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Phenotypic Plasticity of the Lung Surfactant System at High Altitude in Deer Mice, *Peromyscus maniculatus*

A Dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Evolution, Ecology and Organismal Biology

By

Sonia Diaz

December 2012

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ABSTRACT OF THE DISSERTATION

Phenotypic Plasticity of the Lung Surfactant System at High Altitude in Deer Mice, *Peromyscus maniculatus*

by

Sonia Diaz

Doctor of Philosophy, Graduate Program in Evolution, Ecology and Organismal Biology
University of California, Riverside, December 2012
Dr. Kimberly A. Hammond, Chairperson

The pulmonary surfactant system is a multifaceted and highly complex mixture of lipids and proteins that work to reduce the surface tension created at the air-liquid interface within the lung, and provide innate immunity to the lung. Reducing surface tension allows for lung stability, patency and functioning, a crucial task to undertake in oxygen-limiting environments, such as high altitude. The lower partial pressures of oxygen and colder ambient temperatures found at high altitude pose a functional and metabolic challenge to the lung and possibly lung surfactant as well. Therefore, we determined the effects of high altitude hypoxia on the lipids and proteins of the lung surfactant system in adult deer mice. Because deer mice are known to exhibit physiological and genetic adaptations to high altitude, we hypothesized that mice would up-regulate the amounts of lipids and proteins in order to maintain lung stability and function. Specifically, the amounts of saturated lipids will increase to aid in decreasing surface tension, thereby preventing alveolar collapse, and the proteins will have a correlated response given that lipids and proteins work in unison to lower surface tension. Furthermore, since the cholesterol component of lung surfactant is known to increase the
fluidity of surfactant layer, thereby promoting proper function, we determined the
changes in cholesterol amounts under hypoxia and cold temperatures. These questions
were addressed by simple acclimation of mice to low (380 m) and high (3800 m)
altitudes and cold (5°C) or warm (25°C) temperatures. Lung surfactant was obtained via
lung lavage, lipids or proteins analyzed, blood samples taken and organs dissected.
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CHAPTER 1. General Introduction and Review

It is well known that organisms are inseparable from, but also often physiologically challenged by the pervasive biotic and abiotic factors present in their environment (Bartholomew, 1958). Challenging environments, whether they test an organism’s ability to conserve water, acquire oxygen or regulate temperature, are good venues to observe magnifications in physiological responses that result in the maintenance of homeostasis. High altitude presents many physiological challenges including colder ambient temperatures and lower oxygen partial pressures. The lung, being the site of oxygen uptake, is an especially important organ at high altitude. Situated at the interface for oxygen uptake to the blood, the lung must function in spite of impaired gas exchange and air-blood diffusion limitation at high altitude (Schoene, 2001).

The phenotypic plasticity (alteration in phenotype in response to changing environmental conditions) of lung tissue under hypoxic conditions results in numerous mechanisms for accommodating low oxygen levels. Hypoxic challenges to the lung, imposed via environment (e.g., high altitude) or disease, manifest their consequences on all components of the lung (Nicolls and Voelkel, 2007). Generally, lung mass increases when exposed to hypoxia (Bartlett, 1970; Bartlett and Remmers, 1971; Hammond et al., 1999; Hammond et al., 2001), presumably to increase the surface area available for gas exchange. Hypoxia has also been shown to affect cell membrane receptor expression and function (Tuder et al. 1995), promote vascular re-modeling (Stenmark et al., 2006), up-
regulate transcription factors and alter many lung signaling pathways. Hypoxic pulmonary vasoconstriction (HPV), occurring in response to alveolar hypoxia, allows blood flow to be redirected to well ventilated areas of the lung (Von Euler and Liljestrand, 1946), but may also result in pulmonary hypertension. The elevated pressure within the pulmonary capillaries, characteristic of pulmonary hypertension, can ultimately cause fluid to leak into the air spaces and further impair oxygen diffusion (Eldridge et al. 2006; Maggiorini et al. 2001). Pulmonary surfactant, a complex mixture of lipids and proteins found within the lung, has been implicated in preventing pulmonary edema (Gnadet et al., 2012; Luo et al., 2012; Schoene, 2004). More importantly, pulmonary surfactant is responsible for reducing the surface tension in the lung, promoting alveolar stability and increasing lung compliance; factors that are of great importance for increasing oxygen uptake at high altitude.

The importance of lung surfactant is evidenced by the physiological consequences when it is absent. Human preterm infants, not having adequate time to develop their surfactant system, experience respiratory distress syndrome, characterized by the inability to inflate their air sacs properly and thereby impeding their ability to breath. Specifically, the forces that create surface tension in the lung are not overcome and the alveoli within the lung are not distended or allowed to fill with air (Avery and Mead, 1959). This highlights the significance of the lipids and proteins that make up the pulmonary surfactant system and allow for surface tension to be reduced. This complex and dynamic surfactant system, comprised of phospholipids (saturated and unsaturated),
neutral lipids and proteins is necessary for lung stability and functioning, and consequently for life.

Given its absolute necessity, the lung surfactant system is likely to possess some degree of plasticity in order to accommodate changing and/or harsh environments, such as at high altitude. To address this I reviewed the literature to assess past and current understanding of how lung surfactant responds the physiological stress of hypoxia. I then examined the effects of high altitude on the plasticity of the lung surfactant system as a result of simple acclimation at two different altitudes. Mice were either acclimated to high altitude (3800 m, ~100 torr) or remained at low altitude (340 m, ~154 torr) and used as controls. I measured changes in individual phospholipid amounts between high- and low-altitude acclimated mice; including a measure of total phospholipid. Given that colder ambient temperatures are innate at high altitude and require increased oxygen uptake to support a higher metabolic output for thermoregulation, I also determined the change in surfactant lipid amounts at lower temperatures. In particular I measured changing cholesterol levels in response to cold temperatures to determine if it plays a role in fluidizing the surfactant lipids.

Since surfactant lipids and proteins work in unison to lower surface tension, I measured the changes in relative amounts of proteins SP-B and SP-C between high- and low-altitude acclimated mice; including a measure of total protein. In addition to measuring protein and lipid amounts, data on organ masses and hematocrit were collected.
By measuring changes in the amounts of lipids and proteins in lung surfactant I was able to determine if and how the lung surfactant system responds to the physiological stress of high altitude hypoxia and to what capacity the degree of phospholipid saturation plays a role in surfactant changes. Ultimately, the lung’s response to hypoxia involves multiple cell types and requires the integrative response of many systems; all of these processes governed by the genetic complexity that underlies phenotypic plasticity.
Review

Introduction

Anatomically, the lung appears as a simple and delicate branching system, functioning as the interface between internal and external environments, constantly exchanging gases. Despite this, the complexity involved in its development, growth and physiology have been researched and documented at length (Harding, 2004). Given the lung’s primary role in gas exchange, substantial attention has been focused on how the lung functions in differing environmental conditions, particularly where oxygen is limiting. Indeed, studies have demonstrated the numerous effects of hypoxia on parameters such as lung morphology, fluid balance and ion transport (Aarseth et al., 1980; Bartlett and Remmers, 1971; Hammond et al., 1999; Hammond et al., 2001; Heberlein et al., 2000; Papen et al., 2001; Suzuki et al., 1999; Suzuki et al., 1999; White et al. 1994; Weissmann, 2008). However, one aspect of the lung that has not been as extensively studied in hypoxia is the plasticity of the pulmonary surfactant system. This dynamic system is responsible for maintaining lung stability, preventing alveolar collapse, increasing lung compliance and reducing the work associated with breathing, a crucial task that must be accomplished in the face of lowered oxygen pressure. The purpose of this review is to summarize past and current findings on how the surfactant system responds to hypoxia and how these findings can be applied to the study of organisms native to high altitudes and the role surfactant plays in their physiological adaptation to this oxygen-limited environment.
**The Pulmonary Surfactant System**

A complex mixture of proteins and lipids, the pulmonary surfactant system, reduces the surface tension (T) at the air-liquid interface in the lung. The major lipids that make up mammalian surfactant are **phospholipids (PL)**, which include phosphatidylcholine (PC), lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE). Approximately 80% of surfactant is composed of phosphatidylcholine, half of which is in the form of dipalmitoylphosphatidylcholine (DPPC) (Veldhuizen et al., 1998). The major neutral lipid component of surfactant is cholesterol (Veldhuizen et al., 1998) and the four surfactant proteins that work in conjunction with the lipids are known as surfactant proteins (SP)- A, -B, -C and- D (Possmayer, 1988).

Two cell types predominate within the alveoli: alveolar type I and II cells. Type I cells provide structural support to the alveoli and are the principal cells where gas exchange occurs while alveolar type II cells are responsible for secreting the surfactant lipids into the hypophase, the liquid layer that lines the lung (Figure 1.1). The PL are known to be assembled in the endoplasmic reticulum (ER) of the type II cell and transported to the Golgi apparatus where the lipids and proteins are packaged into lamellar bodies (LB) (Buckingham and Avery, 1962); (Daniels and Orgeig, 2003; Dietl and Haller, 2005). The lamellar bodies are secreted from the Golgi apparatus via exocytosis into the hypophase, where they swell and unravel to form tubular myelin. It is
the tubular myelin that delivers the lipids and proteins to the air-liquid interface. The lipids at the interface and those found directly underneath the interface (surface associated phase) can be recycled back into the type II cell via endocytosis (Figure 1.2).

The degree of saturation found in the hydrocarbon chains of phospholipids play an important role in lowering surface tension. Phospholipids that posses disaturated hydrocarbon tails (no double bonds) allow for tight packing between lipids, thereby creating a dense monolayer of lipids at the air-liquid interface with extremely low surface tension (Whitsett, 2004) (Figure 1.3). Disaturated PLs are referred to as DSPs. The major lipid thought responsible for reducing surface tension to a minimum near 0 mN/m is the fully saturated DPPC. Unsaturated PL (containing double bonds), on the other hand, do not allow for the orderly packing of lipids and hence cannot lower surface tension to the same extent as DSPs. However, biophysical studies of surfactant and interfacial films, using scanning and atomic force microscopy, have found that a pure film of DPPC is not required for generating very low surface tensions as previously thought. Additionally, low surface tensions can be achieved with high levels of unsaturated PL, although the mechanism of how this occurs is unknown (Crane et al., 1999; Discher et al., 1996; Discher et al., 1999; Discher et al., 1999; Grunder et al., 1999; Nag et al., 1998; Perez-Gil, 2008; Piknova et al., 2001; Piknova et al., 2002; Smith et al., 2003; Zuo et al., 2008).

Studies on dunnarts, wombats, shrews and Tazmanian devils also show that DPPC is not the major PL in their pulmonary surfactant and that surfactant composition is matched to the biology of each organism (Lang et al., 2005).
The lung and its alveoli are dynamic in the sense that they are consistently undergoing cyclical inflation and deflation thereby increasing and decreasing their volume. Although lung stability is always necessary, it is of particular importance at low lung volumes. Consider the law of Young and Laplace,

\[ P = \frac{2T}{r} \]

where \( P \) is the pressure (dyn/cm\(^2\)) within a distensible sphere, \( T \) is the tension (dyn/cm\(^2\)) and \( r \) is the radius of the sphere (cm). If we regard alveoli as spheres of variable sizes, with equal surface tension, connected by a common airway (as in fact they are), then according to Laplace’s law the pressure of a small alveolus will be greater than that of a larger alveolus and empty into the larger one. This instability within the lungs would be fatal, but the surfactant system works against this instability by reducing the surface tension near zero at very low lung volumes. The retractile forces in the lung are thus equivalent within separate regions of the alveoli and between the respiratory units, creating alveolar stability (Schurch, 1982). This results in a decrease in the elastic recoil of the lung, increased compliance and therefore a decrease in the work required for breathing (Levitzky, 2003) (Figure 1.4).

As with most lipids found in cell membranes, surfactant lipids have critical transition temperatures (\( T_c \)) which determine whether the lipid will exist in one of two states: ordered gel state or disordered liquid state (Possmayer, 2004). This is an extremely important characteristic of the phospholipids found in surfactant in many ways. First, in order for lipids to spread over an area of the alveoli, the lipids must all exist in the fluid
state. Considering that different lipids have different transitions temperatures, (e.g. at a particular temperature one lipid will exist in a gel state while another exists in the fluid state), makes it imperative that many different lipids (unsaturated, saturated and neutral lipids) coexist in the surfactant film at the same time to lower the transition temperature of the mixture enough to permit fluidity. An example of this is seen in DPPC, whose transition temperature of 41°C (Meban, 1981) is above physiological temperature and thus needs other lipids to allow it to exist in a fluid state, allowing it to spread at the interface of the alveoli and reduce surface tension.

Providing lung stability and quick adsorption to the air-liquid interface is not an inherent quality of phospholipids alone. Surfactant proteins, A, B, C, and D (Possmayer, 1988) play an important role in lowering surface tension and providing immunity to the lung. Surfactant proteins make up ~8% of surfactant by mass and are either hydrophilic (SP-A and SP-D) or hydrophobic (SP-B and SP-C) in character. SP-A, the most abundant protein (50% of protein in purified surfactant), is thought to play a role in lipid recycling and is a key player in providing lung immunity (Crouch and Wright, 2001). SP-D shows functional and structural similarity to SP-A, reflecting a similar role in lung defense (Crouch and Wright, 2001; Reid, 1998). SP-B is tightly associated with surfactant phospholipids, enhances the spreading and stability of the surfactant and is the only protein required for proper lung functioning (Figure 1.5). A deficiency of this protein is fatal for both mice and humans (Weaver and Conkright, 2001). SP-C, the most hydrophobic protein, enhances the rate of adsorption both alone and in combination with SP-B (Cockshutt and Possmayer, 1991; Tabor et al., 1990; Van Golde et al., 1988)
(Figure 1.5). These proteins, although different in structure and composition, work together to promote the goal of the surfactant system: stability and proper functioning of the lung.

**General Effects of Hypoxia on the Lung**

It is well known that the lung exhibits a plastic response to hypoxia. The effects of hypoxia on lung growth and morphology present mixed results and depend on whether the hypoxia is simulated or caused by exposure to high altitude. Bartlett (1970), found that simulated, short-term hypoxic exposure had no effect on lung growth or morphology in rats, while Bartlett and Remmers (1971) found that simulated hypobaric hypoxia in young rats resulted in an increase in lung volume and alveolar surface area. Tenney and Remmers (1966) found no difference in lung volume or alveolar size between guinea pigs native to high altitude and those raised at sea level. On the other hand, Kistler et al., (1966) found an increase in lung volume in rats exposed to high altitude hypoxia. The lack of consistency in the direction or nature of changes in lung growth and morphology could be due to differences in the degree of hypoxia and time course of acclimation (Stickney and Van Liere, 1953). Indeed guinea pigs acclimated to high altitude for 1, 3 and 6 months increased their lung volume and alveolar surface area after one month with a continued increase after six months (Hsai et al., 2005). Although changes in lung morphology depend on the degree of hypoxia and acclimation period, the fact that low oxygen availability induces morphological changes in the lung is clear. Being the organ responsible for gas exchange, and therefore dependent on a vast surface area, these
changes presumably help to accommodate and/or accelerate physiological adaptation to hypoxia. Consequently, pulmonary surfactant, being seated at the air-liquid interface of the respiratory units, could inherently be regulated by hypoxia as well.

**Effects of Hypoxia on Lung Surfactant Lipids**

The profound effects hypoxia can have on lipids have been shown across many organ systems. Epididymal fat pads incubated in hypoxic conditions had a significantly greater basal release of free fatty acids (FFA) and glycogen content compared to controls (Alpert, 1970). Yatsu and Moss (1971) found a reduced amount of brain phospholipids in rats exposed to hypoxia. More recently, it’s been noted that hypoxia plays a role in creating a state of insulin resistance in adipose cells by inhibiting insulin signaling pathways (Regazzetti et al., 2009). Baze et al., (2010), using oligonucleotide microarrays, noted that hypoxia results in differential expression of genes involved in glycolysis and lipid metabolism, implicating their involvement in acclimation to hypoxia. As a result, the protein and lipid mixture known as surfactant has been of interest to those who study hypoxia given its absolute necessity for proper lung function and stability.

Due to the complex nature of the surfactant system and the fact that it is situated and functions within the lung, studying lung surfactant requires the use of excised lungs, cultured lung cells or extracted surfactant via bronchoalveolar lavage (BAL). Most studies looking at the effects of hypoxia on lung surfactant have used simulated conditions, utilizing chambers where pressure and oxygen concentrations could be manipulated, or have artificially altered respiration rates to reduce oxygen delivery to
Few studies have focused on environments where hypoxia occurs naturally, including high altitude. One of the first references to hypoxia possibly altering the surfactant system was documented by Valdivia et al., (1966). An accumulation of lipid material and reduced number of lamellar bodies were seen in the pneumocytes of guinea pigs exposed to varying levels of altitude. Greater amounts of lipid vacuoles were observed under more severe hypoxic conditions, though no clear identification of the lipid species was made.

Initial studies on lung surfactant and hypoxia traced the incorporation of radioactively labeled free fatty acids (FFA) into lung tissue to determine changes in lipid metabolism. This question was of special interest since it was known that lung surfactant was housed within the lung and had a prominent phospholipid component. Naimark and Klass (1967) showed that under hypoxia, greater amounts of FFA were incorporated into lung tissue but significantly lower amounts of FFAs were incorporated into triglycerides and phospholipids, the lipid components of surfactant. These findings, although specific to whole lung tissue, suggested that lipid metabolism of the lung was indeed being altered in the presence of hypoxia, and equally as important it created a new level of inquiry as to the physiological importance of the surfactant system in varying environmental conditions, such as high altitude hypoxia. Newman and Naimark (1968) confirmed that hypoxia decreased fatty acid incorporation into lung tissue phospholipid and triglyceride fractions as well as decreased the turnover rate of lipids. It was concluded that hypoxia reduced the rate of lipid metabolism in the lung, as had been previously seen in other tissues (Benson et al., 1961; Chetverikov and Gasteva, 1966; Michal et al., 1959; Sanders
et al., 1965). Similarly, Chander et al., (1975) found that hypoxia decreased total lipid, total phospholipid and PC amounts in lung tissue. Alveolar surfactant lipid amounts were also found to decrease, specifically PC and PE. However, incorporation of $^{32}$P into lung PC increased almost three times as much as controls, suggesting that PC amounts may be metabolized or secreted at a higher rate given its importance in lowering surface tension. Despite finding decreased lipid amounts, the surface activity (ability to lower surface tension) of the lipid material was not affected. When surface activity was indirectly measured using the stability index of the alveolar bubbles (Newmark and Naimark, 1968). In contrast, subsequent studies, found that acute hypoxia did not change lipid quantities in lung tissue or surfactant, but upon superimposing cold temperature total PL, PC and PE amounts were reduced (Kumar et al., 1980). Although these results were not consistent with previous studies, the addition of cold temperature presented another important and physiologically relevant factor found at high altitude that could modify surfactant composition.

Modifications in surfactant lipid amounts could consequently alter surfactant function. Given that interfacial forces found within the lung dictate the elastic behavior of the lung (Beckman and Bean, 1970; Radford and Hunt, 1964) any alterations in lung surfactant can potentially increase the surface tension within the lung. Specifically, a decrease in PC, a major lipid involved in lowering surface tension, can alter the surface tension lowering properties of surfactant. Indeed Castillo and Johnson (1969) found that a simulated altitude of 14,000 ft resulted in reduced surface activity of lipids obtained from lung tissue extracts. The elevated surface tension was attributed to reduced
surfactant amounts, although surfactant amounts were not measured. Similarly, Srivastava et al., (1974) found that hypoxia significantly increased surface tension of lung homogenates in rats as well as significantly reduced lung compliance in mice (Srivastava et al., 1976).

Decreased amounts of surfactant lipids, such as PC, are associated with an increase in surface tension but are also indicative of increased oxidative activity at high altitude. A number of studies have shown that hypoxia increases oxidative stress in the form of lipid peroxidation (Jefferson et al., 2004; Araneda et al., 2005; Behn et al., 2007). Decreased PC levels in hypoxia are generally accompanied by an increase in lysocompounds (derivatives of phosphatidic acid), such as LPC, and have emphasized the possible roles of lipid peroxidation and/or macrophage activity in reducing phospholipid amounts (Prevost et al., 1980; Zaitseva et al. 1981). Prevost et al. (1980) demonstrated that hypoxia decreased PC levels by 20% and increased LPC by 10-fold, indicating the oxidation of PC to LPC. Likewise, Zaitseva et al. (1981) found a significant decrease in PC with a 2-fold increase in the content of LPC, increased surface tension and edematous alveoli. Interestingly, lung edema, a respiratory malady experienced at high altitude, could be attributed to increased surface tension due to altered lipid amounts that ultimately result in facilitating the movement of fluid from the interstitium into the alveoli (Droma et al., 2001).

Environments lacking oxygen require organisms to rapidly modify cell and tissue function to maintain homeostasis. Similarly, due to its essential function, lung surfactant
has been shown to alter its composition rapidly under hypoxic conditions. Lyamtsev and Arbuzov (1981) revealed that surfactant surface activity decreased in rats after 6 hours in simulated hypoxia (6000 m). Parallel changes were seen in the degree of atelectasis (alveolar collapse) and accumulation of lipid material at the periphery and within the lumen of the alveoli. Furthermore, it was noted that surface activity returned to normal at a simulated altitude of 9000 m. These findings suggest that lung surfactant can indeed rapidly alter its function in a manner that suggests compensation mechanisms (changing lipid amounts) are necessary above a critical level of hypoxia (Lyamtsev and Arbuzov, 1981).

Overall, it appears that hypoxia alters surfactant lipid metabolism in such a way that total and individual lipids decrease under hypoxic conditions; either as a consequence of increased lipid peroxidation, macrophage activity or decreased lipid synthesis or secretion. As a corollary the capacity to lower surface tension and maintain adequate lung compliance is compromised. Taken together, it appears hypoxia may result in negative consequences for any organism inhabiting high altitude environments where hypoxia is ubiquitous. However, the enormous diversity of life forms at high altitude suggests otherwise, and stresses the importance of the role of surfactant in physiological adaptation. The varied and sometimes inconsistent results of the previous studies could be a manifestation of the inherent complexity of the lung surfactant system and the current inability to investigate this system in situ. Many factors, of course, could account for these differences: time course of acclimation, the specific experimental animals used, using whole lung versus cultured lung cells, and severity (chronic or acute)
and type of hypoxia (simulated, pathological, and environmental). Nevertheless, it is evident that the lung surfactant system possesses some degree of plasticity that ultimately contributes to maintaining lung function and thus supports a myriad of metabolic processes in oxygen-limiting environments.

**Effects of Hypoxia on Surfactant Proteins**

Due to the fact that surfactant lipids have traditionally been implicated as the major players in reducing surface tension, more focus has been placed on their function, metabolism and composition in hypoxia. However, surfactant proteins (SP-A, SP-B, SP-C and SP-D) provide a different but equally important function to the surfactant system. Surfactant proteins, initially thought not to be involved in reducing surface tension (Metcalf et al., 1980), were soon discovered to serve numerous yet specific roles. SP-A and SP-D provide immunity to the lung (Hawgood and Clements, 1990; Shimizu et al., 1992) while SP-B and SP-C, via interactions with lipids, aid in reducing surface tension (Hawgood and Clements, 1990; Perez-Gil and Weaver, 2010).

The effects of hypoxia on surfactant proteins are not well elucidated and have mostly been determined in cultured lung cells, resulting in contradictory conclusions. Jackson et al., (1996) cultured alveolar type II cells under hypoxic environments (2.5% O\textsubscript{2}) for a period ranging from 24 hours to 3 days and found that SP-A transcript expression remained constant throughout. On the contrary, hypoxia (1% O\textsubscript{2}) down-regulated the expression of surfactant SP-C after 8 hours and nearly abolished protein expression after 24 hours in cultured alveolar epithelial cells (Vaporidi et al., 2005).
Hypoxia was also found to induce apoptosis of alveolar cells after only 8 hours. Since severe hypoxia is known to induce lung injury in the form of epithelial cell damage, inflammation, edema and surfactant abnormalities (Greene et al., 1999; Tomashefski, 1990), the effects of hypoxia on surfactant proteins have been investigated in the context of lung injury and its medical applications. Thus, information on how hypoxia alters surfactant proteins and their ability to aid in lowering surface tension are scarce.

Within the framework of lung development, studies have focused on the spatial and temporal expression of surfactant proteins under hypoxic conditions and demonstrated that oxygen levels modulate surfactant protein mRNA expression. Fetal sheep exposed to hypoxemia significantly increased the amount of SP-A mRNA. SP-B mRNA levels increased but not significantly while SP-C levels did not change (Braems, 2003). Given that SP-A is developmentally regulated via hormones and various transcription factors (Odom et al., 1987), it follows that SP-A would increase as lung development progresses, lung vascularization increases and the need for a fully functional air-breathing lung becomes imminent. Yet, from a molecular stance it has been shown that hypoxia inhibits the expression of the SP-A gene by preventing cAMP and Interleukin-1 from stimulating its expression in fetal lungs (Islam and Mendelson, 2002; Islam and Mendelson, 2006; Benlhabib and Mendelson, 2011). However as lung development advances throughout gestation, increased O₂ availability to the lung sets off a series of molecular events enabling the activation of SP-A transcription (Islam and Mendelson, 2006). It is interesting to note that a lack of oxygen initially suppresses
surfactant protein expression but increasing oxygen levels exerts a permissive effect on the stimulation of SP-A gene expression in the developing lung.

Hypoxia has also been shown to have indirect effects on surfactant proteins. Both SP-B and SP-C expression are known to be regulated by hypoxia-induced mitogenic factor (HIMF), a gene found to be highly upregulated in a hypoxia-induced pulmonary hypertension mouse model (Tong et al., 2006). HIMF enhances and induces surfactant protein production and increases both promoter activity and mRNA stability of SP-B and SP-C in lung epithelial cells, indicating that HIMF may ultimately increase production of these surfactant proteins. Therefore, although hypoxia can induce various pathological conditions not related to the lung, its effects are far-reaching and can ultimately influence surfactant production. These findings illustrate the complexity behind the regulation of lung surfactant but also the importance of cellular mechanisms that allow the proper expression of surfactant proteins in the face of physiological challenges.

Currently, more information is available on the effects of hypoxia on the prenatal development of the pulmonary surfactant system (Orgeig et al., 2011) than on the effects of hypoxia on adult lung surfactant (Orgeig et al., 2011; Orgeig and Daniels, 2004). Lung development studies detail the various mechanisms that control the development, maturity, secretion and maintenance of a functional surfactant system from gestation to birth. Hypoxia is known to alter the developmental trajectory of the lung and increasing the risk of lung and surfactant dysfunction. Furthermore, hypoxia has been proposed to
be the evolutionary driver that accounts for differential surfactant development and maturity among vertebrate species (Orgeig et al., 2011).

Despite the wealth of information regarding the surfactant system, it is still unclear how surfactant proteins, alone and in conjunction with surfactant lipids, are altered, not only in the context of high altitude but also with regards to native inhabitants of oxygen-limiting environments. The dynamic nature of the surfactant system under various environmental conditions suggests high altitude hypoxia could provide further insight into surfactant function, composition and behavior as a surface-active film. These results could ultimately expand across many disciplines, having medical applications for synthetic surfactants currently used with neonatal respiratory distress syndrome and implications for elucidating the various and remarkable physiological adaptations organisms employ to survive and thrive in their environments.

The presence of this lipid and protein complex, termed the lung surfactant system, is absolutely necessary to initiate breathing at birth, for proper lung functioning and stability and for maintaining the alveoli open throughout life. Proper lung function, being directly dependent on lung surfactant, is needed for an organism to obtain oxygen, deliver it to different cells and tissues of the body and ultimately produce the energy needed for its survival. The survival of organisms that inhabit hypoxic environments could be contingent upon a present and properly functioning surfactant system. Thus, determining the plasticity of the surfactant system is crucial and can provide a larger framework under which additional intriguing and fundamental questions regarding respiratory physiology
can be answered. Additionally, since lipids and proteins work in unison to reduce surface
tension and maintain lung patency, a study incorporating both components under high
altitude hypoxia would help to elucidate the response of each and perhaps provide a more
comprehensive representation of how the surfactant system (lipids and proteins together)
responds to hypoxia.
Figure 1.1. An alveolar cell depicting type I and type II cells and an alveolar macrophage. Surfactant is synthesized and secreted from the type II cell and the phospholipids reduce surface tension created by the alveolar fluid (hypophase).
Figure 1.2. A schematic representation of the surfactant life cycle. The lipid and protein mixture is synthesized in the ER, made into lamellar bodies within the Golgi apparatus and secreted into the hypophase as tubular myelin where the lipids adsorb to the air-liquid interface to reduce surface tension.
Figure 1.3. Detailed representation of how saturated and unsaturated phospholipids position themselves at the air-liquid interface to disrupt the molecular forces between water molecules and reduce surface tension. Surfactant proteins are in the vicinity aiding in the positioning and spreading of the phospholipids.
Figure 1.4. The Law of Young and Laplace describes how alveoli of different sizes would experience varying pressures. Under the same tension smaller alveoli (B) would experience a greater pressure than larger alveoli (A), due to its smaller radius, and empty into the larger alveoli. This instability within the alveoli could promote alveolar collapse and be fatal but the surfactant system works against this instability by reducing surface tension to near zero at low lung volumes.
Figure 1.5. Schematic representation of the roles surfactant proteins B and C play in stabilizing the surface film and re-spread lipids upon lung compression and expansion, respectively.


CHAPTER 2. Effects of High Altitude and Cold Temperatures on Surfactant Lipids

Abstract. Lung surfactant consists of a mixture of lipids and proteins that work to reduce surface tension at the air-liquid interface found within the lung. The lipid composition of the surfactant system is known to be influenced by temperature and environmental conditions. Thus, lung surfactant has some degree of plasticity, especially with respect to lipids such as cholesterol that allows for a more fluid surfactant to exist at lower temperatures. We examined the effects of high altitude and cold exposure on the lung surfactant system in deer mice, Peromyscus maniculatus. We tested the prediction that deer mice at high altitude would have greater surfactant lipid content relative to mice tested at low altitude. In addition, the amounts of cholesterol would be greater when cold temperatures posed a threat to the fluidity and functioning of the surfactant system. Mice acclimated to both high altitude and cold temperatures had consistently greater amounts of the major lipids involved in reducing surface tension, including cholesterol. Although these results were not statistically significant using standard statistics, changes in alveolar membrane function may be the result of tiny (potentially not statistically significant) changes in membrane fluidity as a result of increases amounts of cholesterol and other phospholipids. Thus, demonstrating that there may be some plasticity to the pulmonary surfactant system under an environmental stressor such as high altitude is important. High altitude hypoxia can present a respiratory challenge that could ultimately affect various metabolic functions. For small mammals living at high altitude, alterations in lung surfactant system could potentially affect metabolic activities such as foraging. In humans, who experience respiratory maladies such as High Altitude Pulmonary Edema
(HAPE), alterations in lung surfactant can lead to understanding what physiological events lead to this condition and what can be done to prevent it.

**Introduction**

The lower partial pressure of oxygen found at high altitude is restrictive to mammalian vertebrates with high daily metabolic rates because it limits the amount of oxygen that can diffuse into the lung and circulating blood, where binding to hemoglobin is crucial. This effect ultimately cascades down to the mitochondria of metabolizing tissues, presenting energetic and physiological constraints for small mammals inhabiting high altitude environments (Hayes, 1989). Researchers have documented the genetic basis of adaptation to high altitude by comparing populations native to high- and low-altitudes, across many vertebrate taxa (Beall, 2007; Cheviron and Brumfield, 2012; Scheinfeldt and Tishkoff, 2010; Simonson et al., 2010; Storz et al., 2007; Storz and Moriyama, 2008). In addition to these genetic changes, however, phenotypic modifications including changes in hematocrit, concentrations of 2,3 diphosphoglycerate, and organ size, are important in facilitating the delivery of oxygen to respiring cells. The lung, essential for acquiring oxygen, is a particularly interesting organ to study at high altitude where lower partial pressures of oxygen prevail. Although morphological (alveolar size) and functional (ventilation) responses to lower oxygen tensions are commonly studied (Bartlett and Remmers, 1971; Burri and Weibel, 1971; Chiodi, 1957; Tenney and Remmers, 1966), less frequently considered are the contributions of the pulmonary surfactant system to the accommodation of hypoxia. Additionally, given that
hypoxia is not the sole environmental challenge at high altitude, additional factors, such as temperature, are important considerations.

Mice at High Altitude as a Model for Surfactant Plasticity

Deer mice, *Peromyscus maniculatus*, are well suited for studying lung physiology at high altitude due to the fact that they are one of the most widespread groups of North American rodents; found across a wide range of altitudes (below sea level to 4300 m) (Hall and Kelson, 1959). Phenotypic changes to digestive and cardiopulmonary organ size and function in deer mice living at high altitudes have been demonstrated in numerous studies (Hammond et al., 1999; Hammond et al., 2001). These changes may help to maintain functional aerobic capacity while the oxygen-limiting environment they live in dictates otherwise (Hammond et al., 2001; Chappell et al., 2007; Russell et al., 2008). Classic studies on the genetics and physiology of hemoglobin have revealed that deer mice possess specialized adaptations potentially allowing for a higher oxygen binding affinity to accommodate lower oxygen availability at high altitudes (e.f., Snyder et al., 1988, Storz et al. 2007, 2008). Modifications that contribute to higher binding affinity have occurred in both the α- and β- subunits of hemoglobin molecules (Storz et al., 2009). More recent work, however, has shown that the high affinity modifications provide only a modest increase in O₂ binding affinity at high altitudes (Storz et al., 2010a) and that not all high-altitude populations of deer mice express the coded adaptations for hemoglobin function (Storz, personal communication). As a consequence of tighter binding, unloading oxygen at the tissues is more difficult and may negatively
impact performance and require deer mice to rely more on phenotypic changes to accommodate the effects of high altitude. Therefore, deer mice are optimal organisms for investigating lung plasticity at high altitude.

*Plasticity in the Lung*

The increases in lung mass, lung volume and alveolar number of mice living at high versus low altitude (Bartlett, 1970; Bartlett and Remmers, 1971; Hammond et al., 1999; Hammond et al., 2001), imply that the lung itself is plastic in the face of low oxygen pressures. These changes have the potential to help accommodate the lower partial pressures of oxygen inherent to high altitude by creating more surface area for oxygen transport from the atmosphere to the blood (Schoene, 2001). Other phenotypic changes occur in heart, blood (hematocrit and hemoglobin) and muscle regulatory enzymes (Hammond et al., 1999; Hammond et al., 2001; Sheafor, 2003), indicating their involvement in maximizing oxygen delivery to tissues. High altitude may also increase energetic demands on small mammals, due to colder temperatures and lower oxygen availability. To optimize the digestion and delivery of nutrients, the gut (particularly the small intestine) is also larger at high altitudes in *P. maniculatus* (Hammond et al., 1999). The physiological challenges of high altitude, therefore, require integration across many systems at all levels of organization. As a result we predicted that the pulmonary surfactant system, responsible for ensuring lung patency and stability, will exhibit plasticity in hypoxic environments.
Pulmonary surfactant is a complex lipid and protein mixture responsible for lowering the surface tension found within the lung. Surface tension is created between the layer of liquid (hypophase) that lines the lung and the air constantly cycling through the lung. Lung surfactant prevents alveolar collapse, increases lung compliance and reduces the work associated with breathing (Nicholas, 1996). Although surfactant lipids and proteins work in unison to lower surface tension, the lipids, in the form of phospholipids and neutral lipids, are largely responsible for disrupting the forces that create surface tension. The major lipids that make up mammalian surfactant are **phospholipids (PL)** that include phosphatidylcholine (PC), lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylglycerol (PG), phosphatidylinisitol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE). Approximately 80% of surfactant is composed of phosphatidylcholine, half of which is in the form of dipalmitoylphosphatidylcholine (DPPC) (Veldhuizen et al., 1998). Cholesterol (Chol), the second most abundant lipid, is the major neutral lipid component of surfactant (Veldhuizen et al., 1998) (Table 2.1). Cholesterol helps to create and maintain fluidity in the lipid monolayer in addition to improving film re-spreading (Notter et al., 1980). Therefore, via its dynamic nature, it is likely that cholesterol plays an important role in maintaining or improving surfactant fluidity at cold temperatures and hence potentially improve breathing efficiency. The four surfactant proteins that work in conjunction with the lipids are known as surfactant proteins (SP)- A, -B, -C and -D (Possmayer, 1988). Type II cells found in the alveoli synthesize, store and secrete surfactant lipid and
proteins (Buckingham and Avery, 1962; Daniels and Orgeig, 2003; Dietl and Haller, 2005).

In a small number of studies, mammalian models have been used to study the surfactant system under both acute-simulated hypoxia (lowered partial pressure of O₂ for short durations) and natural long-term exposure to high altitude (weeks to months of exposure to low partial pressure of O₂ in altitudes above 3000m). In rats, simulated hypoxic exposure decreases total surfactant lipids, specifically, the amount of PC (Chander et al., 1975; Kumar et al. 1980). Lower levels of PC in hypoxia are accompanied by a five-fold higher minimum surface tension, indicating a much higher surface tension in the lung (Zaitseva et al., 1981). In addition, levels of lysophosphatidylcholine (LPC) were found to be twice as high as PC, indicating that lipid peroxidation and phospholipase activity had increased the conversion of PC to LPC (Zaitseva et al., 1981). Furthermore, mice raised at altitude (3500 m) had a reduced number of macrophages and lung lipids. Hypoxia has been shown to generate reactive oxygen species (Clanton, 2007) that could contribute to cell injury including lipid peroxidation (Behn 2007; Celedon et al 1998; Jefferson et al. 2004) in both cell membranes and the surfactant system.

Overall, it appears that the metabolism of surfactant must be altered in such a way that lipid amounts decrease but few studies address the functional consequences of this. Newman and Naimark (1968) reported that decreased surfactant lipid amounts had no effect on surface activity, the surfactant’s ability to lower surface tension. However,
Castillo and Johnson (1969) found that simulated high altitude reduced surface activity and attributed it to a decreased presence of surface active material of the lung, although amounts of lipids were not measured. Srivastava (1974, 1976) measured lower lung compliance and higher surface tension at altitude, indicating that lower amounts of surfactant lipids cannot minimize the inward forces within the alveoli, leading to higher surface tension, poor lung compliance and potential respiratory difficulties. While these studies are useful, the various hypoxic conditions, study organisms used and parameters measured across studies do not present conclusive results.

The aims of this study were to determine the effects of acclimation to high altitude on the pulmonary surfactant system in adult deer mice, *P. maniculatus*, by characterizing the surfactant lipid profile and assessing if and how the lipid amounts are different relative to low altitude acclimated mice, and to determine how surfactant cholesterol levels change under the cumulative effects of low oxygen tensions and cold temperatures in adult mice acclimated to both high and low altitude. We hypothesized that pulmonary surfactant composition would change at high relative to low altitude. Specifically we predicted that: i) total lipid and individual lipid amounts would be higher in high-altitude mice than in low-altitude mice, ii) the cold ambient temperatures found at high altitude would increase the proportion of cholesterol present in surfactant, relative to low-altitude acclimated mice. If correct, the latter prediction suggests that more cholesterol is involved when environmental temperatures pose a challenge to normal surfactant functioning.
Materials and Methods

Animals and Experimental Design

We used *Peromyscus maniculatus sonoriensis* from a captive colony that was originally trapped in the White Mountains of Eastern California and is now 8-10 generations removed from the wild. The risk of hanta-virus is relatively common in this area. A total of 39 mice from our low elevation colony of deer mice were randomly placed in one of two altitudes (high and low) and temperatures (23°C or 5°C). The high altitude study site was located at the Barcroft Laboratory of the White Mountain Research Station (3800 m elevation, ~100 torr). The low altitude study site was located at the University of California, Riverside (340 m elevation, ~154 torr). Mice in the warm temperature treatments were housed at constant temperature of 23-25°C at either site. Mice in the cold treatment groups were housed in an outdoor enclosure on site at the Barcroft station where the average daily temperature was 5°C or in a temperature controlled environmental chamber held at 5°C at UC Riverside. All mice were given *ad libitum* food and water (23% protein, 4.5% fat, 6% fiber, 8% ash, 2.5% minerals) and housed individually in 27x21x14 cm plastic shoebox cages with bedding and cotton. Mice were maintained on a light:dark (14:10) cycle that resembles the natural cycle at Barcroft during the summer months (Hammond et al., 1999). All mice were allowed to acclimate to both altitudes and temperatures for a period of 5-8 weeks (Rezende et al., 2004). Figure 2.1 illustrates the experimental design we used for testing all hypotheses.

Surfactant Extraction and Lipid Analysis
Mice were euthanized with an intra-peritoneal injection of Euthasol ® (sodium pentobarbitol, 0.03 ml). Lung surfactant was obtained via bronchoalveolar lavage (BAL), the most widely used method of obtaining endogenous lung surfactant. Specifically, the lung was rinsed with a chilled saline solution (0.15 M NaCl) by inserting plastic tubing (Silastic® laboratory tubing .51mm I.D. x .94mm O.D.), connected to a 1ml syringe filled with saline, between the upper cartilage rings in the trachea. A volume (between 0.5- 0.7 ml) of saline solution was inserted into the lung and withdrawn three times. This entire procedure was repeated three times on each individual mouse. The amount of saline solution inserted depended on lung size. The lung was carefully filled to maximum capacity, ensuring the tissue did not rupture or leak. The recovered BAL fluid was centrifuged at 150 g to remove macrophages and cellular debris (Blacker et al., 2004; Langman et al., 1996) and frozen in liquid nitrogen.

Surfactant lipids were lyophilized using a Labconco Centrivap® concentrator system, extracted using the method of Bligh and Dryer (Bligh and Dyer, 1959) and stored in Teflon-capped vials at -20 °C. Total PL content was calculated by measuring total inorganic phosphorus (P) by means of a phosphorous assay (Bartlett, 1959). To separate phospholipid species, lung surfactant aliquots (1.5μg P) were spotted (in duplicate when possible) on thin layer chromatography plates (Whatman TLC Plates, Partisil ® LK5D, Silica gel 150 Å, 20 x 20 cm, 250μm thickness) and run in 15 ml chloroform, 17 ml ethanol, 17.5 ml triethanolamine and 4.25 ml distilled water for 1-1.5 hours. Lipid standards (Avanti® Polar Lipids) were run in duplicate. The TLC plates were heated (160°C for 10 min) to evaporate residual triethylamine. Phospholipid bands were
visualized by charring, using a 9.2% copper sulfate solution, and then heating the plate at 180°C for 10 minutes. The plates were then scanned using a Hewlett Packard scanner and the density of the bands analyzed using the ImageJ image processing program developed by the NIH (http://rsb.info.nih.gov/ij/) (Rasband, 1997-2009). The specific surfactant phospholipids that we identified using thin layer chromatography included: LPC, PC, PI, PG and Chol. All lipid values are presented as µg of lipid per ml per mg of dry lung mass (µg lipid/ml-mg dry lung).

**Blood Sampling and Organ Measurement**

Prior to collecting lung surfactant, blood samples (a maximum of 100 µl) were taken, via a retro-orbital puncture, to measure hematocrit. Immediately following surfactant collection organs (lung, heart, liver, spleen and kidneys) were dissected, cleaned of fat or connective tissue and weighed. Organs were dried for at least 48 hours at 60°C and weighed again to obtain a dry mass.

**Statistical Analyses**

To test the effects of acclimation to high- and low-altitude at two different temperatures on surfactant lipid amounts we used a 2 x 2 factorial ANOVA with two levels each of altitude and temperature. The independent variables included site of acclimation and temperature while the dependent variables consisted of total lipid amounts, individual lipid amounts, cholesterol amounts, organ masses (wet and dry) and hematocrit. Body mass and dry lung mass were used as covariates where appropriate. If
the effect of altitude was not statistically significant multiple mean comparisons were made for temperature using one-way ANOVA. Significance was set at p < 0.05 and values presented as means ± S.E.M. All statistics were performed using SAS Software, version 9.1.3 of the SAS System for Windows. Copyright © 2006 SAS Institute Inc.

Results

Body Mass

Body mass (Fig. 2.2) was 12% greater in mice acclimated to high altitude compared to low-altitude mice (F₃,₉₂=4.18, P=0.0437). Temperature had no effect on body mass.

Cardiopulmonary organs

Because of the differences in body mass between altitudes, mass was used as a covariate for the estimates of differences in cardiopulmonary organ mass. Mice acclimated to high altitude had a 12% greater dry lung mass (Fig. 2.3) than their low altitude counterparts (F₄,₉₁=5.84, P=0.0177) and temperature did not have an effect on dry lung mass. Both temperature and altitude had significant effects on dry heart mass (Fig. 2.4). Dry heart mass was 22% greater in high altitude mice (F₄,₉₁= 22.86, P=<0.0001) and 18% greater at cold temperatures (F₁,₉₀= 16.28, P=0.0001).
Blood parameters

Mice acclimated to high altitude had 12% greater hematocrit than mice at low altitude ($F_{3,67}= 27.58, P=<0.0001$), while mice acclimated to cold temperature had 3% greater hematocrit than mice acclimated to warm temperatures ($F_{3,67}= 2.22, P=<0.1412$) (Fig. 2.5).

Surfactant Components

Because there were significant differences in lung dry mass between high and low altitude mice, lung dry mass was used as a covariate for the surfactant components presented below so that we did not report artifacts of lung mass changes in the lipid results.

Total phospholipid: There was not a statistically significant difference in the total phospholipid amount found in the surfactant of mice relative to either altitude ($F_{4,31}= 0.39, P=0.5363$) or acclimation temperature ($F_{4,31}= 0.56, P=0.4615$) (Fig. 2.6). On average mice acclimated to high altitude had 7% less total phospholipid amounts than low altitude mice.

Individual surfactant components

Phospholipids: Altitude did not have a significant effect on individual PL, although in all cases, except LPC individual PL mean amounts were 9-28% greater in cold-acclimated mice and between 7-20% higher in high altitude animals. Table 2.2 shows the means and statistics of all the individual surfactant lipids. In addition
however, for each individual lipid, except LPC and PA, mice acclimated to both the cold and high altitude had a 12-55% greater content of the individual surfactant lipids than all other treatment groups.

**Cholesterol**

We found no statistically significant differences in the surfactant cholesterol levels associated with both altitude and temperature (Figure 2.7). Mice at high altitude had surfactant cholesterol that was, on average, 15% higher than the surfactant cholesterol levels of mice at low altitude ($F_{4,34}=2.46; P=0.1264$) and mice acclimated to cold temperatures had surfactant cholesterol that was, on average, 12% higher than the surfactant cholesterol levels of mice at warmer temperatures ($F_{4,33}=2.15; P=0.1522$).

**Discussion**

The importance of the lung surfactant system and its possible role in accommodating high altitude hypoxia has been largely understudied and it has been investigated mostly under *simulated* hypoxic conditions. At high altitude, where metabolic activities are costlier and lung function may be altered, the plasticity of the lung surfactant lipids may play a significant role in further maintaining lung stability, increasing lung compliance and reducing the work of breathing. Under natural conditions, however, animals are faced not only with hypoxia but with hypobaric conditions, and some combination of drier air, and low ambient temperatures. Consistent with previous work in our lab (Hammond et al., 1999; Hammond et al. 2001), heart and lung mass and hematocrit were significantly greater at high altitude compared to low
altitude controls. Furthermore individual surfactant phospholipid (PC, PI, PG and Chol) amounts were consistently higher at both high altitude and colder temperatures. Although the lipids amounts were not found to be statistically significant between high- and low- altitude mice, to our knowledge, our findings are the first to report consistent increases in surfactant lipid amounts in response to life at high altitude. Previous studies have shown that hypoxia reduced the rate of lipid metabolism (Newman and Naimark, 1968) as was evidence by decreased total lipid, total phospholipid and PC amounts in lung tissue and decreased PC and PE amounts in alveolar surfactant (Chander et al., 1975). Subsequent studies, on the other hand, found that acute hypoxia did not change lipid amounts in lung tissue or surfactant, but upon superimposing cold temperature total PL, PC and PE amounts were reduced (Kumar et al., 1980).

As with most lipids found in cell membranes, surfactant lipids have critical transition temperatures (Tc) that determine whether the lipid will exist in one of two states: ordered gel state or disordered liquid state (Possmayer, 2004). This is an extremely important characteristic of the phospholipids found in surfactant because in order for lipids to spread over an area of the alveoli, the lipids must all exist in the fluid state. Considering that different lipids have different transitions temperatures, (e.g. at some temperature one lipid will exist in a gel state while another exists in the fluid state), makes it imperative that many different lipids (unsaturated, saturated and neutral lipids) coexist in the surfactant film at the same time to lower the transition temperature of the mixture enough to permit fluidity. Therefore, the fact that individual phospholipid amounts increased
simultaneously at high altitude indicates their importance in lowering surface tension and maintaining fluidity.

Previous studies have found that upon hypoxic exposure, the levels of LPC in surfactant were twice as high as PC indicating that lipid peroxidation and phospholipase activity had increased the conversion of PC to LPC. This suggests that hypoxia alters surfactant lipids and possibly the utility of the surfactant system (Zaitseva et al., 1981). In this study, LPC was found only in trace amounts and not consistently present across all mice. Furthermore, PA, phosphatidic acid, an important lipid intermediate in the synthesis of various phospholipids found in surfactant (van Golde, 1985), was also found inconsistently and in trace amounts. These findings suggest that high altitude hypoxia may not be altering the degradation of surfactant lipids or the synthesis of phospholipid precursor amounts in lung surfactant. However, measuring the enzymatic activity of phospholipases may prove to be more conclusive (Minko et al., 2002).

PC is one of the principal phospholipids responsible for generating a surface tension low enough to allow the lungs to stabilize at low lung volumes, particularly when PC exists in its disaturated form called DPPC. PI and PG, the acidic PLs, may play roles in reducing surface tension as is evidenced by their presence in all pulmonary surfactants. PG amounts have been shown to be lower in patients with acute respiratory disease syndrome, indicating an important role in lowering surface tension (Yu and Possmayer, 1992). The increased levels of PC, PG and PI at high altitude could suggest a greater
need for these phospholipids to be available at the air-liquid interface to promote low surface tension and lung patency.

As mentioned previously, the major neutral lipid found in the surfactant of typical eutherian mammals is cholesterol, (Chol) (Possmayer et al., 1984; Veldhuizen et al., 1998) which helps to create and maintain fluidity in the lipid monolayer in addition to improving film re-spreading (Notter et al., 1980). The greater amounts of cholesterol at high altitude and in the cold temperature reinforce cholesterol’s importance in maintaining surfactant fluidity under hypoxic conditions and more importantly when cold temperatures can threaten the lipid transition temperatures, lipid spreading and ultimately the functioning of the surfactant system. Furthermore, it is known that the types and concentrations of phospholipids present will determine the degree to which cholesterol will lower the transition temperature (Morrow et al., 1996). This stresses the importance of having various phospholipids and lipid combinations to reduce surface tension.

The remaining phospholipids, PE, PS, LPC and SM, although most not present or detected by our thin layer chromatography methods, have been found in small amounts in pulmonary surfactant (Veldhuizen et al., 1998). Although their roles are not clear, it is presumed that their ability to lower surface tension is low and their influence on surface activity is minimal. They may support the formation of structures such as tubular myelin and are implied to be involved in the cell signaling events related to surfactant metabolism (Orgieg et al., 2004).
Taken together, our results suggest that greater individual phospholipid amounts are beneficial to maintaining lowered surface tensions within the alveoli at high altitude and in reduced ambient temperatures. Increased and/or abnormal surfactant amounts can also be observed under certain pathological conditions, such as high altitude pulmonary edema (HAPE) (Schoene et al., 1988; Hopkins, 2010). However, it seems unlikely for that to be the case given that total phospholipid amounts were lower at high altitude, where the risk for HAPE is inevitable and previous research in our lab has shown that lung edema is not evident in our mice that have been acclimated long term (> 4 weeks) at high altitude (Hammond, 2001). Consequently, it appears as though a combination of hypoxia and reduced temperature stress necessitates the up-regulation of individual surfactant lipids in order to maintain lipid fluidity, film spreading and proper surfactant functioning. The up-regulation of lipid amounts could be due to increased synthesis and/or secretion of surfactant from the type II cell or as a response to increased lipid oxidation, although those parameters were either not measured or detected by our methods.

These results also highlight the significant role that surfactant proteins, in conjunction with the lipids, serve in promoting low surface tension by not only transporting lipids to the air-liquid interface but also spreading the surface active film within the alveoli (Perez-Gil and Weaver, 2010). More comprehensive conclusions could be obtained by determining the additive effects of changes in both surfactant lipids and proteins under high altitude hypoxia and cold temperatures. Moreover, having a direct measure of surface tension, or the surfactant’s ability to lower surface tension would help elucidate
whether or not the increase in lipid amounts are in fact helping to lower and/or maintain a reduced surface tension at high altitude. Measuring the surface tension of deer mouse surfactant under various temperature conditions could reveal how well surface tension is lowered at cold temperatures and to what extent the phospholipids and cholesterol are involved. Unfortunately, surface tension was not measured in this study due to the complexity of obtaining these measurements and lack of equipment. It would be informative to determine surfactant lipid amounts using a more quantitative method such as High Performance Liquid Chromatography (HPLC). Mass spectrometry can also be used to determine the significance of greater lipid amounts at high altitude by verifying the molecular species of the lipids and establishing if and how lipid saturation levels help to lower surface tension in challenging environments.

The surfactant system is complex in its nature and functioning. The lipids and proteins that comprise this critically important system, through a coordinated series of molecular events, are being synthesized, secreted and transported to the alveolar space as they are needed to maintain lung function. This lipid composition can be altered at any moment as lipids are degraded and recycled and the lung undergoes its cyclical inflation and deflation pattern. Lipid turnover can create a diverse lipid composition as lipids join and exit the surface active film to maintain fluidity. Furthermore it is well documented that changes in physiological conditions modify lipid amounts. Given this complexity, it is not unreasonable to assume that slight changes in lipid classes or amounts might be functionally significant but not be statistically significant. Although our results were not statistically significant, we take our findings to show that our predictions were correct.
Individual lipid amounts were found to be higher in high-altitude mice than in low-altitude mice, and the cold ambient temperatures found at high altitude increased the amount of cholesterol present in surfactant. The effects of altitude and temperature did not have a significant effect on cholesterol amounts although we did find that mice acclimated to both high altitude and cold temperatures had significantly higher amounts of cholesterol than mice acclimated to low altitude at warm temperatures ($P = 0.0527$). This suggests that cholesterol is one component of surfactant that needs to be investigated more thoroughly and further emphasize the pivotal role the plasticity of the lung surfactant system plays accommodating the stressors of high altitude.
<table>
<thead>
<tr>
<th>Component</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phospholipids:</strong></td>
<td>Principal lipid responsible for lowering surface tension near 0mN/m upon compression</td>
</tr>
<tr>
<td>- Phosphatidylcholine (PC)</td>
<td></td>
</tr>
<tr>
<td>50% of PC in the form of DPPC</td>
<td></td>
</tr>
<tr>
<td><strong>Acidic Phospholipids:</strong></td>
<td>Precise roles unknown, play minor role in surface tension reduction</td>
</tr>
<tr>
<td>- Phosphatidylglycerol (PG)</td>
<td></td>
</tr>
<tr>
<td>- Phosphatidylinositol (PI)</td>
<td></td>
</tr>
<tr>
<td><strong>Other Phospholipids:</strong></td>
<td>May support the formation of tubular myelin or be involved in signaling events in surfactant metabolism</td>
</tr>
<tr>
<td>- Phosphatidylethanolamine (PE)</td>
<td></td>
</tr>
<tr>
<td>- Phosphatidylserine (PS)</td>
<td></td>
</tr>
<tr>
<td>- Lysophosphatidylcholine (LPC)</td>
<td></td>
</tr>
<tr>
<td>- Sphingomyelin (SM)</td>
<td></td>
</tr>
<tr>
<td><strong>Neutral Lipids:</strong></td>
<td>Enhances film adsorption, varies with temperature and regulates phase transitions in PL</td>
</tr>
<tr>
<td>- Cholesterol (Chol)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1. Surfactant Lipid Classes (Orgeig et al. 2004)
**Experimental Design for Acclimation**

<table>
<thead>
<tr>
<th>Birth site</th>
<th>Acclimation site*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riverside, 340 m</td>
<td>Riverside, 340 m</td>
</tr>
<tr>
<td></td>
<td>23 or 5 °C</td>
</tr>
<tr>
<td>Riverside, 340 m</td>
<td>Barcroft, 3800 m</td>
</tr>
<tr>
<td></td>
<td>23 or 5° C</td>
</tr>
</tbody>
</table>

*mice are allowed to acclimate for at least 8 weeks to their respective altitude and/or temperature

Figure 2.1 Experimental design used to acclimate mice to high altitude and determine the phenotypic effects of hypoxia and cold temperatures on lipid and protein amounts. All measurements were taken at the site of acclimation.
### Table 2.2. Effects of altitude and temperature on individual surfactant lipids. Values are represented as means and S.E.M. All phospholipids are in units of μg lipid/mg dry lung.

<table>
<thead>
<tr>
<th>Phospholipid (µg lipid/mg dry lung)</th>
<th>5 °C</th>
<th>Se</th>
<th>23 °C</th>
<th>Se</th>
<th>5 °C</th>
<th>Se</th>
<th>23 °C</th>
<th>Se</th>
<th>Altitude</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>7.546</td>
<td>0.8488</td>
<td>7.437</td>
<td>0.573</td>
<td>8.513</td>
<td>0.969</td>
<td>6.306</td>
<td>0.536</td>
<td>Ns</td>
<td>Ns</td>
</tr>
<tr>
<td>PI</td>
<td>2.731</td>
<td>0.404</td>
<td>2.067</td>
<td>0.273</td>
<td>3.206</td>
<td>0.462</td>
<td>2.556</td>
<td>0.255</td>
<td>Ns</td>
<td>Ns</td>
</tr>
<tr>
<td>PG</td>
<td>2.801</td>
<td>0.411</td>
<td>2.927</td>
<td>0.277</td>
<td>3.599</td>
<td>0.470</td>
<td>2.894</td>
<td>0.259</td>
<td>Ns</td>
<td>Ns</td>
</tr>
<tr>
<td>LPC</td>
<td>3.063</td>
<td>0.489</td>
<td>2.939</td>
<td>0.331</td>
<td>2.921</td>
<td>0.559</td>
<td>2.636</td>
<td>0.309</td>
<td>Ns</td>
<td>Ns</td>
</tr>
<tr>
<td>PA</td>
<td>0.568</td>
<td>0.094</td>
<td>0.563</td>
<td>0.073</td>
<td>0.577</td>
<td>0.094</td>
<td>0.638</td>
<td>0.065</td>
<td>Ns</td>
<td>Ns</td>
</tr>
<tr>
<td>CHOL</td>
<td>3.537</td>
<td>0.344</td>
<td>3.378</td>
<td>0.232</td>
<td>4.338</td>
<td>0.393</td>
<td>3.631</td>
<td>0.217</td>
<td>Ns</td>
<td>Ns</td>
</tr>
</tbody>
</table>
Figure 2.2. Body mass (g) of mice acclimated to high- and low-altitude at two temperatures (5°C and 23°C). Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 2.3. Lung mass (dry) of mice acclimated to high- and low-altitude at two temperatures (5°C and 23°C). Body mass was used as a covariate. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 2.4. Dry heart mass (g) of mice acclimated to high- and low-altitude at two temperatures (5°C and 23°C). Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 2.5. Hematocrit (%) of mice acclimated to high- and low-altitude at two temperatures (5°C and 23°C). Percent signifies percent red blood cells relative to total plasma volume. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 2.6. Total phospholipid amounts of mice acclimated to high- and low-altitude at two temperatures (5°C and 23°C). Body mass was used as a covariate. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 2.7. Cholesterol (Chol) amounts of mice acclimated to high- and low-altitude at two temperatures (5°C and 23°C). Dry lung mass was used as a covariate. Letters above bars signify statistical significance. Error bars are ± 1S.E.M.
Literature Cited


CHAPTER 3. The Effects of High Altitude on Surfactant Proteins

Abstract. A complex mixture of proteins and lipids, known as the pulmonary surfactant system, reduces the surface tension at the air-liquid interface within the lung. While the lipids are primarily responsible for lowering surface tension, the proteins play important roles as well. Surfactant proteins (A,B,C,D), although different in structure and composition, aid in the adsorption and spreading of lipids at the air-liquid interface, modify the properties of the surface film to allow surface tension to reach near 0 mN/m, and are involved in providing immunity to the lung. The effects of high altitude on the lung surfactant system are not well defined and have been mostly investigated in terms of the lipid components. We examined the effects of high altitude on the surfactant proteins in deer mice, *Peromyscus maniculatus* and predicted that mice acclimated to high altitude would up-regulate the amounts of surfactant proteins present in their lung given that previous studies in our lab have shown an increase in lipid amounts (unpublished data). Specifically, since surfactant proteins aid in the recycling of lipids to and from the air-liquid interface, a greater presence of lipids might require greater amounts of protein. Of the four proteins present in surfactant we focused specifically on proteins SP-B and SP-C, since they are primarily involved in lowering surface tension. Mice acclimated to high altitude had lower levels of total protein than did mice acclimated to low altitude. Greater amounts of SP-B were observed in mice at high altitude relative to their low altitude counterparts. SP-C amounts, however, did not change between high- and low-acclimated mice. Taken together with the surfactant lipid results it seems that the lung surfactant system of deer mice acclimated to high altitude up-regulates the amount of
lipids and proteins, presumably to maintain a low surface tension and in turn promote lung stability and proper functioning. These results further promote the idea that the plasticity of the surfactant system is an important component of accommodating environmental stressors, especially when the stress can have consequences that span many physiological systems and ultimately affects an organism’s survival.

**Introduction**

*The Lung at High Altitude*

Of the various organ systems that high altitude hypoxia impacts, the respiratory system is of particular importance since it is the interface connecting the external environment with the internal metabolic processes of the body, where a series of physiological events occur in response to the hypoxia. Hypoxia alters ventilation rates, which in turn increase the respiratory muscle’s metabolic demand. Oxygen must diffuse into the blood despite a decreased driving force, and the binding of oxygen to hemoglobin may be impaired due to the reduced availability of oxygen or ability to match ventilation and perfusion (Schoene, 2001). In addition, the lung surfactant system, located at the interface of both environments, must continue to synthesize and secrete its lipids and proteins to maintain a low surface tension and prevent the alveoli from collapsing. Alveolar collapse could potentially be deleterious given that alveoli must remain inflated to allow for the optimal diffusive exchange of oxygen at limiting partial pressures and deliver it to the blood and metabolizing tissues. As a result, the lung
surfactant system could play a vital role in promoting and maintaining adequate lung function under hypoxic conditions.

Pulmonary surfactant is a complex and dynamic mixture of lipids and proteins with two primary functions of reducing surface tension at the air-liquid interface and providing immunity to the lung. Lung surfactant reduces surface tension, allowing the stabilization and patency of alveoli, permitting the lung to be more compliant and therefore reducing the work of breathing. The lipids that make up the lung surfactant system are responsible for lowering the surface tension within the alveoli while four surfactant proteins (SP) A, B, C and D aid in surface tension reduction and lung immunity. SP-B and SP-C are involved in lowering surface tension by recycling and transporting the lipids to and from the interface as well as facilitating the spreading of lipids across the alveoli (Perez-Gil and Weaver, 2010; Weaver and Whitsett, 1988; Weaver and Whitsett, 1991; Weaver and Conkright, 2001; Whitsett et al., 1995; Whitsett and Weaver, 2002) (Figure 3.1). SP-A and SP-D are primarily involved in proving immunity to the lung (Crouch and Wright, 2001; Korfhagen et al., 1996; Reid, 1998; see also Table 3.1).

SP-B is a hydrophobic protein, synthesized in alveolar type II cells, is required for the formation of tubular myelin in the presence of SP-A, phospholipids and calcium (Suzuki et al., 1989). SP-B is tightly associated with surfactant phospholipids, enhances the spreading and stability of the surfactant, promotes the removal (or squeeze-out) of unsaturated phospholipids (Weaver, 1998) and is the only protein required for proper
lung function. A deficiency of this protein is fatal to both mice and humans (Weaver and Conkright, 2001).

SP-C, the most hydrophobic protein, is also synthesized in the alveolar type II cells and enhances the rate of adsorption both alone and in combination with SP-B (Cockshutt and Possmayer, 1992; Tabor et al., 1990; Van Golde et al., 1988). Studies involving SP-C knockout mice emphasize its role in recruiting PL to the surfactant monolayer and providing stability of the surfactant films upon compression (Whitsett and Weaver, 2002).

*Hypoxia as a Selective Pressure for the Development of Pulmonary Surfactant*

Hypoxia is thought to be one of three major environmental variables (in addition to temperature and pressure) that have provided significant selection pressure for the evolution of the pulmonary surfactant system in vertebrates, particularly in developing lungs (Orgeig and Daniels, 2009). In developing chicken embryos, hypoxia accelerated the release of surfactant lipids that coincided with early hatching of the chicks by 24 hours (Blacker et al., 2004). Fetal growth restriction (FGR), induced by anemia, maternal under-nutrition and hypertension, causes fetal hypoxemia (low levels of blood oxygenation) which is known to affect lung maturation (Tyson et al., 1995). Conflicting studies using growth-restricted fetuses have reported both an increase (Gross et al., 1981) and decrease (Rees et al., 1991; Lechner et al., 1986; Lin and Lechner, 1991) in specific species surfactant lipids. Furthermore, hypoxemia induced in fetal sheep increased levels of cortisol in the latter part of gestation which correlated with elevated levels of SP-A and
SP-B mRNA in lung tissue (Braems et al., 1998). These results have provided evidence for the plasticity exhibited by the surfactant system under various environmental stressors, highlighting its ability to alter its lipid and protein composition in order to regulate the functioning and utility of this dynamic system.

Although it is generally recognized that fetal hypoxia is considered an important selective pressure in the development of lung surfactant (Orgeig and Daniels, 2009), less is known about the effects of hypoxia on surfactant proteins in fully developed lungs (adults) exposed to natural occurring hypoxia, such as at high altitude. A small number of studies have determined the effects of hypoxia on surfactant proteins in cultured lung cells, resulting in contradictory conclusions. Jackson et al. (1996) cultured alveolar type II cells under hypoxic environments (2.5% O_2) for a period ranging from 24 hours to 3 days and found that SP-A transcript expression remained constant throughout. In contrast, hypoxia (1% O_2) down-regulated the expression of surfactant SP-C protein after 8 hours and almost abolished protein expression after 24 hours in cultured alveolar epithelial cells (Vaporidi et al., 2005). Additionally, hypoxia was also found to induce apoptosis of alveolar cells after only 8 hours. Since severe hypoxia is known to induce lung injury in the form of epithelial cell damage, inflammation, edema and surfactant abnormalities (Greene et al., 1999; Tomashesfski, 1990), the effects of hypoxia on surfactant proteins have been investigated primarily in the context of lung injury and its medical applications. Nevertheless, these results demonstrate that while most studies focus on lipid changes, surfactant protein levels are also modified in response to hypoxic exposure.
We examined the effects of acclimation to high altitude on surfactant proteins in adult deer mice, *Peromyscus maniculatus*, by identifying the surfactant proteins SP-B and SP-C and assessing if and how protein amounts vary relative to low altitude acclimated mice. *P. maniculatus* are well suited for studying lung physiology at high altitude due to the fact that they are one of the most widespread groups of North American rodents; found across a wide range of altitudes (below sea level to 4300 m) (Hall and Kelson, 1959). Phenotypic changes to digestive and cardiopulmonary organ size and function in deer mice living at high altitudes have been demonstrated in a number of studies (Hammond et al., 1999; Hammond et al., 2001). These changes may help to maintain functional aerobic capacity while the oxygen-limiting environment they live in dictates otherwise (Hammond et al., 2001; Chappell et al., 2007; Russell et al., 2008). Classic studies on the genetics and physiology of hemoglobin show that deer mice possess specialized adaptations potentially allowing for a higher oxygen binding affinity to accommodate lower oxygen availability at high altitudes (c.f., Snyder et al., 1988, Storz et al. 2007, 2008).

Given that we expected surfactant lipid amounts to increase at high altitude and lipids and proteins work in unison to reduce surface tension, we hypothesized that pulmonary surfactant protein amounts would change at altitude. Specifically we predicted that: 1) total protein amounts would be higher in high-altitude acclimated mice, 2) The relative amounts of SP-B and SP-C would be greater in mice acclimated to high altitude and 3) The changes in surfactant protein amounts would mirror the changes in
surfactant lipid amounts, presenting a coordinated change in the lung surfactant system (lipids and proteins) as a whole at high altitude.

**Materials and Method**

*Animals and Experimental Design*

We used *Peromyscus maniculatus sonoriensis* from a captive colony that was originally trapped in the White Mountains of Eastern California and is now 8-10 generations removed from the wild. We were unable to use a large population of wild mice from the area because of the high risk of hanta virus. A total of 32 mice (n=16 high altitude; n=16 high altitude) from our low elevation colony of deer mice were randomly placed in one of two altitudes (high and low). The high altitude study site was located at the Barcroft Laboratory of the White Mountain Research Station (3800 m elevation, ~100 torr). The low altitude study site was located at the University of California, Riverside (340 m elevation, ~154 torr). All mice were housed at constant temperature of 23-25°C at both sites. All mice were given *ad libitum* food and water (23% protein, 4.5% fat, 6% fiber, 8% ash, 2.5% minerals) and housed individually in 27x21x14 cm plastic shoebox cages with bedding and cotton. Mice were maintained on a light:dark (14:10) cycle that resembles the natural cycle at Barcroft during the summer months (Hammond et al., 1999). All mice were allowed to acclimate to both altitudes for a period of 5-8 weeks (Rezende et al., 2004).

*Surfactant Extraction and Protein Analysis*
Mice were euthanized with an intra-peritoneal injection of Euthasol ® (sodium pentobarbitol, 0.03 ml). Lung surfactant was obtained via bronchoalveolar lavage (BAL), the most widely used method of obtaining endogenous lung surfactant. Specifically, the lung was rinsed with a chilled saline solution (0.15 M NaCl) by inserting plastic tubing (Silastic® laboratory tubing .51mm I.D. x .94mm O.D.), connected to a 1ml syringe filled with saline, between the upper cartilage rings in the trachea. A volume of saline solution was inserted into the lung and withdrawn three times. This entire procedure was repeated three times on each individual mouse. The amount of saline solution inserted depended on lung size. The lung was carefully filled to maximum capacity, ensuring the tissue did not rupture or leak. The recovered BAL fluid was centrifuged at 150 g to remove macrophages and cellular debris (Blacker et al., 2004; Langman et al., 1996) and frozen in liquid nitrogen.

Total protein amounts were determined on lavage samples using the bicinechoninic assay (BCA) (Thermo Scientific, Pierce® BCA protein assay kit). Following the BCA assay all samples were concentrated with a Labconco Centrivap® concentrator system and re-suspended in 4x NuPAGE LDS sample buffer. Aliquots containing 10µg of total protein were then stored at -80°C. A total of 10µg of protein were loaded onto 4-12% gradient gels (Life Technologies, Novex® NuPAGE® 4-12% Bis-Tris Gels, 1.5mm x 10 well) along with pre-stain markers (Bio-Rad Precision Plus Protein™ Dual Xtra Standards) and a protein standard. The gel box (Novex XCell II Sure Lock® Mini Cell) was filled with running buffer and the gel was run @ 200 volts for 2 hours (Bio-Rad Power Pac 200). The proteins were then transferred to PVDF membrane (Bio-Rad Sequi-
Blot™ PVDF membrane, 0.2 µm, 26cm x 3.3m) for 2 hours at 40 volts. The PVDF was immediately incubated in a blocking solution (5% dry milk in TBS and 0.1% Tween) overnight at 4°C on a rocker. The PVDF was cut between markers (15–20 kDa markers) to separate SP-B from SP-C in order to incubate with primary antibody. Primary antibody raised against surfactant protein B (Abcam, Rabbit polyclonal to Surfactant Protein B (Mature)) or surfactant protein C (Seven Hills Bioreagents, Rabbit Anti-Mature SP-C, polyclonal) were diluted to 1:5000 (5% dry milk in TBS and 0.01% Tween) and incubated with the appropriate section of PVDF overnight at 4°C with rocking. To minimize amounts of antibody needed, small Western boxes were used (GenHunter, Perfect Western™ containers). Primary antibody was washed off using 3 volumes of TBS. Secondary antibody, goat anti-rabbit conjugated to horseradish peroxidase was diluted to 1:5000 (5% dry milk in TBS and 0.01% Tween) and incubated with PVDF with gentle rocking for 2 hours at room temperature. Secondary antibody (Sigma-Aldrich, Anti-Rabbit IgG (whole molecule)- Peroxidase antibody produced in goat) was washed off using 3 volumes of TBS with vigorous rocking. A chemiluminescent system (GE Healthcare, Amersham™ ECL Plus Western Blotting Detection System) was used to visualize the protein bands. The solution was placed on the PVDF for 5 minutes in the dark, wrapped in saran wrap and exposed to film (GE Healthcare, Amersham Hyperfilm™ ECL 8 x10 sheets). Exposure times included 0.5, 1, 5, and 10 minutes. The film was developed using an X-ray film processor (AFP Imaging Mini-Medical 90). ImageJ, an image processing program developed by the NIH (http://rsb.info.nih.gov/ij/)
(Rasband, 1997-2009), was used to analyze and quantify the mean pixel density of the bands. (Figure 3.2)

Due to the fact that pure forms of SP-B and SP-C were not run alongside the surfactant samples, reported SP-B and SP-C amounts are relative to a surfactant protein standard that was made by combining the lung lavage of three mice. Briefly, three low altitude mice were lavaged and their surfactant extracted and pooled. Total protein of the mixture was determined using the BCA assay. The remainder of the lavage was concentrated, resuspended in 4x LDS sample buffer and volumes of 10 ug total protein aliquoted and stored at -80°C. This surfactant protein standard was run on all gels and also used as a between gel control. Protein amounts are reported as mg of SP-B or SP-C per mg of dry lung mass.

Statistical Analyses

To test the effects of acclimation to high- and low-altitude on surfactant protein amounts we used a one-way ANOVA. The independent variables included site of acclimation (high or low altitude) and sex (male or female), while the dependent variables consisted of body mass, dry lung mass, total protein amounts, SP-B and SP-C amounts, and hematocrit. Body mass and dry lung mass were used as covariates where appropriate. When testing for the effects of altitude and sex on surfactant proteins, we used a 2x2 factorial, where there were two levels of altitude (high and low) and two levels of sex (male and female). We were unable to determine the effects of temperature on surfactant proteins, as with surfactant lipids, so this variable was omitted in these
analyses. Significance was set at \( p < 0.05 \) and values presented as means ± S.E.M. All statistics were done using SAS Software, version 9.1.3 of the SAS System for Windows.

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**Results**

*Body Mass*

Body mass (Fig. 3.3) was 10% less in mice acclimated to high altitude compared to low-altitude mice (\( F_{1,34}=5.46, P=0.0255 \)).

*Lung mass*

Because of the differences in body mass between altitudes, mass was used as a covariate for the estimates of differences in lung mass. Mice acclimated to high altitude had a 37% greater dry lung mass (Fig. 3.4) than their low altitude counterparts (\( F_{2,32}=10.63, P=0.0026 \)). Wet lung mass was not used in these analyses since lavaging the lungs with saline would artificially inflate these values.

*Blood parameters*

Mice acclimated to high altitude had 14% greater hematocrit than mice at low altitude (\( F_{1,31}=25.22, P=0.0001 \)) (Fig. 3.5).

*Total Protein and Surfactant Proteins*

Mice acclimated to high altitude had 23% less total surfactant protein compared to low-altitude acclimated mice (\( F_{2,28}=10.08, P=0.0036 \)) (Fig. 3.6). With regards to
individual surfactant proteins SP-B and SP-C, mice at high altitude had 83% greater relative amounts of SP-B ($F_{2,26}= 2.44, P=<0.1307$) although not statistically significant (Fig. 3.7). Relative amounts of SP-C essentially remained the same across altitudes with mice acclimate to high altitude displaying only a 0.27% increase in the amounts of SP-C ($F_{2,26}= 0.0, P=<0.992$) (Figure 3.8).

**Effect of Sex on Surfactant Proteins B and C**

We found an effect of gender on individual surfactant protein quantities. Regardless of acclimation altitude, males had greater amounts of both SP-B and SP-C than females. Males had 104% more SP-B ($F_{2,26}= 3.38, P=<0.0776$), although not statistically significant (Figure 3.9), and 88% more SP-C than females ($F_{2,26}= 6.82, P=<0.0148$) (Figure 3.10). When acclimation altitude was accounted for, males at high altitude had 80-400% greater amounts of SP-B than females at high altitude and both males and females at low altitude (Figure 3.11). Similarly, males at high altitude had 64-167% greater relative amounts of SP-C when compared to females both at high and low altitude. However, in the case of SP-C males at low altitude had greater relative amounts of SP-C than high altitude-acclimated males (Figure 3.12).

**Discussion**

Previous results from our lab have shown that surfactant lipid levels increase in the presence of a combination of cold temperatures and high altitude hypoxia. Although the effect of altitude on surfactant lipids was not found to be statistically significant, determining the changes of surfactant protein quantities at high altitude was important for
many reasons: 1) Surfactant lipids and proteins work in unison to lower surface tension and therefore both components must be studied to get a comprehensive view of how surfactant as a system (proteins and lipids) responds to hypoxia; 2) studies focusing on the effects of hypoxia on surfactant proteins are scarce and mostly carried out in cultured lung cells under simulated hypoxia; 3) surfactant protein changes at high altitude in deer mice could provide further evidence for the importance of the plasticity of the lung surfactant system at high altitude. There exist four surfactant-associated proteins (SP-A, B, C, D). However, we focused only on SP-B and SP-C since they are involved in lowering surface tension within the lung.

Consistent with previous results (Hammond et al., 1999; Hammond et al., 2001), mice acclimated to high altitude had 37% greater dry lung mass and 14% greater hematocrit when compared to low altitude-acclimated mice. Increases in hematocrit and lung mass are consistently found with deer mice at high altitude and are presumed to be physiological responses to lowered oxygen tensions. A greater lung mass could be indicative of a greater diffusive surface area to aid in the acquisition of oxygen while a greater proportion of red blood cells could bind more oxygen for delivery to metabolizing tissues. Total protein amounts were found to be 23% lower in mice acclimated to high altitude, relative to low altitude mice. Relative amounts of SP-B were 83% greater under hypoxic conditions while relative amounts of SP-C remained essentially the same between high and low-altitude mice.
The few studies that have focused on the effects of hypoxia on surfactant proteins have demonstrated that surfactant protein mRNA levels either remain constant or are reduced under hypoxic conditions (Jackson et al., 1996; Vaporidi et al., 2005). These results, however, were obtained under different experimental conditions than ours; employing simulated hypoxia, cultured lung tissue, and quantifying protein mRNA expression. Our results showed that relative SP-B amounts, obtained via lung lavage, increased at high altitude. This indicates that greater amounts of SP-B may be necessary to aid with lipid spreading and insertion into the surface active film. The fact that SP-C amounts did not change between high and low altitude mice was surprising given that both SP-B and SP-C interact with surfactant lipids (Almlen et al., 2008; Andersson et al., 1995; Baatz et al., 1990) and independently work to accelerate the spreading and formation of the phospholipid film at the air-liquid interface (Nag et al., 1999; Oosterlakendijksterhuis et al., 1991). However, SP-B is the only surfactant protein that is required for postnatal functioning of the lung and survival (Weaver and Conkright, 2001).

The cyclical compression and expansion of the lung that occurs during breathing directly translates to changes in the surfactant film in terms of its composition and functioning. As the lung compresses (during exhalation) the surface film, consisting of phospholipids, folds on itself, expels lipids from the film and achieves the lowest possible surface tension to prevent the alveoli from collapsing (see Figure 3.1) (Perez-Gil and Weaver, 2010). Studies using SP-B deficient mice have shown that SP-B is required to achieve and sustain the lowest possible surface tension upon surface film compression, as well as provide mechanical stabilization to the lipid film (Cruz et al., 2000).
Insertion of SP-C into a phospholipid membrane is known to disrupt the packing of the lipids and allow for lipid movement into and out of the lipid layer (Horowitz et al., 1993), which could facilitate the folding of the lipid film upon compression as well as expulsion of lipids from the interface (Almlen et al. 2008; Perez-Gil et al., 1992; Taneva and Keough, 1994). Furthermore SP-C may be involved in surfactant catabolism (Horowitz et al., 1996) as well as enhancing the spreading and stability of phospholipids in the surface active film (Whitsett and Weaver, 2002). Upon lung expansion (during inhalation), as the lipid film spreads across the alveoli, it is thought that both SP-B and SP-C promote the re-spreading as well as insertion of phospholipids into the interfacial film (Artemenko et al., 2001; Bachofen et al., 2005). Hence, both SP-B and SP-C are needed to optimize the conditions under which the surfactant film can lower surface tension.

The fact that SP-B is the only surfactant protein absolutely required for survival of an organism and aids in maximally reducing surface tension, could explain why deer mice acclimated to high altitude displayed greater amounts of this protein. Consistent amounts of SP-C between high and low altitude mice could be indicative of SP-C’s involvement in the maintenance of the surfactant film as the lung compresses and expands. Furthermore SP-C enhances the uptake of surfactant phospholipids and plays a role in the catabolism of lipids as they are recycled from the surface film that is actively lowering surface tension. Therefore, SP-C may play a greater role in maintaining the dynamics of the lipid film and lipid recycling and a minor role in reducing surface tension. Nevertheless, cooperation between SP-B and SP-C is essential and highlights the complex interplay
between these two proteins as they share similar but also distinct roles in surfactant functioning.

The multifaceted nature of surfactant proteins could be further complicated at high altitude, where the processing, synthesis and secretion of proteins could be under the influence of altered respiratory mechanics (hyperventilation), hormones and even macrophage activity (Dranoff et al., 1994). Yet, greater amounts of both lipids and proteins found in mice acclimated to high altitude, suggests that hypoxic environments might necessitate the up-regulation of the surfactant system to maintain a low surface tension and therefore adequate lung function.

Interestingly, male mice had 100% and 88% greater relative amounts of SP-B and SP-C, respectively, when compared to female mice without accounting for their acclimation altitude. The effect of gender was more pronounced when the acclimation altitude was accounted for. Males acclimated to high altitude had 80-400% greater amounts of SP-B than all other treatment groups. SP-C amounts were greater in male mice at both high and low altitude when compare to females. These findings are unusual given that most alterations in lung surfactant are attributed to environmental factors, respiratory diseases and age. Studies have shown that lung surfactant pools in the alveoli are known to vary significantly with age, where neonates have larger surfactant pools than adults (Carnielli et al., 2009; Ikegami et al., 2009; Zimmermann et al., 2005). However, since surfactant maturation is affected by hormones and hormonal differences exist between males and females, it could provide an explanation for these results (Carey et al., 2007). In addition
female rats have been shown to have a greater hyperventilatory response to hypoxia than males (Mortola and Saiki, 1996) which suggests a greater surfactant production by females since mechanical stretch of the alveoli stimulates surfactant release. Our results show the opposite trend between males and females, however, and stresses the need for increased sample sizes to further discern differences in amounts of SP-B and SP-C in male and female mice. Establishing the condition of the alveoli at high altitude may be beneficial to rule out any surfactant pathophysiologies triggered by hypoxic exposure and ultimately determine if greater surfactant amounts are beneficial to organisms at high altitude.

Overall, the up-regulation of both surfactant lipid and protein amounts at high altitude provides evidence for the plasticity of lung surfactant. Although the functional consequences of these changes are largely unknown because much is unknown regarding the regulation of surfactant protein and lipid synthesis, secretion, recycling or degradation (Perez-Gil and Weaver, 2010), various other factors can be measured to begin to elucidate the functional meaning of greater surfactant amounts. These include measuring surface tension, determining the distribution of type I and type II cells in mice at high altitude, quantifying the specific molecular species of lipids present, and obtaining an absolute amount of surfactant proteins using purified protein standards. An interesting avenue to pursue would be to further determine the effects of altered oxygen tensions on lipid oxidation and its consequences on the functioning of the surfactant system. Many studies have recognized hypoxia as a trigger for oxidative stress (Magalhaes et al., 2005), which could have major implications for surfactant lipid oxidation. Moreover,
Rodriguez-Capote et al. (2006) revealed that exposing surfactant to oxidative stress, in the form of reactive oxygen species, resulted in an increase of lipid and protein oxidation products as well as on overall inactivation of surfactant functioning.
Figure 3.1. Roles surfactant proteins B and C play in stabilizing the surface film and re-spreadung lipids upon lung compression and expansion, respectively. SP-B (red protein) is involved in stabilizing the lipid layer upon compression (top figure) as well as facilitating the spreading of lipids across the alveoli (bottom figure). SP-C (blue protein) is involved in recycling and transporting the lipids to and from the interface (bottom figure).
<table>
<thead>
<tr>
<th>Protein</th>
<th>Monomeric size (Daltons)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-A</td>
<td>30,000-36,000</td>
<td>Promotes lung immunity; maintains structural integrity of tubular myelin</td>
</tr>
<tr>
<td>SP-D</td>
<td>43,000</td>
<td>Promotes lung immunity; plays a role in surfactant homeostasis</td>
</tr>
<tr>
<td>SP-B</td>
<td>8,000</td>
<td>Enhances adsorption of PL to, and provides stability to the surfactant film; required for tubular myelin formation; essential for lung function</td>
</tr>
<tr>
<td>SP-C</td>
<td>3,800</td>
<td>Enhances adsorption of PL, maintains tight packaging of PL in lamellar bodies and keeps lipids close to surfactant film</td>
</tr>
</tbody>
</table>

Table 3.1. Mammalian Surfactant Proteins. (Orgeig et al., 2004)
Figure 3.2. Representative image of a Western Blot depicting SP-B and SP-C at high-and low-altitude. The protein standard is shown on the last lane.
Figure 3.3. Body mass (g) of mice acclimated to high- and low-altitude. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 3.4. Lung mass (dry) of mice acclimated to high- and low-altitude. Body mass was used as a covariate. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M..
Figure 3.5. Hematocrit (% RBC) of mice acclimated to high- and low-altitude. Percent signifies percent red blood cells relative to total plasma volume. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 3.6. Total protein amounts of mice acclimated to high- and low-altitude. Body mass was used as a covariate. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 3.7. Relative SP-B amounts of mice acclimated to high- and low-altitude. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 3.8. Relative SP-C amounts of mice acclimated to high- and low-altitude. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 3.9. Relative SP-B amounts in male and female mice. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 3.10. Relative SP-C amounts in male and female mice. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 3.11. Relative SP-B amounts in male and female mice at both high- and low-altitude. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.
Figure 3.12. Relative SP-C amounts in male and female mice at both high- and low-altitude. Letters above bars signify statistical significance. Error bars are ± 1 S.E.M.


CHAPTER 4. General Conclusions and Significance

Phenotypic plasticity of the lung surfactant system at high altitude

I measured surfactant lipid and protein amounts at high altitude to determine: 1) if any alterations in surfactant lipids and/or proteins were evident 2) the extent to which these changes (phenotypic plasticity) may aid in accommodating the stress of high altitude hypoxia 3) the functional implications of these changes and the potential consequences (either positive or negative) on the physiological ecology of deer mice. Phenotypic plasticity, by its simplest definition is the ability of a genotype to change its phenotype in response to varying environmental demands. I have provided evidence that the pulmonary surfactant system of deer mice, *Peromyscus maniculatus*, exhibits phenotypic plasticity in response to the stressors found at high altitude. Both surfactant lipids and proteins were found in greater quantities at high altitude, suggesting that increased surfactant amounts are necessary to maintain a reduced surface tension in the face of an oxygen-limited environment. While these results must be corroborated with other data such as lung histology, surface tension measurements and possibly respirometry data, the fact that deer mice are able to live and thrive in hypoxic conditions presents additional evidence to support the importance of the plasticity of the lung surfactant system.

Plasticity in surfactant lipids and proteins

The consistent increase observed in organ mass (e.g., liver, heart, spleen) of deer mice at high altitude emphasizes the importance of phenotypic plasticity in the process of acclimating to hypoxia (Hammond et al. 1999; Hammond et al., 2001). Increases in
cardiopulmonary organs masses are particularly significant since the heart and lung can significantly influence an organism’s aerobic metabolism and ultimately its survival.

Surfactant found within the lung could provide an additional mechanism in accommodating the hypoxic challenge, especially since it is involved in preventing alveolar collapse. Acclimation to high altitude increased the amounts of the majority of surfactant lipids however, acclimation to cold temperature in addition to altitude had a significantly greater effect on the amounts of lipids present, particularly those primarily involved in lowering surface tension. These lipids included PC, PG, PI and Cholesterol. Being that there was a simultaneous increase in all of these lipids indicates that all of these lipids, collectively, must be present and in greater amounts to achieve lung stability. Additionally, increased quantities of cholesterol support previous suggestions that cholesterol is critically important in maintaining the fluidity of the surface film when faced with fluctuating temperature conditions (Codd et al., 2003; Orgeig and Daniels, 2001; Slocombe et al., 2000).

Greater amounts of surfactant lipids could also suggest that a hypoxia-induced pathology may exist. However, most studies have determined the characteristics of lung diseases associated with high altitude and found that lung lavage is infiltrated with various proteins, macrophages and leukocytes (Schoene et al., 1986; Schoene et al., 1988) and not lipids. These findings also put emphasis on changes in respiratory dynamics that may be occurring at high altitude, such as hyperventilation (Ainslie and Burgess, 2008; West, 2006). The secretion of surfactant from the type II cell to the air-liquid interface is enhanced by the mechanical stretch of the alveoli as they are inflated.
(Dietl et al., 2010; Orgeig and Daniels, 2004; Wirtz and Schmidt, 1992). Chappell (1985) measured the effects of altitude and ambient temperature of ventilation and gas exchange in deer mice. Mice exposed to cold temperatures increased their total ventilation by increasing their respiration frequency. This response was similar at high altitude but to a significantly greater extent (Chappell, 1985). Correspondingly, the mice also exhibited a larger minute volume (quantity of gas exhaled from lungs per minute), indicating that the mice compensate for the reduced oxygen availability at high altitude by increasing their total ventilation (inhaled and exhaled gas). Therefore, hyperventilation could also influence the secretion and amounts of surfactant lipids at high altitude and thus respiratory measurements may be necessary to differentiate between the effects of hypoxia, temperature and respiratory dynamics on surfactant lipid amounts.

Relative amounts of SP-B increased while those of SP-C remained unchanged in response to hypoxia. These results are not surprising given that surfactant lipids, responsible for lowering surface tension, rely on a complex interplay with surfactant proteins to accomplish the transport, spreading and recycling of surfactant lipids. Furthermore, through lipid-protein interactions, surfactant proteins provide stability to the lipid film that is actively reducing surface tension as the lung continually expands and compresses (Perez-Gil and Weaver, 2010). An increased presence of lipids would necessitate a greater amount of SP-B to maintain a low surface tension and prevent alveolar collapse, a condition known to occur at high altitude and activate the hypoxic pulmonary vasoconstriction mechanism (Fishman, 2004). Similarly, greater quantities of
SP-C were expected but not observed. Mice acclimated to high and low altitude had similar amounts of SP-C present. This could be due to a small sample size or to the fact that SP-C may not play as critical a role in reducing surface tension as SP-B. Deletion of the SP-C gene in mice results in pulmonary pathologies, such as chronic lung disease, but has minimal effects on surfactant trafficking, packaging and secretion and does not affect survival (Glasser et al., 2001; Glasser et al., 2003; Whitsett and Weaver, 2002). Targeted deletion of the SP-B gene, however, results in lethal respiratory failure immediately after birth (Clark et al., 1995), indicating that SP-B is absolutely necessary to reduce surface tension and keep alveoli open.

**Complexity of the lung surfactant system**

Lung surfactant is composed of multiple components, is inherently complex and can be regulated by many factors, such as hormonal and environmental changes to name a few. This creates a complicated situation from which to ascertain the functional significance of these changes observed at high altitude. Although tremendous progress has been made since 1929 when von Neegaard (Wrobel, 2004) demonstrated that greater inflation pressures were required to expand the lung in the absence of fluid (due to surface tension), there are many questions that are still unanswered. For example, the specific and individual roles of SP-B and SP-C remain unclear since the two proteins work together to support similar but distinct functions. Transcriptional control of surfactant protein expression is uncertain but remains an important point of investigation. The combinations of individual lipids that result in the lowest surface tension are unknown but critical to producing synthetic surfactants. The pathways involved in the
intracellular trafficking of phospholipids remain elusive and the molecular mechanisms that modulate and balance the synthesis, secretion, recycling and degradation of surfactant are unknown (Perez-Gil and Weaver, 2010). These are a few examples that illustrate the difficulties in studying lung surfactant without compounding the effects of high altitude hypoxia. Yet, answers to these and more questions can only be determined by investigating the composition and structure of the surface film under physiologically relevant conditions.

In spite of this, surfactant’s inherent complexity allows for various approaches and techniques to be employed to address these questions. Electron microscopy has provided a means to get a detailed look at the ultrastructure of surfactant lipids and proteins under various conditions, such as disease, thereby advancing the ability to determine the true functional significance of the alterations in surfactant composition (Ochs, 2010). Genetic models, particularly knockout organisms, have greatly enhanced the information currently available on lipid and protein function. Biophysical studies of lipids and lipid-protein interactions have been instrumental in deciphering the formation, behavior and function of surfactant films and their physiological implications (Perez-Gil et al., 1992; Perez-Gil et al., 1995; Perez-Gil and Keough, 1998; Perez-Gil 2001; Perez-Gil 2008). Consequently, studies such as this one are pivotal in developing and enhancing the current data on the physiological effects of environmental stressors on the lung surfactant system and its functional significance.

Conclusion
That the discovery of and work on the surfactant system has revolutionized health care is undisputable. Synthetic surfactants are now developed and administered to neonates with respiratory distress syndrome all over the world. The impact of surfactant research on the maintenance of human life is especially evident in third world countries where nutrition and education are lacking and thus preterm births inevitable. The pulmonary surfactant system is critically important not only in a medical sense but also in terms of physiological ecology. For example, pinnipeds have an altered lung physiology to cope with high pressures associated with deep diving. Their surfactant has high levels of cholesterol and SP-B to increase fluidity and facilitate surfactant re-spreading, respectively. These surfactant characteristics allow the sea lion to better cope with the compression of the monolayer that occurs during deep dives (Miller et al., 2004; Miller et al., 2005). Similarly, studies on microchiropteran bats that readily enter torpor show significant changes in their surfactant composition. Upon entering torpor cholesterol levels, relative to DSP, rise to maintain fluidity and then decrease upon coming out of torpor (Codd et al., 2000). Such changes in the pulmonary surfactant system are apparent in deer mice and demonstrate that plasticity of lung surfactant is an important physiological adaptation to high altitude. These studies are the first, to our knowledge, to report increases in both surfactant lipids and proteins at high altitude in mice that exhibit many physiological adaptations to naturally occurring hypoxia.

Furthermore, these results provide an indication of lung function and stability, that would presumably be critical for these small mice in needing sustained aerobic metabolism. Limits in aerobic metabolism can potentially be manifested in terms of
foraging behavior, social interactions, escape from predators and the ability to effectively go into torpor when needed. Thus, the plasticity of the surfactant system is crucial and future studies can provide a larger framework under which additional intriguing and fundamental questions regarding respiratory physiology and the evolution of the lung surfactant system can be answered.
Literature Cited


