Metabolic Adaptations to Life at High-Altitude: Evolved Increases in Oxidative Capacity Across Many Skeletal Muscles in High-Altitude Deer Mice, *Peromyscus maniculatus*

Metabolic Adaptations to Life at High-Altitude: Evolved Increases in Oxidative Capacity Across Many Skeletal Muscles in High-Altitude Deer Mice, *Peromyscus maniculatus*

By EMILY J. GARRETT, B. Sc.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements of the Degree Master of Science

McMaster University, MASTER OF SCIENCE (2023) Hamilton, Ontario (Biology)

TITLE: Metabolic adaptations to life at high-altitude: evolved increases in oxidative capacity across many skeletal muscles in high-altitude deer mice, *Peromyscus maniculatus*

AUTHOR: Emily J. Garrett

Hons. B. Sc. (Mount Allison University)

SUPERVISOR: Dr. Graham R. Scott

NUMBER OF PAGES: iix, 78

LAY ABSTRACT

High altitude environments are often considered to be harsh environments for animals to inhabit. This stems from the concurrent challenge of low oxygen availability coupled with year-round cold temperatures. Despite these challenges, there exist many high-altitude species including birds, mammals, and humans, whom have each, over time, developed suites of adaptations that aid them in successfully living at high altitude. My thesis focuses on the role of skeletal muscle adaptation in supporting life at high altitude. To address this, I compared the capacity for key steps in oxidative and anaerobic metabolism in the skeletal muscles of low-altitude and high-altitude species of North American deer mice. My thesis contributes to our understanding of how skeletal muscles can support adaptations to life at high altitude.

ABSTRACT

The cold and hypoxic conditions at high altitude necessitate high metabolic O_2 demands to support thermogenesis while hypoxia reduces O_2 availability. Skeletal muscles play key roles in thermogenesis, but our appreciation of muscle plasticity and adaptation at high altitude has been hindered by past emphasis on only a small number of muscles. We examined this issue in deer mice (*Peromyscus maniculatus*). Mice derived from both high-altitude and low-altitude populations were born and raised in captivity and then exposed as adults to normoxia or hypobaric hypoxia (12 kPa O_2 for 6-8 weeks). Maximal activities of citrate synthase (CS), cytochrome c oxidase (COX), β-hydroxyacyl-CoA dehydrogenase (HOAD), hexokinase (HK), pyruvate kinase (PK), and lactate dehydrogenase (LDH) were measured in 20 muscles involved in shivering, locomotion, body posture, ventilation, and mastication. Principal components analysis revealed an overall difference in muscle phenotype between populations but no effect of hypoxia acclimation. High-altitude mice had greater activities of mitochondrial enzymes and/or lower activities of PK/LDH across many (but not all) respiratory, locomotory, and postural muscles compared to low-altitude mice. In contrast, chronic hypoxia exposure had very few effects on metabolic phenotype across muscles. Further examination of CS in the gastrocnemius showed that population differences in enzyme activity stemmed from differences in protein abundance and mRNA expression, but not from population differences in CS amino acid sequence. Overall, our results suggest that evolved increases in oxidative capacity across many skeletal muscles, at least partially driven by differences in transcriptional regulation, may contribute to high-altitude adaptation in deer mice.

ACKNOWLEDGMENTS

I am very fortunate to have had the opportunity to come to McMaster and embark on the adventure that has been the last two years spent working on this thesis. My experience here has been spent surrounded by great mentors and colleagues without whom this experience surely would not have been the same. To my supervisory committee: Rosa, thank you for your thoughtful ideas and guidance during the development and completion of this thesis. Grant, thank you for your insights on the project, and for showing me how to set mouse traps while out on the mountain in Colorado. Graham, thanks for taking a chance on an East Coaster who had never been west of Montreal, these last two years have been everything I was hoping they would be. I have learnt how to be a better critical thinking and researcher during my time here and am very thankful for that.

To the members of Team Deer Mouse, thank you for being one of the best lab groups I've had the opportunity of working with. A special thank you to Sully is in order, starting in lab during lockdown was a very daunting task at the time, but having you to chat with made everything feel like it would work out eventually, you are the best colleague anyone could ask for. To Megan, I'm very glad I had someone going through this degree at the same time I was, thanks for being a great writing partner. Ranim, thanks for the countless chats sitting around the lab, somehow you always found a way to get a smile or laugh out of each of us, even when the assays weren't cooperating. Finally, to my family back home, thanks for supporting me on my adventure in Ontario and for making the long drive out to Hamilton with me.

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LIST OF ABBREVIATIONS

ATP: Adenosine triphosphate

BAT: brown adipose tissue

BSA: bovine serum albumin

COX: cytochrome c oxidase

CS: citrate synthase

DTNB: 5,5'-dithio-bis-(2-nitrobenzoic acid

ECL: enhanced chemiluminescence

EDTA: ethylenediaminetetraacetic acid

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF: Hypoxia-inducible factor

HK: hexokinase

HOAD: β-hydroxyacyl-CoA dehydrogenase

HRP: horseradish peroxidase

HRV: hypoxic ventilatory response

kDa: kiloDalton

kPa: kilopascal

LDH: lactate dehydrogenase

NADH: Nicotinamide adenine dinucleotide

PBS: phosphate buffered saline

PC1: Principal component 1

PCA: principal components analysis

PK: pyruvate kinase

PVDF: polyvinylidene difluoride

RIPA: Radioimmunoprecipitation assay

SDS: Sodium dodecyl sulfate

SEM: standard error of the mean

SNP: singly nucleotide polymorphism

UCP1: uncoupling protein 1

UTR: untranslated region

V_{max}: Maximal enzyme activity

DECLERATION OF ACADEMIC ACHIEVEMENT

This thesis is organized in the sandwich format, as recommended, and approved by members of my supervisory committee and approved by McMaster University. It consists of three chapters. Chapter one is an overview of background material and the objectives of this work. Chapter two is a manuscript that is in preparation for submission to a peer-reviewed scientific journal. Chapter three summarizes the major findings of this thesis and discusses how these findings relate to current knowledge and how they may inform future directions of research.

CHAPTER 1: General Introduction

CHAPTER 2: Evolved Changes in Phenotype Across Skeletal Muscles in Deer Mice Native to Hight Altitude.

Authors: Emily J. Garrett, Srikripa K. Prasad, Rena M. Schweizer, Grant B. McClelland, Graham R. Scott

Status: manuscript in preparation for submission

CHAPTER 3: General Discussion

CHAPTER 1: General Introduction

1.1 BACKGROUND

The study of organisms which inhabit extreme environments has long informed our understanding of life's limits of tolerance. At high-altitude, the main physiological stressors organisms encounter are chronic low partial pressures of O_2 (hypoxia) coupled with year-round low ambient temperatures. Despite these challenges, large and small endotherms have been recorded living at high-altitude. Compared to large endotherms (e.g. yaks and cattle), small endotherms such as rodents and lagomorphs have the added challenge of overcoming the increased pressure of maintaining optimal body temperature as their large body surface area-volume ratio promotes heat loss to the environment. The low ambient temperatures at high-altitude, which are almost always outside a small rodent's thermal neutral zone, necessitates that small endotherms direct a great deal of energy towards aerobically demanding thermogenesis to maintain body temperature, thus, some small endotherms can consistently operate close to their maximal aerobic capacity (Hayes, 1989). This may constrain the capacity for other aerobic processes such as foraging, digestion, predator avoidance and reproduction.

Skeletal muscles comprise the largest proportion of total body mass, accounting for approximately 40% total body mass and are the main consumers of O_2. They are also the effector tissue for locomotion and shivering thermogenesis, two highly aerobic processes. Therefore, it is essential that skeletal muscles maintain a high capacity for oxidative metabolism to meet their energic demands even when faced with low oxygen availability. Aspects of skeletal muscles which are capable of remodeling to maintain high capacities for oxidative metabolism include fuel selection and energy metabolism pathways, relative mass, capillarity, and fiber-type

composition. Given the essential nature of skeletal muscles in daily life, their phenotypes are likely an important target for selection in extreme environments.

Many studies have characterized how muscles adjust to hypoxia acclimation in low-altitude native species as well as how these adjustments compare to the skeletal muscles of high-altitude natives, in their native environments. However, within and across species these studies report conflicting trends. Further, many of these studies give only a perspective on a narrow group of muscles. My thesis aims to gain a better understanding of how a wide array of muscles have evolved in the high-altitude rodent, the North American Deer Mouse.

1.2 PLASTIC TRAITS AT HIGH ALTITUDE

Upon exposure to the hypobaric hypoxia imposed by high-altitude environments, low-altitude humans exhibit a range of homeostatic responses, including changes in ventilation and metabolism. When exposed to chronic hypoxia, low altitude species often increase ventilation (hypoxic ventilatory response: HVR), which remains elevated (Sato et al., 1994; Weil et al., 1971; Zhuang et al., 1993). High-altitude humans such as Tibetans experience similar HVR trends as low altitude humans, while Andeans have comparably lower HVRs compared to sea-level values (Beall, 2007).

Despite the constraint on aerobic metabolism at high-altitude due to hypobaric hypoxia, basal metabolic rate increases initially, then returns to baseline levels over many weeks of hypoxia acclimation (Butterfield et al., 1992). Oxygen delivery is also affected, where the upper range is reduced by about a third of baseline levels initially, which trends back towards baseline levels over the course of a year. Thus, metabolic demand increases, concurrent with a decrease in

maximal oxygen delivery, ultimately constraining aerobic activity (Baker and Little, 1976; Marconi et al., 2006; Wu and Kayser, 2006). However, high-altitude native Andeans and Tibetans, when exposed to similar elevations, maintain normal metabolic rates and oxygen delivery capacities similar to a low altitude individual when at sea level (Beall et al., 1996; Mazess et al., 1969; Picon-Reátegui et al., 1961).

1.3 EVOLVED TRAITS AT HIGH ALTITUDE

High-altitude adaptations have developed independently in many species in high-altitude regions across the globe. Two highly studied regions where we find animals and humans uniquely qualified for life at high-altitude are the native inhabitants of the Andean and Tibetan plateaus. In general, these taxa have evolved traits along the oxygen cascade and in skeletal muscles which enable them to maintain high rates of aerobic metabolism despite the constraint of low oxygen availability at high-altitude. It is important to note that there are also many high-altitude communities in the East African plateaus, however those are less well studied.

Although native high-altitude populations of Andean and Tibetan humans have been exposed to similar levels of hypoxic stress over generations, they have each evolved via different paths, with unique sets of traits which allow them to successfully occupy their high-altitude environments (Beall, 2007). While Andean high-altitude humans have evolved cardiovascular strategies (Beall, 2006; Crawford et al., 2017), Tibetan high-altitude natives have developed a strategy at the molecular level (Beall et al., 2010; Bigham and Lee, 2014; Simonson et al., 2010; Witt and Huerta-Sánchez, 2019; Yi et al., 2010). In the hypobaric hypoxia environment at high-altitude, Andeans maintain lower resting ventilation rates, and have a blunted ventilatory response to hypoxia compared to their Tibetan counterparts (Beall et al., 1997; Brutsaert, 2007;

Moore, 2000). Although counterintuitive, it is believed that this strategy helps maintain blood pH homeostasis and reduces the energetic costs of breathing (Hochachka, 1986; Powell, 2007). Tibetan high-altitude natives on the other hand seemed to have evolved genetic adaptations in the gene involved in the HIF pathway response to hypoxia (Beall et al., 2010; Bigham et al., 2010; Simonson et al., 2010; Witt and Huerta-Sánchez, 2019; Yi et al., 2010). Non-human high-altitude inhabitants such as birds and mammals are also known to have evolved more effective ventilation patterns (Ivy and Scott, 2017; Scott and Milsom, 2007; York et al., 2017), and increased hemoglobin-O_2 affinity (Chappell and Snyder, 1984; Zhang et al., 1996).

The goal of changes in breathing patterns and efficiencies is to transport oxygen to working tissues, of which the largest consumer of oxygen being skeletal muscles, where oxygen is ultimately used to produce energy. In some high-altitude taxa, skeletal muscles have evolved increased capillarity, mitochondrial density and oxidative capacity (Cheviron et al., 2012; Dawson et al., 2016, p. 216; Lui et al., 2015; Mahalingam et al., 2017; Mathieu-Costello et al., 1998; Scott et al., 2015, 2011, 2009a), however other studies do not support these findings (Horscroft et al., 2017; León-Velarde et al., 1993; Sheafor, 2003), some even report skeletal muscles with lower mitochondrial volumes which are believed to have higher metabolic efficiency (Beall, 2007; Gelfi et al., 2004; Hoppeler et al., 2003; Kayser et al., 1996; Marconi et al., 2006). High-altitude taxa have also evolved the attenuation of some maladaptive pathological responses to chronic hypoxia (Brutsaert, 2016; Monge and Leon-Velarde, 1991; Tate et al., 2020; Velotta et al., 2018; West et al., 2021).

One common limiting aspect of the research that has been done to characterize the adaptations of native high-altitude animals is that they are often only studied in their native high-altitude environment, thus making it difficult to determine whether the phenotypes being

measured indeed arise from genetic adaptation or phenotypic plasticity. Similarly, much of what we understand to date on skeletal muscle adaptation to life at high-altitude come from studies which often present a narrow representation of skeletal muscles, often primarily involved in locomotion, we therefore lack a good understanding of how muscles with other functions have evolved.

1.4 ENERGY METABOLISM IN SKELETAL MUSCLES

A key factor in maintaining homeostasis is matching energy (ATP) production to energy demands of working tissues. When oxygen is abundantly available, energy production typically occurs via oxidative phosphorylation, however, under limiting oxygen conditions there is typically a shift towards anaerobic metabolism.

Many studies show evidence of a shift away from fatty acid oxidation and oxidative metabolism, while glycolytic metabolism remains unchanged or increases in low-altitude natives when exposed to high-altitude environments (Green et al., 1992, 1989; Horscroft and Murray, 2014; Howald et al., 1990; Viganò et al., 2008). These findings are often coupled with losses in mitochondrial volume density and respiratory capacity (Hoppeler et al., 1990; Jacobs et al., 2012; Levett et al., 2012). It remains unclear if the changes in energy metabolism are a direct result of the changes in mitochondria physiology.

If energy homeostasis is not properly being maintained under hypoxic conditions, one could expect to observe a downregulation of highly energy demanding processes such as protein synthesis (Murray, 2009; Shaw, 2009; Wullschleger et al., 2006). This may be one of the underlying mechanisms behind the muscle wasting seen in many low altitude species when they

are acclimated to high-altitude hypoxic conditions (Murray, 2009). However, this is not a viable long-term strategy for individuals who live at high-altitude year-round.

1.5 THERMOGENESIS

A hallmark trait of mammals is their capacity to maintain internal body temperature (endothermy). When exposed to ambient temperatures below their thermoneutral zones, these mammals must engage in thermogenesis to stay warm. Thermogenesis is a metabolically costly and aerobically dependent activity, requiring a large increase in oxygen consumption from basal metabolic rate (Scholander et al., 1950). Heat production can be achieved through two distinct mechanisms.

Firstly, non-shivering thermogenesis can occur in brown adipose tissue (BAT) stores located throughout the body. BAT have high mitochondrial densities and primarily use lipids as fuel for oxidative phosphorylation and thermogenesis (Cannon and Nedergaard, 2004; Cinti, 2007). However, in brown adipose tissue, uncoupling protein 1 (UCP1) which is located on the inner mitochondrial membrane, acts as a transmembrane protein, allowing protons to re-enter the mitochondrial matrix, dissipating the electrochemical gradient required for ATP synthesis. This process is also known as futile cycling and results is a significant amount of chemical energy in the form of heat (Cannon and Nedergaard, 2004; Enerbäck et al., 1997; Klingenberg and Huang, 1999).

Secondly, shivering thermogenesis occurs in skeletal muscles where there is the repetitive, involuntary contraction of muscle fibers, ultimately producing heat (Hemingway, 1963). Shivering can either occur as continuous, low intensity contractions, mediated by type I slow

oxidative fibers, or in high-intensity bursts, mediated by type II fibers (Haman et al., 2004a; Meigal, 2002). Continuous low intensity shivering tends to account for most of the heat produced via shivering, while burst shivering accounts than less than 10% of total shivering activity (Haman et al., 2004a), although contribution patterns of each type of shivering have been shown to be quite variable between individuals and between muscles (Haman and Blondin, 2017). It has been suggested that burst shivering may be related to muscle fiber type composition (Blondin et al., 2017). Similarly, some have suggested that fuel selection during shivering may be linked to the pattern of shivering (burst or continuous low intensity) and the muscle fiber types recruited during shivering (Haman et al., 2004b). Skeletal muscle accounts for a larger proportion of body mass (~40%) and thus has a large capacity for thermogenesis across the entire body. Typically, the main areas of shivering thermogenesis include the upper body (Bell et al., 1992; Blondin et al., 2014, 2011; Haman, 2006; Haman et al., 2004b) and the upper legs (Bell et al., 1992; Blondin et al., 2014; Haman et al., 2004a, 2004b), although specific shivering patterns vary largely across individuals and species.

1.6 THE DEER MOUSE MODEL

A common limitation of studies of high-altitude species is that they are often studied in their native high-altitude environments, making it challenging to distinguish the relative importance of phenotypic plasticity and genetic adaptation to their overall success at high-altitude. The North American deer mouse (*Peromyscus maniculatus*) is a powerful model species for the study of physiological adjustments in the face of challenging high-altitude environments. Deer mice have one of the broadest altitudinal ranges of any mammal (Hock, 1964; Natarajan et al., 2015; Snyder et al., 1982) with established genetically distinct high-altitude and low-altitude

populations. Previous studies have characterized an ample number of traits along the oxygen cascade and in skeletal muscles suggesting that the high-altitude populations of deer mice are particularly well adjusted to life at high-altitude. Wild high-altitude deer mice maintain higher field metabolic rates than low-altitude counterparts (Hayes, 1989), which is though to support the greater metabolic demands of thermogenesis required at high-altitude. There is also strong directional selection for increased aerobic capacity for thermogenesis (Hayes and O'Connor, 1999), which has likely led to the evolution of greater oxidative capacity in hypoxia when compared to the low-altitude populations (Cheviron et al., 2012; Tate et al., 2020). At the skeletal muscle level, increased capillarity, oxidative capacity, and mitochondrial volume density in the gastrocnemius (Cheviron et al., 2012; Lui et al., 2015; Mahalingam et al., 2017; Robertson and McClelland, 2019; Scott et al., 2015) underlie the high-altitude populations evolved increase in aerobic capacity. However, our current understanding of how skeletal muscles have evolved to facilitate life at high-altitude is limited as most studies tend to focus on only 1-2 skeletal muscles.

1.7 AIMS AND OBJECTIVES

Given our generally poor understanding of the capacity of many different types of muscles to adjust to support life at high-altitude, the goal of my thesis is to gain a broader understanding of how skeletal muscles have evolved to enable high-altitude deer mice to cope with the challenges posed by their high-altitude environment. By quantifying the maximal capacity of six key metabolic enzymes (Fig. 1.1), I will seek to understanding how the metabolic phenotype of skeletal muscles involved in locomotion, shivering, body posture, breathing and mastication have evolved to life at high-altitude (Fig. 1.2). I hypothesize that high-altitude deer mice have evolved a more oxidative phenotype across skeletal muscles to support an increased aerobic capacity for

shivering and locomotion in high-altitude environments. I predicted that high-altitude deer mice will have greater activities of mitochondrial enzymes across muscles, particularly in hypoxic environments, and that differences in metabolic enzyme activities will be underlain by differences in gene expression.

1.8 FIGURES

Figure 1.1

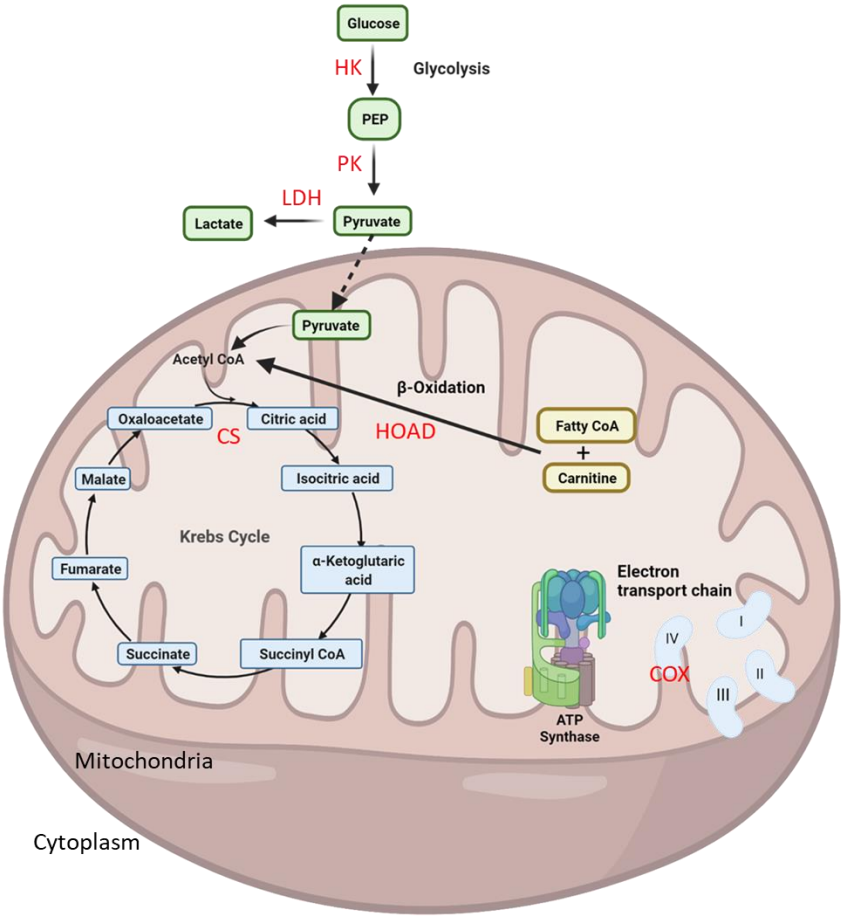

Figure 1.1: Overview of investigated metabolic pathways. Key investigated enzymes shown in red (HK, hexokinase; PK, pyruvate kinase; LDH, lactate dehydrogenase; CS, citrate synthase; HOAD, β-hydroxyacyl-CoA dehydrogenase; COX, cytochrome c oxidase) and their position in subcellular compartmentalization.

Figure 1.2

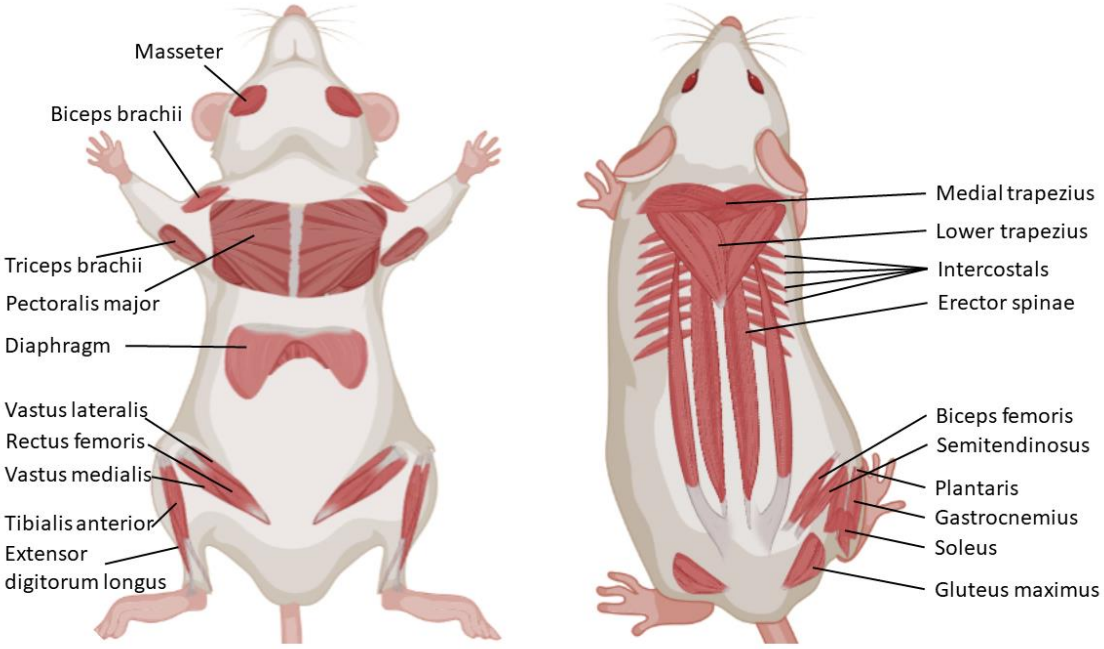
Masseter
Biceps brachii
Triceps brachii
Pectoralis major
Diaphragm
Vastus lateralis
Rectus femoris
Vastus medialis
Tibialis anterior
Extensor digitorum longus
Medial trapezius
Lower trapezius
Intercostals
Erector spinae
Biceps femoris
Semitendinosus
Plantaris
Gastrocnemius
Soleus
Gluteus maximus

Figure 1.2: Skeletal muscle sampling. The location of the 20 sampled skeletal muscles are shown and labeled on a general mouse model.

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CHAPTER 2: Evolved Changes in Phenotype Across Skeletal Muscles in Deer Mice Native to High Altitude

2.1 ABSTRACT

The cold and hypoxic conditions at high altitude necessitate high metabolic O_2 demands to support thermogenesis while hypoxia reduces O_2 availability. Skeletal muscles play key roles in thermogenesis, but our appreciation of muscle plasticity and adaptation at high altitude has been hindered by past emphasis on only a small number of muscles. We examined this issue in deer mice (*Peromyscus maniculatus*). Mice derived from both high-altitude and low-altitude populations were born and raised in captivity and then exposed as adults to normoxia or hypobaric hypoxia (12 kPa O_2 for 6-8 weeks). Maximal activities of citrate synthase (CS), cytochrome c oxidase (COX), β-hydroxyacyl-CoA dehydrogenase (HOAD), hexokinase (HK), pyruvate kinase (PK), and lactate dehydrogenase (LDH) were measured in 20 muscles involved in shivering, locomotion, body posture, ventilation, and mastication. Principal components analysis revealed an overall difference in muscle phenotype between populations but no effect of hypoxia acclimation. High-altitude mice had greater activities of mitochondrial enzymes and/or lower activities of PK/LDH across many (but not all) respiratory, locomotory, and postural muscles compared to low-altitude mice. In contrast, chronic hypoxia exposure had very few effects on metabolic phenotype across muscles. Further examination of CS in the gastrocnemius showed that population differences in enzyme activity stemmed from differences in protein abundance and mRNA expression, but not from population differences in CS amino acid sequence. Overall, our results suggest that evolved increases in oxidative capacity across many skeletal muscles, at least partially driven by differences in transcriptional regulation, may contribute to high-altitude adaptation in deer mice.

2.2 INTRODUCTION

The cold and hypoxic environment at high altitudes presents a significant challenge to aerobic metabolism. In endotherms at high altitude, cold temperatures increase the demands for thermogenesis, while hypoxia concurrently constrains O_2 availability to fuel the metabolic demands of thermogenesis as well as locomotion, reproduction, and other important activities that contribute to fitness (Hochachka, 1985; McClelland and Scott, 2019; West, 2000). Evidence suggests that the ability to cope with these challenges requires physiological adjustments across the O_2 transport pathway, composed of ventilation, pulmonary diffusion, circulation, and the diffusion and utilization of O_2 by mitochondria in active tissues (McClelland and Scott, 2019; Monge and Leon-Velarde, 1991; Storz and Scott, 2021). Plastic changes across the O_2 pathway in response to chronic hypoxia during adulthood or early life can improve aerobic capacity in hypoxia (Brutsaert, 2016; Ivy et al., 2021). In taxa that are genetically adapted to high altitude, evolved changes across the O_2 pathway can further improve aerobic capacity, and can also attenuate maladaptive pathological responses to chronic hypoxia (Brutsaert, 2016; Monge and Leon-Velarde, 1991; Tate et al., 2020; West et al., 2021). However, many previous studies have examined high-altitude taxa in their native environment, so it has often been challenging to distinguish the relative influence of plasticity and genetic adaptation on the phenotype of high-altitude natives (Brutsaert, 2016; Moore, 2017).

Skeletal muscles are dominant consumers of O_2 and metabolic fuels to support shivering thermogenesis, locomotion, and ventilation, such that muscles underpin the function of multiple steps in the O_2 pathway. Many previous studies have found that skeletal muscle phenotypes are adjusted in high-altitude environments, but the role and nature of these adjustments have been controversial (D'Hulst and Deldicque, 2017; Murray, 2016; Scott et al., 2018). In low-altitude

humans, for example, some studies have found that acclimatization to high altitude reduces oxidative capacity of skeletal muscle, reflected by losses of mitochondrial density and/or respiratory capacity (Hoppeler et al., 1990; Jacobs et al., 2012; Levett et al., 2012), but opposite changes have been observed in some other studies (Chicco et al., 2018; Jacobs et al., 2016). Similarly, among mammals and birds native to high altitude, some taxa exhibit higher mitochondrial density or oxidative capacity in skeletal muscles than their low-altitude counterparts (Cheviron et al., 2012; Dawson et al., 2016; Mathieu-Costello et al., 1998; Scott et al., 2009b; Scott et al., 2015), whereas others do not (Horscroft et al., 2017; León-Velarde et al., 1993; Schippers et al., 2012; Sheafor, 2003). Some of these discrepancies between studies could undoubtedly arise from differences in elevation, locomotory activity, thermogenic demands, and/or nutritional status between individuals or species. However, nearly all previous studies of muscle phenotype in high-altitude natives have focussed on only 1-2 skeletal muscles, because of their involvement in locomotion, so differences in the response to high altitude between muscles or muscle types could also lead to discrepancies between studies. This has likely confounded attempts to gain a broad appreciation of skeletal muscle plasticity in hypoxia, and to explain how changes in skeletal muscle phenotype contribute to high-altitude adaptation.

The North American deer mouse (*Peromyscus maniculatus*) is a useful species for examining the physiological adjustments that help endotherms cope with metabolic challenges in high-altitude environments. Deer mice have a broad elevational range from near sea level to over 4300 m in the Rocky Mountains (Hock, 1964; Natarajan et al., 2015; Snyder et al., 1982). High-altitude populations of deer mice maintain higher field metabolic rates in the wild than their low-altitude counterparts (Hayes, 1989), likely to support the greater metabolic demands of thermogenesis at high altitude. High-altitude populations also experience strong directional

selection for increased aerobic capacity for thermogenesis (Hayes and O'Connor, 1999), and have thus evolved greater aerobic capacity in hypoxia than populations from low altitude (Cheviron et al., 2012; Tate et al., 2020). This evolved increase in aerobic capacity is associated with greater capillarity, oxidative capacity, and mitochondrial volume density in the gastrocnemius (Cheviron et al., 2012; Lui et al., 2015; Mahalingam et al., 2017; Robertson and McClelland, 2019; Scott et al., 2015), a large hindlimb muscle involved in shivering and locomotion (Pearson et al., 2005). Similar changes were not observed in the soleus (Mahalingam et al., 2017), a smaller postural muscle in the hindlimb, but few other skeletal muscles have been previously examined.

Here, we investigate the metabolic phenotype of 20 skeletal muscles involved in locomotion, shivering, body posture, ventilation, and mastication to gain a better appreciation of how changes in skeletal muscle phenotype contribute to high-altitude adaptation in deer mice. This was achieved by measuring the maximal activities of six key metabolic enzymes: citrate synthase (CS), cytochrome c oxidase (COX), β-hydroxyacyl-CoA dehydrogenase (HOAD), hexokinase (HK), lactate dehydrogenase (LDH) and pyruvate kinase (PK). Deer mice from populations native to both high altitude and low altitude were raised in common rearing conditions until adulthood and were then acclimated to either normoxia or hypoxia, in order to distinguish hypoxia-induced plasticity and evolved changes in muscle phenotype. We hypothesized that high-altitude deer mice have evolved a more oxidative phenotype across skeletal muscles to support an increased aerobic capacity for shivering and locomotion in high-altitude environments. We predicted that high-altitude deer mice will have greater activities of mitochondrial enzymes across muscles, particularly in hypoxic environments, and that differences in metabolic enzyme activities will be underlain by differences in gene expression.

2.3 MATERIALS AND METHODS

Animals and acclimations

Wild deer mice were trapped at high altitude near the summit of Mount Evans (4350 m above sea level; CO, USA, 39°35’18”N, 105°38’38”W) and at low altitude in the Great Plains (320 m above seal level; Nine-Mile Prairie, NE, USA, 0°52’12”N, 96°48’20.3”W). These wild mice were transported to McMaster University (90 m above sea level; Hamilton, ON, CA) where they were housed in common conditions (25°C, 14:10-h light-dark cycle with unlimited access to water and food) and bred to produce first-generation laboratory-born progeny for each population. Progeny were raised in standard holding conditions (25°C, 12:12-h light-dark cycle) with unlimited access to water and standard rodent chow (Teklad 22/5 Rodent Diet formula 8640; Envigo).

Treatments

Adult mice (6-12 months of age) from both high-altitude and low-altitude populations were randomly assigned to each of two acclimation groups: normobaric normoxia (ambient atmospheric conditions, barometric pressure of 101 kPa) or hypobaric hypoxia (barometric pressure of 60 kPa; roughly equivalent to the atmospheric conditions at 4350 m elevation). Hypobaric hypoxia was maintained using hypobaric chambers that have been previously described (Ivy and Scott, 2017; McClelland et al., 1998). Cage cleaning and replenishment of food and water were carried out twice per week, which required that hypoxic mice be removed from the hypobaric chambers and returned to normobaria for less than 20 min. Mice were otherwise subjected to standard holding and husbandry conditions during acclimation treatments.

Mice in the hypoxia acclimation group were sampled after 6-8 weeks of hypoxia exposure, and mice in the normoxia acclimation group were sampled at overlapping times and ages. All animal procedures were approved by the McMaster University Animal Research Ethics Board following the guidelines establish by the Canadian Council on Animal Care.

Tissue sampling

Twenty skeletal muscles were sampled from across the body, selected to include those involved in locomotion, shivering, body posture, ventilation, and mastication. Mice were deeply anesthetized with isoflurane and euthanized by cervical dislocation. Fifteen muscles were dissected intact and weighed, and for which masses are reported: biceps brachii, biceps femoris, diaphragm, erector spinae, extensor digitorum longus, gastrocnemius, gluteus maximus, pectoralis major, rectus femoris, semitendinosus, soleus, tibialis anterior, triceps brachii, vastus lateralis, vastus medialis. The anatomy of the remaining 5 muscles meant that we could only remove part of the muscle, so we do not report masses: intercostals, lower trapezius, medial trapezius, masseter, plantaris. After dissection and weighing, muscles were frozen in liquid N_2 and stored at -80°C until used for enzyme assays.

Enzyme assays

Apparent maximal enzyme activities (V_{max}) were measured for 6 key metabolic enzymes. Muscle samples were powdered under liquid N_2 and a portion was homogenized in 20 volumes of homogenization buffer (100 mM KH_2PO_4, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton-X-100, pH 7.2) using a glass tissue grinder. The maximal activities of cytochrome c

oxidase (COX), β-hydroxyacyl-CoA dehydrogenase (HOAD) and hexokinase (HK) were assayed from fresh homogenates within an hour of homogenization, and the remaining homogenate was frozen and stored at -80°C. The activities of citrate synthase (CS), lactate dehydrogenase (LDH) and pyruvate kinase (PK) were assayed from these frozen homogenates. These colourimetric assays were performed at 37°C by measuring the rate of change in absorbance over time at the appropriate wavelength (412 nm for CS; 550 nm for COX; 340 nm for LDH, HOAD, HK, and PK) in the following conditions: CS, 0.1 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), 0.22 mM acetyl-CoA, 0.5 mM oxaloacetate (omitted in background control), 40 mM Tris at pH 8.0; COX, 0.15 mM reduced cytochrome c, 100 mM KH_2PO_4 at pH 7.0; HOAD, 0.1 mM acetoacetyl-CoA (omitted in background control), 0.28 mM NADH, 100 mM triethanolamine (TEA)-HCl, pH 7.0; HK, 8 mM ATP, 8 mM $MgCl_2$, 5 mM D-glucose (omitted in background control), 0.5 mM NAD(P), 6 U glucose-6-phosphate dehydrogenase, 50 mM HEPES, pH 7.4; LDH, 0.28 mM NADH, 1 mM sodium pyruvate (omitted in background control), 40 mM Tris-HCl, pH 7.4; PK, 100 mM KCl, 10 mM $MgCl_2$, 5 mM ADP, 0.15 mM NADH, 10 mM fructose-1,6-phosphate, 9.25 U LDH, 5 mM phosphoenolpyruvate (PEP) (omitted in background control), 50 mM imidazole, pH 7.4. Assays were run in triplicate along with a background control in which a key substrate was omitted. Preliminary trials confirmed that substrate conditions were saturating for each enzyme. Sample limitations precluded measurement of HK in 5 muscles (erector spinae, extensor digitorum longus, soleus, tibialis anterior, and triceps brachii).

Western blotting

Citrate synthase protein content was measured in the gastrocnemius by Western blotting using the tissue homogenate that remained from enzyme assays. We first added one volume of ten-fold concentrated RIPA buffer stock (10 % Triton-X-100, 5 % deoxycholic acid, 1 % SDS, 500 mM Tris, pH 8.0) containing protease inhibitor (cOmplete™ Mini Protease Inhibitor Cocktail, Sigma-Aldrich; 1 tablet per 10 ml) to nine volumes of each tissue homogenate. These homogenates containing RIPA buffer were re-homogenized for 30s with a motorized homogenizer, and then centrifuged at 10000 × *g* at 4°C for 20 min. The supernatants were conserved and used in subsequent steps. Protein concentration was determined using a detergent compatible (DC) protein assay (Bio-Rad Canada, Mississauga, ON). Proteins were denatured in Laemmli sample buffer (0.002 % bromophenol blue, 5 % β-mercaptoethanol, 10 % glycerol, 70 mM SDS, 62.5 mM Tris-HCl, pH 6.8) for 5 min at 95°C. 4 µg of protein from each sample was then loaded into precast 12 % polyacrylamide gels (Mini-PROTEAN® TGX™ Precast Gels, Bio-Rad) and run for 30 min at 100 V followed by 45 min at 150 V using the Mini PROTEAN 3 system (Bio-Rad) with running buffer composed of 25 mM Tris, 192 mM glycine, and 0.1 % SDS (pH 8.3). Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Trans-Blot Turbo Midi 0.2 µm PVDF membrane, Bio-Rad) at 2.5 A, 25 V, for 7 min using a Trans Blot Turbo Transfer system (Bio-Rad). Membranes were incubated overnight in blocking solution (1% bovine serum albumin in PBST; PBST-BSA). Following overnight incubation, membranes were briefly rinsed with fresh PBST and then incubated with primary antibody (rabbit anti-CS polyclonal antibody, PA5-22126, Invitrogen; 1:500 dilution in PBST-BSA) for 1 h at 4°C with shaking. Membranes were rinsed well in PBST and then incubated with HRP-conjugated secondary antibody (goat anti-rabbit IgG, 31466, Invitrogen; 1:5000 dilution in

PBST-BSA) for 1 h at 4°C with shaking. Membranes were rinsed well in PBST and then incubated for 1 min in ECL solution (Clarity Western ECL substrate, Bio-Rad). Citrate synthase was identified by a single band at the expected size of 52 kDa (Figure S1) and band intensity was detected and quantified using a ChemiDoc MP Imaging System (Bio-Rad). A common sample was loaded onto each membrane to account for variations in protein transfer efficiency between membranes. Band intensity was normalized to this common sample as well as to total protein as determined by Coomassie staining (Bio-Safe Coomassie Staining Solution, Bio-Rad). CS protein abundance is presented here relative to the mean value of low-altitude deer mice acclimated to normoxia.

Quantitative PCR

Quantitative PCR (qPCR) was used to measure CS gene expression in the gastrocnemius using 12S rRNA as a reference gene. CS primers were designed using Primer3 software based on the CS transcript sequence for *P. maniculatus bairdii* (NCBI accession number XM_042263107): forward primer, 5'-TGGGAAGACGGTGGTGGGCC-3'; reverse primer, 5'-TGTAGCCTCGGAAACGGATGCCC-3'. Primer sequences for 12S have been used successfully and reported previously (Lui et al., 2015). Primers were synthesized by Bio-Rad Canada. Total RNA was extracted from ~5 mg of powdered gastrocnemius tissue using the AllPrep® DNA/RNA Micro Kit (Qiagen, Hilden, Germany) following the protocols outlined by the manufacturer. RNA concentration in the extracts was quantified using a NanoDrop spectrophotometer (Thermo-Fisher Scientific). For each sample, cDNA was synthesized from 65 ng of total RNA using the SuperScript™ IV First-Strand Synthesis System (Invitrogen), while following the manufacturer's instructions and using a 3:1 mix of random hexamer and oligo-dT

primers. A negative control was also created for each sample, in which all components of the cDNA reaction were included except the reverse transcriptase enzyme (which was replaced with RNase/Dnase free water; Thermo-Fisher Scientific), in order to quantify the amount of contaminating genomic DNA in the cDNA for each sample. The resulting cDNA and negative controls were diluted 20-fold using Rnase/Dnase free water and then used as templates for qPCR reactions carried out in a CFX Connect Real-Time System (Bio-Rad). Each qPCR reaction contained 2 µl of the cDNA/control template, 1 µl of forward and reverse primer (CS or 12S), 7 µl Rnase/Dnase free water and 10 µl of qPCR master mix (PowerTrack™ SYBR Green Master Mix, Invitrogen). The cycling conditions were as follows: 95°C for 2 min, 40 cycles of 95°C for 15 s followed by 60°C for 1 min. All qPCR reactions were performed in triplicate. A melt curve was performed at the end of the amplification protocol for each sample to ensure that only a single product was amplified. Primer efficiency was assessed for both primers by creating a standard curve using one sample (CS, 96.8%; 12S, 100.1%), and amplification results were expressed relative to this standard curve. CS levels were then normalized to the levels of the 12S control gene. CS levels measured in negative controls were less than 1% of the CS levels in each cDNA sample and were therefore ignored. For each primer set a no template control was also run and confirmed the absence of any amplified contaminants. CS mRNA expression is presented here relative to the mean value of low-altitude deer mice acclimated to normoxia.

CS gene sequence analysis

We examined whether high-altitude deer mice possess sequence differences in the CS gene that could influence enzyme function or expression. This was motivated by recent findings that CS was a significant outlier in tests of positive selection in high-altitude deer mice

(Schweizer et al. 2021). This previous publication determined sites of single nucleotide polymorphisms (SNPs) across the exome, and identified those sites for which there existed significant changes in allele frequency in the high-altitude population (see Schweizer et al. 2021 for details). The CS gene was thus determined to possess 18 SNPs exhibiting differences in allele frequency between high- and low-altitude populations (Table S1). Here, we map these SNPs onto the CS gene using the most recent assembly of the *P. maniculatus bairdii* genome (assembly 2.1.3; assembly accession number GCF_003704035.1). While looking further at the available CS gene sequence annotation, it was noted that the *P. maniculatus* sequence did not align well to the CS sequence from *Mus musculus* or *P. leucopus*. Specifically, the annotated start codon for *P. maniculatus* was 245 bp upstream of the start codon for *M. musculus* and *P. leucopus*, such that the first exon annotation for *P. maniculatus* was significantly larger than expected. However, there is strong sequence conservation throughout this region between *M. musculus*, *P. leucopus*, and *P. maniculatus*, so the discrepancy between species in the annotated start codon and first exon was likely a mistake of the predictive software used to annotate the CS gene in *P. maniculatus*. We therefore used the site of the start codon identified in *M. musculus* and *P. leucopus* as the true start codon for this gene, and then ran sequence alignments of the protein coding regions from *P. maniculatus* against *M. musculus* and *P. leucopus* to confirm proper exon alignment. We then used computational predictive software, hTFtarget (Zhang et al., 2020) and AnimalTFDB3 (Hu et al., 2019), to identify whether any of the SNPs identified in non-coding regions were located in potential transcription factor binding sites. From the initial list of all potential transcription factor binding sites, we report those with $P<0.05$ and $Q<0.05$, and for which there was also independent evidence from the literature that the transcription

factor is involved in skeletal muscle development and maintenance and/or mitochondrial biogenesis.

Statistics

Statistical analysis was performed using Rstudio software version 4.2.0 (RStudio Team, 2022). Using the inverse non-linear PCA (NLPCA) function from the pcaMethods package (Stacklies et al., 2007), we ran principal components analysis on the entire data set of muscle enzyme activities. The resulting values for principal component 1 (PC1) were used as a metric to assess the overall variation in muscle phenotype. The effects of population and acclimation environment on PC1 and on values of mass, enzyme activity, CS protein expression, and CS mRNA expression for each individual muscle were analyzed using linear mixed-effects models from the lme4 package (Bates et al., 2015). Initial models also included sex and family as random effects as well as body mass as a covariate. In cases where effects of sex, family, and/or body mass did not approach significance (P>0.10), they were removed from the final models reported here. When main effects or interactions were significant, Tukey post-hoc tests were used to make pairwise comparisons between all groups. We also examined the overall variation in the activity of each enzyme across all muscles by comparing between populations in each acclimation environment using Wilcoxon signed-rank tests. Data are presented as individual values and/or mean ± SEM, and we used a significance level of $P < 0.05$.

2.4 RESULTS

Populations differed in the overall metabolic phenotype across skeletal muscles

We first used principal components analysis (PCA) to assess the overall variation in enzyme activity across all muscles and enzymes. The first principal component (PC1) accounted for 19.5% of variation across the entire enzyme activity data set. PC1 was greater in high-altitude populations than their low-altitude counterparts (Fig. 2.1), as reflected by a significant main effect of population ($P < 0.001$) but no effect of acclimation environment. The top 10 loadings on PC1 were measurements of COX, HOAD, CS or HK in the gastrocnemius, gluteus maximus, plantaris, vastus medialis or rectus femoris. PC1 was also affected by body mass ($P < 0.001$), which was 19.9 ± 0.7 g across all groups, but there were no significant differences in body mass between populations or acclimation environments (Table S2). Therefore, much of the overall variation in the metabolic phenotype of skeletal muscles appeared to be driven by differences between high-altitude and low-altitude populations.

High-altitude deer mice exhibited high activities of mitochondrial enzymes across skeletal muscles

We next compared between populations for the overall variation across all muscles for each metabolic enzyme. The overall variation was visualized by plotting the activity in each muscle in high-altitude mice against the activities in the same muscles in low-altitude mice (Figs. 2.2, 2.3), and overall differences between populations were evaluated using Wilcoxon signed-rank tests. High-altitude deer mice exhibited greater overall activities of the mitochondrial enzymes CS, COX, and HOAD in both normoxia ($P = 0.013$, $P < 0.001$, $P = 0.038$) and hypoxia

($P = 0.009$, $P < 0.001$, $P = 0.004$) (Fig. 2.2). High-altitude deer mice also exhibited lower overall activities across muscles of the glycolytic enzymes LDH and PK in normoxia ($P < 0.001$ and $P = 0.005$) and hypoxia (both $P < 0.001$) (Fig. 2.3). In contrast, HK, which catalyzes the conversion of glucose to glucose-6-phosphate in glycolysis, had greater overall activities in high-altitude mice in normoxia and hypoxia (both $P < 0.001$) (Fig. 2.3). Nevertheless, the overall population differences across skeletal muscles appeared to result from high-altitude mice having a more oxidative phenotype than low-altitude mice.

Many different types of muscles contributed to the overall metabolic differences in high-altitude deer mice

To evaluate which muscles contributed to the overall variation in muscle metabolic phenotype between populations, we tested for significant main effects of population and population×environment interactions on the activity of each enzyme in each muscle. The results of these tests are summarized here, and the full data set and *P*-values from statistical tests are shown in Tables S3-S8. For the respiratory muscles, there were several significant population effects on mitochondrial enzymes in both the diaphragm (COX, HOAD) and the intercostals (CS and HOAD), all driven by higher activities in the high-altitude population. High-altitude mice also exhibited higher HK activity in the diaphragm and lower PK activity in the intercostals. Similarly, many hindlimb muscles exhibited higher mitochondrial enzyme and HK activities in the high-altitude population in normoxia and/or hypoxia, as reflected by significant population effects in the plantaris (CS, COX, HOAD, HK), vastus medialis (CS, COX, HOAD, HK), gluteus maximus (COX, HOAD), biceps femoris (COX, HK), gastrocnemius (CS), semitendinosus (HOAD), soleus (COX), and rectus femoris (HK). The high-altitude population

also exhibited higher activities of COX in erector spinae and HOAD in pectoralis major. There were significant population×environment interactions for the two forelimb muscles, biceps brachii (CS, COX) and triceps brachii (CS, HOAD, LDH), which were driven by higher values in the high-altitude population in normoxia but that declined to similar values as the low-altitude population in hypoxia. The trapezius and masseter exhibited significantly lower levels of the glycolytic enzymes (LDH and/or PK) in the high-altitude population in normoxia and/or hypoxia. These population differences in enzyme activity existed without many significant population differences in muscle mass, with the only exception being diaphragm mass, for which there a significant population×environment interaction driven by an increase in lowlanders in hypoxia (Table S2). Only two muscles, tibialis anterior and vastus lateralis, showed no significant population or population×environment effects. Therefore, the overall differences in muscle metabolic phenotype in the high-altitude population results from many muscles involved in locomotion, shivering, body posture, ventilation, and mastication.

Although the overall variation in muscle enzyme activities was not significantly affected by acclimation environment (Fig. 2.1), the metabolic phenotype of some individual muscles changed with acclimation to chronic hypoxia, as reflected by significant main effects of acclimation environment (Tables S3-S8). The activities of some mitochondrial enzymes increased in hypoxia in erector spinae (CS), pectoralis major (COX), and vastus medialis (COX), but decreased in hypoxia in the intercostals (COX). The vastus medialis also experienced a decrease in PK activity in hypoxia. Two muscles, erector spinae and gluteus maximus, increased in mass in chronic hypoxia, but no muscles decreased in mass (Table S2). Therefore, in contrast to the much larger number of population effects on muscle enzyme activities, there were relatively few muscles that changed consistently in chronic hypoxia in both populations.

Population differences in gene expression contributed to variation in CS activity

We examined CS in the gastrocnemius to gain insight into the underlying determinants of variation in metabolic enzyme activity. Consistent with previous findings (Lui et al., 2015; Robertson and McClelland, 2019), we observed a significant main effect of population (P = 0.004) but not acclimation environment on CS activity in the gastrocnemius, with high-altitude mice having 1.6-fold greater CS activity than low-altitude mice on average (Fig. 2.4A). This was mirrored by variation in CS protein abundance (main effect of population, P = 0.031), which was 1.3- and 1.4-fold greater on average in normoxia and hypoxia in high-altitude mice than in their low-altitude counterparts (Fig. 2.4B). Similar variation was observed for CS mRNA expression (main effect of population, P = 0.018), which was 1.4- and 1.2-fold greater in high-altitude mice than in low-altitude mice (Fig. 2.4C).

Potential contributions of population differences in CS gene sequence to variation in CS expression and activity

The CS gene of deer mice contains 18 single nucleotide polymorphisms (SNPs) that exhibit significant differences in allele frequency between high-altitude and low-altitude populations (Schweizer et al. 2021) (Table S1). Only two of these SNPs are found in protein coding regions, both of which are synonymous substitutions within the 7th exon (Fig. 2.5), so there are no population differences in CS amino acid sequence contributing to variation in CS enzyme activity. The remaining non-coding SNPs cluster in two regions of the gene. The first cluster (8 SNPs) is in intron 2 immediately upstream of the 3rd exon, and the second cluster (7 SNPs) is in the 3’ UTR (Fig. 2.5). Predictive software was used to identify potential transcription

factor binding sites overlapping these SNPs, and strict selection criteria were used to identify the strongest candidates for CS regulation in muscle (see Materials and Methods for details). This analysis identified 29 potential transcription factors (Table S9), including regulators of muscle metabolism (e.g., RXR, SRF) and the transcriptional responses to hypoxia (HIF-1α, HIF-2α [encoded by EPAS1], EP300) (Fig. 2.5).

2.5 DISCUSSION

Skeletal muscles are involved in shivering thermogenesis, locomotion, and ventilation, and thus support the function of multiple steps in the O_2 pathway. The importance of muscle plasticity and adaptation in high-altitude environments has been controversial (D'Hulst and Deldicque, 2017; Murray, 2016; Scott et al., 2018), potentially in part because most previous studies have focussed on a very limited number of skeletal muscles. Our study sought to address this limitation to gain a better understanding of how changes in skeletal muscle phenotype may contribute to improving aerobic performance at high altitude. We show that many, but not all, skeletal muscles exhibit a more oxidative phenotype in high-altitude deer mice than their low-altitude counterparts, and this variation exists across muscles of various functions across different parts of the body. The effects of chronic hypoxia on muscle phenotype were modest and limited to only a few muscles. Our findings suggest that evolved increases in oxidative capacity across many skeletal muscles may contribute to high-altitude adaptation in deer mice.

Evolved population differences in enzyme activities were the most significant source of variation across skeletal muscles (Figs. 2.1-2.3). Increased activities of mitochondrial enzymes CS, COX, HOAD were observed in many respiratory, hindlimb, and core muscles of high-altitude mice, as previously observed in the gastrocnemius (Lau et al., 2017; Lui et al., 2015).

Many of these hindlimb and core muscles have been shown to be involved in shivering thermogenesis in humans, rabbits and Japanese quail (Israel and Pozos, 1989; Kosaka et al., 1967; Mekjavic and Eiken, 1985; Stevens et al., 1986), in addition to their roles in locomotion. These findings mirror the differences observed in some high-altitude birds, in which the flight muscles exhibit greater respiratory capacity and/or activity of mitochondrial complexes compared to low-altitude birds (Dawson et al., 2020; León-Velarde et al., 1993; Mathieu-Costello et al., 1998; Parr et al., 2021; Scott et al., 2009b; Scott et al., 2011). Increases in muscle oxidative capacity may be advantageous at high altitude by increasing capacity for muscle thermogenesis or as a strategy to increase overall mitochondrial respiration when intracellular O_2 tensions decrease (Hochachka, 1985; Scott et al., 2009a). Some other high-altitude birds match the pattern in high-altitude deer mice by exhibiting greater muscle HOAD activities than their low-altitude relatives (Dawson et al., 2020). Such increases in HOAD activity may be advantageous for supporting higher rates of lipid oxidation during shivering thermogenesis (Cheviron et al., 2012; Lyons et al., 2021). Future measurements of mitochondrial respiratory capacities for oxidative phosphorylation and lipid oxidation across muscles would be useful for gaining insight into these possibilities, given that changes in enzyme activity may not always lead to clear changes in mitochondrial respiratory capacity (Jacobs et al., 2013).

Despite the overall pattern showing increased mitochondrial enzyme activities across muscles in high-altitude mice, some muscles showed no significant population differences. Similarly, high-altitude populations of the torrent duck exhibit higher mitochondrial respiratory capacities and COX activities than low-altitude populations in the gastrocnemius but not in the pectoralis (the former muscle is the more important for locomotion in this diving species) (Dawson et al., 2016). Such discrepancies emphasize the need to consider several distinct

muscles to fully appreciate muscle adaptations to high altitude. Indeed, one of the muscles that showed no significant population differences in enzyme activity here, vastus lateralis, is a common focus of human studies (Jacobs et al., 2012; Levett et al., 2012; Horscroft et al., 2017; Chicco et al., 2018; Jacobs et al., 2016), suggesting that future studies of other muscles may be needed to fully appreciate muscle plasticity and adaptation in humans at high altitude.

In contrast to the strong differences between populations, enzyme activities were largely unaffected by exposure to chronic hypoxia across most muscles. Of the minority of muscles that were affected by acclimation environment, most had increased mitochondrial enzyme activities in hypoxia in one or both populations. These findings are consistent with previous measurements of mitochondrial respiratory capacity in deer mice, in which chronic hypoxia had no effect (gastrocnemius, soleus) or increased (diaphragm) respiratory capacity (Mahalingam et al., 2017; Dawson et al., 2018). Comparable data in other rodents are quite limited, but a previous study of CD-1 mice also observed no effect of chronic hypoxia on mitochondrial enzyme activities in the gastrocnemius (Beaudry and McClelland, 2010). However, these findings contrast some reports in low-altitude humans, in which mitochondrial volume and/or respiratory capacity in vastus lateralis were reduced by high-altitude acclimatization (Jacobs et al., 2012) (Levett et al., 2012). Effects of high-altitude exposure in humans may depend on the altitude and duration of exposure (D'Hulst and Deldicque, 2017), but reductions in respiratory capacity have been observed after 4 weeks exposure to lower altitudes than those simulated here (Jacobs et al., 2012). Therefore, mice may be resistant to some of the plastic responses of muscle phenotype to chronic hypoxia that occur in humans.

The population differences in enzyme activities suggest that high-altitude deer mice have evolved increased mitochondrial volume and/or respiratory capacity across many skeletal

muscles, as observed in previous studies of the gastrocnemius (Lui et al., 2015; Mahalingam et al., 2017). In these previous studies, increases in mitochondrial volume density in the gastrocnemius were paralleled by increases in CS activity in the high-altitude population. We observed comparable variation in CS activity in the gastrocnemius here, in association with greater CS protein abundance and CS mRNA expression in the high-altitude population (Fig. 2.4). CS is often used as a marker of mitochondrial volume density (Larsen et al., 2012; Reichmann et al., 1985; Scott et al., 2018), so this variation in CS activity and expression could result from coordinated regulation by drivers of fibre-type composition and mitochondrial abundance. However, variation in tissue enzyme activity can have multiple causes across the regulatory hierarchy from gene sequence to enzyme function (Dalziel et al., 2005; Suarez and Moyes, 2012). Indeed, recent exome-wide genomic analysis identified that the CS gene is under selection in high-altitude deer mice (Schweizer et al., 2021), raising the possibility that variation in tissue CS activity also results from genetic differences in coding or regulatory regions of the CS gene. However, our population genetic analysis of the CS gene found no allelic differences that could alter CS amino acid sequence, so variation in CS activity cannot be explained by population differences in the inherent function of the enzyme. Most allelic differences were found in intron 2 and in the 3' UTR (Fig. 2.5). It is possible that these genetic variants reside in *cis*-regulatory regions that affect CS expression, based on our identification of several potential transcription factor binding sites in these regions that include regulators of muscle metabolism and the transcriptional response to hypoxia (e.g., RXR, SRF, HIF1α, HIF-2α, EP300) (Fig. 2.5). Therefore, genetic variants in regulatory regions of the CS gene could contribute to the increased levels of CS gene expression and CS activity in the high-altitude population.

One of the key goals of evolutionary physiology is to uncover the mechanisms underlying adaptive variation in organismal performance (Dalziel et al., 2009; Garland and Carter, 1994; Scott and Dalziel, 2021). Here, we contribute to the emerging evidence that deer mice have adapted to high altitude through evolved changes across transport and utilization pathways for O_2 and metabolic fuels, increasing aerobic performance in hypoxia (McClelland and Scott, 2019; Storz et al., 2019). Skeletal muscles underlie many functions in these pathways in their roles in shivering thermogenesis, locomotion, and ventilation, and our results here suggest that high-altitude deer mice have evolved greater capacities for oxidative phosphorylation and/or lipid oxidation in many muscles across the body. Therefore, the concerted evolution of aerobic processes across many tissues and physiological systems appear to be critical to the process of high-altitude adaptation.

2.6 ACKNOWLEDGEMENTS

The authors would like to thank the members of the Scott and McClelland labs for help and support while completing this work. In particular, the authors thank Cayleih Robertson, Sulayman Lyons, and Claire West for technical support, advice, and mentorship that were essential for carrying out this project.

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2.8 FIGURES

Figure 2.1

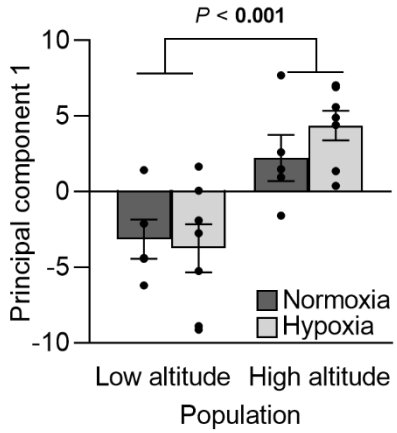

Figure 2.1: The overall metabolic phenotype across skeletal muscles differed between high-altitude and low-altitude populations of deer mice. Principal components analysis was used to compare overall levels of variation in enzyme activity across 20 skeletal muscles in mice from both populations acclimated to normoxia and hypoxia. Principal component 1 (PC1) explained 19.5% of the overall variation. Main effect of population, $P < 0.001$; main effect of acclimation environment, $P = 0.229$; population×environment, $P = 0.601$. Data are shown with circles representing values for individual mice and bars representing mean ± SEM.

Figure 2.2

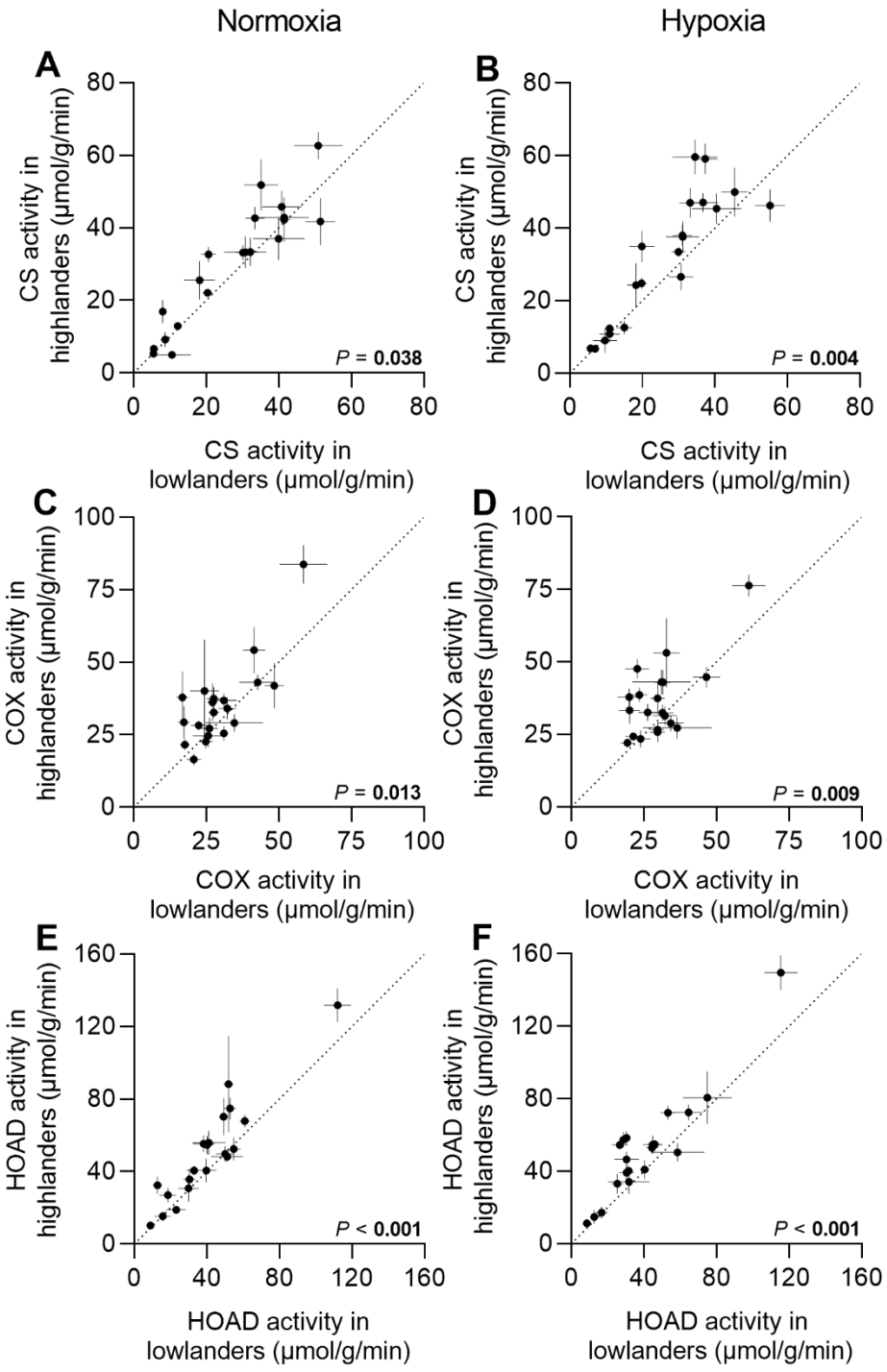
Normoxia
Hypoxia
A
B
C
D
E
F
CS activity in highlanders (µmol/g/min)
CS activity in lowlanders (µmol/g/min)
COX activity in highlanders (µmol/g/min)
COX activity in lowlanders (µmol/g/min)
HOAD activity in highlanders (µmol/g/min)
HOAD activity in lowlanders (µmol/g/min)
P = 0.038
P = 0.004
P = 0.013
P = 0.009
P < 0.001
P < 0.001

Figure 2.2: High-altitude deer mice have greater activities of mitochondrial enzymes across skeletal muscles than low-altitude deer mice. Each data point represents mean ± SEM (μmol substrate per g tissue per min) for a different muscle for citrate synthase (CS) activity (A, B), cytochrome c oxidase (COX) activity (C, D), and β-hydroxyacyl-coA dehydrogenase (HOAD) activity (E, F) from mice acclimated to normoxia (A,C,E) or hypoxia (B, D, F). Diagonal dotted lines represent the line of equality (y = x). There were significant overall differences in enzyme activities between populations using Wilcoxon's signed-rank tests (*P*-values for each test are shown on each corresponding panel).

Figure 2.3

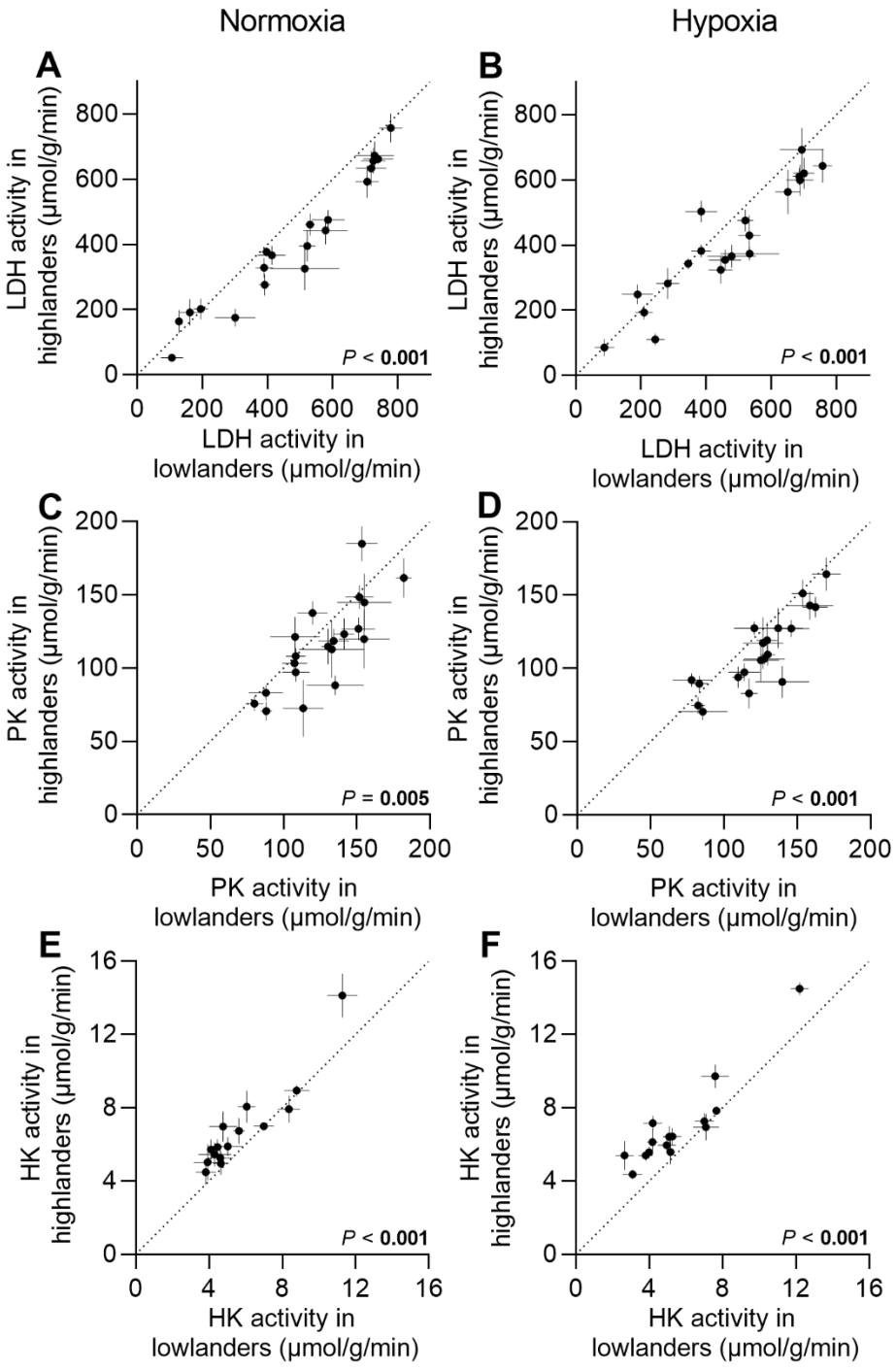
Normoxia
Hypoxia
A
B
C
D
E
F
LDH activity in highlanders (µmol/g/min)
LDH activity in lowlanders (µmol/g/min)
PK activity in highlanders (µmol/g/min)
PK activity in lowlanders (µmol/g/min)
HK activity in highlanders (µmol/g/min)
HK activity in lowlanders (µmol/g/min)
P < 0.001
P = 0.005
0
200
400
600
800
50
100
150
4
8
12
16

Figure 2.3: High-altitude deer mice have lower activities of lactate dehydrogenase (LDH) and pyruvate kinase (PK) but higher activities of hexokinase (HK) across skeletal muscles compared to low-altitude deer mice. Each data point represents mean ± SEM (μmol substrate per g tissue per min) for a different muscle for LDH activity (A, B), PK activity (C, D), and HK activity (E, F) from mice acclimated to normoxia (A, C, E) or hypoxia (B, D, F). Diagonal dotted lines represent the line of equality (y = x). There were significant overall differences in enzyme activities between populations using Wilcoxon's signed-rank tests (*P*-values for each test are shown on each corresponding panel).

Figure 2.4

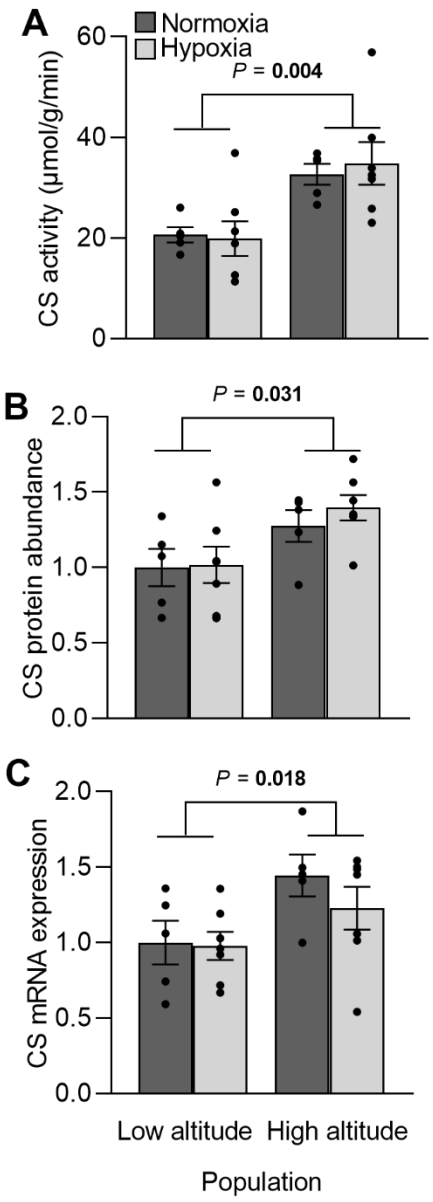
A
Normoxia
Hypoxia
P = 0.004
CS activity (µmol/g/min)
60
40
20
0
B
P = 0.031
CS protein abundance
2.0
1.5
1.0
0.5
0.0
C
P = 0.018
CS mRNA expression
2.0
1.5
1.0
0.5
0.0
Low altitude
High altitude
Population

Figure 2.4: Increased citrate synthase (CS) activity in the gastrocnemius of high-altitude deer mice is associated with increased CS protein abundance and increased CS mRNA expression. (A) CS activity is expressed as μmol substrate per g tissue per min (population effect, $P = 0.004$; environment effect, $P = 0.650$; population×environment, $P = 0.943$). (B) CS protein abundance was quantified by Western blotting (Fig. S1) (population effect, $P = 0.031$; environment effect, $P = 0.381$; population×environment, $P = 0.899$). (C) CS mRNA expression was quantified by qPCR (population effect, $P = 0.018$; environment effect, $P = 0.371$; population×environment, $P = 0.465$). Data are shown with circles representing individual values and bars representing mean ± SEM.

Figure 2.5

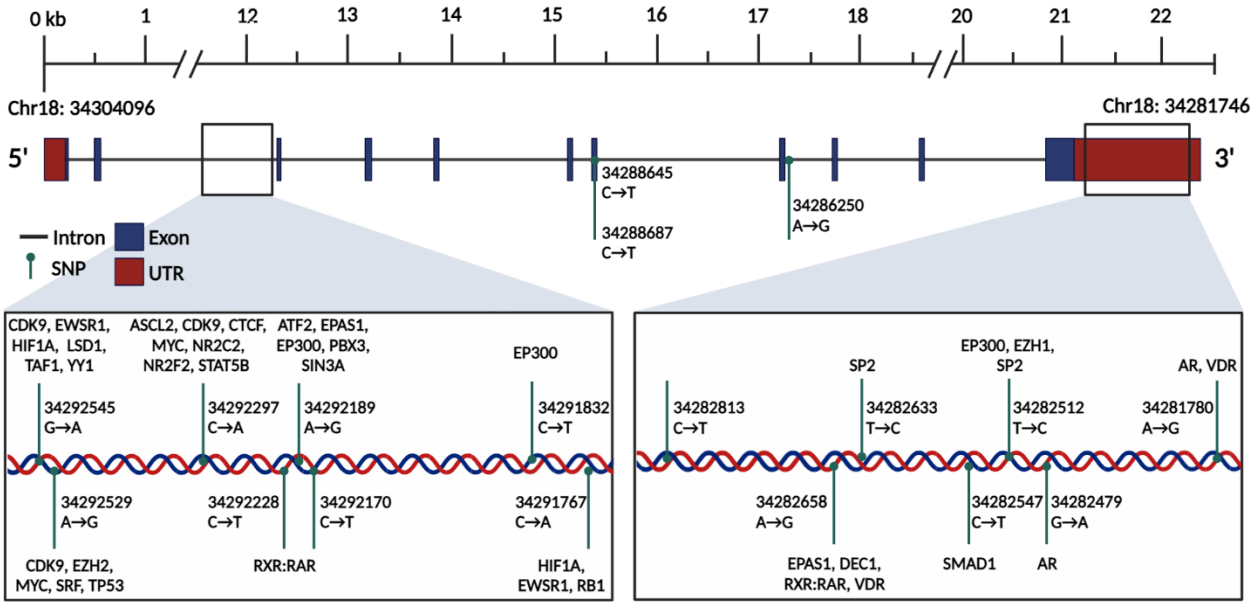
0 kb
1
12
13
14
15
16
17
18
20
21
22
Chr18: 34304096
Chr18: 34281746
5'
3'
34288645
C→T
34288687
C→T
34286250
A→G
Intron
Exon
SNP
UTR
CDK9, EWSR1, HIF1A, LSD1, TAF1, YY1
ASCL2, CDK9, CTCF, MYC, NR2C2, NR2F2, STAT5B
ATF2, EPAS1, EP300, PBX3, SIN3A
EP300
34292545
G→A
34292297
C→A
34292189
A→G
34291832
C→T
34292529
A→G
34292228
C→T
34292170
C→T
34291767
C→A
CDK9, EZH2, MYC, SRF, TP53
RXR:RAR
HIF1A, EWSR1, RB1
EP300, EZH1, SP2
SP2
AR, VDR
34282813
C→T
34282633
T→C
34282512
T→C
34281780
A→G
34282658
A→G
34282547
C→T
34282479
G→A
EPAS1, DEC1, RXR:RAR, VDR
SMAD1
AR

Figure 2.5: Single nucleotide polymorphisms (SNPs) in the citrate synthase (CS) gene of deer mice exhibit population differences in allele frequency. The 22.3 kb CS gene is located on chromosome 18 (Chr18) from nucleotides 34281746 to 34304096, and is composed of 11 exons (blue), 10 introns (black line), and 5' and 3' untranslated regions (red). Of the 18 SNPs in the gene (shown with green lines), 16 occur in non-coding regions, and the two in coding regions are synonymous substitutions. SNP position as well as the allele frequency shift in the high-altitude population is shown at each SNP. Predicted transcription factor binding sites that overlap SNPs are shown above each respective SNP (detailed results of transcription factor binding site analysis can be found in Table S9).

CHAPTER 3: General Discussion

3.1 DISCUSSION

From breathing, to locomotion, shivering thermogenesis and foraging, skeletal muscles are essential to every mammal's daily life. Skeletal muscles have a large capacity for remodeling and can adjust their phenotype in response to environmental pressures. High-altitude hypoxia is an important environmental pressure for high-altitude inhabitants; thus, comparing the skeletal muscle metabolic characteristics of high-altitude and low-altitude populations of deer mice allows us to gain insight into the relative contribution of hypoxia acclimation and genetic adaptation to the overall phenotype of skeletal muscles. I sought to understand how the phenotype of a wide range of skeletal muscles have evolved to cope with chronic hypoxia at high altitude. The primary objective of my thesis was to 1) determine the degree to which phenotypic plasticity and ancestry govern the capacities of metabolically important energy pathways, which likely aid in enabling the highly aerobic phenotype of high-altitude deer mice, and 2) investigate potential mechanisms responsible for the overall differences in metabolic phenotype between low-altitude and high-altitude populations of deer mice. I hypothesized that high-altitude deer mice have broadly evolved skeletal muscles with greater capacity for oxidative metabolism, at the loss of capacity for anaerobic metabolism, ultimately supporting the more aerobic phenotype of high-altitude deer mice. Briefly, I found that high-altitude deer mice have muscles that had an overall metabolic phenotype that was distinct from those of low-altitude deer mice, with high-altitude deer mice expressing a greater overall capacity for many enzymes contributing to oxidative metabolism, with lower capacity for anaerobic glycolysis when compared to low-altitude deer mice. With further investigation into the levels of hierarchical regulation that underlie differences in enzyme capacity, we found that the increased CS activity in the

gastrocnemius of high-altitude deer mice stems from similar increases in CS protein abundance and mRNA expression.

It is well accepted that high-altitude populations are uniquely suited for life at high altitude, although there remains a dispute over the relative importance of the contribution of skeletal muscle phenotypic plasticity and genetic adaptation (D'Hulst and Deldicque, 2017; Murray, 2016; Scott et al., 2018). Here, we show that across many skeletal muscles, the capacity for oxidative metabolism is increased in high-altitude deer mice, and remains generally unaffected by hypoxia acclimation, therefore, evolved adaptation to life at high-altitude are the more important factor in determining skeletal muscle phenotype (see Fig. 3.1 for pattern of variation). Previous studies have reported increased capacity for mitochondrial enzyme activity (CS, COX, HOAD) in the gastrocnemius of high-altitude deer mice compared to their low altitude counterparts (Lau et al., 2017; Lui et al., 2015). Here, we confirm those findings, as well as extend that pattern of variation to include other hindlimb muscles, as well as respiratory and core muscles. In the myocardium of some high altitude adapted species (taruca, llama, alpaca), there is evidence for increased absolute oxidative capacity, and decreased in anaerobic capacity. In skeletal muscles researchers only observed a decreased in anaerobic capacity (Hochachka, 1985). A more oxidative muscle phenotype in muscles of animals at high altitude may facilitate increased O_2 flux during low intracellular O_2 tension (Hochachka, 1985; Scott et al., 2009b). Increased absolute oxidative capacity in muscles at high altitude would result in decreased flux through the electron transport chain per gram as a result of there being a larger amount of electron transport chain per gram present. Therefore, at any given work rate, mitochondria are likely capable of maintain function at lower intracellular O_2 concentrations (Hochachka, 1985). Similarly, greater capacity for enzymes across oxidative metabolic pathways could also provide

added support in maintaining a high aerobic capacity for functions such as shivering and locomotion. Indeed, strong direction selection has been detected for increase aerobic capacity in high-altitude deer mice (Hayes and O'Connor, 1999), which likely underlies greater field metabolic rates of wild high-altitude deer mice (Hayes, 1989), as well increased aerobic capacity of first generation progeny of high-altitude deer mice compared to their low-altitude counterparts (Cheviron et al., 2013; Lui et al., 2015). When comparing the number of muscles where high-altitude deer mice had greater capacities of our three mitochondrial enzymes, we note that there were more instances of significant population differences in HOAD (8) and COX (7) compared to CS (4). This potential disconnect between the Krebs cycles (CS) and the electron transport chain (COX; ETC) may be indicative of a strategy where by increasing COX capacity without matched increases in CS, each COX enzyme may operate at a lower turnover rate, effectively increasing mitochondrial oxygen affinity, which would be favourable under hypoxia (Gnaiger et al., 1998).

Increased capacities for oxidative metabolism (potentially at the expense of anaerobic metabolism) at high altitude is likely part of an extensive suit of biochemical adaptations to high altitude. A longstanding hypothesis on metabolic adaptation to high-altitude proposes that maximizing energy (ATP) production per mole O_2 consumed and per mole fuel used, was of importance (Hochachka, 1994). The oxidation of carbohydrates has a greater ATP/mole O_2 yield than fatty acid oxidation, however fatty acid oxidation provides more energy per gram fuel, but also requires more oxygen. Indeed, in some high-altitude species, highly oxidative muscles phenotypes have been reported (Dawson et al., 2020; Lui et al., 2015; Mahalingam et al., 2017; Scott et al., 2015, 2009a). Many small mammals, including deer mice, preferentially use fatty acids to fuel thermogenesis (Haman et al., 2016; Lyons et al., 2021; Vaillancourt et al., 2009;

Weber and Haman, 2005), however, there is evidence that suggests that with acclimation to hypoxia, they may increase the proportion of carbohydrates used as fuel during activities such as exercise (Lau et al., 2017), potentially as an O_2-saving strategy. Other high-altitude natives also have adjustments in fuel use at high altitude where they exhibit greater use of carbohydrates than fatty acids as fuels during submaximal exercise compared to their low-altitude counterparts (Schippers et al., 2021, 2012). The more oxidative phenotype across many muscles that we report here may support elevated O_2 utilization for aerobic metabolism, ultimately producing more ATP per mole fuel compared to anaerobic glycolysis, of which we see a decreased capacity for in our high-altitude deer mice. Although carbohydrate metabolism provides more ATP per mole O_2 than fatty acid metabolism, the use of fatty acids and oxidative metabolism *versus* carbohydrate metabolisms at high altitude may arise to prevent the depletion of the relatively small carbohydrate stores (McClelland et al., 2017; McClelland and Scott, 2019).

Describing the potential mechanisms which underlie variation in performance has long been a goal in the field of evolutionary physiology. Here we hoped to gain insight into the potential mechanisms across hierarchical levels of regulation which govern the increased oxidative capacity of skeletal muscles in high-altitude deer mice, focussing on CS (a valuable marker of mitochondrial abundance) in the gastrocnemius. Greater CS activity may arise due to a greater abundance of CS protein (as a result of greater mitochondrial abundance or simply greater protein abundance). Previously, increased CS activity in the gastrocnemius of high-altitude deer mice had been associated with increases in mitochondrial volume density, attributed in large part to increases in subsarcolemmal mitochondria (Mahalingam et al., 2017). Indeed, we observed similar population differences here, where CS activity was paralleled by greater CS protein abundance and CS mRNA expression. Given that the degree of variation in CS mRNA

expression and CS protein abundance were very similar, it is unlikely that post-translational regulation is responsible for the population specific differences in CS protein abundance observed. Upon review of the population genetics data available for the CS gene (Schweizer et al., 2021), we found no genetic variation leading to changes in amino acid sequence of the CS protein. However, we did observe many sites of sequence variation in intronic regions which could be acting as transcription factor binding sites. Therefore, transcriptional regulation likely accounts for the majority of the variation in CS activity, and regulation of CS activity has possibly arisen from genetic differences in important *cis*-regulatory regions. CS is certainly not the only gene which shows evidence of positive selection at high altitude, however it is the only enzyme studied here which exhibits clear signs of selection. Many of the other identified genes with evidence of positive selection at high altitude from the Schweizer study have roles in altering oxygen homeostasis and aerobic metabolism (Schweizer et al., 2021). Similarly, gene expression regulation has also been the source of many other high-altitude native traits such as in the regulation of hemoglobin isoforms in high-altitude sheep, goats and antelope (Signore and Storz, 2020).

3.2 IMPLICATIONS AND FUTURE DIRECTIONS

Here we show that skeletal muscles in high-altitude deer mice have generally evolved to have a more oxidative phenotype, and that these differences occur not only in muscles of the hindlimb, but also in the core and forelimb regions (Fig. 3.1). Focusing on determinants of variation in CS, we found that transcriptional regulation is likely one of the mechanisms through which overall oxidative capacity is increase in skeletal muscles of high-altitude deer mice. With the varied pattern of differences in metabolic capacities, my results highlight that the phenotype of a single muscle is not necessarily representative of the larger picture across all muscles. Often, studies characterizing skeletal muscle phenotypes only investigate a very small number of

muscles, in many human studies, the vastus lateralis is a focal muscle often the target for biopsies, and subsequent findings generalized to the rest of the leg muscles. Our results show that this is not necessarily always the case as our findings in the vastus lateralis do not always reflect what is observed in the other hindlimb muscles of the deer mouse. Thus, in future studies attempting to characterize skeletal muscle phenotypes, a more comprehensive muscle sampling should be considered rather than to focus only on a single muscle. In our study, a few muscles stood out where high-altitude deer mice had significantly different capacities for multiple enzymes compared to their low-altitude counterparts, including the gluteus maximus, diaphragm, intercostals, plantaris and vastus medialis. The rectus femoris and semitendinosus also notably had multiple instances of enzyme capacities where population differences neared significance. As such, in future studies aimed at characterising skeletal muscle phenotypes, it may be worth considering including these muscles as part of the investigated muscles.

Given the overall differences in capacity for anaerobic and oxidative metabolisms between populations of deer mice that we report, it would be interesting to evaluate if these differences have implications for muscle performance, that is, whether these metabolic capacity differences have functional consequences in behaviours such as climbing and running. These performance traits could be measured at both the whole animal (sprint tests, grip strength or hanging tests) and the single fiber level where *in situ* muscle stimulation measurements of maximal force production and muscle endurance could be measured (Thomas et al., 2014).

Here we show that increased oxidative (CS) capacity in the gastrocnemius of high-altitude deer mice is consistent with increased gene expression and protein abundance of CS. It is however possible that these trends are a result of variation in fiber type composition as past studies have linked increased oxidative capacity with variation in fiber type composition (Lui et

al., 2015). Future studies could be aimed at determining if fiber type composition is the underlying cause of the variation in enzyme activities between low-altitude and high-altitude deer mice, or if these differences occur without similar shifts in fiber types. Similarly, in my study I was only able to survey the gene expression and protein abundance of one of the assayed enzymes. In the future it would be informative to include gene expression data from the other five enzymes to determine if similar mechanisms which underlie the variation in CS activity also govern COX, HOAD, HK, PK and LDH capacity in muscles. We have also shown here than transcription factors involved in mitochondrial abundance and skeletal muscles could also play a role in regulating the expression of key enzymes in metabolic pathways such as in the case of CS. Understanding whether the expression of these transcription factors differs between high-altitude and low-altitude species, and if they do indeed affect skeletal muscle phenotype is another avenue for future research.

3.3 FIGURES

Figure 3.1

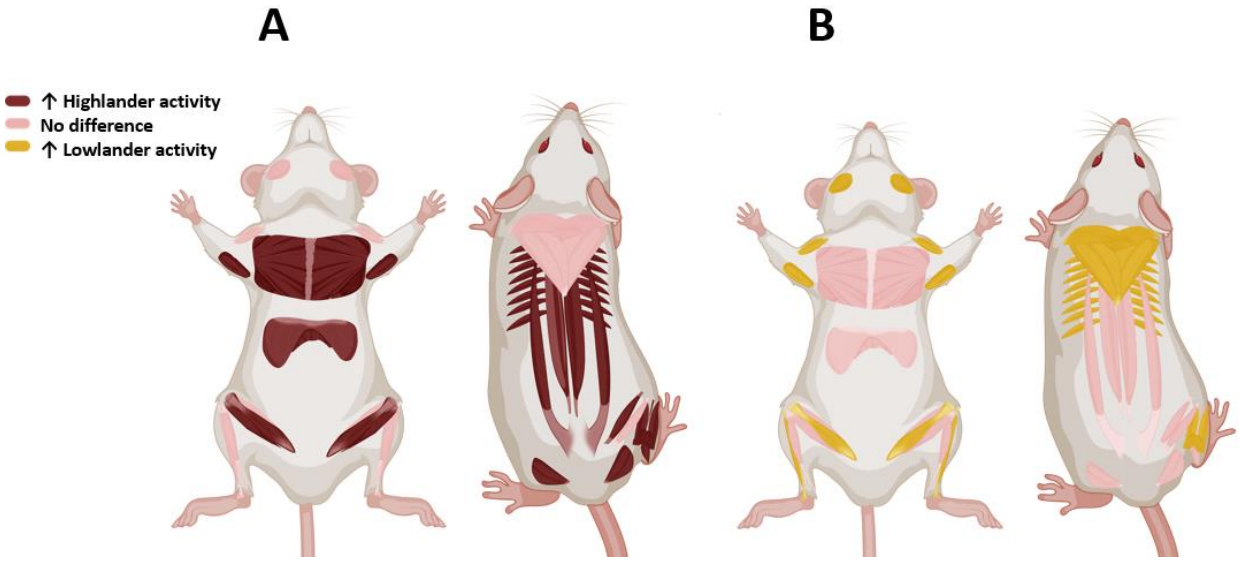

Figure 3.1: Overview of skeletal muscle location and differences in metabolic capacities. Muscles coloured in dark red represent muscles where high-altitude deer mice had significantly greater activity of either CS, COX, or HOAD, while muscles coloured in yellow represent muscles where low-altitude deer mice had significantly greater activity of either PK or LDH. Where there are no significant differences, muscles are coloured in pink.

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APPENDIX A

Supplementary Materials for Chapter 2

Table S1. Single nucleotide polymorphisms (SNP) within the CS gene that exhibit different allele frequencies between high-altitude and low-altitude populations of deer mice.

Nucleotide position	SNP	Allele frequencies	
		Low-altitude population	High-altitude population
34281780	A/G	0.565/0.435	0.145/0.855
34282479	G/A	0.981/0.019	0.016/0.984
34282512	T/C	0.222/0.778	0/1
34282547	C/T	0.963/0.037	0.156/0.844
34282633	T/C	0.37/0.63	0.016/0.984
34282658	A/G	0.426/0.574	0.097/0.903
34282813	C/T	0.963/0.037	0.359/0.641
34286250	A/G	0.852/0.148	0.016/0.984
34288645	C/T	1/0	0.661/0.339
34288687	C/T	0.519/0.481	0.031/0.969
34291767	C/A	1/0	0.022/0.978
34291832	C/T	1/0	0.019/0.981
34292170	C/T	0.423/0.577	0/1
34292189	A/G	0.538/0.462	0.048/0.952
34292228	C/T	0.481/0.519	0.016/0.984
34292297	C/A	0.519/0.481	0/1
34292529	A/G	0.444/0.556	0/1
34292545	G/A	0.907/0.093	0.031/0.969

The CS gene in located on chromosome 18 from nucleotides 34281746 to 34304096 (NW_006501362.1:651105-661326 as NW contig ID and position in *P. maniculatus bairdii* genome assembly 2.1).

Table S2. Body masses and masses of sampled muscles

	Low-altitude population		High-altitude population		Pop	Env	Pop × Env
	Normoxia	Hypoxia	Normoxia	Hypoxia			
Body mass	20.93 ± 1.57 (5)	19.79 ± 257 (7)	21.33 ± 1.02 (5)	17.86 ± 1.03 (7)	0.0776	0.5830	0.4030
Biceps brachii	0.99 ± 0.06 (5)	0.96 ± 0.12 (7)	1.12 ± 0.09 (5)	0.85 ± 0.13 (7)	0.9497	0.2120	0.3444
Biceps femoris	5.50 ± 0.81 (5)	5.64 ± 0.33 (7)	6.20 ± 0.08 (5)	5.65 ± 0.29 (7)	0.4970	0.6399	0.4305
Diaphragm	3.06 ± 0.25 (5)^{a}	4.22 ± 0.29 (7)^{b}	3.52 ± 0.21 (5)^{ab}	3.32 ± 0.22 (7)^{ab}	0.2081	0.0819	**0.0153**
Erector spinae	3.12 ± 0.45 (5)^{a}	4.25 ± 0.52 (7)^{ab}	3.02 ± 0.32 (5)^{a}	4.90 ± 0.25 (7)^{b}	0.4190	**0.0017**	0.3770
Extensor digitorum longus	1.30 ± 1.03 (5)	0.32 ± 0.04 (7)	0.25 ± 0.03 (5)	0.35 ± 0.03 (7)	0.3318	0.3249	0.2250
Gastrocnemius	3.80 ± 0.27 (5)	3.97 ± 0.16 (7)	4.03 ± 0.29 (5)	3.72 ± 0.10 (7)	0.9683	0.7159	0.1583
Gluteus maximus	3.29 ± 0.18 (5)	4.37 ± 0.40 (7)	3.21 ± 0.16 (5)	4.30 ± 0.47 (7)	0.8416	**0.0106**	0.9907
Pectoralis major	3.16 ± 0.47 (5)	3.24 ± 0.38 (7)	3.60 ± 0.25 (5)	3.51 ± 0.32 (7)	0.1018	0.7839	0.8801
Rectus femoris	3.13 ± 0.36 (5)	3.46 ± 0.18 (7)	9.29 ± 5.90 (5)	3.46 ± 0.15 (7)	0.3010	0.2754	0.2234
Semitendinosus	5.09 ± 0.40 (5)	5.97 ± 0.21 (7)	5.11 ± 0.34 (5)	4.97 ± 0.20 (7)	0.3252	0.2613	0.1392
Soleus	0.22 ± 0.04 (5)	0.24 ± 0.03 (7)	0.64 ± 0.23 (5)	0.24 ± 0.04 (7)	0.1101	0.0800	0.1392
Tibialis anterior	1.47 ± 0.17 (5)	1.66 ± 0.13 (7)	1.51 ± 0.10 (5)	1.50 ± 0.13 (7)	0.6345	0.6908	0.7547
Triceps brachii	3.16 ± 0.24 (5)	3.51 ± 0.17 (7)	3.51 ± 0.25 (5)	3.17 ± 0.24 (7)	0.7972	0.9760	0.1406
Vastus lateralis	2.82 ± 0.58 (5)	3.13 ± 0.59 (7)	2.38 ± 0.56 (5)	3.38 ± 0.21 (7)	0.9482	0.2040	0.4956
Vastus medialis	0.83 ± 0.07 (5)	0.96 ± 0.41 (6)	0.86 ± 0.11 (5)	0.90 ± 0.09 (7)	0.6918	0.5200	0.6322

Body masses (g) and skeletal muscle masses (mg per g body mass) are reported as mean ± SEM (N). Masses are only reported for muscles that were sampled intact. Muscles for which we could only obtain partial samples included the intercostals, masseter, plantaris, and trapezius (medial and lower). P-values are also shown for the effects of population (Pop), acclimation environment (Env), and their interaction in linear mixed-effects models (significant effects shown in bold). When a significant effect or interaction was detected, Tukey's multiple comparisons were performed, for which groups not sharing a letter are significantly different ($P<0.05$).

Table S3. Maximal CS activity of sampled muscles

Muscle	Low-altitude population		High-altitude population		Pop	Env	Pop × Env
	Normoxia	Hypoxia	Normoxia	Hypoxia			
Biceps brachii	50.91 ± 6.64 (5)	55.2 ± 4.04 (7)	62.63 ± 3.74 (5)	46.18 ± 4.43 (7)	0.938	0.219	**0.043**
Biceps femoris	40.83 ± 5.4 (5)	40.49 ± 6.78 (7)	45.82 ± 4.44 (5)	45.38 ± 4.14 (7)	0.911	0.788	0.576
Diaphragm	30.11 ± 1.83 (5)	29.95 ± 1.82 (7)	33.17 ± 2.03 (5)	33.46 ± 1.33 (7)	0.072	0.971	0.901
Erector spinae	8.66 ± 1.31 (5)	15.04 ± 2.11 (7)	9.16 ± 2.05 (5)	12.6 ± 1.83 (7)	0.537	**0.021**	0.463
Extensor digitorum longus	10.58 ± 5.12 (5)	9.68 ± 3.31 (5)	4.97 ± 0.67 (4)	9.06 ± 3.33 (7)	0.498	0.674	0.517
Gastrocnemius	20.66 ± 1.54 (5)	19.86 ± 3.43 (7)	32.68 ± 2.05 (5)	34.98 ± 4.22 (7)	**0.004**	0.650	0.943
Gluteus maximus	41.44 ± 6.83 (5)	33.13 ± 2.88 (7)	42.86 ± 1.95 (5)	46.95 ± 4.07 (7)	0.219	0.781	0.246
Intercostals	20.37 ± 1.37 (5)	19.8 ± 1.54 (7)	22.1 ± 0.63 (5)	24.74 ± 1.56 (7)	**0.021**	0.487	0.286
Masseter	51.48 ± 4.06 (5)	36.7 ± 4.21 (7)	41.72 ± 6.43 (5)	47.05 ± 2.56 (7)	0.647	0.287	**0.031**
Pectoralis major	5.58 ± 0.69 (5)	7.01 ± 0.56 (7)	6.62 ± 0.33 (5)	6.71 ± 0.57 (7)	0.656	0.204	0.261
Plantaris	39.97 ± 7.08	34.51 ± 6.14 (6)[a]	37.02 ± 5.79	59.58 ± 4.76	**0.037**	0.441	**0.029**
Rectus femoris	33.47 ± 2.54 (5)	31.06 ± 2.64 (7)	42.67 ± 3.02 (5)	38.00 ± 2.75 (7)	0.064	0.270	0.292
Semitendinosus	12.13 ± 0.81 (5)	10.98 ± 1.01 (7)	12.89 ± 0.78 (5)	12.33 ± 1.19 (7)	0.857	0.548	0.898
Soleus	32.18 ± 2.12 (5)	31.12 ± 4.54 (7)	33.35 ± 3.87 (5)	37.6 ± 4.27 (6)	0.326	0.717	0.526
Tibialis anterior	18.15 ± 4.19 (5)	18.27 ± 2.36 (7)	25.57 ± 5.27 (5)	24.29 ± 6.04 (7)	0.178	0.905	0.886
Trapezius (lower)	30.78 ± 5.81 (5)	30.65 ± 3.34 (7)	33.22 ± 4.34 (5)	26.58 ± 3.65 (7)	0.749	0.433	0.450
Trapezius (medial)	41.52 ± 1.6 (5)	45.45 ± 3.6 (7)	42.28 ± 6.13 (5)	49.92 ± 6.7 (7)	0.580	0.286	0.728
Triceps brachii	8.00 ± 0.95 (4)	10.99 ± 2.78 (7)	16.89 ± 3.11 (5)	10.8 ± 2.26 (7)	0.205	0.497	0.110
Vastus lateralis	5.46 ± 0.58 (5)	5.75 ± 1.08 (6)	5.27 ± 0.43 (5)	6.79 ± 0.82 (7)	0.523	0.281	0.469
Vastus medialis	35.13 ± 4.64 (5)[a]	37.28 ± 3.4 (6)[ab]	51.82 ± 7.01	59.08 ± 4.2 (7)[b]	**0.002**	0.133	0.970

CS activity (µmol per g tissue per min) is presented as mean ± SEM (N). P-values are also shown for the effects of population (Pop), acclimation environment (Env), and their interaction in linear mixed-effects models for each muscle (significant effects shown in bold). Body mass was also a significant covariate for the biceps femoris, gastrocnemius, gluteus maximus, rectus femoris, semitendinosus and vastus medialis. When a significant effect or interaction was detected, Tukey's multiple comparisons were performed, for which groups not sharing a letter are significantly different ($P<0.05$).

Table S4. Maximal COX activity of sampled muscles

	Low-altitude population		High-altitude population		Pop	Env	Pop ×
Muscle	Normoxia	Hypoxia	Normoxia	Hypoxia			
Biceps brachii	27.61 ± 2.36	29.72 ± 2.09 (7)^{ab}	37.45 ± 3.91 (5)^{b}	26.62 ± 2.01 (7)^{a}	0.378	0.106	**0.021**
Biceps femoris	17.32 ± 1.98 (5)	23.84 ± 3.52 (7)	29.29 ± 5.57 (5)	23.5 ± 2.87 (7)	0.650	0.654	**0.018**
Diaphragm	58.55 ± 8.13 (5)	61.12 ± 5.72 (7)	83.7 ± 6.61 (5)	76.32 ± 3.75 (7)	**0.004**	0.692	0.414
Erector spinae	16.74 ± 2.01 (5)	29.71 ± 2.46 (6)	37.91 ± 8.96 (5)	37.48 ± 5.44 (5)	**0.017**	0.230	0.221
Extensor digitorum longus	24.41 ± 5.02 (5)	36.52 ± 11.8 (7)	40.13 ± 17.64 (4)	27.3 ± 3.68 (7)	0.961	0.962	0.235
Gastrocnemius	36.65 ± 4.25 (5)	46.63 ± 6.69 (7)	46.79 ± 4.88 (5)	49.42 ± 5.17 (7)	0.826	0.089	0.294
Gluteus maximus	26.07 ± 2.85 (5)	20.02 ± 3.58 (7)	27.2 ± 3.56 (5)	33.34 ± 4.58 (7)	**0.048**	0.992	0.140
Intercostals	41.50 ± 3.74	31.58 ± 2.67 (7)^{a}	54.22 ± 7.95 (5)^{b}	42.97 ± 4.1 (7)^{ab}	**0.017**	**0.005**	0.826
Masseter	48.44 ± 3.31 (5)	46.56 ± 4.69 (7)	41.86 ± 7.77 (5)	44.73 ± 3.5 (7)	0.445	0.922	0.637
Pectoralis major	24.80 ± 2.38	32.21 ± 4.08 (7)^{b}	22.57 ± 2.35 (5)^{a}	31.38 ± 3.18 (7)^{ab}	0.233	**0.008**	0.818
Plantaris	25.72 ± 5.33 (5)^{a}	22.68 ± 2.86 (6)^{a}	24.67 ± 3.95 (5)^{a}	47.62 ± 3.27 (7)^{b}	**0.008**	**0.008**	**0.004**
Rectus femoris	27.62 ± 1.79 (5)	26.37 ± 3.20 (7)	32.63 ± 1.73 (5)	32.59 ± 2.89 (7)	0.234	0.970	0.833
Semitendinosus	32.28 ± 3.00 (5)	31.38 ± 3.80 (7)	34.02 ± 3.63 (5)	32.43 ± 2.82 (7)	0.070	0.722	0.922
Soleus	31.14 ± 4.38	19.94 ± 4.05 (7)^{a}	36.8 ± 2.36 (5)^{b}	37.87 ± 2.83 (6)^{b}	**0.008**	0.190	0.129
Tibialis anterior	34.64 ± 9.89 (5)	32.79 ± 4.54 (7)	29.04 ± 3.03 (5)	53.1 ± 11.91 (7)	0.709	0.134	0.247
Trapezius (lower)	31.05 ± 1.41 (5)	29.69 ± 2.57 (7)	25.45 ± 2.56 (5)	25.81 ± 3.38 (7)	0.115	0.860	0.765
Trapezius	27.13 ± 1.71 (5)	34.26 ± 4.39 (7)	36.17 ± 6.50 (5)	28.88 ± 2.71 (7)	0.882	0.984	0.099
Triceps brachii	42.70 ± 6.32 (5)	31.05 ± 10.03 (7)	43.05 ± 2.56 (3)	43.01 ± 4.23 (7)	0.368	0.409	0.485
Vastus lateralis	20.73 ± 2.49 (5)	19.29 ± 2.28 (7)	16.49 ± 2.01 (5)	22.07 ± 1.53 (6)	0.923	0.304	0.107
Vastus medialis	22.40 ± 2.60 (5)^{a}	23.50 ± 2.60 (6)^{a}	28.18 ± 1.69 (5)^{a}	38.57 ± 2.50 (7)^{b}	**0.001**	**0.018**	0.121

COX activity (μmol per g tissue per min) is presented as mean ± SEM (N). P-values are also shown for the effects of population (Pop), acclimation environment (Env), and their interaction in linear mixed-effects models for each muscle (significant effects shown in bold). Body mass was also a significant covariate for the biceps femoris, gastrocnemius, plantaris, rectus femoris, tibialis anterior and vastus medialis. When a significant effect or interaction was detected, Tukey's multiple comparisons were performed, for which groups not sharing a letter are significantly different ($P<0.05$).

Table S5. Maximal HOAD activity of sampled muscles

	Low-altitude population		High-altitude population		Pop	Env	Pop ×
Muscle	Normoxia	Hypoxia	Normoxia	Hypoxia			Env
Biceps brachii	40.00 ± 3.29 (5)	40.44 ± 2.49 (7)	54.67 ± 4.96 (5)	40.96 ± 5.1 (7)	0.138	0.131	0.108
Biceps femoris	51.18 ± 8.75 (5)	46.09 ± 3.7 (7)	48.22 ± 2.74 (5)	54.77 ± 2.46 (7)	0.412	0.877	0.224
Diaphragm	112.06 ± 7.33 (5)	115.58 ± 8.99	131.73 ± 9.28	149.64 ± 9.6 (7)	**0.029**	0.177	0.631
Erector spinae	18.55 ± 4.63 (5)	25.33 ± 3.23 (7)	27.04 ± 3.89 (5)	33.06 ± 5.53 (7)	0.294	0.096	0.632
Extensor digitorum longus	15.62 ± 4.33 (5)	12.61 ± 2.12 (7)	15.36 ± 2.52 (4)	14.85 ± 3.75 (7)	0.564	0.876	0.906
Gastrocnemius	30.38 ± 3.22 (5)	30.49 ± 4.06 (7)	35.63 ± 5.72 (5)	39.22 ± 3.66 (7)	0.444	0.449	0.856
Gluteus maximus	41.02 ± 9.14 (5)^{ab}	30.42 ± 2.17 (7)^{a}	55.88 ± 6.54	58.38 ± 3.70 (7)^{b}	**0.003**	0.614	0.388
Intercostals	60.95 ± 2.75 (5)^{ab}	53.36 ± 4.15 (7)^{a}	67.88 ± 3.46	72.31 ± 3.69 (7)^{b}	**0.008**	0.789	0.183
Masseter	54.89 ± 3.59 (5)	44.46 ± 3.01 (7)	52.41 ± 6.24 (5)	52.97 ± 2.39 (7)	0.528	0.193	0.169
Pectoralis major	52.83 ± 3.23 (5)	64.72 ± 7.73 (7)	74.76 ± 6.07 (5)	72.5 ± 4.26 (7)	**0.032**	0.433	0.254
Plantaris	29.91 ± 5.64 (5)^{a}	26.64 ± 4.21 (6)^{a}	30.68 ± 7.30 (5)^{a}	54.53 ± 1.94 (7)^{b}	**0.009**	**0.032**	**0.015**
Rectus femoris	49.23 ± 2.57 (5)	44.90 ± 5.3 (7)	70.26 ± 10.24	54.63 ± 4.96 (7)	0.146	0.139	0.139
Semitendinosus	32.88 ± 3.99 (5)	31.63 ± 2.15 (7)	40.51 ± 2.67 (5)	40.24 ± 2.88 (7)	**0.035**	0.884	0.998
Soleus	39.71 ± 5.52 (5)	30.45 ± 6.69 (7)	40.56 ± 6.48 (5)	46.45 ± 4.36 (6)	0.381	0.857	0.338
Tibialis anterior	23.07 ± 5.31 (5)	31.71 ± 11.48	18.84 ± 1.83 (5)	34.22 ± 6.45 (7)	0.971	0.161	0.687
Trapezius (lower)	50.14 ± 4.98 (5)	58.61 ± 14.68	49.73 ± 4.33 (5)	50.39 ± 4.99 (7)	0.610	0.644	0.692
Trapezius	51.97 ± 2.64 (5)	75.00 ± 13.35	88.23 ± 26.38	80.58 ± 14.39 (7)	0.261	0.638	0.352
Triceps brachii	12.74 ± 2.42 (5)^{a}	16.70 ± 3.23(7)^{b}	32.46 ± 4.66 (5)^{c}	17.14 ± 3.02 (7)^{b}	**0.021**	0.114	**0.011**
Vastus lateralis	8.97 ± 0.79 (5)	8.49 ± 0.82 (6)	10.28 ± 0.92 (5)	11.3 ± 1.56 (7)	0.083	0.808	0.537
Vastus medialis	38.26 ± 5.86 (5)^{a}	28.80 ± 2.45 (6)^{a}	55.30 ± 4.60	57.34 ± 4.23 (7)^{b}	**0.000**	0.555	0.324

HOAD activity (μmol per g tissue per min) is presented as mean ± SEM (N). P-values are also shown for the effects of population (Pop), acclimation environment (Env), and their interaction in linear mixed-effects models for each muscle (significant effects shown in bold). Body mass was also a significant covariate for the diaphragm, erector spinae, EDL, gastrocnemius, gluteus maximus, intercostals, rectus femoris and vastus medialis. When a significant effect or interaction was detected, Tukey's multiple comparisons were performed, for which groups not sharing a letter are significantly different ($P<0.05$).

Table S6. Maximal LDH activity of sampled muscles

	Low-altitude population		High-altitude population		Pop	Env	Pop ×
Muscle	Normoxia	Hypoxia	Normoxia	Hypoxia			Env
Biceps brachii	413.02 ± 42.23 (5)	444.46 ± 36.96 (7)	366.57 ± 28.94	323.67 ± 41.97	0.185	0.611	0.592
Biceps femoris	724.66 ± 37.36 (5)	686.35 ± 20.79 (7)	656.28 ± 39.63	611.07 ± 26.32	0.074	0.141	0.910
Diaphragm	389.71 ± 26.27 (5)	384.65 ± 29.42 (7)	328.51 ± 29.83	382.03 ± 19.59	0.584	0.477	0.165
Erector spinae	300.72 ± 62.26 (5)	282.18 ± 34.66 (7)	174.92 ± 26.95	281.94 ± 48.28	0.254	0.341	0.182
Extensor digitorum longus	106.77 ± 33.77 (5)	87.66 ± 29.64 (5)	52.41 ± 6.83 (4)	85.76 ± 26.20 (7)	0.411	0.800	0.371
Gastrocnemius	577.99 ± 34.02 (5)	519.93 ± 23.78 (7)	475.09 ± 29.64	474.99 ± 34.02	0.064	0.476	0.297
Gluteus maximus	778.36 ± 34.97 (5)	757.27 ± 29.26 (7)	757.18 ± 44.32	643.39 ± 51.57	0.306	0.067	0.448
Intercostals	739.79 ± 46.07 (5)	693.31 ± 66.75 (7)	662.13 ± 15.05	692.48 ± 67.17	0.700	0.637	0.221
Masseter	391.8 ± 17.16 (5)[b]	344.75 ± 15.09 (7)[a]	276.31 ± 33.15	342.37 ± 17.86	**0.026**	0.654	**0.014**
Pectoralis major	706.41 ± 35.24 (5)	650.79 ± 38.00 (7)	592.48 ± 49.05	562.66 ± 67.98	0.073	0.430	0.810
Plantaris	514.78 ± 105.16	384.82 ± 48.93 (6)	325.51 ± 65.37	502.51 ± 33.03	0.081	0.654	**0.025**
Rectus femoris	728.56 ± 59.6 (5)	687.91 ± 41.01 (7)	672.28 ± 43.37	599.14 ± 48.17	0.133	0.257	0.742
Semitendinosus	718.19 ± 46.25 (5)	699.81 ± 32.05 (7)	633.94 ± 53.41	619.93 ± 47.88	0.278	0.549	0.682
Soleus	194.88 ± 22.16 (5)	189.11 ± 47.27 (7)	201.95 ± 31.43	248.33 ± 29.34	0.346	0.609	0.493
Tibialis anterior	161.78 ± 30.7 (5)	209.44 ± 26.68 (7)	191.36 ± 40.14	193.26 ± 21.37	0.197	0.547	0.737
Trapezius (lower)	578.23 ± 67.63 (5)[b]	532.94 ± 34.15	442.56 ± 42.45	429.14 ± 60.94	**0.038**	0.589	0.768
Trapezius	530.18 ± 13.55 (5)[b]	457.67 ± 48.97	461.09 ± 33.85	354.35 ± 31.70	**0.028**	**0.029**	0.658
Triceps brachii	127.86 ± 11.15 (4)[a]	243.76 ± 27.33 (7)[b]	163.93 ± 34.32	110.37 ± 17.97	**0.014**	0.340	**0.004**
Vastus lateralis	521.88 ± 25.08 (5)	533.81 ± 89.77 (6)	395.36 ± 48.21	373.51 ± 20.12	0.073	0.674	0.886
Vastus medialis	396.66 ± 21.48 (5)	478.26 ± 74.38 (6)	376.69 ± 14.81	365.56 ± 34.13	0.384	0.580	0.462

LDH activity (μmol per g tissue per min) is presented as mean ± SEM (N). P-values are also shown for the effects of population (Pop), acclimation environment (Env), and their interaction in linear mixed-effects models for each muscle (significant effects shown in bold). Body mass was also a significant covariate for the biceps brachii, gluteus maximus, intercostals, semitendinosus, tibialis anterior and vastus lateralis. When a significant effect or interaction was detected, Tukey's multiple comparisons were performed, for which groups not sharing a letter are significantly different ($P<0.05$).

Table S7. Maximal PK activity of sampled muscles

	Low-altitude population		High-altitude population		Pop	Env	Pop ×
Muscle	Normoxia	Hypoxia	Normoxia	Hypoxia			Env
Biceps brachii	108.45 ± 6.57 (5)[ab]	117.34 ± 5.61 (7)[b]	108.15 ± 5.28	83.03 ± 10.26	**0.017**	0.313	**0.042**
Biceps femoris	155.18 ± 18.43 (5)	158.82 ± 15.87	144.79 ± 19.58	142.8 ± 9.61 (7)	0.389	0.959	0.860
Diaphragm	80.19 ± 6.03 (5)	82.53 ± 4.72 (7)	75.75 ± 4.95 (5)	74.63 ± 5.53 (7)	0.242	0.912	0.753
Erector spinae	135.17 ± 19.4 (5)	137.25 ± 16.59	88.23 ± 6.6 (5)	127.52 ± 13.73	0.295	0.237	0.156
Extensor digitorum longus	113.42 ± 13.75 (5)	85.59 ± 16.87 (5)	72.61 ± 19.41 (4)	70.41 ± 5.61 (7)	**0.046**	0.281	0.351
Gastrocnemius	156.67 ± 8.90 (5)	153.85 ± 7.43 (7)	148.37 ± 8.02 (5)	151.07 ± 9.4 (7)	0.519	0.950	0.799
Gluteus maximus	153.56 ± 10.6 (5)[ab]	162.56 ± 8.33	184.87 ± 11.83	141.84 ± 6.85	0.918	0.080	**0.011**
Intercostals	151.12 ± 11.34 (5)	146.13 ± 12.32	126.9 ± 7.77 (5)	127.25 ± 5.75	**0.044**	0.818	0.791
Masseter	88.17 ± 3.77 (5)	83.3 ± 5.66 (7)	70.62 ± 6.33 (5)	89.44 ± 5.27 (7)	0.504	0.224	**0.046**
Pectoralis major	130.29 ± 4.43 (5)	129.57 ± 6.93 (7)	114.68 ± 12.08	119.11 ± 12.03	0.211	0.853	0.797
Plantaris	132.95 ± 22.18 (5)	120.8 ± 13.75 (6)	112.83 ± 19.2 (5)	127.5 ± 4.57 (7)	0.742	0.910	0.379
Rectus femoris	155.04 ± 17.22 (5)	127.63 ± 13.89	119.73 ± 20.02	106.39 ± 3.72	0.062	0.159	0.619
Semitendinosus	182.06 ± 5.27 (5)	170.04 ± 9.82 (7)	161.48 ± 13.44	164.21 ± 11.35	0.275	0.672	0.503
Soleus	87.96 ± 11.6 (5)	78.14 ± 12.77 (7)	83.21 ± 5.24 (5)	92.01 ± 4.4 (6)	0.556	0.935	0.363
Tibialis anterior	107.84 ± 16.89 (5)	126.87 ± 13.66	121.35 ± 13.65	117.32 ± 17.67	0.997	0.647	0.483
Trapezius (lower)	108.45 ± 9.15 (5)	109.93 ± 3.96 (7)	97.08 ± 6.43 (5)	94.05 ± 7.29 (7)	**0.050**	0.911	0.743
Trapezius	119.81 ± 10.07 (5)	130.07 ± 4.97 (7)	137.61 ± 7.87 (5)	109.38 ± 7.36	0.538	0.246	**0.019**
Triceps brachii	107.53 ± 8.36 (4)[ab]	139.93 ± 18.15	103.34 ± 4.83	90.8 ± 10.91 (7)[a]	**0.029**	0.554	0.124
Vastus lateralis	141.31 ± 6.64 (5)	125.29 ± 12.21	123.23 ± 10.53	105.56 ± 14.62	0.341	0.496	0.917
Vastus medialis	134.24 ± 10.44 (5)	114.06 ± 10.71	118.62 ± 8.37 (5)	97.42 ± 6.01 (7)	0.210	**0.013**	0.744

PK activity (μmol per g tissue per min) is presented as mean ± SEM (N). P-values are also shown for the effects of population (Pop), acclimation environment (Env), and their interaction in linear mixed-effects models for each muscle (significant effects shown in bold). Body mass was also a significant covariate for the vastus medialis. When a significant effect or interaction was detected, Tukey's multiple comparisons were performed, for which groups not sharing a letter are significantly different ($P<0.05$).

Table S8. Maximal HK activity of sampled muscles

Muscle	Low-altitude population		High-altitude population		Pop	Env	Pop × Env
	Normoxia	Hypoxia	Normoxia	Hypoxia			
Biceps brachii	5.63 ± 0.3 (5)	5.16 ± 0.23 (7)	6.74 ± 0.7 (5)	5.57 ± 0.66 (7)	0.193	0.139	0.522
Biceps femoris	4.46 ± 0.24 (5)	5.09 ± 0.35 (7)	5.86 ± 0.43 (5)	6.4 ± 0.57 (7)	**0.025**	0.162	0.745
Diaphragm	11.27 ± 0.81 (5)[a]	12.2 ± 0.47 (7)[ab]	14.12 ± 1.19	14.49 ± 0.34	**0.001**	0.360	0.691
Erector spinae	ND	ND	ND	ND			
Extensor digitorum longus	ND	ND	ND	ND			
Gastrocnemius	4.65 ± 0.41 (5)	4.96 ± 0.48 (7)	4.96 ± 0.61 (5)	5.96 ± 0.38 (7)	0.879	0.186	0.365
Gluteus maximus	4.62 ± 0.68 (5)	3.8 ± 0.38 (7)	5.25 ± 0.52 (5)	5.39 ± 0.28 (7)	0.075	0.582	0.438
Intercostals	8.79 ± 0.68 (5)	7.59 ± 0.75 (7)	8.94 ± 0.31 (5)	9.71 ± 0.63 (7)	0.064	0.751	0.159
Masseter	8.36 ± 0.59 (5)	7.66 ± 0.13 (7)	7.92 ± 0.74 (5)	7.83 ± 0.24 (7)	0.867	0.366	0.512
Pectoralis major	5.02 ± 0.82 (5)	5.25 ± 0.47 (7)	5.89 ± 0.51 (5)	6.42 ± 0.46 (7)	0.077	0.507	0.791
Plantaris	4.32 ± 0.89 (5)[a]	4.19 ± 0.5 (6)[a]	5.44 ± 0.64 (5)[ab]	7.16 ± 0.38 (7)[b]	**0.006**	0.153	0.187
Rectus femoris	4.11 ± 0.24 (5)[a]	3.99 ± 0.15 (7)[a]	5.72 ± 0.54 (5)[b]	5.56 ± 0.3 (7)[b]	**0.000**	0.744	0.820
Semitendinosus	3.82 ± 0.55 (5)	3.08 ± 0.53 (7)	4.48 ± 0.66 (5)	4.35 ± 0.25 (7)	0.192	0.503	0.728
Soleus	ND	ND	ND	ND			
Tibialis anterior	ND	ND	ND	ND			
Trapezius (lower)	6.05 ± 0.46 (5)	7.09 ± 0.7 (7)	8.06 ± 0.86 (5)	6.93 ± 0.72 (7)	0.315	0.953	0.153
Trapezius	6.99 ± 0.54 (5)	7.01 ± 0.52 (7)	6.98 ± 0.16 (5)	7.26 ± 0.45 (7)	0.758	0.754	0.782
Triceps brachii	ND	ND	ND	ND			
Vastus lateralis	3.92 ± 0.74 (5)	2.65 ± 0.49 (7)	5.02 ± 0.99 (5)	5.4 ± 0.78 (7)	0.074	0.729	0.430
Vastus medialis	4.77 ± 0.75 (5)	4.17 ± 0.28 (6)	6.98 ± 0.82 (5)	6.13 ± 0.67 (7)	**0.028**	0.352	0.594

HK activity (μmol per g tissue per min) is presented as mean ± SEM (N). P-values are also shown for the effects of population (Pop), acclimation environment (Env), and their interaction in linear mixed-effects models for each muscle (significant effects shown in bold). Body mass was a significant covariate for the biceps femoris, gastrocnemius, gluteus maximus, rectus femoris, plantaris, semitendinosus, vastus lateralis and vastus medialis. Maximal HK activity was not determined (ND) for erector spinae, extensor digitorum longus, soleus, tibialis anterior and triceps brachii. Tukey's multiple comparisons were performed when a significant effect or interaction was detected, for which groups not sharing a letter are significantly different ($P<0.05$).

Table S9. Predicted transcription factor binding sites that overlap CS gene SNPs

Transcription factor	Full name	SNP position	P-value	Q-value	Reference
YY1	Yin Yang 1	34292545	4.94E-05	0.00927	[1, 2]
HIF1A	Hypoxia Inducible Factor 1 Subunit Alpha	34292545	4.11E-05	0.0288	[3, 4]
		34291767	8.83E-05	0.0288	
EWSR1	EWS RNA Binding Protein 1	34292545	1.82E-05	0.0149	[5]
		34291767	1.66E-05	0.0271	
TAF1	TATA-Box Binding Protein Associated Factor 1	34292545	8.32E-06	0.0044	[4]
LSD1	Lysine Demethylase 1A	34292545	8.29E-05	0.0452	[6, 7]
CDK9	Cyclin Dependent Kinase 9	34292545	3.25E-05	0.016	[8]
		34292297	3.76E-05	0.0101	
		34292529	3.49E-05	0.016	
EZH2	Histone-Lysine N-Methyltransferase EZH2	34292529	5.08E-05	0.043	[9]
MYC	MYC Proto-Oncogene, bHLH transcription factor	34292529	4.07E-05	0.0342	[10, 11]
		34292297	9.58E-06	0.0163	
SRF	Serum Response Factor	34292529	1.64E-05	0.0287	[12]
TP53	Tumor Protein P53	34292529	4.69E-05	0.04	[13]
CTCF	CCCTC-Binding Factor	34292297	1.90E-05	0.0322	[14]
STAT5B	Signal Transducer and Activator of Transcription 5B	34292297	1.66E-05	0.0273	[15]
NR2C2/TR4	Nuclear Receptor Subfamily 2 Group C Member 2/ Testicular nuclear receptor 4	34292297	2.18E-05	0.0371	[15]
NR2F2	Nuclear Receptor Subfamily 2 Group F Member 2	34292297	1.28E-05	0.0211	[16]
ASCL2	Achaete-Scute Family bHLH Transcription Factor 2	34292297	8.21E-06	0.0141	[17]
RXR:RAR	Retinoid x receptor: retinoic acid receptor complex	34292228	1.86E-05	0.0324	[18, 19, 20]
		34282658	5.48E-06	0.0115	
SIN3A	IN3 Transcription Regulator Family Member A	34292189	2.28E-05	0.0193	[21, 22]
PBX3	PBX Homeobox 3	34292189	2.74E-05	0.048	[23]
ATF2	Activating Transcription Factor 2	34292189	2.43E-05	0.0415	[24]
EP300	E1A Binding Protein P300	34292189	3.69E-06	0.00636	[23, 25]
		34291832	2.60E-05	0.0444	

		34282512	2.80E-05	0.0292	
EPAS1	Endothelial PAS Domain-Containing Protein 1	34292189	5.75E-05	0.0499	26, 27
	(HIF2A)	34282658	1.56E-05	0.033	
RB1	Retinoblastoma-associated protein	34291767	1.62E-05	0.0271	28, 29
VDR	Vitamin D Receptor	34282658	2.37E-05	0.0476	30
		34281780	3.40E-05	0.0488	
DEC1	Differentiated embryo-chondrocyte expressed gene 1	34282658	1.65E-06	0.00345	31, 32, 33
SP2	SP2 Transcription Factor	34282633	2.15E-05	0.0243	34
		34282512	1.04E-05	0.00907	
EZH1	Histone-Lysine N-Methyltransferase EZH1	34282512	1.83E-05	0.0376	9
SMAD1	SMAD Family member 1	34282547	3.05E-05	0.0267	35
AR	Androgen Receptor	34282479	5.22E-05	0.0449	36, 37
		34281780	1.36E-05	0.0283	

Predicted transcription factor binding sites with involvement in skeletal muscles and the SNP position in the CS gene they overlap with. P and Q statistic values. References for the transcription factors involvement in skeletal muscles: (Chen et al., 2019)[1] (Zhou et al., 2015)[2] (Lindholm and Rundqvist, 2016)[3] (Valle-Tenney et al., 2020)[4] (Lee et al., 2019)[5] (Tosic et al., 2018)[6] (Choi et al., 2010)[7] (Anshabo et al., 2021)[8] (Stojic et al., 2011)[9] (Luo et al., 2019)[10] (Zeller et al., 2006)[11] (Li et al., 2005)[12] (Beyfuss and Hood, 2018)[13] (Delgado-Olguín et al., 2011)[14] (Klover et al., 2009)[15] (Lee et al., 2017)[16] (Wang et al., 2017)[17] (Chen and Li, 2016)[18] (Crumbley et al., 2012)[19] (Le and Li, 2012)[20] (Das et al., 2013)[21] (van Oevelen et al., 2010)[22] (Martins et al., 2020)[23] (Vale-Cruz et al., 2008)[24] (Svensson et al., 2020)[25] (Pircher et al., 2021)[26] (Rasbach et al., 2010)[27] (Novitch et al., 1996)[28] (Zacksenhaus et al., 1996)[29] (Girgis et al., 2014)[30] (Huang et al., 2018)[31] (Vercherat et al., 2009)[32] (Hsiao et al., 2009)[33] (Terrados et al., 2012)[34] (Goodman and Hornberger, 2014)[35] (Chambon et al., 2010)[36] (Sinha-Hikim et al., 2004)[37]

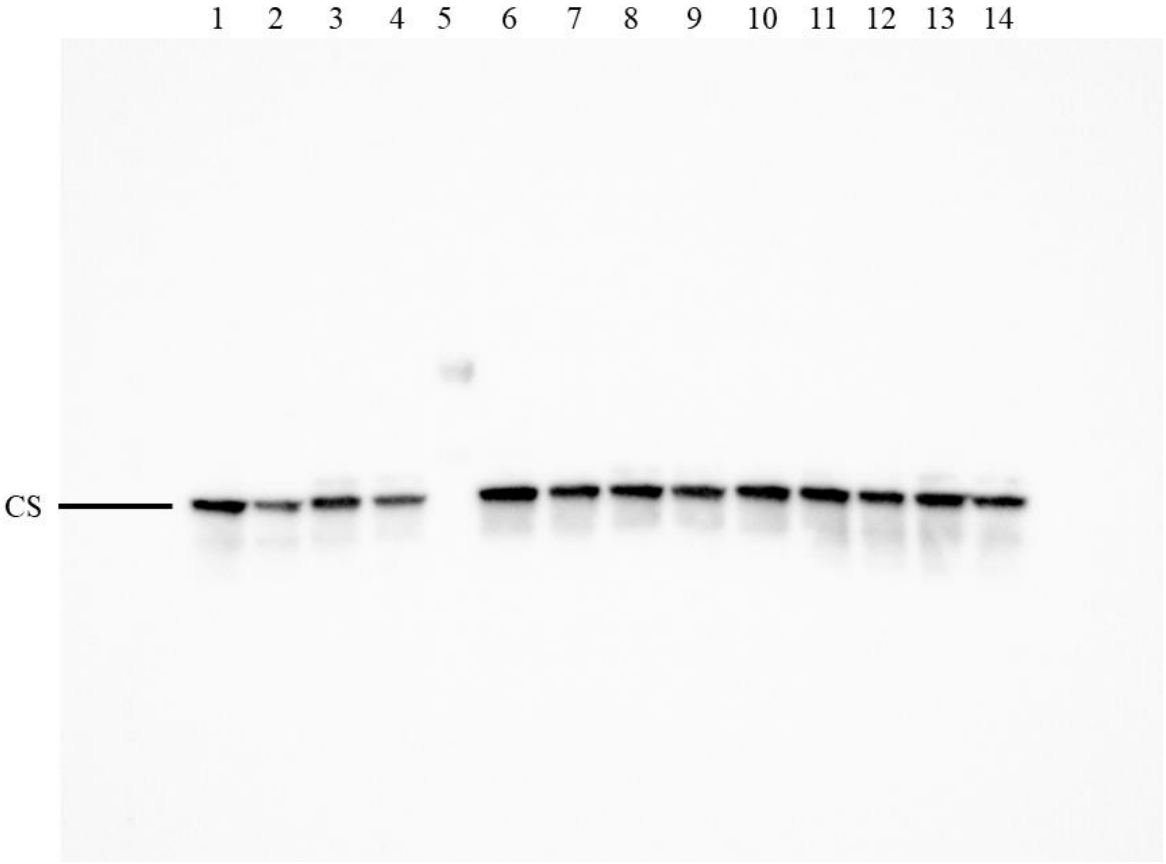

Figure S1. Representative western blot for citrate synthase (CS) in the gastrocnemius. Citrate synthase was identified by a single band near the expected size of 52 kDa. Representative bands are shown for high-altitude mice in normoxia (lanes 7, 9) and hypoxia (lanes 1, 6, 10, 12) and for low-altitude mice in normoxia (lanes 2, 8, 13, 14) and hypoxia (lanes 3, 4, 11).

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