Development of the pulmonary surfactant system in two oviparous vertebrates

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Johnston, Sonya D., Sandra Orgeig, Olga V. Lopatko, and Christopher B. Daniels. Development of the pulmonary surfactant system in two oviparous vertebrates. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R486–R493, 2000.—In birds and oviparous reptiles, hatching is often a lengthy and exhausting process, which commences with pipping followed by lung clearance and pulmonary ventilation. We examined the composition of pulmonary surfactant in the developing lungs of the chicken, Gallus gallus, and of the bearded dragon, Pogona vitticeps. Lung tissue was collected from chicken embryos at days 14, 16, 18 (prepippped), and 20 (postpippped) of incubation and from 1 day and 3 wk posthatch and adult animals. In chicken, surfactant protein A mRNA was detected using Northern blot analysis in lung tissue at all stages sampled, appearing relatively earlier in development compared with placental mammals. Chickens were lavaged at days 16, 18, and 20 of incubation and 1 day posthatch, whereas bearded dragons were lavaged at day 55, days 57–60 (postpippped), and days 58–61 (posthatched). In both species, total phospholipid (PL) from the lavage increased throughout incubation. Disaturated PL (DSP) was not measurable before 16 days of incubation in the chick embryo nor before 55 days in bearded dragons. However, the percentage of DSP/PL increased markedly throughout late development in both species. Because cholesterol (Chol) remained unchanged, the Chol/PL and Chol/DSP ratios decreased in both species. Thus the Chol and PL components are differentially regulated. The lizard surfactant system develops and matures over a relatively shorter time than that of birds and mammals. This probably reflects the highly precocial nature of hatchling reptiles.

lungs; phospholipid; cholesterol; lung fluid; oviparity; pipping; lizard

TO BEGIN PULMONARY VENTILATION, any newborn animal must clear its lungs of fluid and inflate them with air. Pulmonary surfactant is a complex mixture of lipids and proteins that is crucial for the initiation of air breathing by lowering surface tension at the air-liquid interface. It reduces the work of breathing, promotes alveolar stability, and prevents transudation of fluid (38). Pulmonary surfactant is comprised of disaturated phospholipids [PL (DSP)], unsaturated PLs (USP), cholesterol (Chol), and the four surfactant proteins (SP), SP-A, SP-B, SP-C, and SP-D. DSP molecules are the surface active component of surfactant. The structure of the hydrophobic fatty acid tails and hydrophilic head groups enable DSP molecules to aggregate at the air-liquid interface, displacing water and lowering surface tension (32). The USPs and Cholesterol are thought to maintain fluidity of the surface film over varying body temperatures (8, 10), whereas the proteins are important in the uptake, secretion, and homeostasis of the lipids and in innate lung defense (37).

Pulmonary surfactant has been found in gas holding structures from representatives of every vertebrate group, including the single-chambered lung of lizards and the parabronchial lung of birds (8, 10). The lungs of the lizard, Pogona vitticeps, consist of a paired baglike structure with respiratory epithelium spanned between the outer wall of the lung and an inner tracheobronchial network (23). In the avian lung, air is propelled through tiny tubules within the rigid lung with the aid of avascular airsacs, which act as bellows (13, 14). In the reptilian lung, pulmonary surfactant primarily acts as an antiadhesive, whereas it may function to prevent edema in the avian lung (10).

Birds and most reptiles hatch from eggs, an event that may take place over several days, unlike the rapid transition from an intrauterine to an extraterrestrial environment displayed in most mammals. Birds and reptiles commence pulmonary ventilation by “pipping.” Birds pip internally into an aircell within the confines of the shell, whereas reptiles pip directly through the shell. Embryos may remain in this state for some time prior to escape from the egg, retaining a well-vascularized portion of the chorioallantoic membrane, the extra-embryonic respiratory organ. This, in turn, results in a dual respiratory system at the onset of pulmonary ventilation (40).

In eutherians, the pulmonary surfactant system develops late in gestation and is indicated by an increase in the amount of total PL and by the enrichment of DSPs in both tracheal and amniotic fluid (4, 15, 20). Glucocorticoids initiate maturation of the fetal lung, acting directly on the alveolar type II cell and enhancing the synthesis of surfactant PLs via a receptor-mediated response (17). Cortisol is released in response to fetal hypoxia. As the fetus grows, its demand for oxygen surpasses the supply from the placenta, thereby triggering the release of cortisol from the adrenal cortex. The chicken embryo also experiences hypoxia late in incubation as a result of increased oxygen demand and limited gas exchange through the pores of the shell (40). Although some experimental evidence...
corroborates the putative role of fetal corticosteroids in avian lung maturation (21, 26), the exact nature of the cascade of events that results in maturation of the surfactant system in birds remains unclear.

Despite the differences in oviparous and viviparous parturition and the vastly different morphology of respiratory systems, all lungs must be prepared to commence gas exchange. Little is known about the maturation of the pulmonary surfactant system in oviparous vertebrates. Morphological studies have demonstrated the appearance of lamellar bodies, the intra-cellular storage site of surfactant lipids, and an osmophilic surface film in the latter stages of incubation in the embryonic chicken lung (18, 21, 31). Total and saturated PL increase in lung homogenates of the chicken embryo during incubation (18, 21, 26, 36), with males having an elevated content and saturation of PLs throughout development compared with females (26). Although these studies show trends in the development of the pulmonary surfactant system in chickens, they involved the use of homogenates that are significantly contaminated by cell membrane lipids. Surfactant proteins SP-B and SP-A have been detected in the developing chick on the 15th day of incubation and 1 day after hatching, respectively (44). To date, no studies have been carried out on the development of the surfactant system in reptiles. The present study compares the maturation of the pulmonary surfactant system in birds, Gallus gallus, and oviparous lizards, Pogona vitticeps, with this process in a bird, Gallus gallus. We postulate that the development of the pulmonary surfactant system is similar in these animals despite their phylogenetic, morphological, and reproductive differences.

**MATERIALS AND METHODS**

Animals. Adult and 3-wk-old chickens, Gallus gallus, and fertilized chicken eggs were obtained from a commercial supplier (Globe Derby Poultry, Bolivar, SA, Australia). Eggs were incubated under normoxic and normobaric conditions at 39°C in a Bellsouth 100 automatic turner (Bellsouth, Narre Warren, Victoria, Australia).

Six gravid bearded dragons, Pogona vitticeps, were collected from the southern Flinders Ranges in October, 1997. They were housed in cages (1.0 × 0.65 × 0.45 m) and were provided with water ad libitum and fresh fruit, vegetables, calcium supplement, and mealworms (Tenebrio larvae) twice per week. Females chose to lay their eggs in containers filled with damp sphagnum moss situated within the cages. Each egg was marked to indicate its orientation at oviposition. This orientation was maintained throughout incubation. Individual clutches were then transferred to plastic boxes containing fine vermiculite (∼5 mm particles) with a gravimetric water content of 1 g/g dry mass, resulting in a water potential greater than −200 kPa (29). Containers were weighed after the eggs were deposited, then sealed and placed in a constant temperature cabinet at 29°C. Boxes were rotated throughout the cabinet daily to minimize exposure to fluctuations in temperature. To maintain a constant water potential within the substrate, clutches were weighed weekly and distilled water was added to the vermiculite until the initial mass of the container was reached.

Sampling. Chicken eggs were sampled after 14, 16, 18 (prepipped), and 20 days (postpipped) of incubation and during the first 24 h after hatching (hatch group). Pipping occurred on day 19 of incubation. Embryos at 14, 16, and 18 days of incubation were killed by dipping eggs in liquid nitrogen. Animals that had commenced pulmonary ventilation were killed either by CO₂ inhalation (embryos incubated for 20 days and hatchlings) or, in the case of adults, by careful injection of pentobarbital sodium (150 mg/kg body mass (BM) Nembutal, Abbott Laboratories, Sydney, NSW, Australia) into the peritoneal cavity.

Bearded dragon eggs were sampled from each clutch after 55 days of incubation, the time of pipping, 58.83 ± 0.47 days of incubation (mean ± SE) and after hatching, 59.25 ± 0.63 days of incubation (mean ± SE). Embryos and hatchlings were killed by a massive dose of pentobarbital sodium injected intraperitoneally.

Detection of SP-A mRNA. Lungs were removed from chicken embryos after 14, 16, 18, and 20 days of incubation and from hatchlings, a 3-wk-old chick, and an adult. All lungs were snap-frozen in liquid nitrogen and stored at −80°C for further analysis. Total RNA was isolated from lung tissue using TRIReagent (Sigma Chemical, St Louis, MO) following the instructions of the manufacturer. Total RNA was dissolved in sodium acetate and the quantity and purity were determined by absorbance at 260 nm. Viability of total RNA was determined using 1× TAE (40 mM Tris, 20 mM Na acetate, 1 mM EDTA, pH 7.2) agarose minigels stained with ethidium bromide and visualized under ultraviolet light. Total RNA from mouse lung was used as a positive control. Northern blot analysis was performed by a modified method of Sullivan et al. (35). Briefly, 20 µg of total RNA from chicken lung tissue and 5 µg from mouse were denatured and separated on a 1% agarose formaldehyde gel. RNA was then transferred to a nylon membrane and baked at 75°C for 30 min, then incubated in prehybridization solution containing 0.1 mg/ml of denatured salmon sperm DNA (Boehringer Mannheim) overnight at 42°C. Approximately 2 × 10⁶ counts/min 1·ml⁻¹ of 32P-labeled mouse SP-A DNA were added to the membranes and hybridized for 3 days at 42°C. The membranes were washed twice in a solution containing 0.1% SDS in 1× standard sodium citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate) at 42°C for 30 min/wash. The membranes were then dried, sealed in plastic bags, and exposed to a Fuji PhosphorImager screen (FujiBas) for 60 h. RNA for bearded dragons was not available as the number of animals required for an adequate sample was not warranted.

Lavage protocol. Chicken embryos at 16, 18, and 20 days of incubation, hatchlings, and adults were tracheal cannulated, and the lungs were lavaged with three volumes of chilled 0.15 M NaCl (0.04 to 0.07 ml/g BM) instilled and withdrawn three times per volume. Any fetal lung fluid (FLF) present was incorporated into the lavage. Lavage from individuals was centrifuged (Beckman model TJ-6 centrifuge) for 5 min at 800 × g. The supernatant was decanted and withdrawn three times. Volumes ranging from 40 to 80% of the 0.5-ml reservoir were injected as embryos increased in mass. Any lung fluid present was incorporated into the
lavage. Lavage was centrifuged for 5 min at 150 g to remove cellular debris. Because the total lavage was so small, the lavage from two individuals at the same incubation age and from the same clutch were pooled.

Lipid analyses. Lipids were extracted from lavage with a chloroform and methanol mixture (1:2 vol/vol) (5). Phosphorus was measured by the method of Bartlett (3) and total PL was determined by multiplying the phosphorus content by 25, as PLs contain ~4% phosphorus. The DSP fraction was separated from the neutral lipid fraction on aluminum oxide using adsorption chromatography (22). The neutral lipid fraction was dried under nitrogen and reconstituted in isopropanol at 2°C. Chol was quantified using a high-pressure liquid chromatography system (11), comprised of a Waters pumping system (model M-45, Waters, Milford, MA) and an LKB 2157 autosampler (Pharmacia LKB Biotechnology, Uppsala, Sweden). Twenty microliters of either sample or standard were injected onto a Waters C-18 Novopak guard column (3.9 × 50 mm) and a Waters C-18 Novapak analytical column (150 × 4.6 mm ID) packed with 4-µm silica spheres. Isocratic elution of Chol was completed within 32 min at room temperature using a mobile phase consisting of acetonitrile, isopropanol, and water (63:31 vol/vol/vol). A flow rate of 1 ml/min and an operating pressure of 1,600 psi was maintained throughout the elution, and ultraviolet absorbance was recorded at 210 nm. The detector output was digitized using a Delta Chromatography data system for acquisition of data and integration of peaks (Digital Solutions, Brisbane, Australia). Standards were assayed in duplicate and were included at the beginning of each run (5–50 µg/ml).

Statistical analyses. Chol/PL and Chol/DSP are expressed as microgram-to-microgram ratios, whereas DSP/PL is expressed as a percentage. Data were analyzed using a one-way ANOVA followed by paired t-tests where appropriate after arcsin transforming the ratios. Statistical significance was assumed when P < 0.05.

RESULTS

Pulmonary surfactant in the developing chicken lung. BMs of chicken embryos increased significantly between embryos incubated for 16 and 18 days (P < 0.001) and between embryos incubated for 18 and 20 days (P < 0.001); however, there was no difference between embryos incubated for 20 days and hatchlings (P = 0.24) (Table 1). Dry lung weights from chicken embryos increased significantly between adjacent sampling groups (P < 0.05 for all tests; Table 1), yet the ratio of dry lung weight to BM did not differ significantly between chicken embryos incubated for 16 and 18 days (P = 0.31). This indicates that the recovery of material lavaged from the lungs of embryos incubated for 16 and 18 days was equivalent to lavage from older embryos relative to the size of the lungs and body (Table 1). FLF was observed in the fetal chick lung after 16 and 18 days of incubation, however, no lung fluid was observed following the 20th day of incubation.

Northern blot analysis demonstrated that the mouse SP-A cDNA probe hybridized with RNA from chick embryos incubated for 14, 16, 18, and 20 days, hatchlings, 3-wk-old chick, and adult chicken and mouse (Fig. 1). Three transcripts were present in the mouse.

Chicken Chol, expressed as a function of dry lung weight, did not differ during incubation (ANOVA, P = 0.11), however, both total PL and DSP were present in significantly lower amounts in chicken embryos incubated for 16 days (P < 0.05) than in embryos that had pipped and in hatchlings (Table 1). DSP/PL increased from 0 in 16-day incubated chick embryos to 17.74 ± 3.55% in embryos incubated for 18 days (P < 0.01) with a further increase to 30.66 ± 5.65% in 20-day incubated embryos (P < 0.05; Fig. 2A). DSP/PL was present in statistically similar amounts in chicken embryos incubated for 20 days in hatchlings and in adults (Fig. 2A). The Chol/PL ratio declined significantly throughout the latter part of incubation, with a twofold reduction from day 16 (0.40 ± 0.07) to day 18 embryos (0.19 ± 0.04; P < 0.01) and a further reduction after pulmonary ventilation had commenced at day 20 (0.14 ± 0.04) to hatching (0.09 ± 0.01; P < 0.05; Fig. 2B). Hatching Chol/PL ratios were comparable to adults (0.07 ± 0.02; Fig. 2B).

Because DSP was undetectable in chicken embryos incubated for 16 days, the Chol/DSP ratio approached infinity, therefore, no statistical tests could be performed on this stage. The Chol/DSP ratio declined to 1.47 ± 0.50 in embryos incubated for 18 days with a further twofold reduction to 0.49 ± 0.08 in day 20 embryos (P < 0.05). There was no difference between day 20 embryos and hatchlings (P = 0.07). Chol/DSP was significantly lower in adults (0.22 ± 0.05; P < 0.05; Fig. 2C).

Pulmonary surfactant in the developing bearded dragon lung. Fetal lung fluid was observed in one bearded dragon embryo incubated for 55 days, whereas none was detectable in embryos that had pipped. BMs of bearded dragon embryos differed significantly among

| Table 1. BM, DLW, and surfactant lipid composition of embryonic and hatching chickens |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Age             | BM, g           | DLW, mg         | DLW/BM, %       | Total PL, µg/mg DL | DSP, µg/mg DL | Chol, µg/mg DL |
| Day 16          | 18.03 ± 0.40    | 20.23 ± 1.09    | 0.11 ± 0.01     | 0.26 ± 0.09        | NM             | 0.09 ± 0.02     |
| Day 18          | 24.37 ± 0.44    | 26.32 ± 1.62    | 0.11 ± 0.01     | 0.99 ± 0.21        | 0.18 ± 0.08    | 0.15 ± 0.03     |
| Day 20          | 33.77 ± 1.31    | 32.95 ± 1.53    | 0.08 ± 0.004    | 0.69 ± 0.21        | 0.16 ± 0.04    | 0.07 ± 0.02     |
| Hatch           | 38.54 ± 1.06    | 42.36 ± 3.48    | 0.11 ± 0.01     | 0.93 ± 0.24        | 0.25 ± 0.07    | 0.08 ± 0.02     |
|                 |                 |                 |                 |                  |                |                |
| Results are means ± SE; number of chickens in parentheses. DLW, dry lung weight; BM, body mass; PL, total phospholipid; DSP, disaturated PL; Chol, cholesterol; DL, dry lung; NM, not measurable; Hatch, hatching. |
sampling groups (\(P < 0.05\) for all tests), whereas lung masses did not differ (ANOVA, \(P = 0.52\); Table 2). Total PL and DSP, expressed per milligram dry lung were significantly lower in embryos incubated for 55 days than embryos that had pipped and hatchlings, whereas Chol remained unchanged between groups (ANOVA, \(P = 0.52\); Table 2). DSP/PL increased significantly from 0.71 ± 0.71% in bearded dragon embryos incubated for 55 days to 25.87 ± 2.73% in those that had pipped (\(P < 10^{-5}\)) and remained unchanged thereafter (hatch: 26.18 ± 3.40%; Fig. 3A). The Chol/PL ratio of bearded dragons decreased significantly from 0.14 ± 0.05 in embryos incubated for 55 days to 0.05 ± 0.01 in embryos that had pipped (\(P < 0.05\)), with further reduction to 0.03 ± 0.01 in hatchlings (\(P < 0.05\); Fig. 3B). Because DSP was undetectable in bearded dragons incubated for 55 days, Chol/DSP approached infinity. Hence, there was a marked decrease in the Chol/DSP ratio from bearded dragon embryos incubated for 55 days to embryos that had pipped (0.23 ± 0.08) and no difference between pipping and hatchling groups (hatch: 0.15 ± 0.07; \(P = 0.24\); Fig. 3C).

**DISCUSSION**

**SP-A mRNA.** Previously, we demonstrated the presence of two SP-A mRNA transcripts of 3.7 and 1.7 kb in adult chicken lung using a mouse SP-A cDNA probe (35). Here, we identified two SP-A mRNA transcripts in chicken embryos after 14 days of incubation (Fig. 1). The bands were weak and poorly defined, presumably because of the low degree of homology between the chicken samples and the mouse probe (35). Chicken SP-A mRNA was present after 66% of the total incubation time, substantially earlier than that of the rat, rabbit, and baboon (relative to their lengths of gestation). Rat SP-A mRNA is not detectable until the 18th day of gestation (term = 22 days) (33), the 26th day (83%) of gestation in the rabbit (term = 31) (24), and the 150th day (83%) of gestation in the baboon (term = 180) (25). SP-A mRNA is undetectable (2) or is present in very low amounts (41) in the second trimester human fetus. The timing of the appearance of SP-A mRNA is, therefore, substantially different among species.

**PLs.** In the present study, we used the method of Mason et al. (22) to extract DSP. Whereas this method overestimates the dipalmitoylphosphatidylcholine (DPPC) fraction by detecting both DSP and monoenoic species (39), it is acceptable when used to compare values between experimental groups.

The increase in total PL and DSP in surfactant from chicken embryos incubated for 16 (76% of total incubation) to 18 days (86% of incubation; Table 1) resembles the compositional changes of lung fluid and amniotic fluid in mammals. Total PL, phosphatidylcholine (PC), and DPPC increases exponentially between the 19th day (86%) of gestation and the first postnatal day in the fetal rat (20). Likewise, the concentration of total PL in lung liquid and amniotic fluid from fetal sheep in-
creases dramatically with increasing gestational age from low concentrations on the 110th day (75%) of gestation to a 2.5-fold increase between the 125th (86%) and 130th day (90%) of gestation (term 5146 days). This occurs with a concomitant increase in surface activity (15). Benson et al. (4) found PC and DPPC do not reach adult levels in the fetal sheep until 3–4 days before term. Similarly, bearded dragons dramatically increased total PL and DSP during the latter part of incubation from the 55th day (92%) of incubation to pipping (98% of incubation; Table 2). However, relative to incubation time, the timing of release was extremely truncated. In addition, chicken surfactant did not attain a “mature” composition until after pulmonary ventilation had been established, as DSP content continued to increase relative to total PL (Fig. 2A). However, in bearded dragons, adult levels of DSP/PL (measured at a body temperature of 23°C) (9) were achieved at

Table 2. BM, DLW, and surfactant lipid composition from embryonic and hatchling bearded dragons

<table>
<thead>
<tr>
<th>Age</th>
<th>BM, g</th>
<th>DLW, mg</th>
<th>DLW/BM, %</th>
<th>PL, µg/mg DL</th>
<th>DSP, µg/mg DL</th>
<th>Chol, µg/mg DL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 55</td>
<td>2.31 ± 0.13 (11)</td>
<td>3.16 ± 0.20 (11)</td>
<td>0.13 ± 0.01 (11)</td>
<td>1.25 ± 0.86 (5)</td>
<td>0.03 ± 0.03 (5)</td>
<td>0.11 ± 0.06 (5)</td>
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<tr>
<td>Pip</td>
<td>3.17 ± 0.10 (15)</td>
<td>3.44 ± 0.12 (15)</td>
<td>0.11 ± 0.01 (15)</td>
<td>3.75 ± 0.88 (7)</td>
<td>1.04 ± 0.32 (7)</td>
<td>0.18 ± 0.04 (7)</td>
</tr>
<tr>
<td>Hatch</td>
<td>2.85 ± 0.11 (16)</td>
<td>3.17 ± 0.21 (16)</td>
<td>0.11 ± 0.01 (16)</td>
<td>4.42 ± 0.84 (7)</td>
<td>1.19 ± 0.29 (7)</td>
<td>0.13 ± 0.03 (7)</td>
</tr>
</tbody>
</table>

Results are means ± SE; number of bearded dragons in parentheses. Determination of PL, DSP, and Chol required pooling lavage material from 2 individuals.
pipping (Fig. 3A). Therefore, the lizard surfactant system appears to develop and mature over a relatively shorter time than that of birds and mammals.

Chol. In the adult lizard, Ctenophorus nuchalis, and in the marsupial, Sminthopsis crassicaudata, the Chol content of the surfactant increases with decreasing body temperature, presumably to maintain the fluidity of the surface film over fluctuating body temperatures (7, 19). It is unlikely that Chol is important in maintaining fluidity in the developing lung as temperature remained constant throughout incubation. Despite the large amounts of Chol that are present throughout development, the function of Chol in the premature lung is not known. Prior to the commencement of pulmonary ventilation, both Chol/PL and Chol/DSP declined rapidly in the developing chicken (Fig. 2, B and C) and lizard lung (Fig. 3, B and C) during incubation by increasing PL and DSP without altering Chol. In both the chicken and lizard, the content of Chol did not alter throughout development, despite marked changes in the content and saturation of the PLs (Tables 1 and 2). Similarly, Chol content does not change in the developing lung of the tiger salamander, Ambystoma tigrinum. Yet, unlike the chicken and lizard, total PL remains unchanged, and DSP decreases throughout development in this amphibian (28). The reasons and mechanisms by which changes in Chol occur during lung development remain unknown. However, it is likely that the factors regulating Chol are independent of those controlling PLs. Moreover, the Chol and DSP components of surfactant in the alveolar compartment of rats (27) and humans (12) are handled differently and appear to be differentially released.

Surprisingly, to our knowledge the changes in the amount of alveolar Chol and composition prior to birth have never been described for any mammal, thus comparisons cannot be drawn between species. The Chol/PL and Chol/DSP ratios have also not been previously described in birds. Adult chickens have Chol/PL similar to that of mammals and reptiles, and Chol/DSP levels are also similar to those of the other terrestrial tetrapods (8). However, the Chol/PL and Chol/DSP ratios of embryonic chicks (Fig. 2, B and C) are much greater than any previously recorded (8, 10).

General discussion. It appears that the general pattern for the development of the pulmonary surfactant system is similar between reptiles, birds, and mammals. The amount and saturation of the PLs increased late in either incubation or gestation, whereas Chol was independently regulated. However, it is the relative timing of such events that differed considerably between birds and reptiles. Birds exhibited a protracted release and saturation of PL when compared with lizards. This was demonstrated by the presence of an intermediate level of DSP/PL on the 18th day of incubation in the chicken (Fig. 2A), which was not present in the bearded dragon. Although sampling of bearded dragons was infrequent, the sampling periods relative to total incubation time showed clearly that the lizard surfactant system matures very quickly. The composition of lavage from bearded dragons changed from a surfactant low in PL and DSP and high in Chol to a mature surfactant high in PL and DSP and low in Chol over ~6% of the total incubation time (92% at day 55 to 98% at pipping). Chickens, on the other hand, took ~20% of total incubation time for surfactant to change to a mixture high in saturated PLs (76% at day 16 to 95% at day 20).

The transition from in ovo to hatching in chicken and bearded dragon embryos reflects the timing of release of surfactant lipids in the latter part of incubation. Bearded dragons spend ~2% of their total incubation time "pipping," whereas the corresponding time in chickens is 9.5%. Such temporal differences may relate to differences in the lung morphology and lung clearance between birds and reptiles. FLF was observed in the chicken lung after 16 and 18 days of incubation, whereas very little FLF was observed in bearded dragons prior to pipping. Retention of FLF until pipping in the chicken embryo may reflect the relatively large size of the avian respiratory system, or it may be necessary to maintain patency of the airsacs prior to ventilation as they lack a surface film to prevent adhesion of apposing surfaces. Furthermore, FLF present in avascular airsacs is a great distance from the site of clearance within the lung, which may result in an increased time of pipping. Several authors have suggested that avian surfactant prevents accumulation of liquid in the air capillaries (14, 30, 31). After internally pipping, birds may rely on their choioallantoic membrane for gas exchange while titrating their surfactant and aerating the tiny air capillaries and parabronchi. The greatest aeration of the avian lung occurs following internal pipping. This is achieved by proliferation of the air capillaries and removal of fluid from the parabronchi by convection and reabsorption (34). Hence, the presence of pulmonary surfactant would facilitate the reabsorption of fluid within the parabronchi and air capillaries.

The lack of FLF and the rapid release and saturation of the PLs at pipping in bearded dragons suggests the lungs and surfactant system are "prepared" at the onset of pipping. Hatching reptiles are highly precocial and must undergo bouts of digging to free themselves from the nest (1), which are often followed by sprints to avoid predation (6). Their pulmonary surfactant system must be prepared directly after hatching to tolerate temperature fluctuations outside the nest and to undertake intense activity. Body temperature affects the ability of hatching Galapagos land iguanas, Conolophus pallidus, to avoid predation from hawks when dispersing from the nest (6). Total PL from bearded dragon hatchlings surpassed amounts observed in the adult bearded dragon at a body temperature of 37°C (adults: 2.27 ± 0.33 µg/mg dry lung vs. Table 2) (43). This may be attributed to an elevated density of adult lung tissue or to the requirement for greater amounts of PL to flee the nest as total PL content increases with exercise in the adult bearded dragon lung (42). Moreover, temperature profoundly affects breathing frequency and metabolism in agamid lizards (16) further
emphasizing the need for a well-functioning surfactant system following hatching.

In conclusion, the pattern of development of the surfactant lipids in the embryonic chicken and bearded dragon was similar to that of mammals. However, the lipids developed more rapidly in the embryonic bearded dragon relative to total incubation time than in embryonic chickens and fetal mammals, reflecting the highly precocial state of the young. SP-A mRNA appeared earlier in the embryonic chicken lung than in mammals relative to incubation time, which may relate to an uncoupling of the immunological role of SP-A from the function of the lipids, whereas Chol did not change throughout development and, therefore, is independently regulated from the PLs.

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