THE ROLE OF LUNG STRUCTURE AND FUNCTION IN ADAPTATION TO HIGH ALTITUDE IN DEER MICE *(PEROMYSCUS MANICULATUS)*

THE ROLE OF LUNG STRUCTURE AND FUNCTION IN ADAPTATION TO HIGH ALTITUDE IN DEER MICE *(PEROMYSCUS MANICULATUS)*

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LAY ABSTRACT

High altitude is a harsh environment that is characterized by low levels of oxygen and cold temperature, making it a challenging place to live. Regardless, many highland species of animals, including humans, have been living at high altitude for centuries; however, lowland species tend to face a host of detrimental side effects from high altitude exposure. My thesis focuses on the ways that the structure and function of lungs may differ in highland natives who are adapted to the high-altitude environment compared to lowland natives. To address this question, I used high- and low-altitude native North American deer mice, as they are broadly distributed across North America and they have a larger altitudinal range (0-4300 m above sea level). My thesis contributes to our understanding of how highland animals have adapted to high-altitude environments.

ABSTRACT

High altitude environments present two main stressors: 1) low levels of oxygen and 2) low temperatures. For small endotherms, this is especially challenging, as they have to generate heat to maintain their body temperature. Lowland animals that travel to high altitude are often faced with maladaptive responses to hypoxia (*e.g.*, hypoxic pulmonary hypertension), while the same responses are not seen in highland natives. Using North American deer mice *(Peromyscus maniculatus)*, we investigated whether highlanders and lowlanders had different responses to chronic hypoxia exposure (12 kPa O_2, simulating the O_2 pressure at 4300 m for 6-8 weeks). In response to chronic hypoxia, lowlanders show hypoxic pulmonary hypertension (HPH; increases in right ventricle systolic pressure), thickening of pulmonary arterial smooth muscle, and right ventricle hypertrophy. In contrast, highlanders showed attenuated HPH and no associated changes in pulmonary arterial structure or right ventricle hypertrophy. Both highlanders and lowlanders were able to maintain V-Q matching in chronic hypoxia, but highlanders show increased *in vivo* lung volume after chronic hypoxia. Overall, evolved changes in lung function help attenuate maladaptive plasticity and contribute to hypoxia tolerance in high-altitude deer mice.

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LIST OF ABBREVIATIONS AND SYMBOLS

α SMA: α - Smooth muscle actin

ANOVA: Analysis of variance

CO: Carbon monoxide

CT: Computed tomography

Epas1: Endothelial PAS domain-containing protein 1

FVC: Forced vital capacity

Hb: Haemoglobin

HIF: Hypoxia inducible factor

HPH: Hypoxic pulmonary hypertension

HU: Hounsfield units

kPA: Kilopascals

kVp: Peak kilovoltage

mA: Milliampere

MBq: Megabecquerels

MI: Mutual information

MIGET: Multiple inert gas technique

NGS: Normal goat serum

NO: Nitric oxide

O_2: Oxygen

PBS: Phosphate buffered saline

PO_2: Partial pressure of oxygen

Q: Perfusion

RV: Right ventricle

RVSP: Right ventricular systolic pressure

SEM: Standard error of the mean

SPECT: Single-photon emission computed tomography

VO_2: Oxygen consumption rate

VO_{2max}: Maximal oxygen consumption rate

V: Ventilation

Q: Perfusion

^{99m}Tc: Technetium

^{99m}Tc-MAA: ^{99m}Tc macro aggregated albumin

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis is organized in sandwich format, as recommended and approved by members of my supervisory committee and approved by McMaster University. It consists of three chapters. Chapter 1 is an overview of background material and hypotheses tested. Chapter 2 is a manuscript prepared for submission to the Journal of Applied Physiology. Chapter 3 is an overview of the major findings of this thesis, how these findings relate to current knowledge, and suggestions of future directions of research.

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: PULMONARY HYPERTENSION IS ATTENUATED AND VENTILATION-PERFUSION MATCHING IS MAINTAINED DURING CHRONIC HYPOXIA IN DEER MICE NATIVE TO HIGH ALTITUDE

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CHAPTER 3: GENERAL DISCUSSION

CHAPTER 1: GENERAL INTRODUCTION

Extreme environments provide an excellent opportunity to investigate how physiological systems work. Animals native to these environments are able to survive and thrive in conditions that are intolerable to most others. Understanding the physiological basis for such feats can provide valuable insights into how physiological systems can be pushed to their limits, be adjusted to overcome pathology, and evolve across animals.

One such extreme environment is high altitude, where animals are faced with the compounding stressors of a reduced partial pressure of oxygen (*i.e.*, hypoxia) and cold temperatures. These two factors are physiologically stressful because cold temperatures stimulate the metabolic demand for oxygen (O_2) to support heat production (shivering and non-shivering thermogenesis) in an oxygen-limited environment (13). Maintaining body temperature through shivering and non-shivering thermogenesis depends on aerobic performance; however, hypoxia can reduce aerobic performance by constraining O_2 delivery to tissues. Furthermore, demands for thermogenesis are greater at high compared to low altitude (13, 14), so high-altitude animals must find a way to address this potential imbalance between O_2 supply and O_2 demand. The mammalian oxygen cascade supports the passage of O_2 from the external environment to the tissues, and is comprised of ventilation, pulmonary oxygen diffusion, circulation of O_2 in the blood, and tissue diffusion. Changes along this oxygen cascade (*i.e.* phenotypic plasticity and/or genetic adaptations) could foreseeably increase the capacity to transport O_2, allowing animals to cope with the challenges of living at high altitude. However, some forms of phenotypic plasticity in response to chronic hypoxia are maladaptive, so high-altitude animals could

also attenuate such maladaptive plasticity to improve health and fitness. Here, I will focus on these issues in the context of lung physiology, first discussing potential beneficial aspects of lung function in high-altitude natives, and then discussing maladaptive plasticity in lung function.

1.1 Adaptive changes in lung structure in high-altitude natives

O_2 uptake could be improved at high altitude by increases in the morphological O_2 diffusing capacity of the lungs (such as by increasing the volume, alveolar surface density, or capillary volume density of the lungs). Early observations noted that highland native human populations appeared to have large chests relative to their overall body weight (19), suggesting that something about the lungs of highlanders might be unique compared to lowlanders. Highland native human populations (born and raised ~4338m elevation) have larger lung volumes (as measured by forced vital capacity, FVC) compared to lowland-native human populations (born and raised ~ sea level) (4). Increases in lung size in response to chronic hypoxia has also been seen in acclimatized lowlanders. For example, rats (either 3- or 9-weeks old) were subjected to simulated hypoxia (12-13% O_2, equivalent to ~4300 m elevation, for 3 or 6 weeks), and regardless of the age or duration of hypoxia exposure, all animals exposed to hypoxia showed an increase in mass-corrected lung weight and total lung capacity (7). Similarly, developing guinea pigs (3 weeks old) raised at high altitude (3800 m elevation) show an increase in absolute lung volume after one month of hypoxia exposure (18). The same trend was seen in a separate study where weanling guinea pigs were exposed to 2-14 weeks of simulated

hypoxia (PO_2 = 80 Torr, equivalent to ~ 5800 m elevation) (20). After 3 weeks of exposure, mass-specific lung volume was significantly larger in guinea pigs exposed to hypoxia compared to sea level controls (20). This trend has also been observed in highland deer mice (9-11 weeks old) that have been maintained at sea level for 18-20 generations, where highland deer mice had larger lungs after 8-week acclimatization to 3800m elevation compared to highland mice acclimatized to 252 m elevation (8).

Evolved changes in lung microstructure may also contribute to improving O_2 diffusion in the lung. When comparing highland and lowland populations of leaf eared mice (*Phyllotis darwini)* sampled in their native environments, highland mice had a higher ratio of surface area of the alveolar epithelium and volume of the alveolar space compared to lowlanders, suggesting that highlanders have larger lungs that contained more, smaller respiratory units (22). Studies also show that raising animals in hypoxia results in enhanced alveolar growth and remodeling compared to animals raised in normoxia. In developing rats (1 month old) exposed to simulated high altitude (equivalent to ~4200 m elevation) for 20-21 days, alveolar surface area was significantly increased compared to normoxia-exposed controls (1). Bartlett and Remmers (1971) also found that mean alveolar diameter was not significantly different in hypoxia-exposed rats compared to normoxia-exposed rats, indicating that the increase in alveolar surface area was likely due to formation of new alveoli, rather than an increase in size of existing alveoli. Similarly, another study on developing rats (23 days old; one group acclimatized to high altitude, 3450m elevation, and another acclimatized to sea level) showed that mass-specific alveolar and capillary surface area were significantly higher in rats acclimatized

to high altitude (5). These trends were echoed in developing guinea pigs (~3 weeks old) acclimatized to high altitude (3800 m elevation), where alveolar surface area and capillary blood volume were elevated compared to sea level controls after one month of exposure to high altitude (18). Notably, many of these previous studies involved hypoxia exposure in developing animals, thus it is unclear whether hypoxia exposure in adult animals would result in the same observations.

Many previous studies addressing the effect of chronic hypoxia on lung structure often involve highlanders measured at high-altitude or developing lowlanders acclimatized to high altitude, making it difficult to discern whether previous observations are the result of developmental hypoxia or genetically based differences. Comparing lowland animals (*e.g.*, sheep) to highland animals (*e.g.*, llamas) provide indication of genetic influences in traits, but it is confounded by the fact that these two species are distantly related. In order to disentangle the contributions of genetics, phenotypic plasticity, and developmental plasticity, we must evaluate traits in two populations of the same species raised in normoxia and tested in hypoxia.

1.2 Maladaptive responses in the pulmonary vasculature

Although many forms of phenotypic plasticity can be adaptive (*i.e.*, beneficial for fitness by improving survival and reproductive success), some forms of plasticity can be maladaptive (*i.e.*, detrimental for fitness) (30). Maladaptive plasticity is believed to arise when plasticity that is adaptive in the historical context of a species (*e.g.*, physiological adjustments to low O_2 in tissues of low-altitude taxa) acts in a novel environment (*e.g.*, environmental hypoxia at high altitude) where it elicits an off-target response. By

comparing the degree of plasticity in high- and low-altitude native animals, we can begin to understand how high-altitude taxa have altered maladaptive plasticity to chronic hypoxia.

In mammals, pulmonary arteries dilate in response to high O_2 levels (and constrict in response to low O_2 levels) in order to direct blood flow to well-ventilated areas of the lungs. At sea level, an animal may encounter unequal ventilation (V) and/or perfusion (Q) as a result of disease (*e.g.*, changes in posture, regional inflammation, airway obstruction, etc.), resulting in regional vasoconstriction within the lungs to direct blood flow to well-ventilated areas of the lung and maintain V-Q matching (2). This response to hypoxia is intrinsic to the pulmonary vasculature and occurs in the absence of neural and/or hormonal influences (3). However, at high altitude, all of the ventilated air is hypoxic resulting in widespread vasoconstriction throughout the lung (23, 27). After prolonged exposure to hypoxia, the global vasoconstriction throughout the lung results in remodeling of the pulmonary vasculature such that the smooth muscle in the arterial walls thicken and makes the vessels less distensible over time (9, 25). This maladaptive response to hypoxia results in hypoxic pulmonary hypertension (HPH), an increase in pressure within the pulmonary arterial circulation and the right ventricle. Presence of thick smooth muscle in the small pulmonary arterioles is indicative of the vascular remodeling associated with HPH, as smooth muscle in these arterioles is relatively thin under normal conditions (25). Right ventricle hypertrophy is another consequence of HPH, and severe HPH can lead to right heart failure, so the ability to resist this maladaptive plasticity might be essential to life at high altitude.

Some evidence suggests that high-altitude natives attenuate HPH. Tibetan humans, who live above 3658m in elevation, demonstrate resting pulmonary arterial pressure comparable to sea-level values, demonstrating reduced hypoxic pulmonary vasoconstriction and thus reduced HPH (12). Similarly, pikas (*Ochotona curzonia)* native to 4300m elevation show no significant increase in pulmonary arterial pressure across hypoxia levels equivalent to 5000 m elevation and no increase in the ratio of right ventricle to left ventricle and septum, suggesting that pikas have adapted to high altitude by blunting hypoxic pulmonary vasoconstriction (11). The presence of pulmonary hypertension and right heart failure in cattle is termed high mountain or brisket disease, and by comparing offspring of animals with a history of brisket disease, researchers sought to determine whether susceptibility to brisket disease was genetically determined (33). Offspring of cattle that recovered from brisket disease (*i.e.*, susceptible calves) demonstrated progressive brisket disease, whereas calves of cattle that had not previously developed brisket disease (*i.e.*, resistant calves) never did, thus supporting the idea that there is a genetic component to HPH susceptibility (33).

As mentioned above, presence of thickened pulmonary arterial smooth muscle is indicative of HPH, so an absence of thickened pulmonary arterial smooth muscle may suggest an attenuation of HPH. Some highland taxa have thinner pulmonary arterial smooth muscle in compared to lowland animals that were acclimatized to high altitude. For example, llamas born and raised above 4330 m elevation had significantly thinner pulmonary arterial walls compared to a variety of lowland-native animals living at high altitude (*e.g.*, cat, dog, bull, cow), suggesting that the thinner pulmonary arterial walls are

the result of adaptation to high-altitude in llamas (16). Mountain vizcacha (*Lagidium peruanum*), indigenous to the Peruvian Andes (4200 m above sea level), have thin-walled pulmonary arteries, indicating that these animals may have lost the hypoxic vasoconstriction response (17). Similarly, Tibetan snow pigs (*Marmota himalayana)* show pulmonary arterial pressures comparable to mammals at sea level, and they also have thin pulmonary arterial walls, further supporting the idea that highland taxa are adapted to their environment by attenuating hypoxic pulmonary vasoconstriction and thus HPH (26).

It is possible that remodeling of pulmonary arterioles in chronic hypoxia, or the potential evolved changes to attenuate remodeling in high-altitude natives, could prevent effective V-Q matching within the lungs. Effective V-Q matching is essential for pulmonary O_2 extraction and should be particularly important in an oxygen-limited environment like high altitude. Research in humans has used the multiple inert gas elimination technique (MIGET) to investigate V-Q mismatch during exercise and hypoxia exposure. In adult men acutely exposed to a range of simulated altitudes (comparable to ~0, 1500, 3000, and 4500 m elevation), the dispersion of ventilation and blood flow did not change across levels of hypoxia at rest (10). However, when hypoxia and exercise were combined blood flow dispersion increased while ventilation remained unchanged (10). A similar study found that there is no effect of simulated high altitude alone on V-Q mismatch, but that the combination of altitude and exercise results in increasing mismatch with increasing severity (of both hypoxia and exercise), and that this V-Q mismatch is driven by increased perfusion dispersion, suggesting a vascular

mechanism involved in increasing V-Q mismatch during exercise in hypoxia (31). In both of the above-mentioned studies, the V-Q mismatch observed during acute bouts of exercise in simulated high altitude was resolved by breathing 100% O_2, suggesting that hypoxic pulmonary vasoconstriction may be involved and that these effects are reversible during acute challenges. During slow decompression to simulate a climb to the summit of Mount Everest (hypoxia equivalent to 0 to 9150 m elevation over 40 days), researchers found that there was a correlation between blood dispersion (contributing to V-Q mismatch) and mean pulmonary arterial pressure, suggesting a more clear link between HPH and V-Q mismatch (32). Additionally, Wagner and colleagues (1987) found that the increase in V-Q mismatch with increasingly severe exercise and hypoxia was not resolved by breathing 100% O_2, demonstrating pulmonary vascular remodeling that is associated with chronic hypoxia exposure.

Based on this work in humans, researchers have suggested that interstitial and perivascular pulmonary edema that relates to the high pulmonary arterial pressures caused by hypoxia may explain the V-Q mismatch seen during exercise at high altitude (32). Otherwise, there have been very few studies of the effects of chronic hypoxia on V-Q matching in the lungs, particularly in small mammals, in which gravity plays a much smaller role in V-Q distribution (6) and mass-specific resting metabolic rates are proportionately higher (24) compared to humans. Furthermore, it is largely unknown whether the effects of chronic hypoxia on V-Q matching differ in highlanders compared to lowlanders.

1.3 Deer mice (Peromyscus maniculatus)

Deer mice are a powerful model for studying naturally evolved solutions to hypoxia as they are well distributed across North America, and also have the largest altitudinal range of any North American mammal (0-4300 m above sea level; Hayes and O'Connor, 1999). Populations from high- and low-altitude ancestry can be born, raised, and studied in captivity, allowing for controlled environmental conditions and detailed physiological analyses to be conducted. Field metabolic rates are greater in high-altitude populations compared to those at low altitude (13, 14), and that increased thermogenic capacity can be under strong directional selection in high-altitude deer mice (15). High-altitude populations have responded to this selective pressure with evolved increases in VO_2max in chronic hypoxia, which is associated with increases in the lung's ability to extract O_2 from air along with other changes across the O_2 cascade (21, 28, 29). Whether these differences in pulmonary O_2 extraction are associated with evolved changes in lung function is unknown. My thesis sought to shed light on this issue.

1.4 Aims and Objectives

The **purpose** of my thesis was to investigate maladaptive plasticity to chronic hypoxia and determine the contribution of lung structure and function to high-altitude adaptation in North American deer mice (*Peromyscus maniculatus)*. I used a common garden experimental design with first generation deer mice of either high- or low-altitude ancestry to test the **overarching hypothesis** that high-altitude deer mice have evolved

altered responses to chronic hypoxia in pulmonary function that are more favourable for maintaining O_2 uptake at high altitude as compared to low-altitude deer mice.

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CHAPTER 2

PULMONARY HYPERTENSION IS ATTENUATED AND VENTILATION-PERFUSION MATCHING IS MAINTAINED DURING CHRONIC HYPOXIA IN DEER MICE NATIVE TO HIGH ALTITUDE

2.1 ABSTRACT

Hypoxia at high altitude can constrain metabolism and performance and can stimulate physiological adjustments that are maladaptive to health and fitness. Hypoxic pulmonary hypertension is a particularly serious and maladaptive response to chronic hypoxia, which results from vasoconstriction and pathological remodeling of pulmonary arteries and can lead to pulmonary edema and right ventricle hypertrophy. We investigated whether deer mice (*Peromyscus maniculatus)* native to high altitude have attenuated this maladaptive response to chronic hypoxia, and whether evolved or hypoxia-induced changes in lung function might impact ventilation-perfusion (V-Q) matching in chronic hypoxia. Deer mouse populations from both high and low altitudes were born and raised to adulthood in captivity, and various aspects of lung function were measured before and after exposure to chronic hypoxia (12 kPa O_2, simulating the O_2 pressure at 4300 m) for 6-8 weeks. In lowlanders, chronic hypoxia induced pulmonary hypertension (increases in right ventricle systolic pressure), in association with thickening of smooth muscle in pulmonary arteries and right ventricle hypertrophy. In highlanders, hypoxic pulmonary hypertension was attenuated, and chronic hypoxia had no effect on smooth muscle thickness or right ventricle mass. There was no evidence that chronic hypoxia impaired V-Q matching, as reflected by regional V/Q distributions measured at

rest using SPECT-CT imaging. However, *in vivo* lung volume (measured by CT) increased in highlanders (but not lowlanders) after chronic hypoxia. Therefore, evolved changes in lung function help attenuate maladaptive plasticity and contribute to hypoxia tolerance in high-altitude deer mice.

2.2 INTRODUCTION

Hypoxic pulmonary hypertension (HPH) is a maladaptive response to chronic hypoxia that can arise at high altitude and in a range of pathological conditions (*e.g.,* chronic obstructive pulmonary disease, pulmonary fibrosis, sleep-disordered breathing) (46, 63). At high altitude, for example, HPH contributes to disease in humans (*e.g.,* mountain sickness), in other mammals (*e.g.,* brisket disease in cattle), and in birds (*e.g.,* ascites syndrome in poultry) (15, 41, 49, 72). In mammals, a key early step in the progression of HPH is hypoxic pulmonary vasoconstriction, whereby arterioles in the lungs constrict in response to local hypoxia. This mechanism is important at birth for a successful transition to air breathing and it is likely important for regional matching of ventilation (V) and perfusion (Q) in later life, but in high-altitude hypoxia it leads to widespread vasoconstriction of pulmonary arterioles (63). It is eventually associated with vascular remodeling that thickens arterial smooth muscle, makes pulmonary vessels less distensible, and increases pulmonary arterial blood pressure (59, 62, 63). HPH increases load on the right ventricle (RV) of the heart, often resulting in RV hypertrophy, and in severe cases can lead to pulmonary edema, right heart failure, and death (74).

The pulmonary vascular remodeling that occurs as a result of chronic hypoxia exposure might be expected to alter regional V-Q matching in the lungs by altering

pulmonary vascular function. Research in human subjects using the multiple inert gas emission technique (MIGET) suggests that this might be the case (17, 45, 70, 71). Dispersion of the pulmonary blood flow distribution during exercise increased in short-term exposure to hypoxia in a hypobaric chamber, reflecting increased V-Q mismatch (17) that contributed to increasing the alveolar-arterial PO_2 difference (67). This has been confirmed in subsequent studies, which also found that indices of V-Q mismatch were strongly correlated with elevated pulmonary arterial pressures (along with ventilation) (70). Furthermore, subjects with a history of high-altitude pulmonary edema have greater V-Q mismatch and pulmonary arterial pressures during exercise (45), all of which suggests that there may be a relationship between pulmonary hypertension, extravascular fluid accumulation, and V-Q mismatch. Increases in V-Q mismatch and pulmonary arterial pressure during exercise were also observed after longer exposures to hypobaric hypoxia during Operation Everest II, and in this particular study, V-Q mismatch also increased under resting conditions in severe hypoxia (71). Otherwise, there have been very few studies of the effects of chronic hypoxia on V-Q matching in the lungs, particularly in small mammals. The effects of hypoxia on V-Q matching in humans may not accurately reflect the effects in small mammals, in which gravity plays a much smaller role in V-Q distribution (10) and mass-specific resting metabolic rates are proportionately higher (51).

High-altitude natives have often evolved a greater ability to cope with chronic hypoxia than their low-altitude counterparts and present a compelling system for examining how HPH and V-Q mismatch might be overcome. Several high-altitude taxa,

including human populations and mammalian species that are endemic to high altitude (*e.g.*, llama, pika), do not exhibit pulmonary hypertension when studied in their native high-altitude environment (18, 21, 22, 48). Some other high-altitude taxa (*e.g.*, yak, mountain vizcacha, Tibetan snow pigs, etc.) have thinner smooth muscle in pulmonary arteries as compared to low-altitude animals that were acclimatized to high altitude (25–27, 61, 73), along with other changes in lung volume and/or morphology that augment O_2 diffusing capacity (6, 7, 35, 53). However, in many cases, prior studies have examined high-altitude taxa in their native environment, so it has been difficult to distinguish whether plastic responses to developmental hypoxia or genetically based differences between taxa are responsible for previous observations. These possibilities have been distinguished in some previous studies that made controlled comparisons between high- versus low-altitude natives in both normoxia and hypoxia, particularly in comparisons between llama and sheep (48). However, these species are distantly related from different families (Camelidae versus Bovidae), so it is possible that species differences that are unrelated to high altitude could have contributed to these previous observations. Valuable insight into this issue could be provided from comparisons between populations of the same species across altitudinal gradients, in which neutral (non-adaptive) divergence in physiology is less likely, such that adaptive differences in pulmonary physiology can be more easily discerned with greater confidence.

In the current study, we examine these issues in the North American deer mouse (*Peromyscus maniculatus*). This species has the broadest altitudinal range of any North American mammal, from near sea level to over 4300 m in the Rocky Mountains. High-

altitude populations of deer mice sustain high metabolic rates in the wild (23) and are subject to strong directional selection for increased aerobic capacity (VO_2max) (24), presumably as a result of the high O_2 demands for aerobic thermogenesis in cold alpine environments. High-altitude deer mice have thus evolved increased VO_2max in hypoxia as compared to both low-altitude deer mice and white-footed mice (a congener found exclusively at low altitudes) (8, 9). This evolved increase in VO_2max is associated with increases in pulmonary O_2 extraction, arterial O_2 saturation, and cardiac output (64, 65), along with increases in capillarity, oxidative capacity, and mitochondrial volume density in skeletal muscles (14, 34, 38, 50, 55, 56). Furthermore, high-altitude deer mice do not exhibit right-ventricle hypertrophy during chronic hypoxia, in contrast to the robust RV hypertrophy that occurs in white-footed mice (69), but it is otherwise unknown if they exhibit HPH or V-Q mismatch in chronic hypoxia. Here, we studied high- and low-altitude populations of deer mice that were born in captivity and raised to adulthood at sea level, and then compared highlanders versus lowlanders both before and after chronic exposure to hypoxia. We hypothesized that high-altitude deer mice would respond more favourably to chronic hypoxia than low-altitude deer mice, exhibiting 1) little to no HPH, 2) preserved V-Q matching, and 3) morphological changes in lung volume and structure to improve pulmonary O_2 uptake.

2.3 METHODS

2.3.1 Deer mouse populations

Wild deer mice were live-trapped at high altitude (near the summit of Mount Evans, Clear Creek County, CO, USA at 39°35′18″N, 105°38′38″W; 4350 m above sea

level) or low altitude (Great Plains, Nine Mile Prairie, Lancaster County, NE, USA at 40°52′12″N, 96°48′20.3″W; 430 m above sea level) and were transported to McMaster University (50 m above sea level), where they were bred within each population to produce first generation progeny under common sea level conditions. These progeny were raised in standard holding conditions (24-25°C, 12:12 light-dark photoperiod) in normoxia (ambient atmospheric conditions), with unlimited access to water and standard mouse chow, until at least 6 months of age before beginning experiments. All animal protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

2.3.2 Chronic treatments

Animals were studied in the normobaric normoxic conditions in which they were raised and/or following chronic exposure to hypobaric hypoxia for 6-8 weeks (barometric pressure of 60 kPa, an O_2 partial pressure of approximately 12 kPa; simulating ~4300 m above sea level). One subset of animals was used only for terminal measurements (right ventricle systolic pressure, ventricle masses, lung histology) either in normoxia or after chronic exposure to hypoxia. A second subset of animals were used for repeated non-lethal measurements using *in vivo* SPECT-CT imaging, first in normoxia and then again after chronic hypoxia, and were then used for the terminal measurements described above. Hypobaric hypoxia was maintained using hypobaric chambers that have been previously described (29, 39). During chronic hypoxia, animals were returned to normobaric conditions twice per week for less than 20 min for cage cleaning.

2.3.3. Right ventricle systolic pressure and ventricle masses

Right ventricle systolic pressure was measured immediately before euthanasia. Mice were anaesthetised using 3% isoflurane (balance O_2) to a surgical plane of anaesthesia, and then maintained using 1% isoflurane. Mice were positioned supine, the thoracic fur was quickly shaved, and the skin was cut to expose the musculature over the ribcage using blunt dissection. This allowed visualization of the heart beating through the intercostal musculature to assess right-ventricle position. A 27g needle connected to a saline-filled (0.9% sodium chloride solution) catheter (MRE-025; Braintree Scientific, Braintree, MA, USA) was inserted through the intercostal muscle and directly into the lumen of the right ventricle and held there for ~1 min. The other end of this catheter was connected to a calibrated pressure transducer (MLT0699, ADInstruments, Colorado Springs, CO, USA), used to measure right-ventricle pressure data, which were acquired using a PowerLab 8/32 with LabChart 8 Pro Software (ADInstruments). The right-ventricle systolic pressure (RVSP) and associated heart rate data reported here were the averages from >1 s of recording (*i.e.* > 9 beats) during the first 5-10 s of stable pressure measurements. RVSP measurements were completed within 5 min of anaesthesia. Mice were then euthanized with an over-dose of isoflurane followed by decapitation, the thoracic cavity was opened, and the heart ventricles were isolated while taking care to not damage the lungs. The right ventricle was carefully removed, and the right ventricle as well as the left ventricle plus septum were weighed. The primary bronchus of the left lung was then intubated, and the lung was inflated with 10% formalin to a pressure of 30

cm H_2O, fixed in 10% formalin for 72h, and then stored in 70% ethanol until it was embedded in paraffin.

2.3.4 Lung histology

Fixed and paraffin-embedded lungs were sectioned using a microtome at a thickness of 5 µm and were mounted onto slides (Superfrost Plus; Fisher Scientific, Mississauga, ON, Canada). Sections were taken at each of 3-4 different locations along the rostrocaudal axis. One set of sections was stained for α-smooth muscle actin (α-SMA) to measure smooth muscle thickness, and a second set was used for hematoxylin and eosin (H & E) staining to measure alveolar surface density. Immediately before both types of staining, sections were deparaffinized with two 10 min washes in xylene and then washed twice in 100% ethanol and once in 95% ethanol for 5 min each. For α-SMA staining, slides were then washed for 5 min in phosphate buffered saline (PBS; 137 mmol l^{-1} NaCl, 2.68 mmol l^{-1} KCl, 10.0 mmol l^{-1} Na_2HPO_4, 1.76 mmol l^{-1} KH_2PO_4; pH 7.4), incubated for 30 min in 0.5% hydrogen peroxide in distilled water, washed for 5 min in PBS, and blocked by incubating with 1% normal goal serum (NGS) in PBS for 30 min at room temperature. Slides were then incubated overnight for ~12 h with a primary antibody against α-SMA (mouse IgG2a, MA5-11547, Invitrogen, Carlsbad, CA, USA) (28, 66) diluted 1:400 in PBS containing 1% NGS. Slides were then rinsed twice in PBS for 15 min each, incubated for 60 min at room temperature in horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG, 32430, Thermofisher, Waltham, MA, USA) that was diluted 1:30 in PBS containing 1% NGS and washed in PBS for 5

min. Slides were then developed in ImmPACT DAB (SK-4105, Vector, Burlingame, CA, USA) for 5 min, rinsed with distilled water for 5 min, counterstained with methyl green for 10 min, washed with distilled water for 5 min, dehydrated with one change of 95% ethanol and 2 changes of 100% ethanol for 5 min each, cleared with two changes of xylene for 10 min each, and finally cover-slipped with Permount. For H & E staining, slides were then rinsed in distilled water for 5 min, stained with Gills II haematoxylin for 2 min, washed in water for 1 min, stained with eosin for 45 s, quickly rinsed in water, dehydrated with one wash in 95% ethanol and two washes in 100% ethanol for 5 min each, cleared with two washes in xylene for 10 min each, and finally cover-slipped with Permount (Fisher Scientific)

Stained sections were imaged for analysis using an upright brightfield microscope. The thickness of arterial smooth muscle was measured for all positively stained arterial vessels per section, wherein the thickness of each vessel was determined as the average of eight measurements that were evenly distributed around its circumference, and the average thickness for each mouse is reported here. Stereological methods were used to make unbiased morphometric measurements of alveolar surface density, as previously recommended (42). Images for alveolar surface density were taken from 3 different regions within each section, yielding 9-12 images per animal for analysis. This number of images was sufficient to account for heterogeneity across the lungs, determined by the number of images necessary to yield a stable mean value. All measurements were made using ImageJ software (v 2.0.0-rc-6) (52).

2.3.5 SPECT-CT scanning

We used single-photon-emission computed tomography (SPECT) scanning combined with conventional X-ray computed tomography (CT) scanning to examine V-Q matching along with lung and airway volumes (30, 44). SPECT-CT scanning was carried out on individual mice held in standard normoxic conditions and then again after exposure to chronic hypoxia. Animals were anesthetized with an intraperitoneal injection of ketamine/xylazine (50 mg/kg; 5 mg/kg), and a heating lamp was used to maintain body temperature. Once a light but stable level of anaesthesia was reached, the mice breathed Technegas™ (0.04-0.12 Mbq/mL; Cyclomedica, Lucas Heights, NSW, Australia) for 10 min through a nose cone. Technegas™ is an ultrafine aerosol composed of carbon particles (diameter between 30 and 60 nm) that are labelled with ^{99m}Tc and suspended in argon and is used as a marker of the regional distribution of pulmonary ventilation. The Technegas™ was mixed with the desired fraction of oxygen (21% in normoxia acclimated mice, 12% in hypoxia acclimated mice) and supplied to the nose cone at 0.02 l/min using a rodent ventilator (Model 683, Harvard Apparatus, Holliston, MA, USA). We then acquired SPECT scans on a X-SPECT system (Gamma Medica, Northridge, CA, USA) using dual sodium iodide crystals in combination with low energy pinhole collimators with 1 mm aperture and a radius of rotation of 3.5 cm. Mice were secured to warmed bed to maintain body temperature, and they breathed air from a nose cone with the desired fraction of oxygen (21% in normoxia acclimated mice, 12% in hypoxia acclimated mice) along with isoflurane (~1%) to maintain a light level of anaesthesia. An initial SPECT scan to detect ventilation consisted of thirty-two 50s projections. This was

immediately followed by the collection of four rotations of 1024 X-ray projections for CT, also acquired on the X-SPECT system, with x-ray tube characteristics of 75 kVp and 220 mA. Following these ventilation scans, mice were injected with 11-15 MBq of ^{99m}Tc macro aggregated albumin (^{99m}Tc-MAA) via the tail vein as a marker of pulmonary perfusion. Care was taken not to shift the position of the animal during the injection. A second SPECT scan was then taken to detect perfusion, consisting of thirty-two 40s projections, followed by 1024-projection CT scan. The entire imaging procedure took approximately 1h 20min. Mice were recovered from the imaging procedure, monitored until radioactivity had decayed to background levels, and returned to their appropriate chronic treatment. Mice were then used for the terminal measurements described above, 1-2 weeks after their second set of SPECT-CT scans that were obtained after exposure to chronic hypoxia. All imaging work was completed at the McMaster Center for Preclinical Translational Imaging at McMaster University (Hamilton, ON, Canada).

The reconstruction, fusion, and co-registration of three-dimensional images from the SPECT and CT scans was performed as previously described in detail (30). SPECT and CT images were generated by reconstructing their respective projections using FLEX^{TM}-SPECT (Gamma Medica) and COBRA software (Exxim Sofware, Pleasanton, CA, USA), respectively. The high-quality CT images were used in the processing and analysis of V-Q data in order to provide anatomical features for the fusion and co-registration of SPECT scans, and to also allow for a density-based determination of lung and airway structures. We calibrated each CT image for Hounsfield Unit (HU) scaling using empty airspace within the field of view and a water-filled microtube included in

each scan (air and water are defined as -1000 and 0 HU, respectively). This allowed us to define a 'lung' label for the CT images as described previously (30) using Amira 5.1 software (Visage Imaging, Andover, MA, USA) and a separate 'airway' label. These images allowed us to determine lung volume (reflective of end-expiratory lung volume) and airway volume, where the latter extended rostrally from the larynx, down the trachea, to the secondary bronchi. We also produced inspiratory- and expiratory-gated CT images using RespGate software (16) in order to measure tidal volume as previously described in detail (30). We then fused the perfusion CT (ungated) and SPECT with in-house software, developed in Matlab, that maximizes mutual information (MI) within the lung region as defined by the label field, using multimodality image registration techniques that have been previously described (30, 36). When maximized MI was reached, the result was visually inspected to confirm fusion quality. If image co-registration did not meet standards, we repeated the process using an affine transformation which corrects for more substantial differences in location (e.g., due to small movement of the mouse). We then applied the parameters obtained from fusion of the perfusion SPECT and CT images to the ventilation SPECT and CT images as the spatial relationship between these two data sets remains constant. We then repeated the MI maximization process to co-register the ventilation and perfusion data through rigid body transformation of their respective lung CT images.

Co-registration of the 3D SPECT and CT images allowed for the subsequent quantitative analysis of ventilation (V) and perfusion (Q) on a per-voxel basis. Normalized V and Q values were first generated by dividing the radioactivity value of

each voxel that was measured from each respective SPECT scan by the total activity in the lung label (expressed here in percent). We thus collected log(V/Q) values for all voxels within the lung label. From these data, we calculated the mean, standard deviation, and skewness of log(V/Q). Any voxel where only V equaled zero was set to a log(V/Q) ratio of -∞ and any voxel where only Q equaled zero was set to a log(V/Q) ratio of +∞, but these values were not included in the calculation of the mean, standard deviation, or skew. We instead report these data as the lung volume that was perfused but unventilated (representing the volume of the lung receiving shunt perfusion), and the lung volume that was ventilated but unperfused (which reflects dead space volume within the lungs).

2.3.6 Statistics

We performed two-way ANOVA using GraphPad Prism software (version 7.0; La Jolla, CA, USA) to test for main effects of population and environment (normoxia *versus* chronic hypoxia) as well as their interaction. A repeated-measures design was used for all SPECT/CT data. When the main effects or interaction were significant, we performed Tukey post-hoc comparisons to test for effects of chronic hypoxia exposure within each population. Data are reported as individual values and/or means ± SEM. $P<0.05$ was considered to be significant.

2.4 RESULTS

2.4.1 Attenuation of hypoxic pulmonary hypertension in highlanders

Exposure to chronic hypoxia induced pulmonary hypertension and RV hypertrophy in lowlanders, but these responses were attenuated in highlanders (Fig. 1;

Table 1). In lowlanders, RV systolic pressure (RVSP) increased significantly after exposure to chronic hypoxia from 14.2 to 18.7 mm Hg on average ($P = 0.001$; Fig. 1A), which drove the significant main effect of environment ($P = 0.0005$). This increase was not associated with variation in heart rate at the time of measurement in lowlanders, which was similar in normoxia (652 ± 21 min^{-1}) and after exposure to chronic hypoxia (542 ± 34 min^{-1}; $P = 0.0843$). Chronic hypoxia also increased the relative mass of the right ventricle (RV/LV+S; $P = 0.0176$) and the thickness of smooth muscle in pulmonary arteries ($P = 0.0434$) in lowlanders (Fig. 1B-E), driving the significant main effects of environment on these traits ($P = 0.0046$ and $P = 0.0187$ respectively). In highlanders, however, RVSP increased less in response to chronic hypoxia (P = 0.0492), from 12.8 to 15.4 mmHg on average, with no variation in heart rate at the time of measurement (normoxia, 559 ± 24 min^{-1}; chronic hypoxia, 557 ± 25 min^{-1}; $P = 0.9990$). Some highland individuals exposed to chronic hypoxia were within the normal range of pressures exhibited by normoxia-acclimated mice. Highlanders also tended to have lower RVSP overall, as reflected by the significant main effect of population (P = 0.0083). Furthermore, neither relative RV mass (P = 0.5518) nor smooth muscle thickness (P = 0.5120) increased in highlanders in chronic hypoxia, and there was a significant population effect on relative RV mass (P = 0.0195).

2.4.2 Increased lung volume in highlanders in response to chronic hypoxia

Lung volume, measured with CT, increased in highlanders, but not in lowlanders, after chronic exposure to hypoxia (Fig. 2, Table 2). In highlanders, both mass-specific (P

= 0.0252) and absolute (P = 0.0157) lung volumes increased significantly after chronic exposure to hypoxia, which drove the significant main effects of environment on these traits (P = 0.0052 and 0.0060, respectively). However, chronic hypoxia did not affect the mass-specific or absolute volumes of the airways in either highlanders or lowlanders (Fig. 2, Table 2), as there was no significant environment or population effects on these traits. Likewise, chronic hypoxia did not affect tidal volume or body mass in highlanders or lowlanders, but there was a significant population effect on absolute tidal volume (*P*=0.0468) which tended to be larger in highlanders than in lowlanders (Table 2). In addition, there was a significant population×environment interaction for alveolar surface density (*P*=0.0178), reflecting a population difference in the response to chronic hypoxia, which appeared to result from highlanders having more alveolar surface in normoxia, but with lowlanders increasing to their level after chronic hypoxia (Table 3).

2.4.3 Matching of lung ventilation and perfusion was unaltered by chronic hypoxia

We measured V-Q matching using SPECT-CT scanning, which allowed us to evaluate the characteristics of the log(V/Q) curve in highlanders and lowlanders in normoxia and to examine whether the curve changed after exposure to chronic hypoxia. Representative two-dimensional images in axial, coronal, and sagittal planes for the SPECT/CT imaging are shown in Figure 3. CT images were used to create a 'lung label', on which ventilation (V), perfusion (Q), and log(V/Q) were overlaid. Log(V/Q) values generally display a normal distribution for both highlanders and lowlanders (Fig. 4A and 4B respectively), where mean log(V/Q) values generally centered close to around 0. Log(V/Q) of zero reflects perfect matching of V and Q, so the variation of log(V/Q) from

0 reflects imperfect matching, which we quantified using metrics of the log(V/Q) distribution. After exposure to chronic hypoxia, both highlanders and lowlanders maintained similar V/Q matching to what was exhibited in normoxia before hypoxia exposure (Fig. 5). Neither the mean (Fig. 5A) nor the standard deviation (Fig. 5B) of log(V/Q) changed after exposure to chronic hypoxia in highlanders or lowlanders. Although lowlanders appeared to exhibit a slight decrease in skew after chronic hypoxia exposure (indicative of a slight shift towards over-perfusion and under-ventilation), this change was not significant ($P = 0.1892$; Fig. 5C). The volume of the lung receiving shunt perfusion (i.e. the volume that was perfused by unventilated) and the dead space volume within the lungs (i.e. the volume that was ventilated but unperfused) were both 1% or less of total lung volume on average, and did not vary across groups (Tables 1, 2).

2.5 DISCUSSION

Deer mice are abundant at high altitudes, where they are able to reproduce and sustain high metabolic rates for thermogenesis despite the challenge of chronic hypoxia (23). Here, we show that deer mice native to high altitude have attenuated some maladaptive responses to chronic hypoxia that lead to pathology in low-altitude taxa. High-altitude mice exhibit less hypoxic pulmonary hypertension than low-altitude mice in response to chronic hypoxia (Fig. 1). They also avoid the pulmonary arterial remodeling that contributes to HPH, and thus exhibit no hypertrophy of the right ventricle. High-altitude mice instead respond to chronic hypoxia with increases in lung volume (Fig. 2), and neither high- nor low-altitude mice suffer any impairment in

ventilation-perfusion matching (Fig. 5). The evolved changes in high-altitude deer mice likely contributes to the ability of these animals to survive and thrive in chronic hypoxia.

The hypoxic pulmonary hypertension exhibited by low-altitude deer mice in response the chronic hypoxia was akin to previous observations in many other low-altitude taxa. The magnitude of the increase in RVSP, as an index of systolic pressure in pulmonary arteries, was comparable to those observed in some previous studies (3, 47) but not as high as in some others (1, 12, 20, 40). Variations between taxa and methodologies could contribute to these differences between studies, but another potential explanation is the level of hypoxia exposure. The magnitude of hypoxia used for chronic exposures here (12 kPa O_2, hypoxia equivalent to ~4300 m elevation) was not as severe as in some previous studies, and HPH and other associated traits are expected to increase as hypoxia becomes more severe with increasing elevation (48). Indeed, some of the observations of the most severe incidences of HPH in animals were made after chronic exposure to hypoxia equivalent to the levels at ~5500 m elevation (1, 40).

The association between RVSP and pulmonary artery thickness is consistent with the role of important smooth muscle remodeling in the development of pulmonary hypertension. Although hypoxic pulmonary vasoconstriction represents a key early step in the progression of HPH, the subsequent vascular remodeling is a key contributor to increases in pulmonary arterial blood pressure (31, 57, 68). Vascular remodeling occurs because chronic stimulation of hypoxia-induced vasoconstriction leads to the eventual thickening of the arterial smooth muscle and makes the vessels less distensible over time (13, 18, 37, 59). Hypoxia inducible factor 2α (HIF-2α) may play a key role in this

process, by inducing arginase expression and thus dysregulating vascular nitric oxide homeostasis (11).

Our finding that high-altitude deer mice have attenuated or eliminated the maladaptive pulmonary responses to chronic hypoxia adds to previous observations in other high-altitude natives. Previous studies have shown that several mammal taxa that are native to and studied at high altitude exhibit pulmonary arterial pressures that are in the range of lowlanders at sea level (18, 21, 22). However, many previous studies have not compared high- and low-altitude taxa after chronic exposure to both normoxic and hypoxic conditions, and have therefore been unable to discern whether low pulmonary arterial pressures at high altitude results from a blunted response to hypoxia, or from an overall reduction in pressure to offset the otherwise preserved response to hypoxia. Previous work in high-altitude native llama support the former possibility, because they exhibit similar pulmonary artery pressures to low-altitude native sheep when compared in normoxia, but llama appear to have a blunted response to acute and chronic hypoxia (3, 4). Our results may support both possibilities, because high-altitude mice had lower RVSP overall in both normoxia and hypoxia, and they increased RVSP by a smaller magnitude in response to hypoxia (Fig. 1). Our results also suggest that these changes may represent an evolutionary (genetically based) adaptation to high altitude for two reasons. Firstly, all mice were bred in captivity at sea level from parents that were well acclimatized to normoxia, so developmental plasticity did not contribute to our observations. Secondly, there remains a small amount of gene flow between high- and low-altitude populations in the wild (60), so differences in physiology between

populations are more likely a result of spatially varying selection across altitudinal gradients than a result of neutral (non-adaptive) causes.

Our results suggest that neither the pulmonary vascular responses to chronic hypoxia that contribute to HPH in low-altitude mice, nor the evolved changes to avoid them in high-altitude mice, disrupt V-Q matching under resting conditions. This could suggest that hypoxic pulmonary vasoconstriction still functions after exposure to chronic hypoxia to provide good regional matching of perfusion to ventilation. However, it is possible that impairments in V-Q matching in chronic hypoxia only become apparent in deer mice during exercise or other conditions of elevated metabolic rate (such as during prolonged thermogenesis). Indeed, at levels of hypoxia similar to the current study, human subjects only experience V-Q mismatch during exercise (17, 45, 70, 71), and V-Q mismatch was only observed under resting conditions during exposure to more severe levels of hypoxia (71). Measurements of field metabolic rate suggest that high-altitude deer mice maintain high metabolic rates in the wild (continuously at ~60% of maximal metabolic rate on average), likely to support the metabolic costs of thermogenesis and of exercise to forage for food (23). Therefore, it remains to be determined whether V-Q matching may still be a challenge for wild deer mice at high altitude.

2.6 PERSPECTIVES AND SIGNIFICANCE

This study contributes to the emerging evidence suggesting that deer mice have adapted to high altitude through evolved changes in various aspects of cardiorespiratory and metabolic physiology that help augment aerobic performance and attenuate maladaptive plasticity in chronic hypoxia (8, 34, 38, 60, 64, 65, 69). The attenuation of

HPH and avoidance of some associated maladaptive traits (*e.g.*, right-ventricle hypertrophy) may be particularly important for deer mice at high altitude and elucidating the potential mechanisms for this evolved change could inform biomedical interventions for pulmonary hypertension. The genetic basis for the evolved attenuation of hypoxic pulmonary hypertension in high-altitude deer mice has yet to be resolved. Previous studies in neonatal llama suggest that high-altitude natives may have shifted the balance towards increased vasodilatory and/or decreased vasoconstrictive influences on the pulmonary vasculature (*e.g.*, increased nitric oxide and/or carbon monoxide signaling) compared to their lowland counterparts (48). Recent evidence for a potential role of HIF-2α in HPH (11) suggest that evolved changes in hypoxia signaling could underlie such shifts and contribute to attenuating HPH in high-altitude natives. The gene encoding HIF-2α (*Epas1*) is under strong selection in Tibetan humans and in many animals native to high altitude (2, 5, 19, 32, 33, 58), including high-altitude populations of deer mice (54). The genetic variants of *Epas1* in high-altitude taxa are associated with variation in blood haemoglobin content or heart rate in hypoxia (5, 54). However, given the potentially serious pathological consequences of HPH in high-altitude environments (12, 15, 17, 41, 43, 46, 49, 63, 69, 71, 72), it is possible that selection on *Epas1* during high-altitude adaptation was acting directly or indirectly to attenuate maladaptive pulmonary vascular responses to hypoxia.

2.7 TABLES AND FIGURES

Table 1: Results of statistical comparisons using a two-way ANOVA.

Trait	Population effect	Environment effect	Population× Environment
RVSP (mmHg)	0.0083	0.0005	0.3109
Heart rate (min^{-1})	0.2830	0.1277	0.1404
RV/ LV + septum masses	0.0195	0.0046	0.2276
Arterial SM thickness (µm)	0.7296	0.0187	0.2492
Lung volume (µl)	0.1962	0.0060	0.2543
Lung volume (µl/g)	0.9911	0.0052	0.4813
Airway volume (µl)	0.2286	0.7620	0.0653
Airway volume (µl/g)	0.7936	0.7683	0.1383
Tidal volume (µl)	0.0468	0.2381	0.2836
Tidal volume (µl/g)	0.3485	0.3711	0.5181
Alveolar surface density (μm^{-1})	0.1643	0.8485	0.0178
Mean Log[V/Q]	0.4121	0.1324	0.7914
SD Log[V/Q]	0.4784	0.2539	0.8304
Skew Log[V/Q]	0.4706	0.0775	0.5621
Lung shunt volume (µl)	0.6000	0.1748	0.5477
Lung shunt volume (µl/g)	0.4607	0.1467	0.5528
Lung dead space volume (µl)	0.4680	0.1802	0.9179
Lung dead space volume (µl/g)	0.5476	0.1570	0.9391

RVSP, right ventricular systolic pressure; RV/LV+S, ratio of right ventricular (RV) mass to left ventricle (LV) and septum (S) mass; SM, smooth muscle; Mean, SD, and Skew of Log[V/Q], mean, standard deviation, and skew of the distribution of log(ventilation/perfusion) throughout the lungs.

Table 2: Lung and airway volumes, tidal volume, and body mass in highland and lowland deer mice acclimated to normoxia and hypoxia.

Trait	Population	Normoxia	Hypoxia
Lung volume (µl)	Lowlander	359.54 ± 41.10	400.57 ± 25.53
	Highlander	394.84 ± 28.73	479.74 ± 30.11
Airway volume (µl)	Lowland	14.90 ± 1.84	18.11 ± 2.16
	Highland	22.49 ± 2.72	17.11 ± 1.39
Tidal volume (µl)	Lowlander	102.96 ± 13.91	100.57 ± 13.55
	Highlander	170.24 ± 29.71	122.11 ± 20.96
Tidal volume (µl/g)	Lowlander	5.67 ± 0.85	5.42 ± 0.54
	Highlander	7.26 ± 1.11	5.71 ± 1.17
Lung shunt volume (µl)	Lowlander	1.37 ± 0.59	1.65 ± 0.41
	Highlander	1.48 ± 0.68	2.61 ± 0.66
Lung shunt volume (µl/g)	Lowlander	0.07 ± 0.03	0.24 ± 0.16
	Highlander	0.06 ± 0.03	0.12 ± 0.03
Lung dead space volume (µl)	Lowlander	2.59 ± 1.05	0.24 ± 0.20
	Highlander	4.13 ± 3.18	1.4 ± 0.91
Lung dead space volume (µl/g)	Lowlander	0.13 ± 0.05	0.02 ± 0.01
	Highlander	0.18 ± 0.14	0.06 ± 0.04
Body mass (g)	Lowlander	18.78 ± 1.05	18.50 ± 0.82
	Highlander	21.90 ± 1.45	21.49 ± 0.99

Values are represented as mean ± SEM (N as per Fig. 2).

Table 3: Alveolar surface density (μm-1) in highland and lowland deer mice.

	Normoxia	Hypoxia
Lowlander	0.069 ± 0.006 (3)	0.078 ± 0.002 (7)
Highlander	0.082 ± 0.002 (5)	0.074 ± 0.003 (6)

Values are represented as mean ± SEM (N).

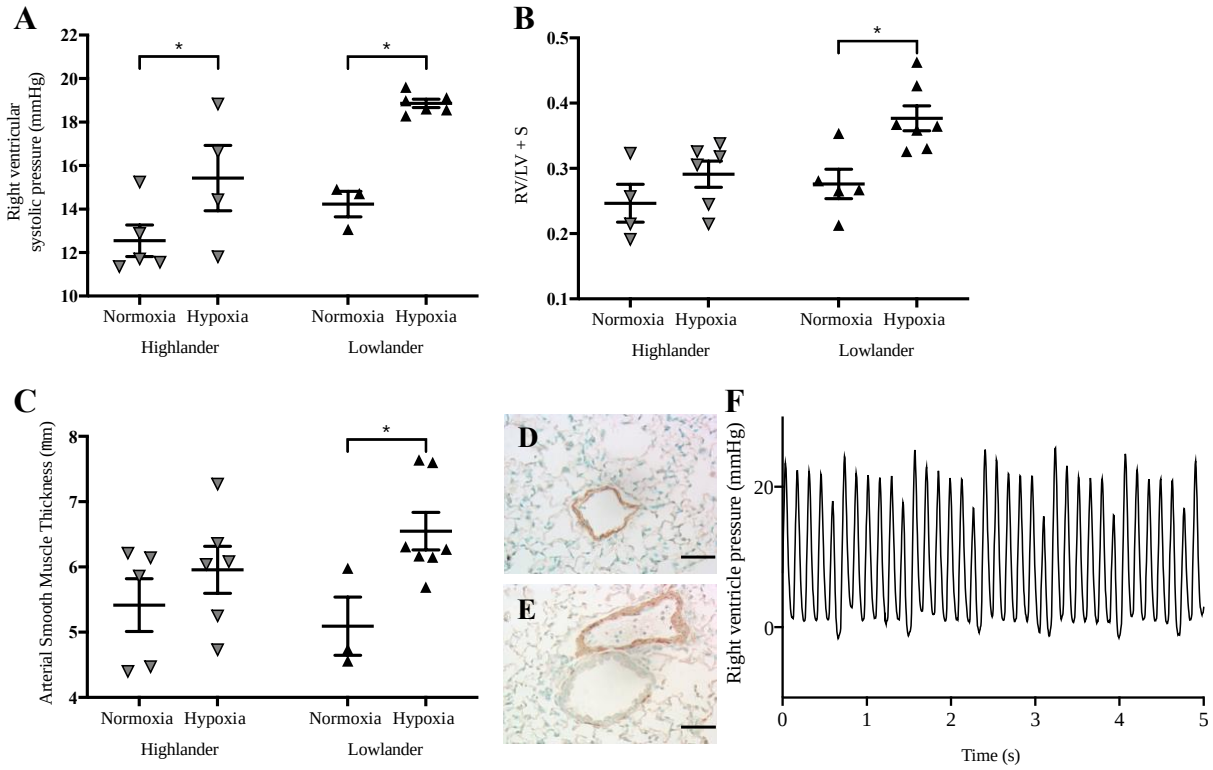

A
Right ventricular systolic pressure (mmHg)
22
20
18
16
14
12
10
*
*
Normoxia Hypoxia
Highlander
Normoxia Hypoxia
Lowlander
B
RV/LV + S
0.5
0.4
0.3
0.2
0.1
*
Normoxia Hypoxia
Highlander
Normoxia Hypoxia
Lowlander
C
Arterial Smooth Muscle Thickness (mm)
8
7
6
5
4
*
Normoxia Hypoxia
Highlander
Normoxia Hypoxia
Lowlander
D
E
F
Right ventricle pressure (mmHg)
20
0
0
1
2
3
4
5
Time (s)

Fig. 1. Increases in right ventricle systolic pressure (A), the mass of the right ventricle relative to the mass of the left ventricle and septum (RV/LV+S) (B), and the thickness of smooth muscle in pulmonary arteries (C) after exposure to chronic hypoxia were attenuated in highlanders. Lines indicate means ± SE and triangles represent individual values. * Significant pairwise difference between environments within a population ($P<0.05$). We stained for α-smooth muscle actin to identify arterial smooth muscle (brown) in the lungs of highlanders (D) and lowlanders (E) (scale bars are 100 μm). In (E), a positively stained artery is shown next to an unstained bronchiole. (F) Representative trace showing right ventricle pressure (mmHg).

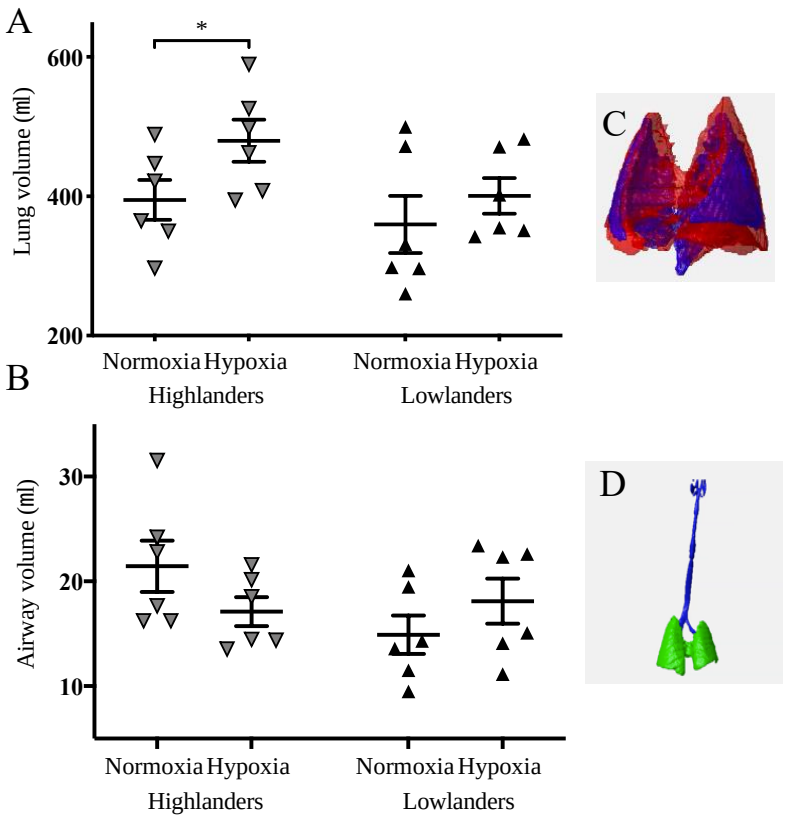

A
*
600
400
200
Lung volume (ml)
Normoxia Hypoxia
Highlanders
Normoxia Hypoxia
Lowlanders
B
30
20
10
Airway volume (ml)
Normoxia Hypoxia
Highlanders
Normoxia Hypoxia
Lowlanders
C
D

Fig. 2. Highlanders increased lung volume (µl/g) after chronic exposure to hypoxia (A,C), but airway volume (µl/g) remained constant in both populations (B,D). (C) Representative CT scans of a highland mouse show the increase in in vivo end-expiratory lung volume from normoxia (blue) to hypoxia (red). (D) Representative CT scan showing the conducting structures (blue) of a lowland mouse in normoxia. Lines indicate means ± SE and triangles represent individual values. * Significant pairwise difference between environments within a population ($P<0.05$).

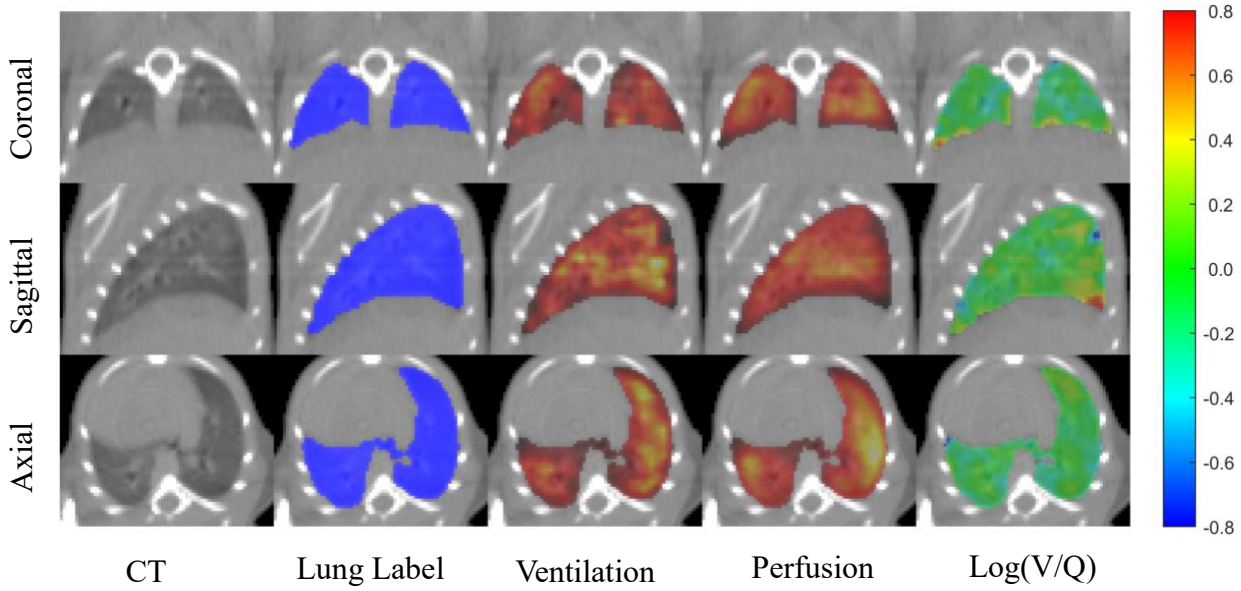

Coronal
Sagittal
Axial
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
CT
Lung Label
Ventilation
Perfusion
Log(V/Q)

Fig. 3. Representative image showing axial, coronal, and sagittal planes of section of SPECT-CT imaging. CT scans were used to create a lung label (blue, second column from left) on which relative ventilation (V), relative perfusion (Q), and log(V/Q) were overlaid.

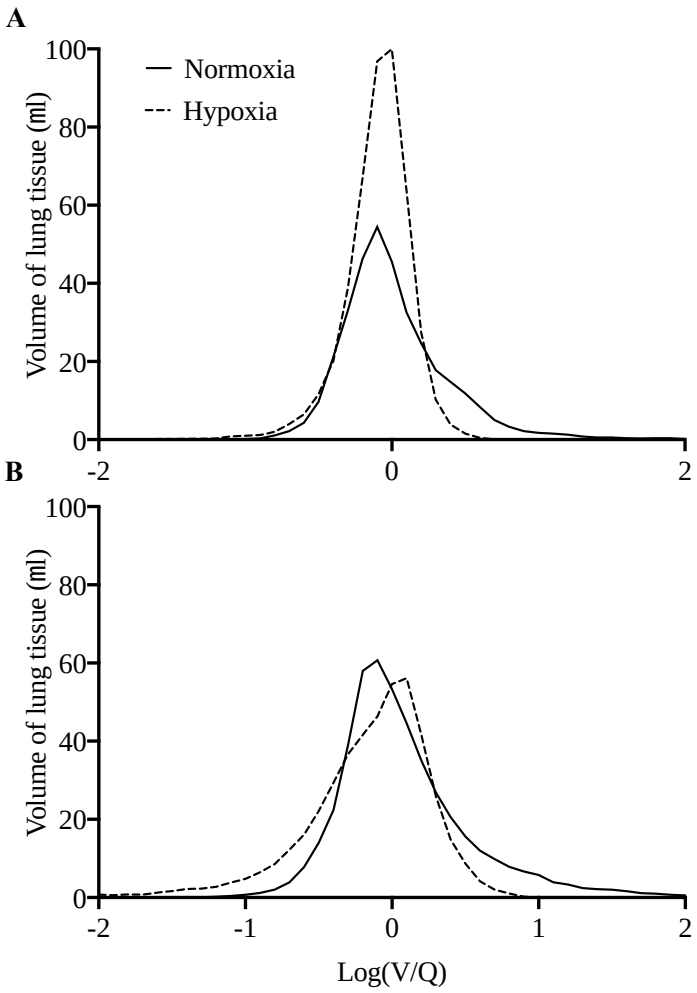
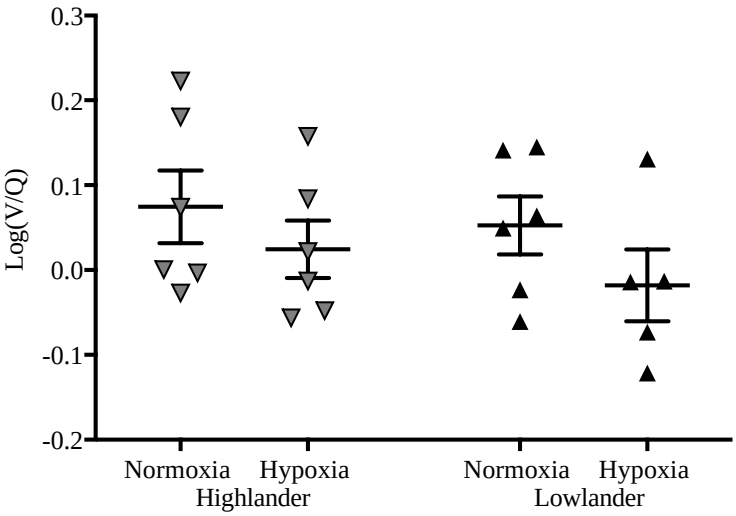
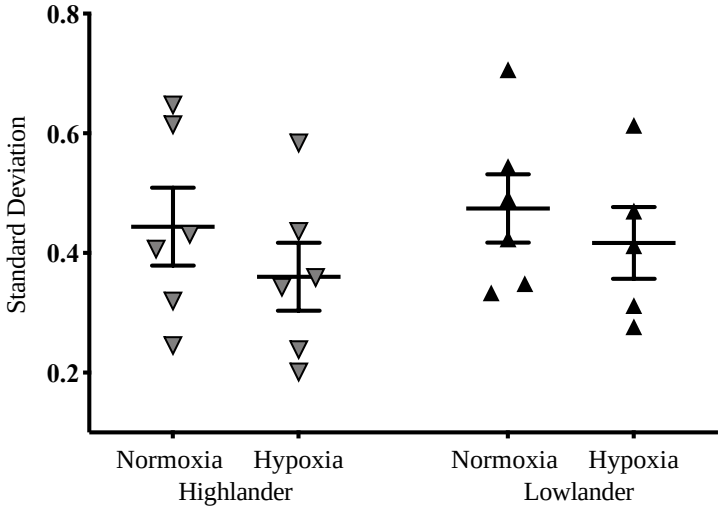

A
Normoxia
Hypoxia
Volume of lung tissue (ml)
100
80
60
40
20
0
-2
0
2
B
Volume of lung tissue (ml)
100
80
60
40
20
0
-2
-1
0
1
2
Log(V/Q)

Fig. 4. Representative distribution of ventilation (V)-perfusion (Q) matching throughout the lungs in highlanders (A) and lowlanders (B) in normoxia (solid line) and after chronic exposure to hypoxia (dashed line). Volumes are based on absolute end-expiratory lung volumes.


0.3
0.2
0.1
0.0
-0.1
-0.2
Log(V/Q)
Normoxia
Hypoxia
Highlander
Normoxia
Hypoxia
Lowlander


0.8
0.6
0.4
0.2
Standard Deviation
Normoxia
Hypoxia
Highlander
Normoxia
Hypoxia
Lowlander

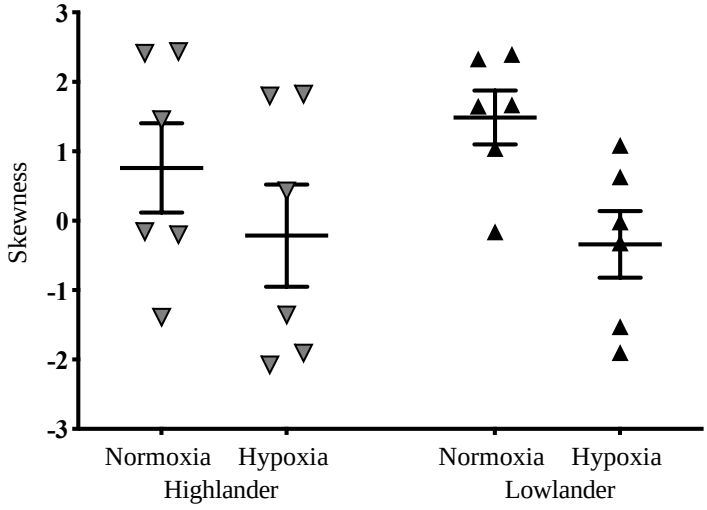

3
2
1
0
-1
-2
-3
Skewness
Normoxia
Hypoxia
Highlander
Normoxia
Hypoxia
Lowlander

Fig. 5. Exposure to chronic hypoxia had little effect on the mean (A), standard deviations (B), and skew (C) of the log(V/Q) distribution in highlanders and lowlanders acclimated to normoxia and hypoxia. Lines indicate means ± SE and triangles represent individual values.

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CHAPTER 3: GENERAL DISCUSSION

High altitude is a challenging environment, yet many animals live there successfully. Life at high altitude is challenging for two main reasons: 1) low temperatures and 2) low partial pressure of oxygen. Low temperatures are challenging because it requires that small endotherms expend appreciable amounts of energy and thus consume oxygen to maintain their body temperature. Small endotherms living at high altitude are therefore faced with the compounded stressor of having to thermoregulate in hypoxia, effectively faced with a decrease in oxygen availability to support the increased oxygen demands. I sought to investigate how deer mice cope with chronic hypoxia at high altitude, focussing on lung function and structure. The **primary objective** of my thesis was to 1) investigate the presence and severity of maladaptive plasticity and 2) to determine potential adaptations in lung function and structure in populations native to high altitude. I tested the **overarching hypothesis** that high-altitude deer mice have evolved altered responses to chronic hypoxia that are more favourable for avoiding maladaptive plasticity and maintaining O_2 uptake at altitude compared to low-altitude deer mice.

3.1 Attenuation of maladaptive plasticity

I have shown that high-altitude deer mice have evolved changes in pulmonary physiology that likely represent adaptations to chronic hypoxia in their native environment. Specifically, I showed that highland deer mice have blunted hypoxic pulmonary hypertension compared to low-altitude mice in response to chronic hypoxia and have eliminated the associated maladaptive changes in pulmonary arteries and the

right ventricle. Considering the potential for the detrimental changes associated with HPH to accentuate the alveolar-arterial PO_2 difference and to thus impair pulmonary O_2 diffusion (31, 32), these putative adaptations in high-altitude deer mice may help maintain O_2 uptake. These findings are consistent with previous work, as many low-altitude native animals demonstrate robust hypoxic pulmonary hypertension in response to chronic hypoxia (28–30), whereas animals that are endemic to high-altitude do not exhibit pulmonary hypertension (9, 11, 12, 25). Many previous observations have been made on high-altitude animals in their native environment, or by comparing lowland and highland animals of different species, making it difficult to disentangle the effects of plasticity, genetics, and interspecies differences. By using two different populations within the same species, I was able to show that highland deer mice have blunted HPH compared to their lowland counterparts after exposure to chronic hypoxia, suggesting the responses to chronic hypoxia have evolved in high-altitude deer mice to reduce the magnitude of maladaptive responses from occurring.

How might highland deer mice reduce HPH in chronic hypoxia? Neonatal llamas do not develop pulmonary hypertension when exposed to chronic hypoxia, nor do they show thickening of smooth muscle in the pulmonary vasculature (25). It appears that neonatal llamas have shifted the balance towards increased vasodilatory and/or decreased vasoconstrictive influences on the pulmonary vasculature (*e.g.*, increased nitric oxide and/or carbon monoxide signaling) compared to their lowland counterparts. *Epas1* is a gene that encodes a hypoxia inducible factor, HIF-2α. HIF-2α is known to play a role in the development of HPH. Cattle that developed HPH (*i.e.*, brisket disease) had a variant

of *Epas1* that is highly associated with HPH and that only expresses disease in the hypoxic state at high altitude, suggesting that *Epas1* plays a major role in the development of HPH in cattle (22). Recent work demonstrates that the rise in pulmonary arterial pressure that usually occurs in response to chronic hypoxia was absent in mice with a HIF-2α deletion and in mice with a arginase 1 deletion, they also found that nitric oxide (NO) concentrations were higher in mice with HIF-2α deletions. Thus, the authors propose that chronic hypoxia results in stabilization of HIF-2α and thus dysregulation of NO homeostasis (5). The genetic variant in *Epas1* in high-altitude deer mice could have led to evolved changes in HIF-2α signaling in the pulmonary endothelium that augments NO production in chronic hypoxia, and thus promotes vasodilation, in absence of any evolved changes in smooth muscle specifically.

Smooth muscle cells in the pulmonary arteries mediate pulmonary vasoconstriction when decreases in alveolar partial pressure of oxygen is sensed directly by smooth muscle cells in the pulmonary arteries, causing inhibition of O_2 sensitive K^+ channels (Kv), membrane depolarization, and Ca^{2+} influx. (1, 14, 21, 27). Previous work in high-altitude Tibetan sheep found that administration of 4-aminopyridine (4-AP), a Kv channel inhibitor, did not alter vascular tone during a hypoxia challenge (20 min at O_2 levels equivalent to elevation of 0, 2260, and 5400 m elevation) (17). Ishizaki and colleagues (2004) suggest that it is possible that dysfunctional and/or down-regulation of oxygen-sensitive Kv channels could be modulating the reduced HPH in highlanders (17). Understanding the mechanisms by which highland-native deer mice attenuate the HPH

exhibited by lowland-native deer mice in response to chronic hypoxia may inform biomedical interventions for pulmonary hypertension.

3.2 Beneficial changes in lung structure in high-altitude natives

The pulmonary vascular remodeling exhibited by lowlanders, but not highlanders, in response to chronic hypoxia might be expected to impair V-Q matching; however, both highlanders and lowlanders were able to maintain ventilation-perfusion matching at rest in chronic hypoxia. In human studies, V-Q matching is maintained in hypoxia until aerobic activity increases (*i.e.*, during exercise) (8, 24, 32, 33), or until severe levels of hypoxia are reached (33). Given that deer mice maintain high field metabolic rates at high altitude (13), it would be beneficial to investigate whether highland and lowland deer mice can maintain V-Q matching during high aerobic activity. However, this would be impossible to do using the SPECT-CT technique, as it requires an unmoving, anesthetized subject. It may be possible to use the multiple inert gas technique (MIGET) to measure global V-Q matching in an active subject; however, MIGET is limited to a measure of global (rather than regional) V-Q matching.

I observed that highland mice responded to chronic hypoxia by increasing lung volume. This is consistent with a previous study in high-altitude deer mice that were raised at sea level for 18-20 generations then exposed to chronic hypoxia (3800 m elevation for 8 weeks) which resulted in significantly larger lungs in acclimatized highlanders compared to sea level controls (7). Most other studies included developmental hypoxia exposure (3, 6, 15), thus making it difficult to determine whether developmental exposure, plasticity, or genetics contribute to the observed increases in

lung volume after chronic hypoxia exposure. By comparing adult deer mice born and raised in normoxia after a chronic hypoxic exposure (equivalent to 4300 m elevation for 6-8 weeks), I was able to remove the effect of developmental hypoxia exposure and show that highland deer mice still increase lung volume in response to chronic hypoxia, while lowlanders do not. While previous studies showed that acclimatized lowlanders responded to chronic hypoxia by increasing lung volume (6, 15, 16, 20), this was typically in response to developmental hypoxia exposure, suggesting that there may be a critical window of hypoxia exposure during development for lowlanders in order for lung growth to be possible.

I found that neither population of deer mice showed changes in alveolar surface density in response to chronic hypoxia; however, since highlanders increase lung volume but maintain alveolar surface density, it is possible that highlanders are increasing alveolar number and/or decreasing alveolar diameter. Adult leaf-eared mice (*Phyllotis darwini)* from a highland population (collected at ~4660 m elevation) had larger lungs with smaller, more densely packed alveoli compared to their lowland counterparts (collected near sea level) (23). Similarly, developing rats acclimatized to hypoxia (3450 m elevation) showed an increase in lung volume; however, their alveolar surface density was not significantly different from low altitude controls, indicating no change in the structure of the alveolar unit in response to developmental hypoxia exposure in rats (4). In my study, we were unable to measure post-mortem lung volume which would permit us to determine whether mass-specific alveolar surface density differed between highland and lowland deer mice after chronic hypoxia exposure, but would be beneficial to

investigate whether highlanders alter alveolar number and/or size in addition to increasing lung volume in response to chronic hypoxia and in the absence of developmental hypoxia exposure.

As briefly mentioned above, lung structure and function have been shown to be altered with developmental hypoxia exposure, suggesting that hypoxia during critical stages of early development can influence pulmonary O_2 diffusion. For example, animals in hypoxia exhibit enhanced alveolar growth and remodeling compared to normoxia-raised animals (2, 4, 15), while other studies have shown no changes in pulmonary structures after exposing adult dogs to hypoxia, but exposing developing pups to hypoxia resulted in increased surface area for diffusion (10, 19). My thesis investigated the effects of chronic hypoxia exposure on adult deer mice who had been raised in normoxic conditions; however, these results may have differed had the animals been exposed to chronic hypoxia throughout development. The developmental period is critical for the survival of deer mice and all animals, so future work could focus on the effect of developmental plasticity in response to chronic hypoxia. Recent work has shown evidence for a delay in the onset of homeothermy and thermogenic capacity in deer mice (26) and that this delay appears to be associated with delays in the development of respiratory phenotypes (18). Therefore, it would be beneficial to examine whether the timeline for lung development is also altered in high-altitude deer mice, and whether developmental hypoxia may lead to adaptive or maladaptive plasticity in these mice.

3.3 Conclusions

Overall, my findings suggest that high-altitude deer mice partially attenuate the harmful consequences of maladaptive plasticity in the lungs. I showed that 1) highlanders attenuate HPH in response to chronic hypoxia (based on measurements of right ventricular systolic pressure), and eliminate the associated changes in the pulmonary vasculature and heart (right ventricle hypertrophy, and pulmonary arterial smooth muscle thickening), 2) V-Q matching at rest is not compromised after chronic hypoxia in either population, and 3) highland mice increase lung volume, while neither population change airway volume or alveolar surface density in response to chronic hypoxia. Overall, my results indicate that highland deer mice have evolved altered responses to chronic hypoxia that appear to be more favourable for maintaining O_2 uptake at high altitude compared to low-altitude deer mice. My thesis focused on the response to chronic hypoxia in adult deer mice of highland or lowland ancestry who were born and raised in common-garden conditions. This was beneficial for determining the role of genetically based changes in the physiological responses to chronic hypoxia exposure. Future research examining the structure and function of the lung in early development when exposed to chronic hypoxia could provide further insight into how deer mice can thrive at high altitude.

3.4 References

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