MONASH University

Prolonged laryngeal adduction at birth in preterm rabbit kittens

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Bachelors of science advanced with honours

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Abstract

Background: Non-invasive ventilation is often unable to provide sufficient respiratory support for very premature infants in the delivery room. While airway obstruction is thought to be a major reason for this failure, the site of obstruction is unknown. In the current thesis, we have investigated whether laryngeal closure at birth is a major cause of airway obstruction and factors influencing laryngeal activity in preterm rabbit kittens. **Methods:** To analyse laryngeal function at birth we used phase contrast X-ray imaging to visualize the upper airways in spontaneously breathing premature rabbit kittens. In the first study, we visualised the larynx immediately after, and at approximately one hour after birth with and without simultaneous intermittent positive pressure ventilation (iPPV). In the 2nd study, kittens received sequential periods of continuous positive airway pressure (CPAP) with air, 100% oxygen, 100% nitrogen, and again with 100% oxygen to oxygenate, or induce hypoxia. **Hypothesis:** In the first study, it was hypothesised that the larynx would be predominantly closed immediately after birth, thus preventing iPPV from successfully ventilating the lungs. After a period of time the larynx would be predominately open, allowing the kitten to breathe freely, and for the successful delivery of iPPV. In the second study, we hypothesised that administering 100% oxygen to the kittens would make them increasingly oxygenated, resulting in a more stable and increased respiratory rate, and thus inducing prolonged laryngeal abduction. We also hypothesised that during the administration of 100% nitrogen the kittens would become hypoxic, resulting in respiratory depression, apnoea, and prolonged laryngeal

adduction, preventing the successful administration of CPAP. **Results:** In the 1st study the glottis and epiglottis were predominantly closed (open 25.5 ± 1.1% and 17.1 ± 1.6% of the time, respectively) in kittens with an unstable breathing pattern over the first 12 minutes after birth. In kittens with a stable breathing pattern, which included the kittens imaged one hour after birth, the glottis and epiglottis were mostly open (90.5±1.9% and 72.3±2.3%, respectively). In the 2nd study after the first 3 minutes of air breathing the respiratory rate was low, yet glottic opening increased rapidly (9.2 ± 4.1 breaths per minute; bpm, and 60.2 ± 24.4% respectively). Following oxygenation, the kittens established a stable breathing pattern with a predominately open glottis (22.7 ± 4.7 bpm and 75.2 ± 7.3%, respectively). During hypoxia, the respiratory rate and glottic opening fell (7.3 ± 4.3 bpm and 11.7 ± 9.0%, respectively). When the kittens were then re-oxygenated the respiratory rate and glottic opening increased again (16.4 ± 3.7 bpm and 62.8 ± 11.5%, respectively) allowing the kittens to breathe freely. **Conclusion:** From the first study, laryngeal closure impeded non-invasive iPPV, which may contribute to the high failure rate of non-invasive ventilation in the delivery room in premature infants. In the second study, non-invasive respiratory support with oxygen promoted the development of a stable breathing pattern and promoted prolonged laryngeal abduction. As such it appears promoting laryngeal opening by stimulating a stable breathing pattern is a promising way to improve the success of non-invasive ventilation for very preterm newborns in the delivery room.

Declaration

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

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Publications during enrolment

Thesis including published works declaration

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

This thesis includes 1 paper accepted for publication. The core theme of the thesis is prolonged laryngeal closure hinders non-invasive ventilation in preterm newborns at birth. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Richie centre under the supervision of Prof. Stuart Hooper and Dr. Marcus Kitchen

In the case of chapter 3 and chapter 4 my contribution to the work involved the following: Assisting with the animal surgery, assisting in the image acquisition, analyzing the images and interpreting the data, and writing the first draft of the submitted paper "Laryngeal closure impedes non-invasive ventilation at birth", complementary to chapter 3.

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution	Co-author, Monash student
Chapter 3	*Laryngeal closure impedes non-invasive ventilation at birth*	*Submitted*	*Surgery 5%* *Data collection 10%* *Data analysis and interpretation 100%* *Wrote first draft*	*1) Marcus Kitchen, Data collection, 40%, Input into manuscript 5%* *2) Caterina Binder-Heschl, Surgery 2.5%* *3) Marta Thio, Surgery 2.5%* *4) Megan Wallace, Surgery 40%, Input into manuscript 5%* *5) Lauren Kerr, Surgery 30%* *6) Charles Roehr, Surgery 5%* *7) Katie Lee, Data collection, 20%* *8) Genevieve Buckley, Data collection, 20%* *9) Peter Davis, input into manuscript 5%* *10) Andreas Flemmer, Surgery 5%* *11) Arjan te Pas, Surgery 5%, Input into manuscript 5%* *12) Stuart Hooper, ventilation, 100%, Input into manuscript 60%, concept design.*	*No*

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Supervisor signature:  Date: 15/05/2017

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Abbreviations

bpm	Breaths per minute
CPAP	Continuous positive airway pressure
CT	Cricothyroid
d GA	Days gestational age
EAct	Electromyography recordings of the cricothyroid
EAdi	Electromyography recordings of the diaphragm
EAta	Electromyography recordings of the thyroarytenoid
EBM	Expiratory braking maneuver
FBM	Fetal breathing movements
FiO_2	Fraction of inspired oxygen
FRC	Functional residual capacity
i.p	Intraperitoneal
I.V	Intravenous
iPPV	intermittent positive-pressure ventilation
LA	Lower airways
LCR	Laryngeal chemoreflex
NICU	Neonatal intensive care unit
nIPPV	Nasal intermittent positive-pressure ventilation

nNAVA	Nasal neurally adjusted ventilatory assist
$PaCO_2$	Partial pressure of arterial carbon dioxide
PaO_2	Partial pressure of arterial oxygen
PAW	Airway pressures
PEEP	Positive end-expiratory pressure
PIP	Positive inspiratory pressure
P_{oesph}	oesophageal pressures
PSV	pressure support ventilation
REM	Rapid eye movement
SEM	Standard error of the mean
SUM	Sum signal of the respiratory inductance plethysmograph
TA	Thyroarytenoid
UA	Upper airways

Chapter 1

Introduction

Approximately 30,000 babies are delivered prematurely (< 37 weeks gestation) in Australia each year (1). Most very preterm newborns (< 32 weeks gestation) are unable to sustain independent respiration at birth and require immediate respiratory support to survive. Traditionally, apnoeic preterm newborns have been intubated and mechanically ventilated in the delivery room; however, this is associated with lung damage, and injury to the upper airways which can lead to life-long morbidity (2, 3). As such there has been a shift in clinical practise towards non-invasive ventilation in the delivery room. Clinical studies from the neonatal intensive care unit (NICU) have shown non-invasive ventilation to be a successful alternative to intubation and mechanical ventilation. However, clinical trials from the delivery room show that non-invasive ventilation is associated with a high, and unexplained failure rate in very and extremely premature newborns (4-9). Non-invasive ventilation is determined to have failed if one or more of the following is evident in the newborn: the newborn requires a fraction of inspired oxygen (FiO_2) greater than 0.6, the partial pressure of carbon dioxide ($PaCo_2$) is greater than 60 mm Hg, the arterial pH below 7.25, apnoeic episodes unresponsive to stimulation, or the newborn experiences metabolic acidosis unresponsive to treatment (6). Subsequent intubation and mechanical ventilation is

unavoidable for the very and extremely premature newborns failing to improve on non-invasive ventilation. The failure rates reported by these clinical studies range from 31% to 100% of preterm newborns, depending on the form of non-invasive ventilation delivered, and the gestation age of the newborn. The cause of this variable failure rate is unknown; however, it has been suggested that mask leak and airway obstructions are major contributors (9, 10).

A critical difference between invasive and non-invasive forms of respiratory support is the involvement of the upper airways, particularly the larynx, yet this important structure is often overlooked. Laryngeal function during fetal life, and from neonatal life (> 1 day old) to adulthood has been extensively studied. However, laryngeal function immediately after birth has yet to be examined, leaving a gap in the current literature. During normal postnatal breathing, the larynx remains abducted throughout the respiratory cycle, only narrowing slightly during expiration. In contrast, the larynx is adducted for the majority of fetal life during fetal apnoea, only opening briefly during periodic fetal breathing movements (FBM). At birth, the larynx must transition from this predominately closed state, to the predominately open state, allowing the newborn to initiate continuous air breathing. Although it is apparent that laryngeal function *in utero* and *ex utero* are very different, it is unknown how, or when the transition from *in utero* laryngeal function to *ex utero* laryngeal function occurs. Furthermore, the interaction between the changing laryngeal physiology at birth and non-invasive ventilation has yet to be examined. It is unclear if we are often trying to ventilate against an adducted "fetal like" larynx in preterm newborns in the delivery room.

1.1 Laryngeal anatomy

The larynx plays a critical role in regulating airflow and protecting the lower airways from foreign substances by controlling the resistance of the upper airways. The larynx also has important roles in a range of other activities, including phonation, swallowing, and the regulation of thoracic pressure. The

body of the larynx is formed by three solitary cartilages; the thyroid, the cricoid and the epiglottis, as well as the three smaller paired cartilages; the arytenoids, the corniculates and cuneiforms (fig.1). The intrinsic laryngeal muscles travel between the laryngeal cartilages, and contract to pull and rotate the laryngeal cartilages, thus altering the dimensions of the glottis (fig.2a).

1.1.1 Laryngeal cartilages

The laryngeal cartilages form the structure of the larynx (fig.1). The largest laryngeal cartilage, the thyroid cartilage, is positioned over the anterior segment of the larynx, and provides protection for the internal structures of the larynx (fig.1a). The cricoid cartilage, located at the tracheal entrance, hosts the important attachment sites for the laryngeal muscles that generate arytenoid rotation (fig.1a). Each arytenoid has a medial vocal process, and a lateral muscular process, providing the attachment sites for the vocal ligament and the muscles generating vocal fold movement, respectively (fig.1b). Three separate muscles attach at the lateral muscular process of each arytenoid, allowing the arytenoids to be rotated and rocked in both the sagittal and the coronal planes. Altering the position of the arytenoids alters the tension and location of the vocal folds, thus opening and closing the glottis. The final laryngeal cartilage is the epiglottis (fig.1 & fig.2). This elastic leaf-shaped cartilage is located behind the hyoid bone, with the narrow posterior edge attaching to the thyroid cartilage. The aryepiglottic and the thyroepiglottic muscles pull the epiglottis down over the laryngeal inlet to protect the lower airways.

1.1.2 Intrinsic laryngeal muscles

The coordinated actions of the intrinsic laryngeal muscles alter the position, length, and tension of the vocal folds. This is achieved by adjusting the orientation and position of the arytenoids with respect to the anterior commissure of the thyroid cartilage. The cricothyroid is the sole tensor muscle of the larynx (fig.2c). Contraction of the cricothyroid tilts the thyroid cartilage down and forward, increasing tension

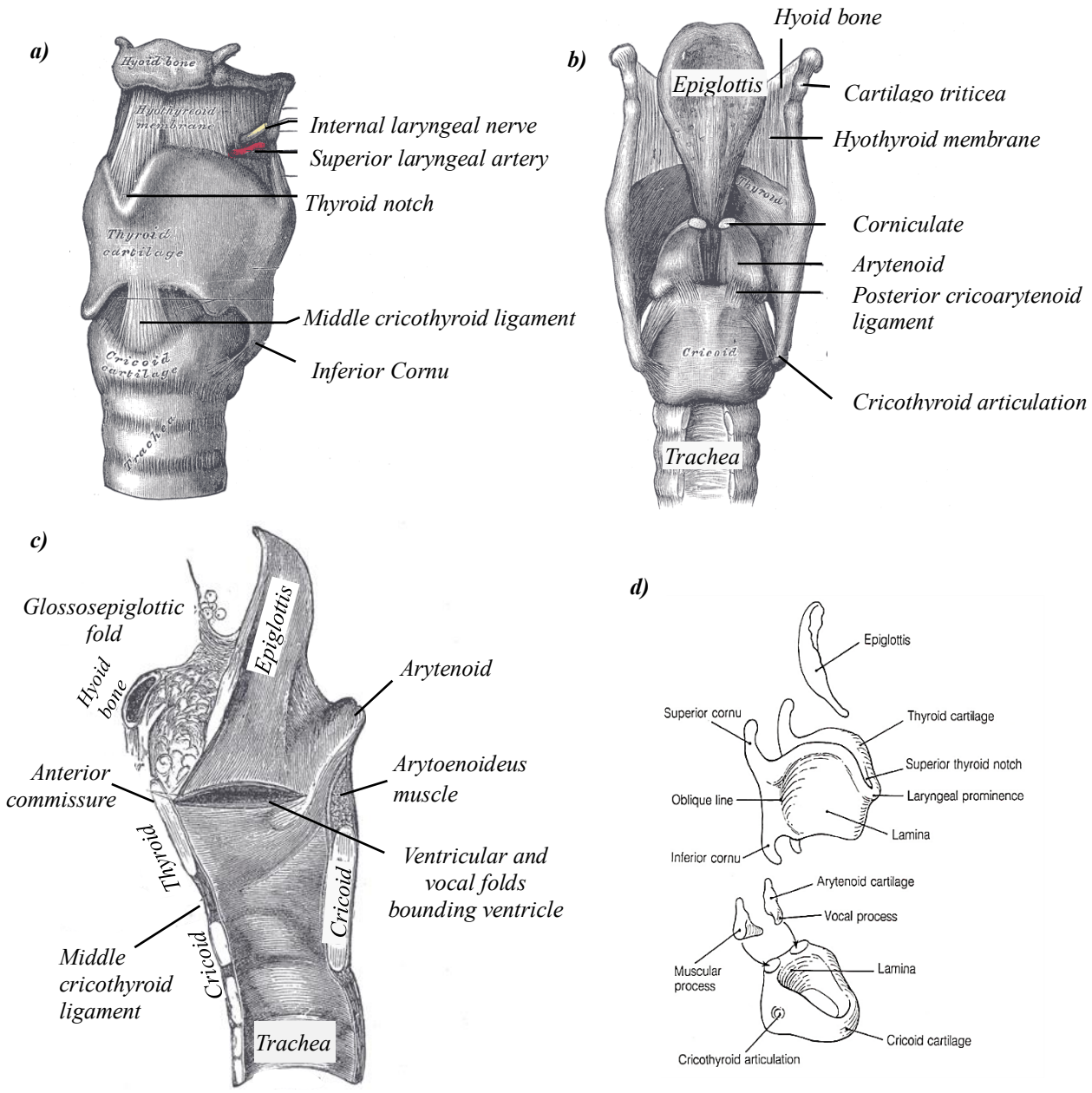

Fig.1 Labelled illustrations of the laryngeal cartilages ***a)*** Anterolateral view, ***b)*** Posterior view, ***c)*** Ventricle cross-section, and ***d)*** Anterolateral view of the laryngeal cartilages. Fig.1a, b and c are sourced from Grey's anatomy pg. 945 - 960, and fig.1d is sourced from http://www.studyblue.com (11, 12).

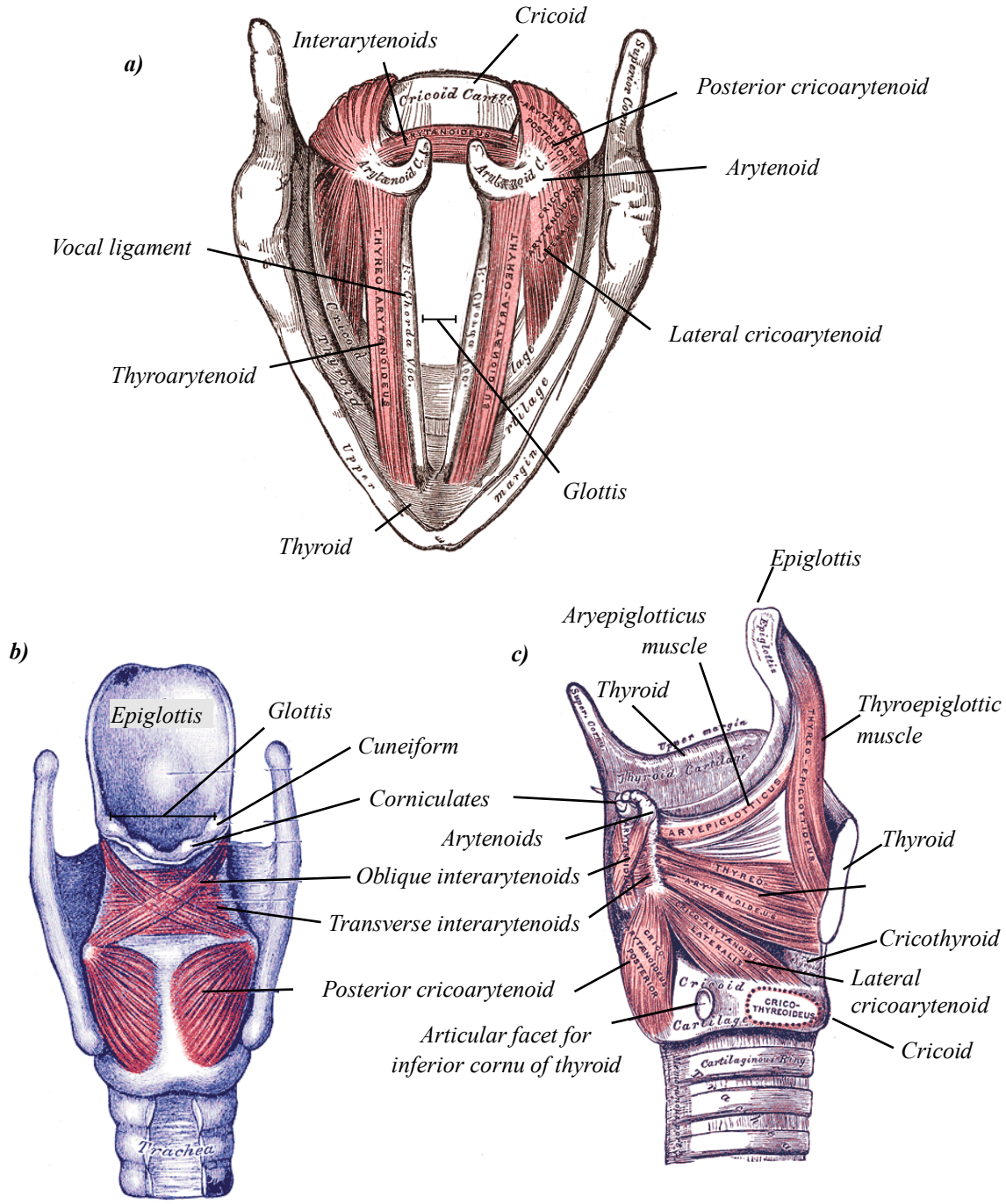

Fig.2 Labelled illustrations of the intrinsic laryngeal muscles from the **a)** superior, **b)** posterior, and **c)** lateral directions. Images sourced from, Gray's anatomy pg. 945-960 (12).

in the vocal folds, and producing higher pitch sounds during exhalation. The posterior cricoarytenoid is the sole laryngeal abductor muscle (fig.2a) and contraction of this muscle generates lateral rotation of the arytenoids, enlarging the glottis. Maximal laryngeal abduction is achieved when the posterior cricoarytenoid contracts simultaneously with the cricothyroid, stretching the vocal folds further and increasing the anteroposterior dimensions of the glottis. The lateral rotation of the arytenoids generated by the posterior cricoarytenoid is counteracted by the lateral cricoarytenoid (fig.2a). Contraction of the lateral cricoarytenoids rotates the arytenoids inwards, adducting the vocal folds and closing the glottis.

The interarytenoids are composed of two separate muscle bellies; the transverse interarytenoid and the oblique interarytenoids (fig.2b). Contraction of the interarytenoids pulls the arytenoids together, and diagonally downwards, narrowing the glottis. The majority of the oblique interarytenoid muscle fibres continue around the apex of the arytenoids and up through the aryepiglottic fold, to attach at the margin of the epiglottis, forming the aryepiglottic muscle.

The final laryngeal muscle attaching to the arytenoids is the thyroarytenoids, composed of the larger thyroarytenoid externus muscle, and the smaller vocalis muscle (fig.2). Contraction of the thyroarytenoid externus pulls the arytenoids anteromedially towards the thyroid, shortening the vocal folds. Simultaneous contraction of the thyroarytenoid externus and the transverse interarytenoid adducts the vestibular folds, creating a sphincter to protect the delicate vocal folds. A group of the uppermost muscle fibres from the thyroarytenoid externus divert caudally, forming the thyroepiglottic muscle (fig.2c). The thyroepiglottic muscle travels through the aryepiglottic fold to join with the fibres of the aryepiglottic muscle and attaches along the margin of the epiglottis. The vocalis is formed from the finer medial fibres of the thyroarytenoid which travel along the vocal folds. The vocalis controls the fine movements of the vocal folds, adjusting the quality and the pitch of the sounds produced.

1.2 *Neural control of respiration*

Phasic activity of the six different types of respiratory neurones located in the reticular formation of the medulla generate rhythmic respiratory drive. The three neural phases of the respiratory cycle (inspiratory, post-inspiratory and expiratory) are coordinated by the six types of respiratory neurons; that is, the early inspiratory, inspiratory, late inspiratory, post-inspiratory, expiratory, and pre-inspiratory neurones. It is believed that phasic inhibition of the respiratory neurones from surrounding respiratory neurones (opposed to phasic excitation) generates the rhythmic nature of respiration. During inspiration, the early inspiratory neurons stimulate the neuronal bodies innervating the laryngeal abductor muscles, to stimulate posterior cricoarytenoid and cricothyroid contraction, thus enlarging the glottis and allowing air to flow into the lungs. In the subsequent 300 ms diaphragmatic contraction pulls the lungs downwards, reducing the pressure within the lungs to subatmospheric pressures, causing air to be drawn down the pressure gradient into the lungs. Post-inspiratory neurons maintain diaphragmatic contraction, while the laryngeal abductors relax. The expiratory neurons inhibit inspiratory neurons, regulate gradual relaxation of the diaphragm, and coordinate the neuronal bodies of the nucleus ambiguous and retrofacial nucleus to maintain tonic laryngeal abductor activity, and occasional activation of the laryngeal adductors. The glottis remains open throughout the respiratory cycle during normal breathing, only narrowing slightly during expiration. In times of increased oxygen consumption, expiratory activity of the laryngeal adductors is enhanced, increasing upper airway resistance with simultaneous activation of the intercostal muscles, thus prolonging expiratory time.

Laryngeal control is complex, and a range of factors can alter the normal pattern of laryngeal activation during the respiratory cycle, including non-invasive ventilation, increased oxygen demands, vocalization, swallowing, postural changes, grunting, defecation, child birth and sleep (13-16). The respiratory neuronal bodies integrate afferent input from several regions, including sensory information from chemoreflexes, pulmonary receptors, and the voluntary control of phonation from the motor cortex.

1.3 The fetal larynx

Before birth the larynx plays a critical role in lung development by controlling lung liquid efflux and pulmonary distention. The fetal larynx is in a state of tonic adduction during periods of fetal apnoea, which are interspersed with episodes of FBM, occurring only 50% of the time (17). During periods of fetal apnoea, tonic activity of the thyroarytenoid, posterior cricoarytenoid and the interarytenoids narrow the larynx, thus increasing laryngeal resistance (18). Elevated laryngeal resistance during fetal apnoea slows lung liquid efflux, resulting in the accumulation of liquid in the lungs. The accumulated liquid opposes the elastic recoil of the pulmonary tissue and maintains the lungs in a hyperdistended state, which is critical for normal lung growth and development. During periods of fetal apnoea, tracheal pressure is usually ~ 1 -2 mmHg greater than that of the amniotic sac owing to the elastic recoil of the pulmonary tissue pushing the lung liquid against the narrowed larynx (19).

Periods of fetal apnoea are interrupted by FBM, which differ from adult breathing in two important ways; FBM are periodic, and are inhibited by hypoxia (20-22). During FBM, rhythmic activity of the neurons in the respiratory centre stimulate coordinated contraction of the diaphragm, the posterior cricoarytenoid and the cricothyroid, replicating *ex utero* breathing movements (23). The larynx abducts and upper airway resistance falls, allowing liquid to flow to and fro within the trachea (18). As liquid flows from the lungs, the elastic recoil of the pulmonary tissue pulls the lung walls inwards, accelerating liquid efflux from the abducted larynx and causing pulmonary pressure to fall (17, 24). Large pressure changes are negated by diaphragmatic contraction, which prevents the loss of significant amounts of lung liquid. In fetal humans, FBM have been observed as early as 10 weeks gestation, and from approximately the same gestational stage in fetal sheep (~ 50 days gestation; d GA) (25, 26). The frequency and duration of FBM increase throughout gestation. Occasionally, in both fetal sheep and fetal humans, coordination between the diaphragm and the larynx is distorted during FBM, resulting in strong

diaphragmatic contractions against an adducted larynx, producing a sharp decrease in pulmonary pressure (27). This discoordination has been likened to hiccups, however the cause, or function is unknown.

Like the breathing patterns of newborns, and mature mammals, FBM vary between different states of electrocortical activity (18). Throughout gestation, the fetus is mostly in a sleep like state. The majority of FBM are rhythmical and shallow (< 5 mmHg), occurring during low-voltage electrocortical activity, resembling rapid eye movement (REM) sleep (28, 29). Fetal apnoea generally occupies periods of high-voltage electrocortical activity, which is associated with non-REM sleep. During this time, fetal apnoea is occasionally interrupted by an irregular, deep FBM (18, 28). Dawes *et al* (1983) demonstrated that caudal brainstem lesions induce continuous FBM by permanently disconnecting this region from the medulla, suggesting FBM are inhibited by descending input from a region above the pons during high-voltage electrocortical activity (23).

The presence of FBM *in utero* indicates that the central respiratory pattern generator regulates and coordinates laryngeal and phrenic motor neurons prior to birth. Electroencephalography and electromyography recordings from fetal sheep have shown that rhythmic activity of the inspiratory neurons in the medulla occurs simultaneously with posterior cricoarytenoid and cricothyroid contraction, resulting in laryngeal abduction during FBM. The thyroarytenoid is essentially quiescent during periods of low-voltage electrocortical activity, and resumes tonic activity at the start of the following period of fetal apnoea, with simultaneous tonic activity in the expiratory neurons (30). Evidence suggests the absence of tonic thyroarytenoid activity during FBM is due to descending inhibition originating from higher regions (31).

1.3.1 *Fetal hypoxia and hypercapnia*

In the fetus decreased circulating oxygen levels (~ 6 - 8 mmHg) trigger a reduction, or the cessation of FBM (20, 21). This inhibitory response to hypoxia in the fetus is a consequence of central inhibition, and is very different to the reflex increase in respiratory drive stimulated by hypoxia in adults. Fetal peripheral chemoreceptors are active and responsive to decreasing partial pressure of oxygen in the arterial blood (PaO_2); however, the afferent signals from the peripheral chemoreceptors to the brain stem are silenced by descending inhibitory input from higher regions (20, 21, 32-34). Caudal lesions through the upper pons eliminates this descending inhibition (23). Following caudal lesions in fetal sheep, hypoxia stimulates respiratory activity, inducing a two-fold increase in respiratory rate and depth, which is more alike the postnatal response to hypoxia (23). Hypoxic stimulation of FBM in fetal sheep with caudal lesions through the upper pons is dependent on the presence of functional peripheral chemoreceptors, and does not occur following arterial and peripheral chemodenervation (35, 36). During hypoxia fetal heart rate falls, blood is redirected towards the heart and brain, and fetal movements are inhibited in an effort to preserve circulating oxygen (37). The inhibition of FBM during hypoxia intensifies with increasing gestational age, and is present for the first few weeks of life (38). As such, it is important that the newborn begins breathing shortly after birth to avoid hypoxic induced respiratory depression.

Fetal hypercapnia stimulates an increase in respiratory function, similar to the postnatal hypercapnic response. Elevated circulating carbon dioxide increases the occurrence and consistency of FBM, with FBM occurring almost continuously during periods of low-voltage electrocortical activity (21, 39). Hypercapnia has not been shown to induce FBM during high-voltage electrocortical activity unless fetal body temperature is reduced by a least 2.1° (40). Recruitment of the intercostal and laryngeal abductor muscles is enhanced during hypercapnia, resulting in increased inspiratory depth (21, 39, 41). As such, FBM become more similar to those of the preterm and surfactant deficient infant suffering respiratory

Table 1 Summary of the effects of hypoxia and hypercapnia on fetal breathing movements.

Fetal hypoxia	Fetal hypercapnia
Inhibitory signals from the respiratory centre inhibit FBM	↑ Incidence and consistency of FBM
↑ Peripheral chemoreceptor stimulation	↑ Recruitment of intercostal and laryngeal abductor muscles
Vasoconstriction of blood vessels in the gut, skin, respiratory muscles and skeletal muscles	↑ Proportion of vigorous FBM
Transient bradycardia	↑ Inspiratory tracheal pressure
Inhibition increases in intensity with increasing gestational age	↑ Proportion of low-voltage electrocortical activity

distress syndrome. During labour, circulating fetal carbon dioxide increases, however FBM are supressed. This is likely to be a consequence of uterine contractions and circulating prepartum hormones, both known to inhibit FBM. Carbon dioxide sensitivity increases following rostral transections, indicating that higher regions of the brain stem may be responsive to circulating carbon dioxide, and act to abridge the response to hypercapnia in intact fetuses. Table 1 summarises the effects of hypoxia and hypercapnia on FBM.

1.4 Initiation of continuous air-breathing at birth

At birth it is imperative that the newborn initiates continuous air-breathing. For a successful transition to air-breathing the lungs must be rapidly cleared of liquid and fill with air, while pulmonary vascular resistance falls, allowing for an increase in pulmonary blood flow (42-44). Liquid clearance is primarily driven by transpulmonary pressure gradients that are generated by inspiration, forcing the lung liquid

from the airways into the interstitial tissue. Studies have shown that an elevated end-expiratory pressure, in conjunction with osmotic gradients generated by transepithelial sodium reabsorption reduces the risk of evacuated liquid re-entering the airways during expiration (45). Expiratory flow and air-volume remaining in the lung at end-expiration (functional residual capacity; FRC) in the newborn are regulated by expiratory braking manoeuvers (EBM). During EBM, the loss of expiratory laryngeal abductor activity results in laryngeal adduction or narrowing, causing the complete cessation of expiratory flow during mid-to-late expiration (46-51). A supra-atmospheric pressure is created in the lungs during EBM by the lungs natural recoil against a closed or partially closed glottis (52-54). Elevated pulmonary pressure is also thought to be important to preserve lung gas volume, increase gas exchange, and prevent alveolar collapse, as well as promoting uniform aeration of the newborn's lungs (50, 55).

Several factors are thought to contribute to the initiation of continuous breathing at birth, however it is difficult to isolate a single factor, and it is likely continuous breathing is stimulated by a combination of factors (56). It has been suggested that continuous breathing is initiated by cord occlusion, owing to the disappearance of placental factors that inhibit continuous FBM throughout gestation, however this theory is controversial (57, 58). Studies examining the effect of cord occlusion in fetal sheep provide conflicting results, with several studies reporting no change in FBM, whilst others report the appearance of continuous fetal breathing, which terminate upon release of the umbilical cord (59-61). Alvaro *et al* (1993) demonstrated that injections of a placental extract inhibited continuous FBM in fetal sheep during umbilical cord occlusion (62). However, in a subsequent study it was shown this decrease in FBM was directly associated with a transition from low to high-voltage electrocortical activity (63). Interestingly, continuous breathing activity at birth does not begin following cord occlusion provided the $PaCO_2$, PaO_2 and core body temperature are maintained constant in newborn lambs supported by an extracorporeal membrane oxygenator system (56). Breathing movements only became continuous when core temperature decreased by 1.2°, provided $PaCO_2$ was maintained constant.

Newborn minute ventilation is proportional to the FiO_2 (64, 65). At birth the newborn is essentially an exteriorised fetus, and similar to the fetus, hypoxia inhibits the newborn's respiratory drive, resulting in prolonged periods of apnoea (64, 65). Failure to initiate air breathing at birth can result in serious apnoea, exacerbate hypoxia, and thus further inhibit respiratory drive. The newborn response to hypercapnia is also similar to that of the fetus. In term and preterm newborns, increased inspired carbon dioxide increases tidal volume, reduces inspiratory time and increases expiratory time (65-67).

At birth and following cord clamping, the newborn is exposed to an environment much cooler than the uterus, with a simultaneous rise in $PaCO_2$, a fall in external pressure and the loss of placental factors known to inhibit continuous FBM. It is likely that a combination of the aforementioned variables stimulate continuous breathing at birth (56). It is important to understand the factors triggering continuous breathing to assist the newborn to make a successful transition to air-breathing at birth.

1.4.1 *Non-invasive ventilation at birth*

Very and extremely premature newborns are often unable to initiate and sustain independent respiration at birth, owing to the immaturity of their respiratory system, and lack of neural development. As such, it is critical that these newborns receive respiratory support to help them survive the transition from *in utero* to *ex utero* life. Although respiratory support at birth is critical for the transition, invasive ventilation can cause lung injury, having negative ramifications throughout the newborns life. To avoid invasive intubation and mechanical ventilation, non-invasive ventilation is becoming increasingly used in the delivery room and during the neonatal period (68-70). Non-invasive ventilation describes any technique that applies a constant or variable pressure to the airways to provide respiratory support without tracheal intubation (fig.3). This is an appealing alternative to invasive intubation and mechanical ventilation, which are associated with severe complications such as pneumonia, tracheal bleeding, tracheal granuloma and bronchopulmonary dysplasia (71, 72). Several different forms of non-invasive

ventilation are frequently delivered to premature newborns in the delivery room, and can be categorised into a constant or variable airway pressure (fig.3). Continuous positive airway pressure (CPAP) provides a constant positive pressure to the airways (fig.4a) via nasal prongs or a facemask (73). Continuous positive airway pressure relies on the spontaneous breathing activity of the newborn. It is thought that CPAP enhances FRC, decreases airway resistance, reduces the work of breathing, splints the airways open, prevents alveolar collapse, and improves gas exchange resulting in an increased PaO_2 (6, 74, 75).

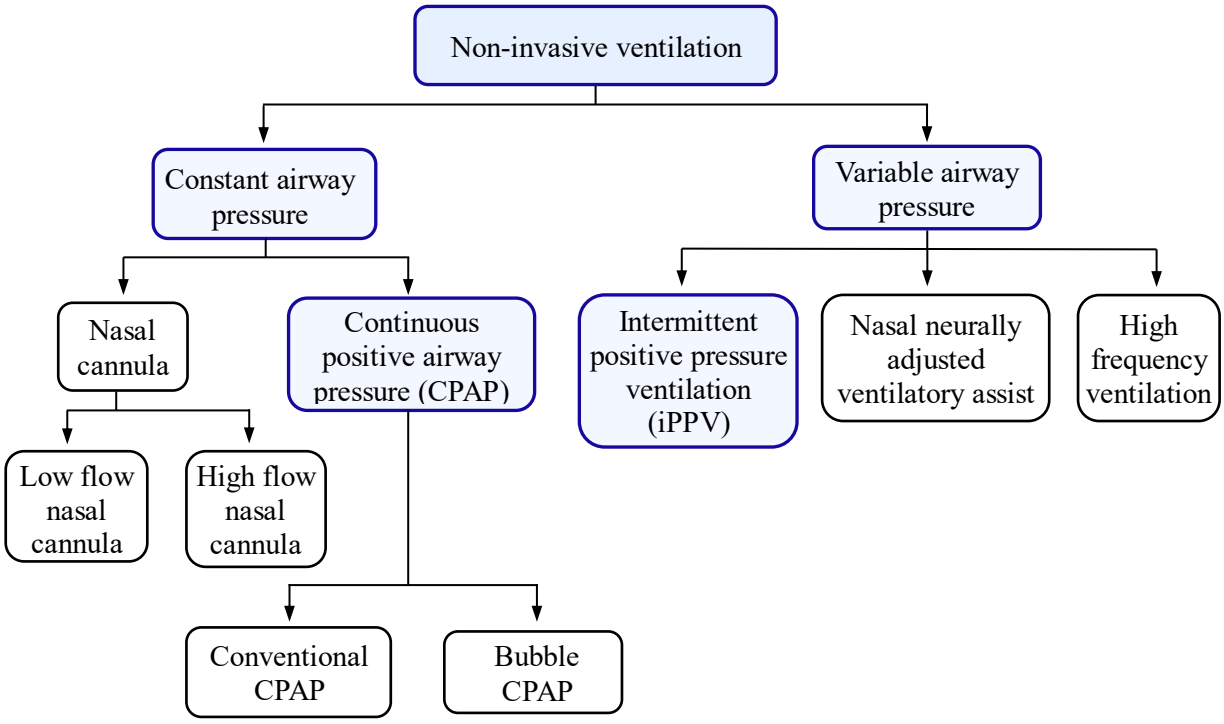

Fig.3 Tree diagram displaying the basic forms of non-invasive ventilation. Continuous positive airway pressure and intermittent positive pressure ventilation, highlighted by blue boxes, are relevant to the studies in the subsequent chapters. Flow chart inspired from Mahmoud *et al* (2011) (71).

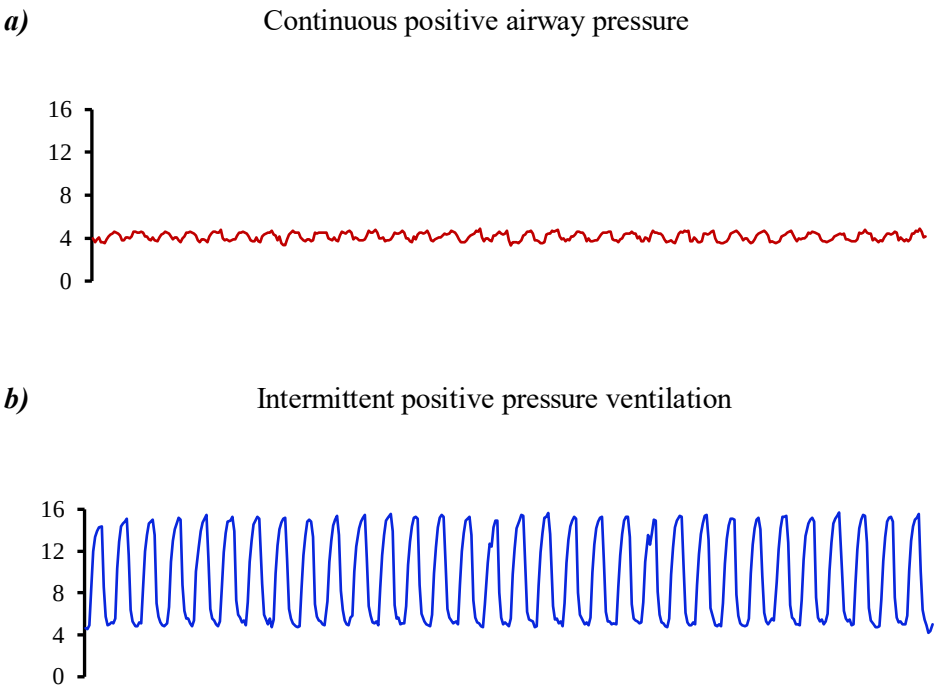

Fig.4 Physiological recordings of airway pressure (Paw) in a preterm rabbit kitten receiving ***a)*** continuous positive airway pressure (4 cmH_2O) and ***b)*** intermittent positive pressure ventilation with a peak inspiratory pressure of 16 cmH_2O and a positive end-expiratory pressure of 4 cmH_2O. The periodic fluctuations seen during CPAP in ***a)*** are generated by the kitten's spontaneous breathing effort.

Retrospective clinical studies report a significant decrease in the incidence of bronchopulmonary dysplasia in institutions with a high usage of CPAP (76, 77). However, the delivery of CPAP is not without its shortfalls, including the potential for over inflation, reduced venous return, and depressed cardiac output owing to increased intra-thoracic pressure. Furthermore, ~ 40 - 60% of premature neonates with respiratory distress syndrome receiving CPAP fail to improve, and require subsequent invasive mechanical ventilation (78, 79).

However, as CPAP relies on the spontaneous breathing efforts of the newborn, CPAP is an inefficient form of non-invasive ventilation for apnoeic newborns. This has prompted the use of non-invasive iPPV in the delivery room and the NICU. An oscillating pressure is delivered during iPPV, mirroring normal respiration, cycling between a peak inspiratory pressure (PIP) and a positive end-expiratory pressure (PEEP, fig.4b). Nasal prongs, nasopharyngeal prongs, nasal cannula and a facemask are commonly used to deliver iPPV (80, 81). Although iPPV has been a popular form of non-invasive ventilation since the early 1970s there is a great deal of uncertainty surrounding its mechanism of action, and how iPPV interacts with the newborn's changing respiratory physiology at birth (82). It has been speculated that iPPV increases alveoli recruitment, and improves FRC; however, this theory has yet to be confirmed in clinical studies (80, 82). Non-invasive iPPV has also been suggested to increase tidal volume, minute volume, and carbon dioxide clearance (83).

Unfortunately, non-invasive ventilation has a high failure rate, with a significant degree of variation in success depending on gestational age, postnatal age, and the form of non-invasive ventilation delivered. The support study reported an 81% failure rate in extremely preterm newborns (24 – 28 weeks gestation) receiving CPAP, while an earlier trial reported only 19% of newborns receiving iPPV failed to improve (5, 84). The cause of this variable failure rate is not known, although several sources have suggested mask leaks, airway obstruction, and laryngeal closure are responsible (9, 10). The upper airways are an important consideration during non-invasive ventilation that are otherwise bypassed in intubated newborns. While laryngeal activity and reflexes during the neonatal period has been well studied in term lambs receiving non-invasive ventilation, the effect of non-invasive ventilation on laryngeal activity during the transition from fetal to newborn life is unknown (15, 85, 86).

Electromyography recordings of the cricothyroid (abductor) and thyroarytenoid (adductor) during quite breathing in neonatal lambs (1 - 20 postnatal days) show that the cricothyroid is active during inspiration, while the thyroarytenoid generally remains silent throughout the respiratory cycle (fig.5).

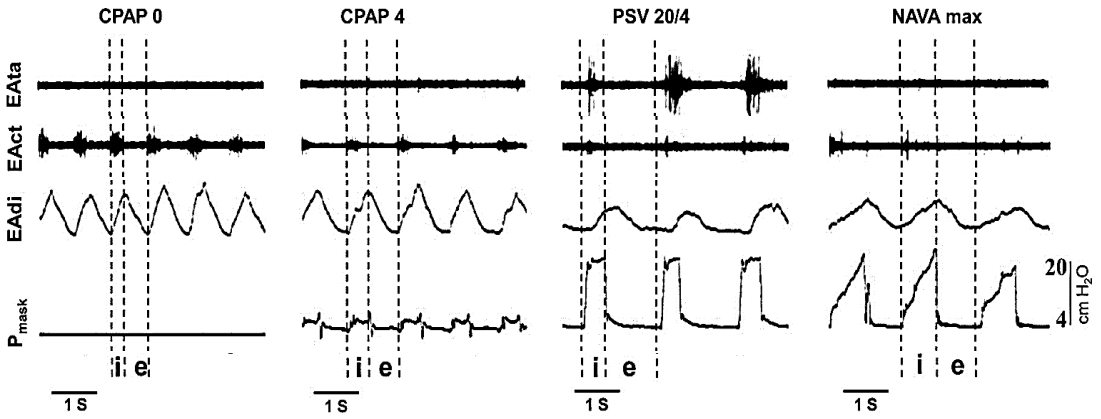

Fig 5 Electromyography recordings of the diaphragm (EAdi), thyroarytenoid (EAta) and cricothyroid (EAct) during normal breathing (CPAP 0), 4 cmH_2O continuous positive airway pressure (CPAP 4), pressure support ventilation (PSV) and neurally adjusted ventilatory assist (NAVA max). Inspiratory cricothyroid activity falls during CPAP 4, PSV and NAVA max. Inspiratory thyroarytenoid activity increases during CPAP 4 and PSV, but not during NAVA. Hadj-Ahmed *et al* 2012 (87).

This indicates that inspiratory laryngeal dilation is active, while expiratory laryngeal narrowing is passive, resulting from the relaxation of the laryngeal abductor muscles (15, 87). Thyroarytenoid activity increases during apnoea, gasping, and EBM, when the larynx actively adducts (88, 89). Thyroarytenoid contraction increases end-expiratory airway pressure and prolongs expiratory time, which is thought to be important to increase gas transfer as well as prevent oxygen desaturation, and airway collapse. Unfortunately, very little is known about the activity of the other intrinsic laryngeal muscles in the neonate, owing to their small size and location.

The normal pattern of laryngeal muscular activity is disrupted during non-invasive ventilation in both neonates and adults (16, 90). Electromyography recordings in neonatal lambs show nasal intermittent positive pressure ventilation (nIPPV) inhibits inspiratory cricothyroid activity, and stimulates inspiratory

thyroarytenoid activity, thus preventing inspiratory laryngeal dilation, and stimulating inspiratory laryngeal narrowing (fig.5) (15, 85). Increasing inspiratory pressures increases the intensity and consistency of cricothyroid inhibition and thyroarytenoid stimulation. Furthermore, maximal thyroarytenoid stimulation occurs simultaneously with the PIP (15). Studies show that nasal CPAP (4 cmH_2O) significantly decreases inspiratory cricothyroid activity, yet causes no observable increase in inspiratory thyroarytenoid activity (fig.5) (87). Inspiratory laryngeal narrowing limits lung ventilation, and increases the risk of gastric distension, thus further compromising respiratory function (91, 92).

Hadj-Ahmed *et al* (2012) has demonstrated in neonatal lambs that nasal neurally adjusted ventilatory assist (nNAVA) failed to induce inspiratory thyroarytenoid contraction, even at maximum inspiratory pressures (right-most panel, fig.5) (87). The absence of inspiratory thyroarytenoid activity during

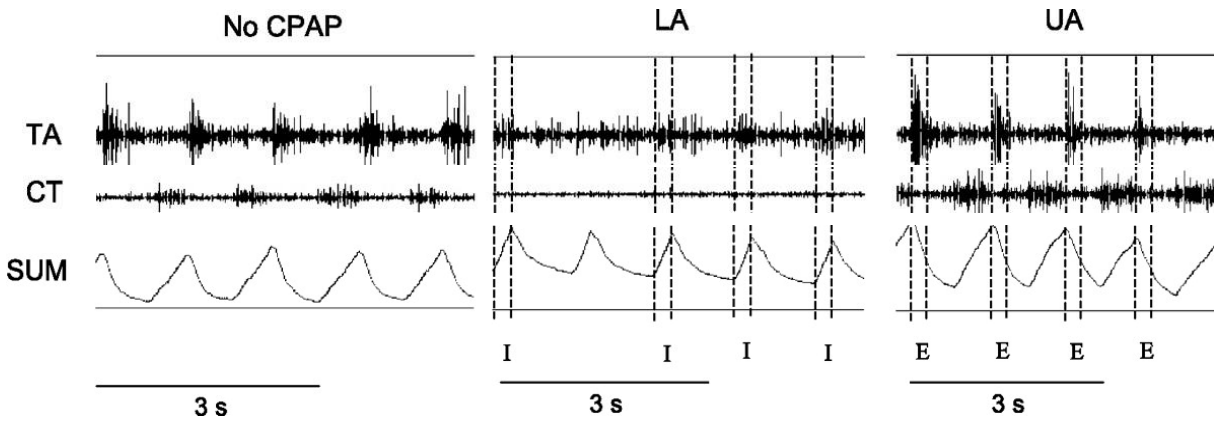

Fig.6 Electromyography and sum signal of the respiratory inductance plethysmography (SUM) recordings from a lamb with a laryngotracheal separation. Inspiratory cricothyroid (CT) and expiratory thyroarytenoid (TA) activity was evident during normal breathing in the intact lamb (left panel). Intermittent positive pressure ventilation (iPPV) applied directly to the lower airways (LA) stimulated inspiratory thyroarytenoid activity and inhibited cricothyroid activity (middle panel). When iPPV was applied to the upper airways (UA) there was no changes in either thyroarytenoid or cricothyroid activity from control, Roy *et al* 2008 (85).

nNAVA suggests an asynchrony between the lamb's central respiratory drive and the non-physiological pressurisation of the airways during inflation may have a significant role in inspiratory laryngeal adduction during nIPPV.

Laryngeal mechanoreceptors and bronchopulmonary receptors are stimulated by changes in pressure, and potentially have a role in ventilator induced inspiratory laryngeal narrowing. Roy *et al* (2008) found that nIPPV delivered directly to the isolated upper airways failed to induce inspiratory thyroarytenoid activity, nor reduce inspiratory cricothyroid activity, as usually seen during nIPPV (fig.6) (85). Furthermore, when iPPV was delivered directly to the lower airways, bypassing the larynx, inspiratory cricothyroid inhibition and inspiratory thyroarytenoid stimulation was evident, as usually seen during nIPPV in neonatal lambs (fig.6). This study went further to show a bilateral vagotomy prevented the inspiratory increase in thyroarytenoid activity during nIPPV, whilst inspiratory cricothyroid activity

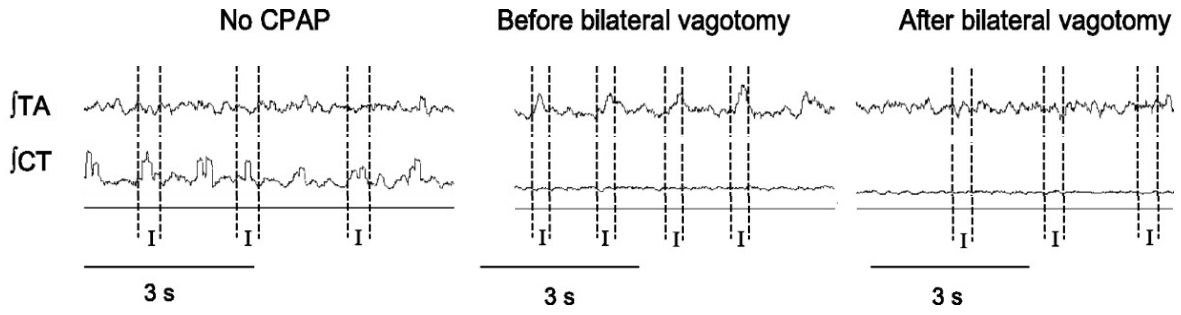

Fig.7 Bilateral vagotomy prevents an increase in inspiratory thyroarytenoid (TA) activity during nasal intermittent positive pressure ventilation (nIPPV), but does not prevent inhibition of inspiratory cricothyroid (CT) activity. The left panel shows the moving time average of the thyroarytenoid and the cricothyroid during normal breathing. Inspiratory thyroarytenoid activity increases and inspiratory cricothyroid activity is reduced in during nIPPV prior to a bilateral vagotomy (middle panel). A bilateral vagotomy does not induce the usual increase in inspiratory thyroarytenoid activity, but does inhibit cricothyroid activity (right panel), Roy *et al* 2008 (85).

persisted (fig.7). These results indicate that the increased inspiratory thyroarytenoid activity induced by nIPPV originates primarily from bronchopulmonary receptor stimulation, with little to no contribution from the upper airway pressure receptors. Furthermore, the results from this study suggests inspiratory cricothyroid inhibition does not originate from the upper airway or bronchopulmonary pressure receptors. However, it must be kept in mind that these studies examined laryngeal activity in neonatal lambs, and not very or extremely preterm human newborns at birth.

The three types of bronchopulmonary receptors responsive to pulmonary tissue distension are the bronchopulmonary C-fibre endings, the slowly-adapting stretch receptors, and the rapidly-adapting stretch receptors. Bronchopulmonary C-fibre endings are stimulated by excessive stretch of the pulmonary tissue, equal to or greater than tidal volume. Stimulation of the pulmonary C-fibre endings triggers active laryngeal adduction and bronchopulmonary constriction in neonatal lambs (93). However, abolition of the C-fibre endings by capsaicin treatment has been shown not to prevent increased inspiratory thyroarytenoid activity during iPPV in neonatal lambs, although further studies are needed to confirm these results (94). Slowly-adapting stretch receptors are stimulated by moderate levels of lung inflation, and continue to discharge for a period of time following the removal of stimulation, playing an important role in the termination of inspiration. Thyroarytenoid activity dissipates prior to the termination of the inspiratory pressure plateau, thus suggesting that the slowly-adapting stretch receptors are not likely to be involved in iPPV induced inspiratory laryngeal narrowing (15). Rapidly-adapting stretch receptors are concentrated in the larger airways and provide positive feed-back for inspiratory drive. These afferent bodies are extremely sensitive to mechanical stimulation and discharge rapidly. Studies in adult animals have shown that stimulation of the rapidly-adapting stretch receptors induces laryngeal narrowing. A process of elimination suggests that the rapidly-adapting stretch receptors may have an important role in stimulating iPPV induced inspiratory laryngeal narrowing, however this has yet to be examined in the newborn.

Interestingly, Carrière *et al* (2015) demonstrated that hypoxia did not induce laryngeal narrowing, and hypercapnia significantly decreased laryngeal narrowing during iPPV in neonatal lambs (95). Hypercapnia increases the risk of brain damage at birth, and as such providing iPPV with high concentrations of carbon dioxide to curtail laryngeal narrowing is not practical. The effects of iPPV in neonatal lambs has been studied in detail, however, the effect of iPPV on laryngeal function at birth has yet to be investigated.

1.4.2 Respiratory inhibition initiated by trigeminal nerve stimulation

The delivery of iPPV via a facemask at birth may stimulate the trigeminal nerve in premature newborns, however no clinical studies have previously addressed this issue. Stimulation of the sensory branch of the trigeminal nerves on the face, either through touch, cold, or pressure, induces a reflex response known as the trigeminocardiac reflex. In adults, stimulation of the sensory branch of the trigeminal nerves results in the sudden onset of apnoea, bradycardia, and elevated cerebral blood flow, amongst other things (96, 97). Observational reports from the delivery room suggest the application of the facemask depresses respiratory drive in preterm newborns; however, this is anecdotal evidence and clinical studies must be conducted. Very few studies have examined this reflex response in neonatal humans. Surprisingly, the reflex respiratory response to trigeminal nerve stimulation in neonates differs greatly from that of the adult. The application of a facemask in term and preterm neonates stimulates an increase in tidal volume and minute ventilation, as well as a small decrease in respiratory rate (98-100). Several studies have separated the response to the increased dead space introduced by the mask, from the stimulation of the trigeminal nerves, showing that the application of the rim of the facial mask accounts for the fall in respiratory rate, while the increased dead space introduced by the mask stimulates an increase in tidal volume (98, 99, 101). This results in an overall increase in minute ventilation when the facemask is applied to the neonate's face. The respiratory response to facemask application is

inversely proportional to gestational and postnatal age. As such, younger infants with a lower gestational age have a more pronounced respiratory response to trigeminal nerve stimulation. No study has examined the respiratory response to trigeminal nerve stimulation by the facemask at birth. As such the effect of the facemask during iPPV at birth is unknown and may be worth consideration. After birth, the external pressure experienced by the newborn decreases significantly following the transition from a pressurised fluid filled intrauterine environment, to the gaseous extra-uterine environment. This may be an important consideration in the reflex response to trigeminal stimulation at birth.

1.4.3 Respiratory inhibition initiated by laryngeal chemoreceptor stimulation

The larynx is the most densely innervated region of the respiratory system, with several types of mechanoreceptors and chemoreceptors. The laryngeal chemoreceptors protect the lower airways from aspiration of liquids and foreign substances. Stimulation of the laryngeal chemoreceptors elicits a protective reflex known as the laryngeal chemoreflex (LCR). The immature LCR is characterised by apnoea, rapid swallowing, bradycardia, hypoxemia, and arousal (102-107). In several studies, prolonged apnoea, induced by stimulation of the laryngeal chemoreceptors, has resulted in mortality in neonatal piglets (104, 105). The LCR matures with postnatal and gestational age, and by adulthood the apnoeic component of the LCR is rarely seen. In adults the LRC generally manifests in coughing and swallowing, effectively removing the foreign substances from the larynx (108). The laryngeal chemoreceptors are stimulated by hypo-osmolar solutions with low chloride concentrations, inciting an LCR that is inversely proportional to the chloride concentration of the foreign substance (109). Therefore, liquids such as amniotic fluid and gastric secretions effectively stimulate the LCR. Fetal laryngeal chemoreceptors are highly sensitive to amniotic fluid, and contact with amniotic fluid results in prolonged laryngeal adduction, inhibition of FBM, and swallowing, which is likely to protect the lower airways from aspiration of amniotic fluid (110). The laryngeal chemoreceptors are slowly adapting receptors, as such

apnoea and reflexive swallowing will persist until the liquid has been removed. It has been suggested that oesophageal pressurisation stimulates relaxation of the lower oesophageal sphincter, thus increasing the risk of gastric reflux during non-invasive ventilation, which can induce a life threatening LCR.

1.5 Conclusion

Despite the lack of clinical evidence supporting the use of non-invasive ventilation at birth, this has become a popular form of respiratory support in the delivery room for preterm newborns unable to sustain independent respiration. However, a very high, and unexplained failure rate is seen in premature newborns receiving non-invasive ventilation at birth. Furthermore, it is unknown how non-invasive ventilation integrates with the preterm newborn's changing respiratory and laryngeal physiology. It has been suggested that upper airway obstructions may hinder the delivery of non-invasive ventilation. Little is known about the larynx in preterm newborns, and assumptions made about the larynx during this time are extrapolated from laryngeal activity during the fetal and neonatal period.

During fetal life the larynx is predominately adducted during fetal apnoea. At birth the larynx must transition to a predominantly abducted neonatal state, allowing for continuous air breathing. The studies presented in this thesis are the first to directly visualise the upper-airways in preterm rabbit kittens receiving non-invasive ventilation at birth. Laryngeal activity in preterm rabbit kittens was visualised using phase contrast X-ray imaging, providing high contrast between abducted and adducted airways. These studies examine laryngeal activity and breathing patterns in preterm kittens with, and without non-invasive ventilation, and with different concentrations of oxygen inhalation. These studies aim to understand laryngeal activity at birth to increase the efficiency of non-invasive ventilation in preterm newborns in the delivery room. It is important to understand newborn laryngeal function and the mechanisms stimulating laryngeal abduction to improve the transition to air-breathing at birth, and

enable successful delivery of non-invasive ventilation.

1.6 Synchrotron based phase Contrast X-ray imaging

In the studies presented in the current thesis we have utilised synchrotron based phase contrast X-ray imaging to visualise the larynx and the lungs in preterm rabbit kittens at birth. Synchrotron based phase contrast X-ray imaging produces high resolution images of the airways, and highlights the boundaries between the aerated airways and the soft tissue. Phase contrast X-ray imaging exploits changes in the refractive indices between adjacent media to enhance image contrast (111). Variations in the refractive indices between the air-filled trachea and the surrounding tissue produce a phase shift in the highly coherent X-ray beam, causing the beam to refract at the boundaries between tissues. The beam also attenuates as it passes through the tissue. The refracted X-rays interfere as they travel from the exit surface of the kitten to the detector, generating dark and bright Fresnel fringes at the air-tissue boundaries in the image. The regional intensity differences and the fringes at the air-tissue boundary allows for the identification of the patent glottis and the epiglottis (fig.8). When open, the larynx appears as bright as the surrounding airways (in comparison to the tissue), while the closed larynx appears darker, similar to the tissue. In the lungs, large phase shifts occur at each air-tissue boundary, generating multiple local refractions of the beam, resulting in a speckle pattern of bright and dark spots in the image, indicating the lung is aerated.

1

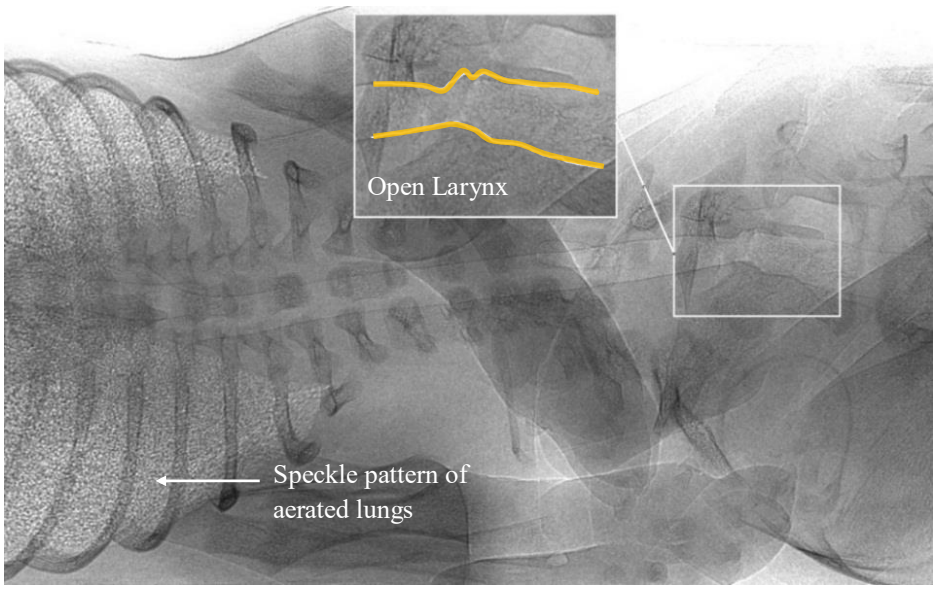

Fig.8 A synchrotron base phase contrast X-ray image of a preterm rabbit kitten with an abducted larynx. The boundaries between the air-filled trachea and the surrounding soft tissue are highlighted due to the phase shift in the X-ray beam as it passes through the adjacent media. As such the patent larynx appears just as bright as the surrounding airways. Within the lungs, a phase shift occurs at the air-tissue boundary in each alveolus, generating a speckled pattern indicating that the lungs are aerated.

1

2

Chapter 2

1st study: Laryngeal closure impedes non-invasive ventilation at birth

2.1 Introduction

At birth most very and extremely premature newborns are unable to sustain independent ventilation and require respiratory support to survive (112). Previously, respiratory support delivered at birth involved intubation and mechanical ventilation; however, intubation can cause damage to the upper airways and mechanical ventilation injures the delicate lungs (112). As such, in recent years there has been a shift in clinical practice towards non-invasive ventilation (113). Although non-invasive ventilation is the preferred form of respiratory support for preterm newborns, it has an unfortunately high failure rate, resulting in the infant being intubated and mechanically ventilated. As it is unknown how non-invasive ventilation integrates with the newborns changing respiratory physiology at birth, it is unclear why non-invasive ventilation is sometimes unable to ventilate very and extremely premature newborns

in the delivery room (4, 5, 78).

The high failure rate of non-invasive ventilation is believed to be the result of airway obstructions (incidence of 26%) and mask leaks (incidence of 51%) (9, 10). Although airway obstruction is less common, the rate of clinical deterioration is four times greater, resulting in the need for intubation (9, 10). When a sustained inflation is delivered at birth, air only passes into the lungs during a spontaneous breath, indicating that the obstruction is located in the upper respiratory tract, and that the larynx may have some involvement (114). The involvement of the larynx in the respiratory circuit is an important, yet overlooked, difference between invasive and non-invasive respiratory support. During fetal apnoea, the larynx is adducted, and at birth laryngeal function must transition to a predominantly open state, allowing for continuous breathing. It is unknown what triggers this transition at birth. We propose that protracted laryngeal adduction in apnoeic very preterm newborns at birth obstructs the upper airways and prevents non-invasive iPPV from aerating the lungs. Furthermore, we suggest that non-invasive ventilation will remain ineffective unless the infant takes a breath. Finally, as a stable breathing pattern is established and the lung aerates, we propose that the larynx will transition to a predominantly open state, allowing for continuous air breathing and for the successful delivery of non-invasively applied iPPV. In this study, we have used phase contrast X-ray imaging to examine laryngeal function immediately after birth, and at the end of the 1st hour of life in preterm rabbit kittens (29 - 30 d GA, term = 32 d GA) receiving non-invasive ventilation via a facemask.

The aim of this study is to characterize laryngeal function immediately after birth, and at the end of the first hour of life in spontaneously breathing premature rabbit kittens. It is hypothesized that immediately after birth, the glottis and the epiglottis will be predominately closed, opening only during a breath, preventing non-invasive ventilation from aerating the lungs. It is also hypothesized that after the onset of a stable breathing pattern the glottis and the epiglottis will have transitioned to a predominantly open state, allowing the lungs to be successfully ventilated non-invasively with iPPV.

2.2 Methods

2.2.1 Experimental procedure

All experimental procedures received approval from SPring-8 Animal Care and Monash University's School of Biomedical Science's Animal Ethics Committees. Experiments were conducted at the Biomedical Imaging Centre at the SPring-8 synchrotron, Japan, in experimental hutch 3 of beamline 20B2.

Pregnant New Zealand white rabbits (n = 21) were housed in the SPring-8/ JASRI holding facility for at least 5 days prior to experimentation. Free access to food and clean water was provided. The rabbits were maintained in an ambient temperature of 18 - 20º with a 12 hour light and dark cycle (7 am - 7 pm). At 29 or 30 d GA, the pregnant doe was restrained and transported to the experimental facility in a custom-made box and the doe's weight was recorded. The external surface of the doe's ear was shaven, exposing the skin overlying the ear veins, and a topical analgesic (5% Lignocaine, Xylocaine, AstraZeneca, Sweden) was applied. A catheter was inserted ~ 5 cm into the ear vein, directed towards the heart. A three-way stopcock was connected to the catheter hub, and secured to the ear using water-proof tape (Leukoplast Sleek tape, BSN Medical Pty. Ltd., South Africa), permitting delivery of both saline and a rapidly acting anaesthetic, Rapinovet. An intravenous (I.V.) bolus of Rapinovet (12 mg/kg I.V.; propofol, Schering-Plough Animal health, USA) followed by heparinised saline (2 ml; 40 mg/100 ml) was delivered via the ear vein catheter to induce the initial anaesthesia. Once anaesthetised, the doe was removed from the box and placed on a heat pad. A continuous infusion of Rapinovet (40 mL/hr; 10 mg/mL I.V.) was administered to maintain sedation. The doe was intubated with an endotracheal tube using a laryngoscope. The endotracheal tube was connected to a mechanical ventilator (Model 683 Rodent Ventilator; Harvard Apparatus Inc., USA) and ventilated for the remainder

of the experiment at a rate of ~ 40 - 45 inflations/minute, with a tidal volume of 25 mL and a flow rate of 1.2 L/min at 60 - 100% oxygen. The Rapinovet infusion ceased, and Isoflurane inhalation (1.5 - 3.0%, Isoflurane, Delvet Pty. Ltd., Australia) was used to sustain sedation. The level of sedation was monitored throughout the experiment from the blink and pedal reflex, and Isoflurane concentrations were adjusted accordingly.

The doe was laid supine on a heat pad, exposing the abdomen. The abdomen was shorn from the mid-ventral region using clippers (Oster Golden A5, Sunbeam Products Inc., USA). A local topical analgesic (Xylocaine) was used to anesthetise the incision site. With the doe laying on her right side, an incision was made along the mid-line of the abdomen through the skin and underlying fat, extending from the umbilicus to the groin region (~ 9 cm). The skin was gently pulled away and a similar incision was made through the linea alba to expose the pregnant uterus. A small portion of the first uterine horn was exteriorised, and supported on a raised heated platform, where the location and orientation of individual kittens was ascertained with gentle palpations. Xylocaine was applied along the uterine surface to anesthetise the incision site. An incision was made through the uterus and fetal membranes overlying the hind limbs, taking care to avoid damaging the placenta. The kitten was exposed and placed supine alongside it's placenta to maintain umbilical blood flow. A custom-made facemask was gently placed over the kitten's head and securely attached using tissue glue. An oesophageal tube was inserted to measure changes in intrathoracic pressure. The umbilical cord was then ligated and cut before 20 mg/kg of caffeine was administered. The kitten was transferred into the imaging hutch, and placed laterally on a heating pad (fig.9a). The facemask was connected to a small animal ventilator and electrocardiograph leads were attached to the kitten. The kittens were given physical stimulation to encourage them to breathe spontaneously before imaging commenced (115). Kittens were imaged during iPPV for ~ 12 minutes. Facemask pressure and oesophageal pressure were continuously recorded (1 kHz, Powerlab; ADInstruments, Sydney, Australia). If during imaging, the kitten was apnoeic, defined as the

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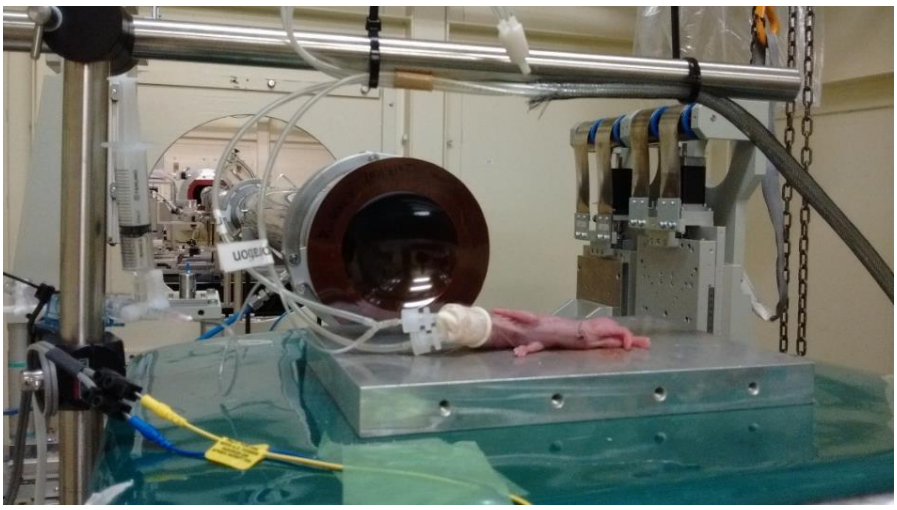

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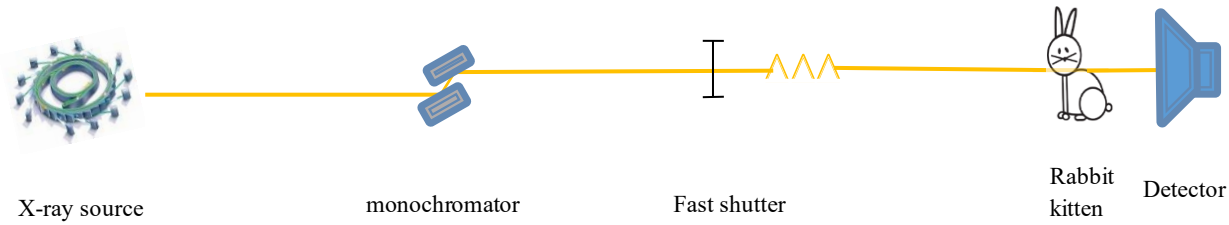

Fig.9 a) A photograph of the experimental set up. A kitten is positioned in the path of the monochromatic X-ray beam upstream of the detector for phase contrast X-ray imaging of the upper-airways during non-invasive ventilation delivered by a facemask secured to the kitten's face. ***b)*** A diagram of the experimental set up for synchrotron based phase contrast X-ray imaging at SPring-8. The source to kitten distance was ~ 210 m and the sample to detector distance was ~ 2 m.

absence of successful breathing efforts for 20 seconds or more, and the heart rate decreased below 100 beats per minute, the hutch was entered to deliver tactile stimulation to the kitten. This was common as most kittens had unstable breathing patterns immediately after birth, and was necessary in 38 kittens. Non-invasive ventilation commenced with an initial CPAP of 5 - 7 cmH_2O (115). To determine whether

non-invasive iPPV could inflate the lung, a PIP of 25 cmH_2O and a PEEP of 5 cmH_2O were used. In previous studies, our group have shown that a PIP of 25 cmH_2O and a PEEP of 5 cmH_2O effectively ventilates the lungs of preterm rabbit kittens at birth (30 d GA) (116, 117).

Following imaging, kittens were removed from the hutch and placed on a heat pad where they received constant tactile stimulation to encourage spontaneous breathing efforts. If a kitten was unable to achieve a stable breathing pattern considered sufficient to maintain viability, the kitten was humanely killed with an overdose of sodium pentobarbitone (intraperitoneal; 100 mg/kg; Somnopentyl, Kyoritsu Seiyaku Co., Ltd., Tokyo). At approximately one hour after birth, viable kittens were returned to the hutch for a second imaging sequence to visualize the larynx and the lungs. During this 2nd sequence, each kitten received a brief period of iPPV (PIP 25 cmH_2O and PEEP 5 cmH_2O) and stepwise increases in CPAP between 0 and 15 cmH_2O. This imaging sequence is depicted in figure 10 below.

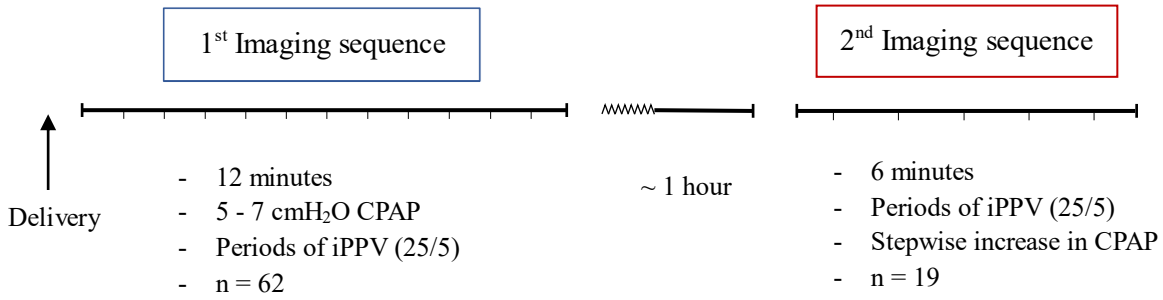

Fig.10 Kittens were delivered and imaged using phase-contrast X-ray imaging with simultaneous non-invasive respiratory support in two sequences. Immediately after birth, during the first imaging sequence, kittens received a constant positive airway pressure (CPAP) between 5 cmH_2O and 7 cmH_2O, as well as periods of intermittent positive pressure ventilation (iPPV) with a peak inflation pressure (PIP) of 25 cmH_2O and a positive end expiratory pressure (PEEP) of 5 cmH_2O. During the second imaging sequence, approximately one hour after the first, kittens received CPAP which increased in a stepwise fashion between 0 and 15 cmH_2O, as well periods of iPPV with a PIP of 25 cmH_2O and a PEEP of 5 cmH_2O.

2.2.2 *Phase contrast X-ray imaging*

Synchrotron-based phase contrast X-ray imaging was used to visualize the upper airways. Kittens were positioned laterally on a heated stage ~ 210 m downstream of the X-ray source with the detector (ORCA Flash 4.0 Hamamatsu C11440-22C, effective pixel size 15.3 μm^2, 2048×2048 pixels) located a further 2 m downstream of the kittens (fig.9b), as previously described (118). The large distance between the X-ray source and the kitten produced a spatially and temporally coherent beam. Broad-spectrum bending magnet radiation was filtered using a Si(111) double-bounce monochromator, producing a monochromatic 24 keV X-ray beam, which we have previously shown to provide the optimal bone/soft tissue contrast and signal-to-noise ratio when imaging rabbit kittens using this beamline (119). The large distance (~ 2 m) between the kitten and the detector allows for the interference of the diffracted X-rays, producing intense edges at the air-tissue interface of the airways. The exposure time was 20 ms and the frame rate was initially set at 5 Hz but was later increased to 10 Hz for greater temporal resolution.

2.2.3 *Data analysis:*

Phase contrast X-ray images were visually analysed to determine if the glottis and epiglottis were open or closed, both immediately and at approximately one hour after birth. This data is expressed as the percentage of time the glottis and epiglottis were open over consecutive 30 second periods. The efficiency of iPPV to ventilate the lung was assessed both before and after the kitten had aerated its lungs and established a relatively stable breathing pattern using images of the lung and intrathoracic oesophageal pressure recordings. A stable breathing pattern was defined as the absence of apnoea, a heart rate above 100 beats per minute, and a spontaneous breathing rate sufficient to maintain viability. The effect of increasing mask pressure on breathing rates was assessed from the intrathoracic oesophageal pressure recordings and the mean percentage of time the glottis and the epiglottis were open. Increasing airway pressure has a direct effect on the distension of the pharynx, and was measured directly from the

images. Pharyngeal area from the two-dimensional cross section of the upper airways in the phase contrast X-ray images was measured using ImageJ (free to access image processing and analysis software, Wayne Rasband, National Institute of Health, USA) at PIP and PEEP. Changes in pharyngeal dimensions measured from the images were matched with the changes in airway pressure generated by the ventilator. As the entire pharynx was not usually included in the field of view, the change in pharyngeal area is expressed as a fraction of the initial pharyngeal area prior to the commencement of iPPV using a constant region of the pharynx.

2.2.4 Statistical analysis

All data is presented as the mean ± the standard error (SEM). Data was compared for significance across time using a one-way ANOVA. An unpaired t-test with a Welch's correction was used to test significance between the sequences for both the glottis and the epiglottis. P-values < 0.05 were considered statistically significant.

2.3 Results

Seventy-one premature rabbit kittens were delivered by caesarean section from 21 does; 38 and 33 kittens were delivered at 29 and at 30 d GA, respectively. Nine kittens were not considered viable at delivery and were not imaged. Immediately after birth, 62 kittens were imaged in the 1st sequence, of which 51 kittens had an unstable breathing pattern and as such were grouped into the **unstable group** (fig.11a). Of the 51 kittens in the unstable group, 25 achieved a stable breathing pattern (with heart rates greater than 100 beats per minute) by the conclusion of the 1st imaging sequence, while the remaining 26 kittens were unable to establish a stable breathing pattern. Of the 62 kittens, 11 were very active after delivery with vigorous and stable respiratory efforts resulting in aeration of the lungs prior to the

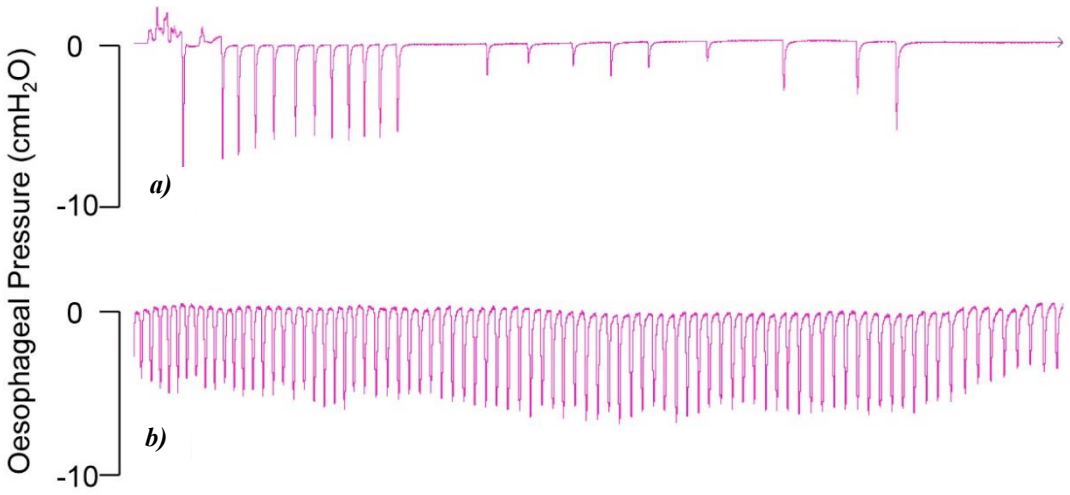

Fig.11 Oesophageal pressure recordings from two kittens showing breathing activity during non-invasive continuous positive airways pressure. Each downwards spike represents a breath. ***a)*** A kitten who is unable to establish a stable breathing pattern and takes very few breaths. ***b)*** A kitten who has established a stable breathing pattern and is breathing rapidly.

commencement of imaging. These kittens were analysed separately from kittens with unaerated lungs and unstable breathing activity when imaging began, and were grouped in the **stable group** (fig.11b). Only 19 of the 36 kittens with a stable breathing pattern remained viable for reimaging during the 2nd sequence at ~ 1 hour after birth. This information is summarised in figure 12. Figure 13a shows a phase contrast X-ray image of a kitten with a closed glottis and epiglottis, while figure 13b shows a phase contrast X-ray image of a kitten at a later time with an open glottis and epiglottis.

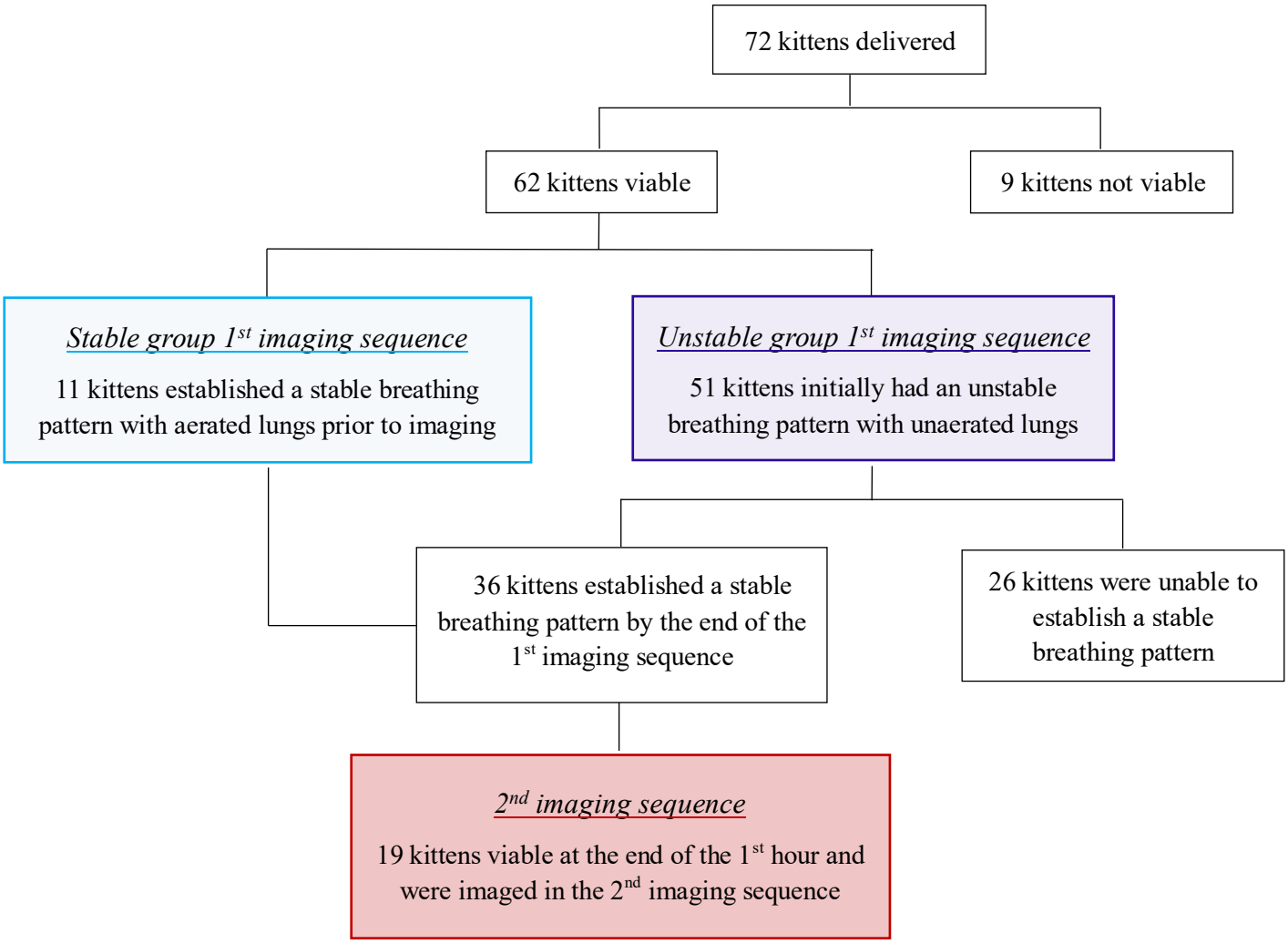

Fig.12 Tree diagram displaying the distribution of kittens in each subgroup.

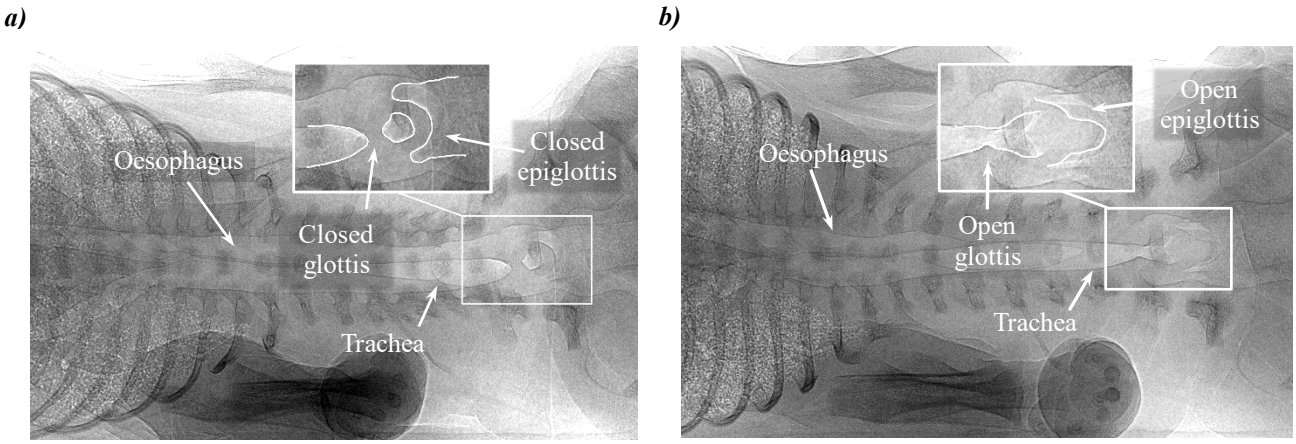

Fig.13 Phase contrast X-ray images of a preterm rabbit kitten receiving non-invasive ventilation after birth. ***a)*** The larynx is adducted, preventing air flowing into the lungs. ***b)*** The larynx is abducted and air can pass freely into the lungs, enabling successful delivery of non-invasive ventilation.

2.3.1 *Image analysis of glottic and epiglottic function:*

Glottis: In kittens with an unstable breathing pattern (n = 51), the glottis was open on average 25.5 ± 1.1% of the time during the first 12 minutes of newborn life (1st imaging sequence, range: 12.0 ± 3.5% to 36.1 ± 12.5%; fig.14a). In contrast, in the kittens with a stable breathing pattern during the 1st imaging sequence (n = 11), the glottis was open on average 76.8 ± 3.7% of the time (range: 36.7 ± 31.8% to 99.1 ± 0.1%; fig.14a). After the 1st hour (2nd imaging sequence), the glottis was open on average 90.5 ± 1.9% of the time (range: 80.3 ± 8.7 to 99.7 ± 0.3%, n = 19, fig.14c) and did not significantly vary throughout this imaging sequence. The glottis was open for a significantly greater percentage of the time during the 2nd imaging sequence compared to the 1st imaging sequence in the kittens with an unstable breathing pattern ($p < 0.0001$), but not in kittens with a stable breathing pattern.

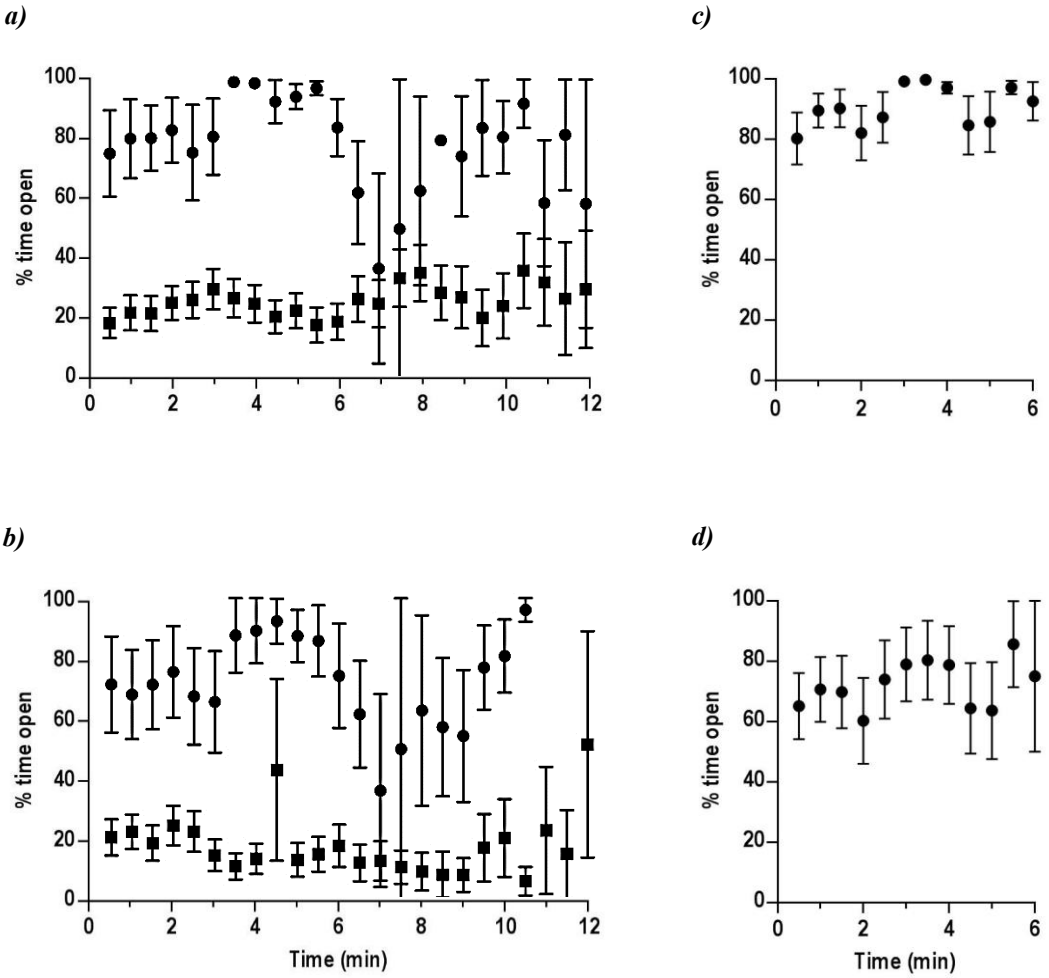

Fig.14 The percentage of time the ***a)*** glottis and ***b)*** epiglottis was open in kittens with stable (circle, n = 11) and unstable (square, n = 51) breathing patterns over the first 12 minutes of life. The percentage of time the ***c)*** glottis and the ***d)*** epiglottis was open after the first hour of life (n = 19). Kittens with a stable breathing pattern established a predominantly abducted glottis and epiglottis rapidly, while those with an unstable breathing pattern had a predominantly adducted glottis and epiglottis. After the first hour of life, the remaining kittens had a predominantly abducted glottis and epiglottis.

1

Epiglottis: During the first 12 minutes after birth in kittens with an unstable breathing pattern (1st imaging sequence n = 51; fig.11a), the epiglottis was open on average 17.1 ± 1.6% of the time (range: 6.2 ± 4.7% to 51.5 ± 27.5%; fig.14b). In contrast, immediately after birth in kittens who were able to establish a stable breathing pattern with aerated lungs (n = 11), the epiglottis was open on average 72.0 ± 3.4% of the time (range: 36.2 ± 31.8% to 96.1 ± 3.9%; fig.14b), again remaining above 70% for the majority of this time. After the 1st hour (2nd imaging sequence), the percentage of time the epiglottis was open significantly increased to an average of 72.3 ± 2.3% (range: 60.3 ± 14.3% to 85.7 ± 14.3%, n = 19; $p < 0.005$ fig.14d). There was no significant variation in the percentage of time the epiglottis was open within the second sequence.

2.3.2 Supplementary movies

Immediately after birth: The majority (51/62) of the kittens had unaerated distal airways (fig.13a) and unstable respiratory patterns (fig.11a). Breathing movements were infrequent and interspersed with apnoeic periods. During these apnoeic periods, the glottis was predominantly closed (Supplementary Movie 1), only opening briefly during spontaneous breaths. During a breath, the glottis and the epiglottis rapidly opened and the pharyngeal wall partially collapsed as air flowed into the lungs, indicating upper airway resistance impeded airflow past the pharynx despite the application of CPAP. Expiration was brief, and laryngeal generated expiratory breaking was absent. The glottis and the epiglottis remain primarily closed between breaths, with no notable changes in lung gas volume. Occasionally the glottis appeared to relax and open, independent of the epiglottis, which remained closed. During periods of apnoea the application of iPPV against a closed glottis and/or epiglottis resulted in distension of the pharynx. Pharyngeal distension appeared to increase proportionally with increasing pressure. In the absence of an oesophageal tube, increased iPPV pressures and the pressurization of the pharynx against a closed larynx caused large amounts of air to pass down the oesophagus into the stomach.

After ~ 1 hour: Following the development of a stable breathing pattern (fig.11b), the glottis and epiglottis remained predominantly open, allowing the kittens to breathe freely and for the successful delivery of iPPV in the majority of kittens. Similar to laryngeal activity in healthy breathing in neonates, the glottis and the epiglottis remained mostly open throughout the respiratory cycle (Supplementary Movie 2). Glottic dilation was enhanced during inspiration, and reduced slightly during expiration; likely due to the reduction in abductor muscle activity during expiration. Complete glottal closure was evident only during the occasional EBM, which were characterized by rapid expansion of the sub-glottic trachea, indicating pressurization of the lower airways (Supplementary Movie 2). In the absence of expiratory breaking, the pharynx periodically dilated during expiration. Expiratory pharyngeal distension was particularly evident at higher CPAP levels, suggesting the upper airways were relatively compliant.

2.3.3 The effect of continuous positive airway pressure level on spontaneous breathing activity

Sixteen kittens with a sufficiently stable respiratory pattern were examined to determine the effects of CPAP levels on spontaneous breathing activity. The majority of these kittens were tested during the second imaging sequence. Variable facemask leaks in several kittens precluded a systematic assessment of individual CPAP levels in some animals. The inhibitory effect of increasing CPAP levels was evident in all kittens, with many showing an almost complete cessation of spontaneous breathing activity (fig.15b). It was noted in kittens with a previously stable breathing pattern that CPAP levels greater than 7 cmH_2O depressed respiratory activity. As such, the effects of CPAP levels above 7 cmH_2O were compared with CPAP levels below 7 cmH_2O (fig.16). Increasing CPAP levels above 7 cmH_2O significantly reduced ($p < 0.001$) respiratory rates from 38.8 ± 3.2 breaths per minute (bpm, range: 11.4 - 80.0 bpm) to 12.6 ± 1.8 bpm (range: 1.3 - 47.7 bpm).

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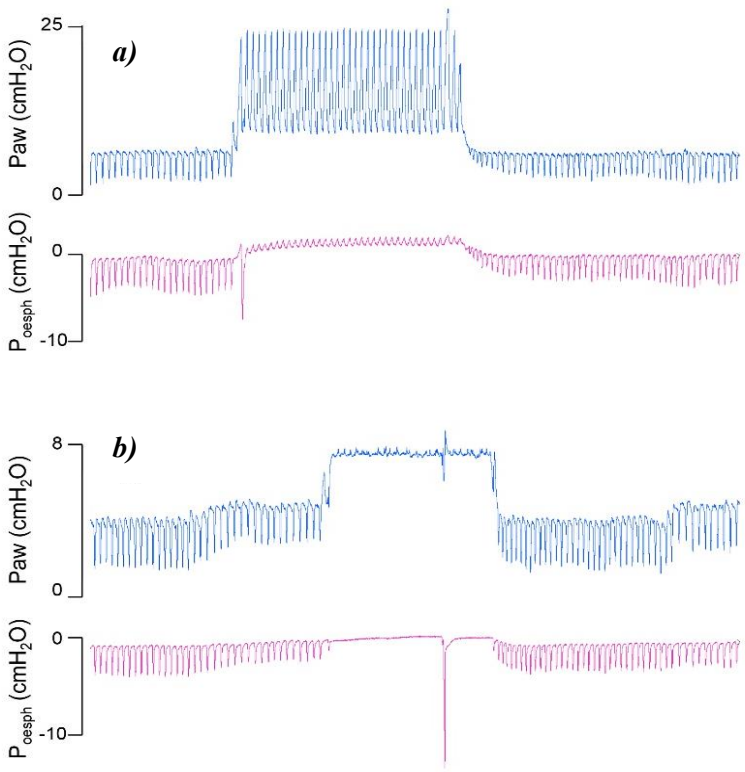

Fig.15 Physiological recordings of airway pressures (Paw) and oesophageal pressures (P_{oesph}) in 2 preterm rabbit kittens that initially had a stable spontaneous breathing pattern. ***a)*** A kitten received intermittent positive pressure ventilation (iPPV) using a peak inspiratory pressure of 25 cmH_2O and an end-expiratory pressure of 5 cmH_2O via a face mask. Non-invasive iPPV caused oesophageal pressure fluctuations, demonstrating transmission of ventilation pressure into the chest that resulted in lung inflations, as confirmed from the phase contrast X-ray images. ***b)*** The kitten received continuous positive airway pressure (CPAP) that when increased to 8 cmH_2O, caused an immediate suppression of spontaneous breathing activity that persisted throughout the elevated CPAP period; only one large deep inspiratory effort was observed. Although Paw increased with increased CPAP, oesophageal pressure did not increase, indicating that the pressure was not transmitted into the chest because the larynx was closed.

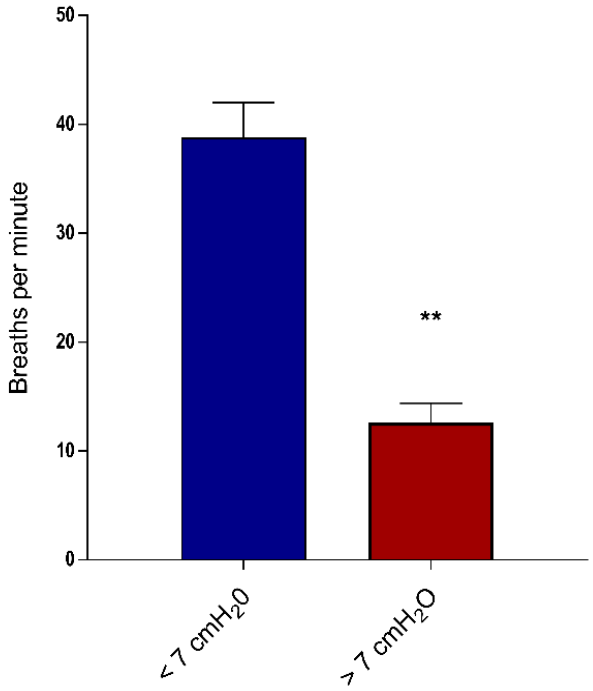

Fig.16 Average respiratory rate in kittens receiving a continuous positive airway pressure (CPAP) less than 7 cmH_2O (blue) and greater than 7 cmH_2O (red). The respiratory rate significantly decreases when the CPAP is increased above 7 cmH_2O ($p < 0.00$, $n = 16$).

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2 ### ***2.3.4*** *The effect of non-invasive intermittent positive pressure ventilation*

3 In kittens that were apnoeic or had unstable breathing patterns, non-invasive iPPV was unable to
4 ventilate the lung due to closure of the glottis and/or epiglottis. This prevented air from entering the
5 trachea, causing the pharynx to expand and deflate in phase with the ventilator, as evident by the periodic
6 pharyngeal expansion and deflation in phase with the ventilator (fig.17, Supplementary Movie 3).

7 In kittens with unaerated lungs and unstable breathing patterns immediately after birth, non-invasive
8 iPPV was unable to ventilate the lung in 16 of 17 attempts (94%; n=12 kittens); facemask leak precluded
9 assessment of iPPV in some kittens. In the one kitten successfully ventilated with non-invasive iPPV,

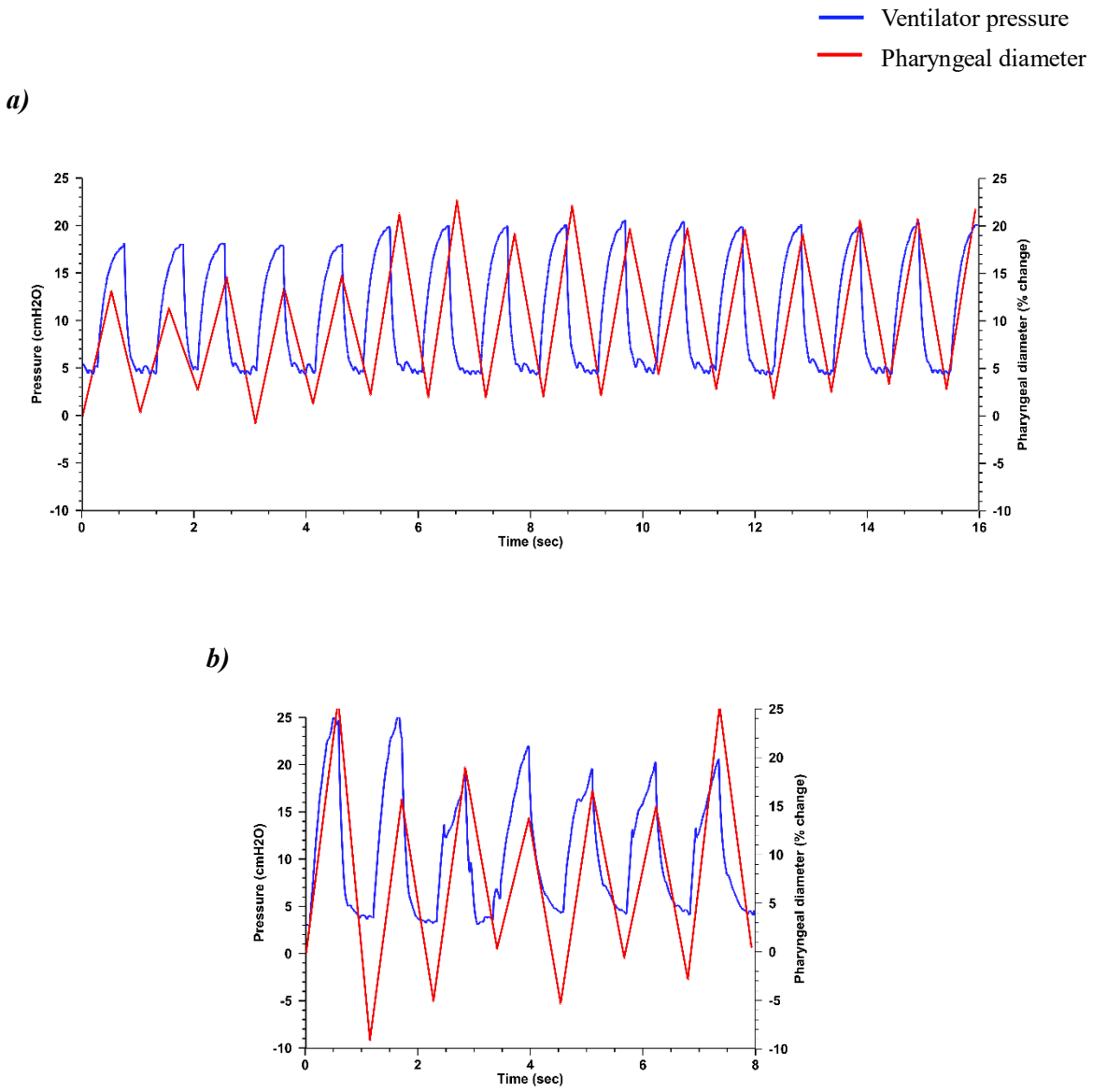


Fig.17 The change in pharyngeal area (red) compared to the change in ventilator pressure (blue) during non-invasive intermittent positive pressure ventilation in a preterm rabbit kitten. The change in pharyngeal area fluctuates in phase with changes in the airway pressure delivered from the ventilator when the larynx is ***a)*** closed and ***b)*** open. The pharynx dilates as much as 25% of initial pharyngeal area at peak inflation pressure.

1

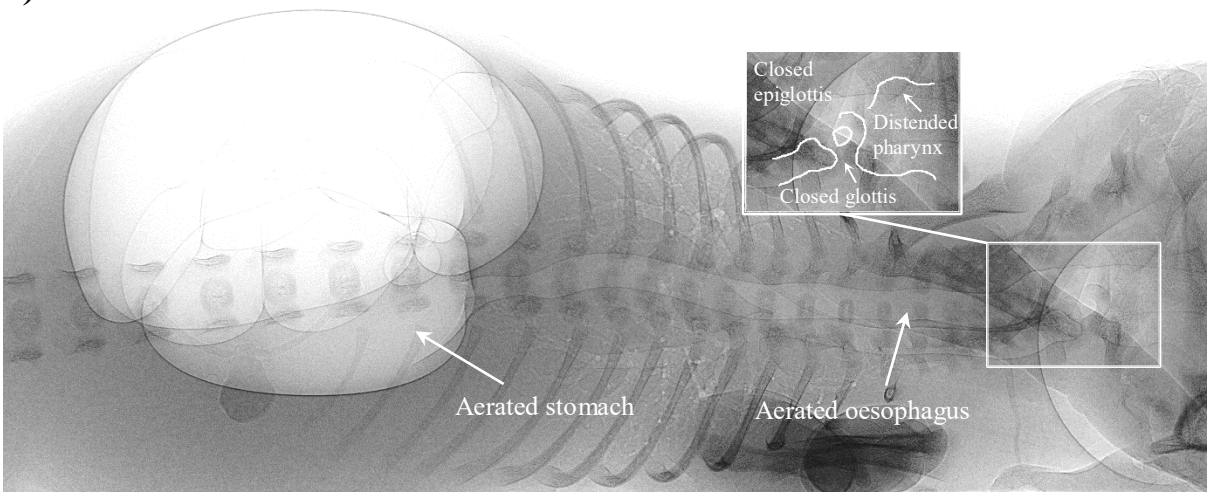
iPPV was initially unsuccessful but after 36 seconds, the kitten took 10 - 15 breaths in synchrony with the ventilator, which aerated the lungs. After this time, the glottis and epiglottis remained opened, allowing non-invasive iPPV to ventilate the lung.

In kittens with a stable respiratory pattern (fig.11b), non-invasive iPPV ventilated the lungs in 17 of 22 attempts (77%; n=14; Supplementary Movie 4). While most (79%) kittens became apnoeic during iPPV, allowing the ventilator alone to ventilate the lung, 21% continued spontaneous breathing in phase with the ventilator (fig.15a). In the four kittens that non-invasive iPPV could not ventilate the lung, spontaneous breathing continued but at a lower rate and the breaths were out of phase with the ventilator. In the four kittens that iPPV was unable to ventilate the lung, spontaneous breathing continued but at a lower rate and the breaths were out of phase with the ventilator.

Non-invasive iPPV caused periodic pharyngeal distension ad deflation in phase with the changing ventilator pressure (Supplementary Movie 3, fig.17). This periodic pharyngeal distension was evident regardless of whether the larynx was open (fig.17a) or closed (fig.17b). Figure 18b shows an image of a kitten at end-expiration superimposed with the negative image of the same kitten seconds later at peak pressure with a closed larynx. It is clear from this image that the pharynx distends to accommodate for the inspiratory flow delivered from the ventilator

Gas that was unable to pass into the lungs due to laryngeal closure was often forced into the digestive system in kittens not fitted with an oesophageal tube (Supplementary Movie 5 and fig.18a). The air in the kitten's stomach can be clearly seen in the phase contrast X-ray images. Kittens with severe gastric distension also tended to be apnoeic.

1 ***a)***



b)

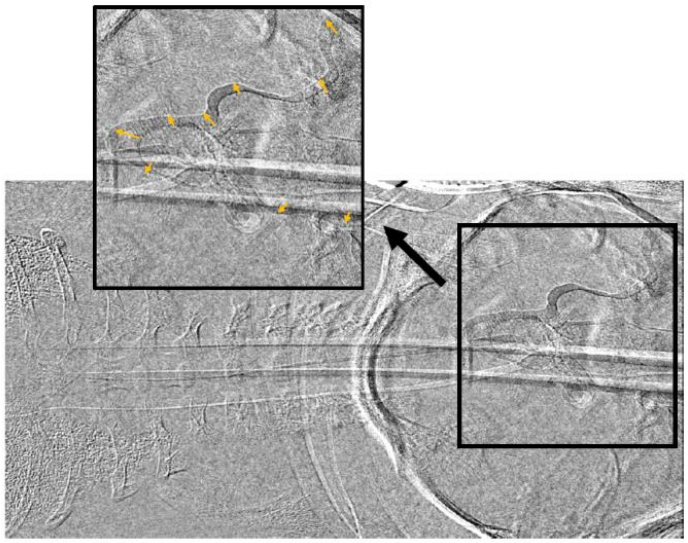


Fig.18a) A phase contrast X-ray image of a preterm rabbit kitten with an adducted larynx causing the gas to be diverted down the oesophagus into the digestive system, resulting in severe gastric distension. It was noted that the inflated stomach often hindered lung aeration, pressing on the diaphragm from below. Pharyngeal distension due to laryngeal adduction during intermittent positive pressure ventilation is also evident. ***b)*** A phase contrast X-ray image of a kitten receiving non-invasive positive pressure ventilation at peak inflation superimposed over a negative image seconds later at end expiration. The increase in pharyngeal area is evident by the dark shadow surrounding the pharynx and highlighted by the yellow arrows in the box.

2.4 Discussion

Although non-invasive ventilation is the preferred mode of respiratory support in the delivery room, it is unknown how non-invasive ventilation integrates with the newborns changing physiology at birth, or why it is often unable to provide sufficient respiratory support for preterm newborns in the delivery room. As a result, invasive intubation and mechanical ventilation is unavoidable for many preterm infants when non-invasive ventilation proves unsuccessful (4, 5, 78). The results in the current study in preterm rabbit kittens show that the glottis and the epiglottis were closed 74.5 ± 1.1% and 82.9 ± 1.6% of the time, respectively, over the first 12 minutes of life prior to the establishment of a stable breathing pattern (fig.14). Laryngeal adduction obstructed the upper airways, preventing CPAP or iPPV from successfully aerating the lungs unless the kitten took a spontaneous breath, thereby opening both its glottis and epiglottis. Due to the obstruction of the upper airways, gas delivered from the ventilator pressurised the pharynx and was diverted to the digestive system, resulting in severe gastric distension in the absence of an oesophageal tube (fig.18a). We propose that the cause of the high failure rate of non-invasive ventilation in the delivery room may be prolonged laryngeal adduction after birth, as seen in the current study

After a period of time (~ 1 hr), following the establishment of a stable breathing pattern, the glottis and the epiglottis were predominantly open (90.5 ± 1.9% and 72.3 ± 2.3% of the time, respectively, fig.14). With a predominately open larynx kittens were able to breathe freely, and non-invasive ventilation successfully aerated and ventilated the lungs. Following the establishment of a stable breathing pattern, iPPV successfully ventilated the lungs in 77% of kittens. Of these kittens, only 21% continued to breathe in synchrony with the ventilator, while the other 79% of these kittens showed a suppression of breathing movements with an open larynx during iPPV. The remaining 23% of kittens, in whom iPPV was unsuccessful, spontaneous breathing movements persisted, however out of synchrony with the ventilator

with an adducted larynx between breaths. This effect on spontaneous breathing has been observed in preterm human infants immediately after birth (10). To the best of our knowledge, this is the first study to directly visualize and examine laryngeal function during the transition to air breathing at birth.

At birth, the premature newborn is very similar to a fetus, and as such, it is important to consider fetal laryngeal function to guide understanding of laryngeal function at birth. Laryngeal adduction during periods of fetal apnoea is sustained by tonic activity of the thyroarytenoid (18). Prolonged periods of fetal apnoea are interrupted by episodes of FBM, when tonic activity of the thyroarytenoid is inhibited and the laryngeal resistance is reduced (31). *In utero* fetal breathing movements are supressed by hypoxia, resulting in prolonged laryngeal adduction (21). This response to hypoxia intensifies with increasing gestational age and persists for several weeks after birth (38, 120, 121). The hypoxia-induced suppression of FBM is in contrast to the increased respiratory drive in adults, and results from a direct inhibitory input into the respiratory centres of the medulla (122).

As preterm newborns are essentially exteriorized fetuses at birth, it is not too surprising that the majority of the apnoeic preterm kittens had an adducted larynx, rendering non-invasive ventilation ineffective. Furthermore, as hypoxia inhibits FBM and causes glottic adduction, it is likely that progressive hypoxia at birth supresses continuous breathing efforts, which may result in prolonged laryngeal adduction, thus obstructing the effective delivery of non-invasive iPPV, as seen in the current study (121). Although it is not yet known if laryngeal adduction at birth in preterm rabbit kittens directly translates to human preterm newborns, it is well established in fetal humans, fetal rabbits and fetal sheep that hypoxia supresses FBM and induces bradycardia, and as such we expect the biology to be similar for the human newborn (121, 123). Thus, we suggest apnoea-induced laryngeal adduction may be a major cause of airway obstruction in preterm infants at birth, preventing the effective delivery of non-invasive ventilation.

After the kittens were able to establish a stable respiratory pattern, the glottis and epiglottis were predominantly open, thus enabling the successful delivery of iPPV in most kittens, but not all. Both laryngeal and respiratory activity are controlled by the respiratory centre in the medulla. The establishment of a stable breathing pattern and laryngeal abduction appeared to be intimately related, as such we suggest that the best way to establish a predominantly open larynx at birth is to establish a stable breathing pattern. We found that if preterm kittens could establish a stable respiratory pattern soon after birth, the glottis and epiglottis were predominantly open, and iPPV was usually able to either augment spontaneous breaths or directly ventilate the lung. As the majority (~ 80%) of preterm newborns display respiratory efforts immediately after birth, one would expect non-invasive ventilation to have a greater success rate than is reported. However, the successful application of non-invasive ventilation (as seen in this study) requires the establishment of a stable breathing pattern, and thus a predominately open larynx (124). The majority (73%) of kittens had a great degree of variation in their respiratory rate (between 1 and 20 bpm). In these kittens, breathing episodes were interspersed with periods of apnoea, during which the larynx was closed and non-invasive ventilation was unable to aerate the lungs. Consequentially, these kittens became increasingly apnoeic and bradycardic and would have required intubation and ventilation if they were not terminated.

These findings indicate that establishing a stable respiratory pattern after birth is the greatest priority in assisting very preterm infants to transition to newborn life when using non-invasive iPPV. Further studies are needed to examine what effect stimulating a stable breathing pattern has on laryngeal opening, and thus the success of non-invasive ventilation. The establishment of a stable breathing pattern may be achieved in several ways including respiratory support with a high oxygen concentration, and caffeine. In this study it was noted that many kittens had poor respiratory activity and were inactive. Although efforts were made to reduce the maternal anaesthetic use to only that was necessary to sedate the pregnant doe, it is of concern that exposure to maternal anaesthetic may have depressed the kitten's respiratory

rate. It was initially suggested that as the maternal anaesthetics are muscle relaxants, that if maternal anaesthetics had an effect on the kittens, it would cause relaxation of the laryngeal muscles, resulting in passive laryngeal abduction, however this was not seen. The maternal anaesthetic used in this study, Propofol and isoflurane, are both known to rapidly cross the placenta and inhibit respiratory drive. As laryngeal abduction appears to be intimately related to respiratory drive, it is possible that the maternal anaesthetics depressed the kitten's respiratory drive, and therefore promoted prolonged laryngeal adduction. As such future studies examining the larynx in preterm newborns should explore ways to further minimise the kittens exposure to maternal anaesthetics.

As the upper airways of the fetus and newborn are highly compliant, the large pressure gradients generated by non-invasive iPPV or a spontaneous breath can cause major distension of the upper airway walls (125-128). Upper airway distension was evident in the current study, particularly during iPPV, where the pharyngeal wall distended in phase with the inspiratory pressure delivered by the ventilator. Inspiratory gas was unable to pass to the lower airways, and therefore accumulated in the upper airways, resulting in pharyngeal expansion (Supplementary Movie 3, fig.17 & fig.18b). This was observed irrespective of whether the larynx was open or closed; however, it was more evident in kittens with a closed larynx. It is thought that CPAP splints open the upper airways, preventing pharyngeal collapse during inspiration, and thus reducing the effort of breathing. In this study, we have confirmed that CPAP distends the pharynx, providing a reservoir for low resistance gas flow into the lungs during inspiration. This gas reservoir in the upper airways may possibly introduce a small error into tidal volume measurements in clinical and experimental settings during non-invasive iPPV. Inspiratory volumes delivered to an apnoeic newborn that appear to successfully inflate the lungs may simply be inflating the upper airways as the newborn becomes increasingly apnoeic. This is consistent with findings in infants and lambs showing tidal volumes measured during iPPV prior to intubation are significantly greater than tidal volumes measured following intubation using the same PIP value (129). However, clinically, when

delivering inspiratory volumes to an apnoeic newborn, tidal volume measurements are not considered in isolation, and additional physiological responses are monitored, such as the rise of the chest wall and an increase in heart rate. The potential damage caused by overexpansion of the pharynx and sinuses at birth has not been investigated. The degree of airway distension is dependent on airway compliance, which decreases with increasing gestational and postnatal age, as such we expect pharyngeal distension to be most notable in preterm newborns at birth (125-128).

Research suggests non-invasive ventilation stimulates active inspiratory laryngeal narrowing in both human adults and neonatal lambs (16, 90). Studies in neonatal lambs show non-invasive ventilation triggers inspiratory laryngeal narrowing by stimulating inspiratory thyroarytenoid activity and inhibiting inspiratory cricothyroid activity (15, 85). In the current study, we noted that when ventilator pressures greater than 7 cmH_2O were delivered, the larynx closed and the kitten became apnoeic (fig.15b). In kittens with a stable breathing pattern, the application of higher levels of CPAP (> 7 cmH_2O) significantly reduced respiratory rates from 38.8 ± 3.2 bpm to 12.6 ± 1.8 bpm ($p < 0.001$, fig.16). When the higher CPAP levels were removed, breathing resumed and the larynx opened (fig.15b). It is likely that the elevated pressures increased the occurrence of laryngeal adduction and apnoea, however, this study was not designed to analyse the pathways responsible for this reflex, or the strength of the relationship between ventilator pressure and laryngeal adduction. Furthermore, variable facemask leak prevented the systematic assessment of the effects of CPAP level on respiratory activity. As such, the effect of CPAP levels on breathing activity, and the pathways responsible for a possible relationship would be an interesting question for future studies. One could speculate that prior to laryngeal adduction, the increased pressure stimulated the bronchopulmonary receptors to initiate the apnoeic response with prolonged laryngeal adduction, trapping the pressure within the lungs. It is also possible that the facemask and the applied ventilator pressure on the kitten's face stimulated the trigeminal nerves, which is known to inhibit breathing. Observations from the delivery room suggest that application of the

facemask and the positive pressure on the newborn's face may contribute to the development of apnoea, however there is a lack of research in this area and further studies need to be conducted to isolate and examine the effect of pressure on the newborn's face.

2.5 Conclusion

Preterm rabbit kittens with an unstable breathing pattern experienced prolonged laryngeal adduction, with a predominately closed glottis and epiglottis, opening only briefly during a breath. Laryngeal function was very similar to that of the fetus and was predominately closed during apnoea, rendering non-invasive ventilation ineffective at aerating and ventilating the lungs. Kittens with a stable breathing pattern, whether immediately after birth, or after the first hour, had a predominately open glottis and epiglottis, allowing for iPPV to be successfully applied non-invasively. Based on the results presented in this study, we propose that laryngeal adduction immediately after birth hinders the ability of non-invasive ventilation to aerate and ventilate the lungs unless the newborn takes spontaneous breaths, which is consistent with observations in human newborns. These findings highlight the importance of establishing a stable respiratory pattern at birth for the effective delivery of non-invasive ventilation.

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Chapter 3

2nd Study: Understanding the effect of oxygenation on laryngeal function at birth to improve non-invasive ventilation

3.1 Introduction

In recent years there has been a transition from invasive to non-invasive respiratory support at birth; however, the success of non-invasive respiratory support in the delivery room is much less than anticipated in very and extremely premature newborns (4). In our previous study, we demonstrated that the larynx was predominately closed, hindering the delivery of non-invasive ventilation in apnoeic preterm kittens at birth. Non-invasive ventilation was only successful in the kittens who were able to develop a stable breathing pattern with a predominately open larynx and aerated lungs. From the previous study, it was evident that laryngeal opening was intimately related to spontaneous breathing efforts. With this in mind, we propose that stimulating a stable breathing pattern will promote prolonged laryngeal

abduction, allowing gas to enter the lungs and for the successful delivery of non-invasive ventilation. Alternatively, factors known to inhibit respiratory drive may result in prolonged laryngeal adduction, thus further hindering non-invasive ventilation.

The newborn response to hypoxia is analogous to that of the fetus. At birth hypoxia inhibits respiratory drive, resulting in prolonged and life threatening apnoea (64, 65). Failure to initiate continuous breathing at birth can result in severe hypoxia, creating a negative feedback loop, and further prolonging apnoea. At birth, it is critically important to avoid hypoxia. In addition to respiratory inhibition, inadequate oxygenation is associated with adverse neurodevelopmental outcomes, long term cardio-respiratory instability, injury to the pulmonary vasculature, and an increased risk of mortality, amongst other negative ramifications (130-132). Apnoeic newborns are often provided respiratory support with greater concentrations of oxygen (> 30%) to avoid hypoxia, and the resulting bradycardia (133). Although it is critically important to avoid hypoxia at birth, it is also important to take care when ventilating with high concentrations of oxygen, as this can also have adverse health consequences (134, 135). Ventilation with supplemental oxygen is thought to stimulate respiratory drive and help prevent hypoxia, which may be related to prolonged laryngeal abduction (64). As such, it is of interest to examine if stimulating a stable breathing pattern in preterm newborn rabbit kittens using high concentrations of supplemental oxygen will result in prolonged laryngeal abduction. Furthermore, it is important to determine if respiratory support with low concentrations of oxygen will induce apnoea, resulting in prolonged laryngeal adduction.

In the current study, using phase contrast X-ray imaging we have examined laryngeal and respiratory activity immediately after birth during CPAP with air, followed by 100% oxygen, 100% nitrogen and again with 100% oxygen. We again used a preterm rabbit model and imaged the airways using phase contrast imaging during their first minutes after delivery via caesarean section. The aim of the present study was to determine if stimulating a stable breathing pattern using a high inspiratory oxygen

concertation would trigger the glottis to transition to a predominately open state at birth in preterm rabbit kittens. We hypothesised that administering 100% oxygen to the kittens would make them increasingly oxygenated, resulting in a more stable and increased respiratory rate, and thus inducing prolonged laryngeal abduction. We also hypothesised that during the administration of 100% nitrogen the kittens would become hypoxic, resulting in respiratory depression, apnoea, and prolonged laryngeal adduction, preventing the successful administration of CPAP.

3.2 Methods

3.2.1 *Experimental procedure*

All experimental procedures were approved by SPring-8 Animal Care and Monash University's School of Biomedical Science's Animal Ethics Committees. Experiments were conducted in experimental hutch 3 of beamline 20B2 of the Biomedical Imaging Centre at the SPring-8 synchrotron.

Pregnant New Zealand white rabbits (n = 11) were housed and prepared as detailed above in the methods section of the first study (Section 2.2.1). As detailed previously, the doe was initially sedated using a propofol bolus (8 mg/kg) delivered via an ear-vein catheter. To minimise the kittens' exposure to maternally administered anaesthetics prior to delivery, does in the current study were sedated with a short acting anaesthetic allowing for the administration of an epidural anaesthetic, as described below. The doe was placed prone, and a small section above the spine was shaven. A facemask was secured to the doe's face to deliver 90% oxygen. After the doe was adequately sedated (as assessed by the loss of blink and pedal reflexes) a 22 GA epidural catheter was inserted into the epidural space on the lower spine to administer 2% lignocaine (4 mg/kg) and 0.5% bupivacaine. Throughout the experiment, analgesia and sedation was maintained with an intravenous infusion of butorphanol (0.5 mg/kg/hr) and midazolam

(1.0 mg/kg/hr) via the ear vein

The kittens were individually exteriorised by caesarean section, as previously described (Section 2.2.1). To measure the kitten's intra-thoracic pressure, a thin tube was passed down the oesophagus, with the tip located within the mid-thoracic region. A custom-made flexible rubber facemask was applied over the kitten's face and secured using tissue glue. The umbilical cord was ligated and severed before the kittens received an intra-peritoneal injection of naloxone (2 mg/kg) to counteract the effects of butorphanol. The kitten was taken into the imaging hutch and placed laterally on a heated platform in the path of the X-ray beam, and electrocardiography leads were attached to monitor heart rate throughout imaging. The facemask was connected to a custom-built mechanical ventilator and CPAP was delivered at the commencement of imaging.

3.2.2 *Ventilation*

The gas concentration delivered was adjusted throughout the experiment via an oxygen and nitrogen blender attached to the ventilator, and delivered to the kitten via the facemask. Figure 19 describes the ventilation sequence to which each kitten was exposed. Throughout these experiments the kittens remained on a CPAP of 5 cmH_2O except during brief periods of iPPV. During the 1st imaging sequence, which lasted approximately three minutes the inspired gas was air. The kittens then received 100% oxygen until the kitten was well oxygenated and had established a stable breathing pattern. After the kittens were breathing freely, the inspired gas was switched to 100% nitrogen until they became hypoxic and apnoeic. At this point, the kittens received physical stimulation and the inspired gas was returned to 100% oxygen (2nd oxygen) to facilitate re-oxygenation and the re-establishment of a stable breathing pattern. The time required for oxygen and nitrogen to stimulate and depress breathing, respectively, differed greatly between kittens, and as such, the laryngeal opening times and respiratory rate were calculated from the final time point in each ventilation sequence.

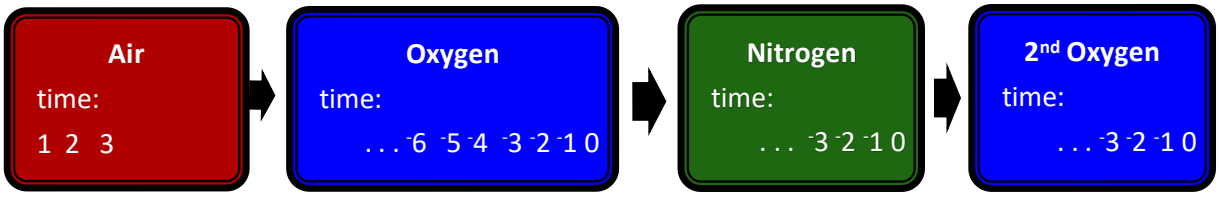

Fig.19 Non-invasive ventilation schedule for each kitten. Kittens were provided CPAP with air for 3 minutes. The gas was then switched to 100% oxygen. After breathing became stable, the gas was switched to 100% nitrogen until the kittens were apnoeic. After the kitten was apnoeic, the gas was returned to 100% oxygen until the kitten had re-established a stable breathing pattern. As the time required to establish a stable breathing pattern or to cease breathing varied considerably between kittens, the common time point was considered to be the final time point (0). Earlier time points are counted with respect to the final time point and are denoted as approaching the final time point from below (-).

3.2.3 *Phase contrast X-ray imaging*

Phase contrast X-ray images were collected as previously described (Section 2.2.2). Briefly, kittens were placed laterally on a heated pad ~ 210 m downstream of the high-power X-ray source and imaging commenced with simultaneous CPAP (fig.9b). Images were collected by a detector (ORCA Flash 4.0 Hamamatsu C11440-22C, effective pixel size 15.3 μm^2, 2048 × 2048 pixels) ~ 2 m from the kitten at 20 Hz with an exposure time of 40 ms. At the conclusion of the imaging period, each kitten was humanely killed by an intraperitoneal injection of sodium pentobarbitone (25 mg). The doe was humanely killed by an intravenous injection of sodium pentobarbitone (150 mg) via the ear vein catheter at the completion of the experiment.

3.2.4 *Image analysis*

Phase contrast X-ray images were visually analysed to determine if the glottis was open or closed, as previously described (Section 2.2.3). This data is expressed as the percentage of time open over 30 second intervals. As the time taken for each kitten to achieve a stable breathing pattern (oxygen) or show significant respiratory depression (nitrogen) varied greatly between kittens, the final time point for each sequence was used as the common time point. The time open over 30 seconds has been averaged from the end of the sequence towards the beginning. The respiratory rate was determined from the oesophageal pressure recordings.

3.2.5 *Statistical analysis*

Data was compared for significance over time using a one-way repeated measures ANOVA with a Tukey multiple comparison. P-values < 0.05 were considered statistically significant.

3.3 Results

Twenty-two premature rabbit kittens were delivered by caesarean section from 11 does at 29 d GA and were administered CPAP with air, oxygen, nitrogen, and again oxygen with simultaneous phase contrast X-ray imaging. There were 15, 22, 15 and 11 kittens in the air, oxygen, nitrogen and 2nd oxygen sequences, respectively. Immediately after birth, 15 kittens received CPAP with air, whereas seven kittens were rapidly switched to oxygen due to bradycardia and respiratory depression. Seven kittens did not receive a nitrogen sequence, because they did not reach a stable breathing pattern despite inspiring oxygen. Four of the remaining 15 kittens could not be resuscitated into a stable breathing pattern following the nitrogen period. The percentage of time the glottis was open during each gas sequence

could be determined from the phase contrast X-ray images, as reported below. Unfortunately, the epiglottis could not be clearly distinguished, and as such, epiglottic abduction could not be accurately determined. Spontaneous breaths were clearly distinguishable by a sharp downwards inflection in the oesophageal pressure recordings, thus providing a measurement for the breathing rates during each gas sequence. For the oxygen and nitrogen sequences, the common time point between all kittens in each sequence was assumed to be the time point at kittens had established a stable breathing pattern, or become apnoeic. This time point in the oxygen and nitrogen sequences is denoted as 0, with earlier time points counted backwards from this (-).

3.3.1 Laryngeal opening and respiratory rates

Air: Of the fifteen kittens receiving air immediately after birth, the average respiratory rate during the first 3 minutes was 9.4 ± 0.2 bpm (range: 8.7 ± 2.6 - 10.1 ± 3.2 bpm) and did not significantly increase (fig.20a). Over the first 30 seconds of air breathing the glottis was open 22.2 ± 9.7% of the time, which increased to 60.2 ± 24.4% after only 3 minutes (fig.20b). A large degree of individual variability was noted in the kittens during air breathing, with some kittens maintaining a predominately open glottis for extended periods, while other kittens struggled to open their glottis and aerate their lungs.

Oxygen: Twenty-two kittens received CPAP with oxygen. The 2nd panel in Figures 20a and 20b demonstrate the effect of oxygenation on laryngeal and respiratory function, respectively, in preterm rabbit kittens. The respiratory rate and glottal opening increased with the application of oxygen, although this was, for the most part, not significant. The respiratory rate at - 6 minutes was 11.7 ± 4.3 bpm, which increased to 22.7 ± 4.7 bpm by the completion of this period. Although the kittens were independently breathing by the completion of oxygen, this increase in respiratory rate was not significant. At -6 minutes the glottis was open 52.2 ± 16.0% of the time, and increased to 75.2 ± 7.3% of the time, enabling the unimpeded delivery of CPAP. Again, this change was not significant to any other time point with any

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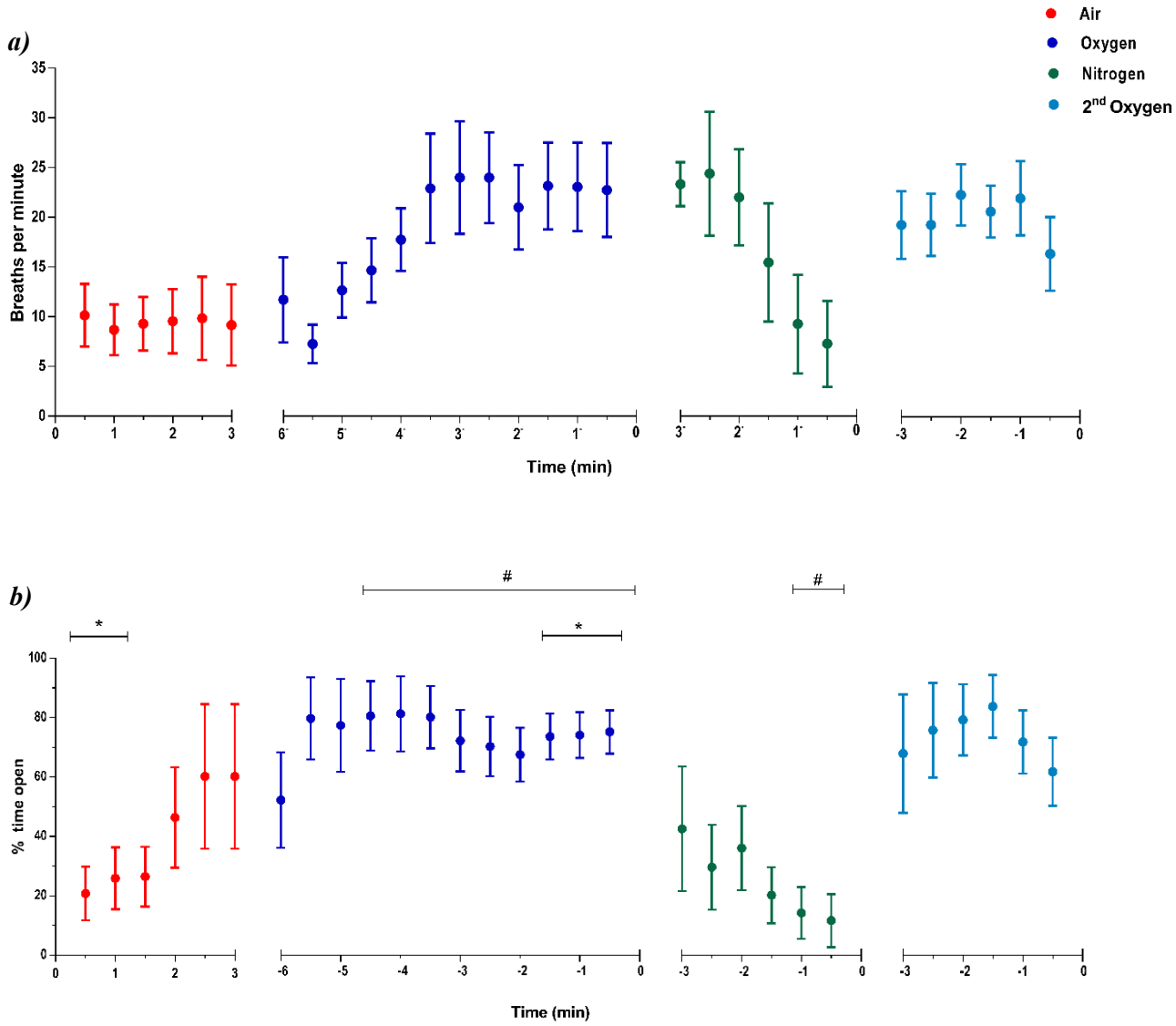

Fig.20 a) Respiratory rate and ***b)*** percentage of time the glottis was open during continuous positive airway pressure (CPAP) with air (n = 15), 100% oxygen (n = 22), 100% nitrogen (n = 15) and again with 100% oxygen (n = 11). ***a)*** The respiratory rate increased during breathing with 100% oxygen, and decreased during breathing with 100% nitrogen, however this was not significant. ***b)*** The glottis was open increasingly often during air, and was open for the greatest proportion of the time during oxygen. Glottic opening time fell during nitrogen and increased again when the inspired gas was switched back to100% oxygen. * denotes a significant difference between air [0.5 - 1 minute] and oxygen [-1.5 - -0.5 minutes]. # denotes a significant difference between oxygen [-4.5 - -0.5 minutes] and nitrogen [-1 - -0.5 minute].

other gas. The variability in the respiratory rate and the proportion of time the glottis was open decreased slightly towards the end of oxygen breathing compared to breathing with air (fig.20). The glottis was open for a significantly greater percentage of the time during the final 90 seconds of oxygen compared to the first 60 seconds of air ($p < 0.05$); however, there was no significant difference between the end of the air sequence and any time during the oxygen sequence.

Nitrogen: Following the establishment of a stable breathing pattern, 11 kittens received nitrogen inhalation until the kittens became apnoeic. The 3rd panel in Figures 20a and 20b demonstrates the effect of progressive hypoxia on the respiratory rate and glottic opening time during nitrogen breathing, respectively. During nitrogen breathing the respiratory rate and glottic opening decreased to less than that seen during air or oxygen breathing. At - 6 minutes the respiratory rate was 23.3 ± 2.2 bpm, which fell to 7.3 ± 4.3 bpm by the completion of the nitrogen sequence. The glottis was open 37.1 ± 31.1% of the time at - 3 minutes of nitrogen breathing. By the completion of nitrogen breathing, the percentage of time the glottis was open had fallen to 11.7 ± 9.0%. Although this decrease was not significant, the increased laryngeal adduction hindered the successful delivery of iPPV. During the final minute of nitrogen breathing, the glottis was open significantly less than during the final 4.5 minutes of oxygen breathing ($p < 0.05$).

2nd Oxygen: Following nitrogen administration, 11 kittens received a 2nd oxygen sequence. The respiratory rate and percentage of time the glottis was open during re-oxygenation is shown in the rightmost panel of Figures 20a and 20b, respectively. When returned to oxygen, the respiratory rate recovered, increasing to 16.4 ± 3.7 bpm; although this increase was not significant. The average respiratory rate and percentage of time the glottis was open during the 2nd oxygen sequence was 20.0 ± .09 bpm and 73.4 ± 3.2%, respectively. This is similar to the average respiratory rate and glottic opening time seen during the 1st oxygen sequence. The glottis was open 62.8 ± 11.5% of the time by the completion of the 2nd oxygen sequence, enabling the kittens to breathe freely.

3.3.2 *Supplementary movies*

Air: Fifteen kittens received air immediately after birth. Initially their lungs were liquid filled, respiratory efforts were infrequent and the glottis was predominately closed (Supplementary Movie 6). The kittens took occasional spontaneous breaths, allowing for air to enter the lungs, and forcing lung liquid into the interstitial fluid. Surprisingly, the proportion of time the glottis was open increased rapidly. The lungs began to aerate, and clear of liquid. The kittens appeared to be successfully making the transition to air breathing with an open glottis (Supplementary Movie 7).

Oxygen: Kittens slowly became increasingly active during oxygen as they became increasingly oxygenated. The respiratory rate increased and the glottis was predominately open. Continuous positive airway pressure successfully aerated the lungs and likely assisted the newborn's respiratory efforts by reducing the effort of respiration. During 100% oxygen, it appeared that the glottis had transitioned to a predominately open newborn state, as seen in Supplementary Movie 8.

Nitrogen: Respiratory efforts slowly dwindled during 100% nitrogen (Supplementary Movie 9). The kittens slowly stopped breathing, as they became increasingly hypoxic. Although the lungs were still well aerated, the glottis became predominately adducted, indicating that the loss of respiratory drive and hypoxia may have induced prolonged laryngeal adduction. During nitrogen, laryngeal activity appeared to revert back to activity more similar to that of the apnoeic fetus, opposed to that seen in healthy newborns. Owing to the increased laryngeal adduction, iPPV was ineffective. The time taken for kittens to become apnoeic varied greatly between kittens. Many kittens appeared to briefly struggle against the facemask during nitrogen respiratory support, before becoming flaccid (Supplementary Movie 9).

2nd Oxygen: When the inspired gas was returned to 100% oxygen, the larynx returned to a predominately open state and the breathing rate slowly increased. With an open larynx, the kittens were able to breathe freely and CPAP successfully assisted breathing efforts (Supplementary movie 10).

3.4 Discussion

Non-invasive respiratory is the preferred form of respiratory support for preterm newborns at birth, however, this form of respiratory support has an unexplained high failure rate. In the current study, we sort to examine the relationship between a stable breathing pattern at birth and the patency of the larynx as it transitions from the predominately closed, to a predominately open state. We chose to alter the oxygen content of the inspired gas to induce either a stable or an unstable breathing pattern. We hypothesised that the kittens would establish a stable breathing pattern with an increased respiratory rate as they became increasingly oxygenated, leading to prolonged laryngeal abduction. We also hypothesized that breathing 100% nitrogen would cause the kittens to become increasingly hypoxic, resulting in respiratory depression, apnoea, and prolonged laryngeal adduction.

During the initial air breathing period, preterm kittens were unable to adequately aerate their lungs and required much physical stimulation to maintain breathing activity. As such, they were unable to increase their respiratory drive, with an average respiratory rate of only 9.4 ± 0.2 bpm, which did not significantly change throughout the sequence (fig.20a). Nonetheless the glottal opening time increased to 60.2 ± 24.4% after only 3 minutes (fig.20b), allowing air to pass into the lungs, despite the low breathing rate. This result appears to contradict the results of the 1st study, where the glottis was not open for greater than 40% over the first 12 minutes in kittens unable to establish a stable breathing pattern (fig.14a). It is possible this difference between the two studies is the consequence of the differing sedatives administered to the does in each study. Furthermore, it is also possible that the stimulation delivered to the kittens prior to the air sequence may have contributed to the differences in glottal opening times between the two studies; however, the amount of stimulation delivered in each study as not recorded and is difficult to quantify retrospectively. Regardless of the tactile stimulation delivered, when in air, the kittens breathing patterns were judged to be unsustainable, and as such, if the switch to oxygen was

delayed, it is also possible that the kittens would have become more hypoxic resulting in more apnoeas and reduced laryngeal opening times.

When breathing oxygen, kittens markedly increased their breathing rate and were able to establish a stable and sustainable breathing pattern as they became increasingly oxygenated. In association with the development of a stable breathing pattern, glottal opening increased to 75.2 ± 7.3%, allowing the kittens to breathe freely. This increased respiratory rate during oxygen confirms previous studies, where a brief period of high supplementary oxygen stimulated respiratory rate and vigour, as PaO_2 increased (136).

Following the switch to nitrogen, which was used to induce hypoxia, the kittens became progressively apnoeic. The respiratory rate during nitrogen inspiration markedly reduced and fell to 7.3 ± 4.3 bpm before ceasing altogether, at which point imaging ceased so that they could be switched back into oxygen (fig.20a). The fall in respiratory rate was accompanied by laryngeal closure, which was only open 11.7 ± 9.0% of the time by the completion of nitrogen, preventing the successful administration of iPPV (fig.20b). Although the lungs appeared to remain aerated with gas trapped within, the larynx was adducted for an increasing proportion of the time, suggesting that hypoxic respiratory inhibition and not lung collapse, was a critical factor prompting laryngeal adduction. *In utero,* fetal hypoxia inhibits FBM, resulting in prolonged fetal apnoea with sustained laryngeal adduction. Tonic thyroarytenoid activity is observed throughout 90% of fetal apnoeas (137). The hypoxic response at birth is very similar to the fetal response to hypoxia. Hypoxia depresses respiratory drive in premature newborns, resulting in a progressively worsening apnoea, as confirmed in the current study. We suggest that the similarities between hypoxia *in utero* and at birth did not simply end with respiratory depression, but may also include prolonged laryngeal adduction.

In this study, we were unable to determine if laryngeal adduction is active or passive during hypoxia. However, in light of the similarities between the fetal and preterm response to hypoxia, it seems likely

that hypoxia induced laryngeal adduction in the newborn is active. Studies in neonatal lambs have shown that hypoxia induces active expiratory glottal adduction (138, 139). However, conflicting results were reported in a later study by Praud *et al* (1995) showing that hypoxia increased inspiratory glottal abduction (140). These studies were conducted on neonatal lambs, and further studies are required to understand the electrophysiology of laryngeal adduction during hypoxia in preterm newborns. It is also possible that prolonged laryngeal adduction serves an important means of preserving lung volume, preventing lung collapse and preventing lung liquid re-flooding the airways.

It is difficult to determine the role, if any, of lung aeration on the establishment of a stable breathing pattern and prolonged laryngeal abduction. The lungs were well aerated during nitrogen when the kittens were apnoeic with an adducted glottis, suggesting lung aeration had very little influence over the respiratory rate and laryngeal opening. However, it is possible that the respiratory inhibition experienced during hypoxia over powered any possible stimulatory effects of lung aeration, thus masking the effects of lung aeration on laryngeal abduction. To understand if lung aeration had a contributing effect on laryngeal abduction at birth, future studies could examine the effect of ventilating with air, opposed to nitrogen, following oxygen ventilation. The beneficial effects of lung aeration on improved oxygenation cannot be overlooked. Experimental studies have shown that lung aeration triggers the fall in pulmonary vascular resistance at birth, with an additional additive effect of oxygenation (116, 141-143). This fall in pulmonary vascular resistance is very important to increase pulmonary blood flow, and thus facilitate the transfer of oxygen across the blood gas barrier and increase PaO_2.

After the kittens had stopped breathing in response to the 100% nitrogen, the kittens received vigorous physical stimulation in addition to 100% oxygen in an attempt to restore a sustainable and stable breathing pattern. This was not always successful and four kittens we discontinued imaging due to an inability to restore a sustainable respiratory pattern. While not significantly different, the respiratory rate increased to 16.4 ± 3.7 bmp by the completion of the 2nd oxygen sequence, which was similar to the

respiratory rate achieved during the 1st oxygen sequence. The glottis returned to a predominately open state, open 62.8 ± 11.5% of the time by the completion of oxygen. The loss of a stable breathing pattern with sustained glottic adduction during hypoxia, and the subsequent return of a stable breathing pattern with an abducted glottis following re-oxygenation further supports the strong relationship between respiratory pattern and laryngeal opening times. It also emphasizes the importance of adequate oxygenation in this process.

Interventions within the initial minutes of life can have long lasting consequences, and it is important to avoid both hyperoxia and hypoxia, which can result in oxygen toxicity or prolonged apnoea, respectively. In past decades, prolonged ventilation with a high FiO_2 was a popular strategy to assist the newborn at birth. However, much evidence has emerged over the past 50 years indicating high oxygen ventilation has adverse health consequences for the newborn. High inspired oxygen concentrations is thought to induce the release of reactive oxygen species, initiating an inflammatory response within the lung. This causes damage to the alveolar-capillary barrier, increases pulmonary permeability, and causes epithelial and endothelial cell death, and predisposes the newborn to chronic lung disease (144, 145). Clinical trials show that resuscitation with room air, as opposed to 100% oxygen, significantly reduces oxidative stress, and the mortality rate, as well as decreases heart, brain, and kidney damage (146-149). Unfortunately, very preterm newborns are at an increased susceptibly to hyperoxic injury, as the natural antioxidant defences develops late in gestation. The optimal concentration of inspired oxygen necessary to avoid both hypoxia and hyperoxia has yet to be determined, and the effects of high oxygen administration in the initial minutes of life is contentious (150).

In previous years, the European consensus guidelines (2010) recommended that resuscitation be initiated with 30 - 60% oxygen and increasing to 100% oxygen if the newborn remains apnoeic (151). However, as excessive oxygen exposure is known to be injurious to the lung, lower oxygen concentrations are now recommended. More recently the 2015 International consensus on

cardiopulmonary resuscitation and emergency cardiovascular care science with treatment recommendations endorsed:

> *"initiating resuscitation with a low-oxygen concentration (21-30%)" and "recommend*(ed) *against initiating resuscitation of preterm newborns (less than 35 weeks of gestation) with high supplementary oxygen concentrations (65% -100%)" (113).*

Although oxygen successfully stimulated respiratory drive, and thus laryngeal abduction in preterm rabbit kittens in the current study, the positive effects of high supplementary oxygen may not outweigh the negative effects, and it is entirely likely that the same effect could have been achieved with a much lower inspired oxygen level. Undeniably, it is essential to establish spontaneous breathing at birth, however, it is also important to avoid long term morbidity in the newborn. As such it is important to tailor the inspired oxygen content to the newborn's needs in order to avoid hyperoxia but also ensure that the newborn does not become hypoxic, which will induce apnoea. It may be a far more practical suggestion to investigate other factors that stimulate spontaneous breathing and prolonged laryngeal abduction at birth, such as caffeine, theophylline, aminophylline and glucose.

Unfortunately, blood gas concentrations were not recorded in the current study due to the complexity of the imaging set up. Future studies should analyse blood gas concentrations during imaging for an accurate correlation between oxygenation and respiratory rate and laryngeal abduction.

3.5 *Conclusion*

In the current study, we have demonstrated that 100% oxygen stimulates the establishment of a stable breathing pattern, resulting in increased laryngeal abduction, thus allowing for the successful delivery of iPPV in most preterm kittens at birth. We have also shown that respiratory depression, as induced by hypoxia resulted in prolonged laryngeal adduction preventing the delivery of iPPV. This suggests that

the establishment of a stable breathing pattern may be an important factoring triggering prolonged laryngeal abduction at birth. In this study, we have demonstrated the importance of the relationship between the establishment of a stable breathing pattern and prolonged laryngeal abduction, and thus allowing for successful non-invasive ventilation.

Chapter 4

General discussion and future directions

The studies presented in this thesis provide evidence to suggest protracted laryngeal adduction is the underlying cause behind the inability of non-invasive ventilation to adequately provide respiratory support for many preterm newborns. Using phase contrast X-ray imaging we have demonstrated that prior to the establishment of a stable breathing pattern, preterm rabbit kittens generally had a predominately closed larynx, and that following the establishment of a stable breathing pattern, the larynx transitioned to a predominantly open state.

At birth, it is essential that laryngeal function transitions from the predominately closed fetal state, to the predominately open newborn state, allowing for a successful transition to air breathing. Although it is assumed this transition occurs smoothly, very little is known about how or when it occurs, or why it fails to occur in some newborns. Approximately 60% of premature newborns are unable to develop a stable breathing pattern at birth, and require some form of respiratory support, often delivered non-invasively (152). Our studies suggest prolonged laryngeal adduction hinders the delivery of non-invasive ventilation, and that laryngeal abduction can be promoted by stimulating a stable breathing pattern, which

may improve the efficiency of non-invasive ventilation at birth. In our second study, we delivered 100% oxygen to stimulate respiratory drive, which resulted in prolonged laryngeal abduction; however, high oxygen concentrations can have negative health ramifications for the newborn. As such, other factors known to stimulate respiratory drive at birth should be analysed in preterm newborns with the aim to stimulate laryngeal abduction and improve the outcomes of non-invasive ventilation in the delivery room. Respiratory drive at birth is influenced by the various forms of non-invasive ventilation, drugs, and environmental factors, as well as inspired and circulating gases. These factors are discussed in the following pages.

Although non-invasive respiratory support has become increasingly used in the delivery room, very little is known regarding how the applied pressure interacts with the transitioning laryngeal function at birth. In the first study, we found in kittens with stable breathing patterns, that increasing the CPAP levels above 7 cmH_2O greatly reduced respiratory activity, often acting like a switch turning off and on breathing (fig.15 & fig.16). Unfortunately, variable facemask leak prevented a systematic assessment of CPAP levels on respiratory function. As such a systematic analysis of the relationship between CPAP levels and respiratory activity may be an interesting focus in future studies. This laryngeal adduction may have been due to the pressurisation of the upper airways, the lower airways, or face, however, our studies were not designed to determine the location trigging a possible reflex. Studies in neonatal lambs show that non-invasive ventilation stimulates inspiratory thyroarytenoid activity, and decreases or inhibits inspiratory cricothyroid activity, resulting in inspiratory laryngeal narrowing and an increased inspiratory laryngeal resistance against the inflation pressure (15, 95). Laryngeal patency is highly influenced by blood gas pressures during non-invasive ventilation in neonatal lambs. A moderate increase in $PaCO_2$ eliminates iPPV induced active laryngeal adduction, whereas hypocapnia promotes active laryngeal adduction during the post-inspiratory phase of the breathing cycle (95, 153-155). Carrière *et al* (2016) found that hypoxia had no effect on inspiratory laryngeal adduction during nIPPV

in neonatal lambs (95). The results presented in our second study are contrary to this finding by Carrière *et al* (2016). When our kittens were administered 100% nitrogen they became hypoxic, stopped breathing, and experienced prolonged laryngeal adduction. However, the difference in the two age groups examined between our study and Carrière *et al* (2016) cannot be overlooked. Furthermore, anaesthetics were not used on the day of experimentation in the study by Carrière *et al* (2016), whereas both studies in the current thesis used anaesthetics.

The maternal anaesthetic administered in the first study; propofol and isoflurane, are both known to rapidly cross the placenta and depress the newborn's respiratory drive and activity (156-160). The kittens in the first study were far less active and had a much less stable respiratory pattern compared to kittens in the second study, where does received an epidural, and sedative (butorphanol) use was kept to an absolute minimum. In the first study the glottis was predominately closed (~ 30%) during the first 12 minutes of life in kittens with an unstable breathing pattern (fig.14a). However, the kittens in the 2nd study had a predominately open glottis (> 60%) after the first 3 minutes. This considerable difference between the studies within the first 3 minutes was evident despite the kittens in each study receiving similar CPAP levels (5 - 7 CPAP in the 1st study compared to 5 CPAP in the 2nd study) with air, were fitted with very similar face-masks, were of similar gestational ages (29 - 30 d GA in the 1st study compared to 29 d GA in the 2nd study) and received similar radiation dosages, and were all delivered by caesarean section. Thus, it seems likely that the maternal anaesthetic used in the first study depressed the kitten's respiratory drive and thereby altered the activity of the laryngeal dilators. Initially it was proposed that if the maternal sedatives were to influence the kitten's upper airways, it would cause relaxation of the laryngeal muscles, resulting in passive laryngeal abduction. However this was not seen, and the anaesthetic appears to have depressed the kitten's respiratory drive, and resulted in irregular breathing and laryngeal adduction. In the second study the maternal anaesthetic was reduced to an absolute minimum, and the kittens were able to establish a more stable breathing pattern, with no evident

respiratory depression observed. As the current studies have shown that the establishment of a stable breathing pattern is important for laryngeal abduction, any respiratory depression caused by anaesthetic exposure may have important implications on laryngeal activity. Furthermore, in the first study respiratory depression consequent to the maternal sedative exposure is likely to have resulted in hypoxia, which is also well established to depress respiratory efforts. This difference between the first and second study may highlight the effect of maternal anaesthetic on the initiation and maintenance of spontaneous breathing and the effects on laryngeal activity in the newborn, and the consequences for non-invasive respiratory support. The predominantly adducted laryngeal activity seen in the first study may suggest exposure to maternal sedatives in the delivery room influences laryngeal activity in the preterm newborn, and thus hinders the delivery of non-invasive respiratory support, however further studies are needed to explore this concept.

Commonly administered opiates (pethidine, diamorphine, morphine, fentanyl) for pain relief during labour are highly lipid soluble, and rapidly transfer across the placenta to the fetus, resulting in neurological and cardiorespiratory depression in the newborn (161-163). Naloxone, used in the second study, is a specific opiate antagonist administered to newborns to counter the respiratory depression caused by intrauterine opiate exposure (164, 165). The combined effects of a minimal sedative exposure and naloxone administration likely contributed to the increased respiratory activity in the kittens in the second study, and therefore help promote laryngeal opening. Despite the reduced anaesthetic exposure, kittens in the second study still required tactile stimulation. When the kittens were switched to 100% oxygen, their breathing became much more regular and they no longer required physical stimulation.

Methylxanthines are another form of pharmacological respiratory stimulant, which inhibit the release of inhibitory neurotransmitters, thus stimulating respiratory drive and promoting the establishment of a stable breathing pattern at birth (166). It is possible that methylxanthine treatments, such as caffeine, may promote laryngeal abduction by promoting the establishment of a stable breathing pattern, and thus

improve the efficiency of non-invasive ventilation at birth, however studies are needed to test this theory.

In the first study, the kittens who possibly experienced respiratory depression owing to maternal anaesthetic exposure are likely to have become hypoxic. Hypoxia is further known to cause respiratory depression and apnoea in preterm newborns, and is likely to have further compounded the respiratory depression observed in the kittens, thus further influencing laryngeal activity. In the second study we showed that respiratory support with nitrogen, which likely resulted in hypoxia, caused respiratory depression and laryngeal adduction in preterm rabbit kittens. In future studies it may be beneficial to assess the FiO_2 in real time using a pulse oximeter to understand the temporal relationship between FiO_2 and laryngeal activity. This may provide an understanding about the relationship between hypoxia and laryngeal activity, however this would add an extra layer of complexity in a time sensitive experiment, and fitting the cuff around the kittens tiny leg may prove too difficult. In future studies it may also be interesting to determine if respiratory support with air or various oxygen concentrations can stimulate the kittens regain a stable breathing pattern with a predominantly open larynx following a period of hypoxic respiratory depression, as seen during respiratory support with nitrogen.

At birth, the newborn is at risk of becoming hypothermic which causes a strong respiratory depression. The newborn experiences a major change in ambient temperature as it transitions from the warm insulated uterine environment, to the highly variable environment of the delivery room. The newborn is generally dried quickly, and if apnoeic or preterm, placed under a heat lamp to keep warm in order to maintain body temperature between 36.5 - 37.5^{c} (167, 168). Anecdotally, we found that if the kittens became cold, they soon became apnoeic, and experienced prolonged laryngeal adduction. As such, we ensured that the kittens were kept warm on a heat pad during and between imaging sequences. Interestingly studies in fetal and newborn sheep suggest that a slight temperature drop (~ 1.2°) is important to initiate continuous breathing efforts at birth (56, 169, 170).

Tactile stimulation within the first 60 seconds of life is recommended by the neonatal resuscitation algorithm to help assist the apnoeic newborn transition to *ex utero* life (171). Although no clinical study has been published assessing the effect of tactile stimulation on respiratory drive at birth, tactile stimulation is often recommended to encourage spontaneous breathing (172, 173). In the delivery room recommended tactile stimulation includes, but is not limited to, rubbing the newborn with the drying cloth, rubbing the newborn's back, and flicking the newborn's feet (172). Tactile stimulation is thought to arouse the newborn, stimulate respiratory drive, and increase the heart rate. In our studies, it was evident that tactile stimulation prompted spontaneous breaths in apnoeic preterm kittens, interrupting periods of prolonged apnoea. In both the first and second study, tactile stimulation was often necessary to incite spontaneous breaths from kittens prior to the imaging sequences. Unfortunately the amount of tactile stimulation delivered to the kittens in each group and in each study was not recorded. In future studies it would be highly beneficial to record the amount and duration of stimulation delivered to the kittens, and the subsequent effect on breathing rates and laryngeal activity. Observations from the delivery room suggests tactile simulation is an important stimulant for the newborn at birth; however, there is a lack of clinical studies investigating the effects of tactile stimulation on respiratory drive during this period (172).

In kittens without an oesophageal tube, laryngeal adduction obstructed the upper airways, causing the insufflated gas to be redirected down the oesophagus to the digestive system, and resulted in severe gastric distension (fig.18b & Supplementary Movie 5). Gas and gastric acid leaked from the kitten's stomach after the positive pressure was removed at the completion of imaging. It is worth noting that gastric acid is a well-documented laryngeal chemoreceptor stimulant, initiating reflex laryngeal adduction, and therefore may have contributed to the laryngeal adduction seen in the kittens with gastric distension. The aerated stomach pushed against the lungs from below, which appeared to hinder respiratory efforts, resulting in respiratory depression and further gastric distension. Gastric distension

was first described by Jaile *et al* in 1992, who coined the term "*CPAP belly*" and suggested the primary contributors to the development of gaseous bowl distension are nasal CPAP, aerophagia, and immaturity of intestinal motility and gastric muscles in very small newborns (174). Elevated gastric distension often parallels improvements in clinical status, suggesting that the redirection of gas to the stomach is an active process of swallowing air in the days after birth, opposed to a passive process. However, it was evident in our studies that gastric distension occurred passively in the immediate period after birth (Supplementary Movie 5).

Promoting laryngeal abduction in preterm newborns at birth by stimulating a stable breathing pattern may increase the success of non-invasive ventilation in the delivery room. Methylxanthines, naloxone, hypercapnia, and hyperoxia are all known to increase respiratory drive in the newborn, while hypothermia, hypoxia, and maternal sedatives have been shown to inhibit respiratory drive at birth. Further studies are needed to explore laryngeal activity at birth in premature newborns, with the end goal of increasing the success of non-invasive ventilation in the delivery room.

1

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Chapter 5

Conclusion

The studies presented in the current thesis show that prolonged laryngeal adduction hinders the successful delivery of iPPV at birth in preterm rabbit kittens. In the first study, it was demonstrated that prior to the establishment of a stable breathing pattern, kittens had a predominately closed larynx, preventing the delivery of iPPV delivered non-invasively. Following the establishment of a stable breathing pattern the larynx was predominantly open, and non-invasive iPPV could be successfully delivered. In the second study we found that kittens receiving CPAP with air quickly established a predominately open larynx with an unstable breathing pattern, but these kittens required near continuous physical stimulation. During breathing with 100% oxygen, kittens established a stable breathing pattern with prolonged laryngeal abduction, allowing for kittens to breathe freely and facilitating the successful delivery of iPPV. The notable increase in laryngeal opening during air and oxygen breathing in the second study compared to the first study may have been a consequence of the reduction in maternal sedatives administered and physical stimulation. Continuous positive airway pressure with 100% nitrogen induced apnoea with prolonged laryngeal closure, preventing iPPV delivery, despite having well aerated lungs. When 100% oxygen was resumed, the breathing rate increased again, and the larynx returned to the

predominately open state. Thus, it appears that the establishment of a stable breathing pattern is an important stimulus for laryngeal opening at birth. Promoting the establishment of a stable breathing pattern may be a beneficial strategy to improve the success rate of non-invasive ventilation for preterm newborns in the delivery room. Future studies are needed to examine the effect of other respiratory stimulants on the success rate of non-invasive ventilation for preterm newborns at birth.

Chapter 6:

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Chapter 7

Appendices

Paper accepted for publication.

Titled: Laryngeal closure impedes non-invasive ventilation at birth

Paper accepted by the archives of childhood disease and has been presented here in the submitted form.

Laryngeal closure impedes non-invasive ventilation at birth

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Key points

- While airway obstruction restricts non-invasive respiratory support in premature newborns at birth, the cause and site of obstruction are unknown.
- We have used phase contrast X-ray imaging to show that the glottis and epiglottis are predominantly closed immediately after birth in spontaneously breathing premature rabbits, opening only briefly during a breath.
- When the glottis and epiglottis were closed, non-invasive PPV was ineffective, unless the kitten took a breath.
- The glottis and epiglottis were predominantly open in premature kittens with aerated lungs and a stable breathing pattern, allowing non-invasive PPV to successfully inflate the lung, irrespective of time after birth.
- Prolonged laryngeal closure at birth may contribute to reduced success rates of non-invasive respiratory support in the delivery room for premature newborns.

Abstract

Background: Non-invasive ventilation is sometimes unable to provide sufficient respiratory support for very premature infants in the delivery room. While airway obstruction is thought to be a major reason for this failure, the site of obstruction is unknown. We investigated whether closure of the glottis is a major site of airway obstruction. **Methods:** We used phase contrast X-ray imaging to visualize laryngeal function in spontaneously breathing premature rabbits immediately after birth and at ~1 h after birth. Non-invasive respiratory support was applied via a facemask and images were analyzed to determine the percentage of the time the glottis and the epiglottis were open. **Hypothesis:** Immediately after birth the glottis is predominantly closed, only opening briefly during a breath, making non-invasive ventilation ineffective, whereas after lung aeration, the glottis is predominantly open allowing non-invasive ventilation to ventilate the lung. **Results:** The glottis and epiglottis were predominantly closed (open 25.5 ± 1.1% and 17.1 ± 1.6% of the time, respectively) in kittens with unaerated lungs and unstable breathing patterns immediately after birth. In contrast, the glottis and the epiglottis were mostly open (90.5±1.9% and 72.3±2.3% of the time, respectively) in kittens with aerated lungs and stable breathing patterns irrespective of time after birth. **Conclusion:** Laryngeal closure impedes non-invasive ventilation at birth and may reduce the effectiveness of non-invasive respiratory support in premature infants immediately after birth.

Abbreviations: CPAP; continuous positive airway pressure, FBM; fetal breathing movements, PC; phase contrast, d GA; days of gestational age, PaO_2; partial pressure of oxygen in arterial blood, $PaCO_2$; partial pressure of carbon dioxide in arterial blood, PPV; positive pressure ventilation.

Introduction

Until recently, most very premature infants requiring respiratory support at birth were intubated and mechanically ventilated. However, as mechanical ventilation increases the risk of lung injury [1], clinical practice has shifted towards non-invasive respiratory support [2] usually administered via a facemask [3, 4]. However, little information is available on how non-invasive respiratory support interacts with the infant's changing physiology at birth and as such, it is unclear why it is sometimes ineffective at birth [4-6].

Airway obstruction and mask leak are common reasons why non-invasive ventilation is sometimes unsuccessful. While mask leak is more common (51%), airway obstruction (26%) increases the rate of clinical deterioration and need for intubation [7, 8]. Although the mechanisms and site of airway obstruction are unclear, incorrect facemask or head positioning are thought to be major causes [7, 8]. However, when preterm infants are given a sustained inflation at birth, air only enters the lung if the infant takes a breath, indicating that the larynx maybe involved [9].

In the fetus, tonic laryngeal adductor muscle activity close the glottis during apnoea to seal the airway [10], whereas during fetal breathing movements (FBM), this activity is absent and dilator activity occurs in phase with diaphragmatic contractions [10]. In the fetus, as laryngeal adduction restricts liquid efflux during apnoea, it helps to maintain lung expansion, which is the primary stimulus for lung growth [11, 12]. We propose that glottic adduction in apnoeic very preterm infants persists after birth and will prevent non-invasive intermittent positive pressure ventilation (iPPV) from ventilating the lung unless the infant takes a breath. Furthermore, as the lung aerates and regular breathing commences, we propose that the glottis will remain open, allowing non-invasive positive pressure ventilation (PPV) to ventilate the lung.

We examined laryngeal activity in premature rabbits immediately after birth using phase-contrast (PC) X-ray imaging [13]. Using this technique, closure or patency of the airway is visible as the air passes

through the glottis (Fig.1), allowing determination of whether it is open or closed. By visualising the lungs, we can also determine whether non-invasive PPV can inflate the lung. We hypothesized that, immediately after birth, the glottis would be predominately closed, only opening during spontaneous breaths, which prevents non-invasive iPPV from inflating the lung. We also hypothesized that the glottis would be predominantly open following the onset of stable breathing, allowing the lung to be ventilated non-invasively.

Methods

Experimental procedure: All experimental procedures received approval from SPring-8 Animal Care and Monash University's School of Biomedical Science's Animal Ethics Committees. Experiments were conducted at the SPring-8 synchrotron in Japan.

At 29d GA (term=32d), pregnant New Zealand white rabbits (n=21) were anesthetized (Rapinovet; I.V.; 12mg/kg bolus), intubated and anaesthesia sustained with isoflurane inhalation (1.5-3.0%). Kittens were exteriorized by cesarean section and a custom-made facemask was placed over the kitten's head and attached using tissue glue. An oesophageal tube was inserted to measure intrathoracic pressure and caffeine was administered (20mg/kg base; i.p.). Kittens were delivered, transferred into the imaging hutch and placed on a heating pad and ECG leads attached. The facemask was connected to a ventilator [14] to give 5-7 cmH_2O of CPAP. Kittens were physically stimulated to encourage spontaneous breathing and were imaged for ~15 minutes. Facemask and oesophageal pressures were recorded (Powerlab; ADInstruments, Sydney, Australia). If the kitten was apnoeic and heart rates were <100 beats per minute, the hutch was entered to deliver tactile stimulation; this was common as most kittens had unstable breathing patterns immediately after birth. To determine whether non-invasive iPPV could inflate the lung, a peak inflation pressure (PIP) of 25 cmH_2O and a positive end-expiratory pressure (PEEP) of 5

cmH_2O were used [14]. Following imaging, kittens were removed from the hutch, placed on a heat pad and received constant tactile stimulation to sustain spontaneous breathing. If kittens were unable to sustain a stable respiratory pattern they were killed with sodium pentobarbitone (i.p. 100 mg/kg; Somnopentyl, Kyoritsu Seiyaku Co., Ltd., Tokyo). At ~1 h after birth, viable kittens were re-imaged and during this second sequence, each kitten received a brief period of iPPV (PIP 25 cmH_2O and PEEP 5 cmH_2O) and stepwise increases in CPAP between 0 and 15 cmH_2O.

PC X-ray imaging: High-resolution PC X-ray imaging was used to visualize the larynx [15] using an ORCA Flash 4.0 Hamamatsu C11440-22C (effective pixel size 15.3 µm, 2048×2048 pixels) located 2 m downstream of the kittens [16, 17].

Data analysis: Imaging was used to determine if the glottis and epiglottis were open or closed immediately after birth and at approximately one hour after birth. Data are expressed as the percentage of time the glottis and epiglottis were open over consecutive 30 second periods. The ability of iPPV to ventilate the lung was assessed using images of the lung and intrathoracic oesophageal pressures, before and after lung aeration and establishment of a stable breathing pattern. The effect of increasing mask pressure on spontaneous breathing was assessed using respiratory rates from oesophageal pressure recordings (Figs.2 & 3). The effect of airway pressures on distension of the pharynx was measured from the images using ImageJ and expressed as a percentage of the initial pharyngeal dimension prior to iPPV. As the entire pharynx was not included in the image field of view, we were unable to assess pharyngeal dimensions in detail.

Statistical analysis: All data are presented as the mean ± standard error of the mean (SEM). Data was compared for significance over time using a two-way repeated measures ANOVA. An unpaired t-test with a Welch's correction was used to test significance between the sequences for both the glottis and the epiglottis. p-values <0.05 were considered statistically significant.

Results

Seventy-one premature kittens were delivered from 21 does; 38 were delivered at 29d GA and 33 at 30d GA; term is 32d GA in rabbits. Immediately after birth, 62 kittens were imaged, with only 36 (of 62) attaining a sustainable breathing pattern and heart rates >100 beats per minute; 9 kittens were non-viable at delivery and were not imaged. Of the 36 kittens with a stable breathing pattern, 17 kittens remained viable for reimaging at ~1 h after birth. Images of the same kitten, initially with a closed glottis and epiglottis (Fig.1A) and then with an open glottis and epiglottis (Fig.1B) are shown in figure 1. Eleven of the 36 kittens were breathing vigorously and were very active after delivery resulting in lung aeration before imaging could commence (Fig.2B). These 11 kittens were initially analyzed separately from kittens with unaerated lungs, unstable respiratory activity, apnoeic periods and bradycardias (Fig.2A); thereafter all kittens were analysed together.

Interpretation of PC X-ray movie sequences:

Immediately after birth: Most (51/62) preterm kittens had unaerated distal airways (aeration restricted to larger airways; Fig.1) and unstable respiratory patterns interspersed with apnoeic periods (Fig.2A). During these apnoeic periods, the glottis was predominantly closed (Supplementary Movie 1) only opening briefly during spontaneous breaths before closing again between breaths. During a breath, the glottis and epiglottis opened and the pharyngeal wall partially collapsed as air flowed into the lungs. This implies that, despite CPAP, upper airway resistance restricted airflow into the pharynx, which acted as a partial reservoir for low resistance gas flow into the lungs during inspiration. Expiration was short in duration and there was no evidence of glottis mediated expiratory braking at this stage. Between breaths, the glottis and epiglottis remained mostly closed, but on occasion the glottis was open while the epiglottis remained closed. At these times, the glottis appeared relaxed and not actively abducted as occurred during inspiration. Between breaths the pharynx was usually distended due to CPAP while the glottis

and/or epiglottis were closed; this distension mostly increased with increasing CPAP. As the oesophageal tube restricted air entry into the stomach, if it was not present, increasing CPAP and pressurization of the pharynx caused air to enter the stomach (Fig.1C).

After lung aeration: In kittens with a stable breathing pattern (Fig.2B), the glottis and epiglottis remained predominantly open and did not open and close in phase with respiratory movements (Supplementary Movie 2), although dilation was enhanced during inspiration. The glottis usually narrowed during expiration, perhaps due to a reduction in abductor muscle activity, but remained open throughout the respiratory cycle. Active transient closure of the glottis was commonly seen during expiration (Supplementary Movie 2). These expiratory braking manoeuvres were characterized by rapid expansion of the sub-glottic, upper trachea indicating pressurization of the airways (Supplementary Movie 2). In the absence of expiratory braking, the pharynx appeared to expand during expiration.

Non-invasive iPPV: In kittens that were apnoeic or had unstable breathing patterns, non-invasive iPPV was unable to ventilate the lung (see below) due to closure of the glottis and/or epiglottis. This prevented air from entering the trachea, causing the pharynx to expand and deflate in phase with the ventilator (Fig.4) (Supplementary Movie 3). In contrast, in kittens with a stable respiratory pattern, the glottis and epiglottis remained mostly open, allowing non-invasive PPV to ventilate the lung in 78% of attempts (17 of 22 attempts in 13 kittens; Fig.6; Supplementary Movie 4). In a small number of kittens (4 of 13 kittens), noninvasive iPPV caused the glottis and/or epiglottis to close, which only opened briefly during a spontaneous breath out of synchrony with the ventilator (5 of 22 or 22% of PPV attempts).

Image analysis of glottis and epiglottis function:

Glottis: In kittens (n=51) with unaerated lungs and unstable breathing patterns immediately after birth (Fig.2A), the glottis was open 25.5±1.1% of the time (range: 12.0±3.5% to 36.1±12.5%; Fig.5A). In contrast, in kittens with aerated lungs and a stable respiratory pattern (Fig.2B) (n=11), the glottis was

open 76.8±3.7% (range 36.7±31.8% to 99.1±0.1%; Fig.5). After the first hour (2nd imaging sequence), the glottis was open on average 90.5±1.9% of the time (range: 80.3±8.7 to 99.7±0.3%, Fig.6) and did not significantly vary over time (n=17 kittens). Glottic opening times were significantly greater during the 2nd imaging sequence compared to the 1st imaging sequence in kittens with an unstable breathing pattern ($p < 0.0001$), but not in kittens with a stable breathing pattern.

Epiglottis: In kittens (n=51) with unaerated lungs and unstable breathing patterns immediately after birth (Fig.2A), the epiglottis was open 17.1±1.6% of the time (range 6.2±4.7% to 51.5±27.5%; Fig.5B). In contrast, in kittens (n=11) with aerated lungs and a stable respiratory pattern (Fig.2B), the epiglottis was open 72.0±3.4% of the time (range 36.2±31.8% to 96.1±3.9% Fig.5B). After the first hour (2nd imaging sequence), the percentage of time the epiglottis was open was 72.3±2.3% (range 60.3±14.3% to 85.7±14.3%; n=17, Fig.6).

Effect of non-invasive iPPV on pulmonary ventilation:

In kittens with unaerated lungs and unstable breathing patterns immediately after birth, non-invasive iPPV was unable to ventilate the lung in 16 of 17 attempts (94%; n=12 kittens); facemask leak precluded assessment of iPPV in some kittens. In the one kitten successfully ventilated with non-invasive iPPV, iPPV was initially unsuccessful but after 36 sec, the kitten took 10-15 breaths in synchrony with the ventilator, which aerated the lungs. After this time, the glottis and epiglottis remained opened, allowing non-invasive PPV to ventilate the lung.

In kittens with a stable respiratory pattern (Fig.2B), non-invasive iPPV ventilated the lungs in 17 of 22 attempts (77%; n=14). While most (79%) kittens became apnoeic during iPPV, allowing the ventilator alone to ventilate the lung, 21% continued spontaneous breathing in phase with the ventilator (Fig.3A). In the four kittens that non-invasive iPPV could not ventilate the lung, spontaneous breathing continued but at a lower rate and the breaths were out of phase with the ventilator.

Effect of CPAP level on spontaneous breathing activity

Sixteen kittens had a sufficiently stable respiratory pattern to determine the effects of CPAP on spontaneous breathing respiratory rates. Variable facemask leak precluded a systematic assessment of CPAP levels on respiratory function in some animals. As such, CPAP levels >7 cmH_2O were compared with CPAP levels <7 cmH_2O. CPAP levels >7 cmH_2O reduced ($p < 0.001$) respiratory rates from 38.8±3.2 breaths per minute to 12.6±1.8 breaths per minute. The inhibitory effect of CPAP was evident in all kittens, causing an almost complete cessation of spontaneous breathing in most kittens (Fig.3B)

Effect of PPV on pharyngeal dimensions:

Non-invasive iPPV caused periodic distension of the pharyngeal wall that was in phase with the change in airway pressure delivered by the ventilator (Fig.4). This periodic pharyngeal distension was evident regardless of whether iPPV occurred when the glottis was closed (lung not ventilated) or open (lung ventilated).

Discussion

While non-invasive ventilation, applied via a facemask, is the preferred mode of respiratory support for very preterm infants, it is unclear how it interacts with the infant's physiology at birth. As such, it is unclear why non-invasive iPPV is sometimes insufficient, requiring these infants to be intubated immediately after birth [4-6]. Our results show that both the glottis (closed 74.5±1.1%) and epiglottis (closed 82.9±1.6%) were predominantly closed at birth, opening briefly only during a breath. Thus, non-invasive iPPV was unable to ventilate the lungs unless the preterm kittens took a breath, thereby opening their glottis and epiglottis. However, after lung aeration and a stable breathing pattern was established, both the glottis (open 90.5±1.9%) and epiglottis (open 72.3±2.3%) remained predominantly open. As a

result, non-invasive iPPV could ventilate the lungs in most (77%) kittens, with the majority (79%) becoming apnoeic during PPV; the other 21% breathed in synchrony with the ventilator. Kittens (23%) that couldn't be ventilated using iPPV, mostly breathed spontaneously out of synchrony with the ventilator and closed their glottis between breaths. This effect of PPV on spontaneous breathing has also been observed in preterm human infants immediately after birth [7].

During development, glottic adduction during apnoea plays a vital role in fetal lung growth by restricting airway liquid loss [11, 12]. This helps to maintain a high degree of fetal lung expansion, which is the primary stimulus for fetal lung growth [11, 12]. As hypoxia suppresses FBMs [18], it also causes glottic adduction [19] and this effect of hypoxia persists well into newborn life [20]. The hypoxia-induced suppression of FBM contrasts with the increased respiratory drive observed in adults and also persists after birth [20, 21]. As preterm newborns are essentially exteriorized fetuses at birth, it is not surprising that most apnoeic preterm kittens had adducted glottises and could not be ventilated non-invasively. Furthermore, as hypoxia inhibits FBM and causes glottic adduction, one would also expect that hypoxia will suppress breathing activity and stimulate glottic adduction in newborns [20], restricting the effectiveness of non-invasive iPPV. While it is unclear whether this occurs in preterm human infants, we would expect the biology to be similar as both preterm rabbits and sheep [20] display the same responses and hypoxia is known to inhibit FBM and cause bradycardia in humans [22].

In contrast to apnoeic kittens, the glottis and epiglottis were predominantly open in kittens with aerated lungs and a stable respiratory pattern, allowing most to be ventilated non-invasively. During FBM periods, tonic activity in the glottic adductor muscles cease and the diaphragm and glottic abductor muscles contract in synchrony [10]. While it is unclear whether the fetal glottis is "predominantly open" during FBM, it probably is because the resistance to airway liquid efflux through the glottis is greatly reduced during FBM compared with apnoeic periods [23]. Furthermore, as preterm kittens with aerated lungs and a stable breathing pattern had a "predominantly open" glottis, irrespective of when they were

imaged after birth, a "predominantly open" glottis during breathing activity likely occurs both before and after birth and is not a uniquely postnatal event that requires lung aeration. Indeed, it appears to be closely related to a stable breathing pattern, rather than other variables such as the degree of lung aeration.

These findings indicate that establishing a stable respiratory pattern after birth is the greatest priority in assisting very preterm infants to transition to newborn life when using non-invasive iPPV. We found that if preterm kittens had a stable respiratory pattern, as the glottis and epiglottis were predominantly open, non-invasive PPV was able to either augment spontaneous breaths or directly ventilate the lung. As most (~80%) preterm infants display visible breathing efforts at birth [24], one would expect that non-invasive PPV should be highly successful. However, this assumes that the respiratory efforts and/or PPV are effective at aerating the lung and establishing effective pulmonary gas exchange. All preterm kittens imaged in our study displayed visible breathing efforts, but only 17 (out of 62; 27%) kittens were able to aerate their lungs and establish a sustained stable respiratory pattern. The majority (73%) of kittens had a respiratory rate that varied widely (between 1 and 20 breaths per minute), but these breathing episodes were interspersed with apnoeic periods and were insufficient to aerate their lungs. During apnoea, as the glottis was closed we were unable to ventilate the lung with iPPV and so these kittens gradually became more apnoeic and bradycardic and would have required intubation and ventilation if they were not terminated.

As the upper airways of the fetus and newborn are highly compliant [25-28], large pressure gradients generated during spontaneous breathing or iPPV can cause major distortion of the upper airway walls. The pharynx either partially or totally collapsed during inspiration, indicating that resistance within the airways upstream of the pharynx restricted gas flow into the lung, increasing the effort of breathing. It is commonly thought that CPAP reduces the effort of breathing by splinting the upper airways open and preventing pharyngeal collapse during inspiration. We can now confirm that CPAP distends the pharynx, allowing it to act as a reservoir for low resistance gas flow into the lung during inspiration. We also

found that during intermittent iPPV, irrespective of whether the glottis was open or closed, the pharynx distended in phase with the increase in airway pressure delivered by the ventilator. This is to be expected because, as regardless of the volume of the system, the ventilator will pressurize all open airways to the set PIP value. This will involve pressurization and distension of just the upper airways when the glottis is closed or both the upper and lower airways when the glottis is open. As the upper airways visibly expand with each inflation, the gas volume entering the lung during non-invasive iPPV is likely to be over-estimated. This is consistent with the findings that tidal volumes measured during iPPV in infants and lambs before intubation are significantly higher than tidal volumes measured following intubation, despite the same PIP [29].

Hypoxia, resulting from inadequate lung aeration, was probably a major contributor to the apnoea and bradycardia observed and likely dominated the breathing response in our preterm kittens [30]. Increasing $PaCO_2$ levels and reducing core body temperature are thought to play an important role in stimulating continuous breathing at birth [30, 31]. However, we found that kittens with a poor breathing response became increasingly apnoeic despite increasing $PaCO_2$ levels that must have accompanied the hypoventilation. Similarly, while we attempted to keep the preterm kittens warm, anecdotally, we found that if kittens were cool, they rapidly became apnoeic.

We also found that placement of the facemask (anecdotal finding) and application of facemask pressures >7 cmH_2O also inhibited breathing. In apnoeic kittens, as the glottis was closed CPAP simply pressurized the pharynx, which promoted the movement of air into the stomach in kittens without an oesphageal tube (Fig.1C). In kittens with a stable breathing pattern, CPAP levels > 7 cmH_2O greatly reduced respiratory activity, often acting like a switch turning off and on breathing (Fig.3B). We consider it likely that application of pressure to the face or pressurization of the pharynx activated receptors that caused reflex closure of the glottis. Indeed, activation of facial receptors that signal via the trigeminal nerve are known to inhibit breathing, including the diving reflex, which is triggered by cold water and is

associated with a bradycardia [32]. Studies in sleeping term infants provide conflicting results, with application of the facemask rim depressing respiratory rate, and the additional dead space introduced by the mask increasing tidal volume, resulting in a significant overall increase in minute ventilation [33, 34].

Conclusion

At birth, the glottis and the epiglottis are predominantly closed in preterm kittens that have an unstable respiratory pattern, opening only briefly during a breath, making non-invasive iPPV ineffective at ventilating and aerating the lung. However, in kittens with a stable breathing pattern, irrespective of when this occurred after birth, the glottis and epiglottis were mostly open, allowing non-invasive PPV to successfully ventilate the lung. We propose that glottic closure immediately after birth greatly restricts the ability of non-invasive PPV to aerate and ventilate the lung unless the infant assists by attempting to breathe, which is consistent with observations in humans. These findings underline the importance of stimulating breathing and establishing a stable respiratory pattern at birth. Avoiding factors that inhibit breathing such as hypoxia are vital to avoid the need for intubation.

What is already known on this topic

- Non-invasive ventilation often fails to provide adequate respiratory support to premature newborns at birth, however the reason for this is unknown.
- Little is known about laryngeal function at birth in term or preterm newborns.

What this study adds

- We have used phase contrast X-ray imaging to show the glottis and epiglottis are predominantly closed immediately after birth in spontaneously breathing premature rabbits.
- When the glottis and epiglottis were closed, non-invasive ventilation was ineffective, unless the kitten took a breath.
- The glottis and epiglottis were predominantly open following lung aeration and the establishment of stable breathing pattern, allowing for successful non-invasive ventilation.

Figures

Figure 1

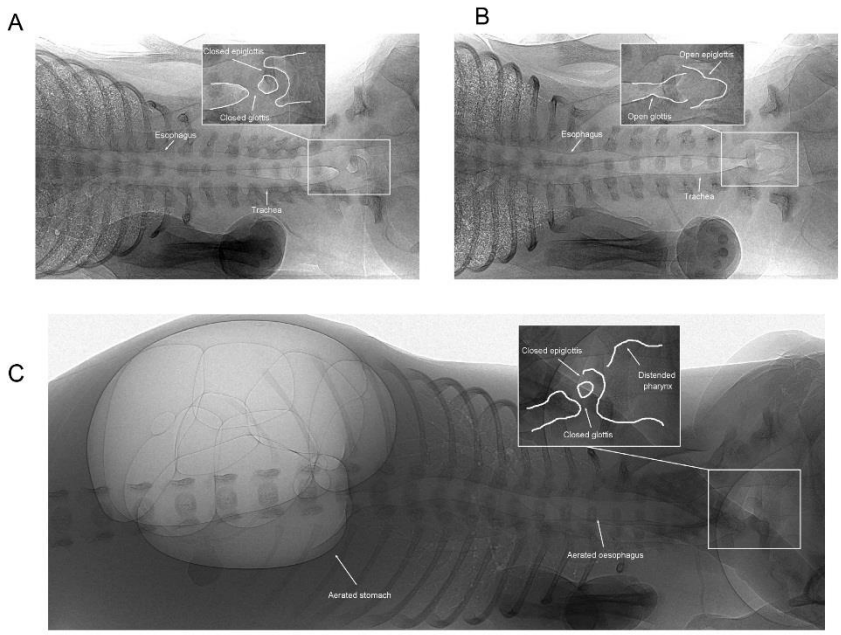

Figure 2

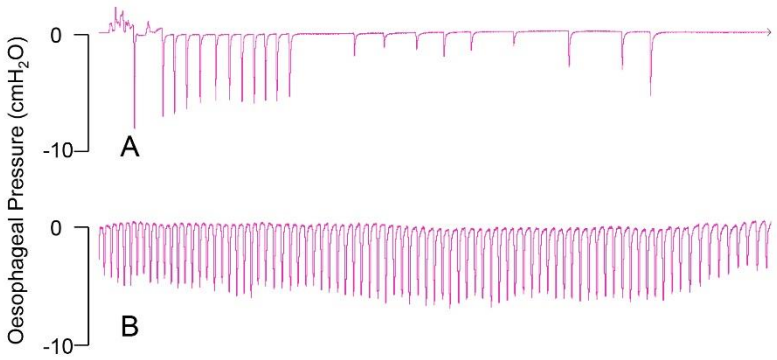

Figure 3

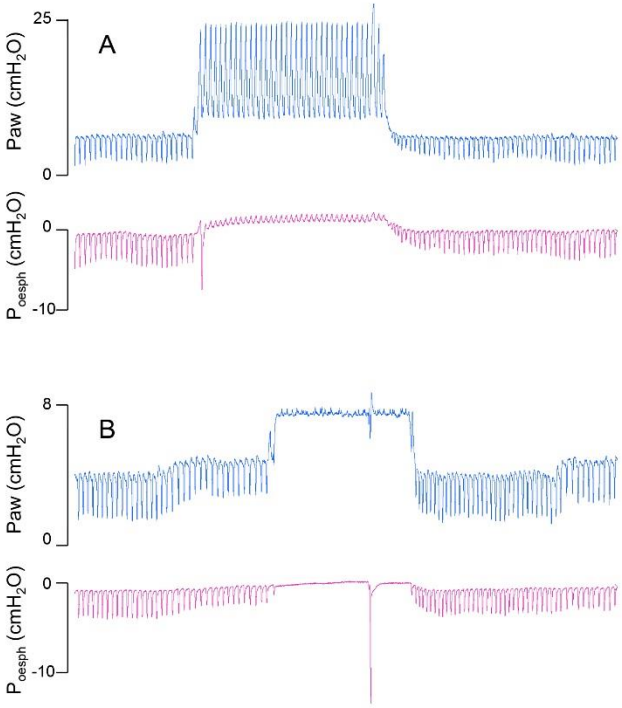

Figure 4

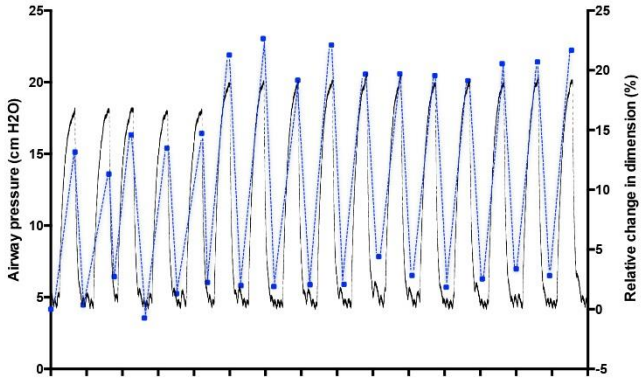

Figure 5

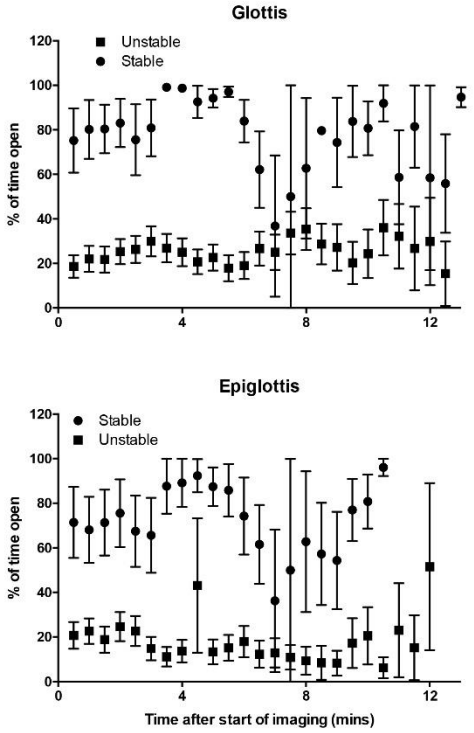

Figure 6

(1h after birth)

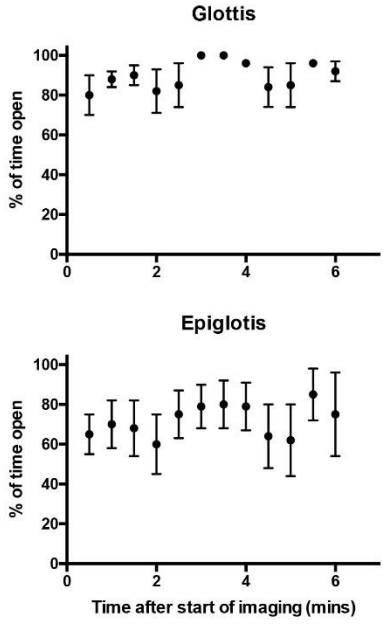

Figure legends:

Figure 1.

Phase contrast X-ray images of a spontaneously breathing newborn preterm rabbit kitten with (A) a closed glottis and epiglottis and (B) an open glottis and epiglottis; the inserts are magnifications of the regions shown within the white boxes. (C) shows air accumulation in the stomach of a rabbit kitten that didn't have an oesphageal tube and received CPAP levels greater than 7 cmH_2O while the glottis was closed.

Figure 2.

Intra-thoracic oesophageal pressure recordings from preterm rabbit kittens displaying (A) an unstable breathing pattern or (B) a stable continuous breathing pattern. Each reduction in pressure represents a breath. Both recordings were obtained within a few minutes of birth. The unstable breathing pattern was characterized by breaths that differed in amplitude, varied in rate and were interspersed with apnoeic periods; these profiles were accompanied with a bradycardia of <100 beats per minute. In contrast, the stable breathing pattern was characterized by regular, continuous breathing that was relatively consistent in amplitude.

Figure 3.

Physiological recordings of airway pressures (Paw) and oesophageal pressures (P_{oesph}) in preterm rabbit kittens that initially had a stable spontaneous breathing pattern. In (A), the kitten received intermittent positive pressure ventilation (PPV) using a peak inspiratory pressure of 25 cmH_2O and an

end-expiratory pressure of 5 cmH_2O. Note that the PPV resulted in positive pressure fluctuations in oesophageal pressure, demonstrating transmission of ventilation pressure into the chest that resulted in lung inflations; lung inflation was confirmed from X-ray imaging. In (B), the kitten received continuous positive airway pressure (CPAP) that when increased to 8 cmH_2O, caused an immediate suppression of spontaneous breathing activity that persisted throughout the elevated CPAP period; only one large deep inspiratory effort was observed. Note that although Paw increased with increased CPAP, oesophageal pressure did not increase, indicating that the pressure was not transmitted into the chest because the glottis closed.

Figure 4.

Simultaneous changes in airway pressure (blue) and percentage change in pharyngeal diameter (red) measured during intermittent positive pressure ventilation in a preterm rabbit kitten. Measurements of pharyngeal diameter were obtained from consecutive phase contrast X-ray images and were measured at both peak inflation and near end-expiration at precisely the same point in the pharynx.

Figure 5

The percentage of time that the glottis (top panel) and epiglottis (bottom panel) were open in preterm rabbit kittens measured within minutes of birth. Kittens were divided into two groups depending on whether they had a stable (closed circles) or unstable breathing pattern after birth (see figure 2).

Figure 6

The percentage of time that the glottis (top panel) and epiglottis (bottom panel) were open in preterm rabbit kittens measured at approximately 1 hr after birth. All kittens had a stable breathing pattern at this stage after birth (see figure 2).

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