DEVELOPMENT OF THE PULMONARY SURFACTANT SYSTEM IN NON-MAMMALIAN AMNIOTES

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In memory of my grandparents, William Henry and Mary Ellen Johnston, who inspired and nurtured my interest in the marvels of nature.

ABSTRACT

Pulmonary surfactant is a complex mixture of phospholipids, neutral lipids and proteins that lines the inner surface of the lung, where it modulates surface tension thereby increasing lung compliance and preventing the transudation of fluid. In mammals, the pulmonary surfactant system develops towards the end of gestation, characterised by an increase in the saturation of phospholipids in lung washings and the appearance of surfactant proteins in amniotic fluid. Birth, the transition from *in utero* to the external environment, is a rapid process. At this time, the pulmonary surfactant system is important in opening and clearing the lungs of fluid to initiate pulmonary ventilation. In oviparous vertebrates, escape from an egg can be a long and exhausting process. The young commence pulmonary ventilation and hatching by "pipping" through the eggshell, where they remain for some time, presumably clearing their lungs. This study describes the development of the pulmonary surfactant system within the non-mammalian amniotes and relates changes in development in response to birth strategy, lung morphology and phylogeny in order to determine the extent of conservation within this developmental process. Total phospholipid (PL), disaturated phospholipid (DSP) and cholesterol (Chol) were quantified from lung washings of embryonic and hatchling chickens, oviparous bearded dragons, viviparous sleepy lizards, snapping turtles and green sea turtles throughout the final stages of incubation and gestation.

In addition to the lipids, another specific parameter was measured for each species to further describe the development of the system. In all cases, the pattern of development of the pulmonary surfactant lipids was consistent with that of mammals. PL and DSP increased throughout the latter stages of development and Chol was differentially regulated from the PLs. Maximal secretion of both PL and DSP occurred at "pipping" in oviparous reptiles, coincident with the onset of airbreathing. Similarly, the amount of DSP relative to total PL was maximal immediately after the initiation of airbreathing in chickens. The

relative timing of the appearance of the lipids differed between groups. In the oviparous lizard, surfactant lipids were released over a relatively shorter time than that of the viviparous lizard, turtles, the chicken and mammals.

The morphology and maturation of the type II cells from bearded dragons matched that of mammals. Surfactant protein (SP)-A messenger RNA was detected in chicken lung tissue throughout development, appearing relatively earlier in development compared to mammals. Unlike the surfactant lipids, the antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase, did not differ appreciably throughout gestation in the viviparous lizard, suggesting that the pulmonary surfactant system and antioxidant enzyme system develop independently of each other. Expression of SP-B and thyroid transcription factor-1 (TTF-1), a mammalian regulator of cell differentiation and gene expression of surfactant proteins was similar between mammals and the freshwater turtle. Environmental cues, such as hypoxia, did not affect incubation time, absolute, nor relative abundance of the surfactant lipids in sea turtles, demonstrating that the development of the system is robust in this species.

Despite temporal differences and vastly different lung morphologies, birth strategies and phylogenies, the overall development and maturation of the pulmonary surfactant lipids and proteins are highly conserved amongst the amniotes. However, the stimuli for secretion and the development of other systems crucial to airbreathing, such as the antioxidant enzyme system, show greater plasticity throughout evolution.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text. The author consents to this thesis being made available for photocopying and loan when deposited in the University Library.

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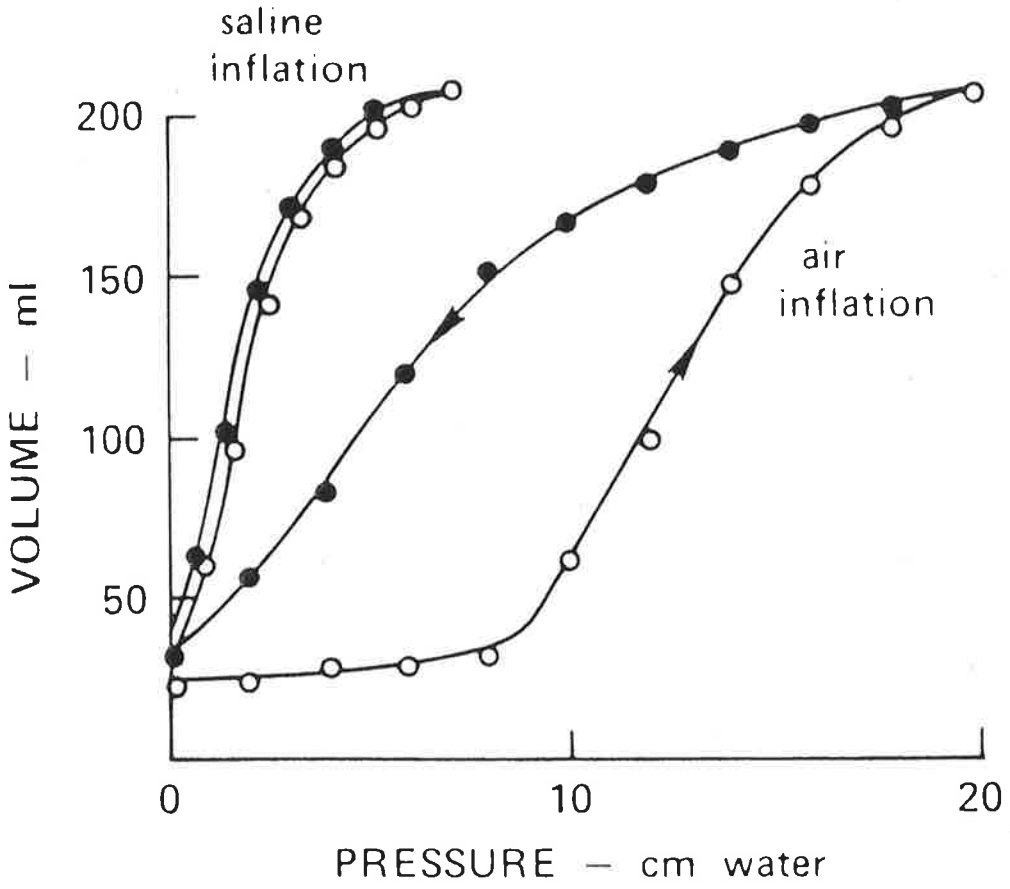
CHAPTER 1 DEVELOPMENT, REGULATION AND EVOLUTION OF THE PULMONARY SURFACTANT SYSTEM IN VERTEBRATES

1.1 PROLOGUE

One of the primary functions of pulmonary surfactant is its ability to lower surface tension to remarkably low values. In doing so, surfactant reduces the work of inspiration by increasing lung compliance. The history of pulmonary surfactant research spans the last 7 decades, commencing with lung compliance discoveries of von Neergaard (1929). von Neergaard (1929) demonstrated that the surface forces of the air/liquid interface of the mammalian lung made a considerable contribution to the retractile pressure of the lung, or the tendency of the lung to collapse at any given volume. This function was determined by comparing the pressure-volume curves from air-filled lungs, where the surface forces were intact, with those of liquid-filled lungs, where surface forces had been eliminated from an array of different mammals (for example, the cat, Figure 1.1). The resultant differences between the curves led von Neergaard (1929) to conclude that approximately 66-75% of the retractile pressure of the lung was due to surface forces. Furthermore, differences in the pressure-volume curves between inspiration and expiration of air-filled lungs indicated that surface tension *varied* during ventilation. Thus, the surface tension within the alveoli had to be lower than that of the fluids in the surrounding tissue, possibly resulting from the accumulation of a surface-active material at the alveolar/air interface; a surfactant. This discovery also led von Neergaard (1929) to suggest that alveolar collapse (atelectasis), observed in some newborns, may have resulted from the considerable force of surface tension, inhibiting the initial expansion of the lungs following birth. While the presence of a surface-active material had been suggested, its existence was not demonstrated for a further 26 years. Pattle (1955) observed the first stable bubbles within pulmonary oedema fluid from rabbit lungs. These bubbles, unlike bubbles in blood, maintained their size over

Figure 1.1 Air-filled and saline-filled pressure-volume curves from excised cat lungs during inflation (○) and deflation (●). Reproduced from West (1995).


saline
inflation
air
inflation
200
150
100
50
VOLUME – ml
0
10
20
PRESSURE – cm water

longer periods and were impervious to the action of silicone antifoams. Therefore, it was believed that the stability of the bubbles resulted from the presence of a surface-active material with an extremely low surface tension originating from the lining layer of the pulmonary air spaces (Pattle, 1955). Concurrently, Clements (1957) detected and quantified the surface-active properties of lung extracts using a Langmuir-Wilhelmy balance. With this instrument, he was able to determine the changes in surface tension by surfactant during compression or expansion of the surface film. Whilst both scientists realised the ability of surfactant to rapidly adsorb to the air/liquid interface to form a surface film, the composition of such a film was unknown. Shortly after, a lipid-protein complex was isolated from lungs and phospholipids were implicated as the surface-active agent within the mixture (Klaus *et al.*, 1961; Pattle & Thomas, 1961). The major constituent of pulmonary surfactant is disaturated lecithin or, dipalmitoylphosphatidylcholine (DPPC) which makes up approximately 40% of the phospholipid fraction (Klaus *et al.*, 1961; Pattle & Thomas, 1961).

Prior to the discoveries of Pattle and Clements, an entomologist named V.B. Wigglesworth (1953) suggested that a surface-active agent, such as a lipid or wax, was necessary to control surface tension within the tiny tracheal respiratory system of insects to prevent oedema (Wigglesworth, 1953). However it was Pattle & Hopkinson (1963) who pioneered the comparative aspect of surfactant research in their paper outlining the presence of a surfactant in the lungs of an amphibian, reptile and bird. Although fraught with mammalian bias, comparative studies on morphology (Okada *et al.*, 1962; Nagaishi *et al.*, 1964; Petrik, 1967; Petrik & Reidel, 1968; Marin & Dameron, 1969; Dameron & Marin, 1970; Hughes, 1973; Hughes *et al.*, 1973; Meban, 1973; Marin & Dameron, 1974; Hughes & Weibel, 1978), biochemistry (Miller & Bondurant, 1961; Harlan *et al.*, 1966; Baxter *et al.*, 1968; Ellison *et al.*, 1969; Fujiwara *et al.*, 1970; Pattle, 1973; Pattle *et al.*,

1977; Dameron & Marin, 1978; Marin *et al.*, 1978; Marin *et al.*, 1979) and evolution (Pattle, 1976; Clements *et al.*, 1970) of the surfactant system followed.

Prior to the discovery of the proteins, the lipids were the only component of surfactant to study. Thus, the primary focus during the 1960s and 1970s involved the descriptions of the morphological and biochemical properties of the lipids in pulmonary surfactant. Of particular interest at this time, and inspired by the observations of von Neergaard and Pattle, was the role of the system during the initiation of airbreathing and the understanding of infant death associated with a dysfunctional surfactant system. Avery and Mead (1959) discovered that respiratory distress syndrome was caused by a deficiency in surfactant. This discovery, in conjunction with the latter development of positive airway pressure (Gregory *et al.*, 1971) revolutionised diagnosis and treatment of infant respiratory diseases and drastically reduced the elevated foetal mortality rate of the time. Moreover, during this time, factors influencing the control of the system, particularly during development, became the predominant research objective, with the comparative aspect of surfactant research being largely confined to morphological descriptions of the surface film and its intracellular components (Okada *et al.*, 1962; Nagaishi *et al.*, 1964; Petrik, 1967; Petrik & Reidel, 1968; Marin & Dameron, 1969; Dameron & Marin, 1970).

The focus on surfactant research shifted from the lipids in the late 1970s and throughout the 1980s to the surfactant proteins following their discovery by King *et al.* (1973) and their subsequent naming by Possmayer (1988); a focus that persists today. To this day the discovery of mutations in surfactant protein genes (Nogee *et al.*, 1993; Klein *et al.*, 1998) continues to improve our understanding of neonatal morbidity and to enhance treatments for children suffering from such congenital diseases.

In this thesis, the primary emphasis will be the compositional changes of the surfactant lipids in the developing lung of different vertebrate species.

1.2 Pulmonary Surfactant in Mammals

To place the development of the surfactant system in non-mammals into context, an initial overview of the morphological, compositional and functional aspects of pulmonary and surfactant biology in mammals has been provided.

1.2.1 Morphology and Composition of Pulmonary Surfactant

1.2.1.1 Morphology of the Lung

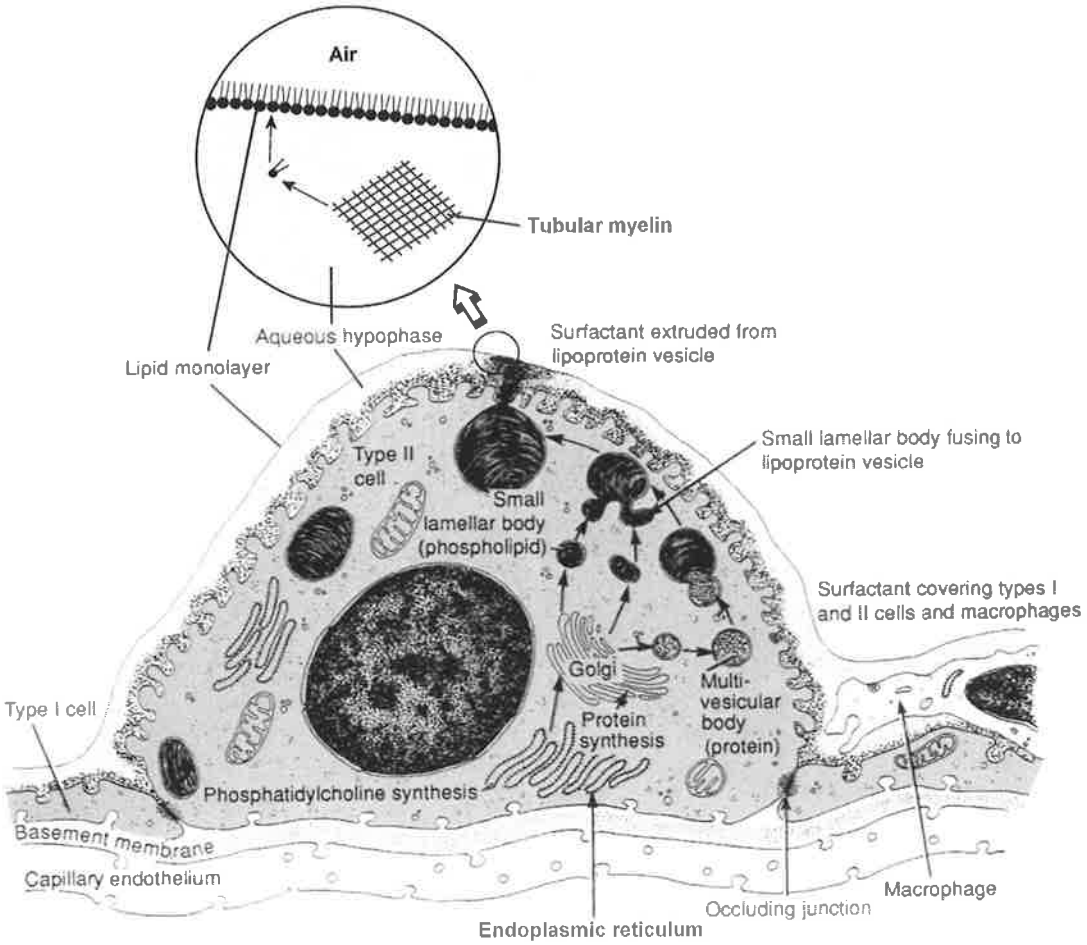
The bronchoalveolar lung of mammals arises from the cartilaginous trachea which bifurcates into two primary bronchi, that subsequently divide, through dichotomous branching, into lobar then segmental bronchi (West, 1995). Branching continues in this manner to the terminal bronchioles. Smooth muscle surrounds the bronchi and bronchioles, which are innervated by both branches of the autonomic nervous system, allowing relaxation or contraction of the smooth muscle, which in turn alters the resistance to airflow. Terminal bronchioles give rise to the respiratory bronchioles, from which some alveoli may emanate. Most alveoli, however, arise from the walls of the alveolar ducts. The parenchyma comprises respiratory bronchioles, alveolar ducts and alveoli (Burri, 1999). A delicate tissue framework, comprising alveolar septa separates the distal airspaces. Both sides of a septum are lined by a thin squamous epithelium and contain a dense capillary network within connective tissue. The respiratory epithelium is comprised of at least two phenotypes (Burri, 1999), specifically the type I cell and the type II cell. Type I cells occupy ~95% of the alveolar surface area (Meyrick & Reid, 1977). These elongate cells, possess extensive cytoplasmic projections that measure ~0.2μm in thickness. It is through these cells that diffusion of respiratory gases occurs.

1.2.1.1.1 Type II Cells

Alveolar type II cells are the primary sites for synthesis, storage and secretion of pulmonary surfactant. These cuboidal epithelial cells are approximately 9 μm in diameter with microvilli along their free edge (Meyrick & Reid, 1977) and are located within the corners, crevices and seams of the alveoli (Nicholas, 1993). Surfactant is stored within characteristic lamellated inclusions termed lamellar bodies. The lamellae form parallel or concentric rings of tightly packed phospholipid bilayers surrounding a proteinaceous core (Wright & Clements, 1987). Surfactant proteins, SP-B, SP-C and to a lesser extent SP-A have been isolated from such inclusions (Possmayer, 1997). However, SP-A is also secreted from type II cells via a constitutive secretory pathway that bypasses lamellar bodies (Rooney *et al.*, 1993; Ikegami *et al.*, 1994).

Type II cells are capable of *de novo* synthesis of all the major phospholipid classes and gene expression of the surfactant proteins has been localised in the case of SP-C, exclusively to this cell type (Haagsman & van Golde, 1991; Johansson & Curstedt, 1997). Synthesis of both surfactant lipids and proteins occurs via the endoplasmic reticulum and Golgi apparatus. Figure 1.2 demonstrates the processing pathways for pulmonary surfactant. The proteins are packaged in multivesicular bodies and/or composite bodies containing both lipid and proteins, which are then transferred to lamellar bodies (Rooney *et al.*, 1994). Upon secretion of surfactant, the lamellar bodies move towards the apical plasma membrane, where they fuse with the membrane and their contents are exocytosed into the alveolar hypophase. After secretion, the phospholipids reorganise within the hypophase to form a tubular cross-hatched structure known as tubular myelin (Possmayer, 1997). Phospholipids are cleaved from the tubular myelin where they quickly adsorb to the air/liquid interface to generate the surface film (Wright & Clements, 1987), which lowers surface tension within the alveoli. Bilayer lipid structures are attached to the lipid

Figure 1.2 Diagrammatic representation of pulmonary surfactant metabolism by the type II pneumocyte. Lipids and proteins are synthesised within the endoplasmic reticulum and Golgi apparatus, then transferred to multivesicular bodies and stored within lamellar bodies. The lamellar bodies are secreted by exocytosis into the alveolar hypophase, where they unravel and arrange into a cross-hatched structure termed tubular myelin. Lipids are cleaved from the tubular myelin and aggregate at the surface of the aqueous hypophase to form a continuous lipid monolayer. Alveolar macrophages lie within the aqueous hypophase. Occluding junctions around the epithelial margins prevent leakage of tissue fluid into the alveolar lumen. Modified from Junqueira *et al.* (1992).


Air
Tubular myelin
Lipid monolayer
Aqueous hypophase
Surfactant extruded from lipoprotein vesicle
Small lamellar body fusing to lipoprotein vesicle
Type II cell
Small lamellar body (phospholipid)
Surfactant covering types I and II cells and macrophages
Golgi
Multi-vesicular body (protein)
Protein synthesis
Type I cell
Phosphatidylcholine synthesis
Basement membrane
Capillary endothelium
Macrophage
Occluding junction
Endoplasmic reticulum

monolayer, serving as a reservoir of lipids and possibly proteins for the monolayer during the respiratory cycle (Veldhuizen & Haagsman, 2000). Spent lipids are forced back into the aqueous hypophase following one or more ventilatory cycles where they form stable bilayer vesicles, which are subsequently degraded or recycled into lamellar bodies within the type II cell.

As the progenitor for type I cells, type II cells are important in the regeneration of the epithelial wall after injury (Meyrick & Reid, 1977; Haagsman & van Golde, 1991). Moreover, these cells are enriched in antioxidant enzymes, protecting the tissue and extracellular elements from oxidative damage (Forman & Fisher, 1981). They also regulate the transepithelial movement of fluid via the Na^+/K^+ ATPase pump and therefore may directly control the composition of the aqueous hypophase (Voelker & Mason, 1989).

1.2.1.2 Lipids

The composition and relative abundance of the surfactant lipids and proteins is remarkably similar among eutherian mammals. Mammalian pulmonary surfactant is a complex mixture of lipids and proteins, comprising approximately 90% lipids and 10% surfactant proteins by weight (Akino, 1992).

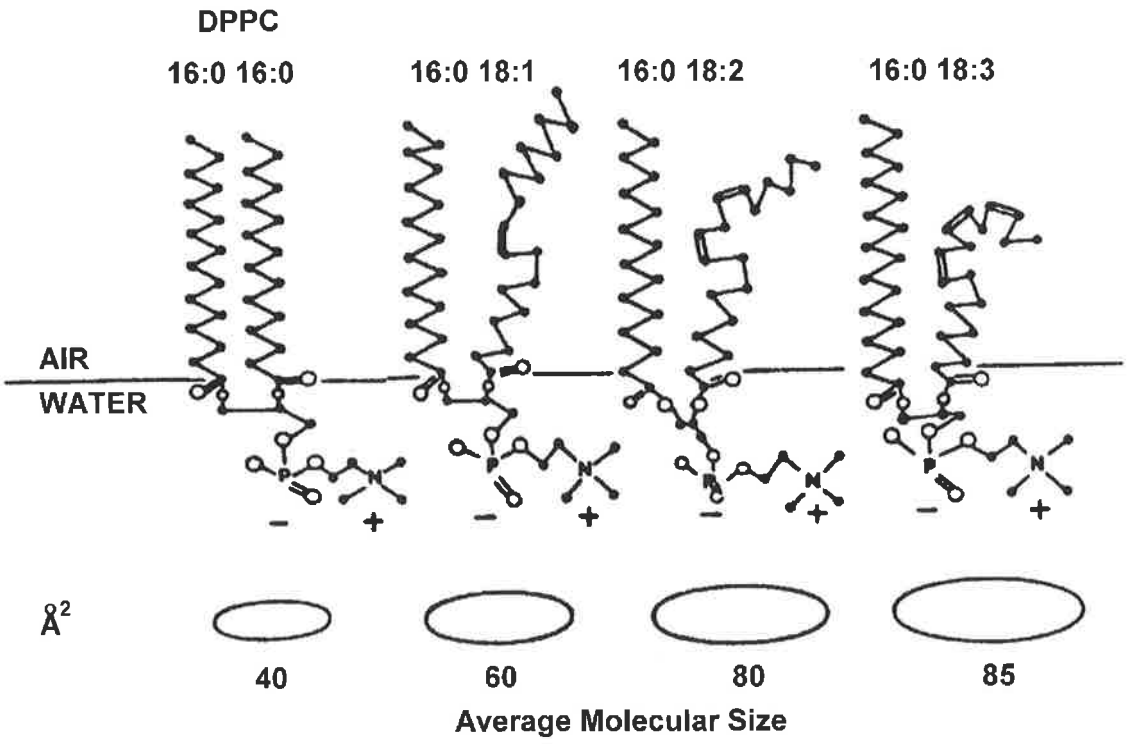
1.2.1.2.1 Phospholipids

Of the lipids within mammalian surfactant, 80-90% are phospholipids (King, 1984). Approximately 70-80% of the phospholipid fraction is phosphatidylcholine (PC), of which about 50% is disaturated with palmitate (Akino, 1992). As a result, DPPC is the most abundant molecule within pulmonary surfactant, comprising approximately 40% of mammalian surfactant. Most tissues and other cellular compartments do not have such a high proportion of DPPC and therefore high quantities of this molecule are a hallmark of

pulmonary surfactant (Veldhuizen *et al.*, 1998). DPPC is primarily responsible for the reductions in surface tension that are observed upon compression of a surfactant monolayer. At an air/liquid interface, DPPC molecules align such that the hydrophilic polar head groups interact with the aqueous layer and the paired hydrophobic fatty acid acyl groups protrude into the air space. Due to the saturation of the fatty acid tails, DPPC has a smaller average molecular size than the kinked tails of unsaturated phospholipids (Figure 1.3), allowing molecules to compress closely together (Possmayer, 1997). When compressed to extremely low surface tensions, the surface film comprises at least 90% DPPC (Hildebran *et al.*, 1979), existing in a gel state at 37°C. In this state the headgroups bind a greater proportion of water at the air/liquid interface, effectively eliminating water from the surface film and thereby lowering surface tension (Possmayer, 1997). However, in its pure form, DPPC does not readily adsorb to an air/liquid interface (Veldhuizen *et al.*, 1998), nor does it readily respread following compression (Notter *et al.*, 1980b). It is the other components such as the unsaturated phospholipids, cholesterol and the surfactant proteins that are required for such functions (Veldhuizen *et al.*, 1998; Orgeig & Daniels, 2001).

Phosphatidylcholine is also present in unsaturated forms, comprising monoenoic and dienoic species. Unsaturated PCs are thought to be involved in either, the formation of the monolayer reservoir, the initial adsorption of lipids to the interface, or the regulation of surface tension during the respiratory cycle (Veldhuizen & Haagsman, 2000). The latter occurs following compression of the lung, when the surface area is reduced. The unsaturated PCs are "squeezed out" of the monolayer, thereby increasing the concentration of saturated phospholipids within the surface film. Upon inflation, the surface area increases and the unsaturated phospholipids and neutral lipids re-enter the surface film to improve its fluidity and spreadability (Nag *et al.*, 1998). The acidic phospholipids, phosphatidylglycerol (PG) and phosphatidylinositol (PI), are the second most abundant

Figure 1.3 Orientation and average molecular size of various molecular species for PC at the air/liquid interface. Note that the disaturated phosphatidylcholine, DPPC, has a smaller average molecular size than unsaturated PCs.


DPPC
16:0 16:0
16:0 18:1
16:0 18:2
16:0 18:3
AIR
WATER
Å2
40
60
80
85
Average Molecular Size

phospholipids, comprising 8-15% of the phospholipid fraction (Veldhuizen *et al.*, 1998). Surface properties of surfactant are not affected by substituting PG with PI or by inducing PG deficiency (Hallman & Epstein, 1980; Beppu *et al.*, 1983). Consequently, PI and PG may substitute each other presumably because the hydroxyls in their head groups are required for intermolecular interactions within surfactant (Keough, 1992). Due to the high degree of unsaturated acyl chains, PG is believed to improve fluidity of the lipid films, thereby increasing adsorption to the interface (Hallman & Gluck, 1976; Johansson & Curstedt, 1997).

The remaining phospholipids, including sphingomyelin (SM), phosphatidylserine (PS) and phosphatidylethanolamine (PE) are generally found in low quantities. Such minor lipid components might induce structures of curvature such as in tubular myelin (Veldhuizen & Haagsman, 2000).

1.2.1.2.2 Neutral Lipids

The neutral lipid fraction is arguably the least studied component of pulmonary surfactant. In mammals, this fraction comprises cholesterol, cholesteryl esters, diacylglycerol, triacylglycerol and some free fatty acids (Possmayer, 1997). Cholesterol is the second most abundant lipid in pulmonary surfactant after DPPC (Yu *et al.*, 1983). By weight, cholesterol accounts for 80-90% of the neutral lipid fraction. It is believed to facilitate adsorption and spreading of the monolayer at the air/liquid interface and improve fluidity of surfactant lipids (Notter *et al.*, 1980a; Fleming & Keough, 1988). This is achieved by the ability of cholesterol to alter the phase transition temperature of the surface film (Presti, 1985).

Lipids exist in either of two phases, an ordered gel phase or a disordered liquid-crystalline phase. The transition between these phases is dependent on ambient temperature (Hadley, 1985). When the phase transition temperature of a lipid is reached,

the lipid changes from the rigid gel phase to the fluid liquid-crystalline phase. Heat is absorbed during the phase change, breaking the tertiary bonds that hold molecules in the rigid structure. The phase transition temperature is dependent upon the length and saturation of the fatty acid chains, the nature of the headgroups, the hydration of the film, the homogeneity of the lipid constituents and the presence and/or proportion of cholesterol (Hadley, 1985). Dipalmitoylphosphatidylcholine has a phase transition temperature of 41-42°C (Goerke & Clements, 1985). At temperatures below its phase transition temperature DPPC will exist in the gel state (Hadley, 1985). Addition of cholesterol to phospholipid mixtures below their phase transition temperature, will reduce the overall phase transition of the film, thereby increasing fluidity of the mixture by minimising the van der Waals forces within and between fatty acid acyl chains (Orgeig & Daniels, 2001). Moreover, because of its size, cholesterol separates phospholipid molecules, thereby disrupting intermolecular forces between the phospholipid headgroups and increasing their motility (Presti, 1985). In addition to the improved fluidity, cholesterol improves spreadability of DPPC enriched films by substantially decreasing surface viscosity despite minor elevations in surface tension (Evans *et al.*, 1980). Thus, it is likely that cholesterol primarily controls the spreadability and fluidity of the surface film in the mammalian lung.

Unlike the phospholipids, the origin of surfactant-associated cholesterol remains obscure. Generally, the composition of pulmonary surfactant from the lamellar body fraction does not differ appreciably from secreted material (Hallman *et al.*, 1976; King & Clements, 1985), suggesting that all the surfactant components are released concurrently when lamellar bodies are secreted. Hass and Longmore (1979) showed that 95% of alveolar cholesterol was derived from lamellar bodies. However, surfactant cholesterol and phospholipid pools differ in their relative turnover rates (Davis *et al.*, 1987; Jones *et al.*, 1993) and appear to be differentially released (Orgeig *et al.*, 1995; Langman *et al.*, 1996). In the alveolar compartment, the rate of cholesterol clearance does not differ from

that of PC (Pettenazzo *et al.*, 1989). Nevertheless, the clearance of cholesterol from tissue (Pettenazzo *et al.*, 1989), and its appearance in lymph (Davis *et al.*, 1987) and various surfactant fractions (Jones *et al.*, 1993) are slower than that of PC and DPPC. Moreover, the turnover of alveolar cholesterol appears to be faster than the phospholipids from heterothermic mammals arousing from torpor (Lopatko *et al.*, 1999). Furthermore, the release of surfactant cholesterol appears to be independently regulated from the phospholipids. Ventilation alters the relative abundance of cholesterol in lavage from the isolated perfused lung of rats (Orgeig *et al.*, 1995). Likewise, exercise results in the differential release of cholesterol and phospholipids (Doyle *et al.*, 1994; Orgeig *et al.*, 1995). Fitter individuals demonstrate a decrease in the content of cholesterol relative to saturated phospholipid, whereas less fit individuals demonstrate an opposite trend (Doyle *et al.*, 1994).

Cholesterol is supplied to the type II cells when they bind and take up high density, low density and very low density lipoproteins (Guthmann *et al.*, 1997). Radiolabel studies demonstrate that blood-borne cholesterol is stored within lamellar bodies without being secreted into the alveolar space. This phenomenon arises because ~76% of total lamellar body cholesterol is confined to the limiting membranes of lamellar bodies (Orgeig & Daniels, 2001). Upon exocytosis, most cholesterol from the lamellar bodies remains within the plasma membrane of the alveolar type II cell. Therefore, although lamellar body cholesterol appears to be derived from serum cholesterol, the majority is not available for release into the alveolus (Orgeig & Daniels, 2001). Furthermore, intraperitoneal injection of radiolabelled cholesterol results in the accumulation of cholesterol in the alveolar space, but not within lamellar bodies, suggesting that alveolar surfactant is not derived from lamellar bodies (Darrah & Hedley-Whyte, 1971; Darrah *et al.*, 1971). In summary, there is evidence mounting that surfactant cholesterol is packaged separately from the phospholipids, however the source of the alveolar cholesterol remains unknown.

1.2.1.3 Proteins

To date, four surfactant proteins, SP-A, SP-B, SP-C and SP-D have been described, comprising ~10% of pulmonary surfactant by weight (Possmayer, 1997). SP-A, SP-B and SP-D are produced by both alveolar type II cells and non-ciliated bronchiolar cells, termed Clara cells (Mason & Voelker, 1998). Moreover, SP-A, SP-B and SP-D have been detected in the gastrointestinal tract and the Eustachian tube of the ear (Rubio *et al.*, 1995; Crouch, 1998; Paananen *et al.*, 1999; Paananen *et al.*, 2001). In addition, SP-D has been isolated from several glands, including salivary, lacrimal, sweat and mammary gland (Crouch, 1998). SP-C therefore is the only protein exclusively synthesised by type II cells.

Of the surfactant proteins, SP-A is the most abundant. It has a molecular mass of 26-36 kDa under reduced conditions. Under non-reduced conditions, the molecular mass is 700 kDa due to extensive oligomerisation of 18 similar sub units (Pison *et al.*, 1994) which form a bouquet structure. The primary structure of SP-A is highly conserved among the eutherian mammals (Creuwels *et al.*, 1997). SP-A and SP-D belong to the collectin subgroup of the C-type lectin super-family because they have an N-terminal collagen-like and C-terminal lectin-like domain, comprising a carbohydrate recognition domain (Kuroki & Sano, 1999). The carbohydrate recognition domain of the SP-A molecule is involved in lipid binding and aggregation (Kuroki & Akino, 1991; Hynsjö *et al.*, 1995) and virus recognition (Pison *et al.*, 1994; Creuwels *et al.*, 1997; Johansson & Curstedt, 1997). Apart from being instrumental in the formation of tubular myelin (Voorhout *et al.*, 1991), SP-A, in the presence of calcium, is believed to enhance the formation of tubular myelin from lamellar bodies and the adsorption of lipids from surfactant films containing the hydrophobic proteins, SP-B and SP-C (Wright & Clements, 1989; Venkitaraman *et al.*, 1990). SP-A binds phospholipids in the gel phase and possesses a high affinity for DPPC (Kuroki & Akino, 1991), suggesting that this protein is involved in selective adsorption of DPPC to the surface film (Creuwels *et al.*, 1997; Veldhuizen & Haagsman, 2000). SP-A

knockout mice are susceptible to infection by some pathogens and lack tubular myelin, however no change in the phospholipid composition or any other respiratory differences are observed (Korfhagen *et al.*, 1996). Therefore, while the role of SP-A in monolayer formation may not be crucial for airbreathing, it may significantly improve pulmonary lipid homeostasis (Veldhuizen & Haagsman, 2000). SP-A also enhances lipid uptake via the type II cells themselves (Wright & Clements, 1987) and lipid clearance by alveolar macrophages (Wright & Youmans, 1995). SP-A also acts as an opsonin. The carbohydrate recognition domain of SP-A, binds to pathogens, augmenting aggregation and activation of alveolar macrophages (Pison *et al.*, 1994; Pikaar *et al.*, 1995). The protein interacts with the surface of the macrophage to induce the release of reactive oxygen species from the macrophage when SP-A is properly presented to the target cell (Pison *et al.*, 1994). SP-A is also chemotaxic, causing alveolar macrophages and neutrophils to migrate toward, and phagocytose pathogens (Pison *et al.*, 1994; van-Iwaarden *et al.*, 1995).

The hydrophobic surfactant proteins, SP-B and SP-C, have molecular masses of 18-20 kDa and 5-6 kDa under non-reduced conditions, respectively. Under reduced conditions the proteins have nominal molecular masses between 5 and 8 kDa (Pison *et al.*, 1994). SP-B has a precursor of approximately 42 kDa, whereas SP-C has one of approximately 21 kDa (Pison *et al.*, 1994). The SP-B proprotein precursor (proSP-B) contains a hydrophobic leader sequence, which directs the protein to the lumen of the endoplasmic reticulum and signals its entry into the secretory pathway of the type II cell. The 79 amino acid human SP-B monomer is produced by proteolytic cleavage of the precursor protein (Whitsett *et al.*, 1995). ProSP-C is a 197-residue polypeptide that acts as a transmembrane protein, where it inserts into the endoplasmic reticulum membrane for the processing and transport of mature SP-C (Johansson & Curstedt, 1997). SP-B and SP-C are chiefly involved in the generation of low surface tensions within the lung, by

selectively removing non-DPPC components from the monolayer (Possmayer & Yu, 1990; Veldhuizen & Haagsman, 2000). When squeezed out of the monolayer, SP-C is accompanied by 7-10 lipid molecules, whereas SP-B is not accompanied by lipid (Taneva & Keough, 1994a; Taneva & Keough, 1994b). In the case of SP-C, insertion of lipid back into the film is believed to rely upon the paired palmitoyl acyl chains of the SP-C molecule that bridge the reservoir bilayer with the monolayer. Therefore, SP-B appears to be important in adsorption whereas SP-C seems to be instrumental in lipid reservoir formation (Possmayer & Yu, 1990; Schürch *et al.*, 1995; Veldhuizen & Haagsman, 2000). In addition, SP-B promotes formation of lamellar bodies and tubular myelin, respreading of the film after collapse phase, reuptake of lipid by type II cells, stabilisation of the monolayer, membrane binding, membrane fusing and lysis. SP-C appears to share many of the functions of SP-B. It is primarily involved in homeostasis of the surface film by promoting adsorption of lipids to the film, aiding the respreading of collapsed films and stabilising the film (Veldhuizen & Haagsman, 2000).

SP-B deficiency in humans (Nogee *et al.*, 1993) and knockout of the SP-B gene in mice (Clark *et al.*, 1995) causes massive respiratory dysfunction. SP-B (-/-) mice lack lamellar bodies and die shortly after birth, reflecting the vital role of this surfactant protein in respiration (Clark *et al.*, 1995). The processing of SP-C is also affected in SP-B deficient children (Klein *et al.*, 1998) and in SP-B null mice (Clark *et al.*, 1995).

Surfactant protein D (SP-D) is 43 kDa under reduced conditions. SP-D is a member of the collectin family of collagenous lectin domain-containing proteins that is expressed in epithelial cells of the lung (Korfhagen *et al.*, 1998). This hydrophilic protein forms a dodecamer composed of four trimers, such that the molecule forms an X-shape. SP-D is not processed with surfactant phospholipids and is not present in lamellar bodies or tubular myelin (Mason & Voelker, 1998) and as a result, it is not believed to be instrumental in the surface-active properties of pulmonary surfactant (Mason & Voelker, 1998; Veldhuizen &

Haagsman, 2000). It may be of prime importance in lung defense (Reid, 1998). Like SP-A, the carbohydrate recognition domain binds to carbohydrate structures present on a range of viruses, bacteria, yeast and fungi (Pikaar *et al.*, 1995; Kishore *et al.*, 1996; Reid, 1998). Since the binding sites of the SP-D trimers are oriented in the same direction, upwards from the originating surface of the molecule, they are perfectly positioned to bind carbohydrates arrayed on a large surface such as the outer wall of bacteria or fungi (Johansson & Curstedt, 1997). However, SP-D gene-targeted mice have 4-8 times more phospholipid in their air spaces and lung tissue, they have hyperplastic type II cells with large lamellar bodies, and numerous foamy macrophages, suggesting a role for the protein in both lipid homeostasis and host defense (Botas *et al.*, 1998; Korfhagen *et al.*, 1998; Ikegami *et al.*, 2000).

1.2.2 Function of Pulmonary Surfactant

1.2.2.1 Lung Compliance

At an air/liquid interface, molecules within the liquid are attracted to each other more than to the air surrounding them, thereby producing surface tension. Liquids with high intermolecular forces have high surface tensions. Due to its hydrogen bonding, water has the highest surface tension of the organic solvents. Therefore, any substance that disturbs the intermolecular forces of water at the surface will substantially lower surface tension (Daniels *et al.*, 1998a).

Alveoli are lined with a continuous aqueous layer of fluid, termed the hypophase (Crapo, 1993). Elevated surface tensions due to water within the hypophase will cause the alveoli to contract and occupy the smallest surface area, causing an overall elastic contractile force on the lung. Lung compliance refers to the effort required to distend the lungs or the change in lung volume per unit of pressure. As previously discussed (Section

1.1), the first static lung compliance studies determined that two thirds to three quarters of the lung's collapse pressure was attributed to the contractile surface forces that were presumably overcome by a surface-active agent (von Neergaard, 1929). By lowering surface tension within the alveoli directly the elastic recoil force is drastically reduced, both directly and indirectly by deforming lung tissue (Bachofen & Schürch, 2001). This in turn, reduces the work required to inflate the lung.

1.2.2.2 Promotion of Alveolar Stability

Pressure within the aqueous hypophase lining the alveoli relies upon two factors; the radius of curvature of the lining fluid and the surface tension at the fluid's surface, expressed by the law of Young and LaPlace

$$P = \frac{-2\gamma}{r}$$

where P is the pressure of the fluid, γ is the surface tension of the fluid and r is the radius of the curvature of the fluid. For two adjacent alveoli with different radii, the smaller alveolus with a smaller radius will have a higher internal pressure than its larger counterpart if surface tension is held constant. Consequently, the smaller alveolus will empty into the larger alveolus, resulting in alveolar collapse, termed atelectasis (Clements *et al.*, 1958). Varying surface tension within individual alveoli, equalise pressures within different sized alveoli, thereby preventing collapse and maintaining stability (Clements *et al.*, 1958; Clements *et al.*, 1961). This is believed to be a primary function of pulmonary surfactant in the bronchoalveolar lung of mammals (Clements & Tierney, 1965; Scarpelli, 1968). However, because of their wall sharing and the organisation of the fibrous network of the lung, alveoli are not individual units. Such models do not accurately represent the anatomical or physiological condition of the lung (Bachofen & Schürch, 2001). Moreover, at fixed lung volumes, surface tension does not vary between alveoli of different sizes

(Schürch, 1982). While alveolar stability remains an important function of mammalian surfactant, new models encapsulating the interdependence of the tissue elements and their surface forces have been postulated to better represent the *in vivo* situation (Bachofen & Schürch, 2001). At high lung volumes, the interdependence of the tissue elements ensures the stability of the alveolar units regardless of surface tension. At low lung volumes, however, alveolar septa crumple in the alveolar corners, generating recoil pressure from both surface forces and tissue structures other than the alveolar walls (Bachofen *et al.*, 1979). It is here that instability may occur. Therefore, surfactant is crucial at low lung volumes where unfolding of the alveoli is the primary mode of deformation (Sanderson *et al.*, 1976). At a low fixed lung volume, alveolar surface area will decrease with increasing surface tension. Hence, the reduction of surface area is a gradual process, rather than simply being opened or collapsed, where the sum of tissue energy and surface energy is minimal (Bachofen & Schürch, 2001). It is apparent that not only surfactant, but also the architecture of the fibrous scaffold and the alveoli contribute to stability of the lung at low lung volumes.

1.2.2.3 Control of Fluid Balance

Lungs are potentially very susceptible to fluid disturbances because they have a large surface area, high blood flow and relatively leaky capillary endothelial cells (Daniels & Orgeig, 2001). Fluid build up in either the alveolus or the interstitium, termed oedema, limits the diffusion of gases, resulting in severe respiratory insufficiency (Smits, 1989). Consequently, pulmonary fluid is tightly regulated. Due to Starling forces, there is a small continual flow of fluid from the capillaries into the alveolar space via the interstitium (West, 1995). The pressure within this fluid may be determined by the law of Young and LaPlace (Section 1.2.2.2). Negative pressures will be large within the fluid lining areas that are highly curved, such as the alveolar seams, corners and crevices and as a result,

attract liquid from other parts of the alveolus or across the interstitium (Gil *et al.*, 1979). However, if surface tension increases or the radius of curvature decreases pressure within the hypophase will become more negative than the surrounding tissue, resulting in alveolar flooding.

By lowering the surface tension of the fluid lining in the corners of the alveoli, surfactant reduces the negative pressure in the hypophase to equivalent or lower values than that of the interstitium (Guyton *et al.*, 1984). Greater negative pressure within the interstitium draws fluid from the airspace into the tissue (Guyton *et al.*, 1984), where it is carried away by the lymphatic system.

1.2.2.4 Prevention of Epithelial Adherence

During inflation, the alveoli expand in a complex manner, best described as unfolding or unpleating (Sanderson *et al.*, 1976). The seams, crevices and corners of the alveoli are filled with fluid. It is likely that walls of the alveoli within these areas may adhere at low lung volumes. The work required to separate parallel apposing tissue is proportional to the surface tension of the fluid and to the area of the exposed walls, originally at a distance x from each other. If the area of the walls is A, then the work, W required to separate them will be the integral of the force, F attracting each to the other over the distance, x separating them.

Now, as

$$F = \frac{dW}{dx} \qquad ①$$

and as the liquid between the surfaces is curved, the law of Young and LaPlace holds, such that,

$$F = \frac{2\gamma(x)A}{x} \qquad ②$$

therefore substituting ① and ② gives

$$dW = \left(\frac{2\gamma(x)A}{x} \right) dx$$

where $2\gamma(x)$ allows for variable surface tension. Separation of adhering surfaces constitutes a considerable portion of the work required to inflate the lung (Sanderson *et al.*, 1976). Upon inflation, phospholipids are believed to adsorb to the expanding air/liquid interface as the radius of curvature increases to overcome high surface forces (Daniels *et al.*, 1998a).

1.2.2.5 Immune Functions

The lung is in direct communication with the external environment. Accordingly, it is susceptible to infection by viruses, bacteria, fungi and damage from inhaled foreign particles. Pulmonary surfactant is believed to improve lung immunity in several ways. Firstly, it improves mucociliary clearance by lowering the surface tension of the fluid bathing the cilia. The viscosity of this fluid is substantially reduced, which in turn reduces the work required for the cilia to beat (Kilburn, 1969) and increases their beat frequency (Kakuta *et al.*, 1991). In doing so, more particles are transferred to the upper respiratory tract and subsequently removed by swallowing. Surfactant also covers the mucus droplets and periciliary gel preventing adhesion between mucus and improving cilia-mucus coupling (Allegra *et al.*, 1985). Administration of exogenous surfactant increases the velocity of mucus clearance from the trachea (De Sanctis *et al.*, 1994) and particulate clearance from the nasal cavities (Outzen & Svane Knudsen, 1993).

Secondly, as discussed previously (Section 1.2.1.3), the hydrophilic surfactant proteins, have been implicated in innate lung defense. Pulmonary surfactant acts by inhibiting the proteolytic activation of viruses, inhibiting the capacity of monocytes and granulocytes to release H_2O_2 or possibly by preventing an excessive immune response (Pison *et al.*, 1994). SP-A and SP-D bind and aggregate an array of structures, ranging

from bacteria and fungi to allergens and environmental inorganic substrates (Lawson & Reid, 2000). These collectins modulate allergic reactions by binding certain glycosylated allergens (Reid, 1998). Both proteins opsonise macrophages and neutrophils, enhancing phagocytosis and death of pathogens (Pison *et al.*, 1994; Pikaar *et al.*, 1995). SP-A knockout mice are susceptible to bacterial, viral and fungal attack. Despite a lack of tubular myelin, the processing of surfactant lipids remains unchanged in SP-A null mice (Korfhagen *et al.*, 1996), further emphasising the primary role of SP-A in innate lung defense. SP-D deficient mice have multiple imbalances in the reuptake and metabolism of surfactant lipids and the form of lung constituents, neither of which can be attributed to a single mechanism (Ikegami *et al.*, 2000). Given that macrophages provide the primary site of attack for pathogens, their foamy appearance and large numbers in SP-D null mice, may suggest dysfunction in pulmonary host defense. Moreover, that the hydrophilic surfactant proteins have been located in the gastric mucosae and other sites prone to microbial attack (Crouch, 1998), further supports their immunological role.

1.2.2.6 Additional Functions

In addition to their immune functions, the hydrophilic surfactant proteins SP-A and SP-D act as antioxidants by protecting the surfactant lipids and macrophages from free radical damage (Bridges *et al.*, 2000). SP-A and S-D show no conformational changes during lipid oxidation, however they instantaneously arrest oxidation of low density lipoproteins, suggesting that they act by directly interfering with the liberation of lipid radicals or as free radical chain terminators (Bridges *et al.*, 2000).

Airway patency is also aided by pulmonary surfactant. The narrow airways that are closest to the alveoli lack the cartilaginous reinforcement of the upper airways. Therefore, like alveoli, they will participate in volume changes during ventilation. The model by Enhörning (2001) bests describes the function of surfactant in the maintenance of airway

patency. It states that during expiration, airways would narrow and fluid would accumulate in the narrowest section by the law of Young and LaPlace (Section 1.2.2.2). At a given surface tension, narrower portions of an airway with smaller radii of curvature will experience greater pressures than wider regions. Since air pressure in communicating sections is equal, the pressure of the liquid lining the narrow part must be more negative than it is in the wide section. Therefore, liquid will be drawn to the narrow section, further reducing the size of the airway and potentially blocking the lumen. By lowering surface tension during expiration, pressure within the narrower section of the airway will be lower than that of the wider portion, which in turn, would prevent the accumulation of fluid in narrow regions and maintain airway patency (Enhörning, 2001).

1.2.3 Development of the Respiratory System

1.2.3.1 Lung Development – Morphology

To ensure rapid uptake of O_2 and release of CO_2, the lung must provide a large surface area for gas exchange coupled with intimate contact between the air and blood. In mammals, this is achieved by the elaborate branching of airways and vasculature to form the lung parenchyma. The development of the mammalian lung can be divided into five morphologically distinct stages, which are the embryonic phase, the glandular phase, the canalicular phase, the terminal sac phase and the alveolar phase. Early developmental studies only included the glandular, canalicular and terminal sac stage. Two additional stages have been incorporated. An embryonic stage precedes the glandular phase and the differentiation of the alveoli within an alveolar phase proceeds the terminal sac phase. The timing of these stages may differ among the eutherian mammals (Table 1.1). The following section on the morphological development of the lung provides a brief overview of these stages to act as a reference and also describes the action of one of the primary

regulatory factors, thyroid transcription factor –1 (TTF-1) in lung morphogenesis and surfactant protein gene expression.

Table 1.1 *Appearance of lamellar bodies and chronological stages of lung development in common eutherian mammals. Modified from Cockshutt and Possmayer (1992).*

Species	**Term**	**Embryonic/ Glandular period**	**Canalicular period**	**Terminal sac period**	**Appearance of mature LB**
	(days)		**(days [% gestation])**		
Mus musculus (Mouse)	19	0 – 16.6 [0-87]	16.7 – 17.4 [88-92]	17.5 – neo [>92]	17 [89]
Rattus norvegicus (Rat)	22	0 – 18 [>82]	18 – 20 [86 – 92]	21 – neo [>92]	19 [88]
Oryctolagus cuniculus (Rabbit)	31	0 – 24 [0-77]	24 – 27 [77 – 87]	27 – neo [>87]	25 [81]
Cavia porcellus (Guinea Pig)	68	0 – 40 [0-59]	40 – 50 [59 – 74]	50 – term [74-100]	55 [80]
Ovis aries (Sheep)	148	0 – 95 [>64]	95 – 120 [64 – 81]	120 – neo [>81]	115 [78]
Macaca mulatta ***M. nemestrina*** (Macaque)	168	0 – 80 [0-48]	80 – 140 [48 – 83]	140 – neo [>83]	133 [79]
Homo sapiens (Human)	280	0-112 [0-40]	112 – 168 [40 – 60]	168 – neo [>60]	165 [60]

Gestational age in days; percentage of gestation in square brackets; Neo, neonate; LB, lamellar bodies.

1.2.3.1.1 Embryonic

The respiratory system develops as an extension of the primitive foregut. Paired endodermal lung buds arise proximally from the stomach. They then grow in a caudocranial direction. In rats and mice, as the lung buds elongate, the tubular foregut is pinched to form the dorsal oesophagus and the ventral trachea (Keÿzer & Post, 1999). In humans, the lung is derived from the intended oesophagus by the laryngotracheal grooves

which subsequently divide to give rise to the left and right lungs (Burri, 1999; Keÿzer & Post, 1999). The airway tree arises from the rapid growth and dichotomous branching of the lung buds. At this stage of development the vascular system is still rudimentary (Burri, 1984; Burri, 1999).

1.2.3.1.2 Glandular

The classification of the remaining stages is based on morphological criteria. The glandular stage, also known as the pseudoglandular stage, was so named because the lung resembles a primitive gland. During this phase, the lung appears as loose mesenchymal tissue surrounding buds of endodermal tissue (Meyrick & Reid, 1977). Repeated dichotomous branching of the developing airways continues forming the bronchioles, respiratory bronchioles and the alveolar ducts. Whilst branching of the airways occurs, the epithelial cells remain undifferentiated as high columnar epithelial cells, comprising large masses of glycogen with few organelles. By the end of the glandular stage, the epithelium decreases in height, giving rise to cuboidal epithelia at the periphery of lung tips. The vascular tree closely follows the development of the bronchial tree (Burri, 1984; Burri, 1999).

1.2.3.1.3 Canalicular

The canalicular phase is marked by the differentiation of future gas exchange tissue. During this stage of lung development, the airway branching is complete and the rapid rate of growth diminishes. The cuboidal epithelial cells lining the alveolar ducts begin degrading glycogen stores, before differentiating into primitive type II cells, which are characterised by the appearance of lamellar bodies (Schellhase *et al.*, 1989; Gomi *et al.*, 1994). The type II cells differentiate into type I cells, which contribute to the formation of

the air-blood barrier (Meyrick & Reid, 1977; Burri, 1984; Burri, 1999). Shortly after differentiation of the epithelial cells, surfactant and tubular myelin are found in the developing airspaces (Schellhase *et al.*, 1989; Gomi *et al.*, 1994). Accompanying this phase, is a massive surge in capillary growth within the interstitium (Burri, 1984; Burri, 1999).

1.2.3.1.4 Terminal Sac

In the terminal sac phase the lung acquires its alveolar appearance, but still lacks alveoli. The distal airspaces undergo expansion, giving the lung a spongy appearance. Remodelling of the interstitial connective tissue occurs, providing structural support for the forthcoming alveoli. Capillaries continue to invade the interstitium, forming a sleeve around each airspace (Burri, 1984; Burri, 1999). At this time, alveolar macrophages begin populating the airspace surfaces and the parenchyma consists of smoothly contoured transitory ducts and saccules. The walls of the saccules are thick and lined with a capillary network on either side of the interstitial connective tissue (Burri, 1984).

1.2.3.1.5 Alveolar

As the name suggests, the alveolar stage marks the appearance of the alveoli. Differentiation of the alveoli occurs quickly in the mammalian lung, with over 90% of all alveoli forming after birth (Burri, 1984). The newly differentiated saccules develop ridges from which secondary alveolar septa emanate. The secondary septa grow and partition the saccules into smaller shallow alveoli, thereby increasing respiratory surface area. Each septum contains a double capillary layer, which undergoes further differentiation to form a single capillary layer in the mature lung. The interalveolar septa increase in height, causing the alveoli to increase in depth and volume (Burri, 1984; Burri, 1999).

1.2.3.1.6 Transcription Factors

Regulation of lung branching morphogenesis and epithelial cell differentiation is complex and involves many factors. During lung development mesenchymal tissue interacts with epithelial cells derived from the embryonic foregut, initiating branching morphogenesis of the lung and differentiation of specialised cell populations (Mendelson, 2000). The commencement of cellular differentiation is signalled by the expression of differentiated gene products. In the lung, such gene products include the surfactant proteins and Clara cell secretory protein (Bohinski *et al.*, 1994). The control of specific protein expression in the developing lung involves interactions between the promoter regions of genes and several nuclear protein ligands, collectively termed transcription factors (Bourbon, 1999; Mendelson, 2000). Transcription factors contain trans-acting elements that bind to the cis-acting elements of the DNA. Several transcription factors have been implicated in lung development and lung-specific gene expression, including thyroid transcription factor-1 (TTF-1) and hepatocyte nuclear factors-3 (HNF-3)/forkhead factors (Bourbon, 1999; Mendelson, 2000). In addition to the activation of tissue specific genes, transcription factors such as TTF-1 (Minoo *et al.*, 1995) and others (for review see Mendelson (2000)) are necessary for lung epithelial morphogenesis. Given the central role that TTF-1 plays in both surfactant protein gene expression and lung morphogenesis, TTF-1 will be the primary focus of this section.

Initially identified as an important regulator of thyroid-specific gene expression, TTF-1 belongs to the NKx class of nuclear factors that are characterised by the presence of a highly conserved homeodomain, capable of binding to regulatory regions of target genes (Bruno *et al.*, 1995; Ikeda *et al.*, 1995). The 60 amino acid homeodomain is encoded by a 180 nucleotide domain termed the homeobox (Gehring *et al.*, 1994). Apart from thyroid, TTF-1 is also expressed in embryonic forebrain and lung (Lazzaro *et al.*, 1991).

Both the structure and the function of this transcription factor have been highly conserved within the mammals (Ikeda *et al.*, 1995). Deletion of the TTF-1 gene in mice results in gross morphological deformities. The stillborn foetuses lack forebrain, thyroid and pituitary gland. Furthermore, the parenchymal tissue is absent in the lung despite the presence of proximal conducting airways, confirming the role of TTF-1 in branching morphogenesis of the lung (Kimura *et al.*, 1996).

Specific binding sites for HNF-3 family members have been detected on the TTF-1 gene, suggesting that HNF-3 family members are involved in transcriptional regulation of the TTF-1 gene (Ikeda *et al.*, 1996; Korfhagen & Whitsett, 1999). With respect to lung-specific gene expression, both HNF-3β, and TTF-1 bind to two distinct regions within the promoter region of the SP-B gene activating surfactant protein expression (Bohinski *et al.*, 1994). Likewise, TTF-1 interacts with multiple complex protein/DNA binding sites located in the 5'-flanking region of the murine SP-A (Bruno *et al.*, 1995) and SP-C (Kelly *et al.*, 1996), enhancing lung epithelial cell-specific expression *in vitro*. Moreover, SP-B, SP-C, and SP-A gene promoters are specifically activated by co-transfected TTF-1 (Bohinski *et al.*, 1994). The role of transcription factors in lung-specific gene expression is further evidenced by immunohistochemical studies. TTF-1 is expressed within the distal epithelium and type II cells of the adult and in the developing respiratory epithelium in the early glandular stage of lung development, preceded by the expression of HNF-3β. The pattern of expression of TTF-1 overlaps the cellular expression of the surfactant protein genes (Zhou *et al.*, 1996). Therefore, the correct expression of each of the lung specific genes requires a combined interaction between TTF-1, HNF-3 family members and other nuclear transcription proteins (Korfhagen & Whitsett, 1999). TTF-1 is the only transcription factor known to commonly regulate all of the known lung-specific genes and is involved in the co-localisation, timing of expression, and transactivation of the surfactant

protein genes, and therefore plays a central role as a molecular regulator within the developing lung (Korfhagen & Whitsett, 1999).

1.2.3.2 Lung Development – Physiology

Associated with the drastic changes in morphology, are the biochemical changes that appear towards the end of gestation. The remaining sections focus on the development of these biochemical changes within the lung during the perinatal period.

1.2.3.2.1 Fluid Balance

The foetal lung secretes liquid from the developing respiratory epithelium at an astonishing rate. Such secretion is driven by secondary active transport of Cl^- from the interstitium to the alveolus. Sodium ions and water follow Cl^- passively. The resultant liquid is essentially sodium chloride (150mM), containing low levels of potassium, bicarbonate and protein. Foetal lung fluid (FLF) causes positive pressures within the lung and therefore promotes lung growth by maintaining the luminal volume (Pitkänen & O'Brodovich, 1999).

During labour, the lung transforms from a secretory to an absorptive organ. The absorption of lung fluid occurs in two phases. During birth, most, but not all of the lung fluid is expelled during spontaneous vaginal delivery. The remaining fluid must be removed quickly for gas exchange to commence. Initially lung fluid is absorbed through pores in the pulmonary capillaries. During birth, these pores may be stretched by the expansion of the lung upon inspiration, allowing the influx of lung liquid without permitting the movement of plasma proteins in the opposite direction (Egan *et al.*, 1975). This mechanism is short lived and is followed by the active transport of Na^+. Elevated levels of circulating catecholamines (Padbury *et al.*, 1982), prevent secretion and initiate

adsorption of foetal lung fluid at birth (Brown *et al.*, 1983). Such actions are blocked by amiloride, a Na^+ channel blocker, indicating that the capacity to absorb fluid results from the maturation of a cellular process, permitting the active transport of Na^+ from the luminal to interstitial side of the respiratory epithelium (Pitkänen & O'Brodovich, 1999). Sodium transport occurs when Na^+/K^+ ATPase pumps are stimulated by the first respiratory movements, generating a gradient for Na^+ entry into the type II cell (Pitkänen & O'Brodovich, 1999). The removal of Na^+ from the lung fluid establishes an osmotic gradient between the lung fluid and the interstitium, causing fluid to be rapidly reabsorbed. The absorptive response of catecholamines is augmented by the simultaneous administration of thyroid hormones and glucocorticoids (Barker *et al.*, 1990; Barker *et al.*, 1991; Wallace *et al.*, 1995). At this time, the foetal lung is also responsive to the elevated partial pressures of oxygen (pO_2), which occur at birth. Increases in ambient pO_2, augment Na^+ transport and increase mRNA levels of amiloride-sensitive epithelial sodium channels in the foetal rat lung (Pitkänen *et al.*, 1996). Tidal breathing excursions progressively increase lung volume and stimulate surfactant secretion. Pulmonary surfactant quickly adsorbs to the surface of the lung fluid, reducing surface tension and further promoting lung fluid reabsorption by maintaining the patency of the opened alveoli and the functional residual capacity of the lung. Administration of exogenous surfactant to premature fluid-filled lungs improves the permeability of the epithelium (Egan *et al.*, 1984). The pulmonary vessels dilate in response to the surge in pO_2 and prostaglandin release, draining away the lung fluid (Hislop & Reid, 1977).

1.2.3.2.2 Development of the Pulmonary Surfactant System

Initiating pulmonary ventilation is a complicated and difficult procedure as the fluid-filled lungs are resistive to inflation and the walls of the pulmonary arteries are constricted prior to birth, resulting in high resistance and therefore poor perfusion (Hislop & Reid, 1977).

Clearance of lung fluid, the resistance to inflation, and the elevated arteriolar resistance of the pulmonary vessels must be simultaneously overcome for airbreathing to begin. In eutherians, the pulmonary surfactant system is involved in the first two of these actions (Possmayer, 1997; Pitkänen & O'Brodovich, 1999). The pulmonary surfactant system develops near term. The amount and saturation of the phospholipids, and the appearance of surfactant proteins in lung fluid, amniotic fluid and lavage increase with advancing gestation (Gluck *et al.*, 1967; Rooney *et al.*, 1976b; Egberts *et al.*, 1981; Torday & Nielson, 1981; Benson *et al.*, 1983).

1.2.3.2.2.1 Lipids

It is well established that the content and saturation of surfactant phospholipid increase throughout the final stages of development in mammals, with the primary release occurring during birth or labour. The timing of appearance of lipids in mammalian lungs is surprisingly uniform, despite precocial maturation of the lung parenchyma in some species such as the guinea pig (Sosenko & Frank, 1987). Foetal lambs aged between 99-119 days (~67-80% gestation) have 100-fold less phospholipid in lavage than term animals (Fujiwara *et al.*, 1968). Fujiwara *et al.* (1968) found that the quantity of PC in the alveolar space increases 50-fold between lambs aged between 99-119 and those aged 120-134 days (~80-90% gestation), with a further 4-fold increase towards term (term =146-148 days). The content of PC increases ~2-fold in lung fluid from foetal lambs aged between 120 (~80%) and 145 days (~99%) of gestation (Ikegami & Jobe, 1981) and between 125 days (~85%) and term (Benson *et al.*, 1983). The disaturated PC does not appear until 120-134 days of incubation in the foetal sheep and increases 4-fold to term (Fujiwara *et al.*, 1968), which is accompanied by an increase in surface activity (Egberts *et al.*, 1981). Similarly, during the 27th day (~87%) of gestation and term (term = 31 days), phospholipid content increases 4-fold and PC content increases 10-fold in lavage harvested from foetal rabbits

(Rooney *et al.*, 1976b) and the disaturated PC content increases 10-fold (Rooney, 1984). Following birth there is an additional 8-15-fold increase in the content of alveolar phospholipid, PC and saturated PC in rabbits (Rooney *et al.*, 1976b; Rooney, 1984). Likewise, there is a marked increase in the PL content of subfractionated lavage following birth in this species (Oulton *et al.*, 1986). Relative to the content of sphingomyelin, both PC and saturated PC increase in lavage and lung fluid from rabbits with advancing gestation, such that the relative saturation of PC increases 4-5-fold in the lavage and amniotic fluid between the 24th (~77%) and 30th day (~97%) of gestation (Torday & Nielson, 1981). In lung tissue there is a ~15-fold increase in the content of phospholipid in rabbits in the final 4 days of gestation, which is maximal at birth (Oulton *et al.*, 1986) and a ~2.5-fold increase in the content of disaturated PC between the 25th day (~81%) of gestation and birth in this species (Frank & Sosenko, 1987). In lung tissue from rat, there is approximately a 1.5-fold increase in total phospholipid, a 2-fold increase in PC and a 3-4-fold increase in DPPC between the 19th day (~86%) of gestation and the first day after birth, which is accompanied by a 10-fold increase in the rate of incorporation of labelled choline into both PC and DPPC (Maniscalco *et al.*, 1978; Frank & Sosenko, 1987). Similarly, in the hamster lung, there is ~3.5-fold increase in the content of disaturated PC between the 13th day (~81%) of gestation and birth (term = 16 days) (Frank & Sosenko, 1987).

Despite the precocial development of the alveoli in the guinea pig lung, the content of phospholipids increases at the same time relative to the gestational length as other more altricial species. The amount of total PL increases 3-fold between foetuses aged at 60 days (~88%) of gestation and newborn guinea pigs (term = 68 days) (Lin & Lechner, 1991). Similarly, the quantity of disaturated PC increases 2-3-fold between day 60-61 and birth in lung tissue from guinea pigs (Sosenko & Frank, 1987; Lin & Lechner, 1991). In the macaque, the content of phospholipids doubles in the lung with advancing gestation due to

the increasing content of PC, comprising 45% at day 135 of gestation and 70% at birth (term = 168 days) (Cockshutt & Possmayer, 1992). The content of DPPC increases ~10-fold in macaque lung tissue from 145 days (~86%) of gestation throughout the remainder of gestation, which is accompanied by an increase in surface activity (Farrell, 1982). Surfactant appears earlier in the human lung than other mammalian species. There is a sharp rise in the amount of disaturated PC at approximately week 24-25 (~60%) of gestation in lung tissue, whereas the primary surge in alveolar disaturated PC occurs mainly at birth (Clements & Tooley, 1977).

The composition of the acidic phospholipids changes in most mammalian species with development. Typically, there is an increase in the relative proportion of PG and a decrease in the proportion of PI (Kulovich *et al.*, 1979; Ikegami & Jobe, 1981; Benson *et al.*, 1983; Oulton *et al.*, 1986; Sosenko & Frank, 1987). The remaining phospholipids, including sphingomyelin do not alter appreciably during development. Hence, the PC/S ratio and the PG/PI ratio have been used to assess foetal lung maturity in mammals (Kulovich *et al.*, 1979).

1.2.3.2.2.2 Proteins

Surfactant proteins are developmentally regulated in the mammalian lung. In humans, SP-A and its messenger RNA have been detected in isolated cells of the conducting airway epithelium, including non-mucus and bronchial glands, during the second trimester of pregnancy (Khoor *et al.*, 1993). However SP-A mRNA is undetectable in human foetuses at 16-20 weeks (Ballard *et al.*, 1986) or is present at extremely low levels (Weaver & Whitsett, 1991; Khoor *et al.*, 1993). Despite these observations, SP-A has been detected in amniotic fluid as early as the 19th week (~48%) of gestation (Pryhuber *et al.*, 1991). Most studies do not find SP-A in lung fluid until the 30th week of gestation (for review see Mendelson and Boggaram (1991)). SP-A mRNA is first detectable in the foetal lung of the

baboon on the 150th day (~83%) of gestation (Minoo *et al.*, 1991). In the mouse, SP-A expression occurs in the alveolar epithelial cells during the canalicular phase of lung development on the 18th day of gestation, in concert with the differentiation of the type II cells (Jaskoll *et al.*, 1984). In the rabbit, both messenger RNA and peptide are present in the lung after the 26th day (~84%) of gestation. SP-A mRNA reaches maximal levels immediately prior to birth in this species (Mendelson *et al.*, 1986; Boggaram *et al.*, 1988). In the rat, like other mammals, SP-A increases with advancing gestation. Both SP-A mRNA (Schellhase *et al.*, 1989) and the protein (Schellhase *et al.*, 1989; Ogasawara *et al.*, 1991) are first detected on the 18th day (~82%) of gestation in the rat and continue to increase throughout pregnancy, with the protein reaching a maximum in the one-day old neonate (Ogasawara *et al.*, 1991).

SP-B and SP-C are expressed in the lung at an earlier stage in foetal development than SP-A. SP-B mRNA, SP-C mRNA and the preproteins proSP-B and proSP-C are present in the lung of the human foetus by the 15th week of pregnancy. In addition to the messenger RNAs and the preproteins, the expression of the active peptides occurs in the distal respiratory epithelia following 25 weeks of gestation (Khoor *et al.*, 1994). The content of both proteins and their messengers increase during development. SP-B is first detected in amniotic fluid after 31 weeks of pregnancy and its content increases significantly to term (Pryhuber *et al.*, 1991). In the developing rat lung, SP-B mRNA is initially detected on the 18th day of gestation and increases to adult levels by the 20th day of gestation (Schellhase *et al.*, 1989). In mouse, proSP-B is initially expressed on the 11th day of gestation in airway epithelial cells. By the 17th day of gestation, proSP-B and the mature peptide are co-localised to type II cells within the distal lung, with staining increasing prior to birth (Zhou *et al.*, 1996).

In both human and mouse, SP-C follows a similar temporal pattern of development to that of SP-B. However, the spatial distribution of this protein differs from SP-A and SP-

B as the proprotein and the mature protein are detected exclusively in type II cells of the postnatal lung (Khoor *et al.*, 1994; Zhou *et al.*, 1996). Expression of SP-C mRNA precedes that of SP-A and SP-B in the foetal rat (Schellhase *et al.*, 1989) and rabbit lung (Xu *et al.*, 1995). SP-C mRNA is detected prior to the appearance of the differentiated type II cells (Weaver & Whitsett, 1991; Cockshutt & Possmayer, 1992).

SP-D appears in very low quantities on the 18th day of gestation in rat lung tissue and increases with increasing gestation (Ogasawara *et al.*, 1991). In humans, SP-D mRNA is first detected in the second trimester with expression increasing in the foetal and postnatal lung (Dulkerian *et al.*, 1996).

Differences in the temporal pattern of development between the different proteins is indicative that the genes encoding the surfactant proteins SP-A, SP-B, and SP-C are independently regulated during foetal and postnatal development (Mendelson, 2000). More studies on the development and expression of SP-D are required before a conclusion can be reached for this protein.

1.2.3.2.2.3 Respiratory Distress Syndrome

Prematurely born infants are at risk of developing respiratory distress syndrome (RDS) after birth due to inadequate development of the pulmonary surfactant system. In these infants, lung fluid clearance is compromised (Barker *et al.*, 1997) resulting in elevated diffusion distances which may cause hypoxaemia, with or without hypercapnia. The low pO_2 may not sufficiently stimulate arteriolar dilatation resulting in pulmonary hypertension. The elevated work of inspiration, coupled with the elevated elastic recoil of the lung results in a high frequency, shallow breathing pattern. The weak thoracic cage of the newborn cannot sustain the decreased lung compliance and therefore the sternum collapses, the diaphragm rises and reduced lung volume ensues. The infant uses much more energy to breathe and audible grunting occurs during expiration caused by prolonged

respiratory effort against a partially closed glottis. The saccules and developing alveoli close upon expiration causing severe atelectasis throughout the lung. If the surfactant system is not activated within several days, the lungs become rigid, which leads to inadequate inflation and often death (Meyrick & Reid, 1977). RDS is the leading cause of neonatal morbidity and mortality in developed countries (Mendelson, 2000). Antenatal administration of corticosteroids reduces the incidence of RDS (Merrill & Ballard, 1998), however RDS is primarily treated by the administration of exogenous surfactants.

1.2.3.2.3 Antioxidant Enzyme System

During birth, the alveolar oxygen tension dramatically rises within the alveoli from 25–30 mmHg in the foetus to >100 mmHg in the newborn (McElroy *et al.*, 1990), with a concomitant increase in the production of oxygen free radicals (Frank *et al.*, 1996). The principal biochemical defenses of the lung against oxygen-induced damage are the antioxidant enzymes (AOE), superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). Toxic by-products of oxygen metabolism, including superoxides, hydrogen peroxide and hydroxyl radicals are liberated when oxygen is reduced to water (Fridovich, 1978). They target pulmonary tissue and surfactant, significantly impairing the ability of the latter to control surface tension (Gilliard *et al.*, 1994). Unsaturated phospholipids such as phosphatidylserine and phosphatidylinositol are particularly sensitive to oxidative damage, whereas DSPs will protect the delicate inner lung from such injury (Goldstein, 1978). As with lung tissue, protection of surfactant phospholipids has been attributed to urate, ascorbate and the antioxidant enzymes (Bridges *et al.*, 2000). These enzymes evolved at least a billion years ago in bacteria and are highly conserved. They are indispensable components of the antioxidant system and make aerobic life possible (Fridovich, 1978). The chronological development of the AOEs is similar to the prenatal development of the pulmonary surfactant lipids in the mammalian lung (Frank &

Groseclose, 1984; Frank & Sosenko, 1987; McElroy *et al.*, 1990; Walther *et al.*, 1991b) and alveolar type II cells have the highest antioxidant enzyme activity of any pulmonary cells (Forman & Fisher, 1981). Therefore, both the pulmonary surfactant and antioxidant enzyme systems are crucial to the initiation of pulmonary ventilation. The synergistic relationship between these two crucial, highly conserved systems determines the optimal development and preparation of the lung prior to birth. It is possible that the antioxidant system was a prerequisite for the evolution of the surfactant system, which in turn enabled the evolution of lungs, airbreathing and terrestriality.

1.2.4 Regulation of the Pulmonary Surfactant System during Development

In eutherian mammals, foetal lung maturation is under multihormonal control, predominated by the effect of corticosteroids and thyroid hormones. During birth, the principal stimuli for surfactant release appear to be the first deep breaths, input from the sympathetic nervous system and possibly an increase in alveolar pO_2. Distortion of the type II cell, mediated by the dramatic increase in tidal volume and the simultaneous elevation in alveolar pO_2, may act synergistically to cause surfactant secretion.

1.2.4.1 Glucocorticoids

Towards the end of gestation, the foetal demand for oxygen surpasses the supply from the placenta, resulting in foetal hypoxia, which in turn stimulates the release of cortisol from the adrenal cortex. Endogenous corticosteroids influence the maturation of foetal organs, predominantly those derived from endodermal origin, including the lung (Post & Smith, 1992). Corticosteroids induce the synthesis of all known components of surfactant. Both endogenous and exogenous corticosteroids induce synthesis of pulmonary surfactant in the mammalian foetal lung by stimulating enzymes involved in phospholipid synthesis,

accelerating lamellar body development and influencing intracellular and extracellular concentrations of surfactant proteins (Merrill & Ballard, 1998). Furthermore, glucocorticoids upregulate antioxidant enzyme activity (Frank *et al.*, 1985; Walther *et al.*, 1991a; Sosenko *et al.*, 1995; Walther *et al.*, 1996) and augment lung fluid clearance (Wallace *et al.*, 1995) prior to, and during birth. However, the mechanisms behind the actions of glucocorticoids are poorly understood.

1.2.4.2 Thyroid Hormones

During the final trimester of pregnancy, levels of triiodothyronine increase in the umbilical cord blood of eutherian mammals. This elevation is accompanied by the surge in pulmonary surfactant content within the lung (Post & Smith, 1992). Administration of the thyroid hormones during pregnancy accelerates the morphological maturation of the lung (Rooney *et al.*, 1975) and enhances the synthesis of surfactant lipids (Gross *et al.*, 1980). Since thyroid hormones do not readily cross the placenta, maternal administration of thyrotropin releasing hormone has been used to elevate foetal triiodothyronine without causing a stress response in the foetus (Rooney *et al.*, 1979). Thyrotropin releasing hormone increases alveolar PC in foetal rabbits (Rooney *et al.*, 1979) and sheep (Liggins *et al.*, 1988), which is accompanied by an increase in lung distensibility. However, the majority of studies indicate that thyroid hormones do not stimulate the production of the surfactant proteins. In fact, triiodothyronine decreases surfactant protein mRNA levels in the rat (Post & Smith, 1992). When administered with glucocorticoids, thyroid hormones appear to augment the effects of glucocorticoids on surfactant lipid synthesis (Smith & Sabry, 1983; Liggins *et al.*, 1988), lung fluid reabsorption (Barker *et al.*, 1990; Barker *et al.*, 1991; Wallace *et al.*, 1995) and antioxidant enzyme activity (Walther *et al.*, 1991a).

1.2.4.3 Ventilation

Ventilation stimulates surfactant secretion in both adult (Oyarzun & Clements, 1978; Nicholas & Barr, 1981) and newborn mammals (Lawson *et al.*, 1979; Oulton *et al.*, 1986), by mechanical distortion of the type II cell (Wirtz & Dobbs, 1990), mediated by an increase in tidal volume (Nicholas & Barr, 1981). Inflation of the lung elevates the amount of phospholipid recovered from lavage (Oyarzun & Clements, 1977; Oyarzun & Clements, 1978; Nicholas *et al.*, 1982). Moreover, the onset of airbreathing promotes surfactant secretion at birth (Gluck *et al.*, 1967; Rooney *et al.*, 1976b; Lawson *et al.*, 1979; Oulton *et al.*, 1986). The changes in surfactant pool sizes before and after birth also suggest that expansion of the lung with gas stimulates the release of large amounts of surfactant from storage sites within the type II cells into the alveoli (Clements & Tooley, 1977). However, distension-induced secretion in newborn rabbits can be depressed by the β-adrenergic antagonist, propranolol, and is therefore mediated, in part, by the sympathetic nervous system (Corbet *et al.*, 1983). In addition to direct distortion of the type II cells, ventilation-induced secretion may also result from release of mediators from other lung cells which stimulate secretion, a reduction in intracellular pCO_2 that produces intracellular alkalosis, or membrane polarisation due to an opening of ion channels by stretch (Mason & Voelker, 1998). During the perinatal period, the rise in pO_2 may contribute to lung maturation. Increases in alveolar pO_2 augment lung fluid reabsorption by increasing sodium transport across the respiratory epithelia (Pitkänen *et al.*, 1996; Ramminger *et al.*, 2000). Furthermore, elevated pO_2 increases surfactant protein gene expression (Acarregui *et al.*, 1993), surfactant protein mRNAs and phospholipid synthesis (Acarregui *et al.*, 1995) in the human foetal lung *in vitro* and may stimulate surfactant secretion in the newborn.

1.2.4.4 β-Agonists

During the period leading up to birth, β-adrenergic influences appear to be most important in surfactant secretion (Post & Smith, 1992). Many *in vivo* and *in vitro* studies have demonstrated that β-adrenergic agonists stimulate surfactant secretion in the adult, foetus and newborn (Abdellatif & Hollingsworth, 1980; Brown & Longmore, 1981; Mettler *et al.*, 1981; Corbet *et al.*, 1983). The adrenal medulla releases catecholamines. Circulating catecholamines are believed to provide the physiological source of β-adrenergic stimulation (Oyarzun & Clements, 1977; Marino & Rooney, 1981; Massaro *et al.*, 1982). During birth, catecholamine levels increase (Padbury *et al.*, 1982) and are thought to stimulate lung fluid reabsorption from the lumen of the alveoli into the interstitium (Brown *et al.*, 1983) and stimulate surfactant secretion from alveolar type II cells (Marino & Rooney, 1981).

1.2.4.5 Other Factors

Many other factors affect surfactant secretion in the newborn and adult lung. Cholinergic agonists act indirectly to stimulate surfactant secretion by initiating contraction of intrapulmonary smooth muscle, which in turn distorts the type II cell (Massaro *et al.*, 1982). Prostaglandins and other lipid mediators may be involved in surfactant secretion in preparation for birth. Prostaglandin E2 increases surfactant secretion from lung slices of newborn rabbits, whereas infusion of prostaglandin synthetase inhibitors during late pregnancy decreases secretion (Marino & Rooney, 1980; Marino & Rooney, 1981). Indeed circulating levels of prostaglandin E2 rise in the foetus immediately prior to birth. In addition, purinergic compounds, calcium ionophores, vasopressin, endothelin-1 and lipoproteins also stimulate surfactant secretion *in vitro* (for review see Mason and Voelker (1998)). Moreover, the composition and synthesis of surfactant phospholipids can be

influenced by the sex of the foetus (Nielsen, 1986), diet (Viscardi, 1995) and exercise (Doyle *et al.*, 1994; Orgeig *et al.*, 1995).

1.3 Evolution and Conservation of the Pulmonary Surfactant System

The previous sections have been solely based on data from mammals. However, a growing body of evidence has arisen demonstrating differences and astounding similarities in the composition, morphology and function of the pulmonary surfactant system between animals with remarkably different lung structures and phyletic origins. Probably the most notable difference between mammals and non-mammals, which contributes to the differences in the composition and function of the surfactant system, is body temperature. Given the high phase transition temperature of DPPC, it is unlikely that ectothermic vertebrates that rarely experience body temperatures above 30°C, could support a surfactant highly enriched with DPPC. Without the addition of other lipids such as unsaturated phospholipids and cholesterol to fluidise the surface film, a surfactant high in DPPC would never enter the liquid crystalline state at low body temperatures and would therefore be useless (Daniels *et al.*, 1995a). Lung structure also differs markedly among the vertebrates. In mammals, pulmonary surfactant chiefly functions to stabilise the tiny respiratory units and aid lung compliance. In non-mammals with simple, compliant lungs that have extremely large respiratory units, or lack them all together, the surfactant system is believed to function as an anti-adherent (Daniels *et al.*, 1995a; Daniels *et al.*, 1998b). Surfactant in these vertebrates would not be required to vary surface tension to the same degree as mammals. Therefore, the function and composition of surfactant in different species may differ according to the thermal preference and lung architecture of the animal (Daniels *et al.*, 1995a; Daniels *et al.*, 1998b).

Moreover, during development, many species hatch from eggs. The hatching process is lengthy and energetically costly when compared to birth. In addition, the

availability of hormones and nutrients differs tremendously during development among species with different reproductive modes.

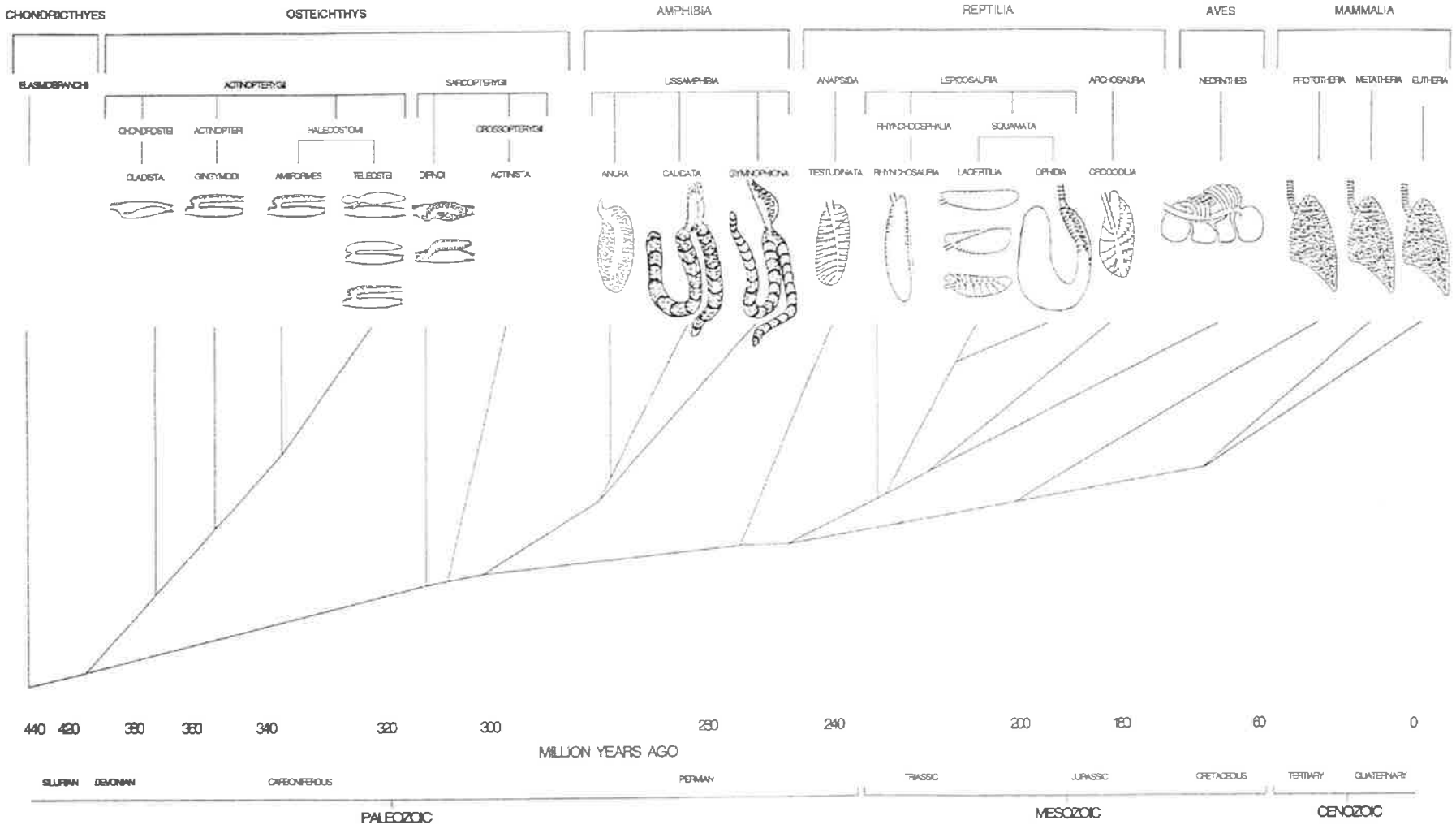
1.3.1 Lung Morphology of Non-Mammals

Lungs have evolved independently many times since the Devonian (Figure 1.4). On the whole, it is the simple saccular lung of many fish, amphibians and reptiles that represent the predominant structure. The bronchoalveolar lung of mammals, therefore, does not denote the archetypal lung structure within the vertebrates. It is possible that the development and regulation of the mammalian lung may differ from that of other vertebrates.

1.3.1.1 Unicameral Lung

Unicameral lungs are simple saccular lungs, comprising a tissue-free central lumen, surrounded by a parenchymal region containing gas exchange tissue and air spaces (Perry, 1989). These lungs are present in most fish, amphibians, lizards and snakes. The parenchyma forms a honeycomb-like network, which is supported by trabeculae on the inner surface of the lung. The trabeculae are comprised of smooth muscle and elastic tissue. Generally, the respiratory epithelium, capillaries, connective tissue and nerves span the trabecular network and the outer wall of the lung forming polygonal cubicles. These cubicles, termed faveoli, act as the respiratory unit of the unicameral lung (Perry, 1989). The respiratory units of many species that possess unicameral lungs are 100-1000 times larger than those of similar sized mammals (Daniels *et al.*, 1995b).

Figure 1.4 Evolution of vertebrate lungs, demonstrating the saccular unicameral and multicameral lungs of fish, amphibians and reptiles, the parabronchial lungs of birds, and the bronchoalveolar lungs of mammals.


CHONDRICTHYES
OSTEICHTHYS
AMPHIBIA
REPTILIA
AVES
MAMMALIA
ELASMOBRANCHI
ACTINOPTERYGII
SARCOPTERYGII
CHONDROSTEI
ACTINOPTERI
HALECOSTOMI
CROSSOPTERYGII
CLADISTA
GINGYMODI
AMIIFORMES
TELEOSTEI
DIPNOI
ACTINISTA
LISSAMPHIBIA
ANAPSIDA
LEPIDOSAURIA
ARCHOSAURIA
NEORNITHES
PROTOTHERIA
METATHERIA
EUTHERIA
RHYNCHOCEPHALIA
SQUAMATA
ANURA
CAUDATA
GYMNOPHIONA
TESTUDINATA
RHYNCHOSAURIA
LACERTILIA
OPHIDIA
CROCODILIA
440
420
360
360
340
320
300
250
240
200
160
60
0
MILLION YEARS AGO
SILURIAN
DEVONIAN
CARBONIFEROUS
PERMIAN
TRIASSIC
JURASSIC
CRETACEOUS
TERTIARY
QUATERNARY
PALEOZOIC
MESOZOIC
CENOZOIC

1.3.1.2 Multicameral Lung

The multicameral lung is present in turtles and some lizards (Duncker, 1978b; Perry, 1989). It is a more complex structure than that of the unicameral lung, comprising a cartilaginous primary intrapulmonary bronchus that communicates with several distinct chambers from which the shallow respiratory units, termed ediculae, are derived (Pastor *et al.*, 1989; Perry *et al.*, 1989; Fleetwood & Munnell, 1996). The degree of complexity differs within this grouping, with sea turtle lungs possessing the greatest partitioning and septation (Duncker, 1978b; Perry, 1989).

1.3.1.3 Parabronchial Lung

The morphology of the avian respiratory system differs markedly from the bronchoalveolar lung of mammals and the archetypal saccular lung of fish, amphibians and reptiles. Avian lungs are dense non-compliant structures with nine avascular, thin-walled airsacs emanating from them (Duncker, 1972; Duncker, 1978b). The airsacs function as bellows, passing air through the unidirectional lungs. The volume of the entire respiratory system of an adult chicken, *Gallus gallus domestica,* is between 298 and 502 ml (resin cast), whereas the volume of the lungs alone range from 35-70 ml. The trachea branches into two extrapulmonary primary bronchi that enter the lungs. The bird lung can be divided into three subdivisions, the primary bronchus, secondary bronchi and parabronchi. The parabronchi never exceed 20-25% of the total lung volume. In the chicken lung, there are 300-500 parabronchi, between 1 and 4 cm in length, with a constant diameter of 500 μm. Extremely thin tubules emanate from the parabronchial walls. These tubules, or air capillaries, range from 3-10 μm in diameter depending on the species and provide the site for gas exchange (Duncker, 1972). They are in close apposition to blood capillaries, which

lie perpendicular to the parabronchi, resulting in an efficient cross current exchange system.

1.3.1.4 Type II cells

Despite gross differences in lung architecture, all vertebrate lungs contain pulmonary surfactant and, hence, the cellular machinery for its manufacture. Type II cells, or analogous cell types, have been described in representatives from each vertebrate group. Moreover, lamellar bodies, an osmiophilic surface film and tubular myelin have been demonstrated in most taxa (for review see Daniels and Orgeig (2001)) (Table 1.2). Surfactant from non-mammalian vertebrates therefore appears to be synthesised, stored and released in a similar manner to mammalian surfactant.

1.3.2 Composition of the Pulmonary Surfactant System in Non-Eutherians

1.3.2.1 Lipids

Pulmonary surfactant has been identified in the gas holding structures of representatives of every vertebrate group (Daniels *et al.*, 1995b; Daniels *et al.*, 1998b; Daniels & Orgeig, 2001) and one invertebrate, the pulmonate snail, *Helix aspersa* (Daniels *et al.*, 1999). The lipid composition of pulmonary surfactant has been reviewed extensively (Daniels *et al.*, 1995a; Daniels *et al.*, 1998b; Daniels & Orgeig, 2001). Therefore, only the predominant changes in composition will be highlighted in the present section. The composition and efficacy of surfactant from non-eutherian vertebrates relates primarily to influences of temperature on the physiochemical nature of lipids, structural differences in respiratory units within the vertebrates, and habitat selection of the different species (Daniels *et al.*, 1995a; Daniels *et al.*, 1995b; Daniels *et al.*, 1998a).

Table 1.2 *Morphology of the pulmonary surfactant system within the vertebrates. Reproduced from Daniels and Orgeig (2001).*

Species	Type II cells (+ analogues)	Lamellar Bodies	Tubular Myelin	Other forms and comments
Mammalia				
Eutheria				
Rattus norvegicus [a]	+	+	+	Lamellar bodies (LBs) numerous – originally called cytosomes; Vesicular forms; common myelin figures
Metatheria				
Sminthopsis crassicaudata [b]	+	+	?	Type II cells can be isolated; secrete phospholipid
Aves				
Gallus gallus domestica [c]	+	+	?	LBs in epithelial cells of atria; non-uniform distribution; exocytosis observed
" [d]	?	+	?	Trilaminar substance in air spaces;
" [e,f,g,h,i]	+	+	?	Lamellar bodies in epithelial cells
" [j,k]	+	+	?	Continuous osmiophilic bilaminar layer on the surface of the air capillaries (~100-150Å). Fully formed by day-20 of incubation. LBs rare in cells of air capillaries. Most in atria.
" [l,m]	+	+	-	Three cell types in respiratory epithelium: granular pneumocytes (type II cells; LBs; short microvilli), squamous atrial cells (produce trilaminar substance) and squamous respiratory cells (form blood-gas barrier). Trilaminar substance overlying granular cells in atria.
Anser anser [n]	?	+	?	
Columba livia [o]	?	+	?	
[l,m]	+	+	-	Three cell types; trilaminar substance in atria
Ferinus canarius [o]	?	+	?	
Coturnix coturnix [l,m]	+	+	-	Three cell types; trilaminar substance in atria
Tyto tyto alba [l,m]	+	+	-	Three cell types; trilaminar substance in atria
Melopsittacus undulatus [p]	+	+	-	Granular (type II) pneumocytes confined to linings of airsacs, parabronchi, and atria; surfactant secretions cover the surfaces of the infundibula and respiratory space (air capillaries).

Table 1.2 continued

Species	Type II cells (+ analogues)	Lamellar Bodies	Tubular Myelin	Other forms and comments
Reptilia				
Ophidia				
Elaphe climacophora [q]	+	+	-	
Elaphe quadrivirgata [q]	+	+	-	
" [f]	+	+	?	
Elaphe obsoleta obsoleta [r]	+	+	?	Internal configuration of lamellar bodies not clear
Ophidia *continued*				
Crotalus viridis oreganus [s]	+	+	+	
Dendroaspis polylepis [t]	+	+	?	Two cell types
Lacertilia				
Anguis fragilis [u]	+	+	+ or ?	Membranous material in airsacs (whorls, stacks of lamellae, lattices); LBs have variable amounts of osmiophilic material
Gekko japonicus [q, f]	+	+	?	Lamellar bodies looked like vacuoles, i.e. no lamellae
Ctenophorus nuchalis [v, w]	+	+	+	
Rhacodactylus leachianus [x]	+	-	-	Only one very lightly stained lamellar body, many vacuolated granules and some extracellular membranous material
Lacerta viridis [y]	+	+	-	
Tupinambis nigropunctatus [z]	+	+	-	Lamellar bodies present in the alveolar space between type II cells
Pogona vitticeps [aa]	+	+	?	Type II cells can be isolated; secrete phospholipid
Testudinata				
Testudo graeca [bb]	+	+	?	Lamellated membranous material in airsacs
[cc]	+	+	?	Type I & II cells, classical LBs, multivesicular bodies, lipid droplets
Chelonia mydas [dd]	+	+	?	

Table 1.2 continued

Species	Type II cells (+ analogues)	Lamellar Bodies	Tubular Myelin	Other forms and comments
Amphibia				
Anura				
Xenopus laevis [ee]	?	+	?	
" [ff]	+	+	?	One cell type
Bufo bufo [gg]	+	+	+	One cell type; LBs common, also in airspace; tubular myelin common; membranes become fuzzy and have irregularly spaced rod-like particles (8-13nm diameter). Used tannic acid
Rana esculenta [ee]	?	+	?	
" [hh]	+	+	+	
Rana nigromaculata [ii]	+	+	-	Osmiophilic bodies found in both type I
Bufo vulgaris japonicus [ii]	+	+	-	and type II cells
Rana temporaria [jj]	+	+	?	One cell type
Rana pipiens [r]	+	+	?	
Rana catesbeiana [b]	+	+	?	Cells can be isolated; secrete phospholipid; LBs lack lamellae, appear as electron-dense bodies
Hyla arborea [gg]	+	+	+	One cell type; LBs common, also in airspace; tubular myelin rare, irregular, unstable; membranes 4-7.2nm thick. Used tannic acid
" [kk]	+	+	?	3 types of LBs: lamellar, dense & multivesicular; surface of lining coated by thin film
Gymnophiona				
Ichthyophis orthoplicatus [jj]	?	+	-	No distinction between type I and II cells
Caudata				
Triturus pyrrhogaster [ii]	+	-	-	Both type I and II cells; neither contained osmiophilic bodies
" [f]	+	+	?	
Triturus vulgaris [ll]	?	-	-	Lamellar bodies were absent
" [jj]	?	+	-	Lamellar bodies present, but rare

Table 1.2 continued

Species	Type II cells (+ analogues)	Lamellar Bodies	Tubular Myelin	Other forms and comments
Caudata *continued*				
Triturus cristatus [mm, nn]	+	+	?	No distinction between type I and II cells; Multivesicular and lamellar bodies in adult and large juveniles
Triturus alpestris [oo]	?	-	?	
Notophthalumus viridescens [pp]	+	+	?	No distinction between type I and II cells
Ambystoma punctatum [qq, rr]	?	+	?	Masses of fibrils ca. 12nm, similar to trilaminar substance
Necturus maculosus [r]	?	+	?	
Proteus anguinus [ss, tt]	?	-	?	
Salamandra salamandra [uu]	+	+	?	Myelin-like bodies; one type of pneumocyte
" [vv]	+	+	-	One cell type; "surfactant" present
" [ww]	+	+	-	LBs at different developmental stages, i.e. electron-dense bodies, no lamellae, paracrystalline structure. Despite using tannic acid, no tubular myelin seen.
Hynobius nebulosus [xx]	+	+	?	Single cell type (features of type I & II); LBs evenly lamellate, double membrane
[yy]	+	+	+	Ciliated epithelial cells also contain LBs – contain loosely lamellated inner core and highly dense, compact outer layer; Tubular myelin in airspaces derived from unravelling LBs.
Osteichthys				
Dipnoi				
Protopterus annectens [zz]	-	-	?	
Protopterus annectens [aaa]	?	+	?	
Protopterus sp. [bbb]	+	+	-	
" [qq]	+	+	?	Osmiophilic inclusion bodies in cells and in alveolar space
Lepidosiren paradoxa [tt]	+(*)	+	?	(*) Only one cell type
" [hh]	+	+	-	Extracellular material also present

Table 1.2 continued

Species	Type II cells (+ analogues)	Lamellar Bodies	Tubular Myelin	Other forms and comments
Dipnoi *continued*				
Neoceratodus forsteri [ff]	+(*)	+	?	
[b]	+	+	?	Type II cells can be isolated; secrete phospholipid
Teleostei				
Salmo gairdneri [ccc]	+(*)	+	-(*)	Non-ciliated (columnar or cuboidal ?) cells that secrete mucus-like material into lumen of swimbladder
Anguilla vulgaris [ddd]	?	+	?	
Lota lota [eee]	+	+	?	Very isolated LBs in cells of the gas gland
Acerina cernua [eee]	?	+	?	
Coregonus lavaretus [fff]	?	+	?	
Fundulus heteroclitus [ggg]	?	+	?	
Gadus callarias [ggg]	?	+	?	
Opsanus tau [ggg]	?	+	?	
Amphipnous cuchia [hhh]	+(*)	-	-	(*)Cuboidal cells; airsac instead of lung
" [iii]				
Anabas tetudineus [hhh]	-	-	-	Labyrinthine organ
" [iii]				
Channa punctatus [hhh]	+(*)	-	-	(*)Cuboidal cells; suprabranchial chamber
" [iii]				
Channa striatus [hhh]	+(*)	-	-	(*)Cuboidal cells; suprabranchial chamber
" [iii]				
Clarias batrachius [hhh]	+(*)	-	-	(*)Cuboidal cells with vesicles; airsac
" [iii]				
Heteropneustes fossilis [iii]	+(*)	-	-	(*)Cuboidal cells; airsac
" [iii]				
Misgurnus fossilis [kkk]	+(*)	+	-	(*)Goblet epithelial cell of respiratory intestine
Amiiformes				
Amia calva [ff]	?	+	?	

Table 1.2 continued

Species	Type II cells (+ analogues)	Lamellar Bodies	Tubular Myelin	Other forms and comments
Cladista				
Polypterus senegalensis [zz]	-	-	-	Electron-opaque granules in rare cells in secretory crypts
Polypterus ornatipinnis [iii]	+	+	-	"Classical-appearing" type II cells
Arthropoda				
Mollusca				
Helix aspersa [lll]	+	+	?	Lamellar body exocytosed into airspace of gas mantle

'+' indicates presence, '-' indicates absence, '?' indicates unresolved or not attempted. *a* (Sorokin, 1967), *b* (Wood *et al.*, 2000), *c* (Akester, 1970), *d* (Tyler & Pangborn, 1964), *e* (Petrik, 1967), *f* (Nagaishi *et al.*, 1964), *g* (Lopez *et al.*, 1984), *h* (Bhattacharyya *et al.*, 1976), *i* (Jones & Radnor, 1972), *j* (Petrik & Reidel, 1968), *k* (Petrik & Reidel, 1968), *l* (Scheuermann *et al.*, 1997), *m* (Klika *et al.*, 1997), *n* (Lambson & Cohn, 1968), *o* (Bargmann & Knoop, 1961), *p* (Smith *et al.*, 1986), *q* (Okada *et al.*, 1963), *r* (Brooks, 1970a), *s* (Luchtel & Kardong, 1981), *t* (Maina, 1989), *u* (Meban, 1978a), *v* (Daniels *et al.*, 1990), *w* (McGregor *et al.*, 1993), *x* (Perry, 1989), *y* (Meban, 1978b), *z* (Klemm *et al.*, 1979), *aa* (Wood *et al.*, 1999), *bb* (Meban, 1977b), *cc* (Pastor *et al.*, 1989), *dd* (Solomon & Purton, 1984), *ee* (Bargmann & Knoop, 1956), *ff* (Meban, 1973), *gg* (Goniakowska-Witalinska, 1984), *hh* (Hughes & Weibel, 1978), *ii* (Okada *et al.*, 1962), *jj* (Pattle *et al.*, 1977), *kk* (Goniakowska-Witalinska, 1986), *ll* (Pattle, 1976), *mm* (Meban, 1977a), *nn* (Goniakowska-Witalinska, 1980b), *oo* (Goniakowska-Witalinska, 1980a), *pp* (Hightower *et al.*, 1974a; Hightower *et al.*, 1974b), *qq* (De Groodt *et al.*, 1960), *rr* (Sorokin, 1963), *ss* (Hughes, 1970), *tt* (Hughes, 1973), *uu* (Goniakowska-Witalinska, 1978), *vv* (Meban, 1979), *ww* (Goniakowska-Witalinska, 1980c), *xx* (Matsumura & Setoguti, 1984), *yy* (Matsumura, 1984), *zz* (Klika & Lelek, 1967), *aaa* (Klika & Janout, 1967), *bbb* (Hughes *et al.*, 1973), *ccc* (Brooks, 1970b) *ddd* (Dorn, 1961), *eee* (Jasinski & Kilarski, 1964), *fff* (Fahlen, 1967), *ggg* (Copeland, 1969), *hhh* (Munshi, 1976), *iii* (Hughes & Munshi, 1973), *jjj* (Marquet *et al.*, 1974), *kkk* (Jasinski, 1973), *lll* (Daniels *et al.*, 1999).

Despite differences in the surface-active properties and the quantity of pulmonary surfactant among the vertebrates, the main constituents have been highly conserved throughout evolution (Daniels *et al.*, 1995a; Daniels *et al.*, 1996; Daniels *et al.*, 1998b; Sullivan *et al.*, 1998). In general, pulmonary surfactant is comprised of surfactant proteins and varying amounts of both saturated and unsaturated phospholipids and neutral lipids, predominantly cholesterol (Daniels *et al.*, 1995a; Sullivan *et al.*, 1998).

1.3.2.1.1 Phospholipids

When expressed on the basis of wet lung mass, the total amount of surfactant increases from low levels in fish to intermediate levels in amphibians to high levels in reptiles and mammals (Daniels *et al.*, 1995a). Similarly, during the course of evolution, the saturation of the surfactant phospholipids has increased. Despite having 7-100-fold more surfactant per unit of respiratory surface area when compared to mammals (Smits *et al.*, 1994), teleost swimbladders, actinopterygiian fish lungs and the lungs of the ancient dipnoan, *Neoceratodus forsteri*, possess the least saturated pulmonary surfactant (Daniels *et al.*, 1995a; Orgeig & Daniels, 1995; Daniels *et al.*, 1998b). The lungs of the derived dipnoans contain phospholipids that are 2-4-fold more saturated than the actinopterygiians and *N. forsteri* (Orgeig & Daniels, 1995; Daniels *et al.*, 1998b). Phospholipids from amphibian lungs contain an intermediate level of saturation whereas the reptiles and mammals have the most highly saturated surfactant (40-50%) (Daniels *et al.*, 1995a). Generally, all taxa possess similar phospholipid profiles. Phosphatidylcholine is the dominant phospholipid headgroup in vertebrate surfactant, contributing 60-80% of the total phospholipid content (Daniels *et al.*, 1996; Veldhuizen *et al.*, 1998). Furthermore, palmitate appears to be the principal fatty acid of PC in pulmonary surfactant from species belonging to every class examined (Harlan *et al.*, 1966; Clements *et al.*, 1970; Lau & Keough, 1981; Daniels *et al.*, 1989; Daniels *et al.*, 1995b; Daniels & Orgeig, 2001). DPPC contributes a large

proportion of the surface-active saturated phospholipids in all types of vertebrate pulmonary surfactant; a feature that appears to be highly conserved (Clements *et al.*, 1970; Daniels *et al.*, 1995a; Daniels *et al.*, 1996; Daniels *et al.*, 1998b). This particular fatty acid chain length is believed to represent the ideal compromise between the ability to pack tightly at low lung volumes, thereby lowering surface tension, and the ability to respread during inspiration (Daniels *et al.*, 1995a). Indeed, DPPC has lower surface viscosities than both dipalmitoyl phosphatic acid and dipalmitoylphosphatidylglycerol (Evans *et al.*, 1980). Thus, this molecule may represent the ideal blend of high surface activity and low surface viscosity necessary for generating a monolayer at lower body temperatures (Daniels *et al.*, 1995a; Daniels *et al.*, 1998b).

Vertebrates from each class contain combined phosphatidylserine and phosphatidylinositol (PS/PI) as well as sphingomyelin (Daniels *et al.*, 1995b). Generally, sphingomyelin is present in low amounts except in the rattlesnake, where it represents between 20-25% of the total phospholipid. The reasons for the high levels of sphingomyelin in this species are unknown (Daniels *et al.*, 1995c). Phosphatidylserine and phosphatidylinositol are present in relatively abundant amounts throughout the vertebrates, contributing to approximately 10-25% of the total phospholipid pool (Daniels & Orgeig, 2001). It seems that surfactants rich in PI, lack PG. Only the snake, *Thamnophis ordinoides* (Daniels *et al.*, 1995c), anurans and mammals have detectable levels of PG within their surfactant (Hallman & Gluck, 1976; Phleger & Saunders, 1978; Vergara & Hughes, 1980; Lau & Keough, 1981; Daniels *et al.*, 1996). Given that PI and PG are capable of substituting each other, they may share a common role in pulmonary surfactant (Keough, 1992). The high levels of PI in species lacking PG are therefore believed to compensate for the absence of PG (Daniels *et al.*, 1995a). Whereas PG is thought to function to maintain fluidity in mammalian surfactant (Hallman & Gluck, 1976), its role in the non-mammalian lung has not been described. The remaining "trace" phospholipids, PS

and PE, demonstrate some minor differences among the vertebrates, however, the significance of these differences is not known (Daniels *et al.*, 1995a).

1.3.2.1.2 Neutral Lipids

The fraction of surfactant cholesterol decreases across the vertebrate classes from fish to mammals (Daniels *et al.*, 1995a). Relative to total phospholipid, the actinopterygiian fish and *Neoceratodus forsteri* have 3-fold more cholesterol than any other vertebrate class (Orgeig & Daniels, 1995). The relative increase in the saturation of phospholipids and decrease in cholesterol during evolution, leads to a 10-15-fold decrease in the amount of cholesterol relative to disaturated phospholipid across the vertebrate classes from fish to mammals (Daniels *et al.*, 1995a). The primitive lungfish and actinopterygiian fish have a surfactant high in cholesterol and unsaturated phospholipids and low in disaturated phospholipids, whereas the more derived lungfish and remaining tetrapods have a surfactant high in DSP and low in cholesterol (Daniels *et al.*, 1995a). Taken together, this data suggests that pulmonary surfactant evolved once and the composition present in actinopterygiians represents the ancestral surfactant (Daniels *et al.*, 1995a). Moreover, the presence of surfactant in the gas mantle of the pulmonate snail, suggests a more ancient system than previously realised (Daniels *et al.*, 1999).

1.3.2.2 Proteins

Surfactant proteins are also conserved throughout evolution. SP-A and its messenger RNA have been found in the lungs of representatives from every vertebrate group (Sullivan *et al.*, 1998). Similarly, SP-A has been detected in the swimbladder of carp, *Cyprinus carpio* (Rubio *et al.*, 1996), eel, *Anguilla anguilla,* perch, *Perca fluviatilis* (Prem *et al.*, 2000) and the lung of the chicken in both adults (Bhattacharyya *et al.*, 1976; Sullivan *et al.*, 1998) and

embryos (Zeng *et al.*, 1998). Interestingly, Bernhard *et al.* (2001) failed to detect SP-A in lavage from ducks and chickens, whereas the presence of SP-B was confirmed in these species. Likewise, SP-B is expressed in the developing chicken lung (Zeng *et al.*, 1998) and the adult axolotl lung, *Ambystoma mexacanum* (Miller *et al.*, 2001). Furthermore, SP-B has recently been discovered in the lung of the teleost fish, the tarpon, *Megalops cyprinoides* and pulmonate snail, *Helix aspersa*, by ELISA (Sullivan, unpublished data). To date, SP-C has not been detected in the lungs of any non-mammalian vertebrate, despite efforts to detect its presence in avian lungs using gel filtration HPLC (Bernhard *et al.*, 2001) and the axolotl lung by immunostaining with an antiserum that was generated against human proSP-C (Miller *et al.*, 2001). However, all surfactant proteins are expressed in the developing lung of the marsupial, the tammar wallaby, *Macropus eugenii* (Miller, unpublished data). The reason for the absence of SP-C remains unclear at this time. The remaining hydrophilic surfactant protein, SP-D is expressed in the amphibian lung (Miller *et al.*, 2001). Collectively, these studies and those of the lipids, confirm that the pulmonary surfactant system evolved once and has subsequently undergone modifications throughout evolution by a range of selection pressures (Daniels *et al.*, 1995b; Daniels *et al.*, 1998b; Daniels & Orgeig, 2001).

1.3.3 Regulation of the Pulmonary Surfactant System in Non-Eutherians

1.3.3.1 Determinants of Lipid Composition

Selection pressures such as temperature, terrestriality, habitat preference and lung morphology have contributed to the variation in the relative proportions of lipids within the lungs of the different vertebrates. However, the primary determinant of surfactant composition among the vertebrates appears to be temperature-dependant fluidity of the surfactant phospholipids and the need to maintain homeoviscosity (Daniels *et al.*, 1998b).

Since the phase transition temperature of DPPC exceeds the range of temperatures that most ectothermic vertebrates would experience, a surfactant rich in DPPC would always be in the gel state and therefore only homeotherms and the most heliothermic of reptiles would be able to tolerate surfactants high in saturated phospholipids. Species with lower body temperatures, such as the amphibians and the derived lungfish, support surfactants with lower degrees of saturation (Daniels *et al.*, 1995a; Daniels *et al.*, 1998b).

Preferred body temperatures affect the composition of surfactant in reptiles. Both the central netted dragon, *Ctenophorus nuchalis* and the rattle snake, C*rotalus atrox* have preferred body temperatures in excess of 35°C and both possess mammalian levels of DSP in their surfactant when measured at 37°C (Daniels *et al.*, 1995b). The remaining species studied, spanning all orders within the class Reptilia, possess 23-33% DSP relative to total phospholipid (Daniels *et al.*, 1995b; Daniels *et al.*, 1996). Furthermore, map turtles, *Malaclemys geographica*, maintained at 5°C for three months have less saturated PC than animals maintained at 32°C (Lau & Keough, 1981). Also, the content of surfactant that can be harvested from the lungs of this turtle (Lau & Keough, 1981) and the bearded dragon, *Pogona vitticeps* (Wood *et al.*, 1995) increases with increasing temperature.

Habitat selection is believed to affect the degree of saturation of the phospholipids in amphibian surfactant. Terrestrial amphibians have a lower saturation of surfactant phospholipids than their aquatic counterparts, which is believed to relate to the more variable temperature ranges encountered by terrestrial species (Daniels *et al.*, 1994b). By possessing higher levels of DSP, surfactant from aquatic amphibians can act as a splint and, therefore, the lungs are able to withstand high hydrostatic compression forces without collapsing when the animals are submerged in deep waters (Daniels *et al.*, 1994b; Orgeig *et al.*, 1994). However, habitat preference does not affect surfactant composition of aquatic reptiles, presumably because of the presence of ribs and/or shells which may

protect the lungs from the elevated hydrostatic pressures (Daniels *et al.*, 1995b; Daniels *et al.*, 1996).

Relative to total phospholipid, the proportions of cholesterol and unsaturated phospholipids appear to relate to the ability of the mixture to adsorb and respread over the air/liquid interface at different body temperatures. Cholesterol lowers the normal phase transition temperature of DSP (Hadley, 1985). Similarly, unsaturated phospholipids have lower phase transition temperatures than saturated phospholipid, and therefore both are able to maintain the surface film in a fluid state at lower temperatures. Ectotherms experience rapid changes in body temperature during their daily cycles. In addition, ectotherms and heterotherms tolerate depressed body temperatures for protracted periods during torpor or hibernation. Cold turtles, hibernating for 2-3 months, produce a surfactant that is higher in unsaturated phospholipids than warm, active animals (Lau & Keough, 1981). When exposed to step-wise decreases in temperature, *C. nuchalis* responds by rapidly increasing the content of cholesterol (Daniels *et al.*, 1990). Likewise, reduced body temperatures during torpor in the marsupial, *Sminthopsis crassicaudata* (Langman *et al.*, 1996) and the microchiropteran bat, *Chalinolobus gouldii* (Codd *et al.*, 2000) are associated with the production of a surfactant enriched in cholesterol.

1.3.3.2 Control of Lipid Secretion

Despite the conservation of the surfactant system both morphologically and compositionally, there appear to be differences in the control of secretion of lipids among the classes. These changes are primarily related to the mode of ventilation of the lungs and the metabolism of the animals.

1.3.3.2.1 Phospholipids

Unlike mammals, changes in ventilatory pattern in the isolated lizard lung do not affect secretion of phospholipids (Wood *et al.*, 1995). However, both sympathetic and parasympathetic agonists trigger phospholipid release in the isolated perfused lung of lizards (Wood *et al.*, 1995; Wood *et al.*, 1997) and from isolated type II cells of lizards (Wood *et al.*, 1999), frogs and heterothermic marsupials (Wood *et al.*, 2000), suggesting that phospholipid secretion is under autonomic control in the non-eutherian lung (Wood *et al.*, 1997; Wood *et al.*, 1999; Wood *et al.*, 2000). Low metabolic rates, coupled with intermittent breathing patterns and differences in lung inflation, may lead to differences in surfactant secretion between eutherian mammals and non-mammals (Wood *et al.*, 1995). Likewise, in the heterothermic marsupial, depressed ventilation and metabolic rate in conjunction with reduced body temperatures may necessitate parasympathetic control of the surfactant secretion during torpor (Daniels *et al.*, 1998b; Wood *et al.*, 2000). Like the majority of taxa, the bearded dragon ventilates its lungs by changing the volume of the large central lumen without appreciably distorting the respiratory region (Daniels *et al.*, 1994a; Wood *et al.*, 1995). Such ventilatory patterns may result in irregular distortion of the type II cell and therefore ventilation would not be a reliable mechanism for continued surfactant secretion in non-mammalian species (Wood *et al.*, 1995; Wood *et al.*, 2000). However, only the cholinergic agonist, acetylcholine, elicits surfactant secretion from type II cells isolated from the primitive lungfish, *N. forsteri*, suggesting that the parasympathetic nervous system represents the phylogenetically primitive control mechanism of the surfactant system in the vertebrates. Sympathetic and ventilatory control of surfactant secretion may have developed subsequent to the development of the stem ancestor of the vertebrates (Wood *et al.*, 2000). The control of phospholipid release at birth in non-eutherians may, therefore, differ substantially from that of eutherian mammals.

1.3.3.2.2. Cholesterol

Release of cholesterol from both the isolated lizard lung (Wood *et al.*, 1995; Wood *et al.*, 1997) and from isolated type II cells of lungfish, frogs, lizards and heterothermic marsupials (Wood *et al.*, 1999; Wood *et al.*, 2000) is not stimulated by either sympathetic or parasympathetic agonists. In contrast to rats (Orgeig *et al.*, 1995), ventilation does not elicit changes in the relative abundance of cholesterol in surfactant harvested from the isolated perfused lung of lizards (Wood *et al.*, 1995). Cholesterol, therefore, is not under autonomic or ventilatory control and is regulated independently from the phospholipids.

1.3.4 Function of the Pulmonary Surfactant System in Non-Eutherians

1.3.4.1 Lung Compliance

Whereas pulmonary surfactant increases lung compliance in eutherian mammals, comparative studies show that lung compliance is not affected by the presence of surfactant in any other vertebrate group (Hughes & Vergara, 1978; Perry & Duncker, 1978; Daniels *et al.*, 1993; Wood *et al.*, 1995). Moreover, lung compliance in the saccular lungs of reptiles (Pages *et al.*, 1990; Daniels *et al.*, 1993; Daniels *et al.*, 1995a) and the airsacs of birds (Perry & Duncker, 1980) is one to two orders of magnitude greater than that of mammalian lungs. The contribution of non-mammalian surfactants to the overall lung compliance in partially inflated *in situ* lungs is small (Daniels *et al.*, 1993) or negligible in isolated lungs (Wood *et al.*, 1995).

1.3.4.2 Promotion of Alveolar Stability

In mammals, pulmonary surfactant promotes alveolar stability. Conversely, maintenance of alveolar stability does not appear to be a crucial function of pulmonary surfactant in non-mammalian vertebrates. The reasons for this are two-fold. Firstly, for any surfactant to stabilise the respiratory units, the units themselves must be extremely small. As discussed in Section 1.2.2.2, the fibrous structure of the bronchoalveolar lung of mammals is crucial for promotion of alveolar stability. Secondly, the surface activity of the surfactant must be extremely high. Therefore, at end expiration an extremely low surface tension is required. Whereas the bronchoalveolar lung of mammals meets such requirements, the saccular lungs of reptiles and the parabronchial lungs of birds do not. Avian lungs do not expand and contract cyclically and the respiratory units form rigid tiny tubules that are reinforced with connective tissue and, therefore, unlikely to collapse (Duncker, 1978a). Moreover, reptiles possess extremely large respiratory units that are up to 1000 times larger than an equivalent sized mammal (Daniels *et al.*, 1995a). The size of the faveoli, coupled with an enriched content of elastin and collagen in the walls of the faveolus and a muscular trabecular network lining the lumen of the faveolus provide greater structural support than the alveoli of mammalian lungs (Daniels *et al.*, 1995b; Daniels *et al.*, 1998b).

In general, the surface activity of pulmonary surfactant from non-mammals is low. Thus, the minimum surface tension that can be reached by such surfactants is considerably higher than that of mammals (Daniels *et al.*, 1998a; Daniels *et al.*, 1999). In these instances, the surfactant acts more like a detergent, as it does not vary surface tension considerably when compared to lung volume. Hence, it is unlikely that non-mammalian vertebrates require a surfactant that is able to vary surface tension greatly, to maintain faveolar stability.

1.3.4.3 Control of Fluid Balance

The parabronchial lung of birds is susceptible to oedema because the tiny radius of the parabronchi leads to large negative pressures within the tubules (Petrik & Reidel, 1968; Duncker, 1972; Pattle, 1978). Moreover, hydrostatic capillary tension would also cause fluid to be drawn into the parabronchi like fluid into a sponge. Characteristically, reptilian lungs have scarce amounts of interstitial tissue, causing capillaries to bulge into the airspace at low lung volumes. This, coupled with a relatively higher pulmonary capillary filtration rate, caused by both higher capillary pressures and leakier capillary membranes (Burggren, 1982; Smits, 1989), may lead to faveolar flooding at low lung volumes. It is likely therefore, that lowering surface tension within the lung of non-mammalian vertebrates would reduce the accumulation of fluid within the airspaces (Orgeig *et al.*, 1997). Indeed, surfactant retards the flux of fluid into the lumen of the adult bearded dragon lung, *Pogona vitticeps*, and augments the uptake of faveolar fluid into the lymphatic and venous system of the turtle, *Trachemys scripta* (Orgeig *et al.*, 1997).

1.3.4.4 Prevention of Epithelial Adherence

Many non-mammalian species routinely collapse their lungs as part of the respiratory cycle. Therefore, an anti-adhesive function for pulmonary surfactant may be of prime importance in these species (Daniels *et al.*, 1993; Daniels *et al.*, 1995a), particularly at low body temperatures. Breathing patterns are dramatically affected by temperature. When cooled, the metabolic rate of ectotherms decreases, reducing the need for frequent breaths, causing lung collapse over long periods (Frappell & Daniels, 1991a; Frappell & Daniels, 1991b). At low body temperatures, the lizard *Ctenophorus nuchalis* experiences periods of apnoea which are accompanied by lung collapse where the respiratory surfaces may come into contact (Frappell & Daniels, 1991a; Daniels *et al.*, 1994a). During lung deflation in

C. nuchalis, the epithelial tissues, which are strung between the outer lung wall and the inner trabecular network, fold in on each other like a concertina. This results in large portions of epithelial tissue coming into contact, a situation in which the anti-adhesive function of surfactant may be critical (Daniels *et al.*, 1994a). Moreover, lizards lack a diaphragm which results in distortion and compression of the lungs by the remaining viscera, particularly in the caudal and ventral regions (Daniels *et al.*, 1994a). In animals that collapse their lungs, it is possible that surfactant reduces the work of separating apposing surfaces and also reduces the shear forces that could damage the delicate respiratory epithelium as the faveoli unfold upon inflation (Daniels & Orgeig, 2001). During birth in these species, surfactant could be equally important in reducing the opening pressures of the collapsed or fluid-filled lungs.

1.3.4.5 Immune Functions

Like mammals, pulmonary surfactant enhances airway clearance in amphibians by removing particles via the mucociliary escalator (Kilburn, 1969; Allegra *et al.*, 1985). Reptiles (McGregor *et al.*, 1993; Fleetwood & Munnell, 1996) and birds (Crespo *et al.*, 1998) have extensive mucociliary tracts along their airways and airsacs, respectively. Low pH significantly impairs mucociliary transport. In pigeons, exogenous administration of surface-active phospholipids reduces the acid-induced inhibition of mucociliary clearance from tracheal epithelium, suggesting that surfactant may have a protective role in mucociliary clearance in birds (Kai *et al.*, 1989). It is likely that pulmonary surfactant would be crucial for lung clearance in other species which lack airways, a diaphragm or an ability to cough (Daniels *et al.*, 1995a; Daniels *et al.*, 1995b). This function may also be important for animals exposed to particulate pollutants. Indeed, birds exposed to urban pollutants have depleted intracellular surfactant stores and higher numbers of macrophages within their lungs than birds from rural areas (Lorz & Lopez, 1997). The snake, *Boa*

constrictor, increases surfactant secretion in response to inhaled particles (Grant *et al.*, 1981). It is possible, therefore, that diminished intracellular stores in birds relate to the increase in surfactant secretion observed in snakes.

The role of surfactant proteins in host defense of non-mammals has not been investigated, however because SP-A is expressed in the primary bronchus of chickens and not in the atria of the tiny parabronchi, a host defense mechanism has been postulated for this species (Zeng *et al.*, 1998).

1.4 Development in Non-Mammalian Amniotes

The vertebrates are further segregated into two groups, the amniotes and the anamniotes, on the basis of the dependence on water for growth and development of their eggs. Extant amniotes, comprising reptiles, birds, monotremes and therian mammals, are defined by the presence of three extra-embryonic membranes during development, the amnion, chorion and allantois. These membranes and the external envelopes, in conjunction with the yolk sac, provide the developing embryo with protection, nutrients and water whilst allowing movement of respiratory gases and removal of nitrogenous waste within an internal environment. The evolution of the amniotic egg contributed to the explosive radiation of the vertebrates onto land (Thompson, 1992). Likewise, pulmonary surfactant was a prerequisite for the evolution of the vertebrate lung and airbreathing and the subsequent radiation within the terrestrial environment (Daniels *et al.*, 1995a). Amniotes, therefore, are extremely diverse and, whilst unified by the presence of the extraembryonic membranes, they demonstrate many phyletic, structural and reproductive differences. Both ectothermic and endothermic amniotes may bear live young or lay eggs. In each case, the young may be born in a developmentally advanced (precocial) state or an altricial state where the offspring must undergo significant developmental changes after birth. As mentioned previously, lung structure and habitat preferences differ among different species

and all these differences may impact on the development of the pulmonary surfactant system.

1.4.1 Lung Development of Non-Mammalian Amniotes

Few studies have focussed on lung maturation in species other than birds and mammals. As Perry (1989) observes "I was unable to find a single original publication on foetal lung development in reptiles more recent than 1942". Since that time only two others have appeared in the literature (Maloney *et al.*, 1989; Perry *et al.*, 1989). Moreover, many of the papers to which Perry (1989) refers, are written in German. Therefore, the majority of this section is based upon his interpretation and that of Ewert (1985) of the early work on reptilian lung development. This previous work chiefly focussed on the manner in which lung bud formation occurred, what influenced the development of the lung to give rise to multicameral or unicameral ultrastructure and the use of the lung and its development as a phylogenetic tool. These studies, therefore, encompassed the early stages of lung development, prior to the development of the lung parenchyma.

1.4.1.1 Unicameral and Multicameral Lungs

In sea turtles, the formation of the lung chambers occurs by dichotomous branching, whereas in the crocodilians chambers form by polar branching (Broman, 1939; Broman, 1940). The lungs arise from the ventrolateral surface of the foregut and project outwards in a caudolateral direction. The lung buds grow and branch from the developing trachea as two primary bronchi in the turtle *Chrysemys picta* (Hesser, 1906). From the primary bronchi, the first two chambers bud. These chambers grow quite large before the next pair of chambers arise (Broman, 1940). Unlike *C. picta*, in the sea turtles, *Caretta caretta*, *Chelonia mydas* and *Dermochelys coriacea*, several chambers bud simultaneously from the

primary bronchi (Broman, 1939). Embryonic sea turtles, like adults, have many more chambers within the lungs than do freshwater turtles, *Chelydra serpentina* and *C. picta* of similar size (Broman, 1939; Broman, 1940). Foetal lung fluid is secreted into the lungs of *C. mydas* (Maloney *et al.*, 1989) and is essential for lung bud formation in reptiles (Hesser, 1906; Broman, 1942). In the unicameral lung of the gecko, *Tarentola* sp. (Hesser, 1906), and the multicameral lung of the chameleon, *Chameleo bitaeniatus* (Broman, 1942), the appearance of smooth muscle in the lung coincides with the formation of the respiratory units, suggesting that contraction of the smooth muscle would generate sufficient pressure within the fluid-filled lungs to promote growth of the respiratory units. However, the differentiation of the chambers occurs prior to the formation of smooth muscle in the multicameral lung of the chameleon, suggesting that chamber formation is not subject to intrapulmonary pressure and is therefore genetically programmed (Broman, 1942; Perry *et al.*, 1989). The ionic composition of this lung fluid suggests that, like mammals, ions are actively pumped across the respiratory epithelium. Lung fluid reabsorption has been observed prior to hatching in sea turtles (Maloney *et al.*, 1989). Ciliated epithelium covering the trabeculae of the lung is also believed to aid lung fluid reabsorption (Perry, 1989).

To date only one study explores the final stages of lung development in a reptile. Perry *et al.* (1989) observed histological changes in the lung of the loggerhead sea turtle, *Caretta caretta*, before and after hatching. Eight days prior to hatching (hatching = 50-52 days of incubation), the lungs are filled with lung fluid and the parenchyma appears finer than that of hatchling turtles. At this stage, the respiratory epithelium consists of a single cell type that resembles the type II cells of the adult (Perry *et al.*, 1989). This stage is believed to correlate with the canalicular stage of lung development in mammals. The lungs do not differ histologically between one day before hatching and five days post-hatching, however the respiratory surfaces are more vascularised than those eight days

prior to hatching. One day before hatching the lungs possess both type I and type II pneumocytes (Perry *et al.*, 1989). Therefore, the lung of the turtle undergoes similar developmental changes to that of mammals.

1.4.1.2 Parabronchial Lung

The lung buds appear on the 3rd day of incubation in the chicken embryo. On the 5th day of incubation, the buds elongate craniodorsally to invade the mesodermal lung primordia attached to the lateral walls of the oesophagus. The primitive endodermal tubes form the primary bronchus within each lung (Zeng *et al.*, 1998). By the 7th day of incubation, the secondary bronchi sprout from the primary bronchus reaching their final number and position by the 10th day of incubation in the chicken (Duncker, 1978a). At this time, the first parabronchi originate from the secondary bronchi as epithelial buds, elongating and obtaining a tubular lumen. The airsacs appear at the distal ends of the secondary bronchi. Between the 14th and 15th day of incubation, the parabronchi elongating from ventral and dorsal bronchi anastomose along the medial plane, forming tubules of uniform calibre (Duncker, 1978a; Zeng *et al.*, 1998). During this time the parabronchi have reached their final number and position. Cuboidal epithelial cells that are surrounded by mesenchyme initially line the parabronchi. Lamellar bodies are first observed in developing type II cells on the 16th day of incubation (Dameron & Marin, 1970). Over the next 2 to 3 days of incubation, the parabronchial epithelium evaginates into the surrounding mesenchyme to form atria, in which the type II cells are located (Petrik, 1967; Jones & Radnor, 1972) and from which the future gas exchange regions, the air capillaries, arise (Duncker, 1978a). With the initiation of lung ventilation on the 19th day of incubation, capillaries proliferate within the parabronchial mesenchyme. Likewise, air capillaries sprout as open tubules from the atria and surround the developing blood capillaries. As a result of their tiny diameter and the consequent high surface tensions within the air capillaries, they cannot be

inflated out of a collapsed state (Duncker, 1978a). Aeration of the lungs occurs in part by proliferation of the air capillaries (Jones & Radnor, 1972; Seymour, 1984).

1.4.2 Reproductive Mode

In addition to morphological and phylogenetic differences, the reproductive mode differs among the amniotes. Many lizards and snakes exhibit viviparous reproduction, whereas all birds, turtles and crocodilians are oviparous. The process and timing of parturition differs between oviparous and viviparous species. When compared to birth, hatching from an egg can be a lengthy and energetically costly process. To commence hatching, oviparous species must first perforate the extraembryonic membranes and/or the shell, a process termed pipping. They draw their first breath and begin aerating their lungs before struggling free of the shell (Duncker, 1978a). In avian eggs, an air cell develops in the blunt end of the egg as the shell membranes dry out during incubation. Initially the bird pips internally into the aircell to commence pulmonary ventilation. At this time, a vascularised portion of the chorioallantois (the extraembryonic respiratory organ) is maintained, resulting in a dual respiratory system at the onset of pulmonary ventilation (Visschedijk, 1968; Duncker, 1978a). After the shell is pipped the chorioallantoic circulation is withdrawn and respiration occurs entirely through the lungs (Visschedijk, 1968). The presence of this dual respiratory system may afford the animals more time to clear their lungs of fluid and aerate their tiny parabronchi and air capillaries. In contrast, oviparous reptiles do not routinely develop an air cell during development. However, air pockets can occur under the shell in some species (Ewert, 1985; Ferguson, 1985). Ferguson (1985) contends that because alligators vocalise prior to hatching, that the developing young may internally pip into air pockets under the shell to allow lung clearance before the eggshell is perforated. However, the development of airspaces reflects states of hydration of the egg (Ewert, 1985). It is likely that most reptiles do not

commence pulmonary ventilation until the eggshell and shell membranes have been ruptured and fluids have drained from around the head (Ferguson, 1985; Booth & Thompson, 1991). The transition between pipping and hatching is highly variable among reptiles. Nevertheless, the time between pipping and hatching in reptiles is shorter than most birds, ranging from 12-48 hours for crocodilians and turtles (Booth & Thompson, 1991). Whether the chorioallantoic membrane supplements gas exchange during this time is unknown, however hatchlings frequently swallow during this period, presumably clearing fluid from their lungs (Thompson, unpublished data, cited in Booth and Thompson (1991)). In contrast, the transition from the intra- to extra-uterine environment is considerably rapid in mammals and viviparous reptiles.

The stresses placed on oviparous embryos may differ substantially from those of viviparous species. Unlike mammals, oviparous species lack the *constant* maternal-foetal transfer of hormones and nutrients during development. Recent evidence suggests that maternal steroid hormones are present in freshly laid bird (Schwabl, 1993) and reptile eggs (Janzen *et al.*, 1998). In addition, maternal triiodothyronine and thyroxine are found in avian albumin and yolk (Prati *et al.*, 1992; Wilson & McNabb, 1997), while maternal antibodies are transferred via the egg to the developing chicken embryo (Katz *et al.*, 1986). Deposition of such hormones and antibodies occurs prior to laying and, therefore, the action of such hormones is under embryonic control. Such differences in the availability of hormones may further impact on the development of the pulmonary surfactant system.

In chickens, the only, non-mammalian amniote in which the development of the surfactant system has been investigated, lung maturation appears to be influenced by both thyroid hormones (Wittmann *et al.*, 1984; Wittmann *et al.*, 1987) and glucocorticoids (Hylka & Doneen, 1983; Nielson & Torday, 1985). Furthermore, oviparous embryos also experience hypoxia late in incubation as a result of increased oxygen demand and limited gas exchange through the pores of the shell, providing the trigger for external pipping

(Visschedijk, 1968). This process is accelerated by thyroid hormones and retarded by antithyroid drugs (Wittmann *et al.*, 1984). In addition, the hypoxia which is generated during development appears to induce increased catecholamine secretion towards the end of incubation (Wittmann & Prechtl, 1991). Likewise, in reptiles, hypoxia appears to promote hatching (Ferguson, 1985; Webb *et al.*, 1986; Packard, 1991). An additional stimulus for hatching includes the hydration of the substrate in which the eggs are incubated. Embryos incubated in moist environments tend to remain in the egg longer before hatching than do embryos incubating in drier conditions (Packard *et al.*, 1987; Packard, 1991). Furthermore, temperature profoundly influences incubation duration in all oviparous reptiles, such that duration of incubation increases with decreasing temperature. Mechanical disruption and vocalisation also initiate hatching in species that hatch synchronously (Ferguson, 1985). Unlike birds, oviparous reptiles are exposed to variable ambient conditions. Moreover, many species hatch underground in hypoxic and hypercapnic environments (Ackerman, 1977; Booth & Thompson, 1991). Hatching in an hypoxic environment may exert additional stresses on the process and control of surfactant release and initiation of airbreathing. Despite differing birth strategies, at the time of birth the lung of every newborn animal must transform from a fluid-filled, non-functional organ to an operative air-filled, respiratory organ (Frank & Massaro, 1980).

1.4.3 Development of the Pulmonary Surfactant System in Non-Mammalian Amniotes

Surfactant lipids are present in the functionless lungs of the fully-aquatic juvenile salamander (Orgeig *et al.*, 1994) and frog tadpole (Oguchi *et al.*, 1994). In these anamniotes the mixture of lipids differs in composition from "mature" surfactant associated with functional respiring lungs (Oguchi *et al.*, 1994; Orgeig *et al.*, 1994). The content of PC increases throughout metamorphosis in tadpoles, *Rana catesbeiana*, with maximal

levels of the phospholipid occurring during metamorphic climax when the lungs assume a respiratory function (Oguchi *et al.*, 1994). Such changes are accompanied by increases in hysteresis throughout metamorphosis and increases in static lung compliance at metamorphic climax (Dupre *et al.*, 1985). Moreover, PC is the primary phospholipid, and palmitate is the dominant fatty acid acyl chain, in metamorphosing frogs (Oguchi *et al.*, 1994). Conversely, in the metamorphosing salamander, *Ambystoma tigrinum*, the content of extracellular phospholipids and cholesterol do not differ, while the relative abundance of disaturated phospholipids declines (Orgeig *et al.*, 1994). The elevated saturation in the juvenile salamander is thought to relate to habitat selection, reflecting their generally higher and thermally stable environments and the elevated hydrostatic pressures placed on the lungs (Orgeig *et al.*, 1994).

To date, no detailed study on pulmonary surfactant in immature, developing or functionless lungs in reptiles has been undertaken. However, a handful of studies have investigated some aspects of the morphological and biochemical development of the pulmonary surfactant system in birds. Of those, most have involved the use of lung homogenates, which pollute the surfactant fraction with phospholipids from blood and membranes. Lamellar bodies first appear on the 16^{th} day of incubation in the chicken and their numbers increase rapidly over the remaining days of incubation (Dameron & Marin, 1970). The composition of the lamellar bodies changes with advancing incubation, where the phospholipid/protein ratio of lamellar bodies decreases in the final days of incubation in the chicken (Marin *et al.*, 1979). A continuous surface film is present in the air capillaries and atria of the lung following 20 days of incubation (Petrik & Reidel, 1968; Jones & Radnor, 1972). Like mammals and adult birds, palmitate is the primary fatty acid of PC in lamellar bodies and whole lung during development of the chicken (Marin *et al.*, 1979). During the final stages of incubation, the content of PC (Tordet & Marin, 1976; Hylka & Doneen, 1982; Nielson & Torday, 1985) and saturated PC (Hylka & Doneen,

1982; Nielson & Torday, 1985) increase in lung tissue from chickens. The greatest elevation in PC and saturated PC occurs during the transition to pulmonary respiration between the 18th and 20th day of incubation (Hylka & Doneen, 1982; Nielson & Torday, 1985), with males having more phospholipid than females (Nielson & Torday, 1985). Similarly, total phospholipid increases 6-fold in lung homogenates from chicken embryos incubated for 14 days until hatching (hatching = 21 days), with the primary elevation occurring on day-18 prior to the onset of airbreathing (Hylka & Doneen, 1982). Although some experimental evidence corroborates the putative role of foetal corticosteroids in avian lung maturation (Hylka & Doneen, 1983; Nielson & Torday, 1985), the exact nature of the cascade of events which results in maturation of the surfactant system in birds remains unclear. Hypophysectomy delays differentiation of the respiratory epithelium in the chick (Marin *et al.*, 1978). Moreover, dexamethasone increases the amount of saturated PC in lung homogenates from chicken embryos, whereas glucocorticoid inhibition decreases the content of saturated PC (Nielson & Torday, 1985). Thyroid hormones accelerate embryonic development by accelerating the development of the structural components of the lung and increasing lung fluid absorption (Wittmann *et al.*, 1987). SP-A and SP-B are expressed in the developing chicken lung from the 15th day of incubation with expression increasing in hatchlings, whereas TTF-1 and HNF-3β are expressed on the 4th day of incubation at the onset of lung bud formation (Zeng *et al.*, 1998).

1.5 Aims

Despite vastly different lung morphologies, phylogenies and reproductive strategies, the vertebrate pulmonary surfactant system appears to have remained conserved both morphologically, compositionally and to a lesser extent, functionally. Whether conservation of the system extends to the development of the system among the amniotes is not known. Moreover, differences in metabolism, breathing pattern, habitat and

regulation of the surfactant system may lead to differences in the secretion of surfactant at birth or hatching. Therefore, this thesis aims to answer a number of questions relating to the development of the pulmonary surfactant system during development in the non-mammalian amniotes. I have chosen species based on their position in the phylogenetic tree, their reproductive strategy and their lung morphology in order to construct an evolutionary framework for the development of the surfactant lipids and to determine which factors have an impact on the development of this system. I will then determine the degree of conservation within this developmental process. In addition to the lipids, one other specific parameter was measured for each species to further describe the development of the system and how it relates to the biology of the specific organism.

In all species, the major lipid components, namely total and disaturated phospholipid and cholesterol were examined from lavage of the various species throughout the latter portion of incubation or gestation. These components were chosen because they have previously been identified in lung washings from representatives of every class, they change during development in mammals and their regulation has been examined in many taxa.

This thesis addresses the following aims:

1. To describe the developmental pattern of the pulmonary surfactant lipids in non-mammalian amniotes.

    1.1 To determine the evolutionary relationship, if any, between the developmental pattern of the surfactant lipids within the amniotes.

    1.2 To relate changes in development of the surfactant lipids to different reproductive modes.

1.3 To relate changes in development of the surfactant lipids to different lung morphologies.

2. To determine the developmental profile of an additional surfactant marker or system for each species to further explain the development of the system.

2.1 To determine the presence of SP-A mRNA in the lung of a non-mammalian amniote during the latter portion of development.

2.2 To describe the maturation of the type II cells in the lung of a non-mammalian amniote during the final stages of development.

2.3 To determine whether the development of the antioxidant enzyme system parallels the development of the surfactant lipids in the lung of a non-mammalian amniote.

2.4 To determine the presence of transcription factors and mature SP-B in the lung of a non-mammalian amniote during the latter portion of development.

2.5 To determine whether environmental cues, such as hypoxia, stimulate maturation of the surfactant system in a non-mammalian amniote.

3. Collectively, these aims will enable the determination of the extent of conservation of the development of the surfactant system among the amniotes.

CHAPTER 2 DEVELOPMENT OF THE PULMONARY SURFACTANT SYSTEM IN A BIRD, *GALLUS GALLUS DOMESTICA*

2.1 INTRODUCTION

Morphological studies have demonstrated the appearance of lamellar bodies, the intracellular storage site of surfactant, and an osmiophilic surface film in the latter stages of incubation in the embryonic chicken lung (Petrik & Reidel, 1968; Dameron & Marin, 1970; Jones & Radnor, 1972). Total phospholipid and saturated phospholipid increase in lung homogenates of the chicken embryo during incubation (Tordet & Marin, 1976; Marin *et al.*, 1979; Hylka & Doneen, 1982; Nielson & Torday, 1985), with males having an elevated content and saturation of phospholipids throughout development compared with females (Nielson & Torday, 1985). Although these studies show trends in the development of the pulmonary surfactant system in chickens, they have involved the use of homogenates, which are significantly contaminated by cell membrane lipids. Moreover, lung homogenates contain both intra- and extracellular surfactant compartments. The present chapter demonstrates, for the first time, the development of surfactant lipids in the extracellular compartment of the avian lung.

Of the surfactant proteins, SP-A (Bhattacharyya *et al.*, 1976; Sullivan *et al.*, 1998) and its messenger RNA (Sullivan *et al.*, 1998) have been identified in the adult chicken lung. Recently, Bernhard *et al.* (2001) confirmed the presence of SP-B in adult chicken and duck, however mammalian polyclonal antibodies for SP-A and SP-C failed to cross-react with purified lavage. Despite some contradictions, both SP-B and SP-A have been detected in the developing chick following the 15^{th} day of incubation (Zeng *et al.*, 1998). The initial appearance of surfactant protein messenger RNA has not been determined in birds. Therefore, the present chapter describes the ontogeny of the pulmonary surfactant lipids and SP-A mRNA in the domestic fowl, *Gallus gallus domestica*.

2.2 Materials and Methods

2.2.1 Animals and Incubation of Eggs

Adult and three-week-old chickens, *Gallus gallus domestica*, and fertilised 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 electronic incubator equipped with a Bellsouth 100AT automatic turner (Bellsouth, Narre Warren, Vic, Australia).

2.2.2 Tissue Sampling

Morphological studies (Marin & Dameron, 1969; Dameron & Marin, 1970; Marin *et al.*, 1978) describe the timing of development of the respiratory epithelium in birds. The first lamellar bodies appear on day-16 of incubation and by day-18 of incubation lamellar bodies are mature and plentiful with the type II cells (Marin & Dameron, 1969; Dameron & Marin, 1970; Marin *et al.*, 1978; Hylka, 1989). In keeping with these previous studies, chicken eggs were sampled after 14, 16, 18 (pre-pipped) and 20 days (post-pipped) 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 sacrificed by dipping eggs in liquid nitrogen. Animals that had commenced pulmonary ventilation were killed either by CO_2 inhalation (embryos incubated for 20 days and hatchlings) or, in the case of adults, by careful injection of sodium pentobarbitone (150 mg/kg body mass [BM] Nembutal, Abbott Laboratories, Sydney, NSW, Australia) into the peritoneal cavity.

2.2.3 Embryonic Staging

To compare between species, data have been normalised to days of incubation, however for completeness embryos were staged according to the physical characters assigned by Hamburger and Hamilton (1951). Day-14 corresponded to stages 39 and 40, day-16 to stage 42, day-18 to stage 44, day-20 to stage 45 and day-21 (hatch group) to stage 46 (Hamburger & Hamilton, 1951).

2.2.4 Lavage Protocol

Chicken embryos at 16, 18 and 20 days of incubation, hatchlings and adults were tracheal-cannulated and the lungs lavaged with three volumes of chilled 0.15M NaCl solution (0.04-0.07 ml/g [BM]), instilled and withdrawn three times per volume. Any foetal lung fluid (FLF) present was incorporated into the lavage. Lavage from individuals was centrifuged (Beckman model TJ-6 centrifuge) for 5 min at 150 *g* at 4°C to remove cellular debris. After lavage, lungs were carefully removed, snap frozen and lyophilised to obtain dry lung weights. Given the complexity of the avian lung and to ensure that the lungs were thoroughly lavaged, a blue dye was added to the saline in two animals from each stage of development. The lungs were excised, sectioned and observed macroscopically. In all instances, the entire lung had taken up the dye. These animals were not used for lipid analyses.

2.2.5 Lipid Analyses

All reagents used for biochemical analyses were of analytical grade or higher. Lyophilised lavage was reconstituted in 2 ml of deionised (Millipore) water and lipids were extracted using chloroform:methanol (1:2 vol/vol) (Bligh & Dyer, 1959) (Appendix 2, Section 1.1).

Total phosphorus was quantified using the method of Bartlett (1959) (Appendix 2, Section 1.2) and total phospholipid (PL) was calculated by multiplying the total phosphorus by 25, as phospholipid comprises 4% phosphorus (Daniels *et al.*, 1989). Disaturated phospholipids (DSP) and neutral lipids were separated by absorption chromatography on activated aluminium oxide columns (Mason *et al.*, 1976) (Appendix 2, Section 1.3). DSP content was measured using the phosphorus assay (Bartlett, 1959). The neutral lipids were dried under nitrogen, reconstituted in isopropanol at 2°C. Cholesterol (Chol) was quantified using a high pressure liquid chromatography system (Daniels *et al.*, 1999), comprising a Waters pumping system (model M-45, Waters, Milford, MA) and an LKB 2157 autosampler (Pharmacia LKB Biotechnology, Uppsala, Sweden). Twenty µl of either sample or standard were injected onto a Waters ^{18}C Novopak guard and analytical column (150 × 4.6 mm ID) packed with 4 µm silica spheres. Isocratic elution of cholesterol was completed within 32 min at room temperature using a mobile phase consisting of acetonitrile, isopropanol and water (6:3:1 vol/vol/vol). A flow rate of 1 ml/min and an operating pressure of 1600 psi were maintained throughout the elution and ultraviolet absorbance was recorded at 210 nm. The detector output was digitised using a Delta Chromatography data system for acquisition of data and integration of peaks (Digital Solutions, Brisbane, QLD, Australia). Standards were assayed in duplicate and were included at the beginning of each run (5-50 µg/ml).

2.2.6 Additional Measures

2.2.6.1 Northern Blot Analyses

Lungs were removed from chicken embryos after 14, 16, 18 and 20 days of incubation and from hatchlings, a three-week-old chick and an adult. All lungs were snap frozen in liquid nitrogen and stored at -80°C for further analyses. Total RNA was isolated from lung tissue

using TriReagent™ (Sigma Chemical Co., St Louis, MO, USA) following the instructions of the manufacturer (Appendix 2, Section 1.4). Total RNA was dissolved in sterile water 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 sodium acetate, 1mM EDTA, pH 7.2) agarose minigels stained with ethidium bromide and visualised 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.* (1998). Twenty 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 overnight at 42°C in prehybridisation solution containing 0.1 mg/ml of denatured salmon sperm DNA (Boehringer Mannheim, Adelaide, SA, Australia). Approximately 2×10^6 cpm/ml of ^{32}P-labelled mouse SP-A cDNA was added to the membranes and hybridised for 3 days at 42°C. The membranes were washed twice in a solution containing 0.1% SDS in 1 × SSC (0.15M NaCl, 15mM sodium citrate) at 42°C for 30 min per wash. The membranes were then dried, sealed in plastic bags and exposed to a FujiBas Phosphor-Imager screen (Fuji-Film Medical Systems, Stamford, CT, USA) for 60 h.

2.2.7 Statistical Analyses

Chol/PL and Chol/DSP are expressed as μg/μg ratios, whereas DSP/PL is expressed as a percentage. Data were analysed using a one-way ANOVA followed by paired Student's t-tests where appropriate after Arcsin transforming the ratios. Statistical significance was assumed when $P < 0.05$.

2.3 Results

2.3.1 Physical Parameters

Body masses 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 2.1).

Table 2.1 Body mass and dry lung weight of embryonic and hatchling chickens.

Age (days)	BM (g)	DLW (mg)	DLW/BM (%)
Day-16	18.03 ± 0.40 (8)	20.23 ± 1.09 (8)	0.11 ± 0.01 (8)
Day-18	24.37 ± 0.44 (8)	26.32 ± 1.62 (8)	0.11 ± 0.01 (8)
Day-20 (Post Pip)	33.77 ± 1.31 (7)	32.95 ± 1.53 (7)	0.08 ± 0.004 (7)
Hatch	38.54 ± 1.06 (9)	42.36 ± 3.48 (9)	0.11 ± 0.01 (9)

Results are mean ± SEM; *n* in parentheses; Post pip, animals that had pipped; DLW, dry lung weight; BM, body mass.

Dry lung weights from chicken embryos increased significantly between adjacent sampling groups ($P < 0.05$ for all tests) (Table 2.1), yet the ratio of dry lung weight to body mass 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 2.1). The percentage of dry lung relative to body mass decreased from day-18 to day-20 of incubation ($P < 0.01$), due to elevated body masses in day-20 embryos that had internalised yolk. Foetal lung fluid was observed in the foetal chick lung

after 16 and 18 days of incubation, however no lung fluid was observed following the 20th day of incubation.

2.3.2 Development of the Surfactant Lipids

Chicken Chol expressed as a function of dry lung weight did not differ during incubation (ANOVA, $P = 0.11$) (Figure 2.1-C), 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 (Figure 2.1-A,B). DSP/PL increased in embryos aged between day-16 and day-18 of incubation ($P < 0.01$) with a further increase following 20 days of incubation ($P < 0.05$) (Figure 2.2-A). DSP/PL was present in statistically similar amounts in chicken embryos incubated for 20 days, in hatchlings and in adults (Figure 2.2-A). The Chol/PL ratio declined significantly throughout the latter part of incubation, with a 2-fold reduction from day-16 to day-18 embryos ($P < 0.01$) and a further reduction after pulmonary ventilation had commenced at day-20 to hatching ($P < 0.05$) (Figure 2.2-B). Hatchling Chol/PL ratios were comparable to adults (Figure 2.2-B).

Since 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 (Figure 2.2-C). The Chol/DSP ratio declined in embryos incubated for 18 days with a further 2-fold reduction in day-20 embryos ($P < 0.05$) (Figure 2.2-C). There was no difference between day-20 embryos and hatchlings ($P = 0.07$) (Figure 2.2-C). Chol/DSP was significantly lower in adults ($P < 0.05$) (Figure 2.2-C).

Figure 2.1 The absolute amount of phospholipids and cholesterol in pulmonary surfactant from the developing lung of the chicken, demonstrating total PL (**A**), DSP (**B**) and Chol (**C**) per mg of dry lung (DL). Data expressed as mean ± SEM. Paired symbols indicate significant difference between adjacent groups. **A**: * P = 0.019, # P = 0.05, **B**: * P = 0.005, # P = 0.016.

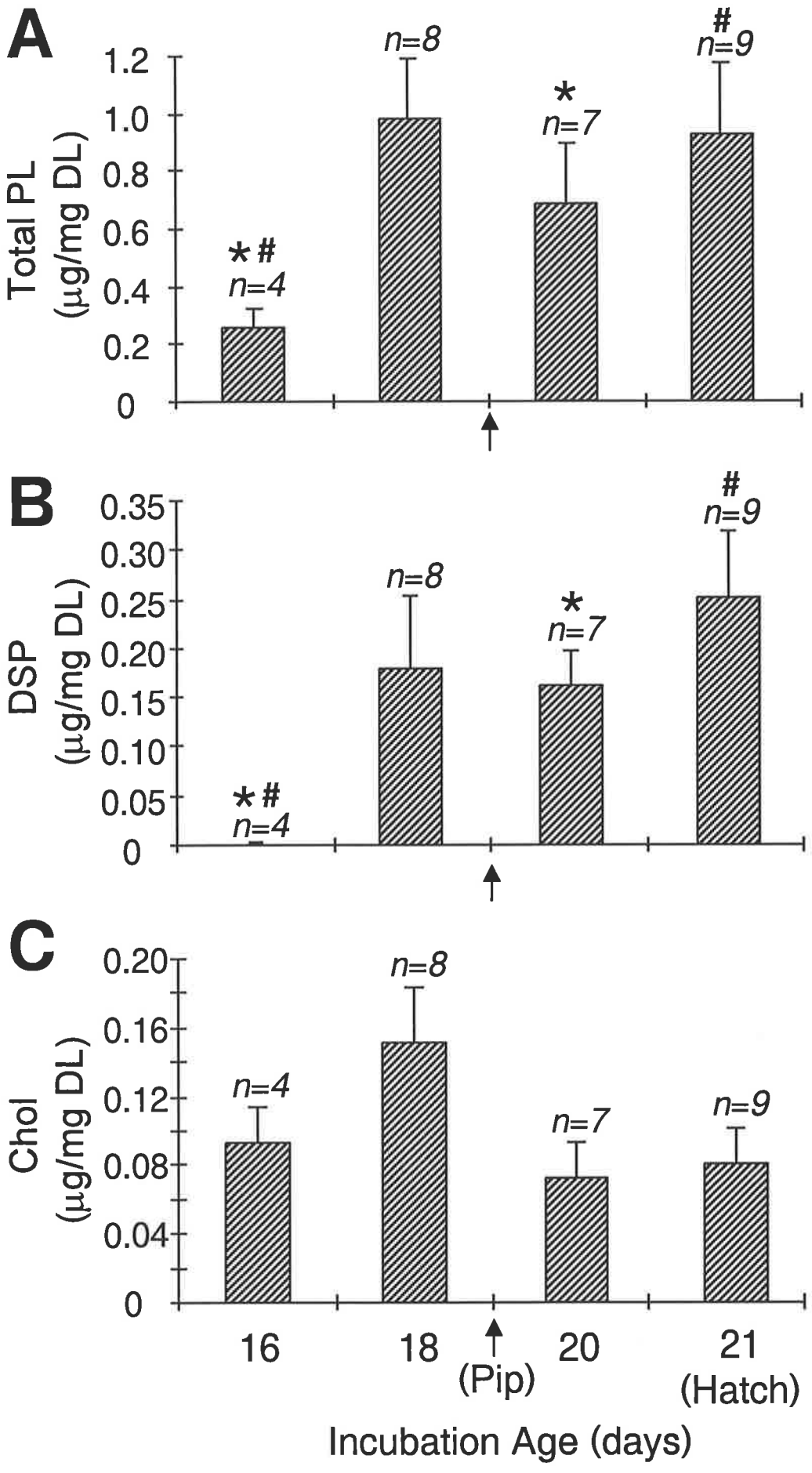
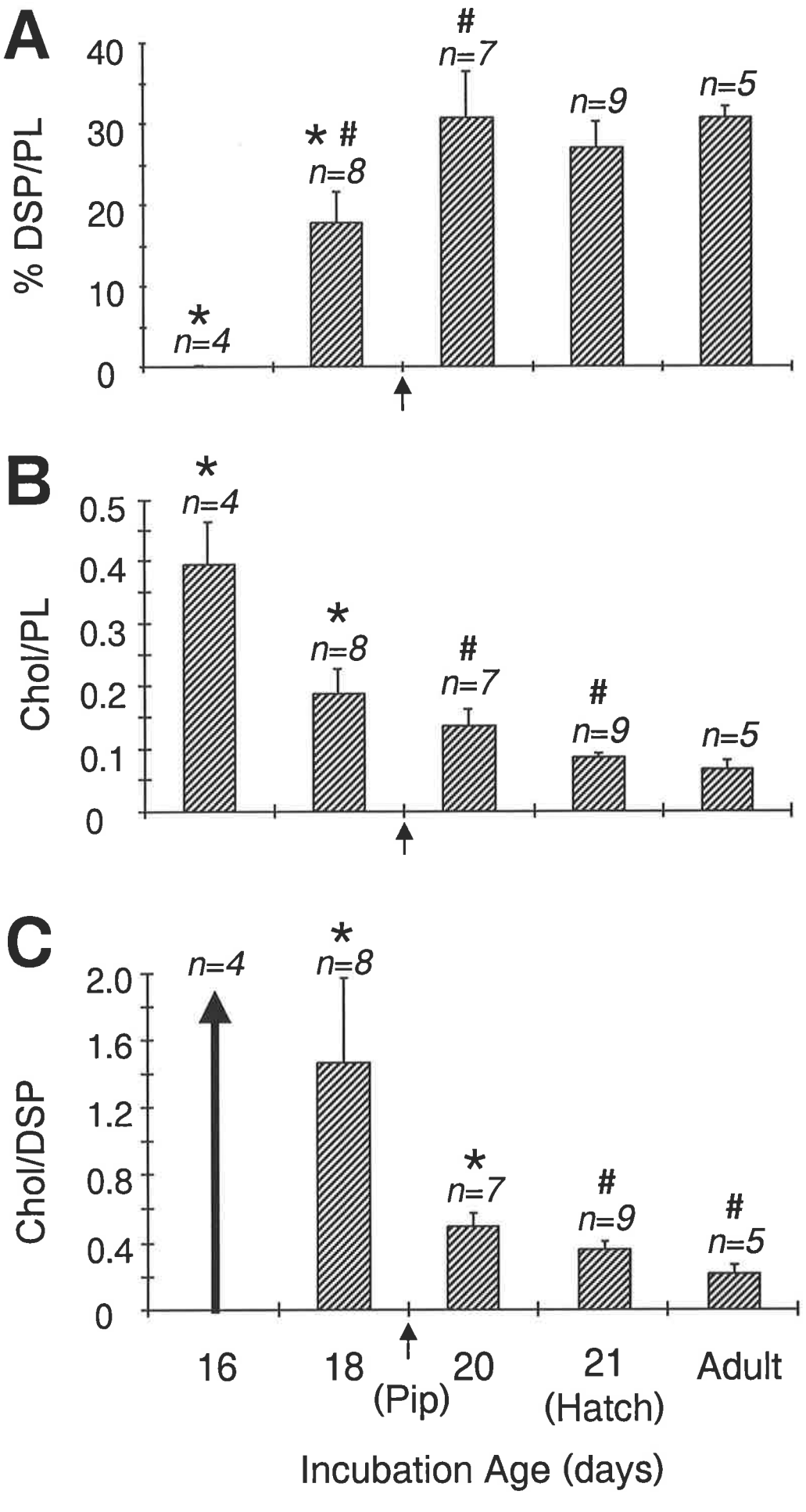

A
Total PL (µg/mg DL)
1.2
1.0
0.8
0.6
0.4
0.2
0
*#
n=4
n=8
*
n=7
#
n=9
B
DSP (µg/mg DL)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
*#
n=4
n=8
*
n=7
#
n=9
C
Chol (µg/mg DL)
0.20
0.16
0.12
0.08
0.04
0
n=4
n=8
n=7
n=9
16
18
(Pip)
20
21
(Hatch)
Incubation Age (days)

Figure 2.2 The relationship between phospholipids and cholesterol in the developing and adult chicken lung, demonstrating DSP expressed as a percentage of total PL (**A**), the Chol/PL ratio (**B**) and the Chol/DSP ratio (**C**). Data expressed as mean ± SEM. Paired symbols indicate significant difference between adjacent groups. As DSP was not measurable in embryos incubated for 16 days, Chol/DSP approached infinity demonstrated by an arrow (**C**). **A**: * $P = 0.003$, # $P = 0.034$; **B**: * $P = 0.008$, # $P = 0.023$; **C**: * $P = 0.048$, # $P = 0.045$.


A
% DSP/PL
40
30
20
10
0
*
n=4
* #
n=8
#
n=7
n=9
n=5
B
Chol/PL
0.5
0.4
0.3
0.2
0.1
0
*
n=4
*
n=8
#
n=7
#
n=9
n=5
C
Chol/DSP
2.0
1.6
1.2
0.8
0.4
0
n=4
*
n=8
*
n=7
#
n=9
#
n=5
16
18
(Pip)
20
21
(Hatch)
Adult
Incubation Age (days)

2.3.3 Additional Measures

2.3.3.1 Detection of SP-A mRNA

Northern blot analysis demonstrated that the mouse SP-A cDNA probe hybridised with RNA from chick embryos incubated for 14, 16, 18 and 20 days, hatchlings, 3 week old chick, adult chicken and mouse (Figure 2.3). Three transcripts were present in the mouse.

2.4 Discussion

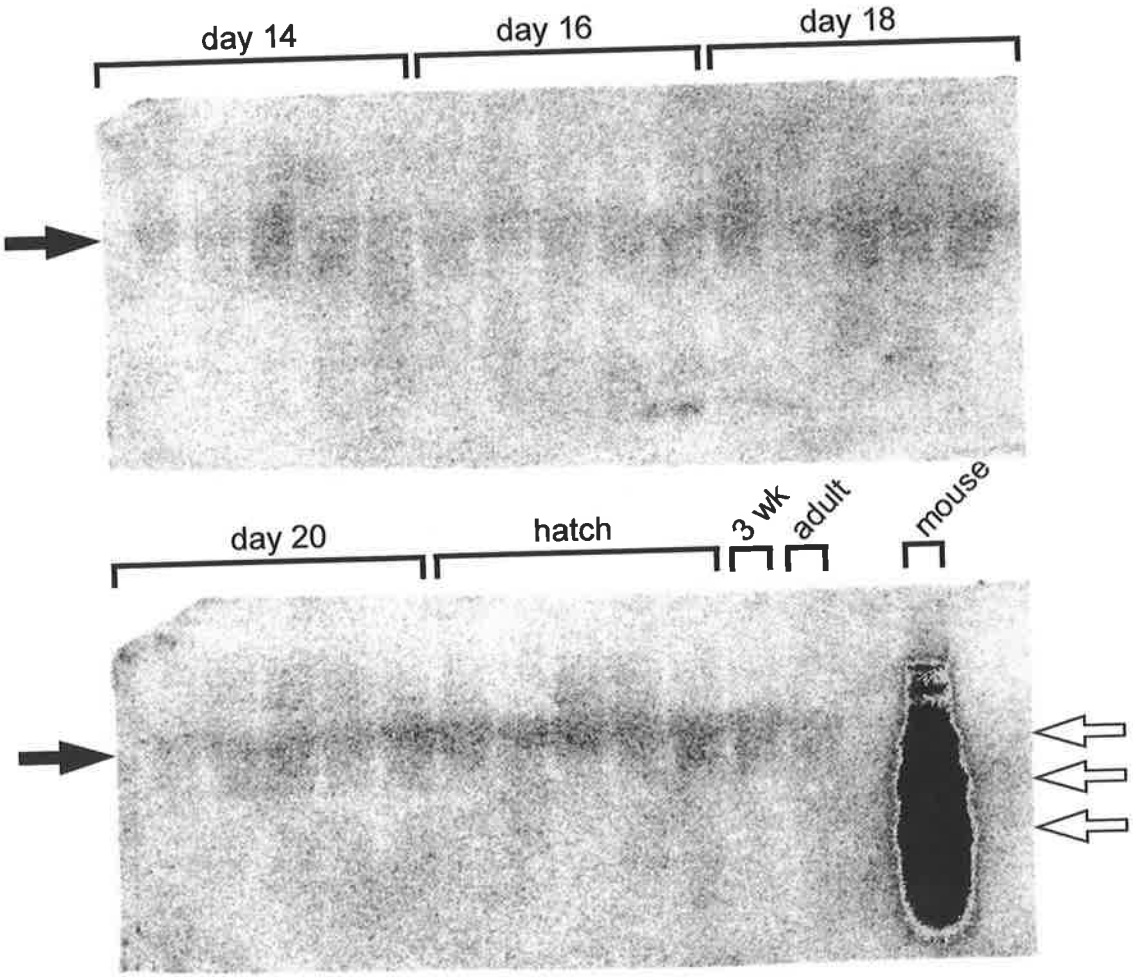
2.4.1 Development of the Surfactant Lipids

2.4.1.1 Phospholipids

The present study used the method of Mason *et al.* (1976) to extract DSP. While this method may overestimate the DPPC fraction by detecting both DSP and monoenoic species (Veldhuizen *et al.*, 1998), it is acceptable when used to compare values between experimental groups.

The increase in total PL and DSP during the latter portion of incubation in chickens resembles the compositional changes observed in lavage, lung fluid and amniotic fluid from mammals. Total phospholipid, PC and DPPC increase exponentially between the 19th day of gestation and the first postnatal day in the foetal rat (Maniscalco *et al.*, 1978). In the developing rabbit, the content of total and saturated PC (relative to sphingomyelin) from lavage increase 4- and 15-fold respectively between the 26th and 30th day of gestation (term = 31 days) (Torday & Nielson, 1981). Likewise, the concentration of total phospholipid in lung liquid and amniotic fluid from foetal sheep increases dramatically with increasing gestational age from low concentrations on the 110th day of gestation to a 2.5-fold increase between the 125th and 130th day of gestation (term = 146 days).

Figure 2.3 Northern blot analysis of chicken lung tissue following incubation with a ^{32}P-labelled mouse SP-A cDNA probe. The mouse SP-A cDNA probe hybridised with chicken RNA from each sampling period (➡). Three transcripts were present for mouse (bottom panel, ⇦). The blot was overexposed to ensure the earliest detection of SP-A mRNA.


day 14
day 16
day 18
day 20
hatch
3 wk
adult
mouse

This occurs with a concomitant increase in surface activity (Egberts *et al.*, 1981). Benson *et al.* (1983) found PC and DPPC do not reach adult levels in the foetal sheep until 3-4 days before term. 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 (Figure 2.2-A).

Given that pulmonary surfactant is instrumental in lung clearance during development in mammals, it is possible that changes in surfactant composition may alter lung fluid clearance in the avian lung. Retention of FLF until pipping in the chicken embryo may also reflect the relatively large size of the avian respiratory system. Alternatively, the presence of FLF at pipping may prevent the adhesion of airsac walls prior pulmonary ventilation and the development of a functional surface film. 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 for lung clearance prior to hatching. Several authors have suggested that avian surfactant prevents accumulation of liquid in the parabronchi and air capillaries (Petrik & Reidel, 1968; Duncker, 1978a; Pattle, 1978). After internally pipping, birds may rely on their chorioallantoic membrane for gas exchange while titrating their surfactant and aerating the tiny parabronchi. The greatest aeration of the avian lung occurs following internal pipping, achieved by proliferation of the air capillaries and removal of fluid from the parabronchi by convection and reabsorption (Seymour, 1984). Hence, the presence of pulmonary surfactant would facilitate the reabsorption of fluid within the parabronchi from embryos that had pipped.

2.4.1.2 Cholesterol

In the adult lizard, *Ctenophorus nuchalis*, and in the marsupial, *Sminthopsis crassicaudata*, the cholesterol content of the surfactant increases with decreasing body temperature, presumably to maintain the fluidity of the surface film over fluctuating body temperatures

(Daniels *et al.*, 1990; Langman *et al.*, 1996). Despite the large amounts of cholesterol that are present throughout development, the function of cholesterol 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 (Figures 2.2-B,C) during incubation by increasing PL and DSP without altering Chol. The content of cholesterol did not alter throughout development, despite marked changes in the content and saturation of the phospholipids (Table 2.1), suggesting that extracellular cholesterol appears relatively earlier in development than the phospholipids. Similarly, cholesterol content does not change in the developing lung of the tiger salamander, *Ambystoma tigrinum*. Yet, unlike the chicken, total PL remains unchanged and DSP decreases throughout development in this amphibian (Orgeig *et al.*, 1994). The reasons and mechanisms by which changes in cholesterol occur during lung development remain unknown. However, it is likely that the factors regulating cholesterol are independent of those controlling phospholipids. Moreover, the Chol and DSP components of surfactant in the alveolar compartment of rats (Orgeig *et al.*, 1995) and humans (Doyle *et al.*, 1994) are processed differently and appear to be differentially released.

Surprisingly, to my knowledge, changes in the amount of alveolar cholesterol and composition prior to birth have never been described for any mammal, thus comparisons cannot be drawn between species. However, Suzuki *et al.* (1978) measured PC/Chol in newborn rats. The reciprocal of the approximated value gives a Chol/PC of ~0.034 in newborn rat lungs, approximately 2.5-fold lower than hatchling chickens. Chol/PL and Chol/DSP have not been previously described in birds. Adult chickens have similar Chol/PL ratio to that of mammals and warm reptiles, and Chol/DSP levels are also similar to those of the other terrestrial tetrapods (Daniels *et al.*, 1995a). However, the Chol/PL

and Chol/DSP ratios of embryonic chicks (Figure 2.2-B,C) are much greater than any previously recorded (Daniels *et al.*, 1995a; Daniels *et al.*, 1998b).

2.4.2 Additional Measures

2.4.2.1 Detection of SP-A mRNA

Previously, the presence of two SP-A mRNA transcripts of 3.7 and 1.7 kb have been demonstrated in adult chicken lung using a mouse SP-A cDNA probe (Sullivan *et al.*, 1998). Here, two SP-A mRNA transcripts were identified in chicken embryos after 14 days of incubation (Figure 2.3). The bands were weak and ill-defined due to a low degree of homology between the chicken samples and the mouse probe (Sullivan *et al.*, 1998). Chicken SP-A mRNA was present after 2/3 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 18^{th} day of gestation (term = 22 days) (Schellhase *et al.*, 1989), the 26^{th} day of gestation in the rabbit (term = 31) (Mendelson *et al.*, 1986) and the 150^{th} day of gestation in the baboon (term = 180) (Minoo *et al.*, 1991). SP-A mRNA is undetectable (Ballard *et al.*, 1986) or is present in very low amounts (Weaver & Whitsett, 1991) in the second trimester human foetus. The timing of the appearance of SP-A mRNA is therefore substantially different among species.

SP-A has been implicated in the role of innate lung defense in mammals (van Golde, 1995). Zeng *et al.* (1998) detected SP-A in subsets of cells in the posterior primary bronchus of hatchling chicks, but not at the site of gas exchange, the air capillaries of the parabronchi. Therefore, SP-A is less likely to be instrumental in the storage, processing or function of surfactant in the developing chicken lung, but may be of prime importance in lung defense (Zeng *et al.*, 1998). Moreover, secretion of SP-A can occur independently of lamellar body secretion (Rooney *et al.*, 1993; Ikegami *et al.*, 1994) and, in the present

study, the appearance of SP-A mRNA precedes the appearance of lamellar bodies in developing chick lung by at least two days (Dameron & Marin, 1970; Marin *et al.*, 1978). Thus, in the chicken, SP-A mRNA may not be required to develop in tandem with the surfactant lipids, suggesting that it too is independently regulated from the surfactant lipids.

2.4.3 Conclusions

The pattern of development of the surfactant lipids in the embryonic chicken was similar to that of mammals, where the amount and saturation of phospholipid increased with advancing incubation. The development of the surfactant lipids therefore appears to be conserved between chickens and mammals. 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. Unlike phospholipids, large quantities of cholesterol were present throughout development, indicating that cholesterol is independently regulated from the phospholipids.

2.4.4 Future Directions

From the present study it is clear that the surfactant lipids develop in a similar manner to that of mammals, whereas developmental patterns of the surfactant proteins show greater lability. As previously mentioned, the role of the surfactant proteins in non-mammalian lung has not been described. From the present study, it appears that SP-A does not develop in tandem with the surfactant lipids. It is possible therefore that SP-A is involved in host defense in the avian lung, which opens an exciting avenue for future research.

The greatest secretion of surfactant lipids appears after pulmonary ventilation has been established, however the chicken is a precocial bird. It is possible that the surfactant

lipids and proteins are developmentally regulated and, therefore, the pattern of development may differ in altricial species. This may be of particular interest given that the patterns of circulating thyroid hormones, triiodothyronine and thyroxine, differ between precocial and altricial birds during development (McNabb *et al.*, 1999). Alternatively, given that lipid secretion was maximal after the initiation of airbreathing, it is possible that the commencement of breathing is the primary cue for the maturation of the system.

The presence of a dual respiratory system at the time of pipping may significantly alter the timing and maturation of the surfactant system in oviparous species. Megapode birds develop in mounds of soil or rotting vegetation. Elevated humidity within the mound reduces evaporative water loss to levels where the eggs do not develop an air cell (Seymour & Ackerman, 1980) and the young do not commence pulmonary ventilation until they have hatched (Seymour, 1984). An examination of this "viviparous" (Seymour, 1984) form of hatching would allow one to determine the development of the surfactant system between oviparous endotherms (birds) with similar lung morphology and phylogeny, yet distinct birth strategies. In contrast, the Wedge-tailed Shearwater, *Puffinus pacificus*, experiences a prolonged interval between pipping and hatching of 5-6 days. What effect this interval has on the development of pulmonary surfactant and lung clearance has not been elucidated.

CHAPTER 3 DEVELOPMENT OF THE LUNG AND PULMONARY SURFACTANT LIPIDS IN AN OVIPAROUS LIZARD, *POGONA VITTICEPS*

3.1 INTRODUCTION

The bearded dragon, *Pogona vitticeps*, like other dragons (Family: Agamidae), has unicameral lungs (Daniels *et al.*, 1994a). Their lungs consist of a paired bag-like structure with respiratory epithelium spanned between the outer wall of the lung and an inner trabecular network (Perry, 1985). In mammals, pulmonary surfactant chiefly aids lung compliance and patency of the tiny bronchioles (Enhörning, 2001) and alveoli (Bachofen & Schürch, 2001). The lungs of the adult bearded dragon however are highly compliant and the respiratory units exceed the size of alveoli of similar sized mammals by 100-1000 times (Daniels *et al.*, 1994a; Daniels *et al.*, 1995b). Indeed, removal of pulmonary surfactant does not alter lung compliance however, escalating temperature and autonomic agonists increase phospholipid secretion in this species (Wood *et al.*, 1995; Wood *et al.*, 1997; Wood *et al.*, 1999). Pulmonary surfactant is believed to prevent adhesion of adjacent epithelial walls in lizards (McGregor *et al.*, 1993; Daniels *et al.*, 1994a; Daniels *et al.*, 1995b), a role which may be of prime importance during the initiation of airbreathing when the agamid embryo must overcome the work of inflating the lung for the first time.

The development of the surfactant system has never been described in a reptile. Despite the highly conserved nature of the pulmonary surfactant system throughout the vertebrate phylogenetic tree, the surfactant system of the bearded dragon has a different primary role. It responds differently to autonomic and ventilatory stimuli and the lungs are morphologically distinct from that of mammals. To determine whether these differences extend to the development of the surfactant system, the present chapter describes the development of the pulmonary surfactant lipids and the maturation of the pulmonary type II cells during the final stages of incubation.

3.2 Materials and Methods

3.2.1 Collection of Animals and Incubation of Eggs

Six gravid bearded dragons, *Pogona vitticeps*, were collected from the southern Flinders Ranges, South Australia in October, 1997 and housed in 1.0 × 0.65 × 0.45 m cages. Lizards 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 (ca. 5 mm particles) with a gravimetric water content of 1 g/g dry mass, resulting in a water potential of -200 kPa or wetter (Packard *et al.*, 1987). 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 minimise exposure to fluctuations in temperature. In order to maintain low 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.

3.2.2 Tissue Sampling

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 ± SEM) and after hatching, 59.25 ± 0.63 days of incubation (mean ± SEM). Embryos and hatchlings were sacrificed by an intraperitoneal injection of sodium pentobarbitone (150 mg/kg body mass [BM] Nembutal, Abbott Laboratories, Sydney, NSW, Australia).

3.2.3 Embryonic Staging

In addition to the time of development, embryos were staged according to the morphological characters of the lacertid lizard, *Lacerta vivipara* (Dufaure & Hubert, 1961). Embryos from day-55 did not differ physically from pipped nor hatchling animals and therefore were ranked at stage 40. For this reason and for subsequent comparisons with other species, the data have been normalised to incubation age.

3.2.4 Lavage Protocol

Bearded dragon embryos at the 55^{th} day of incubation, at the time of pipping and after hatching were tracheal-cannulated with a blunt 26G needle and lavaged with three volumes of isotonic saline (0.08-1.0 ml/ g [BM]) instilled and withdrawn three times. Volumes ranging from 40 to 80% of a 0.5 ml reservoir were injected as embryos increased in mass. Any lung fluid present was incorporated into the lavage. Lavage was centrifuged (Beckman model TJ-6 centrifuge) for 5 min at 150 *g* to remove cellular debris. Since the total lavage volume was so small, the lavage from two individuals at the same incubation age and from the same clutch were pooled.

3.2.5 Lipid Analyses

Total phospholipid, disaturated phospholipid and cholesterol were extracted and quantified from lavage in the same manner as described for the chicken (Section 2.2.5).

3.2.6 Additional Measures

3.2.6.1 Electron Microscopy

Lungs were removed from two embryos at day-55 of incubation, two at the time of pipping and two following hatching from a single clutch and fixed overnight in a routine fixative (4% paraformaldehyde, 1.25% glutaraldehyde, 4% sucrose, in 0.1 M phosphate buffered saline, pH 7.4). The lungs were diced into ca. $1mm^3$ pieces and washed in 0.1 M phosphate buffered saline, pH 7.4, for 10 min. Tissue was post-fixed overnight at 4°C in a 1% osmium tetroxide solution (1% OsO_4 in 0.1 M phosphate buffered saline, pH 7.4) (McGregor *et al.*, 1993) and then rinsed three times in 0.1 M phosphate buffered saline, pH 7.4, for 10-15 min per rinse. Samples were stained *en bloc* in 1.5% uranyl acetate for 90 min at 4°C in the dark. Following staining, tissue was rapidly dehydrated in ascending concentrations of acetone (70, 80, 90, 100%) for 2 min at 4°C. Samples were infiltrated with acetone:epoxy resin (1:1 vol/vol) for 2 h then transferred to pure resin overnight. Tissue was embedded in fresh resin and polymerised under vacuum for 24 h at 60°C. After polymerisation, 5 μm thick sections were cut on an ultramicrotome (Leica Microsystems, Vienna, Austria) and stained with toluidine blue to locate respiratory epithelia. Once an appropriate area was chosen, 0.1 μm thin sections were cut per specimen and mounted on copper/palladium grids (ProSciTech, Kelso, QLD, Australia), stained with lead citrate and viewed under a Philips CM 100 transmission electron microscope. Six grids, containing at least five sections, were viewed per specimen.

3.2.7 Statistical Analyses

Lipid data were analysed in the same manner as chicken data (Section 2.2.7). Statistical significance was assumed when $P < 0.05$.

3.3 Results

3.3.1 Physical Parameters

Body masses of bearded dragon embryos differed significantly between sampling groups ($P < 0.05$ for all tests), whereas lung masses did not differ throughout development (ANOVA, $P = 0.52$). Consequently, the percentage of dry lung mass, relative to body mass, decreased between embryos incubated for 55 days and those that had pipped (Table 3.1)

Table 3.1 *Body mass and dry lung weight from embryonic and hatchling bearded dragons.*

Age (days)	BM (g)	DLW (mg)	DLW/BM (%)
Day-55	2.31 ± 0.13 (11)	3.16 ± 0.2 (11)	0.13 ± 0.01 (11)
Pip	3.17 ± 0.10 (15)	3.44 ± 0.12 (15)	0.11 ± 0.01 (15)
Hatch	2.85 ± 0.11 (16)	3.17 ± 0.21 (16)	0.11 ± 0.01 (16)

Results are mean ± SEM; *n* in parentheses; DLW, dry lung weight; BM, body mass.

3.3.2 Development of the Pulmonary Surfactant Lipids

Total PL and DSP, expressed per mg dry lung were significantly lower in embryos incubated for 55 days than embryos that had pipped ($P < 0.05$, both tests), with no change thereafter ($P = 0.29$, 0.37, respectively) (Figure 3.1-A,B), whereas Chol remained unchanged between groups (ANOVA, $P = 0.52$) (Figure 3.1-C). DSP/PL increased significantly between day-55 of incubation and pipping ($P < 10^{-5}$) and remained unchanged thereafter (Figure 3.2-A). The Chol/PL ratio declined markedly between embryos

Figure 3.1 The absolute amount of phospholipids and cholesterol in pulmonary surfactant from the developing lung of the bearded dragon, demonstrating total PL (**A**), DSP (**B**) and Chol (**C**) per mg of dry lung (DL). Data expressed as mean ± SEM. Paired symbols indicate significant difference between adjacent groups. **A**: * P = 0.038, **B**: * P = 0.013.

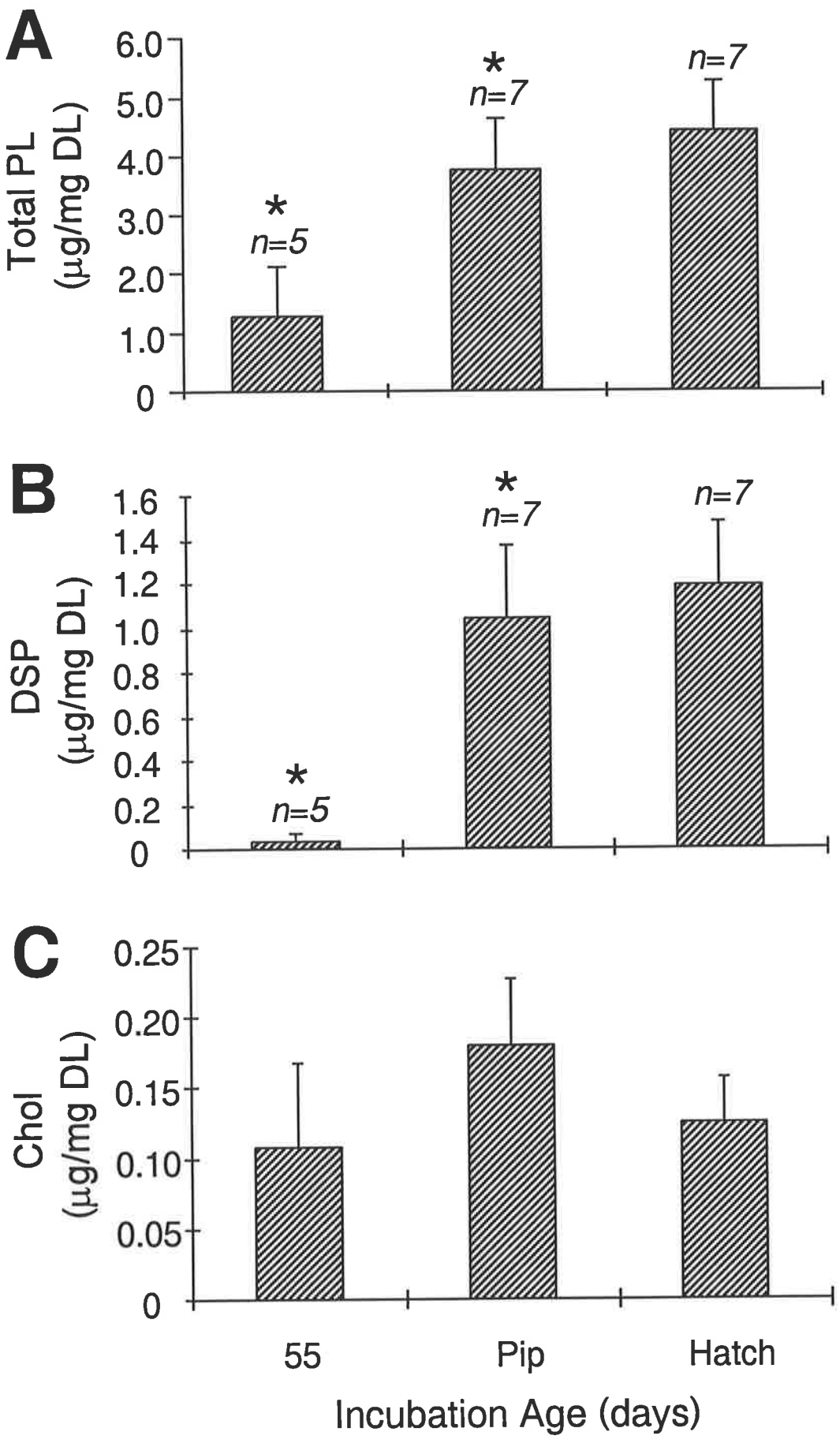
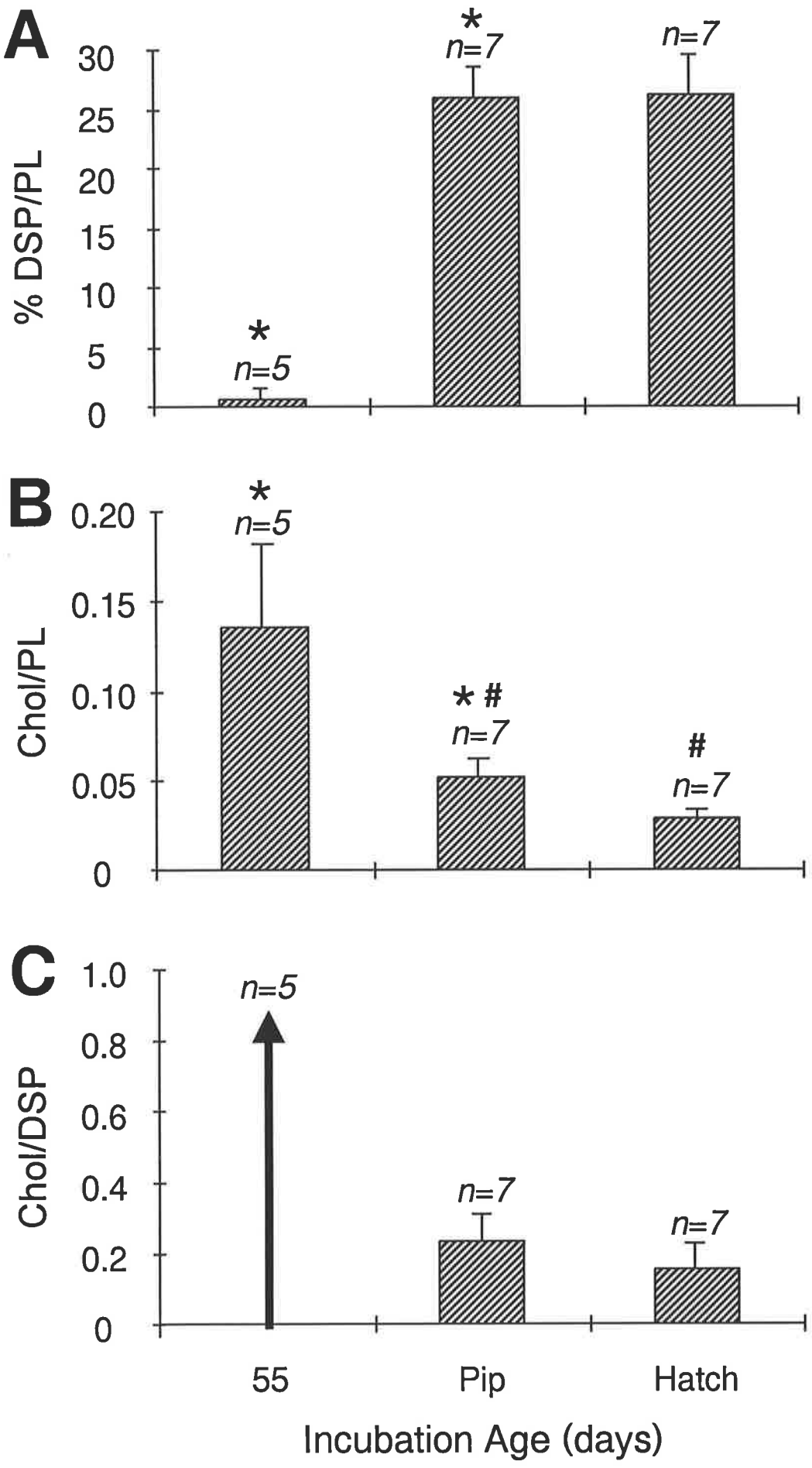

A
Total PL (µg/mg DL)
6.0
5.0
4.0
3.0
2.0
1.0
0
*
n=5
*
n=7
n=7
B
DSP (µg/mg DL)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
n=5
*
n=7
n=7
C
Chol (µg/mg DL)
0.25
0.20
0.15
0.10
0.05
0
55
Pip
Hatch
Incubation Age (days)

Figure 3.2 The relationship between phospholipids and cholesterol in the developing bearded dragon lung demonstrating DSP expressed as a percentage of total PL (**A**), the Chol/PL ratio (**B**) and the Chol/DSP ratio (**C**). Data expressed as mean ± SEM. Paired symbols indicate significant difference between adjacent groups. As DSP was not measurable in embryos incubated for 55 days, Chol/DSP approached infinity demonstrated by an arrow (**C**). **A**: * $P = 9.7 \times 10^{-6}$; **B**: * $P = 0.033$, # $P = 0.046$.


A
% DSP/PL
30
25
20
15
10
5
0
*
n=5
*
n=7
n=7
B
Chol/PL
0.20
0.15
0.10
0.05
0
*
n=5
* #
n=7
#
n=7
C
Chol/DSP
1.0
0.8
0.6
0.4
0.2
0
n=5
n=7
n=7
55
Pip
Hatch
Incubation Age (days)

incubated for 55 days and those that had pipped ($P < 0.05$), with a further reduction in hatchlings ($P < 0.05$) (Figure 3.2-B). Since 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 embryos incubated for 55 days to embryos that had pipped yet there was no difference between pipping and hatchling groups ($P = 0.24$) (Figure 3.2-C).

3.3.3 Additional Measures

3.3.3.1 Development of the Type II Pneumocytes

The lungs of the developing bearded dragon lie within a single pleuroperitoneal cavity. At all stages sampled, the lungs were firmly attached posterolaterally to the ribcage by pigmented connective tissue. Foetal lung fluid was observed in one bearded dragon embryo incubated for 55 days, whereas no lung fluid was detected in embryos that had pipped or hatched. The lungs were poorly perfused at day-55 of incubation. At this stage, the respiratory epithelium was characterised by cuboidal epithelial cells, with features of type II cells (Figure 3.3-A,B). These type II cells contained large stores of intracellular glycogen that were closely associated with lamellar bodies (Figure 3.3-A),. However, several cells had less glycogen and large quantities of lamellar bodies (Figure 3.3-B). Surfactant was rarely present in the airspace at this stage (Figure 3.3-A,B). Following pipping the type II cells contained numerous assemblages of lamellar bodies. After pipping, intracellular glycogen was either absent, or present in extremely low amounts in the type II cells (Figure 3.3-C). Qualitatively, there appeared to be less glycogen in embryos that had pipped than those at day-55 of incubation. At this time, large aggregates of surfactant were also observed in the airspaces (Figure 3.3-C). The ultrastructure of the respiratory epithelium following hatching did not appear to differ substantially from the time of pipping (Figure 3.3-D). Type II cells contained small or non-existent

accumulations of glycogen and large quantities of mature lamellar bodies (Figure 3.3-D). Numerous large aggregates of surfactant were present in the airspace (Figure 3.3-D).

3.4 Discussion

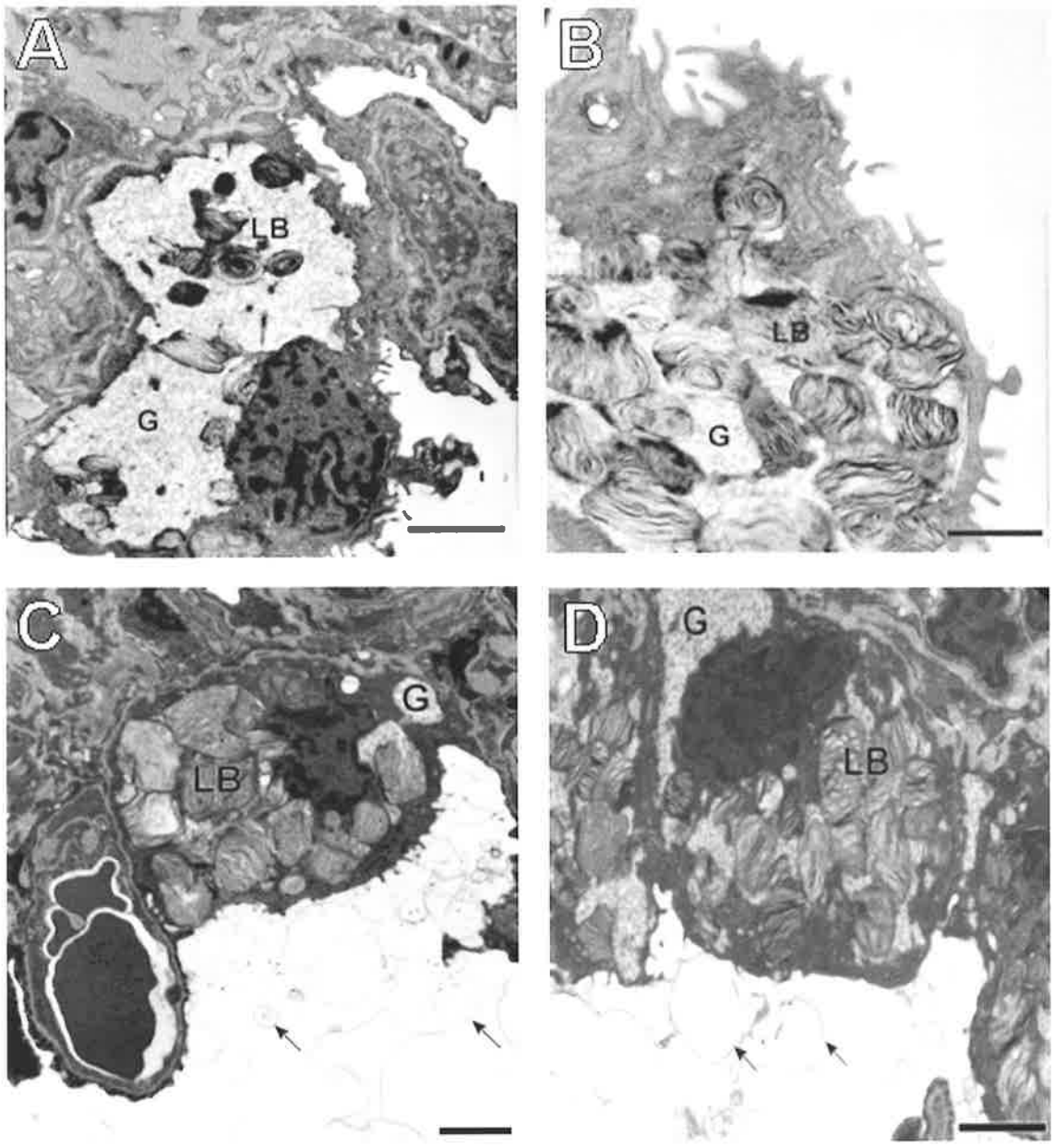
3.4.1 Development of the Pulmonary Surfactant Lipids

3.4.1.1 Phospholipids

Bearded dragons dramatically increased total PL and DSP during the latter 10% of incubation from the 55th day of incubation to pipping (hatching = ~59 days). However, relative to incubation time, the timing of release of surfactant was extremely truncated. Adult levels of DSP/PL, measured from agamid lizards at a body temperature of 23°C, (Daniels *et al.*, 1996) were achieved at pipping (Figure 3.2-A). Therefore, the lizard surfactant system appears to develop and mature over a relatively shorter time than that of birds and mammals.

The lack of FLF and the rapid release and the elevated level of saturation of the phospholipids at pipping, suggests the lungs and surfactant system are "prepared" at the onset of airbreathing. Foetal lung fluid was largely absent from the lungs of embryos at day-55 of incubation, suggesting absorption of lung fluid had occurred prior to the initiation of airbreathing. Similarly, in the rabbit lung, significant quantities of lung fluid are absorbed during labour, prior to the commencement of airbreathing (Bland *et al.*, 1980). Indeed, fluid clearance has been observed in embryonic sea turtles prior to hatching (Maloney *et al.*, 1989). In mammals, lung fluid reabsorption (Brown *et al.*, 1983) and surfactant secretion (Marino & Rooney, 1981) are accelerated by escalating levels of circulating catecholamines at birth. Furthermore, circulating catecholamines are believed to stimulate phospholipid secretion *in vivo* in the adult bearded dragon (Wood *et al.*, 1997).

Figure 3.3 Electron micrographs depicting respiratory epithelial cells from bearded dragons after 55 days of incubation (**A, B**), the time of pipping (**C**) and after hatching (**D**). Note the cuboidal epithelial cells that can be identified as type II cells by the presence of lamellar bodies (LB). Glycogen (G) was present in relatively high quantities in day-55 embryos (**A**), however some cells contained low amounts of glycogen, with large assemblages of lamellar bodies (**B**). Less glycogen was observed in pipping and hatchling lizards. Conversely, the content of lamellar bodies increased throughout the developmental period with a concomitant increase in the amount of large aggregates of surfactant (➡) in the air space from animals that had pipped and hatched. Bar = 2 μm for panels A, C, D. Bar = 1 μm for panel B.


A
LB
G
B
LB
G
C
G
LB
D
G
LB

Circulating catecholamines may therefore trigger surfactant release in bearded dragons at the time of pipping.

Hatchling reptiles are highly precocial and must undergo bouts of digging to free themselves from the nest (Ackerman, 1977). This is often followed by sprints to avoid predation (Christian & Tracy, 1981). 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 hatchling Galapagos land iguanas, *Conolophus pallidus*, to avoid predation from hawks when dispersing from the nest (Christian & Tracy, 1981). Given that total PL content increases with exercise in the lung of the adult bearded dragon (Wood *et al.*, 1997), and total PL was maximal following pipping, greater amounts of PL may be required in order for hatchlings to flee the nest. Moreover, temperature profoundly affects breathing frequency and metabolism in agamid lizards (Frappell & Daniels, 1991a), further emphasising the need for a well-functioning surfactant system following hatching.

3.4.1.2 Cholesterol

In agamid lizards, cholesterol content increases rapidly *in vivo* with increasing body temperature, presumably to maintain the homeoviscosity of the surface film (Daniels *et al.*, 1990). It is unlikely that cholesterol is important in maintaining fluidity in the developing bearded dragon lung as temperature remained constant throughout incubation. Moreover, temperature does not affect cholesterol secretion from isolated type II cells of adult bearded dragons (Wood *et al.*, 1999). Like chickens (Chapter 2), the content of cholesterol did not differ throughout the final stages of incubation (Figure 3.1-C) despite vast increases in total PL and DSP (Figure 3.1-A,B). Consequently, the Chol/PL and Chol/DSP declined during incubation. The development of cholesterol in the lizard lung therefore precedes that of the surfactant lipids, suggesting that the phospholipids and cholesterol have

independent mechanisms for control and release during development in this species. Similarly, the control of cholesterol secretion differs from that of the phospholipid in the isolated perfused lung (Wood *et al.*, 1995; Wood *et al.*, 1997) and isolated type II cells (Wood *et al.*, 1999) of adult bearded dragons. Whereas both adrenaline and acetylcholine stimulate phospholipid release, neither agonist affects cholesterol release (Wood *et al.*, 1995; Wood *et al.*, 1997; Wood *et al.*, 1999).

3.4.2 Additional Measures

3.4.2.1 Development of the Type II Pneumocytes

This is the first study to simultaneously describe the biochemical and morphological development of the pulmonary surfactant system in a reptile. Type II cells differentiate in the canalicular stage of lung development in mammals (Meyrick & Reid, 1977; Sosenko & Frank, 1987; Schellhase *et al.*, 1989; Gomi *et al.*, 1994) and on the 16^{th} day in the chicken, maturing by the 18^{th} day of incubation (Dameron & Marin, 1970; Hylka, 1989). At this time, the respiratory epithelium of mammals is comprised of cuboidal epithelial cells, characteristic of developing type II pneumocytes, with large accumulations of glycogen and scant lamellar bodies (Schellhase *et al.*, 1989; Gomi *et al.*, 1994). Similarly, after 55 days of incubation in the developing lizard lung, many type II cells contain intracellular glycogen and few lamellar bodies (Figure 3.3-A). However, some cells were virtually free of glycogen (Figure 3.3-B). Biochemical and radiolabelling studies have demonstrated that glucose stored as glycogen is a vital precursor for surfactant phospholipid synthesis toward the end of gestation in mammals (Maniscalco *et al.*, 1978; Bourbon *et al.*, 1982) and chickens (Hylka, 1989). Morphological studies demonstrate that the content of glycogen decreases in type II cells from the rat (Schellhase *et al.*, 1989), guinea pig (Sosenko & Frank, 1987) and chicken (Hylka, 1989) with advancing gestation, which is

coupled with a concomitant increase in the number of lamellar bodies. Furthermore, biochemical studies have demonstrated that a fall in the content of foetal lung glycogen coincides with the incorporation of choline into PC and DPPC (Maniscalco *et al.*, 1978) and precedes the surge of saturated PC in lung tissue (Sosenko & Frank, 1987). Similarly, in the bearded dragon the amount of glycogen appears to decline with advancing incubation (Figure 3.3). Lamellar bodies were intimately associated with glycogen throughout development (Figure 3.3). Furthermore, hatchlings appeared to have more lamellar bodies than embryos incubated for 55 days. Indeed, the content of lamellar bodies increases in the foetal rat lung following 21 days of gestation, corresponding to the terminal sac stage of lung development (Schellhase *et al.*, 1989; Gomi *et al.*, 1994). Such qualitative evidence suggests that, as with mammals and chickens, glycogen provides a substrate for surfactant lipid synthesis in lizards.

Morphology of the bearded dragon lung following 55 days of incubation (92% of incubation) therefore appears to correspond with the late canalicular stage of lung development in mammals (86% of gestation in rats, 60-75% in guinea pigs) and day-16 to -18 (76-86% of incubation) in chickens, whereas pipping appears to correlate with the terminal sac (Schellhase *et al.*, 1989; Gomi *et al.*, 1994) or alveolar stage (Sosenko & Frank, 1987) of mammalian lung development. It seems therefore that the final maturation of the respiratory epithelium in lizards mirrors that of mammals and birds. However, the maturation of the type II cells occurs later in development and over a shorter period of time, relative to the length of incubation, when compared with most eutherian mammals. Moreover, the developing lung of the loggerhead sea turtle, *Caretta caretta*, like that of mammals, passes through distinct embryological stages of development, including lung bud formation, chamber proliferation and finally differentiation of the respiratory units (Hesser, 1906; Broman, 1939; Perry *et al.*, 1989). During the final week of development (~84 -100% of incubation) in the sea turtle, the respiratory epithelium transforms from an

epithelium comprising a single cell type with characteristics of type II pneumocytes, to a mature epithelium comprising both type I and type II cells (Perry *et al.*, 1989). There is no difference in the histological appearance between the lungs of embryos one day prior to hatching, coincident with pipping, and those that have hatched (Perry *et al.*, 1989). Similarly, in the bearded dragon lung, there is no difference in the histological appearance of the lung between embryos that have pipped and hatchlings. Therefore, the lungs of both lizards and turtles appear to mature immediately prior to hatching, reflecting the highly precocial nature of the young and the need to flee the nest shortly after hatching.

Lamellar bodies were observed in type II cells from embryos incubated for 55 days, (Figure 3.3-A) and biochemical lipid profiles (Section 3.3.2) demonstrated that minute quantities of DSP were present in the airspace at the same stage of development (Figure 3.1-B, 3.2-A). Since lamellar bodies are the intracellular storage site for pulmonary surfactant and their composition does not differ from the extracellular compartment (Akino, 1992), it is clear that, despite the presence of considerable surfactant stores at day-55, the majority of surfactant is not secreted into the airspace until the time of pipping, coincident with the initiation of airbreathing. That the agamid type II cells mature over a briefer period than those of mammals, further exemplifies the swiftness of maturation and secretion of surfactant in this species.

3.4.3 Conclusions

In the bearded dragon, the development of the surfactant lipids follow the same developmental path as mammals and birds (Chapter 2), suggesting that the pattern of development is conserved between mammals, chickens and oviparous lizards. Cholesterol appears earlier than the phospholipids. Thus, like chickens, the mechanisms controlling the release of surfactant cholesterol differ from those controlling the phospholipids. Like mammals, maximal secretion of the phospholipids occurred at the onset of pipping,

suggesting that the maturation of the system occurs at this time, presumably to aid inflation of the collapsed lung in preparation for airbreathing.

The type II cells follow the same developmental pattern as mammals, birds and turtles, further emphasising the highly conserved nature of this system. Likewise, glycogen appears to be instrumental in the biosynthesis of phospholipids in the developing lizard lung. Relative to incubation, the lipids and type II cells develop more rapidly, and over a shorter time in the embryonic bearded dragons than do those of mammals and birds, and to a lesser extent, turtles, reflecting the highly precocial state of the young and the need for a fully functional surfactant system at hatching in order to flee the nest.

3.4.4 Future Directions

While the biochemical pathways for surfactant secretion and synthesis can be inferred for reptiles (Wood *et al.*, 1997; Wood *et al.*, 1999), they have never been described. From the present study it is clear that glycogen plays a vital role in the synthesis and maturation of the surfactant system in lizards. It would be of considerable interest to confirm the role of glycogen as a substrate for phospholipid biosynthesis in lizard lung. This could be achieved by the method of Maniscalco *et al.* (1978) and Hylka (1989). By biochemically measuring the amount of glucose, glucose oxidation, glycogen and enzymes involved in both glucose metabolism and phospholipid synthesis in the developing lizard lung and comparing them with the incorporation of ^{14}C-glucose into DPPC and PC from lung slices one could ascertain whether glycogen provides a substrate for DPPC and PC synthesis. Bourbon *et al.* (1982) demonstrated that the developing rat lung liberated glucose from endogenous glycogen stores as opposed to exogenous glucose in culture media.

Due to the highly truncated pattern of both structural and biochemical development of the pulmonary surfactant system, it would be important to determine whether such a pattern is a trait of this particular agamid or whether it is a universal phenomenon among

lizards. The experiment should be repeated on other closely related and distantly related oviparous lizards.

CHAPTER 4 DEVELOPMENT OF THE PULMONARY SURFACTANT AND ANTIOXIDANT ENZYME SYSTEMS IN THE VIVIPAROUS LIZARD, *TILIQUA RUGOSA*

4.1 INTRODUCTION

All lungs must be cleared of fluid, inflated and prepared for large increases in the partial pressure of oxygen at birth. Therefore, crucial to lung function are both the pulmonary surfactant and antioxidant enzyme (AOE) systems. In mammals, pulmonary surfactant increases lung compliance and promotes the reabsorption of fluid at birth by modulating surface tension at the air/liquid interface (Possmayer, 1997). The AOE system is required to protect both pulmonary surfactant and lung tissue from oxidative damage that can occur after the initiation of airbreathing (Putman *et al.*, 1997). Therefore, viviparous animals require the release and activation of pulmonary surfactant and AOEs in order to establish ventilation after birth.

During birth, the alveolar oxygen tension dramatically rises within the alveoli from 25–30 mmHg in the foetus to >100 mmHg in the newborn (McElroy *et al.*, 1990), with a concomitant increase in the production of oxygen free radicals (Frank *et al.*, 1996). The oxygen radicals target pulmonary surfactant and lung tissue, impairing the ability of the former to control surface tension (Gilliard *et al.*, 1994). Consequently, antioxidant enzymes may be of particular importance to non-mammalian vertebrates as their pulmonary surfactant is dominated by oxidisable cholesterol and unsaturated phospholipids (Daniels *et al.*, 1995b; Veldhuizen *et al.*, 1998).

In mammals, the activity of AOEs increases markedly in the developing lung to protect the lung from oxidative damage that might occur with the onset of airbreathing (Frank & Groseclose, 1984; Walther *et al.*, 1991b). The temporal development and maturation of the AOEs are similar to the prenatal development of the pulmonary surfactant system in mammals (Frank & Groseclose, 1984; Frank & Sosenko, 1987;

McElroy *et al.*, 1990; Walther *et al.*, 1991b). Moreover, the alveolar type II cells have the highest antioxidant enzyme activity of any pulmonary cells (Forman & Fisher, 1981) and are the primary site of pulmonary surfactant synthesis (Possmayer, 1997). Furthermore, the maturation of both systems is influenced by glucocorticoids and thyroid hormones (Frank *et al.*, 1985; Sosenko *et al.*, 1986; Ramadurai *et al.*, 1998). Thus, the interplay between the two systems is crucial for normal lung function, especially at birth.

Nothing is known about the development or maturation of either the pulmonary surfactant or the AOE systems in ectothermic viviparous vertebrates. Fluctuating body temperatures during birth may impair the control of the composition of pulmonary surfactant. Moreover, the transition from *in utero* to the external environment is extremely brief in viviparous skinks, including the Australian lizard, *Tiliqua rugosa* (Bull *et al.*, 1993). Thus, a functional pulmonary surfactant and AOE system are of paramount importance for the initiation of airbreathing. In the adult of a closely related species, *Tiliqua nigrolutea*, surfactant comprises 71.7% unsaturated phospholipid (Daniels *et al.*, 1996). Thus, the relative abundance of the unsaturated phospholipids may heighten the possibility for lipid peroxidation, as the double bonds within unsaturated phospholipids are the primary site of free radical damage (Goldstein, 1978). This chapter describes the development of both the pulmonary surfactant lipids and the antioxidant enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) in the viviparous skink, *Tiliqua rugosa*.

4.2 Materials and Methods

4.2.1 Collection and Maintenance of Animals

The sleepy lizard, *Tiliqua rugosa*, is a large diurnal lizard that is widely distributed across the southern expanse of continental Australia. A member of the Scincidae, it is highly

specialised as an 'armoured' lizard, with thick, bony scales, blunt head and tail and short legs (Cogger, 1986). Like other *Tiliqua* species, *T. rugosa* give birth to live young, however they are distinctive in having small clutches, usually comprising 1-4 exceptionally large offspring following a lengthy gestation (~150 days). This species possesses a simple chorioallantoic and yolk sac placenta (Weekes, 1935), and the young are completely independent of their mother within minutes of birth (Bull *et al.*, 1993).

Gravid lizards were obtained from the Flinders Ranges, South Australia in October 1998 and maintained individually in pens at 22-24°C throughout their five-month gestation. Animals were fed three times per week on a diet of fruit and vegetables, which was supplemented with cockroaches, snails, calcium and vitamins. Lizards had access to water *ad libitum*. Gestation was monitored by weighing post-absorptive animals twice per week (Bourne *et al.*, 1986). Towards the end of gestation, cages were observed closely to record the time and process of birth.

4.2.2 Tissue Sampling

Females, either gravid (M) or non-gravid (NG) were selected randomly throughout the sampling period and killed with an intraperitoneal dose of sodium pentobarbitone (150 mg/kg [BM] Nembutal, Abbott Laboratories, Sydney, NSW, Australia). Prior to sacrifice, adults were weighed and the snout-vent length was recorded. Foetuses were excised from gravid females, sacrificed and snout-vent lengths and yolk-free body masses were obtained. Foetal and adult lungs were either lavaged with isotonic saline and the lungs were retained to obtain dry lung weights, or they were snap frozen in liquid nitrogen and stored at –80°C for AOE analyses. Newborn animals were killed within the first 24 hours of birth (1d) for both AOE and surfactant analyses and 5 days after birth (5d) to determine the postnatal development of the antioxidant enzymes alone.

4.2.3 Embryonic Staging

As the exact date of conception could not be determined, foetuses were staged *post hoc* using a modified version of Dufaure and Hubert (1961) (hereafter referred to as D&H) adapted from Smith and Shine (1997). The stages observed in this viviparous skink fell between D&H stages 36 to 40, however the stages observed in *T. rugosa* did not conform precisely to the staging system of embryonic *Lacerta vivipara* (Dufaure & Hubert, 1961). For example, scalation was highly advanced in stage 36 (D&H) *T. rugosa* foetuses, despite the majority of other characters adhering to the staging parameters. In most cases, the appearance of scales, claws and pigmentation, which are usually associated with the final stage of development (D&H stage 40), were present in foetuses that were less than half the mass of a standard newborn (Table 4.1). Therefore, stage 40 (D&H) was extremely protracted in this species, allowing the foetus to grow inside the mother during the final ~6 weeks of gestation, without gross differentiation in external characters. For this reason, the majority of individuals had already reached stage 40 (D&H) despite a further ~30% of gestation remaining. In order to stage foetuses relative to each other, additional staging methods were applied. Criteria for ranking foetuses within stage 40 (D&H) were based on the degree of differentiation of 1) the lower eyelid scales, 2) the superior digital scales, 3) the hind- and fore-limb foot pads, 4) the degree of elevation of postorbital scales and 5) the overall pigmentation of the animal. Mass and snout-vent length, in conjunction with these physical characters provided the final ranking of foetuses within stage 40 (D&H) (Table 4.1).

4.2.4 Lavage Protocol

Foetuses and adults were tracheal-cannulated with blunted 18–20G needles depending on the size of the animal, and the lungs were lavaged with three volumes of ice-cold isotonic

saline (0.15M NaCl), each instilled and withdrawn three times. When present, foetal lung fluid was incorporated into the lavage. Volumes of saline ranging from 1.5-3.0 ml were injected into the lungs as foetuses increased in mass, whereas 50 ml was injected into adults, resulting in a lavage volume of 0.02 to 0.11 ml/g BM. The lavage was centrifuged (Beckman model TJ-6 centrifuge) at 150 *g* for 5 min at 4°C to remove cellular debris. The supernatant was snap frozen in liquid nitrogen and freeze-dried. Lyophilised lavage was stored at -80°C for further analyses. Lavaged lungs were blotted dry on paper towel, snap frozen, freeze-dried and weighed.

4.2.5 Lipid Analyses

Total phospholipid, disaturated phospholipid and cholesterol were extracted and quantified from lavage in the same manner as described for chickens (Section 2.2.5).

4.2.6 Additional Measures

4.2.6.1 Tissue Preparation for Antioxidant Enzyme Analyses

Frozen lung tissue was rapidly weighed in pre-cooled tubes and minced for 2 min on ice with fine scissors in 1:20 (w/vol) of ice-cold 10 mM potassium phosphate buffer (pH 7.4, supplemented with 30 mM KCl and 1 mM EDTA). The fine tissue suspension was sonicated (MSE sonicator equipped with an exponential probe, 20kHz, medium power, amplitude 16μm) on ice for a total of 45 sec (1 sec burst/ 4 sec rest) (DelMaestro & McDonald, 1985). Ice was replaced midway through the sonication of each sample to prevent heating of the homogenate. The homogenate was centrifuged at 12 000 *g* for 30 min and the supernatant was divided into four aliquots for catalase, total SOD, GPx and protein assays and stored at -80°C prior to biochemical analyses.

4.2.6.2 Antioxidant Enzyme Analyses

Purified standards for catalase, SOD and GPx (from bovine erythrocytes) were purchased from Sigma Chemical Co. (Sigma Chemical Co. St Louis, MO USA). All other chemicals used were of analytical grade or higher. The activities of catalase, SOD and GPx were analysed using standard spectrophotometric assays performed with a double-beam spectrophotometer (Hitachi-U 2000) equipped with a constant temperature chamber.

Catalase activity was determined by measuring the rate of hydrogen peroxide (10 mM) decomposition in 50 mM potassium phosphate buffer (pH 7.0), at 240 nm and at 25°C (Aebi, 1974). The activity was calculated using the molar extinction coefficient of 0.0436 $mM^{-1}cm^{-1}$ (Aebi, 1974).

Total SOD activity was quantified by monitoring the SOD-induced inhibition of cytochrome C reduction by the superoxide radical generated in a xanthine/xanthine oxidase system (McCord & Fridovich, 1969). Aliquots of supernatant were added to an assay mixture, containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.01 mM cytochrome C, 0.05 mM xanthine and 0.005 units xanthine oxidase in a final volume of 3 ml. The rate of increase in absorbance was continuously recorded spectrophotometrically at 550 nm and at 25°C for 5 min and the maximum linear rate was employed to calculate the activity of SOD. One unit of SOD was defined as the quantity of SOD required to produce 50% inhibition of the rate of reduction of cytochrome C in the system described above.

Total GPx activity was determined indirectly using a coupled enzyme method of Paglia and Valentine (1967) modified by Gunzler and Flohe (1985). GPx catalyses the glutathione-dependent reduction of hydroperoxides, where reduced glutathione (GSH) is converted to its oxidised form (GSSG). In this method, GSSG is continuously reduced to GSH by NADPH in the presence of glutathione reductase. NADPH is continuously oxidised to NADP while the concentration of GSH is kept constant. The amount of GPx in

the sample is proportional to the amount of NADPH oxidised per unit time. In this study, the rate of NADPH consumption was continuously monitored spectrophotometrically at 340 nm and at 37°C and used to calculate the amount of GPx. In summary, 100 µl of supernatant was added to 400 µl of 50 mM potassium phosphate buffer (pH 7.0), containing 1 mM EDTA and 100 µl of 10 mM sodium azide, followed by 100 µl of glutathione reductase (2.4 units/ml) and 100 µl of 10 mM GSH. The mixture was incubated for 10 min at 37°C and 100 µl of 1.5 mM NADPH in 0.1% $NaHCO_3$ was added. The solution was mixed and the hydroperoxide-independent NADPH consumption was recorded at 340 nm and 37°C for 5 min. Subsequently, 100 µl of 1.5 mM hydrogen peroxide was added, mixed and the decrease in absorbance was recorded for a further 5 min. Sodium azide was added to inhibit catalase. One unit of GPx was defined as the amount of GPx causing the oxidation of 1 µmole of GSH per min in the aforementioned system. Values were corrected for both the hydroperoxide-independent decomposition of β-NADPH and for the non-enzymatic oxidation of GSH.

In all cases, the AOE activity from blood was included in the results. Blood increases the activity of catalase, without appreciably affecting SOD or GPx activity (Cross *et al.*, 1979; Gerdin *et al.*, 1985). However, the contribution made by blood is an important part of the development of the antioxidant enzyme system in the lung, particularly as the lung is increasingly perfused with advancing gestation. In the rat, the endothelial cells lining the capillaries are the primary site of oxidative attack at birth (Forman & Fisher, 1985) and, as the perfused lung is in intimate contact with this delicate lining, the pulmonary antioxidant defense system is not complete unless the contribution from blood is included.

4.2.7 Statistical Analyses

The activity of each antioxidant enzyme was calculated in units/ml of homogenate and standardised against mg protein/ml of homogenate such that the final units are units/mg protein. DSP/PL is expressed as a percentage, whereas Chol/PL and Chol/DSP are expressed as μg/μg ratios. All ratios were Arcsin transformed. Data are expressed as mean ± SEM. Curve fitting was performed for stage 40 foetuses. One-way ANOVA followed by Student's t-tests were performed where appropriate. Statistical significance was set at $P < 0.05$.

4.3 Results

4.3.1 Physical Parameters

The mass of intact maternal lizards (M) (640.9 ± 29.11 g, n = 14) did not differ from that of non-gravid females (NG) (591.49 ± 22.42 g, n = 14) (P = 0.095). Similarly, snout-vent length did not differ between adults (M: 29.47 ± 0.38 cm, n = 13; NG: 29.36 ± 0.28 cm, n = 13) (P = 0.41), nor did dry lung weight when expressed in mg (M: 431.40 ± 19.83 mg, n = 11; NG: 423.04 ± 26.34 mg, n = 8) (P = 0.40) or as a percentage of body mass (M: 0.067 ± 0.004%, n = 11; NG: 0.075 ± 0.006%, n = 8) (P = 0.11). In foetal lizards, mass (r^2 = 0.8095, $P < 0.001$), snout-vent length (r^2 = 0.8329, $P < 0.001$) and dry lung weights (r^2 = 0.3635, $P < 0.05$) increased with increasing gestation (Table 4.1). However, when expressed as a percentage of body mass, lung weights declined (r^2 = 0.6944, $P < 0.001$) throughout development, presumably as a result of overall growth and increased scalation and ossification of the integument (Table 4.1). Highly viscous lung fluid was observed in foetal lungs throughout gestation until birth.

4.3.2 Development of the Pulmonary Surfactant Lipids

When expressed as a function of dry lung weight, total PL greatly increased throughout the latter stages of development ($r^2 = 0.9823$, $P < 0.001$), where it was maximal immediately prior to birth (Figure 4.1). Newborn and non-gravid adults had comparable amounts of total PL ($P = 0.34$), whereas maternal lizards had significantly less total PL than both non-gravid and postnatal young ($P < 0.01$ for both tests) (Figure 4.1). The DSP content of foetal lungs remained unchanged until stage 40-6, and then increased exponentially until birth ($r^2 = 0.9945$, $P < 0.001$) (Figure 4.2). As with total PL, the amount of DSP was significantly depressed in mothers when compared to both non-gravid and newborn lizards ($P < 0.05$ and $P < 0.001$, respectively). The lungs of postnatal and non-gravid adults had similar amounts of DSP ($P = 0.14$) (Figure 4.2). The content of cholesterol in the lizard lung increased with advancing gestation ($r^2 = 0.9110$, $P < 0.001$), where it was maximal immediately prior to birth (Figure 4.3). Chol was present in similar quantities between newborn and non-gravid lizards ($P = 0.38$), yet mothers had significantly less Chol than postnatal and non-gravid animals ($P < 0.05$, for both tests) (Figure 4.3). The relative saturation of the phospholipids increased during the final stages of gestation ($r^2 = 0.9584$, $P < 0.001$) (Figure 4.4). The saturation level of PLs in foetuses at stage 40-8 matched those of newborns. There was no difference in the DSP/PL ratio between maternal and non-gravid adults ($P = 0.31$), nor between mothers and newborns ($P = 0.12$). However, non-gravid lizards had a higher DSP/PL ratio than postnatal young ($P < 0.05$) (Figure 4.4). The Chol/PL ratio declined exponentially to stage 40-6 and remained constant for the rest of gestation, matching values from newborn lizards ($r^2 = 0.9176$, $P < 0.001$) (Figure 4.5). Postnatal, maternal and non-gravid lizards demonstrated similar Chol/PL ratios (ANOVA, $P = 0.56$) (Figure 4.5). Like Chol/PL, the Chol/DSP ratio of developing skinks declined dramatically until stage 40-6 ($r^2 = 0.9712$, $P < 0.001$), whereupon it was unchanged for the

Table 4.1 *Stage, body mass, snout-vent length and dry lung weights for foetal sleepy lizards, T. rugosa during late gestation.*

Stage	BM (g)	SVL (cm)	DLW (mg)	DLW/BM (%)
36	3.09 ±0.12 (2)	5.1 ± 0.1 (2)	---	---
38/39	8.75 ± 0.30 (2)	7.7 ± 0.2 (2)	---	---
40-1	36.84	11.8	---	---
40-2	31.98	11.6	42.48	0.133
40-3	30.77 ± 2.16 (3)	11.4 ± 0.2 (3)	30.73 ± 6.76 (3)	0.99 ± 0.019 (3)
40-4	32.91	10.8	---	---
40-5	28.09	10.9	---	---
40-6	48.03	12.5	34.42	0.072
40-7	39.18	12.6	---	---
40-8	55.37	13.5	47.30	0.085
40-9	43.93	12.9	---	---
40-10	86.18 ± 0.08 (2)	14.9 ± 0.6	60.04 ± 4.96 (2)	0.069 ± 0.006 (2)
40-11	85.95	16.0	---	---
40-12	78.50	15.5	---	---
40-13	103.33	16.7	43.60	0.042
≤ Day-1	98.21 ± 6.31 (6)	15.9 ± 0.3 (6)	53.10 ± 3.29 (5)	0.055 ± 0.002 (5)
Day-5	106.34 ± 3.67 (3)	16.0 ± 0.3 (3)	---	---

Results are mean ± SEM; *n* in parentheses; BM, body mass; SVL, snout-vent length; DLW, dry lung weight; ---, data unavailable.

Figure 4.1 The absolute amount of total phospholipid in pulmonary surfactant from the lungs of perinatal, maternal (M) and non-gravid (NG) skinks, *T. rugosa*, normalised against dry lung (DL) weight. All pre-birth individuals were classified as stage 40 (D&H) animals. Numbers 1-14 represent the rankings within this developmental stage. Data for pre-birth stages represent either individual data points or mean ± SEM of an n = 2-3. Postnatal and adult data are expressed as mean ± SEM. * significantly different from non-gravid lizards, # significantly different from one-day-old lizards (1d).

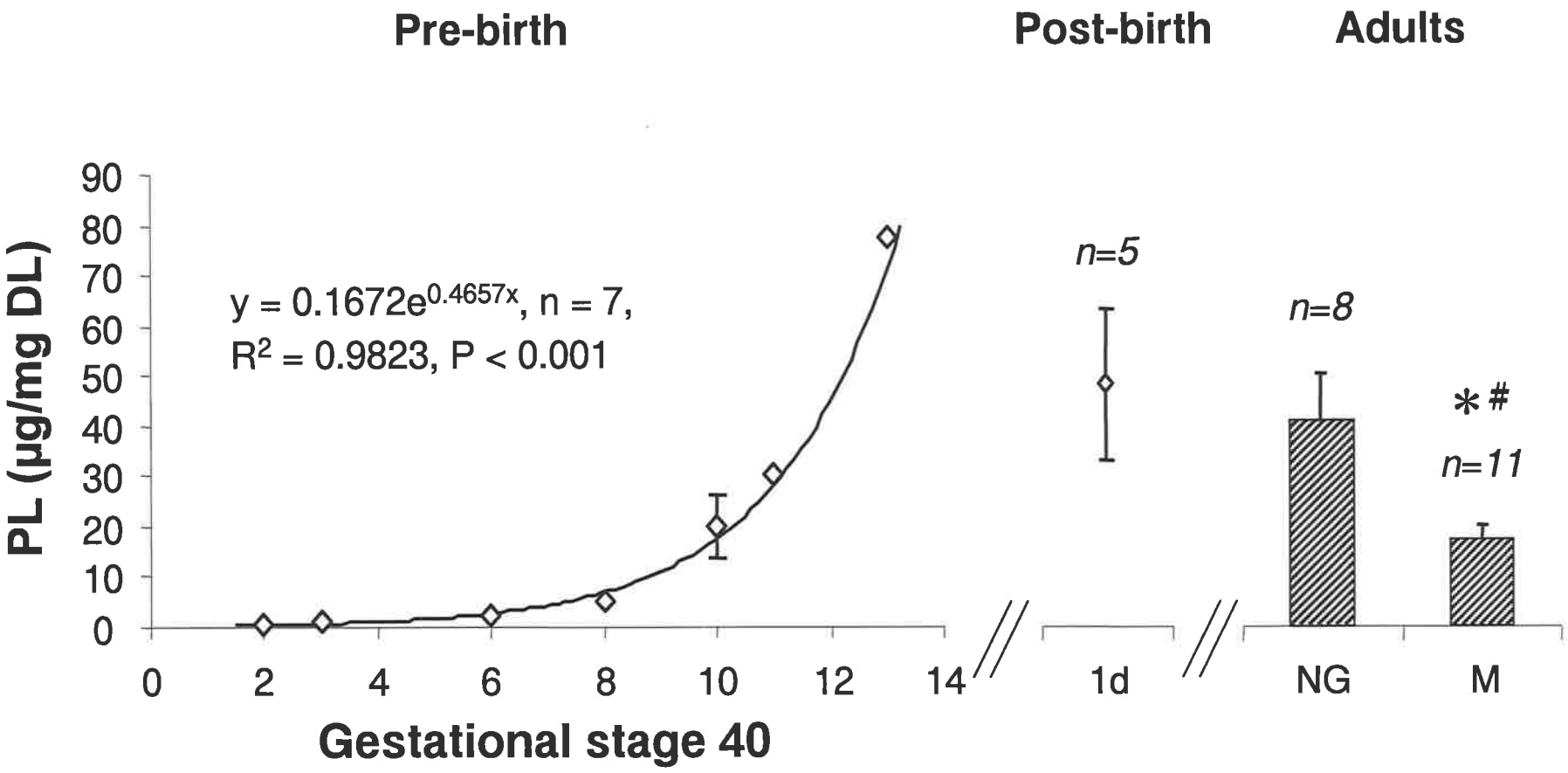

Pre-birth
Post-birth
Adults
PL (µg/mg DL)
90
80
70
60
50
40
30
20
10
0
y = 0.1672e0.4657x, n = 7,
R2 = 0.9823, P < 0.001
n=5
n=8
*#
n=11
0
2
4
6
8
10
12
14
1d
NG
M
Gestational stage 40

Figure 4.2 The absolute amount of disaturated phospholipid in pulmonary surfactant from the lungs of perinatal, maternal (M) and non-gravid (NG) skinks, *T. rugosa*, normalised against dry lung (DL) weight. All pre-birth individuals were classified as stage 40 (D&H) animals. Numbers 1-14 represent the rankings within this developmental stage. Data for pre-birth stages represent either individual data points or mean ± SEM of an n = 2-3. Postnatal and adult data are expressed as mean ± SEM. * significantly different from non-gravid lizards, # significantly different from one-day-old lizards (1d).

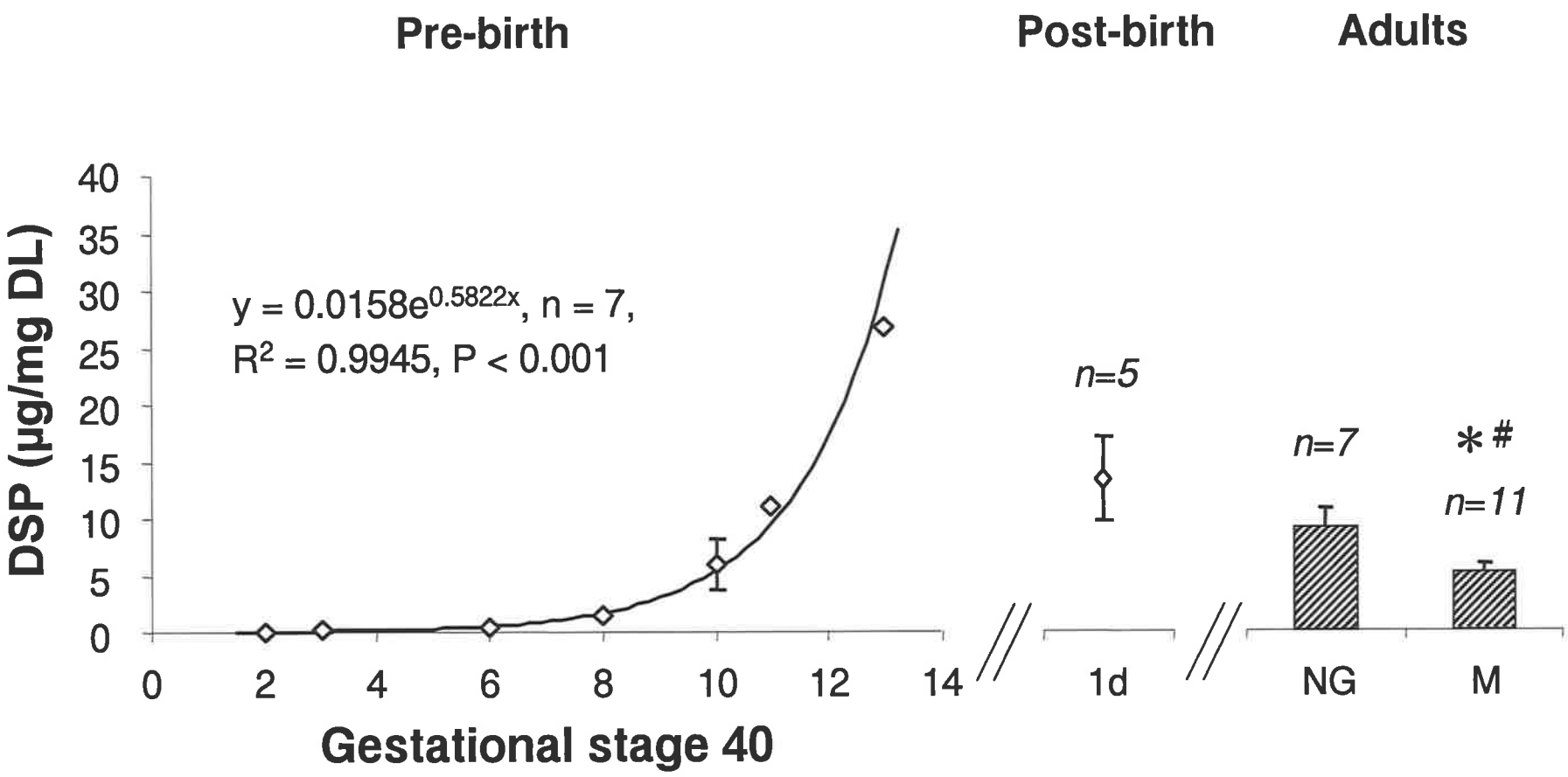

Pre-birth
Post-birth
Adults
DSP (µg/mg DL)
40
35
30
25
20
15
10
5
0
y = 0.0158e^0.5822x, n = 7,
R² = 0.9945, P < 0.001
0
2
4
6
8
10
12
14
Gestational stage 40
n=5
1d
n=7
NG
*#
n=11
M

Figure 4.3 The absolute amount of cholesterol in pulmonary surfactant from the lungs of perinatal, maternal (M) and non-gravid (NG) skinks, *T. rugosa*, normalised against dry lung (DL) weight. All pre-birth individuals were classified as stage 40 (D&H) animals. Numbers 1-14 represent the rankings within this developmental stage. Data for pre-birth stages represent either individual data points or mean ± SEM of an n = 2-3. Postnatal and adult data are expressed as mean ± SEM. * significantly different from non-gravid lizards, # significantly different from one-day-old lizards (1d).

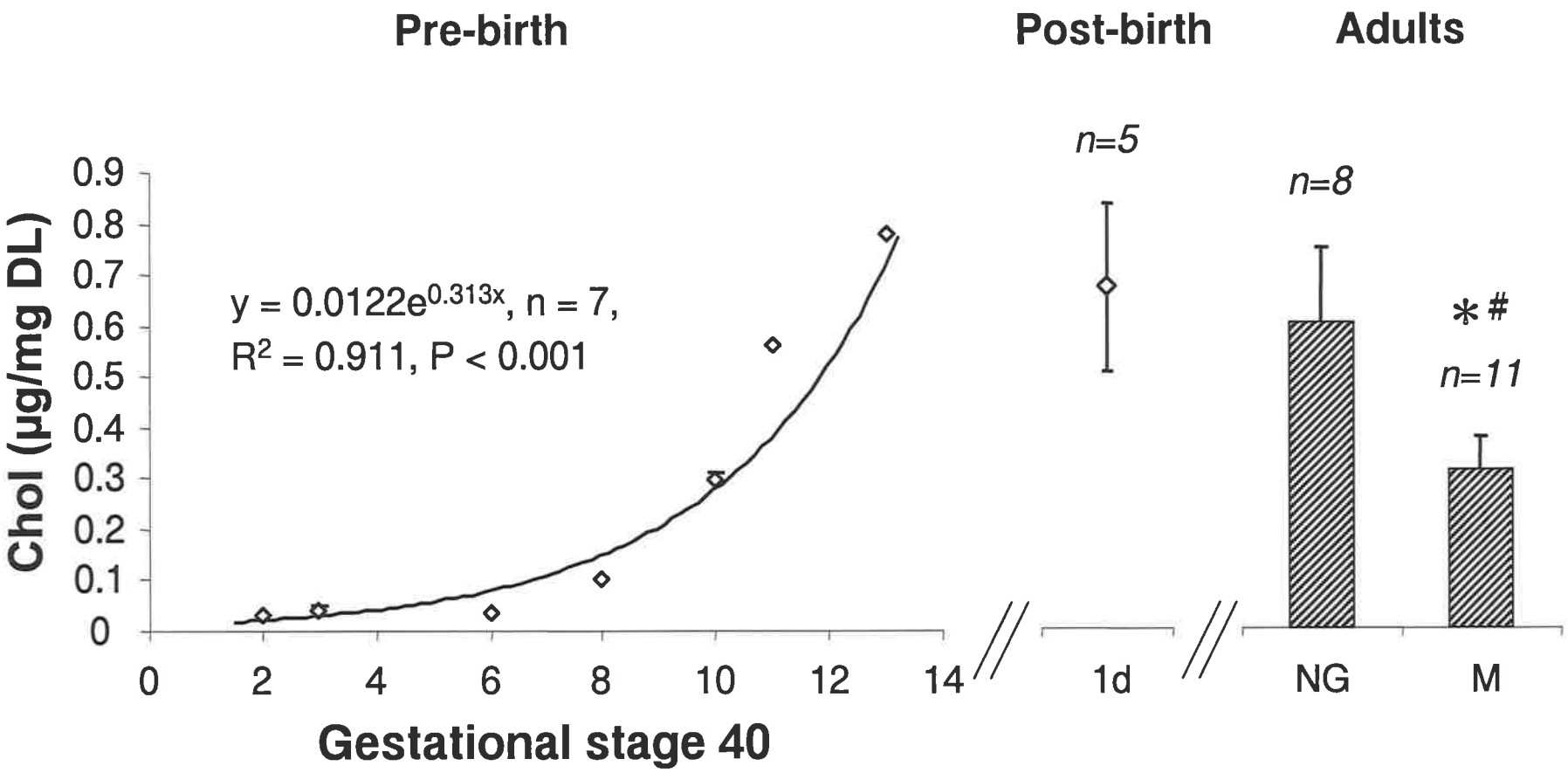

Pre-birth
Post-birth
Adults
Chol (µg/mg DL)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
y = 0.0122e0.313x, n = 7,
R2 = 0.911, P < 0.001
n=5
n=8
*#
n=11
0
2
4
6
8
10
12
14
1d
NG
M
Gestational stage 40

Figure 4.4 The percentage of disaturated phospholipid relative to total phospholipid in pulmonary surfactant from the lungs of perinatal, maternal (M) and non-gravid (NG) skinks, *T. rugosa*. All pre-birth individuals were classified as stage 40 (D&H) animals. Numbers 1-14 represent the rankings within this developmental stage. Data for pre-birth stages represent either individual data points or mean ± SEM of an n = 2-3. Postnatal and adult data are expressed as mean ± SEM. # significantly different from one-day-old lizards (1d).

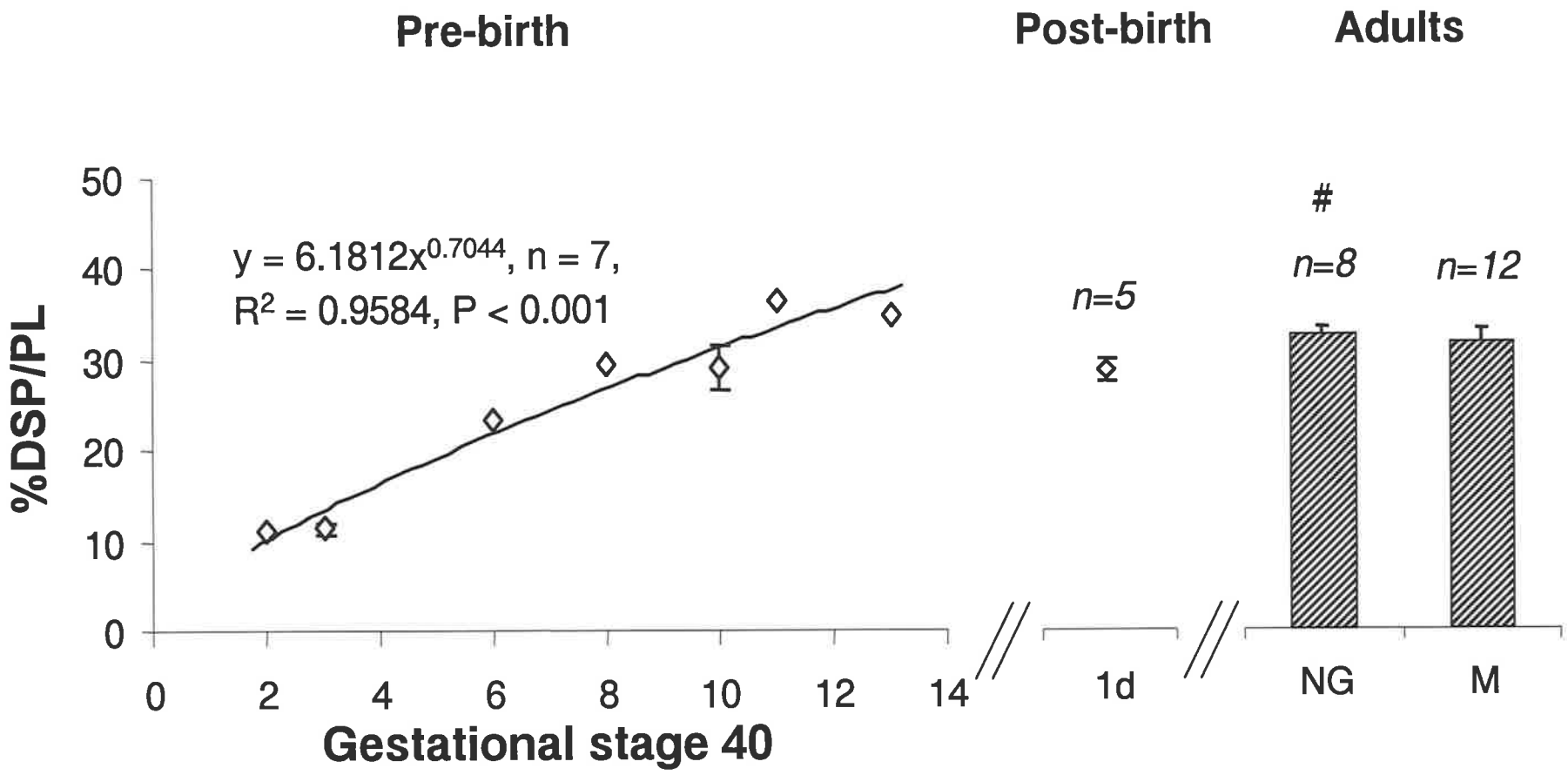
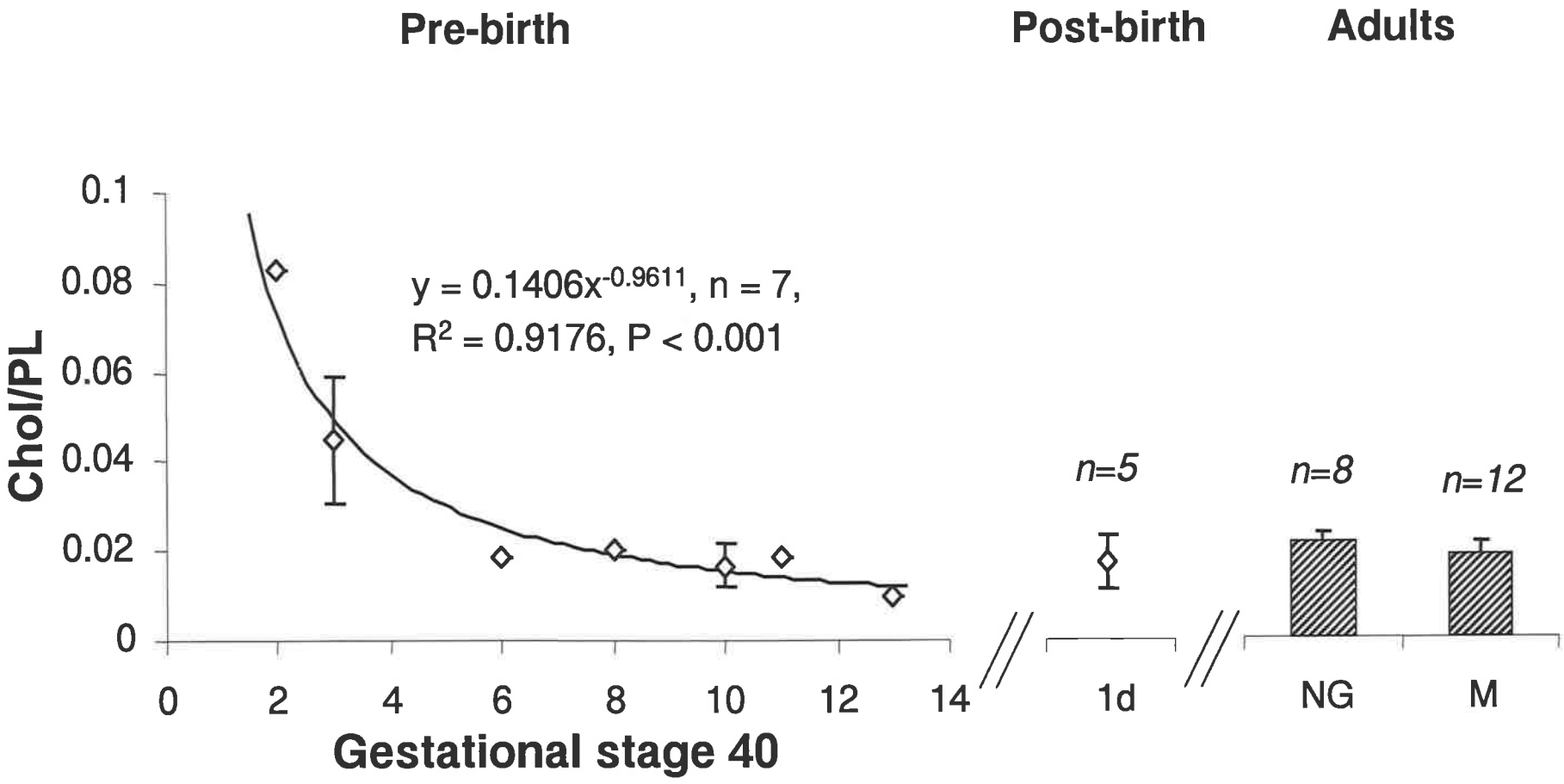

Pre-birth
Post-birth
Adults
%DSP/PL
50
40
30
20
10
0
y = 6.1812x^0.7044, n = 7,
R² = 0.9584, P < 0.001
0
2
4
6
8
10
12
14
Gestational stage 40
n=5
1d
#
n=8
n=12
NG
M

Figure 4.5 The amount of cholesterol relative to total phospholipid in pulmonary surfactant from the lungs of perinatal, maternal (M) and non-gravid (NG) skinks, *T. rugosa*. All pre-birth individuals were classified as stage 40 (D&H) animals. Numbers 1-14 represent the rankings within this developmental stage. Data for pre-birth stages represent either individual data points or mean ± SEM of an n = 2-3. Postnatal and adult data are expressed as mean ± SEM.


Pre-birth
Post-birth
Adults
0.1
0.08
0.06
0.04
0.02
0
Chol/PL
y = 0.1406x-0.9611, n = 7,
R2 = 0.9176, P < 0.001
0
2
4
6
8
10
12
14
Gestational stage 40
n=5
1d
n=8
n=12
NG
M

remainder of development (Figure 4.6). Chol/DSP did not differ between newborn and adults lizards (ANOVA, $P = 0.86$) (Figure 4.6).

4.3.3 Additional Measures

4.3.3.1 Development of the Antioxidant Enzymes

Catalase activity increased between stage 36 (D&H) and stage 40 (D&H) (Figure 4.7). Activity declined initially in stage 40 (D&H) and remained constant throughout the remainder of gestation ($r^2 = 0.4283$, $P < 0.05$). Catalase activity did not differ after one- or five-days postpartum ($P = 0.34$), however maternal catalase activity was higher than that of one-day-old neonates and non-gravid females ($P < 0.05$, for both tests) (Figure 4.7). In the developing lung, total SOD activity varied throughout the latter portion of gestation, with activity lowest just prior to birth (Figure 4.8). SOD activity was elevated above foetal levels in one- and five-day-old neonates yet did not differ from adults (Figure 4.8). GPx activity remained constant throughout gestation, and following one- and five-days postpartum (Figure 4.9). Adult activity of GPx was not significantly elevated above those of neonates ($P = 0.092$) (Figure 4.9).

4.4 Discussion

4.4.1 Development of the Pulmonary Surfactant Lipids

4.4.1.1 Phospholipids

Both the amount and the saturation of the surfactant phospholipids increase during the latter portion of mammalian gestation. These changes are accompanied by increases in the

Figure 4.6 The amount of cholesterol relative to disaturated phospholipid in pulmonary surfactant from the lungs of perinatal, maternal (M) and non-gravid (NG) skinks, *T. rugosa*. All pre-birth individuals were classified as stage 40 (D&H) animals. Numbers 1-14 represent the rankings within this developmental stage. Data for pre-birth stages represent either individual data points or mean ± SEM of an n = 2-3. Postnatal and adult data are expressed as mean ± SEM.

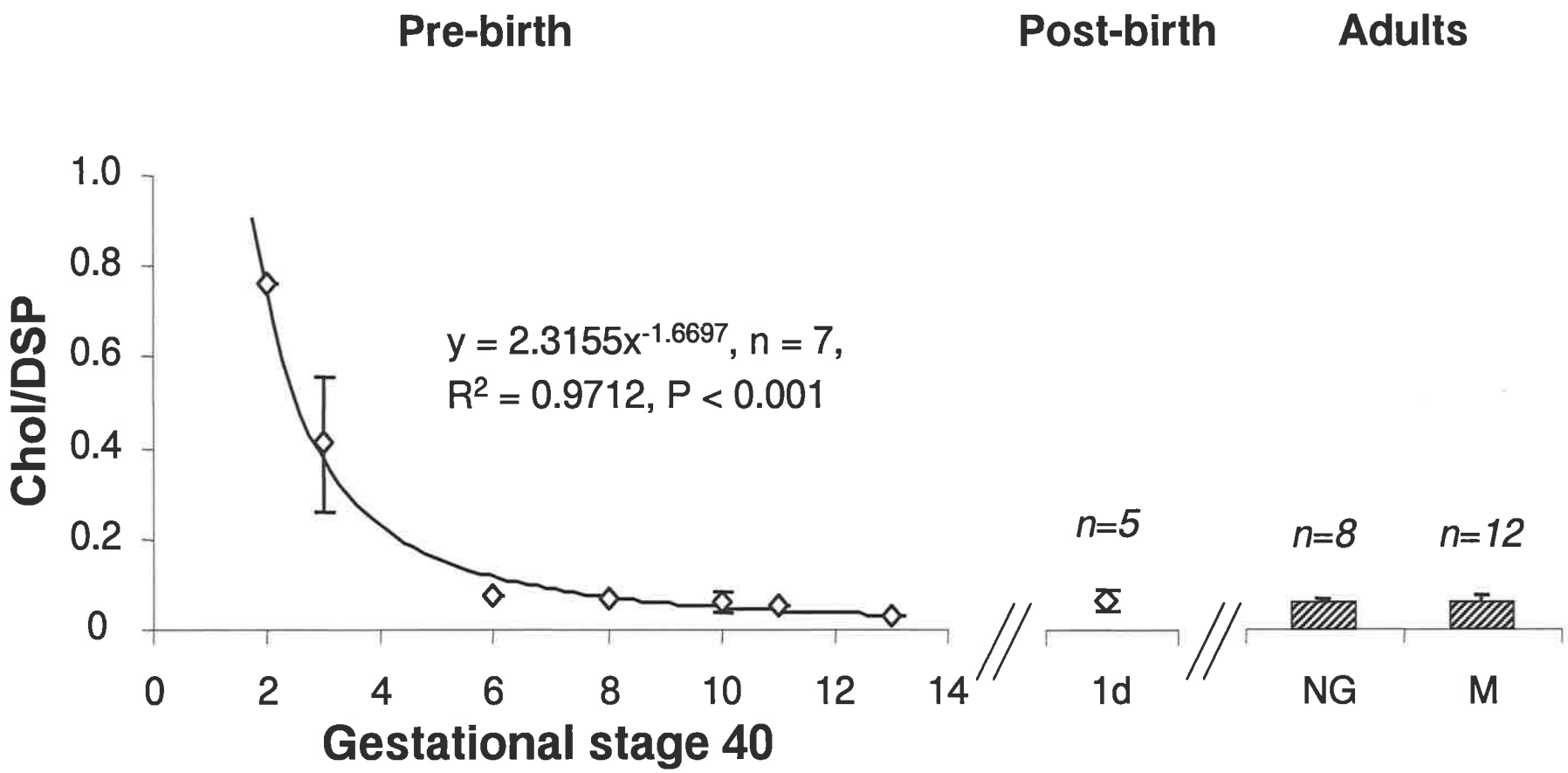

Pre-birth
Post-birth
Adults
Chol/DSP
1.0
0.8
0.6
0.4
0.2
0
y = 2.3155x-1.6697, n = 7,
R2 = 0.9712, P < 0.001
n=5
n=8
n=12
0
2
4
6
8
10
12
14
1d
NG
M
Gestational stage 40

Figure 4.7 The antioxidant enzyme activity of catalase in the lungs of perinatal, maternal (M) and non-gravid (NG) skinks, *T. rugosa*, expressed in mg protein. All pre-birth individuals were classified between stage 36 (D&H) and stage 40 (D&H). Numbers 1-14 within stage 40 represent the rankings within this developmental stage. Data for pre-birth stages represent either individual data points or mean ± SEM of an n = 2-3. One-day-old (1d), five-day-old (5d) and adult data are expressed as mean ± SEM. * significantly different from non-gravid lizards, # significantly different from 1d lizards.

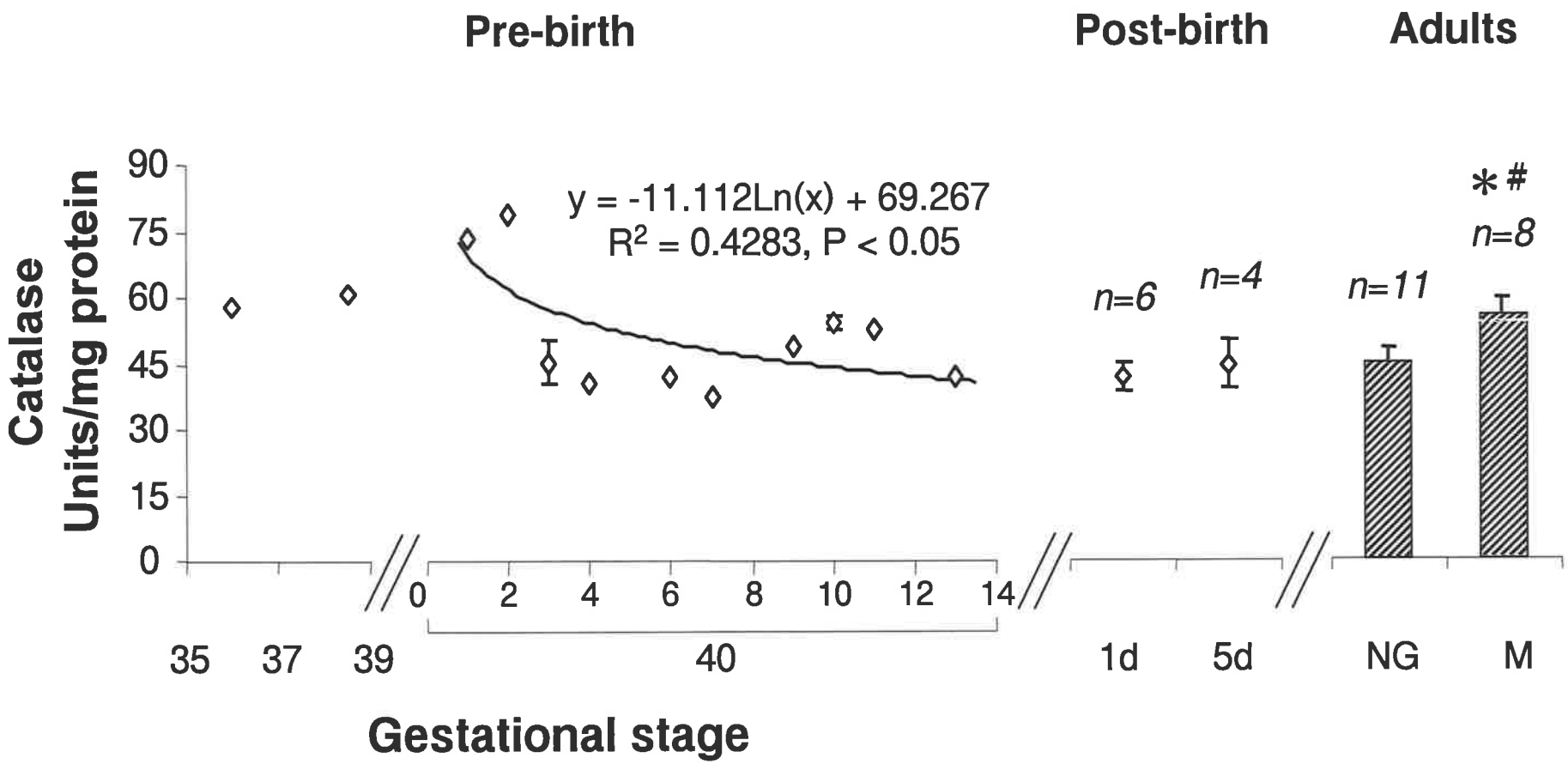

Pre-birth
Post-birth
Adults
Catalase
Units/mg protein
90
75
60
45
30
15
0
y = -11.112Ln(x) + 69.267
R2 = 0.4283, P < 0.05
n=6
n=4
n=11
n=8
*#
0
2
4
6
8
10
12
14
35
37
39
40
1d
5d
NG
M
Gestational stage

Figure 4.8 The antioxidant enzyme activity of total superoxide dismutase in the lungs of perinatal, maternal (M) and non-gravid (NG) skinks, *T. rugosa*, expressed in mg protein. All pre-birth individuals were classified between stage 36 (D&H) and stage 40 (D&H). Numbers 1-14 within stage 40 represent the rankings within this developmental stage. Data for pre-birth stages represent either individual data points or mean ± SEM of an n = 2-3. One-day-old (1d), five-day-old (5d) and adult data are expressed as mean ± SEM.

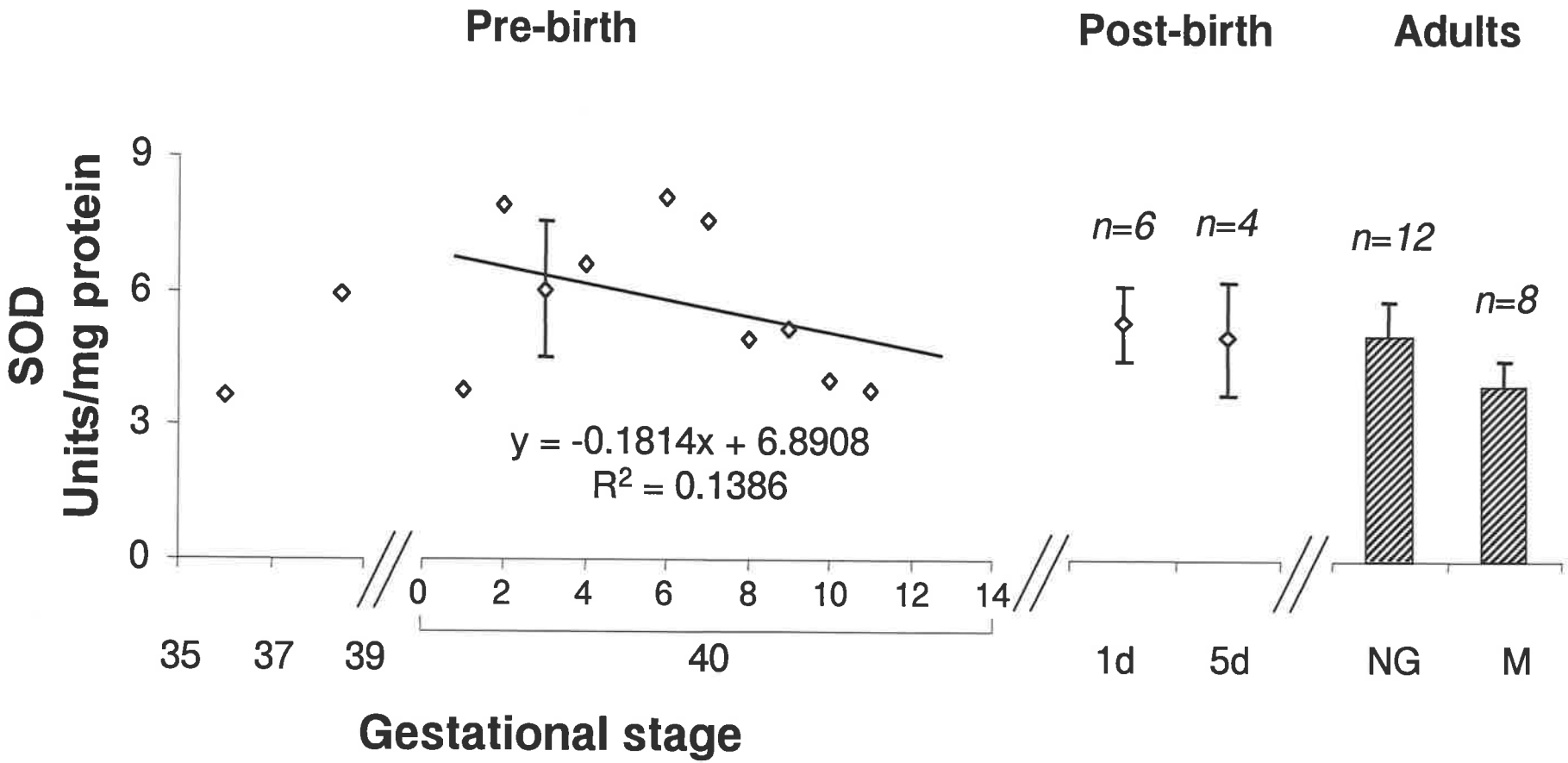
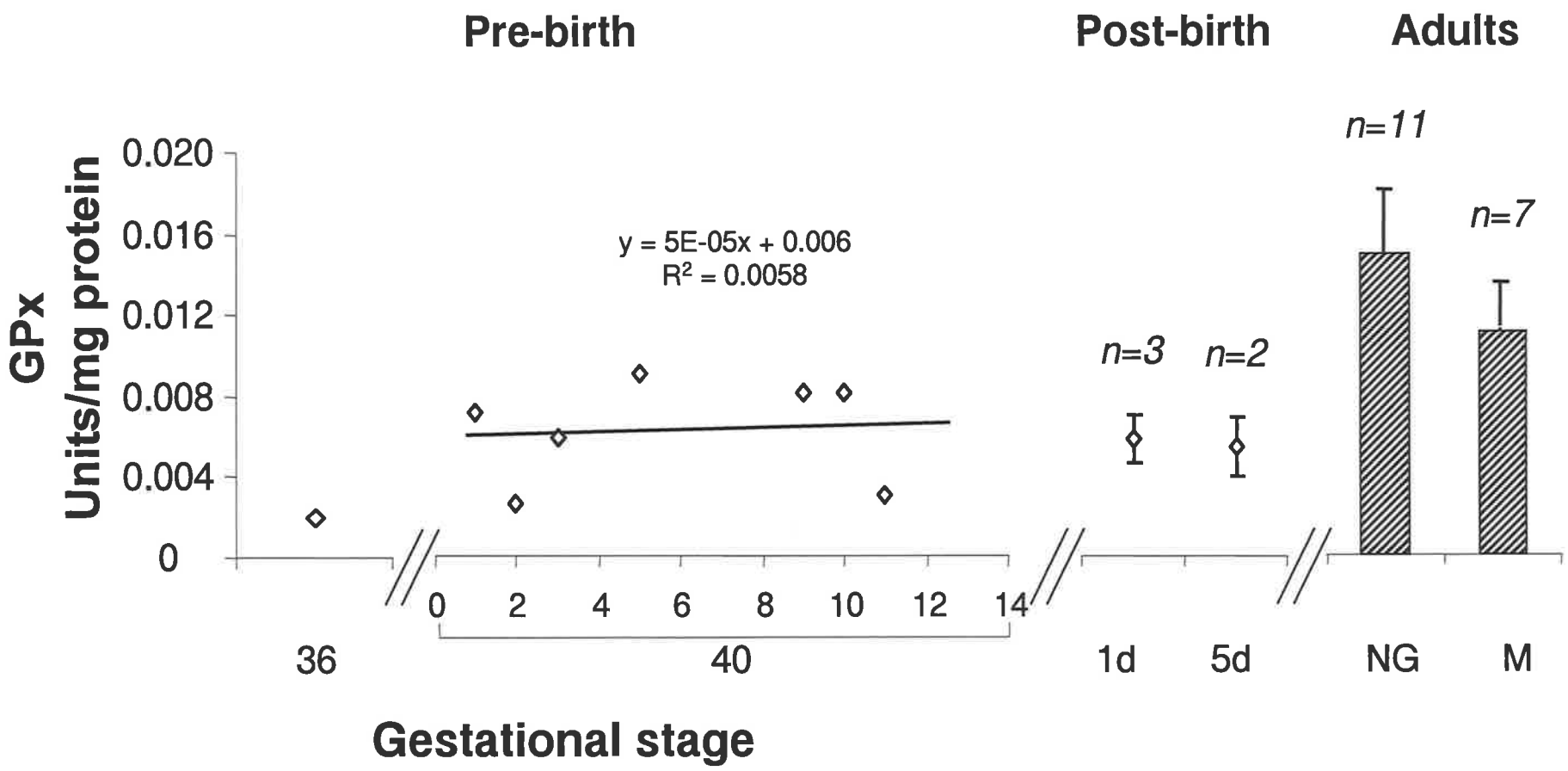

Pre-birth
Post-birth
Adults
SOD
Units/mg protein
9
6
3
0
y = -0.1814x + 6.8908
R² = 0.1386
n=6
n=4
n=12
n=8
0
2
4
6
8
10
12
14
35
37
39
40
1d
5d
NG
M
Gestational stage

Figure 4.9 The antioxidant enzyme activity of glutathione peroxidase in the lungs of perinatal, maternal (M) and non-gravid (NG) skinks, *T. rugosa*, expressed in mg protein. All pre-birth individuals were classified between stage 36 (D&H) and stage 40 (D&H). Numbers 1-14 within stage 40 represent the rankings within this developmental stage. Data for pre-birth stages represent either individual data points or mean ± SEM of an n = 2-3. One-day-old (1d), five-day-old (5d) and adult data are expressed as mean ± SEM.


Pre-birth
Post-birth
Adults
GPx
Units/mg protein
0.020
0.016
0.012
0.008
0.004
0
y = 5E-05x + 0.006
R² = 0.0058
n=3
n=2
n=11
n=7
0
2
4
6
8
10
12
14
36
40
1d
5d
NG
M
Gestational stage

surface-activity of the surface film (Fujiwara *et al.*, 1968; Benson *et al.*, 1983). Similarly, the amount of PL, DSP and cholesterol harvested from the viviparous lizard lung increased with advancing gestation. Since the dry lung weights also increased throughout development (Table 4.1), the elevation in the total amount of surfactant lipid present in the final stages of gestation was especially pronounced. Like mammals, the relative saturation of the phospholipids increased throughout development, so that newborns and stage 40-6 foetuses had comparable levels of saturation of their surfactant phospholipids.

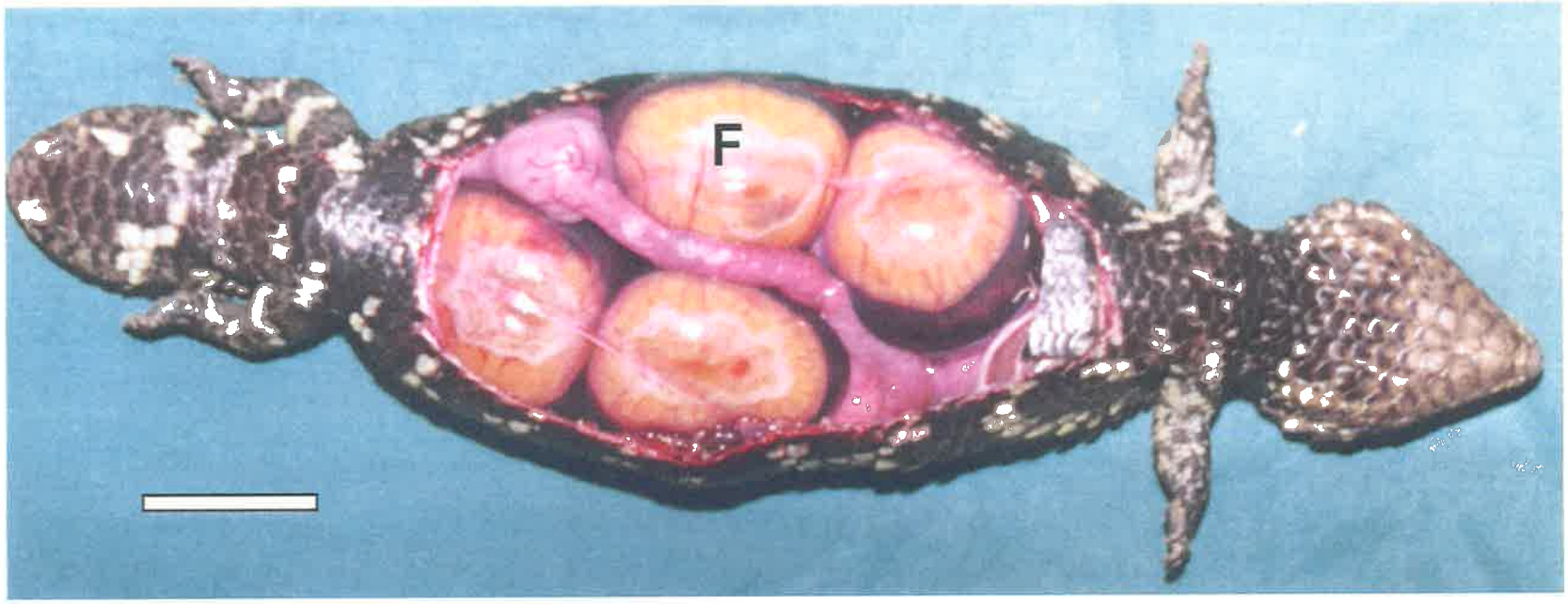
In mammals, the primary stimuli for secretion of surfactant at birth include elevated levels of circulating catecholamines (Marino & Rooney, 1981; Padbury *et al.*, 1982), lung expansion (Lawson *et al.*, 1979), prostaglandins (Marino & Rooney, 1980; Marino & Rooney, 1981) and stress (Rooney *et al.*, 1976a). In *T. rugosa*, secretion of the surfactant lipids was maximal immediately prior to birth, presumably resulting from sympathetic stimulation of the type II pneumocytes. Ventilation does not stimulate surfactant secretion in the isolated perfused lung of the bearded dragon (Wood *et al.*, 1995) and therefore is unlikely to be an important secretagogue in lizards such as *T. rugosa*. The lungs of this skink demonstrate numerous sympathetic ganglia along the major nerve bundles (Burnstock & Wood, 1967) and the postganglionic fibres are likely to directly release noradrenaline onto receptors of the type II cells.

Immediately prior to birth, the amount of phospholipid and the level of saturation of the PL within the lungs of foetal skinks surpass those of every other embryonic reptile studied (Chapters 3,5 and 6). Moreover, newborn and non-gravid lizards have respectively ~2- and ~1.5-fold more total PL than adult bearded dragons maintained at 37°C (Wood *et al.*, 1995). As the amount of phospholipid recovered from reptilian lungs increases with increasing temperature (Lau & Keough, 1981; Wood *et al.*, 1995) and these skinks were maintained at 22-24°C, values in this study could underestimate the content of PL in animals at 37°C. The reason for such heightened levels of surfactant lipids in the foetal

and adult lung is unknown. Perhaps elevated levels of surfactant lipids may result from the accumulation of highly viscous lung fluid in the developing lung, trapping the lipids and retarding the rate of lipid turnover. Additionally, elevated surfactant lipids may be required for liquid-liquid interactions between the lung and amniotic fluid with differing viscosities.

The amount of surfactant lipid in maternal lizards was lower than that in non-gravid and newborn animals, yet the DSP/PL, Chol/PL and Chol/DSP ratios did not differ between adults and newborns. Skinks lack a diaphragm and their simple unicameral lungs traverse almost the entire length of the peritoneal cavity. During gestation, the developing foetuses occupy most of the peritoneal cavity of the mother, placing substantial pressure on the viscera and hence, the lungs (Figure 4.10). It is possible, therefore that such compression forces prevent the lungs from being completely inflated and, as a result, the surface film required to permit lung function may be considerably reduced. Thus, the amount of surfactant required to form a monolayer would be decreased without modifying the composition. Indeed, when scanned using computerised tomography, the ventral surface of the lungs of the adult bearded dragon is greatly compressed and distorted by the gut, potentially causing respiratory epithelia to come into contact (Daniels *et al.*, 1994a). When the lizard lung is under compression by the abdominal contents, pulmonary surfactant is believed to function as an anti-adherent (Daniels *et al.*, 1989; Daniels *et al.*, 1990; Daniels *et al.*, 1994a), a function, which may be of great importance in the gravid animal. However, it is also possible that the reduction in the amount of lavageable surfactant results from diminished rate of secretion. It is likely that the reduced activity of the gravid lizard influences the secretion of phospholipid as exercise elevates secretion of total PL in adult bearded dragons (Wood *et al.*, 1997). Alternatively, it is possible that a greater proportion of lipid is utilised by the developing embryos, potentially depleting maternal lipid stores or the precursors for surfactant biosynthesis.

Figure 4.10 Dissection of gravid sleepy lizard, demonstrating four large foetuses (F) beneath their yolksacs. Note that the developing young occupy the entire peritoneal cavity, which may hinder digestion and breathing in the adult. Bar = 4cm.


F

4.4.1.2 Cholesterol

In mammalian surfactant, cholesterol is the second most abundant lipid, following DPPC (Yu *et al.*, 1983). All the functions of Chol in surfactant are not clearly defined. However, it facilitates spreading of the monolayer at the air/liquid interface and improves fluidity of surfactant lipids (Notter *et al.*, 1980a; Fleming & Keough, 1988). In heterothermic animals, cholesterol is a dynamic component of pulmonary surfactant, possibly maintaining homeoviscosity of the surface film during fluctuations in body temperature (Daniels *et al.*, 1990; Langman *et al.*, 1996). During the final stages of gestation, the cholesterol content increased in tandem with the phospholipids. Previously it has been shown that the content of cholesterol remains unaltered during development of non-mammals despite increases (Chapters 2 and 3) or decreases (Orgeig *et al.*, 1994) in the content of the phospholipids. Moreover, unlike the phospholipids, cholesterol secretion is unaffected by sympathetic and parasympathetic agonists in the isolated perfused lung and cultured type II cells from adult bearded dragons (Wood *et al.*, 1997; Wood *et al.*, 1999). It appears, therefore that the mechanisms for cholesterol regulation differ from those regulating the phospholipids among the vertebrates. Despite increases in the content of cholesterol throughout development of the viviparous skink, the Chol/PL and Chol/DSP declined demonstrating that relatively more cholesterol is present in early stage 40 foetuses than either total PL or DSP. This suggests different rates of secretion, or possibly turnover, further exemplifying differences in control of metabolism among the phospholipids and this neutral lipid. The amounts of cholesterol and the Chol/PL ratio of the material lavaged from the lung of adult *T. rugosa* are comparable to other reptilian species (Daniels *et al.*, 1996).

Unlike surfactant from oviparous vertebrates (Chapters 2, 3, 5 and 6), *T. rugosa* appears to have a compositionally "mature" surfactant in the latter stages of foetal life, well in advance of the initiation of airbreathing. The DSP/PL, Chol/PL and Chol/DSP ratios at

stage 40-6 resembled those of newborns and adults, suggesting that the surfactant system would be capable of functioning at this time. Nevertheless, this developmental stage arises substantially earlier than the initiation of birth and airbreathing.

The early completion of the development of the surfactant system may be attributed to a number of factors. The young of this species are born in a highly advanced state. The foetuses of *T. rugosa* appear to be physically complete several weeks prior to parturition. By extending the gestation period, the offspring are able to grow larger and are born in a highly precocial state, which may reduce predation, provide greater locomotory skills and stamina and, therefore, lead to improved survivorship. Furthermore, in many viviparous species, gravid females that are near term may respond to trauma or stress by spontaneously releasing young (Lombardi, 1998). Indeed, the timing of parturition in *T. rugosa* may be advanced or delayed in direct response to adverse environmental conditions (Fergusson & Bradshaw, 1991). Consequently, those young capable of surviving a premature birth, have an obvious advantage.

4.4.2 Additional Measures

4.4.2.1 Development of the Antioxidant Enzymes

Contrary to the surfactant lipids, antioxidant enzyme activity did not change appreciably throughout development in the lung of *T. rugosa*. In mammals, both the AOEs and surfactant lipids display similar temporal patterns of development (Frank & Groseclose, 1984; Frank & Sosenko, 1987; McElroy *et al.*, 1990; Walther *et al.*, 1991b). Generally, the activity of mammalian AOEs increases with advancing gestation. In mammals, oxygen consumption, and therefore metabolism, increase throughout development, which is accompanied by an elevation in the production of oxygen free radicals (Frank *et al.*, 1996). The mammalian foetus responds to elevations in oxygen radicals by up-regulating its

antioxidant enzyme activity (Frank *et al.*, 1996). However, reptiles have a metabolic rate of approximately 10% that of similar sized mammals. While oxygen consumption increases throughout development in reptiles (Thompson, 1989; Thompson & Stewart, 1997; Booth, 1998; Booth *et al.*, 2000), it is possible that the lower metabolic rate of *T. rugosa* may not be sufficient to increase antioxidant enzyme activity.

To date, only one other study has focussed on the development of the AOEs in a reptile; the oviparous bearded dragon, *Pogona vitticeps* (Starrs *et al.*, Submitted). In bearded dragons, SOD activity declines prior to hatching (Starrs *et al.*, Submitted). Similarly, in the lung of *T. rugosa*, SOD activity was lowest immediately before birth. GPx activity did not change throughout development in the viviparous skinks. In the bearded dragon, GPx activity was unaltered during development, but adults possessed 3-fold greater activity than hatchlings (Starrs *et al.*, Submitted). For both dragons and *T. rugosa*, GPx may not be of prime importance against hydrogen peroxide damage in the foetus (Starrs *et al.*, Submitted). Unlike bearded dragons however, catalase activity decreased in foetal *T. rugosa* and progressively increased after birth, such that maternal lizards had significantly higher catalase activity than non-gravid and postnatal lizards. It is possible, therefore, that the foetal lung of *T. rugosa* does not require the same degree of protection from hydrogen peroxide damage as adult animals. Furthermore, as catalase activity in the foetus does not deviate from postnatal and non-gravid adult activity levels, it may have a greater protective role in the scincid foetus than that of GPx. It appears, therefore that, despite differing birth strategies, the two species of lizards exhibit similar developmental patterns for both SOD and GPx, but differ in their catalase activity. It is unclear why the levels of catalase decrease during development in *T. rugosa*, especially as the perfusion of the lungs increases during this time.

The activity of SOD, catalase and GPx in foetal *T. rugosa* was comparable to those of adult lizards, suggesting that foetal lungs were prepared for oxygen tensions

experienced after birth. This, coupled with the precocial nature of the young and the accelerated maturity of the surfactant lipids, suggests that foetal skinks are prepared for airbreathing some weeks prior to birth, supporting the readiness for premature birth.

4.4.2.2 Developmental Patterns of Surfactant Lipids and Antioxidant Enzymes

This is the first study to describe the simultaneous development of the surfactant lipids and antioxidant enzyme activities in a viviparous lizard. In many mammalian species, the development of the pulmonary surfactant system closely matches that of the AOE system (Frank & Groseclose, 1984; Frank & Sosenko, 1987; McElroy *et al.*, 1990; Walther *et al.*, 1991b). In *T. rugosa*, the developmental pattern of the surfactant lipids contrasts from that of the antioxidant enzymes. The surfactant lipids increase in late gestation, within the prolonged stage 40 period, whereas the AOE system is in place and active prior to this developmental stage. The surfactant and AOE systems therefore are developing independently of each other, maturing at different times and rates throughout development within the type II pneumocytes.

In mammals, there is strong evidence that the two systems are functionally linked as, both are synthesised in a common cell type (Forman & Fisher, 1981; Possmayer, 1997), both display chronologically similar developmental patterns (Frank & Groseclose, 1984; Frank & Sosenko, 1987; McElroy *et al.*, 1990; Walther *et al.*, 1991b) and both share similar control mechanisms (Frank *et al.*, 1985; Sosenko *et al.*, 1986; Ramadurai *et al.*, 1998). For example, administration of glucocorticoids accelerates both the appearance of pulmonary surfactant and the activity of AOE activities in the developing rat lung (Frank *et al.*, 1985). Similarly, metyrapone, an adrenal steroid inhibitor, delays the maturation of both systems (Sosenko *et al.*, 1986). Furthermore, the AOE have been shown to protect the unsaturated phospholipids from lipid peroxidation (Gilliard *et al.*, 1994). Whether the highly unsaturated surfactant lipids of the lizard are in fact protected by the antioxidant

enzymes is not known. Nevertheless, the relatively earlier development of the AOE system in *T. rugosa* may be essential for the highly unsaturated lipid mixture of this species. However, the possibility that other antioxidants are important in the reptilian lung cannot be discounted. Differing concentrations of other antioxidants such as vitamin E and C and oxidant-susceptible polyunsaturated fatty acids may be of equal importance in determining tolerance to oxidative injury (Gerdin *et al.*, 1985). Furthermore, recent evidence suggests that the hydrophilic surfactant proteins, SP-A and SP-D, themselves protect the lung from oxidative attack (Bridges *et al.*, 2000). Interestingly, SP-A and its messenger RNA have been found in the lung of adult *T. rugosa* (Sullivan *et al.*, 1998). It is probable, therefore, that both the proteinaceous antioxidants and the surfactant proteins protect both mammalian and reptilian lung tissue and surfactant lipids from oxidative damage. Hence, while both the AOE (Perez-Campo *et al.*, 1993) and surfactant systems (Daniels *et al.*, 1995a; Sullivan *et al.*, 1998) appear to be highly conserved among the vertebrates, their developmental patterns differ markedly between reptiles and mammals.

4.4.3 Conclusions

Like mammals, the content and saturation phospholipids increase in lung washings from foetal lizards with advancing gestation. Similarly, the relative saturation of the phospholipids increases throughout gestation, suggesting that the developmental pattern of the surfactant lipids is conserved between viviparous lizards and mammals. Unlike mammals, the composition of surfactant from foetal lizards was equivalent to newborns and adults, indicating that the composition of surfactant was complete some weeks prior to parturition, probably to enable improved survivorship of the precocial young in the event of premature birth. Unlike surfactant cholesterol from other non-mammals, cholesterol from *T. rugosa* increased in tandem with the phospholipids, however the Chol/PL and Chol/DSP ratio declined throughout development, indicating that cholesterol and

phospholipids have differing regulatory mechanisms. Despite no differences in the composition of surfactant, maternal lizards have less lavageable surfactant than non-gravid and newborn lizards, presumably due to compression of the lungs under the bulk of the developing foetus. Unlike the surfactant lipids, the AOEs, catalase, superoxide dismutase and glutathione peroxidase, did not differ appreciably throughout gestation. It appears therefore that, like the surfactant lipids, the antioxidant enzyme system is in readiness for airbreathing throughout the latter stages of gestation, possibly in preparation for premature birth. In contrast to those of mammals, the pulmonary surfactant system and the antioxidant enzyme system develop independently from one another.

4.4.4 Future Directions

From the present study, it appears that both the antioxidant enzyme system and surfactant system are prepared for pulmonary ventilation some weeks prior to birth. It would be of interest to deliver foetuses prematurely and assess their ability to breathe in order to verify this prediction.

To confirm the prediction that the lungs of maternal lizards are partially collapsed or compromised by the developing foetuses during late pregnancy, the lungs of gravid females could be viewed using computerised tomography. This, coupled with metabolic measurements would provide insight into the effect of compression on the mechanics of breathing and surfactant composition and secretion in these animals. I would expect that females would experience prolonged periods of apnoea, interspersed with single large breaths, or a series of shallow breaths. Such a study may be of equal importance in oviparous species, which also experience substantial compression of the abdominal organs prior to oviposition. Additional variables such as exercise and temperature may have profound affects on system already under extreme physiological stress.

The integument of *T. rugosa* is extensively ossified. It is possible that the exceptionally large offspring of this species, coupled with the heightened ossification of the scales limits the movement of the rib cage thereby increases compression of the lungs. Therefore, testing the above prediction on other viviparous lizards, without highly specialised integument may explain whether this phenomenon is a result of the specific adaptations of this particular species or is common to viviparous lizards.

CHAPTER 5 DEVELOPMENT AND REGULATION OF THE PULMONARY SURFACTANT SYSTEM IN THE FRESHWATER TURTLE, *CHELYDRA SERPENTINA*

5.1 INTRODUCTION

Reptilian pulmonary surfactant prevents adherence of apposing respiratory epithelia (Daniels *et al.*, 1996), regulates fluid balance (Orgeig *et al.*, 1997), may offer resistance to evaporative water loss with decreasing temperature (Meban, 1981) and may have important roles in innate lung defense in the chelonian lung (Sullivan *et al.*, 1998). Despite extensive research on the function of surfactant in turtles (Meban, 1980; Lau & Keough, 1981; Meban, 1981; Daniels *et al.*, 1995b; Daniels *et al.*, 1996; Orgeig *et al.*, 1997) the development of the system has never been described. In contrast, the development of the pulmonary surfactant system has been well documented in eutherians. Both endogenous and exogenous corticosteroids induce synthesis of pulmonary surfactant in the mammalian foetal lung by stimulating enzymes involved in phospholipid synthesis, accelerating lamellar body development and influencing intracellular and extracellular concentrations of surfactant proteins (Merrill & Ballard, 1998). Unlike viviparous species, oviparous species, such as the snapping turtle, *Chelydra serpentina*, lack dynamic maternal-foetal transfer of nutrients and steroid hormones throughout development. Moreover, hatching is a much lengthier process than that of birth. Therefore, the development and regulation of the surfactant system in the snapping turtle may differ substantially from those of mammals.

Previously, lamellar bodies and an osmiophilic surface film have been observed in the adult turtle lung (Meban, 1977b; Solomon & Purton, 1984; Pastor *et al.*, 1989). Since SP-B is instrumental in the generation of lamellar bodies in mammals (Clark *et al.*, 1995) and SP-A (Sullivan *et al.*, 1998; Zeng *et al.*, 1998), SP-B (Zeng *et al.*, 1998; Miller *et al.*,

2001) and SP-D (Miller *et al.*, 2001) are highly conserved within the vertebrates, it is predicted that SP-B will also be present in the turtle lung.

Transcription of SP-A, SP-B and SP-C genes is regulated by several transcription proteins, including thyroid transcription factor 1 (TTF-1), which binds to the DNA within the promoter region of the target gene (Bohinski *et al.*, 1994; Bruno *et al.*, 1995; Kelly *et al.*, 1996). TTF-1 activates SP-B gene expression by binding to several cis-acting elements in the 5'-flanking region of the gene (Bohinski *et al.*, 1994). TTF-1 is important for lung morphogenesis (Lazzaro *et al.*, 1991; Minoo *et al.*, 1995) and respiratory epithelial cell differentiation (Bohinski *et al.*, 1994) during lung development and injury. Immunohistochemical studies have shown that TTF-1 is expressed in the nuclei of respiratory epithelial cells in the early stages of lung formation in both mammals (Stahlman *et al.*, 1996; Zhou *et al.*, 1996) and birds (Zeng *et al.*, 1998) and is subsequently restricted to peripheral respiratory units throughout development (Zhou *et al.*, 1996; Zeng *et al.*, 1998), whereas staining for the SP-B peptide occurs later in development, being confined to the cytoplasm of type II cells in mammals (Khoor *et al.*, 1994; Zhou *et al.*, 1996) and epithelia of the parabronchi in birds (Zeng *et al.*, 1998). To date, the factors regulating lung development in reptiles have not been described. As a result, the present chapter describes the development of the surfactant lipids and the temporal-spatial location of TTF-1 and SP-B throughout the latter part of incubation in the developing multicameral lung of the snapping turtle.

5.2 Materials and Methods

5.2.1 Collection and Incubation of Eggs

Six clutches of snapping turtle eggs were collected from nests in the Valentine National Wildlife Refuge, Cherry County, Nebraska, USA in mid June 1998. Nests less than 12 hours old were carefully excavated and eggs were placed in coolers containing damp moss and transported to Colorado State University for incubation. Individual clutches were divided in half and transferred to plastic boxes containing fine vermiculite (ca. 5 mm particles) with a gravimetric water content of 1 g/g dry mass, resulting in a water potential of -200 kPa or wetter (Packard *et al.*, 1987). Containers were weighed after the eggs were deposited, then sealed and placed in two constant temperature cabinets at 26°C, producing a male cohort (Yntema, 1981). Boxes were rotated throughout the cabinets daily to minimise exposure to fluctuations in temperature within the cabinets. Containers were weighed weekly and distilled water was added to the vermiculite until the initial mass of the container was reached.

5.2.2 Tissue Sampling

Eggs were sampled after 56 and 61 days of incubation, the time of pipping, which was 64.5 ± 0.4 days of incubation (mean ± SEM), and following hatching, 66 ± 0.5 days of incubation (mean ± SEM). Embryos and hatchlings were sacrificed by intraperitoneal injection of lidocaine hydrochloride (150 mg/kg [BM] ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA).

5.2.3 Embryonic Staging

In addition to days of incubation, embryos were staged according to the criteria of Yntema (1968). Embryos from day-56 corresponded to stage 24, embryos from day-61 corresponded to stage 25, embryos that had pipped corresponded to stages 25 and 26 and hatchlings corresponded to stage 26 (Yntema, 1968).

5.2.4 Lavage Protocol

Turtles were cannulated via the trachea with a 26G needle and the lungs were lavaged with three volumes of chilled isotonic saline (0.15M NaCl) which was injected into, and withdrawn from the lungs three times per volume. Volumes ranging from 0.2 ml to 0.5 ml of a 0.5-ml reservoir were injected into the lungs as embryos increased in mass, resulting in an increased lavage volume of 0.05 to 0.13 ml/g BM. The lavage was centrifuged (Beckman model TJ-6 centrifuge) at 150 *g* for 5 min at 4°C to remove cellular debris. The supernatant was snap frozen in liquid nitrogen and freeze-dried. Lyophilised lavage was stored at -80°C for further analyses. Lavaged lungs were blotted dry on paper towel, snap frozen, freeze-dried and weighed.

5.2.5 Lipid Analyses

Lyophilised lavage was reconstituted in 2 ml of demonised (Millipore) water and lipids were extracted using chloroform:methanol (1:2 vol/vol) (Bligh & Dyer, 1959) (Appendix 2, Section 1.1). The lavage volume from embryos at day-56 of incubation was extremely small. Therefore, lavage from two siblings was pooled. Individual lavage was maintained for all other sampling times. Each sampling point contained equivalent numbers of embryos from each clutch. Total phospholipid, disaturated phospholipid and cholesterol

were extracted and quantified from lavage in the same manner as described for the chicken (Section 2.2.5).

5.2.6 Additional Measures

5.2.6.1 Antibodies

The rabbit polyclonal antibody, generated against a synthetic peptide of rat TTF-1, spanning amino acid residues 110-122 was provided by Dr Roberto Di Lauro (Stazione Zoologica 'Anton Dohrn', Naples, Italy), whereas the rabbit polyclonal antibody raised against the mature SP-B peptide isolated from bovine lung (R28031) was generated in the laboratory of Dr J.A. Whitsett (Children's Hospital Medical Center, Cincinnati, USA). The TTF-1 antibody was used at a dilution of 1:1000, while the mature SP-B antibody was used at a dilution of 1:2000. Specificity of the TTF-1 and SP-B antibodies has been previously established (Clark *et al.*, 1995; Zhou *et al.*, 1996).

5.2.6.2 Immunohistochemistry

Embryos and hatchlings were sacrificed by intraperitoneal injection of lidocaine hydrochloride (150 mg/kg body mass [BM] ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA). Lungs were excised from turtle embryos after 61 days of incubation, at the time of pipping and after hatching. Lungs were fixed in neutral buffered 4% paraformaldehyde for 20 h at 4°C. The tissues were dehydrated in a graded series of ethanol and embedded in paraffin. Five μm transverse sections of the thoracic cage of mouse embryos from 18.5 days of gestation and 5 μm sagittal sections of turtle lung were placed on polylysine-coated slides. Antigen retrieval was performed to enhance immunohistochemical staining for TTF-1 by unmasking antigens. Sections were dewaxed and microwaved in 0.01 M citrate

buffer (0.1 M citric acid, 0.1 M sodium citrate, pH 6.0) for 7.5 min on the high power setting to boil, then microwaved at 60% power for a further 15 min to simmer using a conventional 900 watt microwave oven (Gown *et al.*, 1991).

All sections, including those for SP-B staining, were placed in 0.5% hydrogen peroxide for 15 min to remove endogenous peroxidase activity, blocked with 2% normal goat serum at room temperature for 2 h, then incubated overnight at 4°C with primary antibody and developed using a Vector Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Following incubation with secondary antibodies, localisation of antigens was developed using nickel-diaminobenzidine, followed by incubation with Tris-cobalt and counterstained with Nuclear Fast Red. The absence of immunostaining in slides incubated without the primary antibody was used as a negative control, whereas the foetal mouse lung was used as a positive control for every immunostaining. Slides were viewed and photographed on Kodak 64T tungsten film. Visualisation and interpretation was carried out in the same manner as Zhou *et al.* (1996) and Zeng *et al.* (1998).

5.2.7 Statistical Analyses

Lipid data were expressed and analysed in the same manner as chicken data (Section 2.2.7). Statistical significance was set at $P < 0.05$.

5.3 Results

5.3.1 Physical Parameters

Dry lung weight (DLW) and body mass did not differ on day-56 (DLW: $P = 0.062$; BM: $P = 0.71$) nor on day-61 (DLW: $P = 0.12$; BM: $P = 0.23$) from embryos incubated in the different temperature cabinets. Similarly, there were no differences between the time of

pipping (P = 0.69) and the time of hatching (P = 0.49) across temperature cabinets. Therefore, temperature cabinet effects were dismissed and data were combined.

Body masses increased significantly between embryos incubated for 56 and 61 days ($P < 10^{-6}$) with an additional increase in turtles that had pipped ($P < 0.01$) and hatched ($P < 0.05$) (Table 5.1). Dry lung weights did not differ among sampling groups (ANOVA, P = 0.11) (Table 5.1). When represented as a percentage of body mass, the dry lung weights declined between embryos incubated for 56 and 61 days of incubation ($P < 10^{-5}$), with a further decrease in embryos that had pipped ($P < 0.001$) and no difference in hatchlings (P = 0.16) (Table 5.1). Escalating body masses produced the decreases in the percentage of dry lung weights.

Table 5.1 *Body mass and dry lung weight of embryonic and hatchling snapping turtles.*

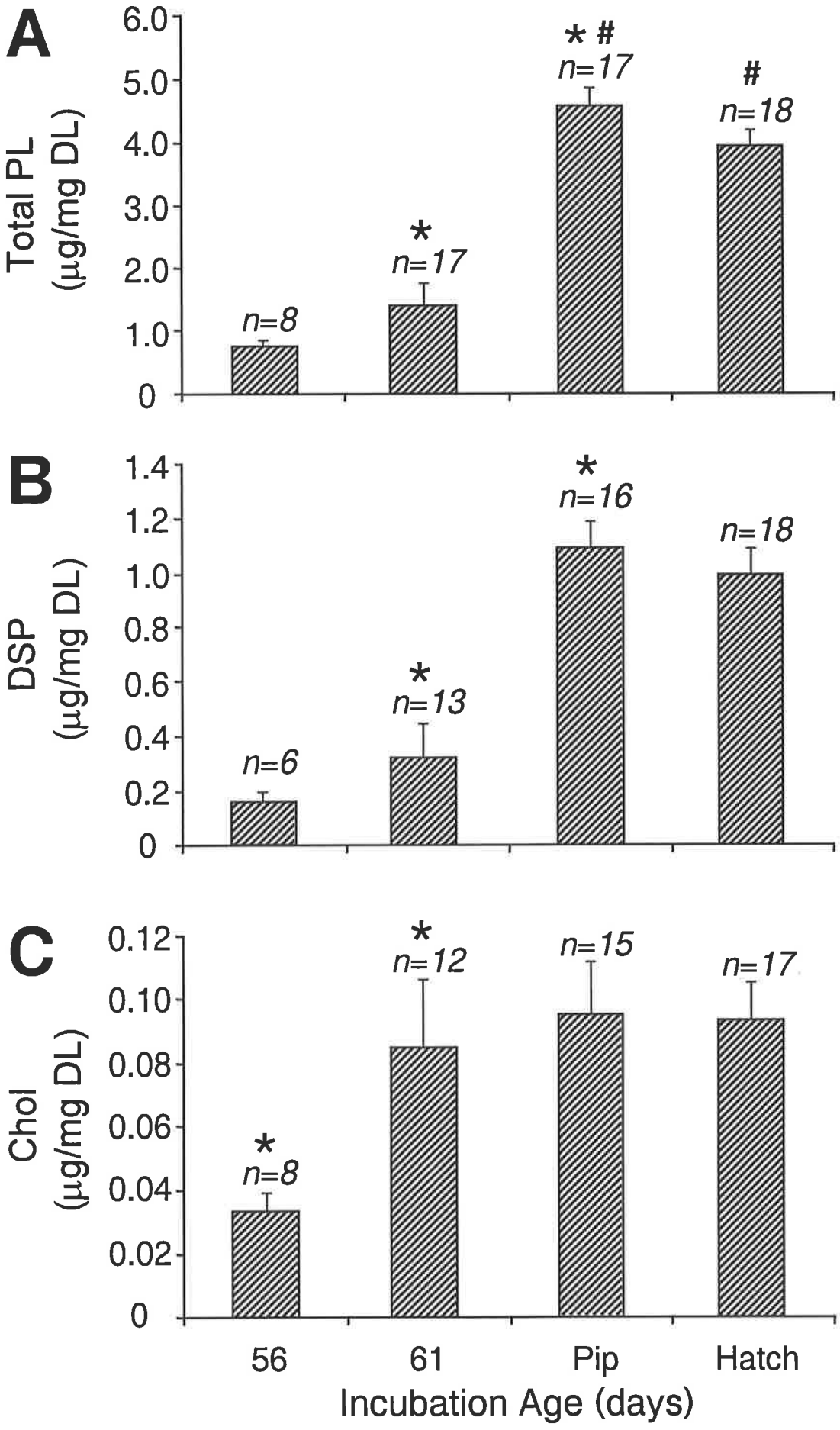
Age (days)	BM (g)	DLW (mg)	DLW/BM (%)
Day-56	6.01 ± 0.18 (18)	5.64 ± 0.17 (16)	0.094 ± 0.002 (16)
Day-61	7.20 ± 0.14 (17)	5.57 ± 0.16 (17)	0.078 ± 0.002 (17)
Pip	7.92 ± 0.19 (18)	5.21 ± 0.15 (18)	0.066 ± 0.002 (18)
Hatch	8.30 ± 0.11 (16)	5.75 ± 0.16 (16)	0.069 ± 0.002 (16)

Results are mean ± SEM; *n* in parentheses; DLW, dry lung weight; BM, body mass.

5.3.2 Development of the Pulmonary Surfactant Lipids

Total PL, expressed per mg dry lung (DL) did not differ between day-56 and day-61, yet significantly increased after pipping ($P < 10^{-8}$) and then decreased marginally following hatching ($P < 0.05$) (Figure 5.1-A). Coincident with the onset of airbreathing, DSP

Figure 5.1 The absolute amounts of phospholipids and cholesterol in pulmonary surfactant from the developing lung of the snapping turtle, demonstrating total PL (**A**), DSP (**B**) and Chol (**C**) per mg of dry lung (DL). Data expressed as mean ± SEM. Paired symbols indicate significant difference between adjacent groups. **A**: * $P = 7.6 \times 10^{-8}$, # P = 0.05, **B**: * $P = 1.5 \times 10^{-5}$; **C**: * P = 0.033.


A
Total PL
(µg/mg DL)
6.0
5.0
4.0
3.0
2.0
1.0
0
n=8
*
n=17
* #
n=17
#
n=18
B
DSP
(µg/mg DL)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
n=6
*
n=13
*
n=16
n=18
C
Chol
(µg/mg DL)
0.12
0.10
0.08
0.06
0.04
0.02
0
*
n=8
*
n=12
n=15
n=17
56
61
Pip
Hatch
Incubation Age (days)

increased ~3.5-fold from day-61 to pipping ($P < 10^{-4}$) and did not change thereafter ($P = 0.22$) (Figure 5.1-B), whereas Chol increased markedly between embryos incubated for 56 days and 61 days ($P < 0.05$), prior to the initiation of pulmonary ventilation and remained constant following pipping and hatching (Figure 5.1-C).

The percentage of DSP relative to total PL was comparable between pre-pipped animals (day-56 vs. day-61, $P = 0.42$) (Figure 5.2-A). However, following pipping, the DSP/PL ratio increased ~1.5-fold ($P < 0.05$) from day-61 of incubation and did not differ after hatching ($P = 0.46$) (Figure 5.2-A). Both Chol/PL and Chol/DSP were elevated prior to pipping, then significantly declined after the initiation of airbreathing ($P < 0.001$ for both tests), remaining unchanged in hatchlings ($P = 0.32$) (Figure 5.2-B,C).

5.3.3 Additional Measures

5.3.3.1 Detection of TTF-1 and SP-B

Positive staining of the TTF-1 antibody occurred in lung tissue of the snapping turtle at all stages sampled (Figure 5.3). TTF-1 stained the nuclei of trabecular and respiratory epithelial cells in day-61, pipped and hatchling turtle lung (Figure 5.3). The degree of immunostaining was lower in animals that had pipped than day-61 embryos (Figure 5.3-B), with a further reduction in the staining intensity and the number of cells expressing TTF-1 in the hatchling lung (Figure 5.3-C). The mature bovine SP-B antibody positively stained turtle lung tissue at all sampling points (Figure 5.4). In all cases, immunostaining was confined to subsets of cells within the gas exchange area (Figure 5.4), coinciding with cells that expressed TTF-1. Staining intesified after hatching (Figure 5.4-C).

Figure 5.2 The relationship between phospholipids and cholesterol in pulmonary surfactant from the developing lung of the snapping turtle, demonstrating DSP expressed as a percentage of total PL (**A**), the Chol/PL ratio (**B**) and the Chol/DSP ratio (**C**). Data expressed as mean ± SEM. Paired symbols indicate significant difference between adjacent groups. **A**: * $P = 0.019$, **B**: * $P = 2.6 \times 10^{-4}$, **C**: * $P = 5.7 \times 10^{-4}$.

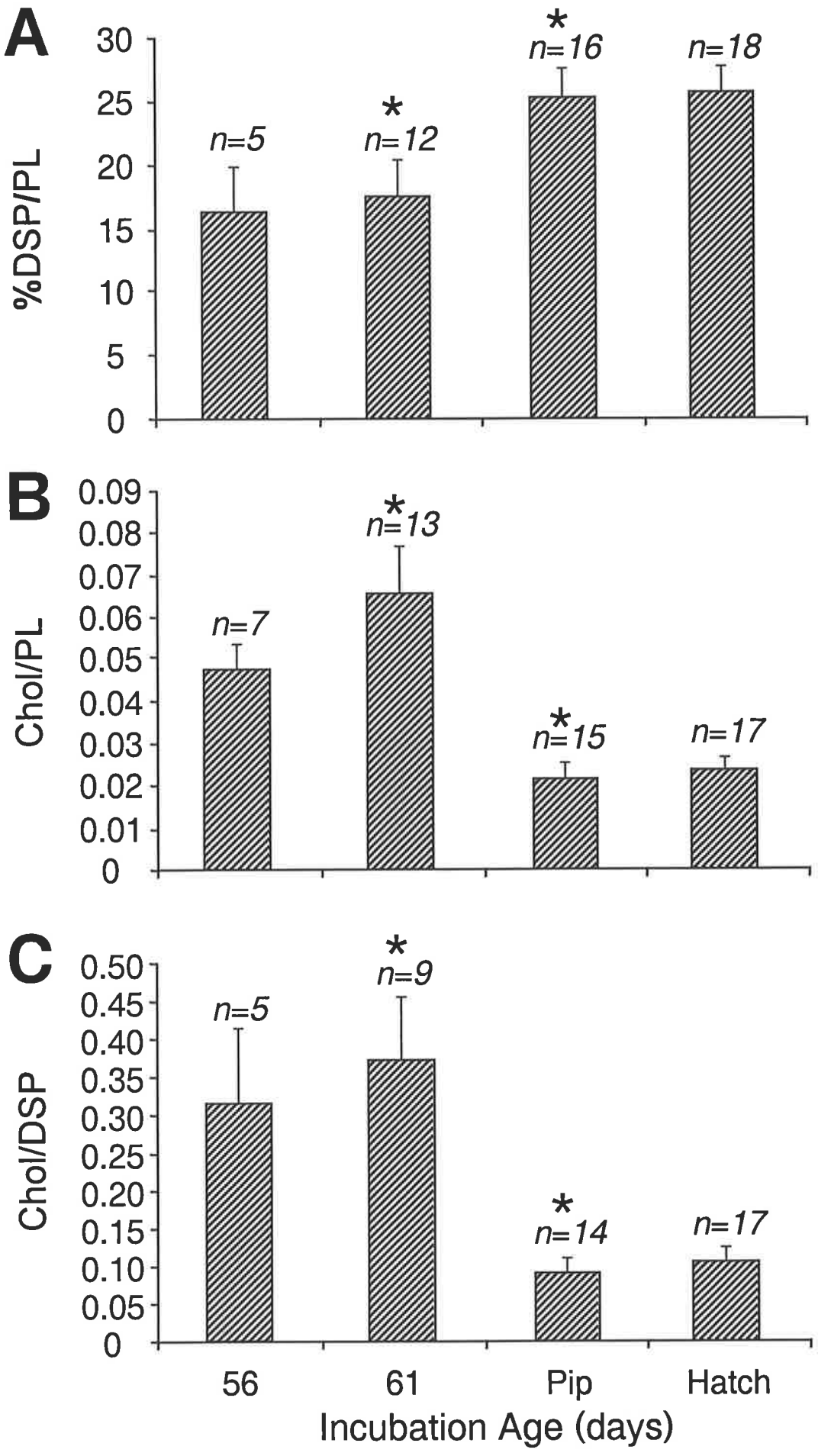

A
%DSP/PL
30
25
20
15
10
5
0
n=5
*
n=12
*
n=16
n=18
B
Chol/PL
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
n=7
*
n=13
*
n=15
n=17
C
Chol/DSP
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
n=5
*
n=9
*
n=14
n=17
56
61
Pip
Hatch
Incubation Age (days)

Figure 5.3 Immunohistochemical staining of TTF-1 in the developing lung of the snapping turtle. TTF-1 was detected in the nuclei of epithelial cells of the trabeculae and gas exchange area (➡) with the greatest prevalence and intensity of staining at day-61 of incubation (**A**), at lower levels after pipping (**B**) and the lowest prevalence and intensity after hatching (**C**). Bar = 50 μm.

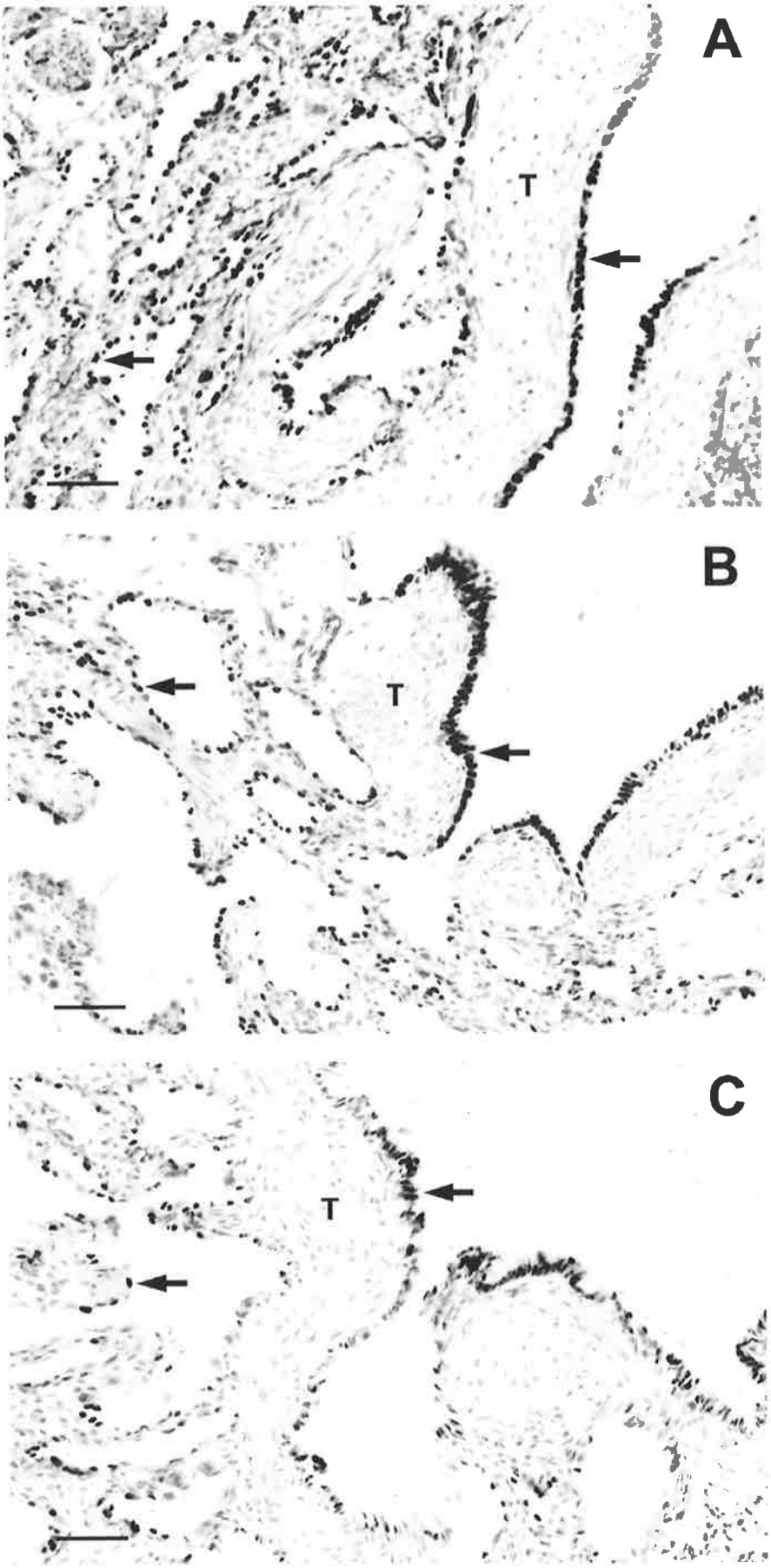
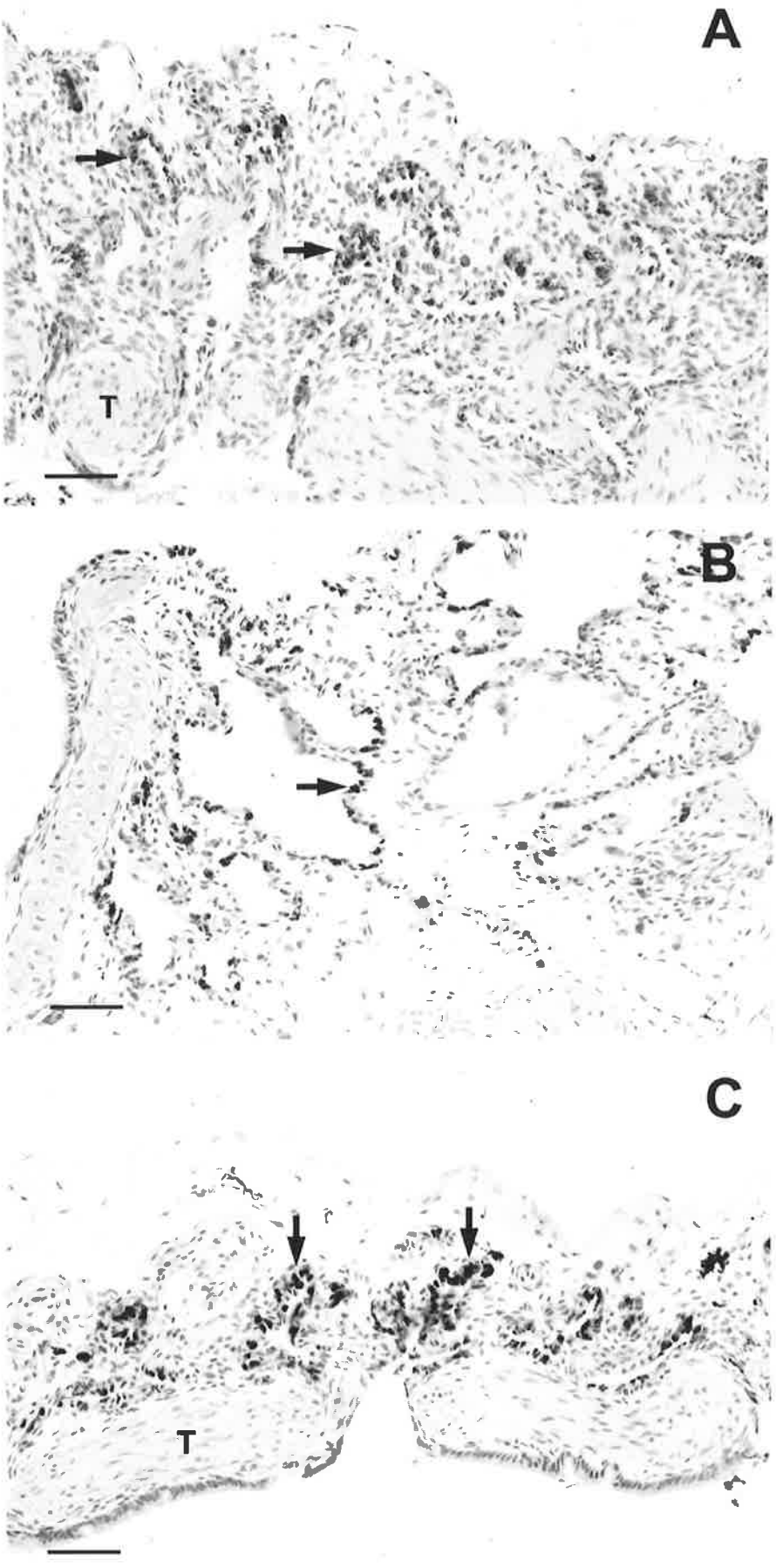

A
T
B
T
C
T

Figure 5.4 Immunohistochemical staining of mature SP-B in the developing lung of the snapping turtle. SP-B was detected in the cytoplasm of subsets of epithelial cells in the gas exchange area (➡) at day-61 of incubation (**A**), after pipping (**B**) and after hatching, when staining intensified (**C**). Bar = 50 μm.


A
T
B
C
T

5.4 Discussion

5.4.1 Development of the Pulmonary Surfactant Lipids

5.4.1.1 Phospholipids

In mammals, the principal stimuli for release of pulmonary surfactant after birth include the first deep breaths and input from the sympathetic nervous system. Distortion of the type II cell (Wirtz & Dobbs, 1990), mediated by the dramatic increase in tidal volume (Nicholas & Barr, 1981) and the simultaneous elevation in alveolar pO_2, may act synergistically to cause the release of surfactant. Similarly, the lung of snapping turtle showed increases in both total PL and DSP with the onset of pulmonary ventilation, suggesting that the stimulation for release at pipping is similar to that of birth in mammals. In contrast, ventilation does not stimulate surfactant secretion from the isolated perfused lung of the adult bearded dragon (Wood *et al.*, 1995), whereas sympathetic agonists trigger release of phospholipids from the isolated perfused lung and isolated type II cells of this species (Wood *et al.*, 1995; Wood *et al.*, 1997; Wood *et al.*, 1999). Thus, is it likely that input from the sympathetic nervous system provides the primary stimuli for secretion at pipping in oviparous vertebrates. Moreover, increases in total PL and DSP may be attributable to the elevated activity of the embryo as it struggles to break the eggshell. Indeed, resting metabolic rate and circulating thyroid hormones are elevated between pipping and hatching in the turkey (Christensen *et al.*, 1982) and oxygen consumption increases before pipping in the green sea turtle, *Chelonia mydas* (Ackerman, 1980). The DSP/PL ratio increased when pulmonary ventilation was established, thus there is greater secretion of DSP relative to the total PL after pipping, presumably to aid in the elimination of lung fluid and the initiation of gas exchange (Figure 5.2-A). Furthermore, the

subsequent depression in total PL after hatching suggests that there is maximal secretion of surfactant during pipping when pulmonary ventilation is first established.

5.4.1.2 Cholesterol

Contrary to the amount of phospholipids, the content of Chol increased significantly in the snapping turtle lung prior to the initiation of airbreathing and remained unchanged thereafter (Figure 5.1-C). In the developing chicken (Chapter 2) and bearded dragon lung (Chapter 3), the amount of Chol does not vary throughout the latter part of incubation, despite increases in the amount and saturation of the phospholipids. Similarly, Chol remains constant during metamorphosis in the tiger salamander, *Ambystoma tigrinum,* while saturation of the phospholipids declines (Orgeig *et al.*, 1994). It therefore appears that neither the sympathetic surge observed at birth in mammals, nor ventilation, nor increases in pO_2 affect Chol secretion in the developing turtle lung. Indeed, adrenaline and acetylcholine do not stimulate Chol secretion in either the isolated perfused lung (Wood *et al.*, 1995; Wood *et al.*, 1997), or isolated type II cells of the adult bearded dragon (Wood *et al.*, 1999). Clearly, factors regulating Chol metabolism are independent from those controlling the phospholipids both within and between species.

Hatchling loggerhead sea turtles, *Caretta caretta* and flatback sea turtles, *Natator depressus*, demonstrate much higher Chol/PL and Chol/DSP ratios than reptiles with unicameral lungs (Daniels *et al.*, 1996). These authors suggested that an elevated Chol/DSP would facilitate spreading and respreading of the surfactant following faveolar compression. The Chol/PL and Chol/DSP ratios of hatchling snapping turtles were approximately three times lower than values obtained for *C. caretta* (Chol/PL: 0.099 ± 0.019, Chol/DSP: 0.360 ± 0.047) and *N. depressus* (Chol/PL: 0.061 ± 0.016, Chol/DSP: 0.223 ± 0.057) (Daniels *et al.*, 1996) (Figure 5.2-B,C). As snapping turtle hatchlings were only hours old, their pulmonary surfactant may not have attained its final composition.

Indeed, newborn rabbits do not acquire the adult composition of subfractionated lavage 24 hours after birth (Stevens *et al.*, 1987). On the other hand, snapping turtles may not exhibit the same lipid profile as sea turtles. Snapping turtles have a considerably reduced plastron, permitting sagging of the visceral structures upon land. This in turn exerts tension on the dorsally located lungs, causing them to inflate. Thus, on land, inspiration in the snapping turtle is largely passive, while expiration is an active process. When in water, the reverse occurs (Gaunt & Gans, 1969). It is possible that differences in the mechanics of breathing on land may result in differences in the composition of the surfactant system between these and other turtles. Moreover, sea turtles from the aforementioned study were highly active (Daniels, pers. comm.). Such activity may drastically alter the composition of the surfactant system since exercise elicits changes in the amount of total PL in pulmonary surfactant from the adult bearded dragon lung (Wood *et al.*, 1997). Despite differences in Chol/PL and Chol/DSP, hatchling snapping turtle DSP/PL was comparable to that of hatchling sea turtles (Daniels *et al.*, 1996). The Chol/PL and Chol/DSP in hatchling turtles matches values obtained for hatchling lizards (Chapters 3 and 4), yet are lower than chickens (Chapter 2), however the reasons for this remain obscure.

5.4.2 Additional Measures

5.4.2.1 Detection of TTF-1 and SP-B

Mammalian-derived antibodies for TTF-1 and SP-B cross-reacted with antigens from snapping turtle lung tissue. Similarly, the same antibodies cross-react with antigens from subsets of cells with distal respiratory epithelium of adult axolotls, *Ambystoma mexicanum* (Miller *et al.* 2001). Hence, immuno-reactive antigens from snapping turtles are believed to represent the mature surfactant protein, SP-B and the transcription factor, TTF-1. Consequently, these results demonstrate the high degree of conservation of both proteins

between mammals and turtles, and between mammals and amphibians (Miller *et al.* 2001). Moreover, the structure of the TTF-1 gene, its polypeptide and function have been conserved between mammals (Ikeda *et al.*, 1995). Since turtles are amongst the most ancient of the amniotes, conservation of TTF-1 and SP-B between testudian and mammalian lung types supports the recent hypothesis for a single origin for vertebrate surfactant (Daniels *et al.*, 1995a; Daniels *et al.*, 1998b; Sullivan *et al.*, 1998).

TTF-1 is expressed within the nuclei of epithelial cells of the developing airways following 11 weeks and 10 days of gestation in the foetal human and rat lung, respectively (Lazzaro *et al.*, 1991; Ikeda *et al.*, 1995; Stahlman *et al.*, 1996), where it is restricted to the type II cells and subsets of bronchiolar epithelial cells after birth (Ikeda *et al.*, 1995; Stahlman *et al.*, 1996). Similarly, TTF-1 stained epithelial cell nuclei in both the conducting airways and the gas exchange area of the developing and hatchling snapping turtle lung. The spatial patterns of both TTF-1 and SP-B expression match those exhibited in the developing mammalian and avian lung. TTF-1 expression precedes and overlaps that of the surfactant proteins, SP-A, SP-B and SP-C in mammals (Stahlman *et al.*, 1996; Zhou *et al.*, 1996) and SP-A and SP-B in birds (Zeng *et al.*, 1998). Likewise, TTF-1 staining occurred concurrently with SP-B staining within the same subsets of cells in the developing lung of the snapping turtle. The prevalence of TTF-1 staining was lower in the hatchling snapping turtle lung when compared with pipping and pre-pipped animals (Figure 5.3). Similarly, immunostaining of TTF-1 declines in the hatchling chick and postnatal mouse and human lung (Ikeda *et al.*, 1995; Stahlman *et al.*, 1996; Zhou *et al.*, 1996; Zeng *et al.*, 1998). As sampling did not encompass the entire developmental period, it is unknown whether TTF-1 staining precedes staining of the surfactant protein, nor whether TTF-1 staining occurs at the onset of lung bud formation. Therefore, an upstream role for TTF-1 in lung specific gene expression (Zhou *et al.*, 1996) can not confirmed in the snapping turtle without further investigation. However, the conservation and

distribution of TTF-1 and SP-B expression in the turtle lung compared with mammals and birds and the adult axolotl (Miller *et al.*, 2001), supports the role of TTF-1 in the regulation of lung development and surfactant homeostasis within the vertebrates.

Ablation of the SP-B gene causes respiratory failure and death immediately after birth in the homozygous mouse due to disturbances in the cycling of the PLs and the formation of lamellar bodies and tubular myelin (Clark *et al.*, 1995), demonstrating the necessity of SP-B for lung function after birth. SP-B expression increases in the mouse lung prior to birth (Zhou *et al.*, 1996), as does SP-B mRNA in the developing rat and human lung (Liley *et al.*, 1989; Schellhase *et al.*, 1989) and the mature peptide in newborn rat lavage (Shimizu *et al.*, 1991). Given that increases in surfactant proteins prior to birth reflect the crucial role they play at the onset of pulmonary ventilation in mammals (Zhou *et al.*, 1996), it is likely that this is also true for snapping turtle lung. In turtles, expression of SP-B appeared to intensify after hatching (Figure 5.4-C) following an elevation in PL and DSP secretion once pulmonary ventilation had commenced (Figure 5.1-A,B).

5.4.3 Conclusions

Snapping turtles, like mammals, dramatically increase the amount of saturated and total phospholipids at the onset of pulmonary ventilation presumably to overcome resistance to inflation and perform lung clearance. Cholesterol increases prior to pulmonary ventilation, demonstrating that cholesterol synthesis and/or secretion are independently regulated from the phospholipids in the developing turtle lung. Hence, the pattern of development of the surfactant proteins and lipids is conserved among these amniotes, with maturation of the system occurring at the time of pipping, in preparation for airbreathing.

TTF-1 was detected in epithelial cells of the gas exchange area and conducting airways through the latter stages of development in the snapping turtle lung and declined in the hatchling lung, whereas SP-B was detected in subsets of cells within the respiratory

epithelium both prior to and subsequent to the onset of pulmonary ventilation. Therefore, this surfactant protein and transcription factor appear to be highly conserved among the vertebrates. Given that both proteins exhibited similar temporal-spatial staining to the patterns observed in the developing mammalian and avian lung, suggests that TTF-1 is involved in lung morphogenesis and surfactant protein gene expression in the vertebrates.

5.4.4 Future Directions

To confirm that the mammalian-derived antibodies are staining TTF-1 and SP-B in the turtle lung, DNA sequencing should be performed. DNA sequencing would yield the amino acid sequence for the proteins. As a result, the degree of homology between the testudian and mammalian proteins could be determined, furthering our knowledge of the conservation of the surfactant proteins and this transcription factor.

To confirm the upstream role of TTF-1 in surfactant protein gene expression studies encompassing the onset of lung bud formation in turtles are required. Given that the structure of TTF-1 is highly conserved throughout evolution, and that the expression of TTF-1 is regulated by HNF-3 family members, this study may also incorporate the control of different nuclear transcription factors in the cytodifferentiation and lung-specific gene expression in reptiles. Results from such a study would demonstrate the role of transcription factors in the organ specific growth of foregut derivatives in reptiles.

In addition, since SP-B is also secreted from Clara cells in the mammalian lung, it would be pertinent to investigate the expression SP-C, which is exclusively expressed in type II pneumocytes. In doing so, one could determine the developmental pattern of the surfactant lipids and proteins within a single cell type (the type II cell) from reptilian lungs. This is especially of interest because mammalian-derived antibodies for proSP-C do not cross-react with antigens in lung tissue from adult axolotls (Miller *et al.* 2001).

CHAPTER 6 DEVELOPMENT OF THE PULMONARY SURFACTANT LIPIDS IN THE MARINE TURTLE, *CHELONIA MYDAS*

6.1 INTRODUCTION

Towards the end of gestation the foetal lung matures and begins to produce pulmonary surfactant. The primary stimuli for surfactant secretion at birth include lung expansion (Lawson *et al.*, 1979; Oulton *et al.*, 1986) mediated by stretch of the basement membrane of the type II cell (Wirtz & Dobbs, 1990) and sympathetic input from elevated levels of circulating catecholamines and labor (Marino & Rooney, 1981). Moreover, the drastic increase in alveolar pO_2 may stimulate surfactant secretion in the newborn. It has been recognised that the elevated alveolar pO_2 associated with birth, increases sodium transport across respiratory epithelia (Pitkänen *et al.*, 1996; Ramminger *et al.*, 2000), increases surfactant protein gene expression (Acarregui *et al.*, 1993), surfactant protein mRNA levels and phospholipid synthesis (Acarregui *et al.*, 1995) in the human foetal lung *in vitro.*

To understand the mechanism of surfactant secretion and its stimuli, it is pertinent to determine the biochemical changes associated with surfactant in the final stages of development. Foetal surfactant composition and synthesis can be altered by maternal diet (Viscardi, 1995) and hormones that cross the placenta (Gross, 1990). The green sea turtle, *Chelonia mydas*, lacks such maternal influences and possesses a lung structure of considerable complexity (Perry *et al.*, 1989). During incubation, sea turtle embryos experience a progressive decline in the oxygen tension within the nest chamber, resulting from limited diffusion of gases through the sand and the escalating oxygen consumption of the respiring embryos (Ackerman, 1977). Therefore, at the time of pipping, when the embryo perforates the shell, the first breaths taken are relatively hypoxic. Similarly, foetal hypoxia, resulting from a mismatching of oxygen supply and demand, initiates the maturation of the surfactant system and birth in the mammalian foetus by the release of

glucocorticoids. Hatching in turtles is also initiated by hypoxia (Webb *et al.*, 1986). It is unknown whether environmental gas tensions affect the maturation of the pulmonary surfactant system in non-mammalian vertebrates. Furthermore, following hatching, hatchling sea turtles undergo highly strenuous bouts of digging to escape the nest followed by sprints to the sea (Ackerman, 1977). Such exertion requires a completely functional surfactant system directly after hatching. Therefore, this species provides an excellent model to determine the limitations of the pulmonary surfactant system during development and the stimuli for its maturation. The effect that hypoxia may have on the lung clearance, pulmonary surfactant secretion and, ultimately the initiation of airbreathing in species which hatch underground have not been elucidated. Therefore, this chapter aims to determine the development of the pulmonary surfactant lipids in *Chelonia mydas* and to investigate whether mild hypoxia affects surfactant development.

6.2 Materials and Methods

6.2.1 Collection and Incubation of Eggs

Three clutches of green sea turtle eggs, *Chelonia mydas*, were collected from Heron Island, QLD, Australia in December, 1999. Eggs were packed in perlite®™ and transported to the laboratory where they were carefully washed in deionised water and dipped in a dilute solution of iodine (1 mg/L), which removes fungal spores that are ubiquitous on Heron Island and cause increased embryonic mortality (Booth, pers. comm.). Eggs were blotted dry on paper towel and weighed. Each clutch was equally divided and transferred to boxes containing hydrated perlite®™ with a gravimetric water content of 1g/g dry mass, with a water potential -200 kPa or wetter (Packard *et al.*, 1987). Containers were weighed after the eggs were deposited, then sealed with tight-fitting lids and placed in constant temperature cabinets at 26°C, which yields male hatchlings (Miller, 1985). At this

temperature, the duration of incubation varies between 68 and 80 days (Miller, 1985). Boxes were rotated throughout the cabinets regularly to minimise exposure to fluctuations in temperature within the cabinets. Containers were weighed weekly and distilled water was added to the substrate until the initial mass of the container was reached.

All eggs were incubated under normoxic (air: 20.94% O_2) and normobaric conditions until day 45 of incubation. At this time, half of the eggs from each clutch were transferred to a separate incubator at 26°C and exposed to 17% O_2 for the remainder of incubation (Section 6.2.6.1).

6.2.2 Tissue Sampling

Sea turtle eggs from the normoxic (N) and hypoxic (H) chambers were sampled after 58, 62, and 73 days of incubation, the time of pipping (N: 77.89 ± 0.72, n = 9; H: 78.00 ± 0.50, n = 9) and after hatching (N: 79.00 ± 0.79, n = 9; H: 79.00 ± 0.50, n = 9). Embryos and hatchlings were sacrificed by an intraperitoneal injection of sodium pentobarbitone (150 mg/kg [BM] Nembutal, Abbott Laboratories, Sydney, NSW, Australia).

6.2.3 Embryonic Staging

In addition to the time of incubation, embryos were staged according to the staging criteria for *Chelonia mydas* described by Miller (1985). Day-58 corresponded to stages 26 and 27, day-62 corresponded to stage 28, day-73 corresponded to stage 29 and pipped and hatchlings turtles corresponded to stage 30 and 31, respectively.

6.2.4 Lavage Protocol

The trachea was cannulated with a blunt 22G needle and the lungs were lavaged with three volumes of chilled isotonic saline (0.15M NaCl), which was injected into, and withdrawn from the lungs three times per volume. Volumes ranging from 0.2-1.5 ml were instilled depending on the age and size of the embryo. Any foetal lung fluid present was incorporated into the lavage. The lavage was centrifuged (Beckman model TJ-6 centrifuge) at 150 *g* for 5 min at 4°C to remove cellular debris. The supernatant was snap frozen in liquid nitrogen and freeze-dried. Lyophilised lavage was stored at -80°C for further analyses.

Following lavage, the lungs were excised from the turtles, blotted dry on paper towel and weighed. Wet lung weights were measured and, when possible, lungs were snap frozen in liquid nitrogen and lyophilised to obtain dry lung weights. Dry lung weights were determined from representative animals at all stages of incubation. Dry lung weights for all experimental animals were calculated from the ratio of wet lung weight to dry lung weight of the representative turtles.

6.2.5 Lipid Analyses

Lyophilised lavage was reconstituted in 2 ml of deionised (Millipore) water and lipids were extracted using chloroform:methanol (1:2 vol/vol) (Bligh & Dyer, 1959) (Appendix 2, Section 1.1). Each sampling point contained equal representation of individuals from each clutch. Total phospholipid, disaturated phospholipid and cholesterol were extracted and quantified from lavage in the same manner as described for the chicken (Section 2.2.5).

6.2.6 Additional Measures

6.2.6.1 Hypoxia Protocol

After 45 days of incubation, half of the eggs from each clutch were transferred from normoxia to a separate incubator, set at 26°C, and they were exposed to 17% O_2 for the remainder of incubation. During the final 20% of incubation, oxygen tension rapidly falls to 100 Torr (13% O_2) in the nest chamber of sea turtles (Ackerman, 1977). To provide an extended (chronic) period of exposure, a gas mixture falling between the two extremes was chosen. Mixed gas (17% O_2 and 83% N_2) was delivered to the incubator at a constant flow rate of 750 cc/min. Vacuum grease was applied to all door seals and thoroughly inspected for leaks. Exhaust gas was monitored regularly throughout incubation using a Helox 2 paramagnetic oxygen analyser, calibrated against nitrogen gas (0.00% O_2) and dry, CO_2-free air (20.94% O_2) to ensure hypoxic conditions were maintained for the duration of the experiment. Opening of both the hypoxic and normoxic chambers was kept to a minimum to avoid temperature and gas tension fluctuations.

6.2.7 Statistical Analyses

Data were expressed and analysed in the same manner as that of the chicken (Section 2.2.7). To demonstrate the overall developmental changes in the surfactant, normoxia data are expressed graphically. To compare changes in the surfactant composition between different incubation regimes, normoxia and hypoxia data are tabulated.

6.3 RESULTS

6.3.1 Physical Parameters

Hypoxia did not alter body mass of turtles at any sampling point (Table 6.1). Similarly, there was no difference in wet lung weights between embryos incubated under normoxic or hypoxic conditions, until animals had hatched (Table 6.1).

Table 6.1 *The effect of hypoxia on body mass and wet lung weight in developing green sea turtles.*

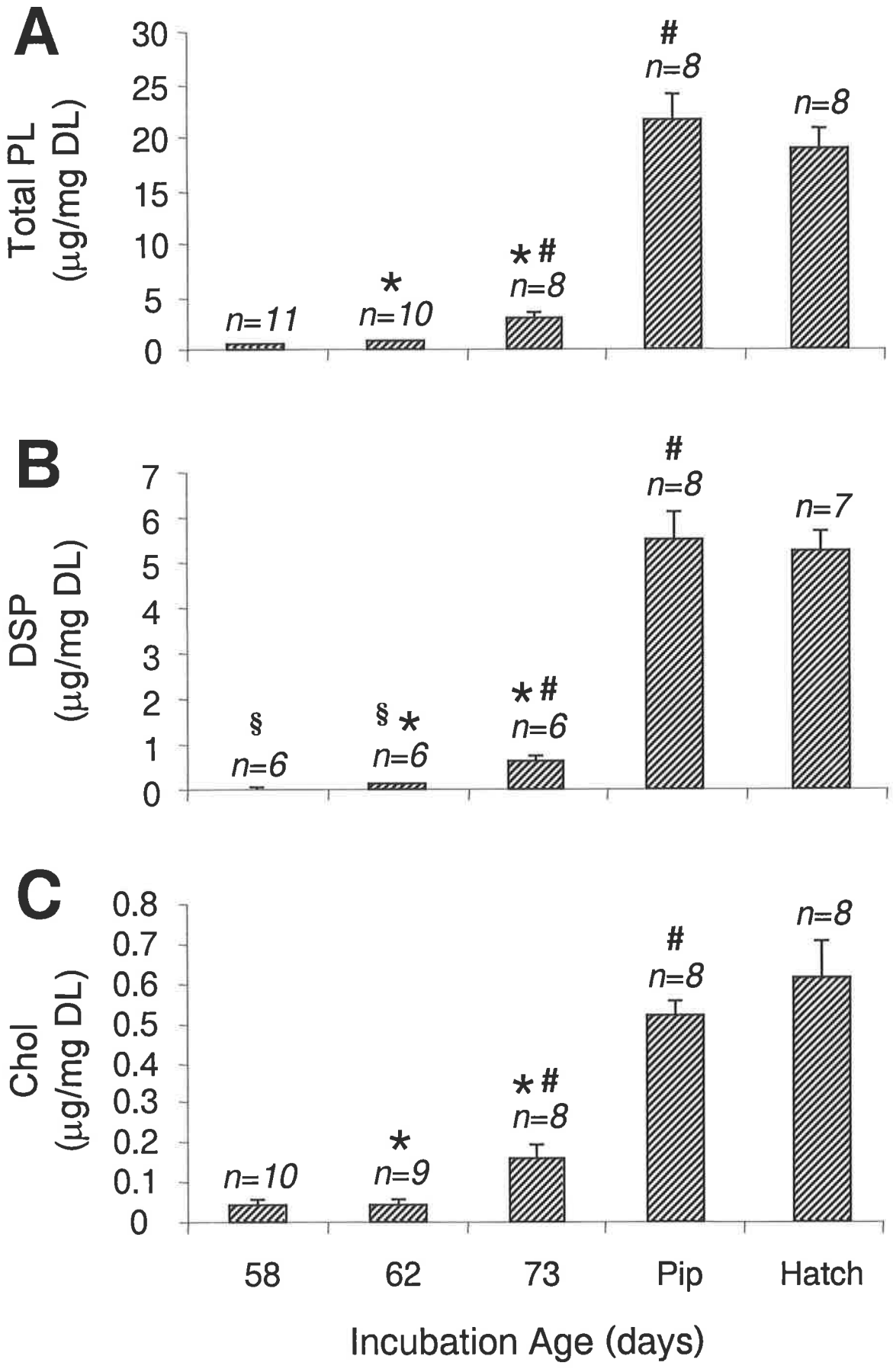
Age (days)	Treatment	BM (g)		WLW (g)	
Day-58	Normoxia	13.55 ± 0.45 (10)	NS	0.221 ± 0.018 (11)	NS
	Hypoxia	13.71 ± 0.37 (10)		0.213 ± 0.007 (10)	
Day-62	Normoxia	17.53 ± 0.39 (10)	NS	0.278 ± 0.018 (10)	NS
	Hypoxia	17.58 ± 0.53 (7)		0.281 ± 0.012 (7)	
Day-73	Normoxia	24.70 ± 0.60 (8)	NS	0.311 ± 0.020 (8)	NS
	Hypoxia	23.75 ± 1.02 (7)		0.283 ± 0.012 (7)	
Pip	Normoxia	26.25 ± 0.72 (8)	NS	0.292 ± 0.016 (8)	NS
	Hypoxia	25.34 ± 0.68 (12)		0.250 ± 0.021 (11)	
Hatch	Normoxia	27.64 ± 1.24 (9)	NS	0.318 ± 0.014 (9)	$P < 0.01$
	Hypoxia	27.75 ± 1.06 (8)		0.258 ± 0.015 (9)	

Results are mean ± SEM; *n* in parentheses; NS, not significant; Pip, pipped embryos; Hatch, hatchlings; WLW, wet lung weight; BM, body mass.

6.3.2 Development of the Pulmonary Surfactant Lipids under Normoxia

Foetal lung fluid was observed in lungs of embryonic turtles until the time of pipping, when pulmonary ventilation was first established. When expressed as a function of dry lung mass, the amount of total phospholipid recovered from the lungs of developing turtles increased throughout incubation (ANOVA, $P = 5.6 \times 10^{-17}$) (Figure 6.1-A). Total PL did

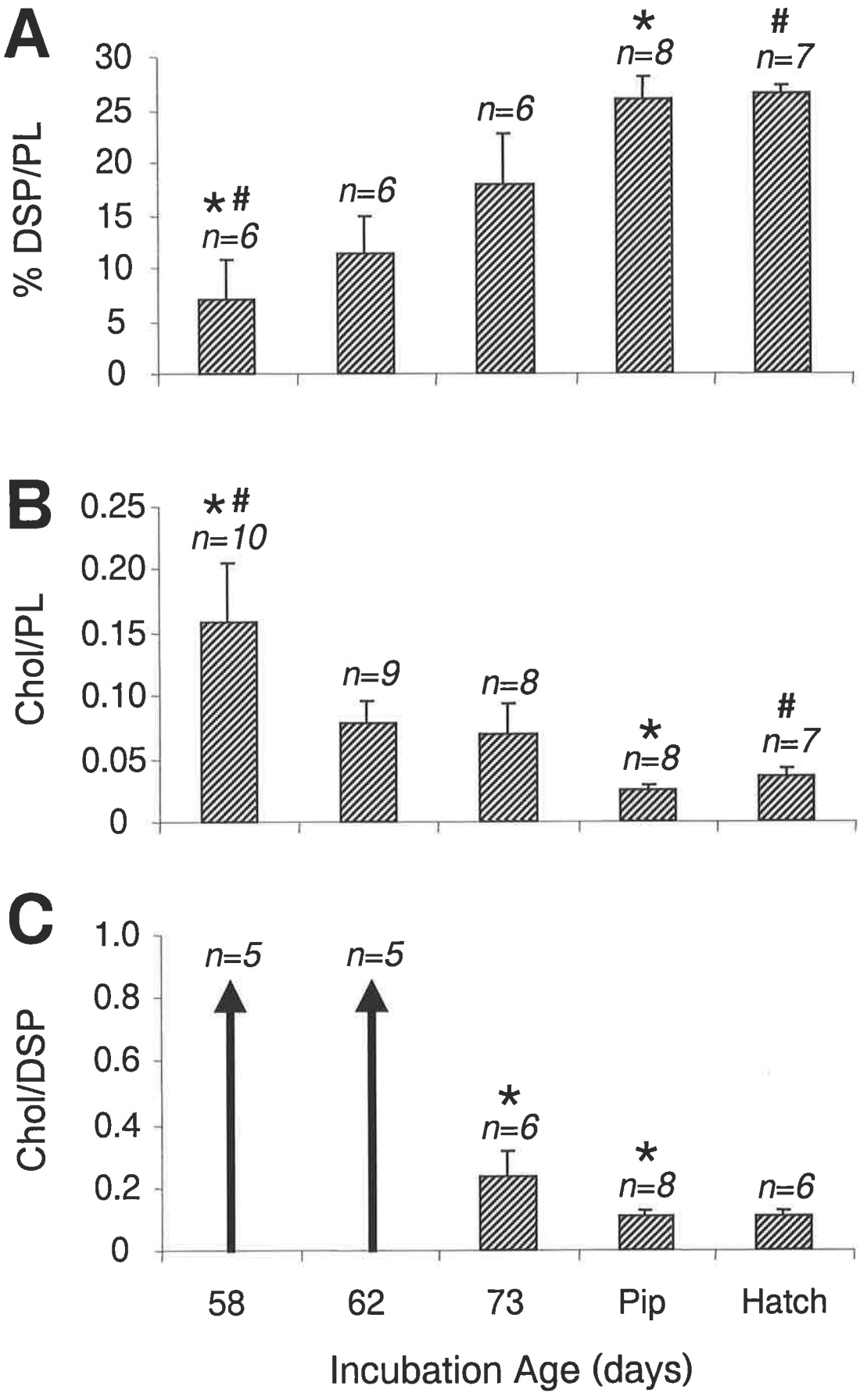
Figure 6.1 The absolute amount of phospholipids and cholesterol in pulmonary surfactant from developing lung of the green sea turtle, demonstrating total PL (**A**), DSP (**B**) and Chol (**C**) per mg of dry lung (DL). Data are expressed as mean ± SEM. Paired symbols indicate significant difference between adjacent groups. **A**: * $P = 2.3 \times 10^{-5}$, # $P = 1.1 \times 10^{-6}$, **B**: § $P = 0.039$, * $P = 0.0084$, # $P = 2.6 \times 10^{-6}$; **C**: * $P = 0.0066$, # $P = 5.2 \times 10^{-6}$.


A
Total PL (µg/mg DL)
30
25
20
15
10
5
0
n=11
*
n=10
*#
n=8
#
n=8
n=8
B
DSP (µg/mg DL)
7
6
5
4
3
2
1
0
§
n=6
§*
n=6
*#
n=6
#
n=8
n=7
C
Chol (µg/mg DL)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
n=10
*
n=9
*#
n=8
#
n=8
n=8
58
62
73
Pip
Hatch
Incubation Age (days)

not differ between day-58 and-62 of incubation ($P = 0.081$), however it increased 4-fold from day-62 to day-73 of incubation ($P < 10^{-4}$) and 7-fold from day-73 of incubation until the time of pipping ($P < 10^{-5}$) (Figure 6.1-A). There was no difference in the content of total phospholipid between pipped and hatchling turtles ($P = 0.18$) (Figure 6.1-A). Like total PL, DSP increased throughout incubation (ANOVA, $P = 4.6 \times 10^{-13}$). There was a significant elevation in the amount of DSP in lung washings between day-58 and -62 of incubation ($P < 0.05$), day-62 and -73 of incubation ($P < 0.01$) and day-73 and the time of pipping ($P < 10^{-5}$), with no change after hatching ($P = 0.37$) (Figure 6.1-B). The content of cholesterol increased in the developing sea turtle lung with advancing incubation (ANOVA, $P = 5.6 \times 10^{-12}$) (Figure 6.1-C). Like total PL, there was no difference in the amount of cholesterol present in lavage from embryos incubated for 58 and 62 days ($P = 0.47$). The amount of cholesterol increased between days-62 and -73 of incubation ($P < 0.01$) and from day-73 until pipping ($P < 10^{-5}$), where it remained constant thereafter ($P = 0.19$) (Figure 6.1-C).

The DSP/PL ratio differed throughout incubation (ANOVA, $P = 0.00025$) (Figure 6.2-A). There was no difference between adjacent time points ($P< 0.05$ for all tests), however the DSP/PL ratio of embryos incubated for 58 days was significantly lower than at the time of pipping ($P < 0.001$) and after hatching ($P < 0.001$) (Figure 6.2-A). The Chol/PL ratio changed over incubation (ANOVA, $P = 0.012$) (Figure 6.2-B). Despite no difference between adjacent sampling groups, day-58 values were significantly elevated above pip ($P < 0.05$) and hatchling values ($P < 0.05$) (Figure 6.2-B). As DSP was not measurable in three sea turtles at day-58 of incubation and one at day-62 of incubation, the Chol/DSP ratio approached infinity at both of these time points (Figure 6.2-C). Following the rapid decline to day-73 of incubation, the Chol/DSP ratio decreased further towards pipping ($P < 0.05$) after which it remained constant ($P = 0.41$) (Figure 6.2-C).

Figure 6.2 The relationship between phospholipids and cholesterol in pulmonary surfactant from the developing lung of the green sea turtle, demonstrating DSP expressed as a percentage of total PL (**A**), the Chol/PL ratio (**B**) and the Chol/DSP ratio (**C**). Data expressed as mean ± SEM. Paired symbols indicate significant difference between adjacent groups. As DSP was not measurable in embryos incubated for 58 and 62 days, Chol/DSP approached infinity demonstrated by an arrow (**C**). **A**: * P = 0.00030, # P = 0.00012, **B**: * P = 0.011, # P = 0.017, **C**: * P = 0.041.


A
% DSP/PL
30
25
20
15
10
5
0
*#
n=6
n=6
n=6
*
n=8
#
n=7
B
Chol/PL
0.25
0.20
0.15
0.10
0.05
0
*#
n=10
n=9
n=8
*
n=8
#
n=7
C
Chol/DSP
1.0
0.8
0.6
0.4
0.2
0
n=5
n=5
*
n=6
*
n=8
n=6
58
62
73
Pip
Hatch
Incubation Age (days)

6.3.3 Additional Measures

6.3.3.1 Effect of Hypoxia on the Development of the Pulmonary Surfactant Lipids

ıere was no difference in the time of pipping (P = 0.45), or hatching (P = 0.50) between hypoxic d normoxic groups. Similarly, hypoxia did not generally alter the amount of total PL, DSP or olesterol harvested from sea turtle lungs throughout the course of development (Table 6.2). Since poxia did not elicit an effect on the absolute amount of surfactant lipids throughout development, ere was no difference in the relative composition of the surfactant lipids between hypoxic and ırmoxic groups (Table 6.3).

6.4 Discussion

6.4.1 Development of the Pulmonary Surfactant Lipids under Normoxia

6.4.1.1 Phospholipids

. mammals, the content and saturation of the phospholipids increase with advancing gestation Looney *et al.*, 1976b; Torday & Nielson, 1981; Benson *et al.*, 1983; Oulton *et al.*, 1986; Sosenko Frank, 1987). Similarly, in the sea turtle lung there is an increase in the amount of total ıospholipid, disaturated phospholipid and cholesterol in the final stages of incubation. The ıntent of each lipid was maximal at the time of pipping, coincident with the initiation of rbreathing, presumably to aid in lung clearance and reduce the resistance to inflation. In addition, e increase in the relative saturation of phospholipids, a hallmark of surfactant maturation, was aximal in the sea turtle at the time of pipping (Figure 6.2-A), suggesting that the onset of eathing stimulates surfactant secretion in this species. However, it has been shown that neither

Table 6.2 The effect of hypoxia on the absolute amount of surfactant lipids in lung washings from developing green sea turtles.

Age (days)	Treatment	Total PL (µg/mgDL)		DSP (µg/mgDL)		Chol (µg/mgDL)	
Day-58	Normoxia	0.49 ± 0.15 (11)	NS	0.029 ± 0.017 (6)	NS	0.045 ± 0.014 (10)	NS
	Hypoxia	0.47 ± 0.12 (10)		0.038 ± 0.020 (7)		0.057 ± 0.014 (7)	
Day-62	Normoxia	0.74 ± 0.09 (10)	$P < 0.01$	0.113 ± 0.039 (6)	NS	0.044 ± 0.011 (9)	NS
	Hypoxia	1.65 ± 0.35 (7)		0.234 ± 0.071 (7)		0.073 ± 0.014 (7)	
Day-73	Normoxia	2.98 ± 0.98 (8)	NS	0.603 ± 0.166 (6)	$P < 0.05$	0.156 ± 0.040 (8)	NS
	Hypoxia	3.95 ± 0.81 (7)		1.11 ± 0.21 (6)		0.170 ± 0.031 (7)	
Pip	Normoxia	21.75 ± 2.40 (8)	NS	5.52 ± 0.61 (8)	NS	0.519 ± 0.036 (8)	NS
	Hypoxia	21.07 ± 2.15 (11)		5.31 ± 0.49 (11)		0.546 ± 0.090 (11)	
Hatch	Normoxia	18.79 ± 2.09 (8)	NS	5.25 ± 0.43 (7)	NS	0.613 ± 0.096 (8)	NS
	Hypoxia	16.52 ± 2.92 (9)		5.14 ± 0.83 (7)		0.555 ± 0.079 (9)	

Results are mean ± SEM; *n* in parentheses; DL, dry lung; NS, not significant; Pip, pipped embryos; Hatch, hatchlings. Total PL differed between treatments on day-62 of incubation, whereas DSP differed between embryos incubated for 73 days. However, overall, hypoxia had no effect on the development of the surfactant lipids.

Table 6.3 *The effect of hypoxia on the relative amount of surfactant lipids in lung washings from developing green sea turtles.*

Age (days)	Treatment	DSP/PL (%)		Chol/PL (µg/µg)		Chol/DSP (µg/µg)	
Day-58	Normoxia	7.08 ± 3.79 (6)	NS	0.159 ± 0.047 (10)	NS	∞ (5)	
	Hypoxia	6.65 ± 3.65 (7)		0.204 ± 0.067 (10)		∞ (7)	
Day-62	Normoxia	11.36 ± 3.52 (6)	NS	0.078 ± 0.019 (9)	NS	∞ (5)	
	Hypoxia	13.80 ± 3.96 (7)		0.055 ± 0.015 (7)		∞ (7)	
Day-73	Normoxia	17.96 ± 4.84 (6)	NS	0.069 ± 0.025 (8)	NS	0.236 ± 0.062 (6)	NS
	Hypoxia	24.82 ± 1.66 (6)		0.046 ± 0.009 (7)		0.218 ± 0.062 (6)	
Pip	Normoxia	25.91 ± 2.13 (8)	NS	0.026 ± 0.003 (8)	NS	0.105 ± 0.018 (8)	NS
	Hypoxia	26.05 ± 1.91 (11)		0.026 ± 0.003 (10)		0.096 ± 0.013 (10)	
Hatch	Normoxia	26.52 ± 0.83 (7)	NS	0.036 ± 0.008 (7)	NS	0.110 ± 0.018 (6)	NS
	Hypoxia	28.16 ± 2.88 (7)		0.042 ± 0.010 (9)		0.111 ± 0.013 (7)	

Results are mean ± SE; *n* in parentheses; NS, not significant; Pip, pipped embryos; Hatch, hatchlings; ∞, infinity. As DSP was not measurable in embryos incubated for 58 and 62 days, the Chol/DSP ratio approached infinity for these time points.

the secretion, nor the composition of pulmonary surfactant is affected by ventilation in the adult lizard lung (Wood *et al.*, 1995). The lack of secretion in response to stretch may be due to differences in breathing pattern, metabolism and the mechanics of breathing between reptiles and mammals (Wood *et al.*, 1995; Wood *et al.*, 1997). Indeed turtles, like lizards, undergo significant non-ventilatory periods interspersed between breaths (Gatz *et al.*, 1987). It is likely therefore that ventilation, mediated via stretch of the type II cell, is not stimulating secretion in the turtle lung. In mammals, β-adrenergic agents are the primary stimuli for surfactant secretion at birth (Cockshutt & Possmayer, 1992). Given that the surfactant system is highly conserved morphologically (Wood *et al.*, 2000), compositionally (Daniels *et al.*, 1995a; Sullivan *et al.*, 1998) and functionally (Daniels *et al.*, 1998b), it is likely that β-adrenergic agents stimulate secretion in the newborn turtle lung. In birds, circulating catecholamines climax immediately prior to hatching (Wittmann & Prechtl, 1991). Furthermore, the autonomic nervous system is believed to be the ancestral control mechanism for surfactant secretion within the vertebrates (Wood *et al.*, 2000).

Hatchling marine turtles *Caretta caretta* and *Natator depressus*, have an order of magnitude more phospholipid than freshwater turtles, *Malaclemys geographica* and *Emydura krefftii* (Daniels *et al.*, 1996). Similarly, in the present study the amount of surfactant harvested from the lung of hatchling green sea turtles surpasses values obtained for other oviparous vertebrates (Chapters 2, 3 and 5). Sea turtles have the greatest partitioning and the most complex primary lung structure of any reptile (Perry *et al.*, 1989) and therefore they may possess greater respiratory surface area than the simple saccular lungs of lizards and snakes. It is conceivable therefore, that sea turtles have both more in total, and a greater proportion of type II cells within their lungs than other reptiles, leading to the elevated levels of surfactant. Alternatively, the heightened activity of sea turtles and any sympathetic surge associated with

hatching may result in greater rates of surfactant secretion. Recent evidence suggests that the amount of phosphatidylcholine secreted from isolated type II cells of this species, increases considerably after hatching (Sullivan *et al.*, 2001).

6.4.1.2 Cholesterol

In heterothermic animals such as reptiles, cholesterol functions to control the fluidity and spreadability of the surface film during changes in body temperature (Daniels *et al.*, 1990). Previously it has been demonstrated that cholesterol is independently regulated from the phospholipids in an array of vertebrates (Daniels *et al.*, 1995a; Orgeig *et al.*, 1995; Wood *et al.*, 1997; Wood *et al.*, 2000), including the metamorphosing lungs of salamanders (Orgeig *et al.*, 1994) and the developing lungs of chickens (Chapter 2), lizards (Chapters 3 and 4) and freshwater turtles (Chapter 5). When compared with the phospholipids, cholesterol is packaged separately (Orgeig *et al.*, 1995) and responds differently to physiological stimuli such as exercise (Doyle *et al.*, 1994), temperature (Daniels *et al.*, 1990) and physiological states such as torpor, which is associated with reduced body temperatures and altered autonomic output (Lopatko *et al.*, 1999; Codd *et al.*, 2000). Likewise, during development cholesterol does not behave in the same manner as the phospholipids. In the salamander, cholesterol does not change during metamorphosis whereas the saturation of the phospholipids decline (Orgeig *et al.*, 1994). In the previous chapters (Chapters 2,3,4 and 5) all species demonstrated a reduction in the Chol/PL and Chol/DSP ratio with advancing incubation or gestation. Here, the Chol/PL ratio did not differ between adjacent sampling points demonstrating that cholesterol and total phospholipids were increasing in tandem. However, the Chol/PL ratio from embryos incubated for 58 days was elevated above those of pipped and hatchling animals, indicating that the Chol/PL ratio declined over development and therefore,

like chickens, lizards and freshwater turtles, cholesterol is under differential control from the phospholipids. The Chol/DSP declined markedly from day-62 of incubation to 73 and continued to decrease towards pipping, confirming the differential regulation of the surfactant lipids in developing sea turtles.

6.4.2 Additional Measures

6.4.2.1 Effect of Hypoxia on the Development of the Pulmonary Surfactant Lipids

The mild, but prolonged hypoxia used in this experiment failed to elicit an effect on the development of the surfactant lipids in the green sea turtle. This suggests that the pulmonary surfactant system is unresponsive to small decreases in pO_2. The lack of effect probably results from the high conductance of chelonian eggshells. Sea turtle eggshells are more permeable to gases and water vapour than avian eggs (Ackerman & Prange, 1972). Moreover, only a minimal oxygen gradient exists across the chelonian eggshell (Prange & Ackerman, 1974) when compared to avian eggs (Wangensteen, 1972). Therefore, turtle embryos can tolerate increasingly hypoxic conditions as they are able to acquire oxygen more readily and, as a result, oxygen may not have been limited in this study. Wood and Wood (1979) showed that *Chelonia mydas* eggs incubated in large numbers experience oxygen tensions of 70 Torr (9% O_2), leading to increased mortality of near-term embryos and abnormal yolk size in hatchlings. However, development progressed normally in embryos that were incubated in fewer numbers and which experienced ambient oxygen tensions of 110 Torr (14% O_2) (Wood & Wood, 1979). Clearly, the severity of hypoxia in this experiment was not great enough to alter the course of development in the sea turtles. Despite this, there were subtle changes in the quantity of lipid between the two groups (Table 6.2). The fact that wet lung weights did

not differ between the two groups throughout embryonic development, yet were significantly lighter in hatchlings incubated in an hypoxic environment when compared to those incubated under normoxic conditions, suggests that the lungs had cleared foetal lung fluid more rapidly than their normoxic counterparts. Indeed, reabsorption of foetal lung fluid has been observed in marine turtles immediately prior to hatching (Maloney *et al.*, 1989). However, this contrasts to the scenario in mammals, whereby elevation in the alveolar pO_2 has been shown to accelerate ion transport across respiratory epithelia (Pitkänen *et al.*, 1996; Ramminger *et al.*, 2000), a necessary process for lung fluid uptake. It is possible that mild hypoxia causes a minor increase in circulating catecholamines in this species, which is sufficient to elicit fluid reabsorption without appreciably altering surfactant secretion, yet this remains to be verified.

6.4.3 Conclusions

It appears that the development of the pulmonary surfactant lipids in marine turtles mirrors that of mammals, birds and other reptiles. There is an elevation in the amount and saturation of the phospholipids during the final stages of development. Secretion of the surfactant lipids is maximal at the onset of breathing, presumably to overcome the resistance to inflation and promote lung clearance. As in other vertebrates, cholesterol from turtles appears to be regulated independently from the phospholipids and its regulation differs between species. The surfactant system of sea turtles is extremely robust and is not influenced by mild hypoxia, however it appears that hypoxia promotes lung clearance in hatchling turtle lungs.

6.4.4 Future Directions

The present study used extremely mild hypoxia to determine the effect of external stimuli on the maturation of the surfactant system in the sea turtle. Due to the high conductance of the eggshell, it would be of interest to repeat this experiment at considerably lower oxygen tensions to test the limitations of the system during development and the stimuli for its maturation. Moreover, given the potential influence of the sympathetic nervous system, it would be valuable to monitor circulating catecholamine levels during incubation.

Furthermore, crocodiles, like sea turtles, experience hypoxic conditions toward the end of incubation, however their lung morphology is unlike that of any other vertebrate group, falling between the saccular lung of lizards and the parabronchial lung of birds in its complexity. Thus, studies on the development of the surfactant system in this species may provide new information regarding the maturation of this system and whether the stimuli for its maturation are environmental or genetically "hard-wired".

CHAPTER 7 GENERAL DISCUSSION

7.1 PHOSPHOLIPIDS

The amount and saturation of total surfactant phospholipid increases in the mammalian lung with advancing gestation (Gluck *et al.*, 1967; Rooney *et al.*, 1976b; Egberts *et al.*, 1981; Torday & Nielson, 1981; Benson *et al.*, 1983). Similarly, the content of PC increases in lung tissue from the chicken (Hylka & Doneen, 1982; Wittmann *et al.*, 1987) and the metamorphosing frog, *Rana catesbeiana* (Oguchi *et al.*, 1994) during the final stages of development. Likewise, when expressed as a percentage of incubation or gestation, the previous chapters demonstrated that both saturated (Table 7.2) and total phospholipid (Table 7.1) increases during the final 10-30% of development in every animal examined, suggesting that the developmental pattern of the surfactant lipids is similar among the vertebrates (Figure 7.1). However, in the larval amphibian, *Ambystoma tigrinum*, the amount of total phospholipid is unaffected by metamorphosis, whereas the saturation of the surfactant phospholipids declines (Orgeig *et al.*, 1994). It is believed that surfactant acts as a splint in this species when the larvae occupy deeper waters (Orgeig *et al.*, 1994). For the surfactant system to be functioning in this manner suggests that the system has already developed, and changes seen during metamorphosis in salamanders are associated with habitat preferences, rather than the transition from a fluid-filled, non-functional lung to an operative respiratory organ.

Both total PL and DSP increased significantly at the time of pipping, coincident with the onset of pulmonary ventilation in all reptiles and the DSP/PL ratio was maximal immediately following pipping in chickens (Tables 7.1,2). Similarly, both total phospholipid and DPPC increase markedly after birth in the rabbit (Oulton *et al.*, 1986) and total DPPC increases

Table 7.1. Absolute amount of total phospholipid from lavage of embryonic, newborn, hatchling and adult non-mammalian amniotes.

	Percentage of total incubation or gestation						
Species	**70-73**	**76-80**	**83-88**	**92-95**	**95-99**	**100**	
					Pip (ovip)	**Hatch/Newborn**	**Adult**
Gallus gallus domestica [a]	---	0.26 ± 0.09	0.99 ± 0.21	---	0.69 ± 0.21	0.93 ± 0.24	---
		(4)	(8)		(7)	(9)	
Pogona vitticeps [c]	---	---	---	1.25 ± 0.86	3.75 ± 0.88	4.42 ± 0.84	27.85 ± 3.33 [b]
				(5)	(7)	(7)	(7)
Tiliqua rugosa [d]	0.37	1.04 ± 0.36	3.58 ± 1.45	23.23 ± 5.02	77.28	48.16 ± 15.11	40.70 ± 9.62
		(3)	(2)	(3)		(5)	(8)
Chelydra serpentina [e]	---	---	0.75 ± 0.10	1.38 ± 0.37	4.54 ± 0.30	3.91 ± 0.24	---
			(8)	(17)	(17)	(18)	
Chelonia mydas [f]	0.49 ± 0.15	0.74 ± 0.09	---	2.98 ± 0.98	21.75 ± 2.40	18.79 ± 2.09	---
	(11)	(10)		(8)	(8)	(8)	

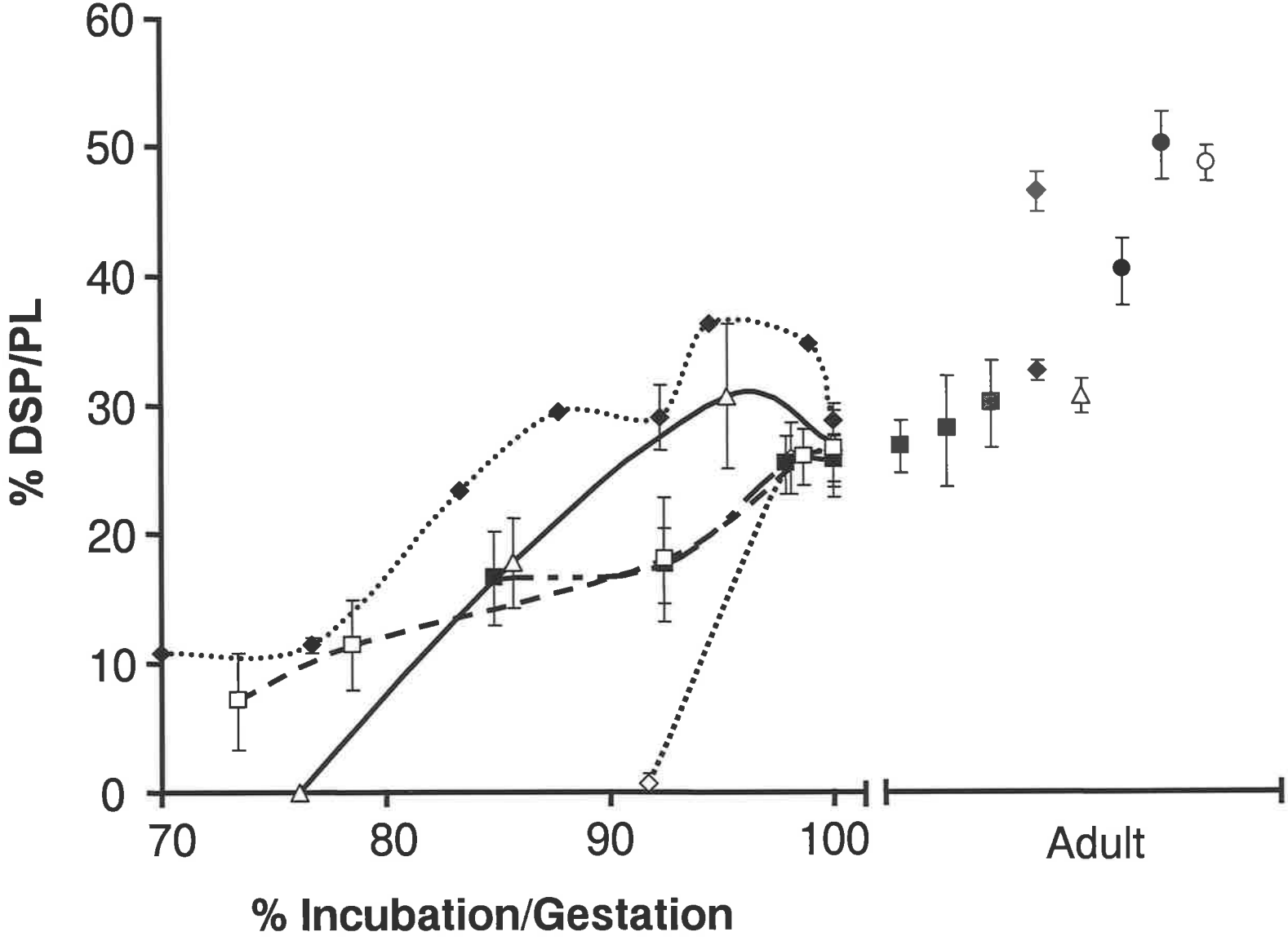
Data are expressed as µg/mg dry lung and presented as mean ± SEM; *n* in parentheses; Pip (ovip), time of pipping in oviparous species; --- data unavailable. [a] Chapter 2, [b] Wood *et al.* (1995), [c] Chapter 3 [d] Chapter 4 [e] Chapter 5, [f] Chapter 6.

Table 7.2. Absolute amount of disaturated phospholipid from lavage of embryonic, newborn, hatchling and adult non-mammalian amniotes.

	Percentage of incubation or gestation						
Species	**70-73**	**76-80**	**83-88**	**92-95**	**95-99**	**100**	
					Pip (ovip)	**Hatch/Newborn**	**Adult**
Gallus gallus domestica[a]	---	NM	0.18 ± 0.08	---	0.16 ± 0.04	0.25 ± 0.07	---
		(4)	(8)		(7)	(9)	
Pogona vitticeps[b]	---	---	---	0.03 ± 0.03	1.04 ± 0.32	1.19 ± 0.29	---
				(5)	(7)	(7)	
Tiliqua rugosa[c]	0.04	0.12 ± 0.04	0.99 ± 0.49	7.58 ± 2.15	26.80	13.34 ± 3.72	9.13 ± 1.69
		(3)	(2)	(3)		(5)	(8)
Chelydra serpentina[d]	---	---	0.16 ± 0.04	0.32 ± 0.12	1.09 ± 0.10	0.99 ± 0.09	---
			(6)	(13)	(16)	(18)	
Chelonia mydas[e]	0.029 ± 0.017	0.113 ± 0.039	---	0.603 ± 0.166	5.52 ± 0.61	5.25 ± 0.43	---
	(6)	(6)		(6)	(8)	(7)	

Data are expressed as μg/mg dry lung and presented as mean ± SEM; *n* in parentheses; Pip (ovip), time of pipping in oviparous species; --- data unavailable; NM not measurable. [a] Chapter 2, [b] Chapter 3, [c] Chapter 4, [d] Chapter 5, [e] Chapter 6.

Figure 7.1. Disaturated phospholipid expressed as a percentage of total phospholipid from lavage of the developing (—△—) and adult (△) chicken, *Gallus gallus domestica* (Chapter 2), the developing bearded dragon, *Pogona vitticeps* (▪▪◇▪▪) (Chapter 3), the developing (▪▪◆▪▪) and adult (◆) sleepy lizard, *Tiliqua rugosa* (Chapter 4), the developing snapping turtle, *Chelydra serpentina* (—■ ▪) (Chapter 5), the developing green sea turtle, *Chelonia mydas* (—□—) (Chapter 6), the mature leatherback sea turtle, *Caretta caretta* (■), the mature flatback sea turtle, *Natator depressus* (■) adult Kreft's turtle, *Emydura krefftii* (▩) (Daniels *et al.*, 1996), the adult central netted dragon, *Ctenophorus nuchalis* (◆) (Daniels *et al.*, 1990), the adult fat-tailed dunnart, *Sminthopsis crassicaudata* (●) (Langman *et al.*, 1996), the adult rat, *Rattus norvegicus* (●) (Orgeig *et al.*, 1995) and the adult human, *Homo sapiens* (○) (Doyle *et al.*, 1994).


60
50
40
30
20
10
0
% DSP/PL
70
80
90
100
Adult
% Incubation/Gestation

during parturition in the guinea pig (Lin & Lechner, 1991). In rabbits, total PL and PC increases 10-fold within one day of birth, suggesting that the onset of breathing triggers the release of surfactant (Rooney *et al.*, 1976b). Thus, it appears that despite phyletic, structural or reproductive differences, the greatest secretion of phospholipid occurs immediately prior to, or during, the transition to postnatal life when airbreathing is first established. Given that ventilation is not a trigger for PL release in the adult bearded dragon (Wood *et al.*, 1995), and avian lungs do not experience mechanical distortion (Duncker, 1972), it likely that secretion at pipping is mediated by adrenergic agents. Indeed, catecholamines promote surfactant secretion at birth in mammals (Marino & Rooney, 1981) and circulating noradrenaline and adrenaline peak immediately prior to pipping in the chicken (Wittmann & Prechtl, 1991). In addition, Sullivan and Orgeig have recently demonstrated that adrenaline stimulates PC secretion from organotypic culture of embryonic chicken lung (Sullivan & Orgeig, Submitted). Hence, while the presence of surfactant appears to be essential for the initiation of airbreathing in reptiles and birds, its secretion does not appear to be caused by lung expansion when airbreathing is first established.

The timing of enrichment of saturated phospholipids differs markedly between species (Figure 7.1). Despite the large variation in the amount of phospholipids recovered from lavage, the DSP/PL ratio was remarkably uniform between species at the onset of pulmonary ventilation and this value was comparable to values obtained for other adult reptiles and birds (Figure 7.1). Thus, neither lung morphology, phylogeny nor reproductive strategy influence the DSP/PL ratio required to establish pulmonary gas exchange. The fact that the DSP/PL ratio is so closely matched between such distantly related species with contrasting lung morphologies and birth strategies indicates that the ratio present at pipping is a essential for the facilitation of lung clearance and the commencement of airbreathing.

7.2 CHOLESTEROL

The composition of surfactant is determined primarily by body temperature in heterothermic vertebrates (Daniels *et al.*, 1998b). In the map turtle, *Malaclemys geographica,* surfactant is enriched in unsaturated phospholipids after long-term exposure to cold (Lau & Keough, 1981). Similarly, in the lizard, *Ctenophorus nuchalis*, the marsupial, *Sminthopsis crassicaudata*, and the bat, *Chalinolobus gouldii*, surfactant cholesterol dynamically increases with decreasing body temperature, presumably to maintain homeoviscosity of the film (Daniels *et al.*, 1990; Langman *et al.*, 1996; Codd *et al.*, 2000). Cholesterol and unsaturated PL therefore, must be present when airbreathing is first established in order to regulate fluidity of the surface film in the fluctuating thermal environment away from the nest or mother. In the present study, cholesterol was present in similar amounts throughout the latter stages of development in the chicken and bearded dragon, however in the snapping turtle, sea turtle and sleepy lizard, it was present in significantly lower amounts at earlier developmental stages (Table 7.3). The temporal appearance of Chol differed from that of PL and DSP both within and between species. Chol/PL and Chol/DSP did not appreciably differ throughout the latter stages of development in the turtles and sleepy lizards, demonstrating that cholesterol is released in tandem with the phospholipids (Figure 7.2,3). In all other species, Chol/PL and Chol/DSP decline with advancing incubation, indicating differential regulation of the surfactant lipids both within and between different species.

Table 7.3 Absolute amount of cholesterol from lavage of embryonic, newborn, hatchling and adult non-mammalian amniotes.

	Percentage of incubation or gestation						
Species	**70-73**	**76-80**	**83-88**	**92-95**	**95-99**	**100**	
					Pip (ovip)	**Hatch/Newborn**	**Adult**
Gallus gallus domestica[a]	---	0.09 ± 0.02	0.15 ± 0.03	---	0.07 ± 0.02	0.08 ± 0.02	---
		(4)	(8)		(7)	(9)	
Pogona vitticeps[c]	---	---	---	0.11 ± 0.06	0.18 ± 0.04	0.13 ± 0.03	2.287 ± 0.309[b]
				(5)	(7)	(7)	(7)
Tiliqua rugosa[d]	0.03	0.04 ± 0.01	0.07 ± 0.03	0.39 ± 0.09	0.78	0.67 ± 0.15	0.61 ± 0.15
		(3)	(2)	(3)		(5)	(8)
Chelydra serpentina[e]	---	---	0.033 ± 0.005	0.085 ± 0.021	0.095 ± 0.017	0.093 ± 0.012	---
			(8)	(12)	(15)	(17)	
Chelonia mydas[f]	0.045 ± 0.014	0.044 ± 0.011	---	0.156 ± 0.040	0.519 ± 0.036	0.613 ± 0.096	---
	(10)	(9)		(8)	(8)	(8)	

Data are expressed as μg/mg dry lung and presented as mean ± SEM; *n* in parentheses; Pip (ovip), time of pipping in oviparous species; --- data unavailable.[a] Chapter 2, [b] Wood *et al.* (1995), [c] Chapter 3, [d] Chapter 4, [e] Chapter 5, [f] Chapter 6.

Figure 7.2 Cholesterol expressed as a ratio to total phospholipid from lavage of the developing (—△—) and adult (△) chicken, *Gallus gallus domestica* (Chapter 2), the developing (··◇··) and adult (◇) bearded dragon, *Pogona vitticeps* (Chapter 3; Wood, 1995 #749], the developing (···◆··) and adult (◆) sleepy lizard, *Tiliqua rugosa* (Chapter 4), the developing snapping turtle, *Chelydra serpentina* (—■ ·) (Chapter 5), the developing green sea turtle, *Chelonia mydas* (—□—) (Chapter 6), the mature leatherback sea turtle, *Caretta caretta* (■), the mature flatback sea turtle, *Natator depressus* (■) (Daniels *et al.*, 1996), the adult central netted dragon, *Ctenophorus nuchalis* (◆) (Daniels *et al.*, 1990), the adult fat-tailed dunnart, *Sminthopsis crassicaudata* (●) (Langman *et al.*, 1996), the adult rat, *Rattus norvegicus* (●) (Orgeig *et al.*, 1995), the newborn rat, *R. norvegicus* (○) (Chol/PC) (Suzuki *et al.*, 1978) and the adult human, *Homo sapiens* (○) (Doyle *et al.*, 1994).

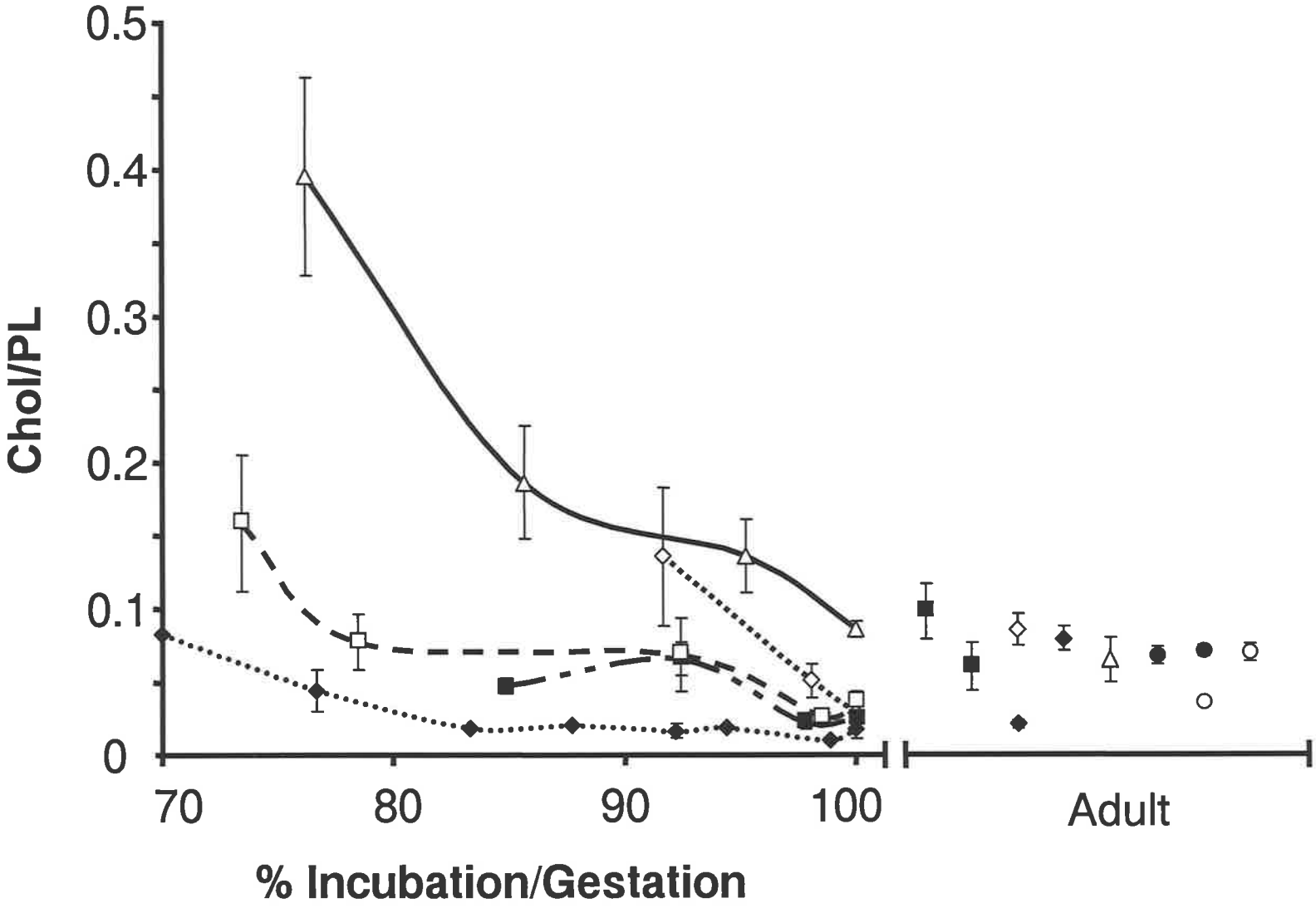
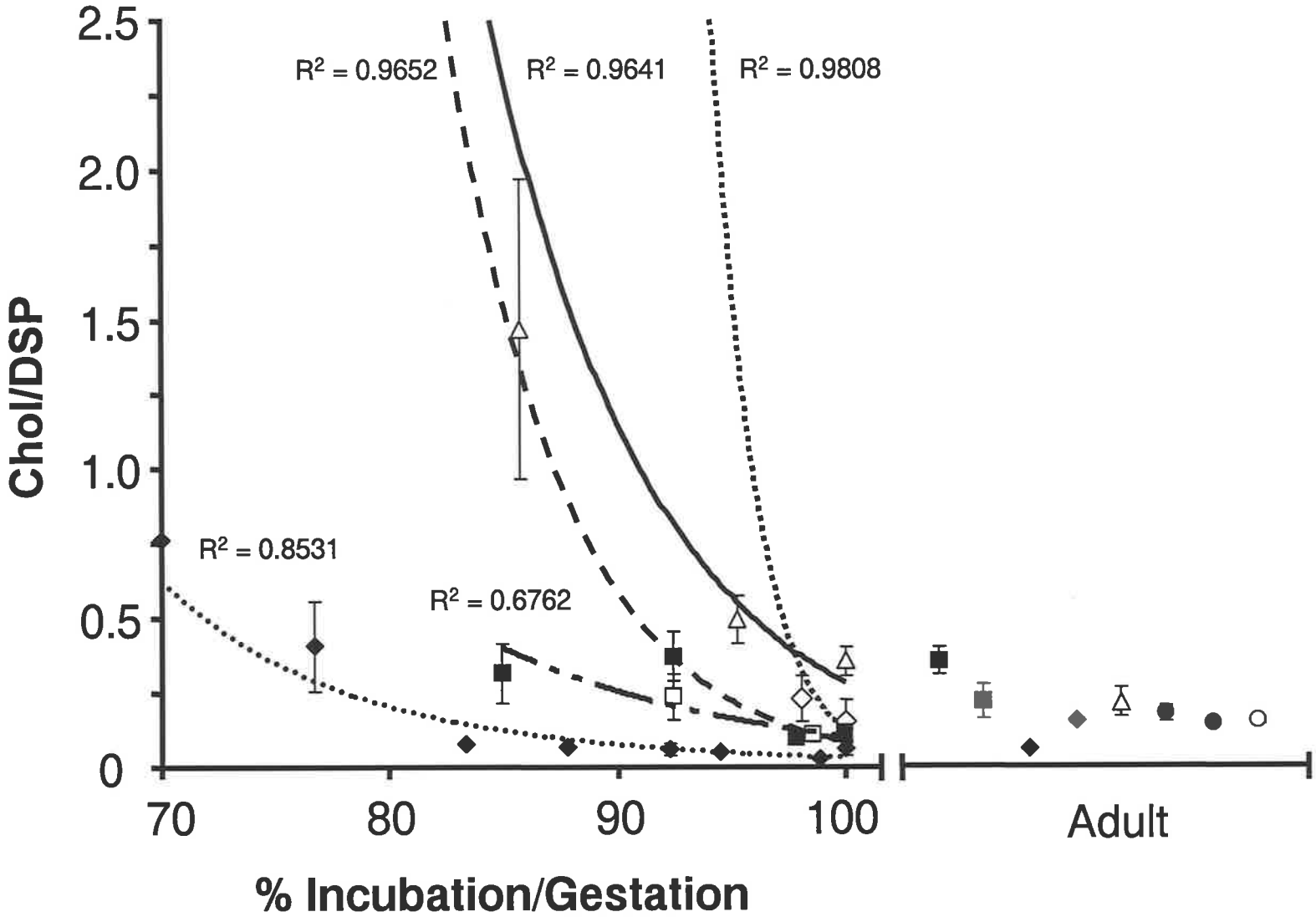

0.5
0.4
0.3
0.2
0.1
0
Chol/PL
70
80
90
100
Adult
% Incubation/Gestation

Figure 7.3 Cholesterol expressed as a ratio to disaturated phospholipid from lavage of the developing (—△—) and adult (△) chicken, *Gallus gallus domestica* [$y = 336907e^{-0.1399x}$, $P < 0.001$] (Chapter 2), the developing bearded dragon, *Pogona vitticeps* (··◇··) [$y = 6E+21e^{-0.5223x}$, $P < 0.001$] (Chapter 3), the developing (··◆··) and adult (◆) sleepy lizard, *Tiliqua rugosa* [$y = 507.49e^{-0.0972x}$, $P < 0.001$] (Chapter 4), the developing snapping turtle, *Chelydra serpentina* (—■ ·) [$y = 791.62e^{-0.0893x}$, $P \leq 0.05$] (Chapter 5), the developing green sea turtle, *Chelonia mydas* (—□—) [$y = 2E+07e^{-0.1932x}$, $P < 0.001$] (Chapter 6), the mature leatherback sea turtle, *Caretta caretta* (■), the mature flatback sea turtle, *Natator depressus* (■) (Daniels *et al.*, 1996), the adult central netted dragon, *Ctenophorus nuchalis* (◆) (Daniels *et al.*, 1990), the adult fat-tailed dunnart, *Sminthopsis crassicaudata* (●) (Langman *et al.*, 1996), the adult rat, *Rattus norvegicus* (●) (Orgeig *et al.*, 1995) and the adult human, *Homo sapiens* (○) (Doyle *et al.*, 1994).


2.5
2.0
1.5
1.0
0.5
0
Chol/DSP
R² = 0.9652
R² = 0.9641
R² = 0.9808
R² = 0.8531
R² = 0.6762
70
80
90
100
Adult
% Incubation/Gestation

Similarly, Chol remains constant during metamorphosis in the tiger salamander, *Ambystoma tigrinum,* while saturation of the phospholipids declines (Orgeig *et al.*, 1994). It is likely therefore that the timing of development of both the phospholipids and cholesterol differ between all species and that Chol is differentially regulated from the phospholipids. It does not appear that the sympathetic surge observed at birth in mammals, changes in ventilation, nor increases in alveolar pO_2 affect Chol secretion in non-mammalian amniotes. In addition, Chol and DSP components of surfactant in the alveolar compartment of rats (Orgeig *et al.*, 1995) and humans (Doyle *et al.*, 1994) are differentially released and appear independently regulated. Moreover, adrenaline and acetylcholine do not stimulate Chol secretion in the isolated perfused lung of lizards, nor from isolated type II cells of the lungfish, frog, lizard and dunnart (Wood *et al.*, 1995; Wood *et al.*, 1997; Wood *et al.*, 1999; Wood *et al.*, 2000). Clearly, the mechanisms regulating secretion of cholesterol differ from those regulating phospholipid homeostasis in both developing and mature lungs and such regulation appears to be species specific.

In all reptiles, the Chol/PL and Chol/DSP are comparable at pipping, despite marked differences in the timing of appearance and the relative proportions of the surfactant lipids (Figure 7.2,3). Thus, the amount of surfactant fluidiser relative to surface-active material present at pipping appears to be crucial for the initiation of airbreathing in all reptiles. Unlike chickens, the Chol/PL and Chol/DSP from hatchling bearded dragon, sea turtle and snapping turtle are lower than levels obtained for other closely related species and the adult bearded dragon (Figures 7.2,3), however they are comparable to the Chol/PC ratio of the newborn rat (Suzuki *et al.*, 1978) (Figure 7.2). It is likely therefore that the ratio necessary for lung clearance and the initiation of pulmonary ventilation differs from the ratio present once regular breathing patterns have been established. Perhaps clearing the lungs of fluid and filling them with air requires a different composition of pulmonary surfactant than adults. Furthermore, upon commencement of pulmonary ventilation, the lung must endure

a 4-5-fold increase in blood oxygen concentration, which may be associated with oxidative damage (Frank & Massaro, 1980). Pulmonary surfactant, is a target for oxidative injury from both endogenous and exogenous radicals, which may significantly impair its ability to control surface tension (Gilliard *et al.*, 1994). The unsaturated phospholipids, phosphatidylserine and phosphatydlinositol are particularly sensitive to oxidative damage, whereas DSP will protect the delicate inner lung from such injury (Goldstein, 1978). Therefore, a robust antioxidant enzyme system and a high level of saturated phospholipids may be crucial at the onset of breathing to protect against the sudden relative hyperoxia. Alternatively, the Chol/PL and Chol/DSP in adults may differ from those of newborns and hatchlings as a result of diet. Exogenous fatty acids modulate the composition of lung, macrophage and surfactant phospholipids in mammals (Palombo *et al.*, 1994). Moreover, maternal diet affects both phospholipid and surfactant protein synthesis in foetal mammal lungs (Viscardi, 1995; Kohri *et al.*, 1996). Thus, the composition of surfactant from the developing lung of oviparous and viviparous species that utilise yolk as the primary source of nutrition may differ from species with matrotrophic placentation.

Why hatchling chickens possess a greater proportion of cholesterol in their surfactant compared to other oviparous species remains unclear. Perhaps the elevated incubation and body temperatures of birds (40°C) affect the physical interactions between cholesterol and phospholipids. While cholesterol will aid fluidity of a surface film comprised of lipids below their phase transition temperature, it will increase the rigidity of the lipids above that temperature (Ladbrooke *et al.*, 1968). Therefore, a high content of cholesterol in birds may serve to stabilise the film where many lipids may be above their phase transition temperature. Given the non-compliant nature of the avian lung and that avian surfactant chiefly functions to prevent parabronchial flooding (Petrik & Reidel, 1968; Pattle, 1978), film stability may be of even greater importance to prevent migration of lipids from their site of action.

7.3 Differences between Species

The amount of the phospholipid and cholesterol recovered from the lungs of sleepy lizards and sea turtles during development greatly surpassed those of the other species. Similarly, sleepy lizards and sea turtles had large quantities of foetal lung fluid (FLF) throughout development. FLF accumulates in the lung over time to aid lung formation and maturation (Egberts *et al.*, 1981; Brown *et al.*, 1983). The FLF from sleepy lizards is highly viscous and this, in conjunction with the large lumen of the simple unicameral lung, may contribute to the accumulation of surfactant lipids within the lung fluid over time by reducing the rate of lipid turnover. Moreover, it is possible that the elevated content of surfactant plays a role in the liquid-liquid interactions between lung fluid and amniotic fluid with differing viscosities (Chapter 4). On the other hand, sleepy lizards and sea turtles may have a greater proportion of type II cells than the other species examined, resulting in larger quantities of total PL, DSP and Chol. Indeed, sea turtles have the most complex primary lung structure of all reptiles (Duncker, 1978b; Perry, 1989).

Although the absolute amounts of surfactant lipids differed between the turtles, the temporal pattern of DSP/PL appeared to be matched throughout development in both species. This matching suggests that the mechanisms regulating phospholipid metabolism are similar in the turtles. Additionally, temperature profoundly affects the behaviour of surfactant lipids. Snapping turtles experience a wider range of temperatures throughout their life history. Sea turtles on the other hand, experience less severe thermal gradients. It is possible therefore that snapping turtles are predisposed to greater temperature fluctuations than sea turtles and, consequently, their surfactant system may exhibit a greater degree of plasticity than their marine relative. The amount of surfactant recovered from marine turtles, *Caretta caretta* and *Natator depressus*, was an order of magnitude greater than the amounts recovered from the freshwater turtles, *Malaclemys geographica*

(Lau & Keough, 1981) and *Emydura krefftii* (Daniels *et al.*, 1996). Thus, while the regulation of the surfactant lipids appears similar between testudines, the differing amounts may be a result of lung morphology and the habitat of the animal.

Unlike the oviparous lizard, the viviparous skink appears to have a "mature" DSP/PL, Chol/PL and Chol/DSP ratio, like that of the adult, between 83% and 88% of gestation, indicating that the surfactant system is capable of functioning prior to the initiation of airbreathing. The lack of change in the composition of the surfactant lipids in the final ~20% of gestation also supports the proposal that surfactant accumulates within the FLF. As previously discussed (Chapter 4), the young of this species appear to be morphologically complete some weeks prior to birth and continue to grow towards the end of gestation. By extending the time spent *in utero*, the young are able to grow larger and therefore are born in a highly precocial state, which may reduce predation, provide greater locomotory skills and therefore lead to improved survivorship. Given that many viviparous species prematurely release young in times of trauma (Lombardi, 1998), the precocious maturation of the surfactant system ensures airbreathing can commence if aborted late in development. In contrast, the developmental pattern of surfactant lipids in the oviparous bearded dragon is highly truncated. Minimal phospholipid is present in the airspaces at 92% of incubation. Thus, the development of the surfactant system in this species is confined to the final 8% of incubation. The reasons for this remain obscure. However, embryonic bearded dragons do not share the same developmental stressors as developing sleepy lizards. Perhaps there is insufficient stimulation for surfactant secretion until the onset of breathing in this species, or the sympathetic surge associated with secretion may only occur at pipping. For whatever reason, it is clear that the development of the surfactant lipids is affected by birth strategy and the necessity to produce a functional surface film at the onset of airbreathing.

Differences in both the quantity and timing of appearance of the surfactant lipids during development are not surprising given the variation in respiratory surface area and respiratory volume between species. The volume of the avian respiratory system, including its associated airsacs, is extremely large when compared with that of the unicameral or multicameral lungs of similar sized reptiles. Moreover, the avascular airsacs are a great distance from the site of lung clearance within the lung. Thus, the temporal differences in the secretion of phospholipids and Chol between species may be a result of differing lung volumes and clearance between the parabronchial lung of birds, the multicameral lung of turtles and unicameral lung of lizards. Furthermore, surfactant aids in lung fluid removal by maintaining the functional residual capacity of the bronchoalveolar lung of mammals. However, this may not be physiologically relevant in the non-compliant lung of birds, nor in reptilian lungs that are routinely collapsed at the end of expiration (Seymour *et al.*, 1981; Daniels *et al.*, 1994a).

7.4 Additional Measures

In addition to the biochemical data, the type II cell exhibits similar morphological patterns of development in mammals, lizards (Chapter 3) and turtles (Perry *et al.*, 1989). Furthermore, the composition of the surfactant lipids, the lipid precursors, the regulation and the function of the system appear to be remarkably similar throughout development within the amniotes. Both avian SP-A mRNA and testudian SP-B cross-reacted with mammalian probes (Chapter 2) and antibodies (Chapter 5), suggesting that these proteins are highly conserved among the vertebrates. While conservation of SP-A has been confirmed previously among the vertebrates (Sullivan *et al.*, 1998), this is the first time that SP-B has been demonstrated in reptiles (Chapter 5). The timing of development of the surfactant proteins, particularly SP-A, appears to differ among mammals and chickens, which may relate to the immune function of this surfactant protein in different vertebrates.

However, it appears that the control of gene expression of the surfactant proteins and lung morphogenesis are conserved by at least one transcription factor, TTF-1, throughout the amniotes. Indeed, recent studies by Miller *et al.* (2001) have shown that mammalian TTF-1 antibodies are expressed in subsets of cells within the respiratory epithelium of axolotl lungs, further supporting the conservation of TTF-1. Antioxidant enzymes, like the surfactant proteins and TTF-1, are highly conserved. In the present study, it has been shown that while the enzymes themselves appear to be conserved in viviparous lizards, their chronological development differs markedly from that of the surfactant lipids, demonstrating that the development of these two ancient systems has been honed throughout evolution to match the specific biology of the animal. Furthermore, environmental cues, such as mild hypoxia, do not affect composition of surfactant in sea turtles, demonstrating that the development of the system is robust in this species and unlikely to be selected against.

7.5 Conclusions

From the findings of the present study, it is clear that the developmental pattern of the pulmonary surfactant system is conserved among the amniotes. This similarity is demonstrated by an increase in the amount and saturation of the phospholipids during the final stages of development and the independent regulation of cholesterol. However, the relative timing of the development of the lipids differs markedly between different species and appears to relate to the specific biology of the animal. Moreover, SP-A mRNA appeared earlier in chicken lung development relative to mammals. The viviparous skink exhibited a precocious composition of the surfactant lipids compared with the oviparous species, yet the bearded dragon demonstrated a highly truncated pattern of development. Thus, birth strategy appears to have a profound affect upon the development of the pulmonary surfactant system in reptiles. Similarly, the onset of breathing appears to

trigger secretion of phospholipids within the amniotes however, the control of release in reptiles and birds differs from that of mammals. Moreover, the composition of surfactant is astoundingly uniform at the onset of airbreathing within the amniotes, despite differences in lung morphology, birth strategy and phylogeny and, therefore, provides the crucial combination of surface-active and fluidising lipids to produce a surface film that promotes the initiation of airbreathing. The necessity to clear the lungs of fluid, coupled with the relative hyperoxia associated with birth and hatching, may lead to differences in the composition of this incredible system between hatchlings and mature animals.

7.6 Future Directions

Given the overall parallelism in the development of the pulmonary surfactant lipids and the overwhelming similarity in the Chol/PL and Chol/DSP ratio in newborn and hatchling amniotes, it would be of interest to determine whether the phospholipid headgroups and molecular species also develop in a similar manner. In turn, the maturity of the non-mammalian foetal lung could be tested using established indices for foetal lung maturity in mammals such as PC/S or PG/PI ratio. Due to the massive surge in PC during the latter portion of mammalian lung development, a PC/S ratio greater than 2 is indicative of foetal lung maturity (Kulovich *et al.*, 1979). Similarly, there is a characteristic fall in the content of PI, with a concomitant rise in PG throughout development (Kulovich *et al.*, 1979). If compatible with previous lung maturity tests, the Chol/PL and Chol/DSP could provide a simple test for foetal lung maturity that is universal among the amniotes.

In all species studied, cholesterol does not appear to be released in concert with the surfactant lipids, suggesting that it is differentially regulated from the phospholipids. In addition, the origin of cholesterol remains poorly understood (Section 1.2.1.2.2). Clearly, more studies are required to determine the origin and mechanism for control of surfactant cholesterol so that we may gain better understanding of its function in the amniote lung.

From the present studies, and others (Orgeig *et al.*, 1994), it would seem that there are substantial differences in the development of the surfactant system in metamorphosing species when compared to the amniotes. The tiger salamander uses its lung to aid buoyancy during early stages of development. Therefore, it would be of interest to test whether the final composition of surfactant is dictated by the transition from a fluid-filled to air-filled organ or by the maturation of structural components of the lung and its subsequent use as a respiratory organ.

The conservation and development of the surfactant proteins provides a new and novel area of research in comparative surfactant physiology. Of the surfactant proteins, only one (SP-A) has been characterised in representatives of every vertebrate lineage (Sullivan *et al.*, 1998). Mature SP-B has been described in the lungs of chickens (Zeng *et al.*, 1998), turtles (Chapter 5), axolotls (Miller *et al.*, 2001) and more recently the tarpon swimbladder and the snail gas mantle (Sullivan, unpublished data). However, SP-C and its preprotein have not been detected in lungs other than the bronchoalveolar lung of mammals. Indeed, SP-C could not be detected in the adult chicken lung (Bernhard *et al.*, 2001). Similarly, immunostaining of proSP-C did not occur in the developing chicken lung (Whitsett, pers comm.), nor adult axolotl lung (Miller *et al.*, 2001), suggesting that the protein did not cross-react with the antiserum that was generated against human proSP-C (Miller *et al.*, 2001) or it is not expressed in these species. Thus, it is possible that lung types other than the bronchoalveolar of mammals do not require SP-C to function, or the synthetic pathway for SP-C differs among the vertebrates. Recently, cDNAs encoding the surfactant proteins, SP-A, SP-B and SP-C, were isolated from the anuran lung (Whitsett, pers. comm.). It most likely therefore that SP-C is synthesised differently among the vertebrates. Despite being an exclusive marker for mammalian type II pneumocytes, and having a high degree of conservation among mammals, SP-C is possibly the least conserved of the surfactant proteins among other vertebrates.

SP-D has been detected in subsets of cells within the respiratory epithelium of the axolotl (Miller *et al.*, 2001), however its presence and function have not been described in the remaining vertebrate groups. SP-D maintains lipid homeostasis in the mammalian lung (Botas *et al.*, 1998; Korfhagen *et al.*, 1998; Ikegami *et al.*, 2000). Thus, its function may be of great importance in non-mammalian vertebrates, which possess 70 times more surfactant per unit surface area than mammals (Daniels *et al.*, 1995a). Thus, it is pertinent to test whether all surfactant proteins are present in the gas-holding structures of vertebrates and invertebrates. The synthesis and the development of these proteins may then be determined, however such experiments are limited by the specificity of antibodies and/or the ability to sequence the DNA and raise appropriate probes.

APPENDICES

APPENDIX 1 ABBREVIATIONS

ANOVA	analysis of variance
AOE	antioxidant enzyme
BM	body mass
Chol	cholesterol
cDNA	complementary deoxyribonucleic acid
D&H	Dufaure and Hubert, (1961)
DL	dry lung
DLW	dry lung weight
DPPC	dipalmitoylphosphatidylcholine
DSP	disaturated phospholipid
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
FLF	foetal lung fluid
GPx	glutathione peroxidase
GSH	glutathione, reduced
GSSG	glutathione, oxidised
H	hypoxia
HNF	hepatocyte nuclear factor
HPLC	high performance liquid chromatography
LB	lamellar body
M	maternal
mRNA	messenger ribonucleic acid
N	normoxia
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NG	non gravid
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PL	phospholipid
pO_2	partial pressure of oxygen
PS	phosphatidylserine
RDS	respiratory distress syndrome
SEM	standard error of mean
SOD	superoxide dismutase
SP	surfactant protein
SSC	saline sodium citrate
SDS	sodium dodecyl sulphate
TEM	transmission electron microscope
TTF-1	thyroid transcription factor-1

1.1 Lipid Extraction from Bligh and Dyer (1959)

Lyophilised lavage was reconstituted in 2 ml of distilled (Millipore) water and transferred to an extraction tube. The initial container was rinsed with 5 ml of methanol to remove any remaining sample and then transferred to the extraction tube. A 2.5 ml volume of chloroform was added to the extraction tube and the sample was vortexed. An additional 2.5 ml of chloroform and 2.5 ml of water was added. The mixture was vortexed again and centrifuged (Beckman model TJ-6 centrifuge) at 800 *g* for 15 min at 4°C to separate the phases. The upper aqueous layer was discarded and the lower organic layer was retained, then dried under nitrogen gas and reconstituted in 1 ml of chloroform. The resultant working solution was stored at –30°C until lipid analyses were performed.

1.2 Phospholipid Analysis from Bartlett (1959)

Sample aliquots from the working solution (Appendix 2, section 1.1) were dried down in test tubes. Two ml of distilled (Millipore) water and 0.25 ml of 10N sulfuric acid was added. The tubes were heated to 160°C for 3 h to break-down phospholipids into inorganic phosphorus. Following the incubation, 100 µl of hydrogen peroxide was added to clear the solution of charred material and the samples were incubated at 160 °C for an additional 90-120 min. Samples were cooled, 2.5 ml of 0.33% ammonium molybdate (0.33%) and 200 µl of Fiske-Subbarrow reagent was added and then heated in a boiling water bath for 10 min to develop the colour. The optical density was measured at an absorbance of 825 nm on double-beam spectrophotometer (Hitachi-U 2000). Standards consisted of known concentrations of potassium dihydrogen phosphate. The amount of phosphorus in the samples was calculated from the regression of the standards. Total phospholipid was

calculated by multiplying the content of phosphorus by 25, as phospholipids comprise approximately 4% phosphorus. Samples were measured in triplicate where possible.

1.3 Disaturated Phospholipid Analysis from Mason *et al.* (1976)

Sample aliquots from the working solution (Appendix 2, section 1.1) were dried under nitrogen gas and reacted with 500 µl of a 6.2% solution of osmium tetroxide made up in carbon tetrachloride for 15 min to bind with unsaturated phospholipids to form a ligand complex. Any unreacted osmium tetroxide was evaporated under nitrogen gas. The remaining precipitate was reconstituted in 200 µl of chloroform:methanol (20:1, vol/vol). Samples were applied to columns containing packed glass wool and 0.8 g neutral aluminium oxide (activity I) which irreversibly binds the complex unsaturated lipids. The neutral lipid fraction was eluted with 10 ml of chloroform:methanol (20:1, vol/vol) and retained at –30°C for further analyses. The disaturated phospholipid fraction was then eluted with 5 ml of choloform:methanol:7M aqueous ammonia (70:30:2, vol/vol/vol). The eluant was dried under nitrogen gas, reconstituted in 400 µl of chloroform and analysed for phosphorus (Appendix 2, section 1.1).

1.4 Isolation of total RNA using Tri Reagent™ (Sigma Chemical Co.)

Between 50-100 mg of frozen lung tissue was homogenised in 1 ml of Tri Reagent™ using a Polytron homogeniser. Samples were allowed to stand for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes. The homogenate was transferred to new tubes and 0.2 ml of isoamyl alcohol free-chloroform (Sigma Catalog No. C-2432) was added to each sample. Tubes were covered and shaken vigorously for 15 sec and allowed to stand for a further 2-15 min at room temperature. The mixture was separated into three phases by centrifugation at 12 000 *g* for 15 min at 4°C. The lower

organic phase contained the proteins, the interface contained DNA and the upper aqueous phase contained the total RNA. The aqueous phase from each sample was transferred to a fresh tube and 0.5 ml of isopropanol was added. The samples were allowed to stand for 5-10 min at room temperature then centrifuged at 12 000 *g* for 10 min at 4°C to pellet the RNA precipitate. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol, vortexed and centrifuged at 7000 *g* for 5 min at 4°C. The ethanol was discarded and the pellet was allowed to air-dry. When dry, the pellet was dissolved in an appropriate volume of sterilised water by repeated pipetting with a micropipette. The concentration and purity of the total RNA was determined by absorbance at 260 nm.

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