THE INFLUENCE OF HEMOGLOBIN-OXYGEN AFFINITY ON AEROBIC CAPACITY IN HIGH ALTITUDE DEER MICE (*PEROMYSCUS MANICULATUS)*

THE INFLUENCE OF HEMOGLOBIN-OXYGEN AFFINITY ON AEROBIC CAPACITY IN HIGH ALTITUDE DEER MICE (*PEROMYSCUS MANICULATUS)*

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TITLE: The Influence of Hemoglobin-Oxygen Affinity on Aerobic Capacity in High Altitude Deer Mice (*Peromyscus maniculatus)*

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Lay Abstract

High altitude is one of the most extreme environments due to extremely cold temperatures and low levels of oxygen. Many high-altitude species have overcome these challenges by increasing hemoglobin-oxygen affinity but the effect that this has on aerobic capacity is not clear. We tested the effects of pharmacologically manipulating hemoglobin-oxygen affinity on aerobic capacity in high- and low-altitude deer mice. Adult mice born and raised in the captivity were acclimated to their natural environments and treated with drugs to increase or decrease hemoglobin-oxygen affinity. Aerobic capacity and tissue phenotypes were then measured. Our findings suggest that the relationship between hemoglobin-oxygen affinity and aerobic capacity in hypoxia is different between high and low-altitude mice. We suggest that increases in hemoglobin-oxygen affinity are only beneficial if the animal has adaptive increases in the ability to extract and consume oxygen at the level of the tissue.

Abstract

High-altitude hypoxia constrains tissue O_2 supply, but several high-altitude populations have evolved adaptations to overcome this challenge. Evolved increases in hemoglobin-O_2 (Hb-O_2) affinity are pervasive across high-altitude taxa, but the influence of such increases on aerobic capacity in hypoxia remains contentious. The influence of Hb-O_2 affinity on aerobic capacity in hypoxia could vary depending on other traits in the O_2 transport pathway, but this possibility is poorly understood. We examined this issue in deer mice (*Peromyscus maniculatus*), which is found from sea level to >4300m altitude in the Rocky Mountains. Mice from populations native to high- and low-altitude were born and raised to adulthood in captivity. Low-altitude mice (n=14) were acclimated to warm normoxia, and high-altitude mice (n=14) were acclimated to cold hypoxia for 6 weeks, creating two groups with distinct capacities for O_2 transport in hypoxia. Aerobic capacity for thermogenesis (VO_2max) was measured in hypoxia after each of three pharmacological treatments to manipulate Hb-O_2 affinity: saline (control); efaproxiral, which decreases Hb-O_2 affinity; and sodium cyanate, which increases Hb-O_2 affinity. Our findings suggest that high-altitude deer mice have an adaptive increase in optimal Hb-O_2 affinity and SaO_2 that likely contributes to greater aerobic capacities in hypoxia. We also suggest that high Hb-O_2 affinity adaptations are species, population, and context specific and highlight the importance of the coordinated changes across the oxygen transport pathway that are needed to take advantage of such increases in affinity.

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Let's do this again sometime, say in 4-5 years?

All the best,
Kayla Marie Garvey, MSc

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I would be remiss if I didn't take the time to thank the mice that went into making this thesis possible. I am indebted to them and grateful for the sacrifice they made for my project. I took the time to name all the mice I used throughout my project as a way to show my respect. Below you'll see all of the names I gave them as well as their family ID's.

Lowlanders

Name	*ID*
Banjo	KNG3345B
Cello	KNG3345C
Space	KNG3331S
Time	KNG3331T
Apples	KNG3343A
Berries	KNG3343B
Data	KNG3339D
Entry	KNG3339E
Sin	KNG3345S
Temptation	KNG3345T
Fill	KNG3339AF
Get Cash	KNG3339AG
Yap	KNG3345Y
Taz	KNG3345Z

Highlanders

Name	*ID*
Hemorrhoids	MEG3340H
IBS	MEG3340I
Jonjo	MEG3342J
Alfonso	MEG3342A
Mitosis	MEG3342M
Necrosis	MEG3342N
Optimism	MEG3342O
Prime	MEG3342P
Nacho	MEG3340N
Olive	MEG3340O
Scotch	MEG3336S
Tequila	MEG3340T
Ultra	MEG3340U
Violet	MEG3340V

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List of All Abbreviations

2,3-DPG	2,3-diphosphoglycerate
ANOVA	Analysis of Variance
β	Effect Size Estimates
BSA	Bovine Serum Albumin
Cl^-	Chloride Ion
CO_2	Carbon Dioxide
COX	Cytochrome C Oxidase
CS	Citrate Synthase
DC	Detergent Compatible
EDTA	Ethylenediaminetetraacetic acid
f_H	Heart Rate
GAMM	Generalized Additive Mixed Models
H^+	Hydrogen Ion
HA	Highlanders
Hb	Hemoglobin
Hb-O_2	Hemoglobin-Oxygen
Hct	Hematocrit
He	Helium
iBAT	Interscapular Brown Adipose Tissue
IgG	Immunoglobulin G
LA	Lowlanders
LV+S	Left Ventricle and Septum

MCHC	Mean Cell Hemoglobin Concentration
N_2	Nitrogen
NaCl	Sodium Chloride
O_2	Oxygen
OEC	Oxygen Equilibrium Curve
P_{50}	Partial Pressure of Oxygen when Hemoglobin is 50% Saturated with Oxygen
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline Containing Tween-20
PVDF	Polyvinylidene Fluoride
pH	Potential of Hydrogen
PO_2	Partial Pressure of Oxygen
RBC	Red Blood Cell
RV	Right Ventricle
S.E.	Standard Error
S.E.M.	Standard Error of the Mean
SaO_2	Arterial Oxygen Saturation
SDS	Sodium Dodecyl Sulfate
UCP1	Uncoupling Protein 1
VEGF	Vascular Endothelial Growth Factor
VO_2	Oxygen Consumption Rate
VO_2max	Maximal Oxygen Consumption

Thesis Organization and Format

This thesis is organized in the “sandwich” format. It contains three main chapters. Chapter one is a general introduction which outlines the relevant background information and discusses the justification for the objectives and hypotheses of my thesis. Chapter two is a manuscript in preparation for submission to a peer-reviewed scientific journal. Chapter three is an overview of the major findings of the thesis and their implications in deer mouse physiology and future directions of research.

CHAPTER ONE:	**GENERAL INTRODUCTION**
CHAPTER TWO:	**THE INFLUENCE OF HEMOGLOBIN-OXYGEN AFFINITY ON AEROBIC CAPACITY IN HIGH ALTITUDE DEER MICE (*Peromyscus maniculatus*)** Authors: Kayla M. Garvey, Graham R. Scott Date of expected submission: September 2024
CHAPTER THREE:	**CHAPTER THREE: OVERVIEW AND GENERAL DISCUSSION**

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

One of the main goals of comparative and evolutionary physiology is to dissect and understand how integrative functions influence complex performance traits. Aerobic capacity (maximal rate of O_2 consumption) is a complex performance trait supported by the integrative function of the O_2 transport pathway and determines an organism's overall fitness. In mammals, hemoglobin (Hb) is the main protein responsible for transporting O_2 from the lungs to metabolically active tissues. Hb is a heterotetrameric protein made of two alpha and two beta subunits which reversibly bind oxygen. There has been longstanding interest in how changes in Hb-O_2 affinity impact aerobic capacity when there are low levels of oxygen in the environment (hypoxia) (Bunn, 1980; Samaja et al., 2003; Dempsey 2020). On one hand, increasing Hb-O_2 affinity in hypoxia will increase O_2 loading at the lungs and safeguard arterial O_2 saturation (SaO_2). Conversely, decreasing Hb-O_2 affinity will enhance O_2 offloading at the tissues to maintain tissue oxygenation. There are trade-offs between which of these changes in Hb-O_2 affinity is more advantageous in hypoxia and it remains contentious whether it is more beneficial to increase or decrease Hb-O_2 in hypoxia.

High altitude natives are a unique tool that can be used to disentangle the influence of Hb-O_2 on aerobic capacity in hypoxia. High altitude environments present a significant challenge to aerobic metabolism due to the low partial pressure of O_2 and extremely cold temperatures. In these conditions it becomes difficult to maintain high rates of O_2 consumption. However, many birds and mammals native to high altitudes have evolved increases in aerobic capacity (Black and Tenney, 1980; Hayes and O'Connor, 1999; Cheviron et al., 2012), which is supported by the integrative function of the cardiorespiratory system. Many high-altitude taxa have evolved increases in Hb-O_2 affinity (Snyder, 1985; Jessen, 1991; Tufts et al., 2013; Storz, 2016) which increases SaO_2 and is often believed to help preserve tissue oxygenation in hypoxia. However, it still remains unclear what the influence of high Hb-O_2 affinity is on aerobic capacity in hypoxia. My thesis examines the influence of Hb-O_2 affinity on aerobic capacity in hypoxia in high- and low-altitude populations of deer mice that have distinct capacities for O_2 transport across the O_2 transport pathway. This project aims to shed light on a decades old debate to uncover the mechanisms by which Hb-O_2 affinity affects aerobic capacity in species adapted to the challenges of high altitude.

1.2 The Oxygen Transport Pathway and the Oxygen Equilibrium Curve

The O_2 transport pathway provides a route for O_2 to take from the air to the tissues in order to match O_2 supply (delivery) with metabolic demands (consumption of O_2 by mitochondria). This pathway starts with (1) ventilatory convection of environmental air in the lungs; (2) diffusion across the alveolar membranes into the pulmonary capillaries (3) circulatory transport where O_2 is reversibly bound to Hb in red blood cells (RBCs), (4) diffusion from the capillaries to the tissue where it is then diffused into the mitochondria and used as the terminal electron acceptor in aerobic respiration (Weibel 1984). The partial pressure of oxygen (PO_2) changes following each of the aforementioned steps and it is the partial pressure gradient of O_2 that drives O_2 diffusion from the blood to the mitochondria. At high altitude there is a decrease in the pressure of O_2 which in turn decreases the amount of O_2 inspired by the lungs and physiological adjustments must be made in order to transport adequate amounts of O_2 to working tissues.

Physiological adjustments can be made throughout the O_2 transport pathway and enhancements to any step may be beneficial for supporting greater O_2 consumption at the tissues. Enhancements in Hb binding affinity are often observed in species that live in or are exposed to hypoxic environments. Hb is composed of two alpha and two beta subunits each containing a covalently bound heme group that reversibly binds a single O_2 molecule. O_2 binding is cooperative in nature so that when one molecule of O_2 binds to Hb there is a change in conformation which make it more likely for more O_2 molecules to bind to a maximum of four O_2 molecules per Hb. The quaternary structure of these subunits shifts Hb between a low-affinity tense "T" state and a high-affinity relaxed "R" state. Hb-O_2 affinity is quantified as the partial pressure of oxygen when hemoglobin is 50% saturated with oxygen (P_{50}). This metric is often calculated using oxygen equilibrium curves (OEC's) which present the partial pressure of O_2 on the x-axis and the percent saturation of Hb on the y-axis. A right shifted OEC represents an increase in P_{50} and a decrease in Hb-O_2 affinity while a left shifted OEC represents a decrease in P_{50} and an increase in Hb-O_2 binding affinity. Within an individual, shifts in the OEC are typically due to changes in the internal environment that Hb is exposed to, but evolutionary shifts can also arise from changes in protein sequence. Common modulators of Hb-O_2 affinity are increases in 2,3-diphosphoglycerate (2,3-DPG), hydrogen (H^+) ions, temperature and carbon dioxide (CO_2). Increases in the previously mentioned modifiers cause a right shift in the OEC, and a decrease in these modulators causes a left shift.

1.3 Changes in Hemoglobin-Oxygen Affinity in High-Altitude Taxa

Changes in Hb-O_2 affinity can be due to evolved changes in the intrinsic capacity for Hb to bind O_2 (genetic adaptation) or through changes in the sensitivity to allosteric modulators. Short term reversible changes in Hb-O_2 affinity involve the modification of Hb by allosteric cofactors such as 2,3- DPG and ions such as H^+ and Cl^- which reduce Hb-O_2 affinity by enhancing the low-affinity "T" state (Mairbäurl et al., 2011). This shift in affinity increases the offloading of Hb at the active tissues. However, many high-altitude taxa have increased Hb-O_2 affinity due to genetic adaptations in Hb conferred by amino acids substitutions which enhance the high affinity "R" state. This increase in affinity enhances O_2-loading in the lungs but has the potential to hinder O_2-offloading at the tissues. This trade off could be compensated by an increased sensitivity to CO_2/pH at the tissue level or an increased Bohr effect however, this is not always observed in high altitude natives (Giardina et al., 2004; Weber, 2007; Natarajan et al., 2015; Jensen et al., 2016).

Many high-altitude taxa have evolved increases in Hb-O_2 affinity, however, the physiological mechanism that induces this increase is not always the same between species. For example, two species of waterfowl that have independently adapted to high-altitude have remarkably high Hb-O_2 affinities compared to their low-altitude counterparts. Strikingly, both of these species have distinct animo acid substitutions in their alpha and beta chain subunits of Hb and both substitution patterns act to destabilize the tense Hb state and promote Hb and O_2 binding thereby increasing Hb-O_2 affinity (Hiebl et al., 1987a.; Jessen et al., 1991; Weber et al., 1993). In comparison, the Rüppell's griffon uses another mechanism to support higher Hb-O_2 affinities at high altitude by expressing different isoforms of Hb. Most vertebrates have genes that encode functionally distinct Hb isoforms during different stages of development, a great example of this is fetal hemoglobin and adult hemoglobin in humans. However, Rüppell's griffons express four unique Hb isoforms at the same stage of development each with a gradient of affinities for O_2. During hypoxic events, their high affinity alpha chain Hb safeguards SaO_2 and their low affinity alpha Hb aids in O_2 offloading at the tissues (Weber et al, 1988a). As another example, Andean camelids such as the vicuña have amino acid substitutions in the alpha chain of Hb that eliminates the binding sites of allosteric modulators such as Cl^- and 2,3-DPG (Kleinschmidt et al., 2009). Uniquely, there are unique high-affinity alleles of alpha-globin genes that are enriched in high-altitude populations (Snyder et al., 1988). The high-affinity allele which is found in higher frequencies in high-altitude natives has amino acid substitutions in the lining of the heme pocket which changes the polarity of the cavity and increases Hb-O_2 binding affinity (Storz et al., 2007; Storz et al., 2010a; Inoguchi et al., 2013). Studies of deer mice have shown that adaptive variation in Hb-O_2 affinity and aerobic performance is associated with genetic variation in the alpha and beta subunits of Hb (Snyder et al., 1982; Chappell and Snyder, 1984). Allelic variation shows that mice with high Hb-O_2 affinity have greater aerobic performance at high altitude than mice with low Hb-O_2 affinity but poorer aerobic performance at low-altitude than low-altitude natives. This suggests that the high Hb-O_2 affinity genotype has higher fitness at high-altitude and the low Hb-O_2 affinity genotype has greater fitness at low-altitude. The patterns of allelic variation of the alpha and beta subunits represent the varying selection between high- and low-altitude populations (Storz et al., 2007, 2008, 2009). Taken together, there are several ways that species evolved to increase Hb-O_2 affinity in hypoxia and given the suite of possible adaptations it seems that an increase in Hb-O_2 affinity is greatly beneficial at high altitudes.

In birds there is a strong positive relationship between Hb-O_2 affinity and native altitude (Jessen 1991; Storz, 2016). In many cases, such changes have arisen in response to strong selection on hemoglobin genes, suggesting that increases in Hb-O_2 affinity are adaptive at high altitude for most high-altitude avian taxa (Weber, 2007; McCracken et al., 2009; Natarajan et al., 2015; Natarajan et al., 2018). For mammals living at high altitude the adaptive trend for increased Hb-O_2 affinity is equivocal. In comparisons between small endothermic mammals native to high-altitude and their low-altitude counterparts such as deer mice and pikas, high-altitude natives have greater Hb-O_2 affinities (Storz et al., 2009; Tufts et al., 2015). However, this trend is not consistent in larger mammals such as marmots and snow leopards which show no distinguishable differences in Hb-O_2 affinities between high- and low-altitude natives (Revsbech et al., 2013; Janecka et al., 2015). Therefore, there's little evidence for an adaptive trend in Hb-O_2 affinity in high-altitude mammals. In high altitude species with increased Hb-O_2 affinity, this increased affinity is presumed to safeguard arterial O_2 saturation and improve circulatory O_2 transfer to enhance aerobic capacity, but this is rarely tested (Chappell and Snyder, 1984; Wearing et al., 2021). However, evolved changes in various other cardiorespiratory traits have also occurred in high-altitude taxa which may contribute to increasing aerobic capacity in hypoxia (Faraci, 1991; Monge and León-Velarde, 1991; McClelland and Scott, 2019; Ivy and Williamson, 2024). Improvements in tissue O_2 extraction would positively impact the influence of Hb-O_2 affinity on aerobic capacity as they would confer an increased ability to take advantage of greater carrying capacity of O_2. Evolved increases in traits such as diffusion distances between blood and active tissues, capillarity, fiber type composition, and oxidative enzyme activity would therefore be beneficial in conferring positive effects of greater Hb-O_2 affinities. Without these improvements in O_2 extraction and consumption there would be much less of an advantage of high Hb-O_2 affinity since less O_2 could be delivered to and consumed by the active tissues.

1.4 Evidence on the Influence of Hemoglobin-Oxygen Affinity on Aerobic Capacity in Hypoxia

Despite the fact that increased Hb-O_2 affinity is often observed in high altitude taxa, there is an ongoing debate over whether an increase or decrease in Hb-O_2 affinity is more beneficial for increasing aerobic capacity in hypoxia (Bunn, 1980; Samaja et al., 2003; Dempsey 2020). In humans, the initial acclimatization response to hypoxia is a suite of hematological changes which increase Hb concentrations in the blood and decrease Hb-O_2 affinity (Monge and Leon-Velarde, 1991; Storz et al., 2010b). Based on these observations, it was originally thought that decreases in Hb-O_2 affinity are more beneficial at high altitude in humans as they enhance O_2 offloading at systemic tissues. The decrease in Hb-O_2 affinity that is often observed in humans exposed to hypoxia is due to an increased concentration of erythrocytic 2,3-DPG (Aste-Salazar and Hurtado, 1944; Lenfant et al., 1968). Hypoxic conditions stimulate 2,3-DPG synthesis because of increase in ventilation experienced at high altitudes which results in respiratory alkalosis that stimulates red blood cell glycolytic activity and increases 2,3-DPG synthesis (Rapoport et al., 1977). 2,3-DPG decreases Hb-O_2 affinity as it preferentially binds and stabilizes deoxy-hemoglobin which shifts the oxygen equilibrium curve to the right and favors low-affinity Hb (Lenfant et al., 1971). Although this is commonly observed in humans as they ascend in altitude, other research in humans indicates that increases in Hb-O_2 affinity are advantageous in hypoxia as they increase pulmonary O_2 uptake and increase SaO_2. Humans that possess mutations in Hb that increase Hb-O_2 affinity exhibit smaller declines in VO_2max in hypoxia compared to those with normal Hb and they have lesser declines in SaO_2 (Hebbel et al., 1978; Dominelli et al., 2020). In addition, theoretical modelling of the O_2 transport pathway using empirical data generated in a small group of low-altitude native humans found that variations in Hb-O_2 have modest effects on VO_2max in hypoxia (Wagner, 1997). Overall, research in humans suggests that the influence of Hb-O_2 affinity on aerobic capacity in hypoxia may be context-dependent and can differ between individuals, populations, and species as a result of genetic and environmental factors. The physiological mechanisms behind these differences are still poorly understood.

1.5 Model Species

The North American deer mouse (*Peromyscus maniculatus*) has the widest altitudinal range of any north American mammal as they are found below sea level to as high as 4350m above sea levels in the Rocky Mountains (Hock 1964). High-altitude populations of deer mice have greater thermogenic VO_2max in hypoxia compared to their low-altitude counterparts due to the combined influence of plastic and evolved responses to hypoxia (Cheviron et al., 2012; Scott et al., 2015, Tate et al., 2020, Ivy et al., 2020). This increased thermogenic VO_2max is associated with evolved increases in Hb-O_2 affinity in high-altitude mice (Snyder et al., 1982; Storz et al., 2009; Storz et al., 2010, Ivy et al., 2020). High-altitude mice also exhibit characteristics that are likely to increase the capacity to extract and consume O_2 from the blood such as increased capacities for circulatory O_2-delivery, tissues O_2-diffusion, and mitochondrial O_2-utilization in skeletal muscles and iBAT (Lui et al., 2015, Mahalingam et al., 2017; Mahalingam et al., 2020; Tate et al., 2020; Lyons et al., 2021, Coulson et al., 2021, Wearing et al., 2022; Scott et al., 2024). These characteristics make it more likely for them to take advantage of increases in Hb-O_2 affinity.

The North American deer mouse presents a unique model to study the effects of single changes in the O_2 transport pathway on aerobic capacity as they have distinct genetic backgrounds and marked differences in the capacity to extract and consume oxygen at the level of the tissues. By studying these populations, we can disentangle environmental and genetic factors that increase thermogenic capacity at high altitude and the suite of adaptations that make high rates survival and fitness possible.

1.6 Objectives and Hypothesis

The objective of this study was to determine whether there are differences in the relationship between Hb-O_2 affinity and aerobic capacity in high- and low-altitude deer mice by pharmacologically manipulating Hb-O_2 affinity and observing the effects on maximal O_2 consumption in hypoxia in both populations. Mice from populations native to high- and low-altitude were acclimated to their natural conditions, creating two groups with very different capacities for O_2-transport in hypoxia. Maximal aerobic capacity for thermogenesis (VO_2max) was then measured in hypoxia after each of three pharmacological treatments to manipulate Hb-O_2 affinity: saline (control); efaproxiral, a negative allosteric regulator that decreases Hb-O_2 affinity; and sodium cyanate, which covalently modifies Hb and increases Hb-O_2 affinity. I hypothesized that the influence of Hb-O_2 affinity on aerobic capacity in hypoxia would be increased in high-altitude deer mice due to their enhanced capacity to uptake, transport, and consume O_2 across the O_2 transport pathway. I predicted that highland mice would have greater capacities to extract and consume O_2 at the level of the tissues by having greater enzymatic activities and a higher abundance of proteins needed for non-shivering thermogenesis. The experiments that follow in Chapter 2 test this hypothesis and these predictions and add to the growing body of work on the importance of coordinated changes across the O_2 transport pathway that are necessary to survive and thrive in challenging environments.

CHAPTER TWO: THE INFLUENCE OF HEMOGLOBIN OXYGEN AFFINITY ON AEROBIC CAPACITY IN HIGH ALTITUDE DEER MICE (*Peromyscus maniculatus*)

2.1 Introduction

Hemoglobin (Hb) plays a vital role in the O_2 transport pathway, and there has been longstanding interest in understanding how changes in Hb-O_2 affinity affect circulatory O_2 transport and aerobic metabolism. In particular, the relationship between Hb-O_2 affinity and aerobic capacity in hypoxia – as reflected by maximal rates of O_2 consumption (VO_2max) – has been debated for decades and remains contentious (Bunn, 1980; Samaja et al., 2003; Dempsey 2020). One common point of view posits that a reduction in Hb-O_2 affinity is advantageous in hypoxia because it augments the release of O_2 at systemic tissues. This view arose from early observations in low-altitude humans, in which exposure to high-altitude hypoxia led to rightward shifts in the Hb-O_2 equilibrium curve due to increased erythrocytic concentrations of 2,3-diphosphoglycerate (2,3-DPG) (Aste-Salazar and Hurtado, 1944; Lenfant et al., 1968). However, some other research in humans suggests that increases in Hb-O_2 affinity are advantageous in hypoxia by augmenting pulmonary O_2 uptake. For example, humans possessing rare mutations that increase Hb-O_2 affinity exhibited smaller declines in VO_2max in hypoxia compared to individuals with normal Hb, in association with lesser declines in arterial O_2 saturation (Hebbel et al., 1978, Dominelli et al., 2020). Furthermore, theoretical modeling of the O_2 pathway in a group of low-altitude human subjects found that variation in Hb-O_2 affinity may have very modest effects of on VO_2max in hypoxia in some cases (Wagner, 1997). Taken together, the previous research in humans suggests that the influence of Hb-O_2 affinity in hypoxia can vary and may be context-dependent, a possibility that is supported by similarly conflicting findings in studies of various other mammal species (Samaja et al., 2003). The influence of Hb-O_2 affinity could thus differ between individuals, populations, or species due to genetic or environmental factors, but the physiological mechanisms underlying such differences remain poorly understood.

High-altitude natives present compelling opportunities to examine the influence of Hb-O_2 affinity on aerobic capacity. High-altitude environments are both cold and hypoxic, which challenges endotherms to maintain high rates of aerobic thermogenesis in thin air with little O_2 available. Many birds and mammals native to high altitude have evolved an increased Hb-O_2 affinity compared to their low-altitude counterparts (Snyder, 1985; Jessen, 1991; Tufts et al., 2013;

Storz, 2016). In many cases, such changes have arisen in response to strong selection on hemoglobin genes, suggesting that increases in Hb-O_2 affinity can be adaptive at high altitude (Weber, 2007; McCracken et al., 2009; Natarajan et al., 2013; Natarajan et al., 2015; Natarajan et al., 2016; Natarajan et al., 2018). The adaptive significance of increases in Hb-O_2 affinity may facilitate safeguarding arterial O_2 saturation in hypoxia, thus helping improve circulatory O_2 transport and aerobic capacity, although this has rarely been tested (Chappell and Snyder, 1984; Wearing et al., 2021). However, evolved changes in various other cardiorespiratory traits have arisen in high-altitude taxa, which likely also contribute to increasing aerobic capacity in hypoxia (Faraci, 1991; Monge and León-Velarde, 1991; McClelland and Scott, 2019; Ivy and Williamson, 2024). Considering the functional integration of Hb within the O_2 transport pathway, the influence of Hb-O_2 affinity on aerobic capacity in high-altitude taxa may be influenced by these evolved changes in other cardiorespiratory traits. For example, the influence of Hb-O_2 affinity on aerobic capacity could depend on the capacity of peripheral tissues to extract and consume O_2 from the blood (Wearing et al., 2021).

The North American deer mouse (*Peromyscus maniculatus*) is a useful species for examining the influence of Hb-O_2 affinity on aerobic capacity and how this influence may change in high-altitude populations. Deer mice have the widest altitudinal range of any north American mammal, stretching from near sea level to >4300m altitude in the Rocky Mountains. High-altitude populations of deer mice have greater thermogenic VO_2max in hypoxia than low-altitude mice due to the combined influence of both plastic and evolved responses to cold hypoxia (Chappell and Hammond, 2004; Rezende et al., 2004; Chappell et al., 2007; Cheviron et al., 2013; Tate et al., 2017; Tate et al., 2020; Ivy et al., 2021; Robertson and McClelland, 2021). These population differences in thermogenic VO_2max are associated with an evolved increase in Hb-O_2 affinity in high-altitude mice (Snyder et al., 1982; Storz et al., 2009; Storz et al., 2010; Ivy et al., 2020). High-altitude deer mice also exhibit increased capacities for circulatory O_2 delivery, tissue O_2 diffusion, and mitochondrial O_2 utilization in skeletal muscles and brown adipose tissue (Lui et al., 2015, Mahalingam et al., 2017; Mahalingam et al., 2020; Tate et al., 2020; Lyons et al., 2021, Coulson et al., 2021, Wearing et al., 2022; Scott et al., 2024). Because these characteristics are likely to increase the capacity of high-altitude mice to extract and consume O_2 from the blood, we hypothesized that the influence of Hb-O_2 affinity on aerobic capacity in hypoxia would be increased in high-altitude deer mice. We tested this hypothesis by pharmacologically manipulating

Hb-O_2 affinity and comparing the resulting effects on thermogenic VO_2max in hypoxia between high- and low-altitude populations of deer mice, each of which were acclimated to conditions simulating their respective natural environments. We predicted that increases in Hb-O_2 affinity and arterial O_2 saturation would increase thermogenic VO_2max up to an optimum, and that this optimum would be greater in high-altitude mice than in low-altitude mice. We also predicted that the thermogenic tissues of high-altitude mice would have greater capacities to extract and consume O_2 and generate body heat compared to those of low-altitude mice, as a potential explanation for population differences in the optimum Hb-O_2 affinity.

2.2 Methods

2.2.1 Animals and Environmental Acclimations

Captive breeding populations were established from wild populations of deer mice native to high altitude near the summit of Mount Blue Sky, CO, USA (39°35′18″N, 105°38′38″W; 4350 m above sea level) and low altitude in the farmed areas surrounding Kearney, NE, USA (40°42′5.4″N, 99°4′58.7″W; 660 m above sea level). Wild mice were transported to McMaster University (near sea level) where they were housed in standard conditions (24-25°C, 12h:12h light: dark photoperiod) and bred within population to produce laboratory-raised progeny. The third generation of these lab mice were raised to 4-8 months of age and each population was then acclimated to conditions simulating its native environment, in which mice from the low-altitude population ('lowlanders') remained in control conditions of warm normoxia (24-25°C, ~20-21 kPa O_2) and mice from the high-altitude population ('highlanders') were acclimated to cold hypoxia (5°C, 12 kPa O_2) for 6 weeks. Acclimation to cold hypoxia was conducted in hypobaric chambers to maintain the barometric pressure at 60 kPa, as previously described (Lui, 2015; McClelland, 1998), and these chambers were held in a room controlled at 5°C. Mice were removed from the chambers 2-3 times per week for <20 minutes for cage cleaning and/or cyanate treatment (see below). All mice were held at a 12h:12h light:dark photoperiod with unlimited access to rodent chow (Teklad 22/5 Rodent Diet formula 8640; Envigo) 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 (Animal Use Protocols 20-01-02 and 23-85).

2.2.2 Pharmacological Treatments

Pharmacological treatments to manipulate hemoglobin-O_2 affinity began after completion of environmental acclimations (i.e., after 6 weeks of cold hypoxia in highlanders, warm normoxia at overlapping times and ages in lowlanders). Efaproxiral (efaproxiral sodium, Fisher Scientific, Whitby, ON, Canada) is a synthetic allosteric modifier of haemoglobin (Hb) that can be administered to acutely reduce Hb-O_2 affinity for short periods of time (<1 h). Cyanate (sodium cyanate, Sigma Aldrich, Oakville, ON, Canada) induces irreversible carbamylation of the Hb protein and can be used to increase Hb-O_2 affinity over several days to weeks. Mice were subjected to three measurements of respirometry and pulse oximetry (see below), once after treatment with each pharmacological agent and once after treatment with control saline. Due to the distinct durations of treatment and effect for each of these pharmacological agents, mice were first subjected to measurements after acute treatments with each of efaproxiral and control saline, in random order and separated by 2 days. This was achieved by giving mice an intraperitoneal injection of efaproxiral (200 mg kg^{-1} body mass in sterile 0.9% NaCl solution) or control saline (each at a volume of 20 ml kg^{-1} body mass) and then performing measurements ~10 min later. Cyanate was then administered by intraperitoneal injections over 2 weeks, with 6 injections spaced 2-3 days apart (each at 100 mg kg^{-1} body mass in sterile 0.9% NaCl solution, given at a volume of 5 ml kg^{-1} body mass), after which mice were subjected to a final set of respirometry and pulse oximetry measurements.

2.2.3 Respirometry and Pulse Oximetry

Thermogenic capacity, as reflected by the maximum rate of O_2 consumption (VO_2max) during acute cold exposure at -5°C, was measured using open-flow respirometry in hypoxic heliox (12% O_2, 88% He). The respirometry chamber (550 ml) was placed in a freezer maintained at a nominal ambient temperature of -5°C, recorded with a thermocouple in the chamber (ADInstruments, Colorado Springs, CO, USA). The chamber was supplied with a constant incurrent flow rate of 1500 ml min^{-1}, regulated using a mass flow controller (MFC-2, Sable Systems, Las Vegas, NV, USA). The excurrent gas was subsampled at 200 ml min^{-1}, dried with pre-baked Drierite, and analyzed for O_2 and CO_2 fractions (FC-10 and CA-10, respectively, Sable Systems, Las Vegas, NV, USA). Chamber temperature, incurrent flow rate, and excurrent O_2 and CO_2 fractions were recorded using a PowerLab 8/32 and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, USA). Baseline measurements of O_2 and CO_2 fractions were first measured before the animal was placed in the chamber. The mouse was given the appropriate intraperitoneal injection as described above, and then instrumented with a pulse oximetry collar to measure and record arterial O_2 saturation (SaO_2) and heart rate (f_H) using a MouseOx Plus pulse oximeter and associated acquisition software (Starr Life Sciences, Oakmont, PA, USA). The mouse was then placed in the chamber while recordings continued. VO_2max was defined as the highest O_2 consumption rate (VO_2) achieved over a 5 second period during the trial. Trials are run until VO_2 peaks at VO_2max and then subsequently declines to <90% of VO_2max, which typically takes 10-20 min. The SaO_2 and heart rate measurements reported here were the average values recorded within 15 s of VO_2max. Core body temperature was measured after the trial using a rectal probe (RET-3, ADInstruments). Pulse oximetry measurements required that the hair be removed from the animal's neck, which was done two days before the first and last respirometry trials under light isoflurane anesthesia (1.5% in O_2) using Nair™ hair removal product.

Each mouse was humanely euthanized following their final set of respirometry and pulse oximetry measurements using gaseous isoflurane overdose (5% in O_2) followed by cervical dislocation. Blood was collected for haematological measurements as described below. The interscapular brown adipose tissue (iBAT) and right gastrocnemius muscle were weighed, frozen in liquid N_2, and stored at -80°C for later use for western blotting and enzyme assays, respectively. The left gastrocnemius was coated in embedding medium, frozen in liquid N_2-cooled isopentane, and stored at -80°C for later use for histology. Several other organs were removed and weighed.

2.2.4 Hematological Measurements

Hemoglobin-O_2 affinity, blood Hb content, and haematocrit were measured in the mice used for respirometry measurements. Small blood samples (< 50 μl) were taken from the tail vein for measurements of Hb-O_2 affinity and blood Hb content before saline/efaproxiral injections (2 days before respirometry measurements), and larger blood samples for all three measurements were taken after cyanate injections and the final respirometry measurements. Hb-O_2 affinity was measured using a Hemox Analyzer (TCS Scientific, New Hope, PA, USA) with 10 μl of whole blood in 5 ml of buffer solution (50 mM Hepes, 10 mM EDTA, 100 mM NaCl, 0.1% bovine serum albumin, 0.2% anti-foaming agent [TCS Scientific], pH 7.4). Oxygen dissociation curves and associated P_{50} values were then determined at 37°C as recommended by the manufacturer. We only report P_{50} values for control and cyanate treatment conditions. This is because the effects of efaproxiral treatment on P_{50} cannot be assessed using a Hemox Analyzer, because the 500-fold dilution of blood in buffer leads to a corresponding dilution of efaproxiral (which is membrane permeable) and thus leads to a dramatic reduction in the number of efaproxiral molecules interacting with Hb. Blood Hb content was measured using Drabkin's reagent as per manufacturer guidelines (Sigma-Aldrich, Oakville, ON, Canada). Hematocrit was measured by centrifuging whole blood in 75 μl capillary tubes at 12,700 g for 5 min. Mean cell hemoglobin concentration was calculated as the quotient of blood Hb content and hematocrit.

2.2.5 Western Blotting

Protein content of uncoupling protein 1 (UCP1) and citrate synthase (CS) were measured in interscapular brown adipose (iBAT) by western blot analysis. Frozen samples were powdered in a mortar and pestle that were cooled with liquid N_2. Powdered tissues were homogenized in cold RIPA buffer (150 mM NaCl, 50 mM Tris HCl, 1% Triton X-100, 0.5% deoxycholic acid, and 0.1% SDS at pH 8) using a glass tissue grinder. Proteins in the homogenate were then denatured at 95°C for 5 min in Laemmli buffer containing 10% 2-mercaptoethanol (Bio-Rad, Mississauga, ON, Canada). Protein concentration was determined using a detergent compatible (DC) protein assay kit (Bio-Rad). Denatured homogenate (6 μg of total protein) was loaded into pre-cast 12% Mini-PROTEAN TGX Protein Gels (Bio-Rad) and separated for 30 min at 100V and then 45 min at 150V using a Mini-Protein Tetra system (Bio-Rad). Proteins were transferred from gel to polyvinylidene difluoride (PVDF) membrane for 7 min at 25V and 1.3A using a Trans Blot Turbo Transfer System (Bio-Rad). Membranes were cut at roughly 37 kDa between the expected protein bands for UCP1 (33 kDa) and CS (45 kDa). Membranes were blocked overnight at 4°C using 5% Skim Milk Powder in phosphate-buffered saline containing Tween-20 (PBST; 136.89 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1% Tween-20 at pH 7.4). Membranes were then incubated for 45 min at 4°C in PBST containing rabbit IgG primary antibodies and 1% Bovine Serum Albumin (BSA). The bottom half of the membrane was incubated with an antibody against UCP1 (1:1000 dilution; UCP1-A, Alpha Diagnostics International, San Antonio, TX, USA), and the top half was incubated with an antibody against CS (1:1000 dilution; PA5-22126, Invitrogen, Thermo Fisher Scientific). Membranes were then washed 3 times in PBST for 10 min each and then incubated for 45 min at 4°C in PBST containing goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (1:5000 dilution, 31466, Invitrogen, Thermo Fisher Scientific) and 1% BSA. Membranes were then washed 3 times in PBST for 10 min and developed in Clarity ECL Substrate (Bio-Rad). Band intensity was detected by chemiluminescence using a ChemiDoc MP Imaging system and Image Lab Software (Bio-Rad). A common protein sample was included on each gel to account for variation in membrane protein transfer and target protein abundance and all other samples were quantified relative to this common protein sample. Protein abundance was normalized to total protein concentration as determined by Coomassie Blue staining.

2.2.6 Enzyme Assays

Maximal activities of cytochrome c oxidase (COX) and citrate synthase (CS) were assayed in the right gastrocnemius muscle. Frozen samples were powdered under liquid N_2 (as above) and stored at -80°C. Powdered samples were homogenized in 20 volumes of homogenization buffer (100 mM KH_2PO_4, 5 mM ethylenediaminetetraacetic acid, and 1% Triton-X-100, pH 7.2) using a glass tissue grinder. COX activity was assayed shortly after homogenization and CS activity was measured after subsequent storage of homogenate at -80°C. Activity was assayed at 37°C by measuring the change in absorbance over time (COX 550 nm; CS, 412 nm) under the following conditions: COX, 50 mM Tris base, 0.15 mM reduced cytochrome c, pH 8.0; CS, 40 mM Tris, 0.5 mM oxaloacetate, 0.23 mM acetyl-CoA, 0.1 mM DTNB, pH 8.0. Preliminary experiments were done to verify that substrate concentrations were saturating. All assays were run in triplicate plus a background control where the driving substrate for the assay was omitted (cytochrome c and oxaloacetate, respectively). Activities were determined by subtracting the background rate from the rates measured in the presence of substrate.

2.2.7 Histology

Oxidative fibres and capillaries were quantified in the left gastrocnemius muscle by histology. The frozen embedded tissues were sectioned transverse to muscle fiber length (10 μm thick) in a cryostat at -20°C. Oxidative muscle fibers (both slow and fast) were identified by staining for succinate dehydrogenase activity (assay buffer concentrations: 0.6 mM nitroblue tetrazolium, 2.0 mM KH_2PO_4, 15.4 mM Na_2HPO_4, and 16.7 mM sodium succinate) for 1 h at room temperature. Capillaries were identified by staining for alkaline phosphatase activity (assay buffer concentrations: 1.0 mM nitroblue tetrazolium, 0.5 mM 5-bromo-4-chloro-3-indoxyl phosphatase, 28 mM $NaBO_2$, and 7 mM $MgSO_4$; pH 9.3) for 1 h at room temperature. Stained slides were coverslipped with Permount (Fisher Scientific), and images were collected systematically across the entire section using light microscopy. Stereological methods were used to make unbiased measurements of the areal and numerical densities of oxidative fibers, the average transverse area of oxidative and glycolytic fibers, capillary density, and capillary to fiber ratio. Image analyses were performed by an experimenter that was blind to population.

2.2.7 Statistical Analysis

Linear mixed-effects models were used for statistical analyses performed in R (version 4.4.0; http://www.R-project.org/) using the lme4 package (Bates et al., 2015). All models tested the fixed effects of population, body mass, and sex, with family and age included as random effects. Data with repeated measurements (VO_2max, SaO_2, heart rate, and some haematology) also tested the fixed effects of treatment and treatment×population and included individual (nested within family) as a random effect to account for repeated measurements. For the statistical analysis of VO_2max and organ masses, tests were carried out on absolute values, but the data reported here are expressed relative to body mass as is conventional in the literature. The lmerTest package was used to generate ANOVA p-values for fixed effects and interactions (Kuznetsova et al., 2017). When the fixed effects and/or interactions were significant, the emmeans package (version 1.8.9; https://CRAN.R-project.org/package=emmeans) was used to make post-hoc pairwise comparisons using the Tukey method. We also report effect size estimates (β) ± s.e. for some key comparisons from statistical models. $p<0.05$ was considered significant. Generalized additive mixed models (GAMM) were used to test for effects of SaO_2, population and SaO_2×population on VO_2max using the gam function in the mgcv package in R (Wood, 2017). Different populations were allowed to have distinct smoothing curves, and smoothing curves could also vary by the random effect of individual (fs smooth term).

2.3 Results

2.3.1 Pharmacological manipulation of hemoglobin-O_2 affinity altered thermogenic capacity in hypoxia

Drug treatments effectively manipulated hemoglobin-O_2 (Hb-O_2) affinity along with *in vivo* measurements of arterial O_2 saturation at thermogenic VO_2max (Fig. 1, Table 1). There was a significant effect of treatment on SaO_2 ($p<0.0001$), in which efaproxiral lowered SaO_2 and cyanate increased SaO_2 (Fig 1A). However, highlanders had greater SaO_2 than lowlanders overall (population effect, $p=0.040$). The magnitude of the effects of treatment also differed between populations (population×treatment, $p=0.0021$), as reflected by lowlanders exhibiting a greater decrease in SaO_2 than highlanders following treatment with efaproxiral ($\beta \pm$ s.e., -15.76 ± 2.02% vs -10.68 ± 2.05%, respectively) and a greater increase following treatment with cyanate ($\beta \pm$ s.e., 16.15 ± 2.07% vs 10.47 ± 2.05%). Mice treated with cyanate also had significantly lower P_{50} values than those treated with saline (treatment, $p<0.0001$), with both lowlanders and highlanders exhibiting lower P_{50} values after treatment with cyanate ($\beta \pm$ s.e., -9.61 ± 0.58 mmHg and -8.97 ± 0.49 mmHg, respectively) (Fig 1B). However, highland mice had lower P_{50} values than lowland mice before cyanate treatment, and this difference tended to persist across drug treatments (population effect, $p=0.054$). Hematocrit was also elevated in highland mice compared to lowland mice (population effect, $p=0.001$), but blood hemoglobin content was similar between populations ($p=0.5385$) and was not significantly affected by drug treatment ($p=0.0979$) (Table 2).

Thermogenic VO_2max was significantly affected by pharmacological manipulation of Hb-O_2 affinity (treatment effect, $p<0.0001$) (Fig. 2, Table 1). This treatment effect was largely driven by efaproxiral, which significantly decreased thermogenic capacity in both populations by ~0.035 ml g^{-1} min^{-1} ($p<0.0001$ for both populations in pairwise comparisons). The effect of sodium cyanate varied between individuals and was not statistically significant ($p>0.4$ for both populations in pairwise comparisons). Across all treatment groups, highland mice had greater thermogenic capacities than lowland mice (population effect, $p=0.0025$). As expected, there was a strong effect of body mass on thermogenic VO_2 max ($p<0.0001$), but body mass was not different between lowland and highland mice (Population effect, $p=0.93$) (Table 2). Heart rate at VO_2max was unaffected by pharmacological treatment ($p=0.2106$) (Table 2), and although highland mice tended to have greater heart rates than lowland mice on average, the effect of population did not reach significance ($p=0.1375$).

The relationship between SaO_2 and thermogenic capacity differed between populations and suggested that the optimal Hb-O_2 affinity is greater in highland mice (Fig. 3). The variation in Hb-O_2 affinity as a result of population and treatment were mirrored by corresponding variation in SaO_2 (Fig. 1), suggesting that the latter can be used as an effective *in vivo* proxy for Hb-O_2 affinity, in which increases in SaO_2 reflect increases in Hb-O_2 affinity (decreases in P_{50}). This is a particular advantage for evaluating the *in vivo* effects of efaproxiral, for which the exact effects on Hb-O_2 affinity cannot be measured using our system of P_{50} measurement (see Methods). While SaO_2 tended to affect thermogenic capacity in hypoxia (SaO_2 effect, $p=0.051$), the relationship between SaO_2 and thermogenic capacity differed appreciably between populations (SaO_2×population, $p<0.0001$) (Fig. 3). Most notable was the effect of SaO_2 on thermogenic capacity above a SaO_2 ~80%. In lowlanders, increasing SaO_2 beyond 80% had no effect on thermogenic capacity, which decreased slightly on average towards higher SaO_2 (resulting from higher Hb-O_2 affinity). In highlanders, by contrast, thermogenic capacity increased to at least a SaO_2 ~88% and appeared to increase further on average with greater increases in SaO_2. These patterns suggest that the optimal Hb-O_2 affinity (and associated SaO_2) of each population is around the normal value in control (saline) conditions, which is greater in highlanders than in lowlanders.

2.3.2 The phenotype of thermogenic tissues differed between highland mice and lowland mice

We next examined the population differences in thermogenic tissues that might alter the relationship between Hb-O_2 affinity and thermogenic capacity. Interscapular brown adipose tissue (iBAT) is a critical contributor to non-shivering thermogenesis, supported by the function of uncoupling protein 1 (UCP1) in mitochondria. Highland mice had greater iBAT mass (population effect, $p<0.0001$) (Fig. 4A) and UCP1 protein abundance in iBAT ($p=0.0162$) (Fig. 4C) compared to lowland mice, but exhibited no difference in citrate synthase (CS; a marker for mitochondrial content) abundance ($p=0.2931$) (Fig 4D). The gastrocnemius is a large hindlimb muscle involved in shivering thermogenesis. Gastrocnemius mass was smaller on average (Fig. 5A) and the activities of cytochrome c oxidase (COX; a marker of mitochondrial oxidative capacity) and CS were greater on average in highland mice (Fig. 5B,C), although the differences did not reach significance ($p=0.1011$, 0.055, and 0.259, respectively). The mass of various other skeletal muscles was similar between populations (Table 2). However, highland mice had greater total ventricle mass (population effect, $p=0.003$) and left ventricle and septum (LV+S) mass ($p=0.005$), but not right ventricle (RV) mass (Population; $p=0.115$), as compared to lowland mice (Table 2). This difference may reflect overall growth of the heart in highland mice to support greater cardiac output, rather than pathological RV hypertrophy, because the Fulton's ratio of RV mass to LV + S mass was similar between populations ($p=0.454$).

Table 2.1. Statistical results for liner mixed-effects models

Trait	Population (P)	Treatment (T)	P × T	Sex	Body mass
P_{50}	0.0538	**<0.0001**	0.4023	0.9501	0.4831
SaO_2	**0.0476**	**<0.0001**	**0.0021**	0.9494	0.6993
VO_2max	**0.0025**	**<0.0001**	0.5859	0.0629	**<0.0001**
Heart rate	0.1375	0.2106	0.2397	0.4979	0.1517
Blood [Hb]	0.5385	0.0978	0.4147	0.3705	**0.0417**
Hct	**0.0011**	-	-	0.9856	0.3759
MCHC	0.9505	-	-	**0.037**	**0.004**
Body mass	0.9291	-	-	**0.0389**	-
Lung Mass	0.2361	-	-	0.0999	**0.0444**
Ventricle Mass	**0.0027**	-	-	0.2167	**<0.0001**
RV Mass	0.1153	-	-	0.1309	0.9535
LV+S Mass	**0.0051**	-	-	0.4196	**0.0004**
RV/LV+S	0.454	-	-	0.3287	**0.025**
Erector Spinae Mass	0.1676	-	-	0.7643	**0.0003**
Vastus Medialis Mass	0.3305	-	-	0.1459	0.9983
Soleus Mass	0.1168	-	-	0.9297	**0.0064**
Plantaris Mass	0.2506	-	-	0.2877	0.1831
Gastrocnemius mass	0.1011	-	-	0.5477	**0.0017**
Gastrocnemius CS	0.2590	-	-	0.4047	0.8096
Gastrocnemius COX	0.0551	-	-	0.3403	0.2385
iBAT mass	**<0.0001**	-	-	0.8949	**0.0005**
iBAT CS	0.2931	-	-	0.2687	0.7999
iBAT UCP1	**0.0162**	-	-	0.8535	0.7778

P_{50}, partial pressure of O_2 at 50% hemoglobin saturation; SaO2, arterial oxygen saturation (%); VO_2max, maximum oxygen consumption (mL g^{-1} min^{-1}); Blood [Hb] (g dL^{-1}), blood hemoglobin concentration; Hct, hematocrit (%); MCHC, mean cell hemoglobin content (g dL^{-1}); RV, right ventricle; LV+S, left ventricle and septum; CS, citrate synthase; COX, cytochrome c oxidase; iBAT, interscapular brown adipose tissue; UCP1, uncoupling protein 1. Sex and body mass were included as co-variates in all analyses.

Table 2.2. Body and tissue masses, haematology, and heart rate

Phenotype	Lowlanders	Highlanders
Body Mass (g)	18.7 ± 1.1	18.7 ± 0.5
Lung Mass (mg g^{-1})	3.69 ± 0.45	4.95 ± 0.52
Total Ventricle Mass (mg g^{-1})	2.03 ± 0.23	2.30 ± 0.15*
Right Ventricle (RV) Mass (mg g^{-1})	0.27 ± 0.03	0.35 ± 0.04
Left Ventricle and Septum (LV+S) Mass (mg g^{-1})	1.76 ± 0.21	1.96 ± 0.13*
RV/LV+S	0.17 ± 0.01	0.18 ± 0.01
Erector Spinae Mass (mg g^{-1})	1.18 ± 0.16	1.03 ± 0.1
Vastus Medialis Mass (mg g^{-1})	0.30 ± 0.04	0.47 ± 0.19
Plantaris Mass (mg g^{-1})	0.19 ± 0.03	0.25 ± 0.03
Soleus Mass (mg g^{-1})	0.063 ± 0.008	0.078 ± 0.009
Hematocrit (%)	46.6 ± 0.9	51.0 ± 0.7*
MCHC (g dL^{-1})	36.8 ± 1.5	38.3 ± 0.8
Blood [haemoglobin] (g dL^{-1})		
Saline	16.6 ± 0.9	17.2 ± 0.7
Cyanate	17.2 ± 0.5	19.3 ± 0.3
Heart rate at VO_2max (min^{-1})		
Efaproxiral	498 ± 16	580 ± 34
Saline	487 ± 20	587 ± 31
Cyanate	542 ± 23	585 ± 24

Values are presented as means ± s.e.m. Masses, haematocrit, and mean cell haemoglobin content (MCHC) were only measured after cyanate treatment. Sample sizes were as follows: masses, N=14 lowlanders (6 females and 8 males) and N=14 highlanders (8 females and 6 males); haematology, N=14 lowlanders (6 females and 8 males) and N=14 highlanders (8 females and 6 males); heart rate, N=14 lowlanders (6 females and 8 males) and N=13 highlanders (8 females and 5 males). Data were analyzed statistically using linear mixed-effects models, the results of which are shown in Table 1 (*$p<0.05$ for population effect).

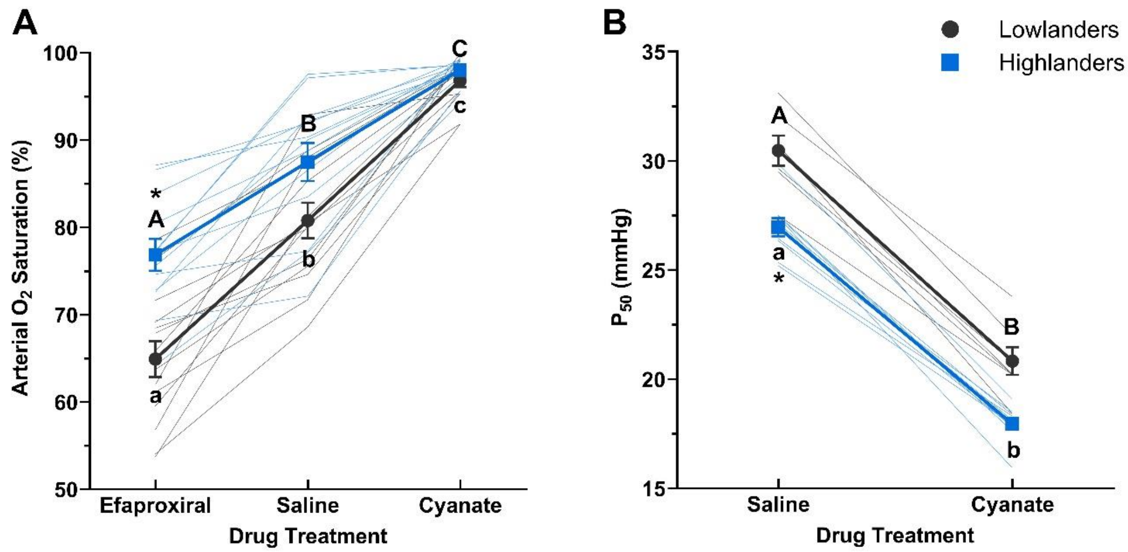

Figure 2.1. Arterial O_2 saturation and hemoglobin-oxygen binding affinity (P_{50}) were altered by drug treatment. (A) Arterial oxygen saturation (N=14 lowlanders, 6 females and 8 males; N=13 highlanders, 8 females and 5 males) and (B) P_{50} (N=7 lowlanders, 3 females and 4 males; N=10 highlanders, 6 females and 4 males), in lowland (grey circles) and highland (blue squares) deer mice following drug treatment. Thin lines represent the data for each individual mouse, whereas thick lines, symbols, and error bars reflect mean ± s.e.m. Statistical analysis was done using linear mixed-effects models, the results of which are shown in Table 1. Pairwise comparisons were performed using the Tukey method, in which asterisks represent significant differences between populations within a treatment group ($p<0.05$), and letters represent comparisons within each population where treatments not sharing a letter are significantly different ($p<0.05$).

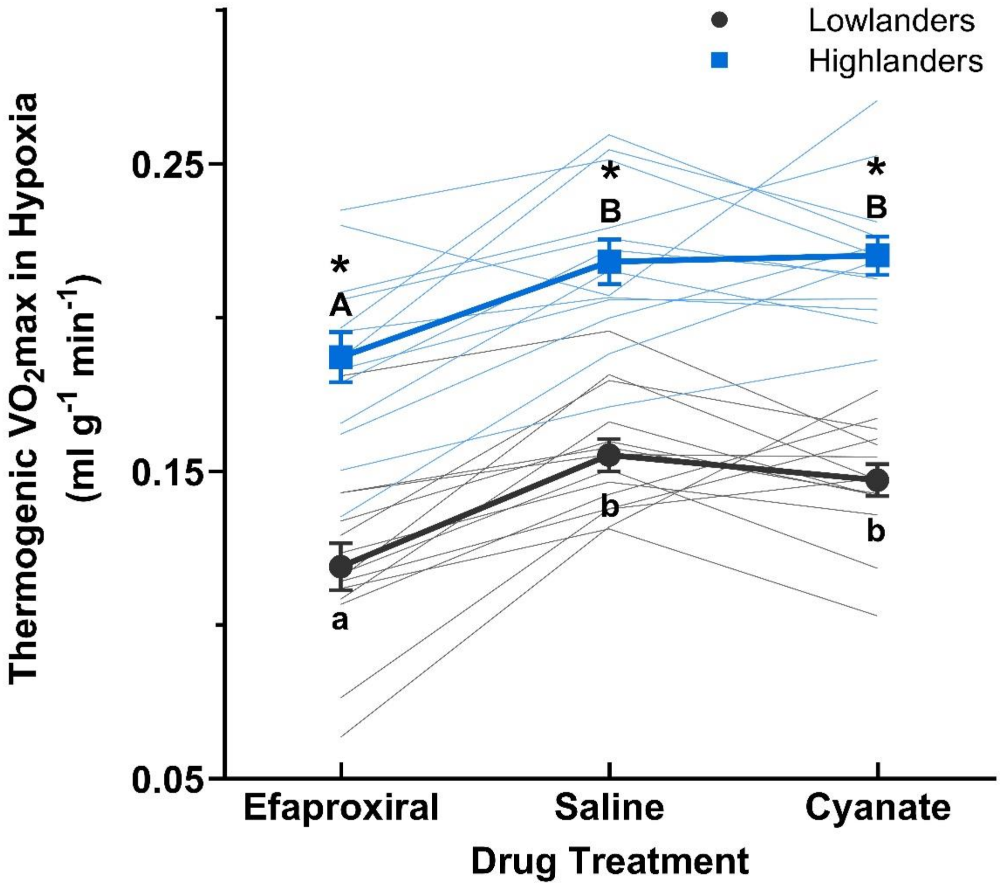

Figure 2.2. Thermogenic capacity in hypoxia is altered by drug treatment. Thermogenic VO_2max in hypoxia in lowland (grey circles) and highland (blue squares) deer mice (N=14 for both populations) following drug treatment. Thin lines represent the data for each individual mouse, whereas thick lines, symbols and error bars reflect mean ± s.e.m. Statistical analysis was done using linear mixed-effects models, the results of which are shown in Table 1. Pairwise comparisons were performed using the Tukey method, in which asterisks represent significant differences between populations within a treatment group ($p<0.05$), and letters represent comparisons within each population where treatments not sharing a letter are significantly different ($p<0.05$).

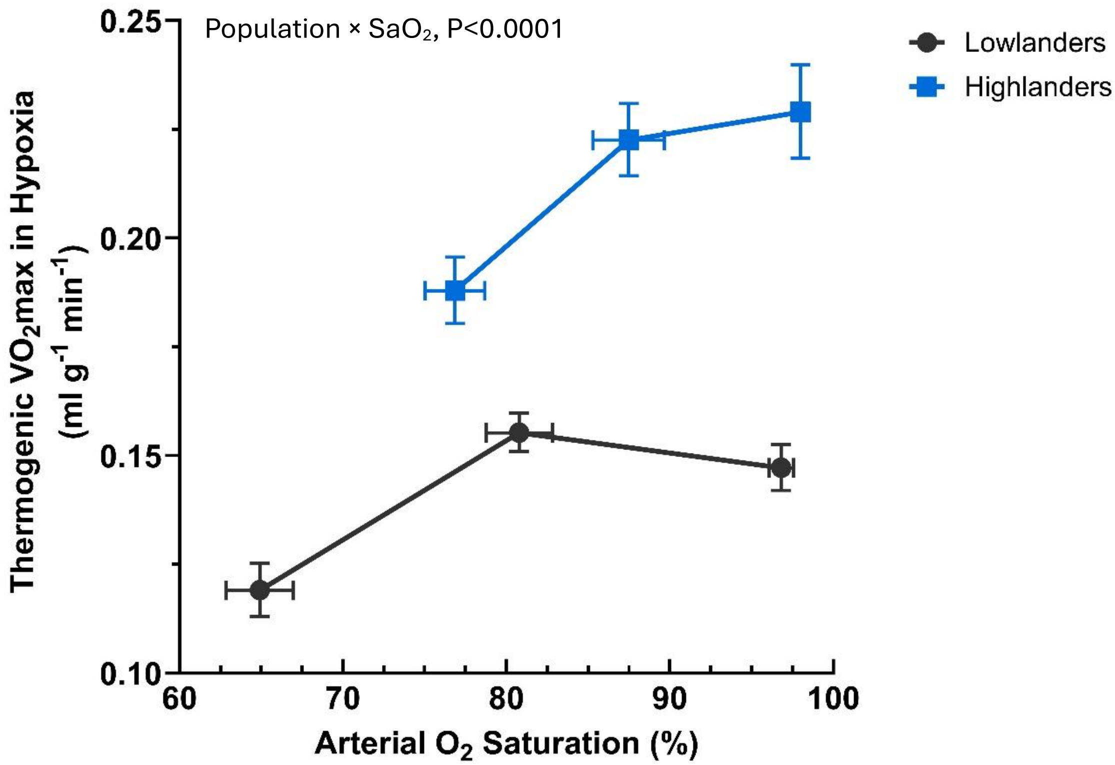

Figure 2.3. The relationship between thermogenic capacity in hypoxia and arterial O_2 saturation (SaO_2) differed between lowland (grey circles) and highland (blue squares) deer mice. This was supported by generalized additive mixed models of thermogenic capacity, which showed that there was a highly significant Population × SaO_2 interaction ($P<0.0001$) (Other statistical results: SaO_2 effect, $P=0.0509$; Population effect, $P=0.3727$; Sex effect, $P=0.2432$; Mass effect, $P<0.0001$). Symbols and error bars reflect mean ± s.e.m. (sample sizes as in Fig. 2).

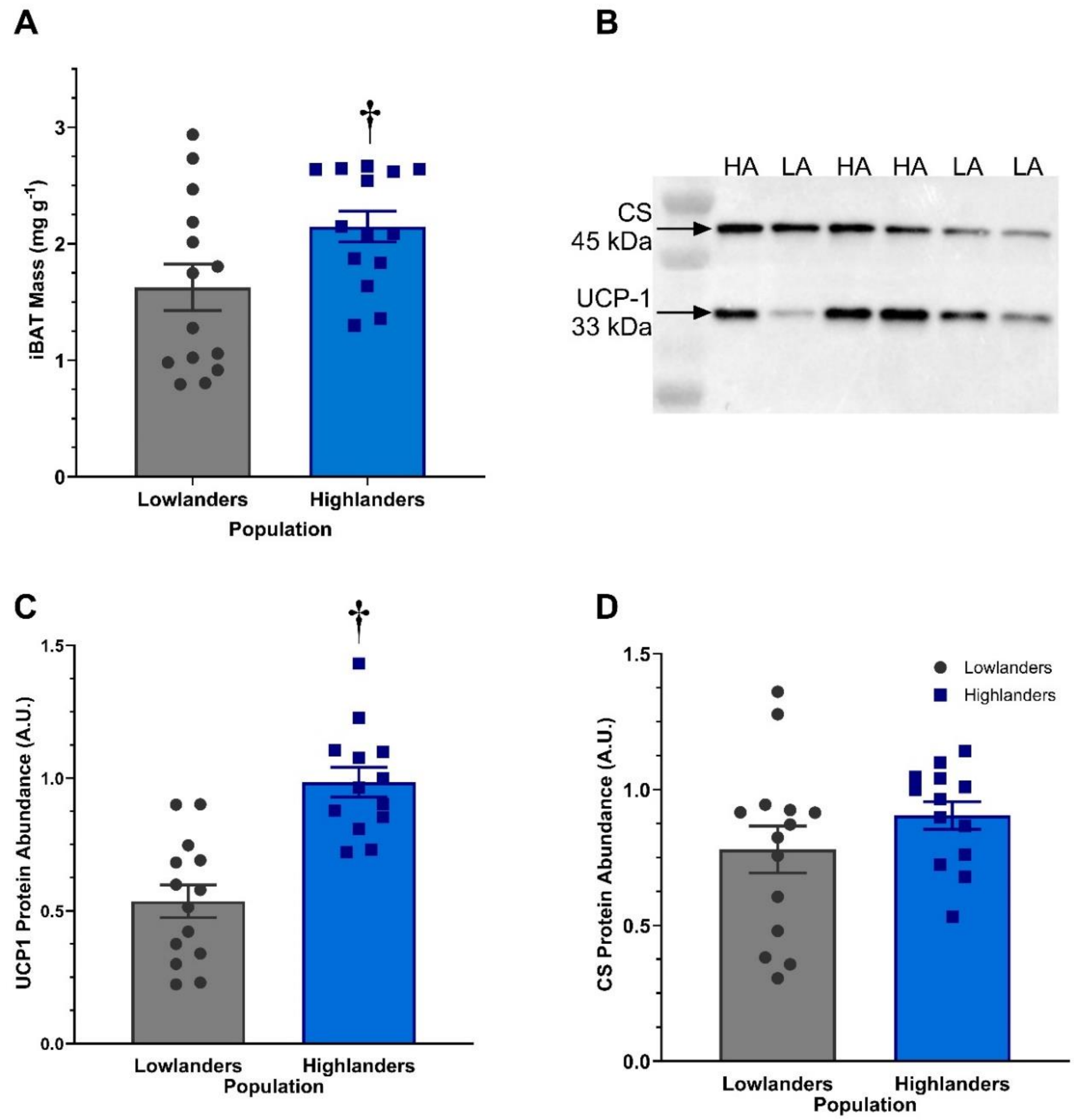

Figure 2.4. Highlanders have greater mass and uncoupling protein 1 (UCP1) protein abundance in interscapular brown adipose tissue (iBAT). (A) iBAT mass in lowland (grey circles) and highland (blue squares) deer mice. (B) Representative western blot of CS and UCP1 proteins in lowlanders (LA) and highlanders (HA). Protein abundance of (C) UCP1 and (D) citrate synthase (CS) in lowlanders and highlanders. Data are shown with symbols representing values for individual mice and bars representing means ± s.e.m., with N=14 lowlanders (6 females and 8 males) and N=14 highlanders (8 females and 6 males). Data was analyzed statistically using linear mixed-effects models, the results of which are shown in Table 1 († $p<0.05$ for population effect).

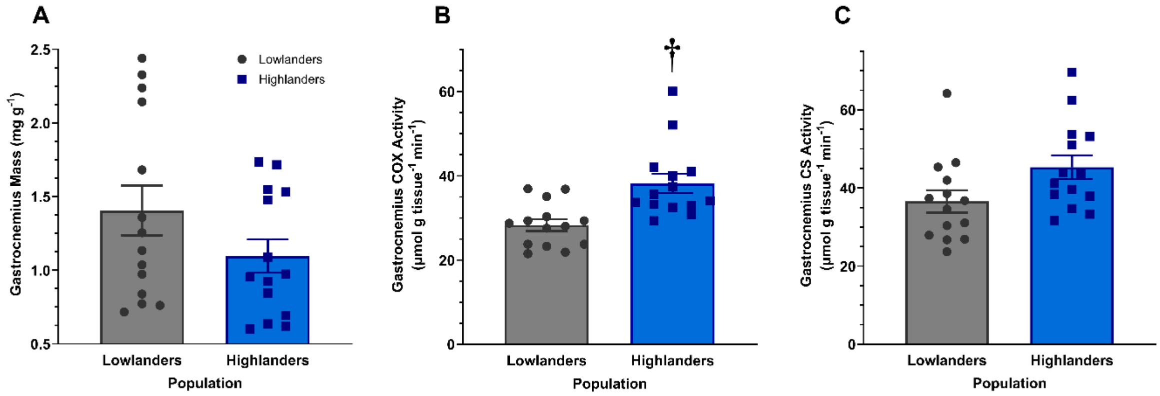

Figure 2.5. (A) Mass, (B) cytochrome c oxidase (COX) activity, and (C) citrate synthase (CS) activity in lowland (grey circles) and highland (blue squares) deer mice. Data are shown with symbols representing values for individual mice and bars representing means ± s.e.m., with N=14 lowlanders (6 females and 8 males) and N=14 highlanders (8 females and 6 males). Data was analyzed statistically using linear mixed-effects models, the results of which are shown in Table 1 († p=0.055 for population effect).

2.4 Discussion

Our findings suggest that the relationship between Hb-O_2 affinity and thermogenic capacity in hypoxia differs between high- and low-altitude populations of deer mice. Thermogenic VO_2max in hypoxia was generally greater in high-altitude mice acclimated to cold hypoxia compared to low-altitude mice acclimated to warm normoxia (Fig. 2), as previously observed (Tate et al., 2020; Lyons et al. 2021). In addition, using arterial O_2 saturation as an *in vivo* index of Hb-O_2 affinity, we observed that the optimal SaO_2 at which VO_2max in hypoxia peaked was also greater in high-altitude mice than in low-altitude mice (Fig. 3). Therefore, our study provides evidence that the relationship between Hb-O_2 affinity and aerobic capacity is malleable and can vary between populations and species, and that this relationship may be shaped by adaptation to the hypoxic environment at high altitude.

Our findings here are also consistent with other findings suggesting that the capacity of thermogenic tissues to extract and consume O_2 from the blood is greater in high-altitude deer mice than their low-altitude counterparts. Our observation that brown adipose tissue was larger and had greater UCP1 content in high-altitude mice (Fig. 4) is consistent with previous findings that the capacity for non-shivering thermogenesis, iBAT UCP1 content, and UCP1-stimulated respiration of iBAT mitochondria are greater in highlanders acclimated to cold hypoxia compared to lowlanders acclimated to warm normoxia (Coulson et al., 2021). Similar comparisons have shown that high-altitude deer mice have greater capillarity, mitochondrial abundance, and oxidative capacity in the gastrocnemius (Lui et al., 2015; Mahalingam et al., 2017; Mahalingam et al., 2020), consistent with the patterns of variation observed here (Fig. 5). In fact, high-altitude mice exhibit greater activities of oxidative enzymes than low-altitude mice across a range of skeletal muscles (Garrett et al., 2024), which could increase the oxidative capacity to support shivering thermogenesis.

We suggest that the greater capacity of high-altitude mice to extract and consume O_2 from the blood augments the influence of an increased Hb-O_2 affinity on thermogenic capacity in hypoxia. Increases in Hb-O_2 affinity augment arterial O_2 saturation in hypoxia (Fig. 1), but they may not manifest to increases in aerobic capacity if the arterial-venous difference in O_2 saturation and the offloading of O_2 at the active tissues is not also increased. However, mathematical modelling of the O_2 transport pathway has suggested that increasing Hb-O_2 affinity has a greater influence on aerobic capacity in hypoxia at higher tissue O_2 diffusing capacities (Wearing et al., 2021). This was because higher O_2 diffusing capacities were associated with lower (near zero) venous O_2 levels. Under these conditions, increases in Hb-O_2 affinity and arterial O_2 saturation led to much greater increases in the arterial-venous difference in O_2 saturation, and thus led to greater improvements in aerobic capacity (Wearing et al., 2021). By this rationale, increases in Hb-O_2 affinity that increase SaO_2 from ~80% to ~90% would not increase VO_2max in lowlanders because they cannot take advantage of this added O_2 and augment blood O_2 extraction (i.e., they cannot widen the arterial-venous difference in O_2 saturation). However, the arterial-venous difference in O_2 saturation is greater in high-altitude deer mice than in low-altitude mice at thermogenic VO_2max (Tate et al., 2020), likely in association with greater O_2 diffusing capacity of the tissues (Scott et al., 2024). These differences arise in large part from genetically-based (evolved) changes in high-altitude populations (Lui et al., 2015; Mahalingam et al., 2017; Tate et al., 2020). As a result, similar increases in Hb-O_2 affinity and SaO_2 *do* increase VO_2max in highlanders, because they are able to extract this additional O_2 from the blood.

In support of the findings here, comparisons of results from multiple past studies suggest that the influence of Hb-O_2 affinity on VO_2max in hypoxia is greater in mice with a highland genetic background (Chappell and Snyder, 1984; Wearing et al., 2021). The increased Hb-O_2 affinity of high-altitude deer mice evolved as a result of amino acid substitutions in the α- and β-chains of the Hb protein (Natarajan et al., 2013; Snyder et al., 1982; Storz et al., 2007, 2009, 2010). The influence of these genetic variants on VO_2max has been examined in two previous studies, in which controlled breeding approaches were used to create inter-population hybrids so distinct Hb genotypes could be compared in a common genetic background. In one study, mice with highland α-globin alleles and higher Hb-O_2 affinity had greater VO_2max in hypoxia than those with lowland α-globins when these comparisons were made in mice with a highland genetic background (Chappell and Snyder, 1984). In the other study, globin genotype and Hb-O_2 affinity had no effect on VO_2max in hypoxia when comparisons were made in mice with a mixed genetic background (i.e., mix of both highland and lowland alleles) (Wearing et al., 2021). Taken together, these studies suggest that the influence of Hb-O_2 affinity on VO_2max in hypoxia is greater in mice possessing the evolved (genetically based) differences in cardiorespiratory function that characterize the high-altitude population.

Although the influence of Hb-O_2 affinity on aerobic capacity may be contingent upon genetic background, increases in Hb-O_2 affinity likely have other important advantages for tolerance of severe hypoxia at rest. For example, treatment of mice with allosteric modifiers that increase Hb-O_2 affinity preserves cardiovascular function, heart rate, blood flow, and aerobic metabolism during severe hypoxia (Dufu et al., 2017). High-affinity Hb may also reduce the level of tissue hypoxia experienced by the heart, preventing tissue damage and enabling greater stroke volume and cardiac output in low O_2 conditions (Lucas et al., 2019). Furthermore, rats treated with cyanate maintain a larger arterio-venous O_2 difference in severe levels of hypoxia than rats treated with saline (Turek et al., 1978). These effects of increased Hb-O_2 affinity could provide an important advantage in high-altitude taxa, particularly in small mammals that may experience severe hypoxia in underground burrows.

A caveat to this study is the possibility that pharmacological treatments may have elicited plasticity and/or had off-target effects that influenced aerobic capacity. There is greater opportunity for this potential effect after cyanate treatment, which required multiple injections over two weeks (see Methods). Chronic manipulation of Hb-O_2 affinity may have altered O_2 levels in systemic tissues and could have thus led to plasticity in some underlying determinants of aerobic capacity (e.g., capillarity, mitochondrial abundance and respiratory capacity). Cyanate treatment itself could have had direct effects on the function of proteins involved in thermogenesis. For example, we have unpublished data showing that four weeks of cyanate treatment decreases the activities of cytochrome c oxidase (COX) and citrate synthase (CS) enzymes in the gastrocnemius compared to untreated controls. This possibility could be tested in future work by using lower doses and/or shorter treatment periods of cyanate. Alternative pharmacological agents that increase Hb-O_2 affinity acutely after a single dose (akin to the effects of efaproxiral) could also be a valuable addition to future work.

In conclusion, our findings suggest that high-altitude deer mice have an adaptive increase in optimal Hb-O_2 affinity and SaO_2 that likely contributes to greater aerobic capacities in hypoxia. This increase is associated with evolved changes in active systemic tissues that should lead to a greater capacity to extract and consume oxygen from the blood. As such, the relationship between Hb-O_2 affinity and aerobic capacity can vary between populations and species, potentially as a result of cardiovascular differences that interact with Hb to determine tissue O_2 transport. This raises the possibility that a high Hb-O_2 affinity may only be beneficial at high altitudes in species that have evolved coordinated changes in other cardiovascular traits in the O_2 transport pathway.

CHAPTER THREE: OVERVIEW AND GENERAL DISCUSSION

3.1 Overview

Haemoglobin is critically important for the efficient transport of O_2 to metabolically active tissues in order to support daily aerobic and metabolic activities. High-altitude species present a useful tool to study how Hb-O_2 affinity may have evolved to provide species an advantage for survival in challenging O_2 limited environments. The aim of this thesis was to investigate the influence of Hb-O_2 affinity on aerobic capacity in hypoxia. Previous research on humans, birds, and small mammals has shown contradicting results on how changes in Hb-O_2 affinity may or may not be advantageous in high altitude environments (Bunn, 1980; Samaja et al., 2003; Dempsey 2020). However, there seems to be strong selection on Hb genes suggesting that increases in Hb-O_2 affinity are adaptive in at least some high-altitude taxa (Weber, 2007; McCracken et al., 2009; Natarajan et al., 2013; Natarajan et al., 2015; Natarajan et al., 2016; Natarajan et al., 2018). High affinity Hb could be beneficial at high altitude as it will increase SaO_2 and potentially help expand the arterial-venous O_2 difference. However, recent mathematical modelling shows that high Hb-O_2 affinity is only beneficial if it is matched with a high tissue O_2 diffusing capacity to facilitate the extraction and consumption of O_2 at the level of the active tissues (Wearing et al., 2021). For my thesis, I hypothesized that increases in Hb-O_2 affinity would augment aerobic capacity in hypoxia across a broader range of affinities in high-altitude deer mice compared to low-altitude mice, and that this would be associated with an enhanced capacity to extract and consume oxygen at metabolically active tissues. I predicted that (1) increases in Hb-O_2 affinity and SaO_2 would increase aerobic capacity up to an optimum, and that this optimum would be greater in high-altitude mice than in low-altitude mice and (2) the thermogenic tissues of high-altitude mice would have greater capacities to extract and consume O_2 and generate body heat compared to those of low-altitude mice. Prediction 1 was supported by my observations that as SaO_2 increases so does thermogenic VO_2max up to a point where it reaches an optimum, and this optimum was about 8% higher in high-altitude mice than it was in low-altitude mice. Prediction 2 was supported by high-altitude mice having greater iBAT masses and UCP1 protein abundance along with greater muscle COX activity compared to low-altitude mice. My experimental data provides strong support for my hypothesis and suggests that Hb-O_2 affinity is an advantage in high-altitude taxa when it is matched with coordinated changes in other steps of the oxygen transport pathway that enhance oxygen extraction and consumption.

3.2 Discussion

Our findings suggest that the relationship between Hb-O_2 affinity and thermogenic capacity in hypoxia is different between high- and low-altitude populations of deer mice. Thermogenic VO_2max is greater in high-altitude mice acclimated to cold hypoxia compared to low-altitude mice acclimated to warm normoxia (Fig. 2.2) as previously observed (Cheviron et al., 2013; Tate et al., 2020; Lyons and McClelland 2024). The difference observed between the populations seems to be driven by optimal SaO_2 being greater in high-altitude mice compared to low-altitude mice. Greater SaO_2 preserves the arterial-venous difference at the tissues and maintains tissue oxygenation to support the greater aerobic capacities observed in high-altitude mice. This suggests the malleability of the relationship between Hb-O_2 affinity and aerobic capacity between species and populations. In addition, our results suggest that highlanders have a greater capacity to extract and consume oxygen at the level of the tissues. This is demonstrated by high-altitude mice having greater iBAT mass, UCP1 protein abundance (Fig. 2.4) and marginally greater enzymatic activities (Fig. 2.5) in comparison to low-altitude mice. These observations are consistent with previous findings that high-altitude mice have greater UCP1-stimulated respiration in iBAT mitochondria, as well as greater capillarity, mitochondrial abundance and oxidative capacity in the gastrocnemius (Coulson et al., 2021, Lui et al., 2015; Mahalingam et al., 2017; Mahalingam et al., 2020, Garrett et al., 2024).

Our findings suggest that the greater capacity to extract and consume O_2 from the blood augments the influence of Hb-O_2 affinity on aerobic capacity in hypoxia, as VO_2max in hypoxia is increased across a broader range of Hb-O_2 affinity and SaO_2 values. Increased Hb-O_2 affinity may not increase aerobic capacity if it isn't matched with effective offloading of O_2 at the tissues and an increase in arterial-venous difference. High-altitude mice have high arterial-venous differences in hypoxia (Tate et al., 2020; Wearing et al., 2021) which may be allowing them to take advantage of increased Hb-O_2 affinity. However, low-altitude mice have low arterial-venous difference in hypoxia (Tate et al., 2020) which likely prevents them from being able to take advantage of increases in Hb-O_2 affinity since they cannot offload sufficient amounts of oxygen.

The greater capacity to extract O_2 from the blood is partly due to genetically based differences in the amino acid composition of Hb in high- and low-altitude deer mice. High-altitude mice have genetic adaptations in alpha and beta-globin alleles of Hb which increase Hb-O_2 affinity. The influence of genetic variations in Hb has been tested in previous experiments and highland alpha-globin alleles are only beneficial for increasing thermogenic VO_2max in mice with highland genetic backgrounds (Chappell and Snyder 1984). There is no benefit of highland alpha-globin alleles on thermogenic VO_2max in mice with an admixed genetic background (mix of high- and low-altitude genes) (Wearing et al., 2021). Our findings agree with this and suggest that increases Hb-O_2 affinity are only advantageous in mice with evolved differences in the O_2 transport pathway.

In conclusion, our findings suggests that high-altitude mice have an adaptive increase in optimal Hb-O_2 affinity and SaO_2 that likely contributes to their greater thermogenic capacities in hypoxia. These greater thermogenic capacities are associated with evolved changes in active skeletal muscle and iBAT which increase the capacity to extract and consume oxygen. This study provides evidence that the relationship between Hb-O_2 affinity and aerobic capacity in hypoxia can vary depending on population, species, and experimental design, potentially due to cardiovascular differences that interact with Hb and determine tissue O_2 transport.

3.3 Future Directions

Given the caveat in this study on the direct and indirect effects of cyanate on the delivery of O_2 to the tissues and the capacity of mitochondrial enzymes to consume O_2 there are some possible avenues in which research can explore this in greater detail. Further efforts should be made to test the effects of increasing Hb-O_2 affinity in deer mice perhaps using an allosteric modifier of Hb and not an irreversible covalent modifier. This way it would be simpler to test various doses and refine the relationship that we see between SaO_2 and thermogenic VO_2max. Moreover, measuring the effects of efaproxiral on Hb-O_2 binding affinity would give us a deeper understanding on the direct binding affinity changes rather than using SaO_2 as an *in vivo* index of Hb-O_2 affinity. Efforts should be made to measure P_{50} in mice following treatment with efaproxiral in order to have a complete understanding of its effects on Hb. Measurements of P_{50} following treatment with efaproxiral were not possible due to the nature of measuring P_{50} in a buffer where efaproxiral would be diluted in the sample instead of in live whole blood where efaproxiral will still be bound to Hb. Future measurements can be made using the Blood Oxygen Binding System (BOBS) rather than the Hemox Analyzer. This system would also be able to provide information on the Bohr effect experienced by hemoglobin after treatment with each of the drugs and deepen our understanding on the relationship between haemoglobin function and thermogenic VO_2max in the deer mice.

This study also provides an interesting avenue for future research on how single changes in the O_2 transport pathway may affect aerobic capacity in hypoxia. It is possible that single changes in other steps of this pathway may give more insight into how aerobic capacity can be regulated by the O_2 transport pathway. The main steps in this pathway are ventilation, pulmonary O_2 diffusion, circulation, tissue O_2 diffusion, and mitochondrial O_2 utilization. This study manipulated the circulation of O_2 as we increased or decreased Hb-O_2 binding affinity. However, it is possible to target other steps in this pathway such as ventilation, heart rate, and angiogenesis. For example, previous work using pharmacological a- and b-adrenergic agonists and antagonists has demonstrated that high-altitude mice had a greater capacity to stimulate β_1-adrenergic receptors and elevate heart rate compared to low-altitude mice and were better able to regulate α-adrenergic receptor stimulation perhaps in order to redistribute blood more effectively (Wearing et al., 2022). This type of pharmacological manipulation could also be exploited to determine the effects of ventilation, heart rate, and blood pressure on aerobic capacity in hypoxia in a similar manner to the pharmacological methods used in this thesis (see methods). This would allow us to probe the effects of another critical step in the oxygen transport pathway on aerobic capacity in hypoxia. In another study examining the effects of a depletion in muscle specific vascular endothelial growth factor (VEGF) it was observed that mice with low levels of VEGF have lower aerobic capacities (Olfert et al., 2009). A depletion in VEGF will decrease the number of capillaries in the muscle which we know are integral to optimal O_2 offloading at the tissues and efficient consumption. This is a possible avenue to test in deer mice as we know high-altitude mice have greater capillary densities and mitochondria closer to the gas exchange surfaces than low-altitude mice (Lui et al., 2015; Scott et al., 2015; Mahalingam et al., 2017; Tate et al., 2020). By observing the effects of a lower or higher number of capillaries on aerobic capacity in hypoxia using deer mice it could provide a further understanding of the coordinated changes needed to increase aerobic capacity at high altitude.

3.4 Implications

The influence of Hb-O_2 on aerobic capacity in hypoxia has been a longstanding question in physiology. My thesis has provided insight into how the relationship between Hb-O_2 affinity and aerobic capacity is malleable and can vary between populations and species. Our findings suggest that high-altitude deer mice have an adaptive increase in optimal Hb-O_2 affinity that is associated with a greater capacity to extract and consume oxygen from the blood. We suggest that a high Hb-O_2 affinity may only be beneficial at high altitudes in species that have evolved coordinated changes in other cardiovascular traits in the O_2 transport pathway.

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