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Phenotypic Plasticity in the Lungs of Deer Mice (*Peromyscus maniculatus*) at High Altitude and the Relationship With Aerobic Performance

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Evolution, Ecology,
and Organismal Biology

by

Nicholas Joseph Shirkey

March 2014

Thesis Committee:
Dr. Kimberly Hammond, Chairperson
Dr. Richard Cardullo
Dr. Mark Chappell

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The Thesis of Nicholas Joseph Shirkey is approved:

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ABSTRACT OF THE THESIS

Phenotypic Plasticity in the Lungs of Deer Mice (*Peromyscus maniculatus*) at High Altitude and the Relationship With Aerobic Performance

by

Nicholas Joseph Shirkey

Master of Science, Graduate Program in
Evolution, Ecology, and Organismal Biology
University of California, Riverside, March 2014
Dr. Kimberly Hammond, Chairperson

The importance of phenotypic plasticity during post developmental acclimation to high altitude has been recognized in the recent years. However, the relationship between morphological changes observed in the lungs resulting from this acclimation process and changes in aerobic performance at high altitude have not been sufficiently investigated. This thesis attempts to elucidate the specific morphological changes associated with changes in the lungs of adult deer mice acclimated to high altitude, and also to discern whether or not there is a relationship between aerobic performance and lung morphology at high altitude. Thus far the results show that adult deer mice have significantly greater surface area of the alveoli, and that this change seems to be the result of hypertrophy of existing alveolar tissue, as opposed to de novo alveolarization. This work also demonstrates that lung volume in deer mice is positively correlated with aerobic performance under hypoxic conditions, suggesting that the increase in lung mass of high

altitude acclimated deer mice is beneficial to aerobic performance. However, this relationship is dependent on heart mass such that mice with greater heart mass receive a greater benefit from possessing larger lungs. This work highlights the importance of phenotypic changes in the lungs resulting from acclimation to high altitude.

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Introduction

Organisms that live in high altitude environments must cope with differences in several abiotic factors relative to low altitude. Correspondingly, organisms living at high altitude may exhibit evolutionary adaptations and/or changes in phenotypic expression in response to these conditions. Perhaps the greater stressor endured by organisms living at high altitude is that of reduced partial pressures of oxygen (pO_2) relative to low altitude. However, animals at high altitude must also deal with low ambient temperatures, low primary productivity (Luo et al., 2004), and increased exposure to ultraviolet radiation relative to low altitude (Blumthaler et al., 1997).

Hypobaric hypoxia at high altitude translates to lower pO_2 in the alveoli of the lungs, resulting in a smaller gradient for oxygen from the environment to the tissues. Though capping oxygen demand by reducing metabolic processes is a solution to transient hypoxia used by some mammals (Ramirez et al., 2007), it is not a useful strategy in the chronically hypoxic high altitude environment. Not only does hypobaric hypoxia present an unrelenting stressor that cannot be avoided behaviorally, but the reduced ambient temperatures at high altitudes means that small endothermic animals must invest metabolic energy into thermogenesis despite reduced oxygen availability.

Exposure to chronic hypoxia can induce a variety of maladaptive or even pathological physiological responses in low living animals. For example while some humans can climb mountains over 8000m without supplemental oxygen, most people experience serious physiological, sensory, and neurological problems (Huey & Eguskitza, 2001). However, animals whose natural history involves life at high altitude often display a mixture of evolved adaptations and physiological acclimation to cope with hypoxia.

The effect of the combined stresses of hypoxia and cold exposure on the physiological processes of animals living under these conditions is likely to have an impact on fitness. Previous studies have even shown that exposure to high altitude conditions can affect life history traits such as fecundity (Bronson, 1979; Moore and Regensteiner, 1983; Badyaev and Ghalambor, 2001). Because of the potential impact of hypoxia and increased thermogenic demand on fitness, species that live at high altitude are of great interest to physiological ecologists who look for examples of evolutionary adaptation and physiological acclimation.

Organismal performance

Negative physiological effects resulting from hypoxia should manifest in the form of reduced organismal performance. Lost performance can be ameliorated either through compensatory genetic changes at the population level, or by phenotypic plasticity at the level of the individual (Garland and Carter, 1994). By measuring organismal performance it is possible to determine whether a specific genotypic or a phenotypic change is associated with a change in performance, the relationship between performance changes and a measure of fitness can then be used to determine if the change is adaptive, maladaptive, or neutral. Maximal oxygen consumption, quantified as the peak oxygen uptake during a bout of intense exercise ($\dot{V}O_2$ max) or cold exposure ($\dot{V}O_2$ sum), is the measure of animal performance most often used in studies at high altitude (Chappell et al., 1988; Chappell et al., 1995; Fulco et al., 1998; Hsia et al., 2007).

Maximal oxygen consumption is governed by a cascade of oxygen as it moves through various compartments in the body (Taylor and Weibel, 1981; Weibel et al., 1981). At the interface of the organism with the environment the amount of oxygen that can be taken into the lungs is determined by bulk flow ventilation. Oxygen then moves across the lungs into the blood by diffusion, relative to a pO_2 gradient. Once in the blood, oxygen bound to hemoglobin is again transported by bulk flow determined by cardiac output, until it reaches the capillaries where it diffuses into the surrounding cells and is used by the mitochondria for cellular respiration. Limiting the flux of oxygen through the body at any given level will restrict the flow of oxygen to all components. Thus environmental hypoxia, which can potentially limit diffusion across the lung due to a reduced gradient, may limit oxygen availability for metabolic activities.

The ecological relevance of maximal oxygen consumption is that it sets the sustainable upper limit for the performance of behaviors with an ecological impact such as thermoregulation or locomotion (Chappell et al., 1995). Indeed some small animals like golden-mantled ground squirrels will travel across their range at speeds close to their aerobic maximum to avoid predation (Kenagy and Hoyt, 1989). At high altitude the effect of lower ambient temperature and partial pressure of oxygen can have a significant impact on maximal oxygen consumption and produce both evolutionary adaptations and physiological acclimation as a result (Snyder, 1981; Chappell and Snyder, 1984; Hammond et al., 2001).

The deer mouse (*Peromyscus maniculatus*) is one of the most well studied organisms in relation to high altitude. This rodent is found across North America at

altitude ranging from below sea level to over 4000m (Hock, 1961). In eastern California populations were historically most dense at 2150m, but evidence of reproduction was found in mice at 3800m despite a significantly shortened breeding season (Dunmire, 1960). This widespread distribution and ability to maintain fitness even at the highest extent of their range makes deer mice an appropriate subject for comparing performance across a wide altitudinal range.

Evolutionary adaptation

Prior research focused on evolutionary responses in deer mice to high altitude in particular in relation to hemoglobin polymorphisms. Hemoglobin binds to oxygen in erythrocytes and consists of four subunits, two α-globin chains and two β-globin chains that each possesses a heme group, which can bind a single oxygen molecule. Oxygen saturation, resulting in four molecules of oxygen bound per hemoglobin molecule, depends on the partial pressure of oxygen (pO_2) in the blood and the cooperative nature of the binding event culminating in a sigmoidal oxygen-dissociation curve. At low and high pO_2, changes in the oxygen availability produce very little change in the degree of binding, however in the middle portion of the curve very small changes in pO_2 can produce significant changes in the amount of oxygen that is bound to hemoglobin. For animals acclimated to low altitude the concentration of oxygen is such that the pO_2 of alveolar air is at the upper portion of the curve. However, if that animal were exposed to a lower ambient pO_2, such as that at high altitude, the region of the curve that animal is working in is shifted, and oxygen loading may be compromised to some degree.

In high altitude populations, however, natural selection should push hemoglobin toward a leftward shift in the oxygen-dissociation curve resulting in improved binding (higher affinity) of oxygen at lower pO_2. Indeed a genetic polymorphism in the two loci that encode the α-globin chain in deer mice hemoglobin was found by Lee Snyder (1978) and showed evidence of being adaptive in this way. The loci are strongly linked such that they always encode the same haplotype, and recombination is rare (Chappell and Snyder, 1984; Chappell et al., 1988). The haplotypes coded for α-globin chains with different p50s (the partial pressure of oxygen at which saturation of the hemoglobin with oxygen is 50%) such that the a0c0 haplotype had a lower p50 than the a1c1 haplotype (Snyder, 1981). Furthermore, these haplotypes correspond with populations at different altitudes, such that populations of deer mice at the highest altitude had a higher frequency of the a0c0 haplotype and populations at low altitudes had a high proportion of a1c1 haplotypes (Snyder, 1981; Snyder et al., 1988). These genotypes had a significant effect on maximal oxygen consumption in mice with known haplotypes from identical-by-descent breeding programs.

Other polymorphisms in the β-globin chain are associated with high altitude populations, and cause reduced sensitivity to 2,3 diphosphoglycerate (DPG) and Cl^- ions resulting in a decrease of the p50 of hemoglobin (Storz et al., 2009; Storz et al., 2010). These changes in the α and β chain of hemoglobin result in higher affinity for oxygen in the pulmonary capillaries of deer mice at high altitude, resulting in improved saturation of hemoglobin in spite of reduced ambient pO_2.

Phenotypic Plasticty

While these hemoglobin polymorphisms are a remarkable example of evolutionary change, animals raised in the lab under warm conditions at low altitude consistently displayed reduced maximal oxygen consumption when tested at high altitude (Chappell and Snyder, 1984; Chappell et al., 1988) relative to wild caught animals, which are exposed to consistently lower temperatures and pO_2 (Hayes, 1989). For example, it was found that despite being derived from a high altitude population that lab bred animals reared at low altitude had a maximal oxygen consumption that was 62% lower than wild caught animals from high altitude when tested under the same low pO_2 conditions (Hammond et at., 2002). This variation in the performance of animals that share a similar genetic history therefore must be due either to the environment or a gene by environmental interaction. Phenotypic plasticity during development and adulthood must therefore play a significant role in the ability of deer mice to maintain their aerobic capacity across an oxygen gradient.

While lab bred low altitude born mice derived from a high altitude population have a sharp reduction in $\dot{V}O_2$ sum when tested under hypoxic conditions (Chappell et al., 2007), wild animals born at high altitude showed virtually no difference in $\dot{V}O_2$ sum from low altitude animals after accounting for temperature (Hayes, 1989). This suggests that changes due to development or acclimation are responsible for maintaining $\dot{V}O_2$ across a gradient of pO_2s. Likewise, low altitude born lab bred mice acclimated to hypoxia show only a 12% reduction in $\dot{V}O_2$ max and a 19% reduction in $\dot{V}O_2$ sum in response to an ~35% reduction in ambient pO_2 (Chappell et al., 1988). While hemoglobin

polymorphisms can account for some of the compensation seen in wild mice, it is clear from these results that plastic changes in the systems involved in the oxygen cascade are also likely involved.

During acclimation to hypoxia a suite of changes occur throughout the circulatory system. Though these changes can be species specific, others are found across multiple taxa. One of the most commonly noted is an increase in hematocrit, or the volume of blood made up of erythrocytes. This trait is known to change in most mammals acclimating to high altitude increasing the carrying capacity of the blood for oxygen (Frisancho, 1975; Snyder et al., 1982; Blake and Banchero, 1984; Hammond et al., 2000; Yilmaz et al., 2007; Tufts et al., 2013). Other hematological traits that exhibit plasticity in deer mice acclimated to high altitude include; increased hemoglobin concentration and increased concentration of 2,3 DPG (Snyder, 1982; Snyder et al., 1982; Tufts et al., 2013). Increased DPG at high altitude is generally considered maladaptive for O_2 loading in the lungs as it results in a rightward shift in the oxygen dissociation curve and an increased p50 for hemoglobin (Mairbaurl et al., 1993), though this can result in greater offloading in the tissues. However, in the context of β-globin chain polymorphisms associated with decreased sensitivity to DPG, plastic increases in the concentration of that compound may not have as great of an impact in high altitude deer mice populations (Storz et al., 2009).

Other changes may have a beneficial effect as an acute response to hypoxia, but ultimately may result in a negative chronic response (Rezende et al., 2005). For example, hypoxia induces the production of proteins like hypoxia-induced mitogenic factor

(HIMF), which has a simultaneous beneficial angiogenic and negative vasoconstrictive effect (Teng et al., 2003). Vasoconstriction in the pulmonary artery caused by hypoxia is the primary cause of pulmonary hypertension and is linked to the pathology known as high altitude pulmonary edema (HAPE) (Bartsch et al., 1991). Chronic vasoconstriction results in thickened arterial walls and elastic lamina in the pulmonary artery (Hunter et al., 1974; Van Bui & Banchero, 1980) and also results in hypertrophy of the right ventricle of the heart (Hunter et al., 1974; Van Bui & Banchero, 1980). This constriction in the pulmonary artery may also reduce aerobic performance at high altitude by restricting blood flow to the lungs, as athletes acclimated to high altitude and treated with a vasodilating agent showed a 10% restoration in $\dot{V}O_2$ max under hypoxic conditions (Naeije et al., 2010). While these negative changes have not been demonstrated in deer mice it is likely that they experience a similar suite of changes when exposed to high altitude. For example, during acute exposure to hypoxia deer mice showed evidence of bloody edematous fluid in the lungs, which is characteristic of pulmonary hypertension (Diaz pers. com.).

Acclimation also results in plastic changes to ventilatory systems as well. Increased bulk flow of air into the lungs by changing ventilation rate and tidal volume can help low altitude animals compensate for reduced oxygen partial pressures at high altitude (Frisancho, 1975; Blake and Banchero, 1985). Indeed, both wild and lab bred deer mice acclimated to high altitude showed increased tidal volume and respiratory rate. Correction for barometric pressure revealed a minute volume in high altitude acclimated animals that was similar to low altitude controls (Chappell, 1985).

Diffusive capacity of the lungs

In addition to increases in bulk flow of air moving into the lungs in high altitude acclimated animals, the diffusive capacity of the lung can change under certain circumstances in mammals acclimating to hypoxia. Oxygen uptake in the lungs ($\dot{V}O_2$) can be quantified by the Bohr equation:

$$\dot{V}O2 = DL_{O_2} \cdot (P\boldsymbol{a}_{O_2} - P\boldsymbol{c}_{O_2})$$

where DL_{O2} refers to the diffusive capacity of the lung, $P\mathbf{a}_{O2}$ refers to the partial pressure of oxygen in the alveolus, and $P\mathbf{c}_{O2}$ refers to the partial pressure of oxygen in the capillaries. In general the partial pressure in the alveoli is determined by the atmospheric pressure and is not able to be changed by the organism, though changes in ventilation can alter $P\mathbf{a}_{O2}$ and $P\mathbf{a}_{CO2}$ to some extent (Cunningham et al., 1963). Likewise, $P\mathbf{c}_{O2}$ is generally controlled by oxygen consumption in the body, and falls as consumption increases (Weibel 1999). Thus increased usage of oxygen by the organism can result in a lower $P\mathbf{c}_{O2}$, and a larger gradient for oxygen to diffuse into the body.

Structural changes in the lung can result in changes in DL_{O2} and thus have an impact on oxygen uptake by the lung. The diffusive capacity of the lung is composed of two parts: the conductance of the membrane (D_M) and the conductance of the erythrocytes (D_E) (*Equation 3*). D_M (Equation 1) is largely determined by the morphology of the lung and consists of the alveolar surface area (S_a), the capillary surface area (S_c), the thickness of the barrier from alveolus to erythrocyte (τ_{hb}), and Krogh's diffusion constant (K) (Weibel et al., 1993; Weibel, 1999). The other term D_E (Equation 2) is

obtained by combining capillary volume (V_c), and the reaction rate of blood with oxygen (θ_{O2}) (Weibel, 1999).

According to these equations, changes in the internal structure of the lungs can produce changes in the capacity for an animal to diffuse oxygen from the lung into the blood, and that these changes are largely dictated by the surface area available for gas exchange and the thickness of the tissue and plasma separating the air from the red blood cells.

$$(1)\ D_M = K \cdot (S_A + S_c)/2\tau_{hb}$$

$$(2)\ D_E = \theta_{O_2} \cdot V_c$$

$$(3)\ \frac{1}{D_L} = \frac{1}{D_M} + \frac{1}{D_E}$$

In response to chronic exposure to high altitude many rodents show increased mass and volume of the lungs (Hunter et al., 1974; Sekhon & Thurlbeck, 1995a; Hammond et al., 1999; Hammond et al., 2001). This same phenomenon also occurs in guinea pigs (Hsia et al., 2005) and dogs (Ravikumar et al., 2009) exposed to high altitude during development. The acclimatory response in the lungs seems to be dictated by the hypoxia experienced at high altitudes as opposed to the hypobaric conditions. For example, several studies showed that normobaric hypoxia produced a similar response in the lungs to hypobaric hypoxia (Cunningham et al., 1974; Lechner & Banchero, 1980; Sekhon & Thurlbeck, 1995a; Sehkon et al., 1995b).

Studies on the response of DL_{O2} to hypoxia have focused on development while somatic growth is still occurring and the number of alveoli can increase by more than ten times the amount present at birth (Dunnill, 1962). Mammals exposed to hypoxia during

development tend to have faster development of lung tissue (Lechner & Banchero, 1980; Lechner et al., 1982), which results in a higher lung volume than in animals exposed to a normoxic environment (Burri and Weibel, 1971; Bartlett and Remmers, 1971; Cunningham et al., 1974; Johnson et al., 1985; Sekhon & Thurlbeck, 1995a; Hsia et al., 2005; Ravikumar et al., 2009). Changes in lung volume and mass alone may not be beneficial if they involved tissue growth in non gas exchange regions, thickening of the barriers to diffusion, or even accumulation of fluids and blood in the lungs. While edema has been found in the lungs of animals acutely exposed to hypoxia, it was not associated with chronic exposure (Bartlett and Remmers, 1971).

Morphological work in rats exposed to hypoxia during development demonstrated that animals had more and larger alveoli and alveolar ducts (Cunningham et al., 1974). This supports other work showing increased alveolar and capillary surface areas in animals raised under hypoxic conditions (Burri & Weibel, 1971; Cunningham et al., 1974; Pearson & Pearson, 1976; Sekhon & Thurlbeck, 1995a; Hsia et al., 2005). Furthermore in developing dogs acclimated to hypoxia a significant reduction of harmonic mean thickness of the blood-gas barrier was found (Ravikumar et al., 2009). Taken together these morphological changes suggest an increased capacity for the uptake of oxygen. These studies as well as others (DeGraff et al., 1970; Guleria et al., 1971; Lomholt and Johansen, 1979; Hsia et al., 2007; McDonough et al., 2005; Yilmaz et al., 2007) demonstrate that the lung diffusive capacity of high altitude natives is significantly greater than low altitude controls in a variety of species of mammals. It is important to note that diffusive capacity is dependent on blood flow (D_E), and that the reduction of

blood flow into the lungs at high altitude due to pulmonary vasoconstriction can reduce the diffusive capacity available to the animal (Yilmaz et al., 2007).

In contrast to work on developing animals, hypoxic acclimation in adult dogs and humans resulted in no significant gains in lung diffusive capacity (West, 1962; Kreuzer & Campagne, 1965; Vincent et al., 1978; Johnson et al., 1985). Adult rats exposed to hypoxia were able to increase alveolar surface area by making alveoli and alveolar ducts larger, but did not significantly change the number of alveoli in their lungs (Cunningham et al., 1974; Blanco et al., 1991). Furthermore, it was found that changes in the lung volume of adult rats exposed to hypoxia were reversible after return to normoxia (Rabinovitch et al., 1981).

Goals of this thesis

Historical work in this field has focused on evolutionary adaptations to explain the ability of deer mice to survive in places with high thermogenic demands and low oxygen availability (Snyder, 1978; Chappell et al., 1988; Storz et al., 2009). However, it is become increasingly apparent that plastic physiological changes occur extensively in the pulmonary and cardiovascular systems during development and acclimation at high altitude. Despite knowledge of these changes, the relative contribution of plasticity during development and adulthood to organismal performance has remained unstudied until recently (Russell et al., 2008; Cheviron et al., 2012). The goal of this current work is to identify the extent of phenotypic changes in the lungs of adult deer mice acclimated to hypoxia, the degree to which those changes impact maximal aerobic capacity, and the

importance of these changes relative to other phenotypic changes (e.g. increased hematocrit) that occur at high altitude.

The importance of this work in relation to conservation is highlighted by the warming effect of climate change. As temperatures warm at lower altitudes many species will find refuge in the mountains where ambient temperatures remain cooler. Thus more and more species will be exposed to conditions that are hypoxic relative to what they were used to. It is important from the perspective of conservation to understand the nature of the phenotypic changes that will permit these animals to continue their existence at higher altitudes. This research will thus help to identify the benefits of those plastic changes in the organs of animals living in this “extreme” environment.

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Chapter 1

The Relationship Between Lung Plasticity
and Aerobic Performance in Deer Mice at High Altitude

Abstract

Animals living in the transition zone of an environment may experience variation in abiotic factors. For example, deer mice (*Peromyscus maniculatus sonoriensis*) in the White mountains of Eastern California are found across a range of partial pressures of oxygen (pO_2). Reduction in pO_2 at high altitude can have a negative impact on aerobic performance. We studied plastic changes in the organs involved in aerobic respiration in response to acclimation to high altitude, and how those changes are matched with aerobic performance measured by $\dot{V}O_2$ max. Adult deer mice born and raised at 340 m were acclimated at either 340 m or 3800 m for a period of nine weeks. Lung volume increased by 9% in mice acclimated to high altitude. $\dot{V}O_2$ max was also significantly higher under hypoxic conditions after high altitude acclimation compared to controls. Body mass corrected residuals of $\dot{V}O_2$ max were significantly correlated with an index of cardiopulmonary size (summed standardized residuals of lung volume and heart mass) under both hypoxic and normoxic conditions These data show that phenotypic plasticity plays an important role in maintaining aerobic performance under hypoxic conditions, and that cardiopulmonary size is potentially responsible for a portion of the maintenance of that trait.

Introduction

Aerobic performance is an emergent trait that is dependent on a cascade of oxygen moving from the environment to the cells via a pathway that involves multiple organ systems (Weibel et al., 1981; Bassett and Howley, 2000). These organs must work together to generate aerobic output, and even small changes in the environment can impact the function of one or more of these systems resulting in a change in organismal performance. Populations of organisms living in areas of transition can be exposed to a range of abiotic factors, and therefore individuals should be equipped to deal with a variety of conditions. For example, altitudinal gradients can produce significant changes in environmental conditions within relatively short distance. Several biotic and abiotic factors vary with altitude including temperature, primary productivity, and UV exposure. However, perhaps the most important change is the reduced ambient partial pressures of oxygen (pO_2) at high altitude.

Organisms that live at high altitudes generally must adapt to the lower levels of oxygen or face reduced aerobic performance as a result, either through evolutionary processes (genetic changes across generations) or phenotypic plasticity (physiological changes during an individual's lifespan) (Garland and Carter, 1994). Although some evolutionary changes have been documented in species that inhabit high altitudes, such as hemoglobin polymorphisms in deer mice (*Peromyscus maniculatus*) (Synder et al., 1988; Storz et al., 2007; Storz et al., 2010), phenotypic plasticity remains an important way to maintain aerobic performance in the face of environmental heterogeneity.

Deer mice have been studied extensively in high altitude physiology. They are widely distributed, both geographically throughout North America, but also across a wide altitudinal range. One subspecies, *P. maniculatus sonoriensis* is found across eastern California and has an altitudinal range that extends from below sea level in Death Valley, CA to over 4000 m in the Sierra Nevada and White Mountains (Sawin, 1970). These mice possess evolutionary adaptations to high altitude such as the aforementioned hemoglobin polymorphisms. However, phenotypic plasticity also plays a major role in acclimation to high altitude both during development and adulthood. Wild caught mice at high altitude tended to have improved thermogenic performance relative to low altitude controls even when seasonal effects are taken into account (Hayes 1989). Likewise mice born at high altitude perform better during exercise tests than low born mice acclimated to high altitude (Chappell et al. 2007). However, previous work has also shown that mice born at high altitude had a decreased acclimatory response when moved to low altitude relative to the reciprocal move in low born mice, indicating that high born mice are constrained by the changes that occur during development (Russell et al., 2008).

The change in whole-animal aerobic capacity resulting from acclimation to high altitude is accompanied by increased hematocrit, hemoglobin concentration, and lung mass compared to animals acclimated to low altitude (Hammond et al., 1999; Hammond et al., 2001). Changes in splenic function have also been noted, as it is generally considered to be associated with storage of red blood cells (Baker and Remington, 1960; Boning et al., 2011). For example in thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*), splenectomy results in a reduced hematocrit in response to low pO_2

(Mcglaghlin et al., 1972). These changes occur in systems that directly impact the oxygen cascade and it is assumed they are at least partially responsible for increased aerobic performance. For example, while it is now generally believed that lung O_2 diffusion capacity is not limiting to aerobic capacity at sea level (Hsia et al., 2007), at high altitudes the lower pO_2 can pose an important constraint to the flow of oxygen into the body, and thus increased lung size could be important in recouping lost performance.

Based on these observations we set about to test the hypothesis that adult deer mice rely on phenotypic changes to maintain performance at high altitude. To evaluate this hypothesis we asked if there was a clear relationship between changes in traits, such as organ morphology and organismal performance. We made three predictions in relation to this hypothesis:

1. Mice acclimated to high altitude will show improved aerobic performance ($\dot{V}O_2$ max during exercise) under both hypoxic and normoxic conditions.
2. Mice acclimated to high altitude will have greater heart mass, lung volume, and hematocrit.
3. Mice with higher cardiopulmonary residuals and hematocrit levels will have higher $\dot{V}O_2$ max residuals.

Materials and Methods

Animals

We used 11 male and 7 female adult deer mice (*P. maniculatus sonoriensis*) for this study. Animals ranged from 382 days to 500 days in age and were captive bred at low altitude (340m) in a colony that was originally removed from the wild in 1995. Mice were acclimated to one of two conditions, high acclimated (HA, n=10) or low acclimated (LA, n=8), for a period of nine weeks. The high altitude study site was the Barcroft Laboratory at the University of California's White Mountain Research Center (Barcroft; 3800 m elevation) and the low altitude study site was the University of California at Riverside Campus (UCR; 340 m elevation).

Animals were housed as individuals or pairs in plastic shoebox cages (27 cm x 21 cm x 14 cm) with aspen shavings for bedding. They were given *ad libitum* food and water, and provided with approximately 1g of cotton for nesting. At Barcroft, cages were housed in a room with an average ambient temperature of 16ºC±2.27 SD (Fig. 1.1), exposed to the natural photoperiod. Ambient temperature was recorded every 30 min with a Stowaway XTI data-logger (Onset Computer Corp, Bourne, MA, USA) placed in an empty cage filled with bedding. Low altitude animals were housed in a vivarium at a near constant ambient temperature of about 22ºC (range 21 – 23ºC). The lights in the vivarium were set to 14 h:10 h light:dark (L:D) photoperiod to approximate the natural photoperiod at Barcroft.

Aerobic performance by maximal oxygen consumption

Maximal oxygen consumption ($\dot{V}O_2$ max), as an estimate of aerobic performance, was measured on all mice on three separate occasions, initially at UCR prior to acclimation (initial run; ambient pO_2~150mm Hg), and twice after the acclimation period ended, once at Barcroft (hypoxia run; ambient pO_2~100mm Hg), and once at UCR (normoxia run; ambient pO_2~150mm Hg). Post acclimation runs were conducted within one day of one another. This was done by transiently (<1 day) moving the low acclimated mice to high altitude for testing and the high acclimated mice to low altitude for testing (See Fig. 1.2). Though it is possible that some deacclimation could occur even in this relatively short period, particularly in characters like hematocrit, it is unlikely that significant morphological changes in the heart or lung size would occur this quickly. Mice were transported between sites in an air conditioned vehicle. The journey took approximately 6 hours, and mice were give slices of apple to maintain hydration.

Maximal oxygen consumption was measured by open flow respirometry during forced treadmill exercise. Air was supplied either by outlet (UCR) or using a positive pressure pump (Barcroft). Incurrent air was dried by Drierite™ (Xenia, OH, USA) and scrubbed of carbon dioxide by soda lime. Flow rate was regulated by Porter mass flow controllers (Hatfeild, PA, USA) upstream of the treadmill. The treadmill's working section was enclosed by Plexiglas with dimensions of 6 cm x 7 cm x 13 cm. Flow rates of 2300 ml min^{-1} and 1550 ml min^{-1} standard temperature and pressure (STP) were used at UCR and Barcroft correspondingly. Approximately 150 ml min^{-1} of excurrent air was

subsampled, then dried and scrubbed of CO_2 before being routed through the oxygen sensor. Oxygen concentration was analyzed with an Ametek/Applied Electrochemistry S-3A analyzers (Pittsburg, PA, USA) and then digitized by Sable Systems UI-2 (Las Vegas, NV, USA) A-D converters and recorded on a Macintosh computer running Warthog Lab Helper software (www.warthog.ucr.edu).

Body mass was measured on animals prior to all runs. Mice were then placed on the treadmill and allowed to adjust for a period of 2-4 minutes. During this time a reference reading of unbreathed air was obtained. The treadmill was then started at a low speed (approximately ~0.1m/s), and speed subsequently increased by increments of 0.1m/s every 30-45 seconds until the mouse could either no longer maintain position on the tread or $\dot{V}O_2$ did not increase with increasing speed. At this time the treadmill was stopped, but $\dot{V}O_2$ measurements continued for several minutes during the animal's recovery period before a second reference reading was recorded.

$\dot{V}O_2$ was calculated from O_2 concentrations using the mode 1 equation in Warthog Lab Analyst software (www.warthog.ucr.edu).

$$\dot{V}O_2 = \dot{V}\frac{(F_IO_2 - F_EO_2)}{(1 - F_EO_2)} \quad (1)$$

In equation (1) $\dot{V}$ is flow rate (ml•min^{-1}STP corrected), and F_IO_2 and F_EO_2 are incurrent (reference) and excurrent fractional O_2 concentrations respectively (F_IO_2 was assumed to be 0.2095). Due to the size of the treadmill the "instantaneous" correction was applied to account for mixing (Bartholomew et al., 1981) and better resolve short-

term metabolic changes. $\dot{V}O_2$ max was calculated as the highest one-minute average during the running bout or post exercise recovery period.

Dissection and organ measurement

All dissections took place at UCR to ensure consistent processing. After post-acclimation metabolic measurements were completed, we euthanized mice by overdose of Euthasol (0.07 ml IP; Vibrac Animal Health, Fort Worth, TX, USA). High altitude acclimated mice were sacrificed within 48 hours of being returned to low altitude. We obtained blood samples by retro-orbital puncture using heparinized microhematocrit tubes. Hematocrit was calculated from centrifuged tubes as the proportion of packed cells over the total volume of blood in the tube. The heart, liver, spleen, and kidneys were subsequently removed from the body, cleaned of any connective tissue and fat and weighed separately (wet mass). Organs were then placed in an oven at 70ºC for at least 72 hours and dried to a constant mass before being reweighed (dry mass).

The lungs were fixed by tracheal instillation of a 2.5% buffered gluteraldehyde solution at a constant airway pressure of 25 cm H_2O above the sternum for a period of 30 minutes. At the end of the 30 minutes the tubing leading to the trachea was tied off to maintain the pressure and the fixative in the lungs. The lungs and tubing were removed from the body and then transferred to a vial and submerged in the glutaraldehyde solution for a period of 24 hours at 4ºC. The fixed lungs were washed twice in 0.1 M cacodylate buffer (pH 7.4) before being placed in vials with the buffer and stored at 4ºC.

Lung volume was measured by immersion displacement directly after removal from the mouse using the method described by Sherle (1970), and again after being separated into lobes (lobar volume; right lung: 4 lobes, left lung: 1 lobe).

Statistical Analysis

We used a 2x2 factorial design with sex and altitude as the independent variables, and five dependent variables; dry heart mass, lung volume, hematocrit, and $\dot{V}O_2$ max (under normoxia and hypoxia). There were no differences between sex for any dependent variable, so we combined males and females for the final analysis. Differences between acclimation groups were determined by analysis of variance (ANOVA) and analysis of covariance (ANCOVA) with body mass as a covariate. We used repeated measures ANOVA to analyze mass corrected $\dot{V}O_2$ max data with acclimation altitude as the between subjects factor, and ambient pO_2 during run as the within subjects factor. A post-hoc Tukey HSD test was used for subsequent pairwise comparisons. An alpha of 0.05 was used for statistical significance, however we report all values that approached the threshold of significance. Treatment and error degrees of freedom are enumerated as subscripts to the F values, and unless otherwise stated all F values come from the aforementioned analyses. In all cases, means are reported with standard error of the means (SEM) and are corrected for body mass by adding least square residuals to the grand mean when appropriate. A list of means for all variables considered in this study can be found in Table 1.1.

In addition to ANOVA, we used a stepwise multiple regression analysis to explore the relationship between $\dot{V}O_2$ max and measures that might have an impact on maximal metabolic output including; lung volume, dry heart mass, and hematocrit. For measures that are typically dependent on body mass ($\dot{V}O_2$ max, lobar volume, heart mass), we used mass residuals. The mass at sacrifice was used for this regression in all cases, including for regression on $\dot{V}O_2$ max. The body mass at sacrifice represented the fully hydrated state of the animals having been moved between sites, and was measured within five days of both the normoxic and hypoxic runs. In all cases except dry heart mass, body mass was a significant covariate, but the residuals of the dry heart mass regression with body mass were still used as it is known that body mass does have an effect on heart mass in general. We checked for multicollinearity by correlation matrix in all variables prior to adding them into the model, but correlations between the predictors were relatively low. Model fit was evaluated by F values and R^2, and individual regression coefficients were evaluated by t value and squared semipartial correlation coefficient.

As an alternative to multiple regression, residuals of dry heart mass and lobar volume were standardized as z-scores and added together to get a value of summed cardiopulmonary size. Residuals of $\dot{V}O_2$ max (for both normoxia and hypoxia) were then regressed on cardiopulmonary size and presented in graphically.

Results

Body mass

Body mass was not significantly different between altitude groups at the start of the acclimation process (HA=24.44±0.78 g, LA=23.97±0.74 g; $F_{1,17}$=0.18, P=0.67), or at the time of sacrifice (Fig. 1.3) (HA=23.52±0.60 g, LA=24.01±0.76 g; $F_{1,17}$=0.27, P=0.61).

Hematocrit

Hematocrit was similar between the HA (46.9±1.55 %) and LA (47.04±0.83 %) groups at the time of sacrifice (Fig. 1.4) ($F_{1,17}$=0.007, P=0.93). Because past studies have consistently shown that deer mice acclimated to high altitude have increased hematocrit (8% higher) and hemoglobin concentrations (10% higher) compared to those at low altitude (Hammond et al., 2001; Hammond et al., 2002; Tufts et al., 2013), we decided to look at the relationship between spleen size and hematocrit in the HA mice. If red blood cells were sequestered in the spleen during their short time at low altitude, it might be expected that animals with a greater spleen mass would have lower values for hematocrit. To test this hypothesis we regressed hematocrit on dry spleen mass in the HA animals, and found a highly significant negative correlation of r = -0.771 ($F_{1,8}$ = 11.71, P = 0.0091).

Cardiopulmonary organs

Body mass was a significant covariate for wet heart mass ($F_{2,16}$=4.47, P=0.05) but not dry heart mass ($F_{2,16}$=1.36, P=0.26), and heart mass did not vary significantly between acclimation altitude in either wet ($F_{2,16}$=1.86, P=0.19), or dry measurements ($F_{2,16}$=1.86, P=0.19). In spite of this result, the body mass corrected mean wet heart mass of the HA group (0.1897±0.0055 g) was 7% higher than the LA group (0.1772±0.0049 g) and corrected dry heart mass was 8% higher in the HA group (0.0415±0.0015 g) compared to the LA group (0.0384±0.0016 g) (Fig. 1.5).

Mice acclimated to high altitude had 8% larger lung volume than low altitude controls (HA 0.91±0.019 ml, LA 0.84±0.017 ml; $F_{2,15}$=6.84, P=0.020). Likewise the summed lobar volume of the lung was 9% greater in the HA group (HA 0.80±0.017 ml, LA 0.73±0.025 ml; $F_{2,15}$=4.55, P=0.050) (Fig. 1.6). In both cases body mass was a significant covariate.

Maximal oxygen consumption

All values of $\dot{V}O_2$ max were corrected for body mass using residuals of linear regression. Initial aerobic performance under normoxic conditions did not differ significantly between the treatment groups (HA=4.59±0.13 ml•min^{-1}, LA=4.42±0.20 ml•min^{-1}; $F_{2,15}$=1.02, P=0.383). A repeated measures ANOVA of post acclimation aerobic performance under normoxic and hypoxic conditions revealed a significant effect of acclimation altitude on $\dot{V}O_2$ max ($F_{1,16}$ = 8.86, P = 0.0089) such that HA mice

performed better than LA controls. In subsequent post-hoc tests we found that HA mice performed significantly better than LA mice under hypoxia ($z = 3.36$, $P = 0.0042$), but not in normoxia ($z = 1.58$, $P = 0.38$). Likewise, mice did better in normoxia than in hypoxia regardless of their acclimation altitude ($F_{1,16} = 32.58$, $P < 0.0001$). Post hoc tests showed that this was true for both HA mice ($z = 3.19$, $P = 0.0075$) and LA mice ($z = 4.99$, $P < 0.0001$). However, HA mice experienced only a 9% reduction in $\dot{V}O_2$ max under hypoxic conditions versus normoxia compared to the 16.5% loss in performance observed in low acclimated mice. There was no significant interaction observed between acclimation altitude and run pO_2 ($F_{1,16} = 2.55$, $P = 0.13$). Under the pO_2 from their respective acclimation regimes, HA under hypoxia and LA under normoxia, mice showed no significant differences in aerobic performance ($z = -0.78$, $P = 0.86$), suggesting that HA mice are able to perform at the same level as LA mice even under reduced pO_2.

Regression of cardiopulmonary size on $\dot{V}O_2$ max

The complete regression model used included either residuals of $\dot{V}O_2$ max under normoxic or hypoxic conditions from post-acclimation runs as the dependent variable and dry heart mass residuals, lobar volume residuals, and hematocrit as the independent variable. We used residuals of $\dot{V}O_2$ max, lobar volume, and heart mass to remove the effect of body mass.

Stepwise analysis showed that hematocrit was not a significant predictor of maximal aerobic capacity in either the hypoxia or normoxia, and thus was removed from subsequent analyses. Inclusion of both heart mass and lobar volume produced the best

model fit in both the hypoxia run ($F_{2,15}$=9.29, P=0.0018) and the normoxia run ($F_{2,15}$=10.71, P=0.0013) with R^2 of 0.570 and 0.588 respectively. For hypoxia $\dot{V}O_2$ max both heart mass (t_{15}=3.55, P=0.0029) and lobar volume (t_{15}=2.58, P=0.024) explained a significant proportion of the variance in $\dot{V}O_2$ max with squared semipartial correlation coefficients of $r^2_{Y(H\bullet L)}$ = 0.361 and $r^2_{Y(L\bullet H)}$ = 0.181 respectively. Both heart mass (t_{15}=4.06, P=0.0010) and lobar volume (t_{15}=2.02, P=0.061) were also important in explaining the variance in the normoxia run $\dot{V}O_2$ max with corresponding squared semipartial correlation coefficients of $r^2_{Y(H\bullet L)}$ = 0.452 and $r^2_{Y(L\bullet H)}$ = 0.112. Further reduction of the model to just heart mass resulted in a reduction of the R^2, and trimming the model to lobar volume alone produced a non-significant result.

Regression of $\dot{V}O_2$ max residuals on the summed standardized cardiopulmonary size gave similar results. The R^2 values for the regression of $\dot{V}O_2$ max under normoxia and hypoxia were 0.534 (Fig. 1.8) and 0.555 (Fig. 1.9) respectively. It was not possible to further partition the variance for lung volume and heart mass. However the summed cardiopulmonary size was a significant predictor of aerobic performance under both normoxia ($F_{1,16}$ = 18.33, P = 0.00057) and hypoxia ($F_{1,16}$ = 19.95, P = 0.00039).

Discussion

Physiological acclimation to high altitude in deer mice is well studied (Cheviron et al., 2012; Hammond et al., 2001; Hammond et al., 2002; Russell et al., 2008; Storz et al., 2010; Tufts et al., 2013). Deer mice acclimated to high altitude had higher aerobic performance than low altitude animals when tested under the same pO_2 conditions (Russell et al., 2008), and the mass of the lungs was also increased in high altitude acclimated mice (Hammond et al., 2001). Other work shows that high altitude hypoxia induces an array of morphological changes in the lungs resulting in a higher diffusive capacity (Burri and Weibel, 1970; Hsia et al., 2005). However, the link between phenotypic changes in specific organs and whole organismal performance under those conditions is not as well documented. This study supplies evidence of a functional connection between acclimatory changes in an organ directly associated with oxygen uptake (the lung) and aerobic performance in deer mice. We also show that this relationship is dependent on the relative size of the heart, such that increased lung volume is only beneficial when coupled with a relatively larger heart.

Our measurements on lung volume are consistent with previous reports of significantly larger lung mass in high altitude acclimated deer mice (Hammond et al., 2001) and the 8-9% change in lung volume we found is consistent with the 9% increase in lung volume documented in guinea pigs (*Cavia porcellus*) developing at high altitude (Hsia et al., 2005). Unlike other studies of mammals measured under similar protocols (Burri and Weibel, 1971; Lechner and Banchero, 1981; Hsia et al., 2005; Ravikumar et al., 2009) at high altitude, the mice used in this study were all well into adulthood,

suggesting that deer mice retain the capacity for substantial morphological changes even after development has ended.

The 8% difference in heart mass between acclimation altitude, though not significant in this study, is consistent with similar changes in guinea pigs exposed to hypoxia (Van Bui & Banchero, 1980). It is likely that the change in mass is due to hypertrophy of the right ventricle resulting from pulmonary hypertension that is induced by hypoxia (Rabinovitch et al., 1981; Reinke et al., 2011). However, it is possible that in our study that the 4ºC temperature difference between the high and low altitude groups may have played a role in changing in heart mass, as cold exposure (5ºC) has been shown to induce hypertrophy of the heart (Van Bui & Banchero, 1980; Hammond et al., 2001; Rezende et al., 2009). Additionally, HA mice experienced higher variation in daily temperatures with highs of up to 24ºC and lows as far as 11ºC (Fig. 1), where low altitude animals were in a climate controlled room.

One unexpected result of this study was the lack of any difference in hematocrit between acclimation groups when measured at UCR at the end of the study. One possible explanation is that HA animals were able to sequester their excess red blood cells into the spleen during the 1-2 days they were at UCR prior to hematocrit determination. The spleen acts as a reservoir for red blood cells in many mammals (Baker and Remington, 1960; Boning et al., 2011). Thus it is possible that mice acclimated to high altitude sequestered excess red blood cells in the spleen upon return to low altitude resulting in lower hematocrit than 1-2 days earlier at high altitude. The strong negative correlation between hematocrit and spleen mass strongly suggests that HA animals did exactly this

after their return to low altitude and potentially explain why HA values of hematocrit approximated those of LA mice.

The 9% reduction in aerobic performance between normoxic and hypoxic runs we observed in the HA group matches closely the difference reported between high and low altitude acclimated mice in previous studies (Chappell et al., 2007). In fact, HA mice had only a 3% lower aerobic performance under hypoxic conditions than LA mice under normoxia. These results demonstrate once again the capacity of these animals to compensate aerobic capacity in spite of a reduction of alveolar pO_2 of up to 37% based on changes in barometric pressure and vapor pressure. Furthermore, the negligible difference in $\dot{V}O_2$ max observed between the HA group compared to the LA group at their respective acclimation pO_2 strongly suggests that the physiological changes resulting from acclimation to hypoxia are responsible for this improvement in performance.

Because aerobic performance is based on a cascade of oxygen throughout the body and therefore is dependent on multiple systems, all measured variables that could influence performance were included in a correlation analyses. The lack of significance of hematocrit in the final model is probably explained by the sequestration of red blood cells by the spleen in high altitude mice. Still, it cannot be said for certain whether hematocrit would have been significant in the final model had it had been measured while the animals were still at high altitude. The high altitude acclimated mice were brought to low altitude to ensure that all animals were processed in the same manner, however, in

future years it would be important to measure hematocrit in mice at their acclimation site before they are moved.

The importance of heart mass in explaining variance in aerobic performance was expected, due to its connection with cardiac output. The delivery of oxygenated blood from the lungs to the rest of the body is key step in the cascade of oxygen and is dependent on bulk flow produced by contraction of the heart. Stroke volume, which is dependent on the volume of the ventricles, is presumably greater in mice with relatively larger heart mass, and therefore for a given heart rate cardiac output should likewise be increased. At least in humans, that maximal cardiac output is likely the key limiting factor in aerobic performance (Bassett and Howley, 2000), thus the fact that heart mass explained up to 45% of the variance in our mice is unsurprising.

Perhaps more interesting is the fact that the significance of lobar volume as a predictor of aerobic performance was dependent on inclusion of heart mass as a predictor. Particularly in the case of the hypoxia run, the importance of the lung as a predictor of aerobic performance seems evident. By the same logic that a larger heart should have a higher stroke volume, a larger lung may have an increased surface for gas exchange and hence a higher diffusive capacity for oxygen. Previous work has supported the idea that animals acclimated to high altitude develop increased surface area for diffusion (Hsia et al., 2005), but have also stated that diffusive capacity is also dependent on the rate of pulmonary blood flow (Yilmaz et al., 2008). Our results are consistent with the idea that large lungs cannot compensate for hypoxic limitations on aerobic performance unless coupled to enhanced cardiac output and hence a larger heart. The significant correlation

between lung volume and aerobic performance in hypoxia indicates that at low pO_2 the diffusive capacity of the lungs may become limiting, and may explain why organisms invest in the growth of gas exchange organs in the presence of hypoxia (Burggren and Mwalukoma, 1983; Burri and Weibel, 1971; Lomholt and Johansen, 1979).

This study demonstrated a functional linkage between organ-level phenotypic changes and whole-animal aerobic performance. As with previous studies we found phenotypic changes (increased lung volume) in response to high altitude acclimation. We also showed that mice acclimated to high altitude have improved aerobic performance under hypoxic conditions compared to low acclimated mice. Lastly, we bridged the gap between organismal performance and subordinate traits, and showed that changes in organ size (lung) resulting from acclimation are related to aerobic performance at the individual level. These results may have increasing significance as climate change continues, and other organisms seek cooler habitats by moving to higher elevations (Moritz et al., 2008). However, a question remains as to whether organisms that evolved under the high pO_2 conditions of low altitude will demonstrate the same level of phenotypic plasticity in response to hypoxia as that shown by this population of deer mouse, which is native to high altitude. Future work might focus on comparing the plastic responses of populations of *P. maniculatus* from low altitude to that of high altitude populations. This could be expanded to test the plastic response of other species of *Peromyscus* that inhabit ranges that are much more restricted that *P. maniculatus*. Such studies would provide a better idea of how plastic response differ across population and species, and how generalized this response to hypoxia is.

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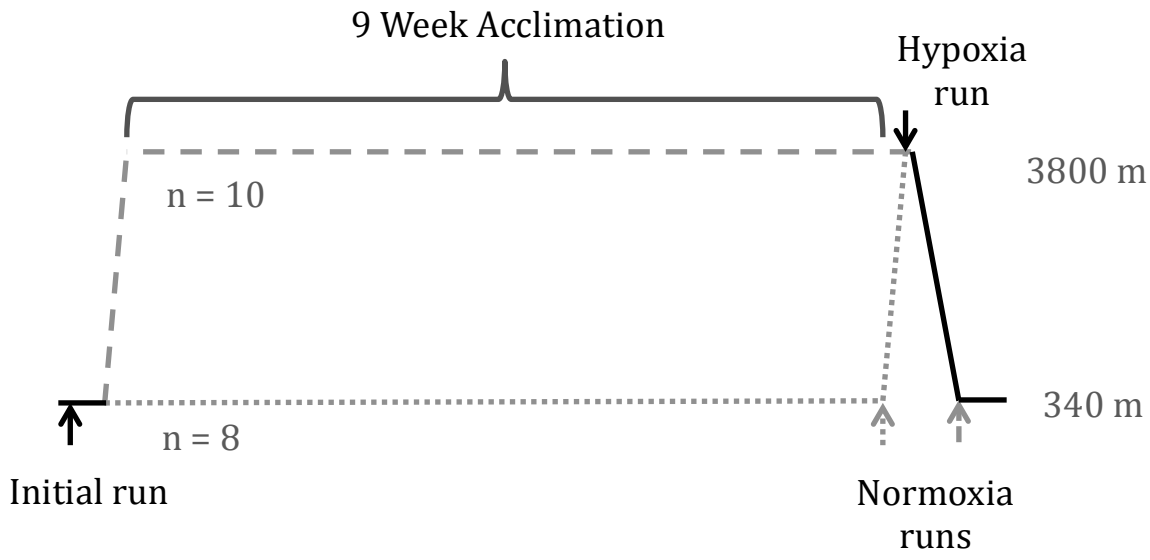

Fig. 1.1. Experimental design employed in this study. Mice were run at low altitude (150 mmHg) initially, and then acclimated either to 3800 m (dashed line; n=10) or 340 m (dotted line; n=8) for a period of 9 weeks. At the end of the acclimation period all mice (solid line) were run at low (150 mmHg) and high (100 mmHg) altitude before being brought to UCR for final processing.

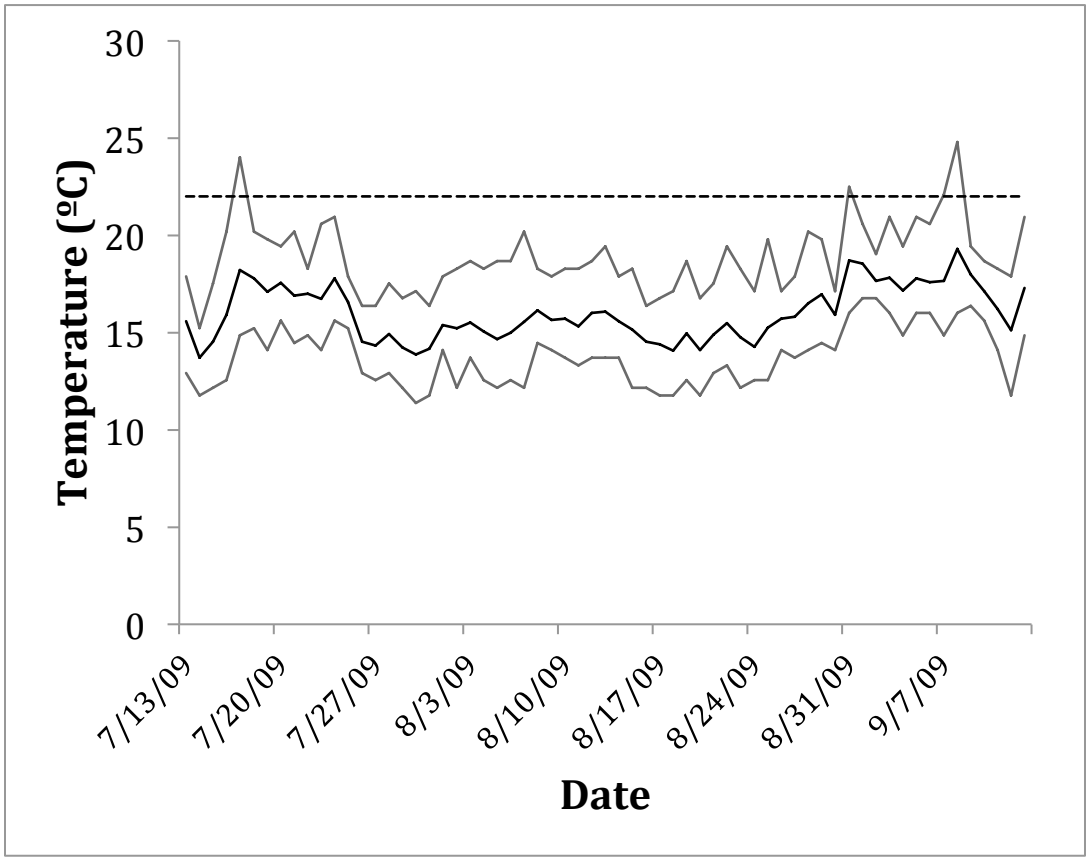

Fig. 1.2. Depicts the daily average (black), low and high (gray) daily temperatures as measured at the field site, Barcroft, compared to the average daily value of the UC Riverside vivarium (dashed line).

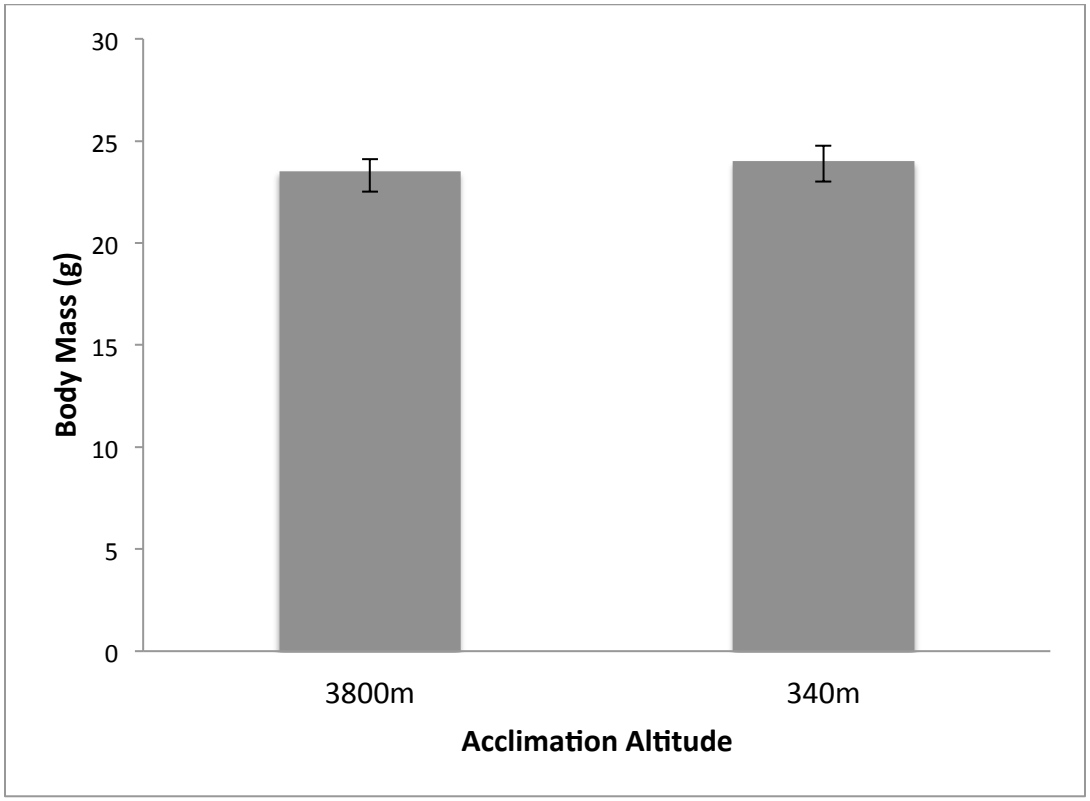

Fig. 1.3. Depicts body mass (g) at time of sacrifice in deer mice from two different altitudes. Asterisks indicate statistically significant differences between acclimation groups. Values are means +1 S.E.M. (*N* as in text).

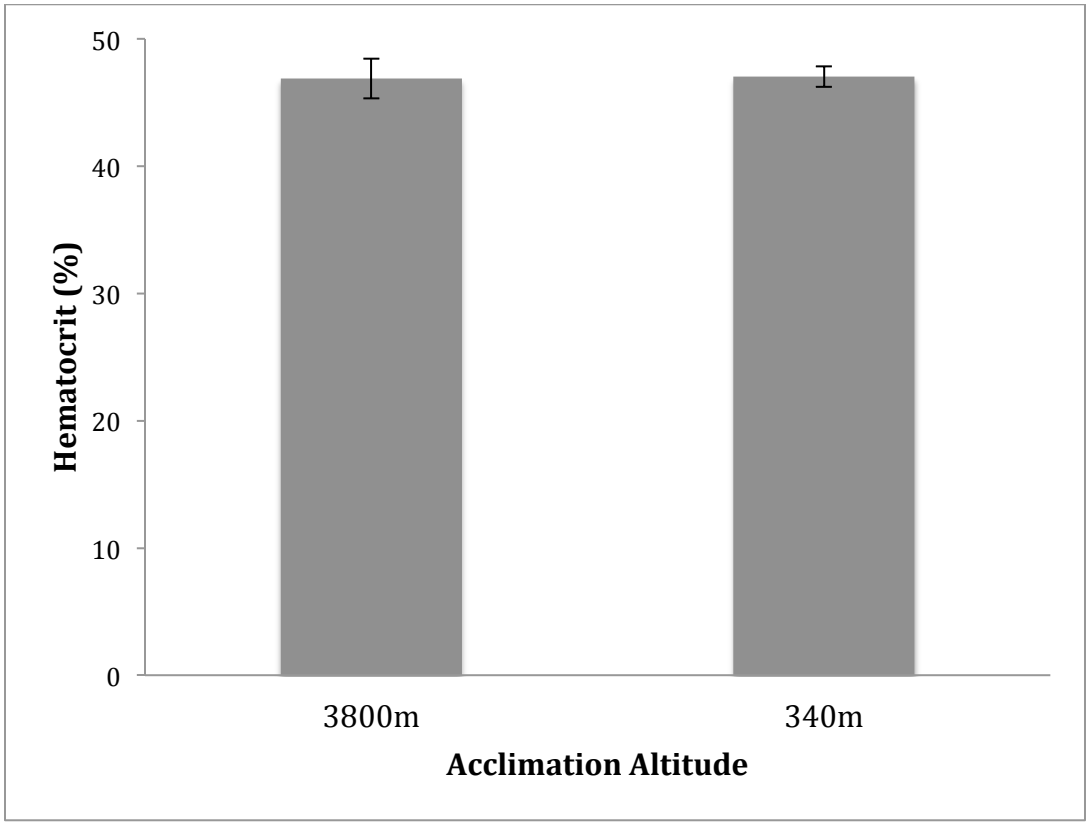

Fig. 1.4. Depicts hematocrit (%) at time of sacrifice in deer mice from two different altitudes. Asterisks indicate statistically significant differences between acclimation groups. Values are means +1 S.E.M. (*N* as in text).

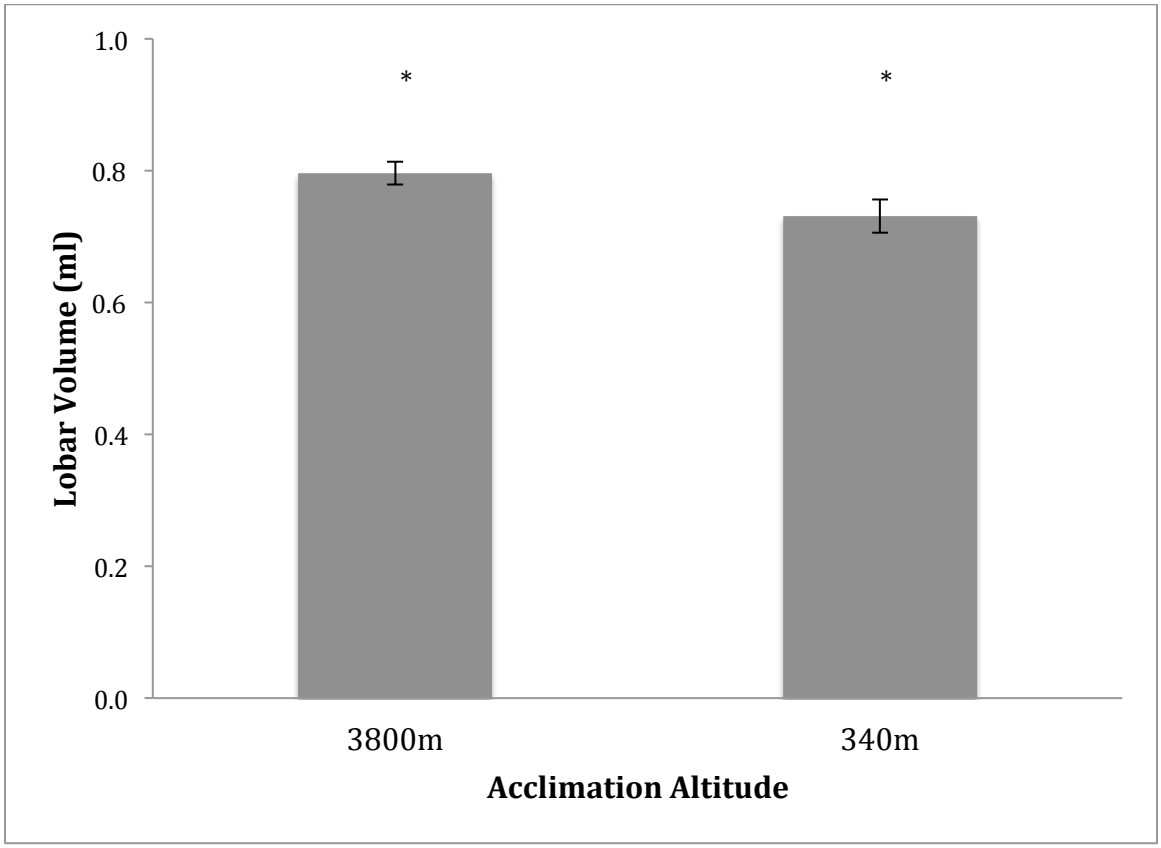

Fig. 1.5. Depicts lobar volume of the lung (ml) in deer mice from two different altitudes. Asterisks indicate statistically significant differences between acclimation groups. Values are mass-corrected means +1 S.E.M. (*N* as in text).

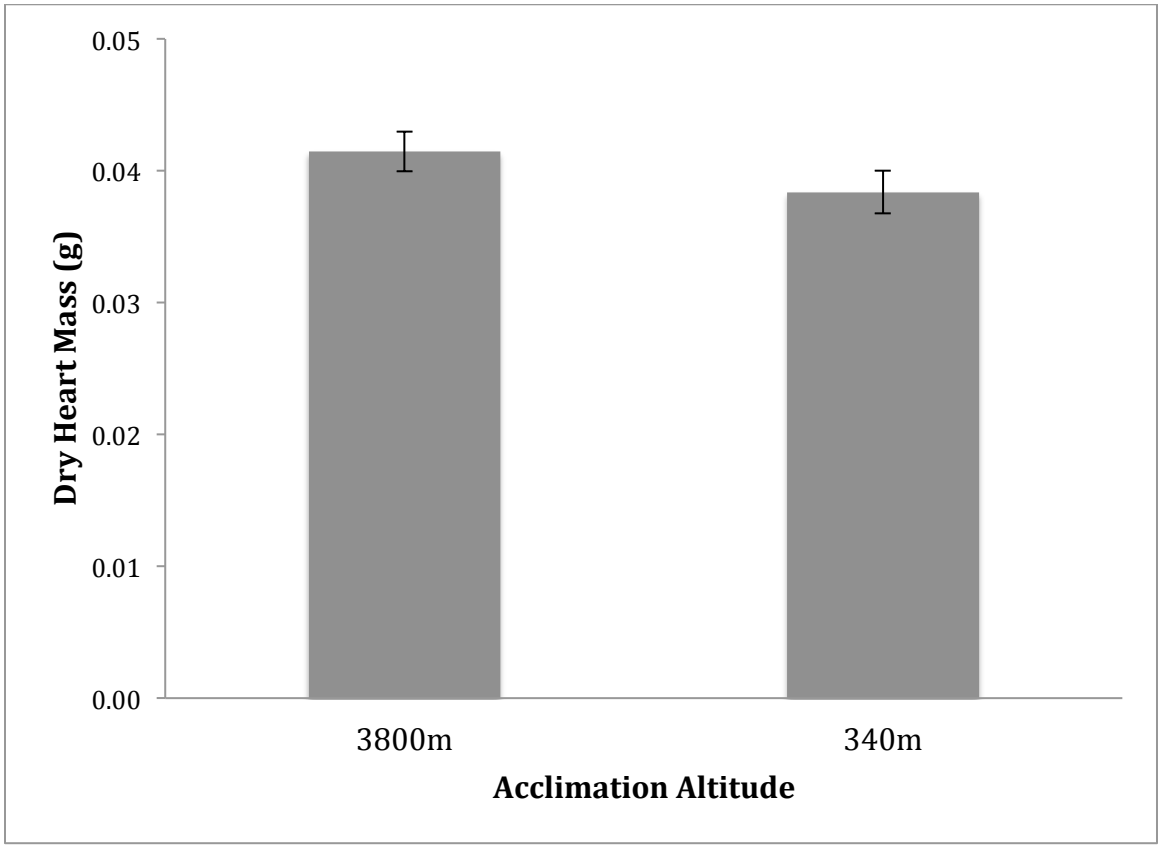

Fig. 1.6. Depicts dry heart mass (g) in deer mice from two different altitudes. Asterisks indicate statistically significant differences between acclimation groups. Values are mass-corrected means +1 S.E.M. (*N* as in text).

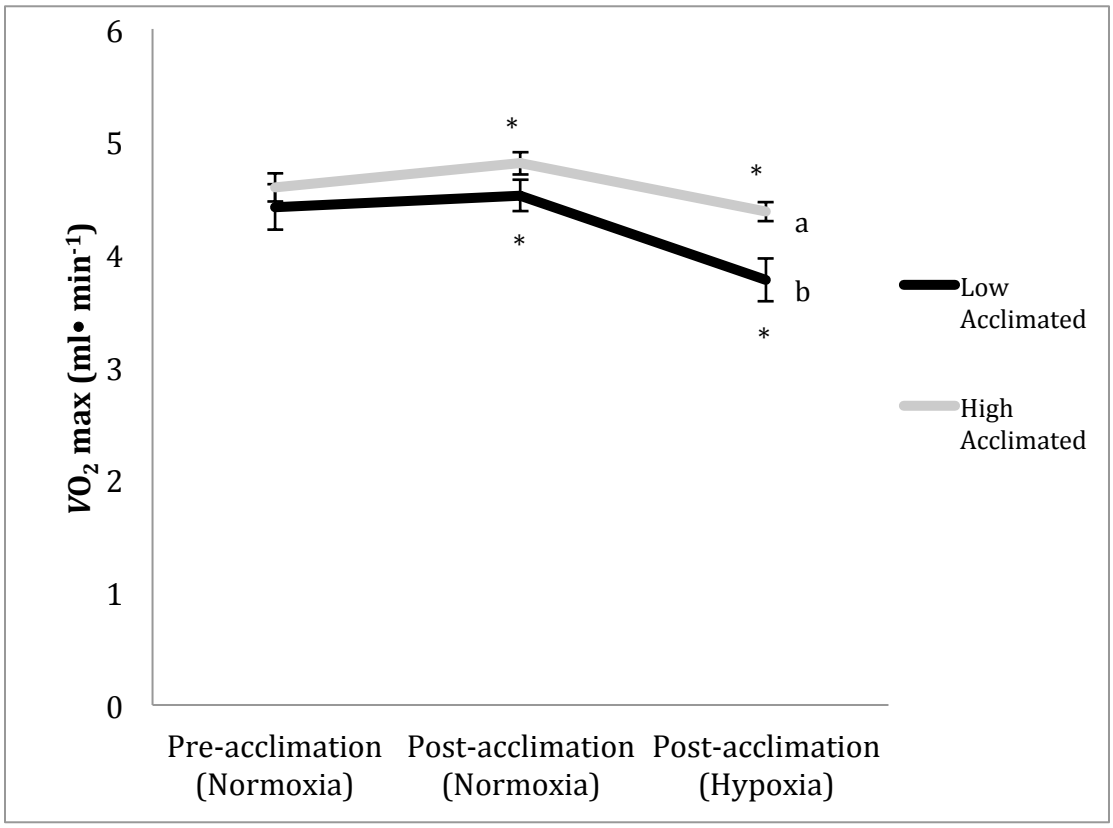

Fig. 1.7. Depicts $\dot{V}O_2$ max (ml/min) in both high acclimated (gray) and low acclimated (black) deer mice from the three $\dot{V}O_2$ max measurements. Letters that are different from each other indicate statistically significant differences between groups, asterisks indicate significant differences within groups. Values are mass-corrected means +1 S.E.M. (*N* as in text).

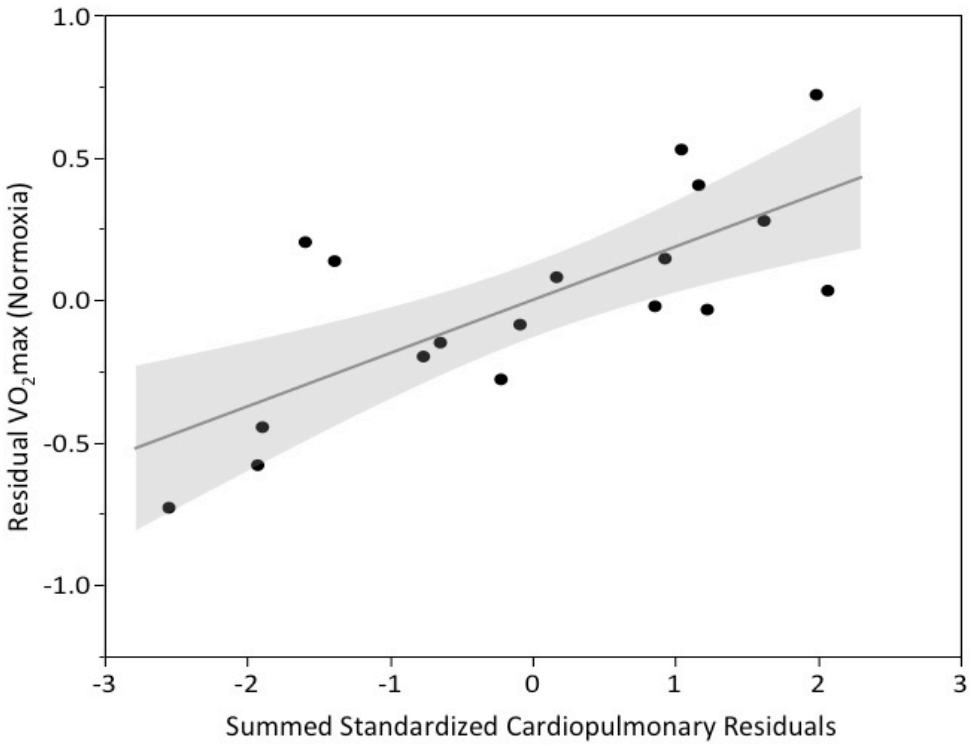

Fig. 1.8. Depicts the regression of $\dot{V}O_2$ max residuals from normoxic conditions regressed against the summed standardized residuals of dry heart mass and lobar volume. Also shown are the line of best fit and the 95% confidence interval of the line.

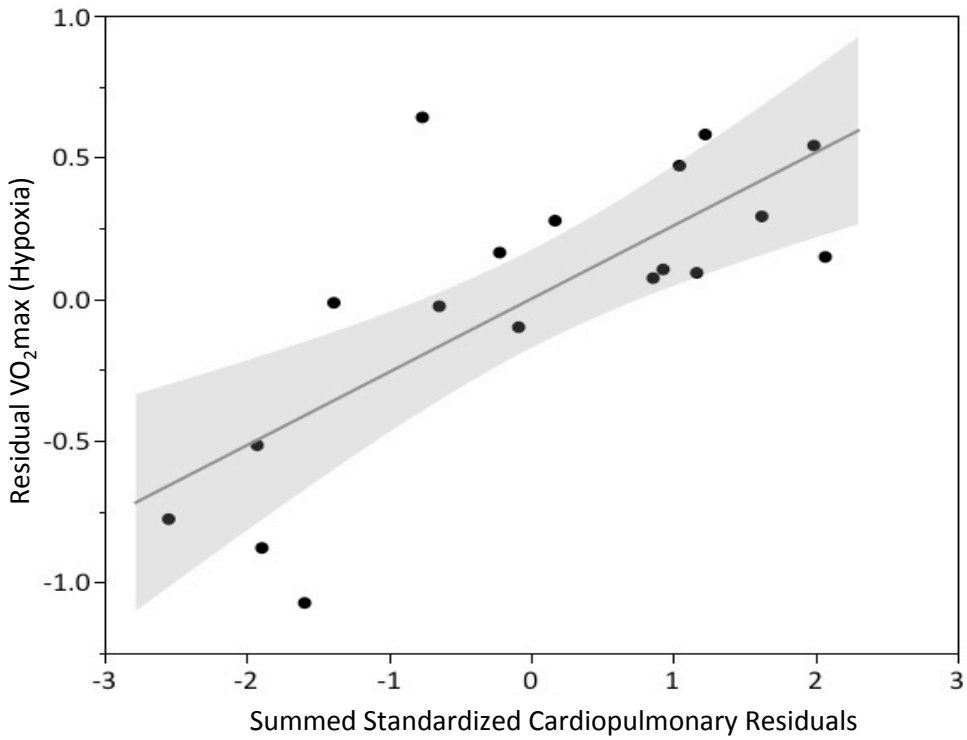

Fig. 1.9. Depicts the regression of $\dot{V}O_2$ max residuals from hypoxic conditions regressed against the summed standardized residuals of dry heart mass and lobar volume. Also shown are the line of best fit and the 95% confidence interval of the line.

Table 1.1	Low acclimated (n=8)		High acclimated (n=10)	
	Mean	±SEM	Mean	±SEM
Body mass (g)				
Pre-acclimation	23.97	0.74	24.44	0.78
Post acclimation	24.01	0.76	23.52	0.60
Hematocrit (%)	47.04	0.83	46.90	1.55
Heart mass (g)				
Wet	0.177	0.005	0.190	0.006
Dry	0.038	0.002	0.042	0.002
Lung volume (ml)				
Whole	0.840*	0.019	0.909*	0.017
Lobar	0.732*	0.025	0.797*	0.018
$\dot{V}O_2$ max (ml•min^{-1})				
Hypoxia	3.77*	0.19	4.38*	0.09
Normoxia	4.52	0.14	4.81	0.10

Values for heart mass, lung volume, and $\dot{V}O_2$ max are mass corrected means.
* Asterisks indicate significant differences between groups

Chapter 2

Phenotypic Plasticity in the Lungs
of Deer Mice Acclimated to High Altitude

Abstract

Hypoxia is known to induce growth in the gas exchange organs of many animals. Previous work has shown that hypoxic exposure during development results in an increased diffusive capacity for oxygen. In this study we acclimated adult deer mice born and reared at low altitude to hypoxic conditions for nine weeks. We predicted that even in adulthood these mice would display increased lung volume and increased alveolar surface area relative to low altitude controls as a result of acclimation. The results showed that adult mice retained the ability to remodel the lungs in spite of somatic maturity. Remodeling was achieved by making the airspaces larger as opposed to de novo alveolarization, but did result in an increase in alveolar surface area. These changes in lung morphology could play an important role in the maintenance of aerobic performance in the face of hypoxia.

Introduction

Aerobic respiration in mammals is dependent on the acquisition of oxygen from the environment, with the movement of oxygen into the bloodstream occurring via diffusion across the alveoli of the lungs. Hypoxia, or reduced oxygen availability, has been shown to stimulate growth in oxygen exchange organs (Burggren and Mwalukoma, 1983; Burri and Weibel, 1971; Lomholt and Johansen, 1979). In a number of mammals, exposure to hypoxia during development causes morphological changes in the lungs resulting in higher diffusive capacity for gas exchange (Burri and Weibel, 1971; Johnson et al., 1985; Hsia et al., 2005; McDonough et al., 2006). However, the ability of the lungs respond to hypoxia after reaching adulthood is less clear. For example rodents can remodel their lungs through hypertrophy of the alveolar spaces after development ends (Cunningham et al., 1979), while adults of other species such as dogs showed no response to hypoxia even after 5 months of exposure (Johnson et al., 1985).

Deer mice (*Peromyscus maniculatus*) are widely distributed throughout North America, and range from below sea level to over 4000m in altitude. Even at the highest altitudes deer mice show resilience to the reduced partial pressures of oxygen. For example, deer mice living at 3800m have higher field metabolic rates and aerobic capacities than low altitude mice during the same time of year despite reduced oxygen availability (Hayes, 1989). Though these mice have genetic polymorphisms in their α-chain hemoglobin that affect aerobic capacity (Snyder et al., 1988; Chappell et al., 1988), phenotypic plasticity also plays an important role in recouping performance lost under

hypoxia (Hammond et al., 2001; Hammond et al., 2002; Chappell et al., 2007; Russell et al., 2008).

Previous work in adult deer mice has shown that high altitude acclimation results in increased lung mass (Hammond et al., 2001). This has led to the hypothesis that deer mice acclimating to high altitude remodel their lungs to maximize the diffusive capacity for oxygen across the lungs. Based on this hypothesis we made the following two predictions:

1. Mice acclimated to high altitude (HA) will have increased volume of the gas exchange regions of the lung relative to low altitude acclimated controls (LA).
2. Mice acclimated to high altitude (HA) will have increased alveolar surface area relative to low altitude acclimated controls (LA).

Materials and Methods

Animals

This study began with 53 mice (33 male, 20 female; *P. maniculatus sonoriensis*), ranging in age from 218 days to 310 days. All were bred at low altitude (340m) in a colony that was originally removed from the wild in 1995. For this study mice were acclimated to one of two altitudes, high (HA; n=23) or low, (LA; n=29) and one of two temperature conditions (cold, warm) for 9 weeks. The high altitude study site was at the University of California's Barcroft Laboratory in the White Mountains (3800 m; Barcroft) and the low altitude study site was at the University of California at Riverside Campus (340 m; UCR).

Animals were housed as individuals or pairs in plastic shoebox cages (27 cm x 21 cm x 14 cm) with aspen shavings for bedding. They were given *ad libitum* food and water, and approximately 1g of cotton for nesting. Cages at Barcroft were placed in one of two conditions, either indoors in a semi-constant temperature room at an average ambient temperature (T_a) of 14.3ºC (range 8ºC to 26ºC; n=12), or in an exterior enclosure that was subject to ambient conditions with an average T_a of 10.9ºC (range -0.6ºC to 40ºC; n=11). At UCR mice were kept at either 20ºC in the vivarium (range 19ºC to 23ºC; n=16), or in an environmental chamber at a constant 5ºC (n=13). The T_a at Barcroft was recorded in 30-minute intervals using an Onset Computer Corporation Stowaway XTI data-logger placed in an empty cage filled with bedding. At UCR T_a were monitored either by staff in the vivarium or the thermostat for the environmental chamber. The lights in the vivarium were set to 14 h:10 h light:dark (L:D) photoperiod to approximate

the natural photoperiod at the high altitude site. At the end of the acclimation period mice were euthanized by overdose of Euthasol (0.7ml IP; Vibrac Animal Health, Fort Worth, TX, USA)

Lung Fixation

All procedures for lung morphological measurements were conducted in accordance with methods described in the official research policy statement published by the American Thoracic Society (Hsia et al. 2010). We used mice whose lungs were fully inflated using gluteraldehyde (as opposed to paraformaldehyde) for morphometric measurements (HA, n = 4 & LA, n = 5). We opened the thoracic cavity along the midline, and inserted a flexible tube into the trachea via an incision between the rings of the tracheal cartilage. We inflated the lungs by instillation of a solution of 2.5% glutaraldehyde in 0.1M cacodylate buffer with a pH of 7.4. Fixation took place at a constant pressure of 25-30 cmH_2O above the sternum, and was maintained for 30 minutes before ligating the tubing with surgical thread. We then removed the lungs and tubing from the body and transferred them into a vial filled with the buffered glutaraldehyde solution for 24 hours at 4ºC. The fixed lungs were washed twice for 10 minutes in 0.1 M cacodylate buffer (pH 7.4) before being placed in vials with the buffer and stored at 4ºC.

Lung volume was measured by immersion displacement directly after removal from the mouse using the method described by Sherle (1970), and again after being separated into lobes (lobar volume; right lung: 4 lobes, left lung: 1 lobe).

Microscopy

We based the selection protocol for sectioning the lungs on a modified version of the systematic uniform random sampling (SURS) procedure (Hsia et al. 2010). Each lobe was serially cut into thick sections (~1mm) using a razor blade. Two sections separated by a distance of 2mm, were selected per lobe. The starting section was chosen at random and determined by a random number generator. This procedure gave us an unbiased sampling of each lobe of the lung.

We prepared lung tissue for microscopy by embedding into plastic resin. Tissue was dehydrated by immersion in an ethyl alcohol series. For light microscopy, the tissue was embedded in glycol methacrylate (JB-4 Embedding Media, Sigma Aldrich, Saint Louis, MO, USA) and sectioned on a motorized Sorvall JB4 microtome using a glass knife. Sections were cut to a thickness of 4 μm, and then stained with 0.01% toluidine blue solution for 5 minutes. We viewed stained sections using a Keyence Biorevo microscope (Itasca, IL, USA) with a motorized stage at three levels of magnification.

Under low magnification light microscopy (40x), multiple images of sections were collected and stitched together using the Keyence software (Itasca, IL, USA) to form a single low magnification image of the entire section. We then superimposed a test grid on the image and used point counting to estimate the volume density of non-parenchyma (bronchi, bronchioles, large blood vessel, cartilage, lymph nodes, etc.) or non-gas exchanging tissue, and the parenchyma (alveoli, alveolar ducts, alveolar septum, capillaries). We calculated volume density of the parenchyma with equation (1):

$$Vv(p, L) = 1 - \frac{P(np)}{P(L)} \quad (1)$$

where P(L) is the number of points falling on lung tissue, and P(np) is the number of points falling on the non-parenchyma (see Fig. 2.1 for representative image).

We determined the volume density of alveolar air space and alveolar septum by point counting of images obtained using higher magnification light microscopy (400x). A series of ten non-overlapping images were obtained for each section, and quantified with the grid system described above. Densities were obtained with equation (2):

$$Vv(p,s) = \frac{P(s)}{P(p)} \qquad (2)$$

where P(s) is the number of points falling on the septum and P(p) is the number of points falling on parenchyma (see Fig. 2.2 for representative image). Volume density of airspace was simply calculated as the remaining proportion of points not falling on the septum.

We estimated the surface density of the alveolus from images obtained at the highest magnification (1000x, using a 100x oil objective). A series of twenty non-overlapping images were taken for each section, and a test grid consisting of test line segments within an unbiased counting frame was superimposed on each image. We used equation (3) to calculate surface density:

$$Sv(a,s) = \frac{2 \cdot I(a)}{\mathrm{K_1} \cdot \mathrm{d} \cdot \mathrm{P(s)}} \quad (3)$$

where I(a) is the number of intersection with the alveolus, K_1 is the length of a single test line, d is the number of test lines in the counting frame, and P(s) is the number of points that fall on the septum (see Fig. 2.3 for representative image).

We performed all image analyses using ImageJ (NIH; http://rsb.info.nih.gov/ij/), and the associated grid plugin (Rasband, 2009; http://rsbweb.nih.gov/ij/plugins/grid.html).

Statistical analysis

Our experimental design began as a 2x2 factorial model with temperature and altitude as independent variables. However, due to the small sample size after lung fixation and because body mass covaried closely with acclimation temperature, we ignored temperature in the final analysis. All results were expressed as means ± SEM. Total lung volume was calculated from the summed volume displacement measurements of individual lobes, and lung volume was normalized to body mass by dividing by the body mass of the individual. Lung morphology parameters were reported both as relative densities (proportion of total lung volume) and as absolute measurements (by accounting for mass corrected lung size). Differences between acclimation groups were evaluated by one way t-tests. An α of 0.05 was used for statistical significance, however all values that approached significance are reported.

Results

Body Mass

We found no differences in body mass between HA (mean 21.81±1.08 g) and LA (mean 21.61±1.49 g) groups at the time of sacrifice ($F_{1,7} = 0.27$, $P = 0.61$).

Lung Morphology

Mice acclimated to high altitude (mean 1.04±0.054 ml) showed a 22.3% increase in lung volume over LA controls (mean 0.85±0.096 ml; $F_{2,6} = 15.77$, $P = 0.0041$) with body mass as a significant covariate. In addition to changes in total lung volume, HA mice also differed in both volume densities of features as well as absolute volume (Table 2.1). In general HA mice had greater volume of gas exchange tissue and increased air space volume relative to LA mice.

Parenchymal tissue volume density, which represents the gas exchange region of the lungs (alveolar ducts and sacs), was higher in HA mice (t_7=2.55 P=0.038) relative to LA mice, and absolute parenchymal volume was 25% higher in HA animals (t_7=4.05 P=0.005) than LA. Additionally, when parenchymal volume was further subdivided into the airspace and septal components, the relative volume density of airspace (air space relative to the individual's parenchymal volume) was higher in HA than in LA mice (t_7=5.05 P=0.0015) while septum volume density (which includes basement membrane, type I and II alveolar cells, and capillaries) was lower in HA mice (t_7=3.23 P=0.014). These findings are consistent with alveolar enlargement in the HA mice. In terms of absolute volume, septa did not differ significantly between altitude groups, but airspace,

in particular alveolar volume (t_7=3.53 P=0.0095), was higher in HA mice. Thus, differences we observed between the HA and LA mice represent a restructuring in the parenchymal components of the lungs rather than growth of additional parenchymal tissue.

Alveolar surface density also differed between groups, with HA mice having 27% higher surface density (t_7=10.66 P=0.00001). The difference in absolute surface area approached statistical significance (t_7=2.37 P=0.055), with the HA mean 22.6% higher than the LA (Table 2.1).

Discussion

Our study demonstrates that high altitude acclimation in adult deer mice elicits an array of morphological changes in the lungs resulting in larger total lung volume and enlarged airspaces, potentially leading to an increased surface area for gas exchange. These results are consistent with previous research that shows hypoxia stimulates growth in gas exchange organ (Burggren and Mwalukoma, 1983; Burri and Weibel, 1971; Lomholt and Johansen, 1979). Unlike the majority of previous work, which focused on morphological changes during development under hypoxic conditions (Burri and Weibel, 1971; Pearson and Pearson, 1976; Lechner and Banchero, 1981; Hsia et al., 2005; Ravikumar et al., 2009; Sekhon and Thurlbeck, 1995), our study demonstrates that plastic changes can occur in fully-developed adult animals. Some previous studies have not found plasticity in the lungs of adult animals in response to hypoxia. For example, adult dogs did not exhibit morphological changes even after an extended time at high altitude (Johnson et al., 1985). Our results of closely match those of Cunningham et al. (1974), which showed that adult rats (9 weeks old) exposed to hypoxia for a period of only 3 weeks had increased alveolar size and increased alveolar surface area relative to controls. This is also consistent with what is known about lung growth resulting from pnuemonectomies. In rodents the removal of a single lobe was sufficient to induce production of genes associate with alveolar growth (Landesberg et al., 2001), while the same genes were not invoked until a 55-58% loss of lung tissue in dogs (Hsia et al., 1994). It appears that the level of stimulus necessary to induce restructuring of the lung is lower in rodents than it is in canines and other large mammals.

High acclimated mice had increased volume density of airspace and a reduction of septal volume density, which is consistent with hypertrophy of existing alveolar spaces rather than the production of de novo alveolarization. Because of the relationship between surface area and volume, any given increase in the radius of the alveolus will result in a greater change in volume than in surface area. Increasing the number of alveoli while keeping the size of the airspaces constant will result in greatly increased surface area. However, adult deer mice apparently rely on alveolar hypertrophy and associated increases in total lung volume to increase diffusive capacity. Surface area is key to the function of the lungs, as diffusion of oxygen into the blood can only occur across the alveolar surface. Diffusive capacity (D_M) can be expressed by equation (5):

$$D_M = K \cdot \frac{(S_A + S_C)}{2 \cdot \tau_{hb}} \qquad (5)$$

where S_A and S_C are alveolar and capillary surface areas, respectively, K is the Krogh permeability coefficient for tissue, and τ_{hb} is the harmonic air-blood barrier thickness.

An increase in diffusive capacity induced by acclimation to high altitude provides a potential explanation for why high acclimated mice had significantly improved performance under hypoxia (Chapter 1) and why mice with greater lung volumes tended to have higher aerobic capacity (Chapter 1). It is also important to note that diffusive capacity is also dependent on pulmonary blood flow (Yilmaz et al., 2007), and that a large surface area for diffusion alone is not sufficient to increase uptake of oxygen. Our results demonstrate that one of the components of total diffusive capacity, alveolar surface area, did significantly increase in HA mice. This indicates a potential for increased total diffusive capacity in HA mice, but future studies should quantify the

values for capillary surface area, harmonic barrier thickness, and pulmonary blood flow in HA and LA mice to fully understand the functional effects of changes in lung morphology on aerobic performance. Regardless, our research demonstrates that mice acclimated to high altitude have increased airspace volume and increased surface area relative to low altitude controls, and that these changes in airspace volume and surface area result from hypertrophy of the airways as opposed to de novo alveolarization. Obviously deer mice maintain the ability to remodel the lungs well into adulthood, something that has not been reported in large mammals (Johnson et al., 1985). This ability may allow deer mice living in zone of transition to move up in elevation without loss of aerobic performance. However, based on the results of this study, adult deer mice exposed to hypoxia do not appear to retain the ability to create novel alveolar tissue, and this remains something that is unique to development under hypoxic conditions, or as a result of extreme loss of gas exchange tissue (Hsia, 2004).

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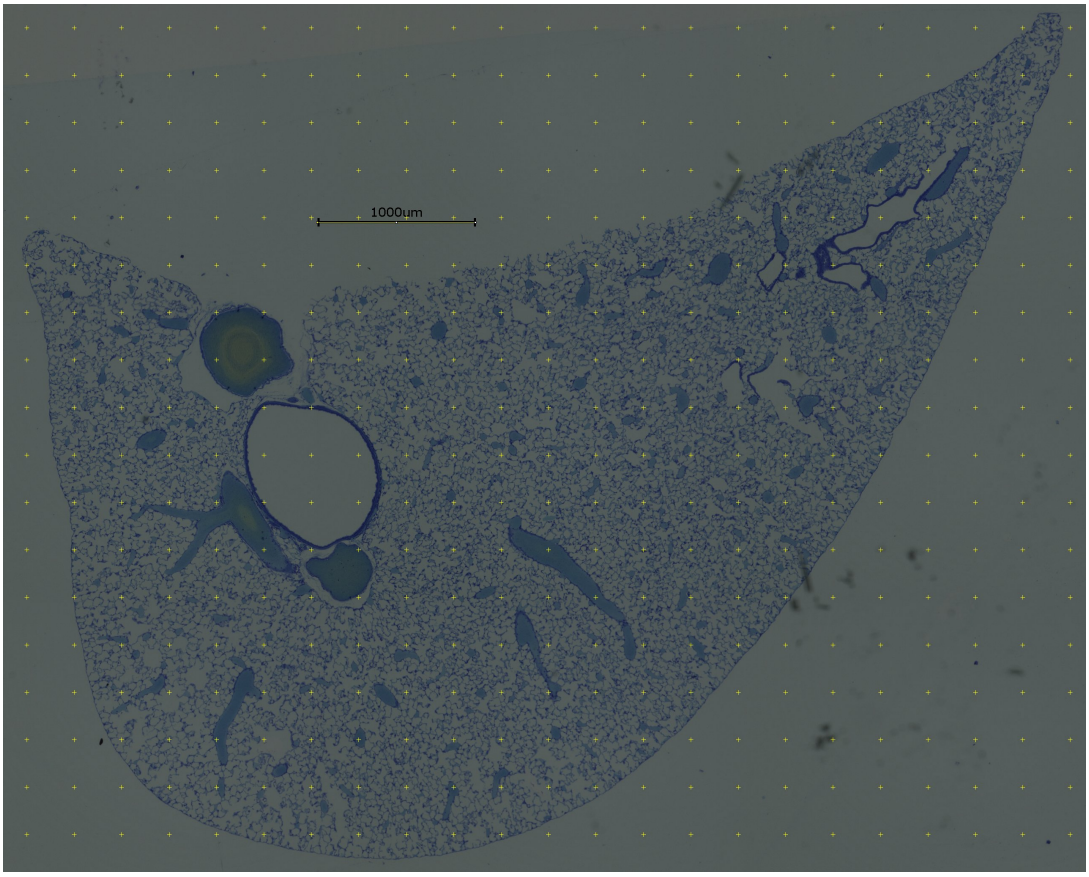

Fig 2.1: A representative section of lung tissue at 40x magnification for quantifying parenchymal tissue volume.

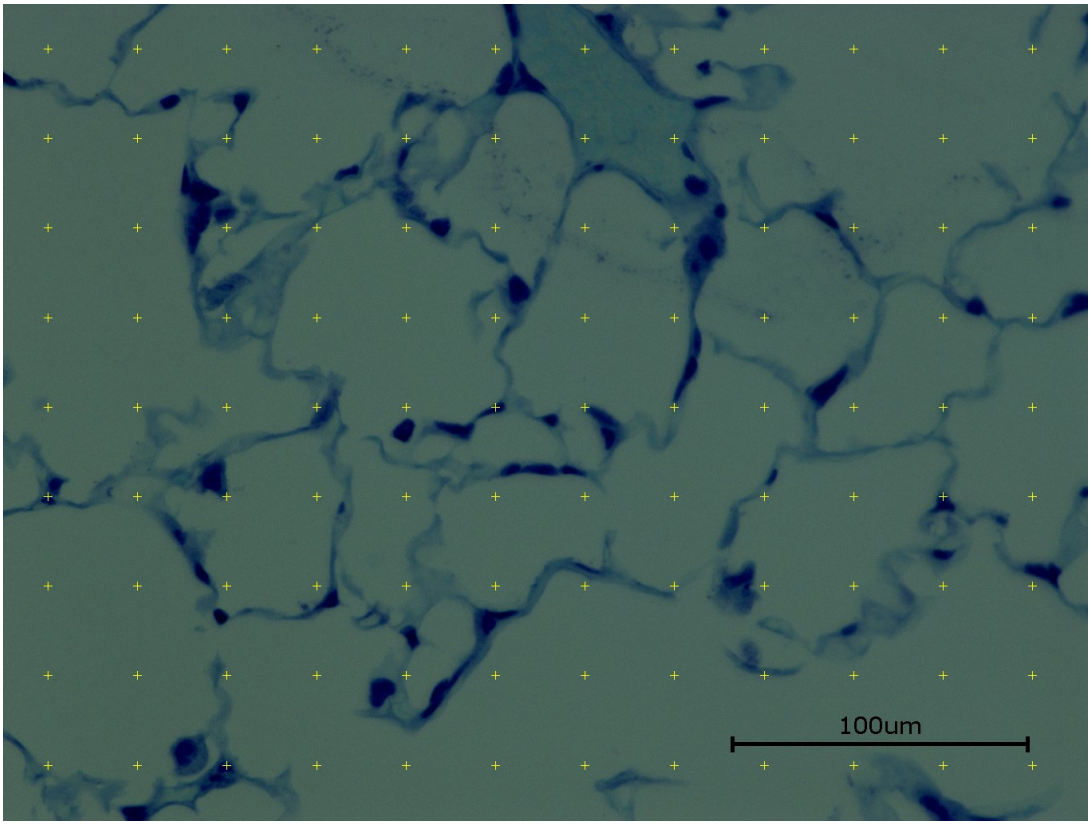

Fig 2.2: A representative section of lung tissue at 400x magnification for quantifying airspace and septal volume.

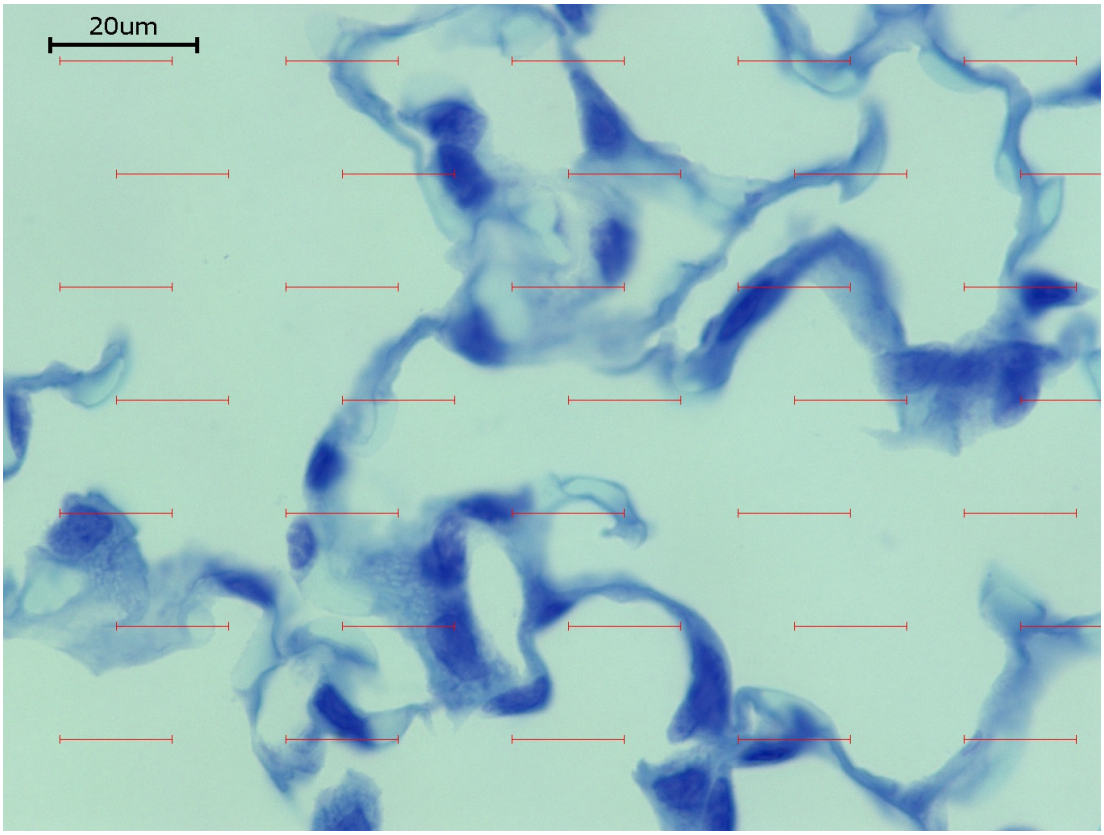

Fig 2.3: A representative section of lung tissue at 1000x magnification for quantifying alveolar surface area.

Table 2.1								
	Low Acclimated		High Acclimated		Low Acclimated		High Acclimated	
	Volume density				Absolute volume			
	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
Parenchyma	0.855*	0.011	0.894*	0.011	0.0340*	0.0057	0.0426*	0.0011
Non-parenchyma	0.145*	0.011	0.106*	0.011	0.0057	0.0002	0.0052	0.0006
Airspace	0.508*	0.014	0.0616*	0.015	0.0196*	0.0009	0.0292*	0.0012
Sacs	0.417	0.017	0.475	0.022	0.0160*	0.0011	0.0226*	0.0016
Ducts	0.091	0.013	0.140	0.030	0.0036	0.0006	0.0066	0.0014
Septum	0.347*	0.006	0.278*	0.023	0.0133	0.0001	0.0131	0.0011
	Surface density				Absolute surface area			
Alveolar Surface	44.62*	0.96	56.51*	0.52	0.603**	0.037	0.739**	0.071

*Asterisk indicates statistical significance $P < 0.05$
** Double asterisk indicates value approaching significance $P = 0.055$

Conclusions

I set out to identify physiological and morphological changes resulting from high altitude acclimation during adulthood, and to see if those changes are associated with differences in whole organism performance at the individual level. The results underline the ability of deer mice (*Peromyscus maniculatus*) to perform at the same aerobic level at high altitude as at low altitudes in spite of a potential 37% reduction (dependent on barometric pressure and vapor pressure) in the alveolar partial pressure of oxygen. They also show that aerobic performance at high altitude is dependent on lung volume when heart size is taken into account. Lastly, this work shows that hypoxia induces significant remodeling of the lungs of adult mice, which may have an impact on diffusive capacity of the lungs, and likely influences maximal aerobic performance.

While previous work on deer mice showed increases in lung mass in response to high altitude acclimation (Hammond et al., 1999; Hammond et al., 2001), this thesis is the first work to show that those mass changes are related to an increased in the volume of the region of the lung associated with gas exchange. We also showed that surface area of the alveoli is increased in mice exposed to hypoxic conditions. Such changes in the structure of the lungs may affect the overall diffusive capacity of the lung, resulting in an increased capacity for gas exchange.

We hypothesized that these changes in the structure of the lung were an "adaptive," in the physiological sense, response to hypoxia, and would result in deer mice at high altitude being able to maintain aerobic performance in spite of pO_2 changes. Indeed we showed that some of the variation in the residuals of $\dot{V}O_2$ max could be explained by residuals of lung volume. However, the significance of lung as a predictor

of aerobic performance was dependent on heart mass residuals. This was an interesting finding as it suggested that increased lung volume was only beneficial to performance when it was associated with a relatively large heart. This is potentially explained by the fact that diffusion of oxygen into the body is dependent not only on the surface area of the lungs, but also the ability of the heart to move red blood cells and therefore hemoglobin through alveolar capillaries of the lungs at a sufficient rate. If blood is not being moved through the lungs quickly enough the hemoglobin will become saturated, and prevent further binding of oxygen. Therefore, simply having a large lungs is not sufficient by itself to maintain aerobic performance at high altitudes.

One interesting point is that we observed a 22% change in lung volume between HA and LA groups in Chapter 2, while a acclimation protocol (Chapter 1) showed a more modest 8% difference in lung volume. This disparity does not seem to have a simple explanation. It could be a result of the small sample size used in Chapter 2. Inclusion of paraformaldehyde fixed lungs, not used in the analyses in Chapter 2, into the average results in a 10% difference, similar to that seen in Chapter 1. However, paraformaldehyde fixation may have been insufficient to maintain the lung tissue fully inflated. Likewise the discrepancy could be due to processing tissue at multiple sites in Chapter 2, as opposed to the uniform processing conducted in Chapter 1. It could also be a result of rapid deacclimation in mice brought to low altitude for processing in Chapter 1, though this seems unlikely. Regardless of the cause of the difference, it should be further explored.

This thesis outlines the importance of phenotypic plasticity in the lungs in relation to high altitude hypoxia. However, more work is needed to 1) identify the generality of this response among other taxa, 2) further explore the specific contribution of changes in lung morphology to aerobic performance, and 3) investigate the importance of changes in lung morphology relative to other plastic changes that take place in response to hypoxia. I believe that the ultimate goal of this field should be to clarify how phenotypic plasticity evolves in a complex system, like the oxygen cascade. The importance of this work is being seen now more than ever. With anthropogenic disturbances changing ecosystems around the world in such a rapid manner the species that survive may be determined more by the capacity for phenotypic plasticity rather than the ability of populations to evolve. By understanding the capacities of various taxa for phenotypic plasticity, we may better understand which taxa will be better equipped to weather the changes to come.

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