will vary with maturational stage as will its susceptibility to fatigue and stretch-induced damage. In addition, these data will provide a baseline to inform subsequent studies investigating how antenatal factors may influence diaphragm function at time of preterm birth.

MATERIALS AND METHODS

Animals, surgery and preparation

Fetuses were delivered from sedated (10 mg/kg ketamine, Parnell Labs; 0.02 mg/kg medetomidine, Pfizer Animal Health, NSW, Australia) date mated pregnant Merino ewes via caesarean section after spinal regional anaesthetic block (2% lignocaine, 3mL, 60 mg) at 75 d (n=5), 100 d (n=8), 125 d (n=14), and 145 d (n=9) gestation (term=147 d). Ewes and their lambs were euthanized at delivery (sodium pentobarbitone, 100 mg/kg, Valabarb®, Pitman-Moore, Australia). Additional date-mated ewes delivered spontaneously at term (~ 147 d gestation) and their offspring were grown to 1 w (154 d postconceptional age; n=5), 3 w (168 d; n=6) and 7 w (200 d; n=4). All lambs were exsanguinated prior to post-mortem. All animal procedures were approved by the Animal Ethics Committee of the University of Western Australia.

Tissue collection

The fetal diaphragm was dissected with the ribs and central tendon attached and hemi-sectioned. Contractile function was assessed on longitudinal strips of muscle fibres isolated from the costal portion of the right hemi-diaphragm. Sections from the costal portion of the left hemi-diaphragm were frozen in liquid nitrogen for analysis of MHC mRNA expression and protein content.

Muscle contractile properties

The hemi-diaphragm was dissected into 3-5 mm wide longitudinal strips and mounted in an *in vitro* muscle test system (model 1205, Aurora Scientific Inc., Canada) containing physiological saline solution (in mM: NaCl, 109; KCl, 5; MgCl₂, 1; CaCl₂,

4; NaHCO_3 , 24; NaH_2PO_4 , 1; sodium pyruvate, 10), continuously bubbled with 95% O_2 / 5% CO_2 . The solution was maintained at 25°C, which is optimal for the maintenance of *in vitro* skeletal muscle force (14).

The preparation was manually adjusted to the optimum muscle length (L_0) at which maximum isometric twitch force (P_t) was recorded. The time course of twitch contractions were analysed for time-to-peak (TTP) and half-relaxation time ($1/2\text{RT}$). The force-frequency relationship was determined between 5 - 80 Hz from which the maximum tetanic force (P_o) was determined. Fatigue resistance was evaluated by stimulating once every second for 330 ms at the optimal frequency for a total of 150 s. The fatigue index was determined from the ratio of the force produced during the 150th contraction relative to the 1st contraction (2), in which a higher number indicates a greater fatigue resistance. Recovery after the fatigue protocol was evaluated by recording P_o at 2, 5, 10, 20, 30, and 40 min after the fatigue protocol or until P_o had recovered to pre-fatigue levels.

The susceptibility to muscle damage was determined from a series of 5 lengthening (eccentric) contractions at 2 min intervals. For each lengthening contraction, a stretch of 10 % of L_0 was applied during the isometric plateau phase of a maximal tetanic contraction. P_o was recorded before and at 2, 5 and 10 mins after the lengthening contraction protocol. The severity of damage was determined by the mean reduction in P_o after the stretch.

Finally, the rib and tendon were removed from the diaphragm strip and the wet muscle weight recorded. Cross sectional area was calculated from the muscle mass (g), L_0 (mm) and density of skeletal muscle ($1.056 \text{ g}\cdot\text{cm}^{-2}$). To account for slight differences in the size of the dissected diaphragm strips, the absolute force was normalized for cross-sectional area and expressed as specific force ($\text{N}\cdot\text{cm}^{-2}$).

MHC Fibre Characteristics

Gene Expression Assay

Total RNA was isolated from 30 mg homogenized diaphragm tissue using the RNeasy Mini kit (Qiagen Pty Ltd., Doncaster, Australia) according to manufacturer's instructions. Contaminating genomic DNA was removed by an on-column DNaseI digestion (DNaseI digestion kit, Qiagen Pty Ltd., Doncaster, Australia). Isolated RNA was reverse transcribed into complementary DNA (cDNA) in a 20 μ L reaction (QuantiTect® Reverse Transcription Kit, Qiagen Pty Ltd., Doncaster, Australia). Primers used for MHC slow and MHC fast were designed on ovine-specific mRNA sequences. Specific products were amplified and detected on the Rotor-gene 3000 real time PCR system (Corbett Life Science) using Rotor-Gene SYBR Green PCR Kit (Qiagen Pty Ltd., Doncaster, Australia) following the manufacturer's instructions. The cycling conditions for all genes were as follows: 5 m at 95 °C, 35-40 cycles of 5 s at 95 °C, 20 s at optimised annealing temperature (Table 1) and 20 s at 72 °C. The expression levels of genes of interest were normalized into 18S RNA (15) using the $2^{-\Delta\Delta CT}$ method (16) and presented as a fold change relative to the full term group (145 d).

Western Blot Analysis

Muscle samples were homogenized in ice-cold lysis buffer containing 20 mM HEPES pH 7.7, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 20 mM β -glycerophosphate, 100 mM NaCl, 0.1% Triton 100, 500 μ M DTT, 100 μ M Na_3VO_4 , 100 mM PMSF, 0.01% NP40 and protease inhibitor cocktail tablet (Roche, Castle Hill, Australia). Homogenates were subjected to 6 cycles of freeze – thaw then centrifuged (10 000 g, 25 min, 4 °C). Total protein concentration in the supernatant was measured by Bradford protein assay (17). Equal amounts of total lysate proteins (50 μ g) were separated by 12 % SDS-PAGE

and transferred to nitrocellulose membranes. After blocking in PBS containing 5% non-fat dry milk, the membranes were incubated with primary antibodies against MHC slow (1:500, Novocastra), MHC fast (1:500, Novocastra), and α -Tubulin (1:1000, Cell Signalling) for 2 h at room temperature. Bound antibodies were detected with 1:1000 dilutions of either anti-rabbit or mouse (Cell Signalling) immunoglobulin conjugated with horseradish peroxidase (HRP). The blots were developed by adding a SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Massachusetts, USA) and quantified by computerized image analysis (ImageQuant™ 350, GE Healthcare). To avoid the variation across membranes arising from different exposure time and transferring/blotting efficiency, a same control sample was added in each test. The values for each protein were standardized with the control sample and then normalized into α -Tubulin abundance.

Statistical Analysis

Parametric data were analysed using one-way or two-way ANOVA as appropriate with post hoc analysis (Holm-Sidak test) to identify differences between independent groups. For non-parametric data, a Kruskal Wallis ANOVA on ranks was employed using Dunn's post-hoc analysis. Correlations between dependent variables and postconceptional age are presented as Pearson correlation coefficients or Spearman r for non-parametric data. An alpha-level of 0.05 was used to compare differences between independent groups and to determine overall significance. Values are means $\pm$ SEM, unless specified otherwise.

RESULTS

Animals

The mean body weight and optimal muscle length (L_o) are presented in Table 2. As expected, both body weight and L_o increased significantly with increasing postconceptional age ($p < 0.01$).

Diaphragm Contractile Properties

Specific force and Force Frequency

There was a significant main effect of postconceptional age on maximum specific force ($p < 0.01$). Maximum force increased by ~2-fold from 128 d to 145 d postconceptional age (Fig 1A). However, there was no significant change in maximum specific force during postnatal development.

The relationship between diaphragm force and stimulation frequency was evaluated between 5 – 80 Hz for each postconceptional age. The force-frequency relationships showed a consistent change with increasing postconceptional age. However for clarity, data are only presented for three gestational ages spanning the pre-term (128 d), near-term (145 d) and early postnatal (154 d) time points (Figure 1B and 1C). The specific force was significantly lower at all frequencies in the pre-term animals (Fig 1B; $p < 0.001$). When normalized to the maximum specific force in each postconceptional age (Fig 1C), the normalized force-frequency relationships were shifted to the right at increasing postconceptional ages reflecting a higher stimulation frequency required to achieve the maximum force production. The effect was most notable at low stimulation frequencies (5 – 15 Hz) at which the relative forces in postnatal (154 d and 200 d) animals were significantly lower than that in pre-term (100 d, 128 d and 145 d) animals ($p < 0.001$).

Twitch contractile properties

The twitch contractile properties for postconceptional ages 100 to 200 d are presented in Table 2; twitch contraction data were not recorded at 75 d. Peak twitch force increased significantly with postconceptional age ($p < 0.01$). At 100 d, P_t was significantly lower than 128 d, 145 d, and 168 d. The time to peak twitch force (TTP) at 154 d was significantly shorter than at 128 d ($p < 0.05$), whereas the relaxation times ($1/2RT$) at late gestation (145 d) and early postnatal ages (154 d, 168 d) were significantly shorter than at 100 d and 128 d ($p < 0.001$). These data reflect a decrease in the contraction and relaxation times in the diaphragm of animals delivered near term and in the first several weeks of life. The twitch:tetanus ratio at 128 d was significantly greater than at all other postconceptional ages ($p < 0.001$).

Susceptibility to fatigue

There was a significant main effect of postconceptional age on the fatigue index ($p < 0.01$). The susceptibility to fatigue increased significantly from 75 d to 100 d. After a decrease in fatigability at 128 d, the susceptibility to fatigue at late gestation (145 d) and all postnatal ages (154 d to 200 d) was significantly greater than at 75 d (Fig 2). Interestingly, the greatest susceptibility to fatigue was observed in the animals examined approximately 1 week after full term delivery (ie at 154 d postconceptional age). The susceptibility to fatigue then decreased progressively with increasing postconceptional age (regression analysis between FI and PCA 154 – 200; $r^2 = 0.34$; $p = 0.03$). In addition, the rate of force recovery after fatigue was significantly slower in older animals (data not shown).

Susceptibility to stretch-induced muscle damage

The susceptibility of the diaphragm muscle to stretch-induced damage at different postconceptional ages is illustrated in Fig 3. The diaphragm of 75 d animals was significantly more susceptible to damage compared to the older (128 d – 168 d) animals ($p < 0.01$). There were no significant differences in susceptibility to muscle damage between any other age groups. The muscle damage protocol could not be conducted on 200 d lambs as the stretch protocol of 110 % L_o was beyond the capacity of the experimental apparatus for fibre length in this age group.

MHC Fibre Characteristics

The levels of mRNA expression for both MHC I and MHC IIa at each postconceptional age are shown in Fig 4A. A regression analysis of these data revealed a significant positive relationship between MHC IIa and postconceptional age ($r = 0.78$; $p < 0.01$). At a protein level, MHC I and MHC II content increased with postconceptional age (Fig 4B). There was a significant positive relationship between MHC I and postconceptional age ($r = 0.77$; $p < 0.01$) and between MHC II and postconceptional age ($r = 0.76$; $p < 0.01$). Furthermore, MHC I and MHC II protein content were also significantly correlated to maximum specific force of the diaphragm muscle ($r = 0.68$; $r = 0.54$; $p < 0.01$, respectively). MHC I protein was not detected in 75 d, 100 d, or 128 d groups thus MHC type proportions at a protein level were only assessed from 145 d to 200 d postconceptional age. The MHC I:MHC II ratio decreased from 145 d to 154 d and then increased again at 200 d postconceptional age (Fig 4C).

DISCUSSION

Our results show that there are significant developmental changes in contractile function and susceptibility to fatigue in the costal diaphragm of neonatal lambs. There were also changes in MHC indices evident at both an mRNA level and at a protein level which support these functional characteristics. Furthermore, the functional development of the diaphragm is likely to influence the susceptibility to damage associated with increased mechanical loading in the spontaneously breathing preterm infant.

Developmental changes in the diaphragm were observed throughout gestation up until seven weeks postnatal age (200 d postconceptional age). As the diaphragm has to be fully functional at birth, it is one of the fastest developing muscles *in utero* (18). We noted a significant and large decrease in the susceptibility to stretch-induced muscle damage at early postconceptional ages (75 d to 100 d) and a large significant increase in specific force in late gestation (between 128 d and 145 d) reflecting the rapid developmental changes in diaphragm function *in utero*. At early postconceptional ages (50 d to 100 d), the limb muscles of sheep are characterised by scattered fibres (19) with poorly defined sarcomeric structure (9) and cytoskeleton. A similar immature myofibrillar structure of the diaphragm would contribute to the significantly greater susceptibility to stretch-induced muscle damage at 75 d postconceptional age. Eccentric diaphragm contractions *in vivo* produce persistent deterioration of contractile function and are associated with sarcomeric and sarcolemmal damage (20). Similar evidence of muscle damage has been observed following periods of inspiratory resistive loading (21,22) which is known to heavily recruit the diaphragm (23). As the extent of diaphragm damage is load dependent (21), the pre-term

diaphragm may be particularly susceptible to this type of damage due to the increased work of breathing and associated mechanical load on the diaphragm.

The maximum specific force in the diaphragm of preterm sheep was significantly lower than those born near term. The marked, 2-fold increase in specific force that we observed between 128 d and 145 d postconceptional age is likely attributed to the neural and hormonal factors that regulate muscle development at this gestation. This is consistent with the establishment of regular and episodic diaphragm contractions (fetal breathing movements) at around 120 d postconceptional age (24,25) and development of the hypothalamo-pituitary-thyroid axis, which are critical in the maturational changes in muscle fibre composition (12,18). The time course of these changes coincide with morphological development of the diaphragm including an increase in myofibre size and a decrease in interstitial space, and hence an increase in myofibrillar density as reported by Ashmore et al. (19) for limb muscles of sheep. In support of this notion, we observed significant increases in MHC mRNA and protein content with increasing postconceptional age and a significant, positive correlation between MHC content and maximum specific force. This increased protein content may reflect a reduction in the level of proteolytic signalling activity at gestational ages > 100d in the fetal sheep (11). Thus the increase in diaphragm strength and resistance to stretch-induced damage are likely to occur in preparation for the transition to postnatal life and to accommodate the increased stress involved in the successful establishment of unsupported spontaneous breathing (26).

Along with increased strength, we also observed significant changes in other contractile parameters including decreased fatigue resistance, shorter twitch contraction times and a rightward shift in the force-frequency relationship with increasing gestational age. Our physiological data suggest a shift from slow to fast

muscle behaviour towards late gestation which is consistent with the observations of West et al. (9) in ovine limb muscles. The resistance to fatigue was lowest at 154 d postconceptional age, but subsequently increased with increasing postnatal maturation. The maturational changes in fatigue resistance were consistent with the changes in twitch contraction and relaxation times which were shortest towards late gestation and the early postnatal period, and then increased again with further postnatal development. Further support for this functional slow to fast transition during late *in utero* development was evident from the rightward shift in the normalized force-frequency relationship reflecting an increase in the stimulation frequency required to produce a tetanic contraction.

A decrease in diaphragm fatigue resistance with increasing maturational age was demonstrated previously in a range of mammalian species including rats (6,27), cats (7), baboons (8) and humans (3). As we showed in the preterm sheep, these functional properties are consistent with *in vitro* contractile measures including shortened twitch contraction times (7,8,28), faster shortening velocities (27) and a rightward shift in the force-frequency relationship (7,28). Considering these contractile features, it is somewhat surprising to note that the early development of the diaphragm muscle is often characterized by an increased proportion of fatigue resistant type I muscle fibres and a corresponding decrease in type II fibres (3,6-8). This apparent discrepancy between contractile properties and histochemical fibre typing was noted previously (7) and may reflect the differential development of oxidative capacity and actomyosin ATPase activity, as reflected by MHC isoform expression (6,29).

Our results indicate an increase in MHC I and MHC II protein content with maturation. However, our analysis does not reflect the neonatal isoform of MHC which is dominant in the developing diaphragm and usually co-expressed with MHC I

and/or IIa isoforms (27). Whilst MHC I protein levels were not detected at very young gestational ages, MHC I and II were detected at higher levels towards the end of gestation which most likely coincides with the disappearance of developmental MHC isoform (2). The increase in MHC I:II ratio beyond 154 d postconceptional age is consistent with an increase in MHC I content during post-natal development and, along with postnatal increases in oxidative capacity (10,11), may account for the increased resistance to fatigue that we observed during this postnatal period.

Although our results suggest an increased susceptibility to respiratory fatigue in term animals, extrapolation of these predictions to the clinical setting should be made with caution. It is important to note that the fatigue protocol used in this study involved maximal isometric contractions of the isolated costal diaphragm. Considering the dramatic increase in maximal force between 128d and 145d animals, the greater force production in the term animals may well account for the significant increase in fatigue in term animals (due to increases in the concentration of contraction-induced metabolites such as phosphate and reactive oxygen species(30)). Furthermore, as this study was restricted to the costal diaphragm, the potential contribution of the crural diaphragm to fatigue resistance is unknown. Although the oxidative capacity of the crural and costal diaphragm is not different in adult sheep (31), it is unclear whether this is true for the developing diaphragm.

Respiratory fatigue in the clinical setting reflects the balance between the work put on the diaphragm, and the diaphragm capacity. With a stable chest wall, advanced alveolar structure, and established surfactant synthesis and secretion, the relative intrinsic workload of the term infant is substantially less than that of the immature preterm infant that has a highly compliant chest wall, and minimal immature (or absent) alveoli and surfactant. The balance of this relation is such that in the absence

of factors limiting breathing efficiency, the term diaphragm is likely to retain adequate function to overcome the intrinsic work of breathing, without needing to exert repetitive maximal contractile function. The preterm, however, is more likely to be operating closer to maximal contractile function (given lower specific force and increased intrinsic work of breathing) and therefore any level of fatigue may result in the development of insufficient spontaneous respiratory effort and respiratory failure.

The fatigue resistance of the diaphragm muscle is critical for maintaining adequate respiratory function when working against high loads. In addition to factors associated with immaturity that increase the intrinsic work of breathing, the fetus and preterm newborn may be exposed to additional environmental and iatrogenic factors that compromise the structure and function of the newborn diaphragm such as nutrition, infection, steroid exposure and mechanical ventilation. Therefore, the functional development of the diaphragm, which varies with gestational age, must be considered when evaluating the susceptibility to diaphragmatic dysfunction and the risk of developing respiratory disorders. Our studies provide an important baseline for assessment of antenatal and postnatal factors that may further compromise postnatal diaphragm integrity in the preterm ovine model.

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Figure 1 – Specific Force and Force-Frequency Relationships: **A.** Mean ($\pm$ SEM) maximum specific force at different postconceptional ages (PCA). * significantly different from 75 d, 100 d, and 128 d; # significantly different from 75 d and 100 d ($p < 0.001$). Vertical line indicates change from fetal to postnatal life. **B.** Mean ($\pm$ SEM) specific force-frequency relationships at three different postconceptional ages, 128 d, 145 d and 154 d. **C.** Mean ($\pm$ SEM) normalised force frequency relationships at 128 d, 145 d and 154 d. * 154 d is significantly different to 128 d and 145 d ($p < 0.05$).

Figure 2 – Fatigue Resistance: Mean ($\pm$ SEM) fatigue index (FI) across different postconceptional ages. The fatigue index was expressed as the force produced during the last fatiguing contraction relative to the first contraction, for which higher number indicates greater fatigue resistance. * significantly different from 75 d and 128 d; # significantly different from 75 d ($p < 0.001$). Vertical dotted line indicates change from fetal to postnatal life.

Figure 3 – Muscle Damage: Mean ($\pm$ SEM) decrease in maximal tetanic force after muscle stretch protocol. Muscles were exposed to five eccentric contractions of 10 % L_0 applied on the plateau of a tetanic contraction. * significantly different from 75 d ($p < 0.01$). Vertical line indicates change from fetal to postnatal life.

Figure 4 – MHC mRNA and Protein: Fold change in MHCI (white) and MHCIIa (grey) mRNA expression (A) and MHCI (white) and MHCII (grey) protein content (B). Ratio of MHCI:MHCII protein content (C) in diaphragm tissue homogenate. Values are median (25th, 75th centile). Error bars show 10th and 90th centile. Black dots represent outlying values. Vertical line indicates change from fetal to postnatal life. Horizontal dotted line in (A) represents median of reference (145d) group.