CIRCULATORY AND METABOLIC PHYSIOLOGY IN HIGH-ALTITUDE MICE

CIRCULATORY AND METABOLIC ADAPTATIONS TO HIGH ALTITUDE IN DEER MICE (*PEROMYSCUS MANICULATUS*)

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LAY ABSTRACT

Deer mice that live at high altitude have developed genetic adaptations to survive in challenging environments. These mammals must maintain high metabolism to generate body heat and stay warm in the cold temperatures at high altitude, despite the air around them being thinner with less oxygen than at sea level. Previous research has shown that high-altitude deer mice have evolved several physiological adaptations that help them transport oxygen from their environment to the tissues that generate body heat. Less is known about how these adaptations interact at a systems level, the regulatory processes that contribute to these adaptations, and whether high-altitude deer mice also possess adaptations to reduce energetic demands in the cold. In my Ph.D. thesis, I aimed to address these gaps in knowledge. Together, my findings demonstrate that both improvements in oxygen supply by the circulatory system and reductions in body temperature help high-altitude deer mice cope with their extreme environment.

ABSTRACT

High-altitude deer mice (*Peromyscus maniculatus*) have evolved to thrive in a persistently cold, low-O_2 (*i.e.*, hypoxic) environment that many low-altitude natives find inhospitable. As a result of strong directional selection on the aerobic capacity for heat generation (*i.e.*, thermogenesis), these small endotherms have evolved an enhanced aerobic capacity in hypoxia. However, the physiological modifications regulating tissue O_2 supply that might underlie this evolved change in aerobic capacity remain unresolved. Furthermore, very little attention has been paid to mechanisms that possibly reduce routine metabolic demands as a means of coping with cold hypoxia. The overarching goals of my Ph.D. thesis were: A) to understand the integration and mechanistic underpinnings of changes in circulatory physiology that underlie evolved increases in aerobic capacity in high-altitude deer mice; B) to elucidate the effects of chronic exposure to hypoxia or cold hypoxia on cardiovascular physiology, and C) to uncover potential strategies for reducing metabolic demands in high-altitude deer mice. I showed that (i) evolved increases in haemoglobin-O_2 affinity and tissue O_2 diffusing capacity likely interact to enhance aerobic capacity in hypoxia, and (ii) that evolved changes in adrenergic control of the heart likely contribute to increase cardiac output and thus help enhance aerobic capacity. I also showed that hypoxia alone has relatively modest but sex specific effects on routine metabolism, body temperature, and cardiovascular function in mice. However, cold hypoxia leads to energy-saving reductions in body temperature setpoint that curb the metabolic costs of endothermy, and high-altitude deer mice have evolved a lower body temperature than their low-altitude counterparts. My thesis shows that both environmentally-induced plasticity

and evolutionary adaptations in circulatory physiology and metabolism help improve O_2 supply and reduce O_2 demands in high-altitude deer mice, to help them cope with the unremitting cold and hypoxic conditions at high altitude.

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Oliver

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

♀ : female

♂ : male

2,3-DPG : 2,3-diphosphoglycerate

α-AR : α-adrenergic receptor

ACR : air convection requirement

β_1-AR : β_1-adrenergic receptor

BAT : brown adipose tissue

BTPS : body temperature and pressure saturated

C : thermal conductance

Cl^- : chloride

CO_2 : carbon dioxide

Δf_H : change in heart rate

D_LO_2 : oxygen diffusing capacity of the lungs

dP/dt_{max} : maximum rate of pressure increase in the left ventricle of the heart

ΔP_{mean} : change in mean arterial blood pressure

$\Delta P_{mean,\alpha\text{-AR}}$: change in mean arterial blood pressure due to α-adrenergic receptor blockade

$\Delta P_{mean,NOS}$: change in mean arterial blood pressure due to nitric oxide synthase blockade

$\Delta P_{mean,Sa}$: change in mean arterial blood pressure due to saline control injection

D_TO_2 : oxygen diffusing capacity of the systemic tissues

EDTA : ethylenediaminetetraacetic acid

E_{es} : end-systolic elastance

EF : ejection fraction of the heart

F_1 : first generation progeny from interpopulation breeding

F_2 : second generation progeny from interpopulation breeding

F_AO_2 : oxygen fraction of alveolar gas

f_H : heart rate

F_IO_2 : oxygen fraction of inspired gas

G_1 : first generation progeny from intrapopulation breeding

G_2 : second generation progeny from intrapopulation breeding

^{H} : highland α- or β-globin haplotype

Hb : haemoglobin

[Hb] : blood haemoglobin concentration

Hct : haematocrit

He : helium

HSD : honestly significant difference

HVR : hypoxic ventilatory response

iBAT : interscapular brown adipose tissue

IP : intraperitoneal

IV : intravenous

kPa : kilopascals

^{L} : lowland α- or β-globin haplotype

L-NAME : Nω-Nitro-L-arginine methyl ester

LV : left ventricle

LV+S : left ventricle and septum

mAChR : muscarinic acetylcholine receptor

mmHg : millimetres of mercury

MSNA : muscle sympathetic nerve activity

n : Hill coefficient

NaCl : sodium chloride

NO : nitric oxide

NOS : nitric oxide synthase

O_2 : oxygen

$[O_2]$: concentration of oxygen, or oxygen content

$[O_2]_a$: oxygen content of arterial blood

$[O_2]_L$: oxygen content in lung capillaries

$[O_2]_T$: oxygen content in systemic tissue capillaries

$[O_2]_v$: oxygen content in venous blood

P_{mean} : mean arterial blood pressure

P_{50} : partial pressure of oxygen at which haemoglobin is 50% saturated with oxygen

P_AO_2 : alveolar partial pressure of oxygen

P_aO_2 : arterial partial pressure of oxygen

P_{dev} : pressure developed during left ventricle contraction

P_LO_2 : lung capillary partial pressure of oxygen

P_{max} : maximum pressure in the left ventricle

P_{min} : minimum pressure in the left ventricle

P_MO_2 : mitochondrial partial pressure of oxygen

PO_2 : partial pressure of oxygen

P_TO_2 : systemic tissue capillary partial pressure of oxygen

P_vO_2 : venous partial pressure of oxygen

P-V : pressure-volume

$\dot{Q}$: cardiac output

Q_{10} : temperature coefficient

RV : right ventricle

SEM : standard error of the mean

STP : standard temperature and pressure

T_a : ambient temperature

T_b : body temperature

t_L : transit time in the lung capillaries

t_T : transit time in the systemic tissue capillaries

$\dot{V}_A$: alveolar ventilation rate

V_{max} : maximum internal volume of the left ventricle

V_{min} : minimum internal volume of the left ventricle

$\dot{V}O_2$: oxygen consumption rate

$\dot{V}O_2max$: maximal oxygen consumption rate

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 six chapters. Chapter 1 is an overview of relevant background material and a summary of the hypotheses tested. Chapters 2 to 5 are manuscripts that are published or have been submitted for publication in peer reviewed scientific journals. Chapter 2 is referred to as 'Wearing et al., 2021', Chapter 3 is referred to as 'Wearing et al., 2022', Chapter 4 is referred to as 'Wearing and Scott, 2022a', and Chapter 5 is referred to as 'Wearing and Scott, 2022b'. Chapter 6 summarizes the major findings of the thesis and places these findings in the context of current knowledge, with a discussion of remaining knowledge gaps that should be addressed with future research.

CHAPTER 1 GENERAL INTRODUCTION

CHAPTER 2 THE ADAPTIVE BENEFIT OF EVOLVED INCREASES IN HAEMOGLOBIN-O_2 AFFINITY IS CONTINGENT ON TISSUE O_2 DIFFUSING CAPACITY IN HIGH-ALTITUDE DEER MICE

Authors: Oliver H. Wearing, Catherine M. Ivy, Natalia Gutiérrez-Pinto, Jonathan P. Velotta, Shane C. Campbell-Staton, Chandrasekhar Natarajan, Zachary A. Cheviron, Jay F. Storz, and Graham R. Scott

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CHAPTER 3 ADRENERGIC CONTROL OF THE CARDIOVASCULAR SYSTEM IN DEER MICE NATIVE TO HIGH ALTITUDE

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CHAPTER 4 EFFECTS OF CHRONIC HYPOXIA ON ROUTINE CARDIOVASCULAR FUNCTION AND METABOLISM IN MICE

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CHAPTER 5 EVOLVED REDUCTIONS IN BODY TEMPERATURE AND THE METABOLIC COSTS OF THERMOREGULATION IN DEER MICE NATIVE TO HIGH ALTITUDE

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CHAPTER 6 GENERAL DISCUSSION

CHAPTER 1: GENERAL INTRODUCTION

1.1 OVERVIEW

Animals that have evolved to survive in extreme environments can offer valuable insights into physiological responses to metabolic challenges that threaten homeostasis. Responses to environmental changes may involve adaptive phenotypic plasticity that helps match metabolic supply to metabolic demand, but may also include maladaptive or even pathological adjustments in physiology. Animals native to extreme environments may also possess genetically based adaptations that help cope with metabolic challenges. Endotherms that live in environments that place constraints on metabolism are perhaps best suited to investigate these plastic and evolved metabolic coping mechanisms. For example, endotherms that live in consistently cold environments face high metabolic demands to support the exaggerated need for heat production (thermogenesis). One such environment – high altitude – can be extremely cold and also exposes animals to O_2-limitation (hypoxia) that is both unavoidable and unremitting. The mammals and birds that have adapted to survive at high altitude therefore provide us with a powerful system in which to test hypotheses about the plasticity and evolution of physiological processes supporting organismal metabolism. In my Ph.D. thesis, I set out to investigate the circulatory and metabolic physiology of high-altitude deer mice (*Peromyscus maniculatus*). I examined the mechanistic underpinnings of evolved increases in aerobic performance in these mice. I also explored how evolved and plastic adjustments in physiology may influence energy demands in hypoxia and/or cold environments.

1.2 PHYSIOLOGICAL RESPONSES TO METABOLIC CHALLENGES

Metabolic homeostasis requires that animals supply O_2 and metabolic fuels to tissues at appropriate rates to maintain their metabolic demands (Ye and Medzhitov, 2019). This balance between energy supply and demand can be challenging in extreme environments that constrain O_2 or food availability and/or that increase metabolic demands. The plastic and evolved mechanisms that protect metabolic homeostasis can therefore take two general forms. They can help increase the supply of O_2 and metabolic fuels needed to support metabolic processes, or they can help to offset the increase in metabolic demands associated with being in the extreme environment (Bickler and Buck, 2007; Burnett, 2015; Dzal et al., 2015; Geiser, 2020; McClelland and Scott, 2019; McKechnie, 2008; Murray et al., 2018; Olsen et al., 2021; Ramirez et al., 2007; Richards, 2010; Staples, 2016; Storz and Scott, 2019; Storz et al., 2010b). The strategies employed in these metabolically challenging environments are often dependent on the duration and severity of the challenge. For example, hibernation and torpor are highly effective at reducing metabolic demands on seasonal time scales (Geiser, 2020; Ramirez et al., 2007; Staples, 2016; Tattersall et al., 2012), but they are not indefinitely sustainable and are incompatible with some life history events (*e.g.*, reproduction, development), usually requiring a period of recovery when conditions become more favourable (Barnes, 1996; Humphries et al., 2003; Ramirez et al., 2007; Tattersall et al., 2012). Environments in which metabolic challenges persist across seasons and years likely require different coping mechanisms that do not sacrifice or put on hold processes involved in reproduction, nutrient digestion and assimilation, immune responses, or a range of other important but costly functions. How the complex

physiological systems that help achieve an effective balance between energy supply and demand have evolved in animals adapted to life in many extreme environments remain poorly understood.

Endotherms in extreme environments offer particularly valuable opportunities to investigate the mechanisms involved in coping with metabolically challenging conditions, due to their high metabolic demands of thermoregulation. The evolution of endothermy has been instrumental to the success and diversification of mammals and birds (Hillenius and Ruben, 2004; Polymeropoulos et al., 2018). By maintaining high body temperatures above ambient temperature, endothermy enables many performance traits important for evolutionary fitness across broad environmental temperature ranges (Bennett and Ruben, 1979; Clarke and Portner, 2010; Farmer, 2003; Hillenius and Ruben, 2004). However, shivering (skeletal muscle) and non-shivering thermogenesis (brown adipose tissue) necessary for maintenance of body temperature is itself energetically demanding, requiring high caloric intake and O_2 uptake (Bennett and Ruben, 1979; Clarke and Portner, 2010). For this reason, food scarcity, hypoxia, and/or severe reductions in environmental temperatures (*e.g.* during winter) can be especially challenging to the ability of endotherms to balance metabolic supply and demand. Some endotherms have evolved the ability to reduce the metabolic demands of endothermy by temporarily depressing body temperature and thermogenesis during times of seasonal hibernation or torpor (Bicego et al., 2007; Carey et al., 2003; Geiser, 2004; Staples, 2016). However, many other endotherms do not hibernate or exhibit extreme bouts of torpor, and must use alternative mechanism for coping with prolonged metabolic challenges.

1.3 HIGH-ALTITUDE ENVIRONMENTS

1.3.1 Metabolic challenges at high altitude

Studies of aerobic performance in endotherms that are native to high altitude can shed valuable insight into the processes by which animals evolve in response to challenging environmental conditions (McClelland and Scott, 2019; Monge and Leon-Velarde, 1991; Storz, 2021; Storz et al., 2015; Wearing and Scott, 2021). High-altitude environments are cold and hypoxic, which challenges the ability of endotherms to maintain sufficient rates of O_2 and metabolic fuel supply to meet the increased aerobic demands of thermogenesis (McClelland and Scott, 2019; Storz et al., 2019; Storz and Scott, 2019; Storz et al., 2010b). Many high-altitude endotherms do not hibernate, and whole-animal metabolism is elevated in small mammals at high altitude as compared to their low-altitude counterparts (Hayes, 1989a; Hayes, 1989b). As a result, a high aerobic capacity for thermogenesis (or thermogenic $\dot{V}O_2max$, quantified as maximal O_2 consumption elicited by acute exposure to extreme cold) has been shown to be associated with increased survival over harsh winters in small mammals at high altitude (Hayes and O'Connor, 1999). In response to such selective pressures, several high-altitude endotherms have evolved increases in aerobic performance relative to their lowland counterparts (Brutsaert, 2007; Monge and Leon-Velarde, 1991; Moore, 2017; Storz and Scott, 2019).

1.3.2 Increased O_2 and metabolic fuel supply in high-altitude natives

To help overcome the metabolic challenges at high altitude, several vertebrate taxa that are native to these environments – including some human populations – have evolved

increased capacities for supplying O_2 and metabolic fuels to tissues during cold hypoxia relative to their low-altitude counterparts. Studies of humans, non-human mammals, and birds have shown that adaptive variation in aerobic performance is underlain by evolved changes across the O_2 transport pathway (Brutsaert, 2007; Burtscher et al., 2018; Faraci, 1991; Gilbert-Kawai et al., 2014; Ivy and Scott, 2015; McClelland and Scott, 2019; Monge and Leon-Velarde, 1991; Storz et al., 2019; Storz and Scott, 2019). This pathway consists of the conceptual steps (ventilation, pulmonary diffusion, circulation, tissue diffusion, and mitochondrial O_2 utilization) involved in transporting O_2 from inspired air to metabolically active tissues, where O_2 is used by mitochondria to support oxidative phosphorylation (Ivy and Scott, 2015; Scott et al., 2015b; Storz and Scott, 2019; Storz et al., 2010b). Evolved changes in pathways involved in the delivery of metabolic substrates to active tissues have also been observed in some high-altitude natives (McClelland and Scott, 2019).

High-altitude environments can also lead to plastic increases in $\dot{V}O_2max$ in response to exposure during adulthood (*e.g.*, acclimatization) or early life (*i.e.*, developmental plasticity) (Ivy and Scott, 2015; McClelland and Scott, 2019; Storz and Cheviron, 2021; Storz and Scott, 2019; Tate et al., 2017; Tate et al., 2020). Therefore, both evolved changes and plasticity in the physiological determinants of O_2 and fuel transport appear to contribute to enhancing $\dot{V}O_2max$ in high-altitude natives (Chen et al., 1997; Lau et al., 2017; Lyons et al., 2021; Tate et al., 2020).

1.3.3 Metabolic O_2 and fuel demands at high altitude

Despite increased capacities for supplying O_2 and metabolic fuels to tissues, metabolic demands may still be extremely difficult to maintain for endotherms living at high altitude. The fact that the metabolic challenges at high altitude are unremitting likely precludes many high-altitude endotherms from undergoing severe metabolic depression similar to seasonal hibernators (Ivy and Scott, 2015; Storz et al., 2010b). However, the possibility that high-altitude endotherms may somehow reduce metabolic O_2 and fuel demands in cold hypoxia has received relatively little attention. There is some limited evidence from plateau pika that metabolic demands may be reduced to cope with chronic cold hypoxia by reducing metabolism (Speakman et al., 2021), but this issue has otherwise been largely unexplored.

1.3.4 Maladaptive cardiovascular responses to high-altitude hypoxia

An additional challenge that must be overcome in high-altitude natives is the potential for chronic hypoxia exposure to cause maladaptive cardiovascular adjustments that lead to pathology. Upon detection of low arterial PO_2 by peripheral chemosensors in the carotid body, the hypoxic chemoreflex leads to activation of the sympathetic nervous system and catecholamine release from the adrenal medulla (Davy et al., 1997; Hainsworth and Drinkhill, 2007; Hainsworth et al., 2007; Heistad and Abboud, 1980; Ivy and Scott, 2015; Johnson et al., 1983). This hypoxia-induced sympathoadrenal activation stimulates cardiac output and tends to cause α-adrenoreceptor-mediated vasoconstriction in peripheral tissues, helping maintain O_2 supply to hypoxia-sensitive tissues like the brain and heart (Heistad and Abboud, 1980; Ivy and Scott, 2015; Slotkin et al., 1988). This response can improve

survival during acute exposure to severe hypoxia, but prolonged sympathoadrenal activation can become detrimental with chronic exposure to more moderate levels of hypoxia, because α-adrenergic vasoconstriction can prevent sufficient blood flow to peripheral tissues and may also lead to systemic hypertension (Calbet, 2003; Calbet et al., 2014; Hansen and Sander, 2003; Kanstrup et al., 1999; Lundby et al., 2018; Parati et al., 2014; Rhodes et al., 2011; Richalet et al., 1988; Schultz et al., 2014; Wolfel et al., 1994). However, most of what is known about the autonomic and cardiovascular effects of chronic hypoxia has been discovered in humans and rats exposed to severe environmental and/or clinical levels of hypoxia (Johnson et al., 1983; Mazzali et al., 2003; Siques et al., 2014; Vaziri and Wang, 1996), and the effects of chronic exposure to the levels of hypoxia typical at high altitude, and how they may differ in other species, remains unresolved.

Some high-altitude natives have evolved mechanisms that attenuate these maladaptive effects of chronic hypoxia, but the presence of such evolved changes appear to be somewhat idiosyncratic between taxa. In humans there appear to be differences between distinct high-altitude populations as to whether evolved mechanisms have arisen to reduce sympathetic activity in chronic hypoxia (Simpson et al., 2021). For example, when considering muscle sympathetic nerve activity (MSNA), which increases in lowland-native humans after 10-20 days at high altitude, MSNA is not increased in highland-native Sherpa residing at high altitude (Simpson et al., 2019) but it is increased in Andeans native to high altitude (Lundby et al., 2018). While these reductions in sympathoadrenal activation may be beneficial for reducing some maladaptive cardiovascular consequences of chronic hypoxia, it is unclear if these evolved changes constrain some other important regulatory functions of the

sympathetic nervous system (*e.g.*, blood flow regulation during thermogenesis or locomotion).

1.4 THE NORTH AMERICAN DEER MOUSE

The North American deer mouse (*Peromyscus maniculatus*) is a powerful model for elucidating the physiological mechanisms underpinning the evolution of aerobic performance and metabolic homeostasis in extreme environments (Storz et al., 2019; Storz and Scott, 2019; Storz and Scott, 2021; Storz et al., 2010b). Deer mice are found across North America (Bedford and Hoekstra, 2015) and have the largest altitudinal range of any North American mammal, ranging from near sea level to montane environments up to approx. 4,350 m above sea level (Hock, 1964). High-altitude populations of deer mice maintain higher field metabolic rates than their low-altitude counterparts (Hayes, 1989a; Hayes, 1989b), likely to meet the increased metabolic costs of thermogenesis. A high aerobic capacity for thermogenesis (*i.e.*, thermogenic $\dot{V}O_2max$) during adulthood imparts a survival advantage and is likely under directional selection during harsh winters at high altitude (Hayes and O'Connor, 1999). High-altitude deer mice have, presumably at least in part due to this selection pressure, evolved a higher thermogenic $\dot{V}O_2max$ in hypoxia compared to low-altitude populations of deer mice and white-footed mice, a congeneric species that is restricted to low altitudes (Fig. 1.1A) (Chappell et al., 1988; Chappell and Snyder, 1984; Cheviron et al., 2012; Cheviron et al., 2013; Tate et al., 2017; Tate et al., 2020). These features make highland deer mice an ideal model in which to test hypotheses

about the evolution of aerobic performance and the mechanisms of metabolic homeostasis in extreme environments.

The high thermogenic $\dot{V}O_2max$ of high-altitude deer mice is supported by both plastic and evolved changes within the O_2 transport pathway (Lau et al., 2017; Lui et al., 2015; Mahalingam et al., 2020; Mahalingam et al., 2017; Storz et al., 2010a; Storz et al., 2007; Tate et al., 2017; Tate et al., 2020; Velotta et al., 2016). Perhaps the best understood mechanism is an evolved increase in Hb-O_2 affinity in high-altitude deer mice, which arises as a result of amino acid replacements in duplicated genes that encode the α- and β-chain subunits of the $\alpha_2\beta_2$ Hb tetramer (Chappell et al., 1988; Chappell and Snyder, 1984; Natarajan et al., 2015; Natarajan et al., 2013; Snyder et al., 1982; Snyder, 1981; Storz et al., 2012; Storz et al., 2010a; Storz et al., 2009; Storz et al., 2007). This evolved increase in Hb-O_2 affinity along with evolved improvements in pulmonary function and O_2 diffusing capacity are believed to contribute to higher arterial O_2 saturation in hypoxia (Fig. 1.1B) (Tate et al., 2017; Tate et al., 2020; West et al., 2021a; West et al., 2021b). Tissue O_2 supply is also augmented by evolved and plastic increases in cardiac output (Fig. 1.1C). Collaborative work that I was involved with early in my Ph.D. showed that high-altitude deer mice have significantly higher heart rates and stroke volumes at thermogenic $\dot{V}O_2max$ in hypoxia compared to low-altitude deer mice and white-footed mice, particularly after acclimation to hypoxia or cold hypoxia (Tate et al., 2017; Tate et al., 2020). This increased cardiac output increases the rate of O_2 supply as well as the rate of lipid and carbohydrate supply to systemic tissues (Lyons et al., 2021; McClelland and Scott, 2019). However, the mechanistic underpinnings of these evolved and plastic increases in cardiac output are

unknown. High capacities for tissue O_2 extraction also appear to play particularly important roles for increasing thermogenic $\dot{V}O_2max$ in highland deer mice, associated with several evolved changes in skeletal muscle phenotype and mitochondrial function (Lui et al., 2015; Mahalingam et al., 2017; Scott et al., 2015a).

While much of the research on high-altitude deer mice has focused on their evolved increases in aerobic performance in cold hypoxia and the physiological mechanisms that support them, relatively little attention has been paid to understanding whether highland mice have also reduced their routine metabolic demands in this extreme environment. Considering that cold-induced increases in the aerobic demand for thermogenesis may be heavily constrained by O_2 limitation and possibly food scarcity at higher elevations (Bears et al., 2009), reductions in metabolic demands could be extremely valuable in high-altitude deer mice. Interestingly, the ontogenetic development of endothermy is delayed in high-altitude deer mice (Robertson and McClelland, 2019; Robertson and McClelland, 2021; Robertson et al., 2019), suggesting that metabolic demands of thermogenesis are reduced in early post-natal life stages. Whether they also exhibit strategies to reduce the metabolic demands of thermogenesis and body temperature regulation in later life remains unresolved.

1.5 THESIS OBJECTIVES/HYPOTHESES

My Ph.D. thesis work has aimed to elucidate evolved and plastic changes in circulatory and metabolic physiology that may have contributed to the successful colonization of deer mice to high altitudes. The **primary objectives** of my Ph.D. thesis were: A) to understand

the integration and mechanistic underpinnings of changes in circulatory physiology that underlie evolved increases in aerobic capacity in high-altitude deer mice, B) to elucidate the effects of chronic exposure to hypoxia or cold hypoxia on cardiovascular physiology, and C) to uncover potential strategies for reducing metabolic demands in high-altitude deer mice. I used a combination of acute and chronic environmental exposures, controlled breeding designs, mathematical modelling of the O_2 transport pathway, pharmacological manipulations, and various surgical approaches to achieve my thesis objectives. The various hypotheses and approaches I used for each study are described below.

1.5.1 Chapter 2: The adaptive benefit of evolved increases in haemoglobin-O_2 affinity is contingent on tissue O_2 diffusing capacity in high-altitude deer mice

In Chapter 2, I examined the contribution of the evolved, genetically based increases in Hb-O_2 affinity in high-altitude deer mice on thermogenic $\dot{V}O_2$max in hypoxia, and also considered whether this contribution is dependent on other cardiorespiratory traits. This was achieved using F_2 lab-raised interpopulation hybrids derived from wild deer mice trapped at high (*Peromyscus maniculatus rufinus*; 4,350 m above sea level, Mt. Evans, CO, USA) and low altitude (*P. m. nebrascensis*; 430 m above sea level, Nine Mile Prairie, Lancaster County, NE, USA) in which the effects of evolved changes in haemoglobin genotype and O_2 affinity could be examined on a mixed genetic background. To account for effects of the environment, adult mice were studied before and after a 6-wk acclimation period to hypobaric hypoxia (12 kPa O_2) resembling the partial pressure of oxygen (PO_2) at 4,350 m above sea level. I **hypothesized** that the increases in Hb-O_2 affinity would

enhance thermogenic $\dot{V}O_2max$ in hypoxia. I therefore predicted that mice with high-altitude Hb variants would have greater arterial O_2 saturation and thermogenic $\dot{V}O_2max$ in hypoxia than mice with low-altitude Hb variants.

1.5.2 Chapter 3: Adrenergic control of the cardiovascular system in deer mice native to high altitude

In Chapter 3, I examined how the function and regulation of the heart has evolved in high-altitude deer mice to support the increases in cardiac output that contribute to increasing thermogenic $\dot{V}O_2max$ in hypoxia. I used first-generation lab-raised mice born of wild deer mice from the same high altitude population described above, and mice born of wild white-footed mice (*P. leucopus*; 430 m above sea level, Nine Mile Prairie, Lancaster County, NE, USA), a species that is exclusively found at low altitudes. I used a common garden experimental design to disentangle the evolved differences in high-altitude mice from the effects of environmentally-induced plasticity induced by environmental exposure, in which mice were raised to adulthood in captivity and acclimated to normobaric normoxia (~20 kPa O_2) in ambient air or to hypobaric hypoxia (~12 kPa O_2) for 6-8 weeks. I tested the **hypothesis** that high-altitude deer mice can achieve higher cardiac outputs by virtue of having evolved greater adrenergic sensitivity and contractility of the heart. I predicted that the scope for increasing heart rate in response to β_1-adrenergic receptor stimulation would be greater in highland mice than in lowland mice. I also predicted that intrinsic cardiac contractility (measured using *in vivo* pressure-volume catheters) would be higher in high-altitude deer mice compared to white-footed mice.

1.5.3 Chapter 4: Effects of chronic hypoxia on routine cardiovascular function and metabolism in mice

In Chapter 4, I carried out a study in CD-1 lab strain of house mice to examine the effects of chronic hypoxia on routine activity, body temperature and cardiovascular function. This study was valuable for laying the groundwork for a larger study in deer mice, and for examining potential sex-specific differences in the responses to chronic hypoxia. Similarly to in Chapter 3, mice were assigned to normoxia or hypoxia for six weeks, and then surgically instrumented with implantable physiological telemeters that allowed for continuous recording of routine activity and physiology in their assigned environments. I **hypothesized** that mice would exhibit systemic hypertension in chronic hypoxia, as observed in humans and rats. I also hypothesized that females would suffer less pronounced effects of chronic hypoxia on metabolism and cardiovascular function than males, based on some evidence in the literature suggesting that female mice exhibit greater cardiorespiratory plasticity in chronic hypoxia (Soliz et al., 2012; Soliz et al., 2008; Soliz et al., 2009). I therefore predicted that normal routine activity and cardiovascular function would be better maintained in hypoxic females than hypoxic males.

1.5.4 Chapter 5: Evolved reductions in body temperature and the metabolic costs of thermoregulation in deer mice native to high altitude

In Chapter 5, I measured body temperature and cardiovascular function in freely behaving deer mice under routine conditions to examine the metabolic and circulatory responses to cold hypoxia. I used second generation lab-raised deer mice derived from

populations at high altitude or low altitude. I again used a common garden experimental design in which adults from each population were acclimated to warm normoxia (25˚C, ~20 kPa O_2) and cold hypoxia (5˚C, ~12 kPa O_2), and then surgically instrumented with physiological telemeters. I **hypothesized** that reductions in body temperature setpoint help high-altitude deer mice reduce the metabolic demands of thermoregulation in cold hypoxia. I predicted that acclimation to cold hypoxia would cause energy-saving reductions in body temperature in deer mice, and that in addition to this plasticity, the high-altitude population would have evolved to operate at a lower body temperature compared to the low-altitude population. I also **hypothesized** that the high-altitude population would not systemic hypertension in cold hypoxia.

1.6 SUMMARY OF DATA CHAPTERS

1.6.1 Chapter 2: The adaptive benefit of evolved increases in haemoglobin-O_2 affinity is contingent on tissue O_2 diffusing capacity in high-altitude deer mice

Chapter 2 is published in *BMC Biology* (Wearing et al., 2021). This study revealed that evolved increases in Hb-O_2 affinity that have arisen in high-altitude deer mice increased arterial O_2 saturation in hypoxia, but this was not sufficient to cause an associated increase in thermogenic $\dot{V}O_2max$. However, by mathematically modelling the O_2 transport pathway, I demonstrated that these increases in Hb-O_2 affinity should improve aerobic performance in hypoxia (*i.e.*, become adaptive) if tissue O_2 diffusing capacity was also increased. As such, this study showed that the adaptive benefit of increasing Hb-O_2 affinity is contingent on the capacity for active tissues to extract O_2 from the blood in high-altitude deer mice.

This study also highlights the importance of considering the interactions between different subordinate traits that together contribute to the evolution of complex organismal traits such as aerobic performance.

1.6.2 Chapter 3: Adrenergic control of the cardiovascular system in deer mice native to high altitude

Chapter 3 is published in *Current Research in Physiology* (Wearing et al., 2022). This study demonstrated that the evolved and plastic increases in cardiac output that underlie increased hypoxic thermogenic $\dot{V}O_2max$ in high-altitude deer mice (Fig. 1.1C) (Tate et al., 2017; Tate et al., 2020) are associated with evolved changes in adrenergic control of the cardiovascular system. Specifically, high-altitude deer mice had a greater capacity to increase heart rate by stimulation of cardiac β_1-adrenergic receptors compared to lowland white-footed mice. White-footed mice reduced α-adrenergic sensitivity in chronic hypoxia, possibly to help circumvent maladaptive vascular responses to chronic hypoxia, but these changes were not observed in high-altitude deer mice. I also found little evidence that intrinsic cardiac contractility is greater in highland deer mice compared to white-footed mice.

1.6.3 Chapter 4: Effects of chronic hypoxia on routine cardiovascular function and metabolism in mice

Chapter 4 is currently under review for publication, and is referred to throughout the thesis as 'Wearing and Scott, 2022a'. This study showed that female mice maintain normal

activity, body temperature and heart rate in hypoxia, in contrast to reductions in these indices in males. Females also exhibit augmented ventilatory acclimatization to hypoxia, greater increases in lung mass, and a blunting of right-ventricle hypertrophy in chronic hypoxia as compared to males. These findings support the growing evidence suggesting that mice are generally more hypoxia tolerant than some other mammals (*e.g.*, humans and rats), avoiding the maladaptive systemic hypertension in chronic hypoxia that has been observed in these species.

1.6.4 Chapter 5: Evolved reductions in body temperature and the metabolic costs of thermoregulation in deer mice native to high altitude

Chapter 4 is nearing submission for publication, and is referred to throughout the thesis as 'Wearing and Scott, 2022b'. This study demonstrated that the increased metabolic demands associated with thermoregulation in cold hypoxia at high altitude are curbed in highland deer mice through evolved and plastic reductions in body temperature setpoint. Both highland and lowland deer mice depressed body temperature after chronic exposure to cold hypoxia, and highlanders consistently operated around 1˚C cooler than lowlanders in both warm normoxia and cold hypoxia. As such, this study provides a rare example of a non-hibernating endotherm using reductions in body temperature to decrease the energetic costs of thermoregulation in metabolically challenging environments.

1.7 FIGURE

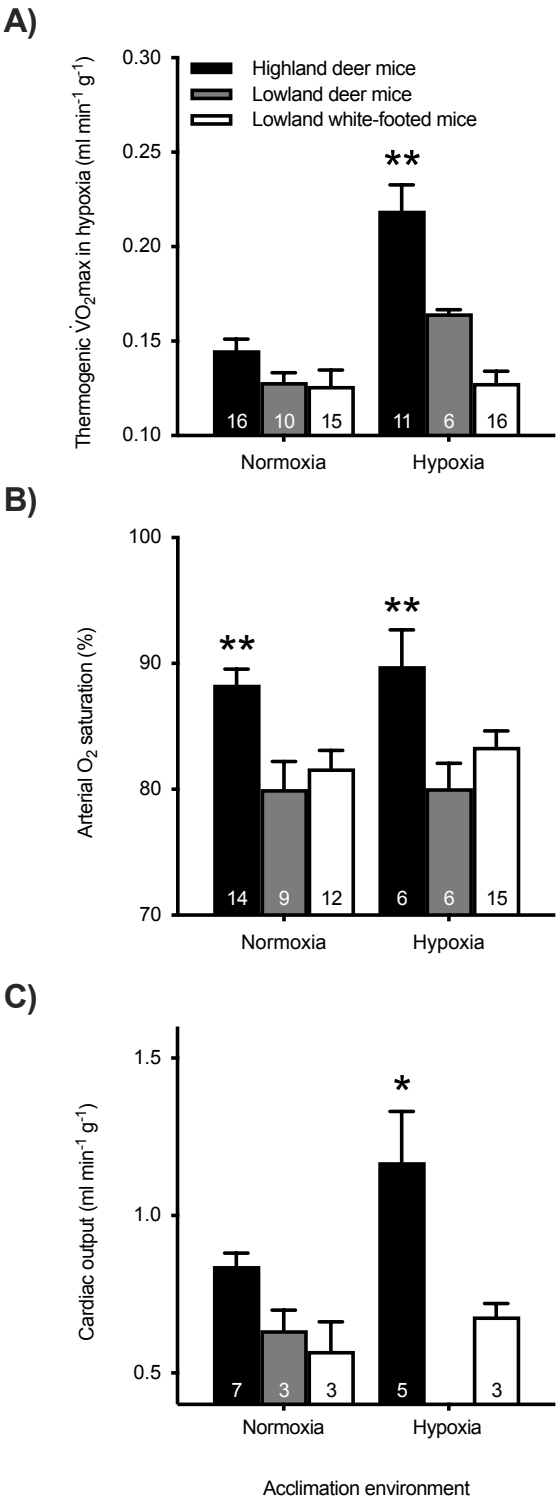
A)
Highland deer mice
Lowland deer mice
Lowland white-footed mice
Thermogenic $\dot{V}O_2$max in hypoxia (ml min^{-1} g^{-1})
0.30
0.25
0.20
0.15
0.10
**
16
10
15
11
6
16
Normoxia
Hypoxia
B)
Arterial O_2 saturation (%)
100
90
80
70
**
**
14
9
12
6
6
15
Normoxia
Hypoxia
C)
Cardiac output (ml min^{-1} g^{-1})
1.5
1.0
0.5
*
7
3
3
5
3
Normoxia
Hypoxia
Acclimation environment

Fig. 1.1 (**A**) Aerobic thermogenic capacity (thermogenic $\dot{V}O_2max$) in hypoxia was found to be higher significantly higher in highland deer mice compared to lowland deer mice and lowland white-footed mice, particularly after acclimation to hypoxia. Arterial O_2 saturation (**B**) and cardiac output (**C**) were also higher in highland deer mice compared to lowland mice. **significant pairwise difference (Dunnett test, GraphPad Prism v.9.3.1) between highland deer mice and both lowland populations within an acclimation environment ($P < 0.05$). *significant pairwise difference (Šídák test excluding data from lowland deer mice, GraphPad Prism v.9.3.1) between highland deer mice and lowland white-footed mice following hypoxia acclimation ($P < 0.05$). Data are means ± SEM, with n for each group indicated within each bar. Modified from Tate et al., 2020.

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CHAPTER 2: The adaptive benefit of evolved increases in haemoglobin-O_2 affinity is contingent on tissue O_2 diffusing capacity in high-altitude deer mice

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2.1 ABSTRACT

Complex organismal traits are often the result of multiple interacting genes and sub-organismal phenotypes, but how these interactions shape the evolutionary trajectories of adaptive traits is poorly understood. I examined how functional interactions between cardiorespiratory traits contribute to adaptive increases in the capacity for aerobic thermogenesis (maximal O_2 consumption, $\dot{V}O_2max$, during acute cold exposure) in high-altitude deer mice (*Peromyscus maniculatus*). I crossed highland and lowland deer mice to produce F_2 inter-population hybrids, which expressed genetically based variation in haemoglobin (Hb) O_2 affinity on a mixed genetic background. I then combined physiological experiments and mathematical modelling of the O_2 transport pathway to examine links between cardiorespiratory traits and $\dot{V}O_2max$. Physiological experiments revealed that increases in Hb-O_2 affinity of red blood cells improved blood oxygenation in hypoxia, but were not associated with an enhancement in $\dot{V}O_2max$. Sensitivity analyses performed using mathematical modelling showed that the influence of Hb-O_2 affinity on $\dot{V}O_2max$ in hypoxia was contingent on the capacity for O_2 diffusion in active tissues. These results suggest that increases in Hb-O_2 affinity would only have adaptive value in hypoxic

conditions if concurrent with or preceded by increases in tissue O_2 diffusing capacity. In high-altitude deer mice, the adaptive benefit of increasing Hb-O_2 affinity is contingent on the capacity to extract O_2 from the blood, which helps resolve controversies about the general role of haemoglobin function in hypoxia tolerance.

2.2 INTRODUCTION

A long-standing goal of evolutionary biology is to understand how the functional integration of traits influences patterns of phenotypic change and adaptation (Gould, 2002). Complex physiological phenotypes often represent an emergent property of functional interactions among different tissues and organ systems, which in turn may be developmentally interrelated and genetically correlated. The functional, developmental, and genetic interdependence of traits may facilitate environmental adaptation if semi-autonomous components of a complex phenotype respond synergistically to selection. Alternatively, functional integration and genetic correlations among components of a trait can limit and channel pathways of phenotypic evolution (Jernigan et al., 1994; Wagner and Altenberg, 1996). Evolutionary questions about phenotypic integration and adaptation can be addressed most profitably by examining well-defined traits with well-characterized functions and well-documented associations with fitness under natural conditions.

The capacity for aerobic thermogenesis in small mammals at high altitude is a complex performance trait that is well suited to experimental studies of how patterns of phenotypic integration affect the process of adaptation. At high altitude, cold temperatures challenge the ability of endotherms to maintain body temperature and activity, which is especially

difficult in smaller animals that have high surface area to volume ratios. Unsurprisingly, aerobic thermogenesis (quantified as maximal O_2 consumption, $\dot{V}O_2max$, during acute cold exposure) in hypoxia is under strong directional selection in some small mammals at high altitude (Hayes and O'Connor, 1999), which have evolved higher thermogenic $\dot{V}O_2max$ (Chappell et al., 1988; Chappell and Snyder, 1984; Cheviron et al., 2012; Cheviron et al., 2013; Tate et al., 2017). Thermogenic $\dot{V}O_2max$ is supported by the integrated function of the O_2 transport pathway, the conceptual steps (ventilation, pulmonary diffusion, circulation, tissue diffusion, and mitochondrial O_2 utilization) involved in transporting O_2 from inspired air to thermogenic tissues where O_2 is used by mitochondria to support oxidative phosphorylation (Ivy and Scott, 2015; Storz et al., 2010b). Therefore, studies of thermogenic $\dot{V}O_2max$ in high-altitude natives are ideal for understanding the mechanisms underlying the adaptive evolution of complex traits.

Evolved increases in the O_2 affinity of haemoglobin (Hb) are pervasive in high-altitude taxa, and have become classic examples of biochemical adaptation (Storz, 2016). However, the nature of the direct adaptive benefit conferred by increases in Hb-O_2 affinity in highland species is controversial. Many highland taxa have evolved increases in Hb-O_2 affinity independently, and in many cases, the molecular mechanisms underlying these changes in protein function are documented in detail (Natarajan et al., 2016; Natarajan et al., 2013; Natarajan et al., 2018; Natarajan et al., 2015b; Storz, 2016; Storz, 2019; Weber, 2007). These increases in Hb-O_2 affinity are often presumed to safeguard arterial O_2 saturation in hypoxia and thus help improve tissue O_2 delivery and aerobic capacity (Chappell et al., 1988; Chappell and Snyder, 1984; McClelland and Scott, 2019; Snyder et al., 1982; Snyder,

1981; Storz et al., 2019; Storz et al., 2012; Storz et al., 2010a; Storz et al., 2009; Winslow, 2007), although this has rarely been tested. Nonetheless, the relationship between Hb-O_2 affinity and $\dot{V}O_2max$ in hypoxia remains contentious (Brauner and Wang, 1997; Dempsey, 2020; Storz, 2016; Wang and Malte, 2011; Winslow, 2007). Theoretical modelling of the O_2 transport pathway in humans suggests that increases in Hb-O_2 affinity do not increase aerobic capacity in hypoxia on their own (Wagner, 1997), because the advantage of increasing Hb-O_2 affinity may be offset by a trade-off in O_2 offloading at tissues (Bunn, 1980; Hebbel et al., 1978; Storz et al., 2010b). A recent study in humans with rare genetic Hb variants found that increases in Hb-O_2 affinity attenuated the hypoxia-induced decline in aerobic capacity, but subjects with high Hb-O_2 affinity also had compensatory polycythaemia (Dominelli et al., 2020). Considering the strong functional integration of Hb within the O_2 transport pathway, the advantages of increasing Hb-O_2 affinity in high-altitude taxa may be contingent on the evolution of other cardiorespiratory traits, but this has not been experimentally investigated.

I sought to determine the effects of evolved increases in Hb-O_2 affinity in high-altitude deer mice (*Peromyscus maniculatus*) on thermogenic $\dot{V}O_2max$ in hypoxia, and to examine whether the adaptive benefit of changes in Hb-O_2 affinity is contingent on other cardiorespiratory changes. Deer mice have the broadest altitudinal range of any North American mammal (Natarajan et al., 2015a), ranging from near sea level to montane environments up to approx. 4,350 m above sea level (Bedford and Hoekstra, 2015), and high-altitude populations have evolved elevated thermogenic $\dot{V}O_2max$ in hypoxia in response to directional selection (Chappell et al., 1988; Chappell and Snyder, 1984;

Cheviron et al., 2012; Cheviron et al., 2013; Hayes and O'Connor, 1999; Tate et al., 2017). In conjunction with a higher $\dot{V}O_2max$ in chronic hypoxia, high-altitude deer mice also exhibit higher pulmonary O_2 extraction, arterial O_2 saturation, cardiac output, and tissue O_2 extraction than their lowland counterparts (Tate et al., 2017; Tate et al., 2020). The latter is associated with several evolved changes in skeletal muscle phenotype and mitochondrial function (Dawson et al., 2018; Lui et al., 2015; Mahalingam et al., 2017; Scott et al., 2015; Scott et al., 2018). Highlanders have also evolved a higher Hb-O_2 affinity as a result of amino acid replacements in duplicated genes that encode the α- and β-chain subunits of the $\alpha_2\beta_2$ Hb tetramer (Chappell et al., 1988; Chappell and Snyder, 1984; Natarajan et al., 2015a; Natarajan et al., 2013; Snyder et al., 1982; Snyder, 1981; Storz et al., 2012; Storz et al., 2010a; Storz et al., 2009; Storz et al., 2007). This evolved increase in Hb-O_2 affinity in highlanders is not complemented by an enhanced Bohr effect to augment O_2 unloading (Jensen et al., 2016). I initially hypothesized that these evolved increases in Hb-O_2 affinity would be responsible for higher thermogenic capacity in highland deer mice, compared to their lowland conspecifics. To investigate the effect of genetically based changes in Hb-O_2 affinity on whole-animal performance in hypoxia, We created F_2 hybrids between high- and low-altitude deer mice (F_2 intercross breeding design) to randomize associations between allelic globin variants, and we then examined the effects of α- and β-globin variants on red blood cell P_{50} (the O_2 pressure, PO_2, at which Hb is 50% saturated), arterial O_2 saturation, thermogenic $\dot{V}O_2max$, and other physiological traits on an admixed genetic background (see Supplemental Figures and Tables, Fig. S2.1 for graphical overview of experimental design). I performed physiological measurements before and after chronic

exposure to hypoxia to test for effects of Hb genotype on trait-specific acclimation responses. I then used my empirical data in an *in silico* model of the O_2 transport pathway to examine the interactive effects of Hb-O_2 affinity and the O_2 diffusing capacity of tissues (D_TO_2) on $\dot{V}O_2max$. My results suggest that increases in Hb-O_2 affinity only contribute to the adaptive enhancement of thermogenic $\dot{V}O_2max$ in hypoxia if accompanied by a corresponding increase in D_TO_2 to augment tissue O_2 extraction.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Wild deer mice (*Peromyscus maniculatus*) 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) 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), and were transported to the University of Montana (elevation 978 m). The wild mice were used to produce one family of first-generation inter-population hybrids (F_1), created by crossing a highland male and a lowland female. These F_1 hybrids were raised to maturity and used for full-sibling matings to produce 4 families of male and female second-generation hybrid progeny (F_2). These F_2 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 University (near sea level) for subsequent experiments (see below). Prior to experimentation, all mice were kept in standard holding conditions (24-25˚C, 12:12-h light-dark photoperiod) under normal

atmospheric conditions, with unlimited access to water and standard mouse chow. All animal protocols were approved by institutional animal research ethics boards.

Each mouse was genotyped for determination of α- and β-globin haplotypes. Tetrameric haemoglobin isoforms 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., 2015a; Storz et al., 2008). A reverse-transcriptase PCR (RT-PCR) approach was used to obtain sequence data for all four of the adult-expressed α- and β-globin transcripts (Natarajan et al., 2015a; Storz et al., 2010a). Total RNA was extracted from red blood cells using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Globin transcripts were then amplified from 1 μg of extracted RNA using the One-Step RT-PCR system with Platinum Taq DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). PCR cycling was performed with 1 cycle at 50 °C for 30 min, 1 cycle at 95˚C for 15 min, 34 cycles at 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 1 min, and then a final extension cycle at 72˚C for 3 min. For the α-globin transcripts, the same primer pair was used for HBA-T1 and HBA-T2 (forward: CTGATTCTCACAGACTCAGGAAG, reverse: CCAAGAGGTACAGGTGCGAG). For the β-globin transcripts, the same RT-PCR primer pair was used for HBB-T1 and HBB-T2 (forward: GACTTGCAACCTCAGAAACAGAC, reverse: GACCAAAGGCCTTCATCATTT). 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 using Big Dye

chemistry (ABI 3730 capillary sequencer; 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 5 distinct combinations of highland (H)- and lowland (L)-associated α- and β-globin haplotypes: n = 5 $\alpha^{LL}\beta^{LH}$, 2 male, 3 female; n = 3 $\alpha^{LL}\beta^{HH}$, 1 male, 2 female; n = 8 $\alpha^{HH}\beta^{LL}$, 5 male, 3 female; n = 5 $\alpha^{HH}\beta^{LH}$, 5 male; and n = 5 $\alpha^{HH}\beta^{HH}$, 5 male.

2.3.2 Acclimation treatments

Physiological measurements (see below) were taken for each mouse both before (mean ± SEM body mass, 23.8 ± 0.9 g) and after (23.7 ± 0.8 g) a six-week acclimation to hypobaric hypoxia (approx. 12 kPa PO_2), approximating the O_2 levels experienced by highland deer mice living at 4,350 m above sea level in the wild (see Supplemental Figures and Tables, Fig. S2.2 for body masses for each genotype before and after hypoxia acclimation). This was achieved by placing mice into custom-made hypobaric chambers inside which barometric pressure was maintained at 60 kPa using a vacuum pump, as previously described (Lui et al., 2015; McClelland et al., 1998). Mice were removed from the chambers for <20 min twice per week for cage cleaning.

2.3.3 Respirometry, plethysmography, and pulse oximetry

I combined open-flow respirometry, plethysmography and pulse oximetry to simultaneously measure aerobic capacity for thermogenesis (thermogenic $\dot{V}O_2max$), pulmonary ventilation, arterial O_2 saturation, and heart rate during acute cold (-5˚C) exposure in heliox (Tate et al., 2017; Tate et al., 2020). I used established methods of measuring thermogenic $\dot{V}O_2max$ that have been shown to elicit values of $\dot{V}O_2max$ that equal or exceed those measured during exercise $\dot{V}O_2max$ in deer mice (Chappell and Hammond, 2004; Rosenmann and Morrison, 1974; Storz et al., 2019). These measurements were performed twice before mice were acclimated to hypoxia: once in normoxic heliox (21% O_2, 79% He) and once in hypoxic heliox (12% O_2, 88% He) in random order. After hypoxia acclimation, both normoxic and hypoxic $\dot{V}O_2max$ trials were repeated. $\dot{V}O_2max$ was measured inside a 530-ml plethysmography chamber that has been previously described in detail (Ivy and Scott, 2017b), and was kept in a regulated freezer to maintain the internal chamber temperature at -5˚C (measured with a PT-6 thermocouple, Physitemp). Two days prior to initial trials, the neck fur of each mouse was removed with Nair^{TM} hair-removal product to facilitate pulse oximetry. Immediately before each trial, each mouse was weighed, fitted with a MouseOx pulse oximetry neck collar (Starr Life Sciences, Oakmont, PA, USA), and placed inside the chamber for 10 min of continuous recording. Incurrent gas flowed through the animal chamber at a rate of 1500 ml min^{-1} (controlled by an MFC-2 mass flow controller, Sable Systems, Las Vegas, NV, USA), and was cooled before entering the plethysmograph by passing through copper coils that were also placed in the freezer. Excurrent gas was subsampled at a rate of 200 ml min^{-1}, and dried with pre-baked

Drierite before passing through O_2 and CO_2 analyzers to determine the fractional concentrations of each gas (FoxBox Respirometry System, Sable Systems). Breathing-induced changes in flow across a pneumotachograph in the chamber lid were measured from pressure oscillations in the animal chamber relative to an identical reference chamber using a differential pressure transducer (Validyne DP45; Cancoppas, Mississauga, ON, Canada) and carrier demodulator (Validyne CD15, Cancoppas), and signals were volume-calibrated before each trial with 300-µl injections using a gas-tight syringe. The core body temperature (T_b) of each mouse was obtained (RET-3-ISO; Physitemp, Clifton, NJ, USA) immediately after being removed from the plethysmograph, and then again at room temperature exactly 24 h afterwards, allowing us to estimate T_b at $\dot{V}O_2max$ for use in tidal volume calculations (see below) by assuming that T_b dropped linearly throughout the trial. All data were acquired and recorded using a PowerLab 8/32 and LabChart 8 Pro Software (ADInstruments, Colorado Springs, CO, USA), with the exception of pulse oximetry data, which were obtained using Starr Life Sciences acquisition hardware and software.

Breathing, heart rate, and arterial O_2 saturation were recorded at thermogenic $\dot{V}O_2max$ for each trial. O_2 consumption rate ($\dot{V}O_2$) was calculated from gas concentration and flow measurements using established equations (Lighton, 2018). $\dot{V}O_2max$ was defined as the maximal $\dot{V}O_2$ measured over a 10-s period during the 10-min trial. $\dot{V}O_2$ usually increased to $\dot{V}O_2max$ within 5-6 min of entering the chamber and would then decline to less than 90% $\dot{V}O_2max$, and all mice had depressed T_b by the end of each 10-min cold exposure. Tidal volume was calculated (in volumes at BTPS) using established equations for the barometric

method in flow-through conditions (Drorbaug and Fenn, 1955; Jacky, 1980). Total ventilation was calculated as the product of tidal volume and breathing frequency.

2.3.4 Haematology

Haematology was measured both before and after hypoxia acclimation. Blood samples were taken from the facial vein 3 days after $\dot{V}O_2max$ measurements. We measured Hb content using Drabkin's reagent (according to instructions from the manufacturer, Sigma-Aldrich) and haematocrit by spinning the blood in capillary tubes at 12,700 *g* for 5 min. The O_2 affinity of intact erythrocytes was measured using 10 µl blood in 5 ml buffer containing 0.1 M Hepes, 0.05 M EDTA, 0.1 M NaCl, 0.1% bovine serum albumin, and 0.2% antifoaming agent at pH 7.4. Oxygen dissociation curves were generated at 37°C using a Hemox Analyzer (TCS Scientific), and red blood cell P_{50} and Hill coefficient (n) were calculated using Hemox Analytic Software.

2.3.5 Statistics

I used linear mixed effects models to test for the effects of Hb genotype and acclimation condition using the lme4 (Bates et al., 2015) package in R (v.3.1.3, R Core Team, 2013). I carried out one set of models to examine the fixed effects of Hb genotype in normoxia-acclimated mice, in absence of the effects of hypoxia acclimation, and with inspired PO_2 as an additional fixed effect. I then carried out a second set of models including data from both before and after chronic hypoxia exposure to examine the effects of Hb genotype, hypoxia acclimation, and their interaction. I used a backwards model selection approach,

in which initial models included sex, family, and individual subject as random factors, as well as body mass as a covariate. If these terms had P values above 0.1, they were removed by stepwise backward deletion (starting with the term with the highest P value) and the model was re-run until all terms in the model (with the exception of fixed factors and individual subject) had P values below 0.1. Family was thus included in only 6 of the models (see Supplemental Figures and Tables, Tables S2.2-S2.5), while the effects of sex were never significant and were removed from all models. Tukey's HSD *post hoc* tests were performed to test for pairwise differences between genotypes within an acclimation/$P\mathrm{O}_2$ treatment, and between acclimation/$P\mathrm{O}_2$ treatment groups within each genotype. Data are presented as individual values and as mean ± SEM, unless otherwise stated.

2.3.6 Modelling the O_2 transport pathway

Mathematical modelling of the O_2 transport pathway of deer mice was used to determine the interactive effects of blood-O_2 affinity and tissue O_2 diffusing capacity on $\dot{V}\mathrm{O}_2\mathrm{max}$ in hypoxia. This was done using established equations that have been used previously to build similar models (Scott and Milsom, 2006; Wagner, 1993; Wagner, 1996a; Wagner, 1996b; Wagner, 1997; Wang and Hicks, 2002). The Fick equation describes the diffusion of oxygen from the alveoli into the blood along capillaries in the lung:

$$\frac{d[O_2]_L}{dt} = \frac{D_L O_2}{t_L \dot{Q}} . (P_A O_2 - P_L O_2) \qquad \text{(Equation 1)}$$

where $[O_2]_L$ and t_L are the instantaneous O_2 content and transit time of the lung capillaries, D_LO_2 is the physiological O_2 diffusing capacity of the lungs, $\dot{Q}$ is cardiac output, and P_AO_2 and P_LO_2 are the PO_2 in the alveoli and instantaneously along lung capillaries, respectively. P_LO_2 began at mixed venous PO_2 (P_vO_2) and the equation was then integrated over the length on the lung capillaries to determine arterial PO_2 (P_aO_2) using the Hill equation (Equation 2) to relate $[O_2]$ and PO_2 in the blood (dependent on blood O_2 affinity and haemoglobin content):

$$[O_2] = 4[Hb].\frac{{PO_2}^n}{{PO_2}^n + {P_{50}}^n} \quad \text{(Equation 2)}$$

where [Hb] is the haemoglobin content of the blood, P_{50} is the PO_2 at which blood is 50% saturated with O_2, and n is the Hill coefficient that describes the cooperativity of blood-O_2 binding. The Fick equation also describes O_2 diffusion from the blood in tissue capillaries to the mitochondria:

$$\frac{d[O_2]_T}{dt} = \frac{-D_TO_2}{t_T\dot{Q}}.(P_TO_2 - P_MO_2) \quad \text{(Equation 3)}$$

where $[O_2]_T$ and t_T are the instantaneous O_2 content and transit time of the tissue capillaries, D_TO_2 is the O_2 diffusing capacity of the tissues, and P_TO_2 and P_MO_2 are the PO_2 instantaneously along the tissue capillaries and at mitochondria, respectively. P_MO_2 was set to zero to facilitate modelling, but mitochondrial PO_2 is likely quite low and relatively close to zero at $\dot{V}O_2$max (Wagner, 1996b). In this case, P_TO_2 begins at P_aO_2 and the equation is integrated along the tissue capillaries to determine P_vO_2. Mass conservation then matches $\dot{V}O_2$max measured from O_2 extraction at the lungs to that at tissues:

$$\dot{V}_A.(F_IO_2 - F_AO_2) = \dot{Q}.([O_2]_a - [O_2]_v) \quad \text{(Equation 4)}$$

where $\dot{V}_A$ is alveolar ventilation, F_IO_2 and F_AO_2 and the O_2 fractions of inspired and alveolar gas, respectively, and $[O_2]_a$ and $[O_2]_v$ are arterial and venous oxygen content, respectively.

The above equations were solved using an iterative approach for the key unknown outcome variables, P_AO_2, P_aO_2, and P_vO_2, from which $\dot{V}O_2max$ was calculated. This was achieved using a F_IO_2 of 0.123, my empirical measurements of total ventilation at hypoxic $\dot{V}O_2max$, red blood cell P_{50} and n, and [Hb] in normoxia acclimated $\alpha^{LL}\beta^{LH}$ mice, a body mass specific lung dead space volume of 6.4 μl g^{-1} (Fallica et al., 2011) to calculate alveolar ventilation from total ventilation, and cardiac output at hypoxic $\dot{V}O_2max$ from our previous measurements in low-altitude deer mice (Tate et al., 2020). Values for D_LO_2 and D_TO_2 were chosen by trial and error to re-produce *in vivo* measurements of P_aO_2, P_vO_2, and $\dot{V}O_2max$. All of the initial values used to solve the model are listed in Supplemental Figures and Tables, Table S2.1. The model was solved iteratively as follows. Using previously recorded *in vivo* P_vO_2 (Tate et al., 2020) and an initial estimate of P_AO_2 as a starting point, Equation 1 was integrated to calculate a predicted value of P_aO_2. This P_aO_2 value was then used to calculate P_vO_2 by integrating Equation 3. The two above steps were repeated until the P_aO_2 and P_vO_2 values became stable to within 0.05% (< 10 iterations). $\dot{V}O_2max$ was then calculated using both the left and right sides of Equation 4. If the values did not agree to within 0.05%, P_AO_2 was adjusted and the above steps were repeated until the left and right sides of Equation 4 were equal to within 0.05%. Reaching a stable solution of the model generally took less than 10 iterations, and the final outcome was independent of the starting estimate of P_vO_2.

I conducted a sensitivity analysis of the effects of increasing D_TO_2 on $\dot{V}O_2max$ using the mean for the most ancestral 'lowland' P_{50} that was measured in $\alpha^{LL}\beta^{LH}$ mice, and then again using the mean for the 'highland' P_{50} that was measured in $\alpha^{HH}\beta^{HH}$ mice, with all other parameters in the model kept constant (including the potential effects of variation in blood pH on P_{50}), with the exception of P_AO_2, P_aO_2, and P_vO_2 (which were under the influence of the changes in D_TO_2 and P_{50}). I also used the mean + SEM and mean - SEM values of P_{50} for each genotype in order to examine the influence of biological variation in P_{50} within each genotype. $\dot{V}O_2max$ and D_TO_2 values are expressed here relative to the initial solution generated using the data here from $\alpha^{LL}\beta^{LH}$ mice or from previous measurements in lowland deer mice (Supplemental Figures and Tables, Table S2.1), which I have termed the 'ancestral' values. The calculations were carried out using spreadsheet software (Microsoft Excel), as in some previous models of the O_2 transport pathway (Wang and Hicks, 2002).

2.4 RESULTS

2.4.1 Overview

I measured thermogenic $\dot{V}O_2max$, arterial O_2 saturation, and other cardiorespiratory traits *in vivo* during acute exposure to cold heliox in normoxia (21% O_2) and hypoxia (12% O_2) in male and female F_2 hybrid mice (mean ± SEM of body mass before hypoxia acclimation, 23.8 ± 0.9 g; see Supplemental Figures and Tables, Fig. S2.2 for body masses for each genotype before and after hypoxia acclimation) that possessed a diverse array of different α- and β-globin genotypes. We also performed *in vitro* measurements of red blood

cell P_{50} using erythrocyte suspensions from the same set of mice. The F_2 hybrids were generated by crossing wild mice from populations at high and low altitudes to produce F_1 inter-population hybrids, followed by full-sibling matings to create 4 families of F_2 hybrid progeny with admixed genetic backgrounds. Measurements of physiological phenotypes were made before and after a 6-week acclimation period to hypobaric hypoxia (12 kPa O_2, simulating ~4,300 m above sea level). In general, hypoxia acclimation was associated with increased $\dot{V}O_2$max in hypoxia, along with increases in pulmonary ventilation, arterial O_2 saturation, heart rate, haematocrit (Hct) and blood Hb concentration ([Hb]), but also increases in red blood cell P_{50} (Fig. 2.1; Supplemental Figures and Tables, Tables S2.2 and S2.3). However, hypoxia acclimation did not affect $\dot{V}O_2$max under normoxic conditions. Below, I describe the effects of Hb genotype on thermogenic $\dot{V}O_2$max and haematological traits in mice acclimated to normoxia, and then I describe how Hb genotype affects acclimation responses to chronic hypoxia.

2.4.2 Genetically based decreases in red blood cell P_{50} improved arterial O_2 saturation in hypoxia

In normoxia-acclimated mice, there was a significant main effect of Hb genotype on red blood cell P_{50} ($P = 0.0048$; Fig. 2.2A; Supplemental Figures and Tables, Table S2.4), which appeared to be largely attributable to the effects of α-globin variants. Mice possessing highland α-globin variants had a lower red blood cell P_{50} compared to those possessing lowland variants, reflecting a higher affinity for O_2. In contrast, Hb genotype did not affect Hct ($P = 0.8339$), [Hb] ($P = 0.9351$), or the Hill coefficient (n) that quantifies

the cooperativity of Hb-O_2 binding (P = 0.8053; Supplemental Figures and Tables, Fig. S2.3 and Table S2.4).

Arterial O_2 saturation varied in association with red blood cell P_{50} in hypoxia, but not in normoxia. There were significant main effects of both Hb genotype (P = 0.0189) and inspired PO_2 (P < 0.0001) on arterial O_2 saturation at $\dot{V}O_2$max, with mice exhibiting reduced saturation in hypoxia (Fig. 2.2B; Supplemental Figures and Tables, Table S2.4). However, the effect of inspired PO_2 on arterial O_2 saturation was influenced by genotype (genotype x PO_2 interaction, P = 0.0389), as mice with the highland α-globin genotype exhibited a smaller reduction in arterial O_2 saturation under hypoxia compared to those with the lowland genotype. Consequently, mice with highland α-globin maintained 9-14% higher arterial O_2 saturation on average than those with lowland α-globin at hypoxic $\dot{V}O_2$max. Higher red blood cell O_2 affinity was associated with higher arterial O_2 saturation in hypoxia, as indicated by a significant negative relationship between arterial O_2 saturation and red blood cell P_{50} (P = 0.0103, R^2 = 0.2441; Fig. 2.2C).

2.4.3 Genetically based variation in red blood cell P_{50} and arterial O_2 saturation had no effect on thermogenic $\dot{V}O_2$max in hypoxia

$\dot{V}O_2$max was significantly reduced in hypoxia compared to normoxia by ~24% on average (P < 0.0001; Fig. 2.3A; Supplemental Figures and Tables, Table S2.4). However, although Hb genotype had a significant main effect on $\dot{V}O_2$max (P = 0.0416), $\dot{V}O_2$max in hypoxia did not follow the pattern of variation seen for arterial O_2 saturation. As such, hypoxic $\dot{V}O_2$max was not correlated with arterial O_2 saturation in hypoxia (Fig. 2.3B). Instead, the

observed variation in $\dot{V}O_2max$ appeared to be associated with variation in heart rate, which was also significantly affected by inspired PO_2 ($P < 0.0001$), though the effect of genotype was only marginally significant ($P = 0.0545$; Supplemental Figures and Tables, Fig. S2.4A and Table S2.4). Total ventilation, tidal volume, and breathing frequency were unaffected by Hb genotype (Supplemental Figures and Tables, Fig. S2.4 and Table S2.4).

2.4.4 Hb genotype influenced the acclimation responses of red blood cell P_{50} and arterial O_2 saturation to chronic hypoxia

There were main effects of hypoxia acclimation that tended to increase both red blood cell P_{50} ($P = 0.0002$) and arterial O_2 saturation measured at $\dot{V}O_2max$ in hypoxia ($P = 0.0005$), but the acclimation response appeared to differ between genotypes (Fig. 2.4; Supplemental Figures and Tables, Table S2.3). Mice with the lowland α-globin variant exhibited no plasticity in red blood cell P_{50} in response to hypoxia acclimation, whereas mice with highland α-globin increased red blood cell P_{50} to values that were comparable to mice with lowland α-globin. Conversely, mice with lowland α-globin showed much greater plasticity in arterial O_2 saturation in hypoxia following hypoxia acclimation, with all individuals increasing saturation (on average by ~13% saturation units). Mice with highland α-globin showed little to no change in saturation after hypoxia acclimation. Hypoxia acclimation increased Hct ($P < 0.0001$) and [Hb] ($P < 0.0001$; Fig. 2.1), but neither these traits nor the Hill coefficient were influenced by Hb genotype (Supplemental Figures and Tables, Fig. S2.3 and Table S2.3).

$\dot{V}O_2$max in hypoxia increased after hypoxia acclimation (P = 0.0013), but this response was not influenced by Hb genotype (P = 0.1764; Supplemental Figures and Tables, Fig. S2.5 and Table S2.3). The magnitude of change in hypoxic $\dot{V}O_2$max following hypoxia acclimation was not associated with the magnitude of change in arterial O_2 saturation (Fig. 2.4C). Hypoxia acclimation also increased heart rate (P = 0.0031), total ventilation, (P < 0.0001), tidal volume (P = 0.0005), and breathing frequency (P < 0.0001) measured at $\dot{V}O_2$max in hypoxia, but none of these traits were affected by Hb genotype (Supplemental Figures and Tables, Fig. S2.6 and Table S2.3). Normoxic $\dot{V}O_2$max was not affected by hypoxia acclimation or Hb genotype, nor were the measurements of heart rate, total ventilation, tidal volume, or breathing frequency at normoxic $\dot{V}O_2$max affected by Hb genotype (Supplemental Figures and Tables, Fig. S2.7 and Table S2.5). However, there was a main effect of genotype on arterial O_2 saturation measured at $\dot{V}O_2$max in normoxia (P = 0.0291) that appeared to result from slightly lower saturation values in mice with characteristic lowland α- and β-globin genotypes ($\alpha^{LL}\beta^{LH}$; Supplemental Figures and Tables, Fig. S2.7B and Table S2.5).

2.4.5 Sensitivity analysis suggested that effects of Hb-O_2 affinity on $\dot{V}O_2$max in hypoxia are contingent on tissue O_2 diffusing capacity (D_TO_2)

I examined the interactive effects of Hb-O_2 affinity and D_TO_2 on $\dot{V}O_2$max in hypoxia using a mathematical model of O_2 flux through the O_2 transport pathway. I generated the initial solutions of the model using empirical data collected for deer mice, and then performed a sensitivity analysis to determine the effects of increasing D_TO_2 on $\dot{V}O_2$max at

each of the red blood cell P_{50} values for mice with characteristic highland ($\alpha^{HH}\beta^{HH}$) and lowland ($\alpha^{LL}\beta^{LH}$) Hb genotypes. Increasing D_TO_2 by 50% increased $\dot{V}O_2max$, but the effect was greater with the P_{50} of the high-affinity $\alpha^{HH}\beta^{HH}$ genotype (11.8%) than with the lower affinity $\alpha^{LL}\beta^{LH}$ genotype (8.5%; Fig. 2.5A). The effect of P_{50} was accentuated when D_TO_2 was increased above 41%, when venous PO_2 (and thus venous O_2 saturation) fell to zero at the higher P_{50} (Fig. 2.5B). These results indicate that an increase in Hb-O_2 affinity only contributes to an enhancement of $\dot{V}O_2max$ in hypoxia if it is paired with an increase in D_TO_2 in thermogenic tissues (*i.e.*, skeletal muscle and/or brown adipose tissue).

2.5 DISCUSSION

This study provides evidence that the adaptive benefit of increasing Hb-O_2 affinity is contingent on the capacity of active tissues to extract O_2 from the blood. In agreement with previous studies (Chappell et al., 1988; Chappell and Snyder, 1984; Jensen et al., 2016; Snyder et al., 1982; Snyder, 1981; Storz et al., 2010a; Storz et al., 2009), my data from F_2 inter-population hybrids demonstrate that Hb variants from high-altitude deer mice confer a higher Hb-O_2 affinity than Hb from lowland conspecifics, and that this evolved increase in affinity augments arterial O_2 saturation in hypoxia by 9-14%. However, these genetically based changes alone did not augment $\dot{V}O_2max$ (*i.e.*, aerobic performance) in hypoxia. Modelling of the O_2 transport pathway revealed that increases in Hb-O_2 affinity would only be expected to enhance $\dot{V}O_2max$ in hypoxia if O_2 diffusing capacity were increased to augment tissue O_2 extraction. Importantly, recent evidence suggests that high-altitude mice have evolved a highly aerobic skeletal muscle phenotype with an enhanced capacity for O_2

diffusion (Dawson et al., 2018; Lui et al., 2015; Mahalingam et al., 2017; Scott et al., 2015; Scott et al., 2018). In particular, the gastrocnemius muscle of highland deer mice has greater capillary density and a redistribution of mitochondria to a subsarcolemmal location that is closer to capillaries, each of which would increase O_2 diffusing capacity. My results therefore suggest that increases in both Hb-O_2 affinity and tissue O_2 diffusing capacity likely contributed to the adaptive increases in $\dot{V}O_2max$ in high-altitude deer mice. These findings suggest the testable hypothesis that other hypoxia-adapted, high-altitude vertebrates that have evolved derived increases in Hb-O_2 affinity will also have evolved increases in tissue capillarity and/or other changes that augment O_2 diffusing capacity.

The genetically based differences in Hb function led to predictable differences in arterial O_2 saturation during acute and chronic hypoxia. Amino acid variation in Hb genes is not always associated with changes in O_2-binding properties (Cheviron et al., 2014; Natarajan et al., 2015b), and even in cases where it has been possible to document causal effects of specific mutations on Hb function (Natarajan et al., 2015a; Natarajan et al., 2013; Poyart et al., 1992; Storz, 2007; Storz and Moriyama, 2008; Storz et al., 2012; Storz et al., 2010a; Storz et al., 2009; Storz et al., 2007; Weber, 2007; Weber and Fago, 2004; Winslow, 2007), the *in vivo* effects on blood oxygenation have rarely been examined. This study suggests that it is critically important to examine how genetic changes in proximal biochemical phenotypes affect higher-level physiological phenotypes (*e.g.*, arterial O_2 saturation and $\dot{V}O_2max$ in hypoxia) to fully understand their potential adaptive significance.

Genetic variation in Hb altered the acclimation response to chronic hypoxia, as highland α-globin genotypes were associated with increased plasticity in Hb-O_2 affinity of

red blood cells. This variation was likely a result of differences in sensitivity to 2,3-diphosphoglycerate (2,3-DPG), an allosteric modulator of Hb-O_2 affinity. Concentrations of 2,3-DPG in erythrocytes are known to increase in response in chronic hypoxia, which tends to reduce red blood cell Hb-O_2 affinity (Lenfant et al., 1968; Lenfant et al., 1971; Mairbaurl et al., 1993; Milles et al., 1987; Savourey et al., 2004). Previous studies have shown that Hb from high-altitude deer mice are more sensitive to 2,3-DPG in the presence of Cl^- than Hb from low-altitude mice (Storz et al., 2010a; Storz et al., 2009). Therefore, in the current study, if red blood cell concentrations of 2,3-DPG were comparable across genotypes, differences in 2,3-DPG sensitivity could explain the differences in plasticity of red blood cell Hb-O_2 affinity. This mechanism may also explain why genotypes differed in the magnitude of plasticity in arterial O_2 saturation in response to chronic hypoxia. Several physiological adjustments contribute to increasing arterial O_2 saturation after hypoxia acclimation, including increases in total ventilation (Fig. 2.1) and adjustments in lung function to augment pulmonary O_2 diffusion (Ivy and Scott, 2017a; Tate et al., 2020), and these effects could potentially be counteracted by reductions in red blood cell Hb-O_2 affinity. Such reductions in affinity did not occur in mice with lowland α-globin, such that they experienced greater improvements in arterial O_2 saturation after hypoxia acclimation.

My results indicate that the adaptive benefit of increasing Hb-O_2 affinity is contingent on the O_2 diffusing capacity of active tissues. This study provides empirical evidence that genetically based increases in Hb-O_2 affinity and arterial O_2 saturation alone are not sufficient to improve aerobic capacity in hypoxia. I also demonstrate that the adaptive benefit of increasing Hb-O_2 affinity is contingent on having a tissue O_2 conductance (D_TO_2)

that is sufficiently high to take advantage of the greater arterial O_2 saturation and extract more O_2 from the blood. The relationship between Hb-O_2 affinity and $\dot{V}O_2max$ in hypoxia is a contentious topic (Dempsey, 2020; Storz, 2016; Winslow, 2007), with different empirical studies and theoretical models providing contradictory results (Brauner and Wang, 1997; Chappell and Snyder, 1984; Dominelli et al., 2020; Wagner, 1997; Wang and Malte, 2011; Woodson and Auerbach, 1982). In fact, previous investigation in deer mice has shown that mice possessing highland α-globin alleles with higher Hb-O_2 affinity did have higher $\dot{V}O_2max$ in hypoxia than mice with lowland α-globin haplotypes (Chappell and Snyder, 1984). However, in this previous study (in which genotyping was based on protein electrophoresis), different α-globin alleles were backcrossed into a highland genetic background (Chappell and Snyder, 1984), unlike the current study in which alternative allelic variants were randomized against an admixed highland/lowland background. As discussed above, highland deer mice appear to have evolved a higher capacity for O_2 diffusion and utilization in skeletal muscles than their lowland conspecifics, comparable to some differences between high-altitude and low-altitude human populations (Gilbert-Kawai et al., 2017). It is therefore possible that the highland mice used in this previous study (Chappell and Snyder, 1984) had a higher D_TO_2 than the F_2 inter-population hybrids used in this study, which would explain the observed differences in the relative influence of Hb genotype on $\dot{V}O_2max$. Indeed, my modelling shows that the adaptive benefits of increasing Hb-O_2 affinity are critically dependent on D_TO_2. Together, my findings suggest adaptive increases in $\dot{V}O_2max$ in high-altitude deer mice may have been facilitated by

evolved increases in D_TO_2, which were required in order for increases in Hb-O_2 affinity to confer an adaptive benefit at high-altitude.

2.6 CONCLUSIONS

Complex organismal traits are often the result of multiple interacting genes and phenotypes, but the role of these interactions in shaping adaptive traits is poorly understood. My findings demonstrate that adaptive increases in thermogenic capacity result from a functional interaction between blood haemoglobin and active tissues, in which the adaptive benefit of increasing haemoglobin O_2 affinity is contingent on the capacity for O_2 diffusion from the blood. This helps reconcile controversy about the general role of haemoglobin in hypoxia tolerance, and provides insight into physiological mechanisms of high-altitude adaptation.

2.7 FIGURES AND TABLES

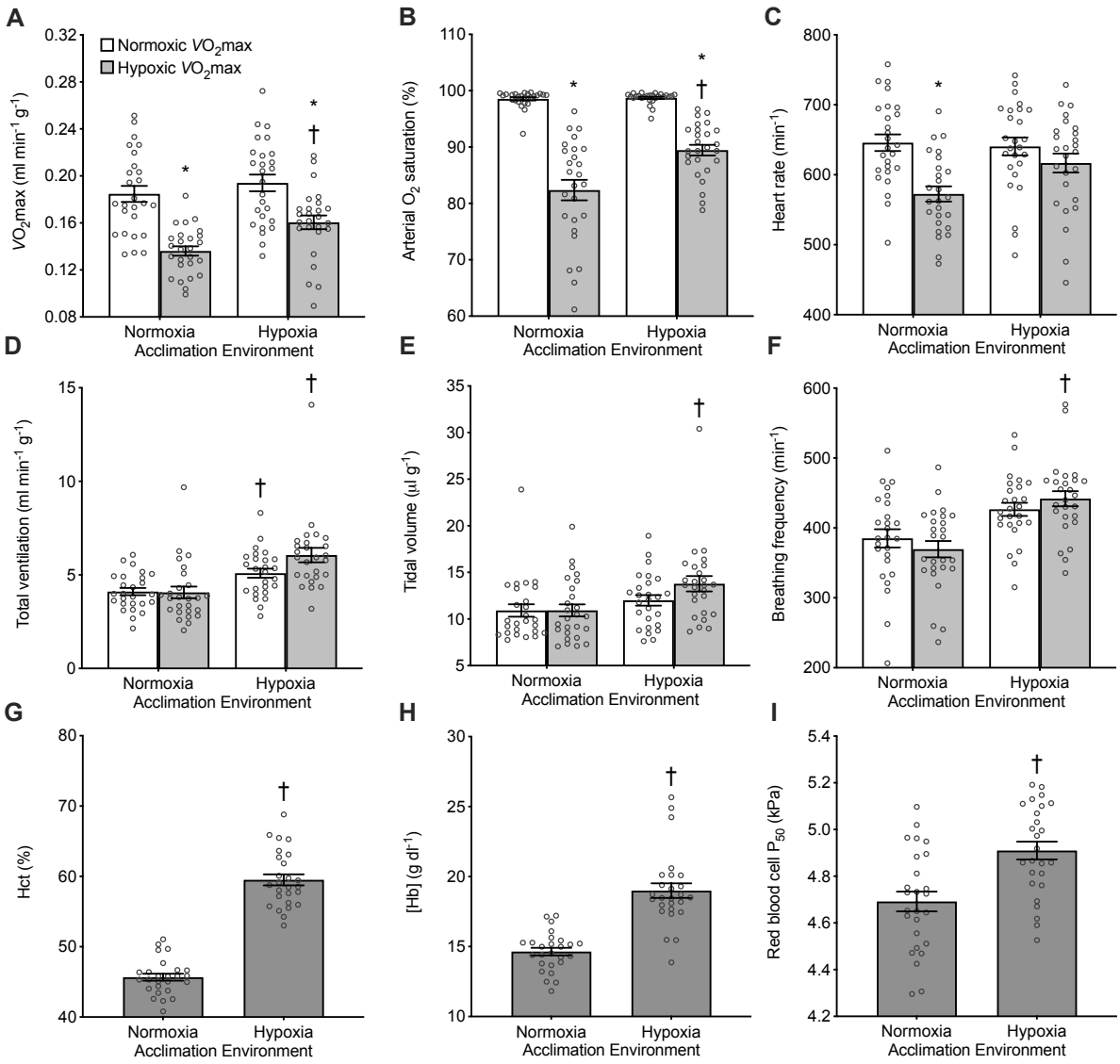

Fig. 2.1 Acclimation responses to chronic hypoxia in all F_2 inter-population hybrid deer mice of all genotypes for measurements at $\dot{V}O_2max$ in normoxia (21 kPa O_2) and hypoxia (12 kPa O_2), and for measurements of haematology. Hct, haematocrit; [Hb], blood haemoglobin content; P_{50}, O_2 pressure at 50% saturation. *$P < 0.05$ between measurements in normoxia versus hypoxia within an acclimation condition. $^{\dagger}P < 0.05$ vs. pre-acclimation value. Bars display mean ± SEM (n = 26) with individual data superimposed (circles).

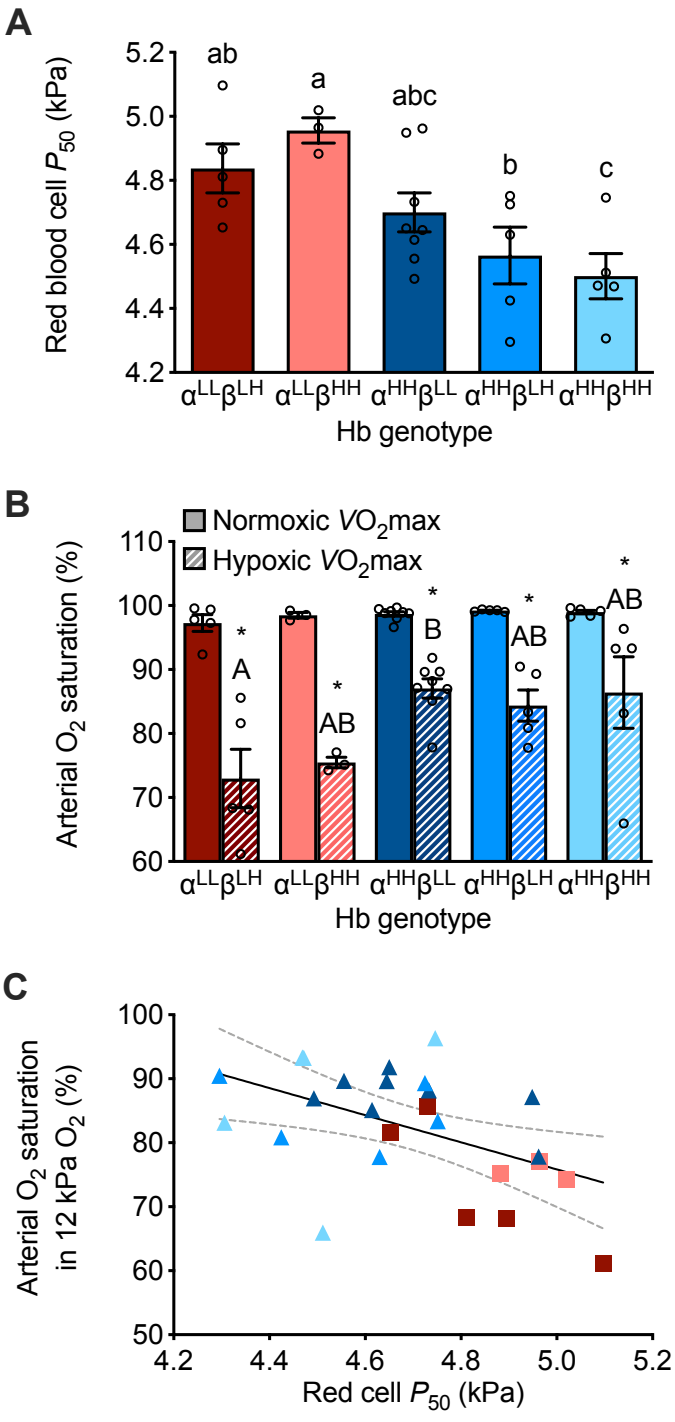
A
Red blood cell P_{50} (kPa)
5.2
5.0
4.8
4.6
4.4
4.2
ab
a
abc
b
c
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
B
Arterial O_2 saturation (%)
Normoxic VO_2max
Hypoxic VO_2max
110
100
90
80
70
60
*
A
*
AB
*
B
*
AB
*
AB
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
C
Arterial O_2 saturation in 12 kPa O_2 (%)
100
90
80
70
60
50
4.2
4.4
4.6
4.8
5.0
5.2
Red cell P_{50} (kPa)

Fig. 2.2 Variation in red blood cell O_2 affinity and arterial O_2 saturation associated with haemoglobin (Hb) genotype in F_2 inter-population hybrid deer mice acclimated to normoxia. **A**) Red blood cell P_{50} (O_2 pressure at 50% saturation). **B**) Arterial O_2 saturation at $\dot{V}O_2max$ measured in normoxia (21 kPa O_2) and hypoxia (12 kPa O_2). Bars display mean ± SEM (n = 3-8) with individual data superimposed (circles). Different α- and β- globin genotypes are shown as superscripts with '^{L}' representing the lowland haplotype and '^{H}' representing the highland haplotype. *P < 0.05, hypoxia vs. normoxia within a genotype. P < 0.05 between genotypes for values not sharing a letter. **C**) Linear regression of arterial O_2 saturation in hypoxia and red blood cell P_{50} for individual data (P = 0.0103, R^2 = 0.2441; dotted line represents 95% confidence interval). Symbol colors reflect Hb genotype, as shown in A and B.

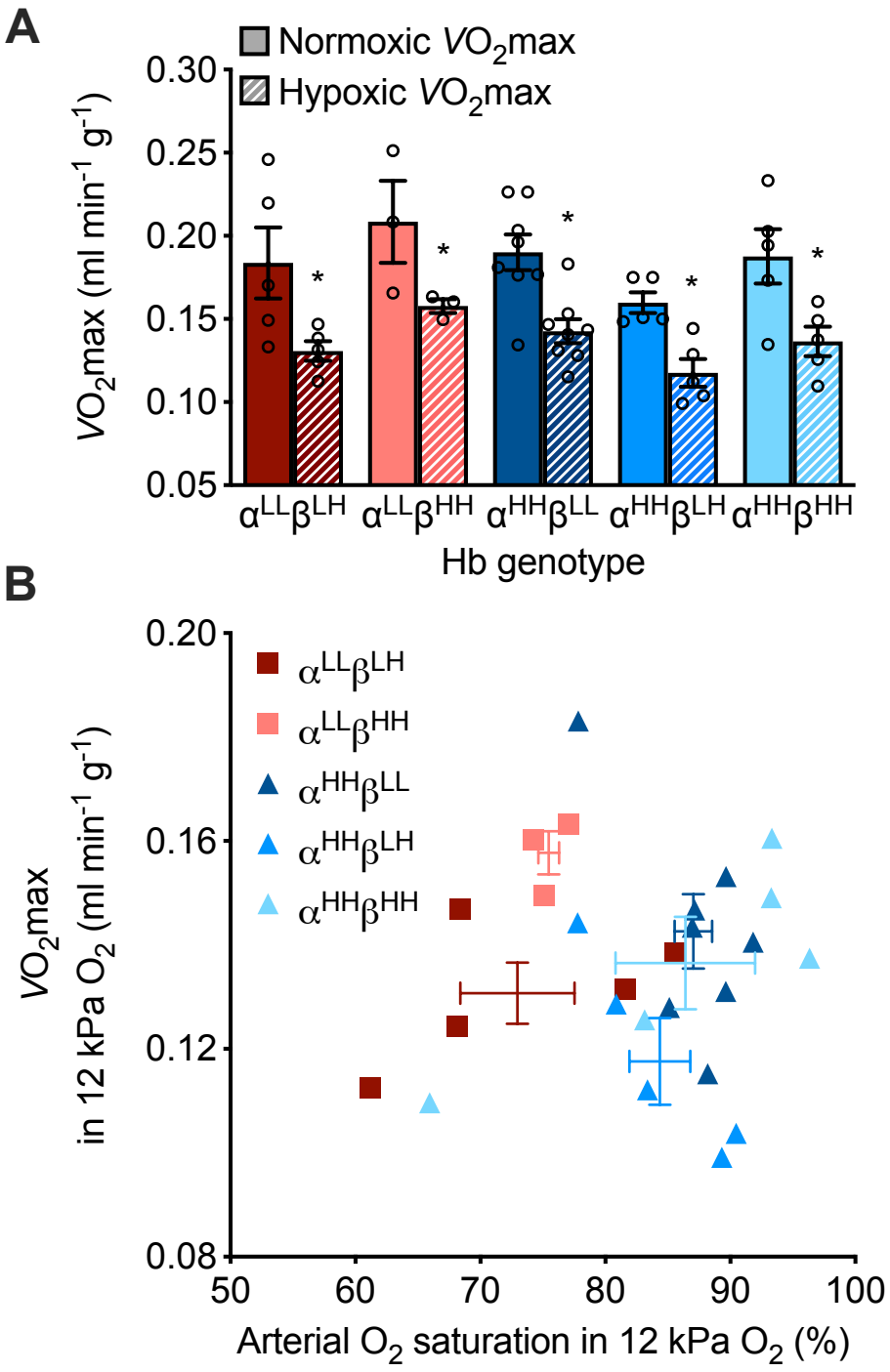
A
Normoxic $\dot{V}O_2$max
Hypoxic $\dot{V}O_2$max
$\dot{V}O_2$max (ml min^{-1} g^{-1})
0.30
0.25
0.20
0.15
0.10
0.05
*
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
B
$\alpha^{LL}\beta^{LH}$
$\alpha^{LL}\beta^{HH}$
$\alpha^{HH}\beta^{LL}$
$\alpha^{HH}\beta^{LH}$
$\alpha^{HH}\beta^{HH}$
$\dot{V}O_2$max in 12 kPa O_2 (ml min^{-1} g^{-1})
0.20
0.16
0.12
0.08
50
60
70
80
90
100
Arterial O_2 saturation in 12 kPa O_2 (%)

Fig. 2.3 Variation in $\dot{V}O_2max$ was unrelated to variation in arterial O_2 saturation in F_2 inter-population hybrid deer mice acclimated to normoxia. **A**) $\dot{V}O_2max$ measured in normoxia and hypoxia. See Fig. 2.2 for details on haemoglobin (Hb) genotypes and symbols. **B**) There was no correlation between hypoxic $\dot{V}O_2max$ and arterial O_2 saturation in hypoxia ($P = 0.8103$) across individuals (mean ± SEM for each genotype are shown as error bars).

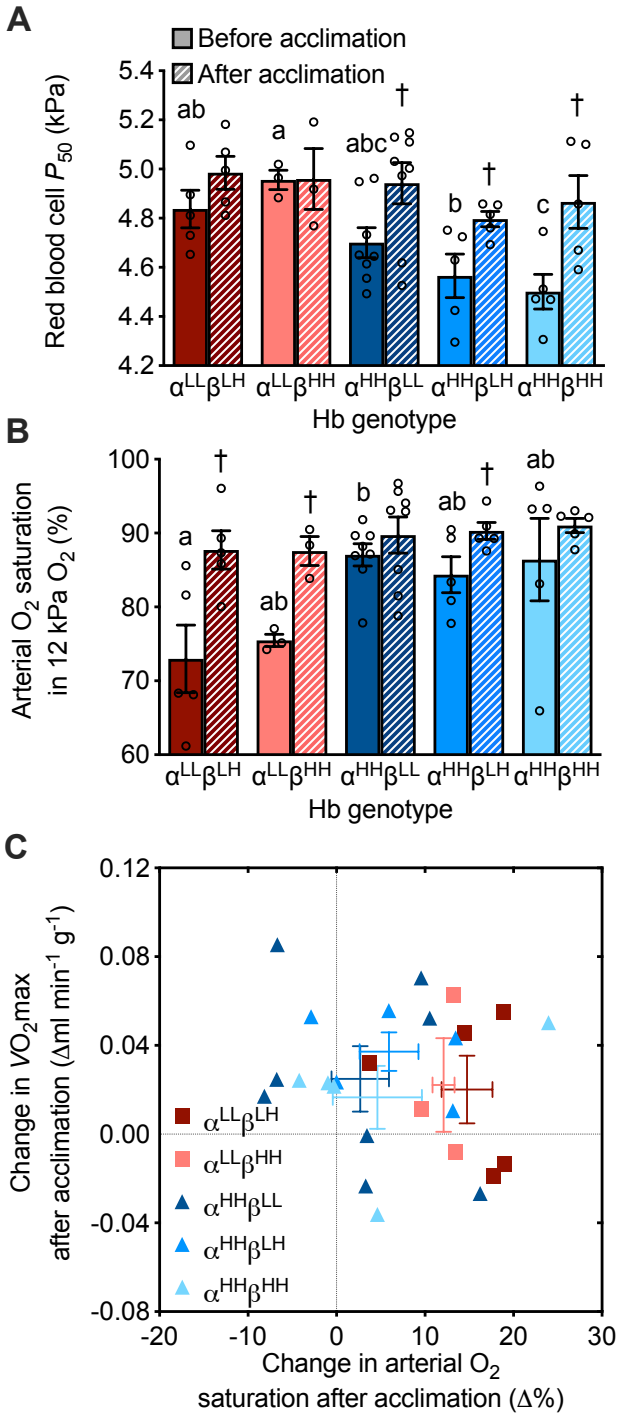
A
Before acclimation
After acclimation
Red blood cell P_{50} (kPa)
5.4
5.2
5.0
4.8
4.6
4.4
4.2
ab
a
abc
b
c
†
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
B
Arterial O_2 saturation in 12 kPa O_2 (%)
100
90
80
70
60
a
ab
b
ab
†
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
C
Change in VO_2max after acclimation (Δml min^{-1} g^{-1})
0.12
0.08
0.04
0.00
-0.04
-0.08
$\alpha^{LL}\beta^{LH}$
$\alpha^{LL}\beta^{HH}$
$\alpha^{HH}\beta^{LL}$
$\alpha^{HH}\beta^{LH}$
$\alpha^{HH}\beta^{HH}$
-20
-10
0
10
20
30
Change in arterial O_2 saturation after acclimation (Δ%)

Fig. 2.4 The effects of hypoxia acclimation on red blood cell O_2 affinity and arterial O_2 saturation differed between haemoglobin (Hb) genotypes in F_2 inter-population hybrid deer mice, but the effects of hypoxia acclimation on $\dot{V}O_2max$ did not. **A**) Red blood cell P_{50} (O_2 pressure at which Hb is 50% saturated) and **B**) arterial O_2 saturation at $\dot{V}O_2max$ in hypoxia, measured before and after a 6-wk acclimation to hypobaric hypoxia (12 kPa O_2). $^{\dagger}P < 0.05$ vs. pre-acclimation value within a genotype. $P < 0.05$ between genotypes within an acclimation condition for values not sharing a letter. **C**) The change in hypoxic $\dot{V}O_2max$ plotted against the change in arterial O_2 saturation in hypoxia in individuals in response to hypoxia acclimation (mean ± SEM for each genotype are shown as error bars). See Fig. 2.2 for other details on Hb genotypes.

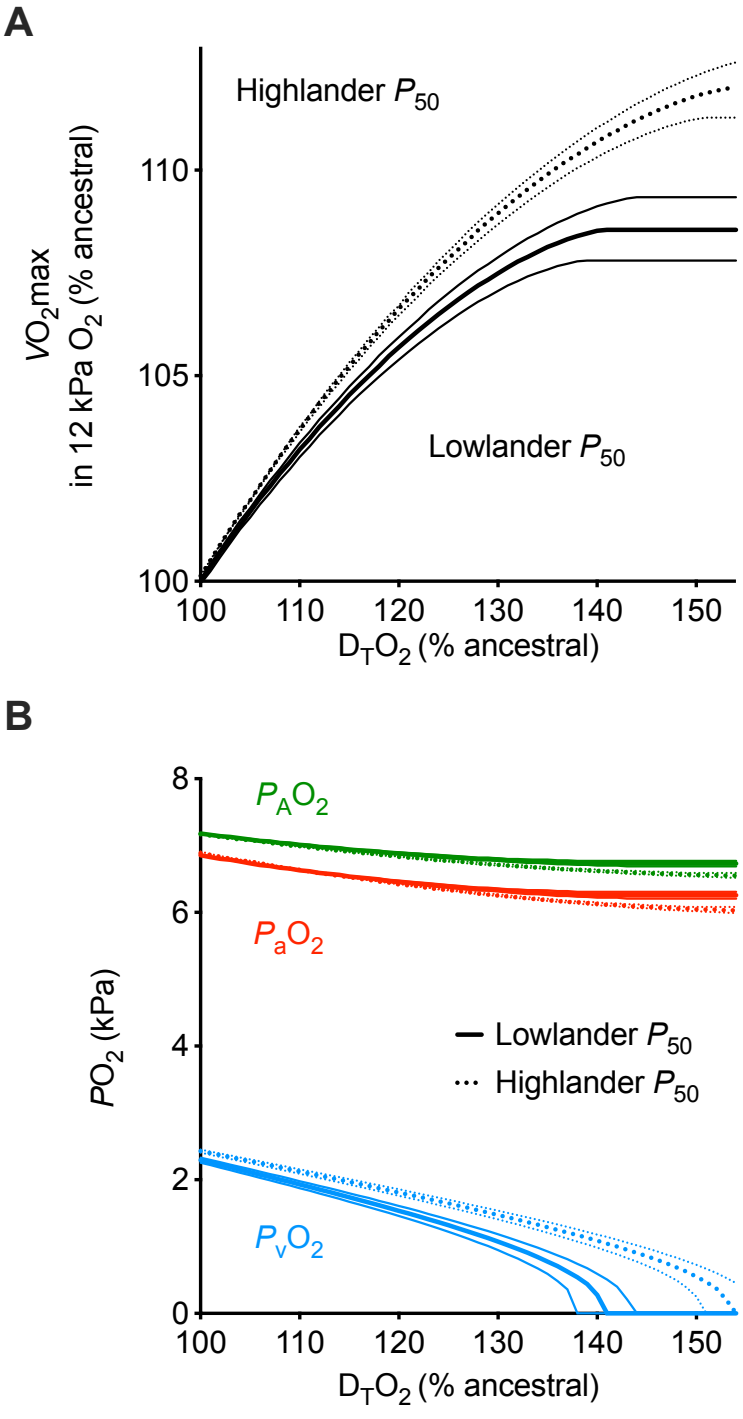
A
Highlander P_{50}
Lowlander P_{50}
$\dot{V}O_2$max in 12 kPa O_2 (% ancestral)
110
105
100
100
110
120
130
140
150
D_TO_2 (% ancestral)
B
P_AO_2
P_aO_2
P_vO_2
Lowlander P_{50}
Highlander P_{50}
PO_2 (kPa)
8
6
4
2
0
100
110
120
130
140
150
D_TO_2 (% ancestral)

Fig. 2.5 Effects of increasing tissue O_2 diffusing capacity (D_TO_2) on hypoxic $\dot{V}O_2max$ and O_2 partial pressures (PO_2) using mathematical modelling of the O_2 transport pathway. **A**) Relative changes in hypoxic $\dot{V}O_2max$ and **B**) changes in alveolar (P_AO_2), arterial (P_aO_2) and venous (P_vO_2) PO_2 in response to relative increases in D_TO_2. Effects were modeled using the mean (bold lines) ± SEM (fine lines) values of red blood cell P_{50} for mice with haemoglobin genotypes that were most characteristic of lowlanders ($\alpha^{LL}\beta^{LH}$) and highlanders ($\alpha^{HH}\beta^{HH}$).

2.8 SUPPLEMENTAL FIGURES AND TABLES

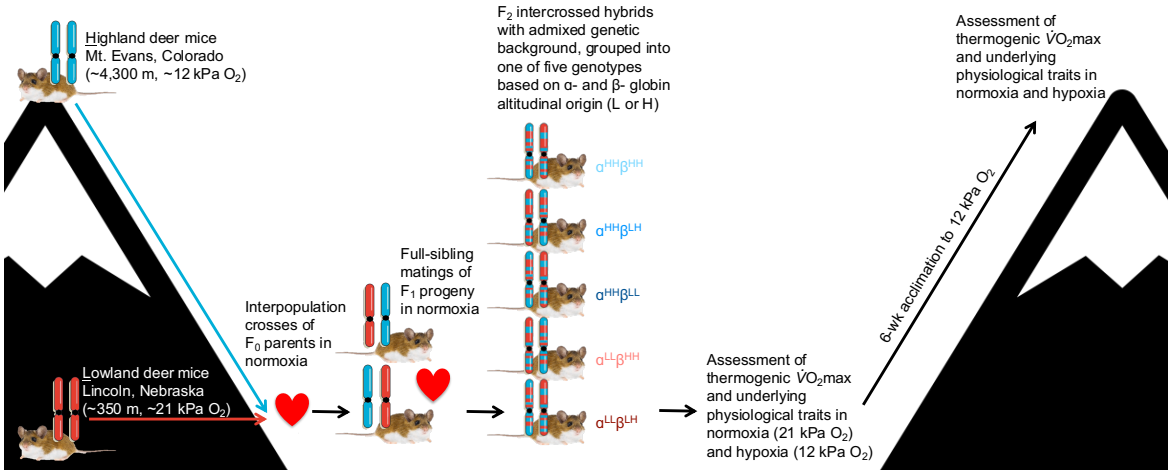

Fig. S2.1 Graphical overview of the experimental design of our study. Deer mice from high- (H) and low- (L) altitude populations were crossed in captivity to produce F_1 interpopulation hybrids that were then mated with siblings to produce the F_2 interpopulation hybrids that were used in our experiments before and after a 6-wk acclimation to hypobaric hypoxia (12 kPa O_2). These hybrids were grouped based on the altitudinal origin of their α- and β- globin genotype.

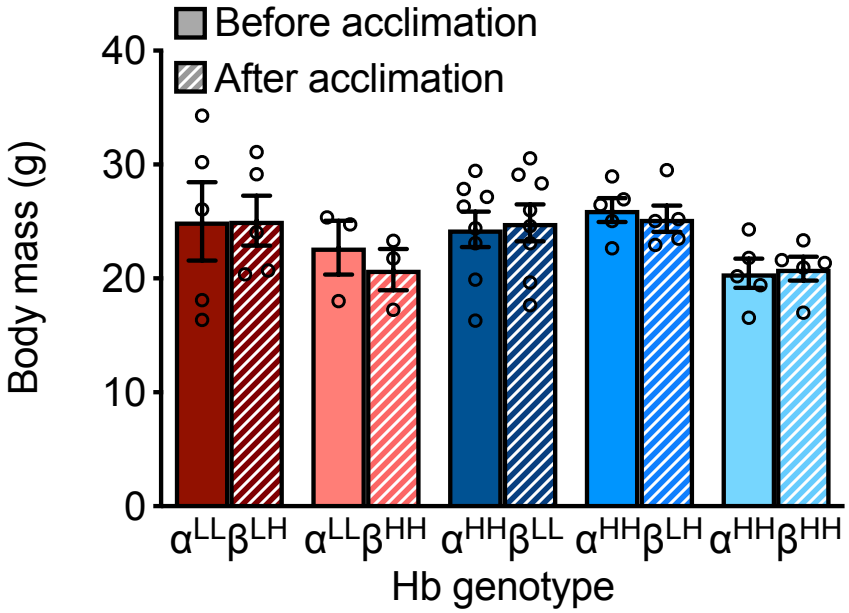
Before acclimation
After acclimation
Body mass (g)
40
30
20
10
0
αLLβLH
αLLβHH
αHHβLL
αHHβLH
αHHβHH
Hb genotype

Fig. S2.2 Body mass of F_2 inter-population hybrids both before and after a 6-wk acclimation to hypobaric hypoxia. Each individual's mass was measured before normoxic and hypoxic $\dot{V}O_2max$ trials, with the mean of these values used to create each individual's data point in the figure. Different α- and β- globin genotypes are shown as superscripts with $^{'L'}$ representing the lowland haplotype and $^{'H'}$ representing the highland haplotype. There was no effect of genotype (P = 0.2977), acclimation (P = 0.4018), or their interaction (P = 0.3362) on body mass. Bars display mean ± SEM (n = 3-8) with individual data superimposed (circles).

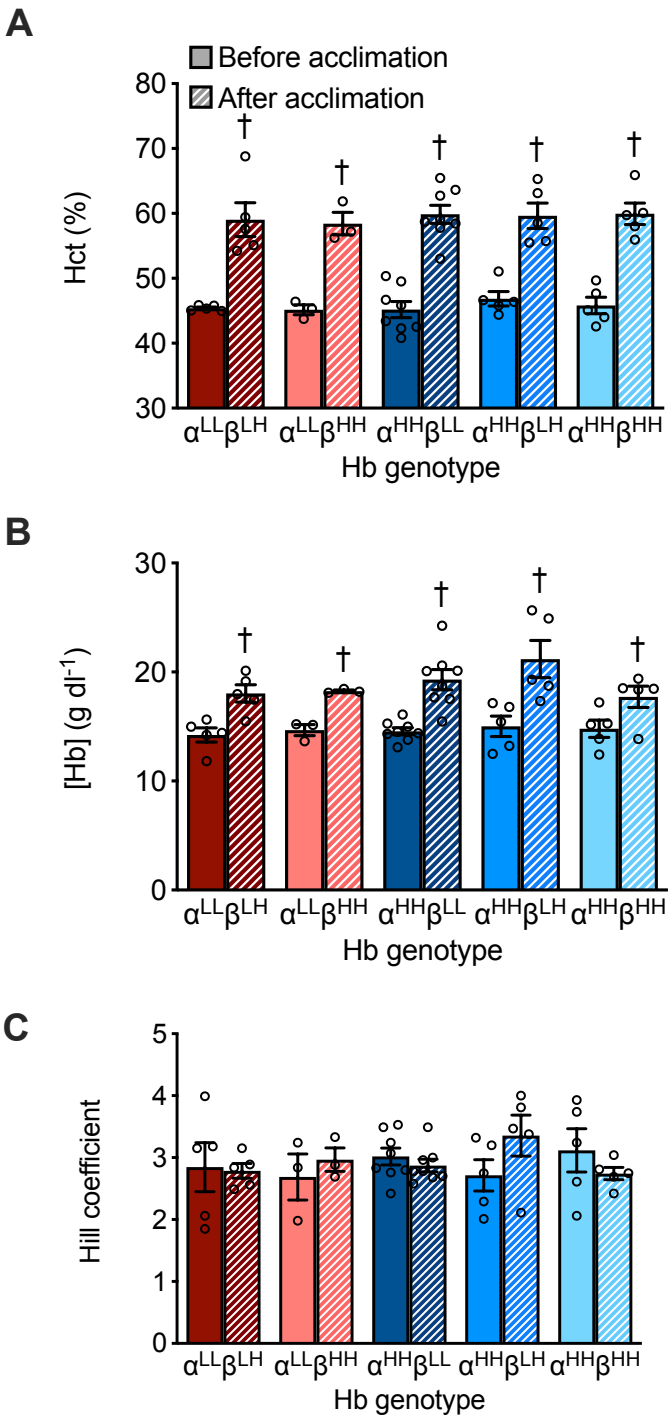
A
Before acclimation
After acclimation
Hct (%)
80
70
60
50
40
30
†
αLLβLH αLLβHH αHHβLL αHHβLH αHHβHH
Hb genotype
B
[Hb] (g dl-1)
30
20
10
0
†
αLLβLH αLLβHH αHHβLL αHHβLH αHHβHH
Hb genotype
C
Hill coefficient
5
4
3
2
1
0
αLLβLH αLLβHH αHHβLL αHHβLH αHHβHH
Hb genotype

Fig. S2.3 Haematology of F_2 inter-population hybrids measured before and after a 6-wk acclimation to hypobaric hypoxia (12 kPa O_2). Hct, haematocrit; [Hb], blood haemoglobin content. Different α- and β- globin genotypes are shown as superscripts with '^{L}' representing the lowland haplotype and '^{H}' representing the highland haplotype. $^{\dagger}P < 0.05$ vs. pre-acclimation value within a genotype. Bars display mean ± SEM (n = 3-8) with individual data superimposed (circles).

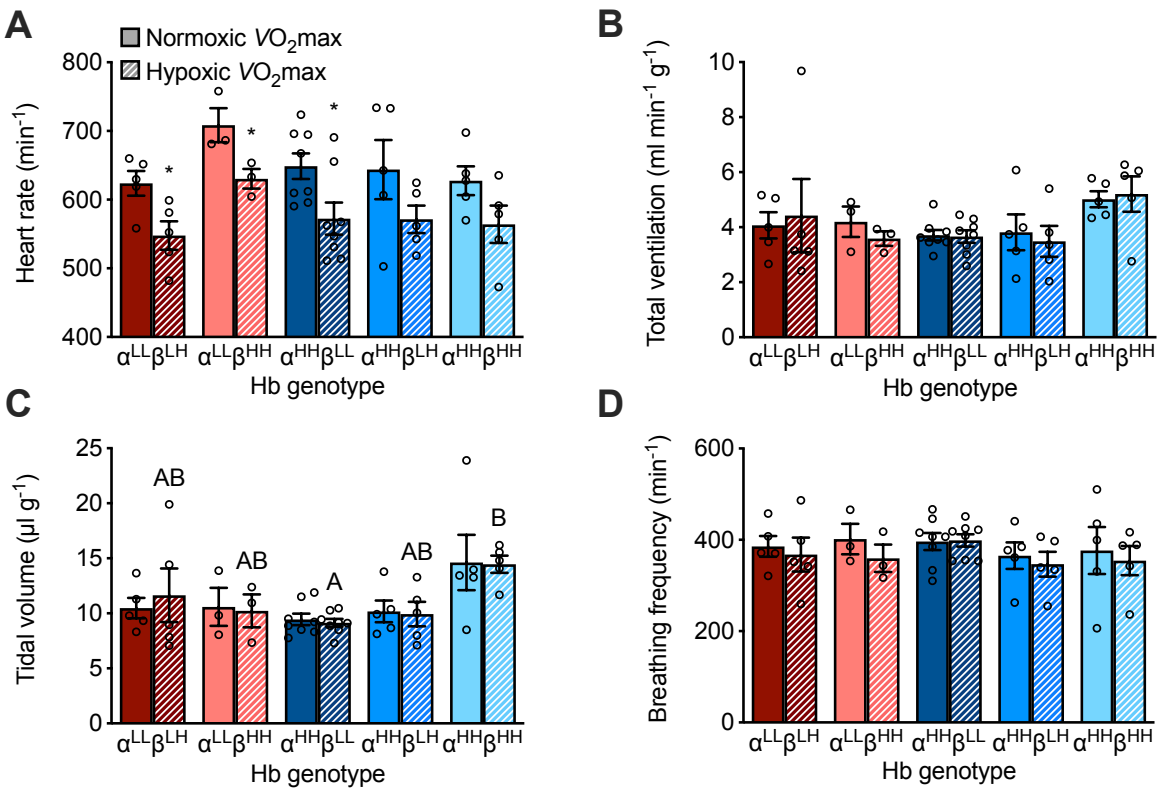
A
Normoxic $\dot{V}O_2max$
Hypoxic $\dot{V}O_2max$
Heart rate (min^{-1})
800
700
600
500
400
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
B
Total ventilation (ml min^{-1} g^{-1})
10
8
6
4
2
0
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
C
Tidal volume (µl g^{-1})
25
20
15
10
5
0
AB
AB
A
AB
B
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
D
Breathing frequency (min^{-1})
600
400
200
0
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype

Fig. S2.4 Physiological parameters for F_2 inter-population hybrids acclimated to normoxia, measured at $\dot{V}O_2max$ in normoxia (21 kPa O_2) and hypoxia (12 kPa O_2). Different α- and β- globin genotypes are shown as superscripts with '^{L}' representing the lowland haplotype and '^{H}' representing the highland haplotype. *P < 0.05 vs. normoxia value within a genotype. P < 0.05 between genotypes for hypoxic values not sharing a letter. Bars display mean ± SEM (n = 3-8) with individual data superimposed (circles).

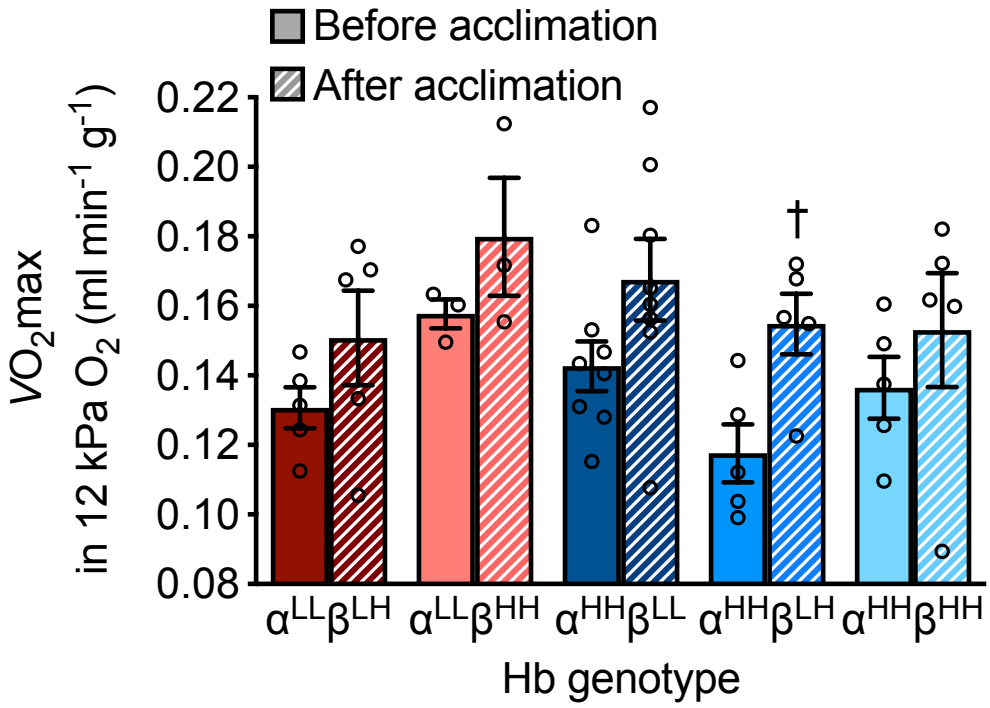
Before acclimation
After acclimation
$\dot{V}O_2max$ in 12 kPa O_2 (ml min^{-1} g^{-1})
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
†
$\alpha^{LL}\beta^{LH}$
$\alpha^{LL}\beta^{HH}$
$\alpha^{HH}\beta^{LL}$
$\alpha^{HH}\beta^{LH}$
$\alpha^{HH}\beta^{HH}$
Hb genotype

Fig. S2.5 Hypoxic $\dot{V}O_2max$ before and after a 6-wk acclimation to hypobaric hypoxia (12 kPa O_2). Different α- and β- globin genotypes are shown as superscripts with '^{L}' representing the lowland haplotype and '^{H}' representing the highland haplotype. $^{\dagger}P < 0.05$ vs. pre-acclimation value within a genotype. Bars display mean ± SEM (n = 3-8) with individual data superimposed (circles).

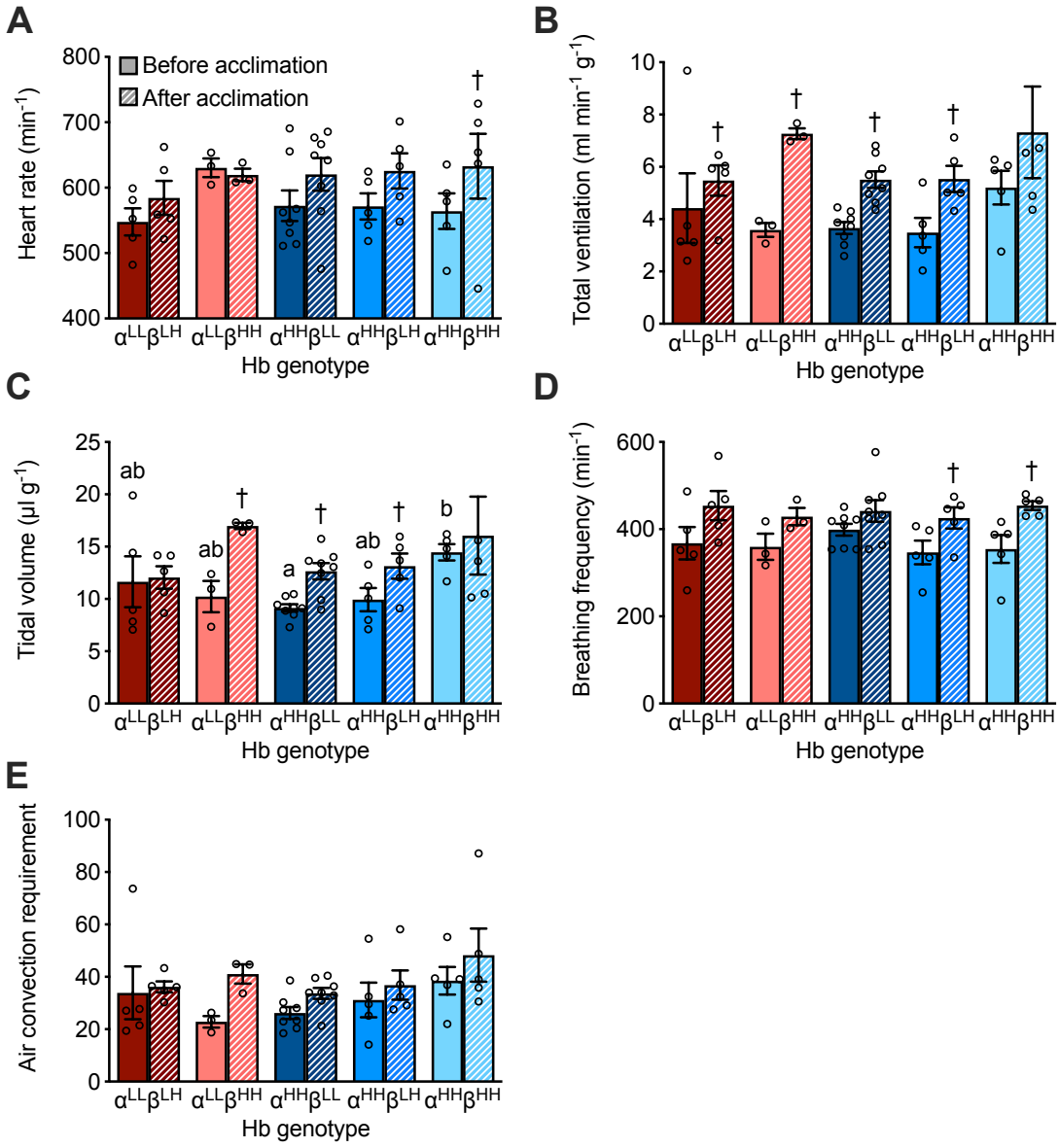
A
Heart rate (min-1)
Before acclimation
After acclimation
Hb genotype
B
Total ventilation (ml min-1 g-1)
Hb genotype
C
Tidal volume (µl g-1)
ab
ab
a
ab
b
Hb genotype
D
Breathing frequency (min-1)
Hb genotype
E
Air convection requirement
Hb genotype
αLLβLH
αLLβHH
αHHβLL
αHHβLH
αHHβHH

Fig. S2.6 Physiological parameters for F_2 inter-population hybrids measured at $\dot{V}O_2max$ in hypoxia (12 kPa O_2) both before and after a 6-wk acclimation to hypobaric hypoxia. Different α- and β- globin genotypes are shown as superscripts with '^{L}' representing the lowland haplotype and '^{H}' representing the highland haplotype. $^{\dagger}P < 0.05$ vs. pre-acclimation value within a genotype. $P < 0.05$ between genotypes within an acclimation condition for values not sharing a letter. Bars display mean ± SEM (n = 3-8) with individual data superimposed (circles).

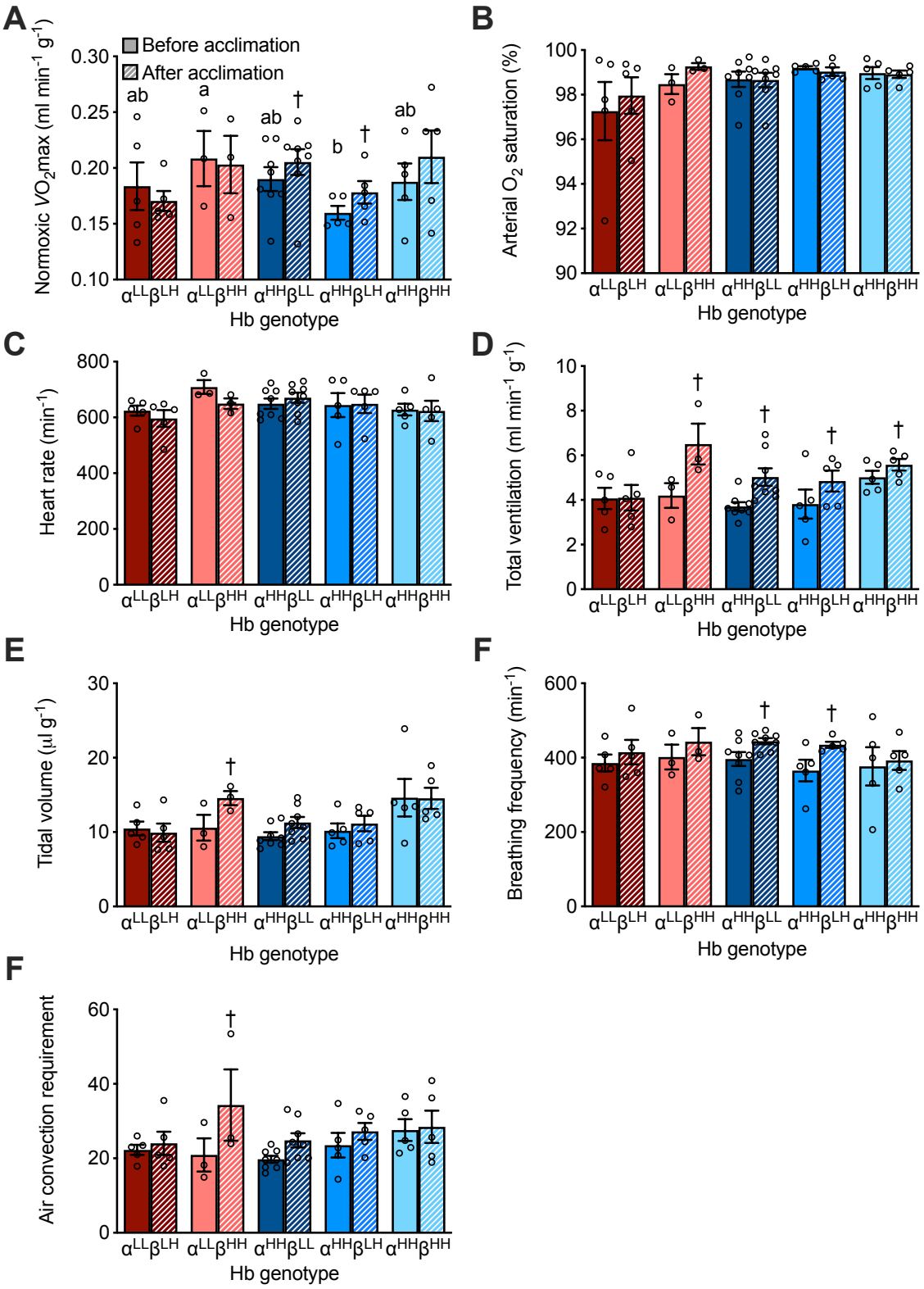
A
Before acclimation
After acclimation
Normoxic $\dot{V}O_2max$ (ml min^{-1} g^{-1})
0.30
0.25
0.20
0.15
0.10
ab
a
ab
†
b
†
ab
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
B
Arterial O_2 saturation (%)
100
98
96
94
92
90
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
C
Heart rate (min^{-1})
800
600
400
200
0
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
D
Total ventilation (ml min^{-1} g^{-1})
10
8
6
4
2
0
†
†
†
†
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
E
Tidal volume (µl g^{-1})
30
20
10
0
†
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
F
Breathing frequency (min^{-1})
600
400
200
0
†
†
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype
F
Air convection requirement
60
40
20
0
†
$\alpha^{LL}\beta^{LH}$ $\alpha^{LL}\beta^{HH}$ $\alpha^{HH}\beta^{LL}$ $\alpha^{HH}\beta^{LH}$ $\alpha^{HH}\beta^{HH}$
Hb genotype

Fig. S2.7 Physiological parameters for F_2 inter-population hybrids measured at $\dot{V}O_2max$ in normoxia (21 kPa O_2) both before and after a 6-wk acclimation to hypobaric hypoxia. Different α- and β- globin genotypes are shown as superscripts with '^{L}' representing the lowland haplotype and '^{H}' representing the highland haplotype. $^{\dagger}P < 0.05$ vs. pre-acclimation value within a genotype. $P < 0.05$ between genotypes within an acclimation condition for values not sharing a letter. Bars display mean ± SEM (n = 3-8) with individual data superimposed (circles).

Table S2.1 Parameters used to generate the initial solution in the model of the oxygen transport pathway representing the 'ancestral condition' with the most lowland P_{50}.

Variable	Value
Measured input parameters	
P_B (kPa)	101
F_IO_2	0.123
$\dot{V}$ (ml min^{-1} g^{-1})	4.96
V_T (µl g^{-1})	13.0
[Hb] (g dl^{-1})	14.2
P_{50} (kPa)	4.84
n	2.85
T_b (˚C)	31.4
Estimated input parameters	
V_D (µl g^{-1})*	6.40
$\dot{Q}$ (ml min^{-1} g^{-1}) †	1.06
Calculated input parameters	
D_LO_2 (ml kPa^{-1} min^{-1})	0.0661
D_TO_2 (ml kPa^{-1} min^{-1})	0.0322
Output parameters (ancestral values shown)	
$\boldsymbol{P_AO_2}$ (kPa)	7.18
$\boldsymbol{P_aO_2}$ (kPa) †	6.85
$\boldsymbol{P_vO_2}$ (kPa)†	2.29
$\boldsymbol{\dot{V}O_2max}$ (ml min^{-1} g^{-1})	0.131

P_B, barometric pressure; F_IO_2, inspired oxygen fraction; $\dot{V}O_2max$, maximal oxygen consumption rate measured during acute cold exposure; $\dot{V}$, total ventilation; V_T, tidal volume; [Hb], blood haemoglobin concentration; P_{50}, PO_2 at 50% O_2 saturation; n, Hill coefficient; P_aO_2, arterial O_2 tension; T_b, body temperature; V_D, dead space volume; $\dot{Q}$, cardiac ouput; P_vO_2, mixed venous O_2 tension; P_AO_2, alveolar O_2 tension; D_LO_2, O_2 diffusing capacity of the lungs; D_TO_2, O_2 diffusing capacity of the tissues. *Indicates value was taken from Fallica et al. (2011). †Indicates value was taken from, or calculated using, data in Tate et al. (2020). Variables in bold were then calculated by the model in sensitivity analyses in response to changes in D_TO_2 and/or P_{50}.

Table S2.2 Effects of inspired P_{O_2} and acclimation to hypoxia on cardiorespiratory physiology of F_2 inter-population hybrid deer mice at $\dot{V}_{O_2}max$, without accounting for effects of genotype.

Trait	Animal mass	Acclimation	P_{O_2}	Interaction[1]
$\dot{V}_{O_2}max$[2]	$F_{1,98} = 37.9184$ **P < 0.0001**	$F_{1,98} = 16.8561$ **P = 0.0001**	$F_{1,98} = 94.4737$ **P < 0.0001**	NS
Arterial O_2 saturation	$F_{1,98} = 3.0602$ P = 0.0909	$F_{1,98} = 14.5282$ **P = 0.0003**	$F_{1,98} =$ **P < 0.0001**	$F_{1,98} = 13.0275$ **P = 0.0006**
Heart rate[2]	$F_{1,97} = 4.3086$ **P = 0.0457**	$F_{1,97} = 4.6517$ **P = 0.0342**	$F_{1,97} = 27.5056$ **P < 0.0001**	$F_{1,97} = 7.6175$ **P = 0.0073**
Total ventilation	NS	$F_{1,99} = 49.9311$ **P < 0.0001**	$F_{1,99} = 3.5126$ P = 0.0648	$F_{1,99} = 5.5837$ **P = 0.0207**
Tidal volume	NS	$F_{1,99} = 22.2705$ **P < 0.0001**	$F_{1,99} = 3.3406$ P = 0.0716	$F_{1,99} = 5.1393$ **P = 0.0263**
Breathing frequency	NS	$F_{1,100} = 24.9573$ **P < 0.0001**	$F_{1,100} < 0.0001$ P = 0.9936	NS

[1]Interaction between acclimation and inspired P_{O_2}. NS denotes no significant effect of the factor, which was removed from the final statistical model. [2]Mouse family was also included as a random factor in the mixed model as $0.05 < P < 0.1$. Significant effects ($P < 0.05$) are shown in bold.

Table S2.3 Effects of acclimation to hypoxia and globin genotype on cardiorespiratory physiology in hypoxia of F_2 inter-population hybrid deer mice.

Trait	Animal mass	Acclimation	Hb genotype	Interaction[1]
Hypoxic $\dot{V}O_2max$	$F_{1,41} = 28.0073$ **P < 0.0001**	$F_{1,41} = 13.0967$ **P = 0.0013**	$F_{4,41} = 1.7576$ P =0.1764	NS
Arterial O_2 saturation	NS	$F_{1,42} = 15.8951$ **P = 0.0005**	$F_{4,42} = 3.0259$ **P = 0.0407**	NS
Heart rate[2]	NS	$F_{1,41} = 10.7106$ **P = 0.0031**	$F_{4,41} = 0.7020$ P = 0.5999	NS
Total ventilation	NS	$F_{1,42} = 34.0654$ **P < 0.0001**	$F_{4,42} = 0.3396$ P = 0.8481	NS
Tidal volume	NS	$F_{1,42} = 15.9684$ **P = 0.0005**	$F_{4,42} = 0.6548$ P = 0.6300	NS
Breathing frequency	NS	$F_{1,42} = 23.7780$ **P < 0.0001**	$F_{4,42} = 0.5053$ P = 0.7323	NS
Air convection requirement	$F_{1,41} = 4.3997$ **P = 0.0469**	$F_{1,41} = 5.7509$ **P = 0.0245**	$F_{4,42} = 1.1485$ P = 0.3634	NS
Haematocrit	NS	$F_{1,42} =$ **P < 0.0001**	$F_{4,42} = 0.2031$ P = 0.9338	NS
Blood Hb concentration	NS	$F_{1,42} = 59.4601$ **P < 0.0001**	$F_{4,42} = 1.3807$ P = 0.2745	NS
Red blood cell P_{50}	NS	$F_{1,42} = 18.8344$ **P = 0.0002**	$F_{4,42} = 3.9622$ **P = 0.0150**	NS
Hill coefficient	NS	$F_{1,42} = 0.0315$ P = 0.8599	$F_{4,42} = 0.2514$ P = 0.9073	NS

[1]Interaction between acclimation and globin genotype. NS denotes no significant effect of the factor, which was removed from the final statistical model. [2]Mouse family was also included as a random factor in the mixed model, for which $0.05 < P < 0.1$. Significant effects ($P < 0.05$) are shown in bold.

Table S2.4 Effects of inspired P_{O_2} and haemoglobin genotype on cardiorespiratory physiology of F_2 inter-population hybrid deer mice acclimated to normoxia.

Trait	Animal mass	P_{O_2}	Hb genotype	Interaction[1]
$\dot{V}O_2$max	$F_{1,41} = 31.2238$ **P < 0.0001**	$F_{1,41} = 61.0119$ **P < 0.0001**	$F_{4,41} = 3.0346$ **P = 0.0416**	NS
Arterial O_2 saturation	NS	$F_{1,41} =$ **P < 0.0001**	$F_{4,41} = 3.7422$ **P = 0.0189**	$F_{4,41} = 3.0674$ **P = 0.0389**
Heart rate[2]	NS	$F_{1,41} = 32.4464$ **P < 0.0001**	$F_{4,41} = 2.8000$ P = 0.0545	NS
Total ventilation	NS	$F_{1,42} = 0.3248$ P = 0.5738	$F_{4,42} = 0.6799$ P = 0.6136	NS
Tidal volume	NS	$F_{1,42} = 0.1428$ P = 0.7087	$F_{4,42} = 1.4425$ P = 0.2551	NS
Breathing frequency	$F_{1,41} = 5.9183$ **P = 0.0242**	$F_{1,41} = 0.8443$ P = 0.3669	$F_{4,41} = 1.0643$ P = 0.3999	NS
Haematocrit	NS	NA	$F_{4,21} = 0.3604$ P = 0.8339	NA
Blood Hb concentration	NS	NA	$F_{4,21} = 0.2008$ P = 0.9351	NA
Red blood cell P_{50}	NS	NA	$F_{4,21} = 5.1298$ **P = 0.0048**	NA
Hill coefficient	NS	NA	$F_{4,21} = 0.4016$ P = 0.8053	NA

[1]Interaction between inspired P_{O_2} and globin genotype. NS denotes no significant effect of the factor, which was removed from the final statistical model. NA, not applicable. [2]Mouse family was also included as a random factor in the mixed model, for which $P < 0.05$. Significant effects ($P < 0.05$) are shown in bold.

Table S2.5 Effects of acclimation to hypoxia and globin genotype on cardiorespiratory physiology in normoxia of F_2 inter-population hybrid deer mice.

Trait	Animal mass	Acclimation	Hb genotype	Interaction[1]
Normoxic $\dot{V}O_2max$[2]	$F_{1,40} = 23.2664$ **P < 0.0001**	$F_{1,40} = 4.2325$ P = 0.0502	$F_{4,40} = 1.6212$ P = 0.2115	NS
Arterial O_2 saturation	$F_{1,41} = 15.3290$ **P = 0.0003**	$F_{1,41} = 0.4014$ P = 0.5296	$F_{4,41} = 2.9739$ **P = 0.0291**	NS
Heart rate[2]	$F_{1,40} = 4.8558$ **P = 0.0381**	$F_{1,40} = 0.1977$ P = 0.6605	$F_{4,40} = 2.6116$ P = 0.0698	NS
Total ventilation	NS	$F_{1,42} = 22.9660$ **P < 0.0001**	$F_{4,42} = 0.3536$ P = 0.8386	NS
Tidal volume	$F_{1,41} = 5.1639$ **P = 0.0317**	$F_{1,41} = 3.1282$ P = 0.0892	$F_{4,41} = 1.5707$ P = 0.2199	NS
Breathing frequency	NS	$F_{1,42} = 8.0995$ **P = 0.0087**	$F_{4,42} = 0.6049$ P = 0.6634	NS
Air convection requirement	NS	$F_{1,42} = 8.0863$ **P = 0.0088**	$F_{4,42} = 1.0302$ P = 0.4149	NS

[1]Interaction between acclimation and globin genotype. NS denotes no significant effect of the factor, which was removed from the final statistical model. [2]Mouse family was also included as a random factor in the mixed model as $P < 0.05$. Significant effects ($P < 0.05$) are shown in bold.

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CHAPTER 3: Adrenergic control of the cardiovascular system in deer mice native to high altitude

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3.1 ABSTRACT

Studies of animals native to high altitude can provide valuable insight into physiological mechanisms and evolution of performance in challenging environments. I investigated how mechanisms controlling cardiovascular function may have evolved in deer mice (*Peromyscus maniculatus*) native to high altitude. High-altitude deer mice and low-altitude white-footed mice (*P. leucopus*) were bred in captivity at sea level, and first-generation lab progeny were raised to adulthood and acclimated to normoxia or hypoxia. I then used pharmacological agents to examine the capacity for adrenergic receptor stimulation to modulate heart rate (f_H) and mean arterial pressure (P_{mean}) in anaesthetized mice, and used cardiac pressure-volume catheters to evaluate the contractility of the left ventricle. I found that highlanders had a consistently greater capacity to increase f_H via pharmacological stimulation of β_1-adrenergic receptors than lowlanders. Also, whereas hypoxia acclimation reduced the capacity for increasing P_{mean} in response to α-adrenergic stimulation in lowlanders, highlanders exhibited no plasticity in this capacity. These differences in highlanders may help augment cardiac output during locomotion or cold stress, and may preserve their capacity for α-mediated vasoconstriction to more effectively

redistribute blood flow to active tissues. Highlanders did not exhibit any differences in some measures of cardiac contractility (maximum pressure derivative, dP/dt_{max}, or end-systolic elastance, E_{es}), but ejection fraction was highest in highlanders after hypoxia acclimation. Overall, my results suggest that evolved changes in sensitivity to adrenergic stimulation of cardiovascular function may help deer mice cope with the cold and hypoxic conditions at high altitude.

3.2 INTRODUCTION

The mechanisms underlying the evolution of complex performance traits is a central and unresolved topic in evolutionary physiology (Dalziel et al., 2009; Garland and Carter, 1994; Garland et al., 2016; Scott and Dalziel, 2021). Studies of aerobic performance in endotherms that are native to high altitude can shed valuable insight into this topic (McClelland and Scott, 2019; Monge and Leon-Velarde, 1991; Storz, 2021; Storz et al., 2015; Wearing and Scott, 2021). High-altitude environments are cold and hypoxic, which challenges the ability of endotherms to maintain sufficient rates of O_2 supply to meet the O_2 demands of thermogenesis and locomotion. To help overcome this challenge, several vertebrate taxa that are native to high altitude – including some human populations – have evolved (genetically-based) increases in aerobic capacity (maximal O_2 consumption, $\dot{V}O_2max$, during exercise or acute cold exposure) in hypoxia relative to their low-altitude counterparts (Brutsaert, 2016; Cheviron et al., 2013; Schippers et al., 2012). High-altitude environments can also lead to plastic increases in $\dot{V}O_2max$ in response to exposure during adulthood (*e.g.*, acclimatization) or early life (*i.e.*, developmental plasticity) (Ivy et al.,

2021; Storz and Cheviron, 2021; Storz and Scott, 2019; Tate et al., 2020). Therefore, both evolved changes and plasticity in the physiological determinants of oxygen transport (*e.g.*, pulmonary, cardiovascular, etc.) appear to contribute to enhancing $\dot{V}O_2max$ in high-altitude natives (Chen et al., 1997; Tate et al., 2020). However, the regulatory mechanisms that control these functional changes are not well understood in many high-altitude taxa.

Cardiac output and the preferential distribution of blood flow to metabolically active tissues are important determinants of aerobic capacity during exercise or thermogenesis, and adrenergic receptor stimulation is an important regulator of these processes. At the onset of exercise, sympathoadrenal activation (*i.e.*, activation of the sympathetic nervous system and/or release of catecholamines from the adrenal medulla) leads to stimulation of cardiac output and relative redistribution of blood flow towards working muscles (Christensen and Galbo, 1983). Similarly, cold exposure leads to sympathoadrenal activation that increases cardiac output and redirects blood flow towards thermogenic muscles and brown adipose tissue (BAT) (Foster and Frydman, 1979; Landsberg et al., 1984). Therefore, sympathoadrenal activation and the resultant tissue responses to adrenergic stimulation are critical for supporting high metabolic rates. However, environmental hypoxia also leads to sympathoexcitation via the hypoxic chemoreflex, and chronic exposure to hypoxia (such as occurs at high altitude) can lead to persistent and prolonged sympathetic activation (Calbet, 2003; Hansen and Sander, 2003; Kuwahira et al., 1993a; Kuwahira et al., 1993b; Richalet, 2016; Saito et al., 1988; Sander, 2016; Simpson et al., 2021; Storz and Scott, 2019). If left unabated, prolonged sympathetic activation due to chronic hypoxia is often associated with reductions in tissue sensitivity to

adrenergic stimulation (Berthelsen et al., 2020; Fischetti et al., 2000; Ueno et al., 1997). However, this desensitization to adrenergic stimulation in response to chronic hypoxia could constrain or disrupt the cardiovascular responses to exercise or thermogenesis. This could have detrimental consequences in high-altitude natives, particularly in small endotherms that must sustain high rates of thermogenesis throughout the year to cope with cold temperatures (Hayes, 1989). Whether the sensitivity to adrenergic stimulation has evolved in high-altitude natives to overcome this issue and maintain appropriate cardiovascular responses to exercise and thermogenesis has yet to be resolved.

Previous studies have shown that evolved changes in autonomic control are idiosyncratic in humans native to high altitude. For example, while muscle sympathetic nerve activity (MSNA) measured at rest increases in lowland-native humans after 10-20 days at high altitude, MSNA is lower in highland-native Sherpa residing at high altitude (Simpson et al., 2019). In contrast, Andeans native to high altitude have resting MSNA resembling that of lowlanders visiting high altitude (Lundby et al., 2018). Therefore, there appears to be taxonomic differences in whether evolved mechanisms have arisen to reduce sympathetic activity in chronic hypoxia and thus help improve tissue blood flow and oxygen delivery at rest (Simpson et al., 2021). However, less is known about sympathetic activation during exercise, and whether the capacities for sympathetic responses have evolved in high-altitude humans. Furthermore, relatively little attention has been paid to adrenergic control in non-human animals native to high altitude. Although there is some evidence that some highland animals have reduced adrenergic sensitivity and/or receptor densities (Leon-Velarde et al., 1996; Pichon et al., 2013), these previous studies did not

control for rearing environment and the results could have been explained by developmental hypoxia exposure.

Deer mice (*Peromyscus maniculatus*) native to high altitude are a powerful model for elucidating the cardiovascular mechanisms underpinning the evolution of aerobic performance. High-altitude populations sustain higher field metabolic rates than their low-altitude counterparts, likely to meet the increased oxygen demands of thermogenesis and the need to move greater distances to find food (Hayes, 1989). Increased thermogenic $\dot{V}O_2max$ imparts a survival advantage and is likely under directional selection during harsh winters at high altitude (Hayes and O'Connor, 1999). As a result of selection, high-altitude deer mice have evolved increased thermogenic $\dot{V}O_2max$ in hypoxia compared to low-altitude populations of deer mice and to white-footed mice, a congeneric species that is restricted to low altitudes (Chappell et al., 1988; Chappell and Snyder, 1984; Cheviron et al., 2012; Cheviron et al., 2013; Tate et al., 2017; Tate et al., 2020). Evolved changes across the oxygen transport pathway contribute to this increased $\dot{V}O_2max$, and high capacities for cardiac output and tissue O_2 extraction at $\dot{V}O_2max$ appear to play particularly important roles (Lui et al., 2015; Mahalingam et al., 2017; Natarajan et al., 2015; Scott et al., 2015; Snyder et al., 1982; Storz et al., 2019; Storz et al., 2010; Storz et al., 2007; Tate et al., 2017; Tate et al., 2020; Wearing et al., 2021; West et al., 2021a; West et al., 2021b). These differences in cardiac output and tissue O_2 extraction could result from changes in adrenergic regulation of the heart and vasculature and/or contractile function of the heart, but these possibilities have yet to be examined.

In this study, I hypothesized that high-altitude deer mice have evolved changes in adrenergic control of cardiovascular function and changes in cardiac contractility to augment cardiac output and blood flow to thermogenic tissues in hypoxia. I predicted that adrenergic control of cardiovascular function would be altered in high-altitude mice in two ways, specifically: 1) β_1-adrenergic receptor stimulation would result in greater increases in heart rate in highland mice than in lowland mice; and 2) changes in α-adrenergic receptor sensitivity that occur in lowland mice in chronic hypoxia would be attenuated in highland mice. I also predicted that highland deer mice would have hearts with greater contractility – assessed from pressure-volume (P-V) relationships of the left ventricle – facilitating greater stroke volumes at $\dot{V}O_2max$ compared to lowland mice.

3.3 MATERIALS AND METHODS

3.3.1 Animals and environmental exposures

Lab-raised breeding colonies were derived from wild adult *Peromyscus* mice caught at high and low altitudes. Deer mice from a high-altitude population (*P. m. rufinus*) were caught around the summit of Mount Evans at 4,350 m above sea level (Clear Creed County, CO, USA at 39°35'18"N, 105°38'38"W), and low-altitude white-footed mice (*P. leucopus*) were caught in the Great Plains of Nebraska at 430 m above sea level (Nine Mile Prairie, Lancaster County, NE, USA at 40°52'12"N, 96°48'20.3"W). Following transportation to McMaster University (near sea level), mice were bred to produce first-generation (G_1) lab progeny. These G_1 progeny of highland deer mice and lowland white-footed mice were raised to 6 months of age in common normoxic conditions before use in experiments. All

mice were held at standard lab temperature (24-25˚C) and photoperiod (12 h light: 12 h dark) with unlimited access to standard rodent chow 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.

Starting at approximately 6 months of age, G_1 mice were exposed for 6-8 weeks to one of two environmental conditions: (i) normobaric normoxia (~20 kPa O_2) in ambient air, or (ii) hypobaric hypoxia (12 kPa O_2, equivalent to hypoxia at ~4,300 m above sea level). Hypoxia was achieved and maintained using hypobaric chambers as described previously (Lui et al., 2015; McClelland et al., 1998). Cages were cleaned twice per week, which required that hypoxic mice experience brief (<20 min) periods of normoxia. After the 6-8 week exposure period, mice were then used for measurements of the capacity for adrenergic control of the cardiovascular system or cardiac contractility. The total number of individuals used in each treatment group in this study were as follows: 10 highland deer mice in normoxia (4 females, 6 males); 9 highland deer mice in hypoxia (3 females, 6 males); 11 lowland white-footed mice in normoxia (6 females, 5 males); 12 lowland white-footed mice in hypoxia (5 females, 7 males).

3.3.2 Adrenergic control of the cardiovascular system

I determined the capacity for adrenergic receptors to modulate cardiovascular function by measuring the difference between maximal stimulation with selective agonists and maximal inhibition with selective antagonists in isoflurane anaesthetized mice. Receptor-specific adrenergic agonists and antagonists were prepared fresh daily. The compounds

were dissolved in sterile physiological saline (0.9% NaCl in deionized water) and prepared for intravenous (IV) injection (receptor agonists, 0.0033 ml per g body mass) or intraperitoneal (IP) injection (receptor antagonists, 0.02 ml per g body mass). Dobutamine hydrochloride (2 mg kg^{-1} IV; Cayman Chemical, Ann Arbor, MI, USA) and metoprolol tartrate (10 mg kg^{-1} IP) were used to stimulate and block the positive chronotropic action of cardiac β_1-adrenergic receptors, respectively. Phenylephrine hydrochloride (0.2 mg kg^{-1} IV) and phentolamine hydrochloride (15 mg kg^{-1} IP) were used to stimulate and block the vasoconstrictive action of vascular α-adrenergic receptors, respectively. Preliminary experiments confirmed that the doses used elicited maximal effects on heart rate (f_H) or mean arterial pressure (P_{mean}). All pharmacological compounds were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada) unless stated otherwise.

Each mouse first underwent surgical catheterization of the right jugular vein and left carotid artery. The mouse was first placed in an anaesthetic induction chamber and anaesthesia was induced using 3% isoflurane balanced with O_2 at a flow rate of 1500 ml min^{-1}. The mouse was then placed supine on a heating pad, and a nose cone was used to administer 1.5% isoflurane in the inspired gas to maintain a stable surgical plane of anaesthesia. This dose of isoflurane (1.5%) is below the concentration at which effects of anaesthesia on heart rate, blood pressure, myocardial contractility, and left ventricular diastolic function have been observed in rats (3% isoflurane) (Yang et al., 2014). The mouse was then instrumented with a rectal thermocouple (RET-3-ISO, Physitemp) and the heating pad was regulated to maintain a core body temperature of 35-40˚C throughout the procedure. The ventral skin of the neck was shaved and wiped clean using an isopropyl

alcohol swab, and a 15-mm incision was made along the midline of the neck. The salivary glands were gently pushed aside by blunt dissection to reveal the trachea and the carotid artery. The artery was carefully isolated from the surrounding tissue (including the vagus nerve) at a location proximal to the carotid bifurcation, and was occlusively cannulated with a microrenathane catheter (MRE025, Braintree Scientific, Braintree, MA, USA) filled with 100 units ml^{-1} heparin dissolved in 0.9% sterile saline. The catheter was advanced approximately 15 mm so the tip was in the aortic arch, the catheter was secured to the carotid artery with suture, and the vessel was ligated distal to the incision used to insert the catheter. The other end of this catheter was connected to a fluid-filled pressure transducer (model MLT0699, ADInstruments) that allowed for acquisition of arterial pressure data (200 Hz) using a PowerLab 8/35 and LabChart 8 Pro software (ADInstruments). From this pressure trace, I recorded mean arterial pressure (P_{mean}) and heart rate (f_H). A second catheter filled with 0.9% saline (without heparin) was similarly inserted approximately 15 mm into the right jugular vein and the other end was connected to a 1-ml syringe with a blunted 27G needle.

Measurements were taken after catheterization once cardiovascular parameters had stabilized. The effects of manipulating α- and β_1-adrenergic receptor stimulation were assessed in a subset of mice in each experimental group. Baseline measurements of P_{mean} and f_H were recorded for 5 min to ensure cardiovascular function was stable. Venous blood was withdrawn into the venous catheter until blood reached the needle hub, and the catheter was clamped using haemostats. A syringe containing phenylephrine was then connected to the needle hub and the drug dose was injected. The response was then recorded and the

maximum P_{mean} achieved over a 1-s recording period was designated as the drug response (typically occurring within 5 s post-injection). Once P_{mean} and f_H had returned to baseline levels, phentolamine was injected IP, and the minimum P_{mean} over 1 s was recorded (typically occurring 10-15 min post-injection). Following this, a similar protocol was carried out using IV dobutamine followed by IP metoprolol to measure maximum and minimum f_H. A separate subset of mice were used to examine the effects of β_1-adrenergic receptor stimulation alone using the same procedure. Samples sizes for all mice that underwent β_1-adrenergic receptor stimulation were as follows: highland deer mice in normoxia, n=7 (3 females, 4 males); highland deer mice in hypoxia, n=6 (2 females, 4 males); lowland white-footed mice in normoxia, n=8 (5 females, 3 males); lowland white-footed mice in hypoxia, n=8 (3 females, 5 males). Samples sizes for the subset of mice used for α-adrenergic receptor stimulation were as follows: highland deer mice in normoxia, n=4 (2 females, 2 males); highland deer mice in hypoxia, n=4 (1 female, 3 males); lowland white-footed mice in normoxia, n=4 (3 females, 1 male); lowland white-footed mice in hypoxia, n=3 (2 females, 1 male). Due to the potential effects of anaesthesia on metabolism and the tone of the autonomic nervous system (Skovsted and Sapthavichaikul, 1977), which could influence baseline cardiovascular values, I focused on the difference between maximal and minimal cardiovascular values elicited pharmacologically to quantify the ability for the targeted adrenergic receptors to regulate cardiovascular function. Upon

completion of the protocol, animals were euthanized under anaesthesia by cervical dislocation, and hearts were excised to determine ventricle masses.

3.3.3 Contractility of the heart

The contractile function of the left ventricle was measured using pressure-volume catheters in each experimental group. Each mouse was first anaesthetized with isoflurane in an anaesthetic induction chamber, moved to a heating pad where body temperature was maintained and they breathed through a nose cone, and then maintained at a surgical plane of anaesthesia with 1.5% isoflurane as described above. The ventral skin of the neck, thorax and abdomen was then shaved and cleaned, and a 50-mm incision was made along the body midline from the chin. The salivary glands and underlying muscles in the neck were gently pushed aside by blunt dissection to reveal the trachea. A small slit was made between the larynx and the first tracheal ring, and a mouse endotracheal tube was quickly inserted into the trachea, secured with suture, connected to a ventilator (VentElite Small Animal Ventilator, Harvard Apparatus, Holliston, MA, USA), and used to initiate artificial ventilation with air (300-µl tidal volume at 130 breaths per min; based on previous ventilation measurements by Ivy and Scott, 2017). Isoflurane (1.5%) in the ventilated air was used to maintained a surgical plane of anaesthesia. A laparotomy was then performed by making an approximately 3-cm midline incision through the skin and abdominal wall from the xyphoid process. The liver was carefully retracted caudad to expose the diaphragm, which was then punctured to make an approx. 1-cm medial incision at the midline to expose the apex of the heart. After carefully peeling back the pericardium from

the apex, the left ventricle was punctured quickly and carefully at the apex using a 21G needle, and a pressure-volume catheter (1.2-F diameter pressure-volume catheter FTH-1212B-3518, Transonic Scisense, London, ON, Canada) was advanced as quickly as possible into the puncture hole. This catheter was connected to a Scisense ADVantage 5.0 control unit (Transonic Scisense) that interfaced with a PowerLab 8/35 (ADInstruments, Colorado Springs, CO, USA), and data was visualised in real-time and recorded using LabChart 8 Pro software (ADInstruments). Once proper catheter placement was confirmed by the production of stereotypical ventricular pressure-volume (P-V) loops (see Fig. 3.1), the preparation was allowed to stabilize before left ventricle parameters were recorded, which occurred within 5 min of catheter placement. Loops were visually inspected to choose representative loops for each animal without catheter placement artifacts, which were then used for parameter calculation using the automated LabChart 8 Pro P-V Loop module (see below). I was thus able to obtain P-V loops for each group with the following sample sizes: highland deer mice in normoxia, n=3 (1 female, 2 males); highland deer mice in hypoxia, n=3 (1 female, 2 males); lowland white-footed mice in normoxia, n=3 (1 female, 2 males); lowland white-footed mice in hypoxia, n=4 (2 females, 2 males). However, given the challenges of this technique, there were additional individuals in each group for which I could not obtain P-V loops and no data are reported. Mice were then euthanized by cervical dislocation, and hearts were excised to determine ventricle masses.

Left ventricle (LV) parameters were calculated using the automated LabChart 8 Pro P-V Loop module. Pressure and volume traces were simultaneously acquired using the P-V catheter, and used to produce P-V loops. The individual LV pressure trace was used to

obtain maximal (P_{max}) and minimal (P_{min}) LV pressures per heart beat, as well as the mean pressure (P_{mean}) calculated as $\frac{P_{max}+2P_{min}}{3}$, and developed pressure, P_{dev} ($P_{max} - P_{min}$). This pressure trace was also used to calculate heart rate (f_H). Stroke volume was calculated as the difference between the minimum and maximum (V_{max}) LV volumes per beat, and was multiplied by f_H to calculate cardiac output. Stroke work was then calculated as the product of P_{mean} and stroke volume. Finally, I acquired three indices of cardiac contractility: dP/dt_{max}, the maximal rate of LV pressure increase per beat acquired from the pressure trace; ejection fraction (EF, %), which was calculated as $\frac{Stroke\ volume}{V_{max}} \times 100$ per beat; and the slope of the end-systolic pressure-volume relationship, the end-systolic elastance (E_{es}), which is calculated as end-systolic pressure divided by end-systolic volume.

3.3.4 Statistical Analysis

I used linear mixed effects models to test for the effects of species, chronic hypoxia exposure, and their interaction using the lme4 package (Bates et al., 2015) in R Studio (Version 1.4.1103, RStudio Public Benefit Corporation, Boston, MA, USA). Initial models were run including effects of body mass, individual (for repeated-measures drug injections), family, and sex as random factors as appropriate. If body mass, family or sex had P values above 0.1 in the initial model, they were removed by stepwise backward deletion (starting with the term with the highest P value) and the model was re-run until all terms in the model (with the exception of fixed factors and individual subject) had P values below 0.1. The full results of final statistical models are included in the supplementary material (Supplemental Tables, Tables S3.1-S3.4) and the salient findings are reported in

the Results (Section 3.4). Family was included in only 4 of the models, and sex was included in only one model. Tukey's HSD post hoc tests were performed to test for pairwise differences between species within an acclimation or injection group, between acclimations within a species or injection group, and between injections within a species or acclimation group. Data are presented as individual values (small circles) and mean ± SEM (bars) unless otherwise stated.

3.4 RESULTS

3.4.1 Highland deer mice had similar sized hearts but relatively small right ventricles compared to white-footed mice

I measured body mass and ventricle masses across all the highland deer mice and lowland white-footed mice used in this study. Highland deer mice were approximately 30% smaller than white-footed mice (main effect of species on body mass, P=0.002) (Table 3.1). This difference was expected based on the known difference in body size between species, and it is not unique to the high-altitude population (within deer mice, low- and high-altitude populations have similar body masses) (Tate et al. 2020). When the effects of variation in body mass was accounted for as a covariate in statistical models, there were no overall differences between species or acclimation environments on total ventricle mass (species effect, P=0.951; acclimation effect, P=0.458), left ventricle plus septum (LV+S) mass (species effect, P=0.481; acclimation effect, P=0.173), or right ventricle (RV) mass (species effect, P=0.088; acclimation effect, P=0.150). Although the species differences were not significant, highlanders tended to have larger total ventricle and LV+S masses and smaller

RV mass when expressed relative to body mass (Table 3.1). As a result, the ventricular mass ratio also known as Fulton's index, RV/(LV+S), was ~22-27% lower in highlanders compared to lowlanders (species effect, $P<0.001$). This difference offset the increase in RV/(LV+S) after hypoxia acclimation (acclimation effect, $P=0.016$), such that RV/(LV+S) was lower on average in hypoxic highlanders than in normoxic lowlanders (Table 3.1).

3.4.2 Highland deer mice have greater scope for adrenergic stimulation of the cardiovascular system

I used pharmacological agents to maximally stimulate and block β_1-adrenergic receptors to determine their ability to increase heart rate (f_H) in anaesthetized mice. There were no significant differences between species nor significant effects of hypoxia acclimation on f_H before pharmacological manipulation (baseline f_H before injections: normoxic lowlander, 516±39 min^{-1}; normoxic highlander, 540±14 min^{-1}; hypoxic lowlander, 509±21 min^{-1}; hypoxic highlander, 477±21 min^{-1}). As expected, β_1-adrenergic receptor drugs had a significant effect on f_H ($P<0.001$), with f_H being 206 min^{-1} to 324 min^{-1} higher after dosing with the β_1-adrenergic receptor agonist dobutamine (Fig. 3.1a) compared to subsequent dosing with the receptor antagonist metoprolol (Fig 3.1b). I then calculated the change in f_H between the β_1-adrenergic receptor agonist and antagonist (*i.e.*, Δf_H) as an indication of the potential scope for adrenergic stimulation of f_H (Fig. 3.1c). Highland deer mice had 34-51% higher Δf_H on average than lowland white-footed mice (species effect, $P=0.023$).

I pharmacologically stimulated and blocked α-adrenergic receptors to determine the ability of these receptors to regulate blood pressure. There were no differences between species nor effects of hypoxia acclimation on mean arterial pressure (P_{mean}) before pharmacological manipulation (baseline P_{mean}: normoxic lowlander, 85.2±8.3 mmHg; normoxic highlander, 91.9±3.1 mmHg; hypoxic lowlander, 101.0±4.6 mmHg; hypoxic highlander, 102.6±9.4 mmHg). As expected, the α-adrenergic receptor drugs had a significant effect on P_{mean} ($P<0.001$), with P_{mean} being 65 to 96 mmHg higher after dosing with the α-adrenergic receptor agonist phenylephrine (Fig. 3.2a) compared to subsequent dosing with the receptor antagonist phentolamine (Fig. 3.2b). Similar to the approach used for β_1-receptor manipulation, I calculated the change in P_{mean} between the α-adrenergic receptor agonist and antagonist (*i.e.*, ΔP_{mean}) as an indication of the potential scope for adrenergic regulation of blood pressure (Fig. 3.2c). There was a significant 30% reduction in ΔP_{mean} after hypoxia acclimation in lowlanders, but no change in ΔP_{mean} in highlanders (species×environment interaction, $P=0.031$). The former was associated with an increase in P_{mean} after phentolamine injection in lowlanders after hypoxia acclimation (Fig. 3.2b). I did not observe any significant effects of sex on α-adrenergic responses (nor on β_1-adrenergic responses; Supplemental Tables, Table S3.3), despite the potential for sex differences in adrenergic control of the cardiovascular system (Joyner et al., 2015; Vizgirda et al., 2002), but I likely lacked sufficient sample sizes of males and females to detect such differences.

3.4.3 Pressure-volume relationships and contractility of the left ventricle

I measured pressure-volume relationships inside the left ventricle in anaesthetized mice. Pressure-volume (P-V) loops exhibited characteristic low-pressure filling phase (bottom), steep isovolumetric contraction phase (right), rising pressure during ejection phase (top), and isovolumetric relaxation phase (left) (Fig. 3.3). After accounting for effects of species differences in body mass as a covariate in statistical models, resting stroke volume, cardiac output, stroke work, and maximum and minimum ventricle volumes were lower overall in highlanders than in lowlanders (species effects, $P \leq 0.05$). The differences in stroke volume, cardiac output, and stroke work were driven primarily by smaller values in highlanders in normoxia, but the species differences were no longer significant after hypoxia acclimation (Table 3.2). Otherwise, hypoxia acclimation significantly increased stroke work (acclimation effect, P=0.023) and the pressure developed by contraction (P_{dev}; acclimation effect, P=0.046), and it also reduced minimum pressure (P_{min}; acclimation effect, P=0.041). There was a reduction in resting f_H in lowlanders after hypoxia acclimation (Table 3.2), but values were still within the ranges measured during the pharmacology manipulations (Fig. 3.1).

I used the aforementioned pressure-volume relationships to obtain load-dependent (maximum pressure derivative, dP/dt_{max}, and ejection fraction, EF) and load-independent (end-systolic elastance, E_{es}) indices of left ventricle contractility (Table 3.2). Neither dP/dt_{max} nor E_{es} differed between species (species effects, P = 0.466 and 0.067, respectively) or acclimation environments (acclimation effects, P = 0.235 and 0.947, respectively). In contrast, whereas ejection fraction was ~35-40% in lowlanders, it rose to

60% in highlanders after hypoxia acclimation (acclimation effect, $P < 0.001$; species×environment interaction, $P = 0.004$) (Table 3.2; Supplemental Tables, Table S3.4). The latter appeared to result from a lower minimum ventricle volume after hypoxia acclimation in highlanders (Table 3.2).

3.5 DISCUSSION

3.5.1 Overview

High-altitude deer mice have evolved a suite of physiological changes across the oxygen transport pathway that aid in supplying oxygen to active tissues in an oxygen-limited environment, but the importance of changes in adrenergic control of cardiovascular function was previously unknown. I found that highland deer mice had a greater capacity than lowlanders to elevate heart rate via stimulation of β_1-adrenergic receptors. Furthermore, whereas chronic hypoxia reduced the capacity for increasing blood pressure in response to α-adrenergic receptor stimulation in lowlanders, this capacity was preserved in chronic hypoxia in highlanders. These differences may help augment cardiac output and preserve the capacity for α-mediated vasoconstriction, to more effectively redistribute blood flow and improve O_2 delivery to active tissues during locomotion or cold stress. High-altitude adaptation does not appear to have caused any substantial changes in the load-independent contractile function of the left ventricle, although highland deer mice exhibited high ejection fraction in chronic hypoxia. Overall, my results suggest that evolved changes in adrenergic regulation of the cardiovascular system may help highland deer mice cope with the cold and hypoxic conditions at high altitude.

3.5.2 Chronic hypoxia reduces vascular responses to adrenergic activation in low-altitude mice

The reduced capacity for responding to α-adrenergic stimulation after hypoxia acclimation in lowland white-footed mice could reflect a plastic vascular response resulting from chronic activation of the hypoxic chemoreflex. The hypoxic chemoreflex, initiated when the carotid bodies detect low arterial O_2 levels, leads to sympathoadrenal activation, and the resulting α-mediated vasoconstriction can increase vascular resistance, restrict blood flow to some tissues, and induce systemic hypertension (Calbet, 2003; Hainsworth and Drinkhill, 2007; Hainsworth et al., 2007; Richalet, 2016; Rimoldi et al., 2016; Sander, 2016). The observed response of lowland white-footed mice to chronic hypoxia should help attenuate these effects by reducing the responsiveness of the systemic vasculature to catecholamines. This response could be explained by a downregulation of α-adrenergic receptor density, as previously described following hypoxia acclimation in other species (Fischetti et al., 2000; Ueno et al., 1997). However, these changes could come at the expense of other homeostatic processes that rely upon autonomic control of the vasculature, such as the baroreflex and the controlled re-distribution of blood flow to active tissues during locomotion and/or thermogenesis (*e.g.*, skeletal muscles, brown adipose tissue).

Lowland white-footed mice demonstrated no plasticity in the capacity for a heart rate response to β_1-adrenergic receptor stimulation, which differs from the expectation from previous studies in some other animals. Chronic hypoxia has been shown to reduce β-receptor sensitivity and density in cardiac tissue in some other species, and has been associated with reductions in maximal heart rate (Favret and Richalet, 2007; Kacimi et al.,

1992; Kanai et al., 2001; Leon-Velarde et al., 1996; Voelkel et al., 1981). My observations suggesting that this does not occur may explain why heart rates measured at thermogenic $\dot{V}O_2max$ (maximal rate of O_2 consumption for thermogenesis) are not reduced after hypoxia acclimation in *Peromyscus* mice (Tate et al., 2017; Tate et al., 2020). My results are therefore supportive of the growing appreciation that interspecific differences between low-altitude mammals (*e.g.*, old world mice versus rats) can alter the responses to and tolerance of chronic hypoxia (Arias-Reyes et al., 2021; Jochmans-Lemoine et al., 2015).

3.5.3 High-altitude deer mice have increased capacity for adrenergic control of the cardiovascular system in chronic hypoxia

My finding that highlanders have an enhanced capacity to increase heart rate in response to β_1-adrenergic stimulation provides a potential mechanism for evolved increases in thermogenic capacity and maximal cardiac output in hypoxia. High-altitude deer mice have evolved greater thermogenic capacity than both low-altitude conspecifics and low-altitude white-footed mice, likely as a result of strong directional selection in cold alpine environments (Chappell et al., 1988; Chappell and Snyder, 1984; Cheviron et al., 2012; Cheviron et al., 2013; Hayes and O'Connor, 1999). We have previously demonstrated that this increase in aerobic capacity is associated with evolved changes across the oxygen transport pathway (Lui et al., 2015; Ivy et al., 2020; Mahalingam et al., 2017; Wearing et al., 2021; West et al., 2021a; West et al., 2021b), including higher maximal heart rate and/or stroke volume at $\dot{V}O_2max$ in chronic hypoxia (Tate et al., 2017; Tate et al., 2020). My results here suggest that the former may be achieved at least in part from a greater response

to stimulation of cardiac β_1-adrenergic receptors upon activation of the sympathetic nervous system during cold exposure (Morrison et al., 2008; Richalet, 2016; Sander, 2016; Storz and Scott, 2019). These findings are in stark contrast to high-altitude pikas and humans, which exhibit lower β_1-adrenergic sensitivity or tone compared to lowlanders, and in the former case this was associated with lower receptor mRNA expression (Pichon et al., 2013; Zhuang et al., 1993). However, such differences may not be surprising in light of previous findings that highland deer mice maintain very high metabolic rates in the wild (Hayes, 1989), in contrast to high-altitude pikas that instead suppress metabolism to cope at high altitude (Speakman et al., 2021). The benefit of an enhanced capacity for cardiac β_1-adrenergic stimulation may be restricted to high-altitude taxa that have high metabolic demands for thermogenesis or locomotion at high altitude.

The lack of any effects of chronic hypoxia on α-adrenergic responses in highlanders may help maintain autonomic vascular control at normal levels. This could be advantageous for preserving the baroreflex and the ability to re-distribute blood flow in response to metabolic need, but it could potentiate systemic hypertension and blood flow restriction if α-receptors are chronically stimulated by the hypoxic chemoreflex. However, we recently found that high-altitude deer mice have lower circulating epinephrine levels than low-altitude mice, due to an evolved reduction in catecholamine secretion from the adrenal medulla (Scott et al., 2019). As such, chronic hypoxia may not lead to chronic adrenergic stimulation via humoral means in highlanders, negating the need to downregulate vascular α-receptors to avoid hypertension and blood flow restriction. It is possible that highland mice have also evolved increased vasodilatory tone on the vasculature, as observed in

highland-native humans from Tibet that show elevated circulating levels of nitric oxide products (Erzurum et al., 2007), but this possibility remains unexamined.

I observed equivocal evidence that the left ventricle of highland deer mice has greater contractility than lowland mice, based on measurements of pressure-volume relationships in anaesthetized mice. On the one hand, there were no species differences (or effects of hypoxia acclimation) on the maximum pressure derivative (dP/dt_{max}) or end-systolic elastance (E_{es}; an index of load-independent contractility). On the other hand, EF was highest in highland mice after hypoxia acclimation. However, given the discordance between these measures of contractility, and the necessity that measurements were made on a relatively small sample size of anaesthetized mice, it remains unclear if highland mice have greater left ventricle contractility at the much higher cardiac outputs and stroke volumes at $\dot{V}O_2max$. Variation in metabolism, systemic vascular resistance, and venous return under anaesthesia could explain some of the observed variation in left ventricle volumes and pressures, all of which would thus be expected to change at the high metabolic rates during intense aerobic thermogenesis. Therefore, although my results suggest there are few differences in cardiac contractility in highland deer mice, future work is needed to determine if this is also the case at higher metabolic rates or if increases in contractility help facilitate increases in stroke volume and cardiac output to augment $\dot{V}O_2max$ in hypoxia.

Bearing in mind the limitations of two-species comparisons for inferring adaptation (Garland and Adolph, 1994), it is possible that some of the species differences observed here reflect overall differences between deer mice and white-footed mice, rather than

derived changes in the high-altitude population. Indeed, deer mice have the widest altitudinal range of any North American mammal, from around sea level to over 4300 m elevation, and low-altitude populations can be found across much of the continent (Bedford and Hoekstra, 2015; Natarajan et al., 2015; Snyder et al., 1982). However, many previous studies of aerobic performance and cardiorespiratory physiology have found that high-altitude deer mice are distinct from both low-altitude conspecifics and low-altitude white-footed mice, reflecting derived changes in physiology in the high-altitude population (Cheviron et al. 2013; Tate et al. 2020; Ivy and Scott 2017; Ivy et al. 2020). Adrenergic control of the cardiovascular system is an important determinant of aerobic performance, so increased capacity for adrenergic control may be a key underlying mechanism for the adaptive increase in thermogenic capacity in highlanders (Cheviron et al. 2013; Tate et al. 2020).

3.6 CONCLUSIONS

This study contributes to the emerging evidence that deer mice have adapted to high altitude through evolved changes in several aspects of cardiorespiratory and metabolic physiology that contribute to augmenting aerobic capacity in chronic hypoxia (Lui et al. 2015; Mahalingam et al. 2017; Tate et al. 2017, 2021; Ivy et al. 2020; Wearing et al. 2021; West et al. 2021a,b). In particular, higher maximal cardiac output and tissue O_2 extraction contribute to augmenting thermogenic $\dot{V}O_2max$ in chronic hypoxia in highland deer mice compared to their lowland counterparts (Tate et al. 2020). Here, I show that this is associated with increased capacity for regulation of the heart and vasculature by adrenergic

receptors. Highlanders had a greater capacity than lowlanders to elevate heart rate *via* stimulation of β_1-adrenergic receptors, which may help augment maximal cardiac output. Highlanders also appeared to maintain the capacity for vascular regulation by α-adrenergic receptor stimulation, potentially to preserve the effective redistribution of blood flow to active tissues and augment O_2 extraction. Therefore, my findings suggest that autonomic regulation of the cardiovascular system has evolved in highland deer mice to help them not only survive but thrive in the challenging environment at high altitude.

3.7 FIGURES AND TABLES

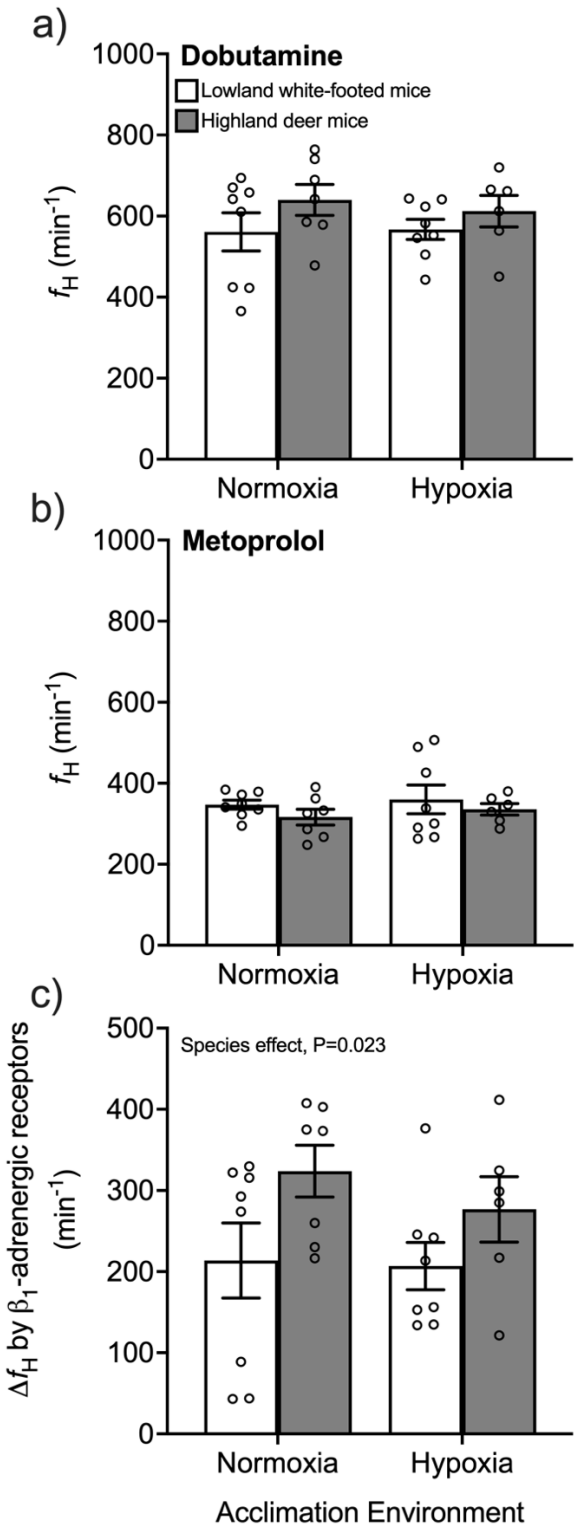

Fig. 3.1 Heart rate (f_H) after pharmacological stimulation of cardiac β_1-adrenergic receptors by dobutamine (a) followed by blockade with metoprolol (b) in white-footed mice (a species restricted to low altitude) and in deer mice from a population native to high altitude. Mice were acclimated to normoxia (21 kPa O_2) or hypobaric hypoxia (12 kPa O_2) for 6-8 weeks. c) Change in heart rate (Δf_H) by stimulation of β_1-adrenergic receptors was calculated as the difference between maximal stimulation with dobutamine and blockade with metoprolol. Bars display mean ± SEM with individual data as circles. *$P < 0.05$ between species within an acclimation environment. $^{\dagger}P < 0.05$ between acclimation environments within a species.

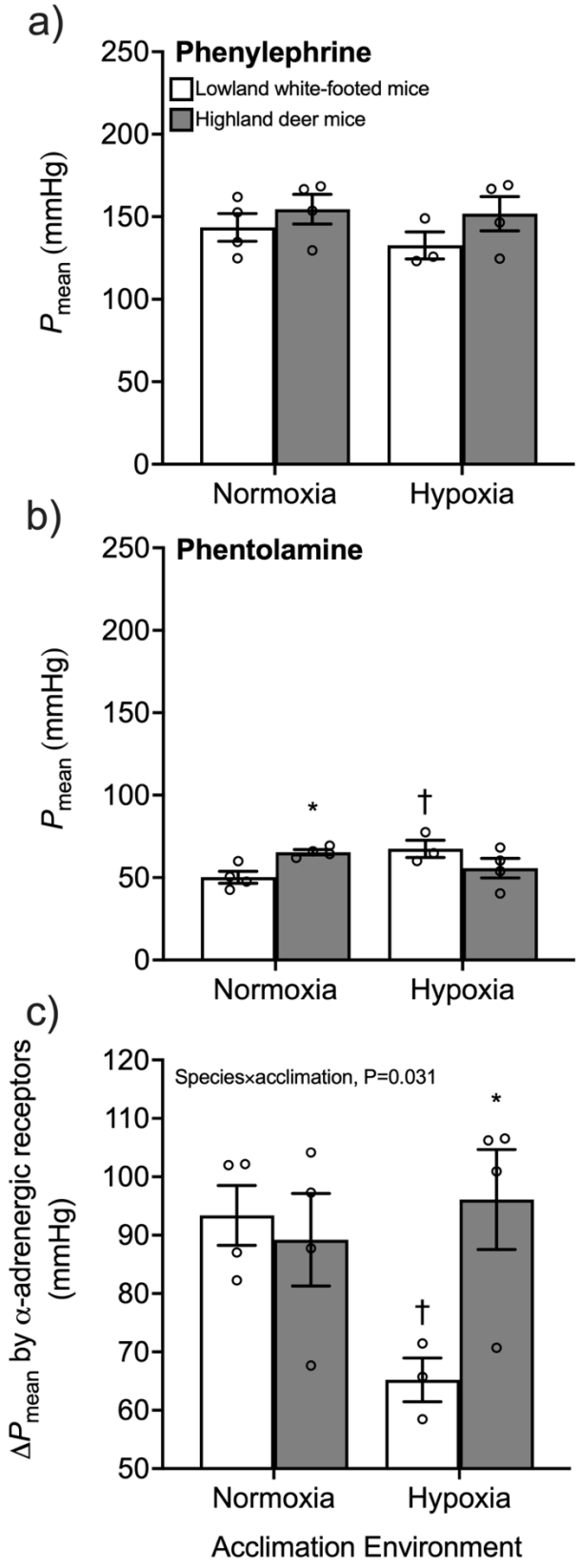
a)
Phenylephrine
Lowland white-footed mice
Highland deer mice
P_{mean} (mmHg)
250
200
150
100
50
0
Normoxia
Hypoxia
b)
Phentolamine
P_{mean} (mmHg)
250
200
150
100
50
0
*
†
Normoxia
Hypoxia
c)
ΔP_{mean} by α-adrenergic receptors (mmHg)
Species×acclimation, P=0.031
120
110
100
90
80
70
60
50
*
†
Normoxia
Hypoxia
Acclimation Environment

Fig. 3.2 Mean arterial pressure (P_{mean}) after pharmacological stimulation of vascular α-adrenergic receptors by phenylephrine (a) followed by blockade with phentolamine (b) in white-footed mice (a species restricted to low altitude) and in deer mice from a population native to high altitude. Mice were acclimated to normoxia (21 kPa O_2) or hypobaric hypoxia (12 kPa O_2) for 6-8 weeks. c) Change in mean arterial pressure (ΔP_{mean}) caused by stimulation of α-adrenergic receptors was calculated as the difference between maximal stimulation with phenylephrine and blockade with phentolamine. Bars display mean ± SEM with individual data as circles. *$P < 0.05$ between species within an acclimation environment. $^{\dagger}P < 0.05$ between acclimation environments within a species.

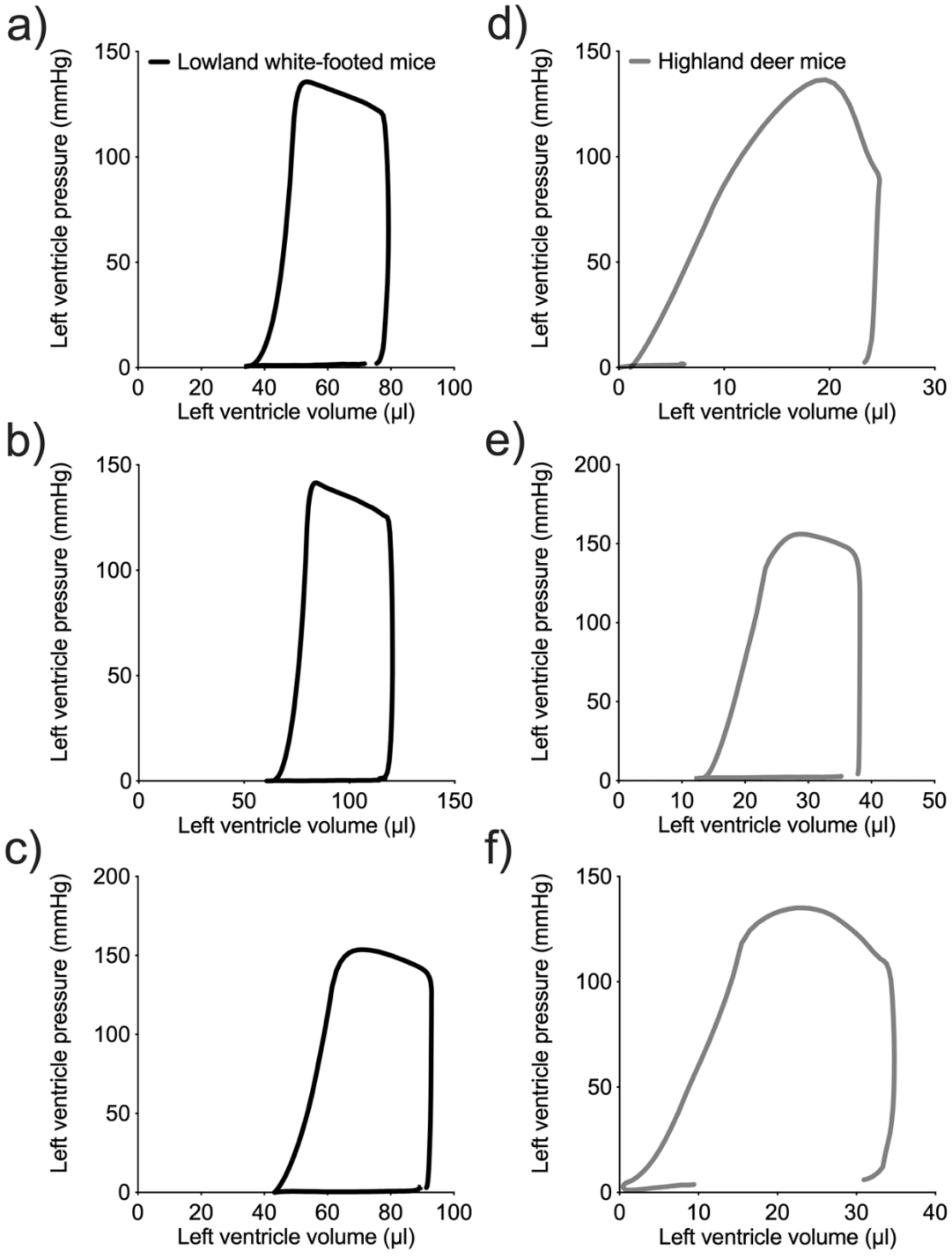
a)
Lowland white-footed mice
Left ventricle pressure (mmHg)
Left ventricle volume (µl)
b)
c)
d)
Highland deer mice
e)
f)

Fig. 3.3 Representative pressure-volume (P-V) loops for the left ventricle of individual white-footed mice (a species restricted to low altitude) (a-c) and individual deer mice from a population native to high altitude (d-f) after hypoxia acclimation.

Table 3.1 Body and heart masses in white-footed mice ('lowlander', *Peromyscus leucopus*) and in deer mice from a population native to high altitude ('highlander', *P. maniculatus*), each of which were acclimated to normoxia (21 kPa O_2) or hypobaric hypoxia (12 kPa O_2) for 6-8 weeks.

	Normoxia		Hypoxia	
	Lowlander (n = 11)	Highlander (n = 10)	Lowlander (n = 12)	Highlander (n = 9)
Body mass, g	28.9 ± 2.2	20.7 ± 1.1*	30.1 ± 1.2	20.6 ± 1.1*
Total ventricle mass, mg g^{-1}	4.21 ± 0.21	4.78 ± 0.15	3.98 ± 0.20	4.61 ± 0.15
RV mass, mg g^{-1}	0.721 ± 0.026	0.631 ± 0.034	0.806 ± 0.087	0.725 ± 0.035
LV+S mass, mg g^{-1}	3.48 ± 0.19	4.15 ± 0.14	3.17 ± 0.15	3.89 ± 0.14
RV/(LV+S)	0.210 ± 0.011	0.153 ± 0.008*	0.255 ± 0.025	0.188 ± 0.010*†

*Significant species effect within acclimation environment, $P < 0.05$. †Significant acclimation effect within a species. RV, right ventricle; LV+S, left ventricle and septum; RV/LV+S, right ventricle to left ventricle and septum ratio. Ventricle masses were obtained from a subset of mice within each group (n = 7).

Table 3.2 Left ventricle parameters measured using intraventricular pressure-volume catheter in white-footed mice ('lowlander', *Peromyscus leucopus*) and in deer mice from a population native to high altitude ('highlander', *P. maniculatus*), each of which were acclimated to normoxia (21 kPa O_2) or hypobaric hypoxia (12 kPa O_2) for 6-8 weeks.

	Normoxia		Hypoxia	
	Lowlander (n = 3)	Highlander (n = 3)	Lowlander (n = 4)	Highlander (n = 3)
f_H, min^{-1}	577 ± 42	543 ± 66	405 ± 39†	530 ± 29
Stroke volume, µl g^{-1}	1.094 ± 0.139	0.582 ± 0.077*	1.221 ± 0.028	1.025 ± 0.209
Cardiac output, ml min^{-1} g^{-1}	0.631 ± 0.090	0.308 ± 0.033*	0.492 ± 0.037	0.544 ± 0.120
Stroke work, mmHg µl g^{-1}	135.2 ± 15.3	61.2 ± 15.4*	165.4 ± 10.3	136.9 ± 25.1
V_{max}, µl g^{-1}	3.145 ± 0.282	1.770 ± 0.317*	3.129 ± 0.159	1.733 ± 0.310
V_{min}, µl g^{-1}	1.552 ± 0.243	0.783 ± 0.345	1.321 ± 0.204	0.225 ± 0.211
P_{max}, mmHg	134 ± 4	113 ± 15	139 ± 6	143 ± 7
P_{min}, mmHg	2.808 ± 1.774	3.729 ± 1.778	0.327 ± 0.479	0.166 ± 0.888
P_{mean}, mmHg	49.7 ± 1.7	39.2 ± 6.6	41.3 ± 4.0	43.5 ± 4.6
P_{dev}, mmHg	131 ± 2	110 ± 14	138 ± 7	142 ± 6
EF, %	34.7 ± 1.8	37.8 ± 11.3	39.4 ± 1.7	60.2 ± 6.7
dP/dt_{max}, mmHg s^{-1}	9314 ± 357	10394 ± 2378	11169 ± 825	12974 ± 2682
E_{es}, mmHg $µl^{-1}$	1.93 ± 0.29	6.73 ± 2.11	3.30 ± 0.44	5.40 ± 2.75

*Significant species effect within acclimation environment, $P < 0.05$. †Significant acclimation effect within a species, $P < 0.05$. f_H, heart rate; V_{max}, maximum left ventricle volume; V_{min}, minimum left ventricle volume; P_{max}, maximum left ventricle pressure; P_{min}, minimum left ventricle pressure; P_{mean}, mean left ventricle pressure; P_{dev}, pressure developed by left ventricle contraction; EF, ejection fraction; dP/dt_{max}, maximum derivative of pressure; E_{es}, end-systolic elastance, which is the slope of the end-systolic pressure-volume relationship.

3.8 SUPPLEMENTAL FIGURES AND TABLES

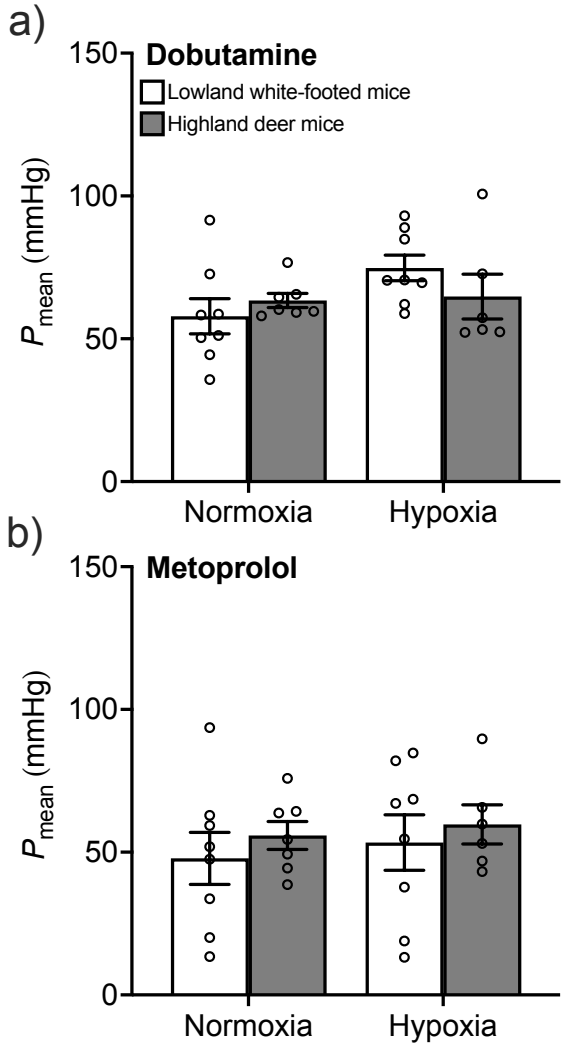

Fig. S3.1 Mean arterial pressure (P_{mean}) after pharmacological stimulation of cardiac β_1-adrenergic receptors by dobutamine (a) followed by blockade with metoprolol (b) in white-footed mice (a species restricted to low altitude) and in deer mice from a population native to high altitude. Mice were acclimated to normoxia (21 kPa O_2) or hypobaric hypoxia (12 kPa O_2) for 6-8 weeks. Bars display mean ± SEM with individual data as circles.

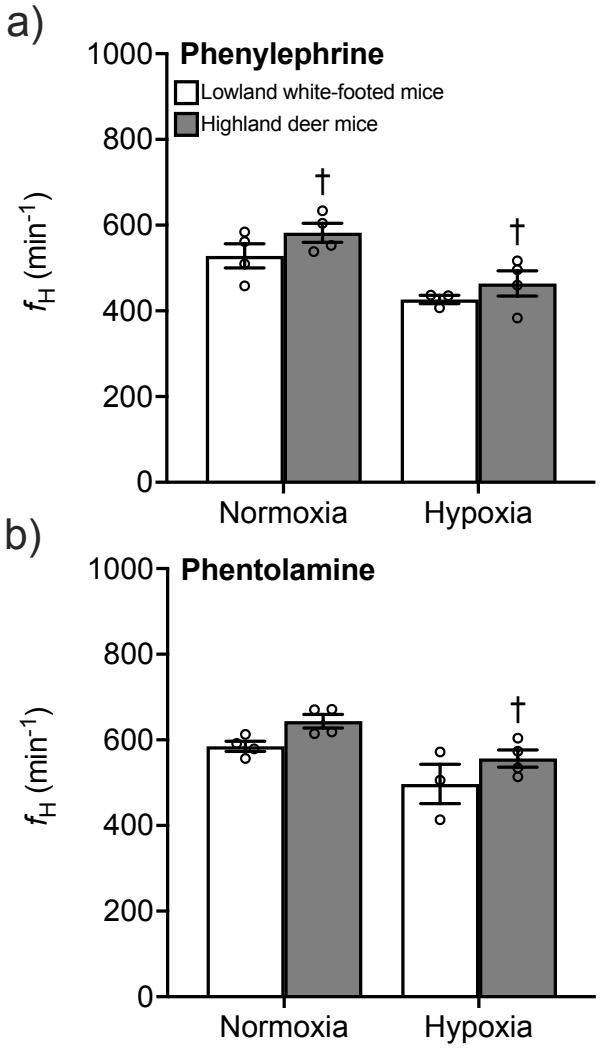
a)
Phenylephrine
Lowland white-footed mice
Highland deer mice
1000
800
600
400
200
0
f_H (min^{-1})
†
†
Normoxia
Hypoxia
b)
Phentolamine
1000
800
600
400
200
0
f_H (min^{-1})
†
Normoxia
Hypoxia

Fig. S3.2 Heart rate (f_H) after pharmacological stimulation of vascular α-adrenergic receptors by phenylephrine (a) followed by blockade with phentolamine (b) in white-footed mice (a species restricted to low altitude) and in deer mice from a population native to high altitude. Mice were acclimated to normoxia (21 kPa O_2) or hypobaric hypoxia (12 kPa O_2) for 6-8 weeks. Bars display mean ± SEM with individual data as circles. $^{\dagger}P < 0.05$ between acclimation environments within a species.

Table S3.1 Results of statistical comparisons using linear mixed models on body and heart mass data.

Trait	Animal mass effect	Species (*s*) effect	Acclimation (*a*) effect	*s* x *a* effect
Body mass[1]	NA	**P = 0.0015**	P = 0.8012	P = 0.8865
		$F_{1,23}$ = 16.7977	$F_{1,23}$ = 0.0644	$F_{1,23}$ = 0.0207
Total ventricle mass	**P < 0.0001**	P = 0.9505	P = 0.4575	P = 0.9618
	$F_{1,23}$ = 41.9719	$F_{1,23}$ = 0.0039	$F_{1,23}$ = 0.5711	$F_{1,23}$ = 0.0023
RV mass	**P < 0.0001**	P = 0.0878	P = 0.1495	P = 0.7476
	$F_{1,23}$ = 39.0714	$F_{1,23}$ = 3.1795	$F_{1,22}$ = 2.2242	$F_{1,22}$ = 0.1061
LV+S mass	**P < 0.0001**	P = 0.4807	P = 0.1726	P = 0.8639
	$F_{1,23}$ = 26.8873	$F_{1,23}$ = 0.5138	$F_{1,22}$ = 1.9814	$F_{1,22}$ = 0.0301
RV/(LV+S)	NA	**P = 0.0004**	**P = 0.0155**	P = 0.7477
		$F_{1,24}$ = 16.5083	$F_{1,24}$ = 6.7955	$F_{1,24}$ = 0.1058

RV, right ventricle; LV+S, left ventricle and septum; RV/LV+S, right ventricle to left ventricle and septum ratio. [1]Family included in model as a significant (P < 0.05) random factor. Statistical tests of ventricle mass data used absolute values and accounted for body mass as a covariate, but the data are reported relative to body mass in Table 1. Significant effects (P < 0.05) are shown in bold.

Table S3.2 Results of statistical comparisons using linear mixed models on the effects of species, hypoxia acclimation and receptor-specific adrenergic drugs on cardiovascular function.

Trait	Species (*s*) effect	Acclimation (*a*) effect	Drug (*d*) effect	*s* x *a* effect	*s* x *d* effect	*a* x *d* effect	*s* x *a* x *d* effect
f_H	P = 0.6697	P = 0.6702	**P < 0.0001**	P = 0.5498	**P = 0.0301**	P = 0.3263	P = 0.6400
	$F_{1,25}$ = 0.1864	$F_{1,25}$ = 0.1857	$F_{2,50}$ = 117.0944	$F_{1,25}$ = 0.3676	$F_{2,50}$ = 3.7601	$F_{2,50}$ = 1.1454	$F_{2,50}$ = 0.4503
P_{mean}	P = 0.4594	P = 0.6383	**P < 0.0001**	P = 0.3620	P = 0.2369	**P = 0.0286**	P = 0.0753
	$F_{1,24}$ = 0.5654	$F_{1,24}$ = 0.2266	$F_{2,24}$ = 255.6394	$F_{1,24}$ = 0.8634	$F_{2,24}$ = 1.5306	$F_{2,24}$ = 4.1371	$F_{2,24}$ = 2.8871

f_H, heart rate; P_{mean}, mean arterial pressure. Significant effects ($P < 0.05$) are shown in bold.

Table S3.3 Results of statistical comparisons using linear mixed models on the effects of species and hypoxia acclimation on the change in cardiovascular variables by adrenergic stimulation.

Trait	Species (*s*) effect	Acclimation (*a*) effect	*s* x *a* effect
Δf_H by β_1-adrenergic receptors	**P = 0.0234** $F_{1,25} = 5.8301$	P = 0.5173 $F_{1,25} = 0.4314$	P = 0.6005 $F_{1,25} = 0.2813$
ΔP_{mean} by α-adrenergic receptors	P = 0.1352 $F_{1,11} = 2.6000$	P = 0.2152 $F_{1,11} = 1.7294$	**P = 0.0308** $F_{1,11} = 6.1287$

f_H, heart rate; P_{mean}, mean arterial pressure. Significant effects ($P < 0.05$) are shown in bold.

Table S3.4 Results of statistical comparisons using linear mixed models on the effects of species and hypoxia acclimation on left ventricle parameters measured using intraventricular pressure-volume catheter.

Trait	Body mass effect	Species (*s*) effect	Acclimation (*a*) effect	*s* x *a* effect
f_H	NS	P = 0.2378	P = 0.0593	P = 0.1157
		$F_{1,9}$ = 1.5988	$F_{1,9}$ = 4.6541	$F_{1,9}$ = 3.0306
Stroke volume	**P < 0.0001**	**P = 0.0003**	P = 0.0666	P = 0.2400
	$F_{1,8}$ = 63.8379	$F_{1,8}$ = 35.4430	$F_{1,8}$ = 4.5027	$F_{1,8}$ = 1.6114
Cardiac output	**P = 0.0004**	**P = 0.0100**	P = 0.4752	**P = 0.0079**
	$F_{1,8}$ = 34.5960	$F_{1,8}$ = 11.2374	$F_{1,8}$ = 0.5612	$F_{1,8}$ = 12.3722
Stroke work	**P = 0.0002**	**P = 0.0011**	**P = 0.0233**	P = 0.3779
	$F_{1,8}$ = 41.3624	$F_{1,8}$ = 25.0122	$F_{1,8}$ = 7.8259	$F_{1,8}$ = 0.8713
V_{max}	**P < 0.0001**	**P = 0.0043**	P = 0.7546	P = 0.9717
	$F_{1,8}$ = 76.2661	$F_{1,8}$ = 15.4679	$F_{1,8}$ = 0.1047	$F_{1,8}$ = 0.0013
V_{min}	**P = 0.0018**	P = 0.0546	P = 0.2122	P = 0.7929
	$F_{1,8}$ = 20.9681	$F_{1,8}$ = 5.0597	$F_{1,8}$ = 1.8379	$F_{1,8}$ = 0.0737
P_{max}	NS	P = 0.3541	P = 0.1007	P = 0.2018
		$F_{1,9}$ = 0.9543	$F_{1,9}$ = 3.3442	$F_{1,9}$ = 1.8958
P_{min}	NS	P = 0.6659	**P = 0.0409**	P = 0.6763
		$F_{1,9}$ = 0.1993	$F_{1,9}$ = 5.6904	$F_{1,9}$ = 0.1862
P_{mean}	NS	P = 0.4502	P = 0.5919	P = 0.1982
		$F_{1,9}$ = 0.6231	$F_{1,9}$ = 0.3089	$F_{1,9}$ = 1.9295
P_{dev}	NS	P = 0.2929	**P = 0.0456**	P = 0.1573
		$F_{1,9}$ = 1.2479	$F_{1,9}$ = 5.3756	$F_{1,9}$ = 2.3801
[1]EF	NS	P = 0.1764	**P = 0.0094**	**P = 0.0035**
		$F_{1,7}$ = 2.7737	$F_{1,7}$ = 15.4298	$F_{1,7}$ = 24.1286
dP/dt_{max}	NS	P = 0.4657	P = 0.2354	P = 0.8387
		$F_{1,9}$ = 0.5803	$F_{1,9}$ = 1.6172	$F_{1,9}$ = 0.0439
E_{es}	NS	P = 0.0665	P = 0.9468	P = 0.4243
		$F_{1,9}$ = 4.3571	$F_{1,9}$ = 0.0047	$F_{1,9}$ = 0.7005

f_H, heart rate; V_{max}, maximum left ventricle volume; V_{min}, minimum left ventricle volume; P_{max}, maximum left ventricle pressure; P_{min}, minimum left ventricle pressure; P_{mean}, mean left ventricle pressure; P_{dev}, pressure developed by left ventricle contraction; EF, ejection fraction; dP/dt_{max}, maximum derivative of pressure; E_{es}, end-systolic elastance, which is the slope of the end-systolic pressure-volume relationship. [1]Sex and family included in model as significant (P < 0.05) random factors. Statistical tests of all volume data used absolute values and accounted for body mass as a covariate, but the data are reported relative to body mass in Table 2. NS denotes situations in which body mass was omitted as a factor in final linear models because initial tests suggested that its effect did not near significance (P ≥ 0.1). Significant effects (P < 0.05) are shown in bold.

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CHAPTER 4: Effects of hypoxia on routine cardiovascular function and metabolism in mice

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4.1. ABSTRACT

Hypoxia can have significant impacts on cardiovascular physiology, but the effects of chronic exposure to moderate hypoxia and how they differ between sexes remain poorly understood. I used physiological telemetry to examine this issue in CD-1 mice. Adult mice were chronically exposed to normoxia or hypobaric hypoxia (12 kPa O_2) for 6 weeks, and then subjected to telemetry measurements of routine physiology across the diel cycle. Heart rate (f_H), mean arterial blood pressure (P_{mean}), body temperature (T_b), and activity were greater during the night-time active phase than the day-time inactive phase. Chronic hypoxia had no effect on these traits at night but had sex-specific effects during the day, when chronic hypoxia reduced f_H, T_b, and activity in males but not females. These differences existed without any effect of chronic hypoxia on α-adrenergic or nitric oxide tone on the vasculature (assessed as P_{mean} response to pharmacological blockade). Responses to acute hypoxia were then measured during stepwise reductions in inspired O_2 from 21 to 8 kPa O_2. O_2 consumption rate, f_H, P_{mean}, and T_b declined in severe hypoxia, but the O_2 tension at which this began was lower in mice held in chronic hypoxia. However, the hypoxic ventilatory response was augmented by exposure to chronic hypoxia in females

but not in males. Females also exhibited larger increases in lung mass and less right-ventricle hypertrophy than males in chronic hypoxia. My results support the growing evidence that there can be considerable sex differences in the cardiorespiratory responses to hypoxia.

4.2 INTRODUCTION

Exposure to low-O_2 conditions (hypoxia) can lead to several adjustments in cardiovascular physiology. Acute hypoxia stimulates the hypoxic chemoreflex, which can activate the sympathetic nervous system and lead to catecholamine release from the adrenal medulla (Davy et al., 1997; Hainsworth and Drinkhill, 2007; Hainsworth et al., 2007; Heistad and Abboud, 1980; Ivy and Scott, 2015; Johnson et al., 1983). This sympathoadrenal activation tends to stimulate cardiac output and cause α-adrenoreceptor-mediated vasoconstriction in some tissues, helping maintain O_2 supply to hypoxia-sensitive tissues like the brain and heart, and thus promoting survival (Heistad and Abboud, 1980; Ivy and Scott, 2015; Slotkin et al., 1988). However, in humans, sympathoadrenal activation can persist and become detrimental with chronic exposure to hypoxia such that α-adrenoreceptor stimulation can act in opposition to local vasodilatory factors, impede blood flow to some peripheral tissues, increase vascular resistance, and contribute to systemic hypertension (Calbet, 2003; Calbet et al., 2014; Hansen and Sander, 2003; Kanstrup et al., 1999; Lundby et al., 2018; Parati et al., 2014; Rhodes et al., 2011; Richalet et al., 1988; Schultz et al., 2014; Wolfel et al., 1994). Nevertheless, these responses are not always observed (Bernardi et al., 1998; Berthelsen et al., 2020; Dhar et al., 2018; Dhar et al., 2014;

Hooper et al., 2010; Marticorena et al., 1969; Reeves et al., 1987; Rostrup, 1998), likely because cardiovascular effects of chronic hypoxia depend on hypoxia severity (Allwood et al., 2018). Moderate sustained hypoxia is common in high-altitude environments and is typical of many disease states (Amalakanti and Pentakota, 2016; Calverley et al., 1982; DeMarco et al., 1981; Fleetham et al., 1982; Fletcher et al., 1987; Garpestad et al., 1994; Koo et al., 1975; Krachman et al., 2005; McNicholas et al., 2004; Mulloy and McNicholas, 1996; Soguel Schenkel et al., 1996; West, 1996; Wynne et al., 1979), but studies of the cardiovascular responses to moderate sustained hypoxia are relatively rare compared to the responses to severe or intermittent hypoxia.

Recent evidence suggests that mice may be more tolerant of environmental hypoxia than some other mammals, which could lead to species differences in the effects of chronic hypoxia on cardiovascular physiology. Compared to rats (*Rattus norvegicus*), house mice (*Mus musculus*) exhibit more pronounced ventilatory and metabolic responses to hypoxia, greater mass-specific lung volume and surface area, and greater arterial O_2 saturation in subacute or chronic hypoxia (Arias-Reyes et al., 2021; Jochmans-Lemoine et al., 2016). These differences likely lead to corresponding differences in tissue O_2 levels, such that mice exhibit less severe increases in haematocrit and blood haemoglobin content and less right ventricle hypertrophy (Arias-Reyes et al., 2021; Jochmans-Lemoine et al., 2016). Whether these differences are associated with variation in cardiovascular function is poorly understood. Like humans, rats subjected to chronic hypoxia exhibit prolonged sympathoexcitation and consequent increases in arterial blood pressure (Johnson et al., 1983; Mazzali et al., 2003; Siques et al., 2014; Vaziri and Wang, 1996). By contrast, in

studies of male house mice exposed to hypoxia for 24 h, mild hypoxia (~15 kPa O_2) had little effect on heart rate and blood pressure, whereas severe hypoxia (~9 kPa O_2) reduced these variables during the daytime inactive phase (Allwood et al., 2018). However, few studies have examined the cardiovascular responses to longer durations of chronic hypoxia in mice, potentially in part due to the challenges of making such measurements in unrestrained animals of small body size (Kramer and Kinter, 2003; Niemeyer, 2016).

In addition to the effects of variation in hypoxia tolerance between species, there may be significant sex differences in cardiorespiratory responses to chronic hypoxia. In humans, there are sex differences in the risk of developing both acute mountain sickness (Hou et al., 2019) and chronic mountain sickness (Villafuerte and Corante, 2016), suggesting that females and males differ in their sensitivity and responses to chronic hypoxia. Indeed, there appear to be significant sex differences in the chemosensory and cardiorespiratory responses to acute and chronic hypoxia in humans and other mammals (Dart et al., 2002; Gassmann et al., 2009; Soliz et al., 2012). For instance, female rats demonstrate a less pronounced haematological responses to chronic hypoxia than males (Joseph et al., 2000; Pequignot et al., 1997), in association with a stronger hypoxic ventilatory response and greater ventilatory acclimatization to hypoxia (Joseph et al., 2000), and similar differences have been demonstrated in mice (Huey et al., 2000; Jungbauer et al., 2017; Soliz et al., 2008). Therefore, studies investigating the effects of chronic hypoxia on cardiovascular function must properly account for potential variation between sexes.

In this study, I investigated the effects of chronic exposure to moderate levels of sustained hypoxia on cardiovascular function and on metabolic and respiratory physiology

in house mice. I examined these effects in both females and males to uncover potential sex-specific responses to chronic hypoxia. Mice were acclimated to normobaric normoxia (21 kPa O_2) or hypobaric hypoxia (12 kPa O_2) and then implanted with physiological telemeters for continuous remote measurement of heart rate, arterial blood pressure, body temperature, and routine activity in unrestrained and freely behaving animals. I also assessed chemoreflex function by measuring cardiorespiratory and metabolic responses during stepwise exposure to increasingly severe levels of acute hypoxia. I hypothesized that female mice would better resist the constraining effects of chronic hypoxia on metabolism and cardiovascular function than males, in association with sex differences in cardiorespiratory plasticity in response to chronic hypoxia.

4.3 MATERIALS AND METHODS

4.3.1 Animals and environmental treatments

Forty CD-1 IGS mice (20 males and 20 females) were purchased from Charles River Laboratories and held for 2 weeks in standard husbandry conditions (25˚C, 12:12-h light-dark photoperiod, unlimited access to standard rodent chow and water) at McMaster University. Mice were then assigned to one of two chronic exposure groups – normoxia (10 males and 12 females) or hypoxia (10 males and 8 females) – and were held in those conditions for 6 weeks. Normoxia consisted of standard husbandry conditions in normobaria (approx. 100 kPa barometric pressure, 20 kPa O_2). Hypobaric hypoxia (approx. 60 kPa barometric pressure, 12 kPa O_2) was created using previously described hypobaric chambers (Lui et al., 2015; McClelland et al., 1998). Hypoxic mice were briefly (<20 min)

returned to normobaria twice per week for cage cleaning and replenishment of food and water. All animal procedures followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

4.3.2 Surgical instrumentation of physiological telemeters

After 6 weeks of exposure to normoxia or hypoxia, mice were surgically instrumented with physiological telemeters for remote measurement of cardiovascular function, body temperature, and routine activity. Each mouse was placed in an anaesthetic induction chamber and a surgical plane of anaesthesia was induced using 3% isoflurane balanced with O_2 at 1500 ml min^{-1} before being transferred to a nose cone. The mouse was then subcutaneously dosed with 0.1 mg kg^{-1} buprenorphine dissolved in 0.5 ml sterile saline, and eye lubricant was applied to the eyes before the ventral surface of the neck was shaved and scrubbed using iodine and isopropyl alcohol. The mouse was then placed supine on a sterile surgical drape above a heating pad and anaesthesia was maintained at 1-2% isoflurane using a nose cone. A 15-mm incision was made along the midline of the neck, and the left carotid artery was carefully isolated by blunt dissection. The artery was then occlusively cannulated using the fluid-filled pressure catheter of a small-animal radiotelemetry implant capable of measuring blood pressure, temperature, and activity (HD-X11, Data Sciences International Inc., MN, USA). The implant was then tunnelled subcutaneously under the ventral skin (using sterile saline for lubrication) until it was located ventrolaterally to the abdomen. The neck incision was sutured closed using an

interrupted subcuticular suture (6-0 Vicryl with 10 mm reverse cutting needle, Ethicon Inc., NJ, USA) to prevent removal by the animal. Mice were then recovered from anaesthesia, placed and housed individually in cages with cellulose bedding (Teklad diamond dry, Envigo, IN, USA) in their respective chronic exposure condition to recover for 3 days. Recovering mice were subcutaneously provided with a combined dose of 0.1 mg kg^{-1} buprenorphine and 5 mg kg^{-1} carprofen in 1 ml sterile saline 8 h after surgery, and then another 5 mg kg^{-1} carprofen 12 h and 24 h after that. Unfortunately, 2 normoxic and 2 hypoxic male mice did not recover well from the implantation surgery and were humanely euthanized before undergoing further experimental procedures, and were not included in the final dataset. An additional hypoxic male and 3 females (1 normoxic, 2 hypoxic) physically damaged their telemeters before or during the data collection period, and were humanely euthanized as described below. However, mass data from these mice were collected and are included.

4.3.3 Physiological telemetry measurements during routine conditions

Following recovery from surgery, I continuously measured cardiovascular function, body temperature, and routine activity in freely behaving and unrestrained mice in each chronic exposure group. After the 3-day recovery period following surgery (which was sufficient for measured parameters to stabilize), mice were moved in their recovery cage from their chronic exposure condition into a temperature (25˚C) and humidity (50% relative humidity) controlled environmental chamber (O_2 Control In Vitro Glove Box, Coy Laboratory Products Inc., MI, USA) in which telemetry measurements were made.

Inflowing compressed air and nitrogen were regulated to attain normobaric normoxia (~20 kPa O_2) or normobaric hypoxia (~12 kPa O_2) at the same O_2 pressure as in the chronic exposure condition, such that normoxia-exposed mice were measured in normoxia and hypoxia-exposed mice were measured in hypoxia. It was necessary to make telemetry measurements in normobaric hypoxia, rather than in the hypobaric hypoxia used in the 6-week chronic exposure period, due to the limited ambient pressure range of the blood pressure sensors (670 to 800 mmHg). Mice were kept in the environmental chamber for 4 days, after which routine measurements were continuously acquired and recorded for 48 h using a Matrix 2.0 data acquisition system (MX2, Data Sciences International Inc.) and Ponemah® software (v. 6.30, Data Sciences International Inc.). Average values of activity, body temperature (T_b), heart rate (f_H) and mean arterial pressure (P_{mean}) were calculated for each hour over the 48-h measurement period. The daily maximum and daily minimum for each parameter are also reported here.

4.3.4 Pharmacological assessment of vascular control

A subset of instrumented mice from each chronic exposure group was used to determine the level of α-adrenergic and nitric oxide mediated vascular tone by measuring the responses of P_{mean} to pharmacological blockade of α-adrenergic receptors (α-AR) and nitric oxide synthase (NOS), respectively. Following the measurements under routine conditions described above, normoxia-exposed mice (5 males and 5 females) and hypoxia-exposed mice (5 males and 4 females) were given intraperitoneal bolus injections (0.02-ml g^{-1}) of sterile saline, phentolamine (α-AR antagonist; 30 mg kg^{-1} in saline; Sigma-Aldrich,

MO, USA), or L-NAME (Nω-Nitro-L-arginine methyl ester, a NOS antagonist; 5 mg kg^{-1} in saline; Sigma-Aldrich). Each mouse received all three injections, with one injection per day in random order over 3 successive days, with injections occurring between 1 pm and 2 pm on each day. P_{mean} was recorded continuously from an hour before injection until an hour after injection. Baseline (*i.e.*, pre-injection) P_{mean} was determined as the average P_{mean} between 6 and 30 min before injection. For the saline control, post-injection P_{mean} was calculated as the average P_{mean} between 6 and 60 min after injection. For phentolamine, post-injection P_{mean} was calculated as the minimum P_{mean} observed over a one-minute period between 6 and 60 min after injection. For L-NAME, post-injection P_{mean} was calculated as the maximum P_{mean} observed over a one-minute period between 6 and 60 min after injection. The change in P_{mean} (ΔP_{mean}) due to saline ($\Delta P_{mean,Sa}$), phentolamine ($\Delta P_{mean,\alpha\text{-}AR}$) and L-NAME ($\Delta P_{mean,NOS}$) injection was then calculated for each mouse by subtracting post-injection P_{mean} from pre-injection P_{mean} for each treatment. Finally, net responses were calculated for α-AR and NOS blockade in each mouse by subtracting $\Delta P_{mean,Sa}$ from $\Delta P_{mean,\alpha\text{-}AR}$ and $\Delta P_{mean,NOS}$, respectively. As such, any effect of the injection process itself was controlled for as much as possible. Peak responses of each drug always occurred within 60 min after injection, and the doses of phentolamine and L-NAME used were confirmed in preliminary experiments to elicit maximal responses in P_{mean}.

4.3.5 Metabolic and cardiorespiratory responses to acute stepwise hypoxia

I assessed the acute cardiorespiratory responses to progressive stepwise hypoxia in all mice. Each mouse was placed in an open-flow plethysmography chamber (530 ml) and left

for 20-60 min until O_2 consumption rate ($\dot{V}O_2$), f_H, and ventilation became stable (see details of measurements below). During this time, the chamber was supplied with normoxic air (21 kPa O_2) at a total flow rate of 600 ml min^{-1}, produced by mixing compressed O_2 and N_2 using precision flow meters (Sierra Instruments, CA, USA) and a mass flow controller (MFC-4, Sable Systems, NV, USA). Once $\dot{V}O_2$, f_H, and ventilation had stabilized, mice were kept in normoxia for another 20 min, after which the incurrent O_2 partial pressure (PO_2) was reduced in a stepwise manner to 16, 12, 10, 9, and 8 kPa O_2, with a period of 20 min at each PO_2 step.

Measurements of $\dot{V}O_2$, T_b, f_H, P_{mean}, and total ventilation were made at rest during the last 10 min at each PO_2 step. Incurrent and excurrent gas streams were subsampled, scrubbed of water vapour using pre-baked Drierite (W.A. Hammond Drierite, Xenia, OH, USA), and O_2 (FC-10, Sable Systems) and CO_2 (CA-10, Sable Systems) fractions were measured. $\dot{V}O_2$ was calculated using established equations for incurrent flow measurement (Lighton, 2018) and is expressed in volumes at standard temperature and pressure (STP). T_b, f_H and P_{mean} were measured using the radiotelemetry implant via the MX2 data acquisition system. Chamber temperature was simultaneously recorded using a thermocouple (PT-6; Physitemp Instruments, Clifton, NJ, USA), and total ventilation was measured by whole-body plethysmography as previously described [Ivy and Scott, 2017a,b]. All data were acquired (1 kHz sampling rate) using a PowerLab 16/32 data acquisition unit and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, USA)

using PhysioTel Connect (ADInstruments) to receive data from the MX2 system. Air convection requirement (ACR) is the quotient of total ventilation and $\dot{V}O_2$.

4.3.6 Heart and lung masses

Once in vivo physiological measurements were complete, mice were weighed and then euthanized with an overdose of anaesthetic (inhalation of >5% isoflurane vapour) followed by cervical dislocation. The right ventricle (RV), left ventricle and septum (LV+S), and the entirety of the lungs were dissected and weighed. I also supplemented these data with previously unpublished mass data from CD-1 mice used in an earlier study by the Scott lab (Ivy and Scott, 2017) to increase sample sizes for these particular measurements.

4.3.7 Statistics

I used linear mixed models using the lme4 package (Bates et al., 2015) in R Studio (v. 1.4.1103, RStudio Public Benefit Corporation, MA, USA). For the hourly measures of f_H, T_b, P_{mean}, and activity, I first tested for overall effects of chronic exposure environment, sex, time of day, and their interactions. However, this approach did not allow us to examine the effects of chronic hypoxia within each sex on its own. To achieve this, I also tested for effects of chronic exposure environment and time of day separately within females and within males. For daily maximum and minimum values of each of these parameters (f_H, T_b, P_{mean}, activity), for the net responses to α-AR and NOS blockade in P_{mean}, and for all mass data, I tested for effects of chronic exposure environment, sex, and their interaction. For measurements during acute stepwise hypoxia, I tested for effects of chronic exposure

environment and inspired $P\mathrm{O}_2$ separately within each sex. I initially included body mass as a covariate in statistical tests, but removed it from final reported models when P>0.10. The full results of final statistical models are included in Supplemental Tables (Tables S4.1-S4.8) and the salient findings are reported in the Results. Holm-adjusted Tukey's HSD post-hoc tests were performed to test for the pairwise differences between chronic exposure environments within a sex, or between sexes or inspired $P\mathrm{O}_2$ within an acclimation environment. Statistical analyses were carried out on absolute values of traits that were not corrected for body mass (because effects of body mass were accounted for in statistical models when significant), but some data presented here are expressed relative to body mass as is conventional in the literature (organ masses, $\dot{V}\mathrm{O}_2$, total ventilation). Data are generally presented as mean ± SEM, and data in bar graphs also report individual values as small circles.

4.4 RESULTS

4.4.1 Males exhibited greater right ventricle hypertrophy in chronic hypoxia, whereas females exhibited greater increases in lung mass

Chronic hypoxia increased right ventricle (RV) and lung masses, but the magnitude of these changes differed between the sexes. Females were 18-21% smaller than males (Table 4.1; main effect of sex on body mass, $P < 0.001$), so I accounted for variation in body mass as a covariate in statistical analyses of heart and lung masses. As expected, body mass had significant effects on left ventricle and septum (LV+S) mass ($P < 0.001$), RV mass ($P < 0.001$), and lung mass ($P = 0.012$). After accounting for this variation in body mass, LV+S

was unaffected by exposure to chronic hypoxia (environment effect, P = 0.348), although males had 1-5% larger LV+S masses than females overall (sex effect, P = 0.008) (Table 4.1). In contrast, chronic hypoxia increased RV mass (environment effect, P < 0.001), and the effect of chronic hypoxia was greater in males (56% increase compared to normoxic males) than in females (23% increase) (environment×sex, P = 0.013) (Table 4.1). As a result, the Fulton's index (RV / LV+S) – a common index for assessing RV hypertrophy – increased by 59% in chronic hypoxia in males (59% increase) but the effect of chronic hypoxia was not significant in females (environment×sex, P = 0.025) (Fig. 4.1A). Chronic hypoxia also increased lung mass (environment effect, P < 0.001), but in this case females exhibited a greater increase (37%) than males (25%) (Fig. 4.1B).

4.4.2 Chronic hypoxia affected routine heart rate, body temperature and activity in males but not females

In general, there was a strong diel cycle in heart rate (main effects of time of day, P < 0.001), with greater heart rates during the night-time active phase than during the daytime inactive phase (Fig. 4.2). Females tended to have higher heart rates (f_H) than males, leading to significant effects of sex (sex effect, P = 0.018; sex × time of day, P = 0.043) in full statistical tests of the overall effects of chronic exposure environment, sex, time of day, and their interactions. To better appreciate the effects of chronic hypoxia within each sex, I also conducted separate statistical tests within each sex. In males, chronic hypoxia reduced f_H during the daytime inactive phase (Fig. 4.2A, top panel), as reflected by a significant interaction between environment and time of day (P = 0.049). In contrast, chronic hypoxia

had no significant effects on f_H in females (Fig. 4.2A, bottom panel; environment effect, P = 0.570; environment × time of day, P = 0.906). These patterns of variation were also reflected in comparisons of minimum values of hourly f_H (Fig. 4.2B), for which females exhibited significantly higher values than males in hypoxia but similar values in normoxia in Tukey post-hoc tests.

Body temperature (T_b) across the diel cycle (Fig. 4.3) varied between sexes (sex×time of day, P = 0.003) and chronic exposure environments (environment×time of day, P = 0.004) in full statistical tests of the overall effects of chronic exposure environment, sex, and time of day. Within males, chronic hypoxia depressed T_b during the daytime inactive phase (Fig. 4.3A, top panel), as reflected by a significant interaction between chronic exposure environment and time of day (P = 0.026) in statistical tests in males alone. In females, however, T_b was unaffected by chronic hypoxia (Fig. 4.3A, bottom panel; environment effect, P = 0.601; environment×time, P = 0.053). Similar patterns of variation were observed for maximum hourly T_b (Fig. 3B, top panel) and minimum hourly T_b (Fig. 4.3B, bottom panel), although the statistical effects were not significant.

Reductions in heart rate and body temperature in males in chronic hypoxia appeared to be associated with reductions in daytime activity. As expected, activity varied appreciably throughout the daily cycle in both males and females (time of day effects, P ≤ 0.027), with maximum hourly activity roughly coinciding with maximum hourly f_H in the middle of the night (Fig. 4.4A). Chronic hypoxia reduced activity during the daytime inactive phase in males, as reflected by a significant pairwise difference in minimum activity between normoxia and hypoxia (Fig. 4.4B, bottom panel; P = 0.042), but there were no effects of

chronic hypoxia in females. Altogether, these results demonstrate that routine physiology and activity are more sensitive to chronic hypoxia in males than in females.

4.4.3 Blood pressure and vascular tone were maintained in chronic hypoxia

Mean arterial blood pressure (P_{mean}) tended to be highest during the night-time active phase (Fig. 4.5), concurrent with the increases in f_H, T_b, and activity described above, but there were no significant effects of chronic hypoxia (P = 0.301) or sex (P = 0.970) on P_{mean}. Pharmacological blockade of α-adrenergic receptors (α-ARs) or nitric oxide synthase (NOS) during the daytime had strong effects on P_{mean}, reflecting substantial baseline levels of vasoconstrictive and vasodilatory tone on the vasculature, but these levels were unaffected by chronic hypoxia and were similar between sexes (Table 4.2). Specifically, after accounting for the effects of saline injection (see Section 4.3.4), pharmacological blockade of α-ARs using phentolamine caused a net decrease in P_{mean} of 35-49 mmHg, whereas blockade of NOS using L-NAME resulted in a net increase in P_{mean} of 27-49 mmHg. These net responses to pharmacological blockade tended to be lower in females than in males, but neither the sex effects (P=0.405 and 0.106) nor the effects of chronic hypoxia (P=0.505 and 0.505) were significant.

4.4.4 Metabolic and cardiovascular responses to acute stepwise hypoxia at rest

In general, acute exposure to severe hypoxia reduced all metabolic and cardiovascular variables (PO_2 effects, P < 0.001), but there was variation between groups in the PO_2 at which this occurred (Fig. 4.6). The PO_2 at which acute hypoxia changed these variables

was determined from pairwise comparisons between measurements at each level of hypoxia to the measurements at 21 kPa O_2. In both males and females, mice held in normoxia depressed resting O_2 consumption rate ($\dot{V}O_2$), f_H, and P_{mean} at ≤ 12 kPa O_2, or ≤ 10 kPa O_2 for $\dot{V}O_2$ in females. In males, exposure to chronic hypoxia reduced the PO_2 range over which these variables were depressed to ≤ 9 ($\dot{V}O_2$ and f_H) or ≤ 10 kPa O_2 (P_{mean}) (Fig. 4.6, left panels). There was a similar effect of chronic hypoxia in females for f_H and P_{mean}, but not $\dot{V}O_2$ (Fig. 4.6, right panels). However, the qualitative effects of chronic hypoxia on T_b differed between males and females (Fig. 4.6D). For females, T_b was depressed at ≤12 kPa O_2 in normoxic mice but not until ≤10 kPa O_2 in chronically hypoxic mice. For males, in contrast, T_b was not depressed until ≤10 kPa O_2 in normoxic mice but at ≤12 kPa O_2 in chronically hypoxic mice.

4.4.5 Ventilatory acclimatization to hypoxia was more pronounced in females

I also examined whether there were sex differences in the effects of chronic hypoxia on the hypoxic ventilatory response (HVR) (Fig. 4.7). As expected, acute reductions in PO_2 tended to increase total ventilation and air convection requirement (ACR; the quotient of total ventilation and $\dot{V}O_2$) (PO_2 effects, P ≤ 0.003). In males, chronic hypoxia had no effect on total ventilation (P = 0.239) or ACR (P = 0.725). Conversely, in females, chronic hypoxia increased total ventilation and ACR compared to normoxic females (environment effects, P < 0.001 and P = 0.008, respectively), and the response of ACR to reductions in PO_2 was amplified by chronic hypoxia (acclimation × PO_2, P = 0.001). These findings

suggest that ventilatory acclimatization to hypoxia – the increase in total ventilation and the HVR in chronic hypoxia – is more pronounced in females than in males in CD-1 mice.

4.5 DISCUSSION

4.5.1 Overview

Hypoxia can have a range of effects on cardiovascular physiology but the effects of chronic hypoxia that is typical of high-altitude environments, and how they may differ between species and sexes, are poorly understood. Here, I show that the effects of chronic exposure to moderate hypoxia in mice are relatively modest and sex specific. Chronic hypoxia had little effect on heart rate, blood pressure, body temperature, and activity during the night-time active phase, and had no effect on α-adrenergic or NO-mediated tone on the vasculature. Chronic hypoxia tended to reduce f_H and T_b during the daytime inactive phase in association with a reduction in activity, but only in males. Females resisted the effects of chronic hypoxia on these traits, exhibited more pronounced increases in total ventilation and lung mass in chronic hypoxia than males, and suffered less right-ventricle hypertrophy. My results support the growing evidence that there can be considerable sex differences in the physiological effects of hypoxia.

4.5.2 Modest effects of chronic hypoxia on cardiovascular physiology in mice

The relatively modest effects of chronic hypoxia I observed may reflect a greater ability of mice to cope with hypoxia than some other mammals. Chronic hypoxia can be associated with chronic sympathoadrenal activation that leads to hypertension, as has been observed

in humans and rats (Calbet, 2003; Calbet et al., 2014; Hansen and Sander, 2003; Johnson et al., 1983; Kanstrup et al., 1999; Lundby et al., 2018; Mazzali et al., 2003; Parati et al., 2014; Rhodes et al., 2011; Richalet, 2016; Schultz et al., 2014; Siques et al., 2014; Vaziri and Wang, 1996; Wolfel et al., 1994). However, I saw no evidence of increased arterial blood pressure or of any changes in α-adrenergic or NO-mediated tone on the vasculature (Fig. 4.5; Table 4.2). This may result from mice being more effective at pulmonary O_2 uptake in hypoxia than some other small mammals like rats (*Rattus norvegicus*), by virtue of having a more pronounced hypoxic ventilatory response and greater pulmonary O_2 diffusing capacity, which combine to increase arterial O_2 saturation in hypoxia (Arias-Reyes et al., 2021; Jochmans-Lemoine et al., 2016). Mice also exhibit smaller increases in haematocrit in chronic hypoxia than rats (Arias-Reyes et al., 2021; Jochmans-Lemoine et al., 2016), so species differences in blood viscosity could contribute to differences in blood pressure.

The lack of any changes in α-adrenergic tone was unexpected when considering previous observations on the effects of chronic hypoxia on vascular responsiveness to α-adrenergic stimulation. For example, I showed that chronic hypoxia reduces the capacity of white-footed mice (*Peromyscus leucopus*) to increase blood pressure in response to α-adrenergic stimulation (Wearing et al., 2022). Chronic hypoxia has also been shown to downregulate the expression of α-adrenergic receptors in peripheral tissues in other species, such as humans and sheep (Fischetti et al., 2000; Ueno et al., 1997). We also measured NO-mediated vasomotor tone to assess whether compensatory changes in vasodilatory influences may help offset changes in adrenergic vasoconstriction in chronic

hypoxia, but found no evidence of any such responses. It is possible that responses of these traits to chronic hypoxia are less pronounced in laboratory mice than in these other species. However, my findings here may also suggest that changes in the sensitivity and/or capacity for responding to α-adrenergic stimulation may occur without any changes in resting tone. This could arise if reductions in vascular responsiveness to α-adrenergic stimulation are offset by increases in sympathetic drive in chronic hypoxia.

4.5.3 Cardiorespiratory responses to chronic hypoxia in mice are sex specific

Males responded to chronic hypoxia with reductions in heart rate, body temperature, and activity during the daytime that may reflect a general reduction in metabolism and O_2 demands. Two lines of evidence suggest that these responses were facultative rather than a result of tissue O_2 limitation. Firstly, f_H, T_b, and activity during the night-time active phase were unaffected by chronic hypoxia (Figs. 4.2-4.4). Secondly, the level of hypoxia used in chronic exposures (12 kPa O_2) was not severe enough to reduce resting rates of O_2 consumption or f_H (Fig. 4.6). However, exposure to chronic hypoxia increased the PO_2 at which T_b depression began to 12 kPa O_2 (Fig. 4.6D, left panel). This suggests that hypoxia-induced anapyrexia may have led to declines in T_b during the daytime inactive phase, but that this mechanism was over-ridden during the increased activity levels exhibited at night. Hypoxia-induced anapyrexia likely does not arise from the activation of peripheral chemoreceptors, unlike the ventilatory responses to hypoxia (Steiner and Branco, 2002), which may help explain the divergent sex differences in the responses to chronic hypoxia for each of these traits.

Females differed from males in their response to chronic hypoxia. Unlike males, females did not exhibit any reductions in routine f_H, T_b, or activity in chronic hypoxia. Females also demonstrated much more pronounced increases in total ventilation and the hypoxic ventilatory response after chronic hypoxia exposure (Fig. 4.7), reflecting a greater magnitude of ventilatory acclimatization to hypoxia, and they also exhibited much greater increases in lung mass (Fig. 4.1). These sex-specific effects of chronic hypoxia in mice could result from the effects of sex hormones on hypoxia responses. Indeed, work in rats has provided evidence that ovarian steroids help stimulate the hypoxic ventilatory response via a dopaminergic pathway and thus underlie sex differences in ventilation (Huang et al., 2018; Joseph et al., 2000; Joseph et al., 2002). However, ventilatory acclimatization to hypoxia may not differ between sexes in humans, at least after a few hours of hypoxia (Fatemian et al., 2016), suggesting that some sex differences in the response to chronic hypoxia may be species specific.

Female mice also exhibited less right-ventricle hypertrophy in chronic hypoxia than males (Fig. 4.1). Right-ventricle hypertrophy is considered to be a pathological response of the heart to hypoxic pulmonary hypertension, which can lead to changes in gene expression associated with cell stress and inflammation, and in severe cases can lead to right heart failure and death (Bartsch et al., 2005; Sylvester et al., 2012; Velotta et al., 2018; West et al., 2021; Young et al., 2019). As such, a blunting of this response should provide clear advantages to health and fitness in chronic hypoxia. My findings in mice are consistent with previously observed sex differences in rats, in which females showed a less pronounced increase in pulmonary artery pressure in response to chronic hypoxia than male

rats (Rabinovitch et al., 1981). This suggests that the physiological responses to chronic hypoxia in females may be preferable for avoiding some of the pathological effects of hypoxia exposure. By exhibiting greater increases in breathing and lung size, female mice may achieve greater arterial O_2 saturation in chronic hypoxia and thus experience milder levels of tissue hypoxia than their male counterparts. In humans, however, women may be more susceptible to pulmonary hypertension than men (Martin and Pabelick, 2014), again illustrating that at least some effects of sex on hypoxia sensitivity may be species specific.

4.6 CONCLUSIONS

My findings contribute to the growing appreciation that sex is a key determinant of the sensitivity and physiological responses to hypoxia in mammals. The effects of chronic exposure to moderate hypoxia in mice were relatively mild, with no evidence of systemic hypertension. Males responded to chronic hypoxia by reducing activity and metabolic O_2 demands during the inactive phase, whereas females maintained normal activity in association with an augmented capacity for respiratory O_2 uptake. These species and sex differences in the effects of chronic hypoxia should be carefully considered when using mice as a biomedical model for humans. Interestingly, some of the effects of sex on hypoxia responses appear to differ between mice and other species. Further mechanistic understanding of why and how the effects of sex on the cardiovascular responses to chronic hypoxia differ between species would be a fruitful avenue for future research.

4.7 FIGURES AND TABLES

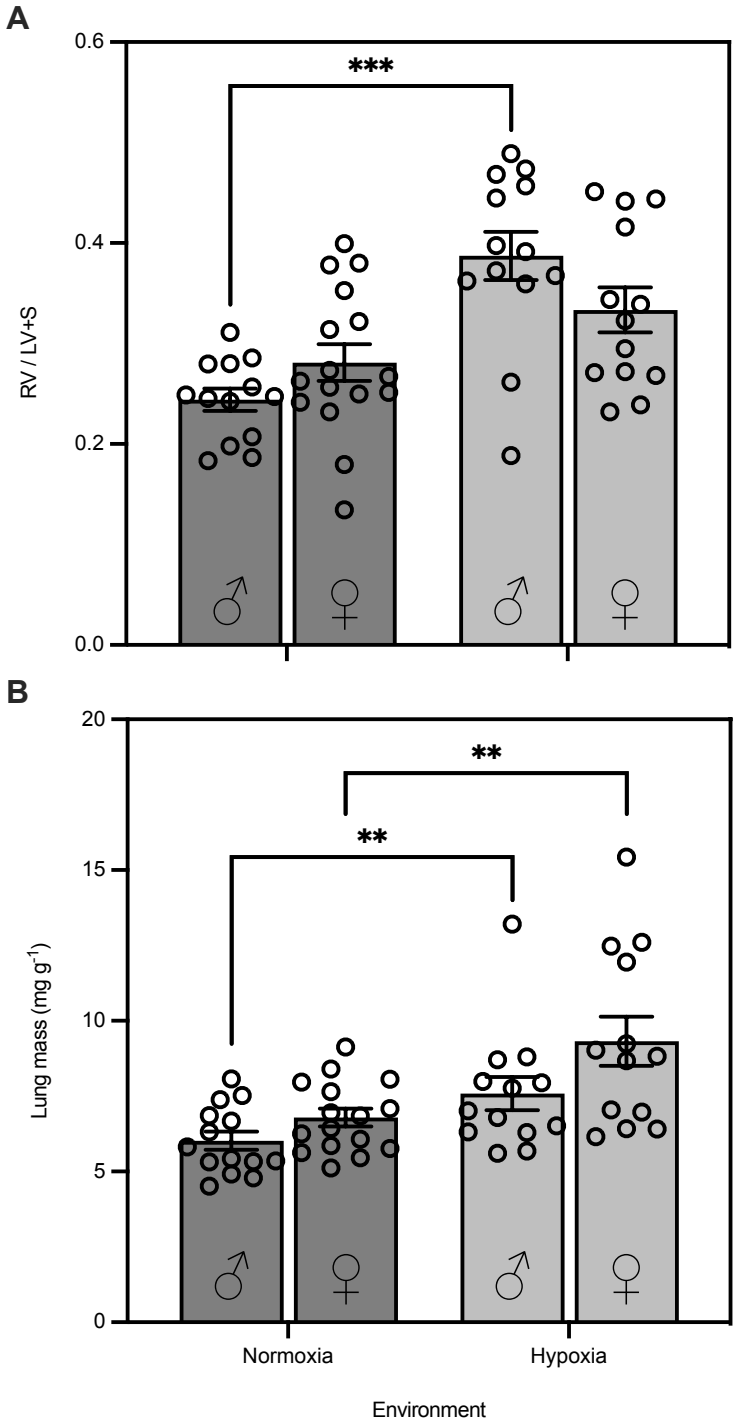

Fig. 4.1 (**A**) Right ventricle mass relative to the combined mass of the left ventricle and septum (RV / LV+S) and (**B**) lung mass relative to body mass in male (♂) and female (♀) CD-1 mice chronically exposed to normoxia (21 kPa O_2) or hypoxia (12 kPa O_2). Bars display mean ± SEM with individual data as circles. ** and *** represent significant pairwise difference between acclimation environments within a sex ($P < 0.01$ and 0.001, respectively).

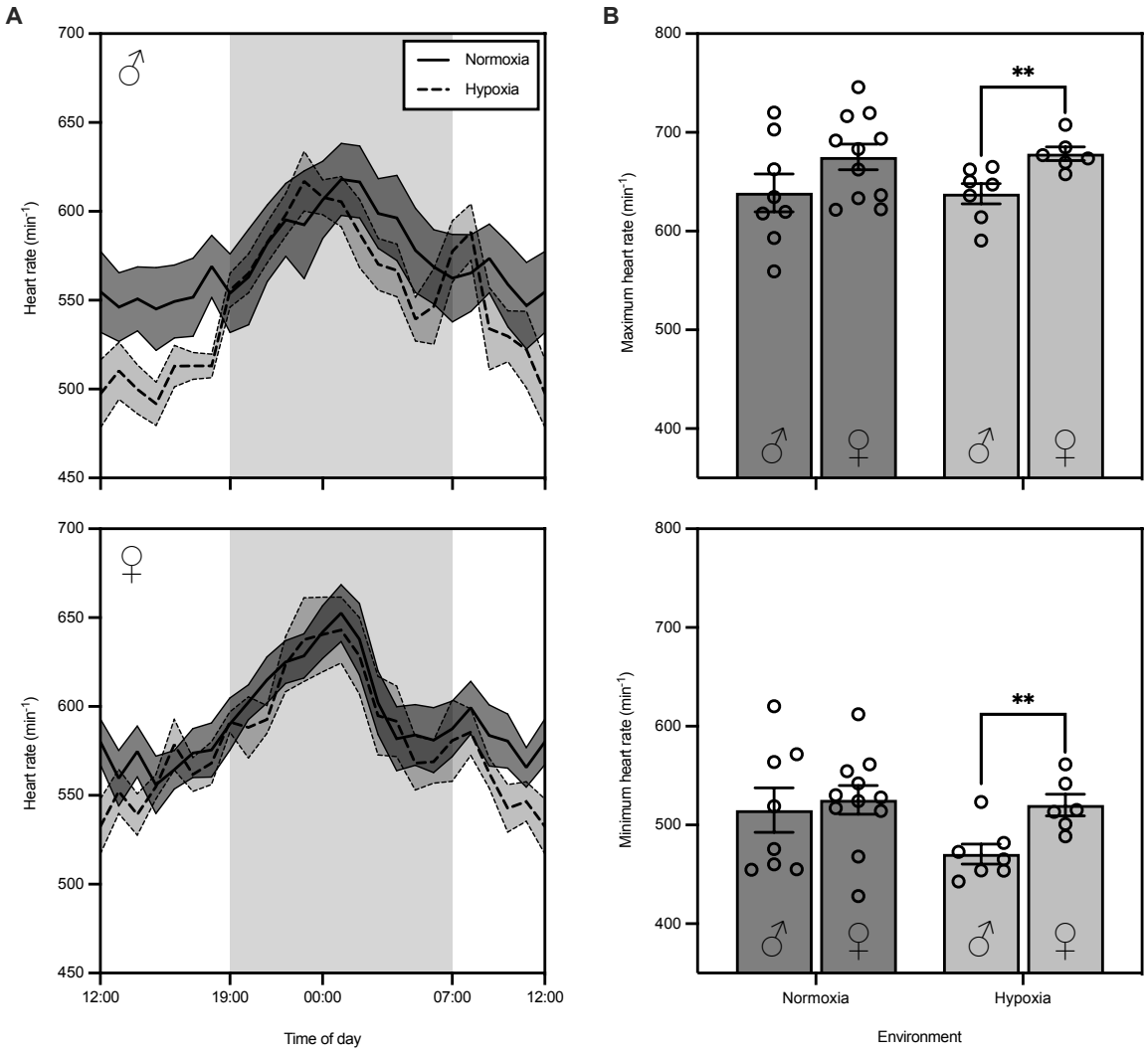
A
B
♂
♀
Normoxia
Hypoxia
Heart rate (min-1)
Time of day
12:00
19:00
00:00
07:00
12:00
Maximum heart rate (min-1)
Minimum heart rate (min-1)
**
Environment
Normoxia
Hypoxia

Fig. 4.2 Routine heart rate (f_H) of male (♂) and female (♀) CD-1 mice that were chronically exposed to and measured in normoxia (21 kPa O_2) or hypoxia (12 kPa O_2). **A**) Routine f_H for each hour of the diel cycle in male (top panel) and female (bottom panel) mice in normoxia (solid line) or hypoxia (dashed line). Data are presented as mean ± SEM (normoxic males, n = 8; hypoxic males, n = 7; normoxic females, n = 11; hypoxic females, n = 6), and the shaded background (19:00-07:00) illustrates when lights were off. **B**) Maximum (top panel) and minimum (bottom panel) hourly f_H. Bars display mean ± SEM with individual data as circles. ** represents a significant pairwise difference between sexes within an acclimation environment ($P < 0.01$).

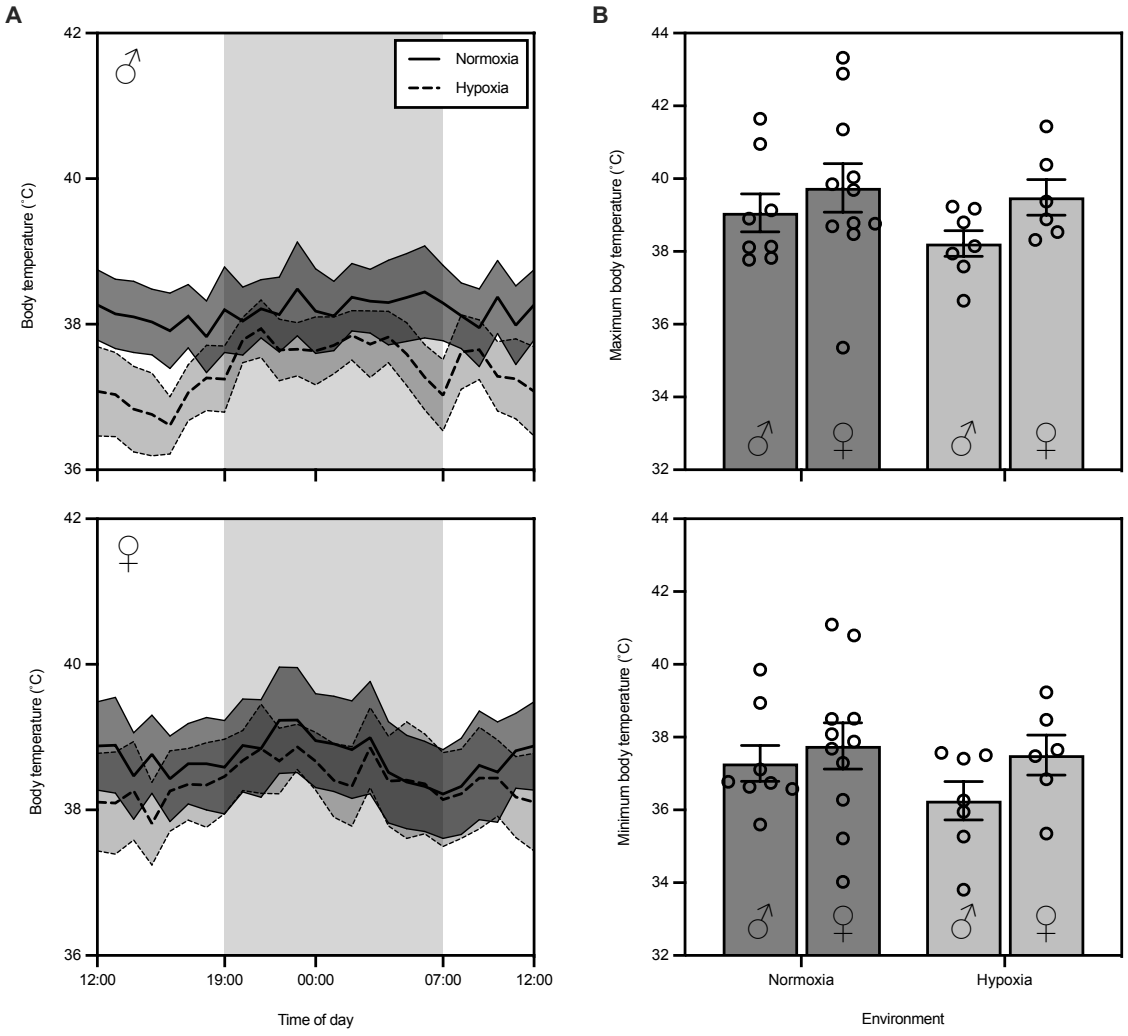
A
B
Normoxia
Hypoxia
Body temperature (˚C)
Maximum body temperature (˚C)
Minimum body temperature (˚C)
Time of day
Environment
12:00
19:00
00:00
07:00
12:00
Normoxia
Hypoxia

Fig. 4.3 Routine body temperature (T_b) of male (♂) and female (♀) CD-1 mice chronically exposed to and measured in normoxia (21 kPa O_2) or hypoxia (12 kPa O_2). **A**) Routine T_b for each hour of the diel cycle in male (top panel) and female (bottom panel) mice in normoxia (solid line) or hypoxia (dashed line). **B**) Maximum (top panel) and minimum (bottom panel) hourly T_b. See Fig. 4.2 for sample sizes and additional details.

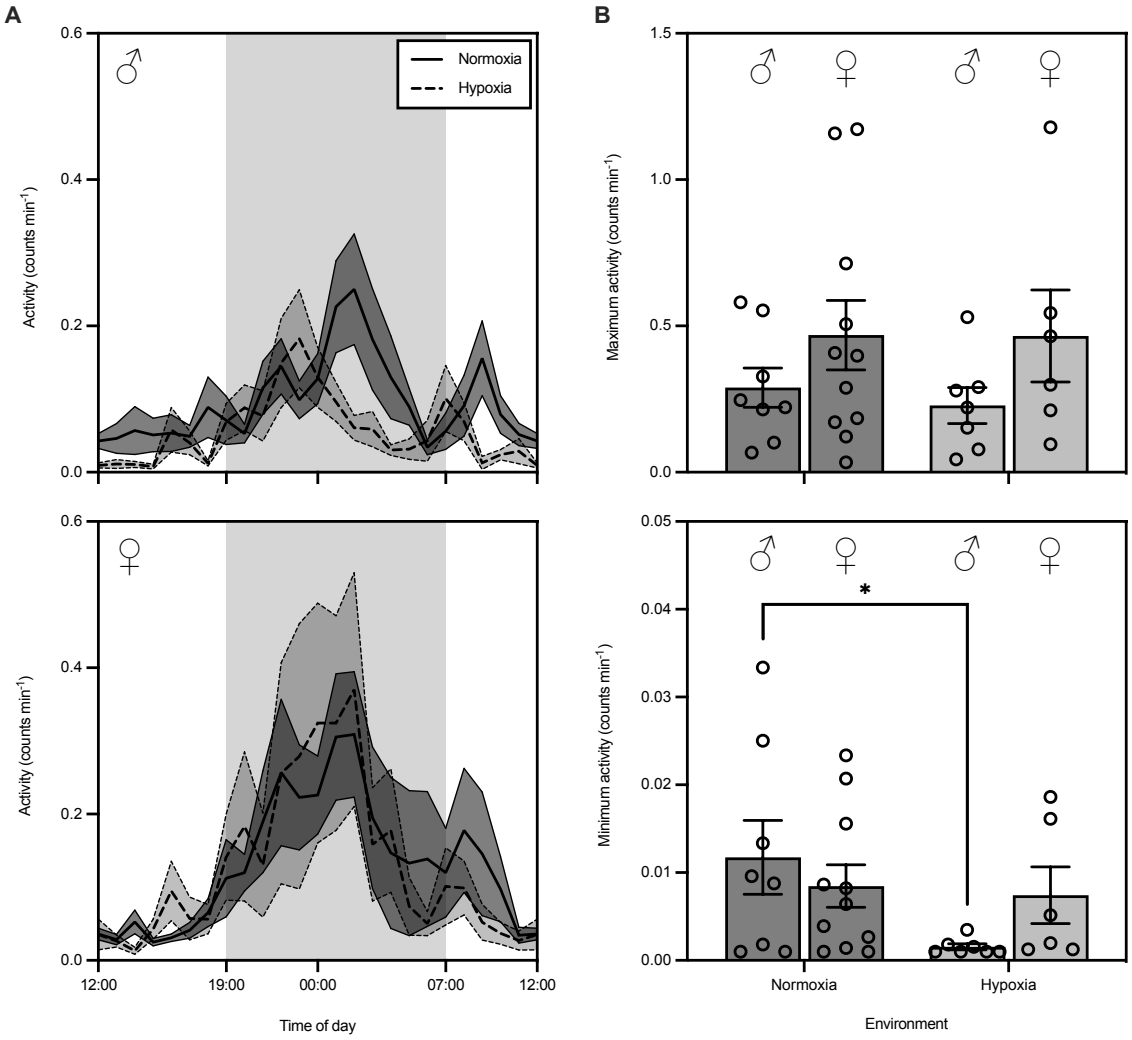
A
B
♂
♀
Normoxia
Hypoxia
Activity (counts min-1)
Maximum activity (counts min-1)
Minimum activity (counts min-1)
0.0
0.2
0.4
0.6
0.5
1.0
1.5
0.01
0.02
0.03
0.04
0.05
*
12:00
19:00
00:00
07:00
12:00
Time of day
Normoxia
Hypoxia
Environment

Fig. 4.4 Routine activity of male (♂) and female (♀) CD-1 mice chronically exposed to and measured in normoxia (21 kPa O_2) or hypoxia (12 kPa O_2). **A**) Routine activity for each hour of the diel cycle in male (top panel) and female (bottom panel) mice in normoxia (solid line) or hypoxia (dashed line). **B**) Maximum (top panel) and minimum (bottom panel) hourly activity. * represents a significant pairwise difference between acclimation environments within a sex ($P < 0.05$). See Fig. 4.2 for sample sizes and additional details.

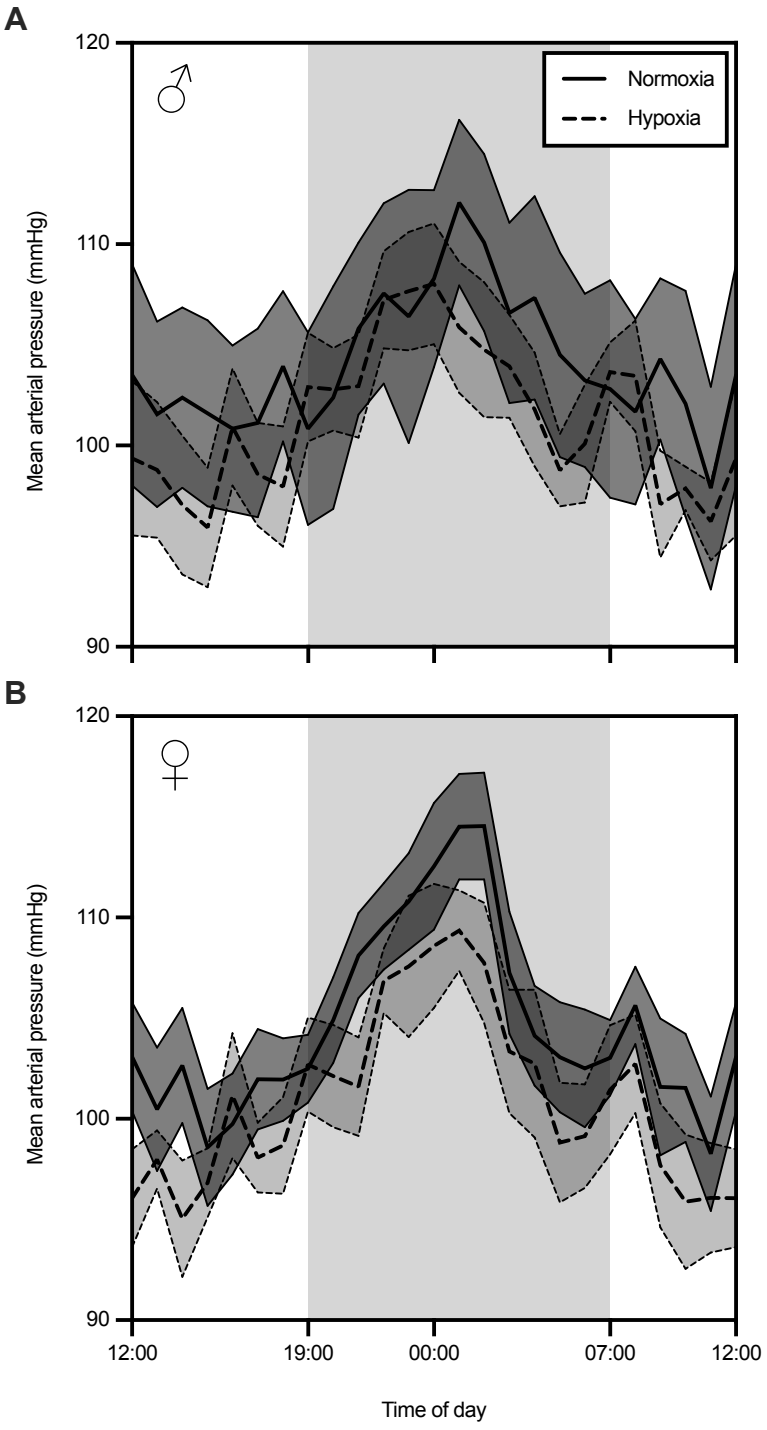
A
B
♂
♀
Normoxia
Hypoxia
Mean arterial pressure (mmHg)
120
110
100
90
12:00
19:00
00:00
07:00
12:00
Time of day

Fig. 4.5 Routine mean arterial pressure (P_{mean}) for each hour of the diel cycle in male (♂, **A**) and female (♀, **B**) CD-1 mice chronically exposed to and measured in normoxia (21 kPa O_2; solid line) or hypoxia (12 kPa O_2; dashed line). See Fig. 4.2 for sample sizes and additional details.

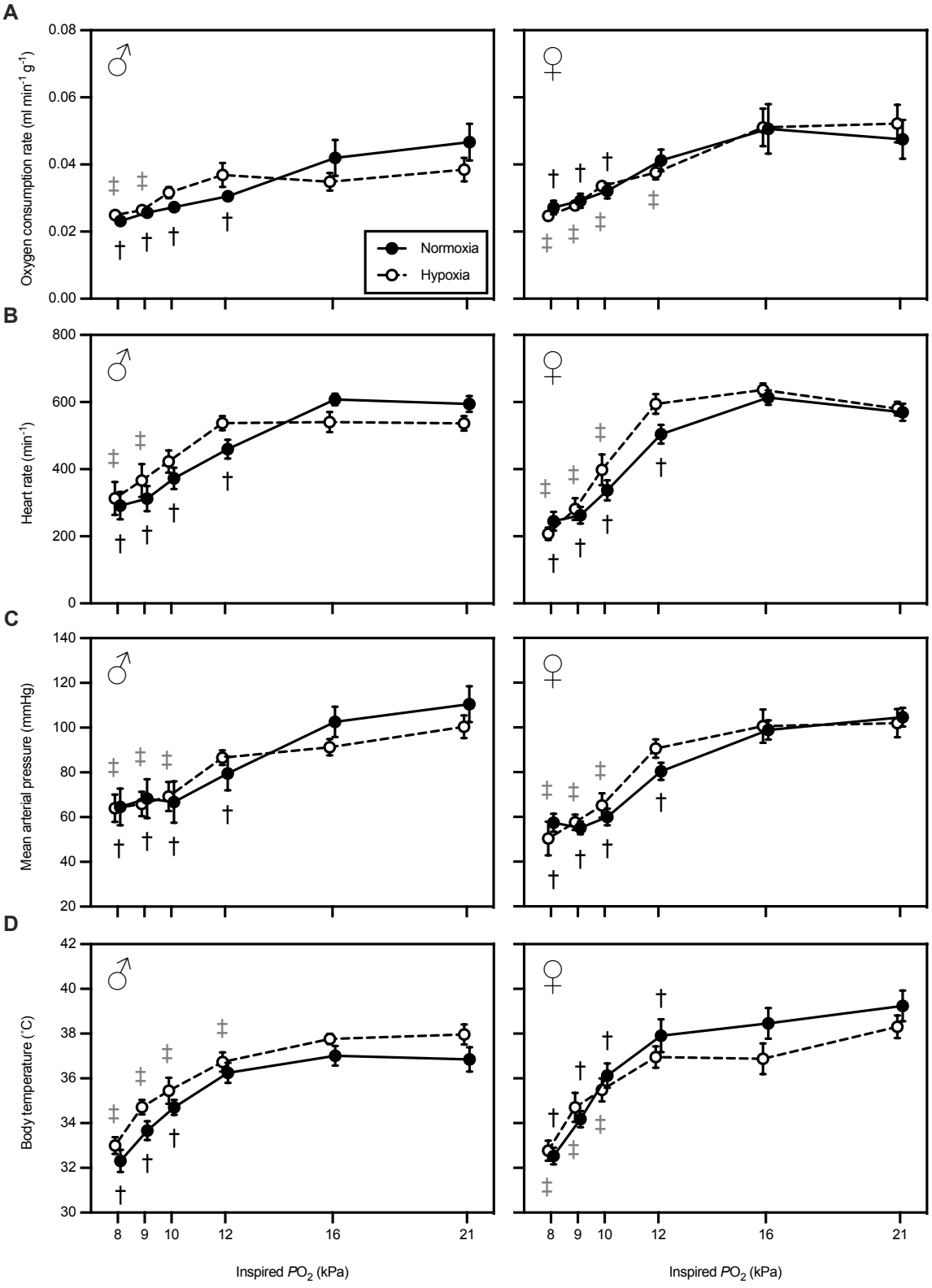
A
B
C
D
♂
♀
Oxygen consumption rate (ml min^{-1} g^{-1})
Heart rate (min^{-1})
Mean arterial pressure (mmHg)
Body temperature (˚C)
Normoxia
Hypoxia
Inspired P_{O_2} (kPa)
Inspired P_{O_2} (kPa)

Fig. 4.6 Effects of acute stepwise hypoxia on (**A**) resting O_2 consumption rate ($\dot{V}O_2$), (**B**) heart rate, (**C**) mean arterial pressure, and (**D**) body temperature of male (♂, left panels) and female (♀, right panels) CD-1 mice chronically exposed to normoxia (21 kPa O_2, black circles) or hypoxia (12 kPa O_2, white circles). Data are presented as mean ± SEM (normoxic males, n = 8; hypoxic males, n = 7; normoxic females, n = 11; hypoxic females, n = 6), with data offset along the x-axis for clarity. † and ‡ represent significant pairwise differences compared to the value at 21 kPa O_2 in mice acclimated to normoxia or hypoxia, respectively ($P < 0.05$).

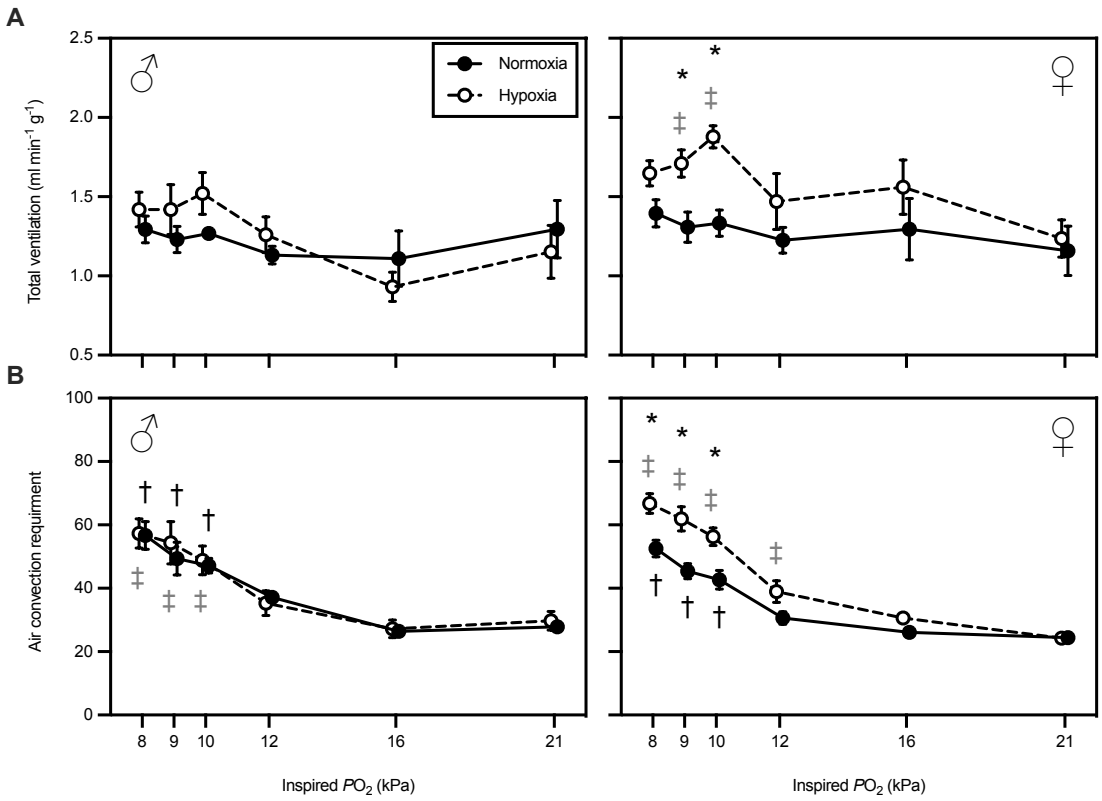
A
B
♂
♀
Normoxia
Hypoxia
Total ventilation (ml min^{-1} g^{-1})
Air convection requirment
Inspired PO_2 (kPa)
Inspired PO_2 (kPa)

Fig. 4.7 Effects of acute stepwise hypoxia on resting total ventilation (**A**) and air convection requirement (ACR, **B**) of male (♂, left panels) and female (♀, right panels) CD-1 mice chronically exposed to normoxia (21 kPa O_2, black circles) or hypoxia (12 kPa O_2, white circles). † and ‡ represent significant pairwise differences compared to the value at 21 kPa O_2 in mice acclimated to normoxia or hypoxia, respectively ($P < 0.05$). * represents significant pairwise differences between acclimation environments within a sex and inspired O_2 partial pressure (PO_2) ($P < 0.05$). See Fig. 4.6 for sample sizes.

Table 4.1 Body and heart masses of male and female CD-1 mice chronically exposed to normoxia (21 kPa O_2) or hypoxia (12 kPa O_2).

	Males		Females	
	Normoxia	Hypoxia	Normoxia	Hypoxia
n	14	13	16	13
Body mass (g)	38.54 ± 2.27	38.52 ± 1.51	30.33 ± 1.07*	31.57 ± 1.23*
LV+S mass (mg g^{-1})	3.289 ± 0.073	3.298 ± 0.132	3.136 ± 0.104*	3.263 ± 0.117†
RV mass (mg g^{-1})	0.806 ± 0.028	1.262 ± 0.083†	0.875 ± 0.062	1.079 ± 0.070†

LV+S, left ventricle and septum; RV, right ventricle (heart masses were not measured in one normoxic male). Data are presented as mean ± SEM. † represents a significant pairwise difference between acclimation environments within a sex in Holm-adjusted Tukey's HSD post-tests ($P < 0.05$). * represents a significant pairwise difference between sexes within an acclimation environment in Holm-adjusted Tukey's HSD post-tests ($P < 0.05$).

Table 4.2 Vascular tone via α-adrenergic receptors and nitric oxide was unaltered by chronic hypoxia in both male and female CD-1 mice.

	Males		Females	
	Normoxia	Hypoxia	Normoxia	Hypoxia
n	5	5	5	4
Saline				
Pre-injection P_{mean}	108.4 ± 3.3	86.7 ± 3.5	105.9 ± 5.2	90.6 ± 5.1
Post-injection P_{mean}	110.2 ± 5.3	103.8 ± 5.0	111.7 ± 1.9	107.5 ± 5.4
$\Delta P_{mean,Sa}$	1.7 ± 3.5	17.1 ± 3.4	5.9 ± 6.1	16.9 ± 8.7
Phentolamine				
Pre-injection P_{mean}	102.8 ± 5.5	90.5 ± 5.2	96.0 ± 3.7	97.3 ± 5.8
Post-injection P_{mean}	55.5 ± 7.1	67.0 ± 8.7	63.3 ± 6.0	79.5 ± 7.0
$\Delta P_{mean,\alpha\text{-}AR}$	-47.3 ± 5.4	-23.4 ± 9.9	-32.7 ± 6.6	-17.8 ± 12.7
L-NAME				
Pre-injection P_{mean}	107.3 ± 2.7	87.7 ± 3.8	103.0 ± 4.3	95.1 ± 6.1
Post-injection P_{mean}	157.6 ± 7.7	140.2 ± 2.6	135.6 ± 4.1	141.7 ± 4.3
$\Delta P_{mean,NOS}$	50.3 ± 6.7	52.5 ± 1.4	32.6 ± 7.5	46.6 ± 3.6
Net responses				
α-AR blockade	-49.0 ± 8.4	-40.5 ± 11.7	-38.6 ± 6.3	-34.7 ± 9.5
NOS blockade	48.6 ± 6.3	35.4 ± 4.8	26.7 ± 12.4	29.7 ± 5.8

L-NAME, N_{ω}-Nitro-L-arginine methyl ester; α-AR, α-adrenergic receptor; NOS, nitric oxide synthase. ΔP_{mean}, change in mean arterial blood pressure due to injection of saline ($\Delta P_{mean,Sa}$), phentolamine ($\Delta P_{mean,\alpha\text{-}AR}$) or L-NAME ($\Delta P_{mean,NOS}$). Data are presented as mean ± SEM. Net responses were calculated for α-AR blockade and NOS blockade by subtracting $\Delta P_{mean,Sa}$ from $\Delta P_{mean,\alpha\text{-}AR}$ and $\Delta P_{mean,NOS}$, respectively. Statistical analyses were performed to assess any effects of sex and acclimation environment on these net responses, but no effects were detected.

4.8 SUPPLEMENTAL TABLES

Table S4.1 Results of statistical comparisons using linear mixed models on mass data for male and female CD-1 mice chronically exposed to normoxia (21 kPa O_2) or hypoxia (12 kPa O_2).

Trait	Body mass effect	Sex (*s*) effect	Acclimation (*a*) effect	*s* x *a* effect
Body mass	NA	**P < 0.001**	P = 0.691	P = 0.691
LV+S mass	**P < 0.001**	**P = 0.008**	P = 0.348	P = 0.559
RV mass	**P < 0.001**	P = 0.353	**P < 0.001**	**P = 0.013**
RV / LV+S	NS	P = 0.572	**P < 0.001**	**P = 0.025**
Lung mass	**P = 0.012**	P = 0.511	**P < 0.001**	P = 0.588

LV+S, left ventricle and septum; RV, right ventricle. Significant effects (P < 0.05) are shown in bold.

Table S4.2 Results of statistical comparisons using linear mixed models on diel activity, body temperature and cardiovascular data for male and female CD-1 mice chronically exposed to and measured in normoxia (21 kPa O_2) or hypoxia (12 kPa O_2).

Trait	Sex (*s*) effect	Time (*t*) effect	Acclimation (*a*) effect	*s* x *t* effect	*s* x *a* effect	*t* x *a* effect	*s* x *t* x *a* effect
Activity	P = 0.372	**P = 0.003**	P = 0.887	P = 0.361	P = 0.529	P = 0.135	P = 0.650
T_b	P = 0.109	**P = 0.024**	P = 0.213	**P = 0.003**	P = 0.685	**P = 0.004**	P = 0.990
f_H	**P = 0.018**	**P < 0.001**	P = 0.127	**P = 0.043**	P = 0.395	P = 0.197	P = 0.145
P_{mean}	P = 0.970	P = 0.081	P = 0.301	P = 0.711	P = 0.886	P = 0.951	P = 0.970

T_b, body temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Significant effects ($P < 0.05$) are shown in bold.

Table S4.3 Results of statistical comparisons using linear mixed models on diel activity, body temperature and cardiovascular data for male CD-1 mice only.

Trait	Acclimation (*a*) effect	Time (*t*) effect	*a* x *t* effect
Activity	P = 0.530	**P = 0.020**	P = 0.232
T_b	P = 0.159	**P < 0.001**	**P = 0.026**
f_H	P = 0.154	**P < 0.001**	**P = 0.049**
P_{mean}	P = 0.611	P = 0.286	P = 0.985

T_b, body temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Significant effects (P < 0.05) are shown in bold.

Table S4.4 Results of statistical comparisons using linear mixed models on diel activity, body temperature and cardiovascular data for female CD-1 mice only.

Trait	Acclimation (*a*) effect	Time (*t*) effect	*a* x *t* effect
Activity	P = 0.666	**P = 0.027**	P = 0.264
T_b	P = 0.601	P = 0.638	P = 0.053
f_H	P = 0.570	**P = 0.033**	P = 0.906
P_{mean}	P = 0.276	P = 0.161	P = 0.948

T_b, body temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Significant effects ($P < 0.05$) are shown in bold.

Table S4.5 Results of statistical comparisons using linear mixed models on maximum and minimum hourly values of routine activity, body temperature and cardiovascular data for male and female CD-1 mice chronically exposed to and measured in normoxia (21 kPa O_2) or hypoxia (12 kPa O_2).

Trait	Acclimation (*a*) effect	Sex (*s*) effect	*a* x *s* effect
Max. Activity	P = 0.624	P = 0.077	P = 0.798
Min. Activity	P = 0.074	P = 0.877	P = 0.141
Max. T_b	P = 0.283	P = 0.129	P = 0.633
Min. T_b	P = 0.239	P = 0.193	P = 0.530
Max. f_H	P = 0.823	**P = 0.013**	P = 0.887
Min. f_H	P = 0.107	P = 0.117	P = 0.249
Max. P_{mean}	P = 0.121	P = 0.249	P = 0.807
Min. P_{mean}	P = 0.965	P = 0.107	P = 0.845

T_b, body temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Significant effects ($P < 0.05$) are shown in bold.

Table S4.6 Results of statistical comparisons using linear mixed models on the net response of mean arterial blood pressure (ΔP_{mean}) to intraperitoneal injection of saline or pharmacological blockade in male and female CD-1 mice chronically exposed to and measured in normoxia (21 kPa O_2) or hypoxia (12 kPa O_2).

Blockade	Sex (*s*) effect	Acclimation (*a*) effect	*s* x *a* effect
α-AR	P = 0.405	P = 0.505	P = 0.803
NOS	P = 0.106	P = 0.505	P = 0.339

α-AR, α-adrenergic receptor; NOS, nitric oxide synthase. Net responses were calculated as the difference between the effect of pharmacological blockade and the effect of saline.

Table S4.7 Results of statistical comparisons using linear mixed models on O_2 consumption rate, body temperature and cardiorespiratory physiology during acute stepwise reductions in O_2 partial pressure (PO_2) in male CD-1 mice only.

Trait	Body mass effect	Acclimation (*a*) effect	PO_2 (*p*) effect	*a* x *p* effect
$\dot{V}O_2$	P = 0.062	P = 0.529	**P < 0.001**	P = 0.074
T_b	NS	P = 0.058	**P < 0.001**	P = 0.964
f_H	**P = 0.033**	P = 0.335	**P < 0.001**	**P = 0.036**
P_{mean}	**P = 0.010**	P = 0.056	**P < 0.001**	P = 0.400
Total ventilation	**P = 0.048**	P = 0.239	**P = 0.003**	P = 0.266
ACR	NS	P = 0.725	**P < 0.001**	P = 0.910

$\dot{V}O_2$, O_2 consumption rate; T_b, body temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure; ACR, air convection requirement = total ventilation / $\dot{V}O_2$. Significant effects ($P < 0.05$) are shown in bold.

Table S4.8 Results of statistical comparisons using linear mixed models on O_2 consumption rate, body temperature and cardiorespiratory physiology during acute stepwise reductions in O_2 partial pressure (PO_2) in female CD-1 mice only.

Trait	Body mass effect	Acclimation (*a*) effect	PO_2 (*p*) effect	*a* x *p* effect
$\dot{V}O_2$	NS	**P = 0.040**	**P < 0.001**	P = 0.381
T_b	NS	P = 0.468	**P < 0.001**	P = 0.077
f_H	NS	P = 0.408	**P < 0.001**	P = 0.054
P_{mean}	NS	P = 0.732	**P < 0.001**	P = 0.306
Total ventilation	NS	**P < 0.001**	**P = 0.001**	P = 0.246
ACR	P = 0.097	**P = 0.008**	**P < 0.001**	**P = 0.001**

$\dot{V}O_2$, O_2 consumption rate; T_b, body temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure; ACR, air convection requirement = total ventilation / $\dot{V}O_2$. Significant effects (P < 0.05) are shown in bold.

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CHAPTER 5: Evolved reductions in body temperature and the metabolic costs of thermoregulation in deer mice native to high altitude

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5.1 ABSTRACT

The evolution of endothermy was instrumental to the diversification of birds and mammals, but the energetic demands of maintaining high body temperature (T_b) could offset the advantages of endothermy in some environments. I hypothesised that reductions in T_b help high-altitude natives overcome the metabolic challenges of cold and hypoxia in their native environment. Deer mice (*Peromyscus maniculatus*) from high-altitude and low-altitude populations were bred in captivity to the second generation, and were acclimated as adults to warm normoxia or cold hypoxia for 6 weeks. Surgically implanted telemeters were used to measure T_b and cardiovascular function throughout the diel cycle. Cold hypoxia increased metabolic demands, as reflected by increased heart rate and food consumption, with the former associated with reduced vagal tone on the heart. These increased metabolic demands were offset by plastic reductions in T_b (~2˚C) in response to cold hypoxia, and highlanders had consistently lower T_b (~1˚C) than lowlanders. Empirical and theoretical evidence suggested that these T_b reductions together reduce metabolic demands by ~10-30%. Therefore, plastic and evolved reductions in T_b can help mammals overcome the metabolic challenges at high altitude, and may be a valuable energy-saving strategy in some non-hibernating endotherms in extreme environments.

5.2 INTRODUCTION

The evolution of endothermy and the ability to maintain high body temperature (T_b) has been instrumental to the success and diversification of mammals and birds (Hillenius and Ruben, 2004; Polymeropoulos et al., 2018). By maintaining high T_b and correspondingly high metabolic rates, endotherms can remain active and support metabolically demanding processes such as locomotion (*e.g.*, for hunting, foraging, competing, and/or evading predators) and reproduction across broad ranges of environmental temperature (Bennett and Ruben, 1979; Clarke and Portner, 2010; Farmer, 2003; Hillenius and Ruben, 2004). However, heat generation (thermogenesis) necessary for maintaining high T_b can itself be energetically demanding, thus leading to high food demands and requiring that O_2 and metabolic fuels be supplied to thermogenic tissues at high rates (Bennett and Ruben, 1979; Clarke and Portner, 2010). Periods of limited food availability and/or high thermogenic requirements (*e.g.*, over winter) can make these demands untenable, such that some endotherms have the ability to temporarily depress T_b and thermogenesis during times of seasonal hibernation or torpor (Bicego et al., 2007; Carey et al., 2003; Geiser, 2004; Levesque and Tattersall, 2010; Staples, 2016). Whether evolved reduction in T_b setpoint may help endotherms reduce metabolic demands in cold environments when they are not hibernating or torpid is less clear. Broad macroevolutionary comparisons suggest that non-hibernating endotherms in cold environments maintain Tb similar to or even slightly greater than their temperate counterparts (Lovegrove, 2003; Moreira et al., 2021; Scholander et al., 1950a). However, such broad comparisons do not consider the possibility that reductions in T_b setpoint may

have arisen within some distinct lineages as a valuable mechanism for coping with prolonged metabolic challenges.

The extreme environment at high altitude provides examples of animals that have evolved to live in metabolically challenging conditions that are both unavoidable and unremitting. Physiological homeostasis requires that animals balance the supply and demand of O_2 and metabolic fuels for tissues, but this balance can be extremely difficult to maintain at high altitude. Cold temperatures persist year-round at high altitude, raising the demands of aerobic thermogenesis, while low O_2 availability (hypoxia) can limit O_2 supply to support aerobic metabolism (McClelland and Scott, 2019; Storz et al., 2019; Storz and Scott, 2019; Storz et al., 2010b). High-altitude natives somehow overcome this challenge, successfully supplying O_2 and metabolic fuels at sufficient rates to meet tissue demands. This is achieved in several high-altitude mammals and birds via environmentally-induced plasticity and evolved modifications across the O_2 transport pathway to augment tissue O_2 supply in cold hypoxia (Burtscher et al., 2018; Faraci, 1991; Gilbert-Kawai et al., 2014; Ivy and Scott, 2015; McClelland and Scott, 2019; Storz and Scott, 2019; Storz et al., 2010b). However, although many high-altitude endotherms do not hibernate, relatively little attention has been paid to whether reductions in T_b setpoint might help offset metabolic demands. This could be a particularly valuable energy-saving strategy in high-altitude environments, where hypoxia can constrain the ability to increase metabolic rate.

Deer mice (*Peromyscus maniculatus*) native to high altitude are a powerful model in which to examine the evolution of T_b regulation (Storz et al., 2019; Storz and Scott, 2019; Storz and Scott, 2021; Storz et al., 2010b). Deer mice are found across North America

(Bedford and Hoekstra, 2015) and have the largest altitudinal range of any North American mammal (Hock, 1964). High-altitude populations of deer mice maintain higher field metabolic rates than their low-altitude counterparts (Hayes, 1989a; Hayes, 1989b), likely due to the heightened costs of thermogenesis at high altitudes. High-altitude deer mice also exhibit a high aerobic capacity for thermogenesis during adulthood, achieved through both plastic and evolved changes in physiological pathways of O_2 and metabolic fuel transport (Lau et al., 2017; Lui et al., 2015; Lyons et al., 2021; Mahalingam et al., 2020; Mahalingam et al., 2017; Storz et al., 2010a; Storz et al., 2007; Tate et al., 2017; Tate et al., 2020; Velotta et al., 2016). Therefore, high-altitude deer mice have a heightened capacity for supplying O_2 and metabolic fuels to tissues, like some other high-altitude taxa. Whether high-altitude deer mice have also reduced the routine demands for O_2 and metabolic fuels is less clear, but such reductions could be highly advantageous when considering that in some instances food availability may also be limited at high altitudes (Bears et al., 2009). The ontogenetic development of endothermy is delayed in high-altitude deer mice (Robertson and McClelland, 2019; Robertson and McClelland, 2021; Robertson et al., 2019), suggesting that metabolic demands of thermogenesis are reduced in early post-natal life stages. Whether they also exhibit strategies to reduce the metabolic demands of thermogenesis and T_b regulation in later life remains unresolved.

In this study, I test the hypothesis that reductions in T_b setpoint help high-altitude deer mice reduce metabolic demands and thus cope with the challenges of life at high altitude. Populations of deer mice native to high altitude and low altitude were each bred in captivity. Second-generation mice from both populations were raised to adulthood, and then

acclimated to warm normoxia and cold hypoxia in a full-factorial design. I predicted that acclimation to cold hypoxia would reduce T_b, and that overlaid upon this plastic response, high-altitude populations would have evolved to operate at a lower T_b than low-altitude populations. I made use of physiological telemetry devices able to measure T_b and other physiological variables throughout the diel cycle, in order to avoid the confounding effects of handling, tethering, or anaesthesia (Meyer et al., 2017). This also enabled measurements of cardiovascular function to provide refined insight into the metabolic demands in cold and hypoxic environments.

5.3 MATERIAL AND METHODS

5.3.1 Animals and environmental acclimations

Wild 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) and at low altitude in the Great Plains of Nebraska (Buffalo County, NE, USA at 40˚41’58’’N, 99˚04’53’’W; approx. 660 m above sea level). These wild adults were transported to McMaster University (Hamilton, ON, Canada; 50 m above sea level), and bred within their respective populations for two generations to produce second-generation (G_2) progeny. These progeny were kept under standard normoxic laboratory conditions (25˚C, ~20 kPa O_2, 12:12-h light-dark photoperiod) until experimentation. At six months of age, mice from each population were assigned to each of two environmental treatments: warm normoxia (25˚C, 20 kPa O_2; 15 lowlanders and 13 highlanders) or cold hypoxia (5˚C, 12 kPa O_2; 12 lowlanders and 15 highlanders). Warm normoxia consisted of standard laboratory

conditions. Cold hypoxia was created using previously described hypobaric chambers (Lui et al., 2015; McClelland et al., 1998) inside a temperature controlled environmental chamber held at 5˚C. Mice were always provided with unlimited access to standard rodent chow and water. Mice in cold hypoxia were briefly (<20 min) returned to normobaria twice per week for cage cleaning and replenishment of food and water.

5.3.2 Surgical instrumentation of physiological telemeters

After 6 weeks of acclimation to warm normoxia or cold hypoxia, deer mice were anaesthetized using isoflurane and surgically instrumented with physiological telemeters (HD-X11, Data Sciences International Inc.) using methods I have previously described (Wearing and Scott, 2022a). Telemetry implants were positioned subcutaneously on the back in the interscapular space for remote measurement of routine body temperature (T_b). The left carotid artery was cannulated with the pressure catheter of the telemeter to measure heart rate (f_H) and arterial blood pressure (P_{mean}). Mice were recovered from surgery for 3 days in their respective environmental conditions.

Anaesthesia was induced in an anaesthetic induction chamber using 3% isoflurane balanced with O_2 delivered at 1500 ml min^{-1}. Once a surgical plane of anaesthesia was reached, mice were transferred to a nose cone delivered with isoflurane for maintaining anaesthesia, given a subcutaneous dose of buprenorphine (0.1 mg per kg body weight; dissolved in 0.5 ml sterile 0.9% saline), eye lubricant was applied, and the ventral surface of the neck was shaved and scrubbed using iodine and isopropyl alcohol. The mouse was placed supine on a sterile surgical drape above a heating pad and a surgical plane of

anaesthesia was maintained using 1-2% isoflurane delivered to the nose cone. A 15-mm incision was made along the midline of the neck, and the left carotid artery was carefully isolated by blunt dissection. The artery was occlusively cannulated using the fluid-filled pressure catheter of a small-animal radiotelemetry implant capable of measuring blood pressure and temperature (HD-X11, Data Sciences International Inc., MN, USA). The main body of the telemetry implant was tunnelled subcutaneously to a dorsal location in the interscapular space (a location that was determined in preliminary surgeries to be best tolerated by deer mice). The neck incision was sutured closed using an interrupted subcuticular suture (6-0 Vicryl with 10 mm reverse cutting needle, Ethicon Inc., NJ, USA). Mice were then recovered from anaesthesia, placed and housed individually in cages with cellulose bedding (Teklad diamond dry, Envigo, IN, USA) in their respective environmental conditions. Recovering mice were provided with a combined subcutaneous dose of 0.1 mg kg^{-1} buprenorphine and 5 mg kg^{-1} carprofen in 1 ml sterile saline 8 h after surgery, and then another 5 mg kg^{-1} carprofen 12 h and 24 h after that. Unfortunately, 1 warm normoxic lowlander, 1 cold hypoxic lowlander and 2 cold hypoxic highlanders recovered poorly from surgery and were immediately euthanized. These mice were omitted from the final telemetry dataset.

5.3.3 Telemetry measurements of routine physiology

Following recovery from surgery, I continuously measured routine T_b and cardiovascular function of freely behaving, unrestrained deer mice. Measurements were made at the same temperature and O_2 levels to which the mice had been acclimated using

a temperature and O_2 controlled cabinet (O_2 Control In Vitro Glove Box, Coy Laboratory Products Inc., MI, USA), in which inflowing air and nitrogen were mixed to create warm normobaric normoxia or cold normobaric hypoxia. It was necessary to carry out the telemetric measurements in normobaria (rather than hypobaria) due to the limited pressure calibration range of the blood pressure sensor (670 to 800 mmHg). Mice were held for 4 d in these conditions to ensure that all telemetry measurements had stabilized at normal values, after which routine physiological measurements were continuously acquired for 48 h using a Matrix 2.0 data acquisition system and Ponemah® software (Data Sciences International), concurrent with measurements of daily food and water consumption. Hourly means of body temperature (T_b), heart rate (f_H) and mean arterial pressure (P_{mean}) were calculated over the 24-h daily cycle, and the maximum and minimum hourly values were determined.

5.3.4 Pharmacological assessment of cardiovascular control

Following measurements of routine physiology, a subset of mice in warm normoxia (6 lowlanders and 5 highlanders) and cold hypoxia (5 lowlanders and 6 highlanders) were used to determine β_1-adrenergic and vagal tone on the heart. This was achieved by measuring the f_H responses to pharmacological blockade of cardiac β_1-adrenergic receptors (β_1-AR) and muscarinic acetylcholine receptors (mAChR), respectively. Metoprolol (β_1-AR blocker) and atropine (mAChR blocker) were administered on separate consecutive days in random order. Each was given in a series of hourly intraperitoneal (IP) injections of increasing concentration, starting at 2 pm local time (metoprolol – 0.04, 0.4, 4, and 40

mg per kg body mass; atropine – 0.05, 0.5, 5, and 50 mg kg^{-1}; each injected at a volume of 20 ml kg^{-1} in sterile saline). Baseline (*i.e.*, pre-injection) f_H was the average between 45 and 15 min before the first injection on each day. The minimum (metoprolol) or maximum (atropine) f_H over a one-minute period was determined 15 to 45 min after each injection. The maximal f_H response (Δf_H) to each blocker was calculated by subtracting post-injection f_H for the dose of blocker eliciting the greatest f_H change from baseline pre-injection f_H. Maximal Δf_H was used as an index of chronotropic tone on the heart.

5.3.5 Physiological responses to acute stepwise hypoxia

I used acute manipulations of inspired O_2 pressure (PO_2) to examine the relationship between body temperature (T_b) and O_2 consumption rate ($\dot{V}O_2$), conducted the day after pharmacological assessments of chronotropic tone on the heart. Each mouse was placed in an open-flow plethysmography chamber (530 ml) and left for 20 to 60 min to become accustomed to the chamber (as reflected by stable resting $\dot{V}O_2$). PO_2 was reduced every 20 min in stepwise increments – 21, 16, 12, 10, 9, and 8 kPa O_2. The desired PO_2 was achieved by mixing compressed O_2 and N_2 using precision flow meters (Sierra Instruments, CA, USA) and a mass flow controller (MFC-4, Sable Systems, NV, USA). Incurrent gas was sub-sampled at 200 ml min^{-1} and used to measure incurrent O_2 with a fuel cell O_2 analyzer (FC-10, Sable Systems). Incurrent flow rate into the chamber (~600 ml min^{-1}) was measured using a precision flow meter (Alicat Scientific, Inc.). Excurrent gas leaving the animal chamber was subsampled at 200 ml min^{-1}, scrubbed of water vapour (Drierite, W.A. Hammond Drierite Co., Ltd., OH, USA), and analyzed for O_2 (FC-10, Sable Systems) and

CO_2 (CA-10, Sable Systems). These data were acquired using a PowerLab 16/32 and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, USA). T_b measured by the telemeter implant was acquired continuously (1 kHz sampling rate) using a Matrix 2.0 data acquisition system, and using PhysioTel Connect (ADInstruments, CO, USA) to record the data in Labchart 8 Pro. $\dot{V}O_2$ was determined at rest during the last 10 min at each PO_2 bout, calculated using established formulas (Lighton, 2018). The relationship between changes in $\dot{V}O_2$ and T_b were examined for each of the four experimental groups using linear regressions of relative $\dot{V}O_2$ (quotient of $\dot{V}O_2$ at reduced PO_2 and $\dot{V}O_2$ at 21 kPa O_2) against T_b (see Supplemental Figure and Tables, Fig. S5.1).

5.3.6 Organ masses and haematology

Once in vivo physiological measurements were complete, mice were weighed and humanely euthanized by overdose of isoflurane followed by decapitation. Blood was collected, haemoglobin content was measured using Drabkin's reagent (according to instructions from the manufacturer, Sigma-Aldrich), and blood was centrifuged at 12,700g for 5 min to measure haematocrit. Masses of heart ventricles, lungs, liver, and interscapular brown adipose tissue were then measured.

5.3.7 Theoretical consideration of the effects of T_b on metabolic rate

I contextualized the empirical measurements of the relationships between T_b and $\dot{V}O_2$ using two approaches. First, I calculated the theoretically expected effects of a 1˚C change

in T_b (ΔT_b) on metabolic rate based on Q_{10} temperature coefficients of 2 and 3, using the following equation for Q_{10}:

$$Q_{10} = \left(\frac{V_1}{V_2}\right)^{\frac{10}{\Delta T_b}}$$

where V_1 is the $\dot{V}O_2$ at the lower temperature and V_2 is the $\dot{V}O_2$ at the higher temperature.

Second, I calculated the expected effects of a 1˚C change in T_b using the Scholander-Irving model of thermoregulation (Scholander et al., 1950b). Stable T_b is maintained when rates of heat loss are balanced by equal rates of metabolic heat production. As a result, $\dot{V}O_2$ at ambient temperatures (T_a) below the thermoneutral zone is a function of the difference between T_b and T_a, and the thermal conductance (C) of the animal (as determined by insulation, etc.):

$$\dot{V}O_2 = C\ (T_b - T_a)$$

Therefore, independent of any changes in C, the effect of a 1˚C change in T_b can be calculated as follows:

$$\left(\frac{V_1}{V_2}\right) = \left(\frac{T_{b1} - T_a}{T_{b2} - T_a}\right)$$

where T_{b1} is the lower T_b and T_{b2} is the higher T_b.

5.3.8 Statistics

Linear mixed models (lme4 package (Bates et al., 2015) in R Studio, v. 1.4.1103, RStudio Public Benefit Corporation, MA, USA) were used to test for effects of population, environment, and time of day on routine physiology data collected across the entire 24-h period. Data were first tested for both populations together to evaluate whether populations

differed in their response to cold hypoxia, after which the response to cold hypoxia was tested separately in each population independent of the other. Models were also run to test for effects of population and environment on maximum and minimum hourly values of routine physiology, food and water consumption, body and organ masses, haematology, and Δf_H for β_1-AR and vagal blockade. Sex and family were included as random factors in all statistical models, but their effects were never significant. Individual was included as a random factor for data with repeated measures across the entire 24-h period. Statistical analyses of organ mass data were carried out on absolute values and included body mass as a covariate, but are presented here relative to body mass as is conventional in the literature. The full results of the linear mixed models are included in Supplemental Figure and Tables (Tables S5.1-S5.7), and the salient findings are reported in the Results. Holm-adjusted Tukey's HSD post-hoc tests were performed to test for pairwise differences between environments within a population, or between populations within an environment. Data are presented as individual values (small circles) and mean ± SEM (bars) unless otherwise stated.

5.4 RESULTS

5.4.1 Body temperature (T_b) is reduced by plastic and evolved responses to cold hypoxia

There was significant diel variation in T_b (time effect, P = 0.013), with increasing T_b during the night-time active phase, and a rapid reduction in T_b within the first few hours of light during the day-time inactive phase (Fig. 5.1a,b). Overlaid upon this diel variation were

significant main effects of environment ($P < 0.001$) and population ($P < 0.001$) on T_b. Exposure to cold hypoxia reduced T_b compared to warm normoxic mice, as reflected by strong effects of environment on T_b within both populations ($P < 0.001$). Maximum (Fig. 5.1c) and minimum (Fig. 5.1d) hourly T_b were 1.9-2.4˚C lower on average in cold hypoxia than in warm normoxia (environment effects, $P < 0.001$). However, highlanders exhibited lower T_b than lowlanders throughout the diel cycle both in warm normoxia and in cold hypoxia (Fig. 5.1a,b), and maximum and minimum T_b were 0.7-1.0˚C lower on average in highlanders (Fig. 5.1c,d). Furthermore, in highlanders only, the reduction in T_b during the early morning hours was more rapid in cold hypoxia than in warm normoxia (environment×time, $P = 0.001$). Therefore, both plastic responses to cold hypoxia and evolved changes in the high-altitude population reduced T_b throughout the diel cycle.

Empirical and theoretical evidence suggested that reductions in T_b likely help reduce the metabolic demands of thermogenesis. To investigate the effect of changes in T_b on metabolism, I measured resting O_2 consumption rate ($\dot{V}O_2$) during acute reductions in inspired O_2 (from 21 kPa O_2 to 8 kPa O_2) to reduce T_b by hypoxic anapyrexia, a well described and pervasive response to severe acute hypoxia across animals (Steiner and Branco, 2002). I then quantified the correlation between relative changes in $\dot{V}O_2$ and T_b. As expected, resting $\dot{V}O_2$ was reduced at lower T_b, and the magnitude of $\dot{V}O_2$ reduction was 5.5-8.1% on average per 1˚C reduction in T_b across groups (Supplemental Figure and Tables, Fig. S5.1). I also used two theoretical approaches to consider the effects of T_b on metabolic rate. First, I calculated the expected effects of temperature on biological rate processes using Q_{10} temperature coefficients of 2 and 3. Indeed, Q_{10} for the effects T_b on

resting $\dot{V}O_2$ is ~2.1-2.2 in humans and small non-torpid mammals (Geiser, 1988; Kampmann and Bröde, 2015). The expected effect of a 1˚C decrease in T_b is a 6.7% reduction in $\dot{V}O_2$ for a Q_{10} of 2 and a 10.4% reduction in $\dot{V}O_2$ for a Q_{10} of 3. Second, I used the Scholander-Irving model of thermoregulation (Scholander et al., 1950b), in which $\dot{V}O_2$ below the thermoneutral zone is a function of thermal conductance and the difference between T_b and ambient temperature. In this case, the expected effect of a 1˚C decrease in T_b on $\dot{V}O_2$ was ~3.4%. Based on the magnitude of these relative effects, the plastic ~2˚C reduction in T_b in response to cold hypoxia combined with the evolved ~1˚C reduction in the high-altitude population could reduce $\dot{V}O_2$ by ~10-30%. These data suggest that reductions in T_b help reduce metabolic demands at high altitude.

5.4.2 Cold hypoxia is associated with higher heart rates and food consumption, and preservation of mean arterial blood pressure

Heart rates were elevated by ~80-200 beats per minute in cold hypoxia, as reflected by significant main effects of environment ($P < 0.001$) on f_H across the diel cycle across populations (Fig. 5.2a,b). Maximum f_H was 17-20% higher and minimum f_H was 25-30% higher in cold hypoxia compared to warm normoxia in both populations (environment effects, $P < 0.001$) (Fig. 5.2c,d). Similarly, daily food consumption was 37-43% greater in cold hypoxia than in warm normoxia in both populations (environment effect, $P < 0.001$), with no significant variation in daily water consumption ($P = 0.122$) (Table 5.1). Unlike T_b, there were no significant effects of population on f_H ($P = 0.920$), food consumption ($P = 0.505$), or water consumption ($P = 0.114$).

Mean arterial blood pressure (P_{mean}) was maintained in cold hypoxia relative to warm normoxia in both populations. P_{mean} exhibited diel variation that mirrored the variation in T_b and f_H (time effects, $P < 0.001$), with 6-12 mmHg higher average pressures during the night-time active phase, but there were no significant effects of environment ($P = 0.926$) or population ($P = 0.829$) (Fig. 5.3a,b). There were 28-37 mmHg differences in pressure between maximum and minimum P_{mean}, but these metrics were also unaffected by population and environment (Fig. 5.3c,d).

5.4.3 Increased heart rates in cold hypoxia were underlain by changes in vagal tone

Pharmacological assessments of autonomic tone on the heart revealed significant decreases in vagal inhibitory tone in cold hypoxia. Pharmacological blockade of muscarinic acetylcholine receptors with atropine increased f_H, and this index of vagal tone was reduced by 44-63% in cold hypoxia compared to warm normoxia across populations (environment effect, $P < 0.001$; Fig. 5.4a). In contrast, whereas pharmacological blockade of β_1-adrenergic receptors with metoprolol reduced f_H, this index of β_1-adrenergic tone was not significantly different between cold hypoxia and warm normoxia (environment effect, $P = 0.172$) (Fig. 5.4b). Consistent with the lack of variation in f_H between populations, there were no population differences in vagal tone (population effect, $P = 0.375$) or β_1-adrenergic tone ($P = 0.420$).

5.4.4 Changes in organ masses and haematology in cold hypoxia

Body mass was similar between populations (population effect, $P = 0.497$) and was unaffected by exposure to cold hypoxia (environment effect, $P = 0.947$), but there were several population-specific changes in organ mass and haematology in chronic hypoxia (Table 5.2). As expected, organ mass was strongly affected by body mass ($P \leq 0.001$), so I accounted for body mass as a covariate in the statistical models for all organ mass data. Cold hypoxia increased total ventricle mass by 19-27% (environment effect, $P < 0.001$), due to increases in mass of the left ventricle and septum (LV+S; $P < 0.001$) and the right ventricle (RV; $P < 0.001$), and cold hypoxia also increased lung mass by 7-24% ($P = 0.011$). However, these traits were not significantly different between populations. Liver mass increased in cold hypoxia (environment effect, $P < 0.001$), but the increase was greater in highlanders (21%) than in lowlanders (11%). Indeed, liver mass was 12% greater in highlanders than in lowlanders in cold hypoxia (but not in warm normoxia), which drove the significant main effect of population on liver mass ($P = 0.002$). Blood haemoglobin content ([Hb]) and haematocrit also increased in cold hypoxia (environment effects, $P < 0.001$), but these effects were largely driven by higher values in lowlanders than in highlanders. This was evidenced by a significant main effect of population on [Hb] ($P = 0.010$), and a significant population×environment interaction for haematocrit Hct ($P = 0.012$) that was associated with a significant pairwise difference between populations in cold hypoxia. In contrast, the mass of interscapular brown adipose tissue (iBAT) did not differ between environments ($P = 0.107$) or populations ($P = 0.561$).

5.5 DISCUSSION

High-altitude endotherms that have adapted to cold hypoxic conditions in their native environment can yield appreciable insight into the evolution of endothermy and body temperature regulation to cope with metabolic challenges. Here, I show that both plastic and evolved reductions in T_b help reduce metabolic demands in cold hypoxia in deer mice native to high altitude. Chronic exposure to cold hypoxia increased metabolic demands, as reflected by increased heart rate and food consumption, with the former associated with significant reductions in vagal tone on the heart. These metabolic demands were offset by plastic reductions in T_b across the daily cycle in response to cold hypoxia. Furthermore, highlanders had consistently lower T_b than lowlanders across environments. Empirical and theoretical evidence suggested that the combined effects of these plastic and evolved reductions in T_b likely helped reduce metabolic demands in cold hypoxia by 10-30%. Therefore, plasticity and further refinement of T_b by natural selection may help some high-altitude endotherms cope with metabolic challenges in their native environment.

My findings emphasize the intense metabolic demands that endotherms can experience in high-altitude environments. Previous studies have shown that field metabolic rates are ~57% greater in wild deer mice at high altitude (~3,800 m elevation in the White Mountains) than in those at lower altitudes at nearby locations (1,230 m to 1,830 m elevation) when measured from July to October (Hayes, 1989b). Part of this difference may arise from the need to forage over greater distances at high altitude, but colder temperatures above ground (daily average of 8˚C in July, -2˚C in October) and in burrows likely increased the metabolic demands of thermogenesis as well (Hayes, 1989b; Hayward, 1965).

My findings here show that the latter effect was likely appreciable, with 21-26% greater f_H (Fig. 5.2) and ~37-43% greater food consumption (Table 5.1) in cold hypoxia. Such changes arise during cold exposure because shivering and non-shivering thermogenesis augment blood flow and nutrient supply to skeletal muscles and brown adipose tissues to meet their increased metabolic demands (Foster and Frydman, 1979; Klingenspor, 2003; Landsberg et al., 1984). Whole-animal rates of aerobic metabolism and O_2 consumption are thus increased, particularly in smaller endotherms in which their higher surface area to volume ratio makes heat retention more difficult (Chappell et al., 1995; Hayes, 1989a; Hayes, 1989b; Hayes and Chappell, 1986; Swanson, 1990). As such, the high demands of thermogenesis at high altitude can amplify the energy and food demands of small endotherms considerably, all while hypoxia may constrain O_2 supply to support increases in aerobic metabolism. Plastic or evolved changes in physiology that help curb these demands should be highly advantageous.

Indeed, my findings suggest that plastic and evolved reductions in T_b can reduce the metabolic demands of thermogenesis at high altitude. Exposure to cold hypoxia led to plastic reductions in T_b of ~2˚C in both populations, and the high-altitude population exhibited a further 1˚C reduction in T_b compared to their low-altitude counterparts (Fig. 5.1). Consistent with the metabolic savings that I estimated from having a lower T_b, high-altitude deer mice exhibit lower O_2 consumption rates than low-altitude deer mice when compared at an ambient temperature of 0˚C (Lyons et al., 2021). Although my findings contrast the observation that many cold-adapted endotherms maintain T_b similar to or even slightly greater than their temperate counterparts (Lovegrove, 2003; Moreira et al., 2021;

Scholander et al., 1950a), high-altitude endotherms must also cope with the concurrent challenge of hypoxia. The potential for hypoxia to constrain aerobic metabolism for thermogenesis may increase the necessity of energy-saving strategies like T_b reduction to cope in high-altitude environments. This raises the interesting question of whether these savings come at the expense of reductions in performance, *e.g.* locomotor activity or sensory function, and how these potential costs may be less important or overcome in high-altitude populations.

My findings provide a potential example of plasticity-led evolution in a natural population. The plasticity-led evolution hypothesis posits that phenotypic plasticity can often precede and facilitate adaptation to novel environments (Braendle and Flatt, 2006; Kelly, 2019; Levis and Pfennig, 2016; Levis and Pfennig, 2019; Storz and Cheviron, 2021; Storz and Scott, 2021; West-Eberhard, 2003). Specifically, plasticity can induce trait changes that enhance fitness in the initial colonists of a novel environment, after which selection then refines the trait further through genetic changes over time. Consistent with this hypothesis, plastic energy-saving reductions in T_b may have improved fitness in the low-altitude deer mice that initially colonized higher altitudes. Selection may have then favoured the individuals with the lowest T_b, thereby leading to further evolved reductions in the high-altitude population. Whether this evolved reduction in T_b is underpinned by a similar mechanism to that which induces the plastic reduction in T_b has yet to be determined.

Although plasticity in T_b is likely adaptive, plasticity of some other traits can be maladaptive in cold hypoxia. For example, chronic hypoxia can induce prolonged

sympathoadrenal activation that leads to systemic hypertension, as observed in some previous studies of low-altitude humans (Calbet, 2003; Calbet et al., 2014; Hansen and Sander, 2003; Kanstrup et al., 1999; Lundby et al., 2018; Parati et al., 2014; Rhodes et al., 2011; Richalet et al., 1988; Schultz et al., 2014; Wolfel et al., 1994) and rats (Johnson et al., 1983; Mazzali et al., 2003; Siques et al., 2014; Vaziri and Wang, 1996), but not in house mice (Wearing and Scott, 2022a). Deer mice did not exhibit this pathological response to chronic hypoxia (Fig. 5.3), which may have played a role in their ability to colonize high-altitude environments. Increases in blood haemoglobin content and haematocrit are another common response to chronic hypoxia across low-altitude mammals and birds, which in this case were also observed in deer mice (Table 5.2). While such changes may seem beneficial by increasing the O_2 carrying capacity of the blood, this benefit is more than offset by the associated increase in blood viscosity, which augments peripheral vascular resistance, can limit cardiac output and aerobic capacity, can contribute to the pathogenesis of chronic mountain sickness, and can increase the risk of stillbirth and adverse birth outcomes (Moore et al., 2011; Simonson et al., 2015; Storz and Scott, 2019). However, this plastic response to cold hypoxia is attenuated in high-altitude deer mice, consistent with previous findings (Lui et al., 2015), suggesting that it was selected against during the process of high-altitude adaptation. Overall, my results and those of many others suggest that phenotypic plasticity is a key determinant of success in high-altitude environments, and that natural selection often reinforces adaptive plasticity and attenuates maladaptive plasticity expressed in response to cold and/or hypoxia (Gilbert-Kawai et al., 2014; Ivy and Scott, 2015; Lui et al.,

2015; McClelland and Scott, 2019; Simonson et al., 2015; Storz and Cheviron, 2021; Storz and Scott, 2019; Storz and Scott, 2021; Storz et al., 2010b; West et al., 2021).

5.6 FIGURES AND TABLES

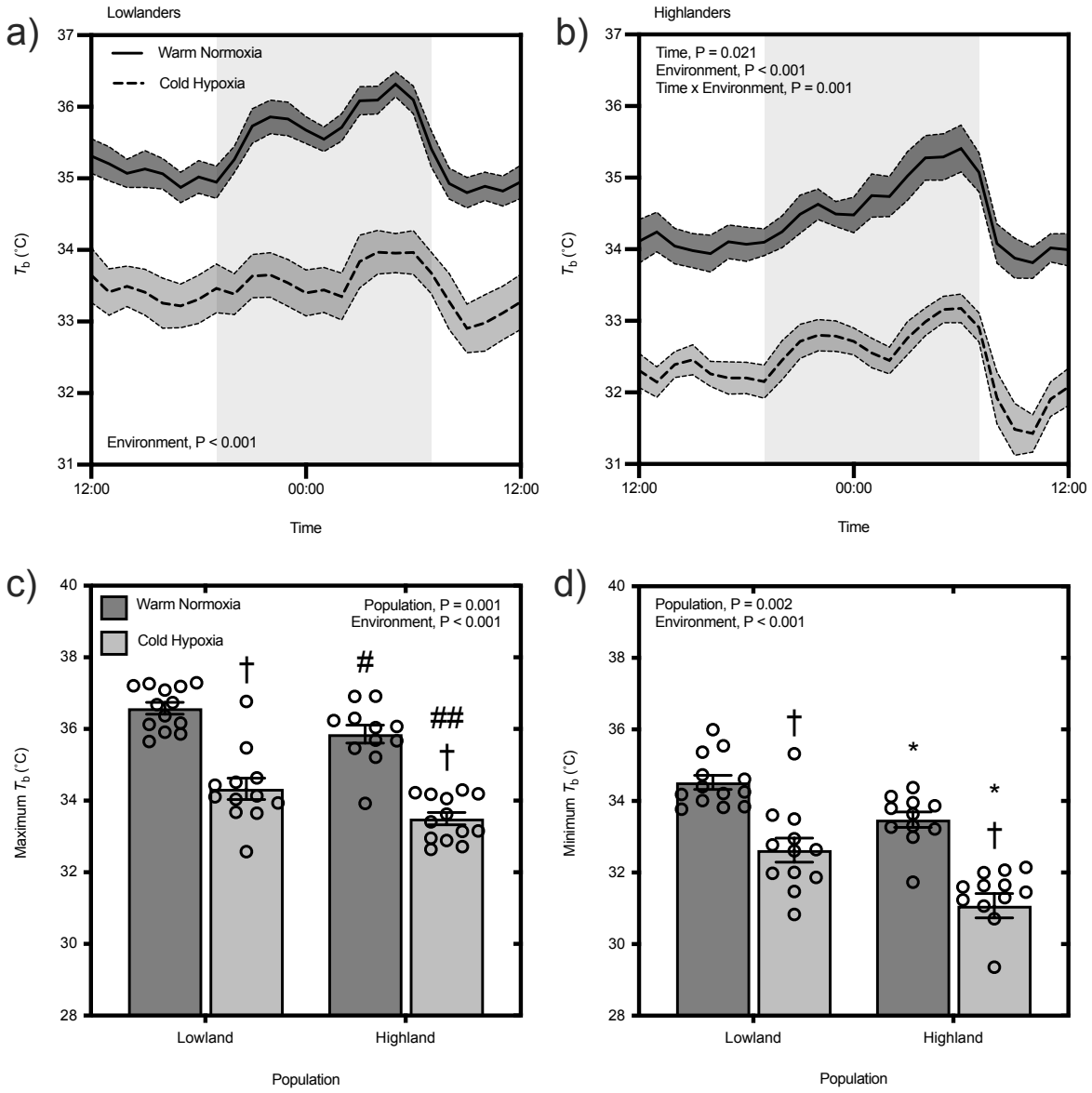

Fig. 5.1 Body temperatures (T_b) of deer mice from the low-altitude population ('lowlanders') and the high-altitude population ('highlanders'), which were chronically exposed to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2). (a,b) Average hourly T_b over the diel cycle (mean ± SEM) for lowlanders and highlanders in warm normoxia (solid line) and cold hypoxia (dashed line). The shaded background (19:00-07:00) illustrates when lights were off. (c,d) Maximum and minimum values of hourly T_b (mean ± SEM as bars, individual data as circles). †, $P < 0.05$ for pairwise differences between environments within a population; *, $P < 0.05$ for pairwise differences between populations within an environment; # and ##, $P = 0.068$ and 0.083 for pairwise differences between populations in warm normoxia or cold hypoxia, respectively.

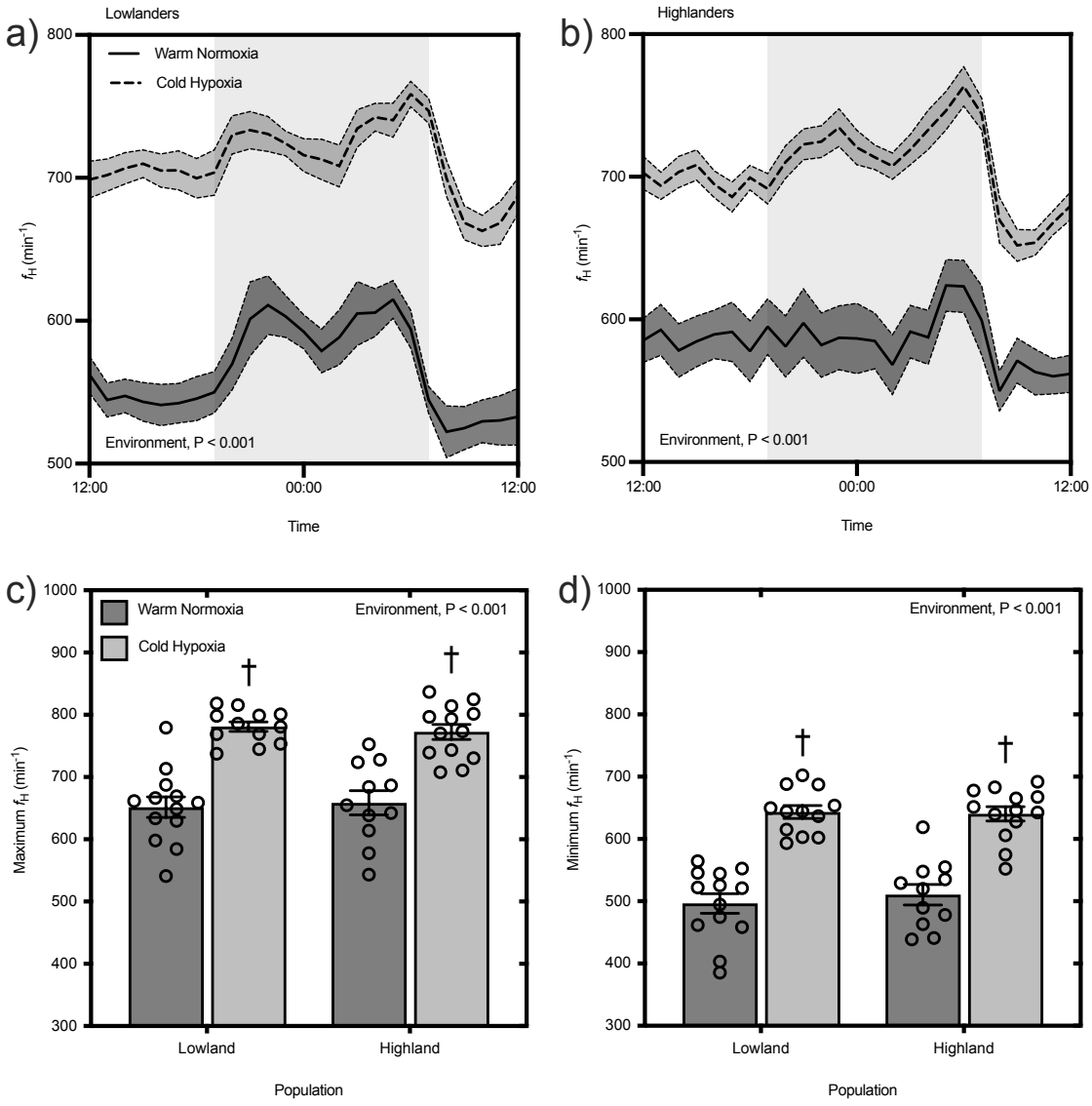
a)
Lowlanders
Warm Normoxia
Cold Hypoxia
Environment, P < 0.001
f_H (min^{-1})
Time
b)
Highlanders
Environment, P < 0.001
Time
c)
Warm Normoxia
Cold Hypoxia
Environment, P < 0.001
Maximum f_H (min^{-1})
Lowland
Highland
Population
d)
Environment, P < 0.001
Minimum f_H (min^{-1})
Lowland
Highland
Population

Fig. 5.2 Heart rate (f_H) of deer mice from the low-altitude population ('lowlanders') and the high-altitude population ('highlanders'), which were chronically exposed to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2). (a,b) Average hourly f_H over the diel cycle (mean ± SEM) for lowlanders and highlanders in warm normoxia (solid line) and cold hypoxia (dashed line). (c,d) Maximum and minimum values of hourly f_H (mean ± SEM as bars, individual data as circles). See Fig. 5.1 for additional details.

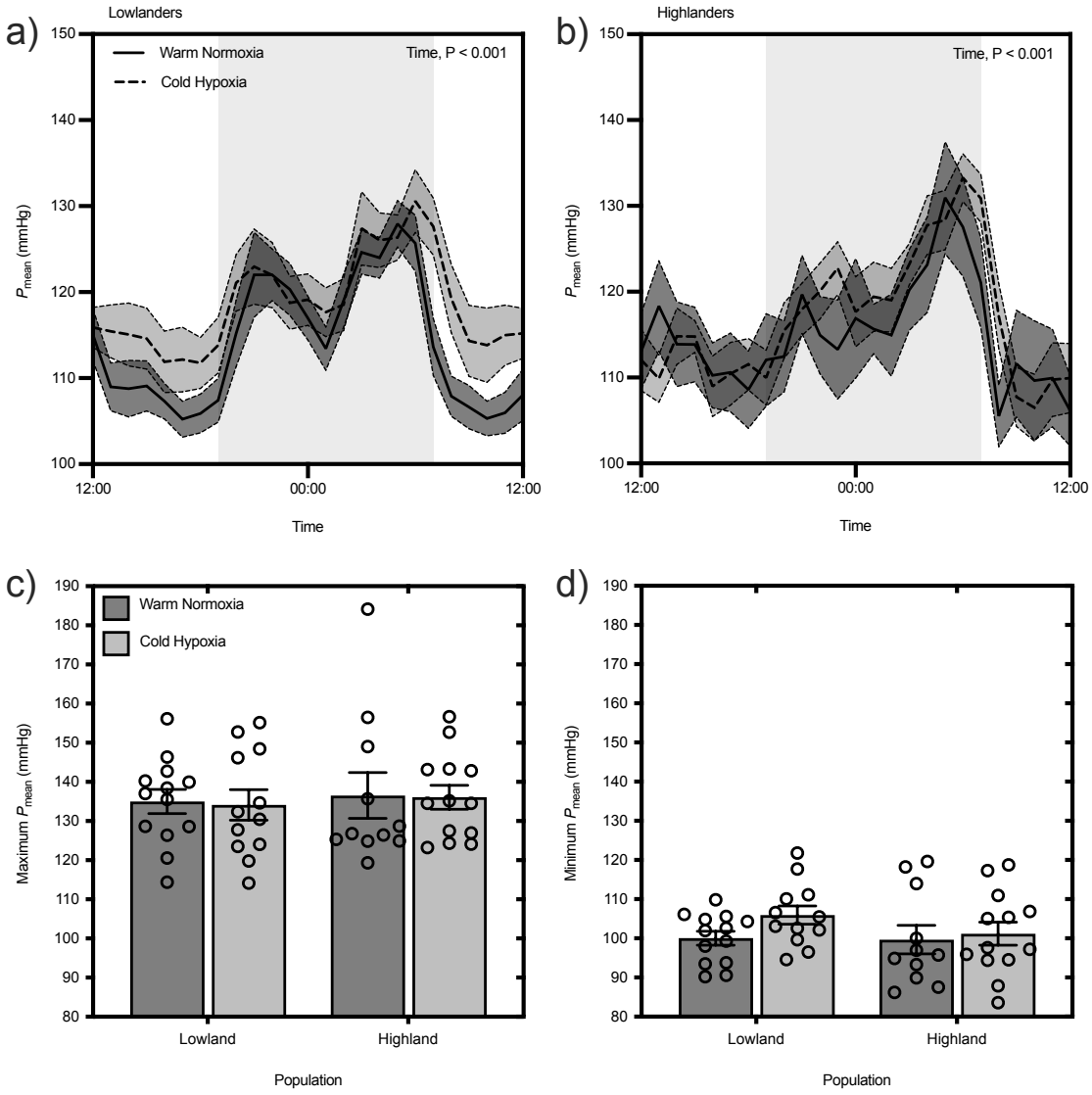
a)
Lowlanders
Warm Normoxia
Cold Hypoxia
Time, P < 0.001
P_{mean} (mmHg)
Time
12:00
00:00
12:00
b)
Highlanders
Time, P < 0.001
c)
Warm Normoxia
Cold Hypoxia
Maximum P_{mean} (mmHg)
Lowland
Highland
Population
d)
Minimum P_{mean} (mmHg)
Lowland
Highland
Population

Fig. 5.3 Mean arterial pressure (P_{mean}) of deer mice from the low-altitude population ('lowlanders') and the high-altitude population ('highlanders'), which were chronically exposed to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2). (a,b) Average hourly P_{mean} over the diel cycle (mean ± SEM) for lowlanders and highlanders in warm normoxia (solid line) and cold hypoxia (dashed line). (c,d) Maximum and minimum values of hourly P_{mean} (mean ± SEM as bars, individual data as circles). See Fig. 5.1 for additional details.

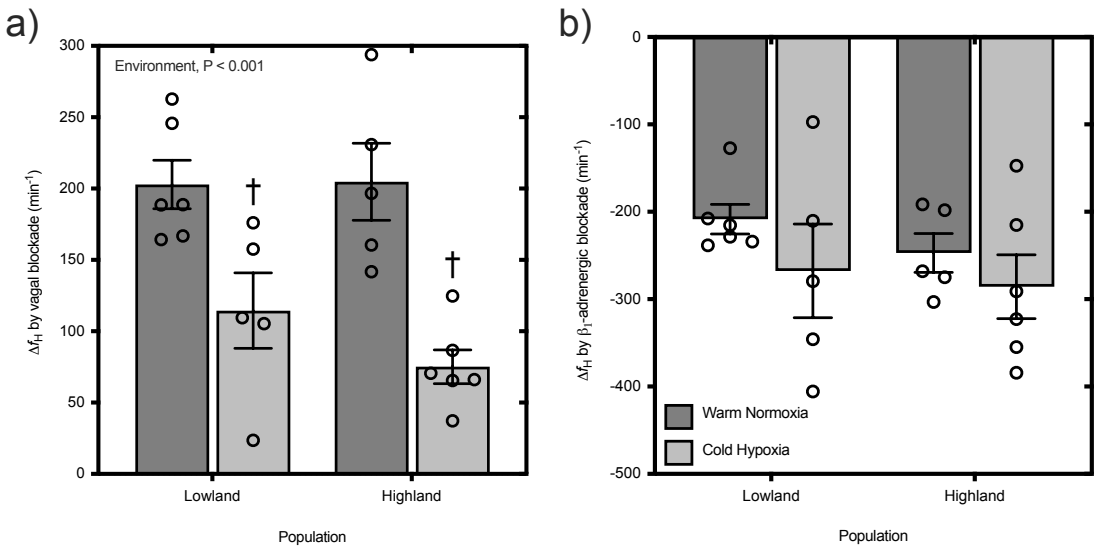
a)
Environment, P < 0.001
Δf_H by vagal blockade (min^{-1})
300
250
200
150
100
50
0
†
†
Lowland
Highland
Population
b)
Δf_H by β_1-adrenergic blockade (min^{-1})
0
-100
-200
-300
-400
-500
Warm Normoxia
Cold Hypoxia
Lowland
Highland
Population

Fig. 5.4 Heart rate responses (Δf_H) to pharmacological blockade of vagal tone (a) and β_1-adrenergic tone (b) in deer mice from the low-altitude population ('lowlanders') and the high-altitude population ('highlanders'), which were chronically exposed to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2). Bars display mean ± SEM with individual data as circles. †, $P < 0.05$ for pairwise differences between environments within a population ($P < 0.05$).

Table 5.1 Daily food and water consumption

	Lowlanders		Highlanders	
	Warm Normoxia	Cold Hypoxia	Warm Normoxia	Cold Hypoxia
N	15	12	13	15
Food (mg g^{-1})	199 ± 17	273 ± 21†	185 ± 10	264 ± 15†
Water (mg g^{-1})	360 ± 36	312 ± 18	307 ± 24	268 ± 15

Data are presented as mean ± SEM and are expressed per g body mass. †, $P < 0.05$ for pairwise differences between acclimation environments within a population in Holm-adjusted Tukey's HSD post-tests.

Table 5.2 Organ masses and haematology

	Lowlanders		Highlanders	
	Warm Normoxia	Cold Hypoxia	Warm Normoxia	Cold Hypoxia
n	14	11	13	13
Animal mass (g)	22.9 ± 1.5	20.8 ± 0.9	19.8 ± 0.6	21.2 ± 0.6
Total ventricle mass (mg g^{-1})	5.65 ± 0.15	7.16 ± 0.27†	5.78 ± 0.15	6.85 ± 0.21†
RV mass (mg g^{-1})	1.33 ± 0.05	1.78 ± 0.09†	1.26 ± 0.05	1.64 ± 0.09†
LV+S mass (mg g^{-1})	4.33 ± 0.15	5.39 ± 0.27†	4.52 ± 0.13	5.22 ± 0.19†
Lung mass (mg g^{-1})	6.79 ± 0.42	8.45 ± 0.50	7.66 ± 0.20	8.20 ± 0.45
Liver mass (mg g^{-1})	40.1 ± 1.4	44.5 ± 1.1†	41.2 ± 1.1	49.9 ± 1.0†*
iBAT mass (mg g^{-1})	5.01 ± 0.31	5.25 ± 0.35	4.54 ± 0.24	5.30 ± 0.51
[Hb] (g dl^{-1})	11.7 ± 0.4	14.3 ± 0.5†	11.2 ± 0.4	12.6 ± 0.4†
Hct (%)	35.7 ± 1.0	45.3 ± 1.7†	35.9 ± 1.2	39.2 ± 0.0†*

RV, right ventricle; LV+S, left ventricle and septum; iBAT, interscapular brown adipose tissue; [Hb], blood haemoglobin concentration; Hct, haematocrit. Data are presented as mean ± SEM, and organ mass data are expressed per g body mass. †, $P < 0.05$ for pairwise differences between acclimation environments within a population in Holm-adjusted Tukey's HSD post-tests. *, $P < 0.05$ for pairwise differences between populations within an acclimation environment.

5.7 SUPPLEMENTAL FIGURE AND TABLES

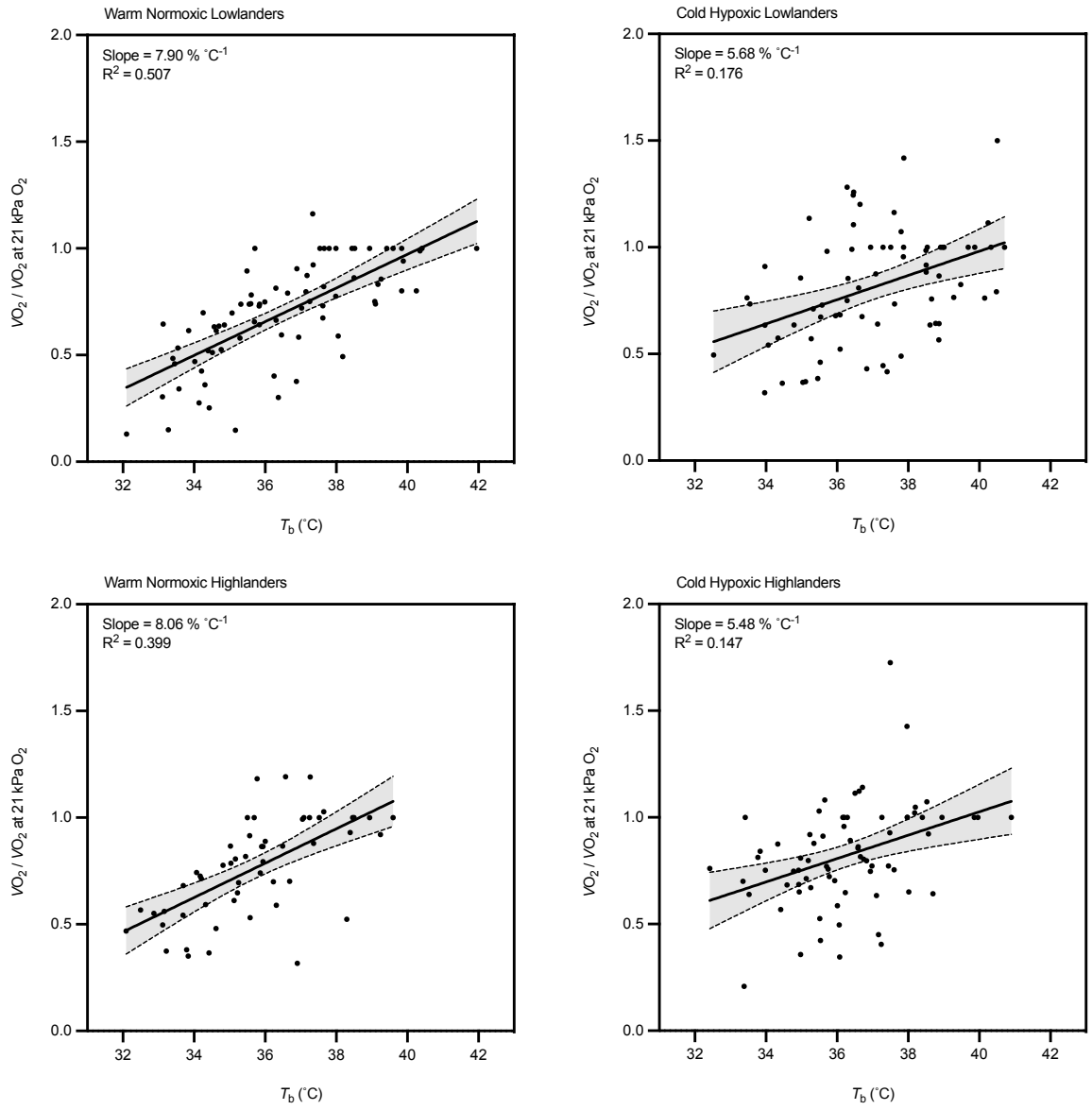

Fig. S5.1 Relationships between body temperature (T_b) and resting O_2 consumption rate ($\dot{V}O_2$, relative to $\dot{V}O_2$ at 21 kPa O_2), assessed by using acute hypoxia to cause reductions in T_b without changes in ambient temperature (25˚C) in lowland and highland deer mice chronically exposed to warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2). Circles represent individual measurements, which were made in each animal across several inspired O_2 levels during stepwise exposure to increasingly severe levels of hypoxia (21, 16, 12, 10, 9, and 8 kPa O_2), and solid lines represent linear regressions (dashed lines are 95% confidence intervals of the regression). The number of animals in each group were as follows: warm normoxic lowlanders, n = 13; cold hypoxic lowlanders, n = 12; warm normoxic highlanders, n = 9; cold hypoxic highlanders, n = 12).

Table S5.1 Results of statistical comparisons using linear mixed models on body temperature and cardiovascular function throughout the diel cycle in lowland and highland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Trait	Population (*p*) effect	Environment (*e*) effect	Time (*t*) effect	*p* x *e* effect	*p* x *t* effect	*e* x *t* effect	*p* x *e* x *t* effect
T_b	**P < 0.001**	**P < 0.001**	**P = 0.013**	P = 0.835	P = 0.318	**P < 0.001**	P = 0.177
f_H	P = 0.920	**P < 0.001**	P = 0.216	P = 0.255	P = 0.347	P = 0.793	P = 0.535
P_{mean}	P = 0.829	P = 0.926	**P < 0.001**	P = 0.538	P = 0.865	**P = 0.031**	P = 0.712

T_b, body temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S5.2 Results of statistical comparisons using linear mixed models on body temperature and cardiovascular function throughout the diel cycle in lowland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Trait	Environment (*e*) effect	Time (*t*) effect	*e* x *t* effect
T_b	**P < 0.001**	P = 0.262	P = 0.064
f_H	**P < 0.001**	P = 0.840	P = 0.546
P_{mean}	P = 0.393	**P < 0.001**	P = 0.193

T_b, body temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S5.3 Results of statistical comparisons using linear mixed models on body temperature and cardiovascular function throughout the diel cycle in highland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Trait	Environment (*e*) effect	Time (*t*) effect	*e* x *t* effect
T_b	**P < 0.001**	**P = 0.021**	**P = 0.001**
f_H	**P < 0.001**	P = 0.109	P = 0.792
P_{mean}	P = 0.652	**P < 0.001**	P = 0.084

T_b, body temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S5.4 Results of statistical comparisons using linear mixed models on maximum and minimum hourly values of body temperature and cardiovascular function in lowland and highland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Trait	Population (*p*) effect	Environment (*e*) effect	*p* x *e* effect
Max. T_b	**P = 0.001**	**P < 0.001**	P = 0.816
Min. T_b	**P = 0.002**	**P < 0.001**	P = 0.368
Max. f_H	P = 0.628	**P < 0.001**	P = 0.553
Min. f_H	P = 0.802	**P < 0.001**	P = 0.619
Max. P_{mean}	P = 0.863	P = 0.689	P = 0.989
Min. P_{mean}	P = 0.399	P = 0.225	P = 0.466

T_b, body temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S5.5 Results of statistical comparisons using linear mixed models on food and water consumption in lowland and highland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Consumption	Population (*p*) effect	Environment (*e*) effect	*p* x *e* effect
Food	P = 0.505	**P < 0.001**	P = 0.885
Water	P = 0.114	P = 0.122	P = 0.938

Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S5.6 Results of statistical comparisons using linear mixed models on the response of heart rate (f_H) to pharmacological blockade in lowland and highland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Blockade	Population (*p*) effect	Environment (*e*) effect	*p* x *e* effect
β_1-AR	P = 0.420	P = 0.172	P = 0.770
Vagal	P = 0.375	**P < 0.001**	P = 0.330

β_1-AR, β_1-adrenergic receptor. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S5.7 Results of statistical comparisons using linear mixed models on mass data for lowland and highland deer mice acclimated to warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Trait	Body mass effect	Population (p) effect	Environment (*e*) effect	*p* x *e* effect
Body mass	NA	P = 0.497	P = 0.947	P = 0.408
Ventricles mass	**P < 0.001**	P = 0.882	**P < 0.001**	P = 0.183
RV mass	**P < 0.001**	P = 0.369	**P < 0.001**	P = 0.470
LV+S mass	**P < 0.001**	P = 0.884	**P < 0.001**	P = 0.243
Lungs mass	**P = 0.001**	P = 0.557	**P = 0.011**	P = 0.360
Liver mass	**P < 0.001**	**P = 0.002**	**P < 0.001**	P = 0.240
iBAT mass	**P < 0.001**	P = 0.561	P = 0.107	P = 0.586
[Hb]	NA	**P = 0.010**	**P < 0.001**	P = 0.165
Hct	NA	**P = 0.017**	**P < 0.001**	**P = 0.012**

RV, right ventricle; LV+S, left ventricle and septum; iBAT, interscapular brown adipose tissue; [Hb], blood haemoglobin concentration; Hct, haematocrit. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects ($P < 0.05$) are shown in bold.

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CHAPTER 6: GENERAL DISCUSSION

6.1 OVERVIEW

The primary goals of my Ph.D. thesis were: A) to understand the integration and mechanistic underpinnings of changes in circulatory physiology that underlie evolved increases in aerobic capacity in high-altitude deer mice (Chapters 2 and 3); B) to elucidate the effects of chronic exposure to hypoxia or cold hypoxia on cardiovascular physiology (Chapters 4 and 5), and C) to uncover potential strategies for reducing metabolic demands in high-altitude deer mice (Chapter 5). In Chapter 2 (Wearing et al., 2021), I showed that the adaptive benefit of evolved cardiorespiratory adjustments in high-altitude mice (specifically, Hb-O_2 affinity) is contingent on changes to additional traits along the O_2 transport pathway, highlighting the importance of integrative approaches to understanding the evolution of complex organismal traits like aerobic performance. In Chapter 3 (Wearing et al., 2022), I showed that evolved changes to adrenergic control of the heart and vasculature underlie evolved and plastic increases in cardiac output and circulatory O_2 supply that underlie enhanced aerobic performance in high-altitude deer mice. In Chapter 4 (Wearing and Scott, 2022a), I used physiological telemetry to examine the effects of chronic hypoxia on routine physiology in house mice, showing that some responses to chronic hypoxia are sex-specific in this species, and laying the groundwork for further studies on deer mice. Finally, in Chapter 5 (Wearing and Scott, 2022b), I showed that the metabolic challenges of cold hypoxia at high altitude are met with evolved and plastic reductions in body temperature setpoint and the metabolic demands of thermogenesis in

high-altitude deer mice. As such, my Ph.D. thesis work has elucidated several key mechanisms involved in high-altitude acclimation/acclimatization and adaptation that highlight the importance of integrative approaches when measuring physiological responses to environmental stimuli, as well as the novel insight that can be gained from making physiological measurements in freely behaving animals in controlled experiments. By leveraging several experimental tools, I have been able to show that high-altitude deer mice have evolved circulatory and metabolic adjustments that likely contribute to their success in the metabolically challenging environment at high altitude. My thesis lays the groundwork for future work to investigate the underlying mechanisms responsible for the evolved and plastic changes in cardiovascular function and metabolism that I have described.

6.2 INTEGRATIVE CARDIORESPIRATORY ADAPTATIONS TO HIGH ALTITUDE

The cold hypoxic conditions at high altitude have potent short- and long-term effects on endotherms, but many high-altitude natives have evolved mechanisms that help balance metabolic supply and demand in cold hypoxia and help attenuate pathological or maladaptive responses exhibited by low-altitude taxa at high elevations. Lowland-native mammals that ascend to high altitude typically demonstrate phenotypic plasticity that often serves to protect O_2 transport to hypoxia-sensitive tissues such as the brain and heart. These responses include polycythaemic increases in blood Hb concentration caused by splenic contraction and upregulation of erythropoiesis, as well as adrenergic-mediated peripheral vasoconstriction due to sympathetic activation by the hypoxic chemoreflex (Calbet, 2003;

Calbet et al., 2014; Hainsworth and Drinkhill, 2007; Hainsworth et al., 2007; Richalet, 2016; Rimoldi et al., 2016; Sander, 2016; Winslow, 2007). While these responses may be beneficial in moderate short-term hypoxia, these responses can turn pathological and therefore become maladaptive in more severe and prolonged hypoxia, limiting O_2 transport to some tissues. For instance, polycythaemia increases blood viscosity and therefore puts additional strain on the heart, limiting cardiac performance (Murray et al., 1962; Richardson and Guyton, 1959; Storz et al., 2010b). Similarly, increased α-adrenergic vasoconstriction that helps redistribute blood flow to sensitive tissues during acute hypoxia may cause chronic systemic hypertension and end up starving other peripheral tissues of much-needed O_2 and metabolic fuels, causing dysfunction (Calbet, 2003; Calbet et al., 2014; Hansen and Sander, 2003; Johnson et al., 1983; Kanstrup et al., 1999; Lundby et al., 2018; Mazzali et al., 2003; Parati et al., 2014; Rhodes et al., 2011; Richalet et al., 1988; Schultz et al., 2014; Siques et al., 2014; Vaziri and Wang, 1996; Wolfel et al., 1994). Indeed, some high-altitude mammals exhibit blunted polycythaemia at high altitude, as well as evolved reductions in adrenergic signalling that may become problematic during chronic sympathoexcitation (Beall and Reichsman, 1984; Black and Tenney, 1980; Leon-Velarde et al., 1996; Lui et al., 2015; Pichon et al., 2013; Storz et al., 2010b). Instead, high-altitude natives have evolved alternative adaptive physiological modifications to the O_2 transport pathway that circumvent the need for these pathological responses. These include increases in Hb-O_2 affinity (Chappell et al., 1988; Chappell and Snyder, 1984; Ivy and Scott, 2015; Jensen et al., 2016; Snyder et al., 1982; Snyder, 1981; Storz et al., 2010a; Storz et al., 2009; Storz and Scott, 2019), enhanced pulmonary function (Beall et al., 1997; Brutsaert, 2007;

Clemens, 1988; Ivy et al., 2020; Ivy and Scott, 2015; Ivy and Scott, 2017; Ivy and Scott, 2018; Ivy et al., 2022; McDonough et al., 2006; Pichon et al., 2009; Scott, 2011; Scott and Milsom, 2007; Storz and Scott, 2019; Tate et al., 2020; Zhuang et al., 1993a), and differences in sympathetic nervous system function in hypoxia (Hainsworth and Drinkhill, 2007; Hainsworth et al., 2007; Ivy and Scott, 2015; Pichon et al., 2013; Scott et al., 2019; Zhuang et al., 1993b).

High-altitude deer mice have evolved a suite of physiological adaptations that improve aerobic performance in this O_2-limited environment and avoid the development of maladaptive pathologies that are observed in low-altitude deer mice and other lowland-native mammals. Presumably as a result of strong directional selection (Hayes and O'Connor, 1999), high-altitude deer mice have evolved enhancements in aerobic performance in hypoxia (Chappell et al., 1988; Chappell and Snyder, 1984; Cheviron et al., 2012; Cheviron et al., 2013; Ivy et al., 2021; Tate et al., 2017; Tate et al., 2020). Previous work by the Scott lab and others have attributed this to several underlying adaptations along the O_2 transport pathway. These include more effective pulmonary function (Ivy et al., 2020; Ivy and Scott, 2017; Ivy and Scott, 2018; Ivy et al., 2022; West et al., 2021a; West et al., 2021b), Hb with a higher affinity for O_2 (Chappell et al., 1988; Chappell and Snyder, 1984; Natarajan et al., 2015; Natarajan et al., 2013; Snyder et al., 1982; Snyder, 1981; Storz et al., 2012; Storz et al., 2010a; Storz et al., 2009; Storz et al., 2007), augmented maximal heart rate and cardiac stroke volume (Tate et al., 2017; Tate et al., 2020), as well as changes in skeletal muscle phenotype that promote evolved increases in tissue O_2 extraction and mitochondrial O_2 utilization (Dawson et al., 2018; Lui et al., 2015; Mahalingam et al., 2017;

Scott et al., 2015). My Ph.D. thesis has not only demonstrated the importance of integration between these steps in realizing the adaptive benefit of each individual trait (Wearing et al., 2021), but also provided mechanistic insight into the regulatory (*i.e.*, adrenergic signalling) mechanisms responsible for evolved and plastic differences in cardiovascular function (Wearing et al., 2022). I also show that, like mice in general (Wearing and Scott 2022a), both low-altitude and high-altitude deer mice do not appear to develop the maladaptive cardiovascular responses to high altitude conditions that are presented by some other mammal species (Wearing and Scott, 2022b), as mentioned above. However, the attenuation of these responses may have resulted in the loss of phenotypic plasticity that could be beneficial in other environmental scenarios (Storz and Scott, 2021). Understanding the ecological consequences of losing physiological plasticity should be a key topic of future research, particularly considering current and future climate change that will likely influence the stability of both high-altitude and low-altitude environments.

A major outcome of my Ph.D. thesis has been demonstrating the importance of an integrative approach and perspective in understanding the evolution of complex traits such as aerobic performance. Aerobic capacity is a complex organismal trait that has important consequences for fitness, determining the capacity to support metabolically demanding processes such as locomotion and thermogenesis (Hayes and O'Connor, 1999; Plaut, 2001; Storz et al., 2019). As discussed above, this complex trait is the emergent property of several subordinate traits along the O_2 and metabolic fuel transport pathways. As such, traditional reductionist approaches that focus on a single step along this pathway in isolation ignore interactions between steps that are likely important for aerobic performance

and its evolution, as I showed in Chapter 2 (Wearing et al., 2021). Instead, a more wholistic, integrative approach that considers multiple systems within these subordinate physiological pathways will likely provide more fruitful and nuanced insight into the evolution of aerobic performance and other complex organismal traits (Wearing and Scott, 2021). As a trait that seems to have complex and context dependent influences on aerobic performance in hypoxia (Dempsey, 2020; Dominelli et al., 2020; Wagner, 1996a; Wagner, 1996b; Wagner, 1997), Hb-O_2 affinity is a good example of how so-called 'hierarchical reductionism' can shed light on the exact conditions for these changes to become adaptive and evolve. Studies of high-altitude adaptation in general can benefit from this experimental framework where possible, allowing researchers to much more confidently draw conclusions about the functional and adaptive consequences of trait variation between populations.

6.3 METABOLIC ADJUSTMENTS TO HIGH-ALTITUDE CONDITIONS

The balance between energy supply and demand is essential to physiological homeostasis, but this can be threatened in extreme environments that constrain O_2 or food availability and/or that increase metabolic demands. Endotherms that have adapted to live in extreme environments offer valuable opportunities to investigate evolved and plastic mechanisms for coping with metabolically challenging conditions. Thermogenesis by shivering (skeletal muscle) and non-shivering thermogenesis (brown adipose tissue) is itself energetically demanding, requiring high caloric intake and O_2 uptake (Bennett and Ruben, 1979; Clarke and Portner, 2010). Periods of limited food availability and/or high thermogenic requirements (*e.g.*, over winter) can make these demands untenable. Some

endotherms have therefore evolved the ability to temporarily reduce this thermogenic metabolic demand by depressing body temperature during times of seasonal hibernation or torpor (Bicego et al., 2007; Carey et al., 2003; Geiser, 2004; Levesque and Tattersall, 2010; Staples, 2016). While examples of such endotherms are well known and relatively well studied, much less is known about the possibility that reductions in body temperature setpoint may have arisen within some distinct lineages of non-hibernating mammals as a valuable mechanism for coping with prolonged metabolic challenges.

High-altitude deer mice have evolved energy-saving reductions in body temperature that help curb the metabolic costs of thermoregulation in this chronically cold and hypoxic environment. High altitude presents endotherms with the combined metabolic challenge of cold and hypoxia. For this reason, small mammals that have evolved to survive in this environment can provide valuable insight into evolved and plastic physiological coping mechanisms in the setting of extreme metabolic challenge (McClelland and Scott, 2019; Monge and Leon-Velarde, 1991; Storz, 2021; Storz et al., 2015; Storz et al., 2019; Storz and Scott, 2019; Storz et al., 2010b; Wearing and Scott, 2021). As expected, chronic exposure to cold hypoxia increased metabolic demands – reflected by increased heart rate and food consumption – in both low-altitude and high-altitude deer mice (Wearing and Scott, 2022b). Such changes arise during cold exposure because shivering and non-shivering thermogenesis augment blood flow and nutrient supply to skeletal muscles and brown adipose tissues to meet their increased metabolic demands (Foster and Frydman, 1979; Klingenspor, 2003; Landsberg et al., 1984). Whole-animal rates of aerobic metabolism and O_2 consumption are thus increased, particularly in smaller endotherms in

which their higher surface area to volume ratio makes heat retention more difficult (Chappell et al., 1995; Hayes, 1989a; Hayes, 1989b; Hayes and Chappell, 1986; Swanson, 1990). As such, the high demands of thermogenesis at high altitude can amplify the energy and food demands of small endotherms considerably, all while hypoxia may constrain O_2 supply to support increases in aerobic metabolism. However, I showed that these increased metabolic demands were offset in deer mice by plastic and evolved reductions in body temperature setpoint across the daily cycle in response to cold hypoxia that could reduce metabolic demands in cold hypoxia by 10-30% (Wearing and Scott, 2022b). Unlike the sex-specific responses to warm hypoxia in house mice (Wearing and Scott, 2022a), the responses of deer mice to cold hypoxia were common to males and females (Wearing and Scott, 2022b). As such, I demonstrated that plasticity and further refinement of body temperature by natural selection may help some high-altitude endotherms cope with metabolic challenges in their native environment.

My findings in deer mice provide several potential examples of plasticity-led evolution in a natural population. The plasticity-led evolution hypothesis posits that phenotypic plasticity can often precede and facilitate adaptation to novel environments (Braendle and Flatt, 2006; Kelly, 2019; Levis and Pfennig, 2016; Levis and Pfennig, 2019; Storz and Cheviron, 2021; Storz and Scott, 2021; West-Eberhard, 2003). Specifically, plasticity can induce trait changes that enhance fitness in the initial colonists of a novel environment, after which selection then refines the trait further through genetic changes over time. Overall, my results and those of many others suggest that phenotypic plasticity is a key determinant of success in high-altitude environments, and that natural selection

often reinforces adaptive plasticity and attenuates maladaptive plasticity expressed in response to cold and/or hypoxia (Gilbert-Kawai et al., 2014; Ivy and Scott, 2015; Lui et al., 2015; McClelland and Scott, 2019; Simonson et al., 2015; Storz and Cheviron, 2021; Storz and Scott, 2019; Storz and Scott, 2021; Storz et al., 2010b; West et al., 2021b).

6.4 CONCLUSIONS

Together, the findings of my Ph.D. demonstrate that both environmentally-induced plasticity and evolutionary adaptations in circulatory physiology and metabolism improve metabolic supply and reduce metabolic demands in highland deer mice, helping them cope with the unremitting cold and hypoxic conditions at high altitude. By increasing the rates of tissue O_2 supply via the O_2 transport pathway (*e.g.*, through evolved increases in Hb-O_2 and tissue O_2 diffusing capacity, as well as evolved and plastic elevations in cardiac output that are underlain by changes in adrenergic stimulation of the heart), high-altitude deer mice have developed circulatory adjustments that help them maintain high aerobic performance in their O_2-limited environment. In addition to these evolved mechanisms that increase O_2 and metabolic fuel supply to active tissues, low-altitude and high-altitude deer mice curb the metabolic demands of chronic cold hypoxia with plastic reductions in body temperature setpoint. Overlaid upon this plasticity, high-altitude deer mice have also evolved further reductions in body temperature setpoint in these unremitting conditions, providing a potential example of plasticity-led evolution and rare experimental evidence that some lineages of non-hibernating small endotherms may utilize subtle reductions in body temperature setpoint to reduce the metabolic costs of endothermy in extreme environments.

As such, my Ph.D. thesis work has highlighted the importance of considering both metabolic supply and demand when investigating the physiological adaptations to extreme environments, even when established changes in one may appear to mitigate the need for changes in another. In addition, this work also demonstrates the importance of understanding how subordinate steps that underlie complex physiological processes interact and together translate to organismal phenotypes that are exposed to selection in ecologically relevant environmental conditions (*e.g.*, thermogenic $\dot{V}O_2max$ in hypoxia). High-altitude deer mice have proved, and should continue to be, a valuable model with which to test hypotheses about the evolution of these integrative physiological mechanisms.

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