

Respiratory adaptation and surfactant composition of unanesthetized male and female lambs differ for up to 8 h after preterm birth

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BACKGROUND: Male preterm infants are more likely to experience respiratory distress syndrome than females. Our objectives were to determine if sex-related differences in physiological adaptation after preterm birth increase with time after birth and if the use of continuous positive airway pressure (CPAP) reduces these differences.

METHODS: Unanesthetized lambs (9F, 8M) were delivered at 0.90 of term. Blood gases, metabolites, and cardiovascular and respiratory parameters were monitored in spontaneously breathing lambs for 8 h. Supplemental oxygen was administered via a face mask at 4 cmH₂O CPAP. At 8 h, lung compliance was determined, and bronchoalveolar lavage fluid (BALF) was analyzed for total protein and surfactant phospholipids. *Surfactant protein (SP)* gene expression and protein expression of SP-A and pro-SP-C were determined in lung tissue.

RESULTS: For 8 h after delivery, males had significantly lower arterial pH and higher PaCO₂, and a greater percentage of males were dependent on supplemental oxygen than females. Inspiratory effort was greater and lung compliance was lower in male lambs. Total protein concentration in BALF, *SP* gene expression, and SP-A protein levels were not different between sexes; pro-SP-C was 24% lower in males.

CONCLUSION: The use of CPAP did not eliminate the male disadvantage, which continues for up to 8 h after preterm birth.

Male preterm infants are at a greater risk of developing respiratory distress syndrome than females of the same gestational age (1–4). Accordingly, male sex is associated with a greater risk of neonatal mortality and respiratory illness including BPD (5–7). Previous studies have indicated that this “male disadvantage” following preterm birth is a result of delayed lung maturation (8), but the basis for the delayed maturation is not completely understood.

Using an ovine model of preterm birth in which survival of females is greater than males (9), we have previously shown that sex-related differences in postnatal adaptation in males become greater with time after delivery; survival rates for male preterm lambs (born at 0.9 of term) were 88% at 4 h, 80% at 8 h, and 55% at 40 h after birth, whereas female survival rates

were 92, 92, and 78%, respectively (9). We recently reported that the poorer respiratory adaptation in male preterm lambs (born at 0.9 of term) was likely due to lower lung compliance than in females and that this may be a result of sex differences in surfactant phospholipid composition and function (10). As our previous study was terminated at only 4 h after delivery, we wished to extend our observation period up to 8 h after preterm delivery to determine if the sex-related differences in postnatal adaptation worsen with time, which could explain the decreased survival rate that we observed in males after 4 h.

The initiation of respiratory support in preterm infants usually involves continuous positive airway pressure (CPAP), which is widely used to improve lung function. CPAP is thought to prevent alveolar collapse and to maintain functional residual capacity, thereby reducing the risk of lung injury and BPD (11,12). The Continuous Positive Airway Pressure or Intubation at Birth (COIN) trial showed that the use of nasal CPAP permitted a significant reduction in the duration of tracheal intubation and mechanical ventilation (13), while a recent review concluded that the use of CPAP from soon after birth, compared with tracheal intubation and positive pressure ventilation, reduces death or BPD in very preterm babies (14). We hypothesized that application of CPAP in the immediate postnatal period would improve the poorer respiratory adaptation in preterm males, thereby attenuating or eliminating the sex difference in cardiorespiratory adaptation following preterm birth.

Thus, the aims of the present study were (i) to determine if sex differences in cardiorespiratory adaptation following preterm birth become greater between 4 and 8 h after birth, at a time when the survival rate in males has been found to decline and (ii) to determine if the use of CPAP after birth attenuates or eliminates the male disadvantage in cardiorespiratory adaptation. We assessed prenatal physiological data to establish whether any sex-related differences were present before birth.

RESULTS

Prenatal Data

Fetal PaCO₂ was significantly higher in males than in females between 128 and 130 d of gestation (DG), but not between 131 and 133 DG (Figure 1d). There were no significant differences

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between male and female fetuses in P_{aO_2} , S_{aO_2} , and pH, blood concentrations of glucose and lactate, mean arterial pressure (MAP), or heart rate (HR; **Figure 1a–c,e–h**).

Postnatal Physiological Data From 0 to 1 h

Following preterm delivery, the lowest arterial pH values were seen in both males (6.965 ± 0.054) and females (7.018 ± 0.018) at 15 min after delivery (**Figure 1c**). Arterial pH was significantly lower in males than in females at each time point between 15 and 60 min (**Figure 1c**). As with arterial pH, P_{aCO_2} reached maximal values at 15 min after delivery in both males (101.3 ± 10.1 mm Hg) and females (81.7 ± 2.6 mm Hg). P_{aCO_2} was significantly higher in males than females at each time point between 15 and 60 min (**Figure 1d**).

Although there was no significant sex difference in S_{aO_2} , females reached the target S_{aO_2} of 80% by 15 min after birth, whereas males took 30 min (**Figure 1b**). During the first hour after birth, there were no significant differences between males and females in P_{aO_2} , plasma concentrations of glucose or lactate, mean arterial pressure, HR, respiratory rate, or inspiratory effort (**Figures 1 and 2**).

After the first hour of the postnatal monitoring period, two male lambs (25%) were killed as their arterial pH was below 7.0 and P_{aCO_2} was above 100 mm Hg for two consecutive 15-min periods; no female lambs were killed prior to the completion of the 8-h monitoring period.

Postnatal Physiological Data From 1 to 8 h

From 90 to 480 min after birth, arterial pH remained significantly lower (**Figure 1c**) and P_{aCO_2} remained significantly higher (**Figure 1d**) in males than in females at each time point. Inspiratory effort was greater in males than in females between 6 and 8 h after birth (**Figure 2b**). Between 90 and 480 min after birth, there were no significant sex differences in P_{aO_2} and S_{aO_2} , blood glucose and lactate concentrations, mean arterial pressure, HR, or respiratory rate (**Figures 1 and 2**).

Supplemental Oxygen Requirements

After delivery, all lambs required supplemental oxygen, delivered with CPAP, to maintain S_{aO_2} above 80%. However, over the 8-h study period, the requirement for supplemental oxygen to maintain S_{aO_2} above 80% was significantly greater in males than in females (**Figure 3**). At 1 h after birth, 38% of females required supplemental oxygen, whereas 100% of males required oxygen. Females did not require any supplemental oxygen after 165 min, whereas 62% of males required supplemental oxygen at this time point. At 8 hours, 50% of males still required supplemental oxygen.

Lung Compliance

The volume of air required to reach a luminal pressure of 40, 30, 20, and 10 cmH₂O was significantly lower in the lungs of males compared to those of females (**Figure 4**).

SP Gene and Protein Expression

The relative gene expression of *SP-D* in lung tissue tended to be higher in males than females (**Figure 5d**; $P = 0.062$), whereas relative *SP-A*, *-B*, and *-C* gene expression in lung tissue was similar in males and females (**Figure 5a–c**). The protein expression of pro-*SP-C* in lung tissue was significantly lower in males than in females (**Figure 5f**). There was no sex difference in *SP-A* protein expression (**Figure 5e**).

Protein Concentration of Bronchoalveolar Lavage Fluid

The concentration of total protein in bronchoalveolar lavage fluid (BALF) was similar in males (2.9 ± 0.3 mg/ml) and females (3.1 ± 0.4 mg/ml).

Surfactant Phospholipid Composition

In BALF collected at 8 h after delivery, the relative proportions of each of the major phospholipid classes (sphingomyelin, phosphatidylcholine (PC), lysophosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylinositol, phosphatidylserine, and phosphatidylglycerol) were not different between males and females (**Figure 6a**). Males had significantly lower proportions of PC 32:1 (**Figure 6c**) and PE 36:2 (**Figure 6e**). However, no sex differences were observed in the proportions of lipid species within sphingomyelin, lysophosphatidylcholine, phosphatidylinositol, phosphatidylserine, and phosphatidylglycerol (**Figure 6b,d,f–h**). The ratios of PC/PE (males: 8.9 ± 0.9 vs. females: 9.7 ± 0.5) and PC/S (males: 11.4 ± 2.3 vs. females: 13.0 ± 1.2) were not significantly different between males and females.

Body and Organ Weights

At preterm delivery, body weights were similar in male and female lambs (**Table 1**). At necropsy, there were no differences in body weight, crown-to-rump length, thoracic girth, hind limb length, or ponderal index (**Table 1**). The absolute and relative (to body weight) dry and wet weights of the lung and the absolute and relative weights of the heart, liver, kidneys, adrenals, and spleen were not different between males and females (**Table 1**). In addition, the volume of the right lung was similar in males and females (**Table 1**).

DISCUSSION

Our major finding was that the impaired respiratory adaptation of male preterm lambs did not appear to worsen between 4 and 8 h after birth and thus could not explain the time-related decline in survival rate of males that was previously described (9). In addition, respiratory support with CPAP did not eliminate or diminish the male disadvantage in respiratory function after preterm delivery. It is likely that the lower lung compliance and altered surfactant composition in male preterm lambs, measured at 8 h after birth, contributed to less effective gas exchange in males, resulting in males being severely hypercapnic and acidemic and a greater proportion of males requiring supplemental oxygen to complete the 8-h study. These factors could contribute to the declining survival rate in male preterm lambs over a longer time-frame after birth.

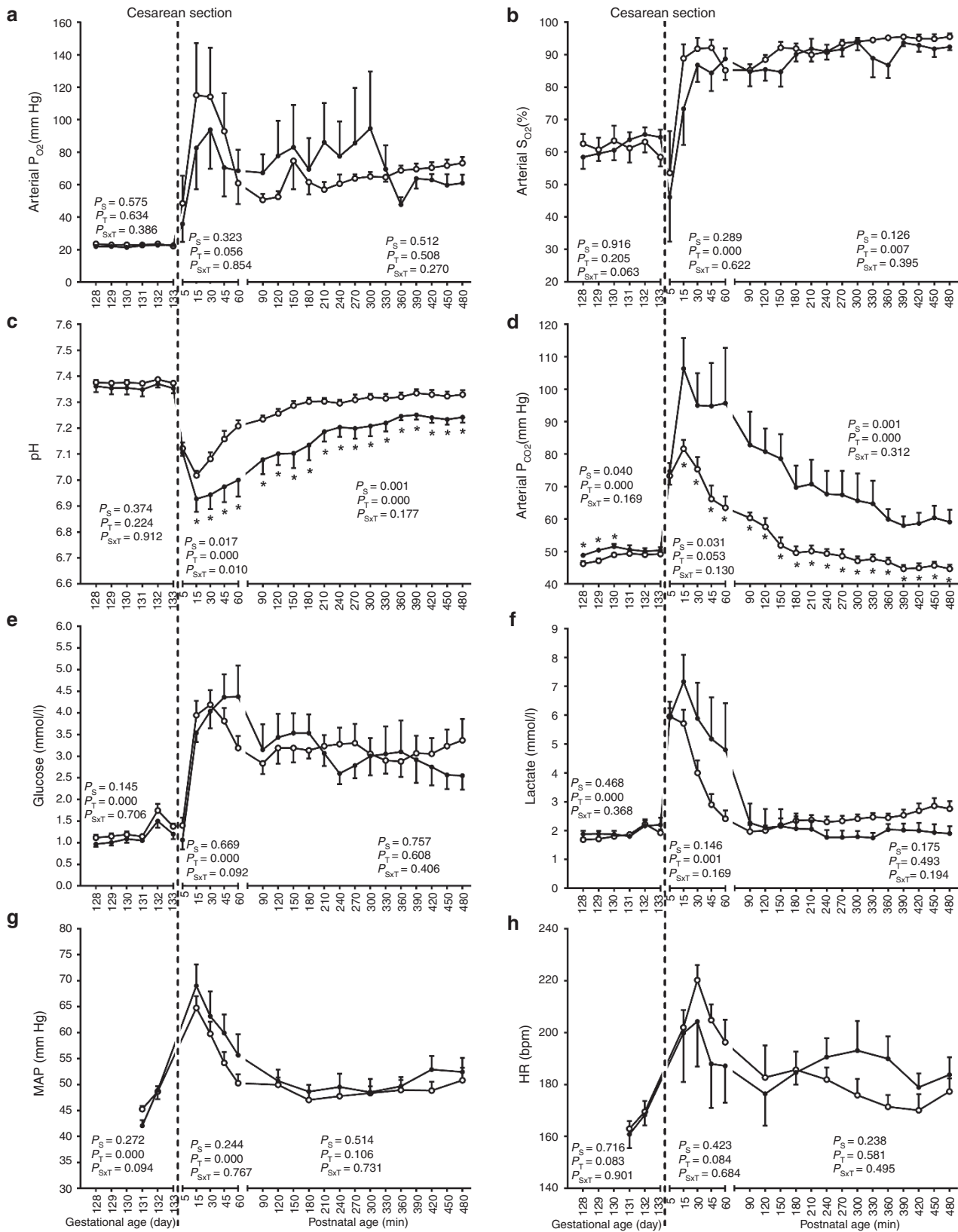


Figure 1. Blood gas measurements before and after birth. (a) Arterial P_{O_2} , (b) arterial S_{O_2} , (c) arterial pH, (d) arterial P_{CO_2} , (e) blood glucose concentration, (f) blood lactate concentration, (g) mean arterial pressure (MAP) and (h) heart rate (HR) measured on 128–133 gestational age (DG) and for up to 480 min following cesarean section at 133 DG in female (open circle) and male (filled circle) fetuses and lambs. Note different time scales before and after delivery. Separate one-way repeated ANOVAs were performed (i) before delivery, (ii) between 0 and 60 min after delivery, and (iii) between 90 and 480 min after delivery. P values: P_S is between factor variable (sex), P_T is repeated factor variable (time), and $P_{S \times T}$ is the interaction term. Data are shown as mean \pm SEM. * $P < 0.05$, female vs. male.

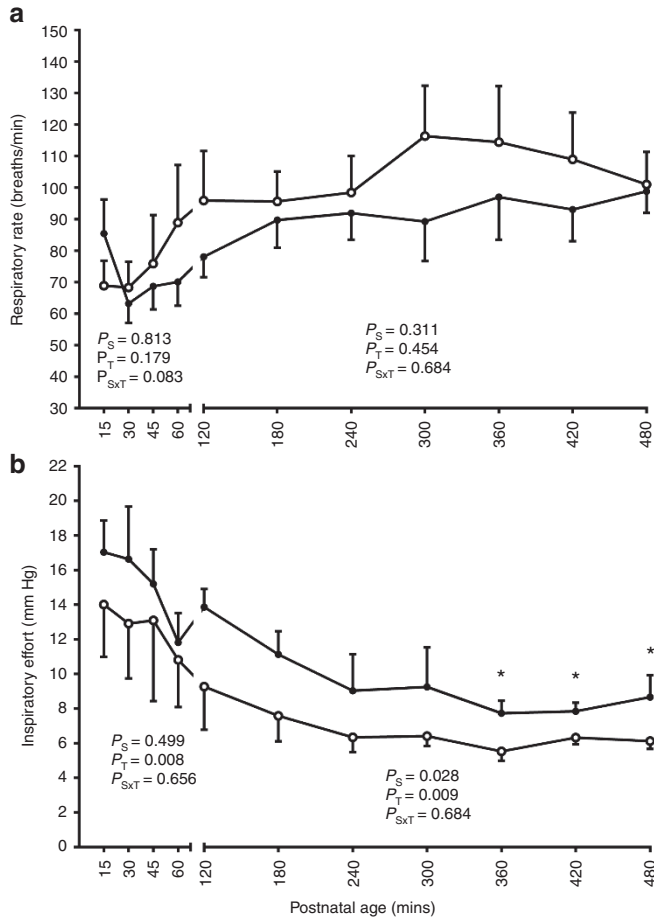


Figure 2. Respiratory rate and inspiratory effort following preterm delivery. (a) Respiratory rate and (b) inspiratory effort following cesarean section at 133 days of gestation in female (open circle) and male (filled circle) lambs. Separate one-way repeated ANOVAs were performed (i) between 0 and 60 min after delivery and (ii) between 120 and 480 min after delivery. *P* values: P_s is between factor variable (sex), P_T is repeated factor variable (time), and $P_{s \times T}$ is the interaction term. Data are shown as mean \pm SEM. **P* < 0.05, female vs. male.

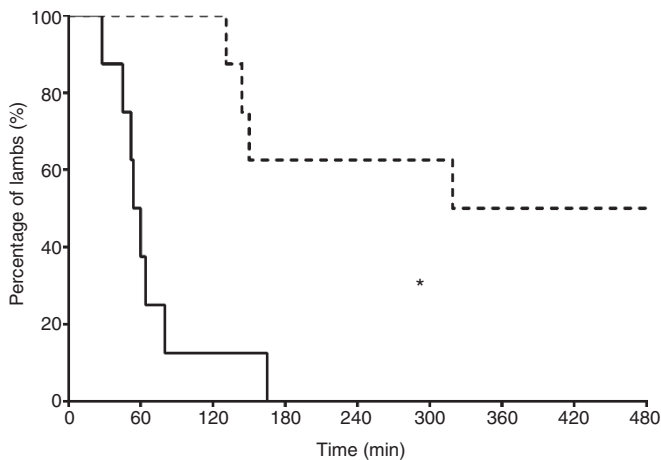


Figure 3. Requirement for supplemental oxygen. Percentage of female (solid line) and male (dashed line) lambs that required supplemental oxygen for up to 480 min after delivery. **P* < 0.05.

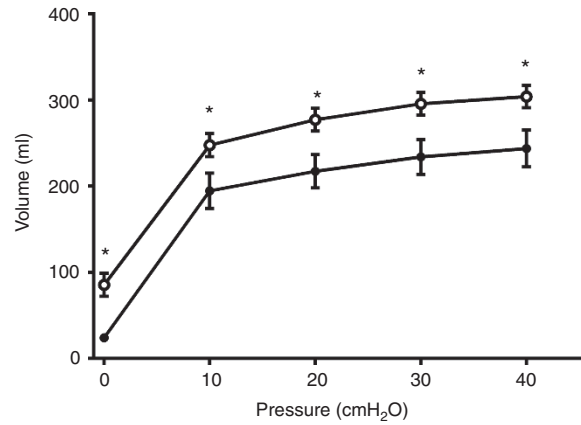


Figure 4. Pulmonary pressure-volume relationship. Pressure-volume curves were created by inflating the lungs with air to a maximal luminal pressure of 40 cmH₂O and then removing the necessary volume (ml) required to maintain pressures of 30, 20, 10, and 0 cmH₂O in female (open circles) and male (filled circles) lambs. Data are shown as mean \pm SEM. **P* < 0.05, female vs. male.

Physiological markers of cardiorespiratory adaptation during the first 4 h of the present study, in preterm male and female lambs, were similar to those seen in our previous study at 4 h after birth (10). We can now add that respiratory adaptation in males does not worsen between 4 and 8 h after birth. Interestingly, both male and female preterm lambs showed a similar improvement in arterial pH and PaCO₂ over the last 7 h. This slow improvement in metabolic parameters may be a result of alveolar recruitment over time and is also observed in the very preterm infant (25–29 wk of gestation) between 2 and 6 h after birth (15).

The initially poorer gas exchange in male lambs, compared to females, may be related to a less compliant lung, which would reduce the ability of males to achieve the same degree of alveolar recruitment as in females. In addition, the stiffer lungs of males may also account for their greater inspiratory effort, which became significant after 6 h and may have consequences for energy expenditure in the longer term. We hypothesized that this initially poorer gas exchange and respiratory adaptation in the male lambs may be overcome with lung recruitment maneuvers such as CPAP; however, the use of CPAP did not improve survival or respiratory dynamics in male preterm lambs. The death of two (out of eight) male lambs within the first hour, in the present study, is a similar survival rate to that seen in our previous studies for preterm lambs (9,10). Two male lambs were killed due to severe hypercapnic acidosis, suggesting impaired pulmonary gas exchange; this indicates that CPAP alone may not be sufficient to improve gas exchange in preterm males. It is possible that lung recruitment maneuvers, such as sustained lung inflation, may be beneficial in recruiting lung volume and improving metabolic status. Studies in preterm lambs at both 127 DG (16) and 139 DG (17) have reported that a single sustained inflation immediately after birth improved circulatory recovery, respiratory status, and lung compliance. As those studies used intubated, mechanically ventilated lambs, the significance of sustained inflation will need to be tested in spontaneously breathing animals, as

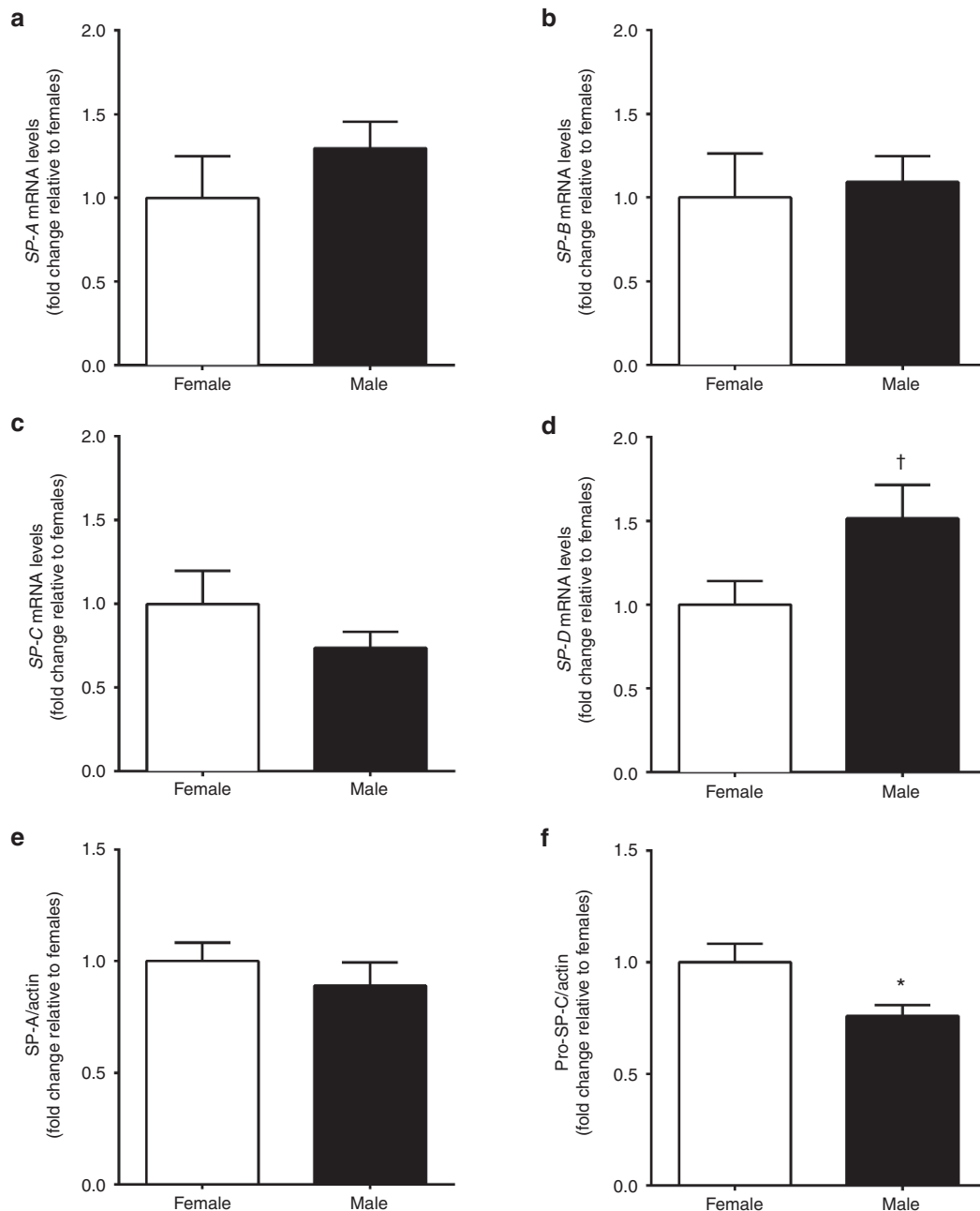


Figure 5. Surfactant protein (SP) gene and protein expression. Relative (a) *SP-A*, (b) *SP-B*, (c) *SP-C*, and (d) *SP-D* mRNA levels and (e) *SP-A* and (f) pro-*SP-C* protein expression in lung tissue of female (open bars) and male (filled bars) lambs. Data are shown as mean \pm SEM. * $P < 0.05$, † $0.05 < P < 0.01$, female vs. male.

a recent study in 70 preterm infants demonstrated that a sustained inflation was not effective due to mask leak, failure of the infant to breathe, or reflex closure of the vocal cords (18).

Of note, female lambs reached the target Sao_2 of 80% earlier than males in both the previous 4-h study (10) and the present 8-h study. In agreement with our findings, spontaneously breathing female preterm human neonates (≤ 32 wk of gestation), receiving CPAP and air, were shown to achieve targeted preductal oxygen saturation levels earlier than males (19). In addition, dependency on supplemental oxygen was greater in males than in females in both our 4- and 8-h studies; this is also observed in preterm infants (5) and is reflective of the higher

incidence of respiratory insufficiency seen in males compared to females of the same gestational age (1,2,6,20,21).

The Paco_2 values in male fetuses were 5–7% higher on days 128–130 DG (3–5 d after surgery) compared to female fetuses, but they remained within the normal physiological range. This finding is consistent with that of our previous study (10) in which we found that male fetuses had a strong tendency ($P = 0.052$) to have a lower arterial pH compared to female fetuses. We cannot rule out the possibility that these differences are a result of male fetuses being less resilient to surgery than female fetuses, or whether they are indicative of an innate male disadvantage during fetal life with respect to acid–base balance.

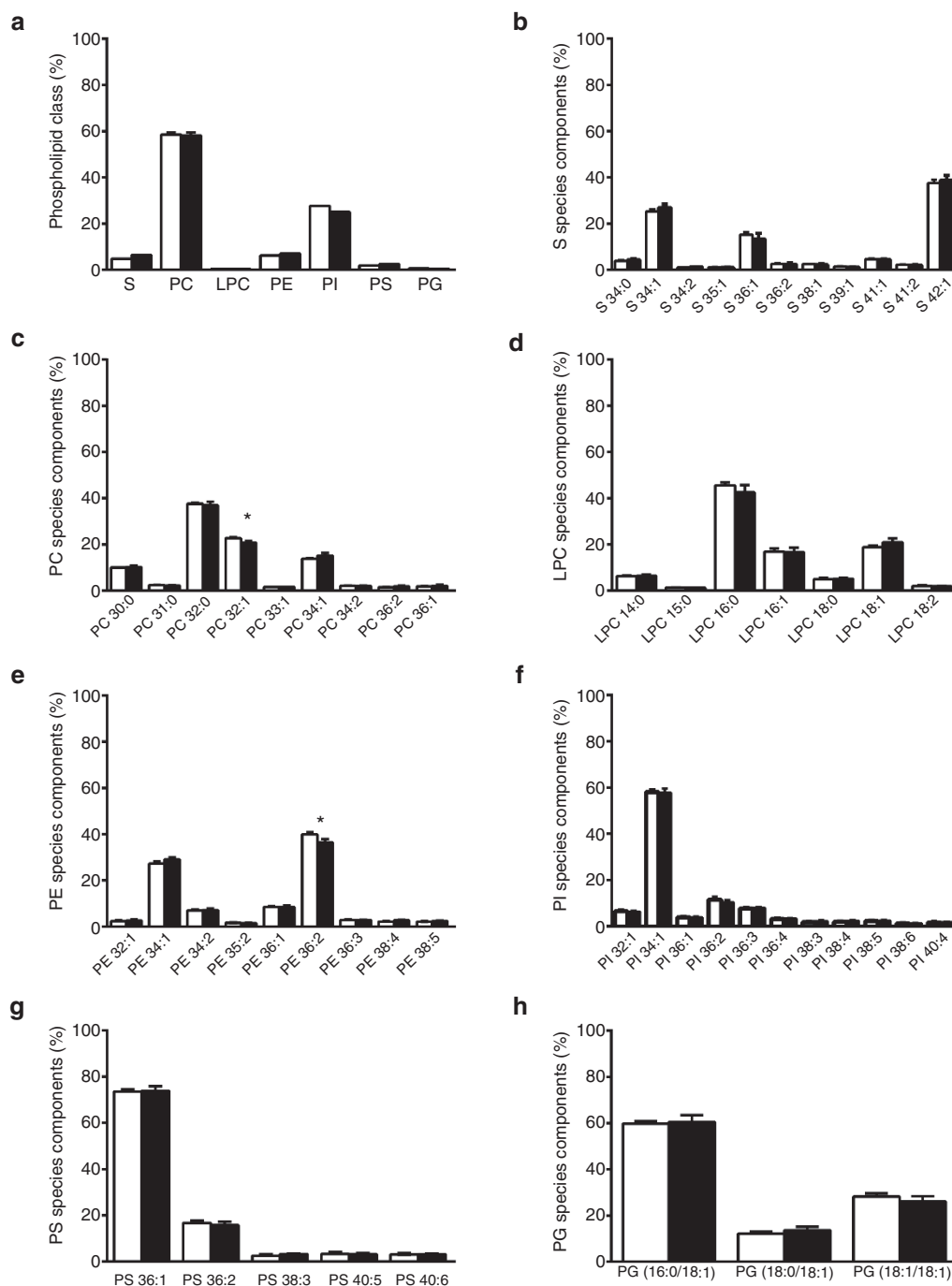


Figure 6. Proportions of major surfactant phospholipid classes and species in bronchoalveolar lavage fluid. **(a)** The proportions of phospholipid classes sphingomyelin (S), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylglycerol (PG) determined in BALF obtained at necropsy in female (open bars) and male (closed bars) lambs. **(b–h)** Proportions of the main phospholipid molecular species of S, PC, LPC, PE, PI, PS, and PG in BALF from female (open bars) and male (closed bars) lambs at necropsy. Data are shown as mean \pm SEM. * $P < 0.05$, female vs. male.

In our previous study (10), we found a 10% reduction in dipalmitoylphosphatidylcholine (PC 32:0) and higher proportions of the plasma phosphatidylcholines, PC 34:2 and PC 36:2, in the BALF of males 4h after preterm delivery, indicating that differences in surfactant phospholipid composition and/or function may, in part, be the cause of the lower lung compliance of males relative to females. Although we did not find differences in these

specific PCs in the present study (at 8h after preterm delivery), males still had a lower lung compliance relative to females; the difference in the pressure–volume relationship suggests that surfactant function is impaired in males compared to females. The total protein concentration of BALF provides an indication of the vascular permeability of the lungs, but more importantly the presence of plasma proteins in the alveolar space is likely to

Table 1. Morphometry and organ weights in female and male lambs

	Female	Male
Morphometric data for postnatal lambs		
Birth weight (kg)	4.70 ± 0.22	4.65 ± 0.22
Weight at 8 h (kg)	4.04 ± 0.15	4.01 ± 0.19
CRL (cm)	51.1 ± 2.1	53.9 ± 1.6
TG (cm)	36.9 ± 0.5	36.4 ± 1.2
HLL (cm)	47.5 ± 1.5	49.9 ± 3.1
PI (g/cm ³ × 100)	2.86 ± 0.42	2.69 ± 0.25
Necropsy organ weights		
Wet lung Wt (g)	133.1 ± 4.9	132.2 ± 7.3
Wet lung Wt/BW (g/kg)	33.9 ± 1.1	33.0 ± 0.9
Dry lung Wt (g)	19.8 ± 1.1	21.2 ± 1.2
Dry lung Wt/BW (g/kg)	5.1 ± 0.2	5.1 ± 0.1
Dry/wet lung Wt	0.15 ± 0.01	0.16 ± 0.00
Lung volume (cm ³)	118.9 ± 5.3	112.6 ± 13.1
Heart (g)	30.6 ± 1.9	30.3 ± 1.1
Heart/BW (g/kg)	7.83 ± 0.28	7.39 ± 0.24
Liver (g)	125.6 ± 10.4	130.7 ± 7.3
Liver/BW (g/kg)	32.4 ± 1.6	31.4 ± 0.9
Kidney (g)	28.5 ± 1.9	31.8 ± 2.5
Kidney/BW (g/kg)	7.39 ± 0.23	7.69 ± 0.41
Adrenal (g)	0.53 ± 0.05	0.51 ± 0.05
Adrenal/BW (g/kg)	0.14 ± 0.01	0.12 ± 0.01
Spleen (g)	6.94 ± 0.71	6.23 ± 0.73
Spleen/BW (g/kg)	1.83 ± 0.19	1.49 ± 0.14

Data represented as mean ± SEM. Lambs were delivered by cesarean section at 133 d of gestational age. Morphometric data and organ weights were collected at necropsy following 8 h of spontaneous breathing. BW, body weight at 8 h; CRL, crown-to-rump length; HLL, hind limb length; PI, ponderal index; TG, thoracic girth; Wt, weight.

reduce the effectiveness of dipalmitoylphosphatidylcholine (and other phospholipids) in lowering surface tension within the lungs (22). In contrast to our previous study of 4 hours' postnatal survival (10), in the present study, we found no difference in total protein concentration in BALF, as well as no difference in the proportions of the plasma PCs 34:2 and 36:2 in BALF, suggesting that the use of CPAP could be less injurious than mandatory positive pressure ventilation.

The lower levels of the pro-SP-C protein observed in the BALF of males compared to females in the present study may contribute to a lower lung compliance. In our previous study, in which BALF was collected at 4 h after delivery, pro-SP-C protein levels were also reduced in preterm males compared to females (unpublished observations). The pro-SP-C protein is synthesized by alveolar type II cells and is the precursor to mature SP-C, which facilitates the adsorption of surfactant lipids onto the surface film lining the alveoli, thereby assisting in preventing alveolar collapse (23,24). Taken together, and in the absence of any difference in lung architecture (25), these results confirm the notion that altered lung surfactant composition in

males likely contributes to the greater incidence of respiratory distress syndrome in male preterm infants.

Conclusions

Male preterm lambs were slightly hypercapnic *in utero* and, after delivery, developed severe hypercapnic acidosis, which slowly ameliorated for up to 8 h after preterm delivery. At 8 h after delivery, the lungs of male preterm lambs were less compliant than in females and had an altered surfactant composition, which most likely contributed to the greater dependency of males on supplemental oxygen. The sex-related differences in postnatal adaptation did not appear to worsen after 4 h, and thus, the decreased survival rate previously reported 8 h after birth (9) cannot be explained. The use of CPAP (4 cmH₂O) did not eliminate the male disadvantage in survival or respiratory function following preterm birth.

Our data suggest that the use of CPAP did not lead to increased vascular permeability in the male preterm lung, unlike the findings of our previous study in which CPAP was not used (10). Taken together, the findings of our two studies suggest that the initial application of CPAP could be effective in maintaining vascular permeability in the preterm lung, which may otherwise be increased by ventilation of incompliant lungs. However, CPAP does not eliminate the innate difference in the composition of pulmonary surfactant. In particular, the ability of male and female preterm infants to achieve acceptable levels of arterial PaCO₂ and pH may be different, with the possibility that males will take longer and require a greater degree of ventilatory support.

We conclude that the male disadvantage in cardiorespiratory adaptation following preterm birth is multifactorial; thus, a combination of ventilatory procedures or higher levels of CPAP, in addition to current treatments (e.g., surfactant administration), may be needed to overcome the increased incidence of respiratory insufficiency observed in males born preterm.

METHODS

All experimental procedures were approved by Monash University Animal Ethics Committee.

Fetal Surgery

Time-mated crossbred ewes underwent surgery at ~125 d after mating (term ≈ 147 d). Using established techniques (10), catheters were chronically implanted into a fetal carotid artery, jugular vein, and amniotic sac. A balloon catheter (~0.5 ml) was implanted into the intrapleural space. A vascular occluder (OC16, In Vivo Metric, Healdsburg, CA) was positioned around the umbilical cord; this was used at delivery to briefly occlude the umbilical cord, thereby preventing anesthetic agents administered to the ewe from entering the fetal circulation.

Fetal Monitoring

Fetal arterial and amniotic catheters were connected to pressure transducers ("DTX Plus" transducer, Becton Dickinson, Singapore, Malaysia), and data were recorded digitally (PowerLab8/30 using LabChart, ADInstruments, Bella Vista, Australia). Fetal arterial pressure and HR were recorded for 1 h on 131 and 132 DG. Following the recording of fetal arterial pressure at 131 DG, betamethasone (5.7 mg i.m., Celestone Chronodose; Schering-Plough, Baulkham Hills, Australia) was administered to the ewe.

Delivery of Lambs

Lambs (nine female; eight male) were delivered at 133 DG, ~14 days before term; this age was chosen as it is the earliest at which independent survival, without the need for mechanical ventilation, is possible (9). The umbilical cord occluder was briefly inflated while sodium thiopentone (25 ml; 50 mg/ml i.v.; Pentothal, Boehringer Ingelheim, Sydney, Australia) was administered to the ewe; this prevented the drug from entering the fetal circulation. The unanesthetized lamb was delivered, together with its catheters, by cesarean section. The time taken from inflating the umbilical cord occluder to cutting the umbilical cord was 110 ± 4 s for male lambs and 115 ± 6 s for females ($P = 0.497$; Student's *t*-test). The lamb was dried, weighed, and placed under a heat source to maintain rectal temperature at 38–39 °C. The ewe was then killed using sodium pentobarbitone (325 mg/ml, i.v.; Lethabarb, Virbac, Milperra, Australia).

Postnatal Monitoring of Lambs

Lambs were monitored for 8 h after delivery, and they were not anesthetized, intubated, or mechanically ventilated. During the 8 h after delivery, lambs were given supplemental oxygen via a Neopuff Infant T-piece resuscitator (Fisher and Paykel, Healthcare, East Tamaki, New Zealand), via a loose-fitting face mask as required to maintain arterial saturation of oxygen (SO₂) above 80%; CPAP was set at 4 cmH₂O. Arterial blood was sampled at 5 min after birth and every 15 min thereafter to measure blood gases, pH, and metabolites (ABL800, Radiometer, DK-2700 Brønshøj, Denmark). Intravenous saline (5 ml) was administered every 15 min for the first hour, while 3 ml of 5% glucose solution (i.v.) was administered 5 min after delivery and every 15 min thereafter if blood glucose concentration fell below 5 mmol/l. Lambs were orally fed colostrum soon after birth, which was milked from the ewe immediately prior to delivery.

Arterial and intrapleural pressure catheters were connected to pressure transducers, and data recorded digitally. During the 8-h postnatal monitoring period, arterial pressure, HR, and intrapleural pressure were continuously recorded. Inspiratory effort was calculated as the maximum amplitude of inspiratory fluctuations in intrapleural pressure. If arterial pH fell below 7.0 and Paco₂ exceeded 100 mm Hg during two consecutive 15-min periods, the lamb was intubated, ventilated, and no longer used in this study. At the end of the 8-h study period, body weight and body dimensions (crown-to-rump length, thoracic girth, hind limb length) were measured.

Pulmonary Pressure–Volume Relationship

At the end of the 8-h postnatal study period, all lambs were anesthetized (sodium thiopentone, 50 mg/ml; Boehringer Ingelheim, Sydney, Australia), intubated, and caused to breathe 100% O₂ for 3 min, after which the endotracheal tube was clamped for 3 min to degas the lungs. Lambs were then killed by an overdose of sodium pentobarbitone (325 mg/ml, i.v.), and the chest wall was opened along the sternum. The volume of air required to inflate the lungs to a maximal luminal pressure of 40 cmH₂O was then recorded. The volume of air removed to maintain luminal pressures of 30, 20, 10, and 0 cmH₂O was also recorded.

Collection of BALF

Lungs were removed and weighed; the left bronchus was then ligated, and the left and right lungs separated. Small portions of the left lung were snap-frozen in liquid nitrogen and stored at –80 °C for molecular analyses. The upper lobe of the right lung was isolated and, the main bronchus cannulated for the collection of BALF. Saline was infused into the right lung via the bronchial cannula and then withdrawn; this was performed three times, and the final sample was collected as BALF. The BALF was centrifuged at 250g (Heraeus Multifuge 3S-R Centrifuge, Thermo Fisher Scientific, Waltham, MA) for 7 min to separate the cellular component. The supernatant was collected and stored at –80 °C for surfactant phospholipid analysis and determination of protein concentration. Other major organs including the heart, liver, spleen, kidneys, and adrenal glands were collected and weighed.

Estimation of Lung Volume

The volume of the right lung was estimated using the Cavalieri method (10,26).

Gene Expression Analysis

Relative surfactant protein (SP)-A, SP-B, SP-C and SP-D mRNA levels in lung tissue were measured using quantitative real-time PCR, as previously described (27). Quantitative real-time PCR was performed using a SYBR green detection method and a Stratagene Mx3000P detection system (Agilent Technologies, Santa Clara, CA). The thermal profile used to amplify the PCR products included an initial 2-min incubation at 50 °C and a 10-min incubation at 95 °C, followed by 45 cycles of denaturation at 95 °C for 20 s and annealing/elongation at 59/60 °C for 60 s; fluorescence readings were recorded after each 60 °C step. Dissociation curves were performed for each quantitative real-time PCR run to ensure that a single PCR product had been amplified per primer set. The relative mRNA level of each gene for each animal was normalized to the mRNA level of the housekeeping gene *ribosomal protein S29* for that animal, using the Δ Ct method (where Ct is cycle threshold). Values were expressed relative to the mean gene mRNA levels in female lambs.

SP Protein Expression

Total protein was extracted from lung tissue by homogenization in radioimmunoprecipitation assay buffer (1% IGEPAL, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1 mmol/l EDTA, 1× PBS pH 7.4, 1× “mini” complete protease inhibitor tablet per 10 ml (Roche Diagnostics, Castle Hill, Australia)). Samples were centrifuged at 16,000g for 20 min at 4 °C. The supernatant was retained, and protein concentrations determined using the Bradford assay (Bio-Rad, Hercules, CA). Total proteins (35 µg for SP-A and 40 µg for pro-SP-C) were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis for SP-A and on a 4–20% Mini-PROTEAN TGX Precast Gel (#456–1093; Bio-Rad, Hercules, CA) for pro-SP-C under reducing conditions. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA), and membranes incubated for 1 h in blocking buffer (5% skim milk powder in PBS containing 0.1% Tween 20 (PBST)) prior to incubation with the primary antibodies, SP-A (1:10,000; rabbit anti-mouse anti-SP-A antibody, Millipore) or pro-SP-C (1:1,000; rabbit anti-mouse anti-pro-SP-C antibody, Millipore), overnight at 4 °C. After washing with PBST, membranes were incubated for 1 h with secondary anti-body (1:15,000; Amersham ECL HRP-linked donkey anti-rabbit IgG, GE Healthcare Life Sciences, Buckinghamshire, UK). Membranes were then washed with PBST, and immunoreactive bands were detected using Immobilon Western Chemiluminescent HRP substrate (Millipore) and x-ray film. Densitometry analysis was performed using ImageQuant TL analysis software (GE Healthcare, Waukesha, WI). To allow for differences in protein loading, membranes were also incubated with rabbit anti-actin polyclonal antibody (1:3,000; Sigma-Aldrich, St Louis, MO). For each protein of interest, the mean protein levels for males were expressed relative to the mean protein levels in females.

Surfactant Phospholipid Analysis

BALF obtained at necropsy was analyzed to assess the effects of sex on surfactant phospholipid composition, as previously described (28). Briefly, phospholipids were extracted from BALF supernatant (10 µl) with 2:1 chloroform-methanol (200 µl) following the addition of internal standards (sphingomyelin 30:1, PC 13:0/13:0, lysophosphatidylcholine 13:0, PE 17:0/17:0, phosphatidylserine 17:0/17:0, and phosphatidylglycerol 17:0/17:0 (Avanti Polar Lipids, Alabaster, AL)). Phospholipid analysis was performed by electrospray ionization tandem mass spectrometry (PE Sciex API 4000 Q/TRAP, Framingham, MA) using a turbo-ion spray source and Analyst 1.5 data system. Prior liquid chromatographic separation was performed on a 1.8-µm, 50% 2.1 mm C18 column (Zorbax, Agilent Technologies, Santa Clara, CA) at 300 µl/min using gradient conditions previously described (28). Individual lipid species were quantified using scheduled multiple reactions monitoring in positive-ion mode (28). Lipid concentrations were calculated by relating the peak area of each species to the peak area of the corresponding internal standard (29). The total lipid content for each class of lipid was calculated by summation of the individual lipid species. The phospholipid class composition was expressed as a molar percentage of the total phospholipids measured. The molecular species composition within each phospholipid class

was expressed as a molar percentage of its respective phospholipid class. Molecular species are denoted as A+B:x+y, where A and B are the number of carbon atoms in the fatty acid chains esterified at the *sn*-1 and *sn*-2 positions, respectively, and x and y are the number of double bonds in the fatty acid chains.

Protein Concentration in BALF

The total protein concentration of BALF supernatant was determined by a protein assay (Bio-Rad), as previously described (10).

Statistical Analyses

Physiologic data were analyzed over three different time periods. Firstly, prenatal data were analyzed between 128 DG and 133 DG. Postnatal data were separately analyzed over (i) the first hour after delivery and (ii) the next 7 h, because physiologic responses differed between these periods. Blood chemistry data, cardiovascular data, respiratory data, and pressure-volume curves were all analyzed using a one-way repeated measures ANOVA, with sex (P_s) and time (P_T – repeated factor) as factors; where appropriate, the Greenhouse-Geisser correction was used. Significant differences detected by ANOVA were further analyzed by the Fisher least significant difference *post hoc* test. Data relating to the requirement for supplemental oxygen were analyzed by a log rank (Mantel-Cox) test. We used the Student's unpaired *t*-test to test for sex-related differences in relative SP mRNA levels, SP protein levels, protein concentration in BALF, surfactant phospholipid composition, body weights and dimensions and organ weights. All statistical tests were performed using IBM SPSS Statistics, Version 22 for Windows (IBM, Armonk, NY). The level of significance was taken at $P < 0.05$; data are presented as mean \pm SEM.

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