COPING WITH THE COLD: HETEROTHERMIC MAMMALS PROVIDE A NEW PARADIGM FOR SURFACTANT COMPOSITION AND FUNCTION

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ABSTRACT

Pulmonary surfactant (PS), a complex mixture of lipids and proteins, lowers surface tension (ST) in the alveoli and is crucial to lung function. Due to the biophysical properties of lipids, temperature potentially can have a profound effect on the function of PS. However, heterothermic mammals regularly endure changes in body temperature (T_b) during torpor without suffering surfactant dysfunction.

The length, depth and frequency of torpor bouts vary in different mammals and may affect the manner in which the PS system responds to the decrease in T_b. Here, I examine the thermal dynamics of PS in three heterothermic mammals that display different torpor patterns. Fat-tailed dunnarts, *Sminthopsis crassicaudata*, enter torpor in response to food shortages and low ambient temperatures (T_a). Gould's wattled bats, *Chalinolobus gouldii*, enter torpor on a daily basis, while golden-mantled ground squirrels, *Spermophilus lateralis*, enter deep and prolonged torpor bouts during hibernation. The composition, structure and function of surfactant isolated from warm-active and torpid mammals was examined using biochemical analyses, mass spectrometry, a Wilhelmy-Langmuir surface balance and epifluorescence and atomic force microscopy.

Torpor had no significant effect on the saturation, or molecular species composition, of phospholipids in surfactant. This suggests that surfactant phospholipid composition is already adapted to the thermoregulatory plasticity experienced by heterotherms. Such adaptations include an enrichment in phosphatidylcholine molecular species 16:0/16:1, 16:0/14:0 and 16:0/18:0, phosphatidylglycerol 16:0/16:1 and phosphatidylinositol 18:0/20:4.

During torpor, cholesterol and surfactant protein C (SP-C) increase significantly. However, changes in film structure, as determined by epifluorescence and atomic force microscopy, between warm-active and torpid surfactants do not correlate with the

fluidising actions typically attributed to cholesterol and sometimes, SP-C. Moreover, a greater number of larger liquid-condensed domains is observed in surfactant from torpid animals, at 23°C. Hence, cholesterol may play a role in the redistribution of phospholipids and alter the structure of the surfactant film so it can function effectively during torpor. In addition, increases in SP-C may improve the formation and stabilisation of surface-associated reservoirs, at lower temperatures. The greater fluidity of warm-active surfactant at 23°C probably hampers the ST-lowering properties and explains observations that surfactant isolated from warm-active mammals is less 'surface-active' than torpid surfactant at 23°C.

The classical dogma for surfactant function is that dipalmitoylphosphatidylcholine (DPPC) is the only significant component of mammalian surfactants capable of reducing ST to low values during compression. However, the low levels of DPPC observed here, in surfactant from dunnarts and the Tasmanian devil, suggest that a DPPC-rich surfactant is not necessarily required for efficient surfactant function in mammals. At the molecular level, there is no single composition of phospholipid molecular species that functions optimally in all mammals. Rather, there is a spectrum of different molecular compositions within mammals, such that the composition of each animal species is unique and optimised to the physiology of that animal.

DECLARATION

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

Carol **Ormond**

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epifluorescence and atomic force microscopy techniques. My sincerest thanks to you all for introducing me to the field of surfactant biophysics and for your patience in teaching a cell biologist and biochemist, about the biophysics of surfactant. I also thank Dr Robert Harbottle and Dr Nils Peterson at the University of Western Ontario, for technical advice and assistance.

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All experiments were performed under the University of Adelaide Ethics Committee, approval number S/54/00, and in compliance with the "Principles of Animal Care" publication number 86–23 of the National Institute of Health and the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes", 6th Edition. In Canada, experiments on ground squirrels were also approved by the University of British Columbia Animal Ethics Committee. Gould's wattled bats were collected with permission from South Australian National Parks and Wildlife (permit number W24091).

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FOREWORD

During my Ph.D., I embarked on two different projects relating to the thermal dynamics of the pulmonary surfactant system in heterothermic mammals. Firstly, using a cell culture approach, I examined the effects of temperature on the regulation of surfactant secretion from heterothermic mammalian type II cells. Secondly, I examined the composition, structure and function of pulmonary surfactant isolated from warm-active and torpid (or hibernating) heterothermic mammals. A review of both these research areas is given in Chapter 1. However, the experiments examining the composition, structure and function of surfactant have yielded some intriguing and exciting results. Consequently, I have chosen to focus on these aspects in the main body of this thesis. The cell experiments, which examined the effects of temperature on surfactant secretion from alveolar type II cells and the regulation of secretion by autonomic neurotransmitters, have already been published (Ormond et al., 2003 *Biochem. Biophys. Res. Comm.* 310, p703; Ormond et al., 2003 *J. Exp. Biol.* 206, p3031) and are included here, in Appendices 3 and 4.

I have chosen to outline the background and aims of this research in the General Introduction (Chapter 1). Materials and methods are outlined in Chapter 2. Following these, there are four results chapters, relating to different animal species. The results for golden-mantled ground squirrels, Gould's wattled bats, fat-tailed dunnarts and other marsupials are given in Chapters 3, 4, 5 and 6, respectively. Each results chapter includes a brief background, hypothesis and summary of results. All results are then discussed together in Chapter 7.

I have chosen this particular thesis style because I believe, in this case, that the most important information is gained from the trends observed in all the animals examined, rather than those observed in particular species. Moreover, there were no major differences between the responses of the surfactant systems with different styles of

torpor. Consequently, the general discussion chapter (Chapter 7) is in two parts. First, I discuss the effect of torpor, or hibernation, on the surfactant system of heterothermic mammals. Secondly, I discuss the comparative aspects of my findings and their importance to our wider understanding of surfactant function.

CHAPTER 1

GENERAL INTRODUCTION

INSIGHTS INTO THE THERMAL DYNAMICS

OF THE SURFACTANT SYSTEM

1.1. RESPONDING TO THE THERMAL ENVIRONMENT

1.1.1. TERMINOLOGY AND DEFINITIONS

Animals display a wide variety of responses to the thermal environment. Endothermic animals maintain their body temperature (T_b) internally through metabolic processes, while ectothermic animals maintain their body temperature by adsorbing heat from, or releasing heat to, the environment (Schmidt-Nielson, 1997). Homeothermic mammals (e.g. humans) maintain almost constant body temperatures (varying by only 1 or 2°C) with internal heat production, regardless of changes in their environment (Schmidt-Nielson, 1997). However, some mammals experience fluctuations in body temperature, such as during torpor or hibernation that reflect changes in ambient temperature, food availability or circadian rhythms. These mammals are known as heterothermic. The term heterothermic is restricted to mammals and birds, although many ectothermic animals, such as reptiles and amphibians, also experience fluctuations in body temperature both daily and seasonally. Heterothermic animals differ from ectothermic animals in that heterotherms are capable of maintaining warm or cold body temperatures, at or above a specific set point, by a proportional increase in metabolic rate, independent of the environment (i.e. to be precise they are heterothermic endotherms). Heterothermic animals can also rewarm themselves from the low body temperature using endogenous heat production while ectothermic animals cannot (Geiser, 1998).

Torpor is defined as a physiological state in animals during which metabolism decreases and body temperature is maintained at a lower level than normal (Geiser and Ruf, 1995). Breathing pattern also often changes during torpor or hibernation from a continuous to an intermittent or arrhythmic pattern (Milsom, 1987). Torpor bouts appear to be triggered by different environmental or physiological cues in different species and can vary in length, depth and duration. As a result, there are many differing viewpoints amongst torpor physiologists concerning the precise determinants and characteristics of

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'true' torpor or hibernation. However, most heterothermic mammals display one of four torpor patterns, which I have defined here as follows. Examples of different animal species with the physiological parameters of the torpor pattern they display are provided in Table 1.1.

1. **Daily Torpor** is a typically shallow torpor pattern that lasts for several hours where body temperature is maintained at a few degrees above ambient temperature. Hence, above a metabolically defended minimum body temperature (usually 15-20°C), the depth of daily torpor (i.e. minimum body temperature) often depends on ambient temperature. Generally, the lower the ambient temperature, the lower the minimum body temperature of the torpid animal and the deeper the torpor pattern. Perhaps the most important distinguishing point of this type of torpor pattern is that entry into torpor occurs on a daily basis in these species exclusively at both low and high ambient temperatures. Hence, entry into daily torpor occurs in most cases, in all seasons and throughout the year. Animals exhibiting 'daily torpor' are also known as 'daily heterotherms' (Geiser and Ruf, 1995).

2. **Stress Torpor** is a pattern of torpor that is similar in depth and duration to that of daily torpor. Consequently, animals displaying stress torpor, as I have defined here, are often grouped with the 'daily heterotherms', as in (Geiser, 1998; Geiser and Ruf, 1995). Stress torpor is similar to daily torpor because it is typically a shallow torpor pattern that lasts for several hours and body temperature drops to a few degrees above ambient temperature, typically, 15-20°C (Geiser and Baudinette, 1987; Godfrey, 1968). The depth of stress torpor, like daily torpor, can also depend on ambient temperature and as such, is often shallower at

warmer ambient temperatures than at colder ambient temperatures. However, unlike daily torpor, entry into the 'stress torpor' pattern occurs predominantly in response to food shortages or low ambient temperatures. Thus, stress torpor often occurs more frequently in winter months when both food availability and ambient temperatures decrease.

Without one, or both, of the environmental cues of cold and food deprivation, these animals may still enter torpor spontaneously, but they do so much less frequently. For example, under winter conditions, when food and water are not provided in a laboratory environment, the Australian dasyurid marsupials, *Sminthopsis crassicaudata* (body mass 14-22 g), *Sminthopsis macroura* (body mass 19-30g) and *Dasyuroides byrnei* (body mass 96-140g) enter torpor with a frequency of 96, 100 and 83 per cent, respectively (Geiser and Baudinette, 1987). However, when food and water are provided ad libitum in a laboratory environment under winter conditions, *S. crassicaudata*, *S. macroura* and *D. byrnei* enter torpor with a frequency of only 14, 30 and 10 per cent, respectively (Geiser and Baudinette, 1987).

Since entry into 'spontaneous' torpor is much less frequent than 'stress-induced' torpor in these animals, 'induced' torpor is more-often examined in the laboratory than 'spontaneous' torpor. However, since factors that trigger the entry into 'induced' or 'spontaneous' torpor in these animals may alter not only the depth and duration of torpor bouts, but also the physiology of the animal in torpor (Geiser and Baudinette, 1987), it is important to distinguish between 'induced' and 'spontaneous' torpor when working with particular species. It is also important to recognise the differences between heterothermic mammals that enter torpor on a regular, daily basis (daily torpor) from those that enter torpor predominantly as a response to food or thermal stress (stress torpor).

3. **Hibernation** is a deep and prolonged period of torpor lasting for approximately 1-3 weeks. Mean minimum body temperature is approximately 6°C (Geiser, 1998) during hibernation. However, some hibernators, such as Artic ground squirrels, *Spermophilus parryi*, can decrease body temperature to just above, or even below freezing temperatures (Toien et al., 2001). During hibernation, metabolic rate is reduced to 1-5% of the basal metabolic rate (Geiser, 1998). Animals that hibernate in response to low ambient temperatures, often display short torpor periods, lasting for less than a day at the beginning of the hibernating season (Geiser, 1998). However, even these shorter bouts have a distinctive physiology. For example, metabolic rate during these short torpor bouts in hibernators is well below that of heterotherms that show daily torpor, even at the same ambient temperature (Geiser, 1998).

4. **Torpor in larger mammals** is a rare, very shallow pattern of torpor that is observed in carnivores such as small bears and badgers. In these animals, body temperature only decreases to 28-30°C and the physiological response differs to that observed in smaller heterothermic mammals (Geiser, 1998).

Table 1.1. Physiological variables of torpor for selected species that represent the four torpor patterns displayed in heterothermic mammals.

Type of torpor pattern and selected species	Mass (g)	T_A °C	$T_{b\,(min)}$ °C	Max. bout length (h)	% BMR
Daily torpor					
Chalinolobus gouldii[a]	10.0	24.0	26.3	~8.0	
Nyctophilus geoffroyi[b]	7.5	24.0	25.0	~7.0	
Nyctophilus geoffroyi[h]	~7.0	1-3°C	2.7	41.3	
Macroglossus minimus[f]	16.0	15.0	22.0	9.0	
Stress-induced torpor					
Sminthopsis crassicaudata[c]	17.3	4-20	16.9	8.9	
Sminthopsis crassicaudata[d]	10.6	10	15.6	8.0[*]	
Sminthopsis macroura[c]	27.0	4-20	18.4	8.8	
Dasyuroides byrnei[c]	115.0	4-20	25.4	4.6	
Antechinomys laniger[e]	27.4	8-30	11.6	15.0	
Hibernation					
Tachyglossus aculeatus	2800	~4.0[g]	4.0	384	20
Cercartetus nanus	24		1.3	552	2
Acrobates pymaeus	12		2.1	192	6
Erinaceus europaeus	700		5.4	231	4
Myotis adversus	7.8		9.0	192	
Eptesicus fuscus	22		5.0	600	
Spermophilus lateralis	200		0.5	384	4
Spermophilus richardsonii	400		2.0	456	4
Marmota marmota	3100		2.0	344	4
Torpor in large mammals					
Meles meles	13000		28.4	1080	
Uras americanus	80000		32.3		19

T_A ambient temperature, $T_{b\,(min)}$ minimum body temperature (°C), Mass (g) body mass in grams, Max bout length, length of the longest torpor bout in hours, %BMR per cent basal metabolic rate (minimum VO_2/BMR x 100). Results selected are from Tables A1 and B1 in (Geiser and Ruf, 1995), unless otherwise indicated as follows: *a* (Codd et al.,

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2000b), *b* (Slocombe et al., 2000), *c* data from (Geiser and Baudinette, 1987) but the classification here is my own, *d* (Langman et al., 1996), *e* data from (Geiser, 1986) but the classification here is my own, *f* (Bartels et al., 1998), *g* (Grigg et al., 1989), * torpid animals sacrificed at this point. Where a range of T_A is given, animals kept under a natural photoperiod and temperature regime, the minimum-maximum T_A is given for the season (or months) in which the longest and deepest torpor bouts were recorded. In hibernators, $T_{b\,(min)}$ is usually similar to T_A.

1.1.2. THE PHYSIOLOGICAL STATE OF TORPOR

At lower body temperatures, basal metabolism is reduced and the conversion of energy such as fatty tissues into body heat is lowered (Randall et al., 1997). Hence, all torpor patterns are considered energy-conserving strategies that evolved in response to the high cost of thermoregulation for animals with high surface area to volume ratios (i.e. small animals) (Randall et al., 1997). Such reductions in body temperature can potentially challenge a number of physiological systems and processes. Moreover, there is now evidence from a diverse range of scientific disciplines indicating that the physiological state of torpor is much more complex than a simple lowering of metabolic rate and a resulting decrease in body temperature. Torpor is a tightly controlled and regulated process involving specific regions in the brain (Ruby et al., 2002; Yu et al., 2002), different molecular (Van Breukelen and Martin, 2002) and biochemical adaptations (Storey and Storey, 2000), as well as changes in physiological mechanisms (Wang and Lee, 2000).

However, there is still a large amount of research needed to understand the physiological state of torpor in, or the impact of torpor on, different biological systems and processes. For example, many researchers have studied breathing patterns, ventilation and oxygen consumption during hibernation and torpor in different species

(reviewed in Mortola and Frappell, 2000), yet there is almost no knowledge of the impact of torpor on the pulmonary surfactant system, which is essential for lung function. The following sections summarise our current knowledge of the thermal dynamics of the surfactant system and in particular, the compositional and biophysical aspects of surfactant that are important to surfactant function. I also review the mechanisms controlling surfactant secretion at the cellular level and although little information is currently available, the effects of temperature on these regulatory factors.

1.2. THE MAMMALIAN SURFACTANT SYSTEM

1.2.1. PULMONARY SURFACTANT

A surface tension is created at any air-liquid interface because the forces of attraction between water molecules are stronger than the forces between water and air. In the lungs, such a surface tension would promote lung collapse and increase the work required to inflate the lung (Possmayer, 1997). Surface tension is usually defined as the force required to stretch a rectangular surface fluid by a known length (usually 1 cm) and is expressed either as dyne/cm (dyn/cm) or milliNewtons/m (mN/m) (where 1 dyne/cm = 10^{-5} N/cm or 1 mN/m). Pure water has a surface tension of 70 dyn/cm (or 70 mN/m) at 37°C. Pulmonary surfactant interferes with the interaction of the surface water molecules and varies the surface tension at the air-liquid interface. This behaviour is termed 'surface activity' (Daniels et al., 1998a). Pulmonary surfactant can reduce surface tension in the lung to less than 5 mN/m in mammals (Schürch et al., 1992).

1.2.2. SURFACTANT COMPOSITION

Pulmonary surfactant is a complex mixture of lipids (90% by weight) and proteins (10% by weight). Phospholipids (PL) comprise 80-90% of the surfactant lipids and are present in different molecular species (molecular forms) that differ from each other in the fatty acid moiety and degree of saturation. Phosphatidylcholine (PC) is the most abundant PL (70-85%) (King, 1982) and the disaturated form, dipalmitoylphosphatidylcholine (DPPC) containing two molecules of the fatty acid, palmitate, is the major contributor to surfactant surface activity (Possmayer, 1997; Wright, 1990). The two other major PL, phosphatidylinositol (PI) and phosphatidylglycerol (PG) have negatively charged, acidic headgroups and vary in their ratio to each other throughout development and between different mammalian and non-mammalian species (Batenburg, 1992; Daniels et al., 1998a). Surfactant also contains minor PL, including sphingomyelin (S), phosphatidylserine (PS) and phosphatidylethanolamine (PE), which make up 1-7% of the surfactant lipids (Goerke, 1998; Veldhuizen et al., 1998; Wright and Clements, 1989).

The remainder of the surfactant lipids include the neutral lipids (primarily cholesterol) and lesser amounts of mono-, di- and tri-acylglycerides and free fatty acids (Wright and Clements, 1989). Cholesterol (CHOL) is the second most abundant lipid in surfactant (Batenburg, 1992) comprising 10% by weight (King, 1982) and 20 mol% of the total lipid (King and Clements, 1985). CHOL and unsaturated phospholipids (USP) increase the fluidity of surfactant and are particularly important in maintaining the spreadability of surfactant over the alveolar surface (Goerke and Clements, 1985; Possmayer, 1991). In addition, four surfactant proteins have been identified. Surfactant protein A (SP-A) and surfactant protein D (SP-D) are hydrophilic, calcium-dependent, carbohydrate-binding proteins (Haagsman et al., 1987; Persson et al., 1990). Surfactant protein B (SP-B) and surfactant protein C (SP-C) are hydrophobic proteins (Hawgood et al., 1998; Johansson, 1998). SP-A is the most abundant surfactant protein and is highly

conserved within the vertebrates (Sullivan et al., 1998). In addition to their roles in the surfactant cycle (Section 1.2.4), SP-A and SP-D are involved in lung defence against pathogens and allergens (McCormack, 1998). SP-A in its delipidated form also regulates surfactant secretion from alveolar type II cells (Mason and Voelker, 1998).

1.2.3. ALVEOLAR TYPE II CELLS

The alveolar epithelium is composed of two main cell types, alveolar type I cells and alveolar type II (ATII) cells. Alveolar type I cells cover 97% of the alveolar surface area (Ward and Nicholas, 1984) and in mammals, ATII cells are located in crevices between the alveoli (Goerke, 1998). ATII cells are cuboidal in shape and contain microvilli and specialised secretory organelles known as lamellar bodies (Wright and Clements, 1989).

A photograph of an ATII cell isolated from the Australian marsupial, *Sminthopsis crassicaudata*, is given in Figure 1.1.
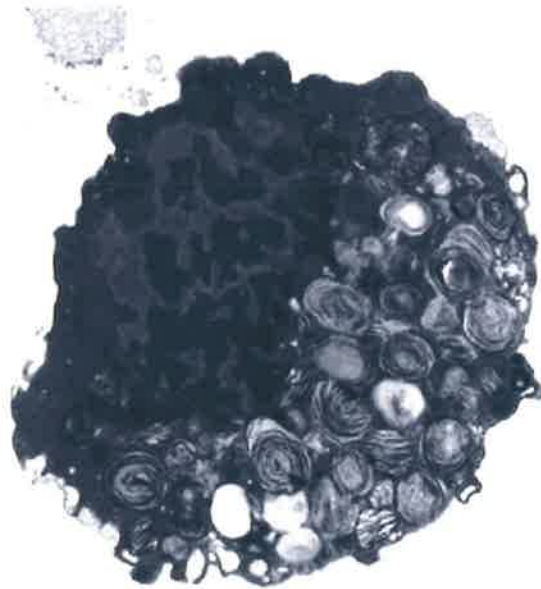
Figure 1.1. Electronmicrograph of an alveolar type II cell isolated from the lungs of an Australian marsupial, the fat-tailed dunnart, *Sminthopsis crassicaudata*. Scale bar = 1 μm. From (Ormond, 1999).

ATII cells are the main site for the synthesis, storage and release of surfactant (Wright and Clements, 1989). ATII cells are capable of *de novo* synthesis of all the major PL of the surfactant system (Haagsman and van Golde, 1991) and the major surfactant proteins. In addition to their crucial function in the surfactant system, ATII cells are involved in the repair of the alveolar epithelium after lung injury (by differentiation into alveolar type I cells) and the regulation of the amount and composition of the fluid lining the alveoli (aqueous hypophase) (Mason and Voelker, 1998). Lamellar bodies are stabilised by the surfactant protein SP-B (Weaver, 1998) and consist of a proteinaceous core, around which are stacked bilayers of lipids within a limiting membrane. The limiting membrane of the lamellar bodies fuses with the plasma membrane of ATII cells to release the surfactant components into the hypophase (Johansson and Curstedt, 1997).

1.2.4. THE SURFACTANT CYCLE

In mammals, lamellar bodies are secreted in response to local biochemical factors (Goerke, 1998), signals from the autonomic nervous system (Massaro et al., 1982) and deep breathing or stretch (Chander and Fisher, 1990; Wirtz and Schmidt, 1992) (Figure 1.2). In the aqueous hypophase, the lamellar body contents hydrate and unravel to form tubular myelin, a characteristic crosshatched structure (Lumb, 1989). Surfactant components are released from the tubular myelin and form a "surface-active" layer at the air-liquid interface (Lumb, 1989). The surfactant layer at the air-liquid interface has long been considered a simple monolayer. However, recent electron microscopy and biophysical studies suggest that several lipid layers are closely associated with the surface film at the air-liquid interface (Johansson and Curstedt, 1997; Schürch et al., 1995). When the alveoli are compressed during expiration, USP and neutral lipids are thought to be excluded from the surface film, and may be recycled or degraded under the

control of SP-A, and possibly SP-D (Kuroki and Voelker, 1994; Wright, 1990). The remaining surface film is rich in DSP, particularly DPPC, and by the end of expiration, the surface tension is almost zero (Haagsman and van Golde, 1991). At this point, the surface film exists in a gel state because DPPC has a phase transition temperature of 41°C (temperature at which it changes from a solid, tightly packed, gel state to a more disordered, liquid-crystalline state). During compression at 37°C, the DPPC-rich surface film is packed tightly together, excluding water molecules and lowering surface tension (Possmayer, 1997). A low surface tension makes it easier to inflate the lungs during inspiration and as the alveoli expand, the DPPC-rich surface film probably pulls apart into rafts (Goerke and Clements, 1985). As the alveoli expand, CHOL and USP are retrieved from the secondary lipid layers or released from tubular myelin and adsorb into the surface layer between the DPPC rafts (Goerke, 1998). SP-B and SP-C promote this rapid adsorption of lipids to the air-liquid interface (Eiijk et al., 1995). Neutral lipids and USP lower the phase transition temperature of the surface layer mixture and therefore, increase the fluidity of surfactant (Possmayer, 1997) so that it can re-spread over the expanding alveolar surface (Goerke, 1998).
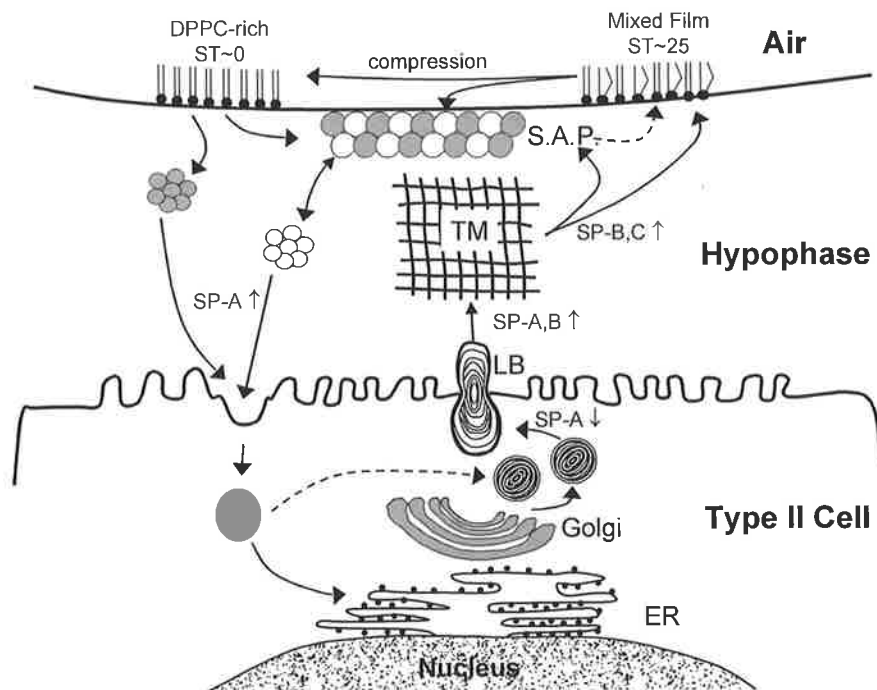
Figure 1.2: Schematic diagram of the life cycle of pulmonary surfactant. Pulmonary surfactant components are synthesised in the endoplasmic reticulum (ER), transported to the Golgi apparatus (Golgi) and packaged into lamellar bodies (LB). Lamellar bodies are secreted into the liquid lining the alveoli (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 as well as the surface-associated phase (S.A.P.). 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 (ST) to near 0 mN/m. It is possible that some lipids from the S.A.P. reenter the surface film (dashed line arrow). Lipids from the surface film and the S.A.P. are eventually recycled and taken back up by the type II cell via endocytosis. The role of some of the surfactant proteins (SP-A, -B and –C) in regulating these processes is indicated with ↑ (stimulation) or ↓ (inhibition). Figure reproduced from Figure 1 in (Orgeig et al., 2003).

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1.2.5. TEMPERATURE

Given the biophysical properties (e.g. phase transition temperature) of the lipid constituents, temperature is of profound importance to the behaviour and function of pulmonary surfactant. However, despite experiencing very large and often very rapid changes in body temperature, both ectotherms and heterothermic endotherms, do not suffer from any apparent respiratory distress, or surfactant dysfunction. While, homeothermic mammals can modify their surfactant composition under certain physiological conditions they do not appear to do so under temperature stress. However, ectotherms and heterothermic mammals alter the composition and surface activity of their surfactant in order to respond to changes in body temperature. The compositional changes that occur at low temperatures appear to facilitate low surface tensions and faster movement of lipids to the air-liquid interface. This suggests that in vivo surfactant function is optimised to body temperature so that surfactant in cold animals functions very similarly to surfactant in warm animals. However, in order for the observed changes in lipid composition and increases in surfactant to occur at cold body temperatures, there must also be changes in the regulatory and secretory pathways controlling surfactant release into the lung.

1.3. TEMPERATURE AND SURFACTANT BIOPHYSICS

1.3.1. COMPARATIVE BIOLOGY OF SURFACTANT FUNCTION

The primary role of surfactant in mammals is to reduce and vary surface tension at the air-liquid interface and thus, increase lung compliance (and therefore, decrease the work of breathing), provide alveolar stability and prevent alveolar oedema (Daniels et al., 1998b). However, in reptiles and amphibians, removal of surfactant has little effect on lung compliance because respiratory units are relatively large and extremely compliant

(Daniels et al., 1998b). In addition, avian lungs are rigid and non-distensible (Daniels et al., 1998b). The different function of surfactant in the lungs of mammals and non-mammals is reflected in differences in surface activity (Table 1.2). In mammals, highly surface-active material is crucial to prevent atelectasis (collapse) of the alveoli, and to increase static compliance. However, the mode of breathing and ventilatory mechanics observed in reptiles, amphibians and fish seems to require a less surface-active surfactant. In these animals, surfactant acts as an 'anti-adhesive' to prevent alveolar surfaces from adhering to each other (Daniels et al., 1994a; Daniels et al., 1994b; Frappell and Daniels, 1991) (Section 1.3.2.). An 'anti-adhesive' function may also be important in heterothermic mammals during torpor when they experience extended non-ventilatory periods (Langman et al., 1996). Furthermore, diving mammals, amphibians and reptiles collapse their alveoli at depth when external compression forces are elevated (Daniels et al., 1998b). Thus, the anti-adhesive function of surfactant, in preventing alveolar surfaces from sticking together, may be essential in cold and diving animals regardless of phylogenetic grouping or lung structure.

In all the vertebrate groups, or more accurately, in all lung types, controlling lung fluid balance and thereby, preventing pulmonary oedema is another important function of surfactant (Daniels et al., 1998b). Filtration, or the movement of fluid from capillaries into the lung tissue, is highest in amphibians and reptiles (Burggren, 1982). Therefore, the tendency for fluid to enter the lungs in amphibians and reptiles is much greater than in mammals. In addition, birds have minute air capillaries and the 'anti-oedema' function may in fact be the only physical function of avian surfactant (Daniels et al., 1998b). Hence, the functions of pulmonary surfactant differ between mammalian and non-mammalian animals and these functional differences are reflected in the surface-activity of the surfactant in their lungs (Table 1.2).

Table 1.2: Summary of surface activity parameters of surfactants from a range of vertebrates.

Species	T_A (°C)	Device	ST_{eq} (mN/m)	ST_{min} (mN/m)	%SA comp	Surface activity
I. Osteichthys						
1. Teleostei						
Carassius auratus [a]	22	BS		26		very low
Hoplias malobaricus [b]	37	WB		22	70[*]	very low
Hoplerythrinus unitaeniatus [b]	37	WB		22	80[*]	very low
Arapaima gigas [b]	37	WB		22	75[*]	very low
2. Ginglymodi						
Lepisosteus osseus [c]	24	WB	25.0±1.3	17.0±1.0	70	very low
3. Dipnoi						
Lepidosiren paradoxa [c]	37	WB		22	70	very low
II. Amphibia						
1. Caudata						
Amphiuma tridactylum [c]	24	WB	27.7±0.4	17.6±1.1	62	low
2. Anura						
Bufo marinus [c]	24	WB	26.7±1.1	18.6±0.5	55	low
III. Reptilia						
1. Ophidia						
Eunectes murinus [d]	37	WB		22	70[*]	very low
2. Crocodilia						
Alligator mississippiensis [c]	24	WB	32.9±1.1	19.6±0.5	70	very low
3. Testudinata						
Trachemys scripta [c]	24	WB	26.7±1.7	18.0±0.4	60	low
Testudo hermanni [e]	24	WB	40.0±6.0	4.0±1.7	65[*]	intermediate
4. Lacertilia						
Pogona vitticeps [c]	24	WB	24.6±1.9	14.4±0.8	30	high
Trachydosaurus rugosus [c]	24	WB	24.4±0.6	13.2±1.5	30	high
IV. Aves						
Gallus gallus domesticus [c]	24	WB	28.6±4.9	18.8±0.2	75.0	very low
Meleagris gallapavo [f]	22	WB		5.6±2.1	57[*]	intermediate

V. Mammalia

Mus musculus [c]	24	WB	25.8±1.5	5.1±1.1	46	very high
Sminthopsis crassicaudata [c]	24	WB	26.3±0.5	5.4±0.3	43	very high
Rattus norvegicus [g]	37	CBS	25	<1	15-30	very high
Chalinolobus gouldii	37	CBS	25	<1	11.8	very high

Data are mean ± SEM. T_A ambient temperature, ST_{eq} equilibrium surface tension, ST_{min} minimum surface tension, % SAcomp % Surface Area Compression, WB Wilhelmy-Langmuir surface balance, BS Enhorning Bubble Surfactometer, CBS Captive Bubble Surfactometer, * values for % SAcomp were recalculated from data in the literature as the compression required to achieve ST_{min} from 100% Area. a (Daniels and Skinner, 1994), b (Phleger and Saunders, 1978), c (Daniels et al., 1998a). d (Phleger and Benson, 1971), e (Meban, 1980), f (Fujiwara et al., 1970), g (Schürch et al., 1992). Table modified from Table 1 in (Daniels et al., 1998a).

1.3.2. FUNCTION OF SURFACTANT AS AN 'ANTI-ADHESIVE' IN ECTOTHERMIC ANIMALS

In ectothermic animals, the surface activity of surfactant is significantly lower than that observed in mammalian lungs (Table 1.2) and this corresponds to differences in surfactant composition (Section 1.4.1). Moreover, most lungs of ectothermic vertebrates are 100-200 times more compliant than mammalian lungs and this alleviates the need to drastically reduce surface tension at the air-liquid interface during inspiration (Daniels et al., 1998a). Thus, the main function of surfactant in ectotherms may be in preventing alveolar surfaces from adhering together at low lung volumes (Daniels et al., 1993). Using, scanning electron microscopy to demonstrate the breathing dynamics of the lizard *Ctenophorus nuchalis*, (Daniels et al., 1994a), showed that during lung deflation, the epithelial tissues fold in on each other like a concertina. This results in large portions of

epithelial tissue coming into contact during low lung volumes. The work required to initially separate these surfaces is dependent upon the distance originally separating the surfaces, the areas of surfaces in contact and the surface tension lining the surfaces (Sanderson et al., 1976). A fluid lining of low surface tension between the surfaces would prevent the adhesion of alveolar surfaces in contact and reduce the work of separating the surfaces (Sanderson et al., 1976). Hence, the pressure initially required to open a completely collapsed lung (opening pressure) can provide an indication of the anti-adhesive function of surfactant (Daniels et al., 1993; Daniels and Skinner, 1994; Smits et al., 1994). Removing surfactant from lungs isolated from lizards (Daniels et al., 1993), actinopterygian fishes (Daniels and Skinner, 1994; Smits et al., 1994), garter snakes (Daniels et al., 1995c) and tiger salamanders (Daniels et al., 1995a) significantly increases opening pressure of the lungs. However, in all cases, filling pressure (i.e. after initial lung opening) was extremely small (1 - 4 cm H_2O) and remained unchanged before and after lavage (Daniels et al., 1998a). This indicates that surfactant is important in these lungs only during the initial phase of inflating a collapsed lung and not during further inflation or deflation, as is the case in mammals.

The anti-adhesive function of surfactant may be particularly important when ectotherms are cold because the normal respiratory cycle of many reptiles and amphibians is characterised by low lung volumes and long non-ventilatory periods (Martin and Hutchison, 1979; Milsom, 1991). Two studies have investigated the effect of changes in body temperature on the anti-adhesive function of surfactant in ectothermic animals. An increase in body temperature from 27 to 37°C in central netted dragons, *Ctenophorus nuchalis*, decreases the opening pressure of the lungs, without affecting lung compliance. This may reflect an increase in fluidity due to the increase in temperature and thus, an increase in the spreadability of surfactant over alveolar surfaces at 37°C (Daniels et al., 1993). In bearded dragons, *Pogona vitticeps*, increasing body

temperature from 18 to 37°C had no affect on opening pressure or filling pressure (Wood et al., 1995). Hence, a lower body temperature does not affect the anti-adhesive properties of surfactant in bearded dragons. Furthermore, the increase in amount of PL observed in bearded dragons at 37°C does not appear to affect opening pressure and thus, surfactant anti-adhesive function. Hence, the observed increases in surfactant PL at 37°C in bearded dragons may be due to increases in metabolic rate and thus, increased secretion of lamellar bodies from ATII cells. Although a critical amount of surfactant may be necessary to serve an anti-adhesive function, increases above this amount do not appear to further promote the non-adherence of epithelial surfaces (Wood et al., 1995).

The anti-adhesive properties of surfactant appear to be dependent on its ability to reduce surface tension to some extent and to remain fluid and readily adsorbed to the air-liquid interface (Daniels et al., 1998a). Hence, the more detergent-like surfactant of ectothermic animals is capable of lowering, but not necessarily varying surface tension to any great extent. However, ectothermic surfactant can spread easily over lung epithelial surfaces to prevent adhesion, even when animals are cold.

1.3.3. SURFACE ACTIVITY MEASUREMENTS

The surface activity of surfactant has been measured using a variety of different procedures (Enhorning, 2001; Keough et al., 1989; Pattle, 1958, 1960; Porter, 1933; Possmayer, 1991; Putz et al., 1994a; Putz et al., 1994b; Wood et al., 1995; Yu et al., 1983) but more recently, using a primed Wilhelmy-Langmuir surface balance (WLB) and a captive bubble surfactometer (CBS). The WLB consists of a half-filled deep Teflon trough fitted with a tight barrier (Goerke and Clements, 1985; Possmayer, 1991). Before application of surfactant material, the Teflon trough and leading side of the barrier are primed with DPPC, to prevent surfactant material from adhering to the walls of the trough and thereby leaking from the surface (Lopatko et al., 1998). Surface-active

materials are placed on the surface of the liquid, where they spread. The mechanically driven barrier compresses the film and a dipping plate attached to a force transducer is then used to calculate surface tension (Possmayer, 1991). In the CBS, a liquid bubble floats (is buoyant) against a hydrophilic roof of 1% agarose gel and the volume of the bubble is controlled by varying the pressure in the chamber (Schürch et al., 1989; Schürch et al., 2001). As volume is reduced, the surface area is reduced and the surface tension of the surfactant film at the bubble surface falls. The bubble shape changes from spherical to oval depending on the surface tension. Surface tension measurements are made through shape analysis of the bubble using digital image processing techniques (Schürch et al., 1992). A temperature-controlled jacket surrounds the chamber containing the bubble so that experiments can be conducted at different temperatures (Schürch et al., 1992). In the CBS, the surface film is not interrupted by barriers such as plastic walls or outlets and therefore, provides a leak proof system, unlike the WLB (Schürch et al., 2001).

When measuring surface activity, a number of biophysical properties are usually examined (Figure 1.3). Adsorption is a measure of the rate at which surface-active material accumulates at the air-liquid interface from the aqueous sub-phase and reduces surface tension (Daniels et al., 1998a). Both the rate of adsorption and the resulting equilibrium surface tension (ST_{eq}) indicate the ability of surfactant to form a surface-active film and are strongly dependent on the concentration of PL in the measured sample. A 'good' surfactant usually has a ST_{eq} of 25 mN/m (Schürch et al., 1992). When the surfactant film is cyclically compressed under dynamic conditions *in vitro,* surface tension decreases below ST_{eq}, to the ST minimum (ST_{min}). A measure of how efficiently surfactant reduces surface tension under compression can be obtained from the change in surface area compression (% SAcomp) required to achieve ST_{min}. The ST_{min} and % SAcomp are therefore, the best indicators of the ability of surfactant to

lower surface tension under dynamic compression. Generally, a "good" surfactant is able to reach an ST_{min} of $< 5mN/m$, and an excellent surfactant < 1 mN/m (Schürch et al., 1992).
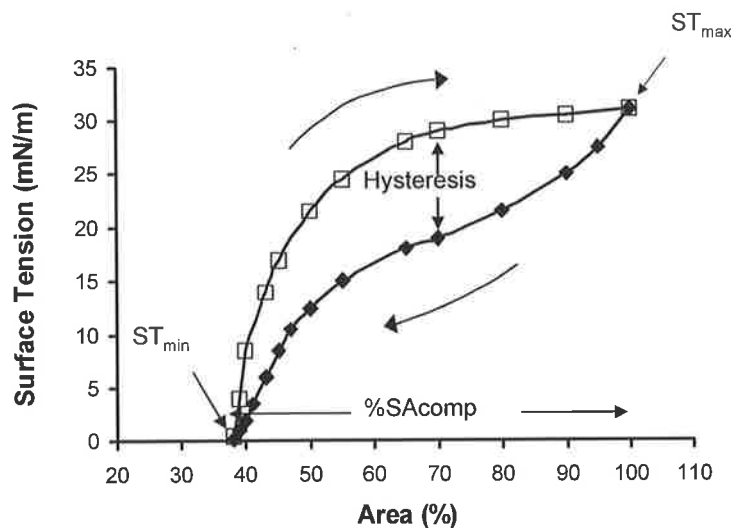
Figure 1.3. Schematic diagram of a typical surface tension-area isotherm during dynamic compression of a surfactant film in vitro. Arrows indicate the expansion and compression limbs of the curve. The difference in surface tension at the same area% between the compression and expansion limbs is termed hysteresis, and represents the irreversible heat loss in the cycle. Upon repeated cycling, the hysteresis rapidly diminishes as the curve is shifted to the right. This has the effect that the % surface area compression (% SAcomp) required to achieve minimum surface tension (ST_{min}) is reduced; i.e. the film is capable of generating low surface tensions more rapidly and efficiently. Furthermore, the maximum surface tension (ST_{max}) frequently decreases with repeated dynamic cycling.

1.3.3.1. HOMEOTHERMIC MAMMALS

The effect of temperature on the surface activity of surfactant isolated from homeothermic mammals appears to be controversial and is largely dependent on the procedures employed to measure surface tension (Avery, 2000; Avery and Mead, 1959; Crosfill and Widdicombe, 1961; Lopatko et al., 1998; Miles et al., 1995; Schürch et al., 1992; Schürch et al., 1985; Schürch et al., 1976) (Table 1.3). The apparent increases in ST_{min} with increasing temperature, seen in some of the earlier studies, are most probably due to surface film leakage in the apparatus used (Lopatko et al., 1998). Recent techniques such as the primed WLB, CBS and the in situ microdroplet technique generally demonstrate that in vitro, temperature does not greatly affect the surface properties of isolated pulmonary surfactant (Lopatko et al., 1998). Miles et al. (1995) reported that ventilating isolated rat lungs at 37°C and 22°C had no effect on the ST_{min} of the surfactants measured using a primed WLB at 22°C and 37°C, respectively. However, ST_{min} did increase significantly when measured on the WLB at 42°C (19 mN/m) (Miles et al., 1995) (Table 1.3). Using an improved microdroplet technique and measuring surface tension over the entire pressure-volume curve at 22°C and 37°C, alveolar surface tension and the surface tension to volume relationship are both essentially identical at the two temperatures (Schürch et al., 1976). Thus, within this temperature range, temperature seems to have little effect on the surface activity of homeothermic surfactant and this may have profound implications in vivo.

Table 1.3. The effects of ambient temperature on surface tension parameters of lavage fluid isolated from lungs of homeothermic mammals.

Animal	T_A	ST_{max}	ST_{min}
	°C	mN/m	mN/m
Dog[a]	22	51.5 + 3.6	7.0 ± 2.2
Dog[b]	22d	55.0± 2.1[*,#]	1.6 ± 1.3[#]
	22w	54.4 ± 3.0[^,∇]	3.2 ± 1.5[^]
	37d	44.8 ± 3.5[*,^]	6.5 ± 4.5[*]
	37w	45.7 ± 6.6[#,∇]	15.3± 4.9[*,^,#]
Human[c]	29	68.0 ± 2.7[*,^,#]	2.0 ± 2.4
	33	62.9 ± 3.6[#]	2.0 ± 1.6
	37	55.6 ± 4.0[^]	1.8 ± 1.6
	41	46.8 ± 4.5[*]	1.8 ± 2.4
Rat[d]	22v	37.0 ± 2.0[*,^]	8.0 ± 6.0[*,^]
	37v	46.0 ± 1.0[*]	9.0 ± 1.0[*,#]
	42v	46.0 ± 3.0[^]	19.0 ± 3.0[^,#]
Bovine[e]	37°C	27.1 ± 01.5	0.5 ± 0.5

Data are mean ± S.D., unless otherwise indicated. T_A ambient temperature, ST_{min} minimum surface tension, ST_{max} maximum surface tension, d ~25% humidified air, w 98% humidified air, v lungs ventilated at different temperatures, but all surfactants were measured on the surface balance at 22°C. Data are from a (Diaz and Gunther, 1984) Wilhelmy-Langmuir balance, b (Wildeboer-Venema, 1980) Wilhelmy-Langmuir balance, c (Meban, 1978) Wilhelmy-Langmuir balance, d (Miles et al., 1995) Kimray Greenfield surfactometer and e (Yu and Possmayer, 1988) For each measurement within a study, pairs of symbols indicate a significant difference.

Breathing requires the contraction of muscles and is therefore, an energy requiring process. The amount of energy required to breathe (work of breathing (W_t)) is dependent on the type of breathing pattern (frequency (f) and tidal volume (V_T)), airways resistance (W_{raw}) and lung compliance (ease of inflation (C)) where

$$W_t = W_{raw} + (f \times \frac{V_T^2}{2C})$$ (Crosfill and Widdicombe, 1961).

Lung compliance (C), or the ease of inflation, is determined by the elasticity of lung tissue and by the surface tension of the fluid lining the alveoli (von Neergaard, 1929). Lung connective tissue is composed of collagen fibres, which are highly sensitive to temperature (Inoue et al., 1982). Decreasing temperature significantly increases the elasticity of lung tissue and therefore, decreases lung tissue compliance (Inoue et al., 1982). A decrease in lung compliance makes it harder to inflate the lungs and thus, increases the work of breathing (Crosfill and Widdicombe, 1961). An increase in the work of breathing leads to an increase in the amount of oxygen required to ventilate the lungs and thus decreases the efficiency of oxygen extraction with each breath (Crosfill and Widdicombe, 1961). However, overall lung compliance could be maintained despite decreases in tissue compliance if the surface tension of the fluid lining the alveoli was further decreased by increases in surfactant surface activity, which are induced by changes in surfactant composition (Langman et al., 1996). Unfortunately, no studies have examined the composition of surfactant from homeotherms at temperatures lower than the relatively mild 20-25°C (Section 1.4.1.3). At temperatures above 20°C, there are also some discrepancies between the lung pressure-volume and hysteresis studies using cats (Horie et al., 1974), dogs (Diaz and Gunther, 1984; Wildeboer-Venema, 1980), rabbits (Inoue et al., 1981, 1982; Lempert and Macklem, 1971; Wildeboer-

Venema, 1980) and rats (Clements, 1977) (Table 1.4). The discrepancies are likely to be due to the different species, methods, temperature regimes and data analyses used. However, although the details and mechanisms remain unclear, it is generally agreed that cooling of lungs from homeothermic mammals is associated with decreases in overall lung compliance (Inoue et al., 1981). This suggests that the surface-active properties of homeothermic surfactant and thus, its composition are not altered by a decrease in temperature. Hence, although homeothermic mammals can modify their surfactant composition under certain physiological conditions (Section 1.4.1.3) they do not appear to do so under temperature stress.

Table 1.4. The effect of temperature on pressure-volume curves and hysteresis in lungs isolated from homeothermic mammals.

Animal	T_A °C	P (cmH$_2$0) during compression, expansion at 50% TLC	P (cmH$_2$0) during compression, expansion at 80% TLC	Hysteresis %
Rabbit[a]	5	7.6$^\wedge$, 19*	19$^\wedge$, 28$^\#$	130 $\pm$ 2.0$^{*\wedge}$
	21	2.4$^\wedge$, 7.5*	5$^\wedge$, 20$^{*\#}$	100*
	37	2.4, 7.4	4.9, 16*	95 $\pm$ 1.6$^\wedge$
Rabbit[b]	4	9*, 21$^\wedge$	19*, 30$^\wedge$	130 $\pm$ 8*
	21	3*, 12$^\wedge$	7$^{*\partial\bullet\delta}$, 18$^\wedge$	100$^{*\wedge\#}$
	37		5$^\partial$, 18	102 $\pm$ 4.0
	47		6$^\bullet$, 17	120 $\pm$ 2.4$^\wedge$
	52	4, 14	6$^\delta$, 17	130 $\pm$ 1.6$^\#$
Cat[c]	22	2.5, 12	6.2, 15	
	37	2.5, 10	6.1, 15	
	47	2.5, 9	6, 13	
Rat[d]	24	2.5, 10	7, 23	0.28$^{*\wedge k}$
	43	2.5, 16	7, 23	0.4^{*k}
	45	4, 17	10, 25	0.42$^{\wedge k}$

T_A ambient temperature, P pressure, TLC total lung capacity, k normalised hysteresis calculated by dividing hysteresis area by the volume and pressure excursions of the loop (cm^2 ml^{-1} cmH$_2$0^{-1}). Data are estimated from pressure-volume curves in a (Inoue et al., 1981), b (Inoue et al., 1982), c (Horie et al., 1974) and d (Jones et al., 1996). For each measurement within a study, pairs of symbols indicate a significant difference.

1.3.3.2. HETEROTHERMIC MAMMALS

In heterothermic mammals, the surfactant system must be very dynamic and respond 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* (Langman et al., 1996; Lopatko et al., 1998; Lopatko et al., 1999; Ormond et al., 2001) and the microchiropteran bats, *Chalinolobus gouldii* (Codd et al., 2000b) and *Nyctophilus geoffroyii* (Slocombe et al., 2000).

Dunnarts are small marsupial mammals that live in the semi-arid regions of Australia. Dunnarts enter torpor in response to food deprivation and low ambient temperatures, i.e. stress torpor. During stress torpor, body temperature decreases from around 35°C in warm-active dunnarts to a few degrees above ambient temperature (often between 10 and 20°C) in torpid dunnarts (Godfrey, 1968). Stress torpor in dunnarts is also accompanied by changes in ventilatory pattern and torpor bouts can last for more than 8 hours (Godfrey, 1968). Dunnarts can be maintained in breeding colonies in the laboratory and thus, like rats and mice, are an ideal research model.

Gould's wattled bats, *Chalinolobus gouldii*, are small insectivorous bats that are widespread and common throughout Australia. Gould's wattled bats enter daily torpor as part of a regular circaddian cycle. During daily torpor in bats, body temperature can drop as low as 5°C (Hosken and Withers, 1997). In torpor, bat ventilation undergoes marked changes, with episodic breathing bouts alternating with long non-ventilatory periods (Morris et al., 1994).

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

metabolic rate (Geiser and Ruf, 1995) and thus, oxygen extraction. Consequently, the work of breathing, as a percentage of metabolic rate, may be expected to rise dramatically (Section 1.3.3.1) as body temperature, falls (Wood and Daniels, 1996). Hence, reducing the work of breathing is likely to be of greater importance in cold ectothermic and heterothermic animals than in homeothermic mammals. However, although tissue compliance decreased at 1 h and 4 h of stress torpor in dunnarts, overall lung compliance measured in the presence of surfactant was unaffected by stress torpor. If tissue compliance decreases, then the surface tension of the alveolar fluid must also decrease in order for overall lung compliance to remain unchanged. Therefore, surfactant appears to counteract the decrease in tissue compliance during torpor in heterothermic mammals and thus, maintains the work of breathing at a minimum. It should be noted however, that this argument does not address any changes in functional compliance that may occur if there are changes in functional residual capacity (FRC). It is extremely difficult to obtain measurements of FRC in animals as small as bats and dunnarts, particularly during torpor, since any disturbance will cause them to arouse. Hence, it is possible that during torpor, tidal ventilation occurs over a flatter portion of the pressure-volume curve and if so, the apparent maintenance of overall lung compliance may be due only to the change in FRC. On the other hand, if tidal ventilation occurs over a steeper portion of the pressure-volume curve during torpor, the role of surfactant in maintaining lung compliance is confirmed. Moreover, ventilatory periods are much more infrequent during torpor (Geiser and Ruf, 1995) and as a result, the work of breathing (as a percentage of metabolic rate) may over time, actually decrease even further (Wood and Daniels, 1996). Therefore, both dunnarts and bats 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. This can be achieved by lowering the phase

transition temperature of the lipid mixture, by changing the lipid composition (Section 1.4.1.2). Consequently, mechanisms that alter and maintain surfactant amount and composition in the lung must function adequately at both warm and cold body temperatures (Section 1.6).

Table 1.5. Effect of temperature on surface activity of pulmonary surfactant from mice and the fat-tailed dunnart, *Sminthopsis crassicaudata*.

Animal/Activity State	ST_{min}	% SAcomp
	Properties measured at 20 °C in WLB	
Mice	5.32 ± 0.30	46.0 ± 3.0
Warm-active Dunnart	5.45 ± 0.2	48.3 ± 0.45
4 h Torpor Dunnart	3.97 ± 0.25	$36.0 \pm 3.2^*$
8 h Torpor Dunnart	4.43 ± 0.19	$26.5 \pm 2.2^\wedge$
	Properties measured at 37 °C in CBS	
Warm-active Dunnart	6.41 ± 0.30	89.1 ± 0.8
8 h Torpor Dunnart	$9.12 \pm 0.3^*$	$84.9 \pm 1.8^*$

Data expressed as mean $\pm$ SE; n = 4-6 animals. Values for surface tension parameters are in mN/m. ST_{min} minimum surface tension, % SAcomp compression required to achieve ST_{min} from 25 mN/m, WLB Wilhelmy-Langmuir Surface Balance, CBS Captive Bubble Surfactometer. * Significantly different from warm-active dunnarts. ^ Significantly different from warm-active and 4 h torpid dunnarts. Data from (Lopatko et al., 1998).

The surface activity properties of surfactant collected from active, torpid and arousing dunnarts and bats are provided in Tables 1.5 and 1.6, respectively. Surfactant collected from active and torpid dunnarts appears to function optimally at the body temperature of the animal from which it was isolated. A surface tension of 9.12 ± 0.3

mN/m at 37°C was found for surfactant obtained from torpid dunnarts killed after being in torpor for 8 h with a body temperature of 15°C. However, a surface tension of 6.41 ± 0.3 mN/m at 37°C was found for surfactant obtained from warm-active dunnarts. Although these changes in surface tension are relatively small (i.e. less than 5 mN/m) and may not seem biologically significant in terms of one breath, the accumulative reductions in surface tension over a period of torpor or hibernation and/or the lifetime of the animal, could potentially be very significant to an animals respiratory function. Furthermore, adsorption (movement to the air-liquid interface) of surfactant collected from torpid dunnarts is significantly slower than surfactant collected from warm-active dunnarts when measured at an ambient temperature of 37°C. Conversely, surfactant from torpid dunnarts adsorbs much faster at 15°C than surfactant from active dunnarts (Lopatko et al., 1998). Surfactant from torpid dunnarts also requires a smaller change in compressibility (% SAcomp) to attain ST_{min} compared with surfactant from active dunnarts measured at either 20°C or 37°C (Lopatko et al., 1998).

Similar changes in surface activity have been observed during torpor in Gould's wattled bats. When surfactants from warm-active and torpid Gould's wattled bats were analysed on a CBS at a temperature matching the body temperature of the bat, equilibrium surface tensions of 25 mN/m and minimum surface tensions of 1 mN/m were achieved. These values are similar to the literature values for other mammals (Codd et al., 2002; Goerke and Clements, 1985). 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 warm-active bats at 37°C yielded lower ST_{min} and % SAcomp than when measured at 24°C. Conversely, surfactant from torpid bats reached a lower ST_{min} and required less % SAcomp to reach low ST_{min} at 24°C than at 37°C (Codd et al., 2002). Hence, in heterothermic mammals,

surfactant from warm-active animals appears to be more suited to function at higher temperatures (37°C) and surfactant from torpid animals appears to function best at lower temperatures.

Table 1.6. The effect of temperature on the surface activity of pulmonary surfactant from Gould's wattled bat, *Chalinolobus gouldii.*

Activity Status	Temp (°C)	n	ST_{min} (mN/m) (mean ± SE)	ST_{max} (mN/n) (mean ± SE)	% SAcomp
Warm-active	37	6	$1.2 \pm 0.008^*$	$27.7 \pm 0.17^*$	$11.8 \pm 0.1^*$
Bat	24	6	$2.5 \pm 0.09^*$	$31.9 \pm 0.15^*$	$17.2 \pm 0.5^*$
Torpid	37	6	$3.76 \pm 0.04^{\#}$	23.36 ± 0.26	$10.1 \pm 0.2^{\#}$
Bat	24	6	$1.2 \pm 0.2^{\#}$	23.03 ± 0.13	$8.75 \pm 0.2^{\#}$
Arousing	37	6	$1.77 \pm 0.51^{\wedge}$	$28.56 \pm 0.3^{\wedge}$	11.9 ± 0.1
Bat	24	6	$2.16 \pm 0.1^{\wedge}$	$26.51 + 1.21^{\wedge}$	$11.3 + 0.3$

Data expressed as mean ± SE for ST_{min}, ST_{max} and % SAcomp for surfactant isolated from warm-active, torpid and arousing bats dynamically cycled at 25 cycles/min and examined at 24°C and 37°C using a captive bubble surfactometer. Pairs of symbols denote values that are significantly different. Bat data from (Codd et al., 2003; Codd et al., 2002).

1.3.3.2.1. Rapid arousals from torpor

Arousal from torpor by heterothermic mammals can be very rapid. During arousal in dunnarts, body temperature increases from between 10°C and 15°C to between 32°C and 35°C at a rate of 0.7–1°C per minute (Geiser and Baudinette, 1990). Bats also arouse very rapidly from torpor with warming rates of up to 0.8°C per minute (Codd et al., 2000a). 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

ST_{min} and % SAcomp) (Codd et al., 2002). Surfactant isolated from dunnarts killed immediately after arousal from torpor, adsorbs slowly at 37°C at a rate similar to that of surfactant from torpid animals. When assayed at 15°C, surfactant from aroused dunnarts adsorbs faster than that of warm-active dunnarts but more slowly than that of torpid dunnarts. However, as in bats, ST_{min} and % SAcomp decrease rapidly in dunnart surfactant immediately after arousal, which results in a pronounced improvement in surface tension lowering ability at 37°C. Thus, surfactant from heterothermic mammals undergoes rapid changes in surface activity that enables the mixture to function effectively at rapidly increasing body temperatures.

At least in dunnarts, but probably also in bats, the surface activity of surfactant undergoes virtually simultaneous changes with increasing body temperature as they rapidly arouse from torpor. Rapid improvements in surface activity occur immediately after arousal and may be necessary to optimise the alveolar surface area for gas exchange when rapid increases in breathing frequency and lung tidal volume occur. Slower changes in surface activity to warm-active levels may reflect a post-arousal stabilisation of the respiratory system at the higher body temperature (Lopatko et al., 1999).

1.4. TEMPERATURE AND SURFACTANT COMPOSITION

1.4.1. LIPID COMPOSITION

The composition of surfactants from a wide range of animal species and their preferred body temperature are given in Table 1.7. The amount of surfactant lipids, when normalised to wet lung mass, is lowest in fish, intermediate in amphibians and highest in reptiles and mammals (Daniels et al., 1995a). However, when normalised to the surface area of the lungs, fish and reptiles contain greater amounts of lipid than occurs in the lungs of mammals (Daniels et al., 1995a). When expressed as a percentage of PL, fish

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contain much greater levels of cholesterol (CHOL) and unsaturated phospholipids (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).

The lowest CHOL levels are found in the highly specialised lungs of microchiropteran bats. Lesser long-eared bats, *Nyctophilus geoffroyi* and Gould's wattled bats, *Chalinolobus gouldii*, have 15 and 6 times less cholesterol, respectively than has been reported in the surfactant of any other mammal (Codd et al., 2000b; Slocombe et al., 2000). The complexity of the bat lung and the minute size of the alveoli may explain the low levels of CHOL observed in bat surfactant. In bats, the air spaces are finely sub-divided such that the alveolar diameter is smaller than that of most other similar sized mammals (Slocombe et al., 2000). The small alveolar surface area may reduce or alleviate the need for a 'spreading' agent in the surfactant of bats. Alternatively, bats may exhibit a decreased CHOL as an adaptive strategy to maximise the surface activity. High surface activity may be necessary to counteract the higher collapse pressures associated with the rapid cycling in volume of small alveoli and the increased ventilation necessary for the high metabolic cost of flight (Codd et al., 2002). Therefore, a high CHOL/low DSP mixture may be a primitive surfactant that was modified through the evolution of the vertebrates by a range of selection pressures, including terrestriality, habitat, lung structure, lung function and temperature (Daniels et al., 1998a).

It is clear from Table 1.7, that temperature has been a powerful evolutionary force on the lipid composition of the surfactant system. 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 up to 42°C) (Daniels and Orgeig, 2001). 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. Conventional theory argues that a cholesterol-rich surfactant can function at low body temperatures because cholesterol lowers the phase transition temperature of the lipid mixture over a broad range of temperatures and acts a fluidiser at the air-liquid interface (Langman et al., 1996; Lopatko et al., 1998; Lopatko et al., 1999). CHOL acts as a fluidiser at low temperatures (Notter et al., 1980), by disrupting the Van der Waals forces between adjacent PL fatty acid chains and forcing the mechanical separation of PL head groups to enhance adsorption and promote surfactant respreading upon inspiration (Presti et al., 1982). USP 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. At low body temperatures, an increase in surfactant fluidity can be achieved by diluting DSP content with CHOL (usually a short-term response) or USP (a long-term response) but these changes will also decrease the surface tension lowering ability (Daniels et al., 1998a; Daniels et al., 1998b; Lopatko et al., 1998; Lopatko et al., 1999). In non-mammalian vertebrates, a decrease in surface tension lowering ability is a feasible evolutionary trade-off, given the primary 'anti-adhesive' function of their surfactant. However, surfactant in heterothermic mammals must be fluid at cold body temperatures, yet still remain surface-active. Heterothermic mammals also maintain fluidity and surface activity, at least in part, by varying the relative proportions of CHOL and USP to DSP during activity and torpor. 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.

Table 1.7. Summary of surfactant lipid composition from a range of vertebrates.

Species	N	$T_{A/B}$ (°C)	PL (mg/ gWL)	PC	PG	PE	SM	PI	PS	LPC/ Unk	Chol (mg/ gWL)	Chol/ PL	DSP (mg/ gWL)	%DSP/ PL
							%PL							
Mammalia														
Eutheria														
Rattus norvegicus [a]	6	37	1.70[b]	75.5	11.0	4.6	0.2	3.0	1.9	ND	0.130[b]	0.071[b]	0.78[b]	45.7[b]
" [c #]	6	37	ND	83.6	8.3	0.7	|---------------4.0--------------|			ND	ND	ND	61[§]
Mus musculus [d]	6	37	16.9*	72.3	18.1	1.9	3.3	4.5		0	1.49*	0.097	7.46*	44.5
Nyctophilus geoffryi [y]	36	32	14.6*	ND	ND	ND	ND	ND		**ND**	0.1*	0.008	6.2*	42.27
Chalinolobus gouldii [z]	37	32	17.3*	ND	ND	ND	ND	ND		**ND**	0.268*	0.019	7.7*	44.1
Homo sapiens [e]	14	37	ND	72.8	9.5	5.3	1.3	11.1		**ND**	15.4[f # *]	0.071[b]	ND	48.5[b]
" [c #]	6	37	ND	80.5	9.1	2.3	|--------------8.1-------------|			ND	ND	ND	68[§]
Metatheria														
Macropus eugenii [g #]	3	37	**ND**	55.0	9.0	13.0	0.0	22.0		0	**ND**		ND	
Sminthopsis	6	~35	21.8*	70.7	6.0	2.1	7.7	13.5		0	1.48*	0.068	9.0*	40.2
crassicaudata [d]	6	~15	38.4*	58.7	6.4	3.9	9.2	21.8		0	4.14*	0.107	18.9*	49.3

Aves

	n	Temp												
Meleagris gallopavo [h]	6	~37	0.17	86.4	ND	5.6	0.3	ND	ND	ND	ND			
Gallus gallus [i]	1	~37	ND	79.2	0	5.2	2.9	8.9	3.0	ND	ND			
Domestica	5	~37									0.065[j]	ND	30.6[j]	
[f, #]	6	RT	15.7*	43.5	**ND**	21.9	10.1	17.7		2.4	18.8*			

Reptilia

Ophidia

	n	Temp												
Eunectes alv.lung [k]	1	~37	ND	54.0	0	12.0	20.0	7.0	8.0	ND	ND	ND	ND	ND
Murinus sac.lung	1	~37	ND	46.0	0	14.0	25.0	5.0	100	**ND**	ND	ND	ND	ND
Thamnophis ordinoides [l]	3	23	0.69	64.0	4.1	0	1.0	16.9		14.2	0.023	0.035	0.21	33.9
Crotalus atrox [l]	3	23	2.42	68.6	0	0	11.7	1.4		17.7	0.17	0.079	1.14	43.6

Lacertilia

	n	Temp												
Ctenophorus nuchalis [m]	8	37	2.16[b]	72.5	0	0	3.1	24.5		ND	0.24[b]	0.080[b]	1.06[b]	46.4[b]
Pogona vitticeps [n]	6-7	37	27.8*	80.1	0	0	3.6	16.2		0	NS	0.086	ND	ND
	4-7	18	20.2*	76.6	0	0	5.4	18.0		0	NS	0.083	ND	ND

Testudinata

	n	Temp												
Malaclemys	4	14	0.19	70.7	0	8.4	3.9	7.6	2.7	ND	ND	ND	ND	ND
geographica [o]	4	32	0.47	77.1	0	6.7	3.5	10.0	2.8	ND	ND	ND	ND	ND

Natator depressus[n]	5	RT	1.47	70.7	0	0	9.9	19.4		0	NS	0.061	NS	27.9
Caretta caretta[n]	5	RT	2.7	66.7	0	0	10.4	22.8		0	NS	0.099	NS	26.7
Pseudemys scriptas[f,#]	8	RT	14.2*	47.5	ND	24.8	8.7	13.2		3.4	11.5*	ND	ND	ND
Crocodilia														
Crocodylus porosus[n]	3	RT	0.96	67.6	0	2.5	4.7	2.5		0	NS	0.067	NS	27.7
Amphibia														
Anura														
Rana catesbiana[i]	1	?	ND	76.3	9.1	6.4	0.8	4.5	1.5	ND	ND	ND	ND	ND
Rana pipiens[f,#]	7	RT	11.7*	49.9	ND	21.5	6.3	14.4		2.4	17.7*	ND	ND	ND
[p]	10	14	1.07	73	11.7	2.8	3.5	1.0	ND	ND	ND	ND	ND	ND
Bufo marinus[q]	3	23	0.33	62.6	13.1	9.0	6.7	8.6		0	0.02	0.035	ND	13.2
Gymnophiona														
Typhlonectes natans[r]	8	23	0.44	66.1	0	0	9.1	13.1	1.2	Unk 10.4	0.043	0.107	0.181	43.4
Caudata														
Amphiuma tridactylum[q]	3	23	0.27	77.5	0	1.2	3.9	17.5	0	0	0.023	0.076	0.075	25.8

Species	n	$T_{A/B}$ (°C)	PL (mg/ gWL)	PC	PG	PE	SM	PI	PS	LPC/ Unk	Chol (mg/ gWL)	Chol/ PL	DSP (mg/ gWL)	% DSP /PL
							%PL							
Osteichthyes														
Dipnoi														
Neoceratodus forsteri [s]	3	23	0.084	59.6	0.5	14.4	15.3	10.2		0	0.022	0.242	0.006	8.9
Lepidosiren paradoxa [t]	1-3	RT	ND	46.0	8.0	19.0	11.0	0	0	0	ND	ND	ND	ND
[s]	3	23	0.22	73.9	1.8	6.9	7.3	9.3		0.8	0.010	0.044	0.059	27.8
Protopterus annectens [s]	4	23	0.61	80.2	3.0	3.0	5.6	7.9		0.4	0.028	0.052	0.126	20.4
Teleostei														
Hoplias malabaricus [t]	1-3	RT	ND	70	0	7	5	0	12	5	ND	ND	ND	ND
Hoplerythrinus Unitaeniatus [t]	1-3	RT	ND	69	0	25	2	0	2	0	ND	ND	ND	ND
Erythrinus erythrinus [t]	1-3	RT	ND	93	0	3	2	2	0	0	ND	ND	ND	ND
Arapaima gigas [t]	1-3	RT	ND	39	6	7	24	8	8	8	ND	ND	ND	ND

Carassius (posterior [u] *Auratus* swimbladder)	9	23	0.14	75	1.2	4.7	15.9	5.1		0	0.051	0.328	0.036	20.8
Ginglymodi														
Lepisosteus osseus [v]	5-8	23	0.50	76.4	1.4	4.6	5.9	7.9		3.0	0.09	0.175	0.069	14.1
Arthropoda														
Mollusca										Unk				
Helix aspersa [x]	1	RT	ND	45.3	2.9	32.4	0	4.3	8.6	6.5	ND	0.041	ND	8.6

$T_{A/B}$ ambient or body temperature, PL phospholipid, Chol cholesterol, DSP disaturated PL, gWL gram wet lung mass, *dry lung mass, RT room temperature (22-25°C), PC phosphatidylcholine, PG phosphatidylglycerol, PE phosphatidyl-ethanolamine, SM sphingomyelin, PI phosphatidylinositol, PS phosphatidylserine (a single value for PI and PS indicates that bands could not be resolved), LPC lysophosphatidylcholine, unkwn unknown PL, ND not determined, # whole lung extracts, § % disaturated PC. *a* (Hallman and Gluck, 1975), *b* (Daniels et al., 1995a), *c* (Shelley, 1984); *d* (Langman et al., 1996), *e* (Neumann et al., 1990), *f* (Harlan et al., 1966), *g* (Ribbons et al., 1989), *h* (Fujiwara et al., 1970), *i* (Hallman and Gluck, 1976), *j* (Johnston et al., 2000) *k* (Phleger and Saunders, 1978), *l* (Daniels et al., 1995b), *m* (Daniels et al., 1989), *n* (Daniels et al., 1996), *o* (Lau and Keough, 1981), *p* (Vergara and Hughes, 1980), *q* (Daniels et al., 1994a), *r* (Orgeig et al., 1994), (Orgeig and Daniels, 1995), *t* (Phleger and Saunders, 1978), *u* (Daniels and Skinner, 1994) *v* (Smits et al., 1994)], *x* (Daniels et al., 1990), *y* (Slocombe et al., 2000), *z* (Codd et al., 2002). Table has been modified from Daniels et al., 1998.

1.4.1.1. ECTOTHERMIC ANIMALS

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 higher in unsaturated fatty acids than surfactant from warm-acclimated turtles. Since then, Daniels et al. (1990) have demonstrated that the central netted dragon, *Ctenophorus nuchalis*, doubles the CHOL/PL ratio in their surfactant after a 4 h decrease in body temperature from 37 to 14°C (Figure 1.4). The overall amount of alveolar surfactant collected from central netted dragons did not change with a 4 h decrease in body temperature (Daniels et al., 1990). In another agamid lizard, the bearded dragon, *Pogona vitticeps*, both CHOL and total PL increased after acclimation for 4 h at 37°C compared to 18°C (Wood et al., 1995). However, the relative proportions of CHOL/PL did not change in these lizards. This may indicate that bearded dragons need longer than 4 h, given their larger size (120.7 ± 5.2 g; mean ± SE) to bring about the changes in CHOL/PL ratio that was observed in the smaller central netted dragons (30.2 ± 4.7 g; mean ± SD) (Wood et al., 1995). Alternatively, CHOL/PL may not be as critical to surfactant fluidity in bearded dragons (Wood et al., 1995) and they may use other methods, such as increasing USP, fatty acids and proteins to increase fluidity when cold. Clearly, more research is needed to determine the full complement of compositional changes and the importance of these changes on surfactant function in ectothermic animals. The consequence of changing surfactant composition on the anti-adhesive function of surfactant isolated from ectotherms has also not been studied.
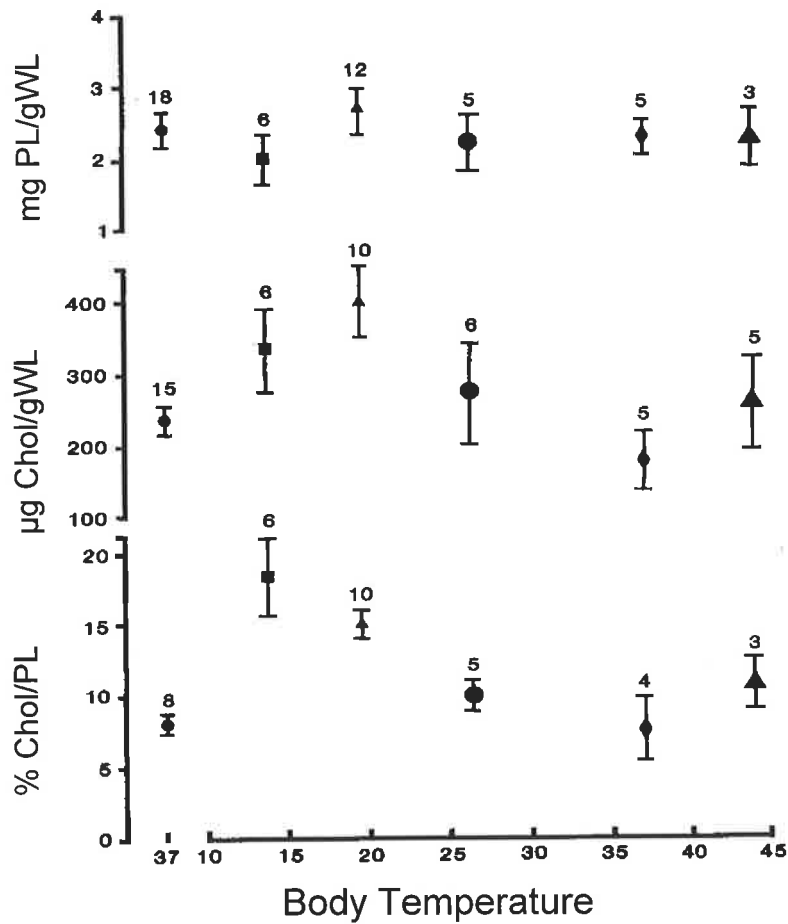
Figure 1.4. Changes in the amount of phospholipid (PL) and cholesterol (Chol) harvested by lavage and expressed per gram wet lung (gWL), together with cholesterol expressed as percentage of phospholipid (Chol/PL), in *Ctenophorus nuchalis* maintained at different body temperatures for 4 h. Results are expressed as means ± SEM. Numbers of lizards given above each data point. Reproduced from (Daniels et al., 1990).

1.4.1.2. HETEROTHERMIC MAMMALS

Surfactant collected from torpid dunnarts (Langman et al., 1996) and cold-acclimated ground squirrels (Melling and Keough, 1981) is higher in total PL than surfactant collected from warm-active dunnarts and warm-acclimated squirrels. During torpor in dunnarts, there are increases in total PL, the relative amounts of DSP, CHOL and PI and a decrease in the predominant phospholipid, PC (Langman et al., 1996). These changes correlate with changes in surface activity (Section 1.3.3.2) and thus enable dunnart surfactant to function effectively at low body temperatures experienced during torpor (Lopatko et al., 1998; Lopatko et al., 1999). In contrast, measures of total surfactant PL and DSP, expressed relative to gram of wet lung, either did not change or decreased during torpor in the microchiropteran bats, *C. gouldii* (Codd et al., 2002) and *N. geoffroyi* (Slocombe et al., 2000), respectively. In both these bats, the DSP/PL ratio also did not change between torpor and activity.

The different response of bats and dunnarts may reflect the different physiological states of the animals during torpor (Codd et al., 2002). Unlike bats, torpor in dunnarts is a stress response to low ambient temperatures and food shortages and may be associated with elevated levels of cortisol, which are known stimuli for surfactant PL synthesis (Haagsman and van Golde, 1991). In addition, in the laboratory studies, torpor in bats (T_b = 24-25°C) was likely to be more shallow than that experienced by dunnarts (T_b = 13-15°C). There is also evidence that the lung of bats may not completely deflate during torpor, even at very low ambient temperatures (Hays et al., 1991; Szewczak and Jackson, 1992) and this may lead to differences in surfactant composition and function during torpor.

Despite differences in the response of their surfactant PL to decreasing body temperature, both *C. gouldii* and dunnarts, increase their CHOL during torpor (Figure 1.5). This suggests a fundamental role for CHOL in cold heterothermic mammals.

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However, CHOL did not increase during torpor in *N. geoffroyi*. Furthermore, the relatively low levels of CHOL in bat surfactant compared to other animals (Table 1.4) suggests that other components, must be acting to aid in the spreading and fluidity of bat surfactant at low body temperatures. Veldhuizen et al. (1998) have demonstrated that the addition of acidic PL, mono-, di- and tri-glycerides and the hydrophobic surfactant proteins, SP-B and SP-C enhance adsorption rate of surfactant to the air-liquid interface. Surfactant proteins are ideal for increasing fluidity because unlike CHOL and other fatty acids, they do not influence surface activity per se, but they do enhance the adsorption rate by interacting with the PL (Veldhuizen et al., 1998). Therefore in bats, and possibly other animals, the surfactant proteins and other fatty acids may be important in maintaining surfactant function at cold temperatures, in preference, or in addition, to increasing CHOL.
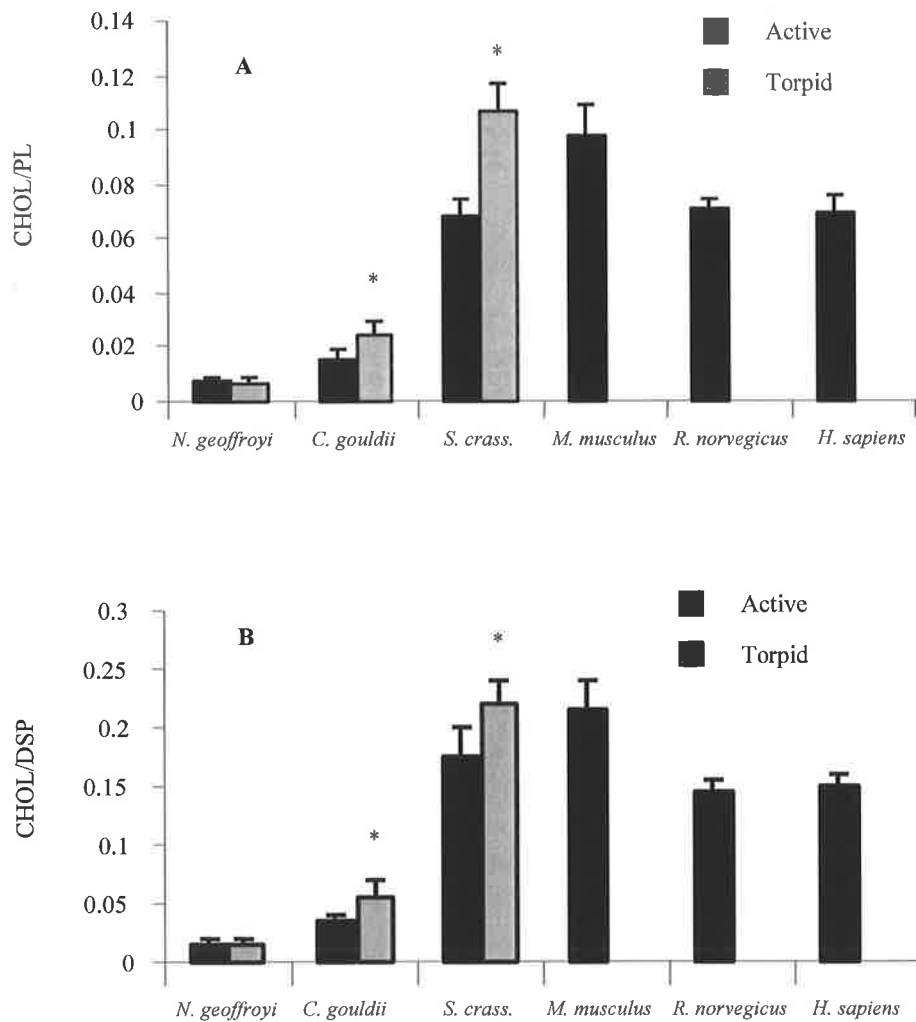
Figure 1.5. Relative amounts of **A** total cholesterol (CHOL) as a fraction of total phospholipid (PL) (μg/μg) for warm-active (active) bats, dunnarts, mice, rats and humans as well as torpid bats and dunnarts. **B** CHOL as a fraction of disaturated phospholipid (DSP) (μg/μg) for warm-active bats, dunnarts, mice, rats and humans and torpid bats and dunnarts. Data are from (Codd et al., 2002; Langman et al., 1996; Slocombe et al., 2000). * indicates a significant difference between lavage isolated from warm-active and torpid animals.

1.4.1.3. HOMEOTHERMIC MAMMALS

Homeothermic mammals rarely experience significant body temperature fluctuations and when they do, as in hypothermia, they can suffer respiratory distress (Inoue et al., 1982; Meban, 1978; Peterson and Davis, 1986). Consequently, experimental manipulations in homeothermic mammals are not ethical or feasible and little is known about the effects of altering body temperature on surfactant composition in these animals. Using isolated perfused rat lungs, (King and Martin, 1981) observed that lowering the incubation temperature to 13°C virtually abolished surfactant PL secretion. However, an increase in total surfactant PL has been observed in isolated perfused rat lungs incubated at 22°C (Miles et al., 1995). Similar increases in the amount of alveolar PL, despite decreases in surfactant secretion, occur during torpor in dunnarts (Ormond et al., 2001) and may act to improve surfactant spreadability and function at cold body temperatures (Langman et al., 1996). Thus, decreases in phospholipid turnover may be one regulatory mechanism that is exploited in hypothermic homeotherms and heterothermic mammals to increase the amount of surfactant phospholipid and thus maintain surfactant function, as body temperature begins to fall.

Alterations in surfactant composition, and particularly CHOL, occur in response to swimming exercise in rats (Orgeig et al., 1995) and cycling exercise in humans (Doyle et al., 1994). In humans, the direction of change in the CHOL/DSP ratio is dependent on the fitness of the individuals and possibly their ventilatory capabilities (Doyle et al., 1994). In perfused rat lungs, the direction of change in the CHOL/DSP ratio is dependent on the type of breathing pattern (high tidal volume or high frequency) (Orgeig et al., 1995). Thus, homeothermic mammals can modify their surfactant composition under certain physiological conditions. This would suggest that the surfactant systems of homeothermic mammals possess the mechanisms needed to alter surfactant composition under conditions of temperature stress. Whether or not these mechanisms occur in

response to temperature changes in vivo, such as those experienced in hypothermia or in isolated lungs transplants, is not known. However, temperature-induced alterations in surfactant composition seem unlikely, given that decreases in temperature decrease lung compliance and have little effect on surfactant surface activity (Section 1.3.3.1).

1.4.2. PROTEIN COMPOSITION

The four surfactant proteins play a very important role in the function of all mammalian lungs and consequently, they have become a dominant research focus over the past 10 years. However, 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 yet to be examined. Other studies, on other proteins, demonstrate that during hibernation gene expression decreases while the half-life of mRNA increases (Van Breukelen and Martin, 2002). This preservation of mRNA is thought to aid the resumption of gene expression during inter-bout arousals and allows for the replenishment of protein pools. During hibernation, there are also changes in differential gene expression and the differential control of enzymatic activity through phosphorylation or sequestering of enzymes (Van Breukelen and Martin, 2002). Hence, it is possible that temperature also influences the rates of surfactant protein synthesis, secretion and the activity of degradation pathways within ATII cells, Clara cells and the alveolar compartment. In addition, temperature may alter interactions between the surfactant proteins and the surfactant phospholipids that are important in the adsorption of surfactant lipids to the air-liquid interface (Veldhuizen et al., 1998). Furthermore, protein clearance in the lung is temperature-dependent and alveolar liquid and protein clearance decreases in isolated goat lungs as temperature is reduced from 30 to 18°C (Serikov et al., 1993). Therefore, as temperature decreases, total protein, and thus, probably the amount of the surfactant proteins, may increase in the lung.

Increases in the surfactant proteins may lead to disruption of the surfactant monolayer (Postle, 2000). Changes to surface activity could increase surfactant fluidity and either improve or hamper the adsorption of lipids to the air-liquid interface. Whether or not the surfactant proteins themselves increase, change in their relative proportions to each other, or alter their function at low temperatures, still remains to be examined. However, there can be no doubt that the findings will be extremely important to our understanding of the thermal dynamics of the entire surfactant system.

1.5. TECHNOLOGICAL DEVELOPMENTS IN SURFACTANT ANALYSIS

Technological developments have greatly improved the precision and accuracy with which we can now measure the composition, structure and function of pulmonary surfactant. Antibodies against all four surfactant proteins are becoming more widely available and ELISA techniques that enable us to quantitate surfactant proteins have now been developed. The technique of electrospray ionisation mass spectrometry (ESI-MS) enables direct measures of molecular species of PL in surfactant mixtures. Epifluorescence microscopy and atomic force microscopy (AFM) have also been modified for surfactant research and provide information about the morphology, domain structure and phase-transitions of surfactant films at an air-liquid interface (Diemel et al., 2002; Ding et al., 2001; Nag et al., 1991; Nag et al., 1998; Piknova et al., 2001).

1.5.1. ELECTROSPRAY IONISATION MASS SPECTROMETRY

Traditional procedures for analysing phospholipid composition include several complicated experimental steps, such as phosphorus assays, thin layer chromatography (TLC), enzymatic removals of headgroups, derivatisation with a UV-absorbing moiety

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and identification by reverse-phase liquid chromatography (LC) or gas chromatography (GC) (Hazel and Landrey, 1988). However, mass spectrometry (MS) enables the direct analysis of PL in complex mixtures and is the most sensitive method for the structural characterisation of PL currently available (Griffiths et al., 2001). The sensitivity of MS, not only enables more precise quantitation of individual molecular species of PL, but also the analysis of samples, where material is limited. In MS, the mass to charge ratio (m/z) of gas-phase ions are determined (Postle et al., 2001). Hence, in order to analyse a surfactant sample, it must first by ionised and vaporised.

The most appropriate method for ionising phospholipids is electrospray ionisation (ESI) (Griffiths et al., 2001). ESI-MS involves dissolving samples in a solvent and pumping it through a thin capillary tube at a high potential (3-4 kV), to create a fine spray of charged molecules (Griffiths et al., 2001). Small droplets of charged molecules are released into a strong electric field and are vaporised with gas, heat or a combination of both (Postle et al., 2001). As the solvent evaporates, the droplets break up into smaller and smaller droplets, eventually releasing ions, which are sampled by the MS (Griffiths et al., 2001).

Phospholipids are ideal molecules for analysis by ESI-MS because they generally only gain one charge. Hence, the mass spectra obtained for PL have peaks directly analogous to their molecular weight (Postle et al., 2001). Analysis of phospholipids using ESI-MS, generally starts with a single-stage MS, which breaks the phospholipids up into those that ionise under positive conditions (e.g. PC and PS) and those that ionise under negative conditions (e.g. PG and PI). Tandem MS/MS analysis enables the unequivocal determination of structural composition of individual species (Postle et al., 2001).

1.5.2. EPIFLUORESCENT MICROSCOPY

Nag and colleagues (1990) developed an epifluorescent microscopic surface balance that allows surfactant monolayers to be observed under compression and/or expansion and thus, provides quantitative information about lipid-lipid interactions and the mixing and refinement of lipids in monolayers (Nag et al., 1991; Worthman et al., 1997). The epifluorescent microscopic surface balance consists of a Wilhelmy-Langmuir surface balance equipped with an epifluorescent microscope (Nag et al., 1991). Fluorescent images are recorded on videotape and can be viewed frame-by-frame using a digital imaging processing system (Nag et al., 1990). Computer programming allows for accurate monitoring of surface tension, temperature and fluorescent intensity distribution during fixed time intervals (Nag et al., 1990). By the partitioning of a fluorescent probe, fluorescent microscopy enables us to detect coexisting phases of lipids in surfactant films. Small amounts of the fluorescent probe, 1-palmitoyl-2-nitrobenzoxadiazole dodecanoyl phosphatidycholine (NBD-PC) are mixed with surfactant samples before they are spread on the surface balance to form a monolayer. The probe dissolves preferentially in the liquid-expanded (LE) phase and hence, gel-like, liquid-condensed (LC) regions appear dark in fluorescent images and regions that are more fluid appear light. Studies with spread films of known composition suggest that the nonfluorescent, dark phase contains mostly DPPC with some cholesterol (Discher et al., 1999b; Nag et al., 1998). Differences in the size and shape of LC domains can be monitored at different surface tensions and yield information about the phase-transition of lipid monolayers.

The probe-excluded domains generated in calf lung surfactant extracts (CLSE) or CLSE lipid fractions using epifluorescent microscopy represent highly enriched DPPC domains (Discher et al., 2002; Discher et al., 1999a) and this has been confirmed by time-of-flight secondary ion mass spectroscopy (Harbottle et al., 2003). DPPG is also

present in these LC domains (Harbottle et al., 2003). Previous studies show that average surface area coverage of the probe-excluding LC phase varies with, and is slightly greater, than the gel phase PL (DPPC and DPPG) content (Nag et al., 1991). These previous studies also showed that the disappearance of intact probe-excluding regions at high surface pressures (low surface tensions) resulted from cholesterol-induced remixing of the domain components with the fluid LE phase, resulting in loss of the probe-insoluble regions (Discher et al., 1999a; Nag et al., 1998).

1.5.3. ATOMIC FORCE MICROSCOPY (AFM)

In order to image surfactant films with AFM, they have to be deposited on a solid, hydrophilic, negatively charged substrate, usually mica. The Langmuir-Blodgett method involves spreading a surfactant monolayer on an aqueous phase in a Langmuir trough. The monolayer is then deposited onto mica by pulling a mica sheet up through the air-water interface, from the aqueous phase into the air (Possmayer, 2003). Atomic force microscopes move a sharp probe over the deposited surfactant films and deflections of the probe are recorded with an optical detector (Fotiadis et al., 2002). Computer imaging provides a reconstruction of the surface topography of the sample (Fotiadis et al., 2002). An AFM image shows the vertical heights of different regions of the surface film. Due to the upright, more perpendicular tilt of the fatty acid chains, gel-like liquid condensed (LC) regions have higher atomic heights than fluid-expanded (LE) regions (Figure 1.6). Hence, LC domains appear brighter in AFM images than LE domains (Possmayer, 2003).

When DPPC films, at a surface tension where LE and LC coexist, are deposited on Langmuir-Blodgett films and analysed using AFM, a distinct kidney bean structure is observed, which corresponds to a LC domain (Figure 1.6A). When, more complex lipid mixtures, such as calf lung surfactant extract (BLES), are examined using AFM, distinct

floral shaped LC domains are observed (Figure 1.6B). The different shapes of the domains are due to differences in line tension (the two-dimensional equivalent of surface tension) between LC and LE phases in the mixtures. High line tensions, such as caused by water, result in spherical domains. However, lower line tensions results in domains with longer perimeters (kidney and floral shapes) (Possmayer, 2003).
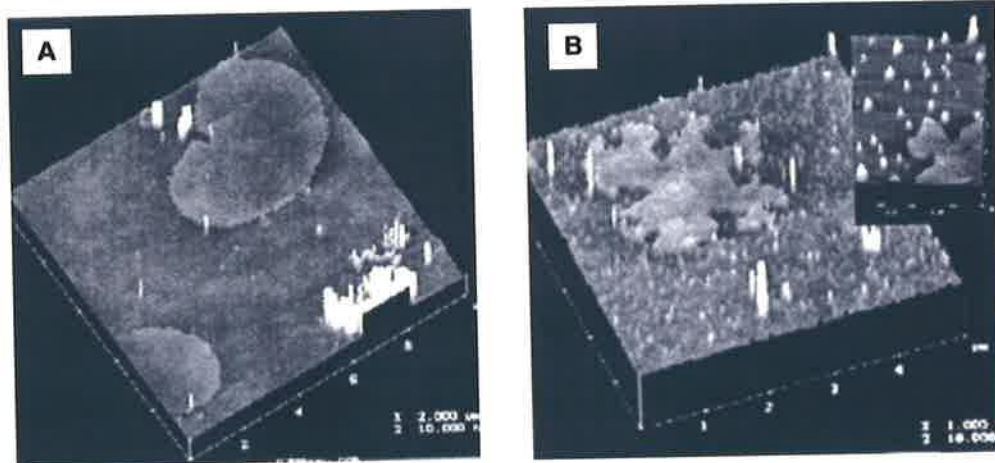
Figure 1.6. Atomic force microscopy of **A** dipalmitoylphosphatidylcholine (DPPC, PC16:0/16:0) and **B** Bovine Lipid Extract Surfactant (BLES) monolayers in the liquid expanded (LE)-liquid condensed (LC) region. The kidney bean LC structure in A is 6 μm long and extends ~ 1-2 nm above the plane of the LE phase. The floral shaped LC structure in B is ~7 nm long and extends ~ 1.7 nm above the LE phase. The nature of the small spikes in the insert is not known. Photographs reproduced from (Possmayer, 2003).

1.6. TEMPERATURE AND CONTROL OF SURFACTANT SECRETION

There are two pools of surfactant in the lungs, the intracellular pool in lamellar bodies of alveolar type II (ATII) cells and the extracellular pool in the alveolar hypophase (Haagsman and van Golde, 1991). The turnover of surfactant is rapid, with 10-40% of the intracellular pool secreted and removed from the extracellular pool per hour (Wright,

1990). Therefore, in order to maintain the composition and amount of alveolar surfactant, these two pools must be tightly regulated by the processes of surfactant synthesis, secretion and turnover (Wirtz and Schmidt, 1992). The effect of temperature on surfactant synthesis and turnover has yet to be examined in detail.

In addition, the neutral lipid, cholesterol, appears to be titrated rapidly and independently of the PL components of surfactant (Wood et al., 1995) and studies using isolated lungs indicate that there may be an alternate (i.e. non-lamellar body) source of cholesterol, which is rapidly mobilised by hyperventilation (Orgeig et al., 1995; Orgeig and Daniels, 2001). Adrenergic and cholinergic agonists control PL release but do not appear to influence CHOL release in isolated lungs of reptiles (Sullivan et al., 2003). Exercise and temperature can also influence CHOL/PL ratios in a variety of species (Orgeig et al., 1995; Orgeig et al., 1996; Ormond et al., 2001; Wood and Daniels, 1996; Wood et al., 1995). However, mechanisms controlling cholesterol release into the lung have so far received only limited experimental attention. Hence, in the following paragraphs I will focus on the effects of temperature on the control of PL secretion from ATII cells. A schematic representation of the factors controlling surfactant secretion and the intracellular signalling pathways they activate is given in Figure 1.7.
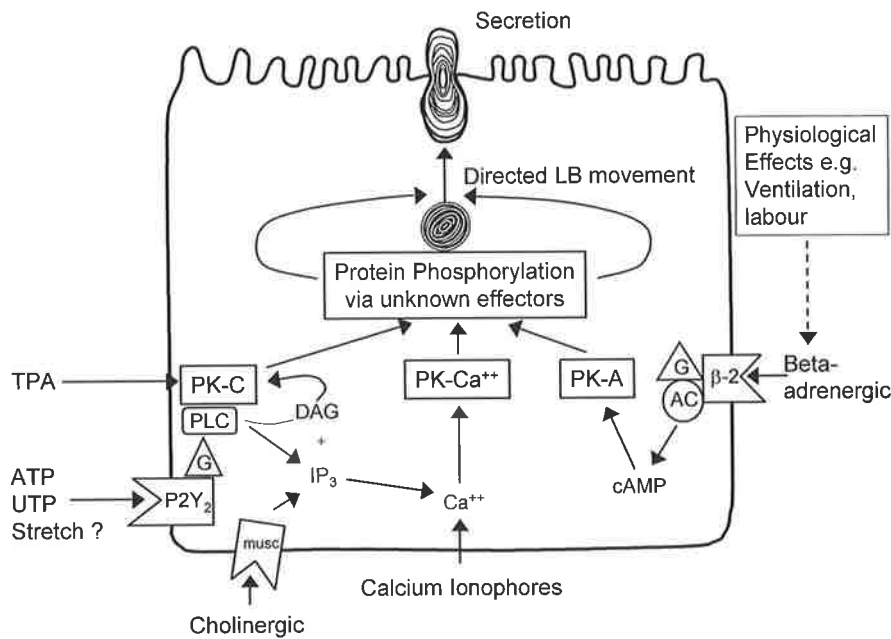
Figure 1.7. Schematic diagram summarising the major factors and signalling pathways that stimulate surfactant secretion from alveolar type II epithelial cells. Several β-adrenergic agonists, including isoproterenol, adrenaline and noradrenaline, stimulate the β2-adrenoreceptor. The receptor is coupled to adenylate cyclase (AC), which produces cyclic AMP (cAMP) via a trimeric GTP-binding protein (G), in order to stimulate cAMP dependent protein kinase (protein kinase, PK-A). Another pathway involves the direct or indirect stimulation of protein kinase C (PK-C). The synthetic surfactant secretagogue tetra-decanoylphorbol acetate (TPA) and cell-permeable diacylglyerols (DAGs) are potent direct stimulators of PK-C. ATP and UTP bind to the purinergic receptor (P2Y2) which is coupled to phospholipase C (PLC) via another G protein. Activation of PLC leads to the formation of inositol triphosphate (IP3) and DAG. The latter stimulates PK-C, while IP3 feeds into the third secretory mechanism, which involves the elevation of intracellular Ca^{2+} levels by IP3 or calcium ionophores. Calcium in turn stimulates calmodulin-dependent protein kinase ($PK-CA^{2+}$). The stimulatory effect of stretch may be mediated via the P2Y2 receptor, and therefore the PK-C and / or $PK-Ca^{2+}$ signalling

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pathways, although physiological stimulation of surfactant secretion by ventilation or labour may be mediated via the β_2 receptor. The exact subsequent mechanisms leading to lamellar body exocytosis are not well understood, but are thought to involve protein kinase-stimulated protein phosphorylations, which presumably activate contractile proteins to move lamellar bodies to the apical surface to fuse with the plasma membrane. (Figure modified from Orgeig et al. (2003), Figures 1 & 2: Mason and Voelker (1998) and Figure 2: Rooney SA (2001)

1.6.1. BASAL SECRETION

Temperature undoubtedly affects the rate of basal secretion from ATII cells. The kinetic effects of temperature on the rate of metabolic processes (such as surfactant secretion), is defined by the Q_{10}. Q_{10} relates the rate of a stimulatory or secretory pathway to temperature. A Q_{10} of 2-3 means that the rate will decrease by one half for every 10°C fall in body temperature (Schmidt-Nielson, 1997). High temperatures increase the metabolic rate of ATII cells and may directly stimulate the rate of synthesis and/or secretion of lamellar bodies (Chander and Fisher, 1990). Low temperatures decrease metabolic rate, and therefore, the rate of surfactant secretion. In ATII cells isolated from homeothermic rats, a decrease in incubation temperature to 5°C virtually abolishes surfactant secretion (Dobbs and Mason, 1979). However, lizard (ectothermic) and dunnart (heterothermic) ATII cells secrete surfactant at both low (18°C) and high (37°C) incubation temperatures (Ormond et al., 2001; Wood et al., 1999). In ATII cells isolated from dunnarts, surfactant secretion is significantly lower at 18°C than at 37°C, but the Q_{10} for the process was 1.3. A Q_{10} of 1.3 indicates that the rate of the metabolic process is not purely dependent on temperature and must also be regulated by other factors (Schmidt-Nielson, 1997). Hence, the secretory pathway in dunnart ATII cells is relatively insensitive to temperature change and is regulated or altered in some way to

counteract the kinetic effects of a decrease in temperature (Ormond et al., 2001). These temperature-resistance mechanisms remain unknown. Whether ATII cells isolated from ectothermic animals are also resistant to temperature change is not yet known. Moreover, Van Breukelen and Martin (2002) suggest that the same biochemical mechanisms that act to counteract the kinetic effects of temperature during torpor and hibernation in heterothermic animals probably also exist in homeothermic mammals.

1.6.2. VENTILATION (MECHANICAL STIMULATION)

1.6.2.1. HOMEOTHERMIC MAMMALS

Ventilation is usually regarded as the major stimulus for surfactant release in homeothermic mammals (Wirtz and Dobbs, 1990). Increasing the volume of air inhaled (ventilatory tidal volume) stimulates surfactant secretion in homeothermic mammals (Wirtz and Schmidt, 1992). A significant decrease in the number of lamellar bodies has been observed in rat lungs inflated by one deep breath in a one-hour period of otherwise normal tidal volume ventilation (Nicholas and Barr, 1983). Ventilation-induced increases in surfactant secretion occur via direct mechanical stimulation of ATII cells and subsequent increases in cytosolic calcium (Edwards, 2001) (Figure 1.7). A deep breath is associated with greater inflation of the alveoli and stretching of the alveolar walls. ATII cells are located in crevices between alveoli where they are exposed to the maximum amount of movement (Wirtz and Schmidt, 1992). Physical stretch of isolated rat type II cells results in an increase in surfactant secretion equivalent to a combination of agonists. This indicates that ventilation is an important stimulus for surfactant release in homeotherms (Edwards, 2001; Wirtz and Dobbs, 1990).

Recent technological advances have made it possible for researchers to mimic different ventilatory patterns in vitro by mechanically stretching ATII cells. Thus, in the near future, researchers should be able to define in detail, the effects of different

ventilatory regimes on mechanical stimulation on homeothermic ATII cells. A much better understanding of the involvement of intracellular calcium and other intracellular signalling pathways, that are activated by the mechanical distortion of ATII cells is also possible with this technology (Edwards, 2001).

1.6.2.2. ECTOTHERMIC ANIMALS

In lizard lungs, ventilatory pattern does not affect surfactant release or composition (Wood et al., 1995) and this is probably because the breathing cycle of reptiles is highly variable and consists of short ventilatory periods, often followed by protracted non-ventilatory periods (Wood and Daniels, 1996). Such a respiratory pattern is discontinuous (Wood et al., 1995) and therefore, does not guarantee a regular mechanical stimulation of the ATII cells (Wood et al., 1995). In addition, the arrangement of ATII cells in the lizard lung may be such that they do not actually experience much mechanical deformation during ventilation (Wood et al., 1995). Thus, the increases in surfactant PL and changes in composition that are observed at warm body temperatures in lizard lungs cannot be attributed to changes in breathing pattern (Wood et al., 1995).

1.6.2.3. HETEROTHERMIC MAMMALS

Given its importance in homeothermic mammals, ventilation is also likely to be an important stimulus in heterothermic mammals, at least, when they are active and warm. However, as body temperature decreases, metabolic rate, and thus the requirement for oxygen also decreases, which reduces the need to breathe. Consequently, both tidal volume and frequency decrease markedly as body temperature decreases during torpor or hibernation in heterothermic mammals (Hays et al., 1991; McArthur and Milson, 1991; Milsom and Reid, 1995). Therefore, mechanical distortion (stretch) of ATII cells also decreases in cold animals and thus, ventilation is unlikely to be an effective regulator of

surfactant release during torpor or hibernation. Yet, surfactant levels remain elevated or even increase in cold animals (Section 1.4.1.2) and this may reflect a decrease in alveolar fluid clearance or surfactant turnover in the lung (Langman et al., 1996). Alternatively, other regulatory mechanisms, such as the parasympathetic nervous system may become more important in regulating surfactant secretion when ventilation decreases during torpor in heterothermic animals.

1.6.3. ADRENERGIC AGONISTS

1.6.3.1. HOMEOTHERMIC MAMMALS

Adrenergic agonists are released from the sympathetic nervous system (SNS) in vivo and are a major stimulus of surfactant release in homeothermic mammals (Brown and Longmore, 1981; Dobbs and Mason, 1978; Wood and Daniels, 1996). In rats, rabbits and humans, stimulation of surfactant secretion by β-adrenergic agonists has been observed in isolated ATII cells, isolated perfused lungs and in vivo (Chander and Fisher, 1990). β-adrenergic agonists bind to membrane-bound β_2-adrenergic receptors on ATII cells and activate the second messenger, cAMP (Figure 1.7). Stereologic analyses demonstrate that this stimulatory response is rapid in mammals, with lamellar body volume density decreasing within 0.5 h after injection (Smith and Griffin, 1987)

1.6.3.2. ECTOTHERMIC ANIMALS

It is not known whether ectothermic animals possess β_2 adrenergic receptors. The sympathetic nervous system (SNS) appears to be poorly developed in lungfish (Fritsche et al., 1993) and consequently, adrenaline does not stimulate surfactant release in ATII cells isolated from the Australian lungfish, *Neoceratodus forsteri* (Wood et al., 2000). However, the SNS is more highly developed in reptiles and amphibians and adrenaline does stimulate surfactant release in ATII cells isolated from bearded dragons, *Pogona*

vitticeps and the North American bull frog, *Rana catesbeiana* (Wood et al., 2000). In addition, perfusing isolated lizard lungs of *P. vitticeps* with adrenaline also stimulates surfactant release (Wood et al., 1995), suggesting that surfactant release is under sympathetic control in these animals. Hence, it is possible that adrenaline acts in ectothermic animals through similar receptors and biochemical pathways to those reported in homeothermic mammals.

Temperature does not appear to affect the overall response of lizard ATII cells to stimulation by adrenergic agonists. Decreasing incubation temperature of isolated lizard ATII cells from 37 to 18°C does not affect the stimulatory response of adrenaline (Wood et al., 1999). Furthermore, increases in body temperature in *P. vitticeps* are accompanied by increases in the amount of surfactant PL (Wood et al., 1995) and plasma levels of the β agonists, adrenaline and noradrenaline (Wood and Daniels, 1996). Hence, the increase in PL found in vivo with increases in body temperature may be triggered by an increase in plasma catecholamines (Wood and Daniels, 1996). However, the observed increases in circulating catecholamines may also reflect an overall increase in sympathetic activity and thus, the activity of sympathetic nerve terminals within the lizard lung. Therefore, PL secretion may be stimulated by direct sympathetic innervation of the ATII cells, or indirectly, by circulating catecholamines. The release of catecholamines by the SNS may result in an increase in local or physiological metabolic activity (Wood and Daniels, 1996). Consequently, regulation by the SNS may be inefficient in cold lizards intent on conserving metabolic energy. Thus, other regulatory mechanisms, with the exception of ventilation, may become more important at cold body temperatures in ectotherms.

1.6.3.3. HETEROTHERMIC MAMMALS

As in homeothermic mammals, adrenergic agonists appear to be central in controlling surfactant release in heterothermic mammals, at least when they are warm. Adrenergic

agonists increase surfactant secretion in ATII cells isolated from fat-tailed dunnarts, *Sminthopsis crassicaudata* at both warm (37°C) and cold (18°C) incubation temperatures (Table 1.8). However, sympathetic activity probably decreases in vivo during torpor in dunnarts. Hence, sympathetic control of secretion in torpid animals is unlikely because the release of adrenergic factors would increase metabolic rate, causing them to arouse from torpor (Wood et al., 2000). However, despite decreases in ventilation and probably sympathetic activity, the amount of surfactant PL increases during torpor in dunnarts (Langman et al., 1996). As in lizards, temperature does not alter the stimulatory response of dunnart ATII cells to adrenergic agonists (Wood et al., 1997). Dunnart ATII cells incubated at both 37°C and 18°C respond equally well to adrenergic stimulation (Ormond et al., 2001). Retaining the capacity to respond to sympathetic stimulation during torpor may allow dunnart ATII cells to respond rapidly to increases in sympathetic activity during the early stages of arousal from torpor, before, or as, body temperature, metabolic rate and ventilation increase. Alternatively, or in addition, regulation by the parasympathetic nervous system (PNS) and cholinergic agonists, which do not increase metabolic rate, may be important in controlling surfactant release during torpor.

Table 1.8. Comparison of agonist-stimulated secretion (expressed as a percentage of basal secretion) for different species at two incubation temperatures.

Animal	Time	°C	% of basal secretion Adrenergic	Cholinergic
Dunnart[a]	4 h	37	141	121
Dunnart[a]	4 h	18	129	136
Dunnart[b]	3 h	37	132	124
Lizard[c]	3 h	37	106 Ad	100
Lizard[c]	3 h	18	113 Ad	108
Rat[d]	3 h	37	147 Tb (10μM)	102
Rat[e]	3 h	37	290 (10μM)	
Rat[f]	1.5 h	37	300 (10μM)	97

Agonist-stimulated secretion is presented as the per cent of basal secretion, where basal secretion is given the value of 100%. *a* (Ormond et al., 2001); *b* (Wood et al., 2000); *c* (Wood et al., 1999); *d* (Dobbs et al., 1986); *e* (Brown and Longmore, 1981); *f* (Dobbs and Mason, 1979). Values from Dobbs and Mason (1979) and Ormond et al. (2001) are calculated from data collected as the per cent of incorporated ^{3}H-choline secreted. Values from Wood et al. (1999, 2000) are calculated from a non-radiolabelled study, where data were presented as the per cent increase in secretion of total phospholipid after a 3 h incubation in the presence of agonist relative to the secretion during a 3 h control period, without agonist. Isoproterenol was used as the adrenergic agonists unless otherwise stated as follows, Ad, Adrenaline HCl and Tb, Terbutaline (10μM). Carbamylcholine chloride was used as the cholinergic agonist. Concentrations of agonists were at 100 μM, unless otherwise indicated in brackets. Reproduced from (Ormond et al., 2001).

1.6.4. CHOLINERGIC AGONISTS

1.6.4.1. HOMEOTHERMIC MAMMALS

There appears to be some controversy about the role of acetylcholine and the PNS in stimulating surfactant release. Acetylcholine has been found to act on muscarinic receptors to increase PL release in intact rabbit (Oyarzun and Clements, 1977) and isolated rat (Brown and Longmore, 1981) lungs. However, Nicholas and Barr (Nicholas and Barr, 1981) failed to find a similar increase in the isolated perfused rat lung. In addition, the cholinergic agonist, carbamylcholine chloride does not stimulate surfactant secretion from ATII cells isolated from humans and rats (Dobbs and Mason, 1979). Although, ATII cells do possess muscarinic receptors, cholinergic nerves have not been found in the alveolar regions of the mammalian lung. Therefore, in homeothermic mammals, cholinergic agonists may not act directly on muscarinic receptors on ATII cells. Wood et al. (1997) postulated that cholinergic factors released by the parasympathetic nervous system (PNS) may influence surfactant secretion in reptiles by stimulating contraction of pulmonary smooth muscle cells and subsequently distorting the ATII cells (mechanical stimulation) (Massaro et al., 1982). This mechanism may also occur in homeothermic mammals because distortion of ATII cells represents the major stimulus for surfactant release (Wirtz and Dobbs, 1990).

1.6.4.2. ECTOTHERMIC ANIMALS

Reptiles, unlike mammals, have lungs richly innervated with both cholinergic and adrenergic nerves (Campbell and Duxson, 1978; Wood et al., 1997). However, regulation by the SNS (adrenergic) is likely to be inappropriate in cold ectotherms because catecholamines will increase metabolic rate locally, regionally and globally within the animal (Wood et al., 1997). It is disadvantageous to increase the metabolic activity and hence oxygen consumption of tissues when torpor is clearly designed to

reduce these processes. Therefore, in ectotherms at low body temperatures and during non-ventilatory periods, the PNS may stimulate activity in pulmonary smooth muscle thereby, promoting gas exchange and ventilation of respiratory surfaces without increasing metabolic rate (Wood et al., 1997). In reptilian lungs, PNS nerve endings are located in the faveolar (respiratory) region (Campbell and Duxson, 1978). Therefore, the release of acetylcholine from parasympathetic nerves could stimulate surfactant secretion by direct interaction with muscarinic receptors on ATII cells. The cholinergic agonist, carbamylcholine chloride, increases PC secretion in ATII cells isolated from Australian lungfish, *Neoceratodus forsteri,* bearded dragons, *Pogona vitticeps* and the North American bull frog, *Rana catesbeiana* (Wood et al., 2000). Furthermore, in isolated perfused lungs of the bearded dragons, the stimulatory activity of acetylcholine is inhibited by atropine, an antagonist (inhibitor) of muscarinic cholinergic receptors (Wood et al., 1997). In addition, the sympathetic ganglion blocker, hexamethonium does not inhibit cholinergically stimulated secretion in isolated lizard lungs (Wood et al., 1997). This suggests that the response to cholinergic agonists is not mediated indirectly through pre-ganglionic stimulation of sympathetic nerve fibres (Wood et al., 1997). Therefore, cholinergic agonists can interact directly with muscarinic receptors, which are located in the smooth muscle and on ATII cells in the lizard lung (Wood et al., 1997).

However, regulation by the PNS is temperature-dependent in bearded dragons, at least at the cellular level. The cholinergic agonist, carbamylcholine chloride stimulated surfactant secretion from bearded dragon ATII cells incubated at 20°C, but not at 37°C (Wood et al., 1999). Since the response of lizard ATII cells to adrenergic agonists is not sensitive to temperature, the switch in the response of ATII cells to cholinergic factors at cold temperatures may be due to temperature-sensitive muscarinic receptors or enzymes that are specifically part of the cholinergic signalling pathway. Alternatively, low temperatures may reduce the activity of enzymes, such as acetylcholinesterase, which

break down acetylcholine and therefore, increase the amount of acetylcholine interacting with the cholinergic receptors on ATII cells at low temperatures (Wood et al., 1997). Consequently, in lizards, adrenergic stimulation is particularly important at warm body temperatures, when the cholinergic pathway is not activated (Wood et al., 1999). In contrast, direct cholinergic stimulation may be more important in cold lizards with decreased metabolic rates.

1.6.4.3. HETEROTHERMIC MAMMALS

Cholinergic agonists also act directly on ATII cells isolated from heterothermic mammals to stimulate surfactant secretion. Carbamylcholine chloride increases PC secretion in ATII cells isolated from fat-tailed dunnarts, *Sminthopsis crassicaudata* (Table 1.8) and juvenile, unfurred, heterothermic tammar wallabies, *Macropus eugenii* (Miller et al., 2001). The cholinergic response may reflect thermoregulatory plasticity, a characteristic of these two marsupials. This finding is in direct contrast to observations made in homeothermic mammals. It is possible that mammals, which maintain a constant body temperature, need only the actions of the SNS (adrenergic) to efficiently regulate surfactant secretion. Heterothermic mammals may have a direct role for the PNS in regulating surfactant secretion at low body temperatures. Studies examining the innervation of the lungs of dunnarts or tammar wallabies have not yet been completed. Therefore, we do not know whether the alveolar regions of these marsupials are innervated with cholinergic nerves (like lizards), or if they lack cholinergic innervation (like homeothermic mammals). Studies examining the effects of cholinergic agonists on surfactant release in vivo or in isolated lungs of heterothermic mammals have also not been completed. However, it is possible, given the observations in both ectotherms and homeotherms, that the PNS of heterotherms also stimulates surfactant secretion

indirectly via stimulation of receptors on pulmonary smooth muscle and thus, distortion of ATII cells.

As in cold lizards, the amount of surfactant PL increases in dunnarts during torpor. However, unlike lizards, carbamylcholine chloride can stimulate surfactant secretion in isolated dunnart ATII cells at both cold (18°C) and warm (37°C) incubation temperatures (Table 1.8) (Ormond et al., 2001). Therefore, both adrenergic and cholinergic control of surfactant secretion is insensitive to temperature in dunnart ATII cells and this further supports the hypothesis that dunnart ATII cells are highly insensitive to temperature changes (Section 1.6.1) (Ormond et al., 2001). However, changes in the adrenergic and cholinergic regulation of surfactant secretion must occur at some physiological level in dunnarts, because the release of catecholamines by the SNS may increase metabolic rate and cause dunnarts to arouse from torpor. Hence, the SNS is probably suppressed during torpor and thus, the PNS may become an important stimulus of secretion in cold animals. Dunnart ATII cells may have lost or not evolved the temperature-sensitive cholinergic switch found in lizard ATII cells and this has a number of advantages (Ormond et al., 2001). Firstly, dunnart ATII cells can respond quickly to a physiological change in autonomic stimulation during the rapid entry into and arousal from, torpor. Secondly, the retention of both parasympathetic and sympathetic stimulation at 37°C may enable the combined action of adrenergic and cholinergic factors on ATII cells, increasing surfactant secretion above that obtained with either factor alone (Ormond et al., 2001).

1.6.5. OTHER FACTORS

A great number of other biochemical factors influence the secretion of surfactant. ATP, through purinergic receptors on ATII cells, is a powerful stimulator for surfactant release (Griese et al., 1991; Rice et al., 1990). Metabolites of arachidonic acid (Mason and

Voelker, 1998), calcium ionophores (Sano et al., 1987), endothelin-1 (Sen et al., 1994), vasopressin (Wright and Dobbs, 1991), lipoproteins (Pian and Dobbs, 1997) and phorbol esters (Wright and Dobbs, 1991) all stimulate surfactant secretion. Once secreted, the surfactant components themselves can also regulate further secretion. At low lung volumes, unsaturated phospholipids and surfactant proteins (especially SP-A) are squeezed out of the surfactant film and these components act to inhibit further secretion (Mason and Voelker, 1998). If delipidated, purified SP-A can inhibit all forms of agonist-induced secretion in vitro, suggesting that SP-A acts to inhibit secretion at a stage shared by all the regulatory mechanisms (Dobbs et al., 1987). Below the phase transition temperatures, saturated PC also inhibits surfactant secretion in vitro (Suwabe et al., 1996). The effects of temperature on the responses of the secretory pathways to these biochemical factors still need to be examined.

1.7. PROJECT RATIONALE

Since the early 20th century, substantial research has focussed on the pulmonary surfactant system in humans and its function in healthy and diseased states. Research concerning the surfactant system of other animals has been largely ignored, except by a few dedicated researchers. Yet, ectotherms and heterothermic mammals are clearly excellent models for investigating how the surfactant system copes with physiological variables, such as temperature. Understanding how the surfactant system can remain functional over a range of body temperatures in heterothermic mammals has the potential to be extremely useful in helping us to understand biochemical, biophysical and regulatory mechanisms involved in surfactant function.

The consequences of decreases in temperature on the function of mammalian surfactant is also likely to be important medically, in hypothermic surgery, lung

transplantation and the development of artificial surfactants for the treatment of respiratory distress syndromes. Hence, the primary aim of this thesis is to improve our understanding of the thermal dynamics of the surfactant system in mammalian animals that enter reduced metabolic states, known as torpor or hibernation.

1.7.1. ANIMAL MODELS

Although highly advantageous in terms of energy conservation, torpor and hibernation by heterothermic mammals have the potential to significantly challenge the surfactant system. The impact of torpor on the surfactant system in mammals is likely to vary with depth, frequency and length of torpor bouts. Consequently, I have examined three heterothermic mammals, that differ in the type of torpor pattern they display, as models in which to examine the thermal dynamics of the mammalian surfactant system.

1.7.1.1. STRESS TORPOR

Fat-tailed dunnarts, *Sminthopsis crassicaudata*, are small marsupial mammals (10-15g) that live in the semi-arid regions of Australia (Figure 1.8). Dunnarts enter stress torpor in response to both food deprivation and low ambient temperatures. During stress torpor, body temperature decreases from around 35°C in warm-active dunnarts to a few degrees above ambient temperature (often between 10 and 20°C) in torpid dunnarts (Godfrey, 1968). Stress torpor in dunnarts is also accompanied by changes in ventilatory pattern and torpor can last for more than 8 hours (Godfrey, 1968). Dunnarts can be maintained in breeding colonies in the laboratory and thus, like rats and mice, are an ideal research model.

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Figure 1.8. Fat-tailed dunnart, *Sminthopsis crassicaudata*. Photograph: Assoc. Prof. Christopher Daniels.

1.7.1.2. DAILY TORPOR

Gould's wattled bats, *Chalinolobus gouldii*, are small insectivorous placental mammals that are widespread and common throughout Australia (Figure 1.9). Characteristics of *C. gouldii* include a soft dense fur that is chocolate brown to black in colour, short, broad ears and loose lobes or 'wattles' hanging down at the corner of the mouth and lower lip (Cronin, 1991). *C. gouldii* are nocturnal and roost by day in tree hollows, under loose bark, in rock crevices and buildings, in colonies of up to 100 bats. Their wings are long and narrow and they can fly fast and direct through open areas and the forest canopy, navigating and hunting for insects using echolocation (Cronin, 1991). Bats enter torpor on a natural daily cycle and body temperature can drop as low as 5°C, but more often 12-15°C (Hosken and Withers, 1997). During daily torpor in bats, ventilation changes to

episodic breathing bouts alternating with long non-ventilatory periods (Morris et al., 1994).

Figure 1.9. Gould's wattled bats, *Chalinolobus gouldii*. Photograph: Stephen Donnellan.

1.7.1.3. HIBERNATION

Golden-mantled ground squirrels, *Spermophilus lateralis*, are rodents that are widespread in Canada and the United States of America (Figure 1.10). During the hibernating season, ground squirrels are capable of reducing their body temperatures to as low as 0-5°C during torpor and torpor bouts usually last for 10-14 days at a time (Milsom et al., 1999). After a torpor bout, the ground squirrels will spontaneously arouse, increasing their body temperature to 37°C for a brief period (hours) before returning to a torpid state. During hibernation, ground squirrels enter a much deeper and more prolonged torpor than that reported for the stress-induced torpor of small marsupials and daily torpor of bats (Geiser and Ruf, 1995). The depth and duration of their torpor bouts makes squirrels an excellent model for studying the thermal dynamics of a mammalian surfactant system. Furthermore, ground squirrels enter hibernation readily under

appropriate laboratory conditions. Virtually nothing is known about the effects of torpor on the surfactant system in deep hibernators.

Figure 1.10. Golden mantled ground squirrel, *Spermophilus lateralis*. Photograph: Carol Ormond.

1.8. AIMS

1. To describe the alterations in surfactant composition, if any, that occur during torpor or hibernation in heterothermic mammals. In particular, the following components of the surfactant system will be examined.

   - Saturation of PL

   - Molecular species composition of lipids

   - Cholesterol

   - Hydrophilic surfactant protein A

   - Hydrophobic surfactant proteins, B and C

2. To determine whether any observed changes are the same or different for each type of torpor pattern (i.e. daily torpor, stress-induced torpor or hibernation).

3. To determine whether changes in surfactant composition, or torpor or hibernation, influence the structure of the surfactant films at an air-liquid interface using epifluorescence and atomic force microscopy.

4. To determine if there are any differences in the saturation of PL, molecular species composition or cholesterol between surfactants isolated from heterothermic and homeothermic mammals that could represent thermal adaptations of the surfactant system in heterothermic mammals.

1.9. HYPOTHESES

- As previously observed in bats and dunnarts, the CHOL to total PL ratio will increase during torpor in ground squirrels.

- The proportion of DSP in total PL will decrease during torpor in ground squirrels.

- There will be changes in the molecular species distribution of the major PL, particularly PC, between warm-active and torpid mammals (i.e. warm-active and torpid squirrels, bats and dunnarts).

- There will be changes in surfactant protein levels in lavage fluid isolated from warm-active and torpid mammals.

- Given differences in torpor pattern and physiology, these compositional changes may differ between squirrels, bats and dunnarts.

- Compositional changes will be accompanied by changes in surfactant film structure at the air-liquid interface that may enable surfactant to function effectively during both activity and hibernation.

- There will be compositional differences between homeothermic and heterothermic mammals that could represent thermal adaptations of the surfactant system in heterothermic mammals.

CHAPTER 2

MATERIALS AND METHODS

2.1. ANIMALS

2.1.1. GOLDEN-MANTLED GROUND SQUIRREL

Adult golden-mantled ground squirrels, *Spermophilus lateralis,* were obtained from a wild-captive colony maintained in the Department of Zoology, University of British Columbia, Canada. Squirrels were housed in a controlled-environment chamber at $T_a =$ 22 $\pm$ 1°C under a 12:12-h light-dark cycle. Squirrels were given water and fed laboratory chow supplemented with sunflower seeds ad libitum throughout the experiments, although they rarely ate during the hibernation season. In September and October (Autumn), warm-active squirrels ($T_b = 35.2 \pm 0.5$°C; mean $\pm$ SE, n = 11) were used. In late November, the temperature in the controlled-environment chamber was reduced to 5°C and the photoperiod altered (8L:18D). Under these conditions, the squirrels entered hibernation within a few weeks. The length of torpor bouts was monitored using the simple, non-invasive method of placing a wood chip on the back of each squirrel and recording whether it was present or absent each day. Hibernating squirrels were used after they had been in a torpor bout for at least 4 days, without a period of arousal. The rectal body temperature of each squirrel was recorded using a thermocouple. Hibernating squirrels recorded body temperatures of 7.7 $\pm$ 0.2°C (mean $\pm$ SE, n = 12). Note: There was an overlap period where some squirrels were kept at summer conditions and some at winter conditions during November. Squirrels maintained under summer conditions did not enter hibernation.

2.1.2. GOULD'S WATTLED BAT

In early March, six male and nine female Gould's wattled bats, *Chalinolobus gouldii,* were trapped, using harp traps, in Glenroy Conservation Park, in the South East of South Australia. The bats were held in a specially designed bat cage (0.5m x 0.15m x 0.35m)

with wire mesh on each long side and calico bags hanging from the centre on which they could roost. Bats were given water ad libitum and fed mealworms (*Tenebrio molitor*) by hand for two days after capture. At sunrise on the third day, seven bats (four females and three males) were placed individually in cloth bags that were tied so they hung vertically in a constant temperature cabinet. Ambient temperature was lowered to 12°C, over a 30 min period and the bats checked after 1 h to ensure they had entered torpor. An ambient temperature of 12°C was used in order that the depth of the daily torpor bout (i.e. minimum body temperature) in bats was comparable to the stress-induced torpor bout of dunnarts (Chapter 5). Torpid bats are easily identifiable by their body posture, degree of alertness and low body temperature (<16°C). All bats entered torpor under these conditions and were kept at T_a = 12°C for 8 h. The rectal body temperature of each animal was recorded using a Fluke 52$^{K/J}$ thermometer. Torpid bats had body temperatures of 12.4 ± 0.3°C (mean ± SEM, n = 7). Warm-active bats were kept at an ambient temperature of 24°C and sacrificed in the evening when they were resting and had rectal body temperatures of 34.8 ± 0.34°C (mean ± SE, n =7).

2.1.3. FAT-TAILED DUNNART

Adult, male fat-tailed dunnarts, *Sminthopsis crassicaudata*, (mass range 8 – 15 g) were purchased from a captive-bred colony at the University of Adelaide, South Australia. Animals were housed individually in standard small animal cages (50 cm x 20 cm x 20 cm) layered with newspaper or a thin layer of sawdust and given toilet rolls or tissue paper as shelter. Dunnarts were kept for up to two weeks in a temperature controlled room set at 25 ± 1°C and exposed to a 8:16 h light-dark cycle that started at 9.00 am and were fed daily with 'Whiskas' cat food (minced meat varieties) and 'Whiskas' cat biscuits dampened in water. Water was provided ad libitum. The body temperatures of "warm-active" dunnarts were 35 ± 0.21°C (mean ± SE, n = 74).

<u>Torpor protocol</u>: Warm-active dunnarts were placed in clean cages layered with newspaper, without food, in a constant temperature cabinet at 20°C overnight. In the morning (~8 am), the temperature in the cabinet was reduced to 12°C. After 1.5 h, the dunnarts were examined to determine if they had entered torpor. Torpid animals are easily identifiable by their body posture and degree of alertness. Those dunnarts that had not entered torpor were fed and returned to the animal house (T_a = 25°C). Dunnarts in torpor were kept at T_a = 12°C for 8 h and monitored regularly to ensure they did not arouse. Eight hours is the typical length of torpor recorded for dunnarts (Godfrey, 1968). The rectal body temperature of each animal was recorded using a Fluke 52$^{K/J}$ thermometer. Torpid dunnarts had body temperatures of 13.14 ± 0.20°C (mean ± SE, n = 59). Each day 1-2 dunnarts were warmed to room temperature (22°C) after the 8 h torpor period and they soon returned to a warm-active state, confirming that they were in torpor and that the protocol did not induce hypothermia.

2.1.4. OTHER MARSUPIALS

An old male Tasmanian devil, *Sarcophilus harrisii*, (age 13 y; mass 13 kg) scheduled for euthanasia, was obtained from a wildlife park in Sydney, Australia. Two southern hairy-nosed wombats, *Lasiorhinus latifrons*, were obtained from a captive colony kept at the University of Adelaide and originally trapped in south western South Australia. One male koala, *Phascolarctos cinereus*, (age ~1-2 y) was passed on to Dr Chris Lee, Department of Anatomy, University of Adelaide for euthanasia, from a local wildlife park, after being involved in a car accident. The koala had a suspected broken jaw, but otherwise appeared in good health and did not appear to have any respiratory injuries. Two tammar wallabies, *Macropus eugenii*, were obtained from a captive colony kept at the University of Adelaide. Due to the difficulty in obtaining lavage from these animals, as most are endangered species, I only have one or two animals.

2.2. MICROSCOPY OF SQUIRREL AND BAT LUNGS

After lavaging, the lungs were excised from squirrels and bats and cut into 1 mm^3 pieces, before fixing in 4% paraformaldehyde, 1.25% glutaraldehyde, 4% sucrose in 0.1M phosphate buffered saline (PBS), pH 7.2 at 4°C for 3-7 days. The fixed material was washed in 0.1M PBS and postfixed in 1% osmium tetroxide overnight. Tissue pieces were then stained en bloc in 1.5% uranyl acetate, dehydrated in 70, 80, 90 and 100% ice-cold acetone, embedded in Araldite resin and polymerised. Cut sections were mounted on grids and photographed using a transmission electron microscope (Philips CM 100 TEM).

2.3. ISOLATION OF PULMONARY SURFACTANT

2.3.1. GOLDEN-MANTLED GROUND SQUIRREL

Squirrels were anaesthetised, with an intraperitoneal injection of pentobarbitone sodium (50-150 mg kg^{-1} body weight). The trachea was cannulated and the ground squirrels placed on a ventilator set to a volume of 10 ml and frequency of 20-30 beats per minute during the perfusion. The thorax was opened and the lungs were perfused, under gravity at 33 cmH$_2$0, via the pulmonary artery with sterile saline solution containing 2 I.U. per ml of heparin sodium, until free of blood. The lungs were lavaged via a tracheal cannula with three separate 10 ml volumes of saline. Each volume was instilled and withdrawn three times. All lavage material was stored immediately on ice, then centrifuged at 150 g for 8 min to remove macrophages and cellular debris. The supernatant was collected, aliquoted into equal fractions and lyophilised.

2.3.2. GOULD'S WATTLED BAT AND FAT-TAILED DUNNART

Warm-active and torpid dunnarts and bats were killed by an intraperitoneal overdose of pentobarbitone sodium (50 mg kg^{-1}). A blunted 14-gauge needle was inserted into the trachea and three volumes (2 ml in syringe, ~ 1 ml lung volume) of ice-cold 0.15M NaCl were infused and withdrawn three times from a syringe. This procedure has been found to remove > 90% of the surfactant in these animals (Daniels et al., 1993; Daniels et al., 1996; Langman et al., 1996; Wood and Daniels, 1996; Wood et al., 1995). Saline from each of the lavage volumes was pooled together and stored on ice, before being centrifuged at 150 g for 10 min at 4°C to remove cell debris. For whole lavage samples, the supernatant was lyophilised on a freeze-drier and stored at –20°C until further analysis.

For analysis of subfractions of dunnart surfactant, the supernatant was spun at 40,000 g for 30 min at 4°C to separate the large aggregate (LA) subfraction (pellet) from the small aggregate (SA) subfraction (supernatant). The LA subfraction contains dense structures that are responsible for the surface-activity of surfactant and hence maintain alveolar stability. Lighter, vesicular forms of surfactant that are undergoing recycling or clearance from the lung and are less surface-active, are present in the SA fraction (Brackenbury, 2002). The LA pellet was resuspended in 2 ml of Hepes buffered saline, for analysis of composition and surface properties, or 2 ml of deionised water, for analysis of surfactant proteins. The LA and SA fractions were then lyophilised and stored at –20°C until further analysis.

2.3.3. OTHER MARSUPIALS

The animals were anaesthetised with an injection of pentobarbitone sodium (150 mg kg^{-1} body weight) via the femoral artery. After removal of reproductive and digestive organs, the diaphragm was punctured in order to deflate the lungs. The trachea was cannulated

with a piece of appropriate sized tubing, which was attached to a three-way valve. Using

a 50 ml syringe, sterile, ice-cold saline was instilled into the lungs and then collected.

Volumes varied for each animal. Final volumes of lavage fluid collected were 310 ml

for Tasmanian devil, 170 ml for wombat (1/2 lung lavaged) and 300 ml for the koala.

Small amounts of mucous were removed from the lavage fluid by skimming the surface

of the fluid with a spatula. The pooled lavage fluid was aliquoted into 50 ml tubes and

spun at 150g for 10 min at 4°C (Beckman GS-6R centrifuge) to remove cell debris and

macrophages. Lavage fluid was then frozen in liquid nitrogen and lyophilised. The

lungs were isolated from the Tasmanian devil, weighed to determine wet lung weight

(99.8 g) and then freeze-dried, to determine dry lung weight (7.35 g).

2.4. LIPID EXTRACTION AND BIOCHEMICAL ANALYSIS

Lipids were extracted from lyophilised lavage fluid using the method of Bligh and Dyer

(1959). Inorganic phosphorus was measured by the method of Bartlett (1959). Total PL

was calculated by multiplying the amount of inorganic phosphorus by 25, because

phosphorus comprises approximately 4% of PL (Langman et al., 1996).

PL concentration was also estimated in the squirrel and large aggregate dunnart

lavage samples using an enzymatic, colorimetric assay kit for PC, supplied by

Boehringer-Mannheim Inc., Germany (Roche Molecular Biochemicals). This assay

measures PC, which accounts for ~ 80% of surfactant PL and sphingomyelin, which

accounts for ~1-2% (Veldhuizen et al., 1998). Hence, samples were multiplied by 80%

to obtain an estimate of total PL. The enzymatic assay employs choline oxidase to

measure choline released from phosphatidylcholine (PC) and sphingomyelin (SM) by

phospholipase D. The assay was conducted according to the manufacturer's protocols.

Samples were compared with standards containing 0-10 μg total PC using bovine lipid

extract surfactant (BLES biochemicals, London, ON, Canada), choline equivalent to 0-10 µg PC. Absorbance was measured at 490 nm in an ELISA reader after 30 min incubation at room temperature.

For squirrel lavage samples, disaturated PL was separated from neutral lipids using adsorption column chromatography as previously described (Orgeig et al., 1995). Briefly, samples were reacted with osmium tetroxide and applied to a column of neutral aluminium oxide. Neutral lipids were eluted first with 10 ml chloroform / methanol (20:1). Disaturated PL was eluted with 5 ml of chloroform / methanol / 7M aqueous ammonia (70:30:2). The eluant was evaporated under nitrogen. Inorganic phosphorus was measured in the disaturated PL fraction by the method of Bartlett (1959). Data were expressed as µg per gram wet lung (µg gWL^{-1}). Squirrel lungs were used for isolated cell experiments and hence, I was not able to determine dry lung weights. Unpaired t-tests assuming equal variance were used to determined statistical significance and significance was set at $p < 0.05$.

2.5. ANALYSIS OF PHOSPHOLIPID MOLECULAR SPECIES

Total lipid extracts were obtained from whole and large aggregate lavage fractions using the method of Bligh and Dyer (1959) and analysed using electrospray ionisation (ESI) mass spectrometry on a triple-quadrupole tandem mass spectrometer (Quattro *Ultima*, Micromass, Manchester, England) equipped with an electrospray-ionisation interface. Total phospholipid extracts were dissolved in 25 µl of methanol: chloroform: water: NH$_4$OH (7:2:0.8:0.2, v:v) for single stage and tandem MS analysis of phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), and were analysed by nanospray ESI-MS. Dry heated nitrogen was used as both the cone and desolvation gas

(70 and 450 L h^{-1} respectively) and dry argon was used as the collision gas (3.5 x 10^{-3} mbar). All data were recorded at mass resolution, as a signal average of 10-20 scans per collection, with a scan time of 2.5 to 12 s.

PC species were detected by positive ionisation, while PI and other acidic phospholipids were preferentially detected using negative ionisation. Following fragmentation with argon gas, PC molecules produced a fragment with m/z = +184, corresponding to the protonated phosphocholine headgroup, and precursor scans of the m/z 184 moiety provided diagnostic determination of PC. Collision gas-induced fragmentation of PI species generated a common dehydrated inositol phosphate fragment with m/z = -241, and precursor scans of this m/z 241 moiety provided diagnostic determination of PI. Identities of PS species were confirmed by constant neutral loss scans of serine (m/z = -87), PE species by neutral loss scans of phosphoethanolamine (m/z = +141), and PG species by precursor scans of glycerophosphate (m/z = -153). Data were acquired and processed using MassLynx NT software. After conversion to centroid format according to area, correction for ^{13}C isotope effects and reduced response with increasing m/z values, the phospholipid species in each class of phospholipid were expressed as percentages of their respective totals present in the sample. The predominant molecular species present for each ion peak resolved was determined by analysis of fatty acyl fragments generated by collision gas-induced fragmentation under negative ionisation. PC was quantified from the parent scan of m/z = +184 under positive ionisation mode, while all other phospholipid classes were quantified from the negative ionisation scans.

The percentages of disaturated phosphatidylcholine (DSPC), disaturated phosphatidylglycerol (DSPG) and disaturated phosphatidylinositol (DSPI) were calculated from the corrected mass spectrometry data by adding the mol% contribution of each of the individual disaturated species. Statistical analyses between lavage isolated

from warm-active and torpid or hibernating animals were only performed on molecular species of PL if they contributed greater than 5 mol% to the PL component. Unpaired t-tests assuming equal variance were used to determine statistical significance, significance at $p < 0.05$.

2.6. CHOLESTEROL ANALYSIS

In lavage samples isolated from golden-mantled ground squirrels, neutral lipids were separated from disaturated PL using adsorption column chromatography as previously described (Section 2.4). The amounts of cholesterol and lathosterol in the neutral lipid fraction were determined by gas chromatography based on a modification of the method described by Wolthers et al. (1991). Briefly, the samples were dried under nitrogen, saponified with KOH at 60°C for 30 min, cooled and extracted with hexane. The extract was evaporated to dryness under a stream of nitrogen and the sterols derivatised with SYLON BTZ (Supelco) for 30 min at 80°C. The silyl derivatives of the sterols were extracted into hexane, concentrated with a stream of nitrogen to 50 µl and 1 µl injected onto the GC column (split ratio 10:1). The gas chromatograph consisted of a DANI 8500 equipped with a split/splitless injector, flame ionisation detector and was coupled to a DELTA computerised chromatography data system. The injector, detector and oven temperatures were set at 265, 260 and 280°C, respectively. The capillary column used was a 25 m x 0.22 mm BPXI (SGE Australia P/L). Cholesterol concentration was calculated from the standard curve using the ratio of the sterol peak area to the peak area of the internal standard (5β-cholestan-3α-ol).

The amount of total cholesterol (free cholesterol and cholesteryl esters) was also determined in lavage samples, isolated from the same squirrels and dunnart large aggregate samples, using an enzymatic colorimetric assay kit supplied by Wako

Chemicals USA Inc. (Richmond, VA). In this assay, free cholesterol is released by cholesteryl esterase and total cholesterol is estimated by cholesteryl oxidase, which yields hydrogen peroxide to react with the colorimetric dye. Samples were compared with standards of bovine lipid extract surfactant (BLES biochemicals, London, ON, Canada), which were equivalent to 0-5 μg cholesterol. Absorbance was measured at 490 nm in an ELISA reader after incubation at room temperature for 15 min. All samples were assayed in duplicate or triplicate, where possible and the mean values reported. Statistical significance was determined using student t-tests, assuming equal variance, significance at $p < 0.05$.

Cholesterol was determined in lavage from the Tasmanian devil as follows. Freeze-dried lavage (1 mg) was made up in 1 ml of deionised water and extracted using the method of Bligh and Dyer (1959). The chloroform layer (2 x 800 μl) was transferred to glass vials and dried down partially under nitrogen gas. The chloroform layer (600 μl) was transferred to a 7 ml glass vial, dried down and stored at −20°C until analysed for phosphorus. Samples were made up in 200 μl of heptane and analysed by normal phase HPLC. The HPLC consisted of a LC1500 HPLC pump, a LKB Bromma variable wavelength UV detector and DP800 data interface (ICI instruments, Australia). A back pressure regulator was also attached to the outflow waste tube. Twenty microliters of sample were injected into a silica column (Zorbax RX-SIL, 5-μm particle size, 4.6 mm ID x 25 cm) using an autoinjector and LC1610 autosampler. The mobile phase was hexane-isopropanol (99:1). Flow rate was 1.3 ml min^{-1}. The wavelength was programmed at 202 nm and cholesterol was eluted at 13 min. Total run-time was extended to 22 min to ensure elution of all components present in the samples.

2.7. SURFACTANT PROTEIN ANALYSIS

Squirrel lavage samples were resuspended in 750 µl of deionised water and diluted 1:2 in ELISA sample buffer for measurement in the surfactant protein ELISAs. Bat lavage samples were resuspended in 250 µl of deionised water and lavage samples from two individual bats were pooled to obtain one sample (i.e. n = 1). Pooled samples were diluted 1:2 in sample buffer for measurement in the ELISAs. Dunnart large aggregate samples were resuspended in 500 µl of deionised water and diluted 1:2 in sample buffer for measurement in the surfactant protein ELISAs.

2.7.1. SURFACTANT PROTEIN B

Reagent suppliers and methods for preparing ELISA solutions are given in Appendix 2. All standards were a generous gift from Prof. Jeffrey Whitsett, Children's Hospital Medical Research Centre, Cincinnati, Ohio. Medium binding 96-well ELISA plates (Griener, Interpath, Australia) were coated overnight at 37°C in 100 µl of 0.1M sodium bicarbonate. Coating buffer was removed from the wells by gently flicking the plates upside down. The plates were washed 3x with ~250 µl of wash buffer and blotted on paper towel. Blocking buffer containing 5% human albumin (100 µl) was added to each well using a 12-well multichannel pipette. The plates were then incubated for at least 15 min at room temperature. SP-B standard was diluted to 500 ng/ml and stored at –20°C. On the day of assay, standards of 0, 5, 10, 25, 50, 75 and 100 ng/ml SP-B were prepared in sample buffer. Freeze-dried lavage samples were resuspended in 750 µl of deionised water and vortexed briefly. Blocking buffer was removed from the plates and the plates blotted dry on paper towel. Each standard (100 µl per well) was added in duplicate to the ELISA plate. Sample buffer (50 µl) was added to each of the sample wells, before the addition of 50 µl of each sample (i.e. 1:2 dilution of sample in sample buffer). Samples were assayed in duplicate, or triplicate, where possible. ELISA plates were

then incubated for 2 h at 37°C. Samples and standards were aspirated from the plates and the plates were washed 3x with wash buffer and blotted dry. The polyclonal antibody, rabbit anti-sheep SP-B (Cat # AB3780; Chemicon Australia Pty Ltd, Australia), was diluted 1:1000 in blocking buffer containing 5% human albumin and 100 µl was added to each well using the multichannel pipette. The plates were then incubated for 1 h at 37°C. Primary antibody was removed from the plates and the plates were washed 3x with wash buffer and blotted dry. The secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (IgG) (Sigma Chemical Corp., MO, USA), was diluted 1:1000 in dilution buffer containing 5% human albumin and 100 µl was added to each well using the multichannel pipette. The plates were incubated for 1 h at 37°C. Secondary antibody was removed from the plates and the plates washed 3x with wash buffer. Plates were blotted dry on paper towel. Just before use, 10x stock of substrate buffer was diluted to 1x with water. 30% hydrogen peroxide solution (185 µl) was added to 25 ml of 1x substrate buffer. Developing buffer was prepared by dissolving one tablet of ortho-phenylenediamine (OPD) (Sigma Chemical Corp., MO, USA) in 25 ml of substrate buffer containing hydrogen peroxide and vortexing until dissolved. Developing buffer (100 µl) was added immediately to each well of the ELISA plate using the multichannel pipette. Plates were shaken at room temperature for 10 min, before stopping the colour reaction with 100 µl of 50% sulphuric acid. Absorbance was read at 492 nm on an ELISA reader. Absorbance was plotted against standard concentration using a linear regression ($y = 0.0043x \pm 0.15$, $r^2 = 0.94$) to estimate the concentration of samples. Statistical significance was determined using Student's t-tests, assuming equal variance, significance at $p < 0.05$.

2.7.2. SURFACTANT PROTEIN C

Surfactant protein C was measured using the method of Schmidt et al. (2002), with slight modifications to the final step of colour development. SP-C standards were prepared by diluting the SP-C stock solution to 400 ng/ml with deionised water (final volume 600 µl). 200, 100, 50, 25, and 12.5 ng/ml standards of SP-C were prepared by 2-fold serial dilutions (300 µl + 300 µl 80% 2-propanol: 20% water (PW) starting from the 400 ng/ml standard. Each standard (100 µl per well) was added to duplicate wells of a polysorp microplate (Nunc Cat #446140, Fisher Cat #12-565-266) immediately after preparing dilutions.

Samples were prepared by diluting lavage samples 1:5 in 80% 2-propanol: 20% water (60 µl + 240 µl PW) and vortexing for 5 s. Samples (100 µl per well) were added to duplicate wells. The solvent was evaporated from the plates by incubating overnight at 37°C. Trifluoroethanol (TFE) (100 µl) was added to each well and the plates incubated for 3 h at 37°C to evaporate solvent. Methanol (200 µl) was added to each well and the plates gently shaken for 20 min at room temperature. Methanol was removed from the wells by flicking the plates. Each well was washed 2x with ~ 250 µl of 1x Tris containing 0.5% (v/v) Tween-20™ (TT solution) and blotted dry. A volume of 200 µl of 1x Tris containing 1% (w/v) bovine serum albumin (BSA) (TA solution) was added to each well, the plates covered and incubated for 2 h at room temperature. The plates were washed 3x with ~ 250 µl per well TT solution. The primary antibody, anti-SP-C (gift from Dr W. Steinhilber, Altana Pharma (formerly Byk-Gulden), Germany) was diluted 1:1000 with TA solution and 200 µl was added to each well. The plate was incubated overnight at room temperature. Anti-SP-C was removed from the plate and the plate was washed 3x with 250 µl per well of TT solution. The secondary antibody, biotinylated donkey anti-rabbit IgG (Sigma Chemical Corp., MO, USA) was diluted 1:1000 with TA solution and 200 µl was added to each well. The plates were

incubated for 2 h at room temperature. The ABC/HRP (DAKO #K0355) stock solution was prepared 1-2 h before use by adding 10 μl reagent A and 10 μl of reagent B to 1 ml 50 mM Tris, pH 7.6. The secondary antibody was removed from the plates and the plates washed 3x with 250 μl of TT solution. Just before use, the ABC/HRP stock solution was diluted 1:50 with TA solution and 200 μl was added to each well. The plates were incubated for 2 h at room temperature. ABC/HRP was removed from the wells and the plate washed 3x with 250 μl/well of TT solution.

Just before use, 20 mg of 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) or ABTS (Sigma Chemical Corp., MO, USA) was dissolved in 30 ml substrate buffer containing 10 μl of hydrogen peroxide. 200 μl of substrate was added to each well and the plate covered and shaken gently at room temperature for 15 min, before reading on an ELISA plate reader at 414 nm. A standard curve was generated from a linear fit of the standard curve optical densities ($y = 0.003x + 0.10$, $r^2 = 0.98$). Sample values were estimated by interpolation from the standard curve. Statistical significance was determined using Student's t-tests, assuming equal variance ($p < 0.05$).

2.7.3. SURFACTANT PROTEIN A

With the exception of the following modifications, the ELISA procedure for measuring SP-A is the same as outlined for SP-B (Section 2.7.1). ELISA plates were coated overnight at 37°C with a 1:100 dilution of goat anti-human SP-A (Cat # AB3422, Chemicon Australia Pty Ltd, Australia) in 0.1M sodium bicarbonate solution. The blocking and dilution buffers contained 2.5% human albumin (Sigma Chemical Corp., MO, USA)) and 2.5% goat serum (Sigma Chemical Corp., MO, USA). The primary antibody was rabbit anti-human SP-A (Cat # AB3420, Chemicon Australia, Australia) used at a 1:1000 dilution. The secondary antibody was HRP-conjugated goat anti-rabbit IgG (Sigma Chemical Corp., MO, USA), used at a 1:1000 dilution. SP-A standard was

diluted to 1 µg/ml in sample buffer and stored at –20°C. On the day of assay, standards

of 0, 5, 10, 25, 50, 75 and 100 ng/ml SP-A were prepared in sample buffer. A standard

curve was generated from a linear fit of the standard curve optical densities ($y = 0.064x +$

0.23, $r^2 = 0.92$).

2.8. EPIFLUORESCENCE MICROSCOPY OF SURFACTANT FILMS

Dunnart large aggregate and squirrel lavage samples were mixed with 1% (w/w) of a

fluorescently labelled phospholipid, 1-palmitoyl-2- [12-{(7-nitro-1, 3-benzooxadiazol-4-

yl) amino} dodecanoyl]-*sn*-glycero-3-phosphocholine or NBD-PC) (purchased from

Avanti Polar Lipids, Pelham, AL USA) before spreading on a Wilhelmy-Langmuir

surface balance attached to an epifluorescence microscope. This probe has been shown

to partition in the fluid or loosely packed phase of surfactant and DPPC films (Discher et

al., 1999b; Nag et al., 1998; Piknova et al., 2001; Piknova et al., 2002) and allows any

non-fluid (or non-fluorescent) phases to be observed in such films directly at the air-

water interface. Films were formed by solvent spreading and were slowly compressed

(0.05 nm^2 molecule^{-1} min^{-1}, with an initial area of 1.2 nm^2 molecule^{-1}). Films were

imaged using a commercial epifluorescence microscope (Axiovert 10, Carl Zeiss, Jena,

Germany) equipped with a charge couple device (CCD) camera (Sony, Japan) attached

to an intensifier (Stardan II, Videoscope International, Japan).

Fluorescence imaging was achieved by exciting the probe at 470 nm (blue) and

observing the fluorescence emission at 530 nm (green). Fluorescence images were

recorded at four different surface tensions (15, 20, 25 and 30 mN m^{-1}), while the pressure

was kept constant for about 5 min. Fluorescence image analysis was conducted using

Northern Eclipse (Empix Imaging Inc., Mississauga, Ontario, Canada, version 6.0

software). The percentage of dark probe-excluding area, presumed to be the condensed

phase, was estimated by measuring the total amount of nonfluorescent area of each image divided by the total area of the image. An average of ten images was analysed at each surface pressure and the data presented as percentage of area covered by the dark phase. The average diameters of the condensed lipid domains were obtained as previously reported (Discher et al., 1999b; Nag et al., 1998). Statistical significance was determined for % area covered and average diameters using one way ANOVA followed by Tukey-Kramer post-hoc test with significance set at $p < 0.05$ using GraphPad Instat statistical programme.

2.9. ATOMIC FORCE MICROSCOPY OF SURFACTANT FILMS

For dunnart large aggregate and squirrel lavage samples, Langmuir-Blodgett films were prepared for atomic force microscopy by depositing the surface monolayer onto a solid substrate (freshly cleaved mica) at 30 mN m^{-1}. This Langmuir-Blodgett method allowed us to deposit the films by a vertical upstroke (10 mm min^{-1}) of the solid substrate (mica sheets) pre-immersed in the subphase, while maintaining a constant surface pressure of 30 mN m^{-1}. The Langmuir-Blodgett films were mounted onto the magnetic steel disc and imaged by atomic force microscopy (AFM) on a Nanoscope III SPM (Digital Instruments, Santa Barbara, CA, USA) in contact mode immediately after deposition.

The AFM images were obtained by laterally scanning the surface topography of Langmuir-Blodgett films using a thin silicon nitride probe, with a nominal tip width of 20 nm (Nag et al., 1998; Takamoto et al., 2001a; Takamoto et al., 2001b) and a force constant of 0.12 mN m^{-1}. This method of imaging allowed for measurements of typical surface heterogeneity of films (due to phase segregation of the different lipids into domains and/or by the tilt of the molecules perpendicular to the plane of the air-water interface) with vertical height profiles of such heterogeneous structures ranging from

0.1-10 nm. All the experiments were performed at a monitored ambient temperature of $23 \pm 1°C$. The images were flattened and analysed using the AFM software to determine the height differences between the observed domains. Values are reported as mean $\pm$ SE. Statistical significance between the groups was determined by Student's unpaired t-tests, assuming equal variance, significance at $p < 0.05$.

CHAPTER 3

RESULTS FOR

WARM-ACTIVE AND HIBERNATING

GOLDEN-MANTLED GROUND SQUIRRELS

Spermophilus lateralis

3.1. BACKGROUND

During hibernation, golden-mantled ground squirrels (*Spermophilus lateralis*) enter a deep and prolonged period of low body temperature during which they experience marked changes in their pulmonary mechanics. Inspiratory reserve volume, inspiratory lung capacity, vital lung capacity and total lung capacity, all increase in hibernation (Milsom and Reid, 1995). Hibernating squirrels also have elevated residual lung volumes, virtually no expiratory reserve volumes, decreased lung compliance, increased hysteresis in pressure-volume curves and greater elastic work required for ventilation (Milsom and Reid, 1995). This suggests that despite an increase in compliance at total lung capacity, there is a decreased compliance at low lung volumes, gas trapping at functional residual capacity and an increase in the work required to breathe in hibernating ground squirrels (Milsom and Reid, 1995). Moreover, surface forces take on more importance at extremely low temperatures (Inoue et al., 1982). However, very little is known about the effects of hibernation on the pulmonary surfactant system, which controls these surface forces, in ground squirrels.

In the early 1980's, Melling and Keough (1981) observed that total PL increased in lavage fluid collected from cold-acclimated, but active, ground squirrels, *Spermophilus richardsoni*. Although 20 years have passed since this study, the surfactant system of ground squirrels, or any hibernating mammal, has not been characterised further. However, the depth and duration of their torpor bouts makes squirrels an excellent model for studying the thermal dynamics of a mammalian surfactant system. Furthermore, ground squirrels enter hibernation readily under appropriate laboratory conditions.

During the hibernating season, golden-mantled ground squirrels are capable of reducing their body temperatures to as low as 0-5°C during torpor and metabolism is reduced to 2-4% (Lyman et al., 1982). Torpor bouts usually last for 10-14 days at a time

(Milsom et al., 1999). After a torpor bout, the ground squirrels will spontaneously arouse, increasing their body temperature to 37°C for a brief period (hours) before returning to the hibernating state. Although hibernation is highly advantageous in terms of energy conservation, the long duration and depth of torpor bouts experienced by ground squirrels is likely to have marked effects on the composition, function and regulation of the surfactant system. These effects may be greater than those reported for the daily, relatively short and shallow torpor, experienced by other heterothermic mammals (Codd et al., 2000a). Alternatively, given the length and depth of their torpor bouts and the annual regularity of the hibernating season, ground squirrels may have adopted novel and unique approaches for maintaining surfactant function at both warm-active (37°C) and very low (0-7°C) body temperatures.

Here I examine the composition, structure and function of surfactant isolated from warm-active (T_b = 37°C) and hibernating (T_b = 7°C) golden-mantled ground squirrels, *Spermophilus lateralis* using mass spectrometry, epifluorescence microscopy and atomic force microscopy. I hypothesise that the CHOL to total PL ratio will increase during torpor in ground squirrels, while the proportion of DSP in total PL will decrease. There will also be changes in the molecular species distribution of the major PL, particularly PC, between warm-active and hibernating squirrels. These compositional changes should act to lower the phase-transition temperature (T_c) of the surfactant mixture and maintain fluidity and function at colder temperatures. Hence, I further hypothesise that these changes in composition will be accompanied by changes in surfactant film structure at the air-liquid interface that may enable surfactant to function effectively during both activity and hibernation.

3.2. RESULTS

3.2.1. SQUIRREL DESCRIPTIVE DATA

Warm-active and hibernating ground squirrels had mean rectal body temperatures of $35.2 \pm 0.5°C$ (mean $\pm$ SE, n =11) and $7.7 \pm 0.2°C$ (mean $\pm$ SE, n = 12), respectively. The wet lung masses recorded for the warm-active and hibernating squirrels were 3.92 ± 0.29 g (mean $\pm$ SE, n = 10) and 3.69 ± 0.16 g (mean $\pm$ SE, n = 10), respectively. There was no significant difference between wet lung mass recorded for warm-active and hibernating squirrels. Sex of the squirrel did not significantly affect wet lung mass, or body temperature.

3.2.2. ELECTRON MICROSCOPY

An electronmicrograph of the alveolar epithelium of the lung of a golden-mantled ground squirrel, *Spermophilus lateralis*, is shown in Figure 3.1. The photograph contains an alveolar type II cell, located in a crevice between alveoli, and demonstrates their cuboidal shape, microvilli and presence of large osmiophilic lamellar bodies.

3.2.3. SURFACTANT COMPOSITION

The amount of PL, as estimated from levels of inorganic phosphorus (P_i), was similar to that determined using the enzymatic colorimetric kit for PC from Boehringer Mannheim (Figure 3.2.). The amount of PL did not change significantly between surfactant isolated from warm-active and hibernating squirrels (P_i: $t_{stat} = 1.197$, df = 7, p = 0.14; enzymatic assay: $t_{stat} = 1.525$, df = 7, p = 0.09) (Figure 3.2). Estimates of percent saturation, as determined by the amount of DSPC per gram wet lung (WL) (ESI-MS) and DSP per gram WL (adsorption chromatography (AC)) also did not change significantly between warm-active and hibernating squirrel lavage (ESI-MS: $t_{stat} = 0.049$, df = 7; p = 0.48; AC:

t_{stat} = 0.684, df = 7; p = 0.26) (Figure 3.3). The ratios of DSPC/PC and DSP/PL did not change significantly in surfactant from hibernating squirrels compared with warm-active squirrels (DSPC/PC: t_{stat} = 1.350, df = 10, p = 0.10; DSP/PL: t_{stat} = 0.037, df = 7, p = 0.49). The percent saturation for PG species in surfactant isolated from warm-active squirrels was 14.8 ± 2.6% (mean ± SE, n = 5) and 17.76 ± 2.2% (mean ± SE, n = 6) for hibernating squirrels. The percent saturation for PI species in surfactant isolated from warm-active squirrels was only 3.1 ± 1.3% (mean ± SE, n = 5) and 1.6 ± 0.8% (mean ± SE, n = 4) for hibernating squirrels. There was no significant difference in the percent saturation of PG (t_{stat} = 0.870, df = 9, p = 0.20) or PI (t_{stat} = 0.945, df = 7, p = 0.19) species in warm-active and hibernating squirrel surfactant (Figure 3.3.).

Amounts of CHOL per gram WL, as determined by gas chromatography (warm-active: 19.7 ± 3.3 µg gWL^{-1}, n = 4; hibernating: 36.9 ± 4.0 µg gWL^{-1}, n = 5), were slightly lower than that determined by the enzymatic, colorimetric method (warm-active: 26.7 ± 10.9 µg gWL^{-1}, n = 4; hibernating 55.1 ± 10.7 µg gWL^{-1}, n = 5) (Figure 3.2.). However, in both cases, cholesterol levels were significantly higher in lavage collected from hibernating squirrels than warm-active squirrels (AC: t_{stat} = 3.188, df = 7, p = 0.008; Enzymatic: t_{stat} = 1.836, df = 7, p = 0.05) (Figure 3.2.). Consequently, the ratio of CHOL/PL, as measured with both methods, was significantly higher in lavage collected from hibernating ground squirrels (AC/Passay: t_{stat} = 3.665, df = 7, p = 0.004; enzymatic: t_{stat} = 1.989, df = 7, p = 0.043) (Figure 3.4). The ratio of CHOL/DSPC, with CHOL measured by gas chromatography and DSPC measured by ESI-MS, was also significantly higher in lavage isolated from hibernating squirrels than warm-active squirrels (AC/ESI-MS: t_{stat} = 5.416, df = 7, p = 0.0007) (Figure 3.4). However, the CHOL/DSP ratio, in which both were separated from each other by adsorption chromatography and CHOL was measured by gas chromatography and DSP by a

phosphorus assay, did not increase significantly between lavage isolated from warm-active and hibernating ground squirrels (AC: t_{stat} = 0.843, df = 7, p = 0.21) (Figure 3.4).

Cholesteryl esters, as measured by the Wako enzymatic assay, contributed only a small proportion (3-10%) to the cholesterol component, but they were significantly higher in surfactant isolated from hibernating squirrels compared with warm-active squirrels (t_{stat} = 3.095, df = 7, p = 0.009) (Figure 3.2). Both the amount of lathosterol (μg gWL^{-1}) (AC: t_{stat} = 4.01, df = 9, p = 0.002) and the lathosterol/CHOL ratio (AC: t_{stat} = 3.8758, df = 9, p = 0.002) were significantly higher in surfactant from hibernating squirrels than warm-active squirrels. Total protein, as determined by the method of Lowry et al. (1951), was significantly higher in lavage from hibernating squirrels (1.86 ± 0.32 mg gWL^{-1}, mean ± SE, n = 5) compared to warm-active squirrels (Lowry/enzymatic: 1.03 ± 0.102 mg gWL^{-1}, mean ± SE, n = 4) (t_{stat} = 2.232, df = 7, p = 0.03).

Figure 3.1. Electronmicrograph of an alveolar type II cell in alveolar epithelium isolated from the golden-mantled ground squirrel, *Spermophilus lateralis*. Scale bar = 2 μm. LB lamellar body, N nucleus, AS air space containing secreted surfactant material.
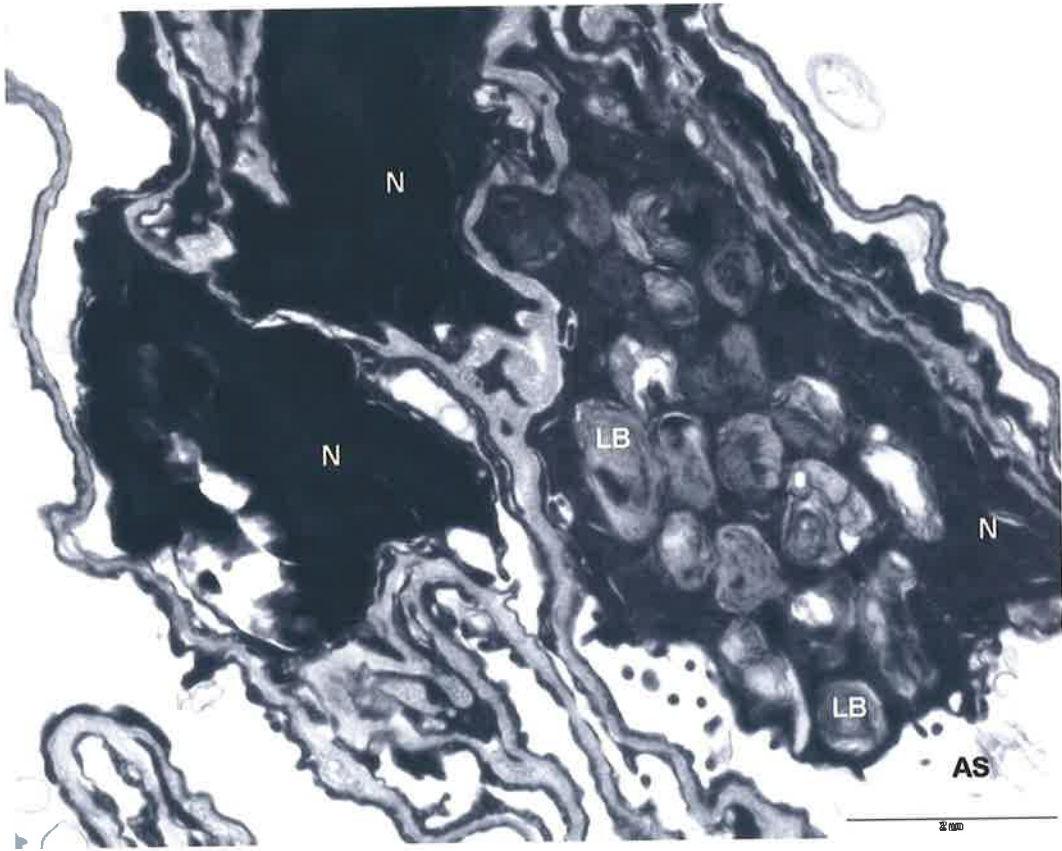
Figure 3.2. Surfactant amounts in lavage fluid isolated from warm-active and hibernating golden-mantled ground squirrels, *Spermophilus lateralis*, measured using different analytical procedures. All data are presented as mean ± SE and expressed as µg per gram wet lung (µg gWL^{-1}). Solid bars represent surfactant isolated from warm-active squirrels. Hatched bars represent surfactant isolated from hibernating squirrels. n = 4 for warm-active squirrels; n = 5 for hibernating squirrels. PL phospholipid; DSP disaturated phospholipid; DSPC disaturated phosphatidylcholine; CHOL cholesterol; LaSL lathosterol. (a) measured using an enzymatic, colorimetric assay kit for PC, supplied by Boehringer-Mannheim Inc., Germany (Roche Molecular Biochemicals) (b) measured using an inorganic phosphorus assay on extracted lipids (Bartlett, 1959); (c) separated from other lipids using adsorption chromatography and measured in eluant using an inorganic phosphorus assay (Bartlett, 1959); (d) calculated by multiplying total PL in each sample, as measured using an inorganic phosphorus assay (Bartlett, 1959), by the fraction of DSPC relative to PC, as determined using electrospray ionisation mass spectrometry (e) CHOL separated from other lipids using adsorption chromatography and measured in eluant using gas chromatography (Wolthers et al., 1991); (f) measured using an enzymatic, colorimetric assay kit for CHOL supplied by Wako Chemicals USA Inc. (Richmond, VA). * Significant difference between lavage fluids isolated from warm-active and hibernating ground squirrels (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

Figure 3.3. Relative proportions of disaturated components in lavage isolated from warm-active and hibernating golden-mantled ground squirrels, *Spermophilus lateralis*. All data are presented as mean ± SE and expressed as ratios. DSP/PL disaturated phospholipid (separated using adsorption chromatography and measured using an inorganic phosphorus assay) as a fraction of total phospholipid (measured using an inorganic phosphorus assay); DSPC/PC disaturated phosphatidylcholine as a fraction of total phosphatidylcholine (measured using electrospray ionisation mass spectrometry (ESI-MS); DSPG/PG disaturated phosphatidylglycerol as a fraction of total phosphatidylglycerol (measured using ESI-MS); DSPI/PI disaturated phosphatidylinositol as a fraction of total phosphatidylinositol (measured using ESI-MS). There were no significant differences between lavage isolated from warm-active and hibernating ground squirrels (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).
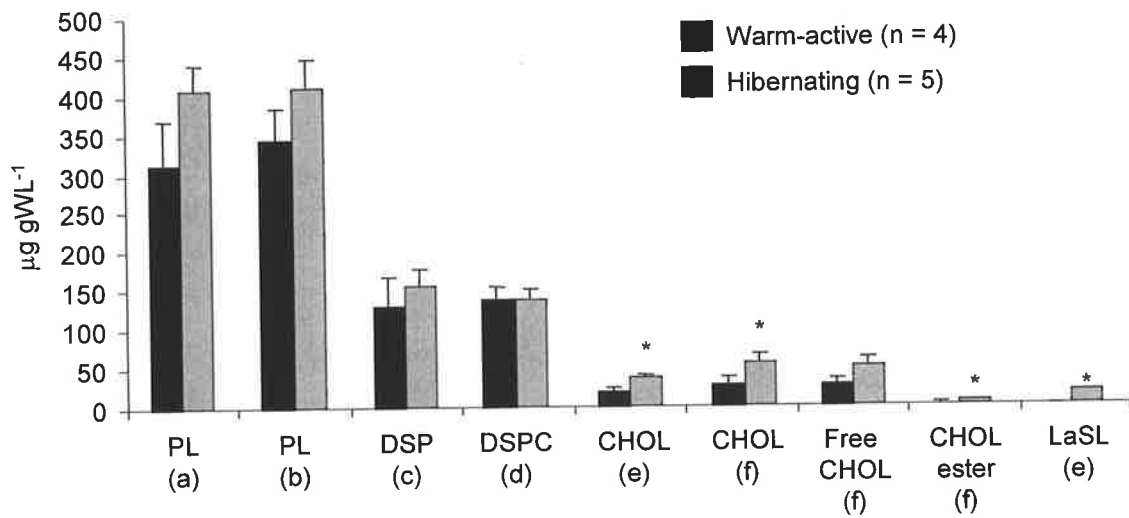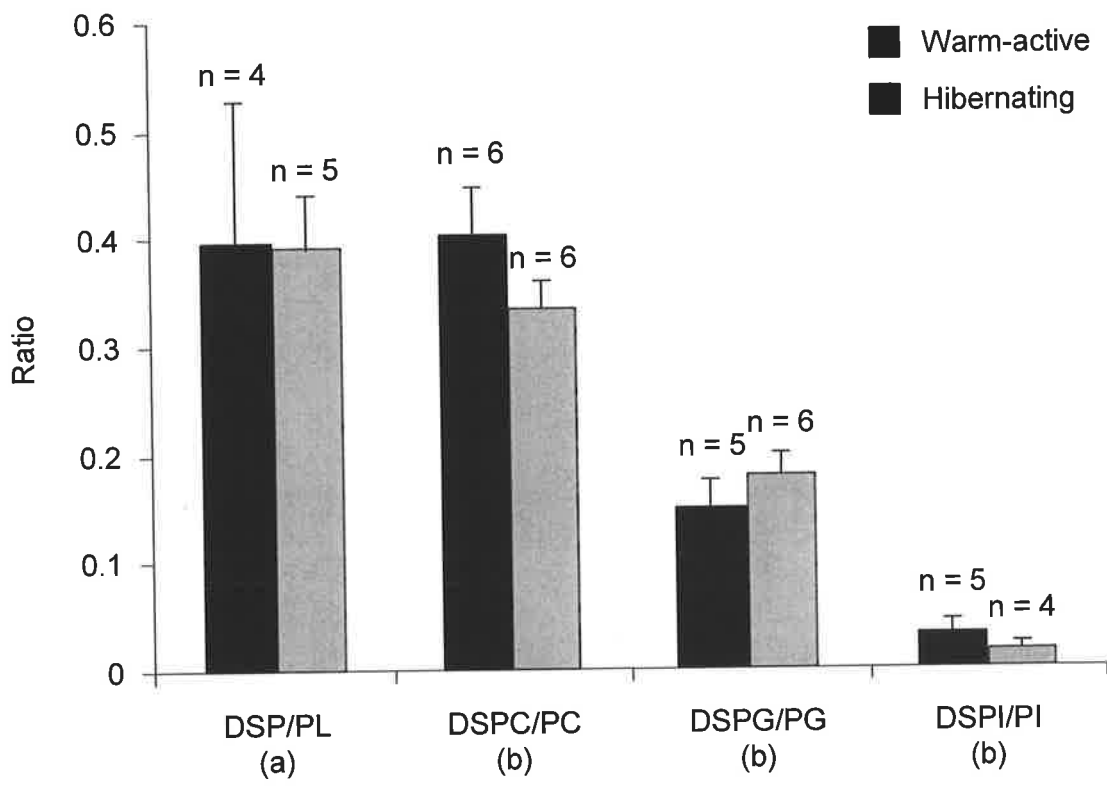
Figure 3.4. Relative proportions of cholesterol to other surfactant components in lavage fluids isolated from warm-active and hibernating golden-mantled ground squirrels, *Spermophilus lateralis*, as measured using different analytical procedures. All data are presented as mean ± SE and expressed as ratios. CHOL/PL cholesterol as a fraction of total phospholipid; CHOL/DSP cholesterol as a fraction of disaturated phospholipid; CHOL/DSPC cholesterol as a fraction of disaturated phosphatidylcholine; CHOL ester/Free CHOL cholesteryl esters as a fraction of free cholesterol; LaSL/CHOL lathosterol as a fraction of total cholesterol. (a) measured using enzymatic, colorimetric assay kits for CHOL and PL supplied by Wako Chemicals USA Inc (Richmond, VA) and Boehringer-Mannheim Inc., Germany (Roche Molecular Biochemicals), respectively (b) CHOL separated from other lipids using adsorption chromatography and measured in eluant using gas chromatography (Wolthers et al., 1991) and compared to total PL, as measured using an inorganic phosphorus assay (Bartlett, 1959); (c) CHOL and DSP separated using adsorption chromatography and measured in eluant using gas chromatography and an inorganic phosphorus assay (Bartlett, 1959), respectively.; (d) CHOL separated from other lipids using adsorption chromatography and measured in eluant using gas chromatography (Wolthers et al., 1991) and compared to DSPC, which was calculated by multiplying total PL in each sample, as measured using an inorganic phosphorus assay (Bartlett, 1959), by the fraction of DSPC/PC determined using electrospray ionisation mass spectrometry; (e) Neutral lipids (CHOL and lathosterol) separated from other lipids using adsorption chromatography and measured in eluant using gas chromatography (Wolthers et al., 1991). * Significant difference between lavage fluids isolated from warm-active and hibernating ground squirrels (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).
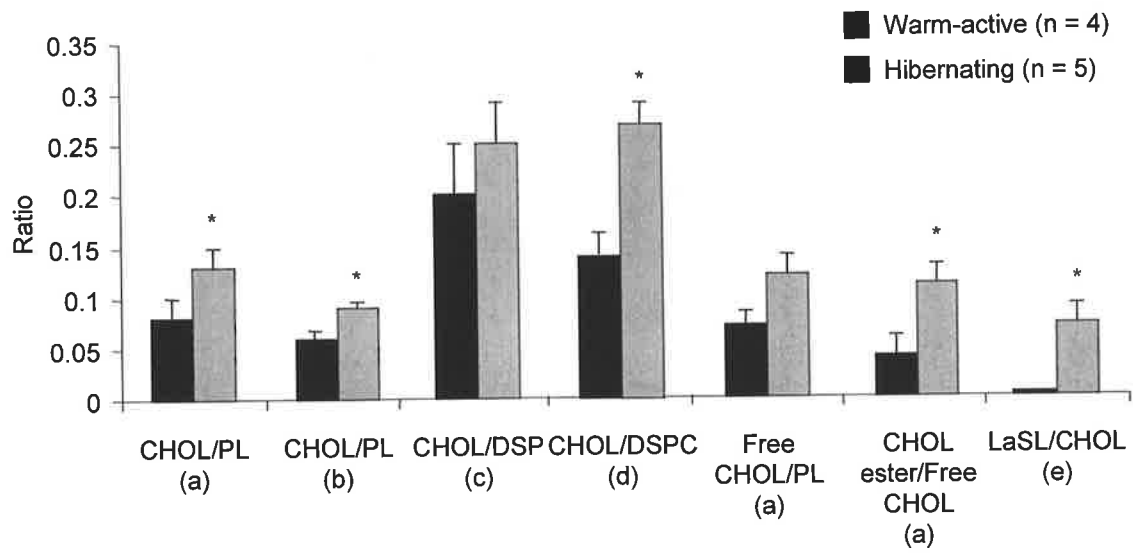
3.2.4. PHOSPHOLIPID MOLECULAR SPECIES COMPOSITION

Examples of typical ESI-MS spectra for squirrel surfactant in positive mode and negative mode are given in Figure 3.5. Typical examples of diagnostic scans of squirrel surfactant are given in Figure 3.6. The molecular species of phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) in surfactant isolated from warm-active and hibernating squirrels are given in Figures, 3.7, 3.8 and 3.9, respectively. There were no significant differences in the molecular species of PC present between surfactant isolated from warm-active and hibernating ground squirrels (Figure 3.7). In both warm-active and hibernating ground squirrels, the composition of surfactant PC was dominated by the disaturated species, PC16:0/16:0, which contributes 30-35 mol% to the PC component. Other major PC components include the unsaturated species, PC16:0/16:1 (25 mol%), PC16:0/18:1 (10 mol%), PC16:0/18:2 (10 mol%) and PC16:0/14:0 (5 mol%). The molecular species PC16:1/14:0, PC16:1/16:1, PC16:0/18:0, PC20:4/16:0, PC18:2/18:1, PC18:1/18:1, PC18:1/18:0 and alkyl-acyl-containing species of PC16:0/16:0 and PC16:0/16:1 are present in small quantities (less than 5 mol% each) and make up the remaining proportion of PC species.

The molecular species of PG did not differ markedly between surfactants isolated from warm-active and hibernating ground squirrels (Figure 3.8). In both warm-active and hibernating squirrels, PG composition was dominated by the unsaturated species PG16:0/18:1, which contributed 25 mol% to the PG component. PG16:0/16:1 comprised from 11-16 mol% and was significantly lower ($t_{stat} = 1.877$, df = 10, p = 0.045) in surfactant from hibernating squirrels (11.5 $\pm$ 1.6 mol%, mean $\pm$ SE, n = 6) compared with warm-active squirrel surfactant (16.3 $\pm$ 2.4 mol%, mean $\pm$ SE, n = 5). PG18:1/18:1 comprised 16 mol% and PG16:0/16:0 and PG16:0/18:2, comprised 14 mol% each. The unsaturated species PG18:1/18:2 comprised 6 mol%. The remaining PG component was

made up of PG16:1/16:1, PG16:1/18:2, PG16:0/18:0, PG18:0/18:1, PG18:0/18:0, PG16:0/22.6 and PG16:0/22.5 (less than 5 mol% each).

In both warm-active and hibernating squirrels, the composition of surfactant PI was dominated by the molecular species PI18:1/18:1, which comprised 30% of the PI component (Figure 3.9). The PI species, PI16:0/18:1 comprised 15-20 mol% of the surfactant PI component, while PI16:1/18:1 and PI18:0/18:1, comprised 12 mol%. PI16:0/16:1, PI16:0/16:0, PI18:1/18:2, PI18:1/20:4, PI18:0/20:4, PI18:0/22:6 and PI20:1/20:4 make up the remaining PI component (less than 5 mol% each).

Figure 3.5. Example of spectra obtained for lavage isolated from **A** warm-active and **B** hibernating golden-mantled ground squirrels, *Spermophilus lateralis* and analysed by electrospray ionisation mass spectrometry under positive-ionisation mode. Examples of spectra obtained for lavage isolated from **C** warm-active and **D** hibernating golden-mantled ground squirrels, *Spermophilus lateralis* and analysed by electrospray ionisation mass spectrometry under negative-ionisation mode. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Only PC molecular species were seen. Mass (m)/charge (z) ratio is equivalent to molecular mass.
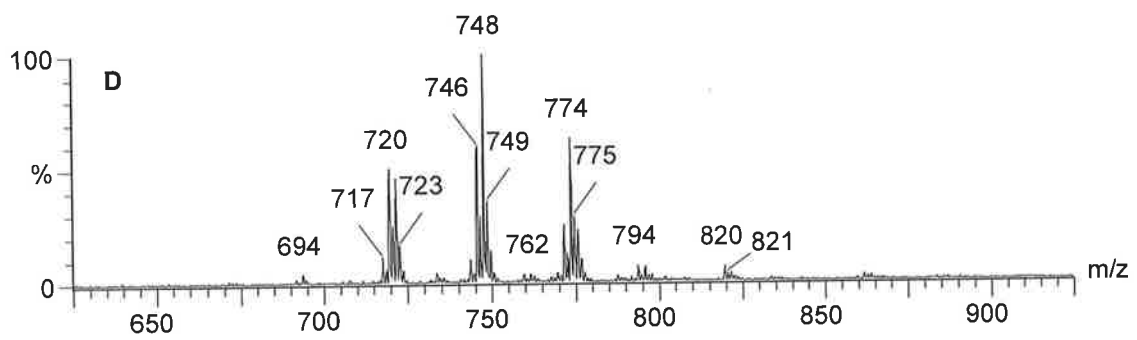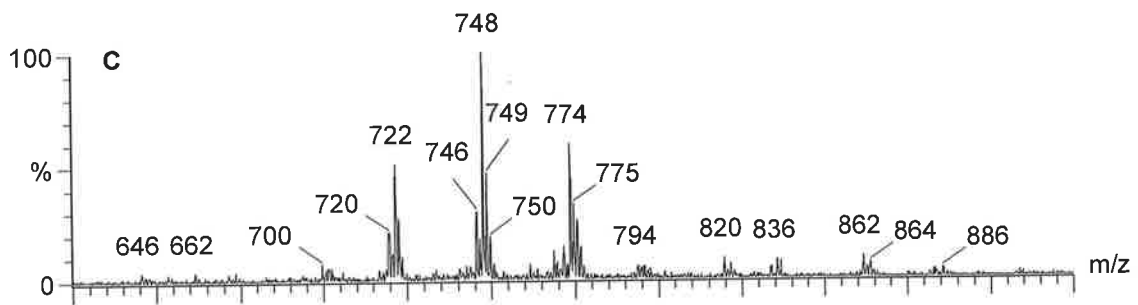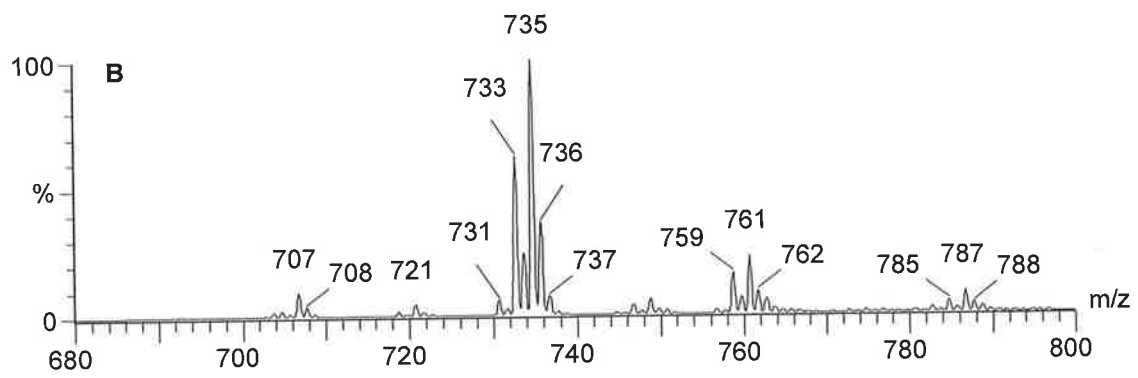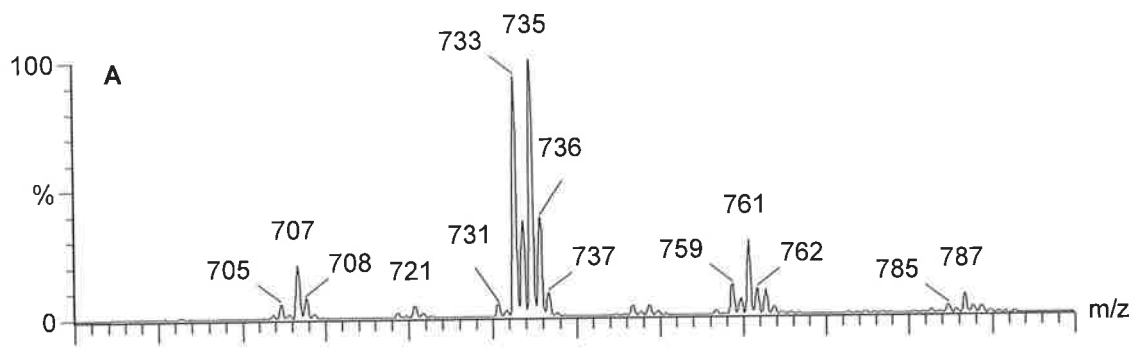
Figure 3.6 This figure shows the typical spectra obtained from diagnostic scans of a lavage sample isolated from golden-mantled ground squirrels, *Spermophilus lateralis*. m/z mass/charge ratio, which is equivalent to molecular mass. **A** phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) (parents of 184^{+}), **B** phosphatidylglycerol (PG) (parents of 153^{-}), **C** phosphatidylinositol (PI) (parents of 214^{-}). Tandem mass spectrometry of the various mass ions was used to confirm the identity of molecular species. **B (INSET)** shows an example of fatty acid fragmentation. The identity of m/z 748 ion was confirmed as PG16:0/18:1 because fragmentation of m/z 748 ion generated an 18:1 fatty acid anion at m/z 281 and a 16:0 fatty acid anion at m/z 255.

Figure A: PC spectrum (m/z 650–900) with peaks labeled PC14:0/16:0, PC16:0/16:1, PC16:0/16:0, PC16:0/18:2, PC16:0/18:1, PC16:0/18:0.

Figure B: PG spectrum (m/z ~710–850) with peaks labeled PG16:0/16:0, PG16:0/18:2, 748, PG16:0/18:0, PG18:0/18:1, 18:0/18:0, PG16:0/22:6, PG16:0/22:5. Inset shows MS/MS of PG16:0/18:1 with fragments 153, 255, 282, 748.

Figure C: PI spectrum (m/z 725–975) with peaks labeled PI16:0/18:2, PI16:0/18:1, PI18:0/18:2, PI18:0/18:1, PI16:0/20:4, PI18:0/22:6, PI18:0/22:5.

Figure 3.7. The composition of individual molecular species of phosphatidylcholine (PC) in lavage isolated from warm-active and hibernating golden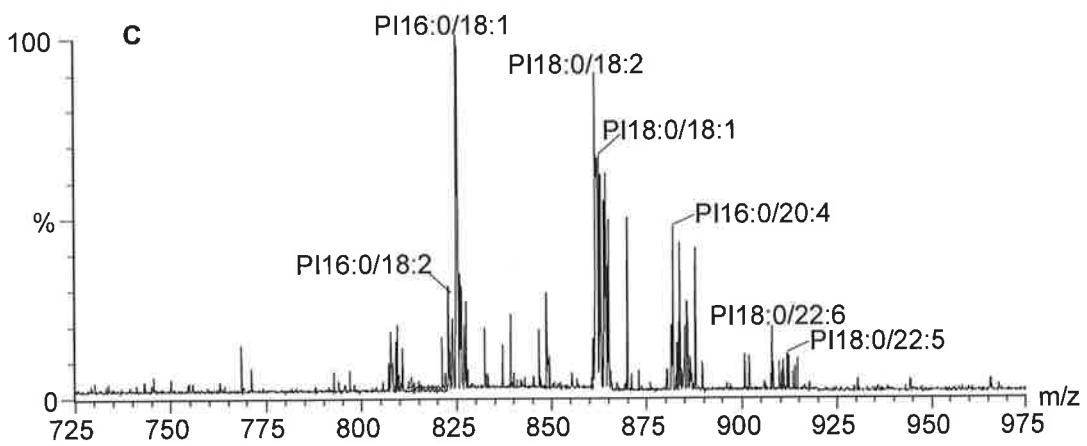-mantled ground squirrels, *Spermophilus lateralis*. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Results are presented as mean $\pm$ SE. There were no significant differences between lavage isolated from warm-active and hibernating ground squirrels (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

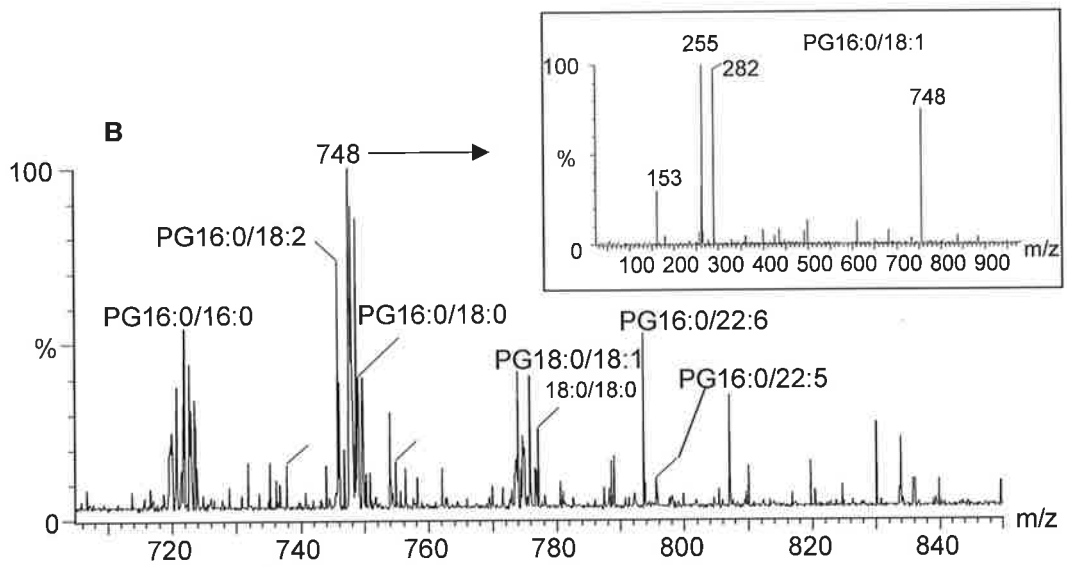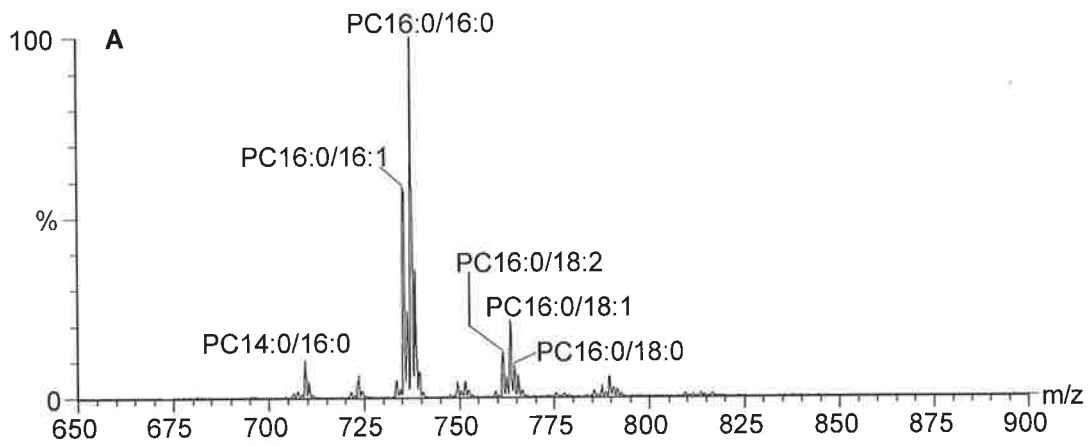Molecular species of phosphatidylcholine

Figure 3.8. The composition of individual molecular species of phosphatidylglycerol (PG) in lavage isolated from warm-active and hibernating golden-mantled ground squirrels, *Spermophilus lateralis.* Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. All molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Results are presented as mean ± SE. * significant decrease in lavage fluid isolated from hibernating ground squirrels (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

Figure showing Phosphatidylglycerol (mol%) for Warm-active (n = 5) and Hibernating (n = 6) across molecular species of phosphatidylglycerol.

Legend:
- Warm-active (n = 5)
- Hibernating (n = 6)

Y-axis: Phosphatidylglycerol (mol%), scale 0 to 30

X-axis (Molecular species of phosphatidylglycerol): 16:1/16:1, 16:0/16:1, 16:0/16:0, 16:1/18:2, 16:0/18:2, 16:0/18:1, 16:0/18:0, 18:1/18:2, 18:1/18:1, 18:0/18:1, 18:0/18:0, 16:0/22:6, 16:0/22:5

Figure 3.9. The composition of individual molecular species of phosphatidylinositol (PI) in lavage isolated from warm-active and hibernating golden-mantled ground squirrels, *Spermophilus lateralis*. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. All molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Results are presented as mean ± SE. There were no significant differences between lavage isolated from warm-active and hibernating ground squirrels (using Student's unpaired t-test, assuming equal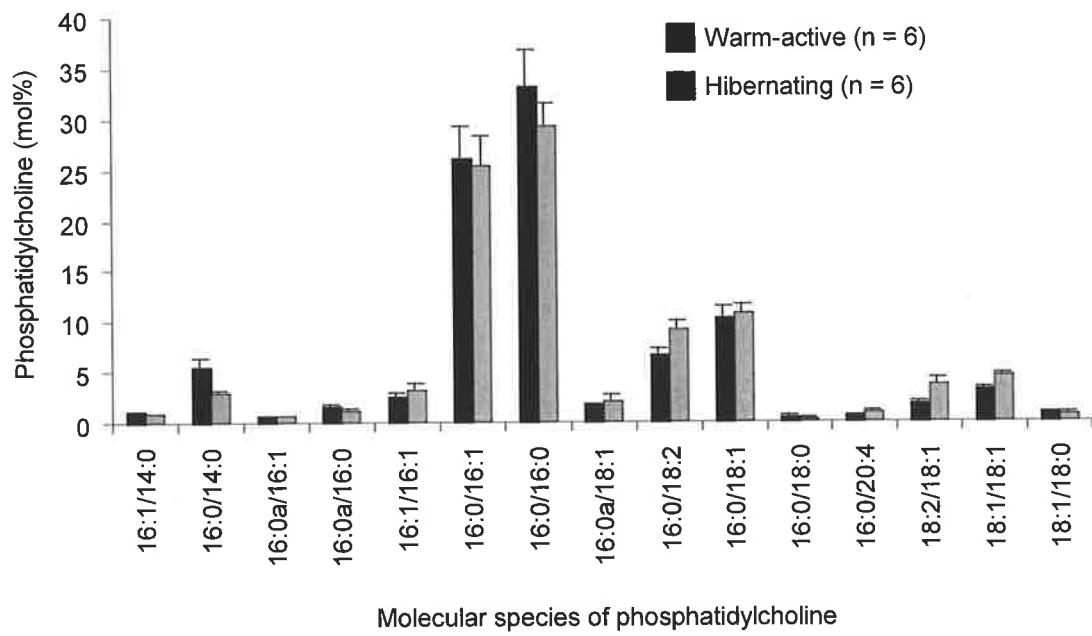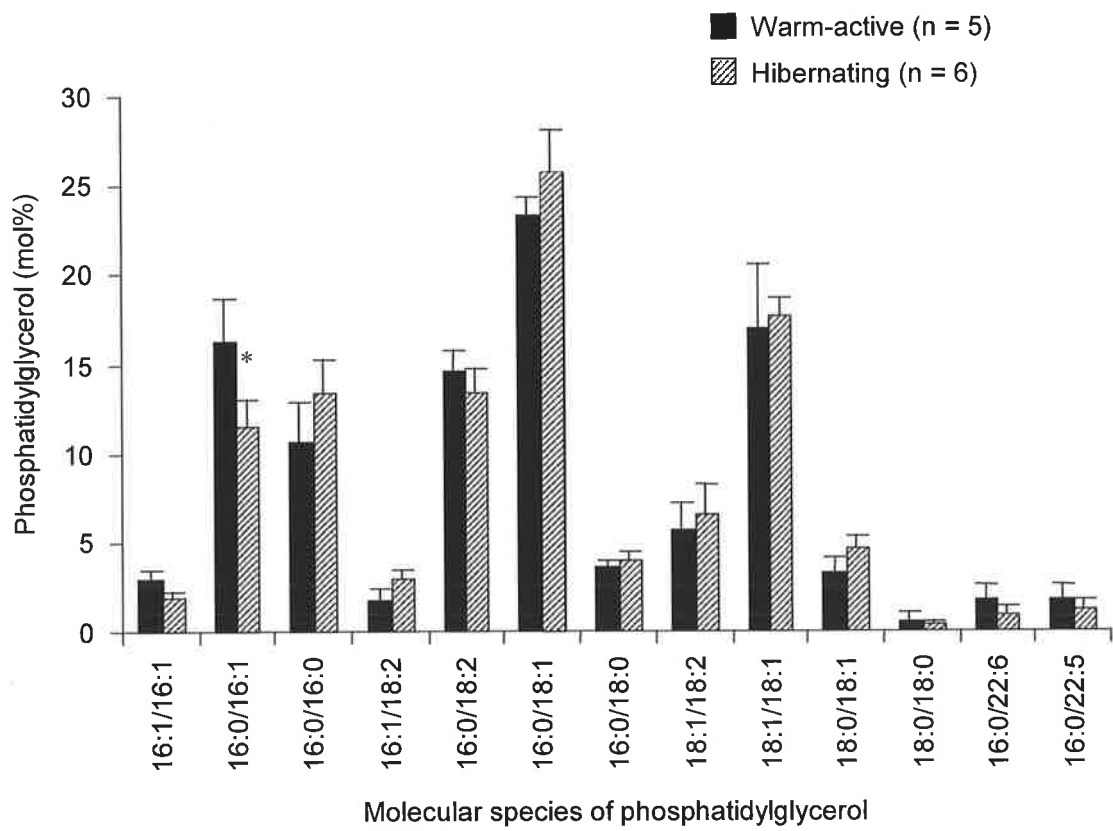 variance, significance at $p < 0.05$). Statistical analysis for PI16:0/18:1 between lavage isolated from warm-active and hibernating squirrels was $t_{stat} = 1.79$, $df = 7$, $p = 0.058$.

Figure showing Phosphatidylinositol (mol%) for Warm-active (n = 5) and Hibernating (n = 4) groups across molecular species of phosphatidylinositol: 16:0/16:1, 16:0/16:0, 16:1/18:1, 16:0/18:1, 18:1/18:2, 18:1/18:1, 18:0/18:1, 18:1/20:4, 18:0/20:4, 18:0/22:6, 20:1/20:4
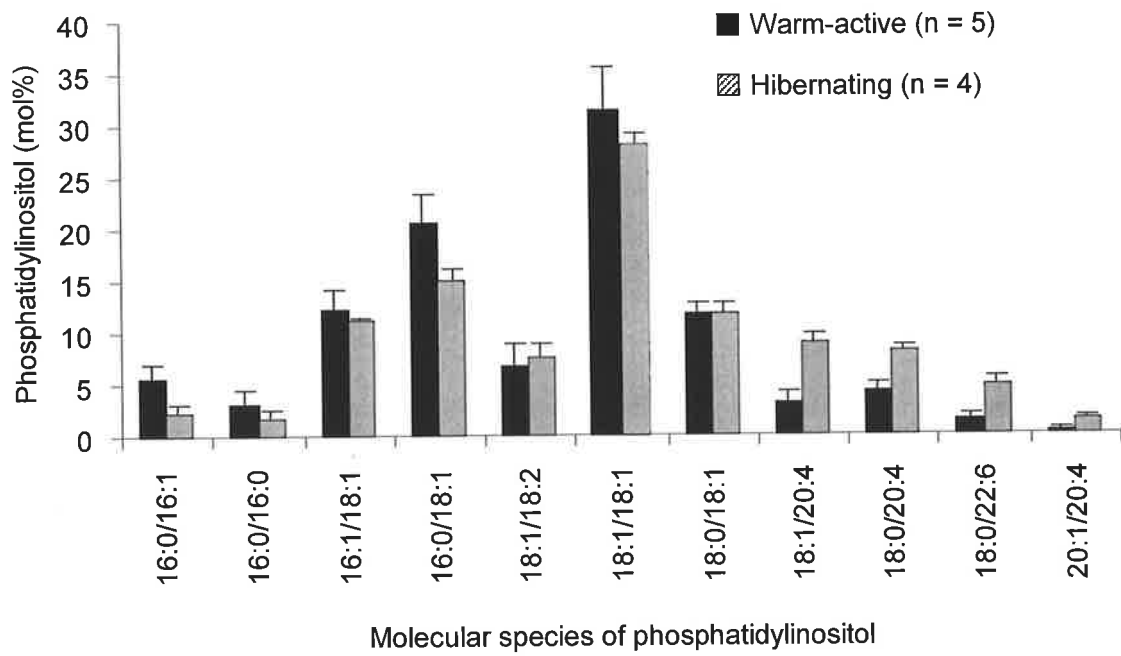
3.2.5. PROTEIN COMPOSITION

The ratio of SP-A/PL was significantly lower in surfactant isolated from hibernating squirrels compared to warm-active squirrels (t_{stat} = 2.906, df = 9, p = 0.009) (Table 3.1). However, the ratio of SP-B/PL did not differ between lavage isolated from warm-active and hibernating animals (t_{stat} = 0.657, df = 11, p = 0.26) (Table 3.1). The ratio of SP-C/PL was significantly higher in surfactant isolated from hibernating squirrels compared to warm-active squirrels (t_{stat} = 1.88, df = 9, p = 0.046) (Table 3.1).

Table 3.1. Surfactant proteins in lavage isolated from warm-active and hibernating ground squirrels, *Spermophilus lateralis*.

	SP-A	SP-B	SP-C
		ng/mg PL	
Warm-active	119.74 ± 21.4	19.51 ± 0.79	606.42 ± 127.4
Hibernating	56.14 ± 9.3*	22.77 ± 3.39	1072.5 ± 245.7^

Surfactant proteins were measured in lavage samples isolated from warm-active and hibernating squirrels using ELISAs outlined in Section 2.7. Values are mean ± SE, n = 6-8. Statistical significance was determined using Student's t-tests, assuming equal variance, significance at p < 0.05. * significant decrease in lavage isolated from hibernating squirrels compared to warm-active squirrels. ^ significant increase in lavage isolated from hibernating squirrels compared to warm-active squirrels.

3.2.6. EPIFLUORESCENCE MICROSCOPY

Examples of the fluorescence images obtained for surface films made of surfactant isolated from warm-active and hibernating squirrels are shown in Figures 3.10 and 3.11, respectively. The relative area of the surface covered by the black, probe-excluded regions and the average diameter of such domains were calculated and the results are

summarised in Figure 3.12. At each surface pressure, there was a significant difference in the relative area covered by the probe between films of surfactant isolated from warm-active and hibernating squirrels (15 mN m^{-1}: q = 0.6.376, p < 0.01; 20 mN m^{-1}: q = 8.410, p < 0.001; 25 mN m^{-1}: q = 12.736, p < 0.001; 30 mN m^{-1}: q = 11.830, p < 0.001) (Figure 3.12A). When comparing the areas between different surface pressures for surfactant isolated from warm-active squirrels, the relative area covered by the probe showed a declining trend after a surface pressure of 15 mN m^{-1}. Conversely, for films of surfactant isolated from hibernating squirrels, the relative area of the probe-covered surface showed an increasing trend after a surface pressure of 15 mN m^{-1}. However, statistically, there were no significant differences in the relative area covered by the probe between different surface pressures in surfactant isolated from both warm-active and hibernating squirrels.

There was a significant difference in diameter of the probe-excluded regions between films of surfactant isolated from warm-active and hibernating squirrels at 20 mN m^{-1} (q = 4.713, p < 0.05), 25 mN m^{-1} (q = 11.924, p < 0.001) and 30 mN m^{-1} (q = 11.503, p < 0.001) surface pressures, but not at 15 mN m^{-1} (q = 0.1732, p > 0.05) surface pressure (Figure 3.12B). Statistically there were no differences in the diameter of the probe-excluded regions between different surface pressures for surfactant films made from both warm-active and hibernating squirrel surfactant. However, the average diameter of the probe-excluded regions showed a decreasing trend as surface pressure increased, from 15 to 30 mN m^{-1} in warm-active surfactant films (Figure 3.12B), but remained fairly constant in films made of surfactant isolated from hibernating squirrels (Figure 3.12B).

Figure 3.10. Epifluorescence images of solvent spread films of surfactant isolated from warm-active golden-mantled ground squirrels, *Spermophilus lateralis* at 15, 20, 25 and 30 mN m^{-1} surface pressures, at 23°C. Surface pressures given in top, left corner of image. Liquid-condensed (LC) domains (or regions) exclude the fluorescent probe and appear dark. Scale bar = 25 μm.

LC domain

15

20

25

30

25 um

25 um

25 um

25 um

LC domain
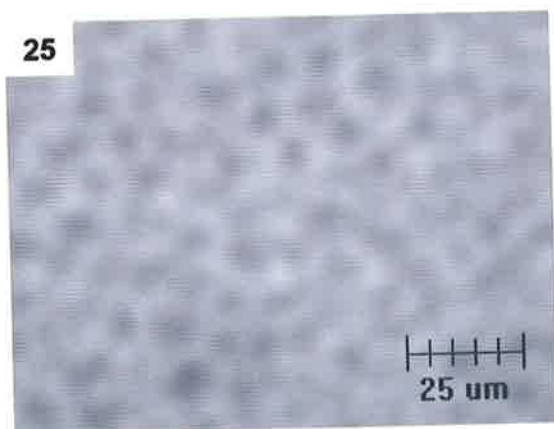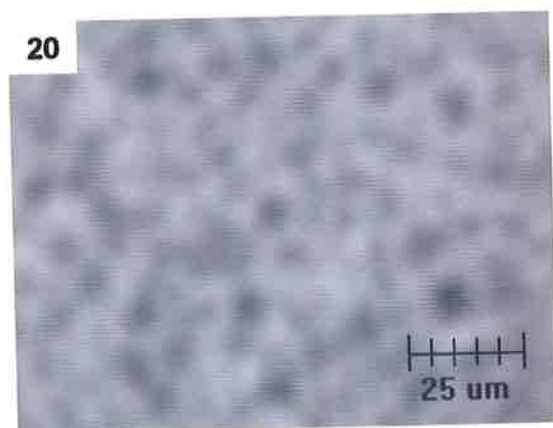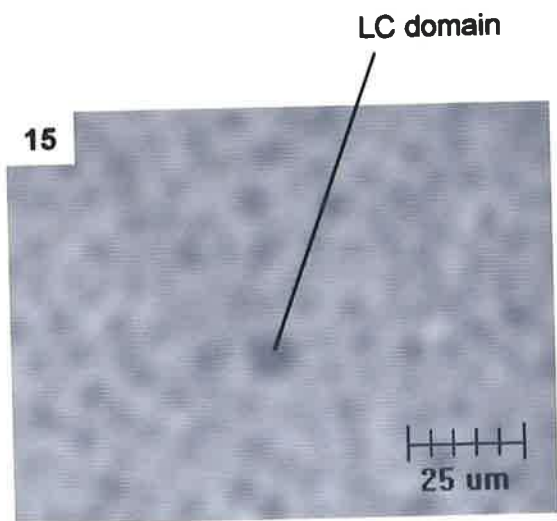
Figure 3.11. Epifluorescence images of solvent spread films of surfactant isolated from hibernating golden-mantled ground squirrels, *Spermophilus lateralis* at 15, 20, 25 and 30 mN m^{-1} surface pressures, at 23°C. Surface pressures given in top, left corner of image. Liquid-condensed (LC) domains (or regions) exclude the fluorescent probe and appear dark. Scale bar = 25 μm.
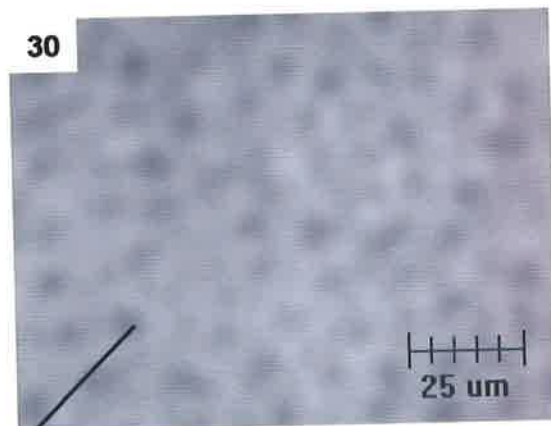
15

20

LC domain

25 um

25 um

25

30

25 um

25 um
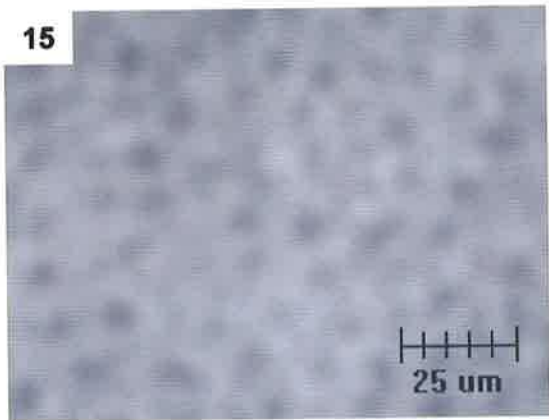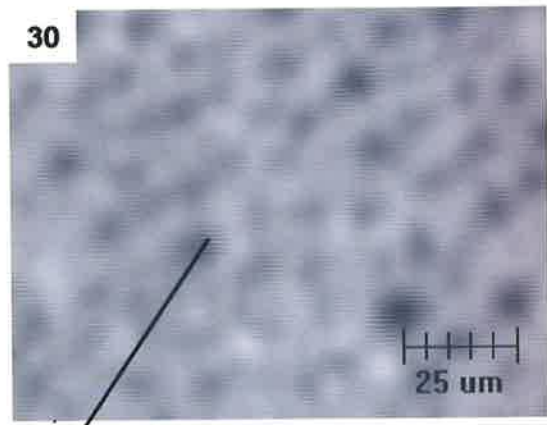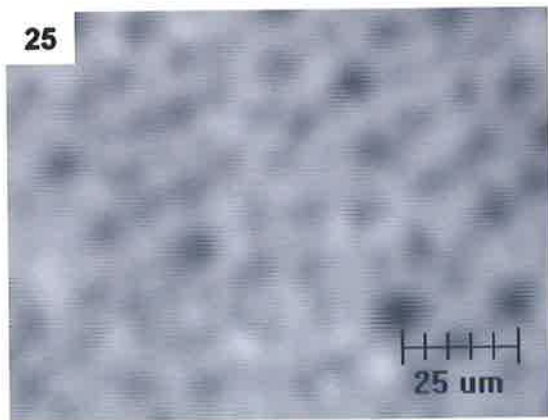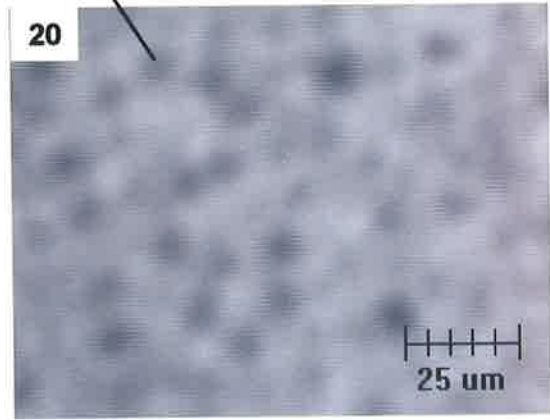
LC domain

Figure 3.12. Fluorescence image analysis of solvent spread films of surfactant isolated from warm-active and hibernating golden-mantled ground squirrels, *Spermophilus lateralis*, at different surface pressures at 23°C. Subphase: 0.15 M NaCl, 1.5 mM CaCl$_2$, 1.0 mM Tris-HCl buffer at pH 7.0. Film compression rate 0.05 nm^{2}mol^{-1}min^{-1} with an initial area of 1.2 nm^2 mol^{-1}. Data are mean ± SE per number of animals (n), multiple measurements per animal. **A** percent of the area covered by probe excluded regions, **B** diameter of the probe excluded regions. * significant difference between surfactant isolated from warm-active and hibernating squirrels at each surface pressure (using one-way ANOVA followed by Tukey post-hoc test, significance at p < 0.05).

A

B
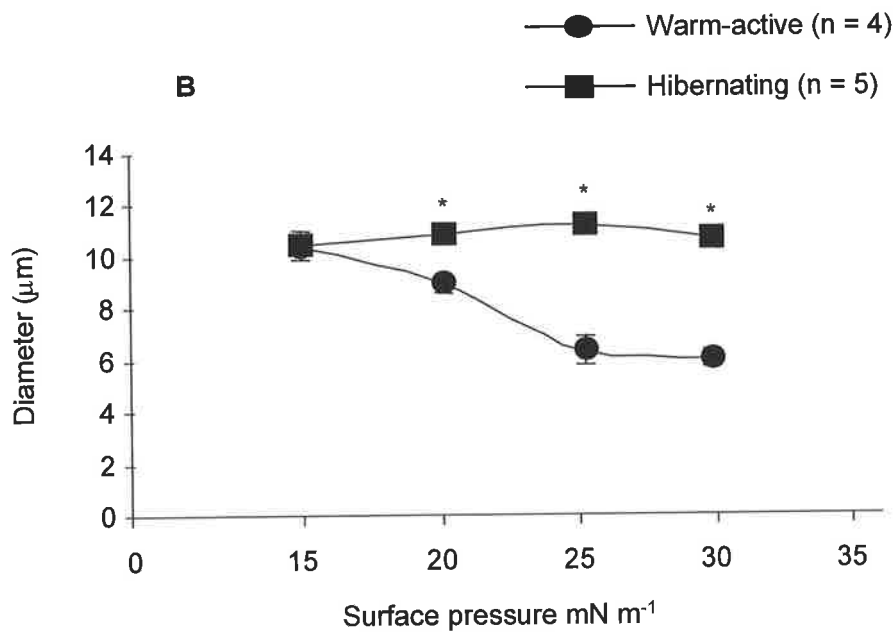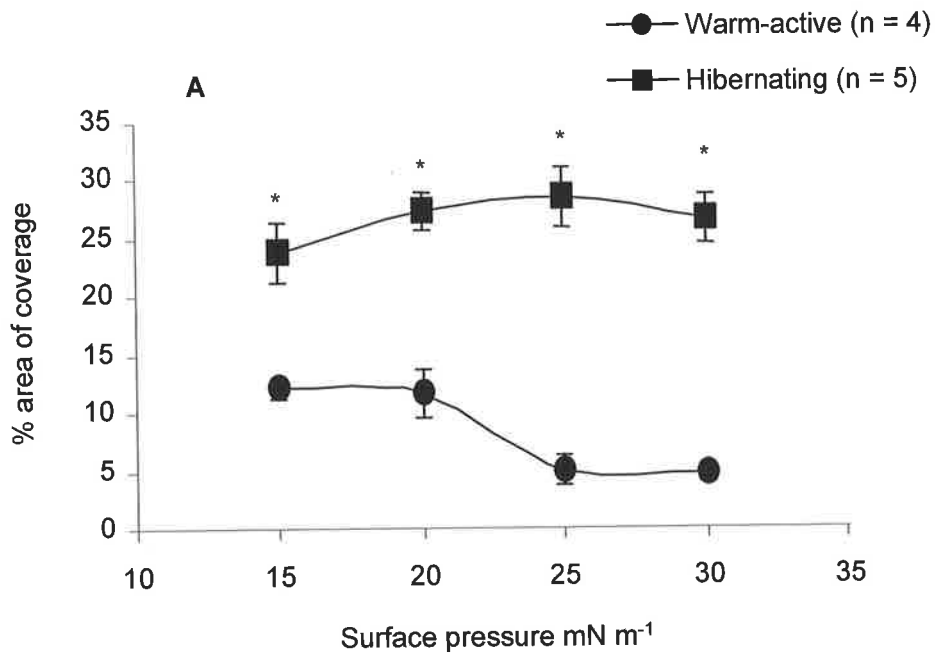
3.2.8. ATOMIC FORCE MICROSCOPY

A surface heterogeneity was observed in AFM images of films prepared from both warm-active and hibernating squirrel surfactant. In both cases, liquid-condensed (brighter) domains were observed. Height analyses showed that this LC domain is enriched with organised molecules with the height characteristics of DPPC (Table 3.2). Phase imaging of the films showed the existence of an even brighter, and hence, an even more condensed and organised region, of greater height inside the organised LC domains. Similar domains were also observed in dunnart surfactant and are shown in Figures 5.17 and 5.18.

The height and size characteristics of different domains in surfactant films isolated from warm-active and hibernating squirrels are given in Table 3.2. The height analysis suggests that there are vertical differences in height between the different domains. The height difference between the liquid-condensed domain (LC) and the even brighter and thus, higher and more condensed domain (2LC), was significantly lower (t_{stat} = 6.816, df = 8, p = 0.000068) in films prepared from hibernating squirrel surfactant than in warm-active squirrel surfactant. The height difference between the highest liquid-condensed domain (i.e. 2LC) and the outside planar region (p) between films of warm-active and hibernating surfactants also changed significantly (t_{stat} = 1.74, df = 33, p = 0.045) between films of warm-active and hibernating squirrel surfactant. The size of the LC domain did not change significantly (t_{stat} = 1.339, df = 33, p = 0.095) between films of warm-active and hibernating squirrel surfactant.

Table 3.2. Height and size characteristics of domains observed using atomic force microscopy, in films of surfactant isolated from warm-active and hibernating golden-mantled ground squirrels, *Spermophilus lateralis*.

Animal State	Mean height difference between domains		Domain Size
	LC-2LC (nm)	*LC-p* (nm)	LC (μm)
Warm-active	0.74 ± 0.05 (n = 5)	1.02 ± 0.06 (n =15)	5.94 ± 0.26 (n=15)
Hibernating	0.33 ± 0.03 (n = 5)*	1.18 ± 0.06 (n =20)*	5.43 ± 0.29 (n=20)

LC-2LC refers to the mean height difference (nm) between the highest liquid-condensed domain (2LC in Figure 3.16) and the larger, liquid-condensed domain (labelled LC in Figure 3.16), *LC-p* refers to the mean height difference between the liquid-condensed domain (LC in Figure 3.16) and the outside planar region (*p* in Figure 3.16). n number of observations. * significant difference between warm-active and hibernating squirrel surfactant (using Student's unpaired t-tests assuming equal variance, significance at $p < 0.05$). Note: Similar domains were also observed in dunnart surfactant and are shown in Figures 5.17 and 5.18.

3.3. SUMMARY OF RESULTS

- There was no difference in the saturation of PL between surfactant isolated from warm-active and hibernating ground squirrels.

- CHOL increased significantly in surfactant isolated from hibernating ground squirrels, which resulted in an increase in the CHOL/PL and CHOL/DSPC ratios.

- There was no change in the molecular species composition of PC between surfactant isolated from warm-active and hibernating ground squirrels

- The molecular species PG16:0/16:1 decreased significantly in surfactant isolated from hibernating squirrels. However, there was no change in any other molecular

species of PG between surfactant isolated from warm-active and hibernating ground squirrels.

- There was no change in the molecular species composition of PI between surfactant isolated from warm-active and hibernating ground squirrels.

- SP-A decreased significantly in surfactant isolated from hibernating ground squirrels.

- There was no difference in SP-B between surfactant isolated from warm-active and hibernating squirrels.

- SP-C increased significantly in surfactant isolated from hibernating ground squirrels.

- At 23°C, epifluorescence studies show that surfactant from hibernating squirrels has a greater number of larger condensed domains than warm-active surfactant i.e. warm-active surfactant has more fluid regions.

- AFM studies confirm that films made of hibernating squirrel surfactant have higher, and thus, more tightly-packed liquid-condensed domains, than films made of warm-active squirrel surfactant.

CHAPTER 4

RESULTS FOR

WARM-ACTIVE AND TORPID

GOULD'S WATTLED BATS

Chalinolobus gouldii

4.1. BACKGROUND

Microchiropteran bats are excellent models for investigating how the pulmonary surfactant system copes with changes in physiological variables, such as temperature and exercise, because their natural daily (24 h) cycle involves both torpor and the most energetically demanding form of exercise, flapping flight. Bats have the most highly specialised mammalian lung, consisting of finely divided air spaces and are the only mammal group to fly. In the warm-active state, bats undergo large fluctuations in physiological variables as the level of activity changes, for example, when feeding (Audet and Brock-Fenton, 1987) or flying (Thomas et al., 1984). During sustained flight, bats hyperventilate with respect to their metabolic requirements while body temperature increases by up to 3°C (Thomas et al., 1990; Thomas et al., 1984). However, Gould's wattled bats, *Chalinolobus gouldii*, also regularly enter into daily torpor, where the body temperature can drop as low as 5°C (Hosken and Withers, 1997). During daily torpor in bats, ventilation changes to episodic breathing bouts alternating with long periods of apnea (Morris et al., 1994). Given the biophysical properties of the surfactant components, the changes in body temperature and ventilation experienced by bats during each daily cycle, have the potential to significantly challenge the pulmonary surfactant system.

Previously we have demonstrated that the amount and composition of surfactant lipids varied over the daily cycle. While the amount of PL, DSP and the proportion of DSP/PL did not change significantly (Codd et al., 2000b), the amount of CHOL did increase by 1.5 fold during daily torpor. The elevated CHOL resulted in an increase in the proportion of CHOL/DSP and CHOL/PL (Codd et al., 2000b). However, compared to other mammalian surfactants, CHOL levels are extremely low in surfactant from both warm-active and torpid bats. Lesser long-eared bats, *Nyctophilus geoffroyi* and Gould's wattled bats, *C. gouldii*, have 15 and 6 times less cholesterol, respectively than has been

reported in the surfactant of any other mammal (Codd et al., 2000a). This suggests that other components, such as surfactant proteins or specific molecular species of PL, must be acting to aid in the spreading and fluidity of bat surfactant at low body temperatures.

Here, I hypothesise that there will be changes in the molecular PL composition, and/or the surfactant proteins, in surfactant during daily torpor in bats. Given the regular, daily pattern of torpor in bats and their flight physiology, these changes may differ from those observed in either dunnarts and/or ground squirrels during stress torpor and hibernation, respectively. Previous observations suggest that torpid bat surfactant has greater surface-tension lowering capabilities than warm-active surfactant, when measured at 24°C (Codd et al., 2000b). Hence, I further hypothesise that changes in surfactant composition will alter the structure of the surface film, in such as way as to enable surfactant to function effectively at cold body temperatures. The molecular PL and protein composition of surfactant isolated from warm-active (T_b = 35°C) and torpid (T_b = 12°C) Gould's wattled bats, *C. gouldii* is examined using electrospray ionisation mass spectrometry and ELISA technology.

4.2. RESULTS

4.2.1. BAT DESCRIPTIVE DATA

The descriptive data for the bats are given in Table 4.1. Rectal body temperature was significantly lower in torpid bats than warm-active bats (t_{stat} = 48.856, df = 11, p < 0.00000001). The mean body mass of warm-active bats did not differ significantly from the mean body mass of torpid bats (t_{stat} = 0.639, df = 10, p = 0.27). There was also no significant difference in wet (t_{stat} = 0.395, df = 13, p = 0.35) or dry (t_{stat} = 11, df = 11, p = 0.18) lung mass between warm-active and torpid bats. The sex of the bats had no significant effect on body temperature, body mass or wet or dry lung mass in either warm-active or torpid bats (df = 4-6, p > 0.05 in all cases).

Table 4.1. Descriptive statistics of warm-active and torpid Gould's wattled bats, *Chalinolobus gouldii*.

Bat	T_b(°C)	Body Mass (g)	WLW (g)	DLW (g)
Warm-active	**34.88 ± 0.34(7)***	**12.95 ± 0.25(6)**	**0.28 ± 0.02(8)**	**0.031 ± 0.001(7)**
Female	35.08 ± 0.53(4)	12.80 ± 0.25(4)	0.29 ± 0.02(5)	0.031 ± 0.001(4)
Male	34.63 ± 0.43(3)	13.27 ± 0.66(2)	0.32 ± 0.02(3)	0.031 ± 0.003(3)
Torpid	**12.42 ± 0.30(6)***	**13.22 ± 0.33(6)**	**0.29 ± 0.01(7)**	**0.032 ± 0.001(6)**
Female	12.00 ± 0.47(3)	13.48 ± 0.26(4)	0.27 ± 0.01(4)	0.032 ± 0.001(3)
Male	12.83 ± 0.24(3)	12.70 ± 0.89(2)	0.31 ± 0.02(3)	0.032 ± 0.002(3)

Values are mean ± SE. Top line (bold) for each group includes both male and female bats. Individual means for males and females are shown below. Numbers in brackets represent the number of animals for each measurement; WLW wet lung weight (grams), DLW dry lung weight (grams). * significant difference between warm-active and torpid

groups (using Student's unpaired t-tests, assuming equal variance, significance at $p <$ 0.05).

4.2.2. ELECTRON MICROSCOPY OF BAT LUNG

Photographs of the alveolar epithelium in lung tissue isolated from Gould's wattled bat, *Chalinolobus gouldii*, are shown in Figure 4.1. The photographs shows the cuboidal shape, microvilli and presence of large osmiophilic lamellar bodies that are characteristic of alveolar type II cells (Ormond, 1999).

4.2.3. SATURATION OF PHOSPHOLIPIDS

Estimates of percent saturation, as determined by the proportion of disaturated PC species, did not change significantly between surfactant isolated from warm-active and torpid bats (t_{stat} = 1.085, df = 15; p = 0.15) (Figure 4.2). However, there was a significant decrease in the percent saturation of PG in the surfactant of torpid bats (t_{stat} = 2.549, df = 13, p = 0.012). The saturation of PI (t_{stat} = 0.909, df = 7, p = 0.2) and phosphatidylserine (PS) (t_{stat} = 1.202, df = 8, p = 0.13) did not change significantly between surfactant isolated from warm-active and torpid bats (Figure 4.2.).

Figure 4.1. Electronmicrographs of alveolar type II epithelial cells located in the alveolar epithelium of lung tissue isolated from Gould's wattled bat, *Chalinolobus gouldii*. Scale bar = 2 µm. AS air space, LB lamellar body, N nucleus.
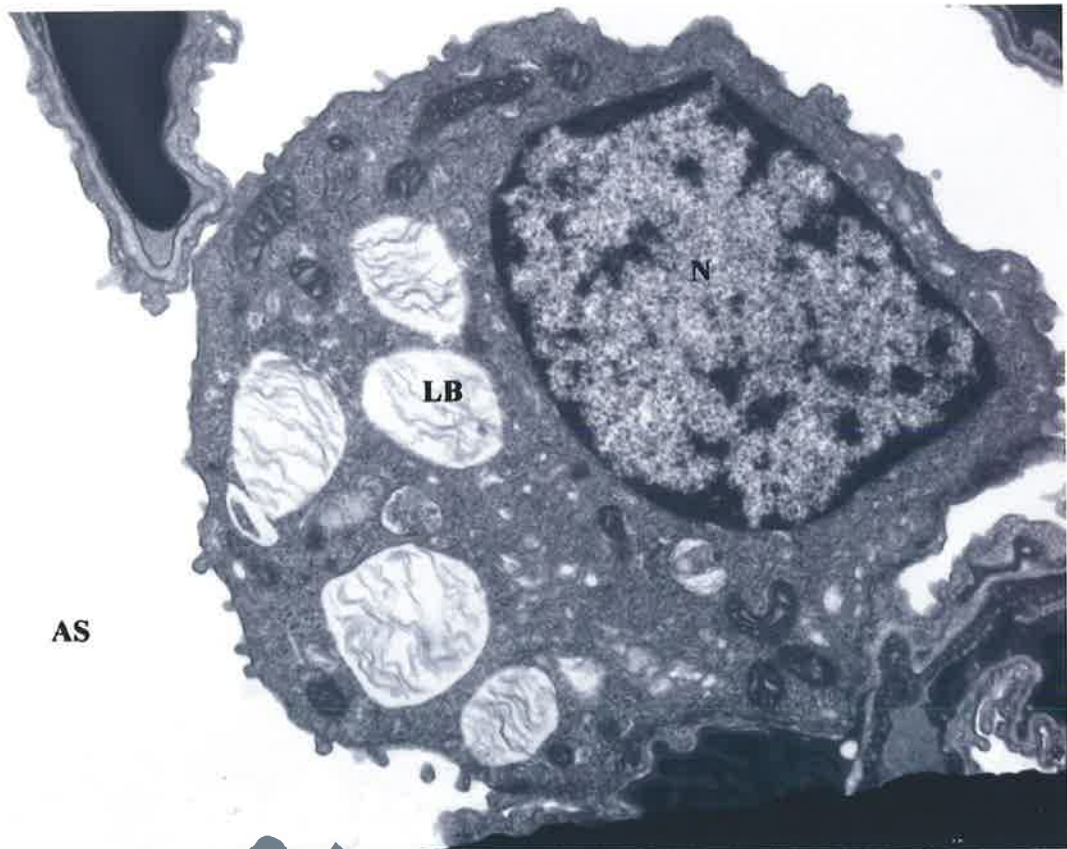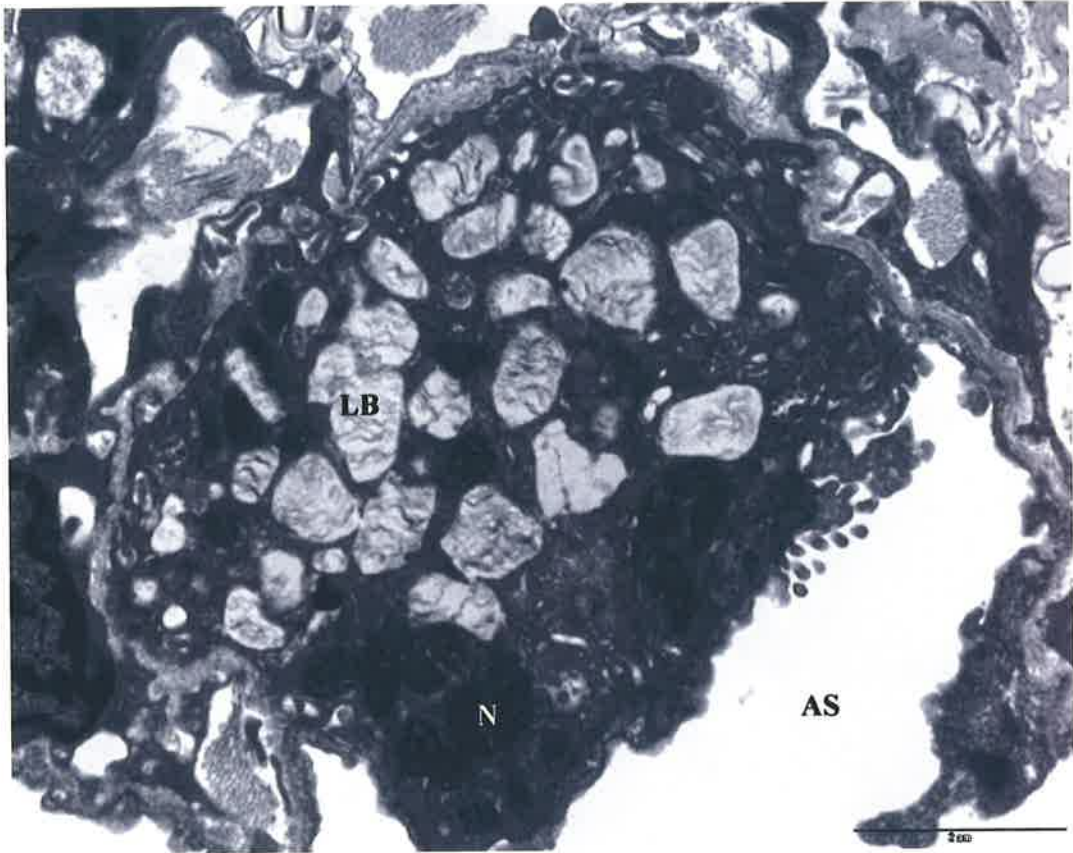
Figure 4.2. Relative proportions of disaturated PL components in surfactant isolated from warm-active and torpid Gould's wattled bats, *Chalinolobus gouldii*. All data are presented as mean ± SE. n number of animals, DSPC/PC disaturated phosphatidylcholine as a fraction of total phosphatidylcholine, DSPG/PG disaturated phosphatidylglycerol as a fraction of total phosphatidylglycerol, DSPI/PI disaturated phosphatidylinositol as a fraction of phosphatidylinositol, DSPS/PS disaturated phosphatidylserine as a fraction of phosphatidylserine, * significant difference between lavage isolated from warm-active and torpid bats (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).
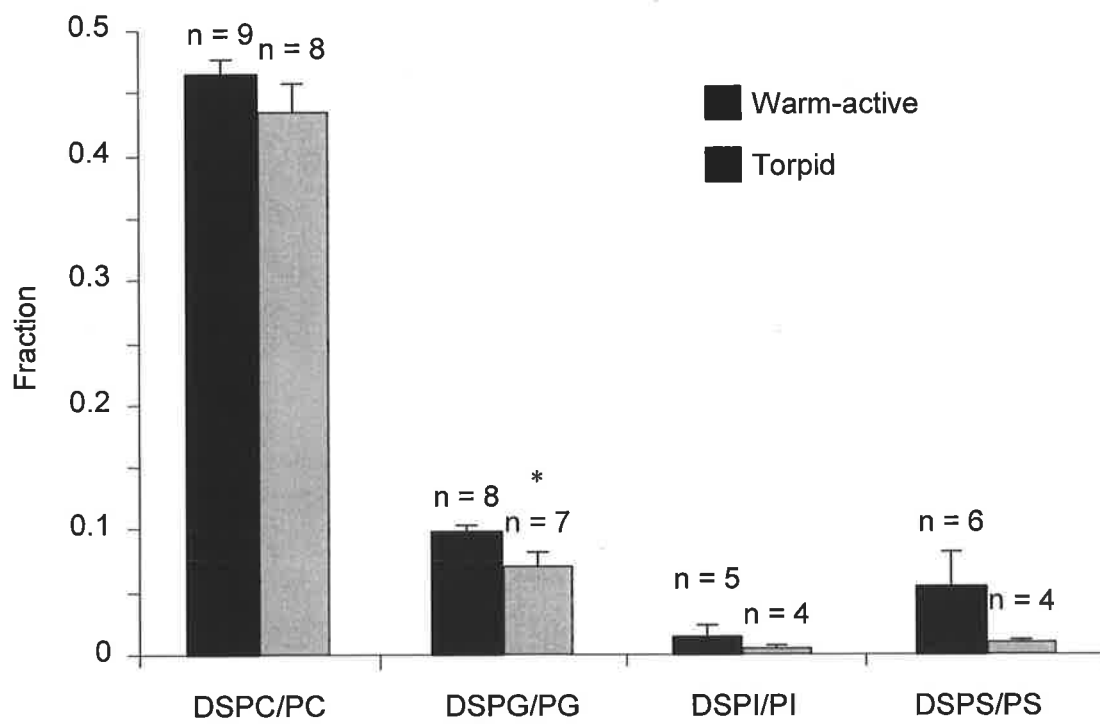
4.2.4. MOLECULAR PHOSPHOLIPID COMPOSITION

Examples of ESI-MS spectra observed for bat surfactant in positive and negative mode are given in Figure 4.3. Typical examples of diagnostic scans for bat surfactant are given in Figure 4.4. The molecular species of phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS) in surfactant isolated from warm-active and torpid bats are given in Figures 4.5, 4.6, 4.7 and 4.8, respectively. There were no significant differences in the molecular species of PC present between surfactant isolated from warm-active and torpid bats. In both warm-active and torpid bats, the composition of surfactant PC was dominated by the disaturated species, PC16:0/16:0, which contributes 40-45 mol% to the PC component. The next major PC components present in bat surfactant are the unsaturated species, PC16:0/16:1 (20 mol%), PC18:1/16:0 (15-18 mol%) and PC18:2/16:0 (6 mol%). The molecular species PC16:1/14:0, PC16:0/18:0, PC20:4/16:0, PC18:2/18:1, PC18:1/18:1, PC18:1/18:0, PC18:2/20:4, PC18:1/20:4, PC18:0/20:4 and alkyl-acyl forms of PC16:0/16:0 and PC16:0/16:1 are present in small quantities (less than 4 mol% each) and contribute to the remaining proportion of PC species.

In surfactant from both warm-active and torpid bats, PG composition was dominated by the unsaturated species PG16:0/18:1, which contributed 40-45 mol% to the PG component. The other major PG components were PG18:1/18:1 (15-20 mol%), PG16:0/18:2 (12 mol%), PG18:0/18:1 (8-10 mol%) and the disaturated species, PG16:0/16:0 (8-10 mol%). There was a significant decrease in PG16:0/16:0 in hibernating squirrel surfactant (t_{stat} = 2.55, df = 13, p = 0.012). The molecular species PG16:0/16:1, PG16:1/18:2, PG16:0/20:4 and PG18:1/18:2 comprised less than 5 mol% each of the PG component.

In both warm-active and torpid bats, the composition of surfactant PI was dominated by the unsaturated species, PI18:0/20:4 (25 mol%) and PI16:0/18:1 (20

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mol%). The other major components of PI in bat surfactant, include PI18:2/18:1 (12-18 mol%), PI18:2/18:2 (10-15 mol%), PI18:0/18:2 (10-20 mol%) and PI16:0/18:2 (10 mol%). There was a significant increase in PI18:0/18:2 in surfactant isolated from torpid bats ($t_{stat} = 3.058$, df = 6, p = 0.01). PI16:1/20:4, PI18:1/18:2, PI18:0/18:1, PI18:2/20:4, PI18:1/20:4, PI18:0/22:6 and PI18:0/22:5 make up the remaining PI component (less than 5 mol% each). The disaturated species, PI16:0/16:0 was present in negligible quantities.

There were no significant differences in the molecular species of PS present in surfactant isolated from warm-active and torpid bats. In both warm-active and torpid bats, the composition of surfactant PS was dominated by the monounsaturated species, PS18:1/18:0, which contributes 20-30 mol% to the PS component. The next major PS components present in bat surfactant are the unsaturated species, PS18:1/18:1 (15-20 mol%), PS18:1/16:0 (15 mol%) and PS18:0/20:4 (5-10 mol%). Other molecular species of PS contribute less than 5 mol% each to the remaining PS component.

Figure 4.3. Examples of spectra obtained for surfactant isolated from **A** warm-active and **B** torpid Gould's wattled bats, *Chalinolobus gouldii* and analysed by electrospray ionisation mass spectrometry in positive mode. Examples of spectra obtained for lavage isolated from **C** warm-active and **D** torpid Gould's wattled bats, *C. gouldii* and analysed by electrospray ionisation mass spectrometry in negative mode. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Mass (m)/charge (z) ratio is equivalent to molecular mass.

A — Phosphatidylcholine

B — Phosphatidylcholine

C — Phosphatidylglycerol, Phosphatidylinositol

D — Phosphatidylglycerol, Phosphatidylinositol
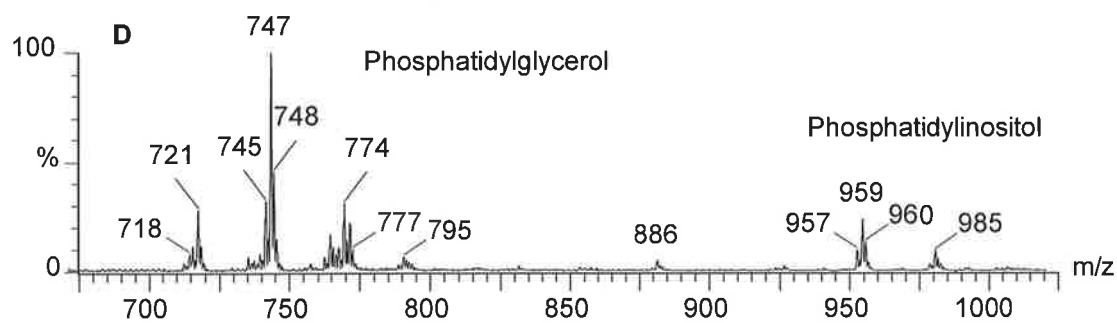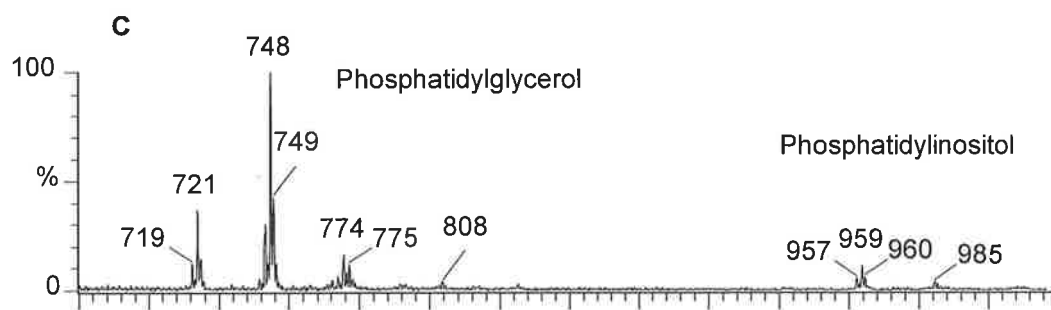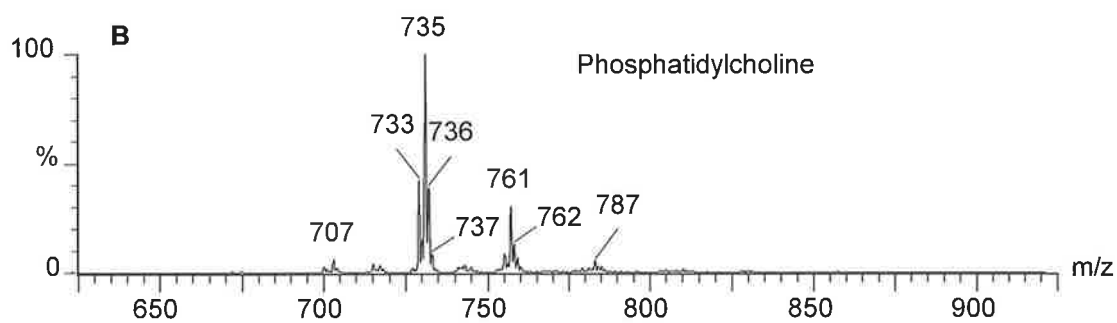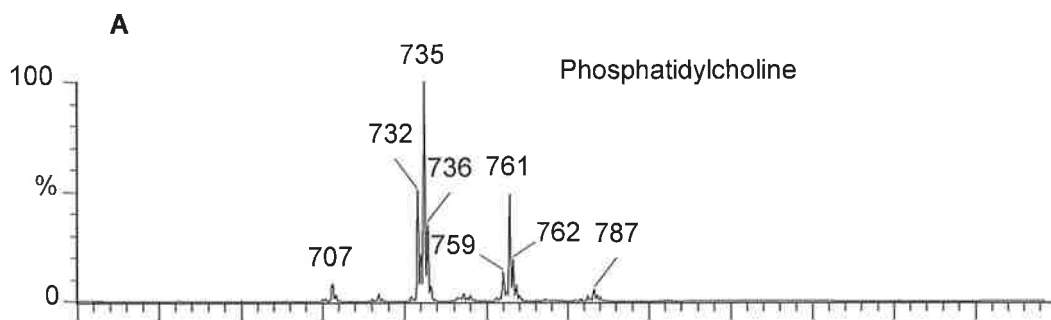
Figure 4.4. Examples of spectra obtained from diagnostic scans of surfactant isolated from Gould's wattled bats, *Chalinolobus gouldii*. **A** phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) (parents of 184^+); **B** phosphatidylglycerol (PG) (parents of 153^-); **C** phosphatidylinositol (PI) (parents of 214^-); **D** phosphatidylserine (PS) (neutral loss of 87^-). Fatty acid fragmentation using tandem mass spectrometry was used to confirm which molecular species are in each ion peak (data not shown). Mass (m)/charge (z) ratio is equivalent to molecular mass.

A — Parents of 184ES+

PC16:0/16:0
PC16:0a/18:1
PC16:0/18:2
PC16:0/16:1
PC16:0/18:1
PC16:0/18:0
PC16:0/14:0

B — Parents of 153ES-

PG16:0/18:1
PG16:0/18:2
PG18:1/18:1
PG16:0/16:0
PG18:0/18:1

C — Parents of 241ES-

PI18:0/20:4
PI18:0/18:2
PI16:0/18:1
16:0/18:2
PI18:0/20:3

D — Neutral Loss 87ES-

PS18:0/18:1
PS18:1/18:1
PS18:0/18:0
PS18:0/20:4
PS18:0/22:6
PS16:0/18:1

Figure 4.5. The composition of individual molecular species of phosphatidylcholine (PC) in surfactant isolated from warm-active and torpid Gould's wattled bats, *Chalinolobus gouldii.* Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Results are presented as mean ± SE. n number of animals. There were no significant differences between surfactant isolated from warm-active and torpid bats (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

Figure showing Phosphatidylcholine (mol%) versus Molecular species of phosphatidylcholine.

X-axis categories: 14:0/6:0, 16:1/16:0a, 16:0/16:0a, 16:1/16:0, 16:0/16:0, 18:2/16:0, 18:1/16:0, 18:0/16:0, 20:4/16:0, 18:2/18:1, 18:1/18:1, 18:1/18:0, 18:2/20:4, 18:1/20:4, 18:0/20:4

Legend: Warm-active (n = 7), Torpid (n = 7)

Figure 4.6. The composition of individual molecular species of phosphatidylglycerol (PG) in surfactant isolated from warm-active and torpid Gould's wattled bats, *Chalinolobus gouldii.* Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. All molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Results are presented as mean ± SE. n number of animals. * significant decrease in surfactant isolated from torpid bats (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

Legend:
- ■ Warm-active (n = 8)
- ■ Torpid (n = 7)

Y-axis: Phosphatidylglycerol (mol%)

X-axis (Molecular species of phosphatidylglycerol): 16:0/16:1, 16:0/16:0, 16:1/18:2, 16:0/18:2, 16:0/18:1, 16:0/20:4, 18:1/18:2, 18:1/18:1, 18:0/18:1
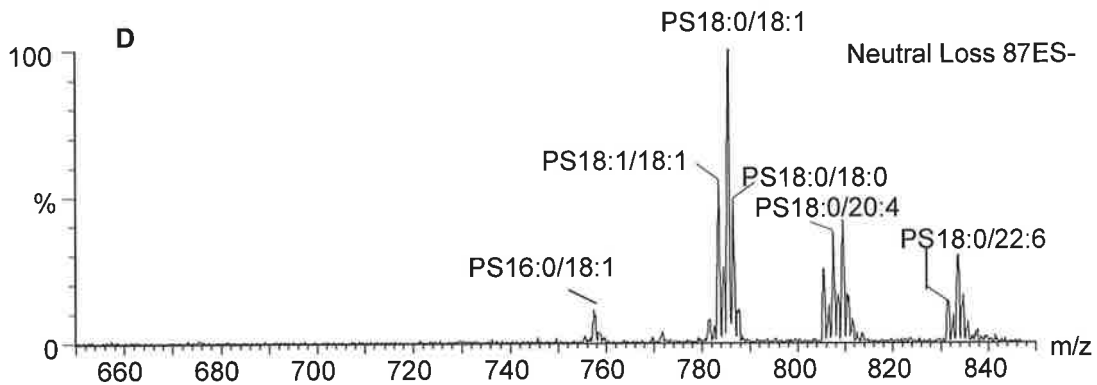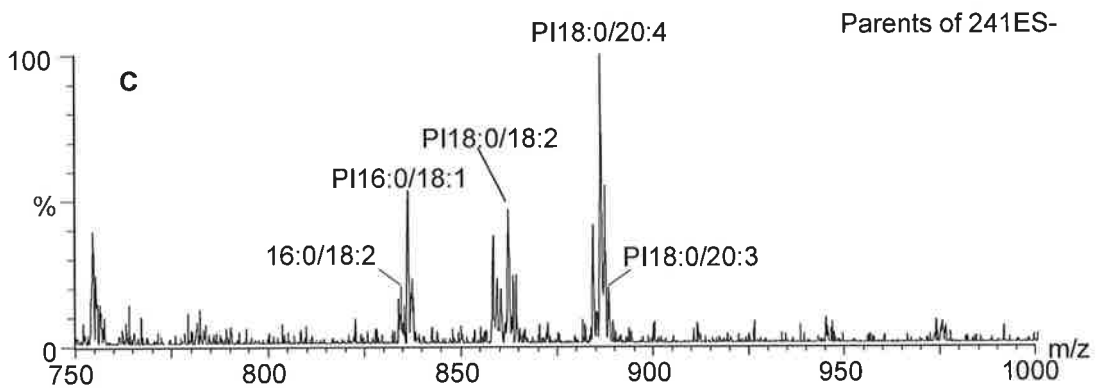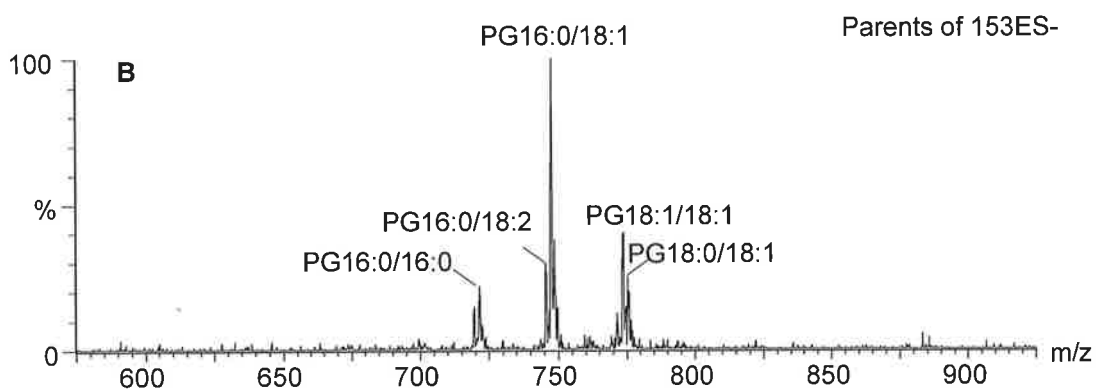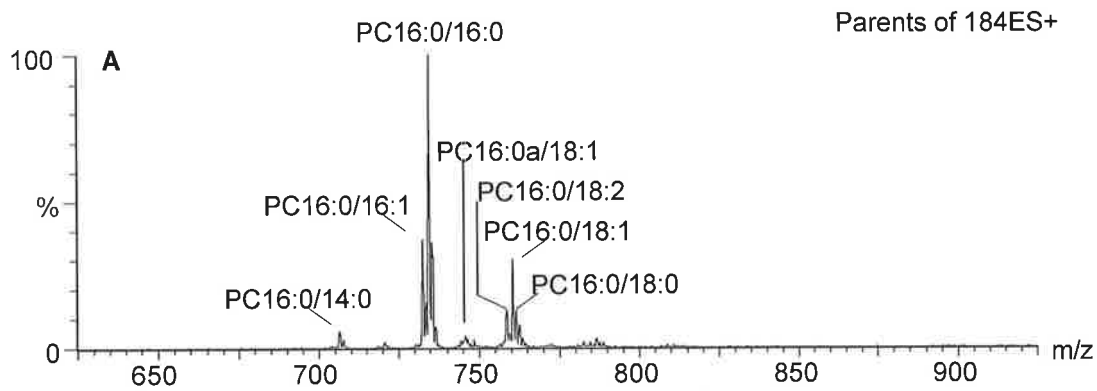
Figure 4.7. The composition of individual molecular species of phosphatidylinositol (PI) in surfactant isolated from warm-active and torpid Gould's wattled bats, *Chalinolobus gouldii.* Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Results are presented as mean ± SE. n number of animals. Significant increase in surfactant isolated from torpid bats (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

Figure showing Phosphatidylinositol (mol%) for Warm-active (n = 4) and Torpid (n = 4) across molecular species of phosphatidylinositol.

Legend:
- Warm-active (n = 4)
- Torpid (n = 4)

Y-axis: Phosphatidylinositol (mol%), 0 to 50

X-axis (Molecular species of phosphatidylinositol): 16:0a/18:1, 16:1/18:2, 16:0/18:2, 16:0/18:1, 16:0/18:0, 18:0a/18:1, 16:1a /20:4, 18:2/18:2, 18:1/18:2, 18:0/18:2, 18:0/18:1, 18:0/18:0, 18:0a/20:4, 16:0/22:6, 18:1/20:4, 18:0/20:4, 18:0/20:3, 18:0/22:6, 18:0/22:5

Figure 4.8. The composition of individual molecular species of phosphatidylserine (PS) in surfactant isolated from warm-active and torpid Gould's wattled bats, *Chalinolobus gouldii*. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Results are presented as mean ± SE. n number of animals. There were no significant differences between surfactant isolated from warm-active and torpid bats (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).
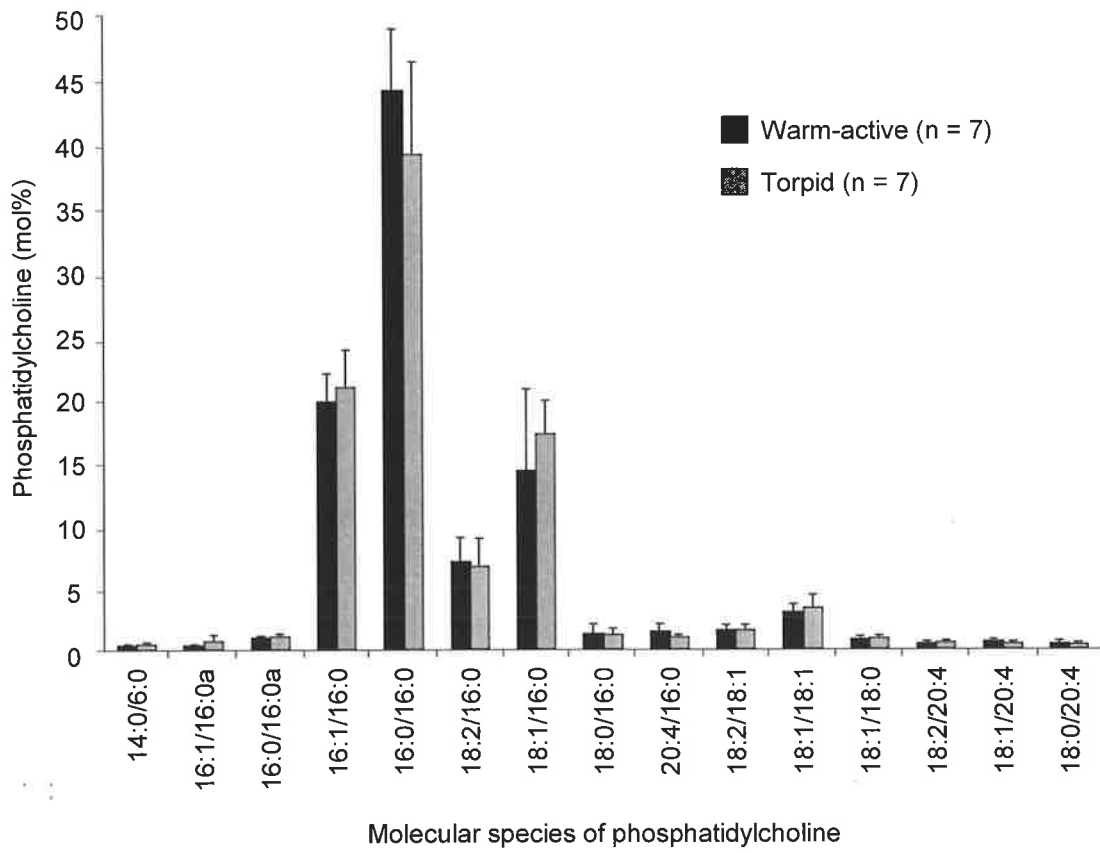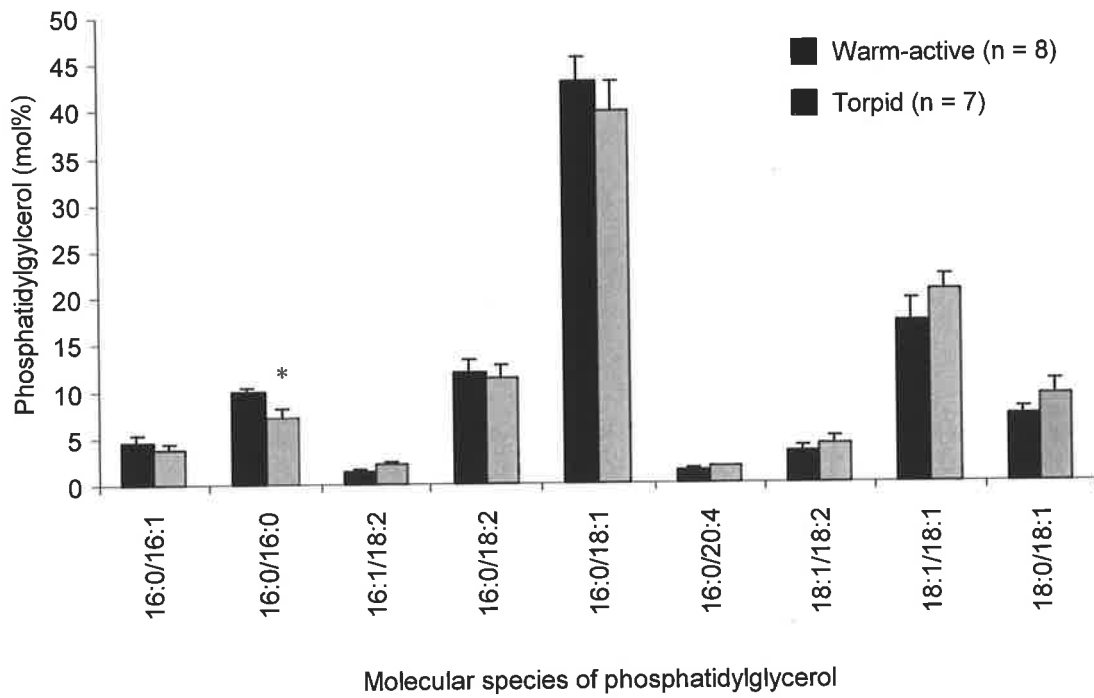
4.2.5. SURFACTANT PROTEIN COMPOSITION

Total protein did not change significantly (t_{stat} = 0.230, df = 9, p = 0.41) between surfactant isolated from warm-active (27.98 ± 4.06 mg gDL^{-1}) and torpid (29.93 ± 7.93 mg gDL^{-1}) bats. In bat surfactant, the ratio of SP-A/PL increased significantly during torpor (t_{stat} = 2.422, df = 4, p = 0.036) (Table 4.2). The ratio of SP-B/PL did not differ between surfactant isolated from warm-active and torpid bats (t_{stat} =0.817, df = 5, p = 0.23). Amounts of SP-C (11-13 µg/mg PL) did not change significantly between surfactant isolated from warm-active and torpid bats, when expressed as a ratio of SP-C/PL (t_{stat} = 1.149, df = 6, p = 0.15) (Table 4.2).

Table 4.2. Surfactant proteins in surfactant isolated from warm-active and torpid Gould's wattled bats, *Chalinolobus gouldii*.

	SP-A	SP-B	SP-C
		ng/mg PL	
Warm-active	728.57 ± 298.5	396.83 ± 102.12	11358 ± 950.2
Torpid	813.4 ± 170.6	553.98 ± 178.82	13945 ± 1972

Surfactant proteins were measured in lavage samples isolated from warm-active and torpid bats using ELISAs described in Section 2.7. Values are mean ± SE, n = 3-4. Each n represents pooled lavage from two individual bats. Statistical significance was determined using Student's t-tests, assuming equal variance, significance at p < 0.05.

4.3. SUMMARY OF RESULTS

- There was no difference in the saturation of PC, PI or PS between surfactant isolated from warm-active and torpid bats.

- There was a significant decrease in the saturation of PG in torpid bats.

- There was no change in the molecular species composition of PC between surfactant isolated from warm-active and torpid bats.

- The molecular species PG16:0/16:0 decreased significantly in surfactant isolated from torpid bats. However, there was no change in any other molecular species of PG between surfactant isolated from warm-active and torpid bats.

- The molecular species PI18:0/18:2 increased significantly in surfactant isolated from torpid bats. However, there was no change in any other molecular species of PI between surfactant isolated from warm-active and torpid bats.

- SP-A increased significantly in surfactant isolated from torpid bats.

- There was no difference in SP-B or SP-C between surfactant isolated from warm-active and torpid bats.

CHAPTER 5

RESULTS FOR

WARM-ACTIVE AND TORPID

FAT-TAILED DUNNARTS

Sminthopsis crassicaudata

5.1. BACKGROUND

Fat-tailed dunnarts are small heterothermic marsupial mammals that enter 'stress torpor' in response to food shortages or cold ambient temperatures. Stress-induced torpor in dunnarts is similar in depth and duration to that observed in animals such as Gould's wattled bats, *Chalinolobus gouldii,* that experience daily torpor. However, the different stimuli leading to stress-induced torpor or daily torpor in these animals may affect the way in which the pulmonary surfactant system responds to, or is affected by, a torpor bout. For example, cortisol can sometimes be elevated in response to external stresses, and is a known stimulus for surfactant synthesis and secretion (Barry, 1983).

During stress-induced torpor in dunnarts, there are increases in total PL, the relative amounts of DSP and CHOL and a decrease in the predominant PL, PC (Langman et al., 1996). These changes correlate with changes in surface activity. Surfactant collected from warm-active and torpid dunnarts appears to function optimally at the body temperature of the animal from which it was isolated (Lopatko et al., 1998; Lopatko et al., 1999).

Here, I determined the molecular composition and film structure of whole lavage and large aggregate (LA) fractions of surfactant isolated from the lungs of warm-active and torpid fat-tailed dunnarts. The large aggregate fraction contains the surface-active components of surfactant. Due to the thermal properties of lipids, I hypothesise, that changes in surfactant PL composition occur during stress-induced torpor in fat-tailed dunnarts. Furthermore, the previously observed changes in surface activity of surfactant isolated from warm-active and torpid dunnarts suggest that there must be changes in the structure of the surface film. Hence, I also hypothesise that changes will occur in the structure of the surface film, that enable surfactant from torpid dunnarts to function better at cold temperatures than surfactant from warm-active dunnarts.

5.2. RESULTS

5.2.1. DESCRIPTIVE DATA

Descriptive data for warm-active and torpid dunnarts is given in Table 5.1. Rectal body temperature was significantly lower in torpid dunnarts than warm-active dunnarts ($t = 2.014$, $df = 45$, $p < 0.000001$). The mean body mass of torpid dunnarts was also lower than warm-active dunnarts ($t = 2.04$, $df = 30$, $p = 0.00001$). However, there were no significant differences between wet ($t = 2.01$, $df = 43$, $p = 0.10$) or dry ($t = 2.01$, $df = 43$, $p = 0.97$) lung mass between warm-active and torpid dunnarts (Table 5.1.).

Table 5.1. Descriptive statistics of warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata.*

Dunnart	N	T_b(°C)	Body mass (g)	WLW (g)	DLW (g)
Warm-active	25	35.54 ± 0.47	17.54 ± 0.58	0.27 ± 0.01	0.036 ± 0.003
Torpid	22	15.4 ± 0.47*	13.59 ± 0.35*	0.25 ± 0.01	0.035 ± 0.003

Values are mean ± SE. N number of animals, T_b body temperature (°C), WLW wet lung weight (grams), DLW dry lung weight (grams). * significant difference between warm-active and torpid dunnarts using a Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$.

5.2.2. BIOCHEMICAL ANALYSIS OF LARGE AGGREGATE FRACTION

The amount of PL in the large aggregate fraction of lavage, as determined using the enzymatic colorimetric assay for PC, did not differ ($t_{stat} = 0.183$, $df = 13$, $p = 0.43$) between large aggregate surfactant fractions isolated from warm-active (11.4 ± 0.96 mg gDL^{-1}; mean ± SE, $n = 9$) and torpid (11.1 ± 1.9 mg gDL^{-1}; mean ± SE, $n = 6$) dunnart lavage (Figure 5.1).

Amounts of total CHOL (mg gDL^{-1}) and free CHOL (mg gDL^{-1}), as measured by the enzymatic assay, did not change significantly between the large aggregate surfactant fractions isolated from warm-active and torpid dunnarts (Total CHOL: t_{stat} = 0.617, df = 13, p = 0.273; Free CHOL: t_{stat} = 0.628, df = 13, p = 0.270) (Figure 5.1). Consequently, the ratios of total CHOL/PL (t_{stat} = 1.285, df = 13, p = 0.11) and free CHOL/PL (t_{stat} = 1.324, df = 13, p = 0.10) were not significantly different in lavage collected from warm-active and torpid dunnarts (Figure 5.2). Cholesteryl esters, as measured by the Wako enzymatic assay, contributed only a small proportion (less than 2%) to the cholesterol component in the large aggregate surfactant fraction and did not change significantly (t_{stat} = 0.189, df = 13, p = 0.43) between large aggregate surfactant fractions isolated from warm-active and torpid dunnarts.

Total protein did not change significantly between large aggregate lavage fractions isolated from torpid dunnarts (30.27 ± 2.53 mg g DL^{-1}; mean ± SE, n = 6) compared to warm-active dunnarts (25.22 ± 3.02 mg gDL^{-1}; mean ± SE, n = 9) (t_{stat} = 1.184, df = 13, p = 0.20). Consequently, the ratio of protein/PL in the large aggregate fraction of lavage fluid did not change significantly (t_{stat} = 1.0996, df =13, p = 0.15) between warm-active (2.38 ± 0.42; mean ± SE, n = 9) and torpid (3.12 ± 0.51; mean ± SE, n = 6) dunnarts.

5.2.3. SATURATION OF PHOSPHOLIPIDS

5.2.3.1. WHOLE LAVAGE

In the whole lavage fractions, the percent saturation of PC (t_{stat} = 0.173, df = 10, p = 0.43) and PI (t_{stat} = 1.268, df = 10, p = 0.12) molecular species, as determined using ESI-MS data, did not change significantly between warm-active and torpid dunnart lavage. However, %DSPG/PG did significantly increase in torpid dunnart lavage (t_{stat} = 2.12, df

=12, p = 0.028) (Figure 5.3). Note: Whole and large aggregate surfactant samples were isolated from different individuals.

5.2.3.2. LARGE AGGREGATE FRACTION

Estimates of percent saturation, as determined by the percent saturation of PC molecular species (ESI-MS) did change significantly between large aggregate fractions of warm-active (47.04 ± 0.5% DSPC/PC; mean ± SE, n = 5) and torpid (43.9 ± 0.17; mean ± SE, n = 5) dunnart lavage (t_{stat} = 5.692, df = 8, p = 0.0002) (Figure 5.3). The percent saturation for PG species in large aggregate surfactant fractions was not significantly different (t_{stat} = 0.392, df = 8, p = 0.35) between warm-active dunnarts (24.03 ± 0.68% DSPG/PG; mean ± SE, n = 5) and torpid dunnarts (23.48 ± 1.2% DSPG/PG; mean ± SE, n = 5) (Figure 5.3).

Figure 5.1. Surfactant phospholipid and cholesterol amounts in the large aggregate fraction of lavage fluid isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata.* All data are presented as mean ± SE and expressed as mg per gram dry lung (mg gDL^{-1}). Solid bars represent surfactant isolated from warm-active dunnarts. Hatched bars represent surfactant isolated from torpid dunnarts. n = 9 for warm-active large aggregate surfactant fractions; n = 6 for torpid large aggregate surfactant fractions. PL phospholipid, CHOL cholesterol. PL measured using an enzymatic, colorimetric assay kit for PC, supplied by Boehringer-Mannheim Inc., Germany (Roche Molecular Biochemicals); CHOL measured using an enzymatic, colorimetric assay kit for CHOL supplied by Wako Chemicals USA Inc. (Richmond, VA). There were no significant differences between lavage fluids isolated from warm-active and torpid dunnarts (using Student's unpaired t-test, assuming equal variance, significance at p < 0.05).
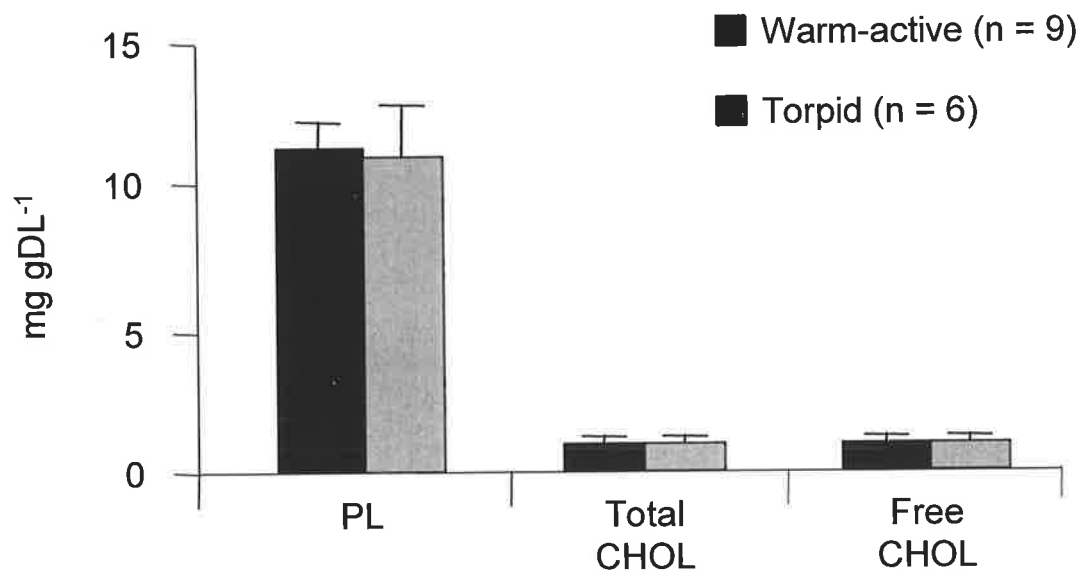
Figure 5.2. Relative proportion of cholesterol (CHOL) to phospholipid (PL) in large aggregate fractions of lavage fluid isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata*. All data are presented as mean ± SE and expressed as ratios. n number of animals, Total CHOL/PL total cholesterol as a fraction of total phospholipid. Free CHOL/PL free cholesterol as a fraction of total phospholipid. CHOL and PL amounts were measured using enzymatic, colorimetric assay kits for CHOL and PL supplied by Wako Chemicals USA Inc (Richmond, VA) and Boehringer-Mannheim Inc., Germany (Roche Molecular Biochemicals), respectively. There were no significant differences between lavage fluids isolated from warm-active and torpid dunnarts (using Student's unpaired t-test, assuming equal variance, significance at p < 0.05).
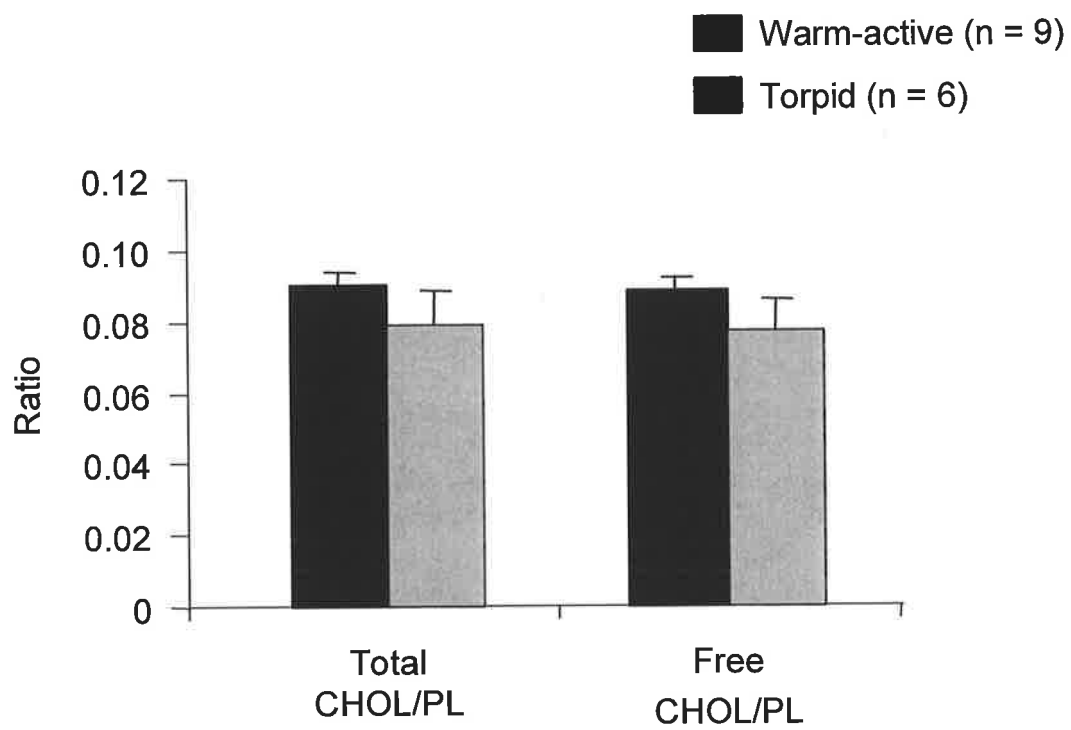
Figure 5.3. Relative proportions of disaturated components in whole and large aggregate fractions of lavage fluid isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata.* All data are presented as mean ± SE and expressed as ratios. n number of animals. DSPC/PC disaturated phosphatidylcholine as a fraction of total phosphatidylcholine, DSPG/PG disaturated phosphatidylglycerol as a fraction of total phosphatidylglycerol, DSPI/PI disaturated phosphatidylinositol as a fraction of total phosphatidylinositol. There were no significant differences between lavage fluid isolated from warm-active and torpid dunnarts (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).
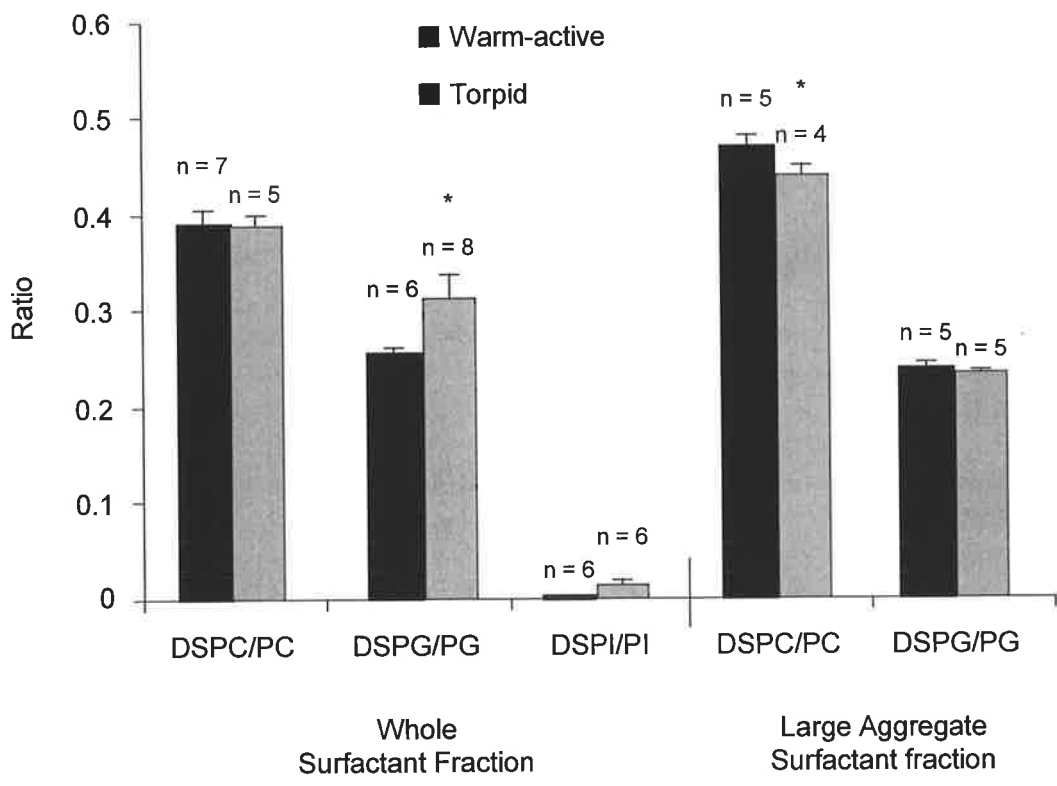
5.2.4. MOLECULAR SPECIES COMPOSITION

5.2.4.1. WHOLE LAVAGE

Examples of ESI-MS spectra in positive and negative ionisation modes for dunnart whole lavage surfactant are given in Figure 5.4. Typical spectra obtained for diagnostic scans and examples of fatty acid fragmentation of dunnart lavage are shown in Figures 5.5 and 5.6. The molecular species of phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) in warm-active and torpid dunnart surfactant are given in Figures 5.7, 5.8, 5.9 and 5.10, respectively. Unlike all other mammalian surfactants studied, DPPC (PC16:0/16:0) is not the major component of dunnart surfactant. In both warm-active and torpid dunnarts, the composition of surfactant PC was dominated by the unsaturated species, PC16:0/16:1, which contributes 30 mol% to the PC component. DPPC is the second major component, comprising 16-18 mol%. Surfactant isolated from dunnarts also contains PC16:0/14:0 (12-15 mol%) and the alkyl-acyl containing forms of PC16:0/16:1 (10 mol%) and PC16:0/16:0 (10 mol%). The remaining PC component was made up of PC14:0/14:0, PC116:1/14:0, PC16:1/16:1, PC16:1/18:2, 16:0/18:2, 16:0/18:1 and acyl-alkyl containing forms of PC14:0/16:0, 16:0/18:1 and 16:0/20:4. There were no significant differences in the molecular species of PC present between surfactant isolated from warm-active and torpid dunnarts.

Lysophosphatidylcholine (LPC) comprised $0.018 \pm 0.005\%$ (mean $\pm$ SE, n = 5) of total PC content in surfactant isolated from warm-active dunnarts and $0.018 \pm 0.007\%$ (mean $\pm$ SE, n = 4) of total PC in surfactant isolated from torpid dunnarts. There was no significant difference between the ratios of LPC/PC between whole lavage fractions isolated from warm-active and torpid dunnarts ($t_{stat} = 0.058$, df = 7, p = 0.48). The fatty acid composition of LPC is given in Figure 5.8. The LPC component was dominated by 16:0 (50 mol%) and 16:1 (30 mol%) fatty acids. Other fatty acids present were 14:0,

15:0, 18:0, 18:1, 18:2 and 20:4 (less than 10 mol% each). There were no significant differences in the headgroup composition of LysoPC between surfactant isolated from warm-active and torpid dunnarts.

In contrast to PC, there was a significant difference in some of the molecular species of PG present between whole lavage fractions of surfactant isolated from warm-active and torpid dunnarts (Figure 5.9). The proportions of PG16:0/14:0 (t_{stat} = 4.464, df = 12, p = 0.0004) and PG16:0/18:2 (t_{stat} = 3.117, df = 12, p = 0.004) increased significantly in the whole lavage fraction of surfactant isolated from torpid dunnarts. The proportion of PG16:0/18:1 decreased significantly (t_{stat} = 3.307, df = 12, p = 0.003) in torpid dunnart surfactant.

In both warm-active and torpid dunnarts, PG composition was dominated by the unsaturated species PG16:0/18:1 and the disaturated species PG16:0/16:0, which contributed 20-25 mol% each to the PG component. PG16:0/16:1 comprised 18 mol% and PG16:0/18:2 comprised 12-15 mol%. The disaturated species, PG14:0/16:0 comprised 6-10% of surfactant PG. The remaining PG component was made up of PG18:0/18:1, PG18:1/18:2, PG16:0/22.6, PG16:0/22.4, PG18:1/20:4 and PG18:0/20:4 (less than 5 mol% each).

In both warm-active and torpid dunnarts, the composition of surfactant PI was dominated by the unsaturated species, PI18:0/20:4 (25-30 mol%) and PI16:0/20:4 (20 mol%) (Figure 5.10). The next major components of PI in dunnart surfactant include PI18:2/20:4 (10 mol%), PI18:1/20:4 (5-10 mol%), PI16:1/18:1 (8-10 mol%) and PI16:0/18:1 (8-10 mol%). As for PC, there were no significant differences in the molecular species of PI present in surfactant isolated from warm-active and torpid dunnarts. PI16:0/16:0 was present only in very low quantities (less than 1 mol%).

Figure 5.4. Example of spectra obtained for whole lavage isolated from **A** warm-active and **B** torpid fat-tailed dunnarts, *Sminthopsis crassicaudata,* and analysed by electrospray ionisation mass spectrometry, in positive ionisation mode. Example of spectra obtained for lavage isolated from **C** warm-active and **D** torpid fat-tailed dunnarts, *S. crassicaudata* and analysed by electrospray ionisation mass spectrometry, in negative ionisation mode. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Mass (m)/charge (z) ratio is equivalent to molecular mass.
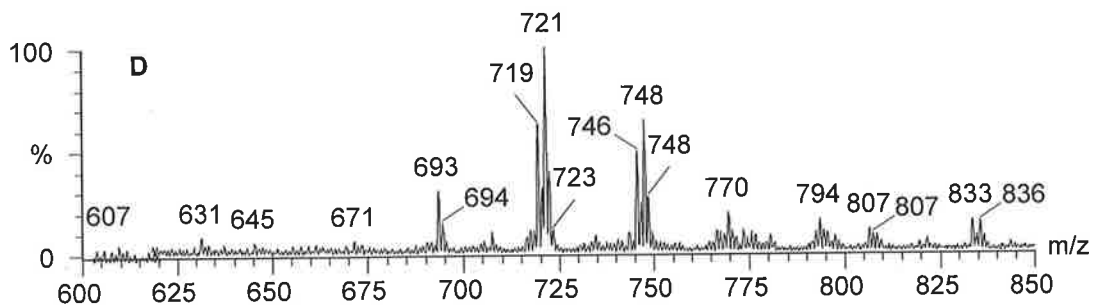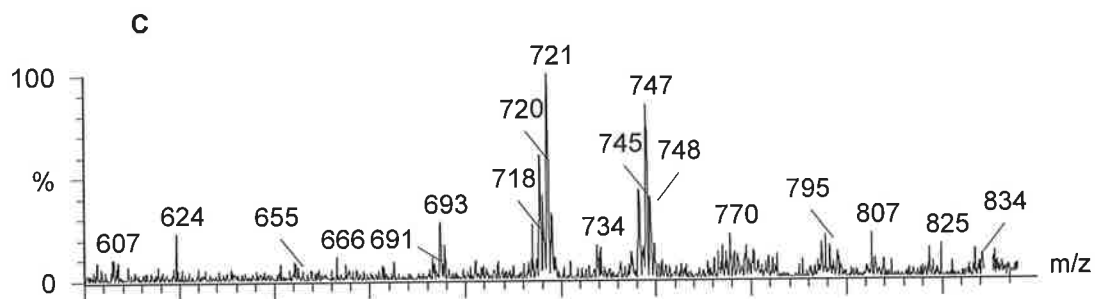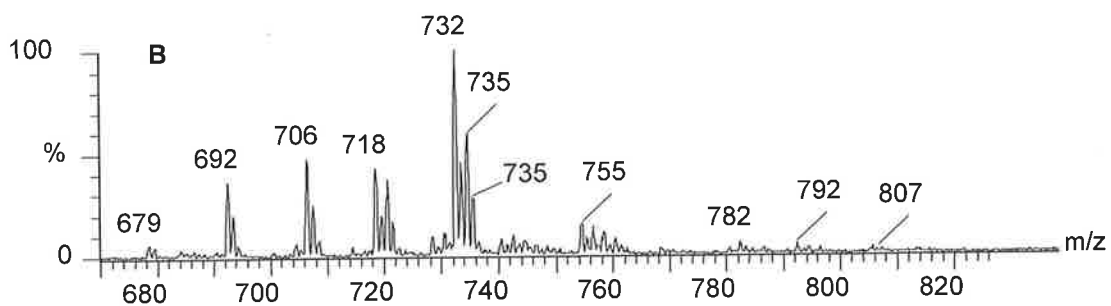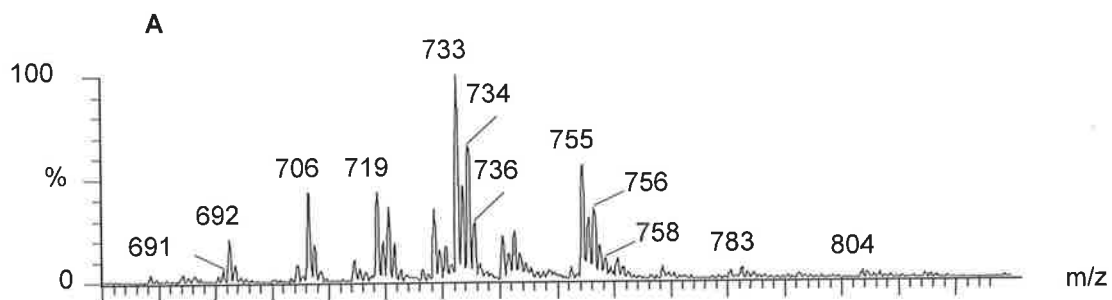
Figure 5.5. This figure shows typical examples of spectra obtained from diagnostic scans of a whole lavage sample isolated from fat-tailed dunnarts, *Sminthopsis crassicaudata*, using electrospray ionisation mass spectrometry. **A** phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) (parents of 184^{+}); **B** phosphatidylglycerol (PG) (parents of 153^{-}) **C** phosphatidylinositol (PI) (parents of 241^{-}). Mass (m)/charge (z) ratio is equivalent to molecular mass. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond.

A

PC16:0/16:1

PC16:1a:16:0

PC16:0/14:0

PC16:0/16:0

PC16:0a:14:0

PC16:0:18:2

PC16:0/18:1

B

PG16:0/16:1

PG16:0/16:0

PG16:0/18:1

PG14:0/16:0

PG16:0/22:6

PG16:0/20:4

PG14:0/14:0

C

PI16:0/20:4

PI18:0/20:4

PI16:0/18:1

PI16:18:2

PI16:0/16:1
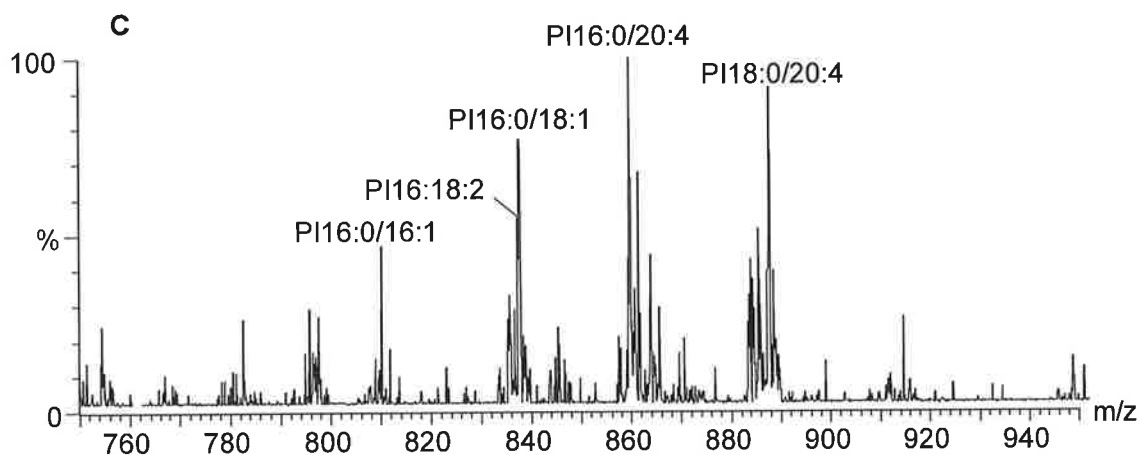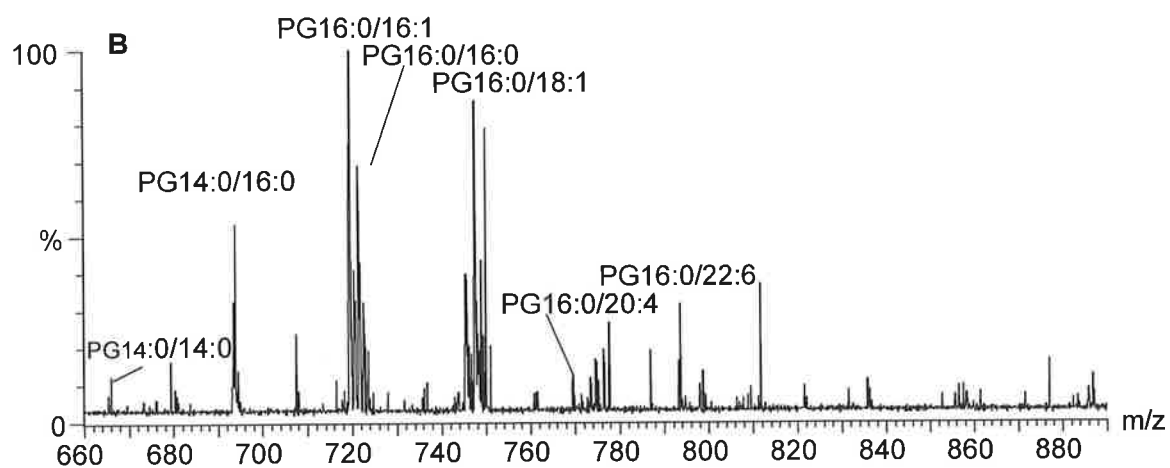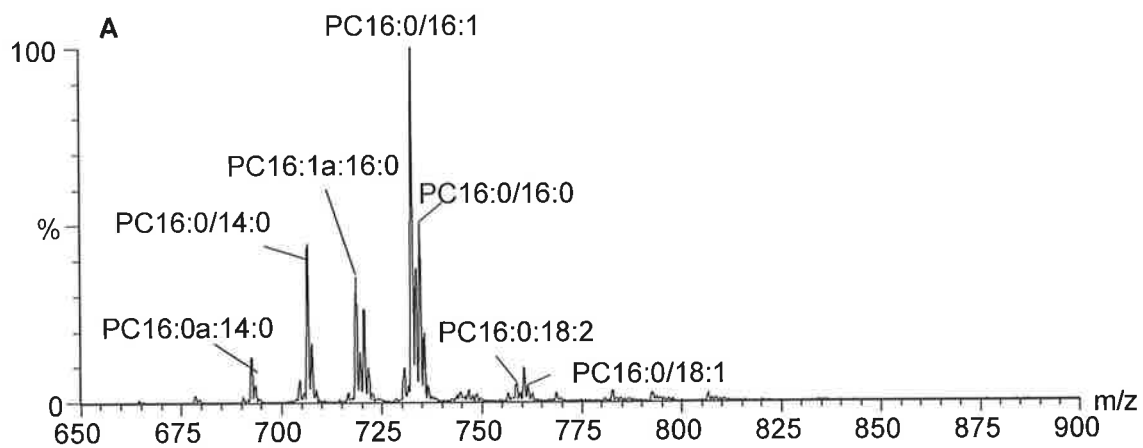
Figure 5.6. Tandem mass spectrometry, of the various mass ions, was used to confirm the identity of molecular species in whole lavage samples isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata*. This figure shows examples of the fatty acid fragmentation pattern observed for various ions. Mass (m)/charge (z) ratio is equivalent to molecular mass. **A** The identity of m/z 734 ion under positive ionisation was confirmed as a phosphatidylcholine species because fragmentation generated a phosphocholine headgroup at m/z 184. **B** The identity of m/z 745 ion under negative ionisation was confirmed as PG16:0/18:2 because fragmentation generated a 16:0 fatty acid anion (m/z 255) and an 18:2 fatty acid anion (m/z 279). **C** The identity of m/z 747 ion under negative ionisation was confirmed as PG16:0/18:1 because fragmentation generated a 16:0 fatty acid anion (m/z 255) and an 18:1 fatty acid anion (m/z 281). **D** The identity of m/z 721 ion under negative ionisation was confirmed as PG16:0/16:0 because fragmentation generated a 16:0 fatty acid anion (m/z 255). **E** The identity of m/z 857 ion under negative ionisation was confirmed as PI16:0/20:4 because fragmentation generated a 16:0 fatty acid anion m/z 255 and an 20:4 fatty acid anion at m/z 303.

Daughters of 733ES+

Daughters of 746ES-

Daughters of 748ES-

Daughters of 821ES-

Daughters of 857ES-
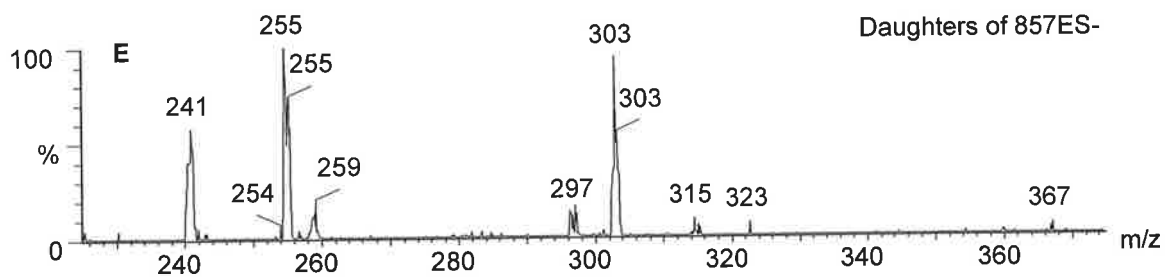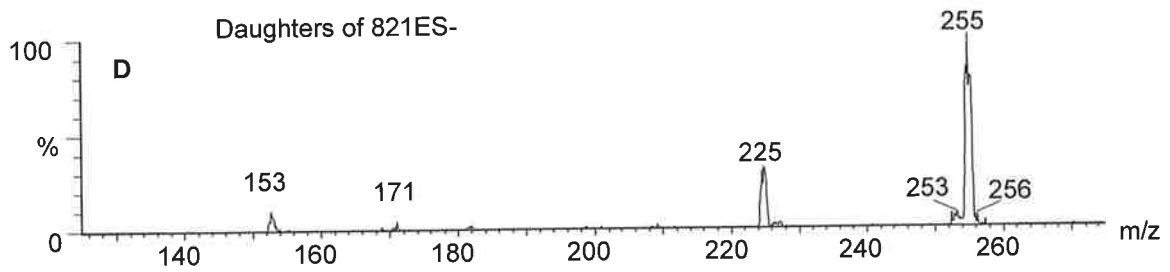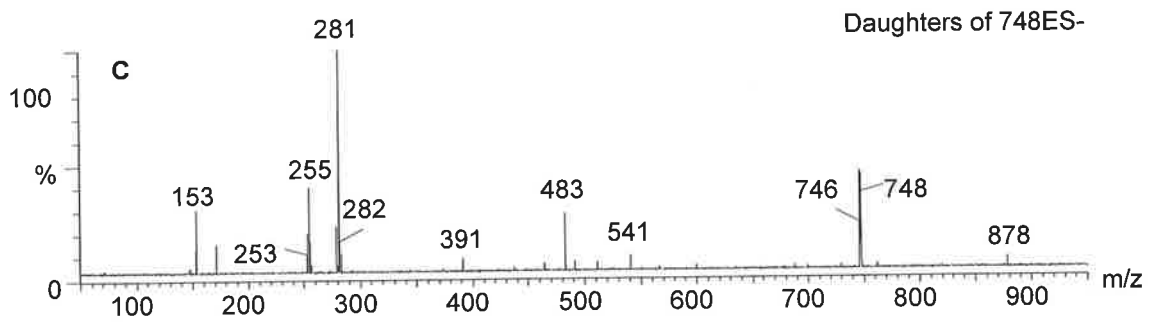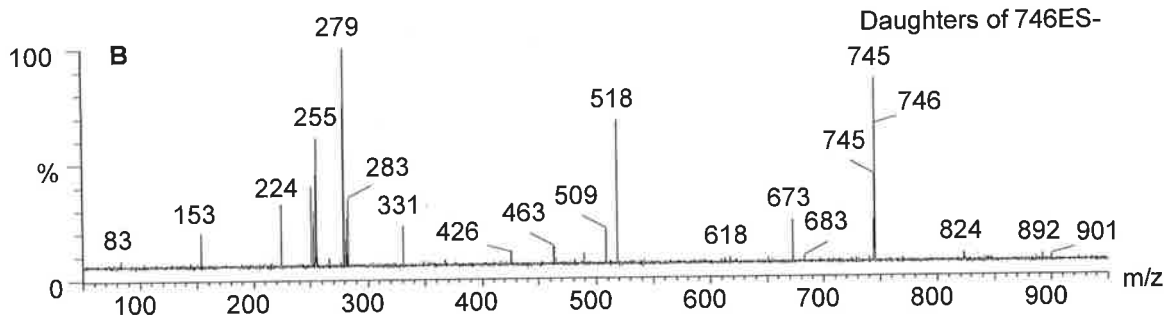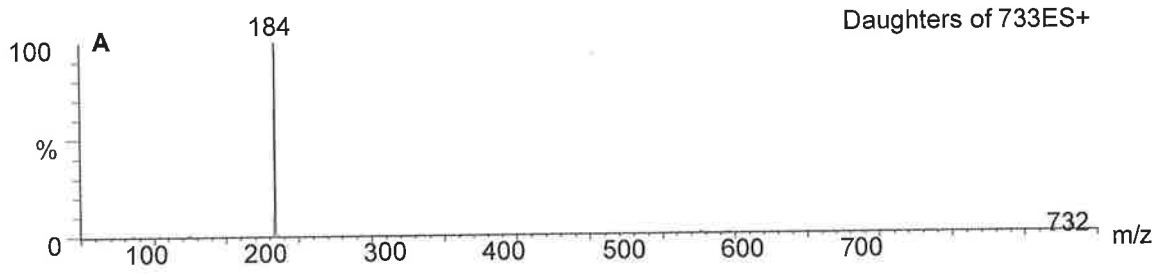
Figure 5.7. The composition of individual molecular species of phosphatidylcholine (PC) in whole lavage isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata*. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Results are presented as mean ± SE. n number of animals. There were no significant differences between lavage isolated from warm-active and torpid dunnarts (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

Molecular species of phosphatidylcholine in whole dunnart lavage

Figure 5.8. The molecular composition of lysophosphatidylcholine (PC) in whole lavage isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata.* Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Results are presented as mean ± SE. n number of animals. There were no significant differences between lavage isolated from warm-active and torpid dunnarts (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

Molecular species of lysophosphatidylcholine in whole dunnart lavage

Figure 5.9. The composition of individual molecular species of phosphatidylglycerol (PG) in whole lavage isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata.* Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. All molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Results are presented as mean $\pm$ SE. n number of animals. * significant increase in lavage fluid isolated from torpid dunnarts (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

Molecular species of phosphatidylglycerol in whole dunnart lavage

Figure 5.10. The composition of individual molecular species of phosphatidylinositol (PI) in whole lavage isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata.* Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. All molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Results are presented as mean ± SE. n number of animals. There were no significant differences between lavage fluid isolated from warm-active and torpid ground dunnarts (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

Molecular species of phosphatidylinositol in whole dunnart lavage

5.2.4.2. LARGE AGGREGATE FRACTION

The molecular species of phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) in large aggregate fractions of warm-active and torpid dunnart lavage are given in Figures 5.11, 5.12, and 5.13, respectively. As for the whole lavage fractions, DPPC (PC16:0/16:0) was not the major component of dunnart large aggregate surfactant fractions. In both warm-active and torpid dunnarts, the composition of surfactant PC was dominated by the monounsaturated species, PC16:0/16:1, which contributes 30 mol% to the PC component. DPPC is the second major component, comprising 20 mol%. The large aggregate surfactant fraction isolated from dunnarts also contained PC16:0/14:0 (15 mol%) and the alkyl-acyl containing forms of PC16:0/16:1 (10 mol%) and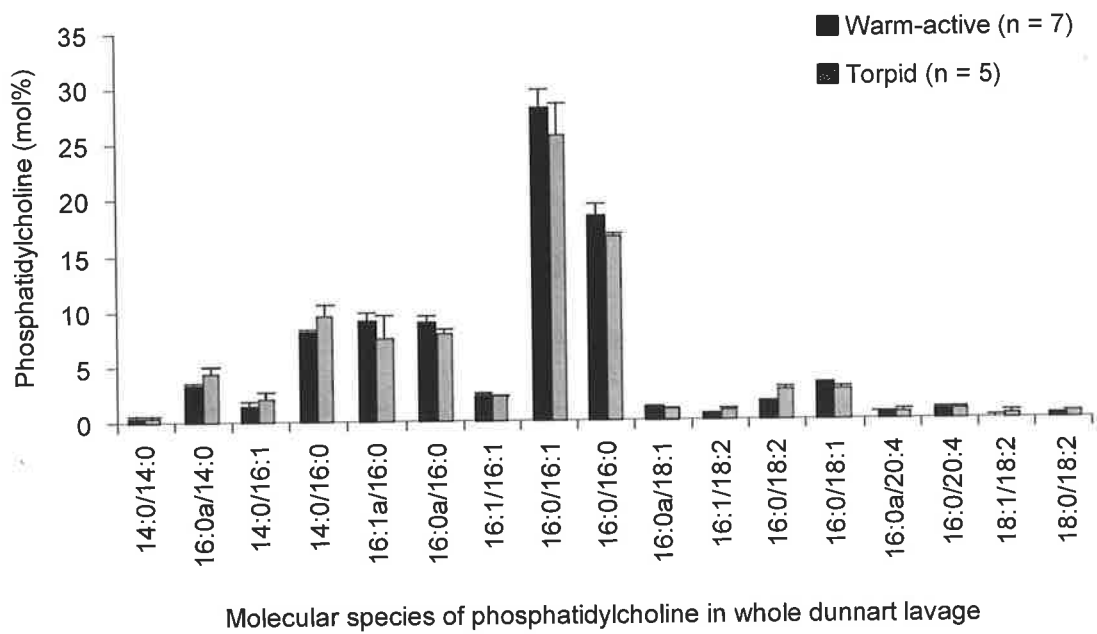 PC16:0/16:0 (8 mol%). The molecular species, PC16:0/18:2, PC16:0/18:1, PC16:0/20:4, PC16:0/22:6 and the alkyl-acyl containing forms of PC16:0/14:0, PC 16:0/20:4 and PC16:0/22:6 are also present in the large aggregate fraction of dunnart surfactant (contributing less than 5 mol% each). There were no significant differences in the molecular species of PC present between the large aggregate surfactant fractions isolated from warm-active and torpid dunnarts.

As for PC, there were no significant differences in the molecular species of PG present in the large-aggregate fraction of surfactant isolated from warm-active and torpid dunnarts. In the large aggregate surfactant fractions isolated from both warm-active and torpid dunnarts, PG composition was dominated by the monounsaturated species PG16:0/18:1, which comprised 23 mol% of surfactant PG. The large aggregate fraction of dunnart surfactant was also enriched in PG16:0/16:1, PG16:0/18:2 and the disaturated species PG16:0/16:0, which contributed 18-20 mol% each to the PG component. The disaturated species, PG14:0/16:0 comprised 6-8 mol% of surfactant PG. The molecular species PG18:0/18:1, PG18:1/18:2, PG18:0/18:2 and PG16:0/20:4 contributed less than 5 mol% each to surfactant PG.

In the large aggregate fraction of surfactant isolated from both warm-active and torpid dunnarts, the composition of surfactant PI was dominated by the unsaturated species, PI18:0/20:4 (20 mol%), PI16:22:6 (20 mol%) and PI 16:0/18:2 (22 mol%). The next major components of PI in dunnart large aggregate surfactant fractions include PI18:1/20:4 (15-18 mol%), PI16:0/20:4 (10-12 mol%) and PI16:0/16:1 (10-11 mol%). The proportion of PI16:0/20:4 decreased significantly (t_{stat} = 4.692.117, df = 8, p = 0.0008) in surfactant isolated from torpid dunnarts.

Figure 5.11. The composition of individual molecular species of phosphatidylcholine (PC) in large aggregate fractions of lavage isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata.* Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Results are presented as mean ± SE. n number of animals. There were no significant differences between lavage isolated from warm-active and torpid dunnarts (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).
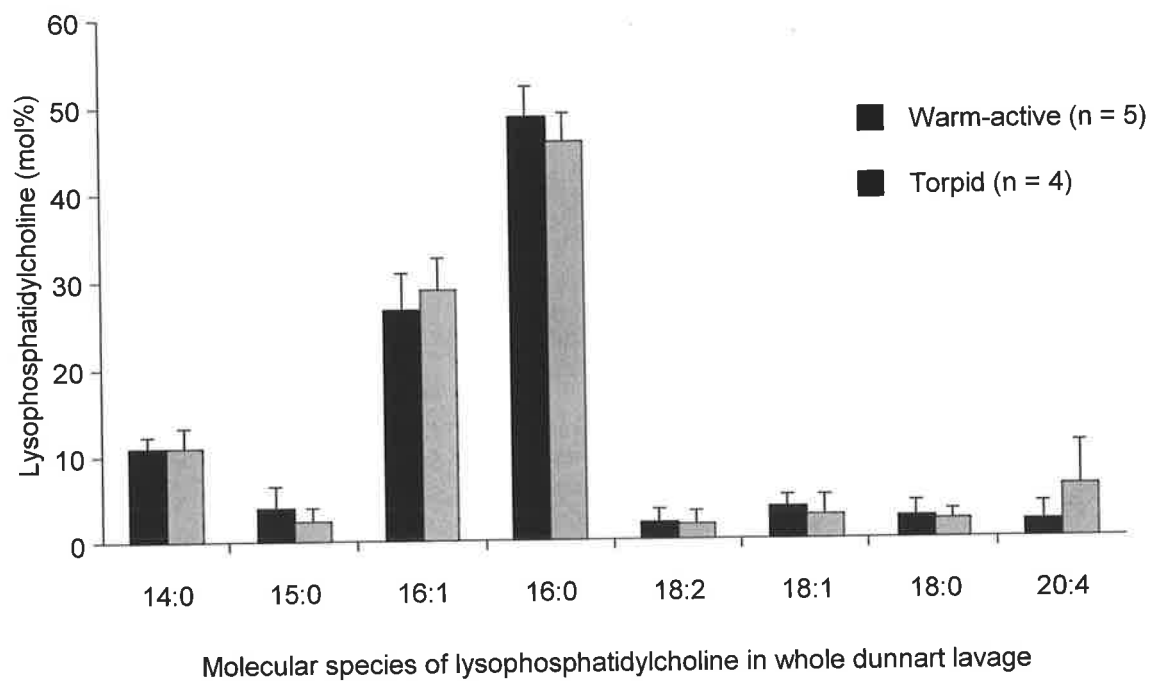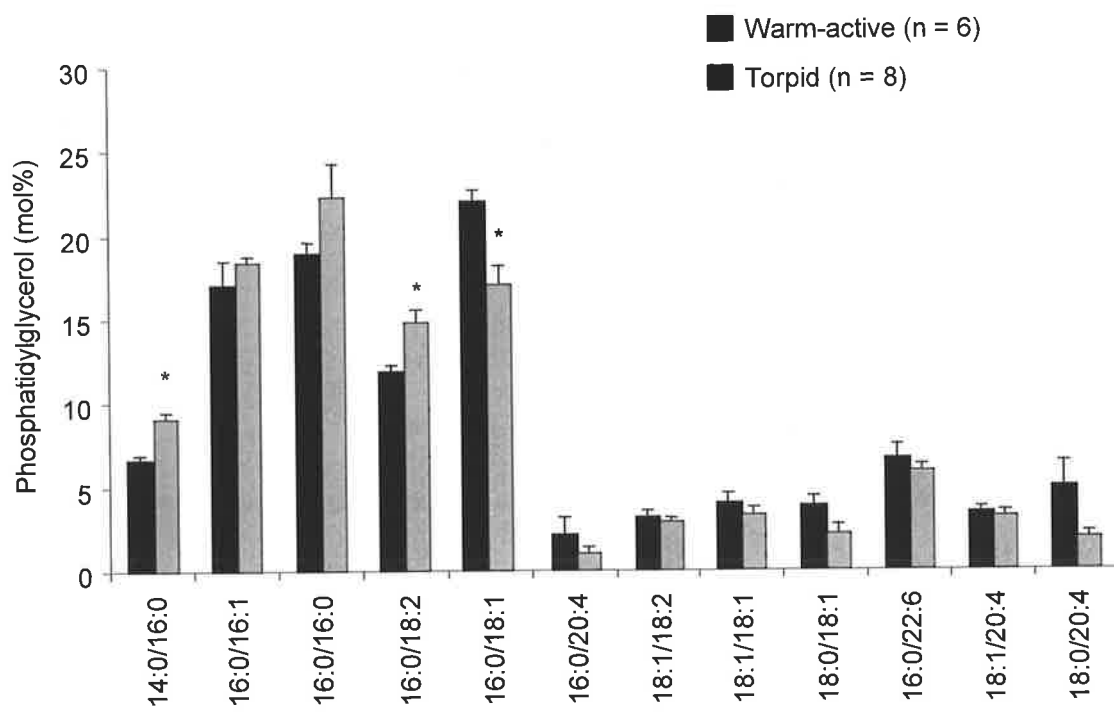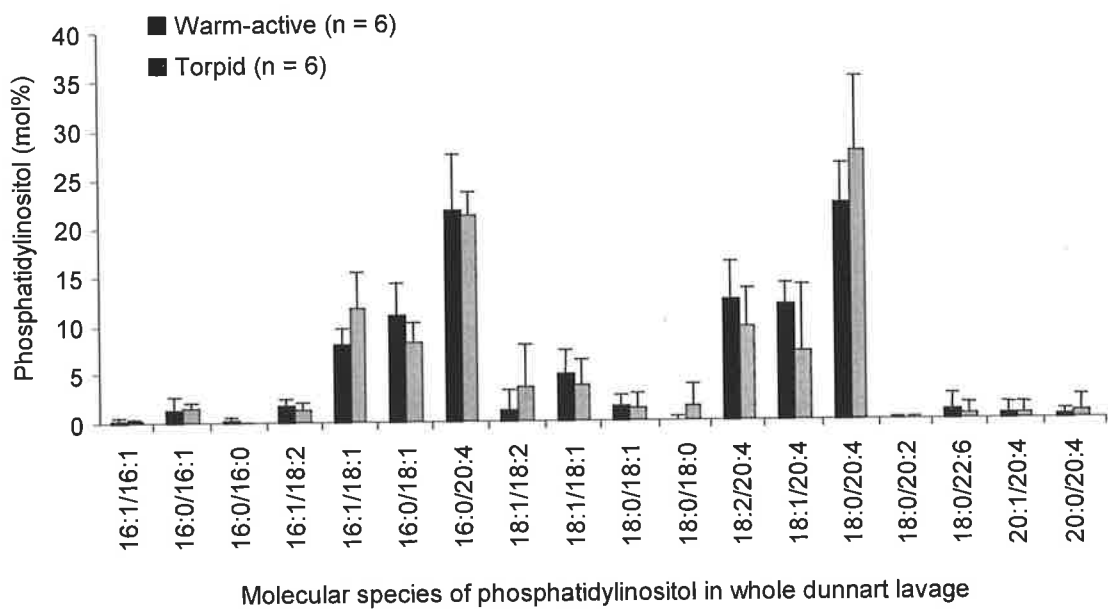
Molecular species of phosphatidylcholine in the large aggregate fraction
of dunnart lavage

Figure 5.12. The composition of individual molecular species of phosphatidylglycerol (PG) in large aggregate fractions of lavage isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata.* Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. All molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Results are presented as mean ± SE. n number of animals. There were no significant differences between lavage fluid isolated from warm-active and torpid dunnarts (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

Molecular species of phosphatidylglycerol in the large aggregate fraction
of dunnart lavage

Figure 5.13. The composition of individual molecular species of phosphatidylinositol (PI) in large aggregate fractions of lavage isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata*. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. All molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Results are presented as mean ± SE. n number of animals. * significant decrease in lavage fluid isolated from torpid dunnarts (using Student's unpaired t-test, assuming equal variance, significance at $p < 0.05$).

Molecular species of phosphatidylinositol in the large aggregate fraction
of dunnart lavage

5.2.5. PROTEIN COMPOSITION IN LARGE AGGREGATE FRACTION

In the large aggregate surfactant fraction, the ratio of SP-A/PL (ng/mg PL) was significantly lower in surfactant isolated from torpid dunnarts compared to warm-active dunnarts (t_{stat} = 2.166, df = 13, p = 0.025). The ratio of SP-B/PL (ng/mg PL) did not differ between the large aggregate surfactant fraction isolated from warm-active and torpid dunnarts (t_{stat} = 0.5296, df = 13, p = 0.30). SP-C levels, relative to PL, were significantly higher in the large aggregate surfactant fraction isolated from torpid dunnarts than warm-active dunnarts (t_{stat} = 2.353, df = 13, p = 0.018) (Table 5.2).

In the small aggregate fractions, SP-C, relative to PL, increased significantly in torpid dunnarts (t_{stat} = 2.99, df = 12, p = 0.006). However, there were no significant differences in the proportion of SP-A (t_{stat} = 0.593, df = 14, p = 0.281) or SP-B (t_{stat} = 1.46, df = 13, p = 0.084) relative to PL, between the small aggregate fractions isolated from warm-active and torpid dunnarts (Table 5.2).

Table 5.2. Surfactant proteins in large and small aggregate surfactant fractions isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata*.

	SP-A	SP-B	SP-C
		ng/mg PL	
Warm-active LA	113.61 ± 23.66*	488.17 ± 64.80	607.3 ± 89^
Torpid LA	56.19 ± 8.17*	444.19 ± 48.67	1009.3 ± 152^
Warm-active SA	14.68 ± 4.13	11.24 ± 4.09	356.2 ± 46.8^
Torpid SA	16.45 ± 2.18	17.71 ± 2.10	658.2 ± 63.22^

Values are mean ± SE. n = 7-8. * significant decrease in torpid dunnarts, ^ significant increase in torpid dunnarts (using Student's unpaired t-test, assuming equal variance, significance at p < 0.05).

5.2.6.   EPIFLUORESCENCE   MICROSCOPY   OF   LARGE   AGGREGATE SURFACTANT FILMS

Examples of the epifluorescence images obtained for solvent spread surfactant films made of large aggregate surfactant fractions isolated from warm-active and torpid dunnarts are shown in Figures 5.14 and 5.15, respectively. Up to a surface pressure of 15 mN m^{-1}, a heterogeneous film morphology was observed for surface films made of surfactant isolated from warm-active dunnarts. At surface pressures of 5, 10 and 15 mN m^{-1}, some black probe-excluded regions were observed in warm-active surfactant films. However, at surface pressures greater than ~15 mN m^{-1}, these probe-excluded domains almost disappeared and a host of 1-2 µm size black, probe-excluded domains (almost beyond the resolution of optical imaging) are see (Figures 5.14). On the other hand, the domain characteristics remained relatively consistent with increasing surface pressure for surface films made of surfactant isolated from torpid dunnarts (Figure 5.15). The relative area of the surface covered by the black, probe-excluded regions and the average diameter of such domains were calculated and the results are summarised in Figure 5.16.

There was a significant difference in the relative area covered by the probe between surfactant films isolated from warm-active and torpid dunnarts at surface pressures of 10 mN m^{-1} (q = 4.714, p < 0.05) and 15 mN m^{-1} (q = 5.796, p < 0.01) (Figure 5.16A). For surfactant isolated from warm-active dunnarts, the relative area covered by the probe shows a declining trend after a surface pressure of 15 mN m^{-1}. However, for films of surfactant isolated from torpid dunnarts, the relative area of the probe-covered surface showed an increasing trend. There was a significant increase, relative to 5 mN m^{-1}, in the area covered by the probe at 20 mN m^{-1} (q = 5.644, p < 0.001) and 30 mN m^{-1} (q = 5.644, p < 0.001) surface pressures in films made of surfactant isolated from torpid dunnarts (Figure 5.16B).

There was a significant difference in the diameter of the probe-excluded regions between surface films made of warm-active and torpid dunnart surfactant at a surface pressure of 10 nM^{-1} (q = 17.36, p = < 0.01). The average diameter of the probe-excluded regions remained fairly constant (~ 8 μm) as surface pressure increased, from 5 to 15 in warm-active dunnart films and from 5 to 30 mNm^{-1} in torpid dunnart films (Figure 5.16).

Figure 5.14. Epifluorescence images of solvent spread films of large aggregate surfactant isolated from warm-active fat-tailed dunnarts, *Sminthopsis crassicaudata* at surface pressures of 5, 10, 15, 20, 25 and 30 nM m^{-1} at 23°C. Surface pressure given in top, left corner of image. Scale bar = 25 μm. Darker (black) regions represent probe-excluded domains in the surface film. At higher surface pressures (>15 nM m^{-1}), the probe-excluded domains almost disappeared and a host of 1-2 μm size black, probe-excluded domains (almost beyond the resolution of optical imaging) were observed.

LC domain

5 10 15 20 25 30

25 microns
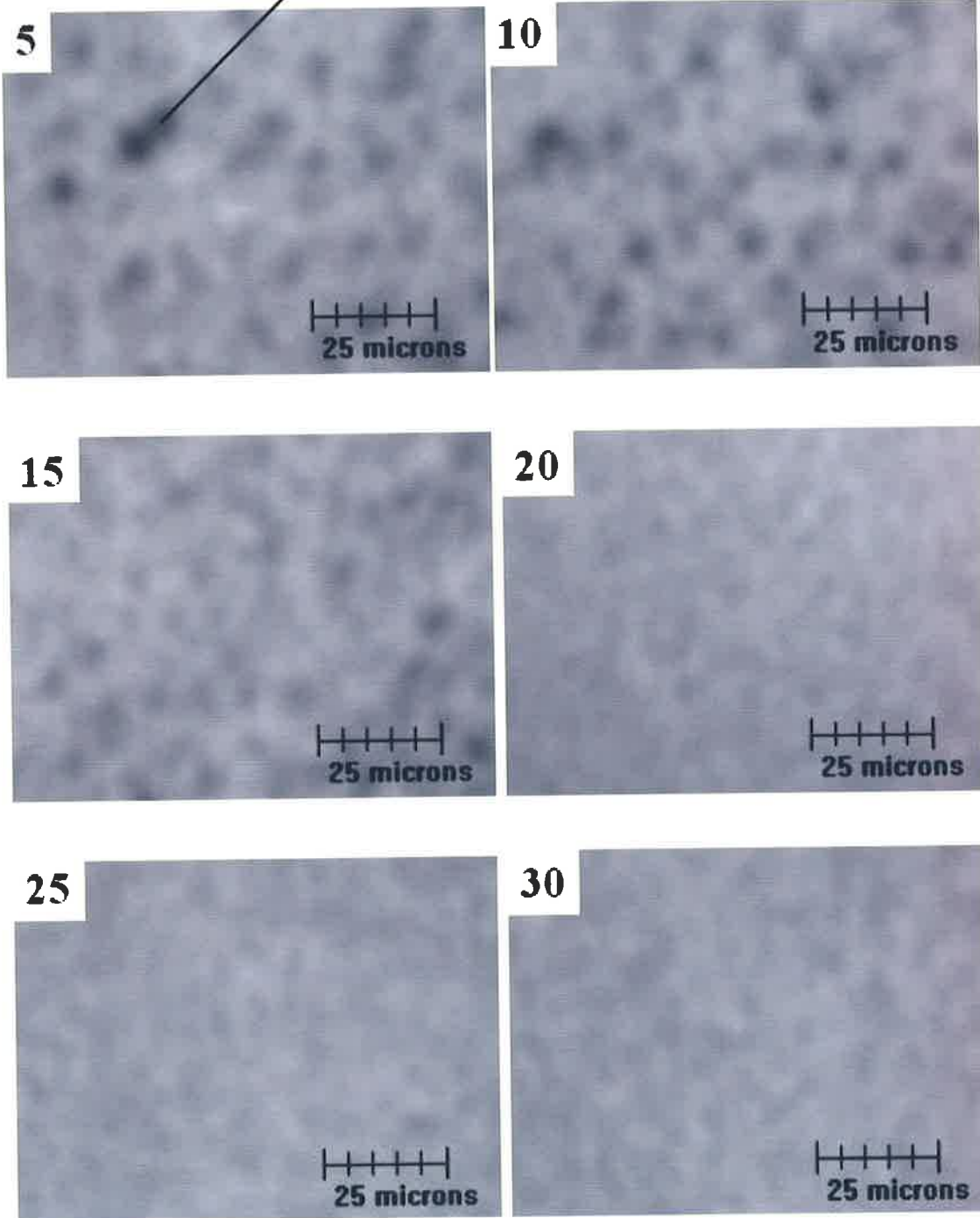
Figure 5.15. Epifluorescence images of solvent spread films of large aggregate surfactant isolated from torpid fat-tailed dunnarts, *Sminthopsis crassicaudata* at surface pressures of 5, 10, 15, 20, 25 and 30 nM m^{-1}at 23°C. Surface pressure (nM m^{-1}) of film is indicated in the top left corner of each image. Scale bar = 25 μm. Darker (black) regions represent probe-excluded, liquid-condensed (LC) domains in the surface film. The characteristics of the domains remain consistent with increases in surface pressure.

LC domain

5

10

15

20

25

30

25 microns

LC domain
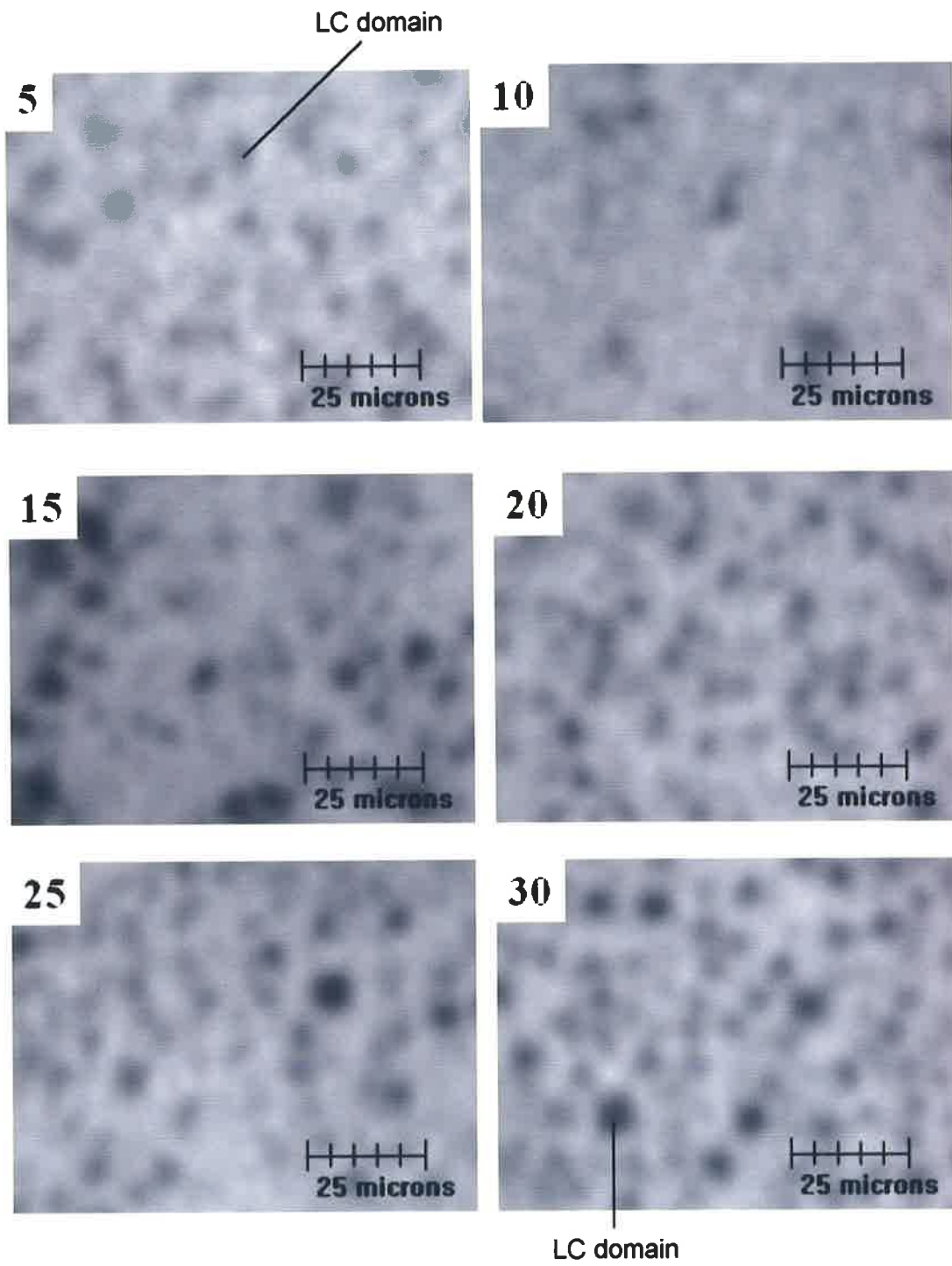
Figure 5.16. Fluorescence image analysis of solvent spread films of large aggregate surfactant isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata* at different surface pressures at 23°C. Subphase: 0.15M NaCl, 1.5 mM CaCl$_2$, 1.0 mM Tris-HCl buffer at pH 7.0. Film compression rate 0.05 nm^2 mol^{-1} min^{-1} with an initial area of 1.2 nm^2 mol^{-1}. **A** percent of the area covered by probe-excluded regions; **B** diameter of the probe-excluded regions. Data are mean ± SE for n number of animals (multiple measurements per image). * significant difference between surfactant isolated from warm-active and torpid dunnarts, at a particular surface pressure, as measured using one-way ANOVA followed by Tukey-Kramer post-hoc test (significance at p < 0.05). ^ significant difference between values obtained at each particular surface pressure, compared to value obtained at 5 nM m^{-1} surface pressure for each group using one-way ANOVA followed by Tukey-Kramer post-hoc test, significance at p < 0.05. **A** Solid line represents $y = 0.22x + 15.4$, $r^2 = 0.2866$. Dashed line represents $y = -0.096x^2 + 1.62x + 6.2$, $r^2 = 1.0$. **B** Solid line represents $y = -0.0067x^2 + 0.20x + 8.18$, $r^2 = 0.22$. Dashed line represents $y = 0.06x + 7.33$, $r^2 = 0.964$.
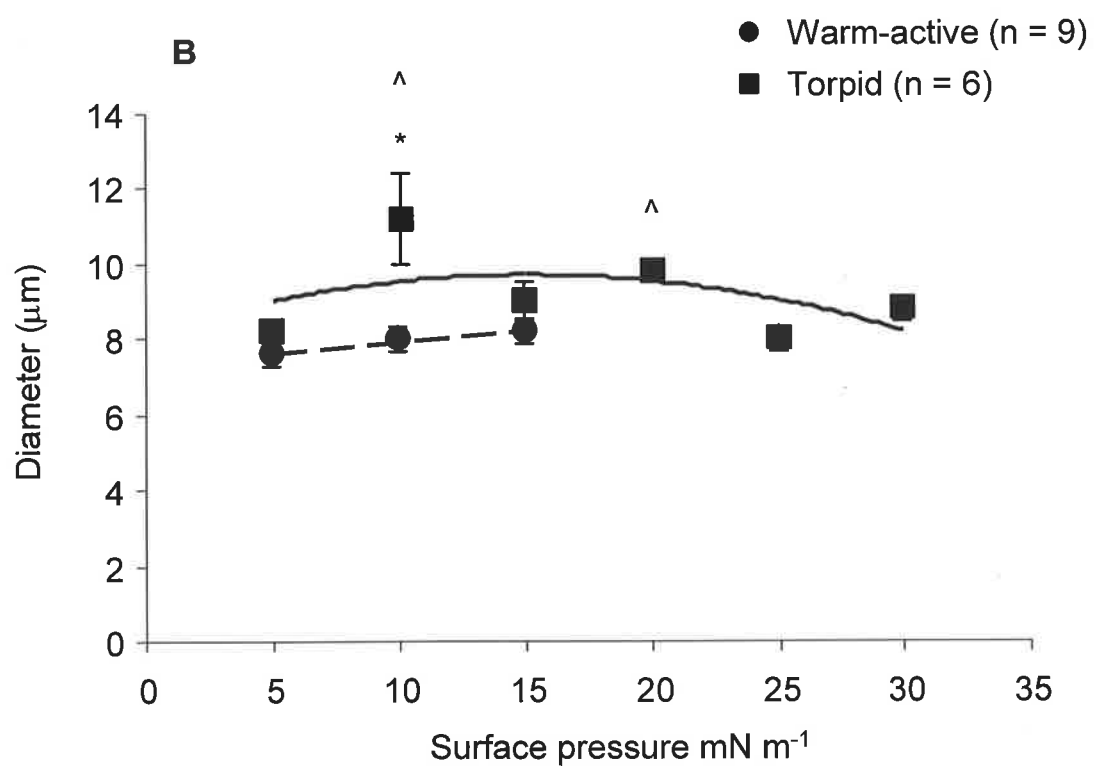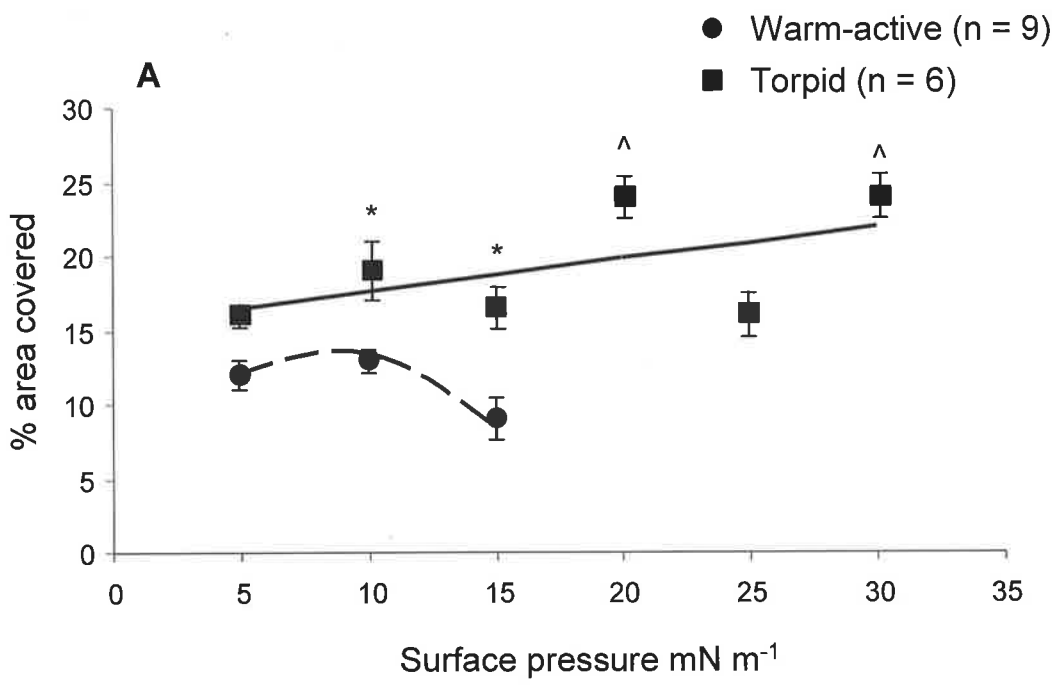
5.2.7. ATOMIC FORCE MICROSCOPY OF LARGE AGGREGATE SURFACTANT FILMS

Atomic force microscopy (AFM) confirmed the observations made using epifluorescence microscopy. Representative AFM images of films prepared from large aggregate surfactant fractions isolated from warm-active and torpid dunnart lavage fluid are shown, in height and phase mode, in Figure 5.17. In contrast to fluorescent images, organised liquid-condensed regions with greater height characteristics appear brighter, while the liquid-expanded regions with lower height characteristics appear less bright. A surface heterogeneity was observed in AFM images of both warm-active and torpid surfactant films. In both cases, organised liquid-condensed (LC) domains were observed, but these are smaller (t_{stat} = 2.993, df =18, p = 0.004) in films made of the LA fraction of warm-active dunnart lavage fluid than in films of torpid dunnart lavage fluid (Figure 5.17, labelled LC). Phase imaging of the films made of the LA fraction of torpid dunnart lavage fluid showed the existence of an even brighter, and hence, an even more condensed and organised region, of greater height inside the organised LC domain (Figure 5.17, labelled 2LC).

Height analyses in both the cases showed that the LC domain is enriched with organised molecules with the height characteristics of DPPC (Figure 5.18.). The height analysis of the torpid dunnart surfactant films suggest that there are vertical differences in height between the different domains observed (Figure 5.18). Height differences between the observed regions are given in Table 5.3, along with the average size of the LC domains. There was a significant difference (t_{stat} = 2.144, df = 18, p = 0.023) between the height of the highest, and thus, most condensed domains (i.e. LC region in warm-active films and 2LC region in torpid films) and the outside planar region (p), between warm-active and torpid dunnarts (Figure 5.18).

Figure 5.17: Atomic force microscopy (AFM) images of solvent spread films of large aggregate surfactant fractions isolated from (**A, B**) warm-active and (**C, D**) torpid dunnarts, at 30 mN m^{-1} surface pressure. The films were transferred onto freshly cleaved mica by Langmuir-Blodgett transfer technique. Note the difference in scale for A,C vs. B,D. Area of scans A, B: 2.5 x 2.5 μm^2; C, D: 10 x 10 μm^2. A and C were taken in the height mode, while B and D were taken in the phase mode. LC, liquid-condensed domain; 2LC, a region of even greater height than LC domain.

157
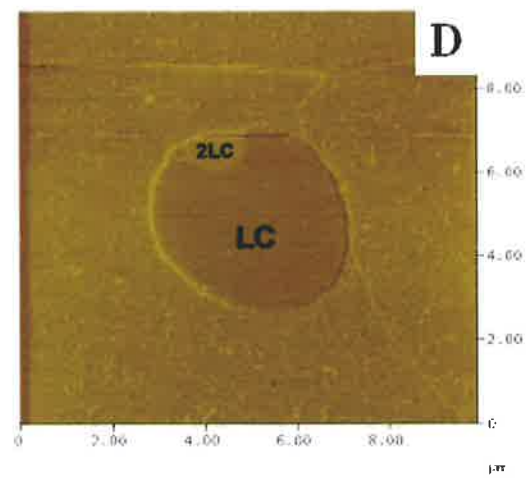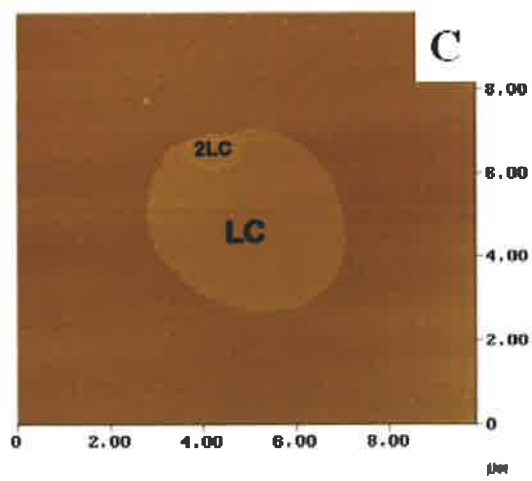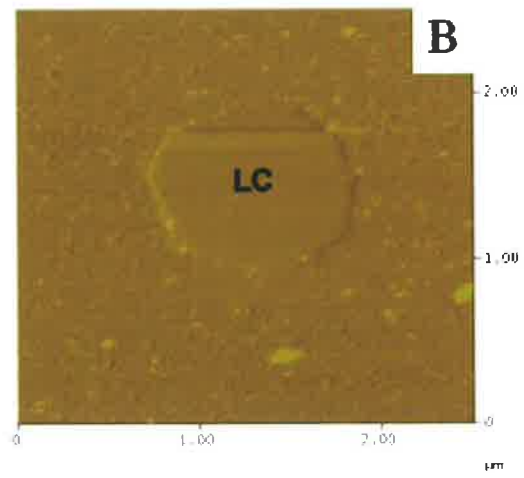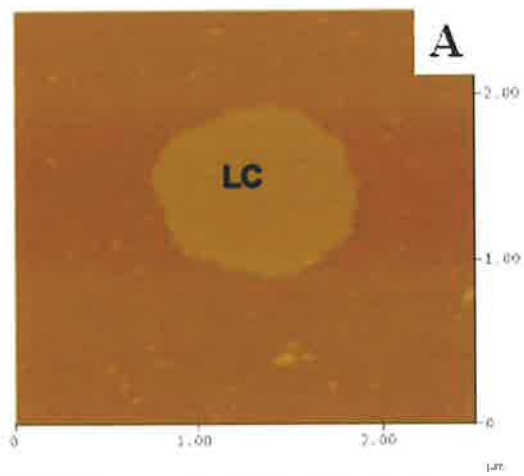
Figure 5.18. Atomic force microscopy (AFM) section analysis of solvent spread films of large aggregate surfactant fractions of lavage fluid isolated from (A) warm-active dunnarts and (B) torpid dunnarts, at 30 nM m^{-1} surface pressure. The films were transferred onto freshly cleaved mica by Langmuir-Blodgett transfer technique. Note the existence of three different phases in the torpid dunnart surfactant films. LC liquid-condensed domain, 2LC a region of even greater height characteristics than LC domain, *p* planar region. Coloured arrows show positions from which the height differences reported in Table 5.3 were measured.

Section Analysis

A

nm
1.00
0
-1.00

LC

p

0   1.00   2.00
μm

Section Analysis

B

nm
1.00
0
-1.00

2LC

LC

p

0   2.00   4.00   6.00   8.00
μm
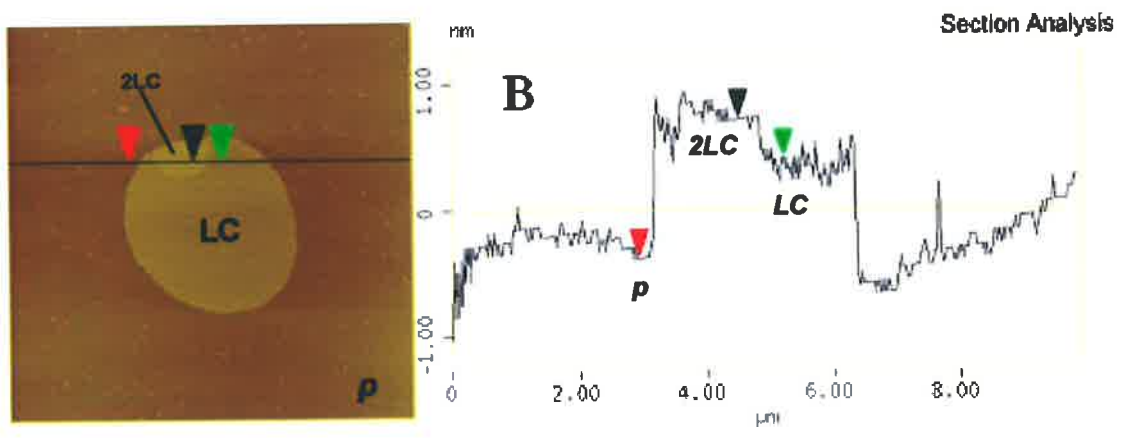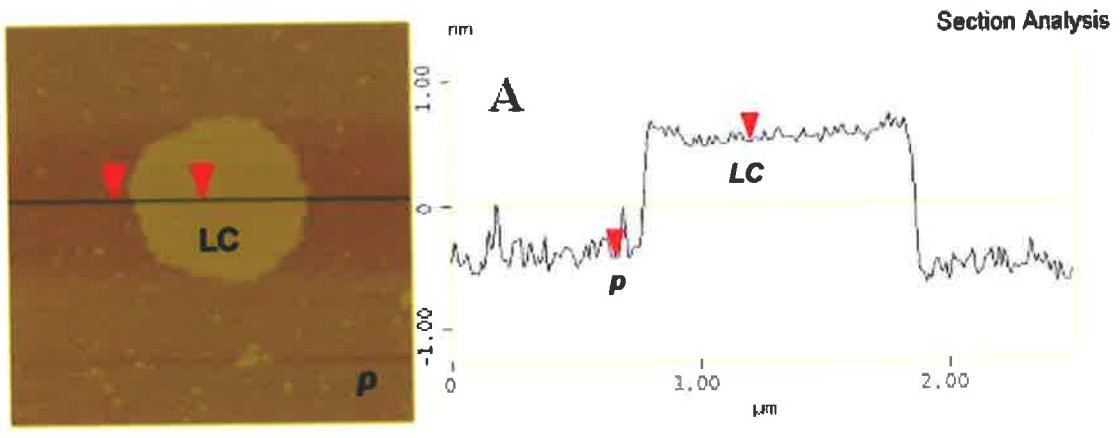
Table 5.3. Height and size characteristics of domains observed using atomic force microscopy, in films of the large aggregate fraction of surfactant isolated from warm-active and torpid fat-tailed dunnarts, *Sminthopsis crassicaudata.*

Animal State	Mean height difference between domains		Domain Size
	LC-2LC (nm)	*LC-p* (nm)	LC (μm)
Warm-active	-	0.83 ± 0.07 (n = 5)	$1.32 \pm .05$ (n = 5)
Torpid	0.55 ± 0.005 (n = 10)	1.05 ± 0.06 (n = 15)*	4.04 ± 0.51 (n = 15)*

LC-2LC refers to the mean height difference (nm) between the highest liquid-condensed domain (labelled 2LC in Figure 5.18) and the larger, liquid-condensed domain (labelled LC in Figure 5.18) observed in torpid dunnart LA surfactant films; *LC-p* refers to the mean height difference between the liquid-condensed domain (labelled LC in Figure 5.18) and the outside planar region (labelled *p* in Figure 5.18). n number of observations. * significant difference between warm-active and torpid dunnart LA surfactant fractions (using Student's unpaired t-tests, assuming equal variance, significance at $p < 0.05$).

5.3. SUMMARY OF RESULTS

- There was no difference in the amount of PL measured in the LA fraction of surfactant isolated from warm-active and torpid dunnarts.

- There was no difference in the amount of CHOL in the LA fractions of surfactant isolated from warm-active and torpid dunnarts. Hence, the ratio of CHOL/PL did not change significantly between warm-active and torpid dunnarts.

- In LA surfactant fractions, there was a small, but significant, decrease in the saturation of PC in surfactant isolated from torpid dunnarts. However, in whole lavage fractions, there was no difference between the saturation of PC between warm-active and torpid dunnart surfactants.

- In LA surfactant fractions, there were no differences in the saturation of PG between lavage isolated from warm-active and torpid dunnarts. However, in whole lavage fractions, there was a small, but significant increase in the saturation of PG in torpid dunnart surfactant.

- There was no difference in the saturation of PI in whole or LA fractions of surfactant isolated from warm-active and torpid dunnarts.

- In both LA and whole lavage surfactant fractions, DPPC (PC16:0/16:0) was not the predominant component of either warm-active or torpid dunnart surfactant. Instead 1-palmitoyl-2-palmitoleoyl phosphatidylcholine (PPPC, PC16:0/16:1) is the dominant molecular species.

- There was no difference in the molecular species composition of PC between warm-active and torpid dunnarts.

- In whole lavage surfactant fractions, the molecular species PG16:0/14:0 and PG16:0/18:2 increased significantly in surfactant isolated from torpid dunnarts. The molecular species PG16:0/18:1 decreased significantly in whole lavage

isolated from torpid dunnarts. There was no change in any molecular species of PG in LA surfactant fractions isolated from warm-active and torpid dunnarts.

- There was no change in the molecular species composition of PI between whole lavage fractions isolated from warm-active and torpid dunnarts. However, in LA surfactant fractions, the proportion of PI16:0/20:4 decreased significantly in torpid dunnarts. There was no change in any other molecular species of PG between either surfactant fraction isolated from warm-active and torpid dunnarts.

- SP-A decreased significantly in surfactant isolated from torpid dunnarts.

- There was no difference in SP-B between surfactant isolated from warm-active and torpid dunnarts.

- SP-C increased significantly in surfactant isolated from torpid dunnarts.

- At 23°C, epifluorescence studies showed that surfactant from torpid dunnarts forms a greater number, of larger condensed domains than warm-active surfactant.

- Atomic force microscopy studies of the surfactant films showed that liquid-condensed domains in films made of surfactant from torpid dunnarts are larger than the equivalent domains in warm-active surfactant films. Surfactant films from torpid dunnarts also have higher and thus, more tightly-packed liquid-condensed regions than surfactant from warm-active dunnarts, in atomic force microscopy images.

CHAPTER 6

MOLECULAR COMPOSITION OF PHOSPHOLIPIDS IN PULMONARY SURFACTANTS OF AUSTRALIAN MARSUPIALS (ORDER: MARSUPIALIA)

6.1. BACKGROUND

In Chapter 5, I observed that pulmonary surfactant isolated from fat-tailed dunnarts, *Sminthopsis crassicaudata*, has an unusual PL composition compared to other mammalian animals. The major component of dunnart surfactant is the mono-unsaturated species, 1-palmitoyl-2-palmitoleoyl phosphatidylcholine or PPPC (PC16:0/16:1) and not dipalmitoylphosphatidylcholine or DPPC (PC16:0/16:0), as in all other mammalian surfactants studied to date. The PG component of dunnart surfactant is also enriched in the molecular species PPPG (PG16:0/16:1) compared to other mammalian animals. There are two possible explanations for the unusual PL molecular species composition observed in dunnart surfactant. The first hypothesis is that the unusual composition of dunnart surfactant is due to their heterothermic physiology. This hypothesis is addressed in Chapter 7, by comparing the compositions of surfactant isolated from heterothermic and homeothermic mammals. The second hypothesis is that the unusual composition of dunnart surfactant is a characteristic of their phylogeny as an Australian marsupial (Order: Marsupialia), or perhaps more specifically, as a dasyurid marsupial (Family: Dasyuridae).

With the exception of the dunnart, most other mammalian animals studied by surfactant researchers, including rats, squirrels, bats, mice, pigs, guinea pigs and humans, are all placental mammals. The major difference between placental mammals and marsupial mammals is their mode of development. Placental mammals possess a placenta that enables young to develop inside the mother. Marsupial mammals are born at a relatively immature stage of development and are kept in a protective pouch, where they suckle and continue their development. Marsupials comprise approximately 270 species of mammals and are divided into 17 extant families (Vickers-Rich and Hewitt-Rich, 1993). The dasyurid marsupials are carnivorous marsupials and include the fat-tailed dunnart, *Sminthopsis crassicaudata*, quolls, antechinuses, planigales and the

Tasmanian devil, *Sarcophilus harrisii*. Koalas (Family: Phascolardidae), wombats (Family Vombatidae), kangaroos and wallabies (Family Macropodidae) are herbivorous marsupials and members of the sub-order Diprotodontia (Vickers-Rich and Hewitt-Rich, 1993).

Here, I isolated pulmonary surfactant from the closest non-heterothermic relative of the dunnart, the carnivorous Tasmanian devil, *Sarcophilus harrisii* (Figure 6.1A) and three homeothermic, herbivorous marsupials. The homeothermic, herbivorous marsupials studied were the southern hairy nosed wombat, *Lasiorhinus latifrons* (Figure 6.1B); the koala, *Phascolarctos cinereus* (Figure 6.1C) and the tammar wallaby, *Macropus eugenii*. All these homeothermic marsupials have body temperatures between 34-36°C. If the unusual PL composition of dunnart surfactant is ubiquitous in the dasyurids or the marsupials in general, I should also observe unusual surfactant profiles in the animals examined here.

Figure 6.1. **A** Tasmanian devil, *Sarcophilus harrisii*. The Tasmanian devil is the closest non-heterothermic relative of the fat-tailed dunnart, *Sminthopsis crassicaudata* (Figure 1.8). **B** southern hairy nosed wombat, *Lasiorhinus latifrons* **C** koala, *Phascolarctos cinereus*. Koalas and wombats are herbivorous, homeothermic marsupials and are members of the sub-order Diprotodontia. Photographs: Carol Ormond.

6.2. RESULTS

6.2.1. PHOSPHOLIPID COMPOSITION

The relative proportions of PC, PG and PI in marsupial surfactants are given in Table 6.1. In all cases, over 90% of the sum of PC, PG and PI components in marsupial surfactants, is composed of PC components.

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Table 6.1. Relative proportions of PC, PG and PI components of pulmonary surfactant isolated from Australian marsupials, *Sarcophilus harrisii*, *Lasiorhinus latifrons*, *Phascolarctos cinereus* and *Macropus eugenii*.

Marsupial	%PC	%PG	%PI	%PG/PC	%PI/PC
Tas. devil	97.93	0.45	1.62	0.5	1.7
Wombat	94.6	4.74	0.66	5.0	0.7
Koala	93.48	5.42	1.10	5.8	1.2
Tam.wallaby	95.97 ± 0.13	3.4 ± 0.15	0.62 ± 0.02	3.5 ± 0.16	0.6 ± 0.02

Data presented are mean ± S.E. (n = 2) for the tammar wallaby and n = 1 for other species. PC Phosphatidylcholine, PG phosphatidylglycerol, PI phosphatidylinositol. PC, PG and PI amounts measured using electrospray ionisation mass spectrometry relative to an appropriate internal standard. %PC calculated by dividing the amount of PC measured in lavage samples by the sum of PC, PG and PI components and multiplying by 100. %PG calculated by dividing the amount of PG measured in lavage samples by the sum of PC, PG and PI components and multiplying by 100. %PI calculated by dividing the amount of PI measured in lavage samples by the sum of PC, PG and PI components and multiplying by 100. %PG/PC calculated by dividing the amount of PG by the amount of PC measured in lavage samples and multiplying by 100. %PI/PC calculated by dividing the amount of PI by the amount of PC measured in lavage samples and multiplying by 100.

6.2.2. SATURATION OF PHOSPHOLIPIDS

Levels of saturation of PC, PG and PI in marsupial surfactants are given in Figure 6.2. The percent saturation of surfactant PC species was lowest in the Tasmanian devil at 34.8%, between 40 and 50% in the dunnart, koala and wombat and highest in the tammar wallaby, at 60.4%. The percent saturation of surfactant PG was very low, only 2%, in surfactant isolated from the Tasmanian devil. For the other marsupials, PG saturation was 14% in the tammar wallaby and koala, 17% in the wombat and 25% in dunnarts. The percent saturation of surfactant PI was very low (less than 5%) in all marsupial surfactants studied.

6.2.3. MOLECULAR SPECIES OF PHOSPHOLIPIDS

6.2.3.1. PHOSPHATIDYLCHOLINE

The molecular species of phosphatidylcholine (PC) in surfactant isolated from the Tasmanian devil, wombat, koala, and tammar wallaby, compared to surfactant isolated from warm-active dunnarts (Chapter 5) are shown in Figure 6.3. In both the tammar wallaby and koala, the composition of surfactant PC was dominated by the disaturated species, PC16:0/16:0, which contributes 35-38 mol% to the PC component. In surfactant isolated from tammar wallabies and the koala, the unsaturated species, PC16:0/16:1 comprised 15 and 19 mol%, respectively and the disaturated species, PC16:0/14:0 comprised 7 and 10 mol%, respectively. Surfactant isolated from the koala also contained PC16:0:18:1 (12 mol%). Surfactant isolated from tammar wallabies also contained the alkyl-acyl-containing species of PC16:0/16:0 and PC16:0/18:1, which contributed 8 mol% each to the PC component. In both the koala and tammar wallaby, other molecular species of PC contributed less than 5 mol% each to surfactant PC.

The molecular species profile obtained for wombat surfactant was the most similar to that obtained for surfactant isolated from fat-tailed dunnarts. Wombat

surfactant, like dunnart surfactant, is enriched in mono-unsaturated 1-palmitoyl-2-palmitoleoyl phosphatidylcholine (PC16:0/16:1) (23 mol% in wombat), such that it contributes a similar proportion to dipalmitoylphosphatidylcholine (PC16:0/16:0) (22 mol% in wombat). The other major components in wombat surfactant are PC16:0/14:0, PC16:0/18:1 and PC16:0/18:2, which contribute 10, 11 and 8 mol%, respectively to the PC component.

The PC composition of surfactant isolated from the Tasmanian devil was substantially different to all other placental and marsupial mammalian surfactants, including the dunnart. Surfactant isolated from the Tasmanian devil consisted largely of alkyl-acyl forms of PC. The alkyl-acyl form of PC16:0/16:1 (i.e. PC16:0a/16:1) contributes 45 mol% to surfactant PC in the Tasmanian devil. The other major components of Tasmanian devil surfactant were the alkyl-acyl form of DPPC (PC16:0a/16:0), which contributed 21 mol% to the surfactant PC and other alkyl-acyl forms of PC, including PC16:0a/14:0 (6 mol%) and PC16:0a/18:1 (6 mol%).

Figure 6.2. Relative proportions of disaturated components in lavage fluid isolated from Australian marsupials. Dunnart *(Sminthopsis crassicaudata),* wombat *(Lasiorhinus latifrons)* and tammar wallaby *(Macropus eugenii)* data are presented as means ± S.E., where possible. An error bar is given when n = 2, but denotes a range. Dunnart, n = 7 for PC, n = 6 for PG and PI; tammar wallaby, n = 2; wombat, n = 1 for PC, n = 2 for PG and PI; Tasmanian devil *(Sarcophilus harrisii)* and koala *(Phascolarctos cinereus)*, n = 1. PC phosphatidylcholine, PG phosphatidylglycerol, PI phosphatidylinositol.

Legend:
- ■ Warm-active dunnart
- ■ Tasmanian devil
- □ Tammar wallaby
- ■ Koala
- ■ Southern hairy-nosed wombat

Y-axis: % saturation

X-axis: Phospholipid class (PC, PG, PI)

Figure 6.3. The composition of individual molecular species of phosphatidylcholine (PC) in whole lavage isolated from Australian marsupials, *Sminthopsis crassicaudata, Sarcophilus harrisii, Lasiorhinus latifrons, Phascolarctos cinereus* and *Macropus eugenii*. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Results are presented as mean ± SE, where possible. n number of animals; when n = 2, error bars denote a range.

Figure showing phosphatidylcholine (mol%) on the y-axis versus molecular species of phosphatidylcholine in marsupial surfactants on the x-axis.

Y-axis: Phosphatidylcholine (mol%), scale from 0 to 50.

X-axis: Molecular species of phosphatidylcholine in marsupial surfactants

X-axis categories: 16:1a/14:0, 16:0a/14:0, 16:1/14:0, 16:0/14:0, 16:0a/16:1, 16:0a/16:0, 16:1/16:0, 16:0/16:0, 16:0a/18:2, 16:0a/18:1, 16:0a/18:0, 16:0/18:2, 16:0/18:1, 18:0a/18:2, 18:0a/18:1

Legend:
- Warm-active dunnart (n = 7)
- Tasmanian devil (n = 1)
- Tammar wallaby (n = 2)
- Koala (n = 1)
- Wombat (n = 2)

6.2.3.2. PHOSPHATIDYLGLYCEROL

The molecular species compositions of phosphatidylglycerol (PG) in surfactants isolated from the Tasmanian devil, wombat, koala and tammar wallaby, compared to surfactant isolated from warm-active dunnart (Chapter 5) are given in Figure 6.4. With the exception of the Tasmanian devil, the predominant PG component of surfactant isolated from all the marsupials studied, was PG16:0/18:1. PG16:0/18:1 contributed 22 mol% in dunnart, 28 mol% in tammar wallaby, 31 mol% in wombat and 47 mol% in koala surfactant. The next major PG components, in all marsupial species, except the Tasmanian devil, were the disaturated PG16:0/16:0 and unsaturated species, PG16:0/16:1. PG16:0/16:0 and PG16:0/16:1 contributed between 10 and 20 mol% each to surfactant PC. The amount of PG16:0/18:2 was higher in dunnart (12 mol%) and wombat (16 mol%) surfactant, than in tammar wallaby (5 mol%) and koala (8 mol%) surfactant. Diacyl and alkyl-acyl containing forms of PG18:0/18:1 comprised 12 and 8 mol% of PG in surfactant isolated from the tammar wallaby, respectively.

As for PC, the PG composition of surfactant isolated from the Tasmanian devil was substantially different to all other placental and marsupial mammalian surfactants, including the dunnart. The major component of surfactant isolated from the Tasmanian devil was the disaturated species, PG18:0/18:0, which contributed only 15 mol% to total PG. The unsaturated species PG16:1/18:2 and PG16:0/16:1 contributed 11 mol% each to surfactant PG in the Tasmanian devil. Other components of Tasmanian devil surfactant include PG16:0/18:1 (9 mol%), PG16:0/18:2 (8 mol%), PG18:1/18:2 (8 mol%), PG16:0/20:4 (7 mol%) and the alkyl-acyl-containing form of PG16:0/18:1 (8 mol%). Phosphatidic acid contributed less than 5 mol%, relative to the PG component of surfactant.

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Figure 6.4. The composition of individual molecular species of phosphatidylglycerol (PG) in whole lavage isolated from Australian marsupials *Sminthopsis crassicaudata, Sarcophilus harrisii, Lasiorhinus latifrons, Phascolarctos cinereus* and *Macropus eugenii*. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Results are presented as mean ± SE, where possible. n number of animals; where n = 2, error bars denote a range.

Molecular species of phosphatidylglycerol in marsupial surfactants

Legend:
- Warm-active dunnart (n = 6)
- Tasmanian devil (n = 1)
- Tammar wallaby (n = 2)
- Koala (n = 1)
- Wombat (n = 1)

Y-axis: Phosphatidylglycerol (mol%)

X-axis categories: 16:0/16:1, 16:0/16:0, 16:0a/18:1, 16:1/18:2, 16:0/18:2, 16:0/18:1, 18:1a/18:2, 18:1a/18:1, 18:0a/18:1, 16:0/20:4, 18:1/18:2, 18:0/18:1, 18:0/18:0

6.2.3.3. PHOSPHATIDYLINOSITOL

The molecular species composition of phosphatidylinositol (PI) in surfactant isolated from the Tasmanian devil, wombat, koala and tammar wallaby, compared to surfactant isolated from warm-active dunnarts (Chapter 5), are given in Figure 6.5. The molecular species composition of PI varied between different marsupial animals.

The major components of PI in the tammar wallaby are the alkyl-acyl-containing forms of PI16:0/18:1 (18 mol%), PI16:0/16:0 (12 mol%) and PI16:0/18:1 (9 mole %). PI16:0/16:1 (7 mol%), PI16:0/18:1 (8 mol%) and the alkyl-acyl-containing form of PI16:1/18:1 (6 mol%) also contribute to surfactant PI in the tammar wallaby.

Wombat surfactant is composed of PI18:0/20:4 (18 mol%) and the alkyl-acyl-containing forms of PI16:0/16:0 (11 mol%) and PI16:0/16:1 (10 mol%). PI16:1/18:0 (9 mol%), PI16:0/18:0 (8 mol%), PI16:0/20:4 (8 mol%) and PI 18:1/18:1 (7 mol%) also contribute to surfactant PI in the wombat. Koala surfactant consisted of mainly PI16:0/18:1, which comprised 26 mol% and PI18:1/18:1 and PI 18:0/18:1, which each comprised 10 mol%. PI16:1/18:1 (8 mol%) and PI16:0/18:1 (6 mol%) were also present in surfactant isolated from the koala. All other molecular species contributed less than 5 mol% to the surfactant PI component in the koala.

As for PC, surfactant PI in the Tasmanian devil consisted mainly of alkyl-acyl-containing molecular species. The spectra obtained from a diagnostic scan for PI species in the Tasmanian devil lavage sample, along with the fatty acid fragmentation pattern confirming the identity of the 821.5 mass/charge PI peak, is shown in Figure 6.5. The alkyl-acyl-containing forms of PI16:0/18:1, PI16:1/18:1 and PI16:0/20:5 contributed 27, 13 and 10 mol%, respectively, to surfactant PI in the Tasmanian devil. All other molecular species contributed less than 5 mol% to surfactant PI in the Tasmanian devil.
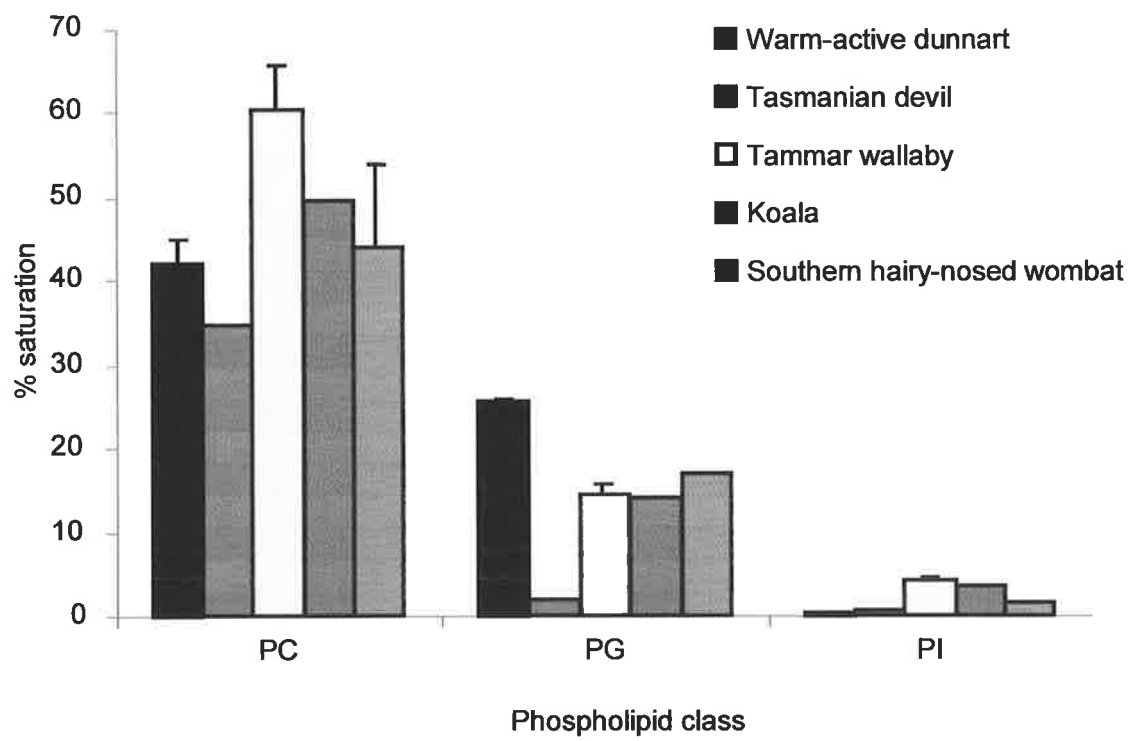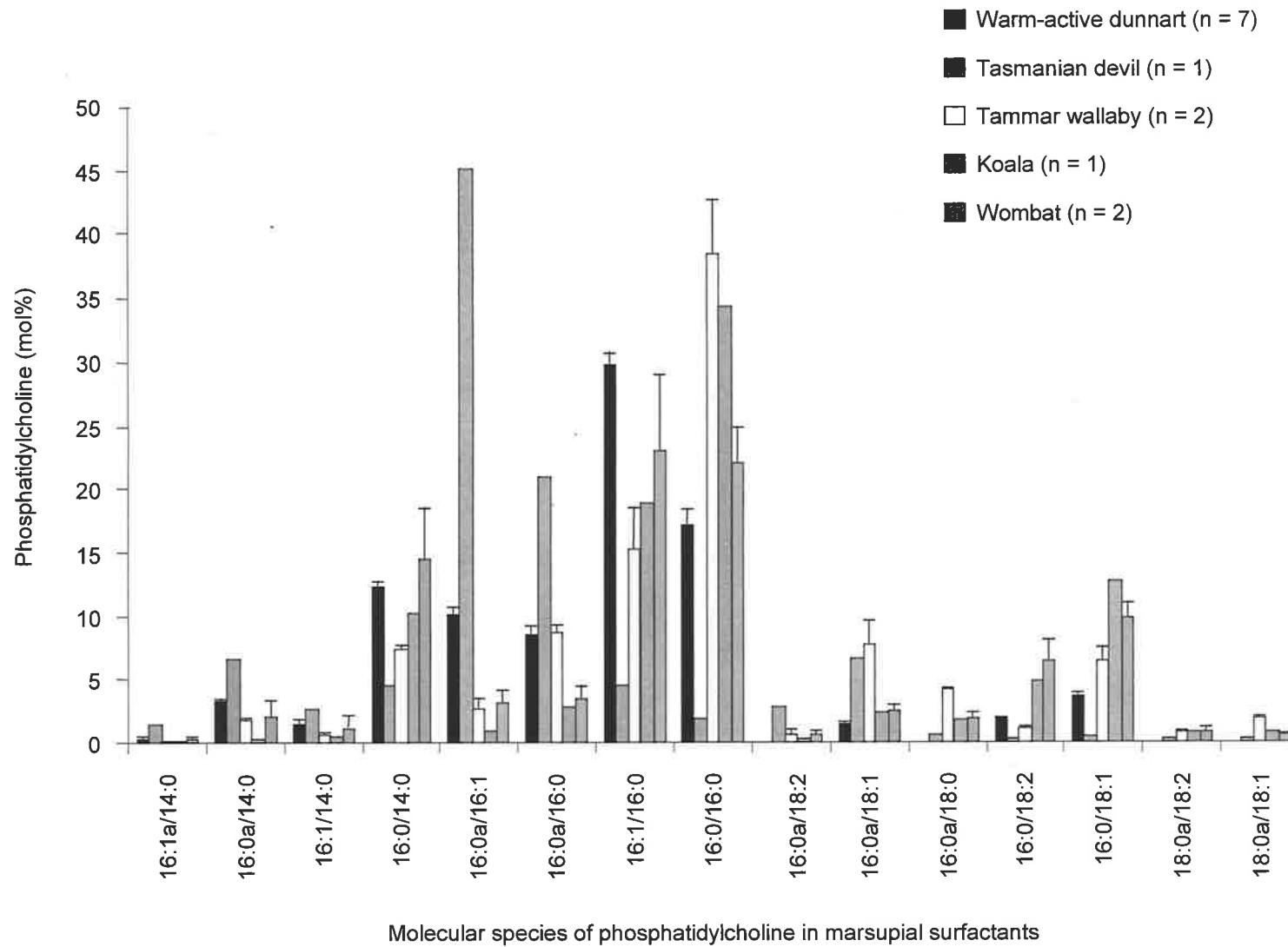
Figure 6.5. The composition of individual molecular species of phosphatidylinositol (PI) in whole lavage isolated from Australian marsupials, *Sminthopsis crassicaudata*, *Sarcophilus harrisii*, *Lasiorhinus latifrons*, *Phascolarctos cinereus* and *Macropus eugenii*. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Results are presented as mean ± SE, where possible. n number of animals; where n = 2, error bars denote a range.
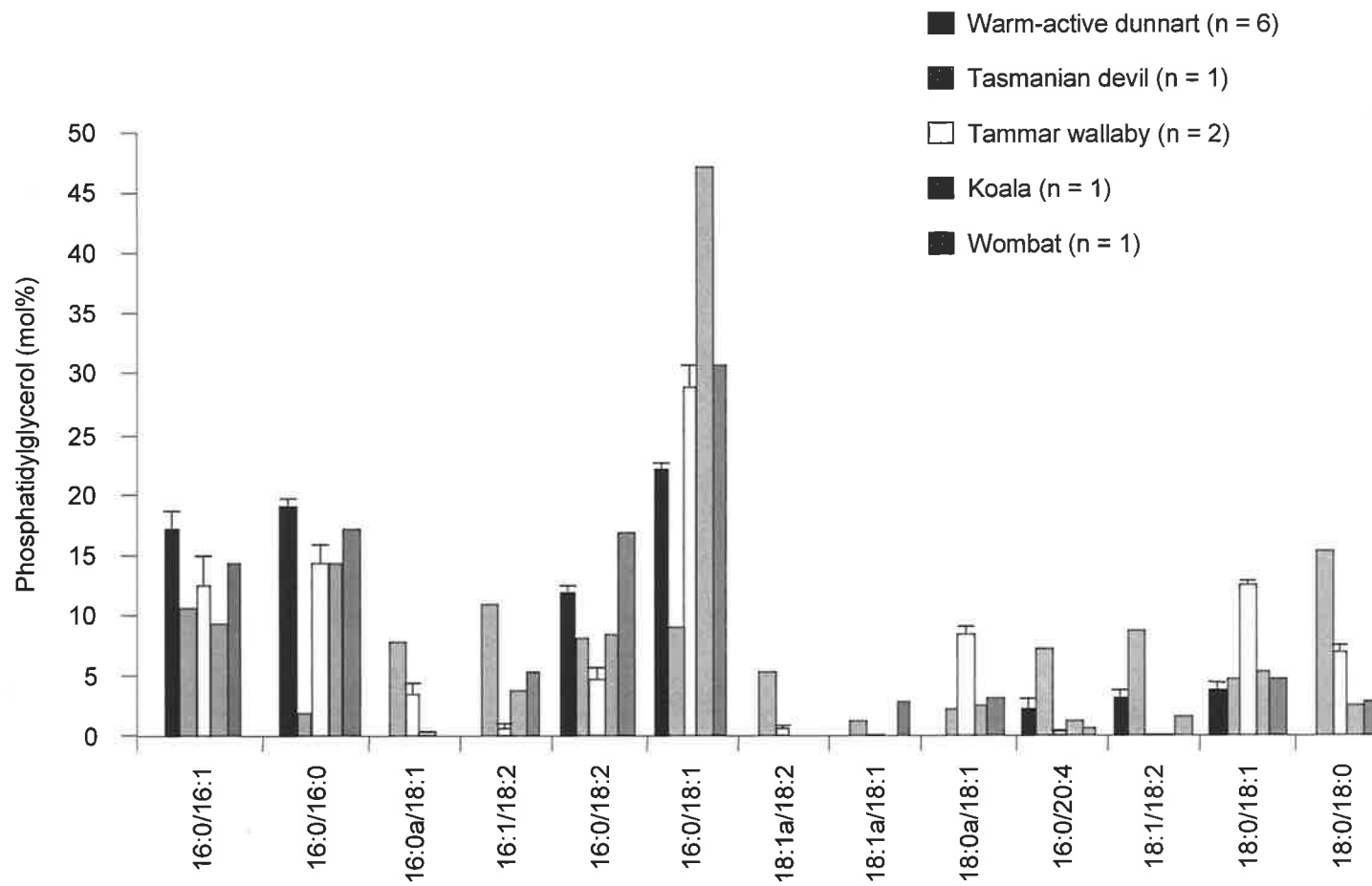
Legend:
- Warm-active dunnart (n = 6)
- Tasmanian devil (n = 1)
- Tammar wallaby (n = 2)
- Koala (n = 1)
- Wombat (n = 1)

Y-axis: Phosphatidylinositol (mol%)

X-axis categories: 16:0a/16:1, 16:0a/16:0, 16:0/16:1, 16:0/16:0, 16:1a/18:2, 16:1a/18:1, 16:0a/18:1, 16:1/18:1, 16:0/18:1, 16:0a/20:4, 18:1a/18:1, 18:0a/18:1, 16:0/20:4, 16:0/20:3, 18:1/18:1, 18:0/18:1, 16:0a/22:6, 18:1a/20:4, 18:0a/20:4, 18:1a/20:1, 18:0a/20:1, 18:0/20:4, 18:0/20:1, 18:0/22:6, 18:0/22:5

X-axis label: Molecular species of phosphatidylinositol in marsupial surfactants

Figure 6.6. The molecular species compositions of marsupial surfactants were confirmed using diagnostic scans and tandem mass spectrometry. For example, the alkyl-acyl nature of the PI component in surfactant isolated from the Tasmanian devil, *Sarcophilus harrisii* is confirmed in this typical spectra obtained from a diagnostic scan of PI species in lavage fluid isolated from the Tasmanian devil. Tandem mass spectrometry of the various mass ions was used to confirm the identity of molecular species. **(INSET)** shows an example of fatty acid fragmentation. The identity of m/z 822 ion was confirmed as the alkyl-acyl form of PI16:0/18:1 (i.e. PI16:0a/18:1). Mass (m)/charge (z) is equivalent to molecular mass.

Parents of 241ES-

PI16:1alkyl/16:1

PI16:0alkyl/18:1

PI18:0alkyl/18:1

Daughters of 822ES-
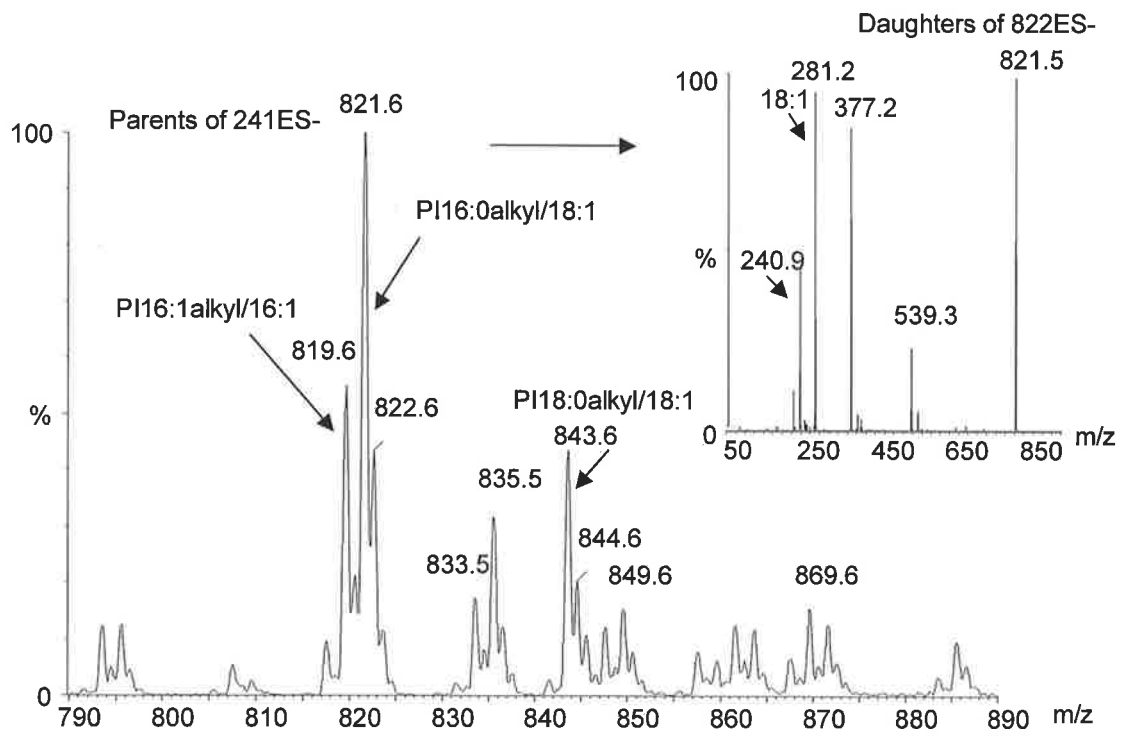
6.2.4. CHOLESTEROL AND PROTEIN IN TASMANIAN DEVIL SURFACTANT

Lavage fluid collected from the Tasmanian devil had 5.59 ± 0.115 μg CHOL mg^{-1} freeze-dried powder (mean $\pm$ SE, n =1, assayed in duplicate), 66.59 ± 0.98 μg PL mg^{-1} powder (mean $\pm$ SE, n = 1, assayed in triplicate) and 39.41 ± 0.84 μg protein mg^{-1} powder (mean $\pm$ SE, n = 1, assayed in triplicate). The CHOL/PL ratio was 0.084 or 8.4%. The protein/PL ratio was 0.59 or 59%. Tasmanian devil lavage fluid had 4.67 ± 0.15 ng SP-A mg^{-1} PL (mean $\pm$ SE, n = 1, assayed in duplicate) and 1.97 ± 0.12 ng SP-B mg^{-1} PL (mean $\pm$ SE, n =1, assayed in duplicate).

6.3. SUMMARY OF RESULTS

- PC contributes over 90% of the sum of PC, PG and PI components of surfactant in all marsupials studied.

- The percent saturation of surfactant PC species was lowest in the Tasmanian devil at 34%, between 40 and 50% in the dunnart, koala and wombat and highest in the tammar wallaby, at 60.4%.

- The percent saturation of surfactant PG was very low (less than 2%) in surfactant isolated from the Tasmanian devil. For the other marsupials, PG saturation was 15-20%.

- The percent saturation of surfactant PI was very low (less than 5%) in all marsupial surfactants studied.

- In both the tammar wallaby and koala, surfactant PC was dominated by DPPC (PC16:0/16:0).

- In the wombat and dunnart, surfactant PC was enriched in PPPC (PC16:0/16:1).

- The composition of surfactant in the Tasmanian devil is substantially different from all other marsupials, including its closest relative, the fat-tailed dunnart.

- Tasmanian devil surfactant consists mainly of alkyl-acyl forms of PC and PI and is largely monounsaturated.

- The molecular composition of PG and PI varied between the different marsupials studied.

- The CHOL/PL ratio of Tasmanian devil surfactant is similar to that reported for other mammalian species (Table 1.7).

CHAPTER 7

GENERAL DISCUSSION

A NEW PARADIGM FOR SURFACTANT COMPOSITION AND

FUNCTION

7.1. TORPOR AND SURFACTANT FUNCTION

Pulmonary surfactant isolated from warm-active and torpid heterothermic mammals appears to function optimally at warm (37°C) and cold (22-24°C) ambient temperatures, respectively. The minimum surface tensions obtained for warm-active and torpid bat and dunnart surfactant in previous studies are summarised in Table 7.1. When measured at 22-24°C, a lower minimum surface tension is consistently recorded for surfactant isolated from torpid animals than from warm-active animals. Conversely, when measured at 37°C in a captive bubble surfactometer, a lower minimum surface tension is recorded for surfactant isolated from warm-active bats and dunnarts than from torpid bats and dunnarts. Adsorption to the air-liquid interface is also faster at 37°C for surfactant isolated from warm-active mammals and faster at 22-24°C for torpid mammals (Table 7.1). Furthermore, surfactant isolated from torpid mammals requires less compression (% SAcomp) to reach low surface tensions (ST) than warm-active surfactant (Table 7.1). Hence, in heterothermic mammals, surfactant from warm-active animals appears to be more suited to function at higher temperatures and surfactant from torpid animals appears to function best at lower temperatures. This phenomenon is observed, irrespective of the type of torpor pattern displayed by the mammals.

Table 7.1. The effect of temperature on the surface activity of pulmonary surfactant isolated from heterothermic mammals.

Animal and activity status	Temp (°C)	ST_{min} (mN m^{-1})	SAcomp %
Active Bat[a]	37_{CBS}	$1.2 \pm 0.008^{*}$	$11.8 \pm 0.1^{*}$
	24_{CBS}	$2.5 \pm 0.09^{*}$	$17.2 \pm 0.5^{*}$
Torpid Bat[a]	37_{CBS}	$3.76 \pm 0.04^{\#}$	$10.1 \pm 0.2^{\#}$
	24_{CBS}	$1.2 \pm 0.2^{\#}$	$8.75 \pm 0.2^{\#}$
Active Dunnart[b]	37_{CBS}	$6.41 \pm 0.3^{\phi}$	$89.1 \pm 0.8^{\bullet}$
	20_{WB}	5.45 ± 0.2	$48.3 \pm 0.45^{\wedge}$
Torpid Dunnart[b]	37_{CBS}	$9.12 \pm 0.3^{\phi}$	$84.9 \pm 1.8^{\bullet}$
	20_{WB}	4.43 ± 0.19	$26.5 \pm 2.2^{\wedge}$

Data expressed as mean $\pm$ SE. ST_{min} minimum surface tension (mN m^{-1}), % SAcomp, percent surface area compression required to reach ST_{min}. Surfactant isolated from warm-active and torpid mammals and examined at 20°C, 24°C and 37°C using a Wilhelmy-Langmuir surface balance (WB) or Captive Bubble Surfactometer (CBS). a data for daily torpor in bats from (Codd et al., 2003; Codd et al., 2002); b data for stress-induced torpor in dunnarts from (Lopatko et al., 1998). Pairs of symbols denote values that are significantly different from each other.

7.2. TORPOR AND SURFACTANT LIPID COMPOSITION

7.2.1. PHOSPHOLIPIDS

7.2.1.1. SATURATION

An increase in the proportion of disaturated phospholipids (DSP) in whole lavage surfactant fractions has been reported previously in dunnarts during stress-induced torpor (Langman et al., 1996). The classical understanding of surfactant function suggests that DSP, and particularly dipalmitoylphosphatidylcholine (DPPC), are the surface-tension reducing components of pulmonary surfactant. Hence, an increase in the saturation of surfactant (i.e. the DSP/PL ratio) is thought to aid in the surface-active reducing function of surfactant during torpor (Daniels and Orgeig, 2001). However, ground squirrels and bats do not alter the overall saturation of surfactant phospholipid (i.e. the DSP/PL ratio) during hibernation (Chapter 3) and daily torpor (Codd et al., 2000b; Slocombe et al., 2000), respectively. Hence, the increases in DSP observed during torpor in dunnarts may be related to the stress-induced nature of their torpor pattern. Cortisol can sometimes be elevated in response to external stresses, such as food deprivation and cold ambient temperatures (Codd et al., 2000b) and is a known stimulus for surfactant synthesis and release (Barry, 1983). Thus, changes in the levels of circulating cortisol may explain the increases observed in DSP during stress-induced torpor in dunnarts, but not daily torpor or hibernation in bats and ground squirrels, respectively.

In the present study, in whole lavage fractions, I did not observe any significant differences in the saturation of phosphatidylcholine (PC) between warm-active and torpid (or hibernating) bats, dunnarts or squirrels. However, in the large aggregate fraction of surfactant isolated from torpid dunnarts there was a significant decrease in the saturation of PC. Hence, although PC comprises 70% of the phospholipid (PL) component in the dunnart, increases in the saturation of PC cannot explain the previously observed increases in DSP in whole lavage after eight hours of torpor (Langman et al.,

1996). However, the saturation of phosphatidylglycerol (PG) did increase significantly in whole lavage samples isolated from dunnarts during stress-induced torpor (Chapter 5). Therefore, increases in PG saturation may account, at least in part, for the increase in DSP/PL previously reported in whole lavage fractions isolated from torpid dunnarts. However, in the present study, I observed no change in the saturation of PG between large aggregate fractions of surfactant isolated from warm-active and torpid dunnarts. Hence differences in PG saturation observed in whole lavage fractions of dunnart surfactant probably originate from the small aggregate (non-surface-active) component of surfactant and thus, may not have any relevance to the surface-active component of pulmonary surfactant.

In contrast, the percent saturation of PG decreased during daily torpor in the bat, *Chalinolobus gouldii* (Chapter 4). Based on our classical understanding of surfactant function, any decrease in saturated molecules might be expected to lower the phase-transition of the surfactant mixture and improve fluidity, and thus, the spreadability of surfactant at low temperatures. Improving the spreadability of surfactant may be particularly important in bats, which have finely divided airspaces and tiny alveoli (Codd et al., 2000b). However, although significant, the observed decrease in %DSPG/PG, and more specifically dipalmitoylphosphatidylglycerol (PG16:0/16:0), in whole lavage fractions isolated from torpid bats was small (only 3 mol%) and is not reflected in the overall saturation of surfactant PL. Moreover, the %DSP/PL ratio did not change in Gould's wattled bats over a 24 hour cycle, which included a period of daily torpor (Codd et al., 2000b). Hence, the functional significance, if any, of the small decrease in PG16:0/16:0 observed here during daily torpor in Gould's wattled bats is not known.

7.2.1.2. MOLECULAR SPECIES

In all three species examined, there were no changes in the molecular composition of the major PL component, PC, between whole lavage surfactant fractions isolated from warm-active and torpid animals. However, small, but significant, differences were observed in one or two of the molecular components that comprise greater than 5 mol% of phosphatidylglycerol (PG) and phosphatidylinositol (PI) in each animal species. As mentioned above, during daily torpor in bats, the contribution of PG16:0/16:0 to the total PG component of surfactant, decreases by 3 mol%. In addition, the contribution of PI18:0/18:2 to the total PI component of surfactant, increases by 8 mol% during daily torpor in bats. In whole lavage fractions from torpid dunnarts, the contributions of PG16:0/18:2 and PG14:0/16:0 increase during torpor, by 4 and 3 mol%, respectively. However, the contribution of PG16:0/18:1 to the surfactant PG component of dunnart surfactant, decreases by 4 mol% in whole lavage fractions. None of these observed changes in PG and/or PI molecular species are consistent across the animal species examined and this may suggest that they are each specific for the type of torpor pattern displayed and/or the particular animal species.

However, PG and PI are two of the more variable components of pulmonary surfactant, varying throughout development and in different animal species (Section 7.4.). The wide variation observed between different animal species could suggest that the acidic headgroups are more important to surfactant function than the precise molecular species compositions of PG and PI (Postle et al., 2001). Moreover, the variation of PG and PI components between individuals of the same species appears to be greater than that observed for PC (Chapters 3, 4 and 5). In addition, since the observed changes are relatively small and occur in components that comprise only 15-20% of the surfactant PL, they are not necessarily reflected in the overall saturation of surfactant PL.

The DSP/PL ratio did not change between warm-active and torpid bats (Codd et al., 2000b) or warm-active and hibernating ground squirrels (Chapter 3). Furthermore, the differences observed in the molecular species PG16:0/14:0, PG16:0/18:1 and PG16:0/18:2 in dunnart whole lavage were not observed in the large aggregate fractions of dunnart surfactant. This suggests that the observed differences in these molecular components originate from the small aggregate, or non-surface-active component of dunnart lavage fluid and not the large aggregate, surface-active component. Hence, the small differences observed in a few of the molecular species of PG and/or PI during torpor in bats and dunnarts and hibernation in ground squirrels, are probably due either to intra-species variation and/or the isolation and preparation of surfactant from whole lavage fluid. Thus, in terms of surface activity, they are unlikely to have any major functional significance and as such, they are probably not thermal adaptations of the surfactant system to stress-induced torpor, daily torpor or hibernation. However, it is possible that these minor PL components do play a role in signalling events related to surfactant metabolism (Veldhuizen et al., 1998). Hence, the variations observed here, in a few of the molecular species of PG and PI, may be related to differences in surfactant metabolism between warm-active and torpid animals.

Under the classical model of surfactant function, the primary role of PL in surfactant is to lower the surface tension, by forming a tightly packed, condensed film at the air-liquid interface that effectively excludes water molecules. In bats and dunnarts, surfactants isolated from both warm-active and torpid animals are capable of effectively reducing surface tension at the appropriate body temperature, without any major changes in PL composition. This suggests that the molecular composition of PL in surfactants of heterothermic mammals is either sufficient, or already modified, to enable surfactant to effectively reduce surface tension at both warm and cold body temperatures.

The entry into a torpor bout, of any type, is accompanied by a substantial decrease in metabolic rate. Furthermore, the modification of PL composition in surfactant is likely to involve complex synthetic, secretory and enzymatic processes (Rooney et al., 1994). Hence, if the phase-transition of the surfactant mixture is not as crucial to surfactant function as previously surmised, modifying the basic composition of surfactant PL so that it can function effectively, if not optimally, over a range of temperatures, would seem an efficient adaptation for heterotherms. Such adaptations are likely to be more efficient than modifying the PL composition each time upon entry into, or arousal from, a torpor bout. A comparative study of homeothermic and heterothermic mammalian surfactant compositions demonstrates that there are comparative differences in PL molecular species composition between homeothermic and heterothermic mammals that may be thermal adaptations. These are discussed in section 7.4.

However, warm-active and torpid surfactant does not function as effectively when measured at different assay temperatures, i.e. cold and warm body temperatures, respectively (Section 7.1). Thus, other aspects of surfactant composition, lipid-lipid interactions, lipid-protein interactions or film structure must be changing between warm-active and torpid mammals to enhance surfactant function at the specific body temperature of the animal.

7.2.2. CHOLESTEROL

In all heterothermic mammals studied, irrespective of the type of torpor pattern displayed, the amount of cholesterol increases significantly during torpor or hibernation (Figure 7.2). Increases in cholesterol have been observed in whole lavage fluid during daily torpor in bats (Codd et al., 2002), stress-induced torpor in dunnarts (Langman et al., 1996) and hibernation in ground squirrels (Chapter 3). These observations suggest that

cholesterol is particularly important to the surfactant system at the cold body temperatures and/or at low lung volumes, experienced during torpor bouts.

In this study, I did not observe a change in cholesterol between the large aggregate fractions of surfactant isolated from warm-active and torpid dunnarts. This may suggest that the increases in cholesterol observed in whole lavage fractions of surfactant, are due to increases in cholesterol in the small aggregate, non-surface active component of lavage fluid. However, it is also likely that some 'surface-active' cholesterol is lost during ultra-centrifugation procedures, which have been optimised with the aim of improving surface-activity and not necessarily, for cholesterol measurement. If this loss of cholesterol is greater from surface-active material containing higher cholesterol levels, and thus, more fluid, less tightly-packed surfactant mixtures, than it is from more tightly-packed surfactant mixtures with lower cholesterol levels, then it may explain why I did not observe an increase in cholesterol in large aggregate surfactant fractions isolated from torpid dunnarts in this study.

Alternatively, it is possible that the differences in the observations of the present study and that of Langman et al. (1996) for dunnart surfactant could be related to the different procedures used to measure cholesterol. Langman et al. (1996) used traditional chromatographic procedures, while here, I used a more direct enzymatic, colorimetric assay purchased from Boehringer Mannheim. However, in the hibernating squirrel whole lavage samples (Chapter 3), similar increases in cholesterol were observed using both the traditional chromatographic procedures and the enzymatic, colorimetric assay. Hence, the differences observed between this and the previous dunnart studies are most likely related to the centrifugation procedures used to isolate large aggregate fractions of surfactant.

Table 7.2. Comparative biology of cholesterol composition in surfactant isolated from mammalian animals.

Animal species	T_b °C	PL mg gDL^{-1}	CHOL mg gDL^{-1}	CHOL/PL
Homeothermic				
Rat, *Rattus norvegicus*[a]	37	1.7*	0.130*	0.071
Mouse, *Mus musculus*[b]	37	16.9	1.49	0.097
Human, *Homo sapiens*[c]	37	ND	15.4#	0.071
Tas.devil, *S. harrisi*[d]	37	ND	ND	0.084
Heterothermic				
Bat,	37	14.6	0.1	0.008
Nyctophilus geoffroyi[e]	24	16^	0.06	0.006
Bat,	37	17.3	0.268	0.019
Chalinolobus gouldii[f]	24	12.5^	0.4^	0.025^
Dunnart[g],	35	21.8	1.48	0.068
Sminthopsis crassicaudata	15	38.4^	4.14^	0.107^
Dunnart[h],	37	26.8	2.6	0.096
Sminthopsis crassicaudata	15	26.7	2.3	0.081
Dunnart LA[h],	37	11.4	1.03	0.090
Sminthopsis crassicaudata	15	11.0	0.91	0.079
Squirrel[i],	37	0.36*	0.01*	0.077
Spermophilus lateralis	7	0.42*	0.02*^	0.134^

T_b body temperature (°C), PL phospholipid, CHOL cholesterol, mg gDL^{-1} milligram per gram dry lung mass, * wet lung mass, ND not determined, # whole lung extracts, LA large aggregate fraction, ^ significant difference between warm-active and torpid or

hibernating animal. Data are from *a* (Hallman and Gluck, 1975); *b* (Langman et al., 1996); *c* (Neumann et al., 1990); *d* Chapter 6; *e* (Slocombe et al., 2000); *f* (Codd et al., 2000b); *g* (Langman et al., 1996); *h* Chapter 5; *i* Chapter 3.

7.2.2.1. CHOLESTEROL SYNTHESIS

Cholesterol comprises approximately 10% by weight of the total surfactant lipids (King, 1982) and of this cholesterol, 41% is located in the intracellular surfactant pool (i.e. in lamellar bodies) and 59% is located in the extracellular pool in the aqueous layer (Hass and Longmore, 1979, 1980; King, 1982). Cholesterol is synthesised de novo in the lung, but is also taken up from the perfusion media in the form of low density lipoproteins (LDLs), very low density lipoproteins (VLDL) and high density lipoproteins (HDL) (Hass and Longmore, 1979, 1980). Hass and Longmore (1979) concluded that most of the exogenous cholesterol ends up in the surfactant fraction and is incorporated into lamellar bodies for secretion with the surfactant phospholipid.

However, this study is the first to demonstrate the presence of lathosterol in lung lavage fluid (Chapter 3). Lathosterol (LaSL) is a precursor of cholesterol (CHOL) and is commonly used as a marker of cholesterol synthesis or metabolism in plasma and other tissues (Larking, 1999). Both the amount of LaSL and the ratio of LaSL/CHOL increased significantly in lung lavage fluid isolated from hibernating ground squirrels. The observed increase in LaSL/CHOL indicates that cholesterol synthesis is significantly higher in the lung during hibernation in ground squirrels. Hass and Longmore (1979) concluded that endogenous cholesterol synthesis accounts for less than 1% of the total cholesterol used by the lung for surfactant synthesis. If this is the case, then the uptake of cholesterol from the blood may also significantly increase during hibernation in ground squirrels and contribute to observed increases in cholesterol in whole lavage fluid. However, there is also evidence that the cholesterol that originates from plasma,

and is incorporated into lamellar bodies, is not available for use by alveolar surfactant (Orgeig and Daniels, 2001). This suggests that lamellar bodies are not the main source of alveolar cholesterol and that other mechanisms exist (Orgeig and Daniels, 2001). It is possible, therefore, that the level of de novo cholesterol synthesis in the lung may be much greater than previously thought.

7.2.2.2. CHOLESTEROL FUNCTION

Under the classical model of surfactant function, cholesterol and unsaturated phospholipids are thought to act as fluidisers, aiding the spreading of surfactant over expanding alveolar surfaces during inspiration. Upon expiration, the alveolar compression is thought to excluded unsaturated phospholipids and probably cholesterol from the surface film (Figure 1.2). The resulting surface film becomes enriched in DPPC molecules that are packed tightly together to effectively exclude water molecules from the air-liquid interface and thus, lowers surface tension. As the alveoli expand during inspiration, cholesterol and unsaturated phospholipids are adsorbed or retrieved from surface-associated reservoirs and the aqueous layer, and reinsert into the surface film (Schürch et al., 2001). Based on lipid bilayer biophysical studies, cholesterol, due to its bulky ring structure, is predicted to force the mechanical separation of phospholipid headgroups as it inserts into the DPPC-rich surface film, thereby, disrupting the Van der Waals forces existing between adjacent fatty acid chains and lowering the phase-transition temperature of the surfactant mixture and thus, increasing fluidity (Orgeig and Daniels, 2001; Presti et al., 1982). Since a greater proportion of the surfactant PL would be below their phase-transition temperatures at colder body temperatures, this fluidising function of cholesterol would seem particularly important in torpid or hibernating animals. However, recent discoveries, together with the observations of the present

study, indicate that this explanation for the role of cholesterol in surfactant is far too simplistic.

Recently, cholesterol has been found to influence the domain structure of surfactant monolayers. Most interesting, is the discovery that cholesterol can generate a critical point in the phase behaviour of surfactant films at an air-liquid interface (Discher et al., 1999a). At low surface pressures, gel-like liquid-condensed (LC) domains appear. However, when a specific critical surface pressure is reached during compression, these gel-like LC domains disappear and do not appear again as surface pressure continues to increase (Nag et al., 2000). Natural extracts of surfactant, containing 2-7% cholesterol, also demonstrate this lipid mixing behaviour at around 45 mN m^{-1} (Nag et al., 2000). The main liquid-expanded (LE)-liquid condensed (LC) phase transition for DPPC itself lacks such a critical point, which suggests that cholesterol is permeating the condensed domains and inducing significant structural changes (Discher et al., 2002). There is also evidence to suggest that cholesterol can redistribute phospholipids in the films and allows phospholipids, other than DPPC, to be part of condensed domains (Discher et al., 2002; Discher et al., 1999a). Hence, the role of cholesterol in surfactant is much more complex than simply the maintenance of fluidity.

Here, I observed significant differences in the structure of liquid-condensed (LC) and liquid-expanded (LE) domains in surfactant films isolated from warm-active and torpid dunnarts and warm-active and hibernating ground squirrels, at 23°C. Surfactant films from torpid dunnarts and hibernating ground squirrels had greater % area coverage of larger LC domains at 10-30 mN m^{-1} surface pressures than surfactant films from warm-active animals. In whole lavage samples from ground squirrels and dunnarts, the greater number and size of LC domains in surface films correlates with higher levels of CHOL. This observation suggests that cholesterol may aid in the sorting of phospholipids, and subsequent formation of LC domains, in the surface film of torpid

and hibernating animals. Current evidence suggests that variation of cholesterol levels may provide a mechanism by which biological systems can control the sorting of its constituents in surface films (Discher et al., 2002). Hence, a difference in cholesterol concentration may explain the different domain structure we observed between surfactant films from warm-active and torpid or hibernating animals.

However, despite the lack of differences in cholesterol levels, a greater % area coverage of larger LC domains was also observed in surface films prepared from large aggregate surfactant fractions isolated from torpid dunnarts than from warm-active dunnarts. Although this observation does not rule out the importance of cholesterol in reorganising the molecular structure of surfactant films, it does suggest that a more complex explanation, than simply a change in cholesterol level, is required to explain the differences in film structure observed. One possible explanation is that changes in body temperature, even without changes in phospholipid or cholesterol composition, alter the complex interactions between cholesterol and phospholipids and thus, the domain structure of the surfactant films. Other factors, such as the level of surfactant proteins (Section 7.3), may also contribute to the differences in film structure and activity observed between surface films prepared from surfactant isolated from warm-active and torpid dunnarts or warm-active and hibernating ground squirrels.

7.3. TORPOR AND SURFACTANT PROTEIN COMPOSITION

Total protein levels did not change significantly in lavage fluid isolated from warm-active and torpid dunnarts and bats. However, total protein was significantly higher in surfactant isolated from hibernating ground squirrels than warm-active squirrels. These increases in protein are probably due to increases in serum proteins during hibernation, which may result from a down-regulation of alveolar protein clearance or alveolar

degradation pathways, during hibernation. Alternatively, increases in protein may be due to inflammation or damage to the airway epithelium. Increases in serum proteins in lavage also occur during viral infection and in lung diseases, such as asthma, that cause inflammation (Heeley et al., 2000; Kerr and Paton, 1999) and these increases in protein probably result from the disruption of airway epithelial cell junctions and the infiltration of plasma proteins into the alveoli (Heeley et al., 2000). However, in asthma, such increases in protein are accompanied by changes in molecular species of PL, due to the infiltration of plasma phospholipids, as well as proteins (Heeley et al., 2000). In hibernating squirrels, the observed increase in lavage protein was not accompanied by major changes in the molecular species of phospholipids, which may indicate that increases in protein in hibernating squirrels are not due to inflammation of respiratory surfaces. Therefore, increases in protein during hibernation are more likely due to decreased protein clearance from the lung, or to a decrease in recycling or degradation of extracellular proteins, which in turn result from the reduced metabolic rate.

In terms of interpreting the in vitro biophysical studies, the observed increase in alveolar protein in lavage of hibernating squirrels is irrelevant, because most of this protein remains in the aqueous layer after lipid extractions. However, the significance of the increase in alveolar protein to surfactant and lung function *in vivo*, during hibernation in squirrels is currently unknown. High levels of protein could potentially affect the behaviour and properties of the surfactant lipids and thus, the function of pulmonary surfactant in the lung. Serum proteins increase significantly during acute respiratory distress syndrome and acute lung injury in humans and rabbits and can inactivate replacement lung surfactants (Warriner et al., 2002). However, transgenic mouse studies indicate that SP-A and SP-B offer protection against the inhibition of surfactant films by plasma proteins (Kerr and Paton, 1999; Palaniyar et al., 2001). Furthermore, hibernating

ground squirrels have no apparent signs of surfactant dysfunction and obviously survive the hibernating season.

The possibility also exists that the differences in protein levels observed between warm-active and hibernating squirrel lavage fluid may have affected the binding of surfactant proteins in their respective ELISAs. Kerr and Paton (1999) investigated whether SP-A, SP-B and SP-D assays were affected by different concentrations of lavage proteins by performing ELISAs in the presence or absence of 1 mg per ml human albumin. They found that significant interference by other proteins was unlikely.

7.3.1. SURFACTANT PROTEINS

Table 7.3 shows the amounts of surfactant proteins in bat, squirrels and dunnarts relative to total phospholipid (PL). However, differences in sample preparation, and possibly antibody reactivity, prevent a direct comparison between protein levels in bats, squirrels and dunnarts. Squirrel samples were collected in Canada, frozen, freeze-dried (as per quarantine guidelines) and transported to Australia, before they were analysed. Whole lavage samples from individual bats had to be pooled to obtain enough surfactant for measurement of surfactant proteins and only the large aggregate, surface-active fractions of dunnart surfactant were analysed for protein content. Preparation of large aggregate fractions essentially concentrates the hydrophobic surfactant proteins relative to PL. However, despite differences in sample preparation between species, differences in protein content in lavage fluid between warm-active and torpid or hibernating animals can be directly compared.

Table 7.3. Surfactant proteins A, B and C in surfactant isolated from warm-active and torpid or hibernating bats, dunnarts and ground squirrels.

Animal	T_b	SP-A	SP-B	SP-C
	°C	ng/mg PL	ng/mg PL	ng/mg PL
Squirrel	37	119.74 ± 21.4	19.51 ± 0.79	606.42 ± 127.4
n = 6-8	7	56.14 ± 9.3[*]	22.77 ± 3.39	1072.5 ± 245.7[^]
Dunnart LA	37	113.61 ± 23.66	488.17 ± 64.80	607.3 ± 89
n = 7-8	15	56.19 ± 8.17[*]	444.19 ± 48.67	1009.3 ± 152[^]
Bat	37	728.57 ± 298.5	396.83 ± 102.12	11358 ± 950.2
n = 3-4	15	813.4 ± 170.6	553.98 ± 178.82	13945 ± 1972

Surfactant proteins were measured in lavage samples isolated from warm-active and torpid or hibernating animals using ELISAs described in Section 2.7. Values are mean ± SE. n number of animals. Statistical significance was determined using Student's t-tests, assuming equal variance, significance at $p < 0.05$. * significant decrease in lavage isolated from torpid or hibernating animals compared to warm-active animals. ^ significant increase in lavage isolated from torpid or hibernating animals compared to warm-active animals.

7.3.2. HYDROPHILIC SURFACTANT PROTEINS

SP-A and SP-D are hydrophilic, carbohydrate binding proteins that play an important role in the innate immune defence of the lung. Studies in knockout mice, suggest that SP-D may also play an important role in surfactant homeostasis (Korfhagen et al., 1998). However, in lung lavage, while SP-A is mostly associated with surfactant lipids, SP-D is not (Haagsman and Diemel, 2001). Hence, SP-D is not generally considered a 'true' surfactant protein (Haagsman and Diemel, 2001) and was not studied here. In addition to its immune functions, SP-A also regulates surfactant release, is essential for formation

of tubular myelin and is thought to reorganise DPPC in adsorbed films of pulmonary surfactant lipid extracts (Yu and Possmayer, 1996). Here I observed that SP-A was significantly lower in surfactant isolated from torpid dunnarts and hibernating squirrels, than warm-active dunnarts and squirrels.

Due to the diverse roles of SP-A in the lung, there are many possible explanations for the observed decrease in SP-A. Decreases in SP-A may indicate the down-regulation of SP-A gene expression or translation during torpor or hibernation. Gene expression has been observed to decrease for some proteins during hibernation (Van Breukelen and Martin, 2002) and increase for others (Gorham et al., 1998) and there are differential changes in the control of enzymatic activity through phosphorylation or via the sequestering of enzymes (Van Breukelen and Martin, 2002). Low temperatures can also destabilise the hydrophobic interactions required to maintain proper protein conformation, and thus increase the potential for protein denaturation (Somero, 1995). Hence, it is possible that temperature can influence the rates of SP-A synthesis, activity and/or degradation without influencing the synthesis, activity and/or degradation of the other surfactant proteins.

SP-A provides the framework for the formation of tubular myelin (Voorhout et al., 1991) and hence, decreases in SP-A, may also indicate a decrease in tubular myelin in the aqueous hypophase. However, the observed decrease in SP-A in surfactant isolated from torpid animals does not appear to hamper the function of surfactant at 23°C or the formation of condensed domains, as determined using the Wilhelmy-Langmuir surface balance, epifluorescence microscopy and atomic force microscopy. Furthermore, SP-A knockout mice (Korfhagen et al., 1996) show no signs of respiratory problems or surfactant dysfunction. Therefore, the main function of SP-A is thought to be its role in innate host defence (Haagsman and Diemel, 2001).

SP-A can interact with a number of viruses, bacteria, fungi and inhaled allergens by targeting them for phagocytosis by alveolar macrophages (Benne et al., 1997; Crouch, 1998; Haagsman, 1998; Reid, 1998; Wang et al., 1996; Wange et al., 1996). Hence, the decrease in SP-A observed during torpor or hibernation, could lead to a reduced lung immunity and increased susceptibility to infection. Decreases in another collectin, the mannose binding lectin (Wright, 1997) have been shown to increase susceptibility to viral infection in the lung. Furthermore, decreases in SP-A also occur in cystic fibrosis and other lung diseases with profound consequences for the host defence response of lungs to bacterial infection (Postle et al., 1999).

Conversely, the reduction in SP-A observed in lavage isolated from torpid or hibernating animals may reflect an increase in the binding of SP-A to viruses, bacteria or allergens and the subsequent destruction of both the particle and SP-A by alveolar macrophages during torpor or hibernation. The epithelial surface of the lungs is large and continuously exposed to the environment (Haagsman and Diemel, 2001). Even during the non-ventilatory periods experienced during torpor and hibernation in dunnarts and ground squirrels, respectively, there is a residual volume of air in the lungs (Milsom and Reid, 1995). This residual volume of air could potentially contain harmful bacteria or allergens. During torpor or hibernation, the immune system of heterothermic mammals may be depressed, along with metabolic rate. Hence, the innate, non-specific defence mechanism of SP-A may be particularly important in torpid or hibernating animals in eliminating microorganisms in the lung before they lead to infections or disease. However, currently, there is insufficient evidence to confirm either of these speculations.

7.3.3. HYDROPHOBIC SURFACTANT PROTEINS

As mentioned above, a direct comparison between species is restricted by differences in sample preparation and potentially, antibody reactivity. However, it is intriguing that levels of the surfactant proteins, SP-A and SP-C appear to be higher in whole lavage fluid isolated from bats than in squirrels or in the dunnart LA fraction, especially given that the hydrophobic protein, SP-C, should be relatively concentrated in the dunnart LA preparations. Although this phenomenon needs further examination (bat samples had to be pooled, and hence sample size is small), this may indicate a specific role for SP-C in bat surfactant. Bat surfactant contains very little cholesterol relative to that of other mammals (Codd et al., 2002), and higher levels of SP-C may indicate that this protein has a particular role in controlling fluidity and film structure in bat surfactant. However, although intriguing, until a more direct comparison can be made, this idea is purely speculative.

SP-B and SP-C are hydrophobic proteins that isolate into the LE phase of surfactant films. SP-B is thought to aid in monolayer collapse and SP-C is thought to aid in the fast respreading of the lipid materials (Krol et al., 2000). In humans, SP-B deficiency syndrome is fatal (Nogee et al., 1993) and SP-B deficient knockout mice die at birth (Cole et al., 2001; Whitsett et al., 1995). Therefore, SP-B is essential for lung function. In most surfactants, SP-B is more effective than SP-C at facilitating the selective adsorption of DPPC into the surface layer and the squeeze-out of more fluid lipid components of the surface film during compression (Possmayer et al., 2001). Hence, I hypothesised that the amount of SP-B would increase significantly during torpor or hibernation in order to improve the formation and stabilisation of surface monolayers at cold temperatures. However, levels of SP-B, relative to PL did not change between surfactant isolated from warm-active and torpid or hibernating mammals and

this implies that SP-B levels in warm-active animals are sufficient for adequate function at the lower temperatures, during torpor or hibernation.

The SP-C levels observed in this study seem extraordinarily high. For warm-active animals, ratios of SP-A : SP-B : SP-C are 1.0 : 0.16 : 5.05 in squirrel lavage, 1.0 : 4.3 : 5.3 in large aggregate dunnart fractions and 1.0 : 0.5 : 15.6 for bat lavage. Most other investigators find SP-C content to be only a fraction of SP-A content (Ballard et al., 2003; Danlois et al., 2003). For example, in human children, SP-A: SP-B : SP-C ratios are 1.0: 0.1 : 0.3 (Friedrich et al., 2003). Hence, SP-C levels in the heterothermic mammals studied here requires further investigation. However, it is possible that high levels of SP-C may be important in maintaining a functioning surfactant system in heterothermic mammals that experience changes in body temperature.

Unlike SP-B, SP-C is not essential for lung function in the short-term. However, SP-C knockout mice do show abnormalities in surfactant function (as demonstrated by abnormal lung hysteresis) at low lung volumes (Glasser et al., 2001). The efficient function of surfactant at low lung volumes would be particularly important in torpid or hibernating animals, because lung hysteresis and the work required to inflate the lung both increase, as compliance decreases (Langman et al., 1996; Milsom et al., 1999). Here we observed that SP-C levels, relative to PL, significantly increased during torpor in dunnarts and hibernation in ground squirrels. Hence, SP-C may have a role in stabilising surfactant films at low lung volumes during torpor or hibernation.

Furthermore, spectroscopic studies of SP-C show that it disorders the acyl chain region of lipid bilayers and unlike SP-B, which can increase the phase-transition (T_c) of disaturated PL (Baatz et al., 1990), SP-C lowers the T_c of several disaturated PL (Horowitz, 1995; Horowitz et al., 1992). Hence, SP-C is thought to be a fluidiser of surfactant and as such, may improve the respreading and adsorption of surfactant films (Wang et al., 1996) and thus, surfactant function in torpid or hibernating animals.

Epifluorescence and atomic force microscopy studies show that SP-C can increase the number of condensed domains without having a major impact on the surface area covered by the domains (Possmayer et al., 2001). Hence, increases in SP-C during torpor or hibernation may act to increase surfactant fluidity and thus, maintain surfactant function at low temperatures.

Another potential role for SP-C is to promote the lateral separation of surface films into DPPC-enriched and fluid lipid-enriched domains and as such, facilitate the squeeze-out of more fluid components. Captive bubble surfactometer (CBS) studies suggest that SP-C is involved in formation of surface-associated reservoirs, which would provide a destination for unsaturated PL excluded from the monolayer during compression (Possmayer et al., 2001). Surface-associated reservoirs also function as a source of lipids for re-adsorption to the surface film during alveolar expansion. Hence, the increases in SP-C during torpor could potentially aid in the squeeze-out of fluid lipids during compression by aiding the formation of surface-associated reservoirs, which may in turn, stabilise the remaining surface film. In addition, it has been postulated that SP-C has a special role in reincorporating surfactant material back into the monolayer, from the surface-associated reservoirs, during expansion (Possmayer et al., 2001). Hence, increasing SP-C during torpor, could also aid the reformation of the surface-active film when the lungs eventually do expand periodically during torpor or during periodic arousals in ground squirrels. However, until SP-C levels are investigated further in these animals, these possible functions of SP-C in heterothermic mammals are purely speculative.

7.4. TORPOR AND SURFACTANT FILM STRUCTURE

Irrespective of the torpor pattern displayed, epifluorescence microscopy and atomic force microscopy of monolayers prepared from surfactant isolated from warm-active and torpid or hibernating dunnarts and squirrels, both demonstrate that warm-active films consist of more fluid or liquid-expanded regions than surfactant from torpid or hibernating animals at 23°C. This was a somewhat surprising result because I hypothesised, given the phase-transition temperatures of lipids (T_c), that warm-active surfactant would be much less fluid at colder temperatures (23°C) than torpid surfactant.

However, this hypothesis was based on the belief that surfactant composition would change during torpor to increase surfactant fluidity at cold temperatures. Yet, I did not observe any major changes in the molecular species, or saturation of each of the PL, between surfactant isolated from warm-active and torpid animals, to indicate an increase in fluidity. Hence, the phase-transition of the surfactant phospholipids may not be as important to surfactant function, as previously surmised.

Levels of cholesterol and SP-C, which are thought to act as fluidisers of surfactant and can lower the T_c of lipid mixtures, did change between surfactant isolated from warm-active and torpid or hibernating dunnarts and squirrels. However, both cholesterol and SP-C were higher in the less fluid surfactant isolated from torpid or hibernating animals, than in the more fluid surfactant isolated from warm-active animals. Thus, increases in cholesterol and SP-C correlate with a greater number of liquid-condensed (LC) domains and not a greater fluidity in surfactant films, when measured at 23°C. Although this observation contradicts the classical understanding of cholesterol as a fluidiser of surfactant, it does provide further evidence that cholesterol plays a role in the redistribution of PL and in altering the structure of the surfactant film during torpor.

Alternatively, or in addition, the increases in SP-C may aid in the formation and stabilisation of surface-associated lipid reservoirs, and hence, provide a destination point

for unsaturated lipids and cholesterol, as they are excluded from the monolayer during compression (Johansson, 1998). In 1972, King and Clements detected that the 'squeeze-out' plateau and surface compressibility of films prepared from canine surfactant, changes abruptly over a temperature range of 30-35°C (King and Clements, 1972). These changes may be due to an effect of temperature on the formation of surface-associated reservoirs through changes in lipid-protein interactions and lipid packing. Consequently, at 23°C, films prepared from surfactant isolated from warm-active animals are more fluid and require greater compression to achieve ST_{min}. Increasing the proportion of SP-C, during torpor or hibernation, may help maintain or improve the formation of surface-associated reservoirs and hence, the 'squeeze-out' of unsaturated lipids and cholesterol, at lower temperatures. The greater fluidity of warm-active surfactant at 23°C probably hampers the surface-tension lowering properties and can explain our observations that surfactant isolated from warm-active animals is less 'surface-active' than torpid surfactant in the Wilhelmy-Langmuir surface balance, at 23°C. On the other hand, films made of surfactant isolated from torpid animals were able to generate large, persistent probe-excluded liquid-condensed domains in the surface film and hence, maintain surface-tension lowering functions at 23°C.

7.4.1 LIQUID-CONDENSED DOMAINS

In atomic force microscopy studies, films prepared from bovine lipid extract surfactant (BLES) generate floral-like domains with a height of approximately 1.2 nm (Figure 1.6). In contrast, although the same conditions and instrumentation were used in the present study, warm-active and torpid dunnart and squirrel surfactant films exhibited small, roughly circular domains with heights of 0.8–1 nm. These intermediate domains (labelled LC in Figure 5.18), were significantly larger in diameter in torpid dunnart surfactant films than in warm-active dunnart surfactant films. Domains of an

intermediate height step of 0.8 nm are also generated in pure DPPC monolayers under the same conditions (Figure 1.6). However, these pure DPPC domains have a distinctive kidney bean-like shape and fluorescent appearance. In dunnart and squirrel surfactant films, the domains were circular in shape. Moreover, torpid dunnart and squirrel surfactant films contained a second condensed region (labelled 2LC in Figure 5.18), of even greater height (~1.6 nm) that was located within the intermediate domains.

To my knowledge, this is the first study to report both the presence of intermediate height domains and the presence of double condensed domains in pulmonary surfactant. Although chemical evidence is lacking, the highest domains are probably similar in nature (A.K. Panda, personal communication) to the 1.2 nm DPPC-enriched domains observed with BLES (Schürch et al., 1998). Hence, it is possible that the intermediate domains represent a monolayer equivalent of the liquid ordered (L_0) phase observed with cholesterol-containing bilayers (F. Possmayer, personal communication). The L_0 domains, which are thought to be the liquid basis of plasma membrane rafts and caveolae, have often been detected in bilayer systems containing saturated PCs and/or sphingomyelin (Yu and Possmayer, 2003). This would suggest that the ~10% cholesterol content of dunnart surfactant has the ability to mix with DPPC-rich domains of ~1.2 nm height and convert them to intermediate phase entirely (warm-active surfactant) or in part (torpid surfactant). Studies with calf lung surfactant extracts (CLSE) have shown that monolayers at 30 mN m^{-1} require at least 11% DPPC to form probe-excluded fluorescent domains, a value not dissimilar to the 17% DPPC content of dunnart surfactant. Whether the smaller size of the liquid-condensed (LC) domains is related to the absence of the second, even higher liquid-condensed (2LC) domains require further study.

Currently, the relationship between the LC domains present in surfactant films and the ability of surfactant to generate the low surface tensions required to stabilise the

lung is not clear (Harbottle et al., 2003). However, Bangham (1987) suggests that the LC regions prevent the alveolar walls from collapsing with decreasing volume or area, by acting as splints in the alveolar wall. This hypothesis is based on the assumptions that the inside surface of each alveolus is made of many small flat surfaces and that each side of the alveolar wall provides a surface on which the splints reside (Nag et al., 1998). The resulting network of solid and fluid regions is finely divided, such that it is reminiscent of a metal alloy. At high pressures, if such a two-dimensional metal alloy exists in the film, then it would simultaneously strengthen the film and give moderate flexibility. Although this hypothesis is purely speculative, such characteristics would be of particular significance to lung stability at low lung volumes (Nag et al., 1998), as occurs under conditions of lung movements and dynamic volume changes (Nag et al., 1998) and during torpor or hibernation.

7.5. COMPARATIVE BIOLOGY OF MAMMALIAN SURFACTANT LIPID COMPOSITION

7.5.1. SATURATION OF PHOSPHOLIPIDS

The overall percent saturation of PLs in surfactant isolated from heterothermic mammals does not appear to differ from that of homeothermic mammals (Table 7.4). %DSP/PL measurements of saturation are generally 40-50% for both heterothermic and homeothermic mammals. Moreover, while %DSPC/PC measurements are up to ~60% in rat, human and tammar wallaby, they are 40-50% for all other homeothermic and heterothermic animals studied. In both heterothermic and homeothermic mammals, the PG and PI components are enriched in unsaturated molecular species. However, the ratio of %DSPG/PG does vary slightly between species. Saturation of PG species is lowest in the Tasmanian devil (2%), human and bat (~8%) and highest in the rat (34%).

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With the exception of the guinea pig (10%), the saturation of PI is very low (< 5%) in both heterothermic and homeothermic mammals. Hence, although there is some variation between individual animal species, there are no clear distinctions in the saturation levels of total PL, PC, PG or PI between homeothermic and heterothermic mammals. Hence, the saturation of surfactant does not appear to be thermally adapted in heterothermic mammals. This suggests that the overall saturation of surfactant PL, and specifically, the saturation of PC, PG and PI components, in all mammals, is sufficient for maintaining surfactant function at both warm and cold body temperatures.

Table 7.4. Comparative biology of mammalian surfactant: Saturation of surfactant PL

Animal	T_b	%DSP/PL	%DSPC/PC	%DSPG/PG	%DSPI/PI
Homeothermic					
Rat,	37	45.7[a]	61.0[#d]		
R. norvegicus			59[g]	34[g]	4[g]
Mouse,	37	44.5[b]			
M. musculus					
Human,	37	48.5[c]	68.0[#d]		
H. sapiens			67[g]	8[g]	2[g]
Rabbit	37		48[g]	25[g]	2[g]
Oryctolagus sp.					
Guinea pig	37			21[g]	10[g]
Cavia sp.					
Tammar Wallaby	37		60.4	14.4	4.3
M. eugenii					
Tasmanian Devil,	37		34.8	1.9	0.7
S. harrisii					
Wombat,	37		44.0	17.0	1.6
L. latifrons					
Koala,	37		49.6	14.2	3.6
P. cinereus					
Heterothermic					
Dunnart	35	40.2[b]	39.2	25.6	0.3
S. crassicaudata	15	49.3[b*]	38.8	31.1[*]	1.3
Dunnart LA	35		47.0	24.0	3.13

S. crassicaudata	15		44.0[*]	23.5	1.9
Bat,	35	44.1[e]	46.4	9.9	1.4
C. gouldii	15	44.0[f]	43.4	7.0[*]	0.5
Bat,	32	45[h]			
N. geoffroyi	24	45[h]			
Squirrel,	37	39.6	42.0	10.9	3.6
S. lateralis	7	39.1	35.1	13.3	3.4

T_b body temperature, PL phospholipid, DSP disaturated PL, DSPC disaturated phosphatidylcholine, PC phosphatidylcholine, DSPG disaturated phosphatidylglycerol, PG phosphatidylglycerol, DSPI disaturated phosphatidylinositol, PI phosphatidylinositol, # whole lung extracts. Data are from Chapters 3, 4, 5 and 6, unless otherwise indicated. *a* (Hallman and Gluck, 1975), *b* (Langman et al., 1996), *c* (Neumann et al., 1990), *d* (Daniels et al., 1995a), *e, f* (Codd et al., 2002) where T_b is *e* 32°C and *f* 24°C, *g* estimated from figures 1, 2 and 3 in (Postle et al., 2001), *h* (Slocombe et al., 2000). * significant difference between warm-active and torpid animals.

7.5.2. PHOSPHATIDYLCHOLINE

Although there are no clear trends in patterns of saturation of the surfactant PL, and specifically PC, PG and PI, between homeothermic and heterothermic mammals, variations in the individual molecular species compositions of these surfactant components may still play an important role in the thermal dynamics of the surfactant system. As mentioned previously, we currently have little understanding of how all the different lipids in surfactant interact with each other and the surfactant proteins, or how they are sorted into different domains in surface-active films. This and later sections discuss the molecular variations in PC, PG and PI composition between different

animals, or groups, and highlights those which may be thermal adaptations of the surfactant system.

Figure 7.1 summarises the molecular components that contribute greater than 5 mol% to surfactant PC, for each homeothermic and heterothermic mammal studied so far. Clearly, dipalmitoylphosphatidylcholine (DPPC or PC16:0/16:0) is the predominant component of surfactant in most mammals studied. However, despite its classically 'important' role in surfactant function, the proportion of DPPC varies widely, from 17-64 mol% in different mammalian animals. At its lowest concentrations (e.g. in surfactant isolated from the marsupial dunnart), DPPC is exceeded by the mono-unsaturated 1-palmitoyl-2-palmitoleoyl phosphatidylcholine (PPPC or PC16:0/16:1), as the dominant molecule.

The enrichment of the unsaturated species, PPPC (PC16:0/16:1), relative to DPPC (PC16:0/16:0) in heterotherms compared to homeotherms is perhaps the most notable trend in the molecular PC compositions of heterothermic and homeothermic mammals. The average proportions of DPPC and PPPC in surfactant isolated from homeothermic and heterothermic mammals are calculated in Figure 7.2. The Tasmanian devil is excluded from these calculations due to its highly unusual molecular composition, which is discussed in section 7.5.4. Given the wide variation between animals, mean DPPC levels were not statistically different in heterothermic mammals compared to homeothermic mammals. However, mean PPPC levels were significantly higher in heterothermic mammals compared to homeothermic mammals (Figure 7.2). Although sample size is currently small in some groups, this trend is also apparent in Figure 7.3, when the homeothermic and heterothermic mammals are split into placental and marsupial mammalian groups. Homeothermic marsupial mammals, which experience a heterothermic stage during early development, also seem to have higher PPPC levels and lower DPPC levels than observed in placental mammals (Figure 7.3).

Figure 7.1. Comparison of molecular phosphatidylcholine (PC) composition for each mammal studied. Molecular species are included if they comprised over 5 mol% in any animal studied. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively; while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Mol% values for rat[b] and pig estimated from (Bernhard et al., 2001). Mol% values for human, rat[c], rabbit and mouse estimated from (Postle et al., 2001) and wombat, koala and tammar wallaby surfactant are taken from Chapter 6. Heterothermic values are calculated from mol% values reported for squirrel (Chapter 3), bat (Chapter 4) and dunnart (Chapter 5) surfactant.

Figure 7.2. Comparison of the two major molecular species of phosphatidylcholine (PC16:0/16:0 and PC16:0/16:1) between homeothermic and heterothermic mammals. Data are mean ± S.E. n number of species. Homeothermic placental mammal values are calculated from mol% values reported for human, pig, rat, rabbit and mouse surfactant (Bernhard et al., 2001; Postle et al., 2001). Homeothermic marsupial mammal values were calculated from mol% values reported for wombat, koala and tammar wallaby surfactant (Chapter 6). Heterothermic values are calculated from mol% values reported for squirrel (Chapter 3), bat (Chapter 4) and dunnart (Chapter 5) surfactant. Statistical significance between homeothermic and heterothermic mammals was determined using Student's unpaired t-tests, assuming equal variance and statistical significance was set at $p < 0.05$. PC16:0/16:1 ($t_{stat} = 1.434$, df = 10, p = 0.091); PC16:0/16:1 ($t_{stat} = 1.976$, df = 10, p = 0.038). * significant difference between homeothermic and heterothermic mammals.
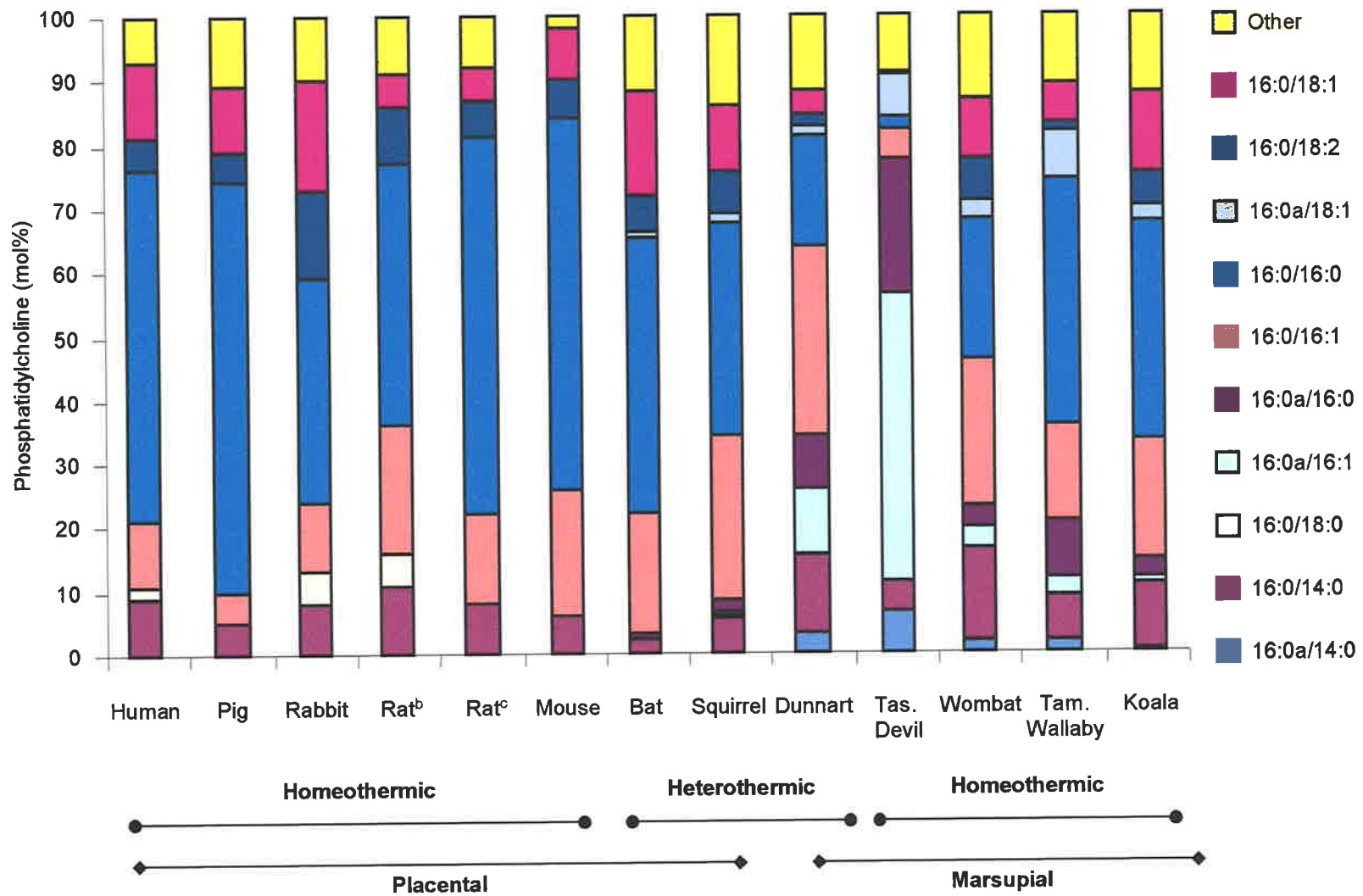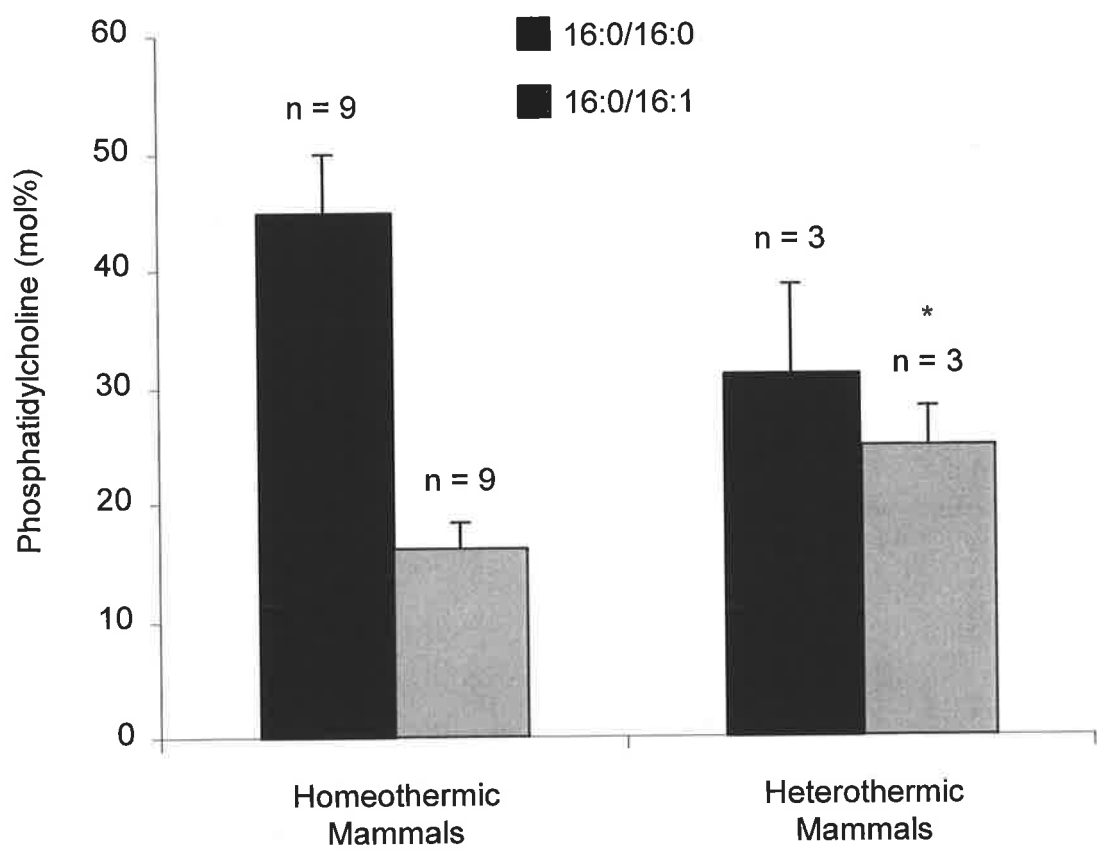
Figure 7.3. Comparison of the two major molecular species of phosphatidylcholine (PC16:0/16:0 and PC16:0/16:1) between different mammalian groups. Data are mean ± S.E. n number of species; when n = 2, error bars denote a range. Homeothermic placental mammal values are calculated from mol% values reported for human, pig, rat, rabbit and mouse surfactant (Bernhard et al., 2001; Postle et al., 2001). Homeothermic marsupial mammal values were calculated from mol% values reported for wombat, koala and tammar wallaby surfactant (Chapter 6). Due to its major differences, the Tasmanian devil is excluded from these analyses. Heterothermic placental mammal values are calculated from mol% values reported for squirrel (Chapter 3) and bat (Chapter 4) surfactant. The value reported for the heterothermic marsupial mammal group represents dunnart surfactant (Chapter 5) only. Statistical significance was determined using Student's unpaired t-tests, assuming equal variance and statistical significance was set at p < 0.05. Pairs of symbols indicate a significant difference between each other. Statistical analyses were as follows for PC16:0/16:0: Homeothermic placental vs. Heterothermic placental (t_{stat} = 1.594, df = 6, p = 0.081); Homeothermic placental vs. Homeothermic marsupial (t_{stat} = 2.735, df = 7, p = = 0.015); Heterothermic placental vs. Homeothermic marsupial (t_{stat} = 0.874, df = 3, p = 0.22); Homeothermic marsupial vs. Heterothermic marsupial (t_{stat} = 1.16, df = 2, p = 0.18). Statistical analyses were as follows for PC16:0/16:1: Homeothermic placental vs. Heterothermic placental (t_{stat} = 1.915, df = 6, p = 0.05); Homeothermic placental vs. Homeothermic marsupial (t_{stat} = 1.742, df = 7, p = = 0.06); Heterothermic placental vs. Homeothermic marsupial (t_{stat} = 0.222, df = 3, p = 0.42); Homeothermic marsupial vs. Heterothermic marsupial (t_{stat} = 1.05, df = 2, p = 0.20).
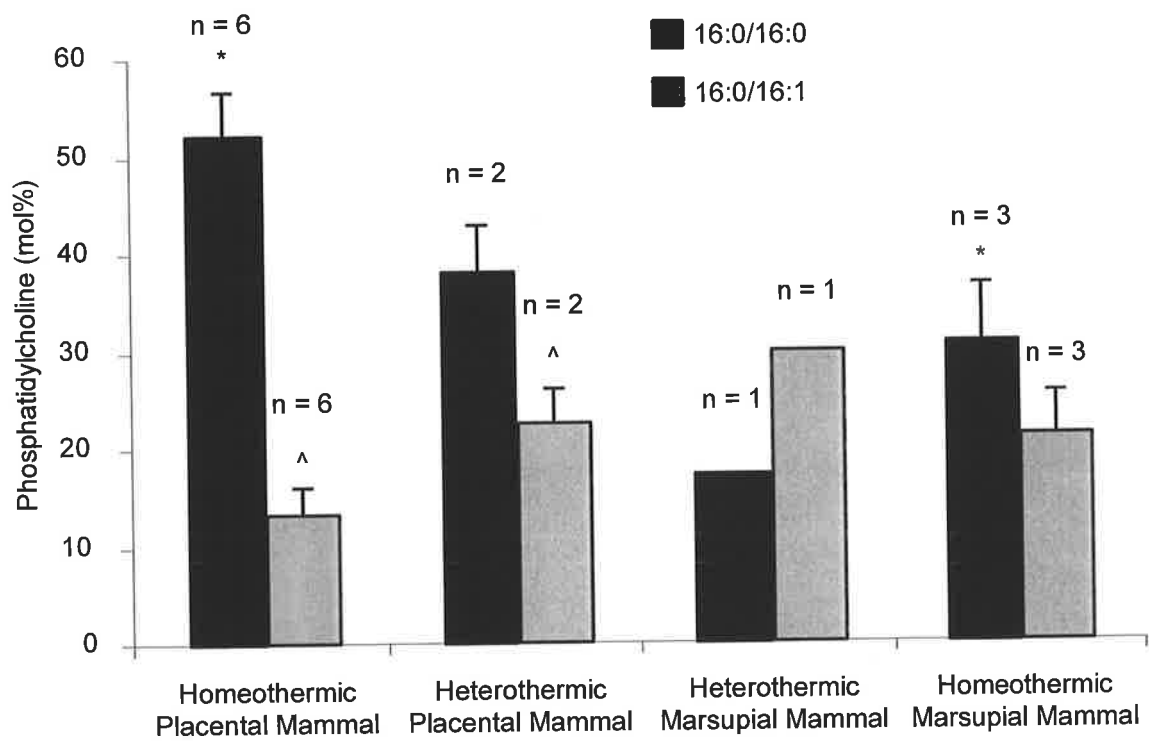
Enrichment of the unsaturated species, PC16:0/16:1, relative to PC16:0/16:0 in surfactants from heterothermic mammals may indicate that this molecule has an important role in the thermodynamics of pulmonary surfactant. From a typical understanding of lipid phase-transition, increases in the unsaturated PL, PPPC, which has a low phase-transition temperature (-7°C), could act to lower the overall phase-transition temperature of a surfactant mixture and thus, maintain surfactant fluidity over a broader range of temperatures. Increasing the fluidity of surfactant would be particularly important in maintaining the spreadability and function of surfactant at the low body temperatures experienced in torpid and hibernating mammals, or those that developing marsupials may be exposed to, before endothermy is fully developed. However, the biophysical aspects of the observed increase in PPPC relative to DPPC in heterotherms and marsupials is probably more complex, than described by typical phase-transition effects, which are based on properties of single component PL films and not complex mixtures, such as surfactant.

Recent studies, such as those by Crane and Hall (2001) and Piknova et al. (2002), suggest that lung surfactant films may possess additional and unusual phase properties, to the typical order-disorder or gel-liquid crystalline transitions which occur for any individual PL component (Nag et al., 2002). Hence, the precise biophysical aspects of increasing a particular molecular species (e.g. PPPC) relative to another (e.g. DPPC) in surfactant and the effect this has on molecular interactions, the formation and structure of domains in the surface films or even how the film responds to changes in temperature, are as yet, unknown. Nevertheless, the significant enrichment in PPPC observed in the surfactant of heterothermic mammals and marsupials, which both experience variations in body temperature at some point in their lives, suggest that increasing the proportion of PPPC may be a particular adaptation of the mammalian surfactant system to thermoregulatory plasticity.

In addition to differences in PPPC (PC16:0/16:1) and DPPC (PC16:0/16:0), the proportions of the other disaturated species, PC14:0/16:0 and PC16:0/18:0 also vary between animals. PC16:0/14:0 and PC16:0/18:0 consistently comprise some 20 mol% of total disaturated PC in rat, rabbit and human surfactants (Postle et al., 2001). However, PC16:0/18:0 is present in negligible quantities in bat, squirrel, dunnart and other marsupial surfactants (Figure 7.1). Moreover, PC14:0/16:0 comprises a much smaller proportion of the total PC component of heterothermic bat (2 mol%) and squirrel (5 mol%) surfactants, than in the surfactants of heterothermic dunnarts (12 mol%) and other homeothermic mammals, including marsupials (7-15 mol%) (Figure 7.1).

The decrease in PC14:0/16:0 and PC16:0/18:0 observed in squirrel surfactant, relative to other homeothermic placental mammals, may account for the slightly lower percent saturation of PC (35-40% DSPC) observed in squirrel surfactant compared to homeothermic placental mammals (45-55% DSPC) (Table 7.4). In dunnarts, however, the negligible proportion of PC16:0/18:0 appears to be counteracted by increases in both diacyl and alkyl-acyl forms of PC16:0/14:0. Furthermore, in bats, the 20% decrease in the disaturates, PC14:0/16:0 and PC16:0/18:0, relative to rats and rabbits, is probably counteracted, at least in part, by a small increase, relative to homeothermic placental mammals (i.e. rat, rabbit), in DPPC (PC16:0/16:0). These differences in the proportion of PC16:0/14:0 and/or PC16:0/18:0, may also be thermal adaptations of the surfactant system in heterothermic mammals.

However, the variations in the molecular components of PC observed here, namely PC16:0/16:1, PC16:0/16:0, PC16:0/14:0 and PC16:0/18:0, are not necessarily restricted to heterothermic mammals. In differentiated foetal human type II cells in culture, enrichments in both PC16:0/14:0 and PC16:0/16:1 have also been reported (Gonzales et al., 2002). The enrichment of PC16:0/14:0 and PC16:0/16:1 also occurs during rat foetal development, and then decreases until adulthood (Bernhard et al., 2001).

Hence, modifications in PC16:0/16:1, PC14:0/16:0 and PC16:0/18:0 are not confined to either heterotherms or homeotherms and thus, variations in these disaturated components are unlikely to be restricted to thermal adaptations of the surfactant system. Rather, as discussed in the following sections, these observations suggest that variations in these PC components are common adaptations to differing requirements in lung function.

7.5.3. PHOSPHATIDYLGLYCEROL AND PHOSPHATIDYLINOSITOL

Phosphatidylglycerol (PG) and phosphatidylinositol (PI) show wide variations in molecular composition and concentration across animal species and during development (Batenburg, 1992; Daniels and Orgeig, 2001). In adult human surfactant, both PG and PI are enriched in the unsaturated species 16:0/18:1, 18:0/18:1 and 18:1/18:1 and there is only a minor proportion of the disaturated species, 16:0/16:0 (Figures 7.4 and 7.5). However, surfactant from adult rats, rabbits and guinea pigs is enriched in the disaturated PG species, PG16:0/16:0 and the unsaturated PG species, PG16:0/18:2 (Figure 7.4). This study demonstrates that the molecular composition of PG and PI in surfactant isolated from heterothermic mammals and marsupials also varies between species.

7.5.3.1. PHOSPHATIDYLGLYCEROL

In this study, as for other homeothermic mammals, surfactants isolated from the squirrel, bat and dunnart were dominated by the unsaturated molecular species, PG16:0/18:1. However, squirrel surfactant is also enriched in PG16:0/18:2 and PG18:1/18:1, while dunnart surfactant, like that of rat, rabbit and guinea pig surfactant is enriched in the disaturated PG species, PG16:0/16:0 and the unsaturated PG species, PG16:0/18:2 and PG16:0/18:1 (Figure 7.4). The PG component of bat surfactant shows closer similarities to human rather than to other placental or marsupial mammals. Both human and bat surfactant have significantly reduced amounts of the disaturated species, PG16:0/16:0 (5-

10 mol%) compared to other mammalian surfactants (20-30 mol%) and greater amounts of the unsaturated molecular species PG18:1/18:1 and PG18:0/18:1 (Figure 7.4). Hence, the PG molecular composition of heterothermic surfactants varies in a similar manner to other homeothermic surfactants.

However, the ratio of the unsaturated species, PG16:0/16:1, relative to the disaturated species, PG16:0/16:0, is higher in squirrel (1.6) and dunnart (1.1) surfactant than reported in homeothermic mammals (0.3-0.6). Similar enrichments of PG16:0/16:1 relative to PG16:0/16:0 are also observed in wombat (0.9) and tammar wallaby (0.9) surfactants, which experience a heterothermic stage during development. Hence, increases in PG16:0/16:1 relative to PG16:0/16:0 may represent a homeoviscous adaptation to the variations in body temperature, experienced by marsupials, squirrels and dunnarts during development, hibernation and stress-induced torpor, respectively.

Postle et al. (2001) noted that increases in concentration of PG16:0/16:0 in rat, rabbit and guinea pig surfactant compared to human surfactant, were accompanied by increases in the concentration of PG species containing more than one double bond, such as PG16:0/18:2 and suggested that this may also represent a homeoviscous adaptation. Increases in the more fluid molecule, PG16:0/18:2 may compensate for the rigidity and tightly packed state of PG16:0/16:0 molecules (Postle et al., 2001). If this is so, then squirrels and bats may take this adaptation further, by increasing concentrations of not only PG16:0/18:2, but also PG18:0/18:1 and PG18:1/18:1, without increasing the concentration of PG16:0/16:0.

However, with the exception of an increase in the ratio of PG16:0/16:1 to PG16:0/16:0, which together comprise ~30 mol% of the PG component, no other variations in molecular species occur specifically in bats, squirrels or dunnarts that do not also occur in homeothermic mammals. Hence, although they may be homeoviscous adaptations, the variations in PG molecular species observed between bats, squirrels and

dunnarts are not necessarily specific adaptations that are restricted to heterothermy or specific torpor patterns. Instead, as for PC, variations in PG composition between species probably represent common adaptations that occur in order to optimise surfactant composition to the particular physiology of each animal. Thermoregulatory plasticity is only one of many factors that may influence the molecular PG composition of an animal's surfactant system.

Figure 7.4. Comparison of molecular phosphatidylglycerol (PG) composition for each mammal studied. Molecular species are included if they comprised over 5 mol% in any animal studied. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Mol% values for human, pig, rat, rabbit and mouse estimated from (Postle et al., 2001), wombat (Chapter 6), koala (Chapter 6) and tammar wallaby (Chapter 6) surfactant. Heterothermic values are calculated from mol% values reported for squirrel (Chapter 3), bat (Chapter 4) and dunnart (Chapter 5) surfactant.
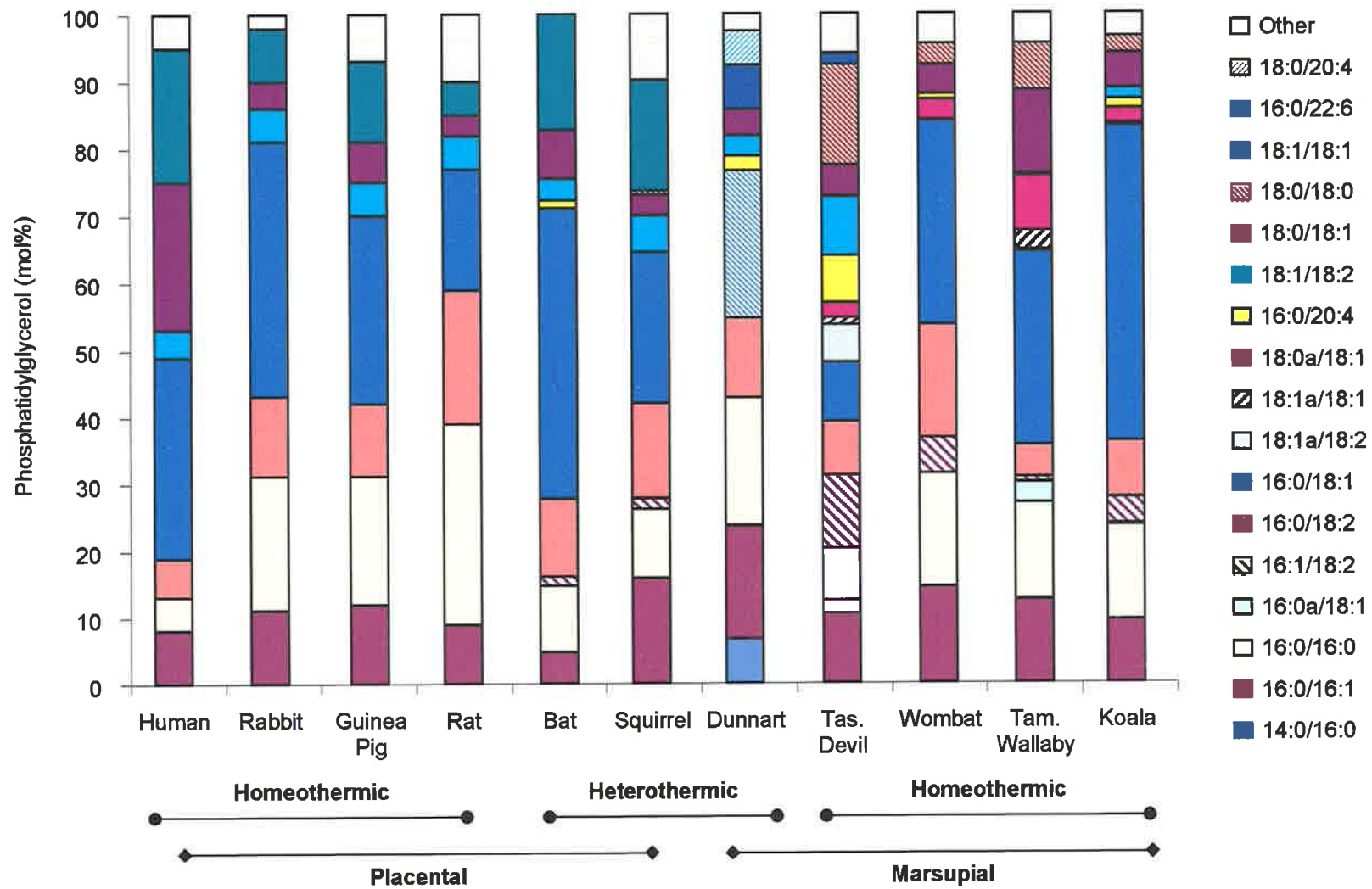
7.5.3.2. PHOSPHATIDYLINOSITOL

In rat, rabbit, human and guinea pig surfactants, the relative proportions of individual molecular species of PI vary between animal species. However, the major PI molecular species present in some, but not all, of these homeothermic surfactants include PI18:1/18:1, PI16:0/18:1, PI16:0/18:2, PI18:0/18:1 and PI18:0/20:4 (Figure 7.5). The molecular species, PI18:1/18:1, PI16:0/18:1 and PI18:0/18:1 are also dominant in squirrel surfactant. However, squirrel surfactant is also enriched in the molecular species PI16:1/18:1 and 18:1/18:2, which may represent adaptations to the thermoregulatory plasticity of ground squirrels or specifically, to hibernation.

The most dominant molecular species of PI in dunnart and bat surfactant is PI18:0/20:4. In dunnarts, PI18:0/20:4, together with PI16:0/20:4, comprise 40 mol% of the PI components. Dunnart surfactant also contains other unsaturated PL, namely, PI18:2/20:4, PI18:1/20:4, PI16:0/18:1 and PI16:1/18:1. In addition to PI18:0/20:4, PI16:0/18:1 is also enriched in bat surfactant. Furthermore, the unsaturated species PI16:0/18:2 (5 mol%) is substantially reduced in both human and bat surfactant compared to other homeothermic placental mammals (20 mol%) (Figure 7.5). However, in bats, longer chained unsaturated species, namely, PI18:2/18:2 (10-15 mol%) and PI 18:0/18:2 (10-15 mol%) may compensate for decreases in PI16:0/18:2. Hence, variations in these PI molecular species may represent thermoregulatory adaptations to heterothermy, or specifically to stress-induced torpor in dunnarts or daily torpor in bats.

However, an enrichment, in PI18:0/20:4 has also been observed in surfactant isolated from rats (Figure 7.5) and a variety of different mouse strains (Postle et al., 2001) and is also observed during inflammation in human lungs because of its presence in macrophages and neutrophils. The enrichment of highly unsaturated PI species observed in the present study in bats and dunnarts (Figure 7.5) and in rats and mice (Postle et al., 2001), may indicate that this may be a general feature of rodent and

marsupial surfactants. Although they are homeothermic, due to their small size, mice and even rats experience variations in body temperature to some degree (Randall et al., 1997). Hence, an increase in highly unsaturated PI species, such as PI18:0/20:4, may also be an adaptation of surfactant for coping with variations in body temperature. However, as for PG, other factors, in addition to temperature, are also likely to influence the molecular composition of PI in an animal's pulmonary surfactant.

As mentioned previously, Postle et al. (2001) suggested that the wide variation observed between molecular species composition of PG and PI in different species suggests that the acidic headgroups are more important to surfactant function than the precise molecular species compositions (Postle et al., 2001). However, as for PC, we have little understanding of how all the different PG and PI species in surfactant interact with other surfactant components, how they are sorted into different domains in surface-active films, or the effect of temperature on these interactions. Hence, the significance of the variation in molecular components of PG and PI should not yet be so easily dismissed. It is possible that the molecular components of PG and PI are more susceptible to the fine-tuning mechanisms (Section 7.8) that presumably act to optimise surfactant function to the specific physiology of the animal, and thus, a greater molecular variation is observed between these species.

Figure 7.5. Comparison of molecular phosphatidylinositol (PI) composition for each mammal studied. Molecular species are included if they comprised over 5 mol% in any animal studied. Molecular species are designated by the abbreviation A:x/B:y, where A and B represent the number of carbon atoms in the fatty acids esterified at the sn-1 and sn-2 positions of the phospholipid, respectively, while x and y represent the numbers of double bonds in each fatty acid. Unless otherwise indicated, all molecules are diacyl forms of molecular species (i.e. both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds). Alkyl-acyl forms of molecular species are designated with an 'a' symbol, after the number of double bonds (y). Alkyl-acyl means that one of the hydrocarbon chains is a fatty alcohol attached by an ether bond. Mol% values for pig, rat, rabbit and mouse estimated from (Postle et al., 2001), wombat (Chapter 6), koala (Chapter 6) and tammar wallaby (Chapter 6) surfactant. Heterothermic values are calculated from mol% values reported for squirrel (Chapter 3), bat (Chapter 4) and dunnart (Chapter 5) surfactant.
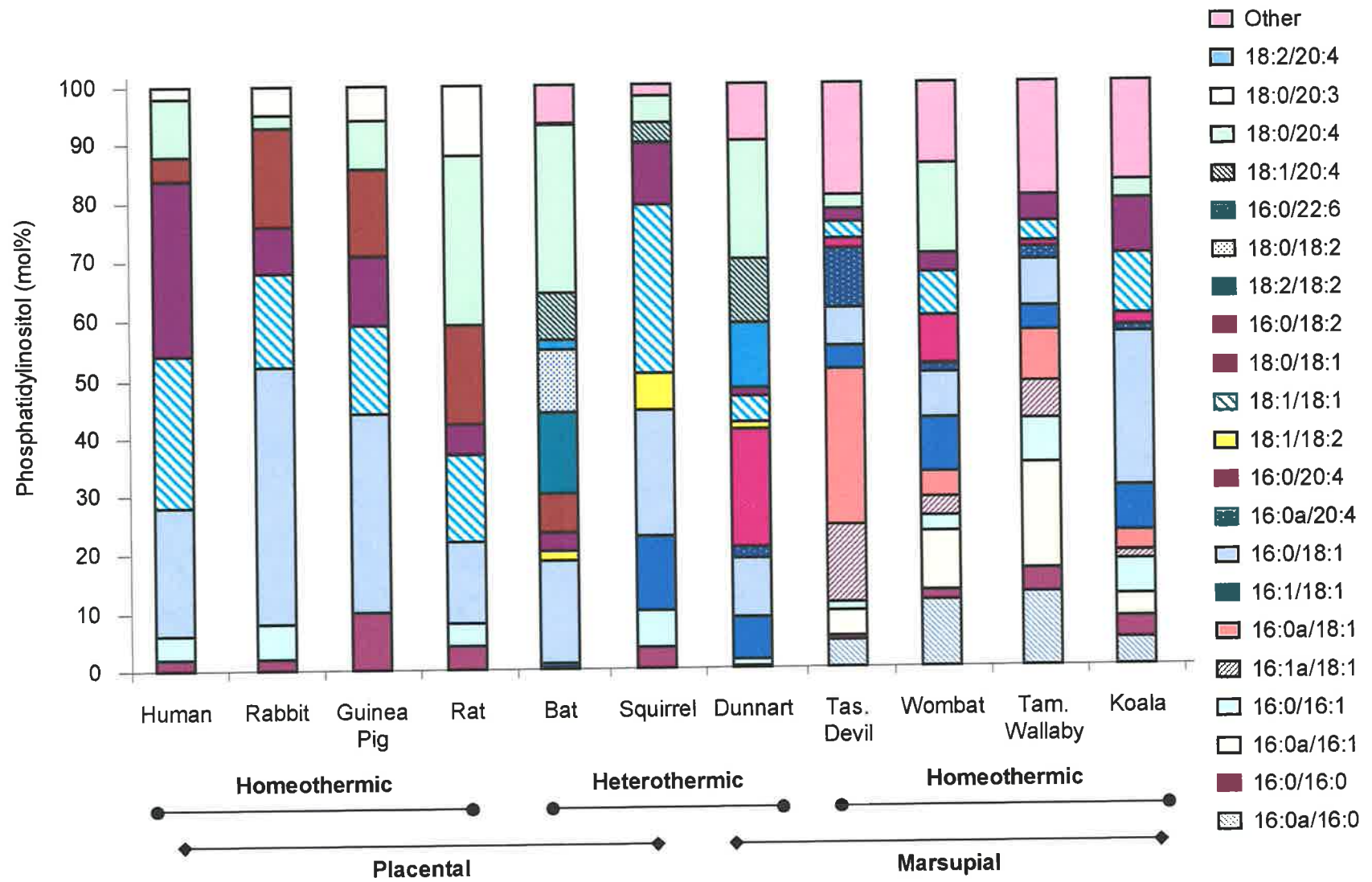
7.5.4. A NOTE ON MARSUPIAL SURFACTANT COMPOSITION

In general, marsupial surfactants are enriched in PC16:0/16:1 and PG16:0/16:1 and have a lower DPPC (PC16:0/16:0) content than placental mammals, and this may reflect the thermoregulatory plasticity they experience during development. In surfactant isolated from dunnarts and wombats, the enrichment of PC16:0/16:1 is such that, PC16:0/16:1, and not PC16:0/16:0, is the major PL component of surfactant (Figure 7.1). Hence, this study is the first to report that some mammalian surfactants do not have DPPC (PC16:0/16:0) as the major surfactant PL. However, the differences observed in dunnart surfactant compared with other heterothermic and homeothermic placental mammals, does not appear to be related to their phylogeny.

Although PC16:0/16:1 is enriched in most marsupial surfactants, in all other respects, the molecular compositions of surfactant isolated from homeothermic tammar wallabies and koalas, are similar to that in homeothermic placental mammals. In addition, the variations observed in the molecular composition of dunnart surfactant are also observed in other systems (i.e. during development) (Section 7.2). Furthermore, the composition of dunnart surfactant shares very few similarities with the composition of its closest non-heterothermic relative, the carnivorous Tasmanian devil, *Sarcophilus harrisii*. Hence, there are no differences in marsupial composition, and particularly that of dunnart composition, that can be specifically related to their phylogeny as an Australian dasyurid marsupial.

7.5.4.1. TASMANIAN DEVIL SURFACTANT

Although we were severely limited in sample size, surfactant isolated from Tasmanian devils appears to have a very different PL composition to that observed in both fat-tailed dunnarts (Chapter 5) and any other mammalian animal (Figures 7.1, 7.1 and 7.5; (Postle et al., 2001). In Tasmanian devil surfactant, the saturation of PC (Table 7.4) is lower

(34%) than reported for other mammalian surfactants (~40-50%). In addition, Tasmanian devil surfactant is comprised primarily of alkyl-acyl forms of PC, PG and PI and not the more common, diacyl forms of PLs (Figures 6.3, 6.4 and 6.5). In diacyl molecular species, both hydrocarbon chains are fatty acids attached to the glycerol backbone by ester bonds. However, in alkyl-acyl forms, one of the hydrocarbon chains is a fatty alcohol attached by an ether bond.

From a biochemical perspective, alkyl-acyl species have different locations and/or patterns of synthesis and turnover than diacyl species and are not susceptible to hydrolysis by lipases (Postle, personal communication). In terms of the biophysical properties, dialkyl species tend to have higher phase-transition temperatures than diacyl species. However, neither the phase-transition properties of alkyl-acyl species nor their role in lung surfactant have been examined. Hence, we do not know whether alkyl-acyl forms of PC function in the same way in surfactant as the more common diacyl forms of PC. Currently I have no explanation as to why the composition of Tasmanian devil surfactant is so different from other mammals. This Tasmanian devil did not show any signs of respiratory distress or surfactant dysfunction. This suggests that devil surfactant functions adequately to reduce surface tension, despite a predominantly alkyl-acyl molecular composition. Thus, alkyl-acyl forms of PC must be functioning similarly, if not as effectively, as diacyl species, in this animal. Such an important observation warrants further study.

7.5.5. CHOLESTEROL

The proportion of CHOL relative to PL in mammalian surfactant does not appear to differ between heterothermic and homeothermic mammals (Table 7.2). The neutral lipid CHOL comprises 7-10% of the PL component in both homeothermic and warm-active heterothermic mammals. Bats are the only exception to this, with very low amounts of

CHOL (0.5-2%). Low cholesterol in bats may be an adaptation to the complexity of the bat lung, their tiny alveoli or the need to maximise surface activity for increased ventilation during flight (Codd et al., 2002). Although there are no apparent differences between homeothermic and heterothermic warm-active mammals, cholesterol is modified during torpor or hibernation in heterothermic mammals. A more detailed discussion of the role of cholesterol in surfactant during torpor was given in Section 7.2.

7.6. CLASSICAL MODEL OF SURFACTANT FUNCTION

The classical dogma for surfactant function is that dipalmitoylphosphatidylcholine, or DPPC (PC16:0/16:0), is the only significant component of mammalian surfactants capable of reducing surface tension to low values during compression. It is also generally accepted that surfactant monolayers composed of nearly pure DPPC are required to stabilise alveoli and maintain lung function (Possmayer, 1991). Moreover, multiple cycles of compression and expansion of surfactant films at an air-liquid interface result in progressively lower surface tensions at minimum surface area (Possmayer, 1997). This observation led to the suggestion that monolayer compression results in squeeze-out of the more fluid PL components, leading to increased surface concentration of DPPC. The role of cholesterol and other unsaturated PL in surfactant is thus restricted to fluidising surfactant, so that it can spread over alveolar surfaces during alveolar expansion (inspiration). This classical model of surfactant function was later modified to include the concept of selective DPPC adsorption because surface area compression required to attain a surface tension near zero was considerably lower than that predicted by surfactant DPPC contents (Goerke, 1998; Perez-Gil and Keough, 1998; Schürch et al., 1998). However, a number of very recent studies challenge the classical model of surfactant function depicted in Figure 1.3.

1. Electron microscopic and surface activity studies suggest that several lipid layers are closely associated with the surface film at the air-liquid interface (Schürch et al., 2001) and therefore, the surface film may function as a multi-layer and not a simple monolayer (Goerke, 1998; Perez-Gil and Keough, 1998; Schürch et al., 1998).

2. Attempts to demonstrate selective DPPC adsorption, for example by transferring surface monolayers from adsorption to spreading surfaces, have proven unsuccessful (Yu and Possmayer, 2001).

3. Epifluorescence monolayer studies have demonstrated surfactant PL films can be compressed to near zero surface tensions with gel phase proportions only slightly higher than that of the fractional DPPC content (Nag et al., 1998).

4. Captive bubble studies have demonstrated rapid compression of fluid phase phospholipids such as PC14:0/14:0 and PC16:0/18:1 can result in near zero surface tensions and such films are sufficiently meta-stable to support normal respiration (Nag et al., 1998).

5. The molar composition of phospholipid molecular species is not static, but can alter with age and possibly respiratory rate, such that the "minor" lipids, such as PPPC (PC16:0/16:1) and POPC (PC16:0/14:0), assume significant proportions (Bernhard et al., 2001).

All of these observations are inconsistent with the classical model of surfactant function. In addition, the wide variation in levels of DPPC between the mammalian animals I have studied suggest that a range of DPPC levels are acceptable for a functioning pulmonary surfactant system. Moreover, I now add the observation that dunnart surfactant contains only 17% DPPC. According to the classical model, this low DPPC content would be insufficient to generate the DPPC-enriched films deemed

essential for maintaining normal lung function. Furthermore, the negligible proportion of diacyl DPPC (PC16:0/16:0) found in surfactant isolated from the Tasmanian devil, *Sarcophilus harrisii*, suggests that surfactants can function with very low levels of this diacyl DPPC molecule (2 mol%), or at least can substitute the common diacyl form of DPPC, with an alkyl-acyl form (PC16:0/16:0a) and still maintain a functioning surfactant system.

In addition, increases in cholesterol during torpor or hibernation in heterothermic mammals correlated with a greater number of liquid-condensed (LC) domains and not with a greater fluidity in surfactant films, when measured at 23°C. This observation suggests that cholesterol is not simply a fluidiser of surfactant at low body temperatures, as depicted by the classical model. Taken together these considerations argue strongly against the formation of almost pure PC16:0/16:0 monolayers favoured by the classical model, thereby emphasising the need for a new paradigm and a shift in the way in which respiratory physiologists view the mammalian surfactant system.

7.7 A NEW PARADIGM OF SURFACTANT FUNCTION

An important shift in our thinking about the function of surfactant system is the realisation that DPPC may not be the only molecule in pulmonary surfactant crucial for lowering surface tension. Other forms of DPPC, such as alkyl-acyl DPPC and even unsaturated PL may also play a crucial role in the surface-tension lowering function of surfactant. Human lungs, therefore, may maintain a high PC16:0/16:0 content, not because it is essential, but because it is optimal for our typical lung function (Postle; personal communication). It would appear, that at the molecular level, there is not a single molecular species PL composition that can function optimally in all mammals. Rather, there is a spectrum of different molecular compositions within mammals, such

that the molecular PL composition of each animal species is unique and optimised to the physiology of that animal. Variable body temperature, as experienced during torpor or hibernation in heterotherms, is one factor that appears to influence the molecular composition of PL in surfactant. Other factors may include development (Bernhard et al., 2001; Johnston et al., 2000), disease (Gibson and McMurchie, 1986; Mander et al., 2002; Meyer and Zimmerman, 2002), altered alveolar size (Daniels and Orgeig, 2001) and respiratory rate (Bernhard et al., 2001). The combination of all these factors, ultimately leads to a molecular PL composition that functions if not optimally, at least effectively, at all physiological variables experienced by an animal in its daily or seasonal cycle, behaviour or development.

Mechanisms resulting in the observed differences in molecular composition, appear to be fine-tuning mechanisms because firstly, the % saturation of PL in mammalian surfactants is fairly constant across the board (typically 40-50%) and secondly, regardless of the precise molecular species composition, the sum of PC16:0/14:0, PC16:0/16:1 and PC16:0/16:0, is 60-70% in all animals studied. Even in the Tasmanian devil, the alkyl-acyl forms of these components contribute 70% to surfactant PC.

The similarities in the extent to which molecular PC composition varies during development and in heterothermic mammals, suggests that similar molecular mechanisms for regulating the packing, synthesis and secretion of surfactant PL are present in all mammalian type II cells (Postle; personal communication). Dunnarts, squirrels and bats may alter these common mechanisms as an adaptive strategy to fine-tune their surfactant composition for efficient function at both warm and cold body temperatures. However, rat and human type II cells may alter these same mechanisms in response to developmental changes in lung structure and function. Common adaptations

to changes in lung function that seem to be emerging include variations in PC16:0/16:1, PC14:0/16:0, PC16:0/18:0, PG16:/16:1 and PI18:0/20:4.

In addition, there is evidence emerging, from this and other studies (Discher et al., 2002; Discher et al., 1999a), suggesting that variation in cholesterol levels may provide a mechanism by which biological systems can control the sorting of its constituents in surface films (Discher et al., 2002). Changes in cholesterol levels occur during torpor and hibernation in heterothermic mammals and may account for differences in surfactant film structure and function. Thus, cholesterol should not be considered simply as a fluidiser of surfactant. Cholesterol may also play an important role in determining the structure of the surface film and thus, is important to the surface-tension lowering functions of surfactant.

7.8. SUMMARY

Torpor in heterothermic mammals is highly advantageous in terms of energy conservation and is characterised by decreases in body temperature (T_b). However, reductions in T_b can challenge other physiological systems. Pulmonary surfactant (PS), a complex mixture of lipids and proteins, lowers surface tension (ST) in the alveoli and is crucial to lung function. Due to the biophysical properties of lipids, temperature potentially can have a profound effect on the function of PS. However, heterothermic mammals regularly endure changes in T_b, during torpor without suffering surfactant dysfunction.

The length, depth and frequency of torpor bouts vary in different mammals and may affect the manner in which the PS system responds to the decrease in T_b. Here, I examine the thermal dynamics of PS in three heterothermic mammals that display different torpor patterns. Fat-tailed dunnarts, *Sminthopsis crassicaudata*, enter torpor in

response to food shortages and low ambient temperatures (T_a). Gould's wattled bats, *Chalinolobus gouldii*, enter torpor on a daily basis, while golden-mantled ground squirrels, *Spermophilus lateralis*, enter deep and prolonged torpor bouts during hibernation. The composition, structure and function of surfactant isolated from warm-active and torpid mammals was examined using biochemical analyses, mass spectrometry, a Wilhelmy-Langmuir surface balance and epifluorescence and atomic force microscopy.

Squirrels and bats did not experience a change to the overall saturation of surfactant PL (i.e. the disaturated phospholipid / phospholipid (DSP/PL)) during torpor. Hence, the increases in DSP previously observed during torpor in dunnarts, may be related to the stress-induced nature of their torpor pattern. In the present study, in whole lavage fractions, I did not observe any significant differences in the saturation of phosphatidylcholine (PC) between warm-active and torpid bats, dunnarts or squirrels. Furthermore, in all three species, the molecular composition of PC, did not change between whole lavage isolated from warm-active and torpid mammals. However, small, but significant, differences were observed in one or two of the molecular components (comprising greater than 5 mol%) of phosphatidylglycerol (PG) and phosphatidylinositol (PI) in each animal. None of these observed changes in PG and/or PI molecular species are consistent across the mammals examined and this may suggest that they are each specific for the type of torpor pattern displayed and/or the particular respiratory physiology of that animal species. However, PG and PI are two of the more variable components of PS. Therefore, the differences observed in these relatively minor components between warm-active and torpid mammals probably do not represent thermal adaptations of the PS system.

Irrespective of the type of torpor pattern, when measured at 22-24°C, a lower minimum ST is recorded consistently for surfactant isolated from torpid mammals than

from warm-active mammals. Hence, surfactants isolated from both warm-active and torpid animals are capable of effectively reducing ST at the appropriate T_b, without a major changes in PL composition. This suggests that the molecular composition of PL in surfactants of heterothermic mammals is either sufficient, or already modified, to enable surfactant to effectively reduce ST at both warm and cold T_b. A comparative study of homeothermic and heterothermic mammalian surfactant compositions demonstrates that there are differences in PL molecular species composition between homeothermic and heterothermic mammals that may be thermal adaptations. These include an enrichment in phosphatidylcholine molecular species 16:0/16:1, 16:0/14:0 and 16:0/18:0, phosphatidylglycerol 16:0/16:1 and phosphatidylinositol 18:0/20:4.

However, surfactant does not function as well when measured at a temperature more than 10°C different to the body temperature of the animal from which the sample was taken. Hence, surfactant from warm-active animals does not function as effectively when measured at a low T_a (regardless of species), while surfactant from torpid animals does not work as well at a warm T_a. Thus, other aspects of surfactant composition, such as lipid-protein interactions or film structure, must enhance surfactant function at warm-active and/or torpid T_b in heterothermic mammals. Increases in cholesterol (CHOL) occur in surfactant during daily torpor in bats (Codd et al., 2002), stress-induced torpor in dunnarts (Langman et al., 1996) and, in the present study, during hibernation in ground squirrels. Levels of surfactant protein B (SP-B) in surfactant did not change between warm-active and torpid mammals. However, SP-C significantly increased during torpor in dunnarts and squirrels, but not bats.

Epifluorescence microscopy and atomic force microscopy of monolayers prepared from surfactant isolated from warm-active and torpid dunnarts and squirrels, both demonstrate that warm-active films consist of more fluid or liquid-expanded (LE) regions than surfactant from torpid animals at 23°C. Surfactant films from torpid

dunnarts and squirrels had greater % area coverage of larger liquid-condensed (LC) domains at 10-30 mN m^{-1} surface pressures than surfactant films from warm-active animals.

The greater number and size of LC domains in surface films correlates with higher levels of CHOL and SP-C. CHOL and in some cases, SP-C, are thought to act as fluidisers of surfactant by lowering the phase-transition temperature of lipid mixtures. However, both CHOL and SP-C were higher in the less fluid surfactant isolated from torpid mammals, than in the more fluid surfactant isolated from warm-active mammals. Thus, increases in CHOL and SP-C correlate with a greater number of LC domains and not a greater fluidity in surfactant films, when measured at 23°C. This observation suggests that the classical understanding of CHOL as a fluidiser of surfactant, is far too simplistic. CHOL must also play a role in the redistribution of PL and in altering the structure of the surfactant film during torpor. In addition, increases in SP-C during torpor may improve the formation of surface-associated reservoirs and hence, the 'squeeze-out' of unsaturated lipids and CHOL, at lower temperatures. The greater fluidity of warm-active surfactant at 23°C probably hampers the ST-lowering properties and can explain observations that surfactant isolated from warm-active mammals is less 'surface-active' than torpid surfactant at 23°C.

In atomic force microscopy studies, surfactant films from warm-active and torpid dunnarts and squirrels exhibited small, roughly circular domains with heights of 0.8–1 nm. These intermediate domains were significantly larger in diameter in surfactant films from torpid dunnarts than surfactant films from warm-active dunnarts. Moreover, torpid dunnart and squirrel surfactant films contained a second condensed region of even greater height (~1.6 nm) that was located within the intermediate domains. To my knowledge, this is the first study to report both the presence of intermediate height domains and the presence of double condensed domains in PS. It is possible that the

intermediate domains represent the monolayer equivalent of the liquid ordered (L_0) phase observed with CHOL-containing bilayers. This would suggest that the ~10% cholesterol content of dunnart and squirrel surfactant has the ability to mix with DPPC-rich domains of ~1.2 nm height and convert them to intermediate phase entirely (warm-active surfactant) or in part (torpid surfactant).

The classical dogma for surfactant function is that dipalmitoylphosphatidylcholine (DPPC) is the only significant component of mammalian surfactants capable of reducing ST to low values during alveolar compression. The role of CHOL and other unsaturated PL in surfactant is thus restricted to fluidising surfactant, so that it can spread over alveolar surfaces during alveolar expansion. However, a number of very recent studies challenge the classical model of surfactant function. In addition, the wide variation in levels of DPPC between mammalian animals observed here, suggest that a range of DPPC levels are acceptable for a functioning PS system. Moreover, I now add the observation that dunnart surfactant contains only 17% DPPC. According to the classical model, this low DPPC content would be insufficient to generate the DPPC-enriched films deemed essential for maintaining normal lung function. Furthermore, I observed a negligible proportion of diacyl DPPC in surfactant isolated from the Australian marsupial, the Tasmanian devil, *Sarcophilus harrisii*. This suggests that surfactants can function with very low levels of this diacyl DPPC molecule (2 mol%), or at least can substitute the common diacyl form of DPPC, with an alkyl-acyl form and still maintain a functioning PS system. In addition, CHOL appears to play an important role in determining film structure at low T_b. Taken together these considerations argue strongly against the theory that almost pure DPPC monolayers are formed during compression, and emphasizes the need for a new paradigm and a shift in the way in which respiratory physiologists view the mammalian surfactant system.

I postulate that human lungs may maintain a high DPPC content, not because it is essential, but because it is optimal for our typical lung function. At the molecular level, the evidence presented here suggests that there is no single PL molecular species composition that functions optimally in all mammals. Rather, there is a spectrum of different molecular compositions within mammals, such that the molecular PL composition of each animal species is unique and optimised to the physiology of that animal. I have demonstrated here that variable T_b, as experienced during torpor in heterotherms, is one factor that influences the molecular composition of PL in surfactant. Other factors may include development, disease, altered alveolar size and respiratory rate. The combination of all these factors, ultimately leads to a molecular PL composition that functions if not optimally, at least effectively, over the range of physiological conditions experienced by an animal in its daily or seasonal cycle, behaviour or development.

7.9. FUTURE DIRECTIONS

An important step in developing this new paradigm of surfactant function is to understand the molecular mechanisms involved in maintaining molecular PL composition. In order to do this, the synthesis, secretion and alveolar recycling of secreted surfactant components should be studied in vivo and in vitro in different animal and lung function models. For example, the research presented here clearly demonstrates the usefulness of both the dunnart and squirrel as laboratory models in which to examine the molecular mechanisms involved in maintaining molecular PL composition in response to temperature requirements. One hypothesis to be tested is 'that mechanisms of adaptation are based on acyl chain length, rather than saturation' (Postle; personal communication). This hypothesis is based on the observation that

longer chain PCs, including PC16:0/18:1 are never enriched in homeothermic surfactants, except in diseased states, when they probably derive from fragments of cell membranes.

This study also highlights the need for further research into the role of cholesterol in surfactant. Hence, an important follow-up experiment from this study will be to determine the effect of removing cholesterol from warm-active and torpid or hibernating surfactants on their film structure, fluidity and function, using epifluorescence, atomic force microscopy and surfactometers.

Currently, there are some effective artificial surfactant mixtures in use for the treatment of respiratory diseases. However, these mixtures have been developed from our classical understanding of surfactant function and in vitro studies of surface-active properties. Cholesterol is typically excluded from artificial surfactants. However, there is now sufficient evidence that cholesterol plays other roles including probably a role in reorganising lipids and determining film structure. Such evidence warrants a further investigation and/or trial of cholesterol in artificial surfactants. Given the observed variations in PL composition in response to temperature, respiratory rate, alveolar structure and disease, it follows that if a lung is under different pressures, due to damage or malformation of alveoli or disease, that the most efficient surfactant mixture may not necessarily be the standard DPPC-rich mixtures commonly in use today. Hence, understanding the precise molecular PL differences between healthy and diseased lungs may enable the development of more efficient artificial surfactants that are tailor-made for specific respiratory conditions or diseases. These conditions include asthma, cystic fibrosis, acute respiratory distress syndromes and both medically and environmentally-induced hypothermia in humans.

APPENDICES

APPENDIX 1 CHEMICAL SUPPLIERS

Abbot Laboratories, Sydney, NSW, Australia

Pentobarbitone sodium, Nembutal™, 60 mg/ml

AJAX Chemicals, Sydney, NSW, Australia

Sodium dihydrogen orthophosphate, $NaH_2PO_4.2H_2O$

Sodium hydrogen carbonate

Asia Pacific Speciality Chemicals Limited, Seven Hills, NSW, Australia

Chloroform

Methanol

David Bull Laboratories, Sydney, NSW, Australia

Heparin sodium, from porcine mucous, 25000 I.U. in five ml.

Fisons Scientific Equipment, Loughborough, England

Calcium Chloride, $CaCl_2.2H_2O$

Disodium hydrogen orthophosphate, monobasic, Na_2HPO_4

May and Baker Pty Ltd, Victoria, Australia

Magnesium sulphate, $MgSO_4.7H_2O$

Sigma Chemical Corporation, St Louis, MO, USA

Sodium Chloride

Tris[hydroxy methyl]aminomethan, TrizmaBase™

Phosphate Buffered Saline, 1L sachets, without calcium and magnesium

Hepes

APPENDIX 2 SURFACTANT PROTEIN ELISA REAGENTS AND SOLUTIONS

Reagents for SP-A and SP-B ELISAs

ELISA plates: Medium binding 96 well ELISA plates (Griener, Interpath Services, Australia)

SP-A Coating Antibody: Goat anti-human SP-A (Chemicon Cat # AB3422)

SP-A Primary Antibody: Rabbit anti-human SP-A (Chemicon Cat # AB3420)

SP-B Primary Antibody: anti-rabbit SP-B (Chemicon Cat # AB3780)

SP-A and SP-B Secondary Antibody: HRP-conjugated goat anti-rabbit IgG (Sigma Chemical Corporation)

SP-A standard: gift from Prof. Jeffrey Whitsett

SP-B standard: gift from Prof. Jeffery Whitsett

Hydrogen Peroxide (30%) (Sigma Chemical Corporation, Cat # H1009)

OPD tablets (Sigma Chemical Corporation)

Bovine serum albumin (BSA) (Sigma Chemical Corporation #A7030)

Goat serum (Sigma Chemical Corporation)

Human serum (Sigma Chemical Corporation)

Solutions for SP-A and SP-B ELISAS

1. Sample Buffer (PBS with Triton X 100) (1x)

Make up 0.05M Na_2HPO_4, 0.15M NaCl, 0.5 ml TritonX100 per 100 ml (Solution 1) and 0.05M NaH_2PO_4 pH 7.4, 0.15M NaCl, 0.5 ml Triton X 100 per 100 ml (Solution 2) as follows: *Solution 1:* To prepare 500 ml, add 3.55 g Na_2HPO_4 and 4.39 g NaCl to 300 ml water and stir until dissolved. Add 2.5 ml Triton X-100. Make up to 500 ml with water. *Solution 2:* To prepare 100 ml, add 0.78 g NaH_2PO_4 and 0.877 g NaCl to 80 ml water and stir until dissolved. Add 0.5 ml Triton X-100. Make to 100 ml with water. Mix

solution 1 and 2 together to pH 7.4. Requires approximately 5:1 $Na_2HPO_4:NaH_2PO_4$.

2. Coating buffer (1x)

To prepare 100 ml, add 0.84 g $NaHCO_3$ to 80 ml water and stir until dissolved. Make up to 100 ml with water. Just before use, add the appropriate dilution of coating antibody.

3. Blocking Buffer (5x)

To prepare 200 ml, add 8.77 g NaCl, 1.2 g Tris base and 5.0 g bovine serum albumin (BSA) to 100 ml water and stir until dissolved. pH to 7.4 with HCl and make up to 200 ml with water. Aliquot into 5 ml aliquots and store at –20°C.

For SP-A ELISA: On day of assay, prepare 1x blocking buffer by adding human albumin to 2.5%, goat serum to 2.5% and water to 5x volume. (i.e. if 5 ml 5x blocking buffer, make up to 25 ml by adding 625 µl human albumin, 625 µl goat serum and 18.75 ml water).

For SP-B ELISA: On day of assay, prepare 1x blocking buffer by adding human albumin to 5% and water to 5x volume. (Take 5 ml of 5x blocking buffer and make up to 25 ml by adding 1250 µl human albumin and 18.75 ml water).

4. Substrate buffer (10x stock)

To prepare 500 ml, add 39.0 g NaH_2PO_4 and 25.0 g sodium citrate to 300 ml water and stir until dissolved. Add 0.05 g thimerosal (be careful, this is toxic!) and stir until dissolved. pH to 6.3 with NaOH or HCl and make up to 500 ml with water. Store at 4°C.

Just prior to use in assay, dilute substrate buffer to 1x with deionised water by adding 5 ml of 10x substrate buffer to 50 ml water. Immediately prior to use add 185 µl of 30% hydrogen peroxide to 25 ml 1x substrate buffer and mix well.

5. Developing Buffer (1x)

Dissolve one tablet of ortho-phenylenediamine (OPD) in 25 ml of substrate buffer (1x) containing hydrogen peroxide (as above). Dissolve as quickly as possible and then add immediately to ELISA plate.

6. Washing buffer (50x stock)

To prepare 500 ml, add 30.275 g Tris base and 2.5 g thimerosal to 300 ml water and stir until dissolved. Add 2.5 ml Tween 20 and pH to 8.0 with concentrated HCl (note: need a lot of HCl). Make up to 500 ml with water. Store at 4°C.

Prepare 1x washing buffer by diluting the 50x stock solution in water to 50x the volume. Store at 4°C and warm to room temperature before use.

7. Dilution Buffer (For secondary HRP-conjugated antibody) (5x stock)

<u>Solution 1</u>: To prepare 500 ml, add 0.25 g thimerosal, 17.75 g Na_2HPO_4 and 21.925 g NaCl to 300 ml water and stir until dissolved. Add 1.25 ml Tween 20 and make up to 500 ml with water. *Solution 2*: To prepare 100 ml, add 0.05 g thimerosal, 3.9 g NaH_2PO_4 and 4.386 g NaCl to 80 ml water and stir until dissolved. Add 0.25 ml Tween 20 and make up to 100 ml with water. Mix solution 1 and solution 2 together to pH 7.4.

SP-A ELISA: Just before use, dilute to 1x solution with 2.5% goat serum, 2.5% human albumin and water to 5x the volume. (i.e to 5 ml of 5x stock, add 625 µl of goat serum, 625 µl of human albumin and 18.75 ml water).

SP-B ELISA: Just before use, dilute to 1x solution with 5% human albumin and water to 5x the volume. (i.e to 5 ml of 5x stock, add 1250 µl of human albumin and 18.75 ml water).

Reagents for SP-C ELISA

Polysorp microplates (Nunc #446140, Fisher #12-565-266)

Recombinant, dipalmitoylated human SP-C (gift of W. Steinhilber, Byk Gulden 9020-002-22)

Polyclonal rabbit anti-recombinant SP-C (gift of W. Steinhilber, Byk Gulden 22/96 v.5.9.96)

Donkey anti-rabbit IgG, biotinylated (Amersham #RPN1004)

ABComplex/HRP (DAKO #K0355)

Bovine serum albumin (BSA) (Sigma #A7030)

Hydrogen peroxide, 30% (Sigma #H1009)

Methanol, certified ACS (Fisher #A412-1)

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma #A1888)

2-Propanol (Fisher #A416-1)

2,2,2-Trifluoroethanol (Aldrich #T6300-2)

Tween-20, low peroxide (Sigma #P1379)

Polypropylene tubes, 2 ml (Eppendorf #22-36-335-2, Fisher #05-402-7)

All other chemicals are reagent grade or better.

Solutions for SP-C ELISA

1. 80% 2-Propanol/20% water (v/v)

Combine 160 ml 2-propanol with 40 ml water in a 250 ml Corning glass bottle. Add 63.2μl of 1N HCl to pH 3.5 and mix well. Store at room temperature.

2. Tris (10X)

Prepare 0.5M Tris, pH 7.6, by dissolving 30.2 g Tris base in a final volume of 500 ml water. Adjust pH with concentrated HCl (about 7 ml). Store at 4°C. Prepare 1x Tris solution by diluting 10x stock with water.

3. TA solution (Tris (1x) containing 1% (w/v BSA))

Dissolve 2 g BSA in 200ml Tris (1x). Sterilise by filtration (0.45μm filter). Divide into 40 ml aliquots. Store at -20°C.

4. TT solution (Tris.(1x) containing 0.5% (v/v) Tween-20)

Prepare fresh on day of use. Dissolve 0.5% Tween-20 in Tris (1x).

5. Substrate buffer

60mM sodium acetate, 50mM sodium phosphate. Prepare 250 ml by dissolving 1.23 g sodium acetate and 1.50 g NaH_2PO_4 (anhydrous) in 250 ml water. Sterilise by filtration (0.45μm filter). Aliquot and store at 4°C.

APPENDIX 3

THERMAL ACCLIMATION OF SURFACTANT SECRETION AND ITS REGULATION BY ADRENERGIC AND CHOLINERGIC AGONISTS IN TYPE II CELLS ISOLATED FROM WARM-ACTIVE AND TORPID GOLDEN-MANTLED GROUND SQUIRRELS, *SPERMOPHILUS LATERALIS*

Thermal acclimation of surfactant secretion and its regulation by adrenergic and cholinergic agonists in type II cells isolated from warm-active and torpid golden-mantled ground squirrels, *Spermophilus lateralis*

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Summary

Homeothermic mammals experience pulmonary surfactant dysfunction with relatively small fluctuations in body temperature. However, ground squirrels survive dramatic changes in body temperature during hibernation, when body temperature drops from 37°C to 0–5°C during prolonged torpor bouts. Using type II cells isolated from both warm-active and torpid squirrels, we determined the effect of assay temperature, autonomic agonists and torpor on surfactant secretion. Basal secretion was significantly higher in type II cells isolated from torpid squirrels compared with warm-active squirrels when assayed at the body temperature of the animal from which they were isolated (4°C and 37°C, respectively). A change in assay temperature significantly decreased surfactant secretion. However, the change in secretory rate between 37°C and 4°C was less than expected if due to temperature alone (Q_{10} range=0.8–1.2). Therefore, the surfactant secretory pathway in squirrel type II cells demonstrates some temperature insensitivity. When incubated at the body temperature of the animal from which the cells were isolated, the adrenergic agonist, isoproterenol, significantly increased surfactant secretion in both warm-active and torpid squirrel type II cells. However, the cholinergic agonist, carbamylcholine chloride, only increased secretion in torpid squirrel type II cells when incubated at 4°C. Torpor did not affect basal cAMP production from isolated type II cells. However, the production of cAMP appears to be upregulated in response to isoproterenol in torpid squirrel type II cells. Thus, at the cellular level, both the secretory and regulatory pathways involved in surfactant secretion are thermally insensitive. Upregulating basal secretion and increasing the sensitivity of type II cells to cholinergic stimulation may be adaptative characteristics of torpor that enable type II cells to function effectively at 0–5°C.

Key words: ground squirrel, *Spermophilus lateralis*, pulmonary surfactant, hibernation, torpor, alveolar type II cell, cholinergic, adrenergic.

Introduction

Pulmonary surfactant is a mixture of phospholipids (80–90%), neutral lipids (10%) and proteins, and is secreted into the alveolar airspace to reduce and vary surface tension in response to changes in lung volume (King, 1982). The most abundant phospholipid (PL) is phosphatidylcholine (PC) (79–85%) (King, 1982), and its disaturated form, dipalmitoylphosphatidylcholine (DPPC), containing two molecules of the fatty acid, palmitate, is the major contributor to surfactant surface activity (Possmayer, 1997). The surfactant components are synthesized in alveolar type II epithelial cells (ATII cells) and are stored in specialised secretory organelles known as lamellar bodies (Goerke, 1998; Wright and Dobbs, 1991). In mammals, lamellar bodies are secreted in response to signals from the autonomic nervous system (Chander and Fisher, 1990; Massaro et al., 1982), ventilation (Wirtz and Schmidt, 1992) and local biochemical factors (Chander and

Fisher, 1990). In the aqueous layer that lines the alveolar epithelium, lamellar bodies unravel to release the surfactant components, which form a surface-active film at the air–liquid interface (Haagsman and Van Golde, 1991).

Homeothermic mammals, such as humans and rats, experience surfactant dysfunction and respiratory distress with small fluctuations in body temperature (Inoue et al., 1981; Meban, 1978; Peterson and Davis, 1986). However, heterothermic mammals such as fat-tailed dunnarts *Sminthopsis crassicaudata* and golden-mantled ground squirrels *Spermophilus lateralis* regularly experience rapid changes in body temperature when they enter a depressed metabolic state, known as torpor or hibernation, respectively (Geiser and Ruf, 1995). Temperature-induced changes in surfactant amount and/or composition have been observed during the stress-induced torpor of fat-tailed dunnarts *S.*

crassicaudata (Langman et al., 1996) and the daily torpor of the microchiropteran bats *Chalinolobus gouldii* (Codd et al., 2000b) and *Nyctophilus geoffroyi* (Slocombe et al., 2000). In dunnarts, after 8 h of torpor, there are increases in the relative amounts of PL, disaturated phospholipid (DSP) and cholesterol (CHOL) (Langman et al., 1996). These changes correlate with changes in surface activity and, therefore, enable dunnart surfactant to function effectively at torpid body temperatures (Lopatko et al., 1998). Similarly, total PL increased in lavage fluid collected from mildly cold-acclimated ground squirrels *Spermophilus richardsoni* (Melling and Keough, 1981). In contrast, surfactant PL decreased or did not change during torpor in the microchiropteran bats *N. geoffroyi* and *C. gouldii*, respectively (Codd et al., 2000b; Slocombe et al., 2000). The different responses of bats, dunnarts and squirrels probably reflect the different physiological states of the animals during torpor (Codd et al., 2000a).

During hibernation, ground squirrels enter a much deeper and more prolonged torpor than that reported for the stress-induced torpor of small marsupials and daily torpor of bats (Geiser and Ruf, 1995). The depth and duration of their torpor bouts makes squirrels an excellent model for studying the thermal dynamics of a mammalian surfactant system. Furthermore, ground squirrels enter hibernation readily under appropriate laboratory conditions. However, very little is known about the effects of torpor on the surfactant system in hibernators. During the hibernating season, golden-mantled ground squirrels *Spermophilus lateralis* are capable of reducing their body temperatures to as low as 0–5°C during torpor, and torpor bouts usually last for 10–14 days at a time (Milsom et al., 1999). After a torpor bout, the ground squirrels will spontaneously arouse, increasing their body temperature to 37°C for a brief period (h) before returning to a torpid state. Although hibernation is highly advantageous in terms of energy conservation, the long duration and depth of torpor bouts experienced by ground squirrels are likely to have marked effects on the composition, function and regulation of the surfactant system. Alternatively, given the different type of torpor and the annual regularity of the hibernating season, ground squirrels may have adopted novel and unique approaches for maintaining surfactant function at both warm-active (37°C) and torpid (0–5°C) body temperatures.

The low temperatures experienced by ground squirrels during torpor bouts are likely to also have a profound effect on the release of surfactant into the lung and the regulatory and secretory pathways controlling surfactant release. High temperatures increase metabolic rate and thus, may directly stimulate the rate of synthesis and/or secretion of lamellar bodies from type II cells (Chander and Fisher, 1990). Conversely, low temperatures decrease metabolic rate, and may therefore lower the rate of surfactant secretion. In ATII cells isolated from homeothermic rats, a decrease in incubation temperature to 5°C virtually abolishes surfactant secretion (Dobbs and Mason, 1979). Basal secretion is also significantly lower in type II cells isolated from warm-active dunnarts when incubated at 15°C compared to 37°C (Ormond et al., 2001). In

dunnart type II cells, however, the decrease in the rate of surfactant secretion has a Q_{10} value of 1.3 (Ormond et al., 2001). The fact that this Q_{10} value is lower than 2, indicates that the secretory pathway in dunnart type II cells is relatively insensitive to temperature and must be regulated or altered in some way to counteract the kinetic effects of decreasing temperature (Ormond et al., 2001; Schmidt-Nielson, 1997). Therefore, we suggest that in ground squirrels, the composition, function and cellular biomechanics of the surfactant system must also be modified to enable efficient functioning at body temperatures of 0–5°C.

The sympathetic nervous system is an important regulator of surfactant release in mammals (Chander and Fisher, 1990). Adrenergic factors are released from the sympathetic nervous system (SNS) and bind to membrane-bound β-adrenergic receptors on type II cells to activate the signalling molecule, cAMP, and enhance surfactant secretion (Brown and Longmore, 1981; Dobbs and Mason, 1978; Wood et al., 1997). Adrenergic agonists stimulate surfactant secretion in type II cells isolated from homeothermic animals such as rat (Brown and Longmore, 1981; Chander and Fisher, 1990), chicken (Sullivan and Orgeig, 2001) and tammar wallaby (Miller et al., 2001), heterothermic animals such as fat-tailed dunnart (Ormond et al., 2001), and ectothermic animals such as bearded dragon, frog, lungfish (Wood et al., 2000) and crocodile (Sullivan et al., 2002). In type II cells isolated from bearded dragons and fat-tailed dunnarts, this response to isoproterenol did not change regardless of assay temperature (Ormond et al., 2001; Wood et al., 1999, 2000). Thus, the regulation of surfactant secretion by the β-adrenergic signalling pathway appears to be relatively temperature-insensitive in lizards and dunnarts. Sympathetic output, however, probably decreases markedly during torpor *in vivo* (Wood et al., 2000). Therefore, regulation by the parasympathetic nervous system (PNS) and cholinergic agonists, which do not increase metabolic rate, may be more important in controlling surfactant release during torpor (Wood et al., 2000).

Carbamylcholine chloride acts through membrane-bound muscarinic receptors to increase PC secretion in ATII cells isolated from fat-tailed dunnarts *Sminthopsis crassicaudata* (Ormond et al., 2001; Wood et al., 1999, 2000), and juvenile, unfurred (heterothermic) tammar wallabies *Macropus eugenii* (Miller et al., 2001). In direct contrast, type II cells isolated from homeothermic mammals such as humans and rats do not respond to cholinergic agonists (Dobbs and Mason, 1979). This suggests that heterothermic mammals may have a direct role for the parasympathetic nervous system in regulating surfactant secretion at low body temperatures. Type II cells isolated from the ectothermic bearded dragon *Pogona vitticeps* (T_b range: 15–40°C; T_b study=25°C) also respond to carbamylcholine chloride, but only at a relatively cold assay temperature of 18°C and not at 37°C (Wood et al., 1999). This switch in the response to cholinergic stimulation in lizard type II cells suggests that the cholinergic signalling pathway is highly sensitive to temperature changes. However, in fat-tailed

dunnarts, the response of isolated type II cells to carbamylcholine chloride remains the same, regardless of assay temperature (Ormond et al., 2001). This may enable dunnart type II cells to respond quickly to a physiological change in autonomic stimulation (adrenergic *vs* cholinergic) during the rapid entry and arousal from torpor (Ormond et al., 2001). Here, we characterise the effect of temperature on the control and release of surfactant from squirrel type II cells isolated from warm-active and torpid ground squirrels. Given the importance of the surfactant system to lung function, we hypothesize that squirrel type II cells will retain the ability to secrete surfactant even at very cold body temperatures. Upregulating their response to adrenergic and cholinergic stimulation may also increase the sensitivity of type II cells to autonomic control during torpor. Understanding how the surfactant system can remain functional over a range of temperatures has important consequences in hypothermic lung transplantation surgery and in the treatment of hypothermia and respiratory distress syndromes (Bernard, 1996; Erasmus et al., 1996; Osanai et al., 1991; Inoue et al., 1981; Meban, 1978).

Materials and methods

Animals

Adult golden-mantled ground squirrels *Spermophilus lateralis* Say 1823 were obtained from a wild-captive colony maintained in the Department of Zoology, University of British Columbia, Canada. Squirrels were housed in a controlled-environment chamber at $T_a=22\pm1°C$ under a 12 h:12 h light:dark cycle. Squirrels were given water and fed laboratory chow supplemented with sunflower seeds *ad libitum* throughout the experiments, although they rarely ate during the hibernation season. In September and October (late summer), warm-active squirrels ($T_b=35.2\pm0.5°C$; mean ± S.E.M., $N=11$) were used in experiments. In late November, the temperature in the controlled-environment chamber was reduced to 5°C and the photoperiod altered (8 h:18 h L:D). Under these conditions, the squirrels entered hibernation within a few weeks. The length of torpor bouts was monitored by placing a wood chip on the back of each squirrel and recording whether it was present or absent each day. Torpid squirrels were used after at least 4 days of torpor without periodic arousal. The rectal body temperature of each squirrel was recorded using a thermocouple. Torpid squirrels recorded body temperatures of $7.7\pm0.2°C$ (mean ± S.E.M., $N=12$). Note that there was an overlap period where some squirrels were kept at summer conditions and some at winter conditions during November. Squirrels maintained under summer conditions did not enter hibernation.

Isolation of type II cells

Procedures for isolating type II cells were modified from the methods of Dobbs et al. (1986a), Wood et al. (1999, 2000) and Ormond et al. (2001). Animals were anaesthetised with an intraperitoneal injection of pentobarbitone sodium (50–150 mg kg^{-1} body mass). The trachea was cannulated and

the ground squirrel placed on a ventilator set to a volume of 10 ml and a frequency of 20–30 breaths min^{-1} during the perfusion. The thorax was opened and the lungs were perfused, under gravity at 33 cmH$_2$O *via* the pulmonary artery, with a sterile saline solution containing 2 i.u. ml^{-1} of heparin sodium, until free of blood. The lungs were lavaged *via* the tracheal cannula with three separate 10 ml volumes of ice-cold saline. After lavaging, the lungs were excised from the squirrels, blotted with sterile gauze to remove excess water and weighed to determine wet lung mass. The lungs were then placed in a sterile 50 ml tube containing saline. Aseptic techniques were used from this point in the isolation procedure and carried out in a laminar flow hood. The lungs were rinsed twice with sterile phosphate-buffered saline containing 10 U ml^{-1} penicillin, 10 µg ml^{-1} streptomycin and 25 ng ml^{-1} amphotericin and transferred to new 50 ml sterile tubes containing 6 U ml^{-1} elastase (120 U per lung), 250 µg ml^{-1} DNase, 10 U ml^{-1} penicillin and 10 µg ml^{-1} streptomycin. The lungs were shaken continuously at room temperature for 30–40 min. The digested lungs were further dissociated mechanically by pipetting up and down with a 1 ml pipette for 2 min and filtered through a sterile 100 mesh filter (Sigma Chemical Company, St Louis, MO, USA) to remove any undigested tissue fragments and large contaminating cells. Cell suspensions were centrifuged at 200 *g* for 10 min at 22°C (Beckman GS-6R centrifuge) and the supernatant discarded.

The cell pellets were resuspended in an appropriate volume of DMEM + 10% foetal bovine serum (FBS) (containing 10 U ml^{-1} penicillin and 10 µg ml^{-1} streptomycin) and incubated on tissue culture plates for 1 h at the appropriate temperature (37°C for cells from warm-active squirrels and 4°C for cells from torpid squirrels) to allow any fibroblasts to attach to the plates. The plates were then gently 'panned' and rinsed twice with 5 ml of culture medium to remove unattached type II cells. All plate washes were collected and pooled. Cell suspensions were centrifuged at 200 *g* for 10 min at 22°C (Beckman GS-6R centrifuge) and the supernatant discarded. Sterile bacteriologic plates (size 60/15, Greiner Laboratories, Austria) were coated with 3–5 ml of bovine IgG solution (500 µg ml^{-1}, Sigma Chemical Corp., St Louis, MO, USA) and incubated at 22°C for a minimum of 3 h. IgG-coated plates were then washed twice with 5 ml of phosphate buffered saline (PBS, Sigma) and once with 5 ml of sterile culture medium (DMEM containing 20 mmol l^{-1} Hepes, 3.7 g l^{-1} sodium bicarbonate, 100 000 U l^{-1} penicillin, 100 mg l^{-1} streptomycin). Cell pellets were resuspended in 4–6 ml per plate of culture medium containing 250 µg ml^{-1} DNase and added to the bacteriological plates. Plates were incubated at the appropriate temperature (37°C for cells from warm-active squirrels, 4°C for cells from torpid squirrels) with 10% CO$_2$ for 1 h to allow macrophages to attach to the IgG-coated plates. After 1 h, the plates were examined to ensure that macrophages had attached to the plates. The plates were then gently 'panned' and rinsed twice with 5 ml of culture medium to remove unattached type II cells. All plate washes were collected and pooled. Final cell suspensions were examined using light and

electron microscopy to confirm cell type and purity. Type II cells have a cuboidal shape and contain lamellar bodies and microvilli. The ability to secrete PC also confirmed that these cells were type II epithelial cells. The final cell suspensions isolated by this method were >90% pure type II cells. The remaining proportion of cells consisted of macrophages and neutrophils that had not adhered to the IgG-coated plates. The occasional erythrocyte was also present.

Cell viability was measured by the exclusion of the vital dye, Trypan Blue (Dobbs et al., 1986b) using a haemocytometer (Neubauer improved, depth 0.1 mm, 0.0025 mm²). Viable type II cells actively exclude the Trypan Blue dye and remain clear. Non-viable type II cells do not exclude the Trypan Blue dye and, therefore, appear blue. A total of 8 cell counts per cell suspension were performed to calculate percentage viability. Cell viability was determined using Trypan Blue on all freshly isolated cells at the time of plating for each experiment. In addition, cell viability was determined using a lactate dehydrogenase (LDH) cytotoxicity assay (Roche Diagnostics, GmbH, Germany) after overnight incubations at each of the temperatures and during the course of the secretion experiments. The assay was performed as per the manufacturer's instructions.

Microscopy of squirrel lung

Freshly isolated squirrel lungs were cut into 1 mm³ pieces and fixed in 4% paraformaldehyde, 1.25% glutaraldehyde, 4% sucrose in 0.1 mol l⁻¹ PBS, pH 7.2, at 4°C for 3–7 days. The fixed material was washed in 0.1 mol l⁻¹ PBS and postfixed in 1% Osmium Tetroxide overnight. Tissue pieces were then stained *en bloc* in 1.5% uranyl acetate, dehydrated in 70, 80, 90 and 100% ice-cold acetone, embedded in Araldite resin and polymerized. Cut sections were mounted on grids and photographed using a transmission electron microscope (Philips CM 100 TEM).

Measurement of PC secretion

Fresh cell isolates from squirrels were centrifuged at $200\,g$ for 10 min at 22°C (Beckman GS-6R centrifuge) and the cell pellet resuspended in culture medium containing 10% FBS (heat inactivated) to give a concentration of 3×10^6 cells ml⁻¹. $2\,\mu l$ ml⁻¹ of [methyl-³H]choline chloride (specific activity 3.00 Tbq mmol⁻¹, 81.0 Ci mmol⁻¹, 1 μCi ml⁻¹, Amersham Pharmacia Biotech, Canada) was added to the final cell suspension. $100\,\mu l$ of ³H-labelled cell suspension were added to each well of fibronectin-coated plates to give a density of 3×10^5 cells well⁻¹ (0.6×10^6 cells cm⁻²). Fibronectin coated plates (5 μg cm⁻²) were prepared by adding $100\,\mu l$ of fibronectin (25 μg ml⁻¹) to each well of a sterile, flat-bottomed 96-well tissue culture plate (Falcon, Becton Dickinson Labware, NJ, USA). The plates were incubated at room temperature for 45 min to allow the fibronectin to bind. The fibronectin solution was removed from the plates by aspiration, immediately before addition of the cell suspension. Plates were incubated for 22 h at the appropriate temperature with 10% CO₂. The type II cells attached to the fibronectin-coated plates during this time.

After 22 h, the cells were examined under the microscope to assess viability and morphology. Cell viability was determined after the 22 h incubations in wells without radiolabel, using both Trypan Blue and a LDH cytotoxicity assay, and during the course of the secretion experiments. Stock solutions (1 mmol l⁻¹) of agonists (adrenergic agonist, isoproterenol and cholinergic agonist, carbamylcholine chloride) were prepared immediately prior to their use in sample medium (culture medium containing 1 mmol l⁻¹ sodium ascorbate and 1% FBS) equilibrated to 4°C or 37°C. A total volume of 100 μl, prepared by the addition of 90 μl of sample medium and 10 μl of 1 mmol l⁻¹ agonist solutions, was added gently to the experimental wells (three replicates of each). 90 μl of sample medium, followed by 10 μl of sample medium was added to control wells. 100 μmol l⁻¹ concentrations of agonists were chosen from the literature (Brown and Longmore, 1981; Dobbs et al., 1986a). Plates were incubated for 30 min and 1 h at either 4°C or 37°C and in 10% CO₂. Following each experiment, the medium from each well was collected, each well was washed twice with 200 μl of culture medium and the washes pooled. 100 μl of 0.25% (w/v) trypsin/0.04% EDTA solution in sterile PBS was added to the wells. Media samples were centrifuged at $3800\,g$ for 2 min in a capsule microcentrifuge (Tomy Seiko, Japan). The supernatant was transferred to fresh Eppendorf tubes, the cell pellet resuspended in 100 μl of fresh culture medium and centrifuged for a further 2 min to wash any secreted phospholipids from the cells. This last 100 μl wash was collected and added to the media samples. Once all the time points had been completed, all plates were incubated at 37°C until the trypsin/EDTA solution had removed all attached cells from the wells. The trypsin/EDTA solution from each well was added to the cell pellets (from medium spins) and each well was washed twice with 200 μl of culture medium and the washes also pooled. Samples were stored at −20°C until extracted. Lipids were extracted from the media and cell samples using the method of Bligh and Dyer (1959). Unlabelled L-α-phosphatidylcholine (250 μg) was added as a carrier molecule to the extractions to improve the recovery of radioactive lipids. Lipids in chloroform were transferred to 8 ml scintillation vials and evaporated in air (16 h). Lipids were reconstituted in 2 ml of ReadyOrganic™ liquid scintillation fluid, vortexed and the radioactivity counted on a liquid scintillation counter (Beckman LS 3801).

Data analysis

c.p.m. values were obtained for the medium and cell fractions of each sample. Results are expressed as the percentage secretion of incorporated ³H-PC, which was calculated, for each sample, as follows:

$$\% \text{secretion} = \frac{\text{c.p.m. of media fraction}}{\text{total c.p.m.}} \times 100 , \qquad (1)$$

where total c.p.m. = c.p.m. of medium fraction + c.p.m. of cell fraction.

Values for the percentage secretion were arcsin-transformed

and between-group differences in %PC secretion between warm-active and torpid groups, the presence or absence of agonist or the two incubation temperatures, were analysed using unpaired Student's *t*-tests (two-sample for means, assuming equal variance, *P*<0.05). For within-group comparisons (i.e. to determine if the agonist affected cells from one particular animal), the control and agonist-treated groups were from the same preparation of cells and incubated and treated in an identical manner and at the same temperature on the same plate. Therefore, the differences in PC secretion between control and agonist-treated wells were analyzed by paired *t*-tests (two-sample for means).

Q_{10} measurements were calculated for each time point at 37°C and 4°C from the mean of the %PC secretion values obtained in each experiment, using the following equation (Schmidt-Nielson, 1997):

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}}, \qquad (2)$$

where R_2=% secretion at 37°C, R_1=% secretion at 4°C, T_2=37°C and T_1=4°C.

cAMP production

The cAMP assay was adapted from McKinley and Hazel (2000). Fresh cell isolates were incubated overnight in DMEM + 10% FBS on bacteriological plates. After 22 h, the cells were centrifuged at 200 *g* and washed twice with PBS. After the final spin, cells were resuspended in PBS containing 0.83 mg ml^{-1} theophylline at 22°C and counted using a haemocytometer. The cell suspension was diluted to approximately 1×10^6 cells ml^{-1}. 300 μl of cell suspension were sampled into Eppendorf tubes (3×10^5 cells per tube). 30 μl of sample medium was added to the control tubes and 30 μl of 1 mmol l^{-1} isoproterenol (prepared in PBS containing theophylline) was added to each Eppendorf tube. The tubes were incubated at either 37°C or 4°C for 15 min. After 20 min, 60 μl of 6% (w/v) trichloroacetic acid (TCA) was added to each tube and the tubes plunged immediately into liquid nitrogen to stop the reaction. The samples were thawed and neutralised with 200 μl of 2 mol l^{-1} KHCO$_3$. The samples were centrifuged for 2 min at 17 000 *g* in an Eppendorf centrifuge and the supernatant collected. The samples were then stored at −80°C for a maximum of 21 days. cAMP was measured using a direct cAMP ELISA kit (Amersham Pharmacia Biotech, Canada). cAMP production (pmole cell^{-1}) was calculated from the data. Data were analysed using paired and unpaired *t*-tests assuming equal variance. Statistical significance was assumed at *P*<0.05.

Results

Descriptive statistics

Warm-active and torpid ground squirrels had mean rectal body temperatures of 35.2±0.5°C (mean ± s.e.m., *N*=11) and 7.7±0.2°C (mean ± s.e.m., *N*=12), respectively. The wet lung mass recorded for the warm-active and torpid squirrels used in these secretion studies were 3.88±0.31 g (mean ± s.e.m., *N*=6) and 3.49±0.25 g (mean ± s.e.m., *N*=6), respectively. There was no significant difference between recorded wet lung mass for warm-active and torpid squirrels.

Cell yield, purity and viability

When analysed using Trypan Blue, yields of 34.5±3.3×10^6 (mean ± s.e.m., *N*=15) and 29.3±3.3×10^6 (mean ± s.e.m., *N*=10) viable alveolar type II cells were obtained from each warm-active and torpid squirrel, respectively. Freshly isolated warm-active squirrel cell suspensions were 96.6±0.4% (mean ± s.e.m., *N*=16) viable. Freshly isolated torpid squirrel cell suspensions were 97.85±0.33% viable (*N*=12). Type II cell suspensions from warm-active squirrels were 93.4±0.5% (mean ± s.e.m., *N*=10) viable after overnight incubations at 37°C. Type II cell suspensions from torpid squirrels were 98.9±0.6% viable (mean ± s.e.m., *N*=10) after overnight incubations at 4°C. As determined by the LDH assay, cell viability remained above 90% after overnight incubations and for the duration of all experiments, at both temperatures. Both the incubation temperature and the presence or absence of agonists had no effect on cell viability, as measured by the exclusion of the vital dye, Trypan Blue and the LDH cytotoxicity assay.

Microscopy

An electron micrograph of the alveolar epithelium of the lung of a golden-mantled ground squirrel *Spermophilus lateralis* is shown in Fig. 1. The photograph shows an alveolar type II cell, located in a crevice between alveoli, and demonstrates the cuboidal shape, microvilli and presence of large osmiophilic lamellar bodies in these cells.

Surfactant secretion
The effect of temperature on phosphatidylcholine secretion

Basal secretion after 0.5 and 1 h of incubation was significantly higher when cells were incubated at a temperature matching the body temperature of the squirrel from which they were isolated (37°C or 4°C), compared to the alternative assay temperature (4°C or 37°C, respectively) (Fig. 2). In type II cells isolated from warm-active squirrels, basal secretion was 1.6- and 2.2-fold higher at 0.5 and 1 h, respectively, at an assay temperature of 37°C compared to 4°C. In type II cells isolated from torpid squirrels, basal secretion was 1.8- and 1.7-fold higher at 0.5 and 1 h, respectively, at an assay temperature of 4°C compared to 37°C. Consequently, agonist-stimulated secretion was also significantly higher at an assay temperature of 4°C than at 37°C in torpid squirrel cells at 0.5 and 1 h time points. The Q_{10} values obtained for the process of surfactant secretion in type II cells isolated from warm-active and torpid squirrels are shown in Table 1. For cells from both warm-active and torpid squirrels, the presence of agonists had no effect on the Q_{10} value of surfactant secretion. Cells from torpid squirrels had a significantly lower Q_{10} value than that observed in cells from warm-active squirrels (1.23 *vs* 0.86); however, both Q_{10} values fell well below a Q_{10} of 2, which
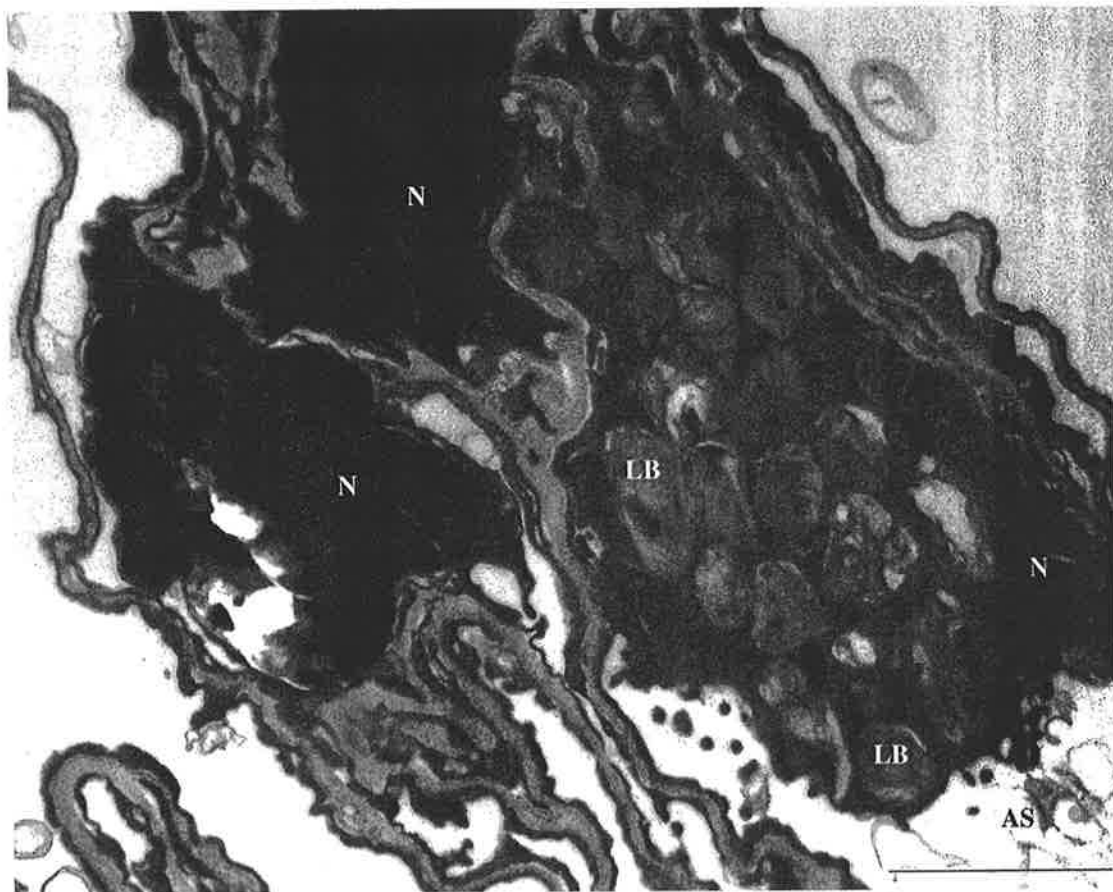
Fig. 1. Electron micrograph of an alveolar type II epithelial cell in lung tissue isolated from the golden mantled-ground squirrel *Spermophilus lateralis*. Scale bar, 2 μm. AS, air space showing surfactant myelin material; LB, lamellar body; N, nucleus.

Fig. 2. Regulation of surfactant phosphatidylcholine (PC) secretion by the adrenergic agonist, isoproterenol (100 μmol l^{-1}) and the cholinergic agonist, carbamylcholine chloride (Carbachol, 100 μmol l^{-1}), in alveolar type II cells isolated from warm-active and torpid ground squirrels. Both warm-active and torpid cells were assayed at 37°C and 4°C for 0.5 and 1 h. *Significant increase above basal secretion for each experimental group (paired *t*-test, $P<0.05$). †Significant difference in secretion between matching experimental groups measured at the two different assay temperatures, i.e. 0.5 h/torpid/4°C *vs* 0.5 h/torpid/37°C (*t*-test, $P<0.05$). ‡Significant difference in % secretion between warm-active cells assayed at 37°C and torpid cells assayed at 4°C (*t*-test, $P<0.05$). Bars represent % of incorporated [^{3}H]choline secreted as phosphatidylcholine (mean ± S.E.M.) for N experiments after 0.5 and 1 h; for warm-active animals, $N=4$; for torpid animals, $N=5$. % secretion = % c.p.m. measured in the medium divided by the total cpm (cells + medium)×100 measured for each sample.
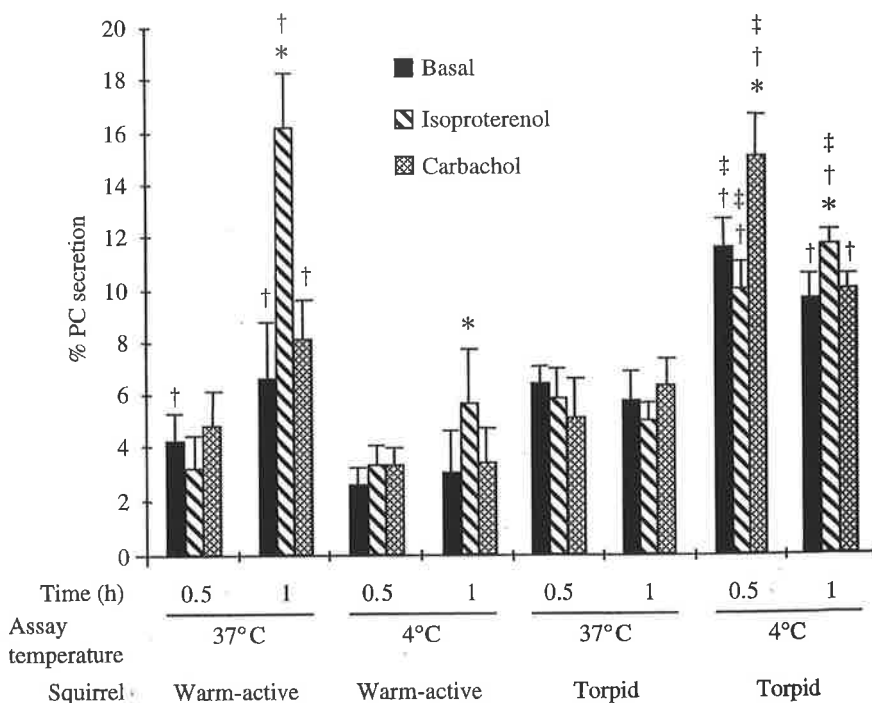
Table 1. *Q10 values for basal and agonist-stimulated secretion of phosphatidylcholine (PC) from type II cells isolated from warm-active and torpid ground squirrels* Spermophilus lateralis

Incubation time	PC secretion (Q_{10})		
	Basal	Isoproterenol	Carbachol
Warm-active squirrel cells			
0.5 h	1.15	1.00	1.12
1 h	1.35	1.36	1.09
Mean	1.25±0.1*	1.18±0.18*	1.11±0.02*
Torpid squirrel cells			
0.5 h	0.87	0.88	0.76
1 h	0.89	0.79	0.89
Mean	0.88±0.01*	0.84±0.04*	0.83±0.06*

Q_{10} values were calculated from the mean rates of secretion given in Fig. 2.

*Significant difference between the Q_{10} values obtained for cells isolated from warm-active and torpid squirrels (*t*-test, $P<0.05$).

There were no significant differences between the Q_{10} values obtained for basal and agonist-stimulated secretion ($P>0.05$), nor between incubation times ($P>0.05$).

suggests that surfactant secretion in squirrel type II cells is insensitive to temperature.

Temperature and the control of phosphatidylcholine secretion by neurochemicals

In type II cells isolated from warm-active squirrels, the adrenergic agonist, isoproterenol, significantly increased surfactant secretion above basal levels after 1 h at 37°C, but not after 0.5 h at 37°C (Fig. 2). When warm-active squirrel type II cells were incubated at 4°C, isoproterenol significantly increased surfactant secretion above basal levels after 1 h. Torpid squirrel type II cells did not respond to the adrenergic agonist, isoproterenol, after 0.5 or 1 h at an assay temperature of 37°C; however, isoproterenol did significantly increase surfactant secretion after 1 h at an assay temperature of 4°C.

The cholinergic agonist, carbamylcholine chloride, did not significantly increase surfactant secretion above basal levels in type II cells isolated from warm-active ground squirrels, at assay temperatures of either 37°C or 4°C (Fig. 2). Moreover, type II cells isolated from torpid ground squirrels did not respond to carbamylcholine chloride when incubated at 37°C; however, carbamylcholine chloride did significantly increase surfactant secretion above basal levels in torpid squirrel cells when the cells were incubated at 4°C for 30 min.

cAMP production

Basal cAMP production was unaffected by either the state of the squirrel from which the cells were isolated, or by the temperature at which the assay was performed (Fig. 3). Isoproterenol significantly increased cAMP production in type II cells isolated from both warm-active and torpid squirrels at both 37°C and 4°C assay temperatures. When assayed at the body temperature of the squirrel from which the cells were isolated, isoproterenol-stimulated secretion was significantly higher in type II cells isolated from torpid ground squirrels compared to warm-active squirrels.

Discussion

The effect of temperature on basal secretion from type II cells

Our laboratory has successfully modified the cell culture method of Dobbs et al. (1986a) to isolate mammalian and non-mammalian alveolar type II cells using different digestive enzymes (Miller et al., 2001; Ormond et al., 2001; Sullivan and Orgeig, 2001; Sullivan et al., 2001; Sullivan et al., 2002; Wood et al., 1999, 2000). In this study, Trypan Blue, microscopy and LDH cytotoxicity assays confirmed the health and viability of the squirrel type II cells during the isolation and assay procedures at both 4°C and 37°C. Warm-active squirrel type II cells assayed at 37°C appeared to secrete 2–3 times the amount of phosphatidylcholine (6% after 1 h) than was secreted from isolated rat type II cells in culture (2% after 1.5 h) (Brown and Longmore, 1981; Dobbs and Mason, 1979). Furthermore, torpid squirrel type II cells assayed at 4°C secreted even higher basal levels of phosphatidylcholine (10%
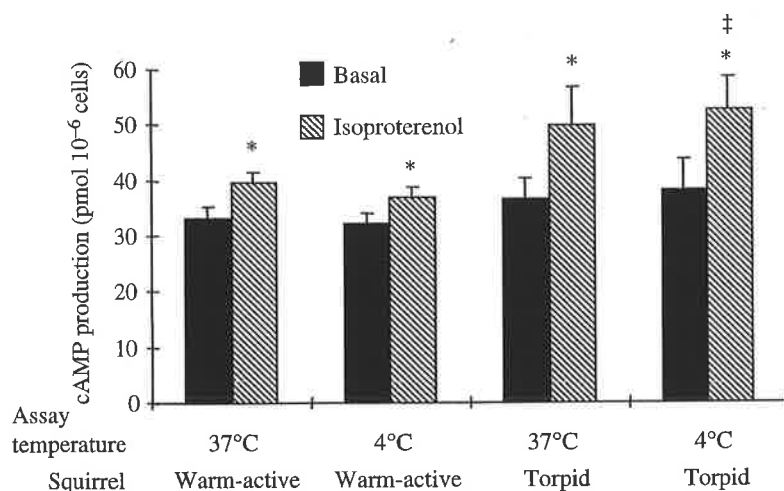
Fig. 3. The production of cAMP (pmoles 10^{-6} cells) in alveolar type II cells isolated from warm-active and torpid ground squirrels and assayed at two assay temperatures (37°C and 4°C). cAMP production was calculated using a direct cAMP ELISA kit (Amersham Pharmacia, Sydney, Australia). Bars represent the amount of cAMP produced (mean ± S.E.M.) in $N=5$ experiments in all cases. *Significant increase above basal cAMP production for each experimental group; †significant difference between warm-active cells assayed at 37°C and torpid cells assayed at 4°C (*t*-test, $P<0.05$).

after 1 h) than warm-active type II cells at 37°C (Fig. 2). High levels of phosphatidylcholine secretion have been observed in type II cells isolated from heterothermic fat-tailed dunnarts *Sminthopsis crassicaudata* (10% after 1 h) (Ormond et al., 2001) and juvenile tammar wallabies *Macropus eugenii* (12–18% after 4 h) (Miller et al., 2001). Hence, a higher rate of basal phosphatidylcholine secretion may be an adaptation to the temperature fluctuations experienced by heterothermic mammals. In addition to reducing the intrapulmonary pressure required to inflate compressed lungs (Possmayer, 1997), larger amounts of surfactant may be required during torpor to prevent the adhesion of alveolar surfaces, which are in contact with each other during long non-ventilatory periods (Daniels et al., 1998).

PC secretion from type II cells isolated from torpid squirrels incubated at 4°C is almost double that measured from type II cells isolated from warm-active squirrels incubated at 37°C. Therefore, despite a dramatic drop in body temperature during torpor, the release of surfactant from type II cells in torpid squirrels is maintained and even upregulated to levels above those of type II cells from warm-active squirrels. Such an observation suggests that squirrel type II cells and, particularly, their pathways of surfactant secretion, are highly adapted to cope with temperature fluctuations. Furthermore, the upregulation of surfactant secretion during torpor highlights the importance of surfactant function to the lung even when ventilatory rate and tidal volume are markedly reduced. The Q_{10} values obtained from type II cells isolated from both warm-active and torpid squirrels and incubated at both 37°C and 4°C are given in Table 1. In all experimental groups, the Q_{10} values were significantly below 2, which confirms that the process of surfactant secretion in squirrel type II cells is highly insensitive to changes in incubation temperature. We reported a similar observation ($Q_{10}=1.2$) in type II cells isolated from warm-active dunnarts and incubated at 37°C and 18°C (Ormond et al., 2001). In addition, the Q_{10} value of 0.85 obtained for type II cells isolated from torpid squirrels is significantly lower than the value of 1.2 obtained for type II cells isolated from warm-active squirrels. This may indicate that during torpor, additional modifications occur at the cellular level to promote the secretion of surfactant from squirrel type II cells. Such modifications may include thermal acclimation of plasma membranes (Hazel and Zerba, 1986), thermal modification of proteins or enzymes (Storey, 1997) or the upregulation of receptors and signalling molecules (Van Breukelen and Martin, 2002) involved in the surfactant secretory and regulatory pathways within the cell.

Although the process of surfactant secretion from squirrel type II cells is relatively insensitive to temperature, short-term changes in assay temperature significantly decreased surfactant secretion. PC secretion from type II cells isolated from warm-active squirrels was significantly higher at an assay temperature of 37°C than at 4°C. Conversely, PC secretion from type II cells isolated from torpid squirrels was significantly higher at an assay temperature of 4°C than at 37°C. Hence, basal surfactant secretion was significantly

higher when type II cells were incubated at a temperature similar to the body temperature of the squirrel from which they were isolated, whether 37°C or 4°C. The decreases in surfactant secretion from warm-active squirrel type II cells incubated at 4°C compared to 37°C could be attributed to a decrease in cellular metabolic rate at 4°C. A similar decrease in PC secretion was observed in warm-active dunnart type II cells incubated at 18°C compared to 37°C (Ormond et al., 2001). We observed a significant increase in PC secretion in type II cells isolated from torpid squirrels and incubated at 4°C, however, and this suggests that squirrel type II cells undergo a process of thermal acclimation, in preparation for or during torpor, which results in the upregulation of surfactant secretion. Therefore, the upregulation of surfactant secretion from squirrel type II cells appears to be an adaptive characteristic of torpor.

Control of phosphatidylcholine secretion by an adrenergic agonist

Warm-active squirrel type II cells appear to respond to isoproterenol at both warm and cold incubation temperatures. This is similar to observations we have previously made in type II cells isolated from bearded dragons and warm-active dunnarts incubated at 18°C and 37°C (Wood et al., 1999). In the present study, the stimulatory response of squirrel type II cells to isoproterenol after 1 h at 37°C (270% after 1 h) is similar to that observed in rat type II cells at 37°C (300% after 1.5 h) (Dobbs and Mason, 1979). In type II cells isolated from torpid squirrels, the response to isoproterenol is small (125% after 1 h at 4°C) compared to that observed in warm-active squirrel cells (270% after 1 h at 37°C). Although the relative roles of the sympathetic and parasympathetic nervous systems during torpor are not yet clear, it is generally accepted that during the deep torpor of hibernators the activity of the sympathetic nervous system is dramatically reduced, if not eliminated (Milsom et al., 1999). Hence, adrenergic agonists may not be an important regulatory mechanism of surfactant secretion during deep torpor. Consequently, squirrel type II cells may downregulate their response to adrenergic stimulation by decreasing receptor number or the activity of enzymes in the β-adrenergic stimulatory pathway. Decreasing receptor number, however, could potentially impair the initiation of arousal, which is accompanied by a large increase in sympathetic activity (Milsom et al., 1999). Hence, in order to enable a rapid response to the return of sympathetic activation, it seems more likely that receptor number on alveolar type II cells is maintained during torpor. Furthermore, we observed an increase in the production of cAMP in response to isoproterenol in torpid squirrel type II cells (Fig. 3), which indicates that isoproterenol is binding to at least some β-adrenergic receptors in the plasma membranes of torpid cells. A decrease in cellular metabolic rate and/or the activity of enzymes involved in β-adrenergic receptor signalling could also account for the smaller response to adrenergic stimulation we observed in torpid squirrel type II cells; however, we have observed that torpid squirrel type II

cells have significantly higher levels of basal PC secretion than warm-active squirrel type II cells, when assayed at the body temperature of the animal from which the cells were isolated. Therefore, the upregulation of basal secretion may be an adaptive response to the decrease in adrenergic stimulation, and hence, agonist-stimulated surfactant secretion, during torpor. Alternatively, the decrease in the response of torpid type II cells to adrenergic stimulation may be due, at least in part, to the upregulation of basal secretion during torpor, and therefore a lower reserve capacity to respond to adrenergic agonists.

Control of phosphatidylcholine secretion by a cholinergic agonist

Carbamylcholine chloride did not appear to stimulate surfactant secretion very effectively in the squirrel, and this finding is consistent with observations made in homeothermic mammalian type II cells. While carbamylcholine chloride can stimulate surfactant secretion *in vivo*, it does not appear to act directly on type II cells isolated from homeothermic mammals (Dobbs and Mason, 1979). However, a significant increase in response to carbamylcholine chloride was observed in torpid squirrels at an assay temperature of 4°C after 1 h and this finding is consistent with observations made in type II cells isolated from heterothermic mammals and ectothermic animals. Type II cells isolated from dunnarts *S. crassicaudata*, bearded dragons *P. vitticeps*, frogs *Rana catesbeiana* and Australian lungfish *Neoceratodus forsteri* respond directly to cholinergic agonists (Wood et al., 1999, 2000). Furthermore, in isolated bearded dragon type II cells, carbamylcholine chloride only stimulated cells that were incubated at cold assay temperatures (18°C) and not at warm assay temperatures (37°C) (Wood et al., 1999). Similarly, in the present study, carbamylcholine chloride only stimulated surfactant secretion in torpid squirrel cells incubated at 4°C, and not at 37°C. Furthermore, warm-active squirrel type II cells did not respond to carbamylcholine chloride at either incubation temperature. Hence, the response to carbamylcholine chloride appears to be highly temperature sensitive in squirrel and lizard type II cells and appears to be only operational at cold temperatures. The switch in the response of type II cells to cholinergic factors at low incubation temperatures may be due to temperature-sensitive cholinergic receptors or enzymes in the cholinergic signalling pathway. Alternatively, low temperatures may reduce the activity of enzymes, such as acetylcholinesterase, which break down acetylcholine and, therefore, may lead to a relative increase in the amount of acetylcholine interacting with the cholinergic receptors on type II cells (Wood et al., 1999).

In this study, type II cells isolated from warm-active squirrels did not respond to cholinergic stimulation, which suggests that parasympathetic control of surfactant secretion, through interactions with muscarinic receptors on type II cells, is not crucial in warm-active animals. However, it should be noted that parasympathetic control of surfactant secretion from type II cells may still occur indirectly in warm-active animals

in vivo. Wood et al. (1997) postulated that the parasympathetic nervous system can also stimulate surfactant secretion *in vivo* via the stimulation of receptors on pulmonary smooth muscle and the subsequent distortion of type II cells (mechanical stimulation). In ground squirrels, the entrance into torpor is controlled by the parasympathetic nervous system, which regulates the initial change in heart rate that occurs before body temperature falls (Milsom et al., 1999). The failure of warm-active squirrel type II cells to respond to cholinergic agonists, at both warm and cold assay temperatures, suggests that parasympathetic control may not be important in controlling surfactant secretion during entry into torpor. Indeed, as body temperature begins to fall, parasympathetic tone appears to be progressively withdrawn (Milsom et al., 1999). Some authors believe that during deep torpor, parasympathetic influence is at a minimum (Lyman and O'Brien, 1963) or completely absent (Milsom et al., 1993). There is also evidence, however, to suggest that both the sympathetic and parasympathetic nervous systems are reduced in proportion to body temperature ($Q_{10}=3$) (Milsom et al., 1993). Furthermore, although the activity of the vagus nerve is low during torpor, other studies support the conclusion that the parasympathetic system still plays some role in cardiovascular control (Milsom et al., 1999). In this study, torpor increased the sensitivity of squirrel type II cells to cholinergic agonists, and this supports observations that some parasympathetic tone is retained during deep torpor. Increasing the sensitivity of type II cells to direct cholinergic stimulation during torpor may enable some regulation of surfactant secretion, despite a reduced autonomic output.

cAMP production

We observed no differences in basal cAMP production between type II cells isolated from torpid squirrels and warm-active squirrels assayed at 4°C and 37°C, respectively. Hence, ground squirrels are still able to maintain cAMP levels during torpor at 4°C. Furthermore, an acute temperature change for the assay period (from 37°C to 4°C in warm-active squirrel cells or from 4°C to 37°C in torpid squirrel cells) had no effect on cAMP production. This is probably due to the short incubation time (15 min) of the cAMP assay which, under culture conditions, may not have been long enough to enable the type II cells to register the temperature change. Isoproterenol significantly increases cAMP levels in type II cells isolated from warm-active squirrels and torpid squirrels at both assay temperatures; however, isoproterenol-stimulated cAMP production is significantly higher in type II cells isolated from torpid ground squirrels compared to cells isolated from warm-active squirrels when assayed at the temperature that matched the body temperature of the squirrels from which the cells were isolated. This indicates that the squirrel type II cells may be upregulating their response to isoproterenol during torpor without increasing basal cAMP levels. Furthermore, isoproterenol-induced cAMP production does not appear to correspond directly to isoproterenol-stimulated PC secretion. cAMP is an important indicator of β-adrenergic receptor activity; however, there is no evidence to suggest that increases

in cAMP are directly linked to increases in surfactant secretion. In fact, this study suggests that cAMP is not directly involved in the stimulation of PC secretion, and the activities of other signalling molecules may play a role here.

Conclusions

In this study, we observed that alveolar type II cells isolated from torpid squirrels demonstrate an increased basal secretion of pulmonary surfactant. These findings are supported by previous observations that surfactant PL increases during cold-acclimation in ground squirrels (Melling and Keough, 1981). The process of surfactant secretion from squirrel type II cells is highly resistant to temperature changes, as demonstrated by Q_{10} values of 0.85–1.2. Furthermore, when assayed at the temperature matching the body temperature of the animal from which they were isolated, type II cells from torpid squirrels demonstrated a higher basal surfactant secretion than those isolated from warm-active squirrels. Therefore, the upregulation of surfactant secretion from squirrel type II cells appears to be an adaptive characteristic of torpor, as does the response of type II cells to cholinergic stimulation. Increasing the sensitivity of type II cells to cholinergic (parasympathetic) stimulation during torpor may enable surfactant secretion to be regulated or enhanced, even when autonomic output is low. The response of squirrel type II cells to cholinergic stimulation during torpor, but not euthermia, supports observations that although parasympathetic tone may be significantly reduced during deep torpor, it is still important in regulating some physiological processes.

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APPENDIX 4

THE EFFECT OF TEMPERATURE ON ADRENERGIC RECEPTORS OF ALVEOLAR TYPE II CELLS OF A HETEROTHERMIC MARSUPIAL.

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