HIGH-ALTITUDE ADAPTATION AND CONTROL OF BREATHING IN DEER MICE (*PEROMYSCUS MANICULATUS*)
HIGH-ALTITUDE ADAPTATION AND CONTROL OF BREATHING IN DEER MICE (*PEROMYSCUS MANICULATUS*)

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TITLE: High-altitude adaptation and control of breathing in deer mice 
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LAY ABSTRACT
High-altitude environments are amongst the harshest on earth, with extremely low levels of oxygen, but some animals not only survive but thrive in these conditions. How these animals do so was previously not well understood. My thesis has uncovered how the evolution of respiratory physiology contributes to high-altitude adaptation in the deer mouse, the species with the broadest altitudinal distribution of any North American mammal, and has elucidated the genetic mechanisms involved. My work contributes to understanding nature’s solutions to oxygen deprivation – an all too common problem in many human and animal diseases.
ABSTRACT

For animals at high altitude, low oxygen (hypoxia) is an unremitting stressor that has the potential to impair metabolism and performance. The hypoxic chemoreflex senses reductions in the partial pressure of O\textsubscript{2} in the arterial blood and thus elicits many of the physiological responses to hypoxia, including increases in breathing and activation of the sympathetic nervous system. The hypoxic chemoreflex is vital to surviving acute exposure to severe hypoxia, but the advantage of this reflex during chronic hypoxia is less clear. The goals of my thesis were to examine how control of breathing by the hypoxic chemoreflex has evolved in high-altitude natives to maintain O\textsubscript{2} transport in chronic hypoxia, and to elucidate the potential genetic mechanisms that were involved. This was accomplished using deer mice (\textit{Peromyscus maniculatus}) native to high- and low-altitudes, in addition to a strictly low-altitude species (\textit{P. leucopus}). I found that high-altitude deer mice breathe with higher total ventilation using preferentially deeper breaths, contributing to higher O\textsubscript{2} saturation of arterial blood, but in contrast to lowland mice highlanders do not exhibit ventilatory plasticity in response to chronic hypoxia. These phenotypes appeared to be uniquely evolved in the highland population and arise during the onset of endothermy in early post-natal development. I then used second-generation inter-population hybrids to evaluate the effects of genetic variation (specifically, in the hypoxia-inducible factor 2a gene \textit{Epas1} and in haemoglobin genes) on an admixed genomic background. The high-altitude variant of \(\alpha\)-globin could completely explain the deep breathing pattern of highland mice, whereas the high-altitude variant of \textit{Epas1} and possibly \(\beta\)-globin contributed to their apparent lack of ventilatory plasticity in response to chronic hypoxia. Together, the physiological changes elicited by these mutations
contribute to maintaining O₂ uptake and metabolism in the cold and hypoxic environment at high altitude.
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LIST OF ABBREVIATIONS AND SYMBOLS

2,3-DPG: 2,3-diphosphoglycerate

ACR: Air convection requirement

ANOVA: Analysis of variance

BTPS: Body temperature and pressure saturated

CNS: Central nervous system

CO₂: Carbon dioxide

DAPI: 4′,6-diamindino-2-phenylindole

EGLN1: Egl-9 homolog 1

EPAS1: Endothelial PAS domain-containing protein 1

F₁: First-generation intercross progeny

F₂: Second-generation intercross progeny

G₁: First generation progeny

G₂: Second generation progeny

GADPH: glyceraldehyde 3-phosphate dehydrogenase

GAP-43: Growth-associated protein-43

GFAP: Glial fibrillary acidic protein

H: High-altitude variant

Hb: Haemoglobin

HbA: α-globin

HbB: β-globin

Hct: Haematocrit

HIF: Hypoxia inducible factor
HVR: Hypoxic ventilatory response

IEF: Isoelectric focusing

isoHb: Haemoglobin isoform

kPa: Kilopascal

L: Low-altitude variant

MS/MS: Tandem mass spectrometry

NF: Neurofilament

NGS: Normal goat serum

NO: nitric oxide

NTS: Nucleus tractus solitarius

O2: Oxygen

P: Post-natal day

P50: PO2 at which haemoglobin is 50% saturated with O2

PCO2: Partial pressure of carbon dioxide

PBS: Phosphate buffered saline

PHD2: Prolyl hydroxylase domain-containing protein 2

PO2: Partial pressure of oxygen

RNA-seq: RNA sequencing

RT-PCR: reverse-transcriptase PCR

SaO2: Arterial O2 saturation

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM: Standard error of the mean
SNS: Sympathetic nervous system
STP: Standard temperature and pressure
Tb: Body temperature
TH: Tyrosine hydroxylase
TX: Triton X-100
UTR: Untranslated region
VAH: Ventilatory acclimatization to hypoxia
VCO2: Rate of carbon dioxide production
VO2: Rate of oxygen consumption
VO2max: maximal rate of oxygen consumption
WGCNA: weighted gene co-expression network analysis
DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis is organized in a sandwich format, as recommended and approved by members of my supervisory committee and approved by McMaster University. It consists of seven chapters. Chapter 1 is an overview of background material and hypotheses tested. Chapters 2 through 6 are manuscripts that are published, accepted or ready to be submitted for publication in peer reviewed scientific journals. Chapter 2 is referred to as Ivy and Scott (2017), Chapter 3 is referred to as Ivy and Scott (2018), Chapter 4 is referred to as Ivy, Cheviron, and Scott (2020), Chapter 5 is referred to Ivy et al., (2020a), and Chapter 6 is referred to as Ivy et al., (2020b). Chapter 7 summarizes the major findings of this thesis, places these findings in a context of current knowledge, and indicates future directions that this research may take.

CHAPTER 1
GENERAL INTRODUCTION

CHAPTER 2
CONTROL OF BREATHING AND VENTILATORY ACCLIMATIZATION TO HYPOXIA IN DEER MICE NATIVE TO HIGH ALTITUDES

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**CHAPTER 4**

**GENETIC VARIATION IN HIF-2α ATTENUATES VENTILATORY SENSITIVITY AND CAROTID BODY GROWTH IN CHRONIC HYPOXIA IN HIGH-ALTITUDE DEER MICE**

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**CHAPTER 5**

**GENETIC VARIATION IN HAEMOGLOBIN ALTERS CONTROL OF BREATHING IN HIGH-ALTITUDE DEER MICE**

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**CHAPTER 6**

**ONTOGENESIS OF EVOLVED CHANGES IN RESPIRATORY PHYSIOLOGY IN DEER MICE NATIVE TO HIGH ALTITUDES**
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**CHAPTER 7  GENERAL DISCUSSION**
CHAPTER 1: GENERAL INTRODUCTION
CONTROL OF BREATHING AND CIRCULATION IN HIGH-ALTITUDE NATIVES


Understanding the roles of phenotypic plasticity and evolutionary adaptation along environmental gradients provides insight into how the environment can shape the phenotypes of organisms. Phenotypic plasticity, broadly defined as phenotypic responses to environmental change that occur within the lifetime of an individual, can in some cases allow individuals to better cope with novel environments. However, phenotypic plasticity is not always beneficial, and can sometimes lead to detrimental outcomes with prolonged exposure (Ghalambor et al., 2007; Storz and Scott, 2019; Storz et al., 2010a; Woods and Harrison, 2002). Furthermore, phenotypic plasticity can facilitate or impede evolutionary adaptation (Chevin and Hoffmann, 2017; Lande, 2015; Pigliucci et al., 2006; Schneider and Meyer, 2017), and phenotypic plasticity can itself evolve (Chevin and Hoffmann, 2017; Nussey et al., 2005). However, the importance of phenotypic plasticity and evolutionary adaptation in challenging environments is not well understood, particularly for complex physiological phenotypes.

High-altitude environments provide fertile ground for examining the roles of phenotypic plasticity and evolutionary adaptation in challenging environments. High-altitude is characterized by multiple stressors, including cold temperatures and hypobaric hypoxia (low O2), posing multiple challenges to the organisms that inhabit them. Unlike temperature, which fluctuates daily and seasonally, the low partial pressure of oxygen
(PO$_2$) at high altitude is unavoidable and unremitting. For example, many high-altitude human and mammal populations live, grow, and reproduce at over 4,000 m elevation where the PO$_2$ is roughly 60% of that at sea level. Some birds are also known to fly at high altitudes as a part of their natural migration, despite the fact that flight is an O$_2$ demanding process that becomes all the more challenging in a hypoxic environment (Hawkes et al., 2013; Ward et al., 2002). Since animals at high altitude still have to perform all the same functions as animals at sea level, this implies that of the two general strategies for coping with hypoxia – reduction of O$_2$ demands by metabolic depression (Boutilier, 2001) and/or increases in the supply of O$_2$ through the O$_2$ transport pathway (Hochachka, 1986) – only the latter is a feasible option for the survival and fitness of animals native to high altitude. Therefore, high altitude is a good environment for studying the role of phenotypic plasticity and genetic adaptations in the O$_2$ transport pathway for coping with O$_2$ deprivation.

Aerobic performance, such as occurs during exercise or sustained thermogenesis, could in principle be enhanced in high-altitude hypoxia by increases in the capacity to transport O$_2$ along the O$_2$ cascade. The O$_2$ cascade is composed of pulmonary ventilation, pulmonary O$_2$ diffusion, circulation of O$_2$ in the blood, tissue O$_2$ diffusion, and mitochondrial O$_2$ utilization (Figure 1.1). Enhancements at any step of the O$_2$ cascade could foreseeably be achieved through phenotypic plasticity or evolved genetically-based adaptations, which could thus augment O$_2$ transport through the entire cascade. The highland phenotype – arising through adaptation or various mechanisms of phenotypic plasticity – can include changes in many morphological traits that enhance the capacity
for $O_2$ diffusion, including increases in the surface area of the pulmonary air-blood interface (Hsia et al., 2005; Hsia et al., 2007; Ravikumar et al., 2009), increases in the capillarity of peripheral tissues (Lui et al., 2015; Scott et al., 2015), and a redistribution of mitochondria towards capillaries to a subsarcolemmal location in the skeletal muscle (Mahalingam et al., 2017; Scott et al., 2009). These components of the highland phenotype are extremely important at high altitudes, and have been well reviewed elsewhere (Scott, 2011; Storz and Scott, 2019; Storz et al., 2010a). Additionally, the $O_2$ cascade relies on adequate rates of convective $O_2$ transport, both pulmonary ventilation (the first step of the cascade) and $O_2$ circulation, in order to maintain tissue $O_2$ supply. Therefore, altering pulmonary ventilation and/or $O_2$ circulation may have a critical influence on $O_2$ transport in hypoxic environments (Birchard and Tenney, 1986; Brutsaert, 2007), and is reviewed in the following sections.

**1.1 Phenotypic plasticity and high-altitude hypoxia**

The response of lowland animals to high-altitude hypoxia provides insight into whether phenotypic plasticity can generally be regarded to facilitate or impede evolutionary adaptation in animals that colonize high altitudes (Storz et al., 2010a). Acute exposure to hypoxia (minutes to hours) initiates the hypoxic chemoreflex, resulting in many rapid physiological responses to better cope with hypoxia in adult animals, such as increases in ventilation (the hypoxic ventilatory response, HVR) and activation of the sympathetic nervous system (SNS). Chronic exposure to hypoxia results in amplification of the hypoxic chemoreflex, with further increases in ventilation (ventilatory acclimatization to hypoxia, VAH) and persistent activation of the SNS. Unlike the acute
response, which increases the likelihood of survival, the advantage of maintaining the hypoxic chemoreflex in chronic hypoxia is less clear, as not all responses are beneficial and can be counterproductive for $O_2$ supply.

1.1.1 The hypoxic ventilatory response

The hypoxic ventilatory response is initiated within one breath of a reduction in arterial $PO_2$, and involves an increase in breathing that helps maintain $O_2$ transport (Brutsaert, 2007; Powell et al., 1998). The carotid body immediately senses the drop in arterial $PO_2$ and stimulates afferent sensory discharge in the carotid sinus nerve to initiate the HVR (Gonzalez et al., 1994; Nurse, 2010; Teppema and Dahan, 2010). Glomus (type I) cells in the carotid body are the primary sensor and release the main excitatory neurotransmitters (e.g. ATP, acetylcholine), but discharge from the carotid body can also be regulated by autocrine and paracrine mechanisms by the release of neuromodulators (e.g. dopamine, adenosine) from type I or type II cells (Nurse, 2010; Piskuric and Nurse, 2013). Recent evidence suggests that mitochondria may play a key role in $O_2$ sensing in glomus cells (Kumar and Prabhakar, 2012; Moreno-Domínguez et al., 2020), but other sensors may also be involved, including membrane bound channels (e.g. $O_2$-sensitive $K^+$ channels or $Na^+$ channels), $O_2$-sensitive enzymes (e.g. NADPH oxidase), heme proteins, and/or kinases (e.g. AMP kinase). Nerve impulses transmitted from the carotid sinus nerve reach the nucleus tractus solitarius (NTS) in the medulla, which then integrates the carotid body signal and delivers information about chemoreceptive drive to the respiratory central pattern generator (Gonzalez et al., 1993; Haxhiu et al., 1995; Milsom,
The resulting increase in efferent motor output in neurons innervating the respiratory muscles stimulates breathing frequency and/or tidal volume, leading to increased total ventilation and alveolar $\text{PO}_2$ at the gas exchange surface, and thus corrects or minimizes the drop in arterial $\text{PO}_2$ and oxygen content sensed by the carotid body.

Increases in ventilation are maintained for prolonged periods at high altitude in mammals (Brutsaert, 2007; Duffin and Mahamed, 2003), but the acute HVR is modulated by numerous neural and physiological factors at different time domains of the response (Barnard et al., 1987; Powell et al., 1998). In particular, prolonged durations at high altitude often lead to further increases in ventilation and enhanced ventilatory sensitivity to hypoxia (Powell et al., 1998; Powell et al., 2000a; Powell et al., 2000b; Slessarev et al., 2010a; Slessarev et al., 2010b; Yilmaz et al., 2005) that should help increase alveolar and arterial $\text{PO}_2$ (Fig. 1.1). This is termed ventilatory acclimatization to hypoxia (VAH) and occurs due to increases in both the chemoreceptor sensitivity of the carotid bodies and the responsiveness of central integration sites to afferent inputs from the carotid bodies (Dwinell and Powell, 1999; Kumar and Prabhakar, 2012; Vizek et al., 1987; Wilkinson et al., 2011). Several mechanisms are implicated in the former, including changes in ion channel densities and neurotransmitter stores in the glomus cells, glomus cell hypertrophy and/or hyperplasia (Wang et al., 2008), cell differentiation (Pardal et al., 2007), and/or neovascularization (Hempleman, 1995; Hempleman, 1996; Kusakabe et al., 1993; Prabhakar and Jacono, 2005). Recent research also proposes that signalling mediated by hypoxia inducible factor (HIF) 2α may underlie VAH and carotid body hypertrophy,
because mice in which HIF-2α was knocked out (either via constitutive heterozygous knockout, inducible conditional knockout across the body, or inducible conditional knockout in only tyrosine hydroxylase-expressing cells) exhibited attenuated VAH and reduced growth of the carotid body and hyperplasia of the glomus cells after chronic hypoxia (Fielding et al., 2018; Hodson et al., 2016). This attenuation appears to be uniquely linked to HIF-2α, as HIF-1α knockout did not exhibit any changes in VAH or carotid body growth (Hodson et al., 2016). In fact, HIF-2α appears to play a key role in inducing the genes responsible for O₂ sensing in the carotid bodies in general (Moreno-Domínguez et al., 2020). The mechanisms underlying the increases in CNS responsiveness to afferent chemoreceptor information from the carotid bodies that contribute to VAH are not as well understood, but probably involves changes in glutamatergic signalling in the NTS (Pamenter et al., 2014; Reid and Powell, 2005).

Changes in arterial carbon dioxide tension (PCO₂) and pH also affect breathing during high-altitude hypoxia (Slessarev et al., 2010a; Slessarev et al., 2010b). The HVR increases CO₂ loss and causes a secondary respiratory hypocapnia (reduced CO₂ in the blood) that can lead to blood alkalosis. Increases in CO₂/H⁺ strongly stimulate breathing via the carotid bodies and the central CO₂ chemoreceptors. Hypocapnia and alkalosis will therefore act at cross-purposes to the HVR, by inhibiting ventilation and impeding oxygen transport (Scott and Milsom, 2009). However, changes in CO₂/H⁺ in arterial blood and cerebral spinal fluid are not sufficient on their own to explain the time-dependent increase in ventilation during acclimatization, because chronic high-altitude hypoxia is associated with further decreases in PCO₂ and the secondary increases in
ventilation with VAH are not associated with recovery of pH in arterial blood or cerebrospinal fluid (Bureau and Bouverot, 1975; Powell, 2007). Nevertheless, it is possible that changes in the CO$_2$/pH sensitivity of breathing could influence the progressive increases in breathing at high altitude (Dwinell et al., 1997; Fatemian and Robbins, 1998).

1.1.2 The autonomic nervous system in hypoxia

Acute hypoxia activates the sympathetic nervous system, which acts to increase heart rate, systemic vascular resistance, and blood pressure (Hainsworth and Drinkhill, 2007). This is partly mediated by direct hypoxic stimulation of signalling from the carotid bodies, via some carotid body afferents that synapse with second order NTS neurons that then innervate the cardiovascular control centre of the medulla (Zera et al., 2019). The sympathetic nervous system can also be activated as a secondary consequence of the indirect effects of hypoxia, including (i) the response of pulmonary stretch receptors to the increase in breathing during hypoxia (Marshall, 1994; Reis et al., 1994) and (ii) baroreceptor responses to hypotension resulting from the direct vasodilatory actions of hypoxia and other local factors on vascular smooth muscle in the systemic circulation (Hainsworth and Drinkhill, 2007). Sympathetic activation is a key protective defense against acute hypoxia, because the strong vasoconstriction in most peripheral tissues helps redistribute blood flow to essential core tissues such as the heart and brain.

However, the importance of sympathetic activation in chronic hypoxia at high altitude is less clear. Sympathetic activity remains high after prolonged periods at high
altitude (Calbet, 2003; Dhar et al., 2014; Mazzeo et al., 1994), at least in humans, but heart rate tends to return to sea level values after acclimatization (Foster et al., 2014; Vogel and Harris, 1967). Although this may result in part from a reduction in chemosensory drive arising from the increase in arterial PO\textsubscript{2} with acclimatization (Gonzalez et al., 1993), it is also strongly contributed to by a desensitization of β-adrenergic receptors and a reduction in β-adrenoreceptor density in the cardiac tissue (Kacimi et al., 1992; León-Velarde et al., 2001; Light et al., 1984; Morel et al., 2003; Reeves et al., 1987; Voelkel et al., 1981). Increases in α\textsubscript{1}-adrenergic and M\textsubscript{2} muscarinic receptor densities, as well as increased parasympathetic activity, have also been observed with high-altitude acclimatization (Kacimi et al., 1993; León-Velarde et al., 1996; León-Velarde et al., 2001; Morel et al., 2003). The resulting hypoxia-induced decrease in cardiac chronotropic function is suggested to limit maximum heart rate (Richalet et al., 1989; Richalet et al., 1992).

Activation of the sympathetic nervous system and the release of local vasoactive substances together regulate systemic vascular resistance and blood flow in hypoxia. Forearm vasodilation can occur in humans in response to acute hypoxia (Dinenno et al., 2003; Halliwill, 2003), which may occur through the activation of locally released nitric oxide (NO) and stimulation of β\textsubscript{2}-adrenoreceptors (Blauw et al., 1995; Markwald et al., 2011; Weisbrod et al., 2001). However, systemic vascular resistance and blood pressure usually increase with acute hypoxia, because the vasoconstrictor actions of α-adrenoreceptor activation tends to outweigh the factors that promote vasodilation (Hainsworth and Drinkhill, 2007). Prolonged acclimatization to high-altitude hypoxia
shifts this balance towards vasodilation and partially alleviates the increased vascular resistance, by a mechanism that does not appear to involve changes in NO signalling (Coney et al., 2004), but α-adrenergic tone on the vasculature can remain elevated (Hainsworth and Drinkhill, 2007). Such persistent effects of sympathetic activation could be maladaptive at high altitude if it limits blood flow and O₂ supply to tissues (e.g., digestive and reproductive organs).

1.2 Evolutionary adaptation and high-altitude hypoxia

Overlaid upon the effects of phenotypic plasticity in species and populations that are indigenous to high altitude are evolved changes in physiology that may have been favoured by natural selection. Genetically based adaptations in respiratory physiology appear to have occurred in numerous highland taxa (Monge and Leon-Velarde, 1991; Scott, 2011; Storz et al., 2010a), but how evolutionary adaptations have changed the extent of phenotypic plasticity is poorly understood. This lack of understanding has arisen in large part because many previous studies of highland natives have not controlled for the influences of developmental plasticity or adult environment. One might expect the nature of evolved changes in plasticity to depend upon whether that plasticity is advantageous, detrimental, or has a neutral influence on survival and fitness in chronic hypoxia. Furthermore, relatively few studies have examined whether parallel evolution of physiological traits has occurred in independent highland lineages, but there are some examples of uniquely derived traits involved in respiratory control that have arisen in multiple highland lineages.
1.2.1 Unique traits of highland taxa

Several high-altitude species have been shown to breathe more effectively for O$_2$ transport (Brutsaert, 2007; McDonough et al., 2006; Pichon et al., 2009; Scott, 2011), which should increase the PO$_2$ at the gas-exchange interface (shifting A to D in Fig. 1.1). For example, plateau pika (*Ochotona curzoniae*) from the Tibetan plateau breathe using larger tidal volumes and lower breathing frequencies than domestic rats raised at low altitudes when compared at a given total ventilation rate (Pichon et al., 2009). This change in breathing pattern should reduce the contribution of dead space gas to alveolar PO$_2$ and thus increase alveolar ventilation (Fig. 1.1D). High-altitude human populations from the Tibetan Plateau and rosy finches (*Leucosticte arctoa*) native to high altitudes above 3,500 m in North America also breathe with larger tidal volumes than comparable lowlanders (Beall et al., 1997; Clemens, 1988). Developmental altitude was not controlled in the above studies, but there is some evidence that similar differences persist between highland and lowland natives when compared after being raised in common garden conditions at sea level. Bar-headed geese (*Anser indicus*), a species that summers on the Tibetan plateau and migrates across the Himalayas, breathe with deeper and less frequent breaths than closely-related lowland geese when raised at low altitude (Scott and Milsom, 2007). Parallel changes in breathing pattern have therefore occurred in multiple lineages of high-altitude animals that should improve pulmonary gas exchange in hypoxia.

Little is known as to how a more effective breathing pattern has evolved in high-altitude taxa. Respiratory groups located in the medulla and pons (the ventral respiratory
group, pre-Bötzinger and Bötzinger complexes, NTS, retrotrapezoid nucleus, and parafacial respiratory group) are known to modulate breathing pattern, but how these respiratory groups have been altered to elicit a fixed change in breathing pattern that is more effective for O$_2$ uptake in hypoxia is not known. It could foreseeably involve changes in how the NTS/respiratory groups integrate signals from the carotid body, changes in NTS/respiratory group signalling to appropriate motor nuclei (e.g., the phrenic motor neurons that innervate the diaphragm) and/or central gain in these motor nuclei, and/or changes in motor unit recruitment at the diaphragm for a given level of phrenic activation. Such changes in signalling and integration could produce the high-altitude breathing pattern, but this still remains to be investigated.

The HVR has also evolved in several highland lineages to change the response of total ventilation to hypoxia. For example, total ventilation in severe poikilocapnic hypoxia is higher in bar-headed geese than in lowland waterfowl when compared after each was raised in similar conditions at sea level (Black and Tenney, 1980; Scott and Milsom, 2007). In contrast, total ventilation during isocapnic hypoxia is similar between species, suggesting that bar-headed geese have a reduced sensitivity to respiratory hypocapnia that allows breathing to increase by a greater magnitude during environmental hypoxia (in which CO$_2$ levels are uncontrolled) (Scott and Milsom, 2007). Highland human populations from both the Himalayas and the Andes also have lower ventilatory sensitivities to CO$_2$ than lowlanders acclimatized to high altitude as adults (Slessarev et al., 2010a; Slessarev et al., 2010b). The blunted CO$_2$ chemosensitivity that is observed in multiple highland lineages should minimize the extent to which ventilatory
responses to hypocapnia undermine the hypoxic ventilatory response. Whether these consistent modifications in CO$_2$ chemosensitivity are complemented by changes in O$_2$ sensitivity is unclear, because findings have been inconsistent across highland taxa. High-altitude human populations from the Tibetan plateau have high resting ventilation and an increased ventilatory sensitivity to isocapnic hypoxia (Beall, 2000; Brutsaert, 2007; Moore, 2000; Slessarev et al., 2010a; Terblanche et al., 2005). In contrast, human populations from the Andes have low resting ventilation and a blunted response to isocapnic hypoxia compared to Tibetans and lowlanders (Brutsaert, 2007; Moore, 2000; Slessarev et al., 2010a). Although somewhat paradoxical with regards to the needs for oxygen transport at high altitudes, this blunted response could help reduce CO$_2$ loss and help maintain blood CO$_2$/pH homeostasis, or could reduce the energetic costs of breathing (Hochachka, 1986; Powell, 2007). Unfortunately, developmental plasticity was not adequately controlled in many of these human studies, but the available evidence suggests that population genetic background (ancestry) appears to have influenced breathing by altering several time domains of the HVR (e.g. hypoxic desensitization) or the presence and magnitude of developmental plasticity (Brutsaert, 2007).

Autonomic regulation of the heart is uniquely altered in many highland taxa, but previous findings have not always been consistent. The effects of sympathetic and parasympathetic nervous systems on heart chronotropic function are blunted in plateau pika raised at high altitudes compared to lowland rats (compared at a common intermediate altitude), in association with reduced expression of $\beta_1$-adrenoreceptors and M$_2$ muscarinic receptors in the heart (Pichon et al., 2013). Andean guinea pigs also have
lower heart rate sensitivity to sympathetic agonists compared to low-altitude lab-strain guinea pigs acclimatized to high altitude (León-Velarde et al., 1996). In contrast, however, cardiac M₂ muscarinic receptor density is increased in highlanders compared to lowland-strain guinea pigs acclimatized to high altitude (León-Velarde et al., 1996), which is associated with enhanced parasympathetic activity and a lower resting heart rate (Hartley et al., 1974; Hopkins et al., 2003; Hughson et al., 1994). These discrepancies highlight the potential differences between highland taxa, and emphasize that caution must be taken in ascribing adaptive significance to the uniquely-derived traits of highlanders. Nevertheless, the consistent finding of a blunted cardiac sympathetic sensitivity in both of these species could be extremely important for avoiding chronic over-excitation of the heart at high altitude.

The regulation of systemic blood flow in highland taxa is also distinct from that in lowlanders, and includes alterations in the sensitivity of the peripheral vasculature to catecholamines and NO. In adult Himalayan natives, activation of the sympathetic nervous system in response to high-altitude hypoxia is significantly blunted compared to sea-level natives after high-altitude acclimatization (Bernardi et al., 1998). All else being equal, this could minimize α-adrenoreceptor mediated restriction of blood flow in peripheral tissues, and could thus help improve the functional capacity for O₂ diffusion (helping to shift C to F in Fig. 1.1). However, fetal and neonatal llamas have increased peripheral vasoconstrictor sensitivity to α-adrenergic stimulation compared to lowland sheep, along with different populations of α₁-adrenergic receptor subtypes expressed in the femoral vasculature (Giussani et al., 1996; Moraga et al., 2011). This unique trait has
obvious benefits for coping with short-term reduction in O$_2$ availability in utero, which are likely magnified at high altitude, because it should facilitate the redistribution of blood to essential tissues during hypoxia. The benefit of this trait after birth is less clear, because hypoxia is encountered chronically and blood flow to peripheral tissues must resume. It is possible that neonates have temporarily retained the characteristics of fetal llamas, but it is unclear if this trait disappears as development continues. Peripheral blood flow in hypoxia could be maintained in llamas by adaptations that enhance arterial oxygenation and/or reduce sympathetic activation or by increases in NO-mediated vasodilation. Inhibition of NO synthase increases femoral artery pressure and total femoral vascular resistance substantially in fetal llamas, suggesting that NO maintains a significant baseline vasodilatory tone (Sanhueza et al., 2005). NO production is also higher in adult Tibetans in association with enhanced systemic blood flow in peripheral tissues (Erzurum et al., 2007).

Haemoglobin-O$_2$ affinity has been altered in high-altitude taxa to optimize blood-O$_2$ transport in hypoxia. Many high-altitude taxa exhibit increased haemoglobin affinity for O$_2$ due to amino acid substitutions which shift the allosteric equilibrium in favour of the relaxed (R)-state oxyHb conformation (enhancing the ability of Hb to bind and hold O$_2$) compared to the tense (T)-state (Storz and Moriyama, 2008). This shift in allosteric equilibrium facilitates O$_2$ loading at the lungs, but could hinder O$_2$ unloading at the tissues. Although it is possible that some high-altitude taxa overcome this trade-off on O$_2$ unloading by augmenting the activity of allosteric modulators of Hb-O$_2$ binding, such as
CO₂/pH (Bohr effect) or 2,3-DPG, but there is not strong support for this possibility (Storz, 2007; Storz, 2016; Storz and Moriyama, 2008; Storz et al., 2010a).

1.2.2 Interactions between evolutionary adaptation and phenotypic plasticity

Studying species across broad altitudinal ranges is a powerful means for understanding the interaction between adaptation and phenotypic plasticity. Species that are restricted to high elevation, such as the llama, plateau pika, and Andean goose, are well-diverged from their closest lowland relatives, and thus well-suited to elucidating the physiological mechanisms underlying the most extreme highland phenotypes. However, mechanistic studies of population-level variation within broadly distributed species provide the greatest experimental power for elucidating the genetic and environmental sources of phenotypic variation and for distinguishing the evolutionary forces involved (e.g., adaptation, drift, etc.) (Storz et al., 2010a).

Evolved differences in transcriptional regulation and/or genetic variants may underlie the interactions between adaptation and plasticity in indigenous highlanders (Appenzeller et al., 2006; Cheviron et al., 2008; Cheviron et al., 2013; Storz et al., 2010a). For example, population, environment, and population-environment interaction effects on gene expression have been observed in comparisons of highland and lowland deer mice, and some of this variation is correlated with variation in VO₂max (maximal cold-induced thermogenic capacity) and muscle phenotype (Cheviron et al., 2013).

Recent research is providing greater insight into the genomic basis of high-altitude adaptation across mammals and birds (Beall et al., 2010; Gou et al., 2014; Graham and
McCracken, 2019; Petousi et al., 2014; Schweizer et al., 2019; Simonson, 2015; Simonson et al., 2012). Many candidate genes involved in O\textsubscript{2} sensing have been uncovered in genome scans in high-altitude populations, such as genes in the HIF pathway that regulate the expression of hundreds of genes throughout development and in several different tissues during acute and chronic hypoxia (Petousi and Robbins, 2014; Scheinfeldt and Tishkoff, 2010; Scheinfeldt et al., 2012; Simonson, 2015; Simonson et al., 2012). For example, genome-wide scans have identified EPAS1 (the gene encoding HIF-2α) and EGLN1 (the gene encoding the O\textsubscript{2} sensing prolyl hydroxylase PHD2, which targets HIF-α subunits for degradation in normoxic conditions) as top candidate genes underlying high-altitude adaptation in Tibetan and Andean humans (Petousi et al., 2014; Scheinfeldt and Tishkoff, 2010; Simonson et al., 2012) as well as several species of highland mammals and birds (Gou et al., 2014; Graham and McCracken, 2019; Li et al., 2013; Schweizer et al., 2019). High-altitude variants of EPAS1 are associated with lower Hb concentrations in Tibetans (Beall et al., 2010), suggestive of hyporesponsive HIF pathways (Simonson, 2015; Storz and Scott, 2019). High-altitude variants of EGLN1 appear to increase HIF degradation under hypoxic conditions in Tibetan humans (Lorenzo et al., 2014), and are associated with increased aerobic capacity (\(\dot{V}O_2\text{max}\)) in hypoxia in high-altitude Andean populations (Brutsaert et al., 2019). Genetic variants in alpha and beta haemoglobins have also been well documented in many high-altitude taxa (Natarajan et al., 2013; Projecto-Garcia et al., 2013; Storz et al., 2009; Storz et al., 2010b; Tufts et al., 2015), and often act to increase Hb-O\textsubscript{2} affinity (Storz, 2016). However, despite increasing information about the genomic basis of high-altitude adaptation, there remains
a poor understand of how such genetic variants alter physiology, and how they may alter phenotypic plasticity in high-altitude taxa.

1.3 Deer mice (*Peromyscus maniculatus*)

Deer mice (*Peromyscus maniculatus*) are an emerging model species for studying high-altitude adaptation. Deer mice are broadly distributed across North America and can be found from sea level to over 4,300 m elevation in the Rocky Mountains (Hock, 1964; Natarajan et al., 2015; Snyder et al., 1982). Highland populations of deer mice are genetically distinct from lowland populations, with low levels of gene flow across the Great Plains-Colorado Front Range transition (Storz et al., 2012), and high-altitude populations are thought to have evolved from low-altitude ancestors (Natarajan et al., 2015; Schweizer et al., 2019). High-altitude populations have also recently been shown to have experienced a history of spatially varying selection on many genes, including *Epas1* and haemoglobins (Schweizer et al., 2019; Storz et al., 2012), resulting in high frequencies of unique high-altitude variants. These features of deer mice make them ideal for elucidating the mechanistic basis of evolutionary adaptation and plasticity in high-altitude environments.

Deer mice are subject to strong directional selection for a high aerobic capacity ($\dot{V}O_{2\text{max}}$) for thermogenesis at high altitude (Hayes and O’Connor, 1999), presumably due to the high premium on thermogenesis in the cold, which has led to evolved increases in $\dot{V}O_{2\text{max}}$ in high-altitude populations (Cheviron et al., 2012; Cheviron et al., 2013; Cheviron et al., 2014; Lui et al., 2015; Tate et al., 2017). Increased Hb-O$_2$ binding affinity has also evolved in high-altitude deer mice, due to molecular and structural changes in the
alpha and beta globin subunits, which are expected to improve arterial O\textsubscript{2} saturation in hypoxia (Chappell and Snyder, 1984; Natarajan et al., 2013; Storz et al., 2009; Storz et al., 2010b). Integrative physiological studies have also noted that adult high-altitude deer mice exhibit altered cardiac function, muscle capillarity and metabolic phenotype, mitochondrial physiology, and tissue gene expression compared to lowland conspecifics and congeners (Dawson et al., 2018; Lau et al., 2017; Lui et al., 2015; Mahalingam et al., 2017; Scott et al., 2015; Tate et al., 2017). Whether there are changes in the control of breathing and O\textsubscript{2} chemosensitivity in high-altitude deer mice to enhance O\textsubscript{2} uptake in hypoxia, and the potential genetic basis for these changes were previously unknown. My thesis sought to shed light on these issues.

1.4 Aims and Objectives

Although the control of breathing has been characterized in many high-altitude taxa (reviewed above), few studies have compared between high- and low-altitude populations of a widely distributed species, and fewer still have investigated the genetic basis for evolved differences. My thesis therefore utilized high- and low-altitude populations of deer mice to disentangle plasticity and evolution using common garden exposures in normoxia and hypoxia. The primary objectives of this thesis were to understand (i) how plasticity and evolution have altered the control of breathing and the hypoxic chemoreflex in high-altitude deer mice, both in normal sea level conditions and after exposure to chronic hypoxia, and (ii) what genes may underlie these observed differences in the control of breathing. A secondary objective was to understand how
these evolved differences in breathing develop within an individual’s lifetime, as this is not well understood.

For my thesis, I used a common garden experimental design to assess population-environment interactions. In these experiments, I used adult mice that were the first-generation (G1) and second-generation (G2) lab-reared descendants of wild-caught deer mice and white-footed mice (*Peromyscus leucopus*). High-altitude deer mice (*P. m. rufinus*) were trapped on the summit of Mount Evans, Colorado (4,350 m above sea level), while low-altitude deer mice (*P. m. nebrascensis*) and white-footed mice (a species found exclusively at low altitude) were trapped at Nine Mile Prairie (430 m above sea level) Lancaster County, Nebraska. To assess the genetic underpinnings of my findings, I used a second-generation population intercross (F2) breeding design to disrupt the linkages between loci that result from population genetic structure, so that the effects of gene variants on the control of breathing could be evaluated on an admixed genomic background. This was conducted by hybridizing wild high- and low-altitude populations of deer mice to produce first-generation intercross (F1) progeny, which were used for full-sibling matings to produce F2 progeny. F2 mice were then genotyped for genetic variants in my genes of interest, *Epas1* and haemoglobins. Using these experimental designs, I conducted a series of studies outlined in the following 5 chapters, to test the over-arching hypothesis that deer mice native to high altitude have evolved changes in the control of breathing and the hypoxic chemoreflex that improve O₂ transport in chronic hypoxia.
1.5 Chapter summaries

Chapter 2: Control of breathing and ventilatory acclimatization to hypoxia in deer mice native to high altitudes

Chapter 2 is published in *Acta Physiologica* (Ivy, C.M. and Scott, G.R. 2017). The objective of this study was to examine evolved differences in the control of breathing by hypoxia and how the control of breathing was modified after chronic hypoxia in high-altitude deer mice. As expected, exposure to chronic hypoxia increased breathing (termed ventilatory acclimatization to hypoxia, VAH) and induced carotid body growth in low-altitude mice (*P. leucopus*). In contrast, high-altitude deer mice did not exhibit VAH or carotid body growth, but they exhibited a fixed increase in breathing that was similar to hypoxia acclimated lowlanders, a deep breathing pattern that is more effective for gas exchange, and they maintained higher arterial O\textsubscript{2} saturation in hypoxia overall. These findings suggest that high-altitude deer mice have evolved high rates of alveolar ventilation without enlargement of the carotid bodies.

Chapter 3: Evolved changes in breathing and CO\textsubscript{2} sensitivity in deer mice native to high altitudes

Chapter 3 is published in the *American Journal of Physiology: Regulatory, Integrative, and Comparative Physiology* (Ivy, C.M. and Scott, G.R. 2018). The objective of this study was to investigate how the control of breathing by CO\textsubscript{2} has evolved in deer mice and to determine whether variation in CO\textsubscript{2} sensitivity might contribute to the differences in breathing and VAH between populations that were observed in Chapter 2. I showed that ventilatory sensitivity to hypercarbia (elevated
inspired CO$_2$) increased after hypoxia acclimation, but was generally similar between highland and lowland populations. There was some modest support for the possibility that high-altitude deer mice have enhanced ventilatory sensitivity to respiratory hypocapnia after hypoxia acclimation, but this could not fully explain their apparent absence of VAH.

**Chapter 4: Genetic variation in HIF-2α attenuates ventilatory sensitivity and carotid body growth in chronic hypoxia in high-altitude deer mice**

Based on findings in Chapter 2 and 3, the objective of Chapter 4 was to investigate whether the attenuation of VAH and carotid body growth after chronic hypoxia in high-altitude deer mice was the result of genetic changes in the HIF-2α gene *Epas1*. I found that the effects of chronic hypoxia on breathing and carotid body growth were strongly attenuated in mice that were homozygous for the high-altitude *Epas1* allele (*Epas1$^{H/H}$), consistent with comparisons between populations in Chapters 2 and 3. My findings suggest that HIF-2α plays an important role in modulating breathing and carotid body growth in chronic hypoxia, and that genetic variation in *Epas1* is at least partially responsible for the absence of VAH in high-altitude deer mice.

**Chapter 5: Genetic variation in haemoglobin alters control of breathing in high-altitude deer mice**

Based on findings in Chapters 2 and 3, the objective of Chapter 5 was to investigate whether the genetic variants of haemoglobin genes in high-altitude deer mice contributed to their fixed increase in effective ventilation (manifest as deeper but less
frequent breaths). Some previous studies have suggested that Hb function could influence breathing (El Hasnaoui-Saadani et al., 2007; Izumizaki et al., 2003; Lipton et al., 2001), but the potential role of Hb in the control of breathing is rarely examined. I found that mice that were homozygous for the highland α globin (HbA) haplotype not only had enhanced arterial O$_2$ saturation in hypoxia, but also breathed with the more effective breathing pattern (slower, deeper breaths) that is typical of high-altitude deer mice. These effects of HbA on breathing pattern persisted after exposure to chronic hypoxia, so genetic variation in α globin does not appear to affect responses to chronic hypoxia. Alternatively, the ventilatory response to hypoxia was augmented in normoxia-acclimated mice that were homozygous for the highland β-globin, with these differences abolished after hypoxia acclimation, suggesting that β-globin may influence the effects of chronic hypoxia on the control of breathing. Efaproxiral injection (reduces Hb-O$_2$ binding affinity) elicited no changes in breathing pattern in normoxia or acute hypoxia, suggesting that the differences in breathing pattern are not the result of acute blood Hb effects.

Overall, my findings uncover a previously unrecognized role of the α globin gene in regulating breathing pattern, and that genetic variation in α and β globin is also partially responsible for the evolved differences in the control of breathing I have described in high-altitude deer mice.

Chapter 6: Ontogenesis of evolved changes in respiratory physiology in deer mice native to high altitude
Chapter 6 is published in the *Journal of Experimental Biology* (Ivy, C.M., et al 2020). Based on the differences in breathing observed between adult highland and lowland mice in Chapters 2 and 3, the **objective** of Chapter 6 was to investigate when the evolved differences in respiratory physiology of high-altitude deer mice arise in early development. I found that increases in total ventilation and a more effective breathing pattern arise in highlanders at a time in early post-natal life corresponding to critical benchmarks in the full development of homeothermy. However, the onset of hypoxia responsiveness and carotid body growth shortly after birth was delayed in highland deer mice compared to lowland deer mice. Nevertheless, my findings suggest that the evolved changes in respiratory physiology in high-altitude deer mice become expressed in association with the post-natal development of increased aerobic requirements.
1.6 FIGURE
Figure 1.1. The oxygen cascade – oxygen tension (PO₂) at each step in the pathway could be increased in high-altitude natives by evolutionary adaptations and/or phenotypic plasticity. PO₂ declines along the length of capillaries as O₂ diffuses into target tissues, so a range of capillary PO₂ drives diffusion into tissues. PO₂ also declines with distances from capillaries, so there should be a range of cellular PO₂ depending on both capillary PO₂ and diffusion distance. Adapted from (Taylor and Weibel, 1981; Scott, 2011).
1.7 REFERENCES


CHAPTER 2

CONTROL OF BREATHING AND VENTILATORY ACCLIMATIZATION TO HYPOXIA IN DEER MICE NATIVE TO HIGH ALTITUDES


2.1 ABSTRACT

Aim: We compared the control of breathing and heart rate by hypoxia between high- and low-altitude populations of Peromyscus mice, to help elucidate the physiological specializations that help high-altitude natives cope with O\(_2\) limitation.

Methods: Deer mice (P. maniculatus) native to high altitude and congeneric mice native to low altitude (P. leucopus) were bred in captivity at sea level. The G1 progeny of each population were raised to adulthood and then acclimated to normoxia or hypobaric hypoxia (12 kPa, simulating hypoxia at ~4300 m) for 5 months. Responses to acute hypoxia were then measured during step-wise reductions in inspired O\(_2\) fraction.

Results: Lowlanders exhibited ventilatory acclimatization to hypoxia (VAH), in which hypoxia acclimation enhanced the hypoxic ventilatory response, made breathing pattern more effective (higher tidal volumes and lower breathing frequencies at a given total ventilation), increased arterial O\(_2\) saturation and heart rate during acute hypoxia, augmented respiratory water loss, and led to significant growth of the carotid body. In contrast, highlanders did not exhibit VAH – exhibiting a fixed increase in breathing that was similar to hypoxia acclimated lowlanders – and they maintained even higher arterial
O₂ saturations in hypoxia. However, the carotid bodies of highlanders were not enlarged by hypoxia acclimation and were similar in size to those of normoxic lowlanders. Highlanders also maintained consistently higher heart rates than lowlanders during acute hypoxia.

Conclusions: Our results suggest that highland deer mice have evolved high rates of alveolar ventilation and respiratory O₂ uptake without the significant enlargement of the carotid bodies that is typical of VAH in lowlanders, possibly to adjust the hypoxic chemoreflex for life in high-altitude hypoxia.
2.2 INTRODUCTION

The hypoxic chemoreflex is a key response to acute hypoxia that helps maintain tissue O\textsubscript{2} supply. The hypoxic chemoreflex is initiated by stimulation of O\textsubscript{2}-sensitive type I (glomus) cells in the carotid bodies, which triggers the hypoxic ventilatory response (HVR) to help offset reductions in arterial partial pressure of O\textsubscript{2} (Ivy and Scott, 2015; Powell et al., 1998). The hypoxic chemoreflex also activates the sympathetic nervous system, which increases systemic vascular resistance and blood pressure, and causes α-adrenoreceptor mediated vasoconstriction in many tissues, such that blood flow is preferentially redistributed towards core hypoxia-sensitive organs (i.e., brain and heart) (Hainsworth and Drinkhill, 2007; Ivy and Scott, 2015).

Chronic hypoxia alters many of the above responses to acute hypoxia that are initiated by the hypoxic chemoreflex. Ventilatory acclimatization to hypoxia (VAH) occurs after days to weeks of hypoxia exposure, leading to further increases in breathing and ventilatory O\textsubscript{2} chemosensitivity, which arise from growth and neovascularization of the carotid bodies (Kusakabe et al., 1993; Pardal et al., 2007; Powell et al., 1998; Wang et al., 2008) and from increases in central gain of the afferent signals transmitted to the brainstem (Pamenter et al., 2014; Reid and Powell, 2005). Sympathetic activation persists with prolonged exposure to hypoxia, which tends to increase systemic vascular resistance and blood pressure through continued α-adrenoreceptor mediated vasoconstriction, and opposes the effects of local factors promoting vasodilation (Bernardi et al., 1998; Calbet, 2003; Hainsworth and Drinkhill, 2007). Therefore, hypoxia acclimation elicits many physiological responses that are clearly beneficial to pulmonary O\textsubscript{2} uptake and tissue O\textsubscript{2}
supply (e.g., increases in ventilation), but certain responses to hypoxia (e.g., persistent sympathetic activation) may become counter-productive in chronic hypoxia by impeding O₂ supply to tissues to which blood flow is not prioritized.

Previous research suggests that the control of breathing is altered in populations or species that are native to high altitudes (Ivy and Scott, 2015; Monge and León-Velarde, 1991; Simonson, 2015; Storz et al., 2010a). High-altitude natives often evolve key adaptations to the hypoxic environment at high elevation, and can therefore provide unique insight into whether hypoxia responses are adaptive and favoured by natural selection, or maladaptive and counter-productive to long-term health and fitness (Carey et al., 2012; Dempsey and Morgan, 2015; Storz et al., 2010a). In some high-altitude populations or species, including Tibetan humans, plateau pika (Ochotona curzoniae), and bar-headed geese (Anser indicus), total and/or alveolar ventilation is similar or enhanced compared to lowlanders (Beall et al., 1997; Brutsaert, 2007; Lague et al., 2016; Moore, 2000; Pichon et al., 2009; Scott and Milsom, 2007). In contrast, some other highland taxa exhibit a blunted HVR, including Andean humans, guinea pigs (Cavia porcellus), and Andean geese (Chloephaga melanoptera) (Beall, 2000; Brutsaert et al., 2005; Hock, 1964; Schwenke et al., 2007). With some exceptions (Schwenke et al., 2007), the underlying mechanisms of these evolved changes in the control of breathing are poorly understood. It is possible that the general differences between high- and low-altitude taxa are associated with adjustments in the mechanisms of VAH, reflecting evolved changes in the hypoxia acclimation response (or other forms of phenotypic plasticity) in highlanders, but studies aimed at addressing this possibility by comparing
highlanders and lowlanders acclimated to both normoxic and hypoxic environments are rare. Further insight into the mechanisms underlying the evolved changes in the control of breathing, and how these mechanisms may impact other facets of the hypoxic chemoreflex, may illuminate the ultimate reasons for why distinct evolutionary paths have been taken by different taxa.

The objective of this study was to examine the control of breathing by hypoxia and how the acute hypoxia response is modified by VAH in high-altitude populations of deer mice (*Peromyscus maniculatus*). Deer mice are an extremely powerful model species for studying high-altitude physiology. They inhabit the largest altitudinal range of all North American mammals, from below sea level to over 4,300 m elevation (Hock, 1964). High-altitude natives in the wild must maintain elevated metabolic rates to support thermogenesis in the cold (Hayes, 1989). There is strong directional selection at high altitudes that favours high aerobic capacity (VO$_2$max) in hypoxia (Hayes and O’Connor, 1999), and high-altitude populations appear to have responded to selection with an elevated VO$_2$max in hypoxia during exercise (Lui et al., 2015) or thermogenesis (Cheviron et al., 2012; Cheviron et al., 2013) compared to low-altitude populations of deer mice and to low-altitude white-footed mice (*P. leucopus*). This appears to be underpinned by adaptive increases in haemoglobin-oxygen affinity (Chappell and Snyder, 1984; Storz et al., 2009; Storz et al., 2010b), and increases in the skeletal muscle in capillarity, oxidative capacity, and the abundance and intracellular distribution of mitochondria (Lui et al., 2015; Mahalingam et al., 2017; Scott et al., 2015). It is unknown
if changes in the control of breathing by hypoxia may help enhance respiratory O$_2$ uptake in high-altitude deer mice.

2.3 MATERIALS AND METHODS

2.3.1 Deer mouse populations

Wild adult mice were live trapped at high altitude on the summit of Mount Evans (Clear Creek County, CO, USA at 39°35’18”N, 105°38’38”W; 4,350 m above sea level) (P. m. rufinus) and at low altitude on the Great Plains (Nine Mile Prairie, Lancaster County, NE, USA at 40°52’12”N, 96°48’20.3”W; 430 m above sea level) (P. leucopus; white-footed mice), and were transported to McMaster University (elevation 50 m). Mice were bred within each population in common-garden lab conditions to produce G1 progeny. These progeny were born and raised in normoxic conditions until 6 months of age before experiments were conducted. All mice were held in standard holding conditions (24-25°C, 12:12 light-dark photoperiod) with unlimited access to food and water. 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 Acclimation treatments

To disentangle the influence of population origin and acclimation environment, mice were acclimated to i) standard normobaric normoxia holding conditions, or ii)
hypobraric hypoxia simulating the pressure at an elevation of 4,300 m (barometric pressure of 60 kPa, an \( O_2 \) partial pressure of approximately 12.5 kPa). This resulted in four treatment groups: normoxia acclimated lowlanders (n=14), normoxia acclimated highlanders (n=9), hypoxia acclimated lowlanders (n=15), and hypoxia acclimated highlanders (n=9). Specially designed hypobaric chambers were used for hypoxia acclimation, as previously described (Ivy and Scott, 2017; Lui et al., 2015). Mice were temporarily returned to normobaric conditions twice per week for <20 min for cage cleaning. After 18-20 weeks of acclimation, mice were subjected to ventilatory experiments.

For a subset of 5 mice in each treatment group, thermo-sensitive passive transponders (micro LifeChips with Bio-therm technology; Destron Fearing, Dallas, TX) were implanted into the abdominal cavity ~2 weeks before acclimation. Mice were lightly anaesthetized using isofluorane and the transponder was injected under the skin on the left side of the abdomen close to the leg using a sterile trocar (Destron Fearing). Mice were monitored closely for 1 week to ensure complete recovery.

2.3.3 Acute hypoxia responses

We used two methods for measuring the responses to acute stepwise hypoxia: (i) the barometric technique on unrestrained and uninstrumented mice; and (ii) double-chamber pneumotachography on restrained mice that were instrumented with pulse
oximetry collars. Most mice were tested using both methods, and were allowed to recover for a minimum of 2 days between experiments.

2.3.3.1 Hypoxia responses using the barometric technique

We measured hypoxia responses in unrestrained mice that were allowed to move freely within a single-chamber plethysmograph that has been previously described (Ivy and Scott, 2017). Mice were given 20-60 min to adjust to the chamber before experiments began (until noticeably relaxed and stable breathing was observed), with 21 kPa O$_2$ (balance N$_2$) supplied to the chamber at 600 ml/min. Measurements were then recorded for an additional 20 min at 21 kPa O$_2$, after which mice were exposed to 20 min step-wise reductions in inspired P$_{O_2}$ to 16, 12, 10, 9, and 8 kPa. Dry incurrent gases were mixed using precision flow meters (Sierra Instruments, Monterey, CA, USA) and a mass flow controller (MFC-4, Sable Systems, Las Vegas, NV, USA), such that the desired P$_{O_2}$ was delivered to the chamber at a constant flow rate of 600 ml/min. Body temperature (T$_b$) was measured using a mouse rectal probe (RET-3-ISO, Physitemp) at the end of the experiment and exactly 24 hours later for normoxic T$_{b}$ (This was used as a proxy for the T$_b$ at the start of the experiment, in order to avoid undue stress to the animals but also control for diurnal variation).

Breathing and metabolism were determined during the last 10 min at each P$_{O_2}$, as previously described (Ivy and Scott, 2017). Incurrent and excurrent air flows were subsampled at 200 ml/min; incurrent air was continuously measured for O$_2$ fraction (FC-
10, Sable Systems), and excurrent air was analyzed for water vapour (RH-300, Sable Systems), dried with pre-baked drierite, and analyzed for O$_2$ and CO$_2$ fraction (FC-10 and CA-10, Sable Systems). These data were used to calculate rates of O$_2$ consumption ($\dot{V}_{O_2}$), CO$_2$ production ($\dot{V}_{CO_2}$), and water loss, expressed at standard temperature and pressure (STP), as recommended by Lighton (Lighton, 2008). Chamber temperature was continuously recorded with a thermocouple (PT-6, Physitemp). Breathing frequency and tidal volume were measured from changes in flow across a pneumotachograph in the plethysmograph wall (which arise from the warming and humidifying of the air as it is inspired by the animal), detected using a differential pressure transducer (Validyne DP45, Cancoppas, Mississauga, ON, Canada). Tidal volume was calculated using established equations (Drorbaugh and Fenn, 1955; Jacky, 1980) assuming a constant rate of decline in T$_b$ with declining P$_{O_2}$ – an assumption that we have previously shown to result in similar tidal volumes to those calculated using direct T$_b$ measurements at each P$_{O_2}$ (Ivy and Scott, 2017) – and is expressed at STP. Total ventilation was determined as the product of breathing frequency and tidal volume, air convection requirement was calculated by dividing total ventilation by $\dot{V}_{O_2}$, and pulmonary O$_2$ extraction (%) was calculated as $\dot{V}_{O_2}$ divided by the product of total ventilation and inspired P$_{O_2}$. All data were acquired using a PowerLab 16/32 and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, USA).

T$_b$ measurements were also made at each P$_{O_2}$ for the subset of mice that were implanted with thermo-sensitive passive transponders. The hand-held scanner (Destron
Fearing) was placed beneath the plethysmograph to collect $T_b$ data as desired. $T_b$ was measured every minute throughout the experimental protocol, and the average value during the last 10 min at each $P_{O_2}$ is reported.

2.3.3.2 Hypoxia responses using double-chamber pneumotachography

We also measured hypoxia responses in mice that were restrained from moving in a double-chamber pneumotachograph (head-out respirometer) (Emka Technologies, Falls Church, VA, USA). The chamber and procedure have been previously described in detail (Ivy & Scott, 2017). Mice were restrained with their mouth and nose projecting into a head chamber (where inspired gases were controlled), separated from the body chamber with a rubber dam. Mice were then exposed to the same step-wise hypoxia exposure as described in 2.3.1, but with an incurrent flow rate of 500 ml/min to the head chamber.

Breathing, $O_2$ consumption, water loss, and rectal temperature were determined as described above (except tidal volume, as described below), and we also measured arterial $O_2$ saturation ($Sao_2$) and heart rate using pulse oximetry. Tidal volume was determined by measuring the direct breathing-induced changes in air flow across a pneumotachograph in the wall of the body chamber, using a pressure transducer (Validyne DP45-14, Cancoppas) calibrated with injections of known volumes into the body chamber. Water loss was entirely from the respiratory system, due to the design of the double-chamber plethysmograph, so we report these measurements in restrained mice as respiratory water loss (we do not report water losses from experiments with unrestrained animals, because
it is elevated from the effects of grooming and urine excretion). \( \text{Sao}_2 \) and heart rate were measured using MouseOx Plus pulse oximeter collar sensors (Starr Life Sciences, PA, USA). This was enabled by removing fur around the neck, ~2 days before experiments, using Nair® hair removal product.

### 2.3.4 Carotid body histology and analysis

Carotid bodies were sampled for morphological analysis after a minimum of 24 h recovery from hypoxia response experiments. Mice were euthanized with an overdose of isoflurane, and the bifurcations of the carotid artery were dissected and removed, fixed in 4% paraformaldehyde for 48 h, and cryoprotected in 24% sucrose solution. Samples were then frozen in embedding medium (Cryomatrix, ThermoFisher Scientific, ON, Canada) and stored at -80°C. Samples were serially sectioned at 10 μm in a cryostat (Leica CM1860, Germany) maintained at -20°C, mounted on slides, air dried, and stored at -80°C. Immunohistochemistry was used to identify type I (glomus) cells (using tyrosine hydroxylase, TH, as a marker), neurons (using neurofilament, NF, and growth associated protein-43, GAP-43 as concurrent markers), and nuclei (DAPI, 4′,6-diamidino-2-phenylindole, to assist with the identification of individual cells) on one set of serially sectioned slides, and type II cells (using glial fibrillary acidic protein, GFAP, as a marker) and nuclei (DAPI) on a second set. Sections were hydrated in phosphate buffered saline (PBS), and treated with blocking solution (PBS containing 0.5% Triton X-100 and 10% normal goat serum) for 1 h. Sections were then incubated overnight in blocking solution
that contained primary antibodies against TH (AB152, Millipore, CA, USA; 1:2000 dilution), NF (MAB1615, Millipore; 1:100 dilution), and GAP-43 (G9264, Sigma Aldrich, ON, Canada; 1:2000 dilution) for the first set of slides, or GFAP (Z033429-2, Agilent Technologies, CA, USA; 1:1000 dilution) for the second set of slides. The following morning, slides were rinsed well in PBS and then incubated in blocking solution that contained secondary antibodies (1:400 dilution) against the TH/GFAP primary antibodies (Alexa Fluor 488, goat anti-rabbit IgG; A11034, Life Technologies, ON, Canada) and (in the first set of slides only) both of the NF and GAP-43 primary antibodies (Alexa Fluor 594, goat anti-mouse IgG; A11032, Life Technologies) for 2 h. Sections were rinsed in PBS and incubated in PBS containing 0.5% TX and DAPI (Sigma-Aldrich; 1:100 000 dilution) for 25 min. Slides were rinsed thoroughly in PBS and mounted with Vectashield (Vector Laboratories, ON, Canada). Sections were imaged using an Olympus microscope with Northern Eclipse software (Elite version 8.0, Empix Imaging Inc., ON, Canada).

The first set of slides was used to measure total projected surface areas in each section of the whole carotid body, neurons, and type I cells. The second set of slides was used to measure total projected surface areas of type II cells. All of these area measurements were made using Nikon NIS Elements documentation software (v. 4.30.02). The number of type I cells in each section was manually counted using ImageJ software (v. 1.47). Carotid body volume, type I volume, type II volume, and neural volume were calculated as the sum of the volumes in each section of the carotid body (the latter was calculated as the product of surface area and section thickness) (Saiki et al.,
2006). The total number of glomus cells in the carotid body was calculated as the sum of all glomus cells counted across every carotid body section. 10 normoxia acclimated lowlanders, 9 hypoxia acclimated lowlanders, 8 normoxia acclimated highlanders, and 9 hypoxia acclimated highlander carotid bodies were used in this analysis.

2.3.5 Statistics

Two-factor ANOVA and Holm-Sidak post-tests were used throughout. The effects of acclimation environment (normoxia versus hypoxia) and acute exposure $P_O_2$ (repeated measure) on hypoxia responses were generally evaluated within each population (low- or high-altitude ancestry) to determine the impacts of hypoxia acclimation. However, the main effects of and interactions between population of origin (lowland versus highland) and $P_O_2$ (repeated measure) on breathing data were also evaluated in mice acclimated to normoxia and tested using the barometric technique, in order to directly compare between populations. The effects of population of origin and acclimation environment on carotid body traits were also analyzed using two-factor ANOVAs and Holm-Sidak post-tests. Values are reported as mean ± S.E.M. All statistical analysis was conducted with SigmaStat software (v. 3.5) with a significance level of $P < 0.05$.

2.4 RESULTS
2.4.1 Hypoxia responses using the barometric technique
Both populations of mice exhibited robust ventilatory responses to hypoxia, but highland deer mice exhibited a much deeper breathing pattern (Fig. 2.1). Total ventilation increased in response to acute hypoxia (Fig. 2.1A; main effect of $P_O_2$: $F_{5,105}=36.65$, $P<0.001$), reaching values in 8% $O_2$ that were 1.4- to 1.8-fold above those in normoxia, and there was no significant difference between populations (main effect of population: $F_{1,105}=2.77$, $P=0.111$). The hypoxic ventilatory response was driven primarily by significant increases in breathing frequency in both populations (main effect of $P_O_2$: $F_{5,105}=170.0$, $P<0.001$). However, highlanders exhibited much lower breathing frequencies overall ($F_{1,105}=5.83$, $P=0.025$), and there was a significant population×$P_O_2$ interaction ($F_{5,105}=5.71$, $P<0.001$) (Fig. 2.1B). Highlanders also took much deeper breaths than lowlanders (population effect: $F_{1,105}=10.43$, $P=0.004$), despite the reduction in tidal volume with increasing hypoxia severity ($P_O_2$ effect: $F_{5,105}=39.62$, $P<0.001$; population×$P_O_2$ interaction: $F_{5,105}=5.86$, $P<0.001$) (Fig. 2.1C).

Hypoxia acclimation augmented breathing in lowlanders, but not in highlanders (Fig. 2.2). Hypoxia acclimation had a strong main effect in lowlanders (acclimation effect: $F_{1,135}=5.69$, $P=0.024$), increasing total ventilation across a range of $P_O_2$ of acute hypoxia exposure ($P_O_2$ effect: $F_{5,135}=51.73$, $P<0.001$) (Fig. 2.2A). Breathing pattern also changed after hypoxia acclimation in lowlanders, which exhibited lower breathing frequencies at the most severe levels of hypoxia (acclimation effect: $F_{1,135}=16.13$, $P<0.001$; $P_O_2$ effect: $F_{5,135}=177.99$, $P<0.001$) and deeper tidal volumes overall (acclimation effect: $F_{1,135}=31.73$, $P<0.001$; $P_O_2$ effect: $F_{5,135}=13.07$, $P<0.001$) (Fig.
In contrast, hypoxia acclimation had no effect in highlanders on total ventilation (acclimation effect: $F_{1,80}=0.607, P=0.447$; $P_O_2$ effect: $F_{5,80}=17.43, P<0.001$), breathing frequency (acclimation effect: $F_{1,80}=0.571, P=0.461$; $P_O_2$ effect: $F_{5,80}=135.50, P<0.001$), or tidal volume (acclimation effect: $F_{1,80}=0.222, P=0.644$; $P_O_2$ effect: $F_{5,80}=62.57, P<0.001$), such that highlanders always responded to acute hypoxia in much the same way as hypoxia acclimated lowlanders (Fig. 2.2B,D,F).

Hypoxia acclimation reduced metabolic and $T_b$ depression in severe hypoxia in highlanders but not in lowlanders (Fig. 2.3). In lowlanders, $\dot{V}_O_2$ declined below 10 kPa $O_2$ ($P_O_2$ effect: $F_{5,135}=5.25, P<0.001$), a response to acute hypoxia that was slightly but not significantly affected by hypoxia acclimation (acclimation effect: $F_{1,135}=4.10, P=0.053$) (Fig. 2.3A). However, there were only small non-significant increases in the air convection requirement after hypoxia acclimation in lowlanders (acclimation effect: $F_{1,135}=1.55, P=0.224$; $P_O_2$ effect: $F_{5,135}=79.80, P<0.001$) (Table 2.1), suggesting that some subtle variation in $\dot{V}_O_2$ was associated with the observed variation in breathing with hypoxia acclimation (Fig. 2.2). Rectal $T_b$ measurements (data points on far left and right in Fig. 2.3C) declined by 3.4°C in normoxia acclimated lowlanders and 2.3°C in hypoxia acclimated lowlanders after acute exposure to 8 kPa $O_2$ ($P_O_2$ effect: $F_{1,27}=80.48, P<0.001$), but the effect of hypoxia acclimation on $T_b$ depression was not significant (acclimation effect: $F_{1,27}=3.83, P=0.061$). Measurements of $T_b$ at each $P_O_2$ (in the subset of mice with temperature-sensing implants; shown as data connected by lines in Fig. 2.3C) suggests that $T_b$ depression began at a $P_O_2$ of 9 kPa ($P_O_2$ effect: $F_{5,30}=26.15, P<0.001$).
P<0.001), and that hypoxia acclimation did not alter this onset of T_b depression (acclimation effect: F_{1,30}=0.073, P=0.796). In highlanders, \dot{V}_O_2 was significantly greater in severe hypoxia after hypoxia acclimation (acclimation effect: F_{1,80}=4.89, P=0.042), with the onset of \dot{V}_O_2 depression (relative to normoxia) occurring at 16 kPa O_2 in normoxia acclimated highlanders but not until 9 kPa O_2 in hypoxia acclimated highlanders (P_O_2 effect: F_{5,80}=12.49, P<0.001) (Fig. 2.3B). The increases in metabolism that occurred without any corresponding change in breathing in highlanders after hypoxia acclimation reduced the air convection requirement (acclimation effect: F_{1,80}=9.03, P=0.008; P_O_2 effect: F_{5,80}=44.29, P<0.001) (Table 1). Hypoxia acclimation also reduced the decline in rectal T_b measurements at 8 kPa O_2 (acclimation effect: F_{1,16}=7.26, P=0.016) in highlanders, from a T_b depression of 1.8°C in normoxia acclimated mice to only 1.0°C in hypoxia acclimated mice (P_O_2 effect: F_{1,16}=2.85, P<0.001) (Fig. 2.3D). Hypoxia acclimation shifted the P_O_2 at which T_b depression began from 12 kPa to 10 kPa O_2 in highlanders (acclimation×P_O_2 interaction: F_{5,30}=5.10, P=0.002) such that T_b was higher after acclimation across a range of P_O_2 (acclimation effect: F_{1,30}=6.31, P=0.048; P_O_2 effect: F_{5,30}=21.10, P<0.001) (Fig. 2.3D).

The population differences in how hypoxia acclimation affected metabolic and T_b depression were associated with similar patterns of variation in pulmonary O_2 extraction (Table 2.1). In lowlanders, O_2 extraction increased in response to acute reductions in P_O_2 (P_O_2 effect: F_{5,135}=3.63, P=0.004) but was not altered by hypoxia acclimation (acclimation effect: F_{1,135}=0.63, P=0.434). In highlanders, O_2 extraction also increased in response to
acute reductions in $P_O_2$ ($P_O_2$ effect: $F_{5,80}=4.38, P=0.001$), but extraction was higher in the most severe levels of hypoxia in hypoxia acclimated mice compared to normoxia acclimated mice (acclimation effect: $F_{1,80}=7.19, P=0.016$; acclimation×$P_O_2$ interaction: $F_{5,80}=5.23, P<0.001$).

### 2.4.2 Hypoxia responses using double-chamber pneumotachography

Hypoxia acclimation had similar effects on the hypoxic ventilatory response of each population in experiments using double-chamber pneumotachography compared to those using the barometric technique (Table 2.2, Fig. 2.4). Total ventilation increased after hypoxia acclimation in lowlanders (Fig. 2.4A), primarily resulting from overall increases in tidal volume. In contrast, total ventilation and breathing pattern were unaffected by hypoxia acclimation in highlanders (Fig. 2.4B), in which breathing was generally very similar to that in hypoxia acclimated lowlanders. We have previously shown that restraint can alter the magnitude of and relationship between breathing, $\dot{V}_O_2$, and pulmonary O$_2$ extraction compared to unrestrained animals, potentially due to the added stress associated with restraint (Wu and Kayser, 2006). Nevertheless, although the pattern of variation in $\dot{V}_O_2$ and pulmonary O$_2$ extraction were somewhat different in double-chamber pneumotachography experiments (Table 2.2) than in barometric technique experiments (see above), $\dot{V}_O_2$ depression occurred at and below 12-10 kPa O$_2$ in all groups, and O$_2$ extraction still increased with decreasing $P_O_2$ of acute hypoxia exposure.
Arterial O$_2$ saturation was consistently higher in highlanders than in lowlanders during hypoxia, and only part of this population difference was offset in lowlanders by hypoxia acclimation (Fig. 2.4). Sa$_O$$_2$ declined during acute hypoxia challenge in lowlanders ($P_O_2$ effect: $F_{5,105}$=574.24, $P$$<$0.001), but the magnitude of this decline was reduced after hypoxia acclimation (acclimation effect: $F_{1,105}$=29.40, $P$$<$0.001; acclimation×$P_O$$_2$ interaction: $F_{5,105}$=9.42, $P$$<$0.001) such that Sa$_O$$_2$ was elevated from 16 to 8 kPa O$_2$. In contrast, hypoxia acclimation had no effect on Sa$_O$$_2$ in highlanders (acclimation effect: $F_{1,60}$=1.74, $P$=0.211; $P_O$$_2$ effect: $F_{5,60}$=149.80, $P$$<$0.001). Sa$_O$$_2$ was ~6.5-13% higher in highlanders compared to hypoxia acclimated lowlanders across the full range of $P_O$$_2$, from 16 to 8 kPa O$_2$. This is supported by a post-hoc two-way ANOVA we performed to assess the effects of population and hypoxia acclimation on Sa$_O$$_2$ at 8 kPa O$_2$ (population effect: $F_{1,33}$=53.53, $P$$<$0.001; acclimation effect: $F_{1,33}$=10.51, $P$=0.003; population×acclimation interaction: $F_{1,33}$=4.72, $P$=0.037). When considering total ventilation as a function of Sa$_O$$_2$ (Fig. 2.4E,F), hypoxia acclimation led to a pronounced upwards and rightwards shift in the hypoxic ventilatory response of lowlanders but had no effect on the HVR of highlanders.

Respiratory water loss increased after hypoxia acclimation, and the effect of hypoxia acclimation appeared to be much greater in lowlanders than in highlanders (Fig. 2.5). In lowlanders, water loss was not influenced by the $P_O$$_2$ of acute hypoxia exposure ($P_O$$_2$ effect: $F_{5,105}$=0.61, $P$=0.691), but was elevated by 22-41% after hypoxia acclimation (acclimation effect: $F_{1,105}$=27.03, $P$$<$0.001) (Fig. 2.5A). Hypoxia acclimation also
increased water loss in highlanders (acclimation effect: $F_{1,60}=7.99$, $P=0.015$; $P_O_2$ effect: $F_{5,60}=1.18$, $P=0.332$) but by a lower magnitude (Fig. 2.5B), such that highlanders did not reach levels that were as high on average as hypoxia acclimated lowlanders.

Hypoxia acclimation increased heart rates in deep hypoxia in both populations (Fig. 2.6). There was a strong main effect of the $P_O_2$ of acute hypoxia exposure in both lowlanders ($F_{5,105}=5.86$, $P<0.001$) and highlanders ($F_{5,60}=5.85$, $P<0.001$), which was generally exhibited as an initial increase in heart rate in response to modest to moderate hypoxia followed by a decline in heart rate when the acute hypoxia challenge became more severe. Hypoxia acclimation altered the way in which heart rate changed in response to progressive increases in the severity of acute hypoxia, in both lowlanders (acclimation×$P_O_2$ interactions: $F_{5,105}=7.89$, $P<0.001$; acclimation effect: $F_{1,105}=5.02$, $P=0.035$) and highlanders (acclimation×$P_O_2$ interactions: $F_{5,60}=7.48$, $P<0.001$; acclimation effect: $F_{1,60}=0.87$, $P=0.370$), such that heart rates were higher after acclimation from 8-10 kPa $O_2$. Based on these results, we did a post-hoc two-way ANOVA on the effects of population and hypoxia acclimation on heart rates at 8 kPa $O_2$, and found that highlanders maintained ~9-18% higher heart rates in deep hypoxia (population effect: $F_{1,33}=9.40$, $P=0.004$; acclimation effect: $F_{1,33}=11.88$, $P=0.002$; population×acclimation interaction: $F_{1,33}=6.02$, $P=0.443$).

2.4.3 Carotid body morphology
Hypoxia acclimation enlarged the carotid bodies in lowlanders, with only a small non-significant increase in highlanders (Fig. 2.7). The total volume of the carotid body, the total number and volume of type I (glomus) cells (which composes ~15-19% of total carotid body volume), the total volume of type II cells (~22-30% of total carotid body volume), and the total volume occupied by nerve fibres (~19-27% of total carotid body volume) were all similar between populations in normoxia. However, carotid body volume increased far more after hypoxia acclimation in lowlanders (312%) than the non-significant increase in highlanders (153%) (population×acclimation interaction: $F_{1,32}=8.01, P=0.008$; population effect: $F_{1,32}=9.48, P=0.004$; acclimation effect: $F_{1,32}=20.22, P<0.001$) (Fig. 2.7I). The enlargement of the carotid body in lowlanders after hypoxia acclimation arose from increases in the number (Fig. 2.7J; population×acclimation interaction: $F_{1,32}=7.83, P=0.01$; population effect: $F_{1,32}=14.77, P<0.001$; acclimation effect: $F_{1,32}=30.13, P<0.001$) and total volume (Fig. 2.7K; population×acclimation interaction: $F_{1,32}=4.13, P=0.054$; population effect: $F_{1,32}=10.53, P=0.004$; acclimation effect: $F_{1,32}=14.16, P=0.001$) of type I cells, the total volume of type II cells (Fig. 2.7L; population effect: $F_{1,32}=0.542, P=0.468$; acclimation effect: $F_{1,32}=26.17, P<0.001$), and the total volume occupied by nerve fibres (Fig. 2.7M; population×acclimation interaction: $F_{1,32}=8.15, P=0.007$; population effect: $F_{1,32}=4.99, P=0.033$; acclimation effect: $F_{1,32}=15.50, P<0.001$). The relative expansion of type I cells and nerve fibres appeared to exceed that of type II cells, as the ratio of total type I cell volume to total type II cell volume increased in lowlanders but not in highlanders after hypoxia acclimation (Fig. 2.7N; population×acclimation interaction: $F_{1,32}=5.14, P=0.035$;
population effect: $F_{1,32}=13.24, P=0.002$; acclimation effect: $F_{1,32}=4.27, P=0.052$). Very few components of the carotid body were significantly altered by hypoxia acclimation in highlanders, with the exception of the small increase in total type II cell volume that was in proportion to the non-significant increase in type I cell volume (Fig. 2.7M,N).

2.5 DISCUSSION

The capacity for respiratory $O_2$ transport is a critical determinant of survival, fitness, thermoregulation, and exercise performance in high-altitude environments (Hayes, 1989; Hayes and O’Connor, 1999). Here, we show that the use of a more effective breathing pattern was fixed in deer mice from high-altitude populations, to a pattern only achieved by closely related low-altitude mice after hypoxia acclimation. This effective breathing pattern existed without any change in the size of the carotid bodies, and was associated with a high $SaO_2$ in hypoxia. Breathing, respiratory gas exchange, and carotid body size were unchanged by hypoxia acclimation in high-altitude mice, unlike the pronounced amplification of the hypoxic ventilatory response and carotid body growth that occurred in low-altitude mice. However, high-altitude mice maintained consistently higher heart rates than low-altitude mice in deep hypoxia. These findings suggest that ventilatory acclimatization to hypoxia does not occur in high-altitude deer mice, but that other mechanisms that are insensitive to hypoxia acclimation augment effective ventilation and safeguard respiratory $O_2$ uptake.
2.5.1 Population differences in $O_2$ transport

The appreciable differences in breathing pattern between populations in normoxia (Fig. 2.1) are consistent with some previous observations in other highland taxa, including plateau pika (Pichon et al., 2009) and bar-headed geese (Scott and Milsom, 2007). The mechanisms responsible for population differences in breathing pattern in normoxic mice are unclear, but they exist without any differences in carotid body size, glomus cell number, or innervation (Fig. 2.7). Similarly, differences in the control of breathing between Tibetan humans and sea level residents (Wu and Kayser, 2006) are not associated with differences in the size or number of glomus cells in the carotid bodies (Khan et al., 1988). Previous results in pikas suggest that pharmacological antagonism of glutamate receptors, inhibition of NO synthase, and inhibition of serotonin reuptake has no effect on resting ventilation and breathing pattern (Bai et al., 2015).

A deeper breathing pattern is expected to increase alveolar ventilation, and may thus be more effective for gas exchange and respiratory $O_2$ uptake, but it is generally considered to be more metabolically costly (Vitalis and Milsom, 1986). Whether this is also true at the lower air densities at high altitudes is unclear, because the influence of air density on breathing mechanics is poorly understood. Nevertheless, it is foreseeable that improvements in respiratory $O_2$ uptake could more than outweigh the small effect of changes in breathing pattern on the metabolic cost of breathing (which is believed to be <10% of basal metabolic rate) (Aaron et al., 1992; Skovgaard et al., 2016).
The population differences in breathing pattern in normoxia likely affected arterial O₂ saturation, but the appreciable improvement in SaO₂ in deep hypoxia in highlanders compared to lowlanders (Fig. 2.4A,B) likely arose in large part from the heightened blood-O₂ affinity of high-altitude deer mice (Chappell and Snyder, 1984; Snyder et al., 1982). Increases in haemoglobin-O₂ affinity have arisen in many high-altitude taxa, presumably as an adaptation to safeguard arterial O₂ loading (Storz, 2016; Weber, 2007). In deer mice, this has resulted from selection for several genetic variants in both α- and β-globin genes that interact to increase affinity in the presence and absence of allosteric effectors (e.g., 2,3-diphosphoglycerate) (Storz et al., 2007; Storz et al., 2009; Storz et al., 2012). These haemoglobin adaptations could increase SaO₂ \textit{in vivo} by up to 6.5-13% in hypoxia, based on the population differences in SaO₂ after hypoxia acclimation (when breathing pattern was similar between populations), but it is still unclear whether there are differences in lung structure or function that could also contribute to augmenting arterial O₂ loading in high-altitude deer mice.

Heart rates in deep hypoxia were consistently higher in highlanders than in lowlanders, in both normoxic and hypoxic acclimation environments (Fig. 2.6), which could indicate a greater capacity for circulatory O₂ delivery. A modest increase in heart rate was observed in all of our mice around 12-16 kPa O₂ (O₂ pressures that span the altitudinal range of high-altitude populations of deer mice in the wild), which likely results in part from the hypoxic chemoreflex (Hainsworth and Drinkhill, 2007; Yasuma and Hayano, 2000). However, heart rates in deep hypoxia were increased by hypoxia acclimation and were greater in highlanders than in lowlanders. It is possible that
differences in arterial $O_2$ saturation and corresponding variation in $O_2$ supply to cardiac tissue accounted for some of this variation in heart rate in deep hypoxia. Differences in sympathetic activation, $\alpha$-adrenoreceptor mediated vasoconstriction, and tissue blood flows could also contribute to the variation in heart rates between populations and with hypoxia acclimation.

### 2.5.2 Population differences in the hypoxia acclimation response

Hypoxia acclimation enhanced the hypoxic ventilatory response of lowland mice, such that it resembled the more effective (but fixed) breathing pattern of highland deer mice (Fig. 2.2), and it augmented arterial $O_2$ saturation in deep hypoxia (Fig. 2.4). This hypoxia acclimation response of lowlanders is typical of ventilatory acclimatization to hypoxia (VAH), a well-known process that occurs in domestic house mice (Bishop et al., 2013; Ivy and Scott, 2017; Soliz et al., 2005), rats (Pamenter et al., 2014; Pichon et al., 2009), guinea pigs (Yilmaz et al., 2005), and humans (Slessarev et al., 2010). Hypoxia acclimation had relatively few effects on breathing and pulmonary gas exchange in highland deer mice (Fig. 2.2), such that breathing pattern and the hypoxic ventilatory response appeared quite similar between populations after hypoxia acclimation. The apparent fixation in highlanders of the phenotypes induced by hypoxia acclimation in lowlanders could have resulted from the evolutionary process of genetic assimilation, in which an originally plastic phenotype becomes genetically fixed (assimilated) (Ehrenreich and Pfennig, 2016; Lande, 2015; Pigliucci et al., 2006).
Although the hypoxic ventilatory response was relatively similar between populations after hypoxia acclimation, the appreciable differences in carotid body morphology (Fig. 2.7) suggest that underlying neural mechanisms were very different. Hypoxia acclimation enlarged the carotid bodies, increased its complement of O$_2$-sensing glomus cells, and expanded innervation in lowland *Peromyscus* mice, consistent with past studies in domestic house mice (Edwards et al., 1971; Kato et al., 2010) and other species (Kusakabe et al., 1993; Pardal et al., 2007). This growth of the carotid body, and the resultant increase in afferent signalling to central respiratory centres, likely contributed to VAH in lowlanders (Barer et al., 1976; Bishop et al., 2013). In highlanders, however, carotid body morphology was not significantly altered by hypoxia acclimation and was similar to normoxic mice (Fig. 2.7). Therefore, the deep breathing pattern of highland deer mice existed without the enlargement of the carotid bodies that underlies a deep breathing pattern in lowlanders, so other neural mechanisms must be involved. One possibility is that the neural mechanisms contributing to VAH in the central nervous system (e.g., changes in glutamatergic signalling in the NTS) (Pamenter et al., 2014; Reid and Powell, 2005) have become fixed in highlanders. Another possibility is that a difference in breathing mechanics in highlanders, as observed in some other high-altitude groups (York et al., 2017), helps augment tidal volume.

Hypoxia acclimation increased respiratory water loss by a greater magnitude in lowlanders than in highlanders (Fig. 2.5). It is possible that the amplification of respiratory water loss was a detrimental consequence of VAH in lowlanders. However, respiratory water loss also increased (albeit by a lesser extent) in highlanders, which did
not exhibit VAH, suggesting that other unmeasured effects of hypoxia acclimation (e.g., changes in lung structure or function) may also be involved. Nevertheless, highlanders acclimated to hypoxia suffer greater water loss than lowlanders acclimated to normoxia, suggesting that improvements in respiratory gas exchange in high-altitude environments may come at the expense of greater stresses on water homeostasis.

Why has the normal hypoxia acclimation response been lost in high-altitude deer mice? VAH improves alveolar ventilation and respiratory gas-exchange in lowlanders, and makes them similar in several respects to hypoxia-acclimated highlanders, so why have high-altitude deer mice not simply maintained the VAH response that occurs in their low-altitude relatives? We believe these questions may be answered by considering population differences in the carotid bodies. The enhancement of the hypoxic chemoreflex that results from growth of the carotid bodies may be associated with persistent sympathetic activation in chronic hypoxia. The vasoconstrictor effects of chronic sympathetic activation would be expected to oppose the effects of local vasodilators and contribute to systemic hypertension and increased vascular resistance at rest and during exercise (Wolfel et al., 1991; Wolfel et al., 1994). Increases in carotid chemoreceptor sensitivity to CO₂ (which might be expected to result from growth of the carotid bodies) can also destabilize ventilation, especially during sleep, and contribute to periodic sleep apnea that results in severe intermittent hypoxia and magnifies sympathetic activation (Dempsey and Morgan, 2015; Xie et al., 2001). These changes can be pathological, and if they occur in deer mice, are probably counter-productive to fitness and reproductive success at high altitudes. High-altitude mice exhibit a more effective
breathing pattern without mounting a hypoxia acclimation response or expanding their carotid bodies, which may reflect an evolved strategy to enhance respiratory O₂ uptake without amplifying other effects of the hypoxic chemoreflex.

The above working hypothesis may provide a common underlying explanation for the divergence in the hypoxic ventilatory response between high-altitude lineages. High-altitude deer mice appear much like Tibetan humans, plateau pika, and bar-headed geese, which breathe as much or more than hypoxia-acclimated lowlanders (Beall et al., 1997; Brutsaert, 2007; Lague et al., 2016; Moore, 2000; Pichon et al., 2009; Scott and Milsom, 2007). This strategy contrasts the blunted HVR of Andean humans, guinea pigs, and Andean geese (Beall, 2000; Brutsaert et al., 2005; Schwenke et al., 2007). These differences have previously been suggested to reflect divergent evolutionary outcomes arising from trade-offs between O₂ uptake versus CO₂/pH homeostasis and the metabolic costs of breathing (Powell, 2007; Storz et al., 2010a). Our results here suggest that high-altitude deer mice may be more similar to the Andean taxa than first appears, insofar as they may all have evolved in such a way as to blunt or minimize the amplification of the hypoxic chemoreflex that occurs in chronic hypoxia. Some other highland taxa exhibit reduced sympathetic activation in hypoxia or a blunted sensitivity to sympathetic stimulation (Bernardi et al., 1998; León-Velarde et al., 1996; Pichon et al., 2013). Therefore, the seemingly divergent hypoxia responses between highland lineages may simply reflect distinct mechanisms for overcoming the common problem of persistent sympathetic activation.
### 2.6 TABLES AND FIGURES

Table 2.1. Air convection requirement and pulmonary O\(_2\) extraction of mice acclimated to normoxia or hypoxia, measured using the barometric method

<table>
<thead>
<tr>
<th>(P_O^2) (kPa)</th>
<th>(\text{Normoxia Acclimated Lowlanders})</th>
<th>(\text{Normoxia Acclimated Highlanders})</th>
<th>(\text{Hypoxia Acclimated Lowlanders})</th>
<th>(\text{Hypoxia Acclimated Highlanders})</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>29.56±4.04</td>
<td>33.10±2.15</td>
<td>40.52±2.33</td>
<td>38.54±2.65</td>
</tr>
<tr>
<td>16</td>
<td>35.85±5.09</td>
<td>44.29±2.50</td>
<td>46.42±3.05</td>
<td>40.26±2.14</td>
</tr>
<tr>
<td>12</td>
<td>44.99±4.81</td>
<td>55.13±3.55</td>
<td>56.41±3.21</td>
<td>45.70±1.72</td>
</tr>
<tr>
<td>10</td>
<td>50.31±5.23</td>
<td>71.96±6.22</td>
<td>68.14±3.65</td>
<td>50.61±2.01†</td>
</tr>
<tr>
<td>9</td>
<td>53.79±6.69</td>
<td>81.86±7.42*</td>
<td>74.23±3.76</td>
<td>58.93±1.72†</td>
</tr>
<tr>
<td>8</td>
<td>56.27±6.11</td>
<td>89.98±8.64*</td>
<td>83.77±5.10</td>
<td>62.68±3.02†</td>
</tr>
</tbody>
</table>

\(P_O^2\), partial pressure of O\(_2\); * represents a significant difference between normoxia acclimated highlanders and lowlanders; † represents a significant effect of hypoxia acclimation.
Table 2.2. Ventilatory and metabolic responses of mice acclimated to normoxia or hypoxia, measured using double-chamber pneumotachography

<table>
<thead>
<tr>
<th>Variable</th>
<th>Population</th>
<th>$P_{O_2}$ (kPa)</th>
<th>Normoxia Acclimated</th>
<th>Hypoxia Acclimated</th>
<th>Main Effect of Acclimation Environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Ventilation (ml g⁻¹ min⁻¹)</td>
<td>Lowland</td>
<td>21</td>
<td>0.85±0.09</td>
<td>1.21±0.08</td>
<td>12.89 0.002</td>
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<td></td>
<td>16</td>
<td></td>
<td>1.03±0.07</td>
<td>1.37±0.09</td>
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</tr>
<tr>
<td></td>
<td>12</td>
<td></td>
<td>1.23±0.08</td>
<td>1.53±0.09</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>1.26±0.10</td>
<td>1.68±0.08</td>
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</tr>
<tr>
<td></td>
<td>9</td>
<td></td>
<td>1.29±0.10</td>
<td>1.72±0.07</td>
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<tr>
<td></td>
<td>8</td>
<td></td>
<td>1.31±0.08</td>
<td>1.79±0.08</td>
<td></td>
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<tr>
<td>Highland</td>
<td>21</td>
<td></td>
<td>1.11±0.05</td>
<td>1.18±0.06</td>
<td>0.753 0.402</td>
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<td></td>
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<td>1.28±0.10</td>
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<tr>
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<td>1.33±0.08</td>
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<td></td>
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<td>1.54±0.08</td>
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<td></td>
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<td>1.65±0.01</td>
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<tr>
<td></td>
<td>8</td>
<td></td>
<td>1.64±0.12</td>
<td>1.85±0.08</td>
<td></td>
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<tr>
<td>Breathing Frequency (breaths min⁻¹)</td>
<td>Lowland</td>
<td>21</td>
<td>161.01±11.36</td>
<td>177.64±5.53</td>
<td>1.835 0.190</td>
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<td></td>
<td>16</td>
<td></td>
<td>194.67±12.35</td>
<td>205.50±4.54</td>
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<tr>
<td><strong>O₂ Consumption</strong> (ml O₂ g⁻¹ min⁻¹)</td>
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<td>9</td>
<td>0.040±0.002</td>
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<td>0.066±0.007</td>
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<td>16</td>
<td>0.062±0.004</td>
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<td>0.90 ± 0.35</td>
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<td>0.067±0.004</td>
<td>0.061±0.004</td>
<td>0.90 ± 0.35</td>
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<tr>
<td></td>
<td>10</td>
<td>0.054±0.004</td>
<td>0.050±0.002</td>
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<td>0.90 ± 0.35</td>
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<td></td>
<td>8</td>
<td>0.048±0.003</td>
<td>0.046±0.002</td>
<td>0.90 ± 0.35</td>
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<td><strong>O₂ Extraction (%)</strong></td>
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<td>23.96±1.53</td>
<td>22.74±1.23</td>
<td>1.72 ± 0.20</td>
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<td>16</td>
<td>27.71±1.29</td>
<td>25.16±0.95</td>
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<tr>
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<td>29.03±1.60</td>
<td>29.03±0.91</td>
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<tr>
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<td>31.48±2.00</td>
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<td>Highland</td>
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<td>29.01±3.60</td>
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<td>34.17±2.35</td>
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<td>34.26±0.97</td>
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<td>35.63±1.95</td>
<td>31.99±1.28</td>
<td>5.69 ± 0.03</td>
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<tr>
<td></td>
<td>9</td>
<td>36.06±1.55</td>
<td>31.59±1.16</td>
<td>5.69 ± 0.03</td>
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<tr>
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<td>35.51±1.39</td>
<td>30.56±1.07</td>
<td>5.69 ± 0.03</td>
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\(P_{O₂}\), partial pressure of O₂
Figure 2.1. High-altitude deer mice exhibited a more effective breathing pattern than low-altitude deer mice, characterized by deeper but less frequent breaths during acute hypoxia challenge. Measurements were made using the barometric technique on unrestrained mice. * indicates significant pairwise differences between populations within each $P_{O_2}$ using Holm-Sidak post-tests. n=14 for lowlanders and n=9 for highlanders.
Figure 2.2. Hypobaric hypoxia acclimation enhanced the hypoxic ventilatory response of lowland but not highland mice. Measurements were made using the barometric technique on unrestrained mice. # represents a significant main effect of hypoxia acclimation in two-factor ANOVA, † represents a significant pairwise difference within each $P_{O_2}$ using Holm-Sidak post-tests. n=14 normoxia acclimated lowlanders, n=15 hypoxia acclimated lowlanders, n=9 normoxia acclimated highlanders, and n=9 hypoxia acclimated highlanders.
Ph.D. Thesis – C.M. Ivy; McMaster University – Department of Biology

![Graphs showing oxygen consumption rate and body temperature across different oxygen partial pressures for Lowlander and Highlander species.](Image)

**Graph A:**
- **X-axis:** O₂ Partial Pressure (kPa)
- **Y-axis:** O₂ Consumption Rate (ml g⁻¹ min⁻¹)
- **Legend:**
  - Lowlander
    - Normoxia Acc.
    - Hypoxia Acc.

**Graph B:**
- **Legend:**
  - Highlander
    - Normoxia Acc.
    - Hypoxia Acc.

**Graph C:**
- **X-axis:** O₂ Partial Pressure (kPa)
- **Y-axis:** Body Temperature (°C)
- **Graph D:**
- **Y-axis:** Body Temperature (°C)
Figure 2.3. Highland deer mice maintained higher rates of $O_2$ consumption and body temperatures ($T_b$) in severe acute hypoxia after hypoxia acclimation. Measurements were made using the barometric technique on unrestrained mice. # represents a significant main effect of hypoxia acclimation in two-factor ANOVA, † represents a significant pairwise difference within each $P_{O_2}$ using Holm-Sidak post-tests. In (C) and (D), connected lines represent $T_b$ measurements taken in the subset of mice with temperature-sensing implants ($n=5$ for each treatment), and the offset points are the $T_b$ measurements taken for all mice using rectal probes (see Materials and Methods). $n=14$ normoxia acclimated lowlanders, $n=15$ hypoxia acclimated lowlanders, $n=9$ normoxia acclimated highlanders, and $n=9$ hypoxia acclimated highlanders.
Figure 2.4. Ventilatory acclimation to hypoxia increases arterial $O_2$ saturation in lowland but not in highland mice. Measurements were made using double-chamber pneumotachography on restrained mice. # represents a significant main effect of hypoxia acclimation in two-factor ANOVA, † represents a significant pairwise difference within each $P_{O_2}$ using Holm-Sidak post-tests. n=9 normoxia acclimated lowlanders, n=14 hypoxia acclimated lowlanders, n=6 normoxia acclimated highlanders, n=8 hypoxia acclimated highlanders.
Figure 2.5. Hypoxia acclimation increased respiratory water loss, but the magnitude was greater in lowlanders (A) than in highlanders (B). Measurements were made using double-chamber pneumotachography on restrained mice. # represents a significant main effect of hypoxia acclimation in two-factor ANOVA. n=9 for normoxic lowlanders, n=14 for hypoxia acclimated lowlander, n=6 for normoxic highlanders, n=8 for hypoxia acclimated highlanders.
Figure 2.6. Heart rates in severe acute hypoxia were increased after hypoxia acclimation in both lowland and highland mice. Measurements were made using double-chamber pneumotachography on restrained mice. † represents a significant pairwise difference within each $P_{O_2}$ using Holm-Sidak post-tests. n=9 for normoxic lowlanders, n=14 for hypoxia acclimated lowlander, n=6 for normoxic highlanders, n=8 for hypoxia acclimated highlanders.
Figure 2.7. Hypoxia acclimation induced carotid body growth in lowlanders but not in highlanders. Fluorescence immunohistochemistry was used to identify type I cells (tyrosine hydroxylase, TH, in green), neurons (neurofilament, NF, and growth-associated protein 43, GAP-43 in magenta), and type II cells (glial fibrillary acidic protein, GFAP, in blue) in (A,B) normoxic lowlanders (n=10), (C,D) hypoxia acclimated lowlanders (n=9), (E,F) normoxic highlanders (n=8), and (G,H) hypoxia acclimated highlanders (n=9). The (I) total volume of a single carotid body, (J) total number of type I cells, (K) total volume of type I cells, (L) neural volume of a single carotid body, (M) total volume of type II cells, and (N) ratio of type I cell volume to type II cell volume was determined for each carotid body. # represents a significant main effect of hypoxia acclimation, * indicates significant pairwise differences between populations within an acclimation environment, and † represents a significant effect of hypoxia acclimation in lowlanders in two-factor ANOVA.
2.7 REFERENCES


CHAPTER 3
EVOLVED CHANGES IN BREATHING AND CO₂ SENSITIVITY IN DEER MICE NATIVE TO HIGH ALTITUDES


3.1 ABSTRACT

We examined the control of breathing by O₂ and CO₂ in deer mice native to high altitude, to help uncover the physiological specializations used to cope with hypoxia in high-altitude environments. Highland deer mice (Peromyscus maniculatus) and lowland white-footed mice (P. leucopus) were bred in captivity at sea level. The first- and second-generation progeny of each population were raised to adulthood and then acclimated to normoxia or hypobaric hypoxia (12 kPa O₂, simulating hypoxia at ~4300 m) for 6-8 weeks. Ventilatory responses to poikilocapnic hypoxia (stepwise reductions in inspired O₂) and hypercapnia (stepwise increases in inspired CO₂) were then compared between groups. Both generations of lowlanders appeared to exhibited ventilatory acclimatization to hypoxia (VAH), in which hypoxia acclimation enhanced the hypoxic ventilatory response and/or made breathing pattern more effective (higher tidal volumes and lower breathing frequencies at a given total ventilation). In contrast, hypoxia acclimation had no effect on breathing in either generation of highlanders, and breathing was generally similar to hypoxia-acclimated lowlanders. Therefore, attenuation of VAH may be an evolved feature of highlanders that persists for multiple generations in captivity. Hypoxia acclimation increased CO₂ sensitivity of breathing, but in this case the effect of hypoxia acclimation was similar in highlanders and lowlanders. Our results suggest that highland
deer mice have evolved high rates of alveolar ventilation that are unaltered by exposure to chronic hypoxia, but they have preserved ventilatory sensitivity to CO$_2$. 
3.2 INTRODUCTION

Animals native to high altitude can provide insight into the evolution of complex physiological systems, because they have often adapted to the stressors associated with this challenging environment. High altitude is both cold and hypoxic, which challenges the ability of endotherms to maintain O$_2$ supply for thermoregulation and exercise. However, many human, other mammal, and bird populations live, reproduce, and exercise at high altitude, and the emerging evidence suggests that they have overcome the challenges of this environment through evolved changes in the O$_2$ transport cascade (Monge and Leon-Velarde, 1991; Storz et al., 2010). The function of this cascade – composed of ventilation, pulmonary diffusion, circulation, tissue diffusion, and cellular O$_2$ utilization – relies on adequate rates of ventilation to maintain tissue O$_2$ supply; therefore, increasing breathing is critical for O$_2$ uptake in hypoxic environments (Storz et al., 2010).

Breathing is stimulated by reductions in arterial O$_2$ levels at high altitude. Ventilation increases in response to acute hypoxia challenge, a process termed the hypoxic ventilatory response (HVR). Peripheral chemoreceptors in the carotid bodies sense reductions in the partial pressure of O$_2$ (PO$_2$) in arterial blood, which initiates the hypoxic chemoreflex that results in the HVR (Gonzalez et al., 1994; Powell et al., 1998). Breathing and ventilatory O$_2$ chemosensitivity is further enhanced with prolonged exposure to hypoxia over days to weeks, a process termed ventilatory acclimatization to hypoxia (VAH). VAH is believed to result from increases in chemosensitivity of the carotid bodies, and from increases in central gain of the afferent signals transmitted from the carotid bodies to the brainstem (Pamenter et al., 2014; Pardal et al., 2007; Reid and
Powell, 2005; Wang et al., 2008). The resulting increases in ventilation improve O\textsubscript{2} uptake by increasing alveolar and arterial PO\textsubscript{2} (Ivy and Scott, 2015).

Breathing at high altitude is also modulated by arterial CO\textsubscript{2} levels. The increases in ventilation that act to minimize the fall in arterial PO\textsubscript{2} also lead to a decline in the partial pressure of CO\textsubscript{2} (PCO\textsubscript{2}) (respiratory hypocapnia) (Powell, 2007; Scott and Milsom, 2006). This can reduce the CO\textsubscript{2} chemoreflex drive to breathe, acting as feedback that inhibits the ventilatory response to environmental hypoxia (Powell, 2007). As a result, the HVR measured in poikilocapnic (uncontrolled CO\textsubscript{2}) conditions is generally lesser in magnitude than when the HVR is measured under isocapnic conditions (when arterial PCO\textsubscript{2} is experimentally maintained) (Moore et al., 1986; Scott and Milsom, 2007). Furthermore, exposure to chronic hypoxia can increase ventilatory CO\textsubscript{2} sensitivity (Fan et al., 2010; Fan et al., 2014; Mathew et al., 1983; Schoene et al., 1990; Somogyi et al., 2005). Therefore, CO\textsubscript{2} sensitivity can have a strong influence on breathing and O\textsubscript{2} uptake in individuals at high altitude.

How has the control of breathing been adjusted in high-altitude natives? The isocapnic HVR as a measure of O\textsubscript{2} chemosensitivity has been examined in some studies of highland human populations, but the HVR of most other highland species has been examined in poikilocapnic conditions. Nevertheless, the literature suggests that highland natives can differ from lowland natives in divergent ways, with some highlanders exhibiting similar or enhanced ventilatory responses (Lague et al., 2016; Pichon et al., 2009; Scott and Milsom, 2007) and others exhibiting a blunted HVR (Ivy et al., 2018; Lague et al., 2017; Schwenke et al., 2007). However, in these studies it has often been
difficult to distinguish uniquely evolved differences in highland taxa from developmental or multigenerational effects of exposure to hypoxia (Brutsaert, 2016; Moore, 2017). Much less is known about CO$_2$ sensitivity of breathing in highland taxa. Highland humans appear to have a reduced ventilatory sensitivity to CO$_2$ (Slessarev et al., 2010a; Slessarev et al., 2010b), but it is unclear whether other high-altitude taxa exhibit a similar or distinct pattern of CO$_2$ sensitivity.

The objective of this study was to examine how the control of breathing by O$_2$ and CO$_2$ has evolved in high-altitude populations of deer mice (*Peromyscus maniculatus*). Deer mice are broadly distributed across North America and can be found from sea level to over 4,300 m elevation in the Rocky Mountains (Hock, 1964; Natarajan et al., 2013; Snyder et al., 1982). High-altitude populations must sustain high metabolic rates in the wild (Hayes, 1989), and they have evolved a higher aerobic capacity (V̇O$_2$max) in hypoxia than their low-altitude counterparts (Cheviron et al., 2012; Cheviron et al., 2013; Lui et al., 2015; Tate et al., 2017) in association with changes in haemoglobin-O$_2$ affinity, cardiac function, muscle capillarity and metabolic phenotype, and tissue gene expression (Dawson et al., 2018; Lui et al., 2015; Mahalingam et al., 2017; Nikel et al., 2018; Scott et al., 2015; Snyder et al., 1982; Storz et al., 2009; Tate et al., 2017; Velotta et al.). We recently found that the control of breathing also differs in high-altitude deer mice compared to a congeneric species from low altitude (white-footed mouse, *P. leucopus*), in a study of animals that were born and raised in captivity at sea level but were the first-generation progeny of wild parents (Ivy and Scott, 2017a). Specifically, we found that highlanders do not appear to exhibit VAH, in contrast to the robust VAH exhibited by
lowlanders, but that highlanders have a fixed breathing pattern that is similar to hypoxia-acclimated lowlanders (Ivy and Scott, 2017a). However, because these observations were made in first-generation progeny, it was unclear whether they resulted from an evolved difference in highlanders or from persistent multigenerational effects of the wild parents being born and raised in different native environments. Here, we sought to examine these possibilities by studying mice from both the first and second generations raised in captivity. We also sought to determine whether variation in CO₂ sensitivity has evolved in high-altitude mice, and whether this might contribute to the apparent differences in breathing during poikilocapnic hypoxia and in VAH.

3.3 MATERIALS AND METHODS
3.3.1 Mouse populations.

Wild adult mice were live trapped at low altitude on the Great Plains (Nine Mile Prairie, Lancaster County, NE, at 40°52’12”N, 96°48’20.3”W, 430 m above sea level) (*P. leucopus*) and at high altitude on the summit of Mount Evans (Clear Creek County, CO, at 39°35’18”N, 105°38’38”W, 4,350 m above sea level) (*P. maniculatus rufinus*), and were then transported to McMaster University (Hamilton, ON, Canada; ~50 m above sea level) and held in captivity. Mice were bred within each population in common conditions to produce first-generation (G1) progeny. This was accomplished by putting breeding pairs in individual cages, removing the male when the female was visibly pregnant, and weaning the born pups and moving them to a separate cage at 21 days after birth. G1 mice were similarly bred within each population to produce second-generation (G2) progeny. The experiments here were conducted on several independent families of
G1 mice (3 lowland and 5 highland families) and G2 mice (4 lowland and 4 highland families). We did not determine the relatedness of the wild mice used to establish our breeding colonies, but deer mice were abundant and population sizes are large at each trapping site, so it is likely that the breeders in our colony were unrelated and represented a good proportion of the genetic diversity of the wild parental populations. All captive-born progeny were held in standard holding conditions (24-25°C, 12:12 light-dark photoperiod) with unlimited access to food and water, and were raised in ambient conditions (sea level normoxia) until 6 months of age before experiments were conducted. All animal protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

3.3.2 Acclimation groups

To assess the influence of native population and acclimation environment, mice were chronically exposed to i) standard normobaric normoxia or ii) hypobaric hypoxia simulating the pressure at an elevation of ~4,300 m (barometric pressure of 60 kPa, PO$_2$ ~12.5 kPa). Specially designed hypobaric chambers were used for exposure to chronic hypoxia, as previously described (Ivy and Scott, 2017a; McClelland et al., 1998). Mice in hypobaric hypoxia were temporarily returned to normobaric conditions twice per week for <20 min for cage cleaning. Ventilatory measurements were carried out after 6-8 weeks of exposure.
3.3.3 Acute hypoxia responses

We examined the effects of hypoxia acclimation on the response to acute hypoxia in both G1 and G2 mice from each population. Breathing and O₂ consumption rates (\(\dot{V}O_2\)) were measured in unrestrained mice using barometric plethysmography and respirometry techniques that are consistent with our previous studies (Ivy and Scott, 2017a; Ivy and Scott, 2017b). Mice were placed in a whole-body plethysmograph with normoxic air (21 kPa O₂, balance N₂) supplied at a rate of 600 ml min⁻¹ and were given 20-60 min to adjust to the chamber until relaxed and stable breathing and metabolism was observed. Measurements were then recorded for an additional 20 min at 21 kPa O₂, after which mice were exposed to stepwise reductions in inspired partial pressure of O₂ (PO₂) at 16, 12, 10, 9, and 8 kPa for 20 min at each step. Incurrent gas composition was set by mixing dry compressed gases using precision flow meters (Sierra Instruments, Monterey, CA, USA) and a mass flow controller (MFC-4, Sable Systems, Las Vegas, NV, USA), such that the desired PO₂ was delivered to the chamber at a constant rate of 600 ml min⁻¹. Body temperature (T_b) was measured at the end of the experiment using a mouse rectal probe (RET-3-ISO, Physitemp). T_b was also measured exactly 24 h later to determine normoxic T_b (this was used as a proxy for the normoxic T_b at the start of the experiment, which was not measured to prevent stress to the animal).

Breathing and \(\dot{V}O_2\) were determined during the last 10 min at each PO₂. Incurrent and excurrent air flows were subsampled at 200 ml min⁻¹. For incurrent air, O₂ fraction was continuously measured using a galvanic fuel cell O₂ analyzer (FC-10, Sable Systems). For excurrent air, we first measured water vapour using a thin-film capacitive
water vapour analyzer (RH-300, Sable Systems), then dried the gas stream with pre-baked drierite and measured O₂ fraction as above and CO₂ fraction using an infrared CO₂ analyzer (CA-10, Sable Systems). These data were used to calculate $\dot{V}O_2$, expressed at standard temperature and pressure (STP), using appropriate equations for dry air as described by Lighton (Lighton, 2008). Chamber temperature was continuously recorded with a thermocouple (TC-2000, Sable Systems). Breathing frequency and tidal volume were measured from changes in flow across a pneumotachograph in the plethysmograph wall, detected using a differential pressure transducer (Validyne DP45, Cancopass, Mississauga, ON, Canada). Tidal volume was calculated using established equations (Drorbaugh and Fenn, 1955; Jacky, 1980) assuming a constant rate of decline in $T_b$ with declining PO₂, which we have previously shown results in similar tidal volumes to those calculated using direct $T_b$ measurements at each PO₂ (Ivy and Scott, 2017b). Total ventilation was determined as the product of breathing frequency and tidal volume. Total ventilation and tidal volume data are expressed at STP (standard temperature and pressure). Ventilatory O₂ equivalent is the quotient of total ventilation and $\dot{V}O_2$. All data was acquired using a PowerLab 16/32 and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, USA).

Mice were returned to their acclimation environment after completing the above protocol and allowed at least 2 days to recover and were then subjected to one of two protocols, to measure either the acute hypoxia response in the presence of elevated inspired CO₂ or acute hypercapnia responses, as described below.
3.3.4 Acute hypoxia responses with elevated inspired CO$_2$

We measured responses to acute hypoxia in the presence of modestly elevated levels of inspired CO$_2$ in G1 mice, in order to examine the HVR under conditions in which respiratory hypocapnia was reduced. We used the same whole-body plethysmograph and the same stepwise hypoxia conditions as was used for the acute hypoxia responses that are described above, except that mice were also exposed to a constant incurrent partial pressure of CO$_2$ (PCO$_2$) of 2 kPa across all acute hypoxia steps. Breathing and metabolism were determined as described above, except that CO$_2$ fraction was measured in dry incurrent air for a few minutes at the beginning of each step (to assure a constant incurrent CO$_2$ baseline at the desired level), after which it was measured in dry excurrent air for the remaining time at each step.

3.3.5 Acute hypercapnia responses

We measured responses to acute stepwise hypercapnia in G2 mice in order to assess ventilatory CO$_2$ sensitivity. We used the same whole-body plethysmograph and the same conditions as was used for the measurements of acute hypoxia response that are described above, except that breathing and metabolism were measured during acute stepwise increases in environmental PCO$_2$ at 0, 2, 4, and 6 kPa CO$_2$. These measurements were made once in normoxia (21 kPa O$_2$) and once in hypoxia (12 kPa O$_2$), in two separate experiments that were conducted in random order and separated by at least 2 days. Breathing and metabolism were determined as described above, except that CO$_2$ fraction was continuously measured in dry incurrent air, and incurrent and excurrent air
was dried and scrubbed free of CO$_2$ with soda lime and ascarite before O$_2$ fraction was measured. We therefore calculated $\dot{V}$O$_2$ using appropriate equations for dry and CO$_2$-free air as described by Lighton (Lighton, 2008).

### 3.3.6 Statistical analysis

Two-factor ANOVA and Holm-Sidak post-tests were used throughout. The main effects of acclimation environment (normoxia vs. hypoxia) and inspired gas composition (repeated measure) were evaluated within each population to determine the impacts of hypoxia acclimation on O$_2$ or CO$_2$ sensitivity of breathing. Values are reported as mean ± SEM. All statistical analysis was conducted with SigmaStat software (v. 3.5) with a significance level of $P < 0.05$.

### 3.4 RESULTS

#### 3.4.1 Acute hypoxia responses

Hypoxia acclimation altered breathing in lowland mice, but not in highland mice (Fig. 3.1, 3.2, Tables 3.1, 3.2). Amongst first generation mice (G1), lowlanders exhibited a robust ventilatory response to hypoxia that was primarily driven by increases in breathing frequency, offset slightly by small decreases in tidal volume, and led to a rise in the ventilatory O$_2$ equivalent (Fig. 3.1A,C,E, Tables 3.1, 3.2). Hypoxia acclimation had an appreciable effect on breathing, reflected primarily by strong increases in tidal volume and reductions in breathing frequency (Fig. 3.1C,E, Table 3.1), which offset each other such that there was no significant change in total ventilation and only a decrease in ventilatory O$_2$ equivalent at 8 kPa O$_2$ after hypoxia acclimation (Fig. 3.1A, Tables 3.1,
3.2). $\dot{V}O_2$ and body temperature ($T_b$) declined in response to hypoxia challenge, but hypoxia acclimation reduced the declines in body temperature (Tables 3.1, 3.2). In contrast, hypoxia acclimation had very little effect on breathing or metabolism in G1 highland mice (Fig. 3.1B,D,F, Tables 3.1, 3.2).

The diminished effects of hypoxia acclimation in G1 highland mice were also observed in second generation (G2) highland mice (Fig. 3.2, Tables 3.1, 3.3). Hypoxia acclimation had a strong effect on breathing in G2 lowlanders, as it did in G1 lowlanders, as reflected by significant increases in tidal volume (Fig. 3.2E) and reductions in breathing frequency (Fig. 3.2C) during acute hypoxia. Interestingly, there appeared to be some variation in breathing across generations in lowlanders, which tended to have lower total ventilation, tidal volume, and ventilatory $O_2$ equivalent in G2 than in G1. Nevertheless, even though there was some subtle variation in the magnitude of breathing and the HVR across generations, the effects of hypoxia acclimation on breathing pattern in lowlanders was generally preserved. In contrast, hypoxia acclimation had no effect on breathing or metabolism in G2 highlanders (Fig. 3.2, Table 3.2), as was observed in G1 highlanders, and the magnitude of breathing and the HVR was very similar across generations.

3.4.2 Hypercapnic ventilatory response

We next sought to determine whether hypoxia acclimation increases $CO_2$ sensitivity of breathing, and whether this effect of hypoxia acclimation is altered in highlanders. We examined these possibilities by measuring the ventilatory response to
stepwise hypercapnia in normoxia and hypoxia. We found that hypoxia acclimation enhanced the hypercapnic ventilatory response in both lowland and highland mice when tested in normoxic conditions (Fig. 3.3, Table 3.4). Both lowlanders and highlanders exhibited similar robust ventilatory responses to increasing CO$_2$ that were driven by increases in both tidal volume and breathing frequency (Fig. 3.3, Table 3.4). This response was augmented similarly after hypoxia acclimation in both populations, particularly at higher CO$_2$ levels, due to further increases in tidal volume in both populations and breathing frequency in lowlanders. VO$_2$ and Tb were not altered by acute hypercapnia or by hypoxia acclimation in either population (Table 3.4, data not shown).

We found that hypoxia acclimation resulted in comparable increases in the hypercapnic ventilatory response when it was tested in hypoxic conditions (12 kPa O$_2$) (Fig. 3.4, Table 3.4). The response to acute hypercapnia was similar to that observed in normoxic conditions, except that ventilation was higher overall due to hypoxia. Hypoxia acclimation augmented the hypercapnic ventilatory response measured in hypoxia in both populations, as observed for the hypercapnic ventilatory response measured in normoxia, but in this case the increases in ventilation were entirely caused by increases in tidal volume (Fig. 3.4E,F). These findings suggest that hypoxia acclimation increases the CO$_2$ sensitivity of breathing in both highland and lowland mice, but in general, there were no apparent differences in the hypercapnic ventilatory response of highlanders compared to lowlanders.

3.4.3 Acute hypoxia responses in 2 kPa CO$_2$
We sought to examine whether the apparent lack of VAH in highlanders could be a by-product of increases in ventilatory sensitivity to respiratory hypocapnia after chronic hypoxia. Given that hypoxia acclimation appears to augment CO$_2$ sensitivity (Fig. 3.3, 3.4), this could foreseeably augment the restraining influence of respiratory hypocapnia on the poikilocapnic HVR, and thus offset other effects of chronic hypoxia that stimulate breathing and would tend to cause VAH. We examined this possibility by measuring the HVR during moderately elevated inspired CO$_2$ (2 kPa CO$_2$) to offset respiratory hypocapnia, with the prediction that increases in CO$_2$ would amplify the effects of chronic hypoxia on breathing. There was some modest support for this prediction, as reflected by the apparent increase in the magnitude of the effects of hypoxia acclimation on average in both populations (compare the variation in tidal volume in Fig. 3.5 to Fig. 3.1). In highlanders in particular, hypoxia acclimation increased tidal volume at the higher PO$_2$s when measured in the presence of 2 kPa CO$_2$ (Fig. 3.5F, Table 3.5). However, hypoxia acclimation still had a much smaller effect on breathing in highlanders than in lowlanders, and there were still no significant main effects of hypoxia acclimation on total ventilation, breathing frequency, or tidal volume in highlanders (Table 3.5). Otherwise the effects of acute hypoxia on breathing and metabolism in the presence of 2 kPa CO$_2$ were quite similar to those observed without CO$_2$ in the inspired gas (Table 3.5, 3.6). Therefore, the apparent lack of VAH in highlanders cannot be explained by variation in the CO$_2$ sensitivity of breathing.
3.5 DISCUSSION

Effective control of ventilation is critically important for small endotherms in the O$_2$-limited environment at high altitude, in order to maintain adequate tissue O$_2$ supply for thermogenesis and exercise. Previously, we observed that hypoxia acclimation had little effect on breathing and the HVR of high-altitude deer mice, at levels of chronic hypoxia that did induce a VAH response in lowland mice (Ivy and Scott, 2017a). Here, we show that the apparent blunting of VAH is observed across multiple generations of lab-raised highland mice, suggesting that this blunting has evolved in response to the challenges of life at high altitude. This blunting was not associated with any evolved change in the effects of hypoxia acclimation on CO$_2$ sensitivity of breathing in highland mice. As a consequence, variation in the ventilatory sensitivity to respiratory hypocapnia does not appear to contribute to the attenuation of VAH in highlanders.

3.5.1 VAH is attenuated in high-altitude deer mice

Our findings suggest that the apparent lack of VAH in high-altitude deer mice may result from an evolved change in the magnitude of hypoxia-induced plasticity of breathing. In previous studies, it has been challenging to establish whether changes in the control of breathing in highland taxa are evolved and genetically based, because it has often been difficult to exclude the influence of developmental and/or parental exposure to different environments (Brutsaert, 2016; Moore, 2017). The blunted VAH we previously reported in first-generation highlanders raised in captivity suggested that this blunting cannot be explained by differences in developmental environment (Ivy and Scott, 2017a). However, it was possible that these differences between populations of first-generation
mice could be explained by exposure of parents and/or germ cells to different environments. For example, exposure of parents and their germline cells to hypoxia has persistent effects on hypoxia tolerance in offspring in zebrafish (Ho and Burggren, 2012). However, the persistent lack of VAH in the second-generation of highlanders raised in captivity cannot be attributed to the exposure of parents and germline cells to the high-altitude environment. There might have been some effect of the native low-altitude environment in lowlanders, based on our observations that breathing tended to decline slightly from G1 to G2, but this did not affect the expression of VAH in lowlanders. The attenuation of VAH in highlanders is more likely to be an evolved trait, and could also be influenced by trans-generational epigenetic effects.

Our understanding of the potential importance of trans-generational epigenetic effects on cardiorespiratory physiology at high altitude is still in its infancy (Brown and Rupert, 2014; Ivy and Scott, 2015; Nanduri et al., 2017a). There is known to be epigenetic regulation of cardiorespiratory physiology and carotid body function in response to chronic exposure to intermittent hypoxia in adulthood or early development (Nanduri et al., 2012; Nanduri et al., 2017b), but it is unknown whether such effects can persist across generations. Effects of some other environmental stressors are known to persist across multiple generations (e.g., transgenerational effects of pollutants on survival and development in zebrafish (Corrales et al., 2014)), but it is unclear whether chronic hypoxia can have a similarly persistent effect. Disentangling the relative importance of genetically- and epigenetically-based changes in adaptive phenotypes at high altitude will be an exciting area for future research.
What are the mechanisms that account for blunting of the VAH in high-altitude deer mice? In lowlanders, VAH arises from adjustments in the carotid bodies and the central nervous system. Chronic hypoxia enhances O\textsubscript{2} chemosensitivity of the carotid bodies, which appears to be associated with neovascularization and growth of the organ, and changes in O\textsubscript{2} signalling by O\textsubscript{2}-sensitive glomus cells (Kusakabe et al., 1993; Prabhakar and Jacono, 2005; Wang et al., 2008). Some of these adjustments appear to be attenuated in highland deer mice, as reflected by our previous observation that highlanders do not exhibit carotid body growth in response to chronic hypoxia (Ivy and Scott, 2017a). Chronic hypoxia also leads to increases in central gain of the afferent signals from the carotid body in lowlanders (e.g. changes in glutamatergic signalling in the NTS) (Pamenter et al., 2014; Reid and Powell, 2005), and it is possible that these mechanisms are also attenuated in highland mice. However, before carrying out the current study, we could not exclude the possibility that the apparent blunting in VAH arose from variation in the effects of CO\textsubscript{2} on breathing, because the HVR was measured under poikilocapnic conditions. Our results here suggest that this is not the case, because highlanders still exhibited a blunted VAH when respiratory hypocapnia was alleviated by exposure to moderately elevated inspired CO\textsubscript{2} (Fig. 3.5). This likely implies that VAH and its underlying peripheral and/or central mechanisms are indeed blunted in highland mice. This does not appear to have altered the effects of chronic hypoxia on hypoxic anapyrexia, because the magnitude of body temperature depression in response to acute hypoxia was still reduced after hypoxia acclimation in highland mice (Tables 3.2, 3.3).
3.5.2 Effects of chronic hypoxia on the CO₂ sensitivity of breathing

Hypoxia acclimation increased CO₂ sensitivity of breathing, driven primarily by larger increases in tidal volume in response to high CO₂. This observation is consistent with previous observations in humans, in which chronic hypoxia increases ventilatory CO₂ sensitivity and/or lowers the recruitment threshold above which blood CO₂ stimulates ventilation (Fan et al., 2010; Fan et al., 2014; Slessarev et al., 2010b). Chronic hypoxia is well known to induce mechanisms of acid-base compensation to counter respiratory hypocapnia and alkalosis (Powell et al., 1978), so it is possible that apparent changes in the CO₂ sensitivity of breathing arise from changes in the relationship between CO₂ and pH or in pH buffering of the blood (Fan et al., 2010; Fan et al., 2014). It is also possible that increases in ventilatory CO₂ sensitivity in response to chronic hypoxia arise from increases in the chemosensitivity of peripheral or central CO₂/pH chemoreceptors, as suggested by previous studies in humans (Fatemian and Robbins, 2001). If this is also the case in deer mice, then the mechanisms likely do not depend upon hypoxia-induced growth of the carotid bodies, which occurs in lowlanders but not in highlanders (Ivy and Scott, 2017a).

Ventilatory sensitivity to CO₂ appeared to be similar in high-altitude mice and their low-altitude counterparts, both before and after hypoxia acclimation. This contrasts previous findings in some other high-altitude taxa. Ventilatory sensitivity to CO₂ and ventilatory recruitment threshold are lower in Himalayans residing at high-altitude than in lowlanders at sea-level (Slessarev et al., 2010a). Similarly, ventilatory sensitivity to CO₂ is lower in Andeans residing at high altitude than in lowlanders acclimatized to the same
altitude for 10 days (Slessarev et al., 2010b). In bar-headed geese, a species that flies over the Himalayas during its biannual migration, ventilatory sensitivity to hypercapnia is unaltered but sensitivity to respiratory hypocapnia is reduced, such that bar-headed geese breathe more than low-altitude birds when exposed to poikilocapnic hypoxia (Scott and Milsom, 2007). Therefore, there appears to be differences across highland taxa, with ventilatory sensitivity to CO₂ having been either unaltered or reduced.

3.5.3 High-altitude adaptation and control of breathing

The emerging evidence suggests that there are a number of changes in the control of breathing by hypoxia in high-altitude deer mice. Our results here and in previous studies suggest that VAH and hypoxia-induced growth of the carotid bodies are attenuated in highlanders, which may represent an evolved loss of plasticity associated with high-altitude adaptation. Highlanders instead exhibit a fixed breathing pattern, characterized by deep but less frequent breaths, which should improve effective (alveolar) ventilation, and thus help increase arterial O₂ saturation in hypoxia (Ivy and Scott, 2017a). These changes exist without any apparent alterations in ventilatory CO₂ sensitivity. An intriguing question to consider is why these putatively evolved changes have taken place? VAH increases ventilation and thus improves respiratory gas exchange, so why have high-altitude mice not maintained the VAH response that is typical of lowlanders? One possibility is that highland mice have undergone the evolutionary process of genetic assimilation, in which a phenotype that originally exhibits adaptive plasticity becomes genetically fixed (assimilated) (Ehrenreich and Pfennig, 2016; Lande,
2015; Pigliucci, 2006). Another possibility that we and others have discussed previously is that there may have been an overall restructuring of the hypoxic chemoreflex in high-altitude deer mice (Ivy and Scott, 2017a; Milsom, 2018). It is possible that by fixing a breathing pattern that is beneficial for O₂ uptake, highlanders may avoid some costs associated with plasticity in response to chronic hypoxia at high altitude (e.g., chronic sympathetic activation, etc.). Given the harshness of high-altitude environments and the correspondingly strong selection favouring respiratory performance (Hayes and O’Connor, 1999), evolved changes in control of breathing may help safeguard O₂ uptake and contribute to the success and high abundance of deer mice in high-altitude environments.
### 3.6 TABLES AND FIGURES

Table 3.1. Statistical results of two-way ANOVA of acute hypoxia responses

<table>
<thead>
<tr>
<th></th>
<th>Acclimation Environment</th>
<th>Acute PO₂</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td><strong>First Generation Mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2.612</td>
<td>0.118</td>
<td>78.61</td>
</tr>
<tr>
<td>Ventilation</td>
<td>1.149</td>
<td>0.294</td>
<td>74.62</td>
</tr>
<tr>
<td>Breathing</td>
<td>11.67</td>
<td><strong>0.002</strong></td>
<td>179.9</td>
</tr>
<tr>
<td>Frequency</td>
<td>1.160</td>
<td>0.291</td>
<td>286.4</td>
</tr>
<tr>
<td>Tidal</td>
<td>18.74</td>
<td><strong>&lt;0.001</strong></td>
<td>10.12</td>
</tr>
<tr>
<td>Volume</td>
<td>0.092</td>
<td>0.764</td>
<td>31.17</td>
</tr>
<tr>
<td>O₂</td>
<td>1.233</td>
<td>0.277</td>
<td>12.4</td>
</tr>
<tr>
<td>Consumption</td>
<td>0.794</td>
<td>0.381</td>
<td>22.92</td>
</tr>
<tr>
<td>Body</td>
<td>6.047</td>
<td><strong>0.021</strong></td>
<td>196.8</td>
</tr>
<tr>
<td>Temperature</td>
<td>12.92</td>
<td><strong>&lt;0.001</strong></td>
<td>61.55</td>
</tr>
<tr>
<td>Ventilatory O₂</td>
<td>0.065</td>
<td>0.801</td>
<td>101.3</td>
</tr>
<tr>
<td>Equivalent</td>
<td>0.015</td>
<td>0.904</td>
<td>161.9</td>
</tr>
<tr>
<td><strong>Second Generation Mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.418</td>
<td>0.251</td>
<td>17.78</td>
</tr>
<tr>
<td>Ventilation</td>
<td>0.043</td>
<td>0.838</td>
<td>24.67</td>
</tr>
<tr>
<td>Breathing</td>
<td>5.799</td>
<td><strong>0.028</strong></td>
<td>59.74</td>
</tr>
<tr>
<td>Frequency</td>
<td>0.486</td>
<td>0.492</td>
<td>236.6</td>
</tr>
<tr>
<td>Tidal</td>
<td>4.830</td>
<td><strong>0.043</strong></td>
<td>6.258</td>
</tr>
<tr>
<td>Volume</td>
<td>0.562</td>
<td>0.460</td>
<td>71.05</td>
</tr>
<tr>
<td>O₂</td>
<td>0.827</td>
<td>0.377</td>
<td>17.84</td>
</tr>
<tr>
<td>Consumption</td>
<td>0.016</td>
<td>0.900</td>
<td>27.12</td>
</tr>
<tr>
<td>Body</td>
<td>0.057</td>
<td>0.815</td>
<td>143.0</td>
</tr>
<tr>
<td>Temperature</td>
<td>6.126</td>
<td><strong>0.020</strong></td>
<td>32.53</td>
</tr>
<tr>
<td>Ventilatory O₂</td>
<td>0.079</td>
<td>0.782</td>
<td>95.14</td>
</tr>
<tr>
<td>Equivalent</td>
<td>0.012</td>
<td>0.913</td>
<td>85.07</td>
</tr>
</tbody>
</table>

One degree of freedom for acclimation environment, 5 degrees of freedom for acute PO₂ and their interaction, 130 degrees of freedom for the residuals of first generation lowland and highland mice and second generation highland mice, and 80 degrees of freedom for the residuals of second generation lowland mice.
Table 3.2. The rate of O$_2$ consumption and body temperature during acute hypoxia exposure in first-generation lab-raised mice

<table>
<thead>
<tr>
<th>Acute PO$_2$ (kPa)</th>
<th>Lowland $P$. leucopus</th>
<th>Highland $P$. maniculatus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia Acclimated</td>
<td>Hypoxia Acclimated</td>
</tr>
<tr>
<td></td>
<td>0.045 ± 0.004</td>
<td>0.045 ± 0.003</td>
</tr>
<tr>
<td>21</td>
<td>0.045 ± 0.004</td>
<td>0.048 ± 0.003</td>
</tr>
<tr>
<td>16</td>
<td>0.043 ± 0.004</td>
<td>0.040 ± 0.002</td>
</tr>
<tr>
<td>12</td>
<td>0.039 ± 0.003</td>
<td>0.041 ± 0.003</td>
</tr>
<tr>
<td>10</td>
<td>0.035 ± 0.003</td>
<td>0.037 ± 0.002</td>
</tr>
<tr>
<td>9</td>
<td>0.031 ± 0.002</td>
<td>0.038 ± 0.002</td>
</tr>
<tr>
<td>8</td>
<td>0.027 ± 0.002</td>
<td>0.036 ± 0.002</td>
</tr>
</tbody>
</table>

**O$_2$ Consumption Rate (ml g$^{-1}$ min$^{-1}$)**

<table>
<thead>
<tr>
<th>Ventilatory O$_2$ Equivalent (ml air ml O$_2$$^{-1}$)</th>
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<tbody>
<tr>
<td>21</td>
</tr>
<tr>
<td>16</td>
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<tr>
<td>12</td>
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<tr>
<td>10</td>
</tr>
<tr>
<td>9</td>
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</tbody>
</table>

**Body Temperature (°C)**

| 21 | 37.29 ± 0.29 | 37.25 ± 0.24 | 36.08 ± 0.24 | 36.49 ± 0.23 |
| 8  | 33.52 ± 0.21*| 34.80 ± 0.21*| 33.60 ± 0.23 | 35.20 ± 0.34*|

PO$_2$, partial pressure of O$_2$; values are mean ± SEM.

* Significant pairwise difference from normoxia acclimated mice of the same population.
Table 3.3. The rate of O$_2$ consumption and body temperature during acute hypoxia exposure in second-generation lab-raised mice.

<table>
<thead>
<tr>
<th>Acute PO$_2$ (kPa)</th>
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<tr>
<td></td>
<td>Normoxia Acclimated</td>
<td>Hypoxia Acclimated</td>
</tr>
<tr>
<td>21</td>
<td>0.045 ± 0.004</td>
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<tr>
<td>16</td>
<td>0.040 ± 0.003</td>
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<tr>
<td>12</td>
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<tr>
<td>10</td>
<td>0.032 ± 0.003</td>
<td>0.037 ± 0.003</td>
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<td>9</td>
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$O_2$ Consumption Rate (ml g$^{-1}$ min$^{-1}$)

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<td>10</td>
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<tr>
<td>9</td>
</tr>
<tr>
<td>8</td>
</tr>
</tbody>
</table>

Body Temperature (°C)

| 21         | 37.63 ± 0.33      | 37.45 ± 0.22      | 36.23 ± 0.29      | 36.58 ± 0.30      |
| 8          | 34.30 ± 0.19      | 34.70 ± 0.30      | 34.50 ± 0.23      | 35.80 ± 0.31      |

PO$_2$, partial pressure of O$_2$; values are mean ± SEM.

* Significant pairwise difference from normoxia acclimated mice of the same population.
Table 3.4. Statistical results of two-way ANOVA of acute hypercapnia responses, measured in normoxia or hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Acclimation Environment</th>
<th>Acute PCO₂</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td><strong>Normoxic Conditions (21 kPa O₂)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>7.437</td>
<td>0.014</td>
<td>92.07</td>
</tr>
<tr>
<td>Ventilation Lowlander</td>
<td>11.68</td>
<td>0.002</td>
<td>249.3</td>
</tr>
<tr>
<td>Breathing Highlander</td>
<td>2.535</td>
<td>0.130</td>
<td>51.60</td>
</tr>
<tr>
<td>Frequency Highlander</td>
<td>0.223</td>
<td>0.641</td>
<td>181.2</td>
</tr>
<tr>
<td>Tidal Lowlander</td>
<td>3.451</td>
<td>0.081</td>
<td>106.0</td>
</tr>
<tr>
<td>Volume Highlander</td>
<td>12.55</td>
<td>0.002</td>
<td>120.8</td>
</tr>
<tr>
<td>O₂ Lowlander</td>
<td>3.022</td>
<td>0.100</td>
<td>1.741</td>
</tr>
<tr>
<td>Consumption Highlander</td>
<td>0.579</td>
<td>0.454</td>
<td>1.404</td>
</tr>
<tr>
<td>Body Lowlander</td>
<td>0.045</td>
<td>0.835</td>
<td>5.147</td>
</tr>
<tr>
<td>Temperature Highlander</td>
<td>0.055</td>
<td>0.817</td>
<td>39.52</td>
</tr>
<tr>
<td><strong>Hypoxic Conditions (12 kPa O₂)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9.610</td>
<td>0.007</td>
<td>87.13</td>
</tr>
<tr>
<td>Ventilation Highlander</td>
<td>4.372</td>
<td>0.047</td>
<td>118.6</td>
</tr>
<tr>
<td>Breathing Lowlander</td>
<td>0.088</td>
<td>0.770</td>
<td>16.40</td>
</tr>
<tr>
<td>Frequency Highlander</td>
<td>1.925</td>
<td>0.178</td>
<td>45.09</td>
</tr>
<tr>
<td>Tidal Lowlander</td>
<td>4.916</td>
<td>0.041</td>
<td>195.6</td>
</tr>
<tr>
<td>Volume Highlander</td>
<td>14.87</td>
<td>&lt;0.001</td>
<td>210.6</td>
</tr>
<tr>
<td>O₂ Lowlander</td>
<td>0.001</td>
<td>0.991</td>
<td>1.020</td>
</tr>
<tr>
<td>Consumption Highlander</td>
<td>0.095</td>
<td>0.761</td>
<td>3.722</td>
</tr>
<tr>
<td>Body Lowlander</td>
<td>1.087</td>
<td>0.312</td>
<td>9.683</td>
</tr>
<tr>
<td>Temperature Highlander</td>
<td>0.444</td>
<td>0.511</td>
<td>24.77</td>
</tr>
</tbody>
</table>

One degree of freedom for acclimation environment, 3 degrees of freedom for acute PCO₂ and their interaction, 51 and 75 degrees of freedom for the residuals of lowland and highland mice, respectively, in normoxic and hypoxic conditions.
### Table 3.5. Statistical results of two-way ANOVA of acute hypoxia responses, measured with elevated inspired CO₂

<table>
<thead>
<tr>
<th></th>
<th>Acclimation Environment</th>
<th>Acute PO₂</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>Total</td>
<td>Lowlander</td>
<td>4.082</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>Highlander</td>
<td>1.672</td>
<td>0.207</td>
</tr>
<tr>
<td>Ventilation</td>
<td>Lowlander</td>
<td>10.09</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Highlander</td>
<td>0.001</td>
<td>0.987</td>
</tr>
<tr>
<td>Breathing</td>
<td>Lowlander</td>
<td>17.93</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Highlander</td>
<td>3.223</td>
<td>0.084</td>
</tr>
<tr>
<td>Frequency</td>
<td>Lowlander</td>
<td>0.656</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Highlander</td>
<td>3.637</td>
<td>0.068</td>
</tr>
<tr>
<td>Tidal</td>
<td>Lowlander</td>
<td>7.929</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>Highlander</td>
<td>7.939</td>
<td>0.009</td>
</tr>
</tbody>
</table>

One degree of freedom for acclimation environment, 5 degrees of freedom for acute PO₂ and their interaction, 130 degrees of freedom for the residuals of lowland and highland mice.
Table 3.6. The rate of O₂ consumption and body temperature during acute hypoxia exposure, measured with elevated inspired CO₂

<table>
<thead>
<tr>
<th>Acute PO₂ (kPa)</th>
<th>Lowland <em>P. leucopus</em></th>
<th>Highland <em>P. maniculatus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia Acclimated</td>
<td>Hypoxia Acclimated</td>
</tr>
<tr>
<td>O₂ Consumption Rate (ml g⁻¹ min⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>0.039 ± 0.003</td>
<td>0.041 ± 0.003</td>
</tr>
<tr>
<td>16</td>
<td>0.037 ± 0.003</td>
<td>0.038 ± 0.002</td>
</tr>
<tr>
<td>12</td>
<td>0.037 ± 0.002</td>
<td>0.041 ± 0.002</td>
</tr>
<tr>
<td>10</td>
<td>0.039 ± 0.003</td>
<td>0.039 ± 0.002</td>
</tr>
<tr>
<td>9</td>
<td>0.037 ± 0.002</td>
<td>0.040 ± 0.002</td>
</tr>
<tr>
<td>8</td>
<td>0.035 ± 0.002</td>
<td>0.038 ± 0.002</td>
</tr>
<tr>
<td>Body Temperature (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>36.94 ± 0.35</td>
<td>37.92 ± 0.28</td>
</tr>
<tr>
<td>8</td>
<td>34.11 ± 0.35</td>
<td>34.79 ± 0.24</td>
</tr>
</tbody>
</table>

PO₂, partial pressure of O₂; values are mean ± SEM.

* Significant pairwise difference from normoxia acclimated mice of the same population
Figure 3.1. Hypoxia acclimation has very little effect on breathing in highland deer mice from the first generation (G1) raised in captivity, unlike G1 mice from low altitude. * Significant pairwise difference between acclimation (acc.) groups within each PO$_2$ using Holm-Sidak post-tests (n as follows: 13 normoxia-acclimated lowlanders, 15 hypoxia-acclimated lowlanders, 15 normoxia-acclimated highlanders, 13 hypoxia-acclimated highlanders).
Figure 3.2. Hypoxia acclimation has no effect on breathing in highland deer mice from the second-generation (G2) raised in captivity, unlike G2 mice from low altitude. * Significant pairwise difference between acclimation (acc.) groups within each PO<sub>2</sub> using Holm-Sidak post-tests (n as follows: 10 normoxia-acclimated lowlanders, 9 hypoxia-acclimated lowlanders, 14 normoxia-acclimated highlanders, 14 hypoxia-acclimated highlanders).
Figure 3.3. Hypoxia acclimation increased ventilatory sensitivity to CO$_2$ in both lowland and highland mice, when measured in normoxic conditions (21 kPa O$_2$). Measurements were made on second-generation (G2) mice. * Significant pairwise difference between acclimation groups within each PCO$_2$ using Holm-Sidak post-tests (n as follows: 10 normoxia-acclimated lowlanders, 9 hypoxia-acclimated lowlanders, 13 normoxia-acclimated highlanders, 14 hypoxia-acclimated highlanders).
Figure 3.4. Hypoxia acclimation increased ventilatory sensitivity to CO$_2$ in both lowland and highland mice, when measured in hypoxic conditions (12 kPa O$_2$). Measurements were made on second-generation (G2) mice. * Significant pairwise difference between acclimation groups within each PCO$_2$ using Holm-Sidak post-tests (n as in Fig. 3).
Figure 3.5. Hypoxic ventilatory responses measured in the presence of moderately elevated levels of inspired CO$_2$ (2 kPa). Measurements were made on first-generation (G1) mice. * Significant pairwise difference between acclimation groups within each PO$_2$ using Holm-Sidak post-tests (n as in Fig. 1).
3.7 REFERENCES


CHAPTER 4

GENETIC VARIATION IN HIF-2α ATTENUATES VENTILATORY SENSITIVITY AND CAROTID BODY GROWTH IN CHRONIC HYPOXIA IN HIGH-ALTITUDE DEER MICE

4.1 ABSTRACT

The gene encoding HIF-2α, Epas1, is under strong selection in many high-altitude taxa, but the functional role of mutations in this gene are still poorly understood. We investigated the influence of the high-altitude variant of Epas1 in North American deer mice (Peromyscus maniculatus) on control of breathing and carotid body growth during chronic hypoxia. We created a hybrid population of deer mice using an F2 intercross breeding design, which disrupts linkages between loci that result from population genetic structure, so the physiological effects of highland and lowland Epas1 variants can be compared on an admixed genetic background. In general, chronic hypoxia (4 weeks of hypobaric hypoxia at 12 kPa O₂, simulating ~4,300 m) enhanced ventilatory chemosensitivity (assessed as the acute ventilatory response to hypoxic-hypercapnia), increased total ventilation during exposure to progressive stepwise hypoxia in poikilocapnic conditions, increased haematocrit and blood haemoglobin content, and improved arterial O₂ saturation in hypoxia. However, the effects of chronic hypoxia on ventilatory chemosensitivity were attenuated in mice that were homozygous for the high-altitude Epas1 allele (Epas1H/H). Carotid body growth and glomus cell hyperplasia, which was strongly induced in Epas1L/L mice in chronic hypoxia, was completely absent in Epas1H/H mice. However, chronic hypoxia increased metabolism and attenuated body temperature depression in hypoxia in Epas1H/H mice, but not in Epas1L/L or Epas1H/L.
mice. These findings confirm the important role of HIF-2α in modulating ventilatory sensitivity and carotid body growth in chronic hypoxia, and show that genetic variation in Epas1 is responsible for evolved changes in the control of breathing and metabolism in high-altitude natives.
4.2 INTRODUCTION

The hypoxic chemoreflex is critically important for coping with acute hypoxia. Reductions in the partial pressure of O\textsubscript{2} (PO\textsubscript{2}) in arterial blood initiates the hypoxic chemoreflex by stimulating the O\textsubscript{2}-sensitive type I (glomus) cells in the carotid bodies. Increases in afferent activity from the carotid bodies leads to increases in ventilation termed the hypoxic ventilatory response (HVR) (Ivy and Scott, 2015; Powell et al., 1998). It also activates the sympathetic nervous system, resulting in α-adrenoreceptor-mediated vasoconstriction in peripheral tissues that tends to increase vascular resistance and preferentially redistribute blood flow towards hypoxia-sensitive organs (i.e. brain and heart), and may also increase blood pressure (Hainsworth and Drinkhill, 2007; Ivy and Scott, 2015). These responses are critical to survival in acute hypoxia (Slotkin et al., 1988).

Many aspects of the hypoxic chemoreflex are amplified by chronic exposure to hypoxia, but the value of these chronic adjustments is less clear. Days to weeks of hypoxia exposure result in further increases in ventilation (ventilatory acclimatization to hypoxia, VAH), resulting from increases in O\textsubscript{2} chemosensitivity of the carotid bodies and increases in central gain of afferent signals transmitted to the brain stem (Pamenter et al., 2014; Reid and Powell, 2005), and it also results in growth and neovascularization of the carotid bodies (Kusakabe et al., 1993; Pardal et al., 2007; Powell et al., 1998; Wang et al., 2008). Sympathetic activation can persist in chronic hypoxia, prolonging the increases in vascular resistance and blood pressure through continued α-adrenoreceptor-mediated vasoconstriction, and opposing local factors promoting vasodilation (Bernardi et al., 1998; Calbet, 2003; Hainsworth and Drinkhill, 2007). The value of maintaining or
amplifying the hypoxic chemoreflex is unclear: some associated responses are beneficial, particularly the increases in ventilation that improve pulmonary $O_2$ uptake (albeit at a metabolic cost and placing greater reliance on mechanisms responsible for maintaining acid-base and water homeostasis); other responses such as chronic sympathetic activation can be counterproductive and impede $O_2$ supply to peripheral tissues.

Recent evidence suggests that hypoxia-inducible factor (HIF) signalling plays a role in the changes in the hypoxic chemoreflex that occur in response to chronic hypoxia. HIFs act as key transcription factors responsible for coordinating many diverse cellular and systemic responses to hypoxia (Prabhakar and Semenza, 2012). HIF-$\alpha$ subunits (of which there are 3: HIF-1$\alpha$, HIF-2$\alpha$, HIF-3$\alpha$) are targeted for degradation in the presence of $O_2$, primarily through $O_2$ dependent hydroxylation by prolyl hydroxylases, but in hypoxia HIF-$\alpha$ accumulates, dimerizes with the HIF-1$\beta$ subunit, and the resulting HIF transcription factor can drive the expression of hypoxia responsive genes (Prabhakar and Semenza, 2012). Some recent research in which HIF-2$\alpha$ was acutely inactivated using Cre-Lox recombination, either generally (Hodson et al., 2016) or only in glomus cells and other cells expressing tyrosine hydroxylase (Fielding et al., 2018), suggests that HIF-2$\alpha$ mediated signalling drives VAH and carotid body hyperplasia during chronic hypoxia. However, heterozygous knockout of HIF-2$\alpha$ and HIF-1$\alpha$ have yielded different results in some other studies (Kline et al., 2002; Peng et al., 2011), potentially due to differences in genetic background. These discrepancies suggest that more research is required to fully appreciate the role that HIF signalling plays in the responses of the hypoxic chemoreflex to chronic hypoxia.
Recent research suggests that the HIF pathway has been a frequent target of selection in high-altitude natives. The gene encoding HIF-2α (*Epas1*) in particular is under strong selection in human populations from the Qinghai-Tibet Plateau and in many animals native to high altitude (Ai et al., 2014; Beall et al., 2010; Buroker et al., 2012; Gou et al., 2014; Graham and McCracken, 2019; Li et al., 2013; Li et al., 2014; Petousi et al., 2014; Qu et al., 2013; Schweizer et al., 2019; Simonson et al., 2012; Song et al., 2016; Yi et al., 2010). Studies have revealed that high-altitude *Epas1* variants appear to be associated with lower haemoglobin concentrations in Tibetans (Beall et al., 2010), and with altered cardiovascular function in hypoxia in high-altitude deer mice (Schweizer et al., 2019). These findings, along with the potential role of HIF-2α in VAH and carotid body hyperplasia, suggest that selection on *Epas1* may lead to some of the evolved differences in breathing and the hypoxic chemoreflex that have been observed in high-altitude natives (Beall et al., 1997; Ivy and Scott, 2018). In one particular study, there was no clear influence of *Epas1* genotype on ventilatory and cardiovascular responses to acute hypoxia in Tibetan humans, but this study investigated adults who lived at sea level for 4 years and did not control for developmental environment (Petousi et al., 2014). It remains to be determined whether the genetic variants in *Epas1* that exist in high-altitude natives affect the breathing and the hypoxic chemoreflex in chronic hypoxia.

Deer mice (*Peromyscus maniculatus*) are an emerging model species for studying high-altitude adaptation. Deer mice are broadly distributed across North America and can be found from sea level to over 4,300 m elevation in the Rocky Mountains (Hock, 1964; Natarajan et al., 2015; Snyder et al., 1982). There is evidence for directional selection for
a high aerobic capacity (\( \dot{V}O_{2\text{max}} \)) for thermogenesis in deer mice at high altitude (Hayes and O’Connor, 1999), which has led to evolved increases in \( \dot{V}O_{2\text{max}} \) (Cheviron et al., 2012; Cheviron et al., 2013; Cheviron et al., 2014; Lui et al., 2015; Tate et al., 2017) along with evolved changes in haemoglobin (Hb)-O\(_2\) affinity and in various other traits that influence O\(_2\) supply and utilization (Dawson et al., 2018; Lau et al., 2017; Lui et al., 2015; Mahalingam et al., 2017; Scott et al., 2015; Snyder et al., 1982; Storz et al., 2009; Storz et al., 2010; Tate et al., 2017). In particular, high-altitude deer mice do not appear to exhibit VAH or carotid body hyperplasia in chronic hypoxia, in contrast to the robust VAH and carotid body growth exhibited by lowlanders (Ivy and Scott, 2017a; Ivy and Scott, 2018). Recent evidence also suggests that there has been a history of spatially varying selection on Epas1 in high-altitude deer mice, which resulted in high frequencies of a unique high-altitude variant with three genetic substitutions – one in the 3’ UTR, one non-synonymous substitution (\( C^{2288T} \)) leading to a polarity-altering amino acid change (threonine to methionine) at site 755 of the 14\(^{th}\) exon, and a synonymous substitution in the 14\(^{th}\) exon (Schweizer et al., 2019). Here, we sought to investigate the influence of this high-altitude variant of Epas1 on VAH and carotid body hypertrophy in chronic hypoxia. This was achieved using a F\(_2\) intercross breeding design to disrupt the linkages between loci that result from population genetic structure, so the effects of Epas1 gene variants on breathing and carotid body morphology could be evaluated on an admixed genetic background.
4.3 METHODS
4.3.1 Deer mice and intercross breeding design

Wild adult deer mice were live-trapped at high altitude on the summit of Mount Evans (Clear Creed County, CO, USA at 39°35’18”N, 105°38’38”W; 4350 m above sea level) (P. m. rufinus) and at low altitude on the Great Plains (Nine Mile Prairie, Lancaster County, NE, USA at 40°52’12”N, 96°48’20.3”W; 430 m above sea level) (P. m. nebrascensis) and were transported to the University of Montana (elevation 978 m). Wild mice from Mount Evans that were homozygous for the derived Epa$1^H$ allele (H, high-altitude variant) at non-synonymous substitution in the 14th exon (C2288T) were crossed with mice from Lincoln that were homozygous for the ancestral Epa$1^L$ allele (L, low-altitude variant) at the same point (see below for genotyping protocols). First-generation hybrid progeny (F1) from two families (one from crossing a highland male and a lowland female, and one from crossing a highland female and a lowland male) were raised to maturity, and intercrossed in full-sibling matings to produce 6 families of second-generation progeny (F2) (2 and 4 families descended from each wild breeding pair, respectively). F2 intercrossed mice included those that were homozygous (Epa$1^{HH}$ or Epa$1^{LL}$) or heterozygous (Epa$1^{HL}$) for the alternative Epa$1$ alleles on an otherwise admixed genomic background. All mice were provided with unlimited access to water and standard mouse chow, and were held in standard holding conditions (~23 °C, 12 h:12 h light:dark photoperiod) under normal atmospheric conditions at the University of Montana until used in the experiments described below at McMaster University (elevation 50 m). All animal protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal
Wild mice and F2 mice were genotyped using an ear clip sample as previously described (Schweizer et al., 2019). DNA was extracted using DNeasy (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions. PCR amplification of Epas1 was then conducted with custom exonic primers (5’-GCACGCCTTCCAAGACAC-3’ and 5’-GGTGGCAGGTGTCTCAGT-3’) designed from the Peromyscus maniculatus bairdii genome (NCBI Accession GFC_000500354.1) under the following conditions: 94 °C for 2 min; 30 cycles of 94 °C for 45 sec, 58 °C for 1 min, 72 °C for 1 min; then 72 °C for 10 min. To improve amplification specificity for some samples, we used modified primers and PCR conditions (5’- AGGGCAGAGATGTAAACAGC-3’ and 5’-GAATGTGGTGCCGTCTGATG-3’): 94 °C for 2 min; 35 cycles of 94 °C for 30 sec, 62 °C for 30 sec, 68 °C for 1 min; then 68 °C for 10 min.

4.3.2 Chronic exposures

To investigate the role of Epas1 genotype on responses to chronic hypoxia, 35 F2 mice were first raised in normobaric normoxia in standard holding conditions until they were 1.5 to 2 years old. Mice were then subjected to a subset of measurements (described below), after which they were subjected to 4 weeks of exposure to either (i) normobaric normoxia (standard conditions) or (ii) hypobaric hypoxia simulating the pressure at an elevation of 4,300 m (barometric pressure of 60 kPa, an O2 partial pressure of approximately 12.5 kPa). This resulted in 6 chronic exposure treatment groups: normoxic
Epas1\textsuperscript{HH} (n=5), normoxic Epas1\textsuperscript{HL} (n=6), normoxic Epas1\textsuperscript{LL} (n=6), hypoxic Epas1\textsuperscript{HH} (n=5), hypoxic Epas1\textsuperscript{HL} (n=7), and hypoxic Epas1\textsuperscript{LL} (n=6). Specially designed hypobaric chambers were used for chronic hypoxia exposures, as previously described (Ivy and Scott, 2017a; Lui et al., 2015; McClelland et al., 1998). Mice were temporarily returned to normobaric conditions twice per week for cage cleaning (which took <20 min).

4.3.3. Acute hypoxia responses

We measured the acute hypoxia responses of all mice at the beginning (normoxia) and end of chronic exposures to normoxia or hypoxia using each of two protocols (conducted in random order and separated by at least 2 days). Measurements were made in unrestrained mice using barometric plethysmography and respirometry techniques that we have used in previous studies (Ivy and Scott, 2017a; Ivy and Scott, 2018). One protocol was used to test acute ventilatory sensitivity to hypoxia. Mice were placed in a whole-body plethysmography chamber (530 ml) that was supplied with normoxic air (21 kPa O\textsubscript{2}, balance N\textsubscript{2}) at 2 l min\textsuperscript{-1}. Mice were given 20-40 min to adjust to the chamber until relaxed and stable breathing and metabolism were observed. Breathing was then measured every minute for an additional 5 min at 21 kPa O\textsubscript{2}, then during 5 min of acute exposure to hypoxia and hypercarbia (10 kPa O\textsubscript{2}, 3 kPa CO\textsubscript{2}, balance N\textsubscript{2}), and then finally during 5 min of recovery in normoxia (21 kPa O\textsubscript{2}, balance N\textsubscript{2}). This protocol was used to avoid the respiratory hypocapnia/alkalosis that can occur during exposure to hypoxia in absence of inspired CO\textsubscript{2}, as in previous studies (Bishop et al., 2013; Fielding et al., 2018;
Hodson et al., 2016). Indeed, the chosen level of hypercarbia has been shown to minimize the fall in arterial PCO$_2$ and rise in arterial pH during exposure to hypoxia in mice (Ishiguro et al., 2006). As a result, the responses in this hypercarbic hypoxia protocol likely provide a good reflection of ventilatory sensitivity to hypoxia in absence of the confounding effects of CO$_2$/pH disruption.

We also used a second protocol to measure the responses to stepwise hypoxia in poikilocapnic (uncontrolled CO$_2$) conditions. Mice were placed in the same plethysmograph as described above, and given 20-40 min to adjust to the chamber with 21 kPa O$_2$ supplied at 600 ml min$^{-1}$. Measurements were then recorded for 20 min at 30 kPa O$_2$ (hyperoxia), after which mice were exposed to 20 min stepwise reductions in inspired PO$_2$ to 21, 16, 12, 10, and 8 kPa. In this stepwise poikilocapnic hypoxia protocol, breathing and metabolism were measured during the last 10 min of exposure at each step when stable measurements had been reached. For each protocol, incurrent gas composition was set by mixing dry compressed gases using precision flow meters (Sierra Instruments, Monterey, CA, USA) and a mass flow controller (MFC-4, Sable Systems, Las Vegas, NV, USA). Body temperature (T$_b$) was measured every 5 min using thermosensitive passive transponders (micro LifeChips with Bio-therm technology; Destron Fearing, Dallas, TX, USA) that were implanted subderminally on the left side of the abdomen close to the leg ∼2 weeks before normoxic measurements were conducted.

Breathing (total ventilation, breathing frequency, and tidal volume) and metabolism were measured as follows. Metabolism was measured in the stepwise poikilocapnic hypoxia protocol as recommended by Lighton (Lighton, 2008). Gas
composition was measured continuously in incident and ecurrent air flows that were subsampled at 200 ml min⁻¹; incident air was continuously measured for O₂ fraction (FC-10, Sable Systems), and ecurrent air was analyzed for water vapour (RH-300, Sable Systems), dried with pre-baked drierite and analyzed for O₂ and CO₂ fraction (FC-10 and CA-10, Sable Systems). These data were used to calculate rates of O₂ consumption (VO₂), expressed at standard temperature and pressure (STP), using established equations (Lighton, 2008). Chamber temperature was continuously recorded with a thermocouple (TC-2000, Sable Systems). Breathing frequency and tidal volume were measured from changes in flow across a pneumotachograph in the wall of the plethysmograph chamber, detected using a differential pressure transducer (Validyne DP45, Cancopass, Mississauga, ON, Canada). Tidal volume was calculated using established equations (Drorbaugh and Fenn, 1955; Jacky, 1980). Total ventilation was calculated as the product of breathing frequency and tidal volume. Total ventilation and tidal volume data are reported in volumes expressed at body temperature and pressure saturated (BTPS). Air convection requirement (ACR) is the quotient of total ventilation and VO₂. All data was acquired using a PowerLab 16/32 and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, USA). Arterial O₂ saturation and heart rate were measured using MouseOx Plus pulse oximeter collar sensors and data acquisition system (Starr Life Sciences, Oakmont, PA, USA). This was enabled by removing fur around the neck ~ 2 days before experiments.

4.3.4. Haematology
Blood was collected for haematology and Hb-O₂ affinity assays, both before and after chronic exposures. Before exposures, small samples of blood (30 µl) were collected from the tail vein. After chronic exposures, blood was collected immediately after mice were euthanized with an overdose of isofluorane followed by decapitation. Blood Hb content was measured using Drabkin’s reagent (Sigma-Aldrich, Oakville, ON, Canada) according to the manufacturer’s instructions, and haematocrit was measured by spinning blood in a heparinized capillary tube at 12,700 g for 5 min. Oxygen dissociation curves were generated at 37 °C for all mice using a Hemox Analyzer (TCS Scientific, New Hope, PA, USA) using 10 µl of whole blood in 5 ml of buffer (100 mmol l⁻¹ HEPES, 50 mmol l⁻¹ EDTA, 100 mmol l⁻¹ KCl, 0.1% bovine serum albumin, and 0.2% antifoaming agent; TCS Scientific). Red cell O₂ affinity (P₅₀, the PO₂ at which haemoglobin is 50% saturated with O₂) was calculated using Hemox Analytic Software (TCS Scientific).

4.3.5. Immunohistochemistry of the carotid bodies

We examined carotid body morphology using similar approaches to those we have used and described previously in deer mice (Ivy and Scott, 2017a), and are described briefly here. Mice were euthanized as described above, and the bifurcations of the carotid artery were dissected, removed, and fixed in 4% paraformaldehyde for 48 h. Tissues were then incubated in 24% sucrose solution for cryoprotection, frozen in embedding medium (Cryomatrix; ThermoFisher Scientific, Waltham, MA, USA) and cryosectioned at 10 µm thickness using a CM-1860 cryostat (Leica, Wetzlar, Germany). Every second section through one entire carotid body was mounted on slides (Superfrost Plus Fisherbrand,
Fisher Scientific), which were then air-dried and stored at -80 °C. Sections were immunostained with rabbit anti-tyrosine hydroxylase antibody (TH; 1:2000 dilution; AB152; Millipore, Billerica, MA, USA), mouse anti-neurofilament (NF; 1:100; MAB1615, Millipore), and mouse anti-growth-associated protein-43 (GAP-43; 1:2000; G9264, Sigma-Aldrich, Mississauga, ON, Canada), followed by detection with the fluorescent secondary antibodies AlexaFluor488 goat anti-rabbit (1:400; A11034, Life Technologies, Mississauga, ON, Canada) and AlexaFluor594 goat anti-mouse (1:400; A11032, Life Technologies) along with DAPI (4’,6-diamidino-2-phenylindole; 1:100 000 dilution, Sigma-Aldrich). Sections were imaged using an Olympus microscope with Northern Eclipse software (Elite version 8.0; Empix Imaging, Mississauga, ON, Canada). Projected areas of the whole carotid body, of neurons, and of type I cells were determined and used to calculate total volumes as previously described (Ivy and Scott, 2017a; Saiki et al., 2006). Type I cell counts were obtained using ImageJ software (v. 1.47) (Ivy and Scott, 2017a) and all other measurements were made using Nikon NIS Elements documentation software (V. 4.30.02).

4.3.6 Statistical analysis

Two-factor ANOVA and Holm-Sidak post-tests were used throughout. Values are reported as individual values and as mean ± SEM. All statistical analysis was conducted with SigmaStat software (v. 3.5) with a significance level of P < 0.05.
4.4 RESULTS
4.4.1 Ventilatory chemosensitivity to hypoxic hypercarbia

Ventilatory sensitivity increased after chronic exposure to hypoxia in deer mice (Fig. 4.1, Table 4.1), as occurs in many other species (Aaron and Powell, 1993; Bishop et al., 2013; Sato et al., 1992). This was determined using a brief exposure to hypoxic hypercarbia that elicited strong ventilatory responses to hypoxia but that was unlikely to be confounded by secondary hypocapnia or changes in metabolism (Bishop et al., 2013; Fielding et al., 2018; Hodson et al., 2016). Control mice that were held in normoxia exhibited only modest variation in the total ventilation response to hypoxic hypercarbia (main effect of PO$_2$, P<0.001) over the course of the experiment, with ~1.1-fold greater responses at the end of 4 weeks of normoxia exposure (main effect of acclimation, P=0.030; Fig. 4.1A,B). In general, ventilatory responses to hypoxic hypercarbia arose from large increases in breathing frequency and sometimes more modest increases in tidal volume (Table 4.1). Chronic exposure to hypoxia increased total ventilation in normoxia and led to a much greater ~1.4-fold increase in the ventilatory response to hypoxic hypercarbia (acclimation, P=0.0001; PO$_2$, P<0.0001; acclimation×PO$_2$ interaction, P=0.001; Fig. 4.1C,D). The effects of chronic hypoxia on breathing and ventilatory chemosensitivity were driven largely by increases in tidal volume along with some potentially subtle (non-significant) increases in breathing frequency (Table 4.1).

The effect of chronic hypoxia on ventilatory chemosensitivity were attenuated in mice with the Epas1$^{H/H}$ genotype (Figs. 4.2, 4.3, Table 4.2). Normoxia-acclimated mice increased total ventilation ~2-fold in response to hypoxic hypercarbia (main effect of PO$_2$, P<0.001), with a similar response between Epas1 genotypes (main effect of
genotype, $P=0.760$; Fig. 4.2A,B). However, there was a small decrease in the breathing frequency response to hypoxic hypercarbia in $EpasI^{H/H}$ mice (~1.7-fold increase) compared to $EpasI^{L/L}$ mice (~2-fold increase), with no significant effect on tidal volume (Table 4.2). Hypoxia acclimation led to further increases in ventilatory sensitivity for all genotypes, but the increase in total ventilation during hypoxic hypercapnia was significantly lower in $EpasI^{H/H}$ mice (~2.1-fold increase) than in $EpasI^{H/L}$ (~2.2-fold) and $EpasI^{L/L}$ (~2.3-fold) mice (interaction, $P=0.045$; genotype, $P=0.044$; $PO_2$, $P<0.001$; Fig. 4.2C,D). This attenuated increase in total ventilation in $EpasI^{H/H}$ mice appeared to result from a lower breathing frequency response to hypoxic hypercarbia, but the tidal volume response was largely uninfluenced by $EpasI$ genotype (Fig. 4.3; Table 4.2).

### 4.4.2 Ventilatory and metabolic responses to progressive hypoxia

We next sought to examine whether $EpasI$ genotype affected the ventilatory response to progressive stepwise hypoxia during poikilocapnic conditions, in which increases in breathing augment $CO_2$ release and thus induce respiratory hypocapnia. This was motivated by previous studies suggesting that $EpasI$ knockout has much less pronounced effects on the hypoxic ventilatory response measured in poikilocapnic conditions compared to hypercarbic conditions (Hodson et al., 2016). All mice increased total ventilation in response to stepwise hypoxia as a result of increases in breathing frequency, offset by small declines in tidal volume (Fig. 4.4, Table 4.3). However, there was a clear difference between genotypes acclimated to chronic hypoxia. Chronic hypoxia augmented the breathing frequency response to severe hypoxia in mice with
Epas1<sup>L/L</sup> (acclimation×PO<sub>2</sub>, P=0.007) and Epas1<sup>H/H</sup> (acclimation×PO<sub>2</sub>, P<0.001) genotypes, but chronic hypoxia had no effect on breathing frequency in Epas1<sup>H/H</sup> mice (acclimation×PO<sub>2</sub>, P=0.413; Fig. 4.4A-C). These changes in the breathing frequency response accounted for the rise in total ventilation after chronic hypoxia in Epas1<sup>L/L</sup> (acclimation×PO<sub>2</sub>, P<0.001) and Epas1<sup>H/L</sup> mice (acclimation×PO<sub>2</sub>, P<0.001) (Fig. 4.4G-I). Chronic hypoxia also led to a rise in total ventilation in severe hypoxia in Epas1<sup>H/H</sup> mice (acclimation×PO<sub>2</sub>, P<0.001), but this appeared to be associated with subtle non-significant increases in tidal volume. Chronic exposure to hypoxia increased arterial O<sub>2</sub> saturation across genotypes (Table 4.3), but the increases occurred across a broader range of inspired PO<sub>2</sub> in Epas1<sup>L/L</sup> and Epas1<sup>H/L</sup> mice than in Epas1<sup>H/H</sup> mice (Fig. 4.4J-M).

Therefore, despite the potential confounding effects of respiratory hypocapnia, the high-altitude Epas1 variant was still observed to have a blunted response to chronic hypoxia.

The high-altitude variant of Epas1 also affected metabolism and thermoregulation in chronic hypoxia, which may have contributed to some of the variation in breathing. Prolonged exposure to hypoxia can elicit metabolic depression that tends to reduce ventilation (Dzal and Milsom, 2019; Ivy and Scott, 2017a; Ivy and Scott, 2018; Olson et al., 2001; Tattersall et al., 2002), as was observed here in normoxia acclimated mice as reductions in metabolism (as reflected by rates of O<sub>2</sub> consumption) in severe acute hypoxia compared to normoxia (Tables 4.3, 4.4). However, metabolism increased after chronic hypoxia in Epas1<sup>H/H</sup> mice (acclimation, P=0.008), which likely contributed to increasing total ventilation, but metabolism was much less affected by chronic hypoxia in Epas1<sup>L/L</sup> and Epas1<sup>H/L</sup> mice (Table 4.3, 4.4). As a result, air convection requirement (the
ratio of total ventilation to metabolism) tended to decline in moderate levels of hypoxia in Epas1\(^{H/H}\) mice, but was largely similar throughout hypoxia in Epas1\(^{H/L}\) mice and Epas1\(^{L/L}\) mice (Table 4.4). Chronic hypoxia also reduced the magnitude of body temperature depression during acute hypoxia in Epas1\(^{H/H}\) mice (acclimation\(\times\)PO\(_2\), P=0.003) but not in Epas1\(^{H/L}\) or Epas1\(^{L/L}\) mice (Table 4.4). Therefore, Epas1 genotype appears to affect metabolic and thermoregulatory control in chronic hypoxia.

### 4.4.3 Carotid body morphology

Hypoxia acclimation enlarged the carotid bodies in Epas1\(^{L/L}\) mice, with no significant increases in Epas1\(^{H/H}\) and Epas1\(^{H/L}\) mice (Fig. 4.5). The total volume of the carotid body and the total number of type I cells (glomus cells) were similar between genotypes in normoxia. However, carotid body volume increased significantly after chronic hypoxia in Epas1\(^{L/L}\) mice (~2-fold; main effect of acclimation, P=0.021; main effect of genotype, P=0.021; Fig. 4.5G), with only a slight non-significant increase in Epas1\(^{H/L}\) mice (~1.25-fold) and no change in Epas1\(^{H/H}\) mice. The enlargement of the carotid body in Epas1\(^{L/L}\) mice arose from significant increases in the number of type I cells (acclimation, P=0.001; genotype, P=0.001; Fig. 4.7H) and a trend for increases in the total volume of neurons (data not shown; acclimation, P=0.121; genotype, P=0.380) after hypoxia acclimation, but these traits were unaffected by hypoxia acclimation in Epas1\(^{H/H}\) and Epas1\(^{H/L}\) mice.

### 4.4.4 Haematology and haemoglobin-O\(_2\) binding affinity
Whole-blood haemoglobin (Hb) content and haematocrit increased after hypoxia acclimation in all mice, with no changes in the O2-binding affinity of red cells (Fig. 4.6). *Epas1* genotype had no effect on whole-blood Hb content and haematocrit in normoxia (P=0.492, P=0.644, respectively), and all genotypes increased Hb content and haematocrit similarly after hypoxia acclimation (P<0.001, for both). Red cell P50 was not influenced by *Epas1* genotype (P=0.404) or hypoxia acclimation (P=0.732), and varied across the range exhibited by populations of deer mice from high and low altitudes (Fig. 4.6C) (Ivy *et al.*, 2020).

### 4.5 DISCUSSION

Recent research suggests that the HIF pathway has been a frequent target of selection in high-altitude natives (Ai *et al.*, 2014; Beall *et al.*, 2010; Buroker *et al.*, 2012; Gou *et al.*, 2014; Graham and McCracken, 2019; Li *et al.*, 2013; Petousi *et al.*, 2014; Qu *et al.*, 2013; Schweizer *et al.*, 2019; Simonson *et al.*, 2012; Song *et al.*, 2016; Yi *et al.*, 2010). Although there is a growing appreciation of the important role played by HIF-2α in the hypoxic chemoreflex and ventilatory responses to chronic hypoxia (Fielding *et al.*, 2018; Hodson *et al.*, 2016), the physiological implications of natural genetic variants in the HIF pathway remain poorly understood. Here, we show that mice homozygous for the high-altitude variant of the HIF-2α gene *Epas1* (*Epas1*H/H) exhibit reduced ventilatory chemosensitivity and no carotid body growth after chronic exposure to hypoxia, in strong contrast to mice with the ancestral low-altitude variant. *Epas1*H/H also maintained higher metabolic rates and suffered less body temperature depression in chronic hypoxia.
However, *Epas1* genotype had no effect on the increases in blood haemoglobin content or haematocrit that were exhibited in chronic hypoxia. These findings provide further support for the key role of *Epas1* in ventilatory chemosensitivity in chronic hypoxia, and the genetic mechanisms underlying the evolution of hypoxia resistance in high-altitude natives.

Mice with *Epas1*<sup>H/H</sup> genotype exhibited blunted ventilatory chemosensitivity after chronic hypoxia. Chronic hypoxia tended to increase the ventilatory response to acute hypoxic hypercapnia by increasing both breathing frequency and tidal volume (Fig. 4.1, Table 4.1). The blunted effect of chronic hypoxia on this ventilatory response in *Epas1*<sup>H/H</sup> mice was explained by lower breathing frequency in hypoxic hypercapnia, while tidal volume was generally conserved across genotypes (Figs. 4.2, 4.3). The breathing frequency response to progressive hypoxia in poikilocapnic conditions was also unaffected by chronic hypoxia in *Epas1*<sup>H/H</sup> mice, unlike the amplified response exhibited by *Epas1*<sup>L/L</sup> and *Epas1*<sup>H/L</sup> in chronic hypoxia (Fig. 4.4). These findings parallel recent research using multiple approaches to knockout *Epas1*. The increase in ventilatory chemosensitivity that occurs with ventilatory acclimatization to hypoxia (VAH) has been shown to be strongly attenuated in mice in which HIF-2α was knocked out, via either constitutive heterozygous knockout or acute inducible knockout using Cre-Lox recombination, but not in mice in which HIF-1α was similarly knocked out (Hodson et al., 2016). Similar results were observed when HIF-2α was acutely knocked out only in cells expressing tyrosine hydroxylase (TH, an enzyme involved in catecholamine synthesis that is highly expressed in the O₂-sensitive type I cells in the carotid body), but
not when knocked out in cells expressing glial fibrillary acidic protein (GFAP, a marker of glial cells, including type II cells in the carotid body) (Fielding et al., 2018). The similarity between these findings and our results here suggest that a reduction in HIF-2α-mediated signalling in glomus cells could explain why ventilatory chemosensitivity increased much less in deer mice that were homozygous for the high-altitude Epas1 allele. Indeed, HIF-2α-mediated signalling appears to be important for inducing the expression of genes responsible for O2 sensing in glomus cells of the carotid body (Moreno-Domínguez et al., 2020). The implications of genetic variation in Epas1 on the transcriptome of glomus cells in deer mice is not yet known, but our other recent findings suggest that the high-altitude variant is associated with reduced expression in the adrenal medulla of genes involved in catecholamine biosynthesis and secretion, potentially reflecting broad effects of the high-altitude variant on the hypoxic chemoreflex and sympathetic activation (Schweizer et al., 2019).

Mice with Epas1H/H genotype exhibited no carotid body growth during chronic hypoxia, unlike the strong carotid body growth and hyperplasia of glomus cells that occurred in Epas1L/L mice (Fig. 4.5). These findings also parallel research showing that inducible knockout of HIF-2α, either broadly or only in cells expressing TH, attenuates carotid body hyperplasia (Fielding et al., 2018; Hodson et al., 2016). The former study also suggested that HIF-2α was required for the hypertrophy and the reduction in dense core vesicle content of glomus cells in chronic hypoxia (Fielding et al., 2018). However, the relationship between carotid body growth and ventilatory chemosensitivity remains unclear. VAH has several time domains that are underpinned by distinct mechanisms.
(Powell et al., 1998; Robbins, 2007), and increases in the O₂ sensitivity of the carotid bodies likely arise in part from mechanisms other than glomus cell proliferation (e.g., increased excitability of individual glomus cells) (Hempleman, 1995; Hempleman, 1996). Therefore, it remains to be determined whether there is a direct link between the effects of HIF-2α on glomus cell proliferation, carotid body O₂ sensitivity, and the hypoxic ventilatory response.

The effects of Epas1 genotype on the hypoxic ventilatory response was less apparent during the progressive stepwise hypoxia protocol (Fig. 4.4), which would have coincided with respiratory hypocapnia and metabolic depression. This is consistent with previous studies, in which variation in the magnitude of the HVR was more clearly seen when there was compensation for the secondary respiratory hypocapnia that occurs with increased ventilation (Hodson et al., 2016; Howard and Robbins, 1995). Ventilation is also strongly influenced by changes in metabolism during prolonged hypoxia exposure (Dzal and Milsom, 2019; Olson et al., 2001; Tattersall et al., 2002). However, this progressive hypoxia protocol was valuable for examining the potential role of Epas1 genotype on the metabolic and thermoregulatory responses to hypoxia. Exposure to severe hypoxia reduced aerobic metabolism and body temperature (Table 4.3), as we have previously observed in highland and lowland populations of deer mice (Ivy and Scott, 2017a; Ivy and Scott, 2018), and as observed in other small mammals (Dzal and Milsom, 2019; Ivy and Scott, 2017b; Tattersall et al., 2002). However, chronic exposure to hypoxia increased metabolism and attenuated body temperature depression in Epas1^{H/H} mice, but not in Epas1^{H/L} or Epas1^{L/L} mice. Genetic variation in Epas1 may therefore
explain the very same differences in the response to chronic hypoxia that we have previously observed between high-altitude and low-altitude populations of deer mice (Ivy and Scott, 2017a). Although the effects of Epas1 genotype on metabolism during progressive hypoxia may have masked some of the differences in ventilatory sensitivity to hypoxia per se, effects of chronic hypoxia on the breathing frequency response were completely absent in Epas1H/H mice. There were also subtle differences between genotypes in the effects of chronic hypoxia on air convection requirement. Chronic hypoxia had no effect on air convection requirement in Epas1H/L and Epas1L/L mice, potentially because increases in hypoxic chemosensitivity were offset by increases in CO2 sensitivity (Ivy and Scott, 2018) and/or changes in pulmonary structure and function that increase arterial PO2 (Dane et al., 2018). Chronic hypoxia appeared to reduce air convection requirement in Epas1H/H mice, which could reflect a shift in the balance between these offsetting factors such that breathing relative to metabolism was reduced.

Haematological responses to chronic hypoxia were not altered by the high-altitude Epas1 variant in deer mice (Fig. 4.6). This contrasts findings in Tibetan humans, in which sequence variants in and around the Epas1 gene are strongly associated with reduced blood haemoglobin content at high altitude (Beall et al., 2010; Yi et al., 2010). It remains unclear whether the erythropoietic pathway was the direct phenotypic target of selection acting on Epas1 in Tibetan humans, or whether the reduced blood haemoglobin content was a secondary consequence of effects of the Epas1 variant on other physiological traits that affect O2 supply and thus affect the hypoxic signal in erythropoietic tissues (Simonson, 2015; Storz, 2010; Storz and Scott, 2019). Nevertheless, the discrepancy
between our results and these findings of previous studies suggest that there may be some differences in the physiological effects of selection on *Epas1* across high-altitude taxa. Broad inducible knockout of *Epas1* blunts hypoxia-induced increases in haematocrit in mice (Hodson et al., 2016), suggesting that the high-altitude *Epas1* variant of deer mice does not completely eliminate HIF-2α-mediated signalling across the body, and its effects may be restricted to a subset of the tissues and/or HIF-responsive genes that are affected by HIF-2α in low-altitude mice.

Our findings suggest that sequence variation in the HIF-2α gene may be partly responsible for the absence of VAH in deer mice native to high altitude (Ivy and Scott, 2017a; Ivy and Scott, 2018). Chronic hypoxia leads to robust increases in total ventilation during acute stepwise hypoxia and in carotid body volume in low-altitude deer mice, but these changes do not occur in high-altitude deer mice (Ivy and Scott, 2017a; Ivy and Scott, 2018). Effects of chronic hypoxia on breathing become apparent in high-altitude deer mice when responses to stepwise hypoxia are measured in the presence of 2% CO₂, suggesting that the apparent absence of VAH in highlanders may be partly explained by increased sensitivity to respiratory hypocapnia after chronic hypoxia (Ivy and Scott, 2018). However, these effects of chronic hypoxia were modest and much smaller than the effects of chronic hypoxia in lowlanders (Ivy and Scott, 2018). Here, we show that the highland variant of *Epas1* may also contribute to reducing VAH in high-altitude deer mice. Our findings therefore provide an environmentally relevant context for the role of *Epas1* in carotid body O₂ sensing, and shed light on the physiological and genetic mechanisms of hypoxia tolerance in high-altitude natives.
4.6 TABLES AND FIGURES

Table 4.1. Breathing frequency and tidal volume responses of all deer mice in normoxia and acute hypoxic hypercapnia, before and after chronic exposure (acclimation) to normoxia or hypoxia.

<table>
<thead>
<tr>
<th></th>
<th>Normoxia acclimated mice</th>
<th>Hypoxia acclimated mice</th>
<th>Tidal volume (µl g⁻¹)</th>
<th>Normoxia acclimated mice</th>
<th>Hypoxia acclimated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Breathing frequency (breaths min⁻¹)</td>
<td></td>
<td>Tidal volume (µl g⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxic hypercapnia</td>
<td>Normoxia</td>
<td>Hypoxic hypercapnia</td>
<td>Normoxia</td>
</tr>
<tr>
<td>Pre-exposure</td>
<td>143.34 ± 5.16ᵃ</td>
<td>268.06 ± 11.70ᵇ</td>
<td>145.38 ± 6.53ᵃ</td>
<td>272.11 ± 10.05ᵇ</td>
<td>10.67 ± 0.46</td>
</tr>
<tr>
<td>Post-exposure</td>
<td>142.53 ± 5.58ᵃ</td>
<td>273.48 ± 11.69ᵇ</td>
<td>157.19 ± 7.12ᵃ</td>
<td>292.76 ± 14.28ᵇ</td>
<td>12.02 ± 0.58</td>
</tr>
</tbody>
</table>

Main effect of exposure:
- F: 0.007
- P: 0.936

Main effect of PO₂:
- F: 272.4
- P: <0.001

Exposure×PO₂:
- F: 0.002
- P: 0.965

Values are mean ± SEM. Values that do not share a letter within an acclimation group are significantly different. See Fig. 4.1 for additional details.
Table 4.2. Breathing frequency and tidal volume responses of deer mice with different *Epas1* genotypes in normoxia and acute hypoxic hypercapnia.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Breathing frequency (breaths min⁻¹)</th>
<th>Tidal volume (µl g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia acclimated mice</td>
<td>Hypoxia acclimated mice</td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxic hypercapnia</td>
</tr>
<tr>
<td><em>Epas1</em>HH</td>
<td>135.97 ± 7.35a</td>
<td>239.91 ± 8.07b</td>
</tr>
<tr>
<td><em>Epas1</em>HL</td>
<td>149.70 ± 7.73a</td>
<td>270.58 ± 10.46bc</td>
</tr>
<tr>
<td><em>Epas1</em>LL</td>
<td>145.74 ± 5.60a</td>
<td>297.01 ± 15.59c</td>
</tr>
</tbody>
</table>

Main effect of genotype:
- F: 4.342, P: 0.021
- PO₂: F: 345.6, P: <0.001

Main effect of PO₂:
- F: 2.004, P: 1.409
- 4.347, P: 0.045

Values that do not share a letter within an acclimation group are significantly different. See Fig. 4.2 for additional details.
Table 4.3. ANOVA of acute responses to stepwise hypoxia in *Epas1* intercross deer mice

<table>
<thead>
<tr>
<th><em>Epas1</em> genotype</th>
<th>Breathing frequency</th>
<th>Tidal volume</th>
<th>Total ventilation</th>
<th>Arterial O(_2) saturation</th>
<th>O(_2) consumption</th>
<th>Air convection requirement</th>
<th>Body temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
<td>P</td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>HH</td>
<td>0.244</td>
<td>0.628</td>
<td>91.02</td>
<td>&lt;0.001</td>
<td>1.018</td>
<td>0.413</td>
<td>4.696</td>
</tr>
<tr>
<td>HL</td>
<td>5.017</td>
<td>0.037</td>
<td>143.5</td>
<td>&lt;0.001</td>
<td>5.182</td>
<td>&lt;0.001</td>
<td>14.80</td>
</tr>
<tr>
<td>LL</td>
<td>0.772</td>
<td>0.393</td>
<td>104.8</td>
<td>&lt;0.001</td>
<td>3.482</td>
<td>0.007</td>
<td>0.118</td>
</tr>
<tr>
<td></td>
<td>0.123</td>
<td>0.730</td>
<td>35.16</td>
<td>&lt;0.001</td>
<td>0.833</td>
<td>0.530</td>
<td>0.123</td>
</tr>
<tr>
<td></td>
<td>1.138</td>
<td>0.302</td>
<td>22.59</td>
<td>&lt;0.001</td>
<td>0.614</td>
<td>0.689</td>
<td>0.123</td>
</tr>
<tr>
<td></td>
<td>0.118</td>
<td>0.735</td>
<td>50.30</td>
<td>&lt;0.001</td>
<td>0.905</td>
<td>0.481</td>
<td>0.123</td>
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<tr>
<td></td>
<td>0.067</td>
<td>0.798</td>
<td>51.32</td>
<td>&lt;0.001</td>
<td>0.540</td>
<td>0.746</td>
<td>0.067</td>
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<tr>
<td></td>
<td>0.063</td>
<td>0.805</td>
<td>33.19</td>
<td>&lt;0.001</td>
<td>2.244</td>
<td>0.058</td>
<td>0.063</td>
</tr>
</tbody>
</table>
Table 4.4. \( \text{O}_2 \) consumption rate, air convection requirement, and body temperature of deer mice with different \( \text{Epas1} \) genotypes to stepwise hypoxia in poikilocapnic conditions.

<table>
<thead>
<tr>
<th>( \text{PO}_2 ) (kPa)</th>
<th>( \text{Epas1}^{H/H} ) Normoxia acclimated mice</th>
<th>( \text{Epas1}^{H/L} ) Normoxia acclimated mice</th>
<th>( \text{Epas1}^{L/L} ) Normoxia acclimated mice</th>
<th>( \text{Epas1}^{H/H} ) Hypoxia acclimated mice</th>
<th>( \text{Epas1}^{H/L} ) Hypoxia acclimated mice</th>
<th>( \text{Epas1}^{L/L} ) Hypoxia acclimated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{O}_2 ) consumption rate (ml ( \text{O}_2 ) g(^{-1}) min(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.036 ± 0.002</td>
<td>0.048 ± 0.003*</td>
<td>0.042 ± 0.002</td>
<td>0.040 ± 0.002</td>
<td>0.042 ± 0.003</td>
<td>0.038 ± 0.004</td>
</tr>
<tr>
<td>21</td>
<td>0.041 ± 0.002</td>
<td>0.049 ± 0.002*</td>
<td>0.045 ± 0.002</td>
<td>0.048 ± 0.003</td>
<td>0.047 ± 0.002</td>
<td>0.047 ± 0.004</td>
</tr>
<tr>
<td>16</td>
<td>0.042 ± 0.001</td>
<td>0.050 ± 0.002*</td>
<td>0.046 ± 0.003</td>
<td>0.050 ± 0.002</td>
<td>0.045 ± 0.002</td>
<td>0.048 ± 0.003</td>
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<td>12</td>
<td>0.041 ± 0.001</td>
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<td>0.042 ± 0.003</td>
<td>0.048 ± 0.002</td>
<td>0.043 ± 0.002</td>
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<td>10</td>
<td>0.038 ± 0.002</td>
<td>0.045 ± 0.003*</td>
<td>0.040 ± 0.003</td>
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<tr>
<td>8</td>
<td>0.035 ± 0.001</td>
<td>0.045 ± 0.003*</td>
<td>0.040 ± 0.003</td>
<td>0.045 ± 0.002</td>
<td>0.039 ± 0.002</td>
<td>0.047 ± 0.003</td>
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| \( \text{Air convection requirement (ml air BTPS ml O}_2\text{ STP}^{-1}\) | | | | | | |
| 30 | 45.69 ± 1.79 | 47.15 ± 6.22 | 47.89 ± 5.18 | 45.09 ± 2.62 | 39.00 ± 1.92 | 45.60 ± 2.59* |
| 21 | 39.80 ± 1.63 | 34.92 ± 2.52 | 39.24 ± 3.26 | 38.92 ± 1.77 | 35.00 ± 1.25 | 36.20 ± 2.84 |
| 16 | 39.03 ± 1.47 | 35.34 ± 1.28 | 37.56 ± 1.27 | 29.83 ± 2.25 | 36.20 ± 1.07 | 36.91 ± 2.26 |
| 12 | 46.64 ± 1.48 | 40.77 ± 0.94 | 44.99 ± 1.72 | 44.53 ± 2.76 | 43.67 ± 1.16 | 39.95 ± 1.88 |
| 10 | 53.60 ± 2.44 | 45.00 ± 1.24* | 51.57 ± 1.86 | 53.76 ± 4.48 | 50.53 ± 1.08 | 48.50 ± 3.20 |
| 8  | 61.13 ± 2.40 | 61.35 ± 2.72 | 58.25 ± 2.30 | 68.12 ± 3.43 | 61.74 ± 1.86 | 62.43 ± 2.36 |

| Body temperature (°C) | | | | | | |
| 30 | 37.82 ± 0.39 | 38.31 ± 0.19 | 37.85 ± 0.39 | 38.08 ± 0.30 | 38.15 ± 0.29 | 37.92 ± 0.51 |
| 21 | 37.45 ± 0.41 | 38.07 ± 0.14 | 37.73 ± 0.36 | 37.69 ± 0.27 | 37.93 ± 0.31 | 37.68 ± 0.53 |
| 16 | 37.15 ± 0.36 | 38.01 ± 0.13 | 37.62 ± 0.33 | 37.52 ± 0.31 | 37.67 ± 0.27 | 37.67 ± 0.44 |
| 12 | 36.57 ± 0.34 | 37.55 ± 0.25 | 37.01 ± 0.34 | 37.07 ± 0.45 | 37.27 ± 0.31 | 37.40 ± 0.37 |
| 10 | 35.70 ± 0.33 | 37.34 ± 0.26* | 36.19 ± 0.38 | 36.42 ± 0.53 | 36.41 ± 0.39 | 36.64 ± 0.54 |
| 8  | 34.82 ± 0.27 | 36.59 ± 0.55* | 35.16 ± 0.41 | 35.61 ± 0.54 | 35.48 ± 0.44 | 36.39 ± 0.31 |

H, highland haplotype; L, lowland haplotype. Values are mean ± SEM. * represents a significant pairwise difference between normoxia- and hypoxia-acclimated mice within a genotype and \( \text{PO}_2 \). See Fig. 4.4 for additional details.
Figure 4.1. Chronic hypoxia increased ventilatory sensitivity to acute hypoxic-hypercapnia in deer mice. There were no changes over time in the ventilatory response to acute hypoxic-hypercapnia in time-matched normoxia acclimated controls (A,B), but the ventilatory response was augmented after 4 weeks of chronic hypoxia (C,D). (A,C) Changes in total ventilation over time in normoxia (no line) and hypoxic hypercapnia (black line), shown as mean ± SEM. (B,D) Total ventilation immediately before (minute 4) and just after (minute 6) the transition from normoxia (21 kPa O$_2$) to hypoxic hypercapnia (10 kPa O$_2$, 3 kPa CO$_2$), with data shown as individual values as well as mean ± SEM. Groups that do not share a letter are significantly different in pairwise comparisons using Holm-Sidak post-tests. N as follows: 17 0-week normoxia-acclimated mice, 17 4-week normoxia-acclimated mice, 18 0-week hypoxia-acclimated mice, 18 4-week hypoxia-acclimated mice.
Figure 4.2. The effects of chronic hypoxia on ventilatory sensitivity to acute hypoxic-hypercapnia was blunted in deer mice with Epas1\textsuperscript{H/H} genotype. (A,C) Changes in total ventilation over time in normoxia (no line) and hypoxic hypercapnia (black line), shown as mean ± SEM. (B,D) Total ventilation immediately before (minute 4) and just after (minute 6) the transition from normoxia (21 kPa O\textsubscript{2}) to hypoxic hypercapnia (10 kPa O\textsubscript{2}, 3 kPa CO\textsubscript{2}), with data shown as individual values as well as mean ± SEM. Groups that do not share a letter are significantly different in pairwise comparison using Holm-Sidak post-tests. N as follows: 10 normoxia-acclimated Epas1\textsuperscript{H/H} mice, 13 normoxia-acclimated Epas1\textsuperscript{H/L} mice, 12 normoxia-acclimated Epas1\textsuperscript{L/L} mice, 5 hypoxia-acclimated Epas1\textsuperscript{H/H} mice, 7 hypoxia-acclimated Epas1\textsuperscript{H/L} mice, 6 hypoxia-acclimated Epas1\textsuperscript{L/L} mice.
Hypoxic hypercapnia

Acclimation
- Normoxic mice
- Hypoxic mice

\[ \text{Total Ventilation (ml BTPS g}^{-1} \text{ min}^{-1}) \]

\[ \text{Tidal Volume (µl BTPS g}^{-1} \]

\[ f_R \text{ (breaths min}^{-1}) \]

Epas1\(^{H/H}\)
Epas1\(^{H/L}\)
Epas1\(^{L/L}\)
Figure 4.3. The blunted effects of chronic hypoxia on ventilatory sensitivity in deer mice with $Epa\hspace{0.1cm}s\hspace{0.1cm}I^{H/H}$ genotype was caused by reductions in breathing frequency. Individual values (small symbols) as well as mean ± SEM (larger symbols) in hypoxic hypercapnia for each group are shown. Chronic hypoxia increased total ventilation by increasing tidal volume (reflected by a rightward shift) and breathing frequency, but the primary difference between genotypes in chronic hypoxia was a lower breathing frequency in $Epa\hspace{0.1cm}s\hspace{0.1cm}I^{H/H}$ mice (i.e., lower total ventilation at a similar tidal volume). N as in Fig. 4.2.
Figure 4.4. The effects of chronic hypoxia on the ventilatory response to stepwise hypoxia under poikilocapnic conditions was blunted in deer mice with $Epas1^{HH}$ genotype. Values are mean ± SEM, and * denotes a significant pairwise difference between acclimation groups within each PO$_2$ using Holm-Sidak post-tests. N as follows: 10 normoxia acclimated $Epas1^{HH}$ mice, 5 hypoxia-acclimated $Epas1^{HH}$ mice, 13 normoxia-acclimated $Epas1^{HL}$ mice, 7 hypoxia-acclimated $Epas1^{HL}$ mice, 12 normoxia-acclimated $Epas1^{LL}$ mice, 6 hypoxia-acclimated $Epas1^{LL}$ mice.
Figure 4.5. The effects of chronic hypoxia on carotid body growth were attenuated in deer mice with either \textit{Epas1}^{H/H} or \textit{Epas1}^{H/L} genotypes. (A-F) Fluorescent immunohistochemistry was used to identify type I cells (tyrosine hydroxylase, TH, in green) and neurons (neurofilament, NF, and growth-associated protein 43, GAP-43, in magenta) in normoxia-acclimated \textit{Epas1}^{L/L} mice (n=6), normoxia-acclimated \textit{Epas1}^{H/L} mice (n=6), normoxia-acclimated \textit{Epas1}^{H/H} mice (n=5), hypoxia-acclimated \textit{Epas1}^{L/L} mice (n=6), hypoxia-acclimated \textit{Epas1}^{H/L} mice (n=7), and hypoxia-acclimated \textit{Epas1}^{H/H} mice (n=5). Scale bar is 100 µm. (G,H) Individual values as well as mean ± SEM are shown for the total volume and the total number of type I (glomus) cells for a single carotid body. * denotes a significant pairwise difference between acclimation environments within a genotype using Holm-Sidak post-tests.
Figure 4.6. *Epas1* genotype did not alter the increases in whole-blood haemoglobin content (A) or haematocrit (B) in response to chronic hypoxia, and there was no significant variation in red cell O$_2$ binding affinity across genotypes or acclimation groups (C). Individual values as well as mean ± SEM are shown. * denotes a significant pairwise difference between acclimation environments within a genotype using Holm-Sidak post-tests. N as in Fig. 4.2.
4.7 REFERENCES


CHAPTER 5

GENETIC VARIATION IN HAEMOGLOBIN ALTERS CONTROL OF BREATHING IN HIGH-ALTITUDE DEER MICE

5.1 ABSTRACT

Haemoglobin (Hb)-O$_2$ affinity has been a pervasive target of selection in many high-altitude taxa and is often presumed to safe-guard arterial O$_2$ saturation in hypoxia, but the effect of genetic variation in globins on other aspects of respiratory physiology is poorly understood. We investigated the influence of the high-altitude variants in α- and β-globins of North American deer mice (Peromyscus maniculatus) on control of breathing. We created a hybrid population of deer mice using a F$_2$ intercross breeding design, which disrupts the linkages between loci that result from population genetic structure, so the effects of globin genotype on breathing, metabolism, and arterial O$_2$ saturation during hypoxia could be compared on an admixed genetic background. α-globin genotype had a significant influence on breathing pattern, with highland homozygotes breathing deeper but less frequently across a range of inspired O$_2$. The ventilatory response to hypoxia was augmented in mice that were homozygous for highland β-globin. These differences in breathing did not appear to be caused by genetically based differences in blood-O$_2$ affinity, because treatment with efaproxiral to reduce Hb-O$_2$ affinity had no effect on breathing in normoxia or hypoxia. Globins were not expressed in the brainstem, and globin genotype had little effect on gene expression in the medulla (assessed using RNA-Seq), so differences in control of breathing were not caused by non-erythroid expression of globins in these key sites of ventilatory control. Our findings suggest that the evolution
of globin genes may have pervasive effects on multiple respiratory phenotypes, and may contribute to environmental adaptation via physiological mechanisms that are not commonly ascribed to this protein.
5.2 INTRODUCTION

High-altitude natives are an exceptional model for understanding the genetic and physiological bases of evolutionary adaptation. Species that are broadly-distributed across altitudes can provide particularly strong insight into the genomic basis of high-altitude adaptation, because they can be used to quantify selection on genetic variation that contributes to fitness. Recent research has identified many genes that appear to have experienced selection in high-altitude taxa (Gou et al., 2014; Graham and McCracken, 2019; Simonson, 2015; Simonson et al., 2012), including genes involved in energy metabolism and the hypoxia-inducible factor (HIF) pathway (Natarajan et al., 2013; Projecto-Garcia et al., 2013; Qu et al., 2015; Schweizer et al., 2019; Zhou et al., 2014), but how these genetic variants affect physiological function and organismal performance is not well understood.

Haemoglobin-O₂ affinity has been a pervasive target of selection in many high-altitude taxa. Haemoglobin (Hb) is a tetramer containing two α and two β subunits, and its O₂-binding affinity is an important determinant of O₂ exchange at the lungs and peripheral tissues. Many high-altitude taxa have increased Hb-O₂ affinity as a result of genetic variants in the α and/or β subunits that increase intrinsic O₂ affinity and/or reduce the influence of negative allosteric modifiers (e.g. 2,3-DPG) (Galen et al., 2015; Natarajan et al., 2015a; Natarajan et al., 2016; Natarajan et al., 2018; Projecto-Garcia et al., 2013; Signore et al., 2019; Storz et al., 2010; Tufts et al., 2015; Zhu et al., 2018). These adaptations are presumed to safeguard arterial O₂ saturation in hypoxia, but this has rarely been tested independent of the other phenotypic differences in high-altitude taxa. Furthermore, it is completely unknown whether genetic variation in Hb genes has
additional physiological effects that are advantageous at high altitude and that contribute to the unique phenotype of high-altitude natives in other ways.

Experiments on lab-strain mice suggest that Hb or its α and β globin monomers may play a role in regulating breathing. On the one hand, pharmacological approaches that increase Hb-O₂ binding affinity have little influence on ventilatory responses to hypoxia in rats and guinea pigs (Birchard and Tenney, 1986; Rivera-Ch et al., 1994). On the other hand, mice possessing the Hb Presbyterian mutation in the β globin subunit, which exhibit a reduced Hb-O₂ affinity, exhibit an attenuated ventilatory sensitivity to O₂ and CO₂ (Izumizaki et al., 2003; Shirasawa et al., 2003). Furthermore, deoxyhaemoglobin-derived S-nitrosothiols appear to be involved in signalling the ventilatory response to hypoxia (Lipton et al., 2001). These findings together suggest that Hb or its globin monomers may affect the control of breathing, by a mechanism that is not directly associated with the role of Hb in circulatory O₂ transport. Haemoglobins have recently been shown to be expressed in various non-erythroid cells, including neurons and vascular endothelium (Biagioli et al., 2009; Newton et al., 2006; Richter et al., 2009; Schelshorn et al., 2009; Straub et al., 2012), so it is foreseeable that globins expressed in non-erythroid tissues could regulate ventilatory phenotypes independent from the function of haemoglobins in the blood.

Deer mice (*Peromyscus maniculatus*) are an emerging model species for investigating the mechanisms of high-altitude adaptation. The species exhibits a broad altitudinal distribution from sea level to over 4,300 m above sea level (Hock, 1964). Hb α and β gene clusters have been targets of selection in high-altitude populations of this
species, and mutations in the α and β subunits are associated with increased Hb-O₂ affinity (Chappell and Snyder, 1984; Natarajan et al., 2015b; Snyder et al., 1982; Storz et al., 2009). Deer mice have also experienced strong directional selection for increased aerobic capacity for thermogenesis (Hayes and O’Connor, 1999), which has led to evolved increases in maximal rates of O₂ consumption (\(\dot{V}O_{2\text{max}}\)) in high-altitude populations (Cheviron et al., 2012; Tate et al., 2017, 2020). In addition, high-altitude mice have evolved an enhanced hypoxic ventilatory response and a deeper breathing pattern (larger tidal volumes but lower breathing frequencies at a given level of total ventilation) under routine conditions, both of which should increase alveolar ventilation and be more effective for gas exchange in hypoxia (Ivy and Scott, 2017; Ivy and Scott, 2018; Ivy et al., 2020). The primary objective of this study was to investigate whether high-altitude variants of Hb genes contribute to these evolved changes in control of breathing in high-altitude deer mice. In doing so, we also examined whether these Hb variants help safeguard arterial O₂ saturation in hypoxia. This was achieved using an F₂ intercross breeding design that disrupted the linkages between loci that result from population genetic structure, such that the effects of α and β globin variants could be evaluated on an admixed genetic background. We then examined whether the physiological effects of variation in Hb genes resulted from changes in blood-O₂ affinity, using efaproxiral (a synthetic drug that acts as a negative allosteric regulator of Hb-O₂ binding) to pharmacologically reduce blood-O₂ affinity, and we also used transcriptomics and western blots to evaluate the expression of α and β Hb in the medulla and pons (brainstem regions responsible for the control of breathing).
5.3 METHODS
5.3.1 Deer mouse populations and breeding designs

Wild adult deer mice were live-trapped at high altitude on the summit of Mount Evans (Clear Creed County, CO, USA at 39°35’18”N, 105°38’38”W; 4,350 m above sea level) (P. m. rufinus) and at low altitude on the Great Plains (Nine Mile Prairie, Lancaster County, NE, USA at 40°52’12”N, 96°48’20.3”W; 430 m above sea level) (P. m. nebrascensis) and were transported to the University of Montana (elevation 978 m) or to McMaster University (elevation 50 m). The wild mice transported to Montana were used to produce one family of first-generation inter-population hybrids (F1), created by crossing a highland male and a lowland female. These F1 hybrids were raised to maturity and were used for full-sibling matings to produce 4 families of second-generation hybrid progeny (F2). These F2 hybrids (N=26) were raised to adulthood (1-1.5 years old), a small volume of blood was obtained for genotyping (sampled from the facial vein and then stored at -80°C), and mice were then transported to McMaster for subsequent experiments (see below). The wild mice transported to McMaster were bred in captivity to produce first-generation (G1) progeny within each population. All mice were held in a standard holding environment (~23°C, 12 h:12 h light:dark photoperiod) under normal atmospheric conditions before experiments, and were provided with unlimited access to water and standard mouse chow. All animal protocols were approved by institutional animal research ethics boards.

Tetrameric haemoglobins of adult P. maniculatus incorporate α-chain subunits that are encoded by two tandem gene duplicates, HBA-T1 and HBA-T2 (separated by 5.0
kb on Chromosome 8), and β-chain subunits that are encoded by two other tandem duplicates, \(HBB-T1\) and \(HBB-T2\) (separated by 16.2 kb on Chromosome 1) (Hoffmann et al., 2008; Natarajan et al., 2015b). We used a reverse-transcriptase PCR (RT-PCR) approach to obtain sequence data for all four of the adult-expressed α- and β-globin transcripts in the full panel of mice (Natarajan et al., 2015b; Storz et al., 2010a). Total RNA was extracted from red blood cells using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). We then amplified globin transcripts from 1 µg of extracted RNA using the SuperScript III Platinum One-Step RT-PCR system with Platinum Taq DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). PCR cycling conditions were as follows: 1 cycle at 50 °C for 10 min, 40 cycles at 94 °C for 15 s and 68 °C for 2 min, and then a final extension cycle at 68 °C for 7 min. For the α-globin transcripts, we used the same primer pair for \(HBA-T1\) and \(HBA-T2\) (forward: CTGATTTCACAGACTCAGGAAG, reverse: CCAAGAGGTACAGGTGCGAG). For the β-globin transcripts, we used the same RT-PCR primer pair for \(HBB-T1\) and \(HBB-T2\) (forward: GACTTGCAACCTCAGAAACAGAC, reverse: GACCAAAGGCCTTCATCATTTT). Gel-purified RT-PCR products were then cloned into pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen), and automated DNA sequencing of cloned PCR products was performed on an ABI 3730 capillary sequencer using Big Dye chemistry (Applied Biosystems, Foster City, CA, USA). For each mouse, we sequenced 6 clones containing products of \(HBA\)-specific RT-PCR, and 6 clones containing products of \(HBB\)-specific RT-PCR. Thus, full-length inserts representing cDNAs of all expressed \(HBA\) and \(HBB\) genes were sequenced at 6-fold
coverage, and the haplotype phase of all variable sites was determined experimentally. We thus identified mice with the following combinations of lowland (L) and highland (H) haplotypes of α-globin and β-globin for use in subsequent experiments: N=5 α^{HH}β^{HH}, N=5 α^{HH}β^{HL}, N=7 α^{HH}β^{LL}, N=4 α^{LL}β^{HH} and N=5 α^{LL}β^{HL}.

5.3.2 Experiments with F2 hybrids to elucidate the physiological effects of haemoglobin genotype on an admixed genetic background

F2 hybrids were subjected to a series of measurements both before and after exposure to chronic hypoxia. Acute hypoxia responses and haematology were first measured in mice held in normoxia. Four days later, the mice were moved into specifically designed hypobaric chambers that have been previously described (Ivy and Scott, 2017; Lui et al., 2015; McClelland et al., 1998) and were thus acclimated to hypoxia for 8 weeks (barometric pressure of 60 kPa, simulating the pressure at an elevation of 4,300 m; O₂ pressure ~12.5 kPa). During this time, mice were temporarily returned to normobaric conditions twice per week for <20 min for cage cleaning. Acute hypoxia responses were then measured again after 6-8 weeks in chronic hypoxia. Mice were finally euthanized after a full 8 weeks of hypoxia acclimation with an overdose of isoflurane followed by cervical dislocation, blood was collected for a second set of haematology measurements, and the medulla was sampled and stored at -80°C for subsequent transcriptomic analyses (see below).

Measurements of acute hypoxia responses were made in unrestrained mice using barometric plethysmography, respirometry, and pulse oximetry techniques that we have
used in previous studies (Ivy and Scott, 2017; Ivy and Scott, 2018; Ivy et al., 2020). Mice were placed in a whole-body plethysmography chamber (530 ml) that was supplied with hyperoxic air (30 kPa O$_2$, balance N$_2$) at 600 ml min$^{-1}$. Mice were given 20-40 min to adjust to the chamber until relaxed and stable breathing and metabolism were observed. Mice were then maintained for an additional 20 min at 30 kPa O$_2$, after which they were exposed to 20 min stepwise reductions in inspired O$_2$ pressure (PO$_2$) to 21, 16, 12, 10, and 8 kPa. Dry incurrent gases were mixed using precision flow meters (Sierra Instruments, Monterey, CA, USA) and a mass flow controller (MFC-4, Sable Systems, Las Vegas, NV, USA), such that the desired PO$_2$ was delivered to the chamber at a constant flow rate of 600 ml min$^{-1}$. At the end of this protocol, mice were removed from the chamber and returned to their home cage in the appropriate acclimation condition.

Breathing (total ventilation, breathing frequency, and tidal volume), rates of O$_2$ consumption ($\dot{V}O_2$), body temperature ($T_b$), heart rate, and arterial O$_2$ saturation were determined during the last 10 min at each PO$_2$ as follows. Incurrent and excurrent air flows were subsampled at 200 ml min$^{-1}$; incurrent air was continuously measured for O$_2$ fraction (FC-10, Sable Systems), and excurrent air was analyzed for water vapour (RH-300, Sable Systems), dried with pre-baked drierite, and analyzed for O$_2$ and CO$_2$ fraction (FC-10 and CA-10, Sable Systems). These data were used to calculate $\dot{V}O_2$, expressed in volumes at standard temperature and pressure (STP), using established equations (Lighton, 2008). Chamber temperature was continuously recorded with a thermocouple (TC-2000, Sable Systems). Breathing frequency and tidal volume were measured from changes in flow across a pneumotachograph in the wall of the plethysmograph chamber.
and detected using a differential pressure transducer (Validyne DP45, Cancopass, Mississauga, ON, Canada). Tidal volume was calculated using established equations (Drorbaugh and Fenn, 1955; Jacky, 1980). Total ventilation was calculated as the product of breathing frequency and tidal volume. Total ventilation and tidal volume data are reported in volumes expressed at body temperature and pressure saturated (BTPS). Air convection requirement (ACR) is the quotient of total ventilation and $\dot{V}O_2$. All of the above data was acquired using PowerLab 16/32 and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, USA). $T_b$ was measured using thermosensitive passive transponders (micro LifeChips with Bio-therm technology; Destron Fearing, Dallas, TX, USA), which were implanted subdermally on the left side of the abdomen close to the leg ~2 weeks before normoxic measurements were conducted, along with a hand-held scanner from the same manufacturer. Arterial $O_2$ saturation and heart rate were measured using MouseOx Plus pulse oximeter collar sensors and data acquisition system (Starr Life Sciences, Oakmont, PA, USA). This was enabled by removing fur around the neck ~2 days before experiments.

Blood was collected into heparinized capillary tubes for haematology, sampled from the facial vein under light anaesthesia (~130 µl) for mice acclimated to normoxia, or by severing the jugular vein for mice that were euthanized and sampled after hypoxia acclimation. Blood Hb content was measured using Drabkin’s reagent according to the manufacturer’s instructions (Sigma-Aldrich, Oakville, ON, Canada). Haematocrit was measured by spinning the blood in the capillary tubes at 12,700 g for 5 min. Oxygen dissociation curves were generated at 37 °C for all mice using a Hemox Analyzer (TCS
Scientific, New Hope, PA, USA) using 10 µl of whole blood in 5 ml of buffer (100 mmol l⁻¹ HEPES, 50 mmol l⁻¹ EDTA, 100 mmol l⁻¹ KCl, 0.1% bovine serum albumin, and 0.2% antifoaming agent; TCS Scientific). Red cell O₂ affinity (P₅₀, the PO₂ at which haemoglobin is 50% saturation with O₂) was calculated using Hemox Analytic Software (TCS Scientific).

5.3.3 Experiments with captive populations from high- and low-altitude to elucidate the physiological effects of manipulating Hb-O₂ affinity with efaproxiral

Captive G₁ populations of deer mice from high- and low-altitude, held in standard holding conditions in normoxia, were used to assess the effects of manipulating Hb-O₂ binding affinity on the control of breathing. Mice were placed in the same plethysmograph that is described above, and were exposed to normoxic conditions (21 kPa O₂) for 40 min in order to make baseline measurements before injection. Mice were then removed and given an intraperitoneal injection of either saline or efaproxiral sodium (Fisher Scientific, Whitby, ON, Canada), and returned to the chamber. Measurements were made for 50 min in normoxia, followed by 20 min of hypoxia (12 kPa O₂). Breathing, VO₂, Tb, heart rate, and arterial O₂ saturation were measured in the last 10 min of each exposure as described above. Saline and efaproxiral injections were conducted in random order for each mouse, and were separated by 1 week. Efaproxiral was prepared in sterile saline (0.9% NaCl solution) on the day of experiments and was administered at a dose of 200 mg per kg body mass, which was determined in preliminary tests to have persistent effects on arterial O₂ saturation for the duration of the experiment.
5.3.4 Statistical analysis of physiological variables

Linear mixed-effects models were used for statistical analyses in the above experiments. They were used in experiments with F2 hybrids to test for effects of α and β globin haplotypes, acclimation environment, and inspired PO$_2$. They were used in the efaproxiral experiments to test for effects of efaproxiral, population, and inspired PO$_2$. We initially tested for the random effects of sex and family in both models, but they did not near statistical significance (P>0.10) and were therefore removed from the final models reported here. Holm-Sidak post-tests were used as appropriate. Values are reported as mean ± SEM. All statistical analysis was conducted using the lme4 package in R (v. 3.6.0) (Bates et al., 2015), with a significance level of P < 0.05.

5.3.5 RNA-Seq library preparation and transcriptomic analysis

We used high-throughput sequencing (RNA-seq; Wang et al., 2009) to test for the effects of haemoglobin genotype on gene expression in the medulla of F2 hybrids, from 5 α$^{HH}$β$^{HH}$ mice and 5 α$^{LL}$β$^{HL}$ mice sampled after hypoxia acclimation. We powdered the medulla under liquid N$_2$ and extracted total RNA from 15 mg of tissue using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), and then assessed RNA quality using TapeStation (RIN > 7; Agilent Technologies, Santa Clara, CA, USA). We generated Illumina sequencing libraries using TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA) beginning with 1 µg of RNA. The libraries were sequenced as 100 nt single-end reads on an Illumina HiSeq2500 platform, with all 10 individuals in one lane using
Illumina index adaptors. We performed a series of filtering steps to remove artifacts generated during sequencing. Reads with an average Phred quality score of less than 30 were removed from each library, after which low-quality bases were removed from the remaining sequences (Trimmomatic function for Illumina sequence data in Galaxy (Bolger et al., 2014)). Adaptor sequences were trimmed from sequence reads. These quality control steps yielded a total of 368.3 million reads with an average 36.8 million reads per individual (range = 33.3-44.5 million), and an average read length of approximately 120 bp.

Reads for each individual were mapped to the *P. maniculatus bairdii* genome (Pman_1.0; GenBank accession: GCF_000500345.1) using bwa (Li and Durbin, 2010), and we then computed transcript abundance values in Galaxy. We used *featureCounts* (Liao et al., 2014) to generate a table of transcript abundances. Sequence reads mapped to a total of 28,722 *P. maniculatus* genes. Since genes with low read counts are subject to increased measurement error (Robinson and Smyth, 2007) we excluded those with less than an average of 20 normalized reads per individual, leaving a total of 16,082 genes.

We compared the level of transcript abundance between $\alpha^{HH}\beta^{HH}$ and $\alpha^{LL}\beta^{HL}$ genotypes using whole-transcriptome differential expression analysis in *edgeR* (Robinson and Oshlack, 2010). The function *calcNormFactors* was used to normalize read counts across libraries, after model dispersion was estimated for each transcript separately using the function *estimateDisp* (McCarthy et al., 2012). We tested for differences in transcript abundance by first fitting a quasi-likelihood negative binomial generalized linear model to raw count data (*glmQLFit* function), which included a single main effect of genotype.
P-values were calculated using a quasi-likelihood F test using the `glmGLFTest` function. We controlled for multiple testing by enforcing a transcriptome-wide false discovery rate correction of 0.05 (Benjamini and Hochberg, 1995).

Weighted gene co-expression network analysis (WGCNA v. 1.41-1; Langfelder & Horvath, 2008) was used to identify modules of co-expressed genes and to then examine whether module expression differed between genotypes. Raw read counts were normalized by total library size and log-transformed using the `edgeR` functions `calcNormFactors` and `cpm`, respectively (Robinson et al., 2010). Module detection was performed using the `blockwiseModules` function in WGCNA with default parameters (Langfelder and Horvath, 2008). This involved calculating Pearson correlations of transcript abundance between pairs of genes, with an adjacency matrix computed by raising the correlation matrix to a soft thresholding power of $\beta = 7$. Soft thresholding was performed to achieve an approximately scale-free topology, an approach that favours strong correlations over weak ones (Zhang and Horvath, 2005). A $\beta$ of 7 was chosen because it represents the value where improvement of scale-free topology model fit begins to decrease with increasing threshold of power. A topological overlap measure was computed from the resulting adjacency matrix for each gene pair. Topologically based dissimilarity was then calculated and used as input for average linkage hierarchical clustering in the creation of cluster dendrograms for the medulla. Modules were identified as branches of the resulting cluster tree using the dynamic tree-cutting method (Langfelder and Horvath, 2008). WGCNA identified clusters of genes with highly correlated expression profiles across all 10 transcriptomes that were considered to be
modules of co-expressed genes. ANOVA on rank-transformed module eigengene values was then used to test for the effects of \( \alpha \)-globin genotype across modules, with \( P \)-values for the pairwise comparisons within a module corrected for multiple testing using a false discovery rate of 0.05 (Plachetzki et al., 2014).

### 5.3.6 Globin detection in the brainstem using Western Blots

A distinct group of mice from captive \( G_1 \) populations from high- and low-altitude were used to measure globin proteins in the medulla and pons. Mice (\( n=4 \) from each population) were acclimated to hypobaric hypoxia as described above for 8 weeks. Mice were then euthanized with an overdose of isoflurane, the chest was quickly opened to reveal the heart, an incision was made on the right atrium, and mice were perfused via the left ventricle with phosphate-buffered saline (PBS; 137 mmol l\(^{-1} \) NaCl, 2.68 mmol l\(^{-1} \) KCl, 10.0 mmol l\(^{-1} \) Na\(_2\)HPO\(_4\), 1.76 mmol l\(^{-1} \) KH\(_2\)PO\(_4\)) to flush blood from the circulation of the brainstem. The medulla and pons were then isolated, sampled, and stored at -80°C.

Western blot analysis was carried out according to Towbin et al., (1979) to assess \( \alpha \) and \( \beta \) globin protein expression in the medulla and pons. Medulla and pons tissues were homogenized in ice-cold radioimmune precipitation assay buffer (RIPA) containing 1 mM phenylmethylsulfonyl fluoride and 1X Protein Stabilizing Cocktail (ThermoFisher Scientific, Cat: 89806, Waltham, MA, USA). The homogenate was centrifuged at 13,000 rpm for 10 min at 4°C and the protein content of the supernatant was quantified using the Bradford assay (following instructions from the manufacturer, BioRad, Hercules, CA, USA). These protein isolates (~25 µg) were separated using 15% SDS-PAGE, along with
a positive control (~25 µg protein isolated from deer mouse blood) and a pre-stained protein marker (PageRuler™ Prestained Protein Ladder 10 to 180 kDa, cat# 26616, ThermoFisher Scientific). The proteins were transferred to nitrocellulose membranes (0.2 µm pore size) at 4 °C overnight using a Mini Trans-Blot cell (Bio-Rad, Hercules, CA, USA) at 30 V. The membrane was blocked by incubating in TBST (20 mM Tris, 150 mM NaCl with 0.1% Tween 20) containing 5% non-fat milk powder for 1 h at room temperature. The membrane was washed three times with TBST for 10 min each. The membrane was then incubated overnight at 4 °C in TBST containing 5% BSA, anti-GAPDH primary antibody as a loading control (1:10000 dilution, ab181603, Abcam, Cambridge, MA, USA), and one of the anti-globin primary antibodies: anti-haemoglobin subunit alpha (1:200, ab92492, Abcam) or anti-haemoglobin subunit beta (1:1000, ab214049, Abcam). The membrane was then washed three times with TBST for 10 min each, and incubated for 2 h at room temperature in TBST containing 5% BSA and secondary antibody (goat anti-rabbit IgG (HRP), 1:2000, ab205718, Abcam). The membrane was again washed three times with TBST for 10 min each, and then developed in 10 ml of 50 mM Tris (pH 7.6) containing 6 mg of 3,3’-diaminobenzidine (DAB) and 10 µl of hydrogen peroxide. Developing time ranged from 3 to 10 min, dependent on the band intensity of the house-keeping protein (GADPH) and the background of the membrane.

5.4 RESULTS
5.4.1 Effects of globin genotype on physiological responses to acute and chronic hypoxia
When F2 hybrids were considered altogether, both acute and chronic hypoxia affected breathing, metabolism, and arterial O\textsubscript{2} saturation (Table 5.1, Fig. S5.1). Total ventilation increased in response to acute stepwise hypoxia, as a result of increases in breathing frequency that were offset by small declines in tidal volume. Chronic hypoxia augmented these increases in total ventilation, particularly in response to severe acute hypoxia (acclimation environment×inspired PO\textsubscript{2}, P<0.0001), which arose from significant increases in breathing frequency (environment×PO\textsubscript{2}, P<0.0001) and subtle non-significant increases in tidal volume at low PO\textsubscript{2}. SaO\textsubscript{2}, O\textsubscript{2} consumption rate, heart rate, and body temperature all tended to decrease in response to acute stepwise hypoxia compared to normoxia, but these declines appeared to be reduced after chronic hypoxia (environment effects: P=0.0003, 0.0087, <0.0001, 0.0006, respectively). Chronic hypoxia also increased haematocrit and whole-blood Hb content (Table 5.2; Fig. S5.2).

Several of these cardiorespiratory and metabolic phenotypes varied across α-globin and β-globin genotypes (Tables 5.1,5.2), for which we discuss the significant effects in the Results but include the full suite of measurements for each genotype in Supplementary Figures (Figs. S5.2,S5.3,S5.4). α-globin genotype, but not β-globin genotype, had strong effects on arterial O\textsubscript{2} saturation (Table 5.1). SaO\textsubscript{2} in severe hypoxia was higher in the α\textsuperscript{HH} genotypes compared to the α\textsuperscript{LL} genotypes in measurements among normoxia-acclimated mice (effect of α-globin genotype, P=0.0115; Fig. 5.1A). However, although chronic hypoxia tended to reduce the decline in SaO\textsubscript{2} across genotypes (environment effect, P=0.0003), this effect was greater in mice with the α\textsuperscript{LL} genotypes, such that the difference in SaO\textsubscript{2} between genotypes was abolished after hypoxia.
acclimation (Fig. 5.1B). This variation in SaO\textsubscript{2} appeared to be associated with variation in red cell O\textsubscript{2} affinity, for which there was also a significant effect of \(\alpha\)-globin genotype (\(P=0.0003\)) but not \(\beta\)-globin genotype (Fig. 5.1C, Table 5.2). In particular, \(\alpha^{HH}\) mice exhibited significantly lower \(P_{50}\) than \(\alpha^{LL}\) mice before exposure to chronic hypoxia. However, \(P_{50}\) increased in response to chronic hypoxia in mice with the \(\alpha^{HH}\) genotype, in contrast to mice with the \(\alpha^{LL}\) genotype (\(\alpha\)-genotype×environment, \(P=0.0347\)), such that red cell \(P_{50}\) was similar between \(\alpha\)-globin genotypes after hypoxia acclimation.

There was also a strong effect of \(\alpha\)-globin genotype on breathing pattern, both before and after exposure to chronic hypoxia (Fig. 5.2, Table 5.1). In measurements before hypoxia acclimation, mice with the \(\alpha^{HH}\) genotype breathed using significantly deeper breaths (\(\alpha\)-globin effect, \(P=0.0003\)) but at a slower frequency (\(\alpha\)-globin effect, \(P<0.001\)) than mice with the \(\alpha^{LL}\) genotype, with no significant differences between \(\alpha\)-globin genotypes in total ventilation. Similar differences in breathing pattern between \(\alpha\)-globin genotypes persisted after exposure to chronic hypoxia, despite the increases in total ventilation that occurred in response to chronic hypoxia.

\(\beta\)-globin genotype affected the hypoxic ventilatory response, as reflected by a significant interaction between \(\beta\)-globin genotype and inspired PO\textsubscript{2} on total ventilation (\(P=0.0091\)). \(\beta\)-globin genotype had a particularly strong influence in mice that were homozygous for highland \(\alpha\)-globin (Fig. 5.3). Among these normoxia-acclimated mice, those that were homozygous for highland \(\beta\)-globin had higher total ventilation than both heterozygotes and lowland homozygotes at 12 kPa O\textsubscript{2}, and higher total ventilation than heterozygotes in more severe levels of acute hypoxia (Fig. 5.3A). However, these
differences between β-globin genotypes disappeared after hypoxia acclimation (Fig. 5.3B), potentially because the effects of hypoxia acclimation were greater in heterozygotes and lowland homozygotes. In contrast, β-globin genotype had no significant effects on O₂ consumption rate either before or after hypoxia acclimation (Fig. 5.3C,D; Table 5.1).

Body temperature was affected by both α-globin and β-globin genotypes (Table 5.1; Fig. S5.4). Tₚ in normoxia was similar across genotypes, ~36-38 °C on average. There appeared to be complex variation across genotypes in the PO₂ at which Tₚ depression began and the full magnitude of Tₚ depression in severe hypoxia, particularly among mice that were acclimated to normoxia, but most genotypes reduced the magnitude of Tₚ depression after hypoxia acclimation. However, neither α-globin nor β-globin genotype had significant effects on O₂ consumption rate before or after hypoxia acclimation (Fig. S5.4; Table 5.1).

5.4.2 Effects of manipulating arterial O₂ saturation on breathing pattern

We next sought to examine whether the effects of globin genotype on respiratory phenotypes could have arisen as a result of changes in blood O₂ affinity using deer mice from high- and low-altitude populations that were raised in captivity in normoxia. Efaproxiral – a synthetic drug that acts as a negative allosteric regulator of Hb-O₂ binding – was used to reduce blood O₂ affinity in vivo, which was expected to manifest as a reduction in arterial O₂ saturation in acute hypoxia. Indeed, efaproxiral reduced arterial O₂ saturation in high-altitude deer mice in hypoxia (Fig. 5.4A) and in low-altitude deer
mice in both normoxia and hypoxia (Fig. 5.4B) compared to saline controls (P<0.0001 for treatment effect and treatment×PO$_2$ interaction; Table 5.3). The magnitude of the effect of efaproxiral on arterial O$_2$ saturation differed between populations (population×treatment, P=0.0019; population×treatment×PO$_2$, P=0.0479), driven by larger effects in lowlanders than in highlanders. However, efaproxiral had no consistent effects on breathing frequency, tidal volume, or total ventilation in each population (no significant treatment or treatment×PO$_2$ effects) (Fig. 5.4, Table 5.3). Efaproxiral also had no consistent effects on oxygen consumption rate, air convection requirement, and body temperature (Tables 5.3, 5.4). However, efaproxiral did affect heart rate, as reflected by a significant treatment×PO$_2$ interaction (P=0.0054) that was driven primarily by increased heart rates in lowlanders after efaproxiral injection (population×treatment, P=0.0119; Tables 5.3, 5.4). Therefore, our treatment was successful in reducing arterial O$_2$ saturation and leading to potential compensatory changes in heart rate, but it had no effect on the control of breathing.

5.4.3 Globin expression and transcriptomics of the brainstem

We next sought to examine whether the effects of globin genotype on respiratory phenotypes could have arisen as a result of globin expression in key brainstem sites of ventilatory control, and whether these effects were associated with changes in gene expression in the medulla. Neither α-globin transcripts ($Hba$) nor β-globin transcripts ($Hbb$) were detected by high-throughput sequencing of the medulla transcriptome. Furthermore, neither α-globin nor β-globin proteins were identified using Western blots
of perfused brainstem tissue from the medulla and pons (Fig. 5.5). Therefore, the effects of globin genotype on control of breathing were not an effect of globin expression in key brainstem sites of respiratory control.

There were also very few differences in gene expression in the medulla between mice with different globin genotypes. There were no significant differences between $\alpha^{HH}\beta^{HH}$ mice and $\alpha^{LL}\beta^{HL}$ mice in gene expression across the medulla transcriptome, when significance was considered at $P<0.05$ after transcriptome-wide correction for false discovery rate (FDR). Furthermore, only 4 genes approached significance – $Ii1rap$, $Trpv3$, $Decr2$, and $Cntrob$ ($P = 0.0503$ for all) – all of which had reduced expression in $\alpha^{HH}\beta^{HH}$ mice compared to $\alpha^{LL}\beta^{HL}$ mice (Table S5.1). Weighted gene co-expression network analysis (WGCNA) identified 69 gene expression modules across the medulla transcriptome, but they were not differentially expressed between globin genotypes (Table S5.2). Therefore, the effects of globin genotype on control of breathing do not appear to be associated with medulla-wide changes in gene expression.

5.5 DISCUSSION

Haemoglobin-O$_2$ affinity has been a pervasive target of selection in many high-altitude taxa and is often presumed to safe-guard arterial O$_2$ saturation in hypoxia, but whether genetic variation in globins contribute to the other unique phenotypes of high-altitude natives is unknown. Here, we show that the genetic variants of $\alpha$-globin and $\beta$-globin in high-altitude deer mice lead to changes in control of breathing that should augment alveolar ventilation in hypoxia. These effects did not appear to result from changes in blood-O$_2$ affinity, nor were they associated with non-erythroid expression of
globins in key brainstem sites of ventilatory control or with appreciable changes in gene
expression in the medulla. Nevertheless, our results suggest the evolution of Hb genes
may have pervasive effects on multiple respiratory phenotypes, and may contribute to
environmental adaptation via physiological mechanisms that are not commonly ascribed
to this protein.

α-globin genotype had a strong influence on breathing pattern, in which α\(^{HH}\) mice
breathed deeper but less frequently, a change that likely augments alveolar ventilation.
These differences in breathing pattern between α-globin genotypes can completely
account for the similar differences we have previously observed in highland populations
of deer mice compared to lowland populations of deer mice and white-footed mice,
measured in wild mice and in mice raised for one or two generations in captivity in
normoxia (Ivy and Scott, 2017; Ivy and Scott, 2018; Ivy et al., 2020). Such changes in
breathing pattern have also evolved in the high-altitude bar-headed goose (Scott et al.,
2007), which also possesses an α-globin variant that contributes to increasing
haemoglobin-O\(_2\) affinity (Natarajan et al., 2018), suggesting that there may be a causal
association between α-globin genotype and breathing pattern in other high-altitude taxa.

β-globin genotype appeared to affect the ventilatory response to hypoxia among
normoxia acclimated mice, with α\(^{HH}\)β\(^{HH}\) mice exhibiting higher total ventilation than
α\(^{HH}\)β\(^{HL}\) and α\(^{HH}\)β\(^{LL}\) mice at 12 kPa O\(_2\). These findings mirror those in lab-strain mice
possessing the Hb Presbyterian mutation in β globin, which exhibit reduced Hb-O\(_2\)
affinity and an attenuated hypoxic ventilatory response (Izumizaki et al., 2003).
Differences in β-globin genotype could thus contribute to the increases in total ventilation
in highland deer mice compared to lowland deer mice and white-footed mice that we have observed among mice acclimated to normoxia (Ivy and Scott, 2017; Ivy and Scott, 2018; Ivy et al., 2020). However, these effects of β-globin genotype were abolished after hypoxia acclimation, suggesting that β-globin genotype may influence the effects of chronic hypoxia on the control of breathing, a process termed ventilatory acclimatization to hypoxia (VAH). If so, β-globin may contribute to the attenuation of VAH that we have previously observed in highland deer mice (Ivy and Scott, 2017; Ivy and Scott, 2018). Indeed, we have recently shown that genetic variants in the HIF-2α gene *Epas1* in highland deer mice can partially but not completely account for this attenuation of VAH (Chapter 4), so genetic variation in β-globin could also play a role.

Effects of globin variants on control of breathing did not appear to result from variation in haemoglobin-O₂ affinity or arterial O₂ saturation. Treatment with efaproxiral to reduce the blood-O₂ affinity had no effect on total ventilation or breathing pattern in normoxia or hypoxia. There were differences in the magnitude of the effects of efaproxiral on arterial O₂ saturation and heart rate between populations, likely because the inherently lower blood-O₂ affinity of lowlanders (Ivy et al., 2020) made them more susceptible to impairments in pulmonary O₂ loading upon further reduction in affinity, but efaproxiral had no effect on breathing in either the lowland or highland population. Furthermore, the differences in breathing pattern between α⁺⁺ and α⁺⁻ mice persisted in hyperoxia (30 kPa O₂) when the blood would have been super-saturated with O₂. These findings suggest that the effects of globin variants on breathing arise from mechanisms that are not directly associated with the role of haemoglobin in circulatory O₂ transport.
These mechanisms do not appear to involve non-erythroid expression of globins in key brainstem sites of ventilatory control, nor are they associated with medulla-wide changes in gene expression, and most likely involve actions of globins at other sites.

The effects of genetic variation in α-globin on arterial $O_2$ saturation and red cell $O_2$ affinity were contingent upon acclimation environment. Highland homozygotes maintained higher arterial $O_2$ saturation in hypoxia and had higher Hb-$O_2$ affinity than lowland homozygotes when comparisons were made among normoxia-acclimated mice, but these differences were abolished after hypoxia acclimation. This discrepancy could be explained by differences in sensitivity to 2,3-DPG, a key negative allosteric regulator of Hb-$O_2$ binding in mammalian erythrocytes that typically increases in concentration after hypoxia acclimation (Lenfant et al., 1968). Indeed, previous studies of $O_2$-binding properties of stripped haemoglobin suggest that 2,3-DPG sensitivity is greater in high-altitude populations of deer mice when measured in the presence of relevant concentrations of $Cl^-$ (Storz et al., 2010). Therefore, highland homozygotes could have been more sensitive to the increases in red cell 2,3-DPG concentration that may have occurred with hypoxia acclimation, and have thus exhibited a more pronounced increase in red cell $P_{50}$ and a less pronounced increase in arterial $O_2$ saturation in hypoxia.
### 5.6 TABLES AND FIGURES

Table 5.1. Results of linear mixed-effects models of physiological responses to acute stepwise hypoxia in F2 hybrid deer mice

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>Env</th>
<th>$P_i O_2$</th>
<th>$\alpha*Env$</th>
<th>$\beta*Env$</th>
<th>Env*$P_i O_2$</th>
<th>$\alpha*P_i O_2$</th>
<th>$\beta*P_i O_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial $O_2$ saturation</td>
<td>6.9202</td>
<td>0.3438</td>
<td>15.406</td>
<td>579.58</td>
<td>0.6002</td>
<td>0.5098</td>
<td>4.6531</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>P</td>
<td>0.0115</td>
<td>0.7109</td>
<td>0.0003</td>
<td>&lt;0.0001</td>
<td>0.5531</td>
<td>0.4789</td>
<td>0.0004</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total ventilation</td>
<td>0.2883</td>
<td>1.2737</td>
<td>12.333</td>
<td>215.46</td>
<td>0.1507</td>
<td>0.2229</td>
<td>13.5741</td>
<td>n.s.</td>
<td>2.4262</td>
</tr>
<tr>
<td>P</td>
<td>0.5938</td>
<td>0.2893</td>
<td>0.0009</td>
<td>&lt;0.0001</td>
<td>0.6997</td>
<td>0.8011</td>
<td>&lt;0.0001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Breathing frequency</td>
<td>20.612</td>
<td>0.1032</td>
<td>4.0450</td>
<td>346.65</td>
<td>0.5260</td>
<td>1.6550</td>
<td>5.1967</td>
<td>6.2340</td>
<td>n.s.</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>0.9021</td>
<td>0.0501</td>
<td>&lt;0.0001</td>
<td>0.4721</td>
<td>0.2028</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>n.s.</td>
</tr>
<tr>
<td>Tidal volume</td>
<td>15.581</td>
<td>1.3451</td>
<td>0.8357</td>
<td>61.040</td>
<td>0.1373</td>
<td>0.1466</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>P</td>
<td>0.0003</td>
<td>0.2703</td>
<td>0.3653</td>
<td>&lt;0.0001</td>
<td>0.7128</td>
<td>0.8640</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Air convection requirement</td>
<td>2.0682</td>
<td>47.003</td>
<td>0.1411</td>
<td>118.69</td>
<td>0.1417</td>
<td>0.3870</td>
<td>3.9349</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>P</td>
<td>0.1570</td>
<td>0.1087</td>
<td>0.7088</td>
<td>&lt;0.0001</td>
<td>0.7084</td>
<td>0.6814</td>
<td>0.0019</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>$O_2$ consumption rate</td>
<td>0.5474</td>
<td>0.8862</td>
<td>7.4953</td>
<td>10.342</td>
<td>0.0001</td>
<td>0.0364</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>P</td>
<td>0.4630</td>
<td>0.4190</td>
<td>0.0087</td>
<td>&lt;0.0001</td>
<td>0.9991</td>
<td>0.9643</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Heart rate</td>
<td>1.0164</td>
<td>1.5240</td>
<td>33.048</td>
<td>10.583</td>
<td>0.6605</td>
<td>0.0057</td>
<td>13.524</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
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<td>P</td>
<td>0.3185</td>
<td>0.2284</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.4208</td>
<td>0.9943</td>
<td>&lt;0.0001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Body temperature</td>
<td>7.8256</td>
<td>3.5823</td>
<td>13.469</td>
<td>76.840</td>
<td>2.1004</td>
<td>0.1326</td>
<td>1.9356</td>
<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>P</td>
<td>0.0074</td>
<td>0.0365</td>
<td>0.0006</td>
<td>&lt;0.0001</td>
<td>0.1543</td>
<td>0.8762</td>
<td>0.0890</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Env, acclimation environment, $P_i O_2$, $O_2$ partial pressure of inspired air during acute stepwise hypoxia.
Table 5.2. Results of linear mixed-effects models of blood responses to chronic hypoxia in F2 hybrid deer mice

<table>
<thead>
<tr>
<th></th>
<th>α</th>
<th>β</th>
<th>Env</th>
<th>α*Env</th>
<th>β*Env</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red cell ( P_{50} )</td>
<td>F</td>
<td>18.220</td>
<td>3.0814</td>
<td>13.191</td>
<td>5.0368</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0003</td>
<td>0.0632</td>
<td>0.0003</td>
<td>0.0347</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>F</td>
<td>0.0182</td>
<td>0.1359</td>
<td>223.80</td>
<td>0.2108</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.8937</td>
<td>0.8736</td>
<td>&lt;0.0001</td>
<td>0.6504</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>F</td>
<td>0.2098</td>
<td>0.0494</td>
<td>64.847</td>
<td>0.6543</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.6527</td>
<td>0.9519</td>
<td>&lt;0.0001</td>
<td>0.4329</td>
</tr>
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</table>

Env, acclimation environment, \( P_{50} \), the partial pressure of O\(_2\) at which haemoglobin is 50% saturation with O\(_2\).
Table 5.3. Results of linear mixed-effects models of the effects of efaproxiral and inspired \( \text{PO}_2 \) in highland and lowland populations of deer mice

<table>
<thead>
<tr>
<th></th>
<th>Pop</th>
<th>Treat</th>
<th>( \text{PO}_2 )</th>
<th>Pop*( \text{PO}_2 )</th>
<th>Treat*( \text{PO}_2 )</th>
<th>Pop*Treat</th>
<th>Pop<em>Treat</em>( \text{PO}_2 )</th>
</tr>
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<tbody>
<tr>
<td>Arterial ( \text{O}_2 ) saturation</td>
<td>F 4.1686</td>
<td>39.146</td>
<td>569.12</td>
<td>4.3395</td>
<td>11.410</td>
<td>10.715</td>
<td>3.2306</td>
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<tr>
<td></td>
<td>P 0.0684</td>
<td></td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0183</td>
<td>&lt;0.0001</td>
<td>0.0019</td>
</tr>
<tr>
<td>Breathing frequency</td>
<td>F 17.898</td>
<td>1.1134</td>
<td>39.657</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
</tr>
<tr>
<td></td>
<td>P 0.0017</td>
<td>0.2958</td>
<td>&lt;0.0001</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
</tr>
<tr>
<td>Tidal volume</td>
<td>F 0.0460</td>
<td>0.5733</td>
<td>6.9964</td>
<td>n.s</td>
<td>n.s</td>
<td>7.8920</td>
<td>n.s</td>
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<tr>
<td></td>
<td>P 0.8344</td>
<td>0.4521</td>
<td>0.0019</td>
<td>n.s</td>
<td>n.s</td>
<td>0.0068</td>
<td>n.s</td>
</tr>
<tr>
<td>Total ventilation</td>
<td>F 5.6065</td>
<td>0.0175</td>
<td>11.034</td>
<td>n.s</td>
<td>n.s</td>
<td>7.8799</td>
<td>n.s</td>
</tr>
<tr>
<td></td>
<td>P 0.0394</td>
<td>0.8953</td>
<td>&lt;0.0001</td>
<td>n.s</td>
<td>n.s</td>
<td>0.0069</td>
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</tr>
<tr>
<td>Air convection requirement</td>
<td>F 1.0930</td>
<td>0.6065</td>
<td>15.154</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
</tr>
<tr>
<td></td>
<td>P 0.3204</td>
<td>0.4393</td>
<td>&lt;0.0001</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
</tr>
<tr>
<td>( \text{O}_2 ) consumption rate</td>
<td>F 26.982</td>
<td>0.288</td>
<td>4.3785</td>
<td>n.s</td>
<td>n.s</td>
<td>7.8473</td>
<td>n.s</td>
</tr>
<tr>
<td></td>
<td>P 0.0004</td>
<td>0.5931</td>
<td>0.0171</td>
<td>n.s</td>
<td>n.s</td>
<td>0.0070</td>
<td>n.s</td>
</tr>
<tr>
<td>Heart rate</td>
<td>F 2.5305</td>
<td>2.5382</td>
<td>9.5632</td>
<td>n.s</td>
<td>5.7649</td>
<td>6.7673</td>
<td>n.s</td>
</tr>
<tr>
<td></td>
<td>P 0.1427</td>
<td>0.1170</td>
<td>0.0003</td>
<td>n.s</td>
<td>0.0054</td>
<td>0.0119</td>
<td>n.s</td>
</tr>
<tr>
<td>Body temperature</td>
<td>F 0.0024</td>
<td>1.8942</td>
<td>5.6830</td>
<td>n.s</td>
<td>n.s</td>
<td>12.483</td>
<td>n.s</td>
</tr>
<tr>
<td></td>
<td>P 0.9619</td>
<td>0.1742</td>
<td>0.0057</td>
<td>n.s</td>
<td>n.s</td>
<td>0.0008</td>
<td>n.s</td>
</tr>
</tbody>
</table>

Pop, population; Treat, treatment (saline or efaproxiral); \( \text{PO}_2 \), partial pressure of \( \text{O}_2 \)
Table 5.4. O\textsubscript{2} consumption rate, air convection requirement, heart rate, and body temperature responses of G\textsubscript{1} deer mice during manipulation of arterial O\textsubscript{2} saturation using efaproxiral (200 mg kg\textsuperscript{-1}).

<table>
<thead>
<tr>
<th>PO\textsubscript{2} (kPa)</th>
<th>Highlanders</th>
<th>Lowlanders</th>
<th>Highlanders</th>
<th>Lowlanders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Efaproxiral</td>
<td>Saline</td>
<td>Efaproxiral</td>
<td></td>
</tr>
<tr>
<td>Pre-injection</td>
<td>21</td>
<td>0.051 ± 0.004</td>
<td>0.038 ± 0.003*</td>
<td>0.062 ± 0.004</td>
</tr>
<tr>
<td>Post-injection</td>
<td>21</td>
<td>0.044 ± 0.003</td>
<td>0.042 ± 0.004</td>
<td>0.054 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.043 ± 0.001</td>
<td>0.041 ± 0.002</td>
<td>0.052 ± 0.003</td>
</tr>
</tbody>
</table>

O\textsubscript{2} consumption rate (ml g\textsuperscript{-1} min\textsuperscript{-1})

<table>
<thead>
<tr>
<th>Air convection requirement (ml air per ml O\textsubscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-injection</td>
</tr>
<tr>
<td>21</td>
</tr>
<tr>
<td>Post-injection</td>
</tr>
<tr>
<td>21</td>
</tr>
<tr>
<td>12</td>
</tr>
</tbody>
</table>

Heart rate (beats min\textsuperscript{-1})

| Pre-injection                                               |
| 21 | 513.2 ± 53.3 | 582.7 ± 32.3 | 447.9 ± 41.7 | 556.9 ± 21.4 |
| Post-injection                                              |
| 21 | 498.7 ± 60.3 | 539.6 ± 27.3 | 525.2 ± 36.1 | 654.1 ± 26.4* |
| 12 | 592.4 ± 35.5 | 568.4 ± 29.7 | 596.9 ± 29.7 | 664.4 ± 39.2 |

Body temperature (°C)

| Pre-injection                                               |
| 21 | 37.83 ± 0.38 | 38.67 ± 0.32 | 38.35 ± 0.42 | 37.28 ± 0.63 |
| Post-injection                                              |
| 21 | 36.87 ± 0.76 | 38.23 ± 0.43 | 37.95 ± 0.40 | 37.12 ± 0.50 |
| 12 | 36.67 ± 0.66 | 37.65 ± 0.12 | 37.22 ± 0.39 | 36.20 ± 0.82 |

PO\textsubscript{2}, partial pressure of O\textsubscript{2}. Values are mean ± SEM (N=6). * denotes a significant pairwise difference between saline and efaproxiral injections within a PO\textsubscript{2} and deer mouse population.
A: Normoxia acclimation

B: Hypoxia acclimation

C: Normoxia acclimation

Hypoxia acclimation

*  

#  

Red cell P50 (kPa)  

\(\alpha\)-globin genotype
Figure 5.1. Arterial O$_2$ saturation during acute hypoxia was affected by α-globin genotype in F2 hybrid deer mice before (A) but not after (B) hypoxia acclimation. $\alpha^{HH}$ represents all mice that were homozygous for the highland α-globin genotype (N=17), and $\alpha^{LL}$ represents all mice that were homozygous for the lowland α-globin genotype (N=9). Values are mean ± SEM. * and # denotes significant pairwise differences using Holm-Sidak post-tests between α-globin genotypes within an acclimation environment and between acclimation environments within an α-globin genotype, respectively.
Figure 5.2. Breathing pattern was altered by α-globin genotype in F2 hybrid deer mice both before (A,B,C) and after (D,E,F) hypoxia acclimation. α\textsuperscript{HH} represents all mice that were homozygous for the highland α-globin genotype (N=17), and α\textsuperscript{LL} represents all mice that were homozygous for the lowland α-globin genotype (N=9). Values are mean ± SEM. † represents a significant main effect of α-globin genotype, * represents a significant pairwise differences between genotypes within a PO\textsubscript{2} using Holm-Sidak post-tests.
Figure 5.3. Total ventilation was influenced by β-globin genotype in F2 hybrid deer mice before (A), but not after (B) hypoxia acclimation. There was no significant effect of β-globin genotype on O2 consumption rate (C,D). αHHβHH represents mice that are homozygous for the highland α-globin and β-globin genotype (N=5), αHHβHL represents mice that are homozygous for the highland α-globin genotype and heterozygous β-globin genotype (N=5), αHHβLL represents mice that are homozygous for the highland α-globin genotype and homozygous lowland for the β-globin genotype (N=7). Values are mean ± SEM. γ and ϕ represent significant pairwise differences within a PO2 between αHHβHH and αHHβHL, or between αHHβHH and both of αHHβHL and αHHβLL, respectively (Holm-Sidak post-tests).
Figure 5.4. Efaproxiral treatment to reduce haemoglobin-O\textsubscript{2} affinity reduced arterial O\textsubscript{2} saturation but did not influence breathing pattern in highland or lowland populations of deer mice. Values are mean ± SEM (N=6). * represents a significant pairwise differences between saline and efaproxiral (200 mg kg\textsuperscript{-1}) treatments within a PO\textsubscript{2} using Holm-Sidak post-tests.
Figure 5.5. Western blots for α-globin (HbA) (A,C) or β-globin (HbB) (B,D) and for glyceraldehyde 3-phosphate dehydrogenase (GADPH; loading control) in protein isolates from medulla (A,B) and pons (C,D) tissues of high- and low-altitude populations of deer mice. B, blood protein (positive control); L, protein ladder.
5.7 SUPPLEMENTARY TABLES AND FIGURES

Table S5.1. Genes that were differentially expressed in the medulla between α\textsuperscript{HH}β\textsuperscript{HH} mice and α\textsuperscript{LL}β\textsuperscript{HL} mice at P<0.10 after correction for the false discovery rate (P\textsubscript{FDR}).

<table>
<thead>
<tr>
<th>Gene</th>
<th>logFC</th>
<th>logCPM</th>
<th>F</th>
<th>P</th>
<th>P\textsubscript{FDR}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Il1rap</td>
<td>-0.759701319</td>
<td>6.446530633</td>
<td>58.77968766</td>
<td>6.17x10\textsuperscript{-6}</td>
<td>0.050329613</td>
</tr>
<tr>
<td>Trpv3</td>
<td>-1.980597470</td>
<td>0.822180505</td>
<td>52.04990319</td>
<td>1.13x10\textsuperscript{-5}</td>
<td>0.050329613</td>
</tr>
<tr>
<td>Decr2</td>
<td>-0.692115018</td>
<td>4.264648126</td>
<td>51.10075100</td>
<td>1.24x10\textsuperscript{-5}</td>
<td>0.050329613</td>
</tr>
<tr>
<td>Cntrob</td>
<td>-0.700340848</td>
<td>4.255780705</td>
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</tr>
<tr>
<td>Slc13a5</td>
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<td>3.564332348</td>
<td>42.00434728</td>
<td>3.17x10\textsuperscript{-5}</td>
<td>0.102179242</td>
</tr>
</tbody>
</table>

Table reports average log\textsubscript{2} fold-change (logFC) in expression, average log\textsubscript{2} read counts per million (logCPM), and the F-value, uncorrected P-value, and false-discovery rate corrected p-value (P\textsubscript{FDR}) of a generalized linear model assessing the effects of globin genotype.
Table S5.2. Comparison of modules from WGCNA between genotypes.

<table>
<thead>
<tr>
<th>Module</th>
<th>P</th>
<th>P_{FRD}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plum</td>
<td>0.189</td>
<td>0.862</td>
</tr>
<tr>
<td>Plum1</td>
<td>0.378</td>
<td>0.905</td>
</tr>
<tr>
<td>White</td>
<td>0.774</td>
<td>0.905</td>
</tr>
<tr>
<td>Honeydew1</td>
<td>0.275</td>
<td>0.862</td>
</tr>
<tr>
<td>Cyan</td>
<td><strong>0.005</strong></td>
<td>0.173</td>
</tr>
<tr>
<td>Skyblue3</td>
<td>0.275</td>
<td>0.862</td>
</tr>
<tr>
<td>Yellow4</td>
<td>0.774</td>
<td>0.905</td>
</tr>
<tr>
<td>Darkturquoise</td>
<td>0.122</td>
<td>0.842</td>
</tr>
<tr>
<td>Lightgreen</td>
<td>0.774</td>
<td>0.905</td>
</tr>
<tr>
<td>Thistle1</td>
<td>0.774</td>
<td>0.905</td>
</tr>
<tr>
<td>Brown4</td>
<td>0.631</td>
<td>0.905</td>
</tr>
<tr>
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<td>0.924</td>
<td>0.924</td>
</tr>
<tr>
<td>Darkgrey</td>
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<td>0.924</td>
</tr>
<tr>
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<td>0.862</td>
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</tr>
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<td>---------------</td>
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</tr>
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<td>Greenyellow</td>
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<td>0.842</td>
</tr>
<tr>
<td>Paleturquoise</td>
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</tr>
<tr>
<td>Lightpink4</td>
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<td>0.862</td>
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<tr>
<td>Grey</td>
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<td>0.905</td>
</tr>
</tbody>
</table>

Table reports uncorrected p-values, and false-discovery rate corrected p-values ($P_{FDR}$).
Figure S5.1. Chronic exposure to hypoxia affected the ventilatory response to acute hypoxia across genotypes of F2 hybrid deer mice. Values are mean ± SEM. # represents a significant pairwise difference between acclimation groups within a PO2 using Holm-Sidak post-tests (N=26).
A

![Bar chart showing Haematocrit (%) for Normoxia and Hypoxia conditions.](image)

B

![Bar chart showing Hemoglobin (g d⁻¹) for different globin genotypes.](image)
Figure S5.2. Haematocrit (A) and whole-blood haemoglobin content (B) were not influenced by α- or β-globin genotype in F2 hybrid deer mice, but increased after hypoxia acclimation. $\alpha^{HH}\beta^{HH}$ represents mice that are homozygous for the highland α-globin and β-globin genotype (N=5), $\alpha^{HH}\beta^{HL}$ represents mice that are homozygous for the highland α-globin genotype and heterozygous β-globin genotype (N=5), $\alpha^{HH}\beta^{LL}$ represents mice that are homozygous for the highland α-globin genotype and homozygous lowland for the β-globin genotype (N=7), $\alpha^{LL}\beta^{HH}$ represents mice that are homozygous for the lowland α-globin genotype and homozygous for the highland β-globin genotype (N=4), $\alpha^{LL}\beta^{HL}$ represents mice that are homozygous for the lowland α-globin genotype and heterozygous β-globin genotype. Values are mean ± SEM. # denotes significant pairwise differences using Holm-Sidak post-tests between acclimation environments within an α-globin genotype.
Figure S5.3. Arterial O₂ saturation (A,B), total ventilation (C,D), breathing frequency (E,F), and tidal volume (G,H) responses of F2 hybrid mice before (A,C,E,G) and after (B,D,F,H) hypoxia acclimation separated by α- and β-globin haplotypes. Values are mean ± SEM (N as in Figure S2).
Figure S5.4. Air convection requirement (A,B), O$_2$ consumption rate (C,D), heart rate (E,F), and body temperature (G,H) responses of F2 hybrid mice before (A,C,E,G) and after (B,D,F,H) hypoxia acclimation separated by $\alpha$- and $\beta$-globin haplotypes. Values are mean ± SEM (N as in Figure S2).
5.8 REFERENCES


CHAPTER 6

ONTogenesis Of Evolved Changes In Respiratory Physiology In deer Mice Native To High Altitude


6.1 Abstract

High-altitude environments are cold and hypoxic, and many high-altitude natives have evolved changes in respiratory physiology that improve O\textsubscript{2} uptake in hypoxia as adults. Altricial mammals undergo a dramatic metabolic transition from ectothermy to endothermy in early post-natal life, which may influence the ontogenetic development of respiratory traits at high altitude. We examined the developmental changes in respiratory and haematological traits in deer mice (Peromyscus maniculatus) native to high altitude, comparing the respiratory responses to progressive hypoxia between highland and lowland deer mice. Among adults, highlanders exhibited higher total ventilation and a more effective breathing pattern (relatively deeper tidal volumes), for mice that were caught and tested at their native altitudes and those lab-raised in normoxia. Lab-raised progeny of each population were also tested at post-natal day (P) 7, 14, 21, and 30. Highlanders developed an enhanced hypoxic ventilatory response by P21, concurrent with the full maturation of the carotid bodies, and their more effective breathing pattern arose by P14; these ages correspond to critical benchmarks in the full development of homeothermy in highlanders. However, highlanders exhibited developmental delays in ventilatory sensitivity to hypoxia, hyperplasia of type I cells in the carotid body, and increases in blood haemoglobin content compared to lowland mice. Nevertheless,
highlanders maintained consistently higher arterial O$_2$ saturation in hypoxia across development, in association with increases in blood-O$_2$ affinity that were apparent from birth. We conclude that evolved changes in respiratory physiology in high-altitude deer mice become expressed in association with the post-natal development of endothermy.
6.2 INTRODUCTION

High-altitude natives are great models for understanding physiological mechanisms of hypoxia adaptation. High-altitude environments are both cold and hypoxic, which challenges the ability of endotherms to maintain adequate \( O_2 \) supply for thermoregulation and exercise. However, many animals are endemic to high altitude, with research suggesting they have overcome the challenges to aerobic metabolism associated with living at high altitude through changes in the \( O_2 \) transport pathway (Monge and León-Velarde, 1991; Storz et al., 2010a; Tate et al., 2017). The function of the \( O_2 \) pathway – composed of pulmonary ventilation (breathing), pulmonary \( O_2 \) diffusion, circulatory \( O_2 \) delivery, tissue \( O_2 \) diffusion, and cellular \( O_2 \) utilization – is a critical determinant of tissue \( O_2 \) supply. As the first step in the \( O_2 \) pathway, pulmonary ventilation must occur at appropriate rates and/or using an effective pattern to safeguard \( O_2 \) uptake in hypoxic environments (Ivy and Scott, 2015; Storz and Scott, 2019; Storz et al., 2010a).

The control of breathing has evolved to enhance \( O_2 \) uptake in many high-altitude taxa. Breathing is stimulated by reductions in arterial \( O_2 \) levels in hypoxia, which is sensed by peripheral chemoreceptors in the carotid bodies that initiate the hypoxic chemoreflex and drive the increases in total ventilation (termed the hypoxic ventilatory response, HVR) (Gonzalez et al., 1994; Powell et al., 1998). Several highland taxa exhibit an enhanced HVR compared to their lowland counterparts, including Tibetan humans, plateau pika (\( Ochotona curzoniae \)), and bar-headed geese (\( Anser indicus \)) (Beall et al., 1997; Lague et al., 2016; Moore, 2000; Pichon et al., 2009; Scott and Milsom, 2006). These evolved changes have not occurred in some other highland taxa (Beall, 2000;
Brutsaert et al., 2005; Ivy et al., 2018; Lague et al., 2017; Schwenke et al., 2007), but in the taxa in which they have arisen, they are likely valuable for augmenting O₂ uptake in the hypoxic environment at high altitude to support aerobically demanding activities. However, these previous observations have been made exclusively in adult animals, and we know very little about the ontogenesis of these traits during early development.

Why is it important to study the ontogenetic development of high-altitude phenotypes? Early post-natal life is characterized by many key transitions that facilitate the maturation of offspring towards independence from its mother. For example, small altricial mammals such as mice are born without the ability to thermoregulate, and thermogenesis and the capacity for homeothermy develop over the first few weeks of post-natal life (Chew and Spencer, 1967; Lagerspetz, 1966). Given this developmental timeline, the unique respiratory phenotypes of high-altitude natives may not be present from birth, but may instead manifest only with the complete development of endothermic homeothermy when the metabolic O₂ costs of life are high. Indeed, the development of several processes important for respiratory O₂ uptake is also occurring during the first few weeks of life in small lowland mammals, such as the maturation of O₂ sensitive cells in the carotid body (Bamford et al., 1999; Carroll and Kim, 2012; Carroll et al., 1993) and the maturation of alveoli in the lungs (Amy et al., 1977). Nevertheless, the timing of these developmental milestones has rarely been studied in high-altitude natives.

The objective of this study was to investigate the post-natal development of the evolved changes in the control of breathing and respiratory O₂ uptake in high-altitude deer mice (Peromyscus maniculatus). Deer mice are broadly distributed across North
America and can be found from sea level to over 4,300 m elevation in the Rocky
Mountains (Hock, 1964; Natarajan et al., 2015; Snyder et al., 1982). Adults at high-
altitude sustain high metabolic rates in the wild (Hayes, 1989), and have evolved a higher
aerobic capacity (\(\dot{V}O_{2\text{max}}\)) in hypoxia compared to low-altitude populations of deer mice
as well as to white-footed mice (\(P.\ leucopus\)), a congeneric species that is restricted to
low altitudes (Cheviron et al., 2012; Cheviron et al., 2013; Cheviron et al., 2014; Lui et
al., 2015; Tate et al., 2017). Evolved changes in the \(O_2\) transport pathway have also arisen
in high-altitude deer mice, including increases in haemoglobin (Hb)-\(O_2\) affinity and
changes in various other traits that influence \(O_2\) supply and utilization by thermogenic
tissues in hypoxia (Dawson et al., 2018; Lau et al., 2017; Lui et al., 2015; Mahalingam et
al., 2017; Scott et al., 2015; Snyder et al., 1982; Storz et al., 2009; Storz et al., 2010b;
Tate et al., 2017). Differences in the control of breathing also exist in high-altitude deer
mice relative to low-altitude white-footed mice, as reflected by higher total ventilation
and a more effective breathing pattern (deeper but less frequent breaths) in the former in
comparisons of adults (Ivy and Scott, 2017a; Ivy and Scott, 2018). We hypothesized that
these differences represent evolved changes that are uniquely derived at high altitude,
which we test here by making intraspecific comparisons between high-altitude and low-
altitude populations of deer mice. Furthermore, recent research has shown that the
ontogenetic development of homeothermy and thermogenesis is delayed in high-altitude
deer mice (Robertson and McClelland, 2019; Robertson et al., 2019). Therefore, we also
hypothesized that the evolved changes in respiratory physiology in high-altitude deer
mice arise in early post-natal life during the developmental transitions to endothermy and the acquisition of high aerobic capacity.

6.3 METHODS
6.3.1 Mouse populations

Deer mice (P. m. rufinus) were live-trapped at high altitude on the summit of Mount Evans (Clear Creek County, CO, at 39°35’18”N, 105°38’38”W, 4,350 m above sea level), and both deer mice (P. m. nebrascensis) and white-footed mice (P. leucopus; a species that is restricted to low altitudes) were live trapped at low altitude on the Great Plains (Nine Mile Prairie, Lancaster County, NE at 40°52’12”N, 96°48’20.3”W, 430 m above sea level). A subset of wild deer mice were transported to the University of Denver Mount Evans field station (3,231 m above sea level) or to the University of Nebraska (430 m above sea level), respectively, and were subjected to measurements of acute hypoxia responses (see below) within 1-2 days of capture. Other wild mice were transported to McMaster University (Hamilton, Canada; ~50 m above sea level) and housed in common laboratory conditions, and were used as parental stock to produce first generation (G1) lab progeny for each mouse population. Breeding pairs were held in individual cages, the male was removed when the female was visibly pregnant, and pups were weaned and moved to separate cages at post-natal day (P) 21. Some G1 mice were similarly used as parental stock to produce second generation (G2) progeny for each mouse population. Experiments on captive mice were conducted on several distinct families of G1 deer mice (2 lowland and 5 highland families) during adulthood (at least 6
months of age) and on G2 deer mice during early development on post-natal day (P) 7, 14, 21, and 30 (6 lowland and 7 highland families). Acute hypoxia responses were measured for high- and low-altitude deer mice only, whereas haematology measurements were made on deer mice and white-footed mice. All captive mice were held in standing holding conditions (24-25°C, 12:12 light-dark photoperiod) with unlimited access to food and water. Animal husbandry and experimentation followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

6.3.2 Acute hypoxia responses

Hypoxia responses were measured in unrestrained mice using barometric plethysmography, respirometry, and pulse oximetry techniques that we have used in our previous studies (Ivy and Scott, 2017b; Ivy and Scott, 2018). Mice were placed in a whole-body plethysmography chamber (530 ml) that was supplied with normoxic air (21 kPa O₂, balance N₂) at flow rates appropriate for the size and metabolic rate of the mouse (200 ml min⁻¹ for P7, 300 ml min⁻¹ for P14, 450 ml min⁻¹ for P21 and P30, and 600 ml min⁻¹ for adults) at room temperature (~ 24 °C). Mice were given 20-40 min to adjust to the chamber until relaxed and stable breathing and metabolism were observed. Mice remained at 21 kPa O₂ for an additional 20 min, followed by exposure to acute stepwise reductions in inspired O₂ pressure (PO₂) for 20 min at each step. For wild-caught adults, these stepwise reductions in PO₂ were at 12, 10, 8, and 6 kPa O₂. Adult G1 mice and young G2 mice at P14, P21, and P30 were exposed to a slightly less severe protocol, with
stepwise reductions in PO\textsubscript{2} at 16, 12, 10, 9, and 8 kPa O\textsubscript{2}. P7 mice were only exposed to one level of mild hypoxia, at a PO\textsubscript{2} of 16 kPa O\textsubscript{2}, and since they are not able to thermoregulate at room temperature at this age (Robertson et al., 2019) we helped maintain their normal body temperature over the duration of the experiment by placing a heating pad under the chamber. Incurrent gas composition was set by mixing dry compressed gases using precision flow meters (Sierra Instruments, Monterey, CA) and a mass flow controller (MFC-4, Sable Systems, Las Vegas, NV). Body temperature was measured at the beginning and end of the experiments using a mouse rectal probe (RET-3-ISO, Physitemp).

Breathing and O\textsubscript{2} consumption rate (\textit{\textsuperscript{\textast}}\textit{\textit{\textit{V}}}\textsubscript{\textit{\textit{\textit{O}}}2}) were determined during the last 10 min at each inspired PO\textsubscript{2}. Gas composition was measured continuously in incurrent and excurrent air flows that were subsampled at 150-200 ml min\textsuperscript{-1}. For incurrent air, the subsampled air was dried with prebaked drierite and then O\textsubscript{2} fraction was measured using a galvanic fuel cell O\textsubscript{2} analyzer (Sable Systems). For excurrent air, water vapour pressure was measured using a thin-film capacitive water vapour analyzer (RH-300, Sable Systems), the gas stream was dried with prebaked drierite, the O\textsubscript{2} fraction was measured as above, and then CO\textsubscript{2} fraction was measured using an infrared CO\textsubscript{2} analyzer (Sable Systems). These data were used to calculate \textit{\textsuperscript{\textast}}\textit{\textit{\textit{V}}}\textsubscript{\textit{\textit{\textit{O}}}2}, expressed in volumes at standard temperature and pressure (STP) using appropriate equations for dry air as described by Lighton (2008), as well as rates of water loss. Chamber temperature was continuously recorded with a thermocouple (TC-2000, Sable Systems). Breathing frequency and tidal volume were measured from changes in flow across a pneumotachograph in the wall of
the plethysmograph chamber, detected using a differential pressure transducer (Validyne DP45, Cancopass, Mississauga, Canada). Tidal volume was calculated using established equations (Drorbaugh and Fenn, 1955; Jacky, 1980) assuming a constant rate of decline in body temperature with declining PO$_2$, which we have previously shown results in similar tidal volumes to those calculated using direct body temperature measurements at each PO$_2$ (Ivy and Scott, 2017b). Total ventilation was calculated as the product of breathing frequency and tidal volume. Total ventilation and tidal volume data are expressed in volumes at body temperature and pressure of saturated air (BTPS). Air convection requirement is the quotient of total ventilation and $\dot{V}O_2$. All of the above data were acquired using a PowerLab 16/32 and Labchart 8 Pro software (ADI Instruments, Colorado Springs, CO). Arterial O$_2$ saturation (SaO$_2$) was measured in adult G1 mice, and in young G2 mice at P14, P21, and P30 mice using MouseOx Plus pulse oximeter collar sensors and data acquisition system (Starr Life Sciences, Oakmont, PA, USA). This was enabled by removing fur around the neck ~2 days before experiments.

### 6.3.3 Immunohistochemistry of the carotid bodies

We examined carotid body development in G2 deer mice at P7, P14, P21 and P30 (n=5 highlanders and n=5 lowlanders at each age, each of which was from a distinct family) using similar approaches to those we have used previously in adult mice (Ivy and Scott, 2017a). Mice were euthanized with an overdose of isoflurane followed by decapitation, blood samples (70-300 µl) were taken for haematology (see below), and the bifurcations of the carotid artery were dissected, removed, and fixed in 4%
paraformaldehyde for 48 h. The tissue was then cryoprotected in 24% sucrose solution, frozen in embedding medium (Cryomatrix; ThermoFisher Scientific, Waltham, MA, USA), and stored at -80 °C. Samples were serially sectioned at 10 µm in a cryostat (Leica CM1860, Wetzlar, Germany) maintained at -20 °C, and were then air-dried and stored at -80 °C. We systematically collected and stained every second section to represent the entirety of each carotid body (Superfrost Plus Fisherbrand, ThermoFisher Scientific). Immunohistochemistry was used to identify type I (glomus) cells (using tyrosine hydroxylase, TH, as a marker), neurons (using neurofilament, NF, and growth-associated protein-43, GAP-43 as concurrent markers) and nuclei (DAPI, 4’,6-diamidino-2-phenylindole, to assist with the identification of individual cells) on one set of carotid body slides; type II cells (using glial fibrillary acidic protein, GFAP, as a marker) and nuclei (DAPI) were identified on the second set of slides. Sections were hydrated in phosphate-buffered saline (PBS; 137 mmol l⁻¹ NaCl, 2.68 mmol l⁻¹ KCl, 10.0 mmol l⁻¹ Na₂HPO₄, 1.76 mmol l⁻¹ KH₂PO₄) and treated with blocking solution (PBS containing 0.5% Triton X-100 and 10% normal goat serum) for 1 h. Sections were then incubated overnight in blocking solution containing primary antibodies targeting TH (1:2000 dilution; AB152; Millipore, Billerica, MA, USA), NF (1:100 dilution; MAB1615, Millipore) and GAP-43 (1:2000 dilution; G9264, Sigma-Aldrich, Mississauga, ON, Canada) for the first set of slides, or GFAP (1:1000 dilution; Z033429-2; Agilent Technologies, Santa Clara, CA, USA) for the second set of slides. The following morning, slides were rinsed well in PBS and then incubated for 2 h in blocking solution that contained secondary antibodies (1:400 dilution) against the TH/GFAP primary
antibodies (AlexaFluor488, goat anti-rabbit IgG; A11034, Life Technologies, Mississauga, ON, Canada) and (in the first set of slides only) both of the NF and GAP-43 primary antibodies (AlexaFluor594, goat anti-mouse IgG; A11032, Life Technologies). Sections were rinsed well in PBS, and incubated in PBS containing 0.5% Triton X-100 and DAPI (1:100 000 dilution; Sigma-Aldrich) for 25 min. Slides were rinsed thoroughly in PBS and mounted with Vectashield (Vector Laboratories, Brockville, ON, Canada). Sections were imaged using an Olympus microscope with Northern Eclipse software (Elite version 8.0; Empix Imaging, Mississauga, ON, Canada).

The first set of slides were used to measure total projected areas in each section of the whole carotid body, neurons, and type I cells. The second set of slides was used to measure total projected areas of type II cells. All of these area measurements were made using Nikon NIS Elements documentation software (v. 4.30.02). The number of type I cells in each section was manually counted using ImageJ software (v. 1.47). Carotid body volume, type I volume, type II volume, and neural volume were calculated as the sum of the volumes in each section of the carotid body (the latter was calculated as the product of projection area and section thickness) (Saiki et al., 2006), multiplied by 2 to account for the fact that we only analyzed every second slide. Carotid body volume was determined based on the boundaries defined by the neural staining and DAPI clustering. The total number of glomus cells in the carotid body was calculated as the sum of all glomus cells counted across every carotid body section, similarly multiplied by 2.

6.3.4 Haematology and Hb-O₂ affinity
Blood was collected from captive deer mice and white-footed mice to measure haematological traits and Hb-O$_2$ affinity. Blood Hb content was measured using Drabkin’s reagent (Sigma Aldrich, Oakville, ON, Canada) according to the manufacturer’s instructions, and haematocrit was measured by centrifuging blood in a heparinized capillary tube at 12,700 g for 5 min. Mean corpuscular Hb concentration was calculated as the quotient of blood Hb content and haematocrit. Oxygen dissociation curves were generated at 37 °C for all mice using a Hemox Analyzer (TCS Scientific, New Hope, PA, USA) using 10 µl of whole blood in 5 ml of buffer containing 100 mmol l$^{-1}$ Tris, 50 mmol l$^{-1}$ EDTA, 100 mmol l$^{-1}$ KCl, 0.1% bovine serum albumin, and 0.2% antifoaming agent at pH 7.4 (TCS Scientific). Oxygen dissociation curves were also generated for adult mice using 100 mmol l$^{-1}$ HEPES (also at pH 7.4) instead of 100 mmol l$^{-1}$ Tris, in order to determine the influence of the assay buffer on P$_{50}$ (the PO$_2$ at which Hb is 50% saturated with O$_2$; Weber, 1992). Hb-O$_2$ affinity (P$_{50}$) was calculated using Hemox Analytic Software (TCS Scientific).

6.3.5 Analysis of Hb isoform composition

We tested for developmental changes in the Hb isoform (isoHb) composition of red blood cells by comparing samples collected at P0/P2 versus P27 from a separate set of G2 highlanders and G2 white-footed mice (n=7-8 samples for each developmental timepoint in each population) from the mice used in the experiments above. Mice were euthanized with an overdose of isoflurane followed by decapitation, and red blood cell samples (65-90 µl) were collected after whole blood was spun in heparinized capillary
tubes (as described above). In the white-footed mice, the samples were from newborns at P0 (n=3) and P2 (n=5), whereas in highlanders the samples were all from newborns at P0. We characterized isoHb composition of hemolysates by means of isoelectric focusing (IEF) in combination with tandem mass spectrometry (MS/MS) (Revsbech et al., 2013; Storz et al., 2010b). We electrophoretically separated each isoHb on the basis of net charge using IEF gels with a 5-8 pH gradient (PhastSystem, GE Healthcare Bio-Sciences, Piscataway, NJ, USA). We then used ImageJ (Abràmoff et al., 2004) to obtain densitometric measurements of IEF band intensities to quantify the relative abundance of each identified isoHb. For representative samples of each developmental stage and population, we excised individual bands from the IEF gel to identify the subunit composition of each tetrameric isoHb via MS/MS. For each individual isoHb, we then separated α- and β-type subunits by means of 20% SDS PAGE followed by staining with Coomassie brilliant blue-G.

The α- and β-chain monomers from the SDS gel were digested with trypsin, and the resultant peptides were identified using MS/MS. The peak list of the MS/MS data was generated by Distiller (Matrix Science, London, UK) using the charge state recognition and de-isotoping with default parameters for quadrupole time-of-flight data. Database searches of the resultant MS/MS spectra were performed using Mascot v1.9.0 (Matrix Science). Specifically, peptide mass fingerprints derived from the MS/MS analysis were used to query a customized reference database of sequences representing the full complement of pre- and post-natally expressed α- and β-type globin genes in deer mice and in white-footed mice (Hoffmann et al., 2008; Natarajan et al., 2015; Storz et al.,
2009; Storz et al., 2010b). The following search parameters were used for the MS/MS analysis: no restriction on protein molecular weight or isoelectric point, and methionine oxidation allowed as a variable peptide modification. Mass accuracy settings were 0.15 Da for peptide mass and 0.12 Da for fragment ion masses. We identified all significant protein hits that matched more than one peptide with P<0.05.

### 6.3.6 Statistics

For acute hypoxia responses, the main effects of population (lowlander versus highlander) and acute exposure PO₂ (repeated measure) and their interactions were evaluated using two-factor ANOVA within wild mice, within G1 adult mice, and within each age of G2 mice. Body mass of wild mice and G1 adult mice was compared between populations using t-tests. For body mass (P7-P30), carotid body traits, Hb-O₂ affinity, and other haematological traits, two-factor ANOVA was used to evaluate the main effects of population and age, and their interaction. The full results of these statistical analyses are included in the electronic supplementary information (Tables S6.1-S6.3) and the salient findings are reported in the Results. Developmental changes in the relative abundance of different Hb isoforms (isoHbs) were tested using two-factor ANOVA. Holm-Sidak post-tests were used throughout to make pairwise comparisons between highlanders and lowlanders or P0/2 and P27, respectively. Values are reported as mean ± SEM. All statistical analysis was conducted with SigmaStat software (v. 3.5) with a significance level of P < 0.05.
6.4 RESULTS
6.4.1 Ventilatory and metabolic responses to acute hypoxia

There were differences in breathing and metabolism between high- and low-altitude mice in the wild, some of which persisted into the first generation of lab-raised adult mice (Fig. 6.1, 6.2, Table 6.1). Wild highlanders had higher total ventilation compared to wild lowlanders across several inspired PO$_2$ (main effect of population: P=0.003, Fig. 6.1A), but they also had lower $\dot{V}O_2$ (P=0.008, Fig. 6.1B). As a result, air convection requirement was 1.4- to 2.6-fold higher in wild highlanders than in wild lowlanders across the full range of inspired PO$_2$ (population effect: P<0.001, Fig. 6.1C). Differences between populations for adult G1 mice were not as large, but highlanders had higher total ventilation (population effect: P=0.007, Fig. 6.1D) and/or lower $\dot{V}O_2$ (population effect, P=0.008; population×PO$_2$ interaction, P<0.001; Fig. 6.1E) than lowlanders, such that air convection requirement was still elevated by 1.2- to 1.5-fold in lab-raised highlanders across the full range of inspired PO$_2$ tested (population effect: P<0.001, Fig. 6.1F). The increases in ventilation in response to acute hypoxia were driven by increasing breathing frequency in both wild and lab-raised mice, and were partially offset by reductions in tidal volume (Fig. 6.2). In both wild and lab-raised adult mice, highlanders maintained deeper tidal volumes and/or lower breathing frequencies compared to lowlanders across most levels of inspired PO$_2$ (Fig. 6.2). These differences were not associated with differences in body mass between highlanders (23.08 ± 0.91 g) and lowlanders (19.06 ± 1.86 g) for wild-caught mice (population effect: P=0.118), nor between highlanders (18.87 ± 1.21 g) and lowlanders (22.26 ± 2.06 g) for lab-raised mice (population effect: P=0.152).
Population differences in the ventilatory and metabolic responses to hypoxia were established between P14 and P30 (Fig. 6.3, Table 6.2). Highland mice did not respond to mild hypoxia at P7, as reflected by a lack of increase in either total ventilation or air convection requirement when exposed to 16 kPa O₂ (Fig. 6.3A, B, C). In contrast, at P7 lowland mice maintained total ventilation despite the fall in \( \dot{V}O_2 \) at 16 kPa O₂, such that air convection requirement increased in response to hypoxia. Both populations had developed ventilatory responsiveness to hypoxia by P14, as reflected by increases in both total ventilation and air convection requirement, and both populations reduced \( \dot{V}O_2 \) in hypoxia. Lowlanders exhibited higher \( \dot{V}O_2 \) than highlanders at P14 (population effect: P<0.001, Fig. 6.3E), which persisted at P21 (P<0.001, Fig. 6.3H) and P30 (P=0.006, Fig. 6.3K), similar to the population differences in adults. Highlanders developed higher air convection requirements than lowlanders by P21 (P=0.032, Fig. 6.3I) and this difference persisted at P30 (P=0.006, Fig. 6.3L). The population differences in ventilatory and metabolic traits did not appear to be associated with population differences in body mass, which increased as expected with age (age effect: P<0.001) but was generally similar between highlanders (P7, 4.59 ± 0.18 g; P14, 7.77 ± 0.25 g; P21, 10.22 ± 0.28 g; P30, 13.45 ± 0.54 g) and lowlanders (P7, 4.09 ± 0.13 g; P14, 6.82 ± 0.28 g; P21, 9.65 ± 0.47 g; P30, 12.47 ± 0.43 g).

The differences in breathing pattern between populations that was characteristic of adult mice appeared at P14 in post-natal development (Fig. 6.4, 6.5, Table 6.2). At P7, highlanders had shallower tidal volumes than lowlanders (population effect: P=0.005, Fig. 6.4B). The onset of ventilatory responsiveness to hypoxia by P14 was caused by
robust increases in breathing frequency in both populations. However, highlanders exhibited lower breathing frequencies than lowlanders at P14 (population effect: $P=0.002$, Fig. 6.4C), and this difference persisted at P21 ($P<0.001$, Fig. 6.4E) and P30 ($P=0.018$, Fig. 6.4G), similar to the population differences observed in adults. Highlanders exhibited deeper tidal volumes than lowlanders at P30 (population effect: $P=0.007$, Fig. 6.4H), but there was also a trend for highlanders to exhibit deeper tidal volumes in the severe hypoxia at earlier ages (Fig. 6.4D, F). Indeed, when breathing data at 12 kPa O$_2$ (roughly equivalent to the PO$_2$ at 4300 m elevation) from all captive mice were considered together, by plotting total ventilation as a function of tidal volume, differences in breathing pattern were clearly evident from P14 to adulthood (Fig. 6.5). There was a progressive reduction in mass-specific rates of total ventilation during development, initially due to declines in mass-specific tidal volume from P14 to P30 (i.e., changes that parallel the isopleths of constant breathing frequency) followed by declines in breathing frequency from P30 to adulthood. Nevertheless, highlanders breathed with relatively deeper breaths than lowlanders at any given total ventilation from P14 to adulthood, as reflected by a rightward shift in the relationship between tidal volume and total ventilation (Fig. 6.5).

6.4.2 Arterial O$_2$ saturation during acute hypoxia

Highland deer mice maintained higher SaO$_2$ during hypoxia than lowlanders from P14 to adulthood (Fig. 6.6, Table 6.1, 6.2). SaO$_2$ fell in deer mice of all ages during acute exposure to hypoxia (PO$_2$ effect: $P<0.001$), but highlanders maintained a higher SaO$_2$
across a range of reduced inspired $\text{PO}_2$ at P14 (population effect: $P<0.001$), P21 ($P=0.044$), and P30 ($P=0.027$), and this difference approached significance in adults ($P=0.064$).

### 6.4.3 Body temperature during acute hypoxia

Body temperature tended to fall in response to acute hypoxia exposure in all mice (Table 6.3). Among adult mice caught in the wild, body temperature in normoxia was lower in highlanders than in lowlanders, but lowlanders exhibited a much greater suppression of body temperature in response to hypoxia. This large population difference was not observed in lab-raised mice, in which there were no clear substantial differences in the body temperature response to hypoxia between highlanders and lowlanders. Body temperature in normoxia was similar between populations, and was in a normal range even though ambient temperature (24 °C) was likely below the thermal neutral zone for all ages (Hill, 1976).

### 6.4.4 Carotid body morphology

Highlanders exhibited a delayed maturation of the carotid body (Fig. 6.7, Table 6.4). The total volume of the carotid body volume was established and remained constant from P7 onwards at a similar volume between populations (highlanders: $27.18 \pm 0.23 \times 10^5 \mu m^3$, lowlanders: $24.75 \pm 0.14 \times 10^5 \mu m^3$), as was the volume occupied by neurons (highlanders: $3.24 \pm 0.28 \times 10^5 \mu m^3$, lowlanders: $2.88 \pm 0.23 \times 10^5 \mu m^3$). However, the developmental increases in the total number and volume of type I cells differed between
populations, occurring between P7 and P14 in lowland mice but not until between P14 and P21 in highland mice (Fig. 6.7C, D). Type II cell volume declined from P7 to P14 in both populations, but it returned to initial levels by P21 in lowlanders but not until P30 in highlanders (Fig. 6.7E). In both populations, an increase in the ratio of type I volume to type II volume occurred in conjunction with the initial proliferation of type I cells (Fig. 6.7F).

6.4.5 Haematology and Hb-O2 affinity

We also sought to examine whether differences in haematology might contribute to population differences in blood O2 content across development. Highlanders exhibited a delayed rise in blood Hb content and haematocrit during development (Table 6.4, 6.5). Hb content and haematocrit decreased from P0 to P7 and then increased steadily thereafter, but these increases appeared to occur later in highland deer mice than in lowland deer mice and in the strictly lowland native white-footed mice (Table 6.5). By adulthood, blood Hb content and haematocrit were similar between populations. Mean corpuscular Hb concentration, the quotient of Hb and haematocrit, was unchanged through development and not significantly different between populations (data not shown).

Highlanders had consistently higher Hb-O2 binding affinity than lowlanders (Fig. 6.8, Table 6.4). Red cell P50 increased between P0 and P7 and then remained stable thereafter, but highlanders maintained lower P50 across ages compared to both lowland deer mice and white-footed mice. The combined IEF and MS/MS analyses suggested that
the increase in \( P_{50} \) from P0 to P7 was unlikely to have resulted from major shifts in red cell isoHb composition. IEF of hemolysates from highland mice revealed some subtle shifts in isoHb composition from P0 to P27, as reflected by an increase in the expression of isoHb C, but all ages expressed the same set of 4 isoHbs (Fig. 6.8C, D). The consistency across ages was greater in white-footed mice, in which the relative abundance of 3 expressed isoHbs did not change between P0/2 and P27 (Fig. 6.8E). The MS/MS analysis revealed that the peptide mass fingerprints of the \( \alpha \)- and \( \beta \)-chain subunits comprising each of the electrophoretically distinct isoHb were perfect matches to those expected from translations of nucleotide sequences from the full complement of adult-expressed globin genes (Natarajan et al., 2015; Storz and Kelly, 2008; Storz et al., 2009; Storz et al., 2010b; Storz et al., 2012). We detected no trace of embryonic \( \alpha \)- or \( \beta \)-type globins in P0/2 or P27 mice from either population.

**6.5 DISCUSSION**

We have shown that evolved changes in the control and pattern of breathing in high-altitude deer mice arise early after birth during the post-natal development of endothermy. Many high-altitude animals exhibit evolved changes in the control of breathing that make breathing more effective and improve respiratory gas exchange in hypoxia (Beall et al., 1997; Moore, 2000; Pichon et al., 2009; Scott and Milsom, 2006), but the timing of the development of these responses during early life was unresolved. Here, we show that the evolved increases in effective ventilation and the hypoxic ventilatory response that are observed in adult deer mice from high altitude arise between 7 and 21 days after birth, concurrent with the development of homeothermy and increases
in thermogenic capacity (Robertson and McClelland, 2019; Robertson et al., 2019). However, the development of ventilatory sensitivity to hypoxia and the post-natal hyperplasia of O₂-sensitive cells in the carotid bodies are delayed in highlanders compared to lowlanders. The evolved increase in the hypoxic ventilatory response in highlanders does not arise until after this early post-natal phase of carotid body maturation is complete. Therefore, the first few weeks of post-natal life are a dynamic period for high-altitude deer mice, when many of the respiratory and metabolic phenotypes associated with high-altitude adaptation first emerge.

Our findings here confirm our other recent findings showing that adult deer mice from high altitude breathe more effectively than their lowland counterparts, with higher total ventilation and preferentially deeper breaths that should increase alveolar ventilation and thus help safeguard SaO₂ in hypoxia (Ivy and Scott, 2017a; Ivy and Scott, 2018). Our previous findings showed that lab-raised highland mice exhibit these differences in comparison to a lowland congener (P. leucopus) and that they persist for at least 2 generations in captivity. However, we had not clearly established in past work whether this trait was unique to highland deer mice or could instead have been a common feature across the P. maniculatus species (Ivy and Scott, 2017a; Ivy and Scott, 2018). In this study, we show highland deer mice do indeed exhibit higher total ventilation and preferentially deeper breaths when compared to lowland deer mice as well. These combined results suggest that the ventilatory phenotype of high-altitude deer mice is a derived trait that was uniquely evolved for life at high altitude.
The evolved ventilatory phenotype of adult highlanders was established between 7 and 21 days after birth, concurrent with the development of endothermic homeothermy. Endothermy begins to develop within a few days of birth, and the high aerobic capacity for thermogenesis that is characteristic of adult deer mice from high altitude (Cheviron et al., 2012; Cheviron et al., 2013; Tate et al., 2017) does not develop until after post-natal day 21 (Robertson and McClelland, 2019; Robertson et al., 2019). This roughly corresponds to the time when juvenile deer mice fully express the enhanced ventilatory sensitivity to O₂ and the breathing pattern that is typical of adults (Figs. 6.3, 6.4). These ventilatory phenotypes may be critical to maintaining the high rates of respiratory gas exchange needed to support the O₂ demands of thermogenesis at high altitude.

However, the developmental onset of ventilatory sensitivity to hypoxia appears to be delayed in high-altitude deer mice. Both the hypoxic ventilatory response (as reflected by hypoxia-induced increases in air convection requirement) (Fig. 6.3) and the post-natal hyperplasia of O₂ sensitive type I cells in the carotid bodies (Fig. 6.7) occurred later in highlanders (P14 and P21, respectively) than in lowlanders (P7 and P14, respectively). Carotid body development also occurred later in highlanders than the developmental timing that has been previously reported for domestic lab-strain mice (Kostuk et al., 2011). Furthermore, highlanders exhibited differences in the timing of the developmental changes in the pool of type II cells, which may act as progenitors that differentiate and give rise to new type I cells during carotid body growth (Pardal et al., 2007). These changes in the developmental onset of ventilatory sensitivity to hypoxia may have occurred in conjunction with the development of endothermy, because recent findings
suggest that the onset of homeothermy and the expansion of thermogenic capacity are also delayed in highland deer mice compared to both lowland deer mice and white-footed mice (Robertson and McClelland, 2019; Robertson et al., 2019). This may also explain why the post-natal changes in blood Hb content were also delayed in highland deer mice (Table 6.2).

Highland mice exhibited stronger blood-O$_2$ affinity from birth to adulthood (Figs. 6.6, 6.8). The persistent differences between populations are consistent with previous functional measurements of Hb from adult mice, and can be explained by genetic differences in the α- and β-chain subunits (Natarajan et al., 2013; Storz et al., 2010b). However, blood-O$_2$ affinity declined appreciably from birth (P0) to P7 in both mouse populations. These changes did not appear to result from a progressive decrease in the expression of embryonic or fetal globins, as occurs in some other mammals which exhibit significant shifts in red cell isoHb compositions in the first few weeks of post-natal life to shift blood-O$_2$ affinity dramatically (Baumann et al., 1972; Blunt et al., 1971; Storz, 2018; Tweeddale, 1973), because both highland deer mice and white-footed mice exhibited adult isoHb compositions from birth. The shift in blood-O$_2$ affinity from P0 to P7 that we observed here may instead result from reductions in the concentrations of allosteric modifiers such as 2,3-diphosphoglycerate (DPG) in the red blood cell. Indeed, in mammals that do not express fetal-specific isoHb, fetal red cells typically have reduced DPG concentration (with a corresponding reduction in blood P$_{50}$), to facilitate placental O$_2$ transfer (Bunn and Kitchen, 1973; Storz, 2018). Blood CO$_2$ tension can increase at birth and then subside over time (Adamson, 1991), so it is possible that these changes and
associated changes in acid-base status between P0 and P7 increase glycolytic DPG production in red blood cells, thereby producing the observed reduction in blood-O$_2$ affinity. Nevertheless, highlanders maintain consistently greater blood-O$_2$ affinity, despite the developmental shifts that occur shortly after birth, which probably acts in concert with the unique ventilatory phenotypes of highlanders to help maintain higher arterial O$_2$ saturation in hypoxia.

ACKNOWLEDGEMENTS

We would like to thank Dr. Colin Nurse for his insight and for allowing us to use his microscope for carotid body imaging.
### 6.6 TABLES AND FIGURES

Table 6.1. ANOVA of acute hypoxia responses in wild and lab-raised deer mice during adulthood with t-tests conducted on body mass to compare mouse populations.

<table>
<thead>
<tr>
<th></th>
<th>Population effect</th>
<th>Acute PO₂ effect</th>
<th>Population×PO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td><strong>Wild mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total ventilation</td>
<td>19.61</td>
<td>0.003</td>
<td>3.534</td>
</tr>
<tr>
<td>O₂ consumption rate</td>
<td>13.44</td>
<td>0.008</td>
<td>14.52</td>
</tr>
<tr>
<td>Air convection</td>
<td>57.94</td>
<td>&lt;0.001</td>
<td>38.51</td>
</tr>
<tr>
<td>requirement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breathing frequency</td>
<td>0.253</td>
<td>0.630</td>
<td>70.28</td>
</tr>
<tr>
<td>Tidal volume</td>
<td>36.43</td>
<td>&lt;0.001</td>
<td>10.55</td>
</tr>
<tr>
<td>Body temperature</td>
<td>0.016</td>
<td>0.903</td>
<td>92.11</td>
</tr>
<tr>
<td>Body mass</td>
<td>1.784</td>
<td>0.118</td>
<td>n.a.</td>
</tr>
<tr>
<td><strong>First generation mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total ventilation</td>
<td>9.883</td>
<td>0.007</td>
<td>58.421</td>
</tr>
<tr>
<td>O₂ consumption rate</td>
<td>0.431</td>
<td>0.521</td>
<td>3.115</td>
</tr>
<tr>
<td>Air convection</td>
<td>18.268</td>
<td>&lt;0.001</td>
<td>71.91</td>
</tr>
<tr>
<td>requirement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breathing frequency</td>
<td>3.680</td>
<td>0.074</td>
<td>94.05</td>
</tr>
<tr>
<td>Tidal volume</td>
<td>18.08</td>
<td>&lt;0.001</td>
<td>13.08</td>
</tr>
<tr>
<td>Arterial O₂ saturation</td>
<td>3.988</td>
<td>0.064</td>
<td>250.5</td>
</tr>
<tr>
<td>Body temperature</td>
<td>0.050</td>
<td>0.826</td>
<td>173.8</td>
</tr>
<tr>
<td>Body mass</td>
<td>1.508</td>
<td>0.152</td>
<td>n.a.</td>
</tr>
</tbody>
</table>
Table 6.2. ANOVA of acute hypoxia responses of lab-raised deer mice during early postnatal development.

<table>
<thead>
<tr>
<th></th>
<th>Population effect</th>
<th>Acute PO$_2$ effect</th>
<th>Population×PO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>Total ventilation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P7</td>
<td>4.417</td>
<td>0.046</td>
<td>7.994</td>
</tr>
<tr>
<td>P14</td>
<td>1.586</td>
<td>0.221</td>
<td>10.03</td>
</tr>
<tr>
<td>P21</td>
<td>0.726</td>
<td>0.404</td>
<td>24.41</td>
</tr>
<tr>
<td>P30</td>
<td>0.421</td>
<td>0.523</td>
<td>18.70</td>
</tr>
<tr>
<td>P14</td>
<td>15.18</td>
<td>&lt;0.001</td>
<td>76.10</td>
</tr>
<tr>
<td>O$_2$ consumption rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>40.14</td>
<td>&lt;0.001</td>
<td>41.09</td>
</tr>
<tr>
<td>P30</td>
<td>9.336</td>
<td>0.006</td>
<td>86.86</td>
</tr>
<tr>
<td>P7</td>
<td>0.027</td>
<td>0.872</td>
<td>11.57</td>
</tr>
<tr>
<td>P14</td>
<td>76.10</td>
<td>&lt;0.001</td>
<td>76.10</td>
</tr>
<tr>
<td>Air convection requirement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P14</td>
<td>1.690</td>
<td>0.206</td>
<td>34.98</td>
</tr>
<tr>
<td>P21</td>
<td>5.255</td>
<td>0.032</td>
<td>114.5</td>
</tr>
<tr>
<td>P30</td>
<td>8.965</td>
<td>0.006</td>
<td>106.3</td>
</tr>
<tr>
<td>P7</td>
<td>0.131</td>
<td>0.721</td>
<td>3.504</td>
</tr>
<tr>
<td>P14</td>
<td>1.936</td>
<td>&lt;0.001</td>
<td>41.09</td>
</tr>
<tr>
<td>Breathing frequency</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>12.30</td>
<td>0.002</td>
<td>108.7</td>
</tr>
<tr>
<td>P30</td>
<td>6.443</td>
<td>0.018</td>
<td>72.90</td>
</tr>
<tr>
<td>P7</td>
<td>9.452</td>
<td>0.005</td>
<td>19.971</td>
</tr>
<tr>
<td>P14</td>
<td>26.62</td>
<td>&lt;0.001</td>
<td>166.3</td>
</tr>
<tr>
<td>P21</td>
<td>1.853</td>
<td>0.187</td>
<td>138.3</td>
</tr>
<tr>
<td>Tidal volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>2.233</td>
<td>0.149</td>
<td>86.21</td>
</tr>
<tr>
<td>P30</td>
<td>8.608</td>
<td>0.007</td>
<td>74.38</td>
</tr>
<tr>
<td>P14</td>
<td>4.559</td>
<td>0.044</td>
<td>501.2</td>
</tr>
<tr>
<td>P21</td>
<td>5.569</td>
<td>0.027</td>
<td>390.1</td>
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<tr>
<td>Arterial O$_2$ saturation</td>
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<td></td>
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<td>P30</td>
<td>8.458</td>
<td>0.008</td>
<td>138.7</td>
</tr>
<tr>
<td>P14</td>
<td>4.559</td>
<td>0.044</td>
<td>501.2</td>
</tr>
<tr>
<td>Body temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>1.853</td>
<td>0.187</td>
<td>138.3</td>
</tr>
<tr>
<td>P30</td>
<td>0.145</td>
<td>0.706</td>
<td>129.1</td>
</tr>
</tbody>
</table>

P, post-natal age (days)
Table 6.3. Body temperature (°C) in normoxia and after acute exposure to stepwise hypoxia in adults and in young mice during early post-natal development.

<table>
<thead>
<tr>
<th>Age</th>
<th>Highland deer mouse</th>
<th>Lowland deer mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>Wild adult</td>
<td>35.63 ± 0.33</td>
<td>34.43 ± 0.55</td>
</tr>
<tr>
<td>Lab-raised adult</td>
<td>38.16 ± 0.18</td>
<td>36.21 ± 0.25</td>
</tr>
<tr>
<td>P7</td>
<td>34.65 ± 0.11</td>
<td>-</td>
</tr>
<tr>
<td>P14</td>
<td>35.50 ± 0.23</td>
<td>32.38 ± 0.32</td>
</tr>
<tr>
<td>P21</td>
<td>36.98 ± 0.20</td>
<td>34.74 ± 0.19</td>
</tr>
<tr>
<td>P30</td>
<td>36.70 ± 0.19</td>
<td>35.18 ± 0.16</td>
</tr>
</tbody>
</table>

Hypoxic body temperature is not provided for P7 mice, because body temperature was maintained throughout hypoxia exposure using a heating pad. * significant pairwise difference at the given time point from highland deer mice (n as in Figs. 6.1 and 6.3). P, post-natal age (days).
Table 6.4. Two-way ANOVA of body mass, carotid body morphology, and haematology

<table>
<thead>
<tr>
<th></th>
<th>Population effect</th>
<th></th>
<th></th>
<th>Age effect</th>
<th></th>
<th></th>
<th>Population×Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
<td>P</td>
<td>F</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Body mass</td>
<td>8.663</td>
<td>0.004</td>
<td>208.1</td>
<td>&lt;0.001</td>
<td>0.193</td>
<td>0.901</td>
<td></td>
</tr>
<tr>
<td>Carotid body volume</td>
<td>0.627</td>
<td>0.434</td>
<td>0.244</td>
<td>0.865</td>
<td>0.795</td>
<td>0.505</td>
<td></td>
</tr>
<tr>
<td>Type I cell number</td>
<td>1.858</td>
<td>0.182</td>
<td>19.70</td>
<td>&lt;0.001</td>
<td>4.608</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>Type II cell volume</td>
<td>0.157</td>
<td>0.694</td>
<td>3.425</td>
<td>0.028</td>
<td>1.088</td>
<td>0.367</td>
<td></td>
</tr>
<tr>
<td>Neural volume</td>
<td>0.652</td>
<td>0.425</td>
<td>1.847</td>
<td>0.158</td>
<td>0.697</td>
<td>0.561</td>
<td></td>
</tr>
<tr>
<td>TH volume : GFAP volume</td>
<td>0.202</td>
<td>0.656</td>
<td>6.704</td>
<td>0.001</td>
<td>7.097</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>P&lt;sub&gt;50&lt;/sub&gt;</td>
<td>15.66</td>
<td>&lt;0.001</td>
<td>173.4</td>
<td>&lt;0.001</td>
<td>2.850</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin Concentration</td>
<td>34.06</td>
<td>&lt;0.001</td>
<td>6.665</td>
<td>0.002</td>
<td>1.903</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td>Haematocrit</td>
<td>42.10</td>
<td>&lt;0.001</td>
<td>7.851</td>
<td>&lt;0.001</td>
<td>3.203</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>IsoHb effect</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Highlnd deer mouse</td>
<td>13.12</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.999</td>
<td>21.07</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Lowland white-footed</td>
<td>126.9</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.999</td>
<td>3.635</td>
<td>0.036</td>
<td></td>
</tr>
</tbody>
</table>

P<sub>50</sub>, haemoglobin-O<sub>2</sub> binding affinity; isoHb, haemoglobin isoform
Table 6.5. Haematology of lab-raised adults and of young mice during early post-natal development

<table>
<thead>
<tr>
<th>Age</th>
<th>Highland deer mice</th>
<th>Lowland deer mice</th>
<th>Lowland white-footed mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haemoglobin Concentration (g/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P0</td>
<td>15.20 ± 0.69</td>
<td>-</td>
<td>17.39 ± 0.42</td>
</tr>
<tr>
<td>P7</td>
<td>11.34 ± 0.47</td>
<td>12.67 ± 0.36</td>
<td>10.99 ± 0.55</td>
</tr>
<tr>
<td>P14</td>
<td>12.40 ± 0.33</td>
<td>13.68 ± 0.32</td>
<td>12.68 ± 0.37</td>
</tr>
<tr>
<td>P21</td>
<td>12.90 ± 0.27</td>
<td>15.13 ± 0.47*</td>
<td>14.61 ± 0.47*</td>
</tr>
<tr>
<td>P30</td>
<td>14.35 ± 0.57</td>
<td>15.32 ± 0.37</td>
<td>14.46 ± 0.56</td>
</tr>
<tr>
<td>Adult</td>
<td>16.63 ± 0.54</td>
<td>15.80 ± 0.41</td>
<td>15.14 ± 0.61</td>
</tr>
</tbody>
</table>

|       | Haematocrit (%)     |                   |                           |
| P0    | 45.34 ± 1.65        | -                 | 53.11 ± 1.05              |
| P7    | 35.05 ± 1.22        | 36.88 ± 0.74      | 35.48 ± 1.26              |
| P14   | 35.51 ± 1.30        | 42.45 ± 0.70*     | 37.43 ± 0.73              |
| P21   | 39.66 ± 0.74        | 45.05 ± 1.46*     | 41.65 ± 1.03              |
| P30   | 42.18 ± 0.80        | 45.63 ± 0.74*     | 45.43 ± 1.01*             |
| Adult | 48.59 ± 1.66        | 43.89 ± 0.85      | 46.93 ± 2.24              |

* significant difference from highland deer mice (n as in Fig. 6.8A). P, post-natal age (days)
Figure 6.1. Wild (A,B,C) and lab-raised (D,E,F) deer mice from high altitude exhibit differences in breathing and metabolism in hypoxia compared to deer mice from low altitude. * Significant pairwise difference between populations within each PO₂ using Holm-Sidak post-tests (n as follows: 5 wild lowlanders, 5 wild highlanders, 12 lab-raised lowlanders, 30 lab-raised highlanders). BTPS, body temperature and pressure, saturated; STP, standard temperature and pressure.
Figure 6.2. Wild (A,B) and lab-raised (C,D) deer mice from high altitude take deeper but less frequent breaths in hypoxia compared to deer mice from low altitude. * Significant pairwise difference between populations within each PO₂ using Holm-Sidak post-tests (n as in Fig. 6.1).
Figure 6.3. Highland deer mice are unresponsive to hypoxia at P7 of post-natal development (A,B,C), and begin to express the population differences in ventilation and/or metabolism that are exhibited by adults at P14 (D,E,F), P21 (G,H,I), and P30 (J,K,L). * Significant pairwise difference between populations within each PO\textsubscript{2} using Holm-Sidak post-tests (n as follows: 12 P7 lowlanders, 14 P7 highlanders, 11 P14 lowlanders, 14 P14 highlanders, 10 P21 lowlanders, 14 P21 highlanders, 11 P30 lowlanders, 17 P30 highlanders). P, post-natal age (days).
Figure 6.4. Breathing pattern during hypoxia diverges between highland and lowland populations of deer mice at P14 of post-natal development (C,D), and differences persist at P21 (E,F) and P30 (G,H). # Significant main effect of population in two-factor ANOVA; * significant pairwise difference between populations within each PO₂ using Holm-Sidak post-tests (n as in Fig. 3). P, post-natal age (days).
Figure 6.5. Highland deer mice consistently maintain deeper tidal volumes at 12 kPa O\textsubscript{2} for any given total ventilation from P14 of post-natal development into adulthood (n as in Figs. 1,3). P, post-natal age (days); A, lab-raised adult; fR, breathing frequency (min\textsuperscript{-1}).
Figure 6.6. Highland deer mice maintain higher arterial $O_2$ saturation in hypoxia than lowland deer mice at P14 (A), P21 (B), P30 (C), and in adulthood (D). # Significant main effect of population in two-factor ANOVA, † $P=0.064$ for main effect of population in two-factor ANOVA; * significant pairwise difference between populations within each PO$_2$ using Holm-Sidak post-tests (n as follows: 11 P14 lowlanders, 14 P14 highlanders, 10 P21 lowlanders, 14 P21 highlanders, 11 P30 lowlanders, 17 P30 highlanders, 12 lab-raised lowland adults, 30 lab-raised highland adults). P, post-natal age (days).
Figure 6.7. Carotid body development is delayed in highland deer mice. Fluorescent immunohistochemistry was used to identify (A) type I cells (tyrosine hydroxylase, TH, in green) and neurons (neurofilament, NF, and growth-associated protein 43, GAP-43, in magenta), or (B) type II cells (glial fibrillary acidic protein, GFAP, in blue). Representative images from a lowland mouse at post-natal age (P)21, scale bar is 100 µm. The number (C) and volume (D) of type I cells increases with age, along with developmental changes in the volume of type II cells (E), and the ratio of type I cell volume to type II cell volume (F). * Significant pairwise difference between populations within an age using Holm-Sidak post-tests (n of 5 for each group).
Ph.D. Thesis – C.M. Ivy; McMaster University – Department of Biology

Graphs and images:  
A) Red blood cell P50 (KPa) vs. Age (days post birth) for Highland deer mice, Lowland deer mice, and Lowland white-footed mice.  
B) P50 in HEPES buffer vs. P50 in Tris buffer (KPa) for Highland deer mice.  
C) Western Blot images for P0 Highlander and P27 Highlander.  
D) Relative abundance (%) of iso-Hb for Highland deer mice.  
E) Relative abundance (%) of iso-Hb for Lowland white-footed mice.
Figure 6.8. Highland deer mice exhibit high haemoglobin (Hb)-O$_2$ binding affinity from birth to adulthood. (A) Red blood cell P$_{50}$ increases from P0 to P7, but is consistently lower in highlanders (n as follows: 5 P0, 12 P7, 14 P14, 14 P21, 17 P30, 10 adults) than in lowland deer mice (12 P7, 11 P14, 10 P21, 10 P30, 7 adults) and lowland white-footed mice (4 P0, 11 P7, 12 P14, 11 P21, 11 P30, 10 adults). * Significant pairwise difference between highlanders and lowlanders of both species within an age using Holm-Sidak post-tests, ϕ significant pairwise difference between ages of both isoHb within a species using Holm-Sidak post-tests. (B) Tris buffer increased P$_{50}$ values (by $\sim$1.37 kPa) compared to HEPES buffer for red blood cells from adult mice, but this effect did not alter the population differences in red blood cell P$_{50}$ (n=10 for all groups). (C) Representative isoelectric focusing (IEF) gel showing the different Hb isoforms (isoHb; labelled A-D) expressed in red blood cells of highland mice. (D) IEF analysis revealed variation in the relative abundance of distinct isoHb with age between P0 (n=7) and P27 (n=7) in highland mice. (E) IEF analysis revealed highly similar isoHb profiles in samples from lowland *P. leucopus* at P0/2 (n=8) and P27 (n=7).
### 6.7 SUPPLEMENTARY TABLES

Table S6.1. ANOVA of acute hypoxia responses in wild and lab-raised deer mice during adulthood with t-tests conducted on body mass to compare mouse populations.

<table>
<thead>
<tr>
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<th>Population effect</th>
<th>Acute PO&lt;sub&gt;2&lt;/sub&gt; effect</th>
<th>Population×PO&lt;sub&gt;2&lt;/sub&gt;</th>
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<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td><strong>Wild mice</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total ventilation</td>
<td>19.61</td>
<td>0.003</td>
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<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt; consumption rate</td>
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<td>0.008</td>
<td>14.52</td>
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<tr>
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<td>0.630</td>
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<tr>
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<td>36.43</td>
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<td>10.55</td>
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<td>0.903</td>
<td>92.11</td>
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<td><strong>First generation mice</strong></td>
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Table S6.2. ANOVA of acute hypoxia responses of lab-raised deer mice during early post-natal development.

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<td>P</td>
<td>F</td>
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<tr>
<td>Total ventilation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P7</td>
<td>4.417</td>
<td>0.046</td>
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<tr>
<td>P14</td>
<td>1.586</td>
<td>0.221</td>
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<tr>
<td>P21</td>
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<td>0.404</td>
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<tr>
<td>O\textsubscript{2} consumption rate</td>
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<td></td>
<td></td>
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<tr>
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<td>Air convection requirement</td>
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<tr>
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<td>0.872</td>
<td>3.504</td>
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<td>34.98</td>
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<td>-</td>
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P, post-natal age (days)
Table S6.3. Two-way ANOVA of body mass, carotid body morphology, and haematology

<table>
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<table>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lowland white-footed</td>
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<td>3.635</td>
</tr>
<tr>
<td>mouse isoHb abundance</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

P50, haemoglobin-O2 binding affinity; isoHb, haemoglobin isoform
6.8. REFERENCES


CHAPTER 7: GENERAL DISCUSSION

High altitude is an extreme environment that challenges small endotherms to extract enough O\textsubscript{2} from the environment to support metabolism, thermoregulation in the cold, and the aerobic scope needed for exercise. Increased breathing in response to low O\textsubscript{2} is critical for maintaining O\textsubscript{2} uptake, with many high-altitude taxa having evolved changes in the control of breathing to enhance O\textsubscript{2} uptake (Brutsaert, 2007; McDonough et al., 2006; Pichon et al., 2009; Scott and Milsom, 2007). For my thesis, I sought to investigate how deer mice, which thrive and survive at altitude, have overcome the challenges of maintaining O\textsubscript{2} uptake in hypoxia. The primary objectives of this thesis were to understand (i) how plasticity and evolution have altered the control of breathing and the hypoxic chemoreflex in high-altitude deer mice, both in normal sea level conditions and after exposure to chronic hypoxia, and (ii) what genes may underlie these observed differences in the control of breathing. A secondary objective was to understand how these evolved differences in breathing develop within an individual’s lifetime, as this is not well understood. These objectives aimed at testing the overarching hypothesis that deer mice native to high-altitude have evolved changes in the control of breathing and the hypoxic chemoreflex that improve O\textsubscript{2} transport in chronic hypoxia.

7.1 Control of breathing in high-altitude deer mice
I found that high-altitude deer mice have evolved a more effective breathing pattern, characterized by deeper but less frequent breaths, which should augment alveolar ventilation and improve respiratory gas exchange in hypoxia. This was observed in
chapters 2, 3, and 6 in adult and juvenile mice raised in normoxia. My findings are consistent with previous observations in other highland taxa, such as plateau pika and bar-headed geese (Beall et al., 1997; Lague et al., 2016; Moore, 2000; Pichon et al., 2009; Scott and Milsom, 2007), and adds to the growing literature showing that evolved changes in control of breathing likely contribute to enhancing O$_2$ uptake to overcome the challenges of life at altitude (Beall, 2000; Brutsaert et al., 2005; Ivy et al., 2018; Ivy et al., 2019; Schwenke et al., 2007). My work extended our understanding of the mechanisms for these changes in breathing pattern, which exist without any changes in carotid body size, glomus cell number, or innervation compared to lowlanders, similar to what has previously been observed between Tibetan and sea-level human residents (Wu and Kayser, 2006).

I also showed in Chapters 2 and 3 that high-altitude deer mice responded much less to chronic hypoxia than lowland mice. Lowland mice exhibited ventilatory acclimatization to hypoxia (increases in total ventilation at a given PO$_2$), through increases in tidal volume that were only partially offset by reductions in breathing frequency, and this response to chronic hypoxia helped augment arterial O$_2$ saturation (SaO$_2$). Lowland mice also exhibited carotid body growth via glomus cell hyperplasia, which is commonly observed with chronic hypoxia in many species (Edwards et al., 1971; Kato et al., 2010; Kusakabe et al., 1993; Pardal et al., 2007); the physiological effects of carotid body growth are controversial, but they could potentially contribute to increasing O$_2$ chemosensitivity and afferent signalling to central respiratory centers (Barer et al., 1976; Bishop et al., 2013). In contrast, high-altitude deer mice did not
exhibit VAH or enlargement of the carotid body with chronic hypoxia, suggesting that the
deep breathing pattern observed in highlanders may arise from distinct mechanisms
from those leading to VAH in lowlanders. In fact, highland deer mice did not exhibit
VAH in response to an even more severe level of hypoxia (9 kPa O$_2$) (unpublished data,
Ivy and Scott), so this evolved difference in highlanders reflects a strong attenuation in
ventilatory plasticity regardless of the level of hypoxia.

In Chapter 3, I also examined the question of whether changes in ventilatory CO$_2$
sensitivity in chronic hypoxia might explain the lack of VAH in high-altitude deer mice.
This could potentially have been the case if highlanders became more sensitive to
respiratory hypocapnia after chronic hypoxia, because such a change would tend to
reduce breathing during poikilocapnic hypoxia. I found that highland mice increased
hypocapnia sensitivity after chronic hypoxia, but this increase was similar in magnitude
to that in lowland mice. These findings are in contrast to some other taxa, in which
ventilatory sensitivity to CO$_2$ is reduced at high altitude (Ivy et al., 2018; Lague et al.,
2017; Schwenke et al., 2007; Scott and Milsom, 2007; Slessarev et al., 2010a; Slessarev
et al., 2010b). However, there appeared to be modest population differences in
hypocapnia sensitivity that supported my hypothesis, because in highlanders (but not
lowlanders) chronic hypoxia did elicit a slight (non-significant) increase in tidal volume
when tested during hypoxia with elevated inspired CO$_2$. Nevertheless, the effects of
chronic hypoxia were still greatly blunted in highlanders compared to lowlanders, so
changes in ventilatory CO$_2$ sensitivity do not appear to play a major role in blunting VAH
in high-altitude deer mice.
In Chapters 2, 3, and 6, the altered breathing pattern and attenuated VAH observed in highlanders persisted for at least two generations in captivity, suggesting that it is a derived trait that has evolved for life at high altitude. Studying high-altitude taxa in their native environment can make it challenging to establish whether changes are evolved and genetically based, because it is difficult to exclude the influence of adult, developmental and/or parental exposure to different environments (Brutsaert, 2016; Moore, 2017). By using captively raised mice in my experiments, I was able to eliminate the influence of developmental environment (as all highland and lowland G1 mice were raised in the same lab environment), and by studying second generation mice further allowed me to eliminate the influence of parental environment (the influence of the high-altitude environment on parents and/or germline cells) (Ho and Burggren, 2012).

Although I cannot completely rule out the potential influence of trans-generational epigenetic effects lasting longer than 2 generations, it is otherwise likely that the unique ventilatory phenotype of high-altitude deer mice is an evolved trait, which may have arisen as a result of natural selection in cold and hypoxic alpine environments.

It is intriguing to consider why these evolved changes in control of breathing have taken place, especially when considering that hypoxia acclimation in lowlanders (which increased total ventilation and deepened breathing pattern) achieved a similar ventilatory phenotype to the fixed phenotype of highlanders (higher total ventilation and deepened breathing pattern, but attenuated VAH). It is possible that high-altitude deer mice have undergone genetic assimilation, thereby fixing a phenotype that originally exhibited adaptative plasticity (Ehrenreich and Pfennig, 2016; Lande, 2015; Pigliucci, 2006).
Highland deer mice may have done so by restructuring the hypoxic chemoreflex, fixing the effective breathing pattern observed in lowlanders after chronic hypoxia, but potentially without the detrimental effects of chronically simulating other efferent pathways in the hypoxic chemoreflex. This possibility may be supported by the attenuated growth of the carotid bodies in highland mice after chronic hypoxia, because growth of this peripheral chemoreceptor could potentially be associated with enhanced afferent signalling that leads to persistent sympathetic activation in chronic hypoxia. By attenuating afferent signalling from the carotid bodies, highlanders may reduce the activity of sympathetic neurons and/or blunt catecholamine release from adrenal medullae. Indeed, there is some support for the latter possibility, as reflected by the observation that highland deer mice have lower levels of catecholamines circulating in the blood (Pranckevicius et al., in prep; Scott et al., 2019). Therefore, high-altitude deer mice may exhibit an evolved strategy to enhance respiratory $O_2$ uptake without amplifying the detrimental effects of the hypoxic chemoreflex.

7.2 What is the genetic basis of the evolved changes in control of breathing in highlanders?

7.2.1 Epas1

Recent studies have identified genes that have frequently been targeted by selection in high-altitude populations (Beall et al., 2010; Gou et al., 2014; Graham and McCracken, 2019; Petousi and Robbins, 2014; Schweizer et al., 2019; Simonson, 2015; Simonson et al., 2012), but few studies have explored how this genetic variation contributes to the unique phenotypes of high-altitude taxa. In Chapter 4, I provided
evidence that the high-altitude variant of the gene encoding HIF-2α (*Epas1*) contributes to attenuating VAH in high-altitude deer mice. *Epas1* is under strong selection in many human populations and animal taxa that are native to high altitude (Ai et al., 2014; Beall et al., 2010; Buroker et al., 2012; Gou et al., 2014; Graham and McCracken, 2019; Li et al., 2013; Li et al., 2014; Petousi and Robbins, 2014; Qu et al., 2013; Schweizer et al., 2019; Simonson et al., 2012; Song et al., 2016; Yi et al., 2010). I found that hybrid mice that were homozygous for the highland *Epas1* variant exhibited smaller increases in ventilatory chemosensitivity after chronic hypoxia than mice with the lowland *Epas1* variant, and homozygotes also lacked the growth of the carotid body that occurred in mice that were homozygous for the lowland *Epas1* variant and that generally occur in many lowland animals in response to chronic hypoxia. My findings are consistent with those of studies on domestic mice with constitutive heterozygous knockout or acute inducible knockout of HIF-2α, in which VAH and carotid body growth after chronic hypoxia are also blunted (Fielding et al., 2018; Hodson et al., 2016). This suggests that a reduction in HIF-2α mediated signalling in the carotid body plays an important role in blunting ventilatory chemosensitivity and eliminating carotid body growth in chronic hypoxia, and can thus partially explain the lack of VAH in highland deer mouse populations.

7.2.2 *Haemoglobin*

Haemoglobin-O₂ affinity has also been a pervasive target of selection in many high-altitude taxa (Galen et al., 2015; Natarajan et al., 2015; Natarajan et al., 2016;
Natarajan et al., 2018; Projecto-Garcia et al., 2013; Signore et al., 2019; Storz et al., 2010; Tufts et al., 2015; Zhu et al., 2018), but whether genetic variation in globins contributes to other aspects of the highlander phenotype, in addition to increased Hb-O\(_2\) affinity, was previously unknown. In Chapter 5, I provided novel evidence that genetic variation in \(\alpha\)-globin genotype, arising from natural selection acting on this gene at high altitude, can completely explain the effective breathing pattern of highland deer mice. The influence of \(\alpha\)-globin genotype on breathing pattern in hybrid mice was not the result of differences in blood-\(O_2\) levels, because the observed differences persisted in hyperoxia (30 kPa \(O_2\)) when the blood would have been fully saturated with oxygen. In fact, the effects of \(\alpha\)-globin genotype on breathing pattern appeared to be independent of Hb-\(O_2\) affinity, because injection of highland and lowland deer mice with efaproxiral (a synthetic drug that pharmalogically reduces Hb-\(O_2\) affinity) had no effect on breathing pattern in normoxic or hypoxic conditions.

\(\beta\)-globin genotype influenced the hypoxic ventilatory response in hybrid mice. Among normoxia-acclimated mice that were homozygous for highland \(\alpha\)-globin, \(\alpha^{HH}\beta^{HH}\) mice exhibited a higher total ventilation compared to \(\alpha^{HH}\beta^{HL}\) and \(\alpha^{HH}\beta^{LL}\) mice at 12 kPa \(O_2\). These findings mirrored those reported in lab-strain mice possessing the Hb Presbyterian mutation in \(\beta\) globin, which exhibited a reduced Hb-\(O_2\) affinity and an attenuated hypoxic ventilatory response (Izumizaki et al., 2003). Therefore, differences in \(\beta\)-globin sequence may contribute to the higher total ventilation of highland deer mice compared to both lowland deer mice and white-footed mice in comparisons among normoxia acclimated individuals (Chapters 2, 3, 6). However, the effects of \(\beta\)-globin
genotype were abolished after hypoxia acclimation, suggesting that the highland β-globin genotype reduces the effects of chronic hypoxia on total ventilation in hybrid mice. If so, the highland β-globin variant may act in conjunction with the high-altitude variant of \textit{Epas1} to elicit the attenuation of VAH that I have reported in high-altitude populations of deer mice (Chapters 2 and 3).

How might globins be having their effects on control of breathing? My findings described above with efaproxiral suggest that the effects of globins on breathing are somewhat independent of the role of haemoglobin in circulatory O$_2$ transport, and could reflect a distinct role of globins expressed in non-erythroid tissues. Although I did not identify any expression of globins in the medulla or pons (key brainstem sites of ventilatory control), nor did I see any appreciable changes in gene expression in the medulla, globins have been identified in many other non-erythroid tissues (Biagioli et al., 2009; Newton et al., 2006; Richter et al., 2009; Schelshorn et al., 2009; Straub et al., 2012). For example, globins have been identified in the endothelium of some blood vessels, where they regulate nitric oxide production (Straub et al., 2012). Furthermore, recent research suggests that nitric oxide and S-nitrosothiols can influence breathing in hypoxia (Kline et al., 1998; Kline et al., 2000; Lipton et al., 2001). Together, these findings suggest that globins expressed in the vascular endothelium could regulate nitric oxide signalling and thus influence breathing; however, whether this is indeed the case in deer mice requires further investigation. Nevertheless, my results suggest that the evolution of haemoglobin genes may have pervasive effects on multiple respiratory
phenotypes, and may contribute to environmental adaptation via physiological mechanisms that are not commonly ascribed to this protein.

7.3 Development of the high-altitude phenotype

Is the ventilatory phenotype of highland deer mice present at birth, or does it develop later in post-natal life with the onset of endothermy and elevated metabolic rates? I made novel findings to answer this question in Chapter 6, in which I found that the evolved ventilatory phenotype of adult highland deer mice arises between 7 and 21 days after birth, concurrent with the increases in aerobic requirements for thermoregulation (Robertson and McClelland, 2019; Robertson et al., 2019). However, the development of the hypoxic ventilatory response and the post-natal hyperplasia of O$_2$-sensitive cells in the carotid body appeared to be delayed in high-altitude deer mice. This may correspond with recent findings suggesting that the onset of homeothermy and thermogenic capacity in highland deer mice is also delayed compared to lowland deer mice and white-footed mice (Robertson and McClelland, 2019; Robertson et al., 2019). These findings suggest that the unique ventilatory phenotype of high-altitude deer mice develops during the time in post-natal development when the high requirement for O$_2$ uptake in hypoxia first arises.

Would hypoxia exposure at critical windows during early development alter the ventilatory phenotype of high-altitude deer mice? Studies in lab-strain mice and rats have shown that chronic hypoxia during prenatal development can lead to long-lasting increases in ventilation in adults (Gleed and Mortola, 1991; Peyronnet et al., 2000; Peyronnet et al., 2007), while neonatal hypoxia exposure can blunt or abolish the HVR of adults (Lumbroso and Joseph, 2009). In chapter 6, I showed that there are differences in
the magnitude of ventilatory responses between wild, G1, and G2 mice within a population, which could be attributed to adult plasticity, but also could be a result of developmental plasticity. Additionally, VO$_{2\text{max}}$ has been shown to decline in highland mice from the wild when they are deacclimated in the lab for 6 weeks, and that G1 highland mice have even lower VO$_{2\text{max}}$ (Cheviron et al., 2013), suggesting that there may be developmental plasticity in VO$_{2\text{max}}$ and some other traits. Therefore, hypoxia exposure during critical windows of development in high-altitude deer mice may influence many traits characteristic of the adult highland phenotype, such as ventilatory phenotype, increasing the aerobic scope of these mice to perform exercise and thermoregulation in hypoxia.

7.4 CONCLUSION

Overall, my findings suggest that control of breathing in high-altitude deer mice is highly effective for O$_2$ uptake but relatively resistant to the effects of chronic hypoxia. This ventilatory phenotype appears to be an evolved trait that develops during the onset of endothermy in early post-natal life, and is the result of genetic variation at 3 or more loci (Epas1, α-globin, and β-globin). The high-altitude variant of α-globin can completely explain the deep breathing pattern of the high-altitude population, whereas the high-altitude variant of Epas1 and possibly β-globin contribute to the apparent lack of ventilatory plasticity in response to chronic hypoxia that characterizes VAH. Additionally, mutations in α-globin contribute to increasing haemoglobin-O$_2$ affinity and improving arterial O$_2$ saturation in hypoxia in high-altitude deer mice. These mutations appear to play a major role in creating the unique respiratory phenotype in high-altitude
deer mice, and likely act in concert with the suite of other physiological and genetic changes in highland populations (Schweizer et al., 2019) to help maintain O\textsubscript{2} uptake and metabolism in the cold and hypoxic environment at high altitude.
7.4 REFERENCES


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The End.