Environmental Selection Pressures Shaping the Pulmonary Surfactant System of Adult and Developing Lungs

S. Orgeig and C.B. Daniels

Abstract Pulmonary surfactant is comprised of lipids and proteins. Environmental variables — temperature, pressure and hypoxia — represent powerful evolutionary selection pressures that have shaped the evolution of the system in adult and developing lungs. We review how the composition, structure and function of surfactant changes dramatically in response to temperature. Physical forces, e.g. stretching of the alveolar basement membrane, fluid distension of the lung during development, or cyclical compression and relaxation of the lipid film also affect the secretion, maturation and physical function of surfactant. Here we also review how high hydrostatic pressures experienced by diving mammals influence the molecular, biochemical, cellular and physiological characteristics of surfactant. Also, hypoxia predominantly exerts its effect on maturation of the surfactant lipids and proteins via the endocrine system in developing organisms. However, the influence of hypoxia on the adult surfactant system is unknown. We summarise the major discoveries concerning how temperature, pressure and hypoxia have influenced the pulmonary surfactant system.

1 Introduction

The pulmonary surfactant system is a complex, integrated, highly conserved, yet dynamic system that has lent itself well to both evolutionary and mechanistic studies. The surfactant system is comprised of the alveolar epithelial type II cell which synthesises, stores and releases the complex mixture of lipids and proteins that form a dynamic lipid–protein film at the air–liquid interface of the lung, to regulate surface tension (Daniels and Orgeig 2001). This system is highly conserved among

S. Orgeig (✉)
Sansom Institute, School of Pharmacy and Medical Sciences, R6-18 City East Campus, University of South Australia, Adelaide SA 5001, Australia, E-mail: sandra.orgeig@unisa.edu.au

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the vertebrates, and we have shown that it probably predated the evolution of lungs and swimbladders (Daniels et al. 2004). We have investigated the evolution of the pulmonary surfactant system from a morphological, molecular (gene, protein and lipid), cellular, developmental and biophysical perspective (Sullivan et al. 1998, 2003; Daniels and Orgeig 2001; Johnston and Daniels 2001; Daniels et al. 2004; Lang et al. 2005a; Foot et al. 2006; Orgeig et al. 2007). In particular we have concentrated on the roles of three specific environmental variables that represent powerful evolutionary selection pressures — temperature, pressure and hypoxia — in shaping the evolution of the system in adult and developing lungs. Interpreting how an environmental selection force, might select for modifications of a physiological system is made difficult by the fact that this system is composed of multiple components each of which may be affected differently by the selection force and they will also interact with each other. Moreover, the possibility of multiple physical or phenotypic effects resulting from the alteration of a single gene, a process known as pleiotropy, may further complicate the elucidation of the evolution of a particular system (Foot et al. 2006). Nevertheless, by careful selection of species and comprehensive analyses of different components, we have built up a significant body of knowledge concerning how the environment has shaped the evolution of the surfactant system in adult and developing vertebrate lungs. In this review we first provide a brief overview of the general biology of the vertebrate pulmonary surfactant system before summarising the major discoveries concerning how temperature, pressure and hypoxia have influenced the pulmonary surfactant system.

1.1 The Structure and Composition of the Pulmonary Surfactant System

Pulmonary surfactant is a complex mixture of phospholipids (PL), neutral lipids, particularly cholesterol (Chol), and proteins. The phospholipids which make up ~80% by weight of surfactant consist predominantly of phosphatidylcholine (PC). The most abundant individual molecular species in most mammalian surfactants is the disaturated phospholipid (DSP), dipalmitoylphosphatidylcholine (DPPC). The lipids and proteins are assembled in the endoplasmic reticulum and the Golgi apparatus of alveolar type II cells, and are stored in lamellar bodies until exocytosis (Fig. 1). The lamellar bodies consist of a dense proteinaceous core with lipid bilayers arranged in concentric, stacked lamellae surrounded by a limiting membrane. After the lamellar bodies have been released into the alveolar space, they swell and unravel into a characteristic cross-hatched structure, termed tubular myelin (Fig. 1). It is this structure that supplies the lipids for the surface film which regulates the surface tension of the liquid lining of the lung (Goerke 1998).

The protein component of pulmonary surfactant represents about 10% by weight, and four surfactant proteins have been described. These are surfactant protein-A (SP-A), SP-B, SP-C and SP-D, all of which are synthesised in alveolar type II cells and are associated with purified surfactant (Haagsman and Diemel 2001). Both the

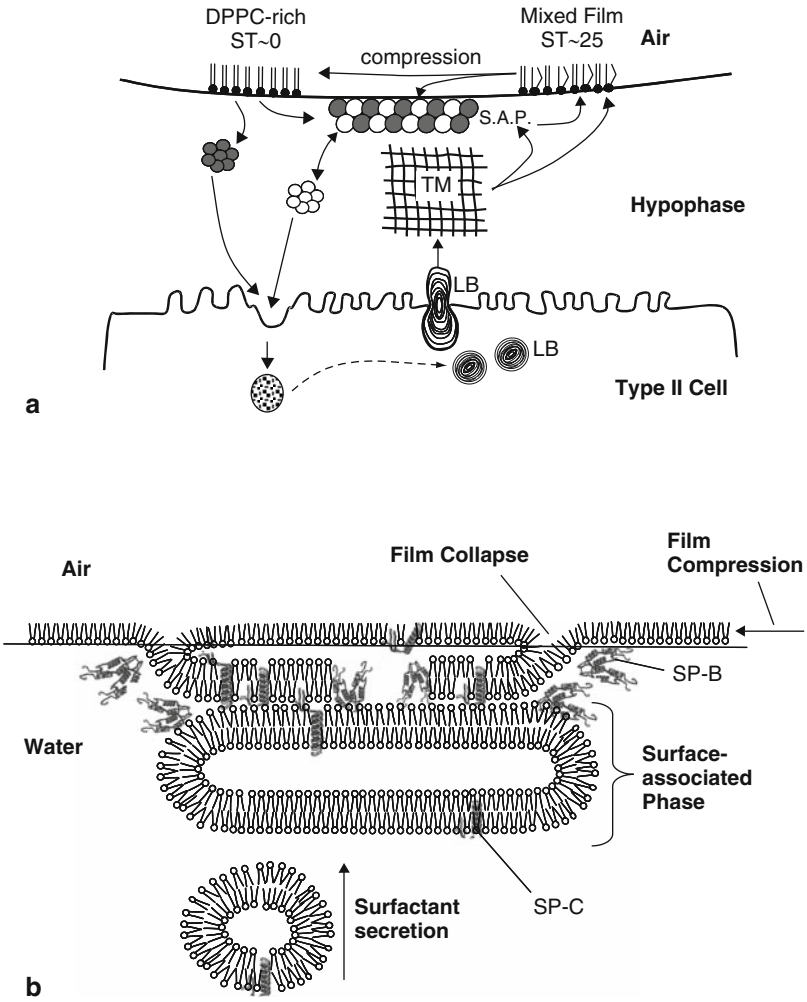

Fig. 1 **a** Schematic diagram of the life cycle of pulmonary surfactant. Pulmonary surfactant is packaged in lamellar bodies (*LB*) that are secreted into the liquid lining the alveoli (*hypophase*) via exocytosis across the type II cell plasma membrane. Here the lamellar bodies swell and unravel, forming a crosshatched structure, termed tubular myelin (*TM*), which consists of lipids and proteins. This structure supplies lipids to the surface film at the air–liquid interface as well as the surface-associated phase (*S.A.P.*), which functions as a reservoir of lipids and proteins. As the mixed molecular film is compressed, lipids are squeezed out of the film into the S.A.P. to produce a DPPC-enriched film, which is capable of reducing surface tension (γ) to near $0\,\mathrm{mN\,m^{-1}}$. Upon reexpansion, some lipids from the S.A.P. re-enter the surface film. Lipids from the surface film and the S.A.P. are eventually recycled and taken back up by the type II cell via endocytosis. **b** Hypothetical model of the surfactant film and surface-associated phase demonstrating film collapse under dynamic compression. The interaction of the two hydrophobic surfactant proteins (*SP-B* and *SP-C*) with the lipid mono- and bilayers is indicated. This interaction aids in the regulation of movement of lipids between the interfacial monolayer and the surface-associated phase. Figure reproduced from Foot et al. 2006 with permission from Elsevier

secretion and the reuptake of surfactant phospholipids into type II cells appear to be regulated by SP-A. Both SP-A and SP-B are essential for the formation and structural integrity of surfactant components. The hydrophobic surfactant proteins, SP-B and SP-C, strongly interact with the lipids and promote the formation and adsorption of the surface film to the air–liquid interface (Haagsman and Diemel 2001). They are integral to the regulation of the movement of the surfactant lipids between the surface-associated phase, the multilayer structure in the hypophase associated with the surfactant film, and the interfacial surfactant film itself (Fig. 1). Hence, they are intricately involved in the surface tension lowering function of the entire system. However, the hydrophilic surfactant proteins, SP-A and SP-D, are predominantly involved in the innate host defense system of the lung (Haagsman and Diemel 2001).

1.2 The Function of the Pulmonary Surfactant System

The main function of the pulmonary surfactant system is to reduce and vary the surface tension at the air–liquid interface. When a film is formed initially, either in vivo or in vitro, as the surface active molecules adsorb to the air–liquid interface, the surface tension is lowered from approximately $70\,\mathrm{mN\,m^{-1}}$ (surface tension, denoted γ, of pure water) to approximately $25\,\mathrm{mN\,m^{-1}}$, defined as the equilibrium surface tension (γ_{eq}). When the film is dynamically compressed, the surface area and hence the surface tension are reduced. The lowest surface tension that can be measured at the lowest surface area is the minimum surface tension (γ_{min}). The extent, expressed as a %, of surface area compression required to reach γ_{min} is known as the % surface area compression (%SAcomp).

The ability to lower and vary surface tension with changing surface area is attributed to the interactions between the disaturated phospholipids (DSP), particularly the most abundant surfactant lipid, dipalmitoylphosphatidylcholine (DPPC) and the other lipids, such as the unsaturated phospholipids (USP) and Chol. Upon expiration, dynamic compression of the mixed surfactant film results in the 'squeezing out' of USP and Chol, resulting in a DPPC-enriched surface film (Fig. 1). The DPPC molecules can be compressed tightly together by virtue of their two fully saturated fatty acid chains. In so doing, they exclude water molecules from the air–liquid interface, thereby virtually eliminating surface tension (Possmayer 2004). Hence, DPPC is regarded as the main surface active component of pulmonary surfactant.

The level to which surface tension reduction occurs in vivo depends very much on the type and function of the particular vertebrate lung (Daniels et al. 1998a; Daniels and Orgeig 2001). For example, in the mammalian lung, with its approximately spherical alveoli, the main function of the surfactant system is to promote alveolar stability by promoting equivalent transmural pressures among interconnected alveoli (Bachofen and Schürch 2001). This alveolar stability function is best served by very low surface tensions (Schürch et al. 2001). Furthermore, these low surface tensions ensure small transmural pressures which reduce the potential for the transudation of interstitial fluid into the alveolus, i.e. the formation of pulmonary edema

(Staub 1983). On the other hand, in animals with much larger and non-spherical respiratory units, e.g. lizards that have faveoli, which are much larger, relative to body size, than alveoli (McGregor et al. 1993; Daniels et al. 1994a), the predominant function of the pulmonary surfactant system is to act as an anti-adhesive (Daniels et al. 1995b,1998a), preventing the delicate epithelial surfaces from adhering to each other during the expiratory phase of the breathing cycle. For such a function it is not essential that surfactant reduces surface tension to extremely low values, as the relative forces are not as great nor the radii of curvature as small, as those in the lung of similar sized mammals (Daniels et al. 1998a). Hence, to fulfil different functions in different animals, the surfactant composition must vary between species.

1.3 The Control of the Pulmonary Surfactant System

The cellular secretion of surfactant is regulated primarily by the autonomic nervous system, via β_2-receptors and by physical forces, particularly stretch of the basement membrane of the alveolar type II cell, associated with ventilation (Mason 1998). The lung distension brought about by the accumulation of fetal lung fluid may also stimulate the production of surfactant lipids during the latter part of gestation (Torday et al. 1998). In addition, there are numerous other biochemical factors that mediate surfactant secretion, including ATP, mediated via purinergic receptors, metabolites of arachidonic acid, calcium ionophores, endothelin-1, vasopressin, lipoproteins and phorbol esters (Orgeig et al. 2004).

There are a number of mechanisms and neurohormonal factors that regulate the maturation of the surfactant system during fetal development. However, the major mechanism is via glucocorticoids which are increasingly produced by the fetal adrenal cortex during late gestation (Winter 2004). Administration in vivo of glucocorticoids to the fetus decreases respiratory distress probably by increasing surfactant phospholipid synthesis via stimulation of the synthetic enzymes and the secretion of phospholipids into the alveolar compartment (Rooney 2004). Administration of exogenous glucocorticoids also stimulates the extent and rate of surfactant protein gene expression (Orgeig et al. 2004). In addition, glucocorticoids enhance or accelerate other indicators of lung maturation, such as alveolarisation (Snyder 2004), the rate of fluid clearance (Wallace et al. 1995) and the density and expression of β-adrenergic receptors (Cheng et al. 1980; Maniscalco and Shapiro 1983). Importantly, it is possible for the expression of glucocorticoids to be altered by environmental stressors, such as hypoxia, which can affect the outcome of lung and surfactant maturation in the fetus, e.g. in growth-restricted fetuses (Orgeig et al. 2004). Other neuro-hormonal and growth factors that stimulate surfactant maturation include adrenergic agonists, thyroid hormones, thyrotropin-releasing hormone, prolactin, estrogen, leptin, parathyroid hormone, insulin-like growth factor, epidermal growth factor and keratinocyte growth factor (Rooney 2004).

1.4 Environmental Influences on the Surfactant System

The function of the pulmonary surfactant system is reliant on the physical interactions of the lipid and protein components. Hence, any environmental force that is capable of altering or influencing these interactions has the potential to exert a long-term selection effect on the pulmonary surfactant system. The three most widely examined environmental forces have been temperature, pressure and hypoxia.

Temperature has a direct and profound effect on the physical state of lipids. Specifically, all lipids can exist in one of two physical states, i.e. either the fluid, disordered (liquid-expanded) state or the ordered, solid (liquid-condensed) state. The transition between these two phases occurs at the phase transition temperature (T_m) of that lipid. In surfactant the major phospholipid, DPPC, has a T_m of 41°C. Hence, at all biologically relevant temperatures, a highly enriched DPPC film at the end of expiration will exist in the ordered gel form. However, in order for the surfactant film to spread over the expanding surface area upon inspiration, the lipids must exist in the disordered, spreadable state. The addition of other lipids, e.g. Chol or USP, into the surface film upon inspiration, lowers the T_m of the lipid mixture, enabling it to exist in the liquid-expanded state at the same body temperature. In this state, the lipids are able to disperse to coat the surface of the expanding fluid layer. Hence, the body temperature of the animal is crucial in determining the physical state of the lipids. Moreover, on an evolutionary scale, it is crucial that the selected lipid composition is appropriate for the thermal biology of the animal, so that surfactant can function optimally. For a general review of phospholipid thermal properties see Chapman (1973, 1975), and for a detailed description of the biophysical properties of pulmonary surfactant see Possmayer (2004).

Physical forces, such as stretching of the alveolar basement membrane (Edwards 2001) or distension of the lung by fetal lung fluid during development (Hooper and Wallace 2004), have profound effects on the secretion and maturation of surfactant, respectively. In addition, the regular compression and relaxation of the lung upon expiration and inspiration exert significant cyclical forces on the lipids of the surfactant film directly (Possmayer 2004). Hence, any environmental force that alters these physical forces, such as changes in the surrounding hydrostatic pressure on an organism, has the potential to alter the function of the pulmonary surfactant system (Foot et al. 2006).

The influence of hypoxia on the function of the pulmonary surfactant system is less intuitively obvious because this factor does not interfere physically with the interaction of the surfactant lipids. Hypoxia exerts its effect on the development and maturation of the surfactant lipids and proteins via the endocrine system (Orgeig et al. 2004). Hence, the effect of hypoxia is most profoundly evidenced in developing organisms, which have been widely used as experimental models.

2 Temperature

Temperature has a profound influence on the structure and function of surfactant lipids (Lang et al. 2005a). Given that the majority of animals have much lower body temperatures than homeothermic mammals, comparative biologists have questioned how animals with low and/or fluctuating body temperatures can regulate the fluidity of their surfactant film. Hence, of all the evolutionary selection pressures, temperature and its effect — short term, long term and on an evolutionary scale — on the pulmonary surfactant system has been the best documented. In the early 1980s, Kevin Keough and his students working with turtles and squirrels investigated the effect of long term (of the order of weeks) thermal changes on the pulmonary surfactant system. Chris Daniels in the late 1980s initiated our long-term research into the comparative thermal biology of pulmonary surfactant by examining the effect of relatively short-term, i.e. of the order of hours, changes in body temperature on the lipid composition of the pulmonary surfactant system in lizards. Hence, temperature has held a long-standing and enduring interest for surfactant researchers for nearly 30 years.

2.1 Temperature: A Selection Force for the Evolution of Surfactant Lipid Composition across the Vertebrates

We have previously discussed and reviewed in detail the pattern of changes in surfactant lipid composition across the vertebrate groups (Daniels et al. 1995b, 1998b; Daniels and Orgeig 2001, 2003; Lang et al. 2005a). Our interpretation of these broad-scale evolutionary changes is that it is the interaction of the body temperature preferenda together with the physicochemical interactions of the major surfactant lipids — the saturated and unsaturated phospholipids and cholesterol — which has placed evolutionary constraints upon the system and hence have driven the evolution of surfactant lipid composition. Briefly, the patterns of surfactant lipid composition across the vertebrate groups are such that, when this composition is expressed as a percentage of total phospholipid (PL), fish contain much greater levels of cholesterol (Chol) and unsaturated phospholipid (USP) than members of the other vertebrate groups, and the percentage of Chol relative to disaturated phospholipids (DSP) decreases 10- to 15-fold in the tetrapods (Daniels et al. 1995a) (Fig. 2).

Animals with lower preferred body temperatures have much higher ratios of Chol/DSP in their surfactant (e.g. fish and amphibians, generally $T_b < 25°C$) than animals with 'warm' body temperatures (some reptiles, birds and mammals, $T_b \sim 37°C$) (Daniels and Orgeig 2001) (Fig. 2). This pattern is consistent across the vertebrates, despite differences in lung structure and phylogeny, and is undoubtedly a result of the thermal and biophysical properties of the surfactant lipids. Cholesterol-rich surfactant can function at low body temperatures because cholesterol lowers the phase-transition temperature of the lipid mixture over a broad

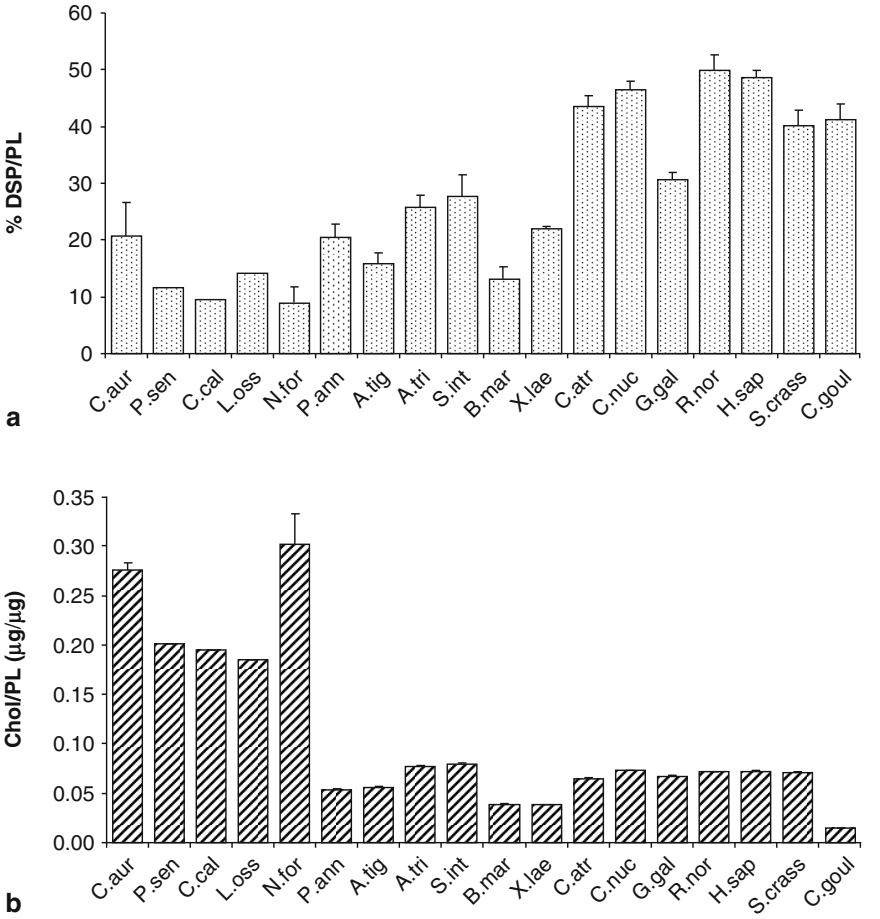

Fig. 2 Relationship of (**a**) disaturated phospholipid as a % of total phospholipid (%DSP/PL) and (**b**) cholesterol as a fraction of total phospholipid (Chol/PL, µg/µg) during the evolution of the vertebrates. Data are presented as mean ± SEM. Animals are grouped into (1) fish: *C. aur* (=*Carassius auratus*, goldfish) (Daniels and Skinner 1994), *P. sen* (=*Polypterus senegalensis*, bichir), *C. cal* (=*Calamoicthys calabaricus*, ropefish), *L. oss* (=*Lepisosteus osseus*, gar) (Smits et al. 1994), (2) lungfish: *N. for (=Neoceratodus forsteri*, Australian lungfish), *P.ann* (=*Protopterus annectens*, African lungfish) (Orgeig et al. 1995), (3) amphibians: *A. tig* (=*Ambystoma tigrinum*, tiger salamander) (Orgeig et al. 1994), *A. tri* (=*Amphiuma tridactylum*, three-toed salamander), *S.int* (=*Siren intermedia*, two-toed salamander), *B. mar* (=*Bufo marinus*, cane toad), *X. lae* (= *Xenopus laevis*, African clawed toad) (Daniels et al. 1994b), (4) reptiles: *C. atr* (=*Crotalus atrox*, rattlesnake) (Daniels et al. 1995c), *C. nuc* (=*Ctenophorus nuchalis*; central netted dragon) (Daniels et al. 1990), (5) birds: *G. gal* (=*Gallus gallus*, chicken) (Johnston et al. 2000), and (6) mammals: *R. nor* (=*Rattus norvegicus*, rat) (Orgeig et al. 1995), *H. sap* (=*Homo sapiens*, human) (Doyle et al. 1994), *S. crass* (=*Sminthopsis crassicaudata*, fat-tailed dunnart) (Langman et al. 1996), *C. goul* (=*Chalinolobus gouldii*, Gould's wattled bat) (Codd et al. 2000b). The lizard, the dunnart and the bat were at their warm-active body temperature (33–37°C)

range of temperatures and acts as a fluidiser at the air–liquid interface (Langman et al. 1996; Lopatko et al. 1998, 1999). Specifically, Chol disrupts the van der Waal's forces between adjacent PL fatty acid chains and forces the mechanical separation of PL head groups (Presti et al. 1982), which is thought to enhance adsorption and promote surfactant respreading upon inspiration (Orgeig and Daniels 2001). USPs also have much lower phase transition temperatures and can increase surfactant fluidity at low temperatures (Daniels et al. 1998a). There is, however, an evolutionary trade-off between temperature and the relative amount of saturated PL, as the addition of USP or Chol at low temperature will also decrease the surface-tension lowering ability of the surfactant mixture (Daniels et al. 1998a, b; Lopatko et al. 1998, 1999). In non-mammalian vertebrates, a decrease in surface activity is a feasible evolutionary trade-off, as pulmonary surfactant in these lungs has primarily an 'anti-adhesive' function which does not require very low surface tensions. However, in heterothermic mammals that are capable of varying their body temperature, as they alternate between periods of torpor and activity, the surfactant must be fluid at cold body temperatures, yet still remain surface-active (Lang et al. 2005a). Hence, the most interesting evolutionary question is: how do heterothermic animals — both ecto- and endotherms — regulate surfactant fluidity and surface activity during periods of activity and torpor?

2.2 Temperature: A Selection Force for Acute Changes in Surfactant Composition, Structure and Function Within Individuals

2.2.1 Surfactant Lipid Composition

Keough and colleagues (Lau and Keough 1981; Melling and Keough 1981) were the first to demonstrate that surfactant lipid composition could alter in response to relatively short-term changes in body temperature. Lau and Keough (1981) observed that surfactant collected from cold-acclimated map turtles, *Malaclemys geographica*, after 2–3 months of hibernation was less lavageable and higher in unsaturated fatty acids than surfactant from warm-acclimated turtles. Since then, our group have demonstrated that the central netted dragon, *Ctenophorus nuchalis*, doubles the Chol/PL ratio in its surfactant after a 4 h decrease in body temperature from 37°C to 14°C (Daniels et al. 1990). Hence, whether individuals increase the relative concentration of Chol or USP, we have hypothesised that these changes reduce the phase-transition temperature, thereby reflecting homeoviscous adaptations in the surfactant of these ectothermic animals. Although the actual effect of these compositional changes on the biophysical properties and behaviour of the surfactant has not been addressed in lizards, we have completed many such studies on heterothermic mammals.

In heterothermic mammals, the surfactant system is very dynamic and responds rapidly to changes in physiological conditions experienced throughout torpor and

activity (Codd et al. 2000a). Most research on the effects of temperature on the surfactant of heterothermic mammals has involved three species: the marsupial fat-tailed dunnart, *Sminthopsis crassicaudata*, the microchiropteran bat, *Chalinolobus gouldii* and the golden-mantled ground squirrel, *Spermophilus lateralis*. Each of these three model animals experiences different patterns of torpor. Dunnarts are small marsupial mammals that live in the semi-arid regions of Australia and enter short-term periods of torpor (up to 8 h) in response to food deprivation and low ambient temperatures (Godfrey 1968). In this species body temperature varies between ~35°C in warm-active dunnarts to a few degrees above ambient temperature (~15–20°C) in torpid dunnarts (Godfrey 1968). Gould's wattled bats are small insectivorous bats that are widespread and common throughout Australia. These species enter torpor spontaneously throughout the year on a daily basis for a period of a few hours even in the face of constant ambient temperatures and food availability. In this bat, body temperature varies from ~37°C during periods of activity to as little as 5°C during torpor (Hosken and Withers 1997, 1999). Ground squirrels on the other hand enter periods of hibernation during the winter, with bouts lasting several weeks and body temperatures decreasing to as low as 2–7°C (Barros et al. 2001).

Amongst these three mammal species, torpor does not appear to be accompanied by any major compositional changes in the composition of the surfactant phospholipids. Using electrospray ionisation mass spectrometry (ESI-MS), we did not observe any differences in phosphatidylcholine (PC) saturation between warm-active and torpid bats, dunnarts or squirrels. Moreover, there were no consistent changes in the molecular species composition of the major phospholipid groups, i.e. PC, phosphatidylglycerol (PG) or phosphatidylinositol (PI) (Lang et al. 2005b). Hence, neither PL saturation nor changes in molecular species composition appear to be a necessary adaptation for torpor in bats, dunnarts or ground squirrels.

However, in all three mammal species, torpor is consistently accompanied by an increase in the amount of cholesterol (Langman et al. 1996; Codd et al. 2002; Lang et al. 2005b). Only in one recent study were we unable to demonstrate the change in cholesterol with torpor in dunnarts (Orgeig et al. 2007). We analyzed the biophysical properties of the large aggregate (LA) fraction of dunnart surfactant, the complex associated with the greatest surface activity and commonly regarded as the 'active' fraction of surfactant, and did not observe changes in the Chol/DSP ratio. It is probable that the increased cholesterol observed in whole lavage is associated predominantly with the small aggregate, less surface-active component of lavage fluid, which is eliminated in the isolation of LAs.

In addition to changes in cholesterol, we have also reported that the amount of lathosterol (LaSL) and the ratio of LaSL/Chol increased significantly in lung lavage fluid isolated from torpid ground squirrels (Lang et al. 2005b). LaSL is a precursor of Chol and is commonly used as a marker of Chol synthesis or metabolism in plasma and other tissues (Larking 1999). Assuming that LaSL is a valid marker for cholesterol synthesis in torpid as well as active animals, the observed increase in LaSL/Chol may indicate that Chol synthesis is significantly higher in the lung during torpor in ground squirrels (Lang et al. 2005b). However, whether this is a common occurrence amongst heterothermic mammals is unknown.

Thus, Chol appears to be very important to the thermal dynamics of surfactant, both in a long-term evolutionary sense and during daily or seasonal changes in body temperature. Given the profound influences of cholesterol on the biophysics of lipids, it is likely that these compositional changes have profound effects on the biophysical behaviour and surface activity of pulmonary surfactant during the different metabolic states of these heterothermic mammals.

2.2.2 Surfactant Protein Composition

The effects of changes in body temperature on the amount, synthesis, behaviour and function of SP-A, SP-B, SP-C and SP-D have not been thoroughly examined. Only one study has investigated the levels of SP-A and SP-B in three species of heterothermic mammal (Lang et al. 2005b). SP-A decreased significantly in surfactants isolated from torpid dunnarts and squirrels. However, the decrease in SP-A was not observed in bats during torpor, suggesting that this response may not be a general phenomenon (Lang et al. 2005b). Due to the diverse roles of SP-A in the lung, the decrease in SP-A could be due to many different mechanisms, including the downregulation of SP-A gene expression. Moreover, low temperatures (Van Breukelen and Martin 2002) can also destabilise the hydrophobic interactions required to maintain proper protein conformation, and thus increase the potential for protein denaturation (Somero 1995). In contrast, SP-B relative to total PL did not change between surfactant isolated from warm-active and torpid mammals (dunnarts, bats and ground squirrels), and this implies that SP-B levels in warm-active animals are sufficient for adequate function at the lower temperatures, during torpor (Lang et al. 2005b). In fast breathing neonatal mammals, SP-B is increased (Rau et al. 2004), but, given that breathing parameters are reduced during torpor, and there is no increase in air–liquid interface dynamics, there would not be a requirement for more SP-B in torpid heterothermic mammals (Lang et al. 2005b). Any changes in SP-C and SP-D (if they occur at all) remain to be described.

2.2.3 Surfactant Protein Structure and Function

In order to determine the possible role of changes in surfactant protein structure on the regulation of surfactant function in animals that experience torpor, we recently tested for the presence of positive (adaptive) selection in the primary sequence of surfactant protein C (SP-C) during evolutionary transitions between heterothermy and homeothermy (Potter et al. 2007). An understanding of the evolutionary pattern of mode of body temperature regulation in mammals was required to determine the direction of selection on SP-C in relation to transitions between homeothermy and heterothermy. Using body mass as a proxy for mode of body temperature regulation, we reconstructed ancestral body masses, to enable an estimation of the pattern and number of transitions in body size during the radiation of the mammals (Potter et al. 2007). The data indicated that ancestral species were generally

$>10\,kg$, suggesting a high frequency of transitions from large homeothermic to small heterothermic species in the mammalian phylogeny. Such transitions enabled us to test for an association between positive selection on SP-C and heterothermy during the transition from large to small body size, which we hypothesised occurs due to selection for a protein that must be able to function under the more physiologically demanding conditions of variable body temperature (Potter et al. 2007). We sequenced SP-C from genomic DNA of 32 mammalian species from groups of closely related heterothermic and homeothermic species (contrasts). We used phylogenetic analysis by maximum likelihood estimates of rates of non-synonymous to synonymous substitutions and fully Bayesian inference of these sequences to determine whether the mode of body temperature regulation exerts a selection pressure driving the molecular adaptation of SP-C (Potter et al. 2007). The results from the maximum likelihood and Bayesian analyses of the *sp-c* gene revealed strong purifying selection in heterothermic and homeothermic species, indicating that constraints are placed on the evolution of SP-C to maintain protein function in surfactant despite variation in mode of body temperature regulation (Potter et al. 2007). The protein sequence of SP-C is highly conserved, with synonymous or highly conservative amino acid substitutions being predominant (Fig. 3).

The evolution of SP-C among mammals is characterised by high rates of transition/transversion and by high codon usage bias. As transitions involve purine–purine or pyrimidine–pyrimidine substitutions (Zhang 2000), they lead either to more synonymous nucleotide substitutions or conservative amino acid substitutions. Similarly, a high codon usage bias suggests that substitutions of amino acids in SP-C are favoured by conservative amino acid replacements. Amongst all the heterothermic/homeothermic contrasts analysed, the only contrast to show evidence of positive selection was that of the bears (*Ursus americanus* and *U. maritimus*). The significance of this result is unclear (Potter et al. 2007). Hence, there is insufficient evidence to suggest that the mode of body temperature regulation has influenced the evolution of SP-C in mammals. Rather, the protein is under strong evolutionary constraints, i.e. purifying selection, to maintain surfactant function despite the variability in mode of mammalian body temperature regulation (Fig. 3). Moreover, in terms of the ability of surfactant from heterotherms to regulate surfactant function under torpid conditions, modulation of SP-C primary sequence does not appear to be the answer. Given the functional redundancy of SP-C and SP-B, it is possible that there are adaptations in the SP-B primary sequence in heterothermic species. Alternatively, it may be possible that there is a differential regulation of the level of SP-C expression in torpid versus warm-active individuals (Potter et al. 2007). This remains to be tested.

2.2.4 Surfactant Biophysical Function During Activity, Torpor and Arousal

As in homeothermic mammals, decreases in body temperature in heterothermic mammals are associated with decreases in tissue elasticity and thus, decreases in lung tissue compliance, at least in the early stages of torpor (Langman et al. 1996).

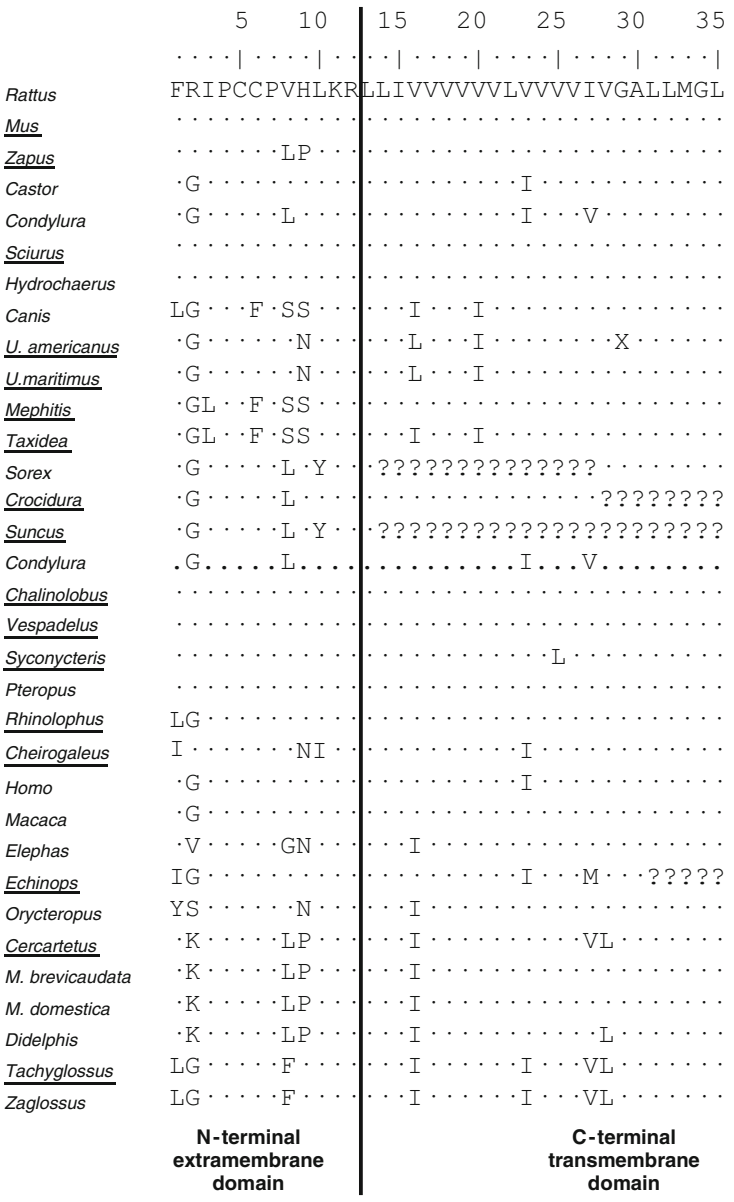

Fig. 3 Aligned amino acid sequences of the mature SP-C protein inferred from the translation of the nucleotide sequences published in Potter et al. (2007). The genus names are listed, except in the case of *U. americanus* and *U. maritimus*, for which the genus is *Ursus* and in the cases of *M. brevicaudata* and *M. domestica*, where the genus name is *Monodelphis*. Heterothermic species are *underlined*. Amino acid position is indicated by the numbers along the *top* of the figure. The *black vertical line* indicates the boundary between the N-terminal extramembrane and the C-terminal transmembrane domains at amino acid position 12. *Dots* indicate conserved amino acids relative to the first sequence, i.e. *Rattus*. *Question marks* indicate residues that were not obtained. '*X*' at site 29 in *Ursus americanus* represents heterozygosity for the amino acids arginine (*R*) or glycine (*G*) (Foot et al. 2007). Figure reproduced from Potter et al. 2007 with permission from Elsevier

However, in order to maintain high lung compliance and hence maintain the work of breathing at a minimum, the surface activity of surfactant must increase to counteract the lower tissue elasticity. Therefore, heterothermic mammals must optimise their surfactant at low temperatures to decrease surface tension in the lung and thus reduce the work of breathing. The fluidity of surfactant must also be maintained at cold body temperatures and this can only be achieved by lowering the phase transition temperature of the lipid mixture, and thus, changing the composition of the surfactant mixture.

We have consistently shown, with a variety of techniques and in both bats and dunnarts, that the in vitro surface activity of surfactant is altered such that it is optimal at that in vitro temperature which matches the body temperature of the animal from which the surfactant was isolated, i.e. whether isolated from either warm-active or torpid animals (Lopatko et al. 1998, 1999; Codd et al. 2002, 2003; Orgeig et al. 2007). These findings are summarised in Table 1. For example, when surfactant from active and torpid Gould's wattled bats were analysed on a captive bubble surfactometer (CBS) at a temperature matching the body temperature of the bat, γ_{eq} of 25 mN m^{-1} and γ_{min} of 1 mN m^{-1} were achieved (Table 1). These values are similar to the literature values for other mammals (Goerke and Clements 1985; Codd et al. 2002). Adsorption was significantly slower when surfactant from active bats was analysed at 24°C compared to 37°C. Conversely, surfactant from torpid bats demonstrated much faster adsorption at 24°C compared with 37°C. Quasi-static and dynamic cycling of surfactant from active bats at 37°C yielded a lower γ_{min} and required a smaller %SAcomp to reach γ_{min} than when measured at 24°C (Table 1). Conversely, surfactant from torpid bats reached a lower γ_{min} and required less %SAcomp to reach low γ_{min} at 24°C than at 37°C (Codd et al. 2002) (Table 1). Hence, in heterothermic mammals, surfactant from active (warm) animals appears to be more suited to function at higher temperatures (37°C) and surfactant from torpid (cold) animals appears to function better at lower temperatures (Lang et al. 2005a).

In addition, we have demonstrated very rapid and precise adjustments in surface activity during arousal from torpor in both bats (Codd et al. 2003) and dunnarts (Lopatko et al. 1999). Arousal from torpor by heterothermic mammals can be very rapid, with rates of 0.7–1°C per minute (Geiser and Baudinette 1990) recorded for dunnarts and up to 0.81°C per minute (Codd et al. 2000a) recorded for bats. Surfactant isolated from bats arousing from torpor (T_b = 28–32°C) adsorbs much faster at 37°C than at 24°C and functions optimally at 37°C (as indicated by a decrease in γ_{min} and %SAcomp) (Codd et al. 2002) (Table 1). Thus, surfactant from heterothermic mammals undergoes rapid changes in surface activity and lipid composition that enable the mixture to function effectively at rapidly increasing body temperatures (Lang et al. 2005a).

2.2.5 Surfactant Film Structure

Recently we have begun an examination of the film structure of surfactants isolated from torpid and warm-active surfactant (Orgeig et al. 2007). We discovered

that differences in film behaviour seen on the Langmuir balance at 23°C were matched with significant differences in film structure as assessed by epifluorescence microscopy of doped phospholipid films and atomic force microscopy (AFM) (Orgeig et al. 2007). Specifically we demonstrated unstable phase partitioning (i.e. coexistence of ordered and disordered phases) in surfactant from warm-active dunnarts suggesting that there was a perturbation to the packing of the surfactant film (Fig. 4a). On the other hand, under the same in vitro conditions, surfactant from torpid dunnarts demonstrated phase coexistence throughout film compression up to 30 mN m^{-1}, indicating stable packing into liquid-condensed regions (Fig. 4b). Moreover, the area of coverage of probe-excluded liquid-condensed regions increased throughout compression and was greater than for surfactant from warm-active dunnarts (Fig. 4c). Atomic Force Microscopy (AFM) confirmed the presence of large circular liquid-condensed domains in surfactant from torpid dunnarts with a mean diameter approximately 5 times greater than those found in surfactant from warm-active dunnarts (Fig. 4d). The height profiles of both types of domains were similar to each other and similar to those that would be obtained with a pure DPPC film (Panda et al. 2004), thereby supporting the fluorescence data by concluding that these were liquid-condensed, DPPC-enriched domains.

Hence, in terms of film structure and behaviour during compression, surfactant from torpid dunnarts was more effective at 23°C than surfactant from warm-active dunnarts (Orgeig et al. 2007). In general, the function and structure of surfactant films from torpid dunnarts were more similar to those expected of natural surfactant isolated from traditional mammalian models that are classed as 'good' surfactants. As these studies were performed at 23°C, a temperature more similar to the body temperature of torpid dunnarts, this supports our previous finding that surfactant function is optimised to function best at that temperature at which it was isolated from the animal. However, future structural studies performed at in vitro temperatures that match the in vivo temperatures are required to confirm this hypothesis (Orgeig et al. 2007).

3 Pressure

We have recently investigated the possibility that high hydrostatic pressure can act as an evolutionary selection pressure resulting in specific adaptations to the surfactant system. Hence, we explored the molecular, biochemical, cellular and physiological characteristics of the surfactant system of diving mammals to determine whether it possesses specific characteristics (adaptations) to cope with the repeated collapse and re-inflation of the lung that occur during diving, in order to provide resistance against the compressive effects of the high hydrostatic pressures (Miller et al. 2004, 2005, 2006b; Miller 2005; Foot et al. 2007). We analysed each component and organisational level of surfactant individually for adaptations (Foot et al. 2006). This series of studies is presented here as an integrated case study for the effect of pressure on all aspects of the pulmonary surfactant system.

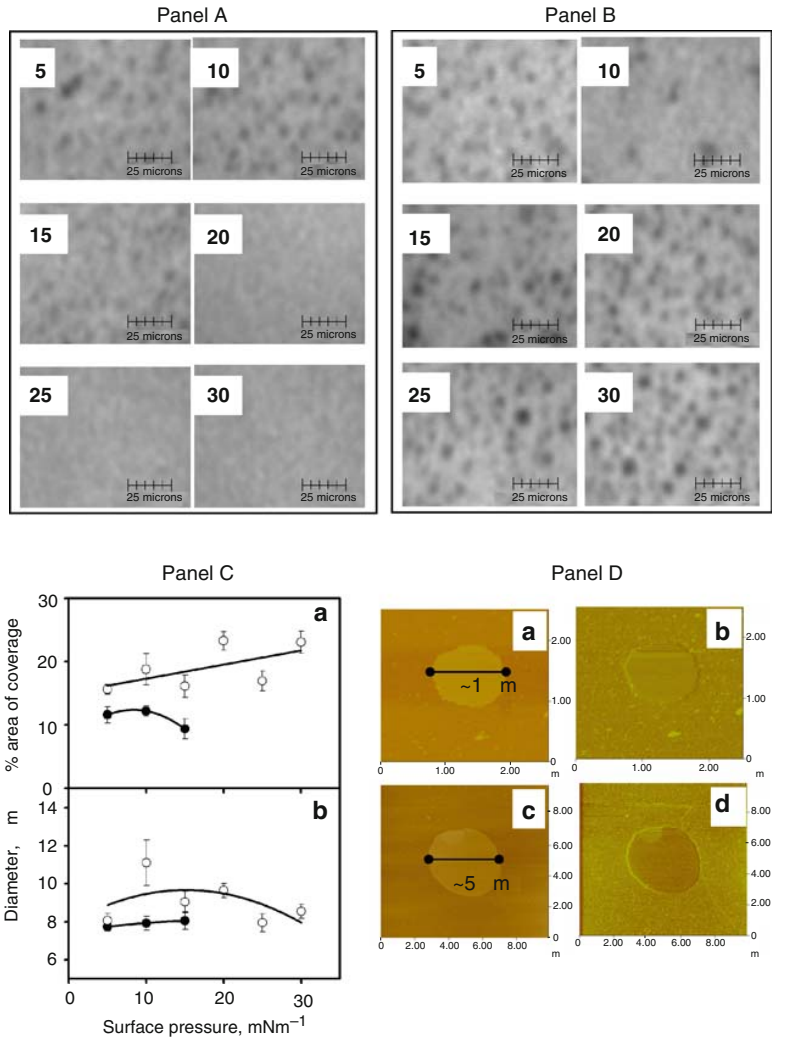

Fig. 4 Fluorescence images taken with a Karl Zeiss epifluorescence microscope of solvent-spread surfactant films containing 1 mol% of the fluorescent PL probe NBD-PC (1-palmitoyl-2-[12-{(7-nitro-1,3-benzooxadiazol-4-yl) amino}dodecanoyl]-sn-glycero-3-phosphocholine) from warm-active (*Panel A*) and torpid (*Panel B*) dunnarts, compressed on a Langmuir-Wilhelmy balance at 23°C to 5, 10, 15, 20, 25 and 30 mN/m surface pressure (indicated inside the images). Subphase: 0.15 M NaCl, 1.5 mM $CaCl_2$, 1.0 mM TRIS-HCl buffer at pH = 7.0. *Dark domains* represent liquid-condensed probe excluded domains. *Panel C*: Quantitative fluorescence image analysis of solvent-spread surfactant films of warm-active (*solid symbols*) and torpid (*open symbols*) dunnarts at different surface pressures. **a** Percentage of area of coverage of the probe-excluded regions. **b** Diameter (μm) of the probe-excluded regions. *Panel D*: Atomic force microscopy images of solvent-spread surfactant films from warm-active (*a*,*b*) and torpid (*c*,*d*) dunnarts at 30 mN m^{-1} surface pressure. The films were transferred onto freshly cleaved mica by the Langmuir–Blodgett transfer technique. Area of scan: *a*,*b* 2.5 × 2.5 μm^2; *c*, *d* 10 × 10 μm^2. *a* and *c* were taken in height mode (the *lighter areas* indicate a greater height profile), while *b* and *d* were taken in phase mode. *LE* liquid expanded phase; *LC* liquid condensed phase. Figure reproduced from Orgeig et al. 2007 with permission from Oxford University Press

3.1 Selection at the Molecular Level

We tested the hypothesis that sequence variations in the SP-C protein of marine mammals have resulted in functional adaptations to cope with the repeated collapse and reinflation of the lung during diving. We examined the ratio (ω) of non-synonymous (amino acid-changing, d_N) to synonymous (silent, d_S) substitution rates among nucleotide and inferred amino acid sequences of the SP-C gene (*sp- c*) (Foot et al. 2007). An excess of non-synonymous over synonymous nucleotide substitutions indicates positive selection (Nielsen 1998; Yang et al. 2000). We also examined the biophysical properties that are associated with the amino acid substitutions in the diving lineages (Foot et al. 2007). To control for lack of phylogenetic independence, we made the comparisons in a series of independent mammal contrasts consisting of semi-aquatic species (i.e. can forage in water, but do not usually dive to depths of more than 5 m) or diving species (i.e. can dive deeper than 70 m) and their nearest terrestrial relatives (i.e. do not forage in water).

SP-C is a highly conserved protein with significant selection constraints, but there was evidence of positively selected sites particularly in the N-terminal domain of SP-C in diving mammals (Foot et al. 2007) (Fig. 5a). Using phylogenetic analysis by maximum likelihood (Yang 1997), site models strongly identified positive selection at different sites in the polar N-terminal extramembrane domain of SP-C in the three diving lineages: site 2 in the cetaceans (whales and dolphins), sites 7, 9 and 10 in the pinnipeds (seals and sea lions) and sites 2, 9 and 10 in the sirenians (dugongs and manatees) (Fig. 5a). Analysis of the biophysical properties that were influential in determining the amino acid substitutions showed that isolelectric point, chemical composition of the side chain, polarity and hydrophobicity were the crucial determinants. Particular sites that demonstrate evolutionary lability are sites 2 and 10, at which there is a tendency for more polar and/or more charged residues, as they are involved in polar interactions with the hydrophilic head groups of the phospholipid layer (Fig. 5b). Stronger binding of the N-terminal domain to the phospholipid layer would be highly desirable during the extreme compression of the lung that occurs during deep diving. The improved binding would prevent squeeze-out of the more fluid USP and neutral lipids, e.g. Chol, which is presumed to occur during normal lung compression on expiration (Fig. 5b). The removal of these lipids from the monolayer results in an enrichment in DSP and increases the surface activity of the monolayer, which is important in preventing the adhesion of respiratory surfaces in terrestrial mammals. However, a monolayer of pure DSP does not respread easily upon reinflation. For the true diving mammals, complete lung collapse is essential for diving. Hence, a more fluid lipid layer may be critical for the rapid reinflation of the lungs during the short excursions to the water surface. In addition, at site 9 there is a tendency for more hydrophobic residues particularly in the pinnipeds, as this site may be involved in hydrophobic interactions with the palmitoylated cysteines (Fig. 5b) and may enable SP-C to adsorb more readily to the air–liquid interface. Such a property would be beneficial when the lungs need to be reinflated rapidly upon resurfacing after a dive. Hence, positive selection in the N-terminal domain

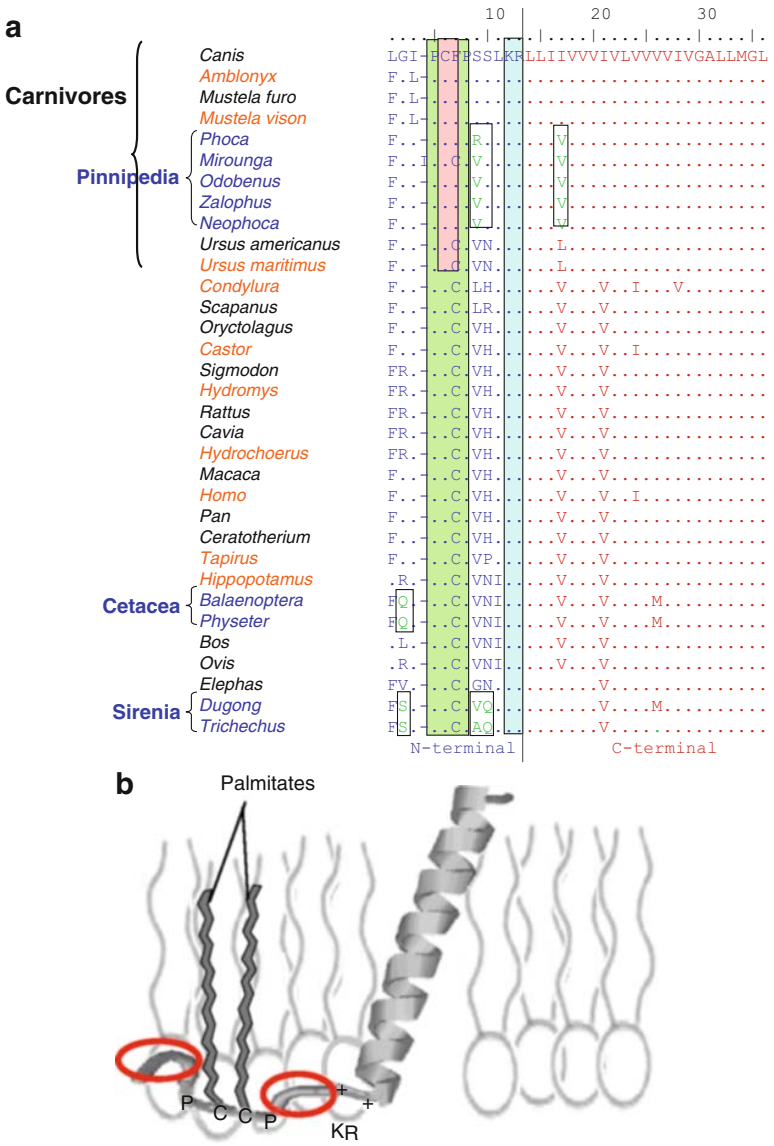

Fig. 5 **a** Aligned amino acid sequences of the mature SP-C protein inferred from translation of the nucleotide sequences published in (Foot et al. 2007). Species indicated in *black* are terrestrial, in *orange* are semi-aquatic and in *blue* are divers. *Dots* indicate conserved amino acids between the sequence of interest and *Canis*. *Dashes* indicate an alignment gap due to the insertion of an extra amino acid in *Mirounga*. Note that due to this insertion, the sequences listed in this figure have one additional amino acid (total 36), and the amino acid position number (from position 4 onwards) is greater by one amino acid relative to the sequences listed in Fig.3. The *light blue box* indicates the two positively charged amino acids, lysine and arginine at the boundary of the N- and C-terminal domains. The *light green box* indicates the PCCP motif, which in the case of the carnivores is a PCFP domain (*pink box*). Amino acids indicated in *light green* with a *black box* surrounding them

of SP-C in diving mammals may reflect adaptations to the repeated collapse and reinflation of the lung upon diving and resurfacing (Foot et al. 2007).

3.2 Selection at the Compositional Level

We hypothesised that the composition of both lipids and proteins will be different in diving mammals compared with terrestrial mammals, to enable the rapid and potentially traumatic expansion of the collapsed lung. We compared the lipid and protein composition of surfactant from different pinniped species (California sea lion, Northern elephant seal and Ringed seal) with those of surfactant isolated from similar-sized terrestrial mammals (cow and sheep) (Miller et al. 2006b). In addition we hypothesised that there will be selection during development, with changes seen in the composition of the surfactant system of a diving mammal as it develops from a terrestrial pup to an aquatic adult. To test this hypothesis we analysed the composition of adult and newborn surfactant from California sea lions (Miller et al. 2005).

3.2.1 Phospholipids and Cholesterol

The relatively minor differences in the phospholipid classes included a reduced level of the anionic surfactant phospholipids, PG and PI, in diving mammals compared with terrestrial mammals (Miller et al. 2006b) (Table 2). Given the potentially

Fig 5 *(Continued)* are those that were identified as being under positive selection in the site models. Positively selected amino acids are only indicated in the diving species of each contrast. Figure reproduced from Foot et al. 2006 with permission from Elsevier. **b** Schematic diagram of an SP-C molecule in a surfactant phospholipid film. The α-helix within the C-terminal transmembrane domain is embedded at an angle in the phospholipid fatty acid tails, and the N-terminal extramembrane domain is associated with the hydrophilic phospholipid head groups. The two palmitate groups that are covalently linked to two cysteine residues (*C*) are anchored within the phospholipid fatty acid tails. On either side of the cysteine residues is a proline residue (*P*) which changes the orientation of the protein chain, enabling correct orientation of the palmitates and the N-terminal segment with the head groups. The *two plus signs* indicate two positively charged residues, Lys (*K*) and Arg (*R*) at the boundary between the more polar N-terminal segment and the hydrophobic C-terminal segment. The *two red circles* indicate the locations of site 2 and sites 9 and 10, which are evolutionarily labile and are under positive selection in the diving mammals. In cetaceans and sirenians, the charge and polarity of these residues is important, possibly leading to stronger binding of the N-terminal tail to the phospholipid headgroups, leading to greater stability of the lipid–protein complex during the high compression forces during diving. In pinnipeds, the hydrophobicity at sites 9 and 10 is crucial, possibly leading to greater interactions with the palmitic acid residues linked to the cysteines (*C*), and possibly aiding in the adsorption of SP-C to the air–liquid interface upon resurfacing after a dive. Figure reproduced from Foot et al. 2006 with permission from Elsevier

destabilizing forces that diving mammal lungs are exposed to, the reduction in PG and PI would appear counterintuitive. However, a reduction in the anionic phospholipids may also lead to an impaired surface activity, which may be better suited to the hypothesised anti-adhesive function of surfactant in these animals. In relation to development within the California sea lion we found that PI is dominant in newborn animals, whereas PG becomes dominant in mature animals, a scenario commonly reported during development of other mammalian species (Hallman and Gluck 1975; Benson et al. 1983; Egberts et al. 1987). In addition, surfactant from the newborn California sea lion was even lower in total anionic phospholipids (i.e. PG + PI) than that from the adult, i.e. 2.9% and 6.8%, respectively.

There were no differences in surfactant phospholipid saturation between diving mammals and their terrestrial counterparts (Miller et al. 2006b). However, surfactant fluidity may also be increased by decreasing the fatty acid chain length (Chapman 1975). Indeed, we discovered that the molecular species of the major phospholipid class, PC, demonstrated a greater percentage of short chain fatty acids (PC16:0/14:0, PC16:0/16:1 and PC16:0/16:0), and a corresponding decrease in longer chain molecular species (PC16:0/18:1) in three species of diving mammal compared with the terrestrial species (Miller et al. 2006b) (Table 2). Similarly, Spragg et al. (2004) demonstrated an increase in fluidic phospholipid species in pinnipeds compared with human and pig surfactant. Hence, this could potentially indicate a diving adaptation in the marine mammals, serving to increase fluidity and therefore enabling surfactant to perform an anti-adhesive function during lung collapse (Miller et al. 2006b).The cholesterol levels were highly variable between diving species, which may be related to the dynamic role of cholesterol (Orgeig 2001) (Table 2). In relation to development within the California sea lion, we described higher levels of the short chain molecular species, PC16:0/14:0, in the adult and an increase in cholesterol relative to the newborn (Miller et al. 2005). These changes in conjunction with the increase in the anionic phospholipids would increase the fluidity of surfactant in the adult, allowing for rapid adsorption to the air–liquid interface, but would result in a poorly surface-active material.

3.2.2 Surfactant Proteins

There were no differences in SP-A levels relative to either total phospholipid or total protein between any of the marine and terrestrial species (Miller et al. 2006b) (Table 2). Similarly, Spragg et al. (2004) reported no significant differences in SP-A between elephant seals and humans. As SP-A does not have a significant role in regulating the biophysical function of surfactant, but is instead involved in host defence functions (Haagsman and Diemel 2001), this finding is not surprising. Although not consistent across all marine mammal species, there was a tendency for a reduced amount of SP-B in the surfactant of diving mammals (Foot et al. 2006) (Table 2). It is likely that the low SP-B levels in some pinniped species may be responsible for the low surface activity and poor equilibrium surface tensions that we have observed (see below). On the other hand, Spragg et al. (2004) observed an increase in SP-B

in elephant seal surfactant relative to human surfactant when analysed by densitometric scanning of electrophoretic gels. However, when analysed by immunologic methods, as in the study by Miller et al. (2006b) and normalised to protein or phospholipid, there was no difference in either SP-B or SP-C (Spragg et al. 2004). Of the three diving species analysed by Miller et al. (2006b), elephant seal also demonstrated the highest levels of SP-B. Hence, it appears that differences in surfactant proteins are not consistent across all marine mammals.

In relation to development within the California sea lion, we described a higher level of SP-B in surfactant from the adult compared with the newborn (Miller et al. 2005). As SP-B functions to facilitate respreading and reformation of the surface film (Possmayer et al. 2001), an increased concentration of SP-B, especially in conjunction with the elevated PC16:0/14:0 and the elevated anionic phospholipids, may ensure that the collapsed surfactant film may respread more easily on lung expansion, a function required by the diving adult, but not the terrestrial newborn.

3.3 Selection at the Functional Level

The function of surfactant must be tailored to the unusual lung structure and breathing mechanics of diving mammals. In marine mammals, the lungs undergo complete collapse during deep dives (Kooyman 1989), resulting in epithelial surfaces coming into contact for extended periods of time. We hypothesised that diving mammals require predominantly an anti-adhesive pulmonary surfactant to prevent adherence of alveolar surfaces following lung collapse. In addition, the surfactant monolayer needs to rapidly reform over the hypophase for efficient lung function. Both of these properties require a highly fluid surfactant mixture, which would demonstrate increased spreadability and adsorption to the air–liquid interface. However, simultaneously such a mixture would demonstrate reduced surface activity compared with terrestrial mammals, as surface tension must only be reduced sufficiently to prevent adhesion of epithelial surfaces, instead of the very low surface tensions that are required to stabilise minute alveoli during a regular inflation/deflation cycle. To further strengthen this hypothesis we analysed the biophysical function of adult and newborn surfactant from California sea lions (Miller et al. 2005) to determine whether there are changes in the function of the surfactant system of a diving mammal as it develops from a terrestrial pup to an aquatic adult.

Supporting this ‘anti-adhesive’ hypothesis drawn from Kooyman’s analysis we discovered that the surfactants of Northern elephant seal, Northern fur seal and Ringed seal were unable to reach typical equilibrium surface tension (γ_{eq}) values ($\sim 25\,mN\,m^{-1}$) after 5 min adsorption (Miller et al. 2006a) (Table 3). However, surfactant from the California sea lion was able to reach the levels of the cow and sheep ($<25\,mN\,m^{-1}$). None of the pinnipeds were able to obtain the very low minimum surface tensions (γ_{min}) achieved by cow ($<2\,mN\,m^{-1}$) (Table 3). Hence, reducing surface tension to very low values is not likely to be the primary

function of surfactant in pinnipeds as it is in terrestrial mammals, but rather a highly fluid anti-adhesive surfactant is likely to be more important to enable the lungs to reopen following collapse during deep diving. These conclusions are further supported by the findings of differences in biophysical function between newborn and adult California sea lions. Although, relative to terrestrial mammals, both adult and newborn surfactant from California sea lions display poor surface-activity (Miller et al. 2006a), it appears that the adult surfactant has better adsorptive properties, as γ_{eq} is lower compared with surfactant from the newborn (Miller et al. 2006a). These increased adsorptive properties would be required for an anti-adhesive type of surfactant and may be better suited to cope with regular lung collapse of diving adults. On the other hand, newborn surfactant had better surface tension lowering properties (Miller et al. 2006a), which appear to be better suited for an alveolar stability function and may reflect the type of tidal volume breathing that occurs in terrestrial mammals.

There are a number of chemical contributors to the poor surface activity in pinniped surfactant, including the overall reduction in the anionic phospholipids, PG and PI, the reduction of SP-B in some pinniped species and an increase in the short chain phospholipid molecular species, PC16:0/14:0 and PC16:0/16:1. All these factors may help explain the very poor surface activity (i.e. γ_{eq} and γ_{min}) and the high surface area compressions that are required to reduce surface tension to γ_{min} (Miller et al. 2006a). Moreover, the relatively greater adsorptive properties of the adult (i.e. the lower γ_{eq} values) compared with the newborn may be explained by the higher level of anionic phospholipids (i.e. PG + PI), the higher levels of the short-chain molecular species, e.g. PC16:0/14:0, higher levels of SP-B, and an increase in cholesterol. These changes may ensure rapid film reformation after lung collapse in the diving adult. On the other hand, these changes would result in a poorly surface-active material, as indicated by the relatively higher γ_{min} compared with the newborn.

Hence, while one particular compositional change in itself may not be highly significant, it is the combination of all of these changes (e.g. lipid and protein composition, as well as protein sequence and structure) that contribute highly significantly to changes in surfactant function in diving compared with terrestrial mammals. Finally, the surfactant of newborn California sea lions is more similar to that of terrestrial newborn mammals, whereas the adult has a ‘diving mammal’ surfactant that can aid the lung during deep dives. Perhaps as the young animals enter the water and begin to forage (and hence dive) for themselves, their surfactant system develops to function more efficiently under these different physiological circumstances.

4 Hypoxia

Despite the fact that hypoxia has significant health consequences, either in adults that ascend to high altitudes, or in the fetus that develops in a compromised uterine environment, the influence of hypoxia on the function of the pulmonary surfactant

system either in adults or during development has not been widely addressed. There is a range of available experimental animal models and experimental protocols that can be utilised to test the effect of hypoxia on the surfactant system. Here we summarise the sketchy information that is available on the role of hypoxia in shaping the pulmonary surfactant system of adult and developing lungs.

4.1 Hypoxia and Altitude: Effects on the Adult Pulmonary Surfactant System

Several studies have examined the effect of hypoxia on lung mass and lung growth, as well as lung function in terms of compliance and alveolar fluid clearance, either in adult or young postnatal animals. In general, hypoxia stimulates lung growth (Sekhon and Thurlbeck 1995, 1996) and lung mass (Hammond et al. 2001), and impairs transalveolar fluid transport (Suzuki et al. 1999) and lung compliance in newborn rats following prenatal hypoxia exposure (Cheung et al. 2000). On the other hand, the information available on the effect of hypoxia on the adult surfactant system, either natural hypoxia at altitude, or induced experimentally, is extremely scant. Acute hypoxia induced experimentally leads to changes in the surfactant lipids (Liamtsev and Arbuzov 1981), including a decrease in phosphatidylcholine (Prevost et al. 1980; Zaitseva et al. 1981) and an increase in lysophosphatidylcholine (Zaitseva et al. 1981) and lysocompounds (Prevost et al. 1980) with a concomitant increase in phospholipase activity (Prevost et al. 1980). Surface activity is impaired (Liamtsev and Arbuzov 1981; Zaitseva et al. 1981; Belov et al. 1985), and edema of the interalveolar septa can occur (Zaitseva et al. 1981). While PC levels of alveolar surfactant decreases, the incorporation of ^{32}P into lung PC and PE increased (Chander et al. 1975), suggesting that the reduced alveolar content may be due to changes in secretion and/or local inactivation, and not due to synthesis (Orgeig et al. 2004).

One interesting but poorly researched area is the response of the surfactant system to chronic hypoxia caused by altitude. Rats raised at an altitude of 3,500 m demonstrated a reduction in the number of macrophages and in the amount of alveolar and lung tissue phospholipid, especially PC (Hegde et al. 1980). Llamas on the other hand, born at an altitude of 4,720 m, demonstrated numerous prominent Clara Cells with large 'apical caps', many of which had been extruded into the terminal bronchioles (Heath et al. 1976). Although the extruded material was not analysed, it is possible that the hyperactivity of the Clara Cells was an adaptive response to chronic hypoxia (Heath et al. 1976). These tantalizing snippets of information should encourage future research into this area (Orgeig et al. 2004).

4.2 Fetal Hypoxia and Growth Restriction: Effects on the Pulmonary Surfactant System in Mammals

Fetal growth restriction (FGR) is a major cause of low birthweight infants and increases the risk of respiratory distress and death in both term and preterm infants

(Tyson et al. 1995; Minior and Divon 1998). Causes of FGR include maternal undernutrition, hypertension, anemia, placental infarction and tobacco smoking. Growth-retarded fetuses suffer from fetal hypoxemia and hypoglycemia, elevated levels of circulating catecholamines and cortisol (Nicolaides et al. 1989; Gagnon et al. 1994; Harding et al. 2001), and decreased levels of insulin-like growth factors and their binding proteins (Owens et al. 1994).

It is generally accepted that in the normal fetus during late gestation, activation of the hypothalamo-pituitary axis (HPA) leads to an increase in the basal levels of adrenocorticotropic hormone (ACTH) and circulating cortisol which functions to facilitate lung maturation (Gross and Ballard 1997). Since the observation by Liggins that fetal exposure to glucocorticoids induced premature delivery and his proposal that glucocorticoids specifically induce surfactant synthesis (Liggins 1969), there has been a plethora of studies investigating the timing, dosage and mechanisms of this pathway (reviewed by Jobe and Ikegami 2000). Although there have been many conflicting results owing to differences in timing, dosage, species choice and whether it is the fetus or the mother that is treated, it does appear that antenatal glucocorticoid treatment reduces the incidence of respiratory distress syndrome by $\sim$50%; it improves fetal lung mechanics after very short treatment-to-delivery times and changes the surfactant system, but only after more prolonged and multiple dosage treatments (Jobe and Ikegami 2000).

Early studies indicated the possibility that growth-restricted infants demonstrated accelerated lung maturation (Gross et al. 1981), suggesting that they may be at lower risk of respiratory distress. As cortisol is increased during periods of physiological stress, such as fetal hypoxemia, it is possible that this represented the mechanism for the stimulation of lung maturation. However, other studies since then have shown that there is no evidence that infants that have been stressed by events such as fetal growth retardation or pre-eclampsia demonstrate a lower incidence of RDS (Tyson et al. 1995; Jobe and Ikegami 2000). However, in addition to such multivariate epidemiological studies, many different experimental animal studies have been performed, using both different species (e.g. sheep, rabbits, guinea pigs, mice) and experimental protocols (placental restriction, undernutrition, knockout) to induce FGR (reviewed by Jobe and Ikegami 2000). Of these only a relatively small number of studies have concentrated on lung and surfactant function, and these have yielded conflicting information.

The hypothesis that FGR leads to enhanced lung maturation stems from the observation that the phosphatidylcholine to sphingomyelin ratio (PC/SM) increases in amniotic fluid of FGR fetuses (Gross et al. 1981). However, this finding was not supported in fetal sheep in which FGR was induced by carunclectomy (Rees et al. 1991); here a decrease in the total PL concentration of luminal liquid occurred. Similar findings were also made in FGR induced by maternal undernutrition in neonatal guinea pigs (Lechner et al. 1986; Lin and Lechner 1991), where substantial reductions in total surfactant phospholipids (including disaturated PC) in lavage and lung tissue occurred, although PL composition, the number of lamellar bodies per type II cell and lung compliance remained normal.

In the fetal sheep, prolonged hypoxemia induced by maternal hypoxia (48 h at gestational days 126–130 or days 134–136) lead to an elevated plasma cortisol level, which was more pronounced later in gestation and correlated with elevations in lung tissue SP-A and SP-B mRNA levels (Braems et al. 1998). SP-C mRNA level was unaffected. The alterations were dependent on the age of the fetus, as only older fetuses (134–136 days) responded to hypoxemia (Braems et al. 2000). Hypoxemia-induced increases in SP-A and -B mRNA occurred in an ovine model of chronic placental insufficiency and FGR (Gagnon et al. 1999). Chronic fetal hypoxemia, maintained for 21 days during late gestation (∼109–130 days) decreased fetal growth and lung growth proportionately, and decreased lung DNA content. Fetal cortisol levels increased, and correlated significantly with increases in SP-A and –B mRNA (but not SP-C mRNA). Although lung morphology and function were not assessed, the decrease in lung DNA content and concentration, with an increase in SP mRNA synthesis, suggests that there was a switch from lung cell proliferation to fetal lung cell maturation (Gagnon et al. 1999).

In direct contrast, chronic placental insufficiency during late gestation (120–140 days) did not change the SP-A, -B or -C mRNA or SP-A protein levels in the lung tissue of fetal sheep (Cock et al. 2001). There was also no correlation between SP mRNA or SP-A protein levels and cortisol levels. Furthermore, although DNA content decreased, relative to lung weight, the DNA concentration was higher in the growth retarded sheep. As DNA concentration decreases during normal gestation (Nardo et al. 1995), the increase found here suggests that the lungs of the growth retarded fetuses were structurally immature.

There is still a relative paucity of information on the effect of fetal hypoxia and growth retardation on the surfactant system, and the data on the levels of SP mRNA expression are inconsistent. The primary difference between the study of Gagnon et al. (1999) and that of Cock et al. (2001) lies in the timing of sampling relative to gestation. Cock et al. (2001) induced FGR between days 120 and 140 days of gestation compared to ∼109–130 days in the study by Gagnon et al. (1999). It is possible that the levels of SP mRNA in the older fetuses had already reached their maximal expression and could not be stimulated further by cortisol. Age exerted a specific effect on SP mRNA expression and cortisol levels after 48 h of mild hypoxia (Braems et al. 2000), so it appears that there is a very narrow window in which surfactant maturation can be perturbed by environmental factors (Orgeig et al. 2004). While this robustness may represent an adaptive advantage during normal development, it may also explain the variable outcomes described (Jobe and Ikegami 2000) in therapeutic interventions such as glucocorticoid administration. Therefore, in order to optimise treatment strategies of fetuses and infants at risk of FGR-related postnatal respiratory complications, it is essential that the mechanisms and timing of lung and surfactant maturation during late gestation are understood, especially in relation to environmental factors which lead to FGR (Orgeig et al. 2004).

4.3 Fetal Hypoxia: Effects on the Pulmonary Surfactant System in Non-mammals

Although the overall pattern of surfactant maturation is similar between vertebrate species, the onset and completion of the development of the surfactant system differ dramatically between species (Johnston and Daniels 2001) (Fig. 6a). These differences do not appear to be directly related to phylogeny, as the most closely related species often have the most different patterns. Instead it appears that the developmental process is determined by birthing strategy, which in turn correlates with the development of relative hypoxia as an embryo develops. For example, green sea turtles, which bury their eggs under wet sand (which can exhibit a high bacterial and fungal load so that the eggs are routinely exposed to low oxygen concentrations during development), the viviparous lizard *Tiliqua rugosa*, as well as placental mammals all complete their surfactant development much earlier (75–80%) than most oviparous species (90–95%) that lay their eggs in a manner that exposes them to normal air (Johnston and Daniels 2001) (Fig. 6a). Therefore, we have proposed that one of the factors that drive the development of the vertebrate surfactant system may be hypoxia (Johnston and Daniels 2001).

When sea turtle eggs were incubated in mild, but prolonged hypoxia (17% O_2) (Johnston et al. 2001), there was no effect on the development of the surfactant, suggesting that the pulmonary surfactant system is unresponsive to mild decreases in PO_2. As sea turtle eggshells are more permeable to gases and water vapour than avian eggs (Ackerman and Prange 1972), the lack of effect probably results from the high conductance of chelonian eggshells. It is probable, therefore, that turtle embryos are tolerant of increasingly hypoxic conditions as they are able to acquire oxygen more readily than other shelled embryos and, as a result, oxygen may not have been limiting in the study by Johnston et al. (2001). Moreover, eggs of *Chelonia mydas* incubated in large numbers experience oxygen tensions as low as 9% O_2, which leads to increased mortality of near-term embryos and abnormal yolk size in hatchlings (Wood and Wood 1979). However, if embryos are incubated in smaller numbers such that they experience ambient oxygen tensions of 14% O_2, development progresses normally (Wood and Wood 1979). Clearly, the severity of hypoxia in this experiment was not great enough to alter the course of development in the sea turtles (Johnston et al. 2001).

On the other hand, under similarly mild hypoxic (17% O_2) conditions, overall development of chicken embryos is accelerated, with hatching brought forward by 24 h (Starrs et al. 2001; Blacker et al. 2004). Moreover, we demonstrated a significant acceleration in the development of the surfactant lipid profile in chicks developing under hypoxic incubation from day 10 of incubation (Blacker et al. 2004). Specifically, hypoxia induced both the early release of PL, DSP and Chol as well as an enhanced maturation of the surfactant lipid ratios (i.e. %DSP/PL, Chol/PL and Chol/DSP), indicating upregulation of the synthetic and secretory pathways of surfactant lipids from day 16 of incubation (Fig. 6b). This premature maturation correlated with the early pipping and hatching, but did not alter the

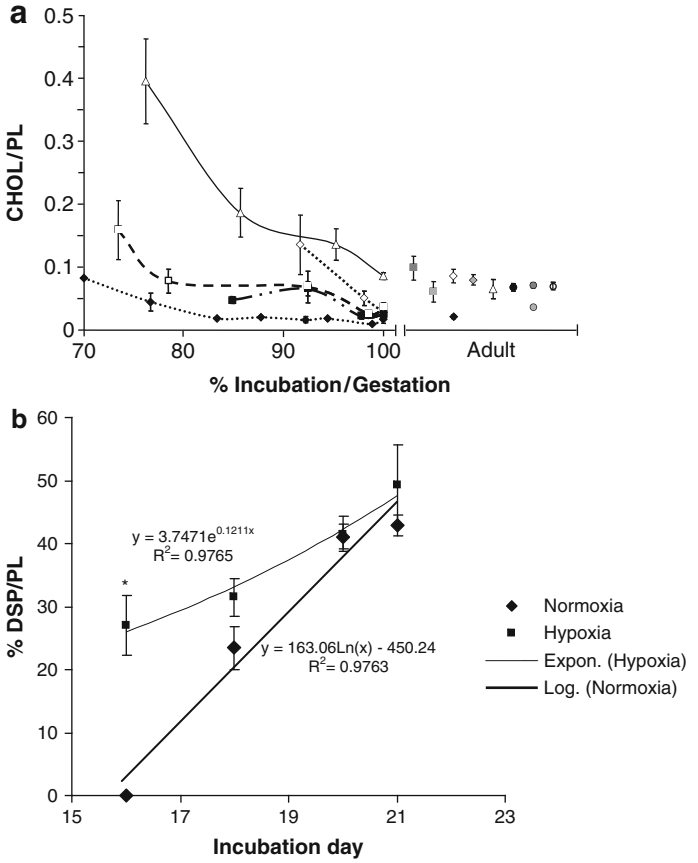

Fig. 6 **a** Cholesterol expressed as a ratio to total phospholipid (μg/μg) from lavage of the developing (*dark line with open triangle*) and adult (*open triangle*) chicken, *Gallus gallus*, the developing (*dotted line with open diamond*) and adult (*open diamond*) bearded dragon, *Pogona vitticeps* (Wood et al. 1995; Johnston et al. 2000), the developing (*dotted line with filled diamond*) and adult (*filled diamond*) sleepy lizard, *Tiliqua rugosa* (Johnston et al. 2002b), the developing snapping turtle, *Chelydra serpentina* (*dark dashed line with filled square)* (Johnston et al., 2002a), the developing green sea turtle, *Chelonia mydas* (*dark dashed line with open square*) (Johnston et al. 2001), the mature leatherback sea turtle, *Caretta caretta* (*dark filled square*), the mature flatback sea turtle, *Natator depressus* (*pale filled square*) (Daniels et al. 1996), the adult central netted dragon, *Ctenophorus nuchalis* (*pale filled diamond*) (Daniels et al. 1990), the adult fat-tailed dunnart, *Sminthopsis crassicaudata* (*dark filled circle*) (Langman et al. 1996), the adult rat, *Rattus norvegicus* (*filled circle with dark edge*) (Orgeig et al. 1995), the newborn rat, *R. novegicus* (*pale filled circle*) (Chol/PC) (Suzuki et al. 1978) and the adult human, *Homo sapiens* (*open circle*) (Doyle et al. 1994). Note: The axis for the adult data points does not represent a timescale, but has been expanded for clarity. Figure reproduced from (Johnston and Daniels 2001) with permission from Elsevier. **b** Effect of incubation under normoxic (21% O_2) or hypoxic (17% O_2) conditions on the percentage of phospholipids (PL) that are disaturated (DSP) (*%DSP/PL*) in lung lavage from embryonic chickens at incubation days 16, 18 and 20 (post-pip) and within the first 24 h of hatching (day 21). Data are expressed as means ± SEM. *Asterisk* = significant differences in %DSP/PL, between normoxic and hypoxic conditions ($P < 0.05$). Figure reproduced from Blacker et al. 2004 with permission from the American Physiological Society

end point, i.e. the lipid composition at hatching was the same in hypoxic and normoxic embryos. The mechanism by which hypoxia could act to trigger surfactant maturation is most likely hormonal. In the chick embryos, plasma corticosterone levels increased in response to hypoxia early in embryonic development. Moreover, in a separate experiment in which we treated developing embryos with the glucocorticoid agonist, dexamethasone, maturation of the surfactant system was also accelerated (Blacker et al. 2004). It is likely, therefore, that the maturation process caused by hypoxia is mediated by glucocorticoids.

5 Conclusions and Future Directions

In this review we have summarised numerous evolutionary and mechanistic studies which show that the evolution of the pulmonary surfactant system has been shaped by environmental selection pressures which include temperature, pressure and hypoxia. Changes in body temperature induce changes in surfactant lipid composition both in the short term, i.e. acute changes within an individual and on an evolutionary time scale between vertebrate groups. However, the evidence to date indicates that changes in either surfactant protein composition or surfactant protein sequence and structure do not appear to be common or consistent. Hence, it is presumably the changes in lipid composition that are responsible for the significant changes in surface activity and surfactant film structure that we have observed with changes in body temperature.

The causal relationship between changes in various lipid components and changes in film structure and behaviour needs to be further characterised. Furthermore, the possibility that the relative expression of SP-C varies with changes in body temperature needs to be examined, as does the possibility that animals with variable body temperatures express an SP-B protein with a different primary sequence. Moreover, the influence of alterations in body temperature on the development of the pulmonary surfactant system has not been examined.

With respect to pressure, we have summarised a series of modifications at numerous organisational levels including modified proteins, lipid components, biophysical function and developmental pattern within a group of mammals experiencing a unique environmental factor, viz. elevated hydrostatic pressure. It appears that diving mammals require an anti-adhesive surfactant with greater fluidity and rapid expansion capabilities to cope with the repeated collapse and reinflation of the lung and large increases in hydrostatic pressure. These functional adaptations are supported by molecular modifications in a key protein (SP-C) and lipid and protein compositional changes. The production of a ‘diving’ type of surfactant may be triggered in part, and in some diving groups, by the onset of diving, lending support to the idea that pressure is the driving force behind the differences observed between terrestrial and diving mammals. An interesting developmental system that deserves investigation from a pulmonary surfactant perspective and that involves changes in pressure, is the metamorphic transition of aquatic larval amphibians to terrestrial adult amphibians.

At this stage there are no clear hypotheses regarding the likely response of the adult surfactant system to hypoxia, which presumably explains and reflects the scarcity of experimental data in this area. Clearly, in the case of developing embryos, hypoxia is a major evolutionary selection pressure, as it appears to trigger various mechanisms to increase maturation, enabling the newborn to escape the potentially dangerous hypoxic environment as quickly as possible. While this is frequently at the expense of the overall growth of the organism, it appears that the lungs and the surfactant system are not only spared, but instead demonstrate accelerated development. This intraspecific plasticity in timing of onset and/or rate of development has recently been termed physiological heterokairy (Spicer and Burggren 2003; Blacker et al. 2004), which has been proposed as a potential mechanism by which environmental factors are able to influence the development of a system within individuals, and which may in some instances over evolutionary timescales result in differences between taxa which are the result of heterochrony (Spicer and Rundle 2007). It will be important to establish whether the heterokairous mechanism involving environmental hypoxia and glucocorticoid hormones is a universal vertebrate phenomenon, hence strengthening the potential link between heterokairy, i.e. intraspecies plasticity and heterochrony, i.e. interspecies differences.

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