THE STRESS OF BEING ON TOP: HIGH-ALTITUDE ADAPTATIONS AND PHENOTYPIC PLASTICITY OF MITOCHONDRIA IN DEER MICE

THE STRESS OF BEING ON TOP: HIGH-ALTITUDE ADAPTATIONS AND PHENOTYPIC PLASTICITY OF MITOCHONDRIA IN DEER MICE, *Peromyscus maniculatus*

By

SAJENI MAHALINGAM, B. Sc., M. Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2017) **McMaster University**

(Biology) **Hamilton, Ontario**

TITLE:	**The stress of being on top: high-altitude adaptations and phenotypic plasticity of mitochondria in deer mice, *Peromyscus maniculatus***
AUTHOR:	**Sajeni Mahalingam, B.Sc., MSc (University of Toronto, Toronto, Ontario)**
SUPERVISORS:	**Dr. G.B. McClelland and G.R. Scott**
NUMBER OF PAGES:	**193**

ABSTRACT

Hypoxia is a major stressor at high altitudes that limits tissue oxygen availability. High altitude environments are also extremely cold which increases thermogenic demand. Small mammals living at high altitude face the competing energetic challenge of maintaining thermogenesis in a hypoxic environment that can impair aerobic ATP supply. It has been suggested that hypoxia-induced impairments in ATP synthesis capacity and cold-induced increases in thermogenic demand could be counteracted by an increase in tissue oxidative capacity and/or fuel selection. As the organelle that consumes oxygen to produce ATP, changes in mitochondrial physiology can help offset physiological impairments at high altitudes. We explored this hypothesis in North American deer mice (*Peromyscus maniculatus)*, from populations native to high and low altitude. We compared mitochondrial volume densities, intracellular distribution, respiratory capacities, enzyme activities of the mitochondrial complexes, capillarity, and fibre-type distribution in skeletal and cardiac muscles. To examine potential changes to mitochondrial physiology at high altitudes deer mice (*P. maniculatus*) were acclimated to: warm (25°C) normoxia; warm hypoxia (simulated altitude of 4300m); cold (5°C) normoxia; and cold + hypoxia. In skeletal muscle, highlanders had higher mitochondrial volume densities than lowlanders, entirely due to an increased abundance of mitochondria in a subsarcolemmal location next to capillaries. Mitochondria from highland mice also had higher mitochondrial respiratory capacities and cytochrome c oxidase activity in control conditions, but these values converged after hypoxia acclimation. Cold acclimation restored pyruvate and fatty acid

respiratory capacity to control levels in highland mice, which also showed an increase in mitochondrial uncoupling. Cold increased respiratory capacities in lowland mice. Acclimation to cold+hypoxia did not change mitochondrial physiology beyond cold alone and appeared to counteract the effects of hypoxia on highland mice. In cardiac muscle highland mice had higher respiratory capacities, but after hypoxia acclimation lowland mice significantly increased respiratory capacities. In response to hypoxia, highland mice increased the relative capacity to oxidize carbohydrates compared to fatty acids. Our results suggest that both highland ancestry and plasticity affect mitochondrial physiology, and likely contributes to performance at high altitudes.

ACKNOWLEDGEMENTS

First, I have to thank my supervisors, Dr. Grant McClelland and Dr. Graham Scott for their support, encouragement and constructive criticism throughout the past four years. Being co-supervised gave me the opportunity to be part of both labs and both research programs. Thank you for all the opportunities you have given me over the past 4 years.

I am also thankful to my awesome labmates (Alex, Gabriele, Sherry, Catie, Brittney, Sulayman, Neal, Nicole, Taylor, Caylieh, Soren, Paras, Nastashya and Oliver). It has been a pleasure working with people that are always supportive, fun and hardworking. All of you made the long hours in the lab enjoyable and the times outside the lab even more enjoyable.

Last, but not least I would like to thank my parents for their unwavering support and unconditional love. I could not be more grateful to have parents that have always encouraged me to pursue my goals. I would also like to thank my brothers for their endless support and for delivering care packages every week. Finally, I'd like to thank my extended family and friends that have helped me stay sane.

PREFACE

This thesis is organized in the sandwich thesis format. Chapter 1 is a general introduction to chapters 2-4. Chapter 2 has been submitted for publication and is currently under review. Chapters 3 and 4 are completed studies that will be submitted for publication. Chapter 5 is a general summary and ties all three data chapters together.

Thesis organization and format

CHAPTER 1	INTRODUCTION
CHAPTER 2	EVOLVED CHANGES IN THE INTRACELLULAR DISTRIBUTION AND PHSIOLOGY OF MUSCLE MITOCHONDRIA IN HIGH-ALTITUDE NATIVE DEER MICE
Authors	Mahalingam, S, McClelland, G.B. and Scott G.R.
Journal	The Journal of Physiology
Contributions	S.M., G.B.M., and G.R.S. were all involved in the conception and design of experiments. S.M. performed the experiments, analyzed the data, and drafted the manuscript. G.B.M. and G.R.S. supervised S.M., interpreted the data, and revised the manuscript.
CHAPTER 3	HIGH-ALTITUDE ANCESTRY ALTERS MUSCLE PHENOTYPE AND THE PLASTICITY OF MUSCLE MITOCHONDRIA IN CHRONIC COLD AND/OR HYPOXIA IN DEER MICE
Authors	Mahalingam, S, McClelland, G.B. and Scott G.R.
CHAPTER 4	HIGH-ALTITUDE ANCESTRY AND HYPOXIA ACCLIMATION AFFECT CARDIAC MITOCHONDRIAL PHYSIOLOGY IN DEER MICE

Authors Mahalingam, S, McClelland, G.B. and Scott G.R.

CHAPTER 5 SUMMARY

Contributions not appearing in thesis

Lui, M.A., **Mahalingam, S.,** Patel, P., Connaty, A.D., Ivy, C.M., Cheviron, Z.A., Storz, J.F., McClelland, G.B., and Scott, G.R. (2015). High-altitude ancestry and hypoxia acclimation have distinct effects on exercise capacity and muscle phenotype in deer mice. *Am. J. Physiol. Reg. Integr. Comp. Physiol.* 308, R779–R791.

Du, S.N.N., **Mahalingam, S.,** Borowiec, B.G. and Scott, G.R. (2016). Mitochondrial physiology and reactive oxygen species production are altered by hypoxia acclimation in killifish (*Fundulus heteroclitus*). *J. Exp. Biol.* 219, 1130-1138.

Lau, D.S., Connaty, A.D., **Mahalingam, S.,** Wall, N., Cheviron, Z.A., Storz, J.F., Scott, G.R. and McClelland, G.B. (2017). Acclimation to hypoxia increases carbohydrate use during exercise in high-altitude deer mice. Am. *J. Physiol. Reg. Integr. Comp. Physiol.* Accepted.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
CAT	carnitine-acyl-carninitine translocase
COX	cytochrome c oxidase
CPT	carnitine palmitoyltransferase
CS	citrate synthase
CI	complex I
CII	complex II
CIII	complex III
CIV	complex IV
CV	complex V
DCPIP	dichlorophenolindophenol
DTNB	dithiobisnitrobenzoic acid
DUB	decylubiquinone
EPO	erythropoietin
ETC	electron transport chain
F1	first generation
FATP	fatty acid transport protein
FABPpm	fatty acid binding protein plasma membrane
GLUT 4	glucose transporters
HA	high-altitude
H	heart type

HK	hexokinase
HIF-1	hypoxia inducible factor one
HOAD	ß-hydroxyacyl-CoA dehydrogenase
I_{mito}	intermyofribillar mitochodnria
L_N	leak respiration in the absence of ATP
L_T	leak respiration in the presence of ATP
LDH	lactate dehydrogenase
M	muscle type
MO_2	rate of O_2 consumption
MPC	mitochondrial pyruvate carriers
NADH	Nicotinamide adenine dinucleotide reduced
NEFA	non-esterified fatty acids
O_2	Oxygen
OXPHOS	oxidative phosphorylation
P_{50}	PO_2 at 50% of inhibition of maximal respiration
P_{PM}	oxphos respiration with pyruvate and malate
P_{MPal}	oxphos respiration with malate, palmitoyl-carnitine
$P_{MPalOct}$	oxphos respiration with malate, palmitoyl-carnitine, and octanoyl-carntine
P_{PMG}	oxphos respiration with pyruvate, malate, and glutamate (CI)
P_{PMGS}	oxphos respiration with pyruvate, malate, glutamate and succinate (CI+II)
$P_{MNad+Lac}$	lactate respiration

$P_{S(Rot)}$	oxphos respiration with pyruvate, malate, glutamate, succinate and rotenone (CII)
P_{Tm}	oxphos respiration with TMPD and ascorbate
PEP	phosphoenolpyruvate
PDK1	pyruvated dehyrdogenase kinase 1
PDH	pyruvate dehydrogenase
PHD1-3	prolyl-hydroxylase enzymes
PK	pyruvate kinase
ROS	reactive oxygen species
RCR	Respiratory control ratio
S_{Mito}	subsarcolemmal mitochondria
SOD	superoxide dismutase
TMPD	N,N,N′,N′-tetramethyl-p-phenylenediamine
VEGF	vascular endothelial growth factor
V_{max}	maximal activity
VO_{2max}	maximum rate of oxygen consumption

CHAPTER 1

INTRODUCTION

1.1 PREFACE

High-altitude (HA) environments are amongst the harshest terrestrial environments inhabited by land animals. The thin air is depleted of oxygen while temperatures can be extremely cold. To maintain body temperatures animals require a continuous supply of oxygen to fuel aerobic metabolism and sustain shivering. However, some animals have adapted to living at high altitudes. Tibetan and Andean highland natives have a lower maximal performance capacity at high altitudes, compared to lowland natives at low altitudes. Maximal performance capacity was measured via maximal oxygen consumption rates (VO_2 max) when running on a treadmill. However, the highland natives have a higher submaximal performance capacity (below VO_2 max) and fatigue resistance than lowlanders (Marconi et al., 2006). Many studies have focused on the physiological adjustments animals have made at different levels of the oxygen cascade to maintain the required oxygen flux in an environment that is oxygen limited. For example, animals can enhance the capacity for O_2 transport by increasing lung size, ventilation, cardiac output, the number of capillaries, hematocrit, hemoglobin concentration, myoglobin concentration or hemoglobin affinity for oxygen (Hurtado, 1960; Storz et al., 2010). Acclimation to laboratory conditions and acclimatization in the wild of lowland native animals to high altitude has provided insight on phenotypic plasticity of components of the oxygen cascade, which may be either adaptive or maladaptive. In addition to changes in the oxygen cascade, the metabolic substrates used for ATP production can affect how

efficiently oxygen is used at high altitudes (Schippers et al., 2012). As the last step in the oxygen cascade, and the organelle that uses oxygen as the terminal electron acceptor to oxidize fuels, mitochondria may be a target of adaptation or phenotypic plasticity that may be beneficial to animals living at high altitudes. An adaptation is a genetic change that helps an organism survive in its environment (Ghalambor et al., 2007). Phenotypic plasticity is the ability of a genotype to produce different phenotypes in response to changes in environmental conditions (Ghalambor et al., 2007). Adaptations or the capacity for phenotypic plasticity in skeletal muscle mitochondria may be advantageous in low oxygen environments because they are important for both locomotion and thermogenesis.

1.2 SKELETAL MUSCLE FUEL USE

Energy is produced in skeletal muscles via oxidation of fuels to support locomotion and for shivering thermogenesis. Skeletal muscles predominantly utilize fuels from the blood (glucose and fatty acids) and from glycogen and lipid stores in the cytosol to produce ATP. In terms of carbohydrates, glucose transported from the blood using glucose transporters (GLUT 4) and glycogen stores within the muscle fibre are converted to pyruvate by the enzymes in glycolysis. The conversion of pyruvate to lactate occurs when rate of glycolysis is greater than the rate of pyruvate oxidation. This can produce ATP anaerobically as an alternative to pyruvate entering the mitochondria and produce ATP aerobically (Weber, 2011). Glycolysis of one glucose molecule results in a net

production of 2 ATP, 2 NADH molecules and 2 pyruvate molecules. If the pyruvate does not continue through oxidative phosphorylation within the mitochondria the 2 pyruvate molecules are converted into two lactate molecules using both NADH molecules. Anaerobic fermentation of glucose only produces 2 ATP molecules (Fox, 2006). When oxidation of glucose occurs aerobically pyruvate enters the mitochondrial matrix via mitochondrial pyruvate carriers (MPC). The pyruvate dehydrogenase (PDH) complex decarboxylation reaction then converts pyruvate to acetyl Co-A which combines with oxaloacetate as the first step in the citric acid cycle. The reducing equivalents produced enter the electron transport chain. When the 2 pyruvate molecules and the 2 NADH molecules produced by glycolysis go through oxidative phosphorylation, the oxidation of one glucose molecule produces 30 ATP molecules. Pyruvate dehydrogenase is inhibited by pyruvate dehyrdogenase kinase 1 (PDK1). When pyruvate dehydrogenase is inhibited metabolism continues via anaerobic respiration.

Free fatty acids (also called non-esterified fatty acids, NEFA) are mobilized from triacylglycerol in adipocytes and myocytes via hormone sensitive lipase. The free fatty acids in blood are transported into the myocyte via transporters (CD36/FAT, fatty acid binding protein plasma membrane (FABPpm) or fatty acid transport protein (FATP). Inside the cytosol free fatty acids are converted to fatty acyl-CoAs and then fatty acid oxidation takes place in the mitochondria (McClelland, 2004). Fatty acid oxidation occurs via ß-oxidation in the mitochondria. Entry of fatty acids into the mitochondria is

controlled by the enzyme carnitine palmitoyltransferase (CPT) I, which is located on the inner side of the outer mitochondrial membrane. CPT I catalyzes the conversion of fatty acyl-CoA to fatty acyl-carnitine. Fatty acyl-carnitine is then transferred into the mitochondrial matrix by carnitine-acyl-carninitine translocase (CAT). Once fatty acyl-carnitine reaches the matrix it is converted back to fatty acyl-CoA by CPT II. Fatty acyl-CoA goes through β-oxidation in the mitochondrial matrix. The final step is the cleavage of a two carbon acetyl-CoA from the remaining fatty acyl-CoA, allowing the acetyl-CoA to then enter the citric acid cycle. Oxidation of acetyl-CoA produces reducing equivalents that enter the electron transport chain (McClelland, 2004). A six carbon fatty acid chain produces a total of 38 ATP molecules. The number of ATP produced by a six carbon fatty acid chain is about 25% higher than a six carbon glucose molecule (Fox, 2006). Furthermore, carbohydrates are heavier than fatty acids; therefore, fatty acids produce more ATP per mole of fuel compared to carbohydrates. Lipids are chemically reduced and due to the high energy density of lipids they are stored in much larger quantities compared to carbohydrates in mammals.

According to the glucose-fatty acid cycle proposed by Randle et al., (1963) oxidation of fatty acids inhibits carbohydrate oxidation. The high ratios of acetyl-CoA to free Co-A and NADH to NAD+ from oxidation of fatty acids is thought to inhibit the active form of PDH, which leads to an accumulation of citrate. The citrate is then transported out of the mitochondria where it can inhibit phosphofructokinase, which leads

to an increase in glucose-6-phosphate. An increase in glucose-6-phosphate inhibits hexokinase. Oxidation of long chain fatty acids can lead to the inhibition of glycolysis.

1.3 CARDIAC MUSCLE FUEL USE

Cardiac muscles produce energy by oxidizing fuels in order to support the high energy demand required to keep the heart beating (Ingwall, 2002). The heart has a very high oxidative capacity and is composed of slow oxidative fibres. Oxidation of carbohydrates and lipids occurs through the same processes in the heart as skeletal muscles. The heart is able to metabolize lipids, carbohydrates, ketone bodies, amino acids and lactate. However, the heart primarily uses lipids as a fuel source (Kolwicz and Tian, 2014).

1.4 MITOCHONDRIA

As the organelle that ultimately oxidizes fuels using oxygen, changes in mitochondrial function at high altitudes can improve performance at high altitudes. Mitochondria have an inner and outer membrane and the electron transport system is embedded in the inner membrane. There are 5 complexes embedded in the inner mitochondrial membrane that are responsible for oxidative phosphorylation. Electrons from reducing equivalents are passed down the electron transport chain to complex four, which uses the electrons to reduce molecular oxygen to water. Complex four is called cytochrome C oxidase (COX) and it is composed of 13 subunits. Many of the subunits have isoforms, but subunit 4 (COX_4) is of particular interest in low oxygen environments

because it can fine tune metabolism under low oxygen conditions. COX_4 has two isoforms in vertebrates, $COX_{4\text{-}1}$ and $COX_{4\text{-}2}$. In mammals, the ratio of $COX_{4\text{-}1}$ to $COX_{4\text{-}2}$ is high under normoxic conditions, but under hypoxia there is an increase in transcription of $COX_{4\text{-}2}$ along with a degradation of $COX_{4\text{-}1}$ by LON protease (Kocha et al., 2015). The P_{50} of COX for oxygen can range from 0.01 to 0.10 kPa. Mitochondrial partial pressure of oxygen in exercising muscles at sea level is predicted to be about 0.13 kPa, but it drops to about 0.016 kPa on the summit of Everst (Murray and Horscroft, 2015). Complexes I, II, and IV pump protons into the intermembrane space, which creates an electrochemical gradient with a more negatively charged matrix and high [H+] in the intermembrane space. Complex V (also called F1Fo ATP synthase) taps into the electrochemical gradient to convert ADP and inorganic phosphate to ATP by allowing protons back into the mitochondrial matrix.

Mitochondria are important sources of reactive oxygen species (ROS). ROS produced by the mitochondria can contribute to mitochondrial or cell damage, but ROS are also important for redox signalling from the mitochondria to the cell (Collins, 2012). ROS are messengers in signalling cascades that control apoptosis, cell differentiation, wound healing and mitochondrial oxidative phosphorylation capacity (Munro and Treberg, 2017). The redox state of a cell depends on the levels of oxidants, antioxidants, and electron carriers. Oxidants create reactive oxygen species, while antioxidants convert reactive oxygen species into less reactive species. Therefore, oxidative stress is high when

the rate of reactive oxygen species production exceeds the capacity ROS detoxification by antioxidants. Superoxide produced by mitochondria is dismutated by superoxide dismutase (SOD) to hydrogen peroxide. Manganese SOD is found within mitochondria and copper and zinc containing SOD is found in the cytosol. Under physiological conditions hydrogen peroxide is detoxified to oxygen and water by glutathione peroxidase, thioredoxin reductase, or catalase (Kowaltwoski and Vercesi, 1999).

1.5 LOCOMOTION

During high intensity aerobic exercise greater than 90% of the oxygen is consumed by skeletal muscle mitochondria (Suarez, 1992; Taylor, 1987). During exercise most of the energy comes from carbohydrates and lipids. Proteins only make up less than 5% of total ATP used during exercise (Rennie et al., 1981; Carraro et al., 1994). The proportion of fuels used is dependent on the exercise intensity; as the intensity increases a greater proportion of carbohydrates is used (Weber, 2011). As exercise intensities increase there is also a progressive increase in the recruitment of fast glycolytic fibres; whereas slow oxidative fibres are recruited at lower intensities (Armstrong and Laughlin, 1985).

1.6 THERMOGENESIS

In small mammals, total thermogenic capacity is the sum of basal metabolic rate, non-shivering thermogenesis and shivering thermogenesis. Skeletal muscle mitochondria provide the ATP required for muscle contractions during shivering thermogenesis. In

larger animals it has been observed that cold acclimations result in skeletal muscle changes similar to that of endurance training (Turner et al., 1995).

Normally non-shivering thermogenesis occurs in brown adipose tissue. Whether skeletal muscle mitochondria are involved in non-shivering thermogenesis continues to be debated (Kinnula et al., 1983; Barre et al., 1985). It has been observed in human skeletal muscle cold exposure leads to uncoupling of mitochondria, which results in heat production rather than ATP (Wijers et al., 2008). However, the extent that skeletal muscle may contribute to non-shivering thermogenesis in animals that are adapted to living at high altitudes has not been investigated.

1.7 HIGH ALTITUDE ADAPTATIONS AND PHENOTYPIC PLASTICITY OF MUSCLE METABOLISM AND MITOCHONDRIA

How animals adapt to living at high altitudes has been of interest for many years. High altitude environments have low oxygen availability and are extremely cold. To survive and function at high altitudes animals will have to deal with the increased oxygen demands using the limited oxygen that is available at high altitudes. At about 4000 m above sea level, every breath of air only contains about 60% of the oxygen molecules compared to a breath at sea level (Beall, 2007). At high altitudes even basal metabolism is difficult to maintain for lowland natives. Long-term activities such as locomotion or

shivering can only be fueled aerobically. Mitochondria are the organelles that consume oxygen to produce energy aerobically; therefore, changes in mitochondrial function that would enhance metabolic capacities in a low oxygen environment would be advantageous at high altitudes. To understand how an animal adapts to living at high altitude, researchers have studied high altitude natives, lowland animals acclimatized to high altitude in the wild, and lowland animals acclimated in the lab to the stressors that would be naturally experienced at high altitude (hypobaric hypoxia, normobaric hypoxia, and/or cold). There are many studies that have looked at skeletal muscle metabolism via enzyme activities, but very few studies measured mitochondrial respiration directly.

1.8 HIGH ALTITUDE NATIVES

Most of the work on high altitude animals to date has been conducted on humans; human highland natives have been repeatedly observed to have a lower skeletal muscle mitochondrial oxidative capacity, but greater oxygen supply to muscle mitochondria compared to lowland natives. An early study by Reynafarje (1962) on Peruvian miners found that the high altitude residents had a higher cytochrome c reductase activity and higher myoglobin content. In a study by Favier et al., (1995) 30 students that were residents of La Paz (3400-4000m) were compared to that of age matched European lowland controls. The muscle biopsies did not show a difference in fibre size, but the high altitude natives had a reduced mitochondrial density, capillary supply, and lower intramyocellular fat content. Another study compared the vastus lateralis muscle of

Sherpas from Nepal to sedentary lowlanders. The number of capillaries per cross sectional area of muscle tissue was significantly higher in the Sherpas, but number of capillaries per fibre did not differ. Although it was not significant the muscle fibres from Sherpas had a lower cross sectional area. The Sherpas had a lower mitochondrial density, more specifically they had a significantly lower volume density of intermyofibrillar mitochondria and a trend towards a lower volume density of subsarcolemmal mitochondria (Kayser et al., 1991). Based on studies on high altitude natives the increase in capillary density results from a decrease in muscle fibre cross sectional area, which may be adaptive because it decreases the diffusion distance for oxygen. Myoglobin gene expression was found to be higher in Andean and Tibetan high altitude natives, which may provide muscles with more oxygen. Tibetan high altitude natives have a lower mitochondrial volume density in the vastus lateralis muscle compared to lowland Nepalese control subjects (Kayser et al., 1991). The vastus lateralis muscle of second generation Tibetans that were born and raised at lowlands had smaller fibre cross-sectional areas, but a similar number of capillaries per muscle fibre compared to lowland Nepalese control subjects. The second generation Tibetans also had lower mitochondrial densities than the control subjects, which were matched by a lower citrate synthase and 3-hydroxyacyl-CoA dehydrogenase activities (Kayser et al., 1996). A smaller fibre cross-sectional area with a constant capillary density and a lower mitochondrial density may be the result of genetic adaptations that help humans sustain sub-maximal rates of aerobic

respiration at high altitudes.

Although the mitochondrial oxidative capacity of human high altitude natives has been reported to be lower, high altitude natives tend to have superior aerobic performance in hypoxia (Favier et al., 1995). Favier et al. (1995) exercised high and low altitude natives under both normoxia and hypoxia. High altitude natives that exercise under acute normoxia have a higher VO_2 max compared to exercise under hypoxia. Furthermore, under normoxia high and low altitude natives have similar oxygen consumption rates. However, under hypoxia the high altitude population have higher oxygen consumption rates compared to the low altitude population.

The few available studies of highland mammals and birds suggest that oxidative capacity can be increased in the muscle of some species, unlike the decreases observed in highland native humans. A study by Valdivia (1958) found that guinea pigs native to high altitudes had higher skeletal muscle capillary densities (number of capillaries per mm^2) and a higher ratio of capillaries to fibres compared to lowland control guinea pigs. An increase in capillary density would increase oxygen supply and decrease diffusion distances of oxygen. Hochachka et al., (1983) studied heart enzymes from Alpaca, Llama and Taruca. They found that there was an increase oxidative capacity and a decrease in anaerobic metabolic capacities. These findings were consistent with a study on high altitude guinea pigs and rabbits having higher oxidative enzyme activities in skeletal muscles compared to lowland counterparts (Barrie et al., 1974). Based on findings at that

time, Hochachka et al. (1983) put forth the following hypothesis, that animals would increase the rate at which oxygen and substrates can be fluxed through the system (i.e. increase enzyme activities/g of tissue) rather than to simply increase oxygen concentration in response to hypoxia.

Bar headed geese skeletal muscles have higher oxidative capacity and oxygen supply compared to lowland birds, which should be adaptive because a reduction in metabolic capacities would be maladaptive since they must maintain high rates of aerobic respiration during flight. Bar headed geese have a higher proportion of fast oxidative fibres and more capillaries than low altitude birds (Scott, 2009a). In permeabilized muscle fibres respiratory capacities and cytochrome c oxidase excess capacity of bar headed geese was higher in the presence of creatine compared to low altitude birds (Scott, 2009b). They also have a greater proportion of subsarcolemmal mitochondria (S_{Mito}) compared to intermyofibrillar mitochondria (I_{mito}) (Scott, 2009a). Subsarcolemmal mitochondria are located closer to the capillaries, which reduces the distance that oxygen needs to diffuse. Bar headed geese increase maximal mitochondrial respiration and re-distribute mitochondria to decrease oxygen diffusion distances.

A study by Cheviron et al., (2012) reported that HA populations of deer mice have overcome the environmental stressors by increasing their thermogenic capacity via increased aerobic, oxidative phosphorylation and lipid oxidation capacities. Thermogenic capacity is an adaptive trait in high altitude populations of deer mice (Hayes and

O'Conner, 1999).

1.9 FUEL USE BY HIGH ALTITUDE NATIVES

At high altitudes oxygen is limiting which makes oxidation of fuels by mitochondria challenging. Carbohydrates produce ~15% more ATP per mole of oxygen than lipids (McClelland, 2004). Therefore, the use of carbohydrates would be beneficial in an environment in which oxygen is limited (Weber, 2011). However, carbohydrate stores are limited and cannot fuel long-term activities. There are large lipid stores in the body and they fuel low intensity, long-term activity.

It was hypothesized that high altitude animals would improve the efficiency of energy production per mole of oxygen by increasing their capacity for oxidative phosphorylation of carbohydrates (Hochachka, 1992, Schipper et al., 2012). This suggests that high altitude animals may increase carbohydrate use to economize oxygen. The skeletal muscle of high altitude populations of Tibetans (Kayser et al., 1991; Kayser et al., 1996) and Quechas (Rosser and Hochachka, 1993) is composed of predominantly slow twitch fibres, which use more oxidative phosphorylation to produce energy rather than anaerobic respiration. High altitude native humans have lower blood lactate, which is a product of anaerobic respiration, compared to lowlanders after VO_2 max tests (Hochachka et al., 2002). This suggests that human high altitude natives use more carbohydrates via oxidative phosphorylation versus glycolysis since lactate levels are low. Oxidative phosphorylation improves the yield of ATP per carbon molecule since oxidative

phosphorylation produces more ATP per molecule of glucose compared to glycolysis. The enzyme activities of high altitude populations also support the hypothesis as they have lower anaerobic glycolytic capacities and the glycolytic pathway results in more oxidation of carbohydrates rather than fermentation (Hochachka, 1992). The hypothesis was also supported in a study Schippers et al. (2012), in which high altitude species of Andean mice utilized a greater proportion of carbohydrates during exercise compared to low altitude species under both normoxia and hypoxia. The metabolism of high altitude populations is shifted towards aerobic carbohydrate oxidation in order to use oxygen efficiently.

Using carbohydrates at high altitudes is more oxygen efficient, but lipids have to be used for long-term activities, such as sub-maximal exercise and shivering. A study by Cheviron et al., (2012) reported that HA populations of deer mice have a higher thermogenic capacity compared to lowland deer mice. Maximal thermogenesis is fuelled primarily by lipid oxidation in both low and highland deer mice, but lipid oxidation rates are twice as high in the high altitude deer mice. This suggests that during shivering high altitude deer mice use lipids and have a greater capacity for lipid oxidation. The two studies on high altitude mice suggest that animals use a greater proportion of carbohydrates during exercise (Schippers et al., 2012), but have a higher capacity to oxidize lipids at a faster rate during thermogenesis (Cheviron et al., 2012). This implies that high altitude natives may need to increase their capacity for both lipid oxidation and

carbohydrate oxidation in order to use oxygen and fuels efficiently. Having more intramuscular and/or intracellular lipids and having them closer to mitochondria might allow an animal to exercise for a longer period of time at high altitudes where oxygen supply is limited. Endurance exercise and long-term activities are fueled by lipids. Endurance training increases intramuscular lipid stores and metabolic capacities; such as, increases in cardiac output and membrane transporters for fats and carbohydrates (Howald et al., 1985; Tarnopolsky et al., 2007). More specifically, the contact area of mitochondria and lipids stores is greater in endurance trained athletes (Tarnopolsky et al., 2007).

1.10 ACCLIMATIZATION TO HIGH ALTITUDES

In addition to studying high altitude natives it is useful to study acclimatization responses of lowland natives to high altitudes to gain further insight about phenotypic plasticity of an animal and whether it will be beneficial at high altitudes (Storz et al., 2010). If a phenotypic response is maladaptive, selection can attenuate the response in high altitude adapted animals.

Based on earlier findings by Validiva (1958) and Reynafarje (1962) it was hypothesized that climbing expeditions to high altitudes would increase mitochondrial and capillary densities in low altitude humans. In an expedition to the Himalayas, the vastus lateralis muscle was sampled from mountaineers before and after their sojurn at high altitude for 8 weeks. The capillary densities in skeletal muscle increased; however, it

was due to a loss of muscle fibre cross sectional area not an increase in new capillary formation (Hoppeler et al., 1990). There was also a decrease in mitochondrial density, with the 43% loss in subsarcolemmal mitochondria and a 13% loss in intermyofibrillar mitochondria. The mitochondrial volume density decreased by 30% after an expedition to the Himalayas, which was matched by a decrease in maximal oxygen consumption. Acclimatization to Mt. Everest also resulted in a decrease in mitochondrial volume density (Howald et al., 1990). A decrease in mitochondrial density with acclimatization to high altitude was also supported by a two-fold increase in lipofuscin, which is a degradation product of mitochondria (Martinelli et al., 1990). The decrease in fibre cross-sectional area and decrease in mitochondrial density from the human acclimatization studies were similar to findings in Tibetan high altitude natives (Kayser et al., 1991).

More recent studies have investigated mitochondrial function in response to high altitude acclimatization. Jacobs et al. (2012) found no significant change in mitochondrial function or respiratory capacity in humans that were acclimatized to 9-11 days of exposure to high altitude (4559m). However, after acclimatization to high altitude there were significant decreases in enzymes in the citric acid cycle and in oxidative phosphorylation. Citrate synthase activity is often used as a marker of mitochondrial density (Larsen et al., 2012), which suggests that mitochondrial density decreased, but tissue respiratory capacities are maintained in humans are acclimatized for 9-11 days. This suggests that mitochondrial phenotype is changing and that each individual

mitochondrion is increasing its respiratory capacity. Therefore, measuring mitochondrial function directly is important because measuring other mitochondrial characteristics, such as enzyme activities and inferring mitochondrial function can be misleading. In the same study, high resolution respirometry on human skeletal muscle mitochondria that were acclimatized to high altitude for 28 days showed a reduced respiratory capacity in complex I, II and a loss of state 3 oxidative phosphorylation capacity (Jacobs et al., 2012). This loss in respiratory capacity was matched by enhanced mitochondrial efficiency. In another study, after 66 days at high altitude there was a decrease in protein expression of transport chain complexes I and IV, PGC-1a, citrate synthase, and UCP3 levels (Levett et al., 2012). Based on human studies, acclimatization to high altitudes results in a decreased mitochondrial density that is matched by a decreased respiration capacity and oxidative phoshorylation capacity, but an increased efficiency in ATP production.

1.11 FUEL USE IN RESPONSE TO HIGH ALTITUDE ACCLIMATIZATION

Acute acclimatization of animals to high altitude was expected to shift metabolism towards glycolysis due to the lack of oxygen, since carbohydrate oxidation uses less oxygen to produce a mole of ATP compared to lipids. High-altitude acclimatization during an expedition to Mt. Everest increased the enzyme activity of glycolytic enzymes, and decreased enzyme activity of citric acid cycle, fatty acid oxidation, and respiratory

chain enzymes (Howald et al., 1990). Brooks et al., (1991) found glucose utilization increased significantly after acclimatization to high altitude for three weeks. These studies suggest a shift towards anaerobic glucose oxidation after acute acclimatization to high altitude and a shift towards aerobic glucose oxidation after chronic high altitude acclimatization due to reduced oxygen availability

1.12 ACCLIMATION TO SIMULATED HIGH ALTITUDES

High altitude environments have multiple stressors. Hypoxia was thought to stimulate an increase in oxidative capacity. Banchero (1987) disagreed that hypoxia alone stimulates changes in muscle structure; he suggested that changes occurred when hypoxia was coupled with cold or exercise. Many acclimation studies that attempt to simulate high altitude have focused on hypoxia or cold and the effects of one of these stressors on animals. Only a couple of studies have looked at the effects of cold and hypoxia simultaneously (Beaudry and McClelland, 2010). Furthermore, most acclimation studies have only been conducted on lowland animals. Studying offspring of high altitude animals that are born and raised at low altitudes will provide insight on genetic adaptations. Since the offspring would have never been exposed to high altitudes if they have the same phenotype as their parents then the phenotype expressed likely has a genetic basis. The acclimation response of the offspring of highland natives that were born and raised at low altitudes would provide greater insight regarding adult phenotypic plasticity of highland natives to specific stressors since they share the same genetic

background.

1.13 ACCLIMATION TO HYPOXIA

Acclimation to hypoxia activates the hypoxia inducible factor one (HIF-1). HIF-1 is the master regulator of responses to hypoxia. It is a heterodimeric transcription factor composed of HIF-1α and HIF-1ß subunits. HIF-1α is continuously synthesized and post-transcriptionally regulated. Hydroxylation of two residues on HIF-1α occurs when oxygen is available by prolyl-hydroxylase enzymes (PHD1-3). Hydroxylation promotes the binding of von Hippel-Lindau protein, which leads to ubiquination and degradation of HIF-1α (Semenza, 2007, Murphy, 2009). When oxygen is low HIF-1α dimerizes with HIF-1ß, the HIF-1 complex then binds to the hypoxia response elements of a number of genes (Jaakkola et al., 2001, Murphy, 2009). HIF-1 regulates many genes that can improve oxygen delivery to muscles; such as, vascular endothelial growth factor (VEGF), erythropoietin (EPO), glycolytic enzymes, pyruvate dehyrdogenase kinase 1 (PDK1), and the subunit 4-2 of mitochondrial cytochrome c oxidase (Murphy, 2009). HIF-2 has also been implicated in adaptations to high altitude. It has been suggested that HIF-2 α can mediate changes in erythropoiesis, iron homeostasis and metabolism (Tissot van Patot and Gassmann (2011).

Acute acclimation to hypoxia shifts metabolism towards anaerobic respiration, activating HIF-1 which increases enzymes involved in glycolysis and activates PDK1, which inhibits pyruvate dehydrogenase (Murray, 2009). CD-1 mice that were exposed to

24 hours of hypobaric hypoxia had higher proteins levels of HIF-1α and PDK1, which was matched by a lower level of pyruvate dehyrdogenase activity compared to normoxic mice. They also had higher levels of lactate after exercise (Le Moine et al., 2011). This suggests that after one day of hypobaric hypoxia CD-1 mice shift metabolism towards glycolysis via HIF-1 activation. CD-1 mice that were acclimated to hypobaric hypoxia for 1 week had similar pyruvate dehydrogenase activity and similar protein levels of HIF-1α and PDK-1 compared to normoxic mice (Le Moine et al., 2011). Chronic hypobaric hypoxia seems to desensitize the HIF-1 pathway to hypoxia and allows metabolism to continue via oxidative phosphorylation.

With chronic hypoxia acclimations metabolism is expected to shift towards aerobic carbohydrate oxidation rather than lipid oxidation in order to conserve oxygen. Rats that were acclimated to hypoxia did not increase the proportion of carbohydrates used during exercise (McClelland et al., 1998). However, F1 progeny of high altitude deer that have never been exposed to high altitude increase GLUT 4 transporter mRNA in skeletal muscle and hexokinase activity in response to acclimation to hypoxia (Lau et al., 2017). However, there was no increase in citrate synthase or isocitrate dehyrdogenase activity in high or low altitude deer mice. This suggests that carbohydrate oxidation capacity in skeletal muscles of deer mice may be increased in high altitude deer mice in response to hypoxia acclimation.

In humans extreme hypobaric hypoxia decreases oxidative capacities.

MacDougall et al., (1991) simulated an expedition to Mt.Everest in a pressure chamber. The simulated ascent led to a decrease in muscle volume and mitochondrial oxidative capacity, and it did not induce capillary neoformation. The simulation to high altitude led to a significant decrease in succinate dehydrogenase, citrate synthase and hexokinase.

There is still controversy whether hypoxia causes an increase or a decrease in ROS production (Hoffman et al., 2007). Mice that were acclimated to two days of severe hypobaric hypoxia had a significantly higher level of protein oxidation (Magalhaes, 2007). Rats that are more resistant to hypoxia had lower levels of ROS production (Venediktova, 2013). Although it seems counterintuitive that there would be an increase in ROS production when there is less oxygen available to produce oxygen oxides, it may be explained by a more reduced state of the ETC due to a lower rate of reduction of oxygen by COX (Hoppler et al., 2003).

1.14 ACCLIMATION TO COLD

Cold exposure elicits shivering and non-shivering thermogenesis in order to maintain body temperature. Total thermogenic capacity is the sum of basal metablic rate, shivering thermogenesis and non-shivering thermogenesis. First generation highland deer mice born and raised at low altitude acclimated to cold have higher thermogenic capacities and fur densities compared to lowland deer mice acclimated to cold. The differences in thermogenic capacity were not due to differences in mass specific brown adipose tissue size. In these mice, shivering does contribute a significant amount to total

thermogenic capacity (Wall, unpublished). Long-term shivering requires a continuous supply of ATP. Due to the limited carbohydrate stores, lipids should be the preferred fuel during shivering thermogenesis. Acclimation to cold increases oxidative phosphorylation capacity (Ballantyne and George, 1977). There is usually an increase in mitochondrial content, indicated by an increase in CS activity and capillarity increases (Behrens and Himms-Hagen, 1977). Acclimation to cold results in a phenotype that is similar to endurance training. Endurance training increases intramuscular lipid stores (Howald et al., 1985; Tarnopolsky et al., 2007).

1.15 HYPOTHESES AND PREDICTIONS

In the preceding sections I have summarized the current understanding of how mitochondrial physiology and muscle phenotype is altered in high altitude environments. There is very limited knowledge on muscle physiology of non-human high altitude animals. Furthermore, apart from the studies from our lab there aren't any studies that have examined evolved changes and phenotypic plasticity in high altitude natives by examining progeny of high altitude natives that have been born and raised at low altitude. In the following chapters I have addressed three main hypotheses.

1) Skeletal muscle of high altitude mice will have a more oxidative phenotype compared to low altitude deer mice. Acclimation to hypoxia will not alter the muscle phenotype or mitochondrial physiology of high altitude deer mice, but low altitude deer mice will increase the oxidative capacity of skeletal muscle.

2) Acclimation to cold or cold + hypoxia will not alter the muscle phenotype or mitochondrial physiology of high altitude deer mice. Acclimation to cold will increase the oxidative capacity of skeletal muscles and acclimation to cold + hypoxia will further increase the oxidative capacity of skeletal muscle in low altitude deer mice.
3) Cardiac muscle of high altitude mice will have a more oxidative phenotype compared to low altitude deer mice. Acclimation to hypoxia will not alter the oxidative phenotype in high altitude deer mice, but it will increase oxidative phenotype in low altitude deer mice. High altitude deer mice acclimated to hypoxia will increase carbohydrate oxidation capacity relative to fatty acid oxidation capacity and increase their capacity to oxidize lactate as a substrate, unlike low altitude mice.

1.16 REFERENCES

Armstrong, R. B., & Laughlin, M. H. (1985). Metabolic indicators of fibre recruitment in mammalian muscles during locomotion. *Journal of Experimental Biology*, *115*(1), 201-213.

Ballantyne, J.S., and George, J.C. (1977). An ultrastructural and histological analysis of the effects of cold acclimation on vertebrate skeletal muscle. *J Thermal Biology*. 3, 109-116.

Banchero, N. (1987). Cardiovascular responses to chronic hypoxia. *Ann. Rev. Physiol.* 49, 465-476.

Barre, H., Geloen, A., Chatonnet, J., Dittmar, A., & Rouanet, J. L. (1985). Potentiated muscular thermogenesis in cold-acclimated muscovy duckling. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *249*(5), R533-R538.

Barrie, E., Heath, D., Stella, J. A., & Harris, P. (1974). Enzyme activities in red and white muscles of guinea-pigs and rabbits indigenous to high altitude. *Environmental physiology & biochemistry*, *5*(1), 18-26.

Beaudry, J. L., & McClelland, G. B. (2010). Thermogenesis in CD-1 mice after combined chronic hypoxia and cold acclimation. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *157*(3), 301-309.

Behrens, W.A., and Himms-Hagen, J. (1977). Alteration in skeletal muscle mitochondria of cold-acclimated rats: association with enhanced metabolic response to noradrenaline. *Journal of Bioenergetics and Biomembranes*. 9, 41-63.

Brooks, G. A., Butterfield, G. E., Wolfe, R. R., Groves, B. M., Mazzeo, R. S., Sutton, J. R., ... & Reeves, J. T. (1991). Increased dependence on blood glucose after acclimatization to 4,300 m. *Journal of applied physiology*, *70*(2), 919-927.

Carrari, F., Naldini, A., Weber, J.-M. and Wolfe, R.R. (1994). Alanine kinetics in humans during low-intensity exercise. *Med. Sci. Sports Exerc.* 26, 348-353.

Cheviron, A. Z., Bachman, G. C., Connaty, A. D., McClelland, G. B., and Storz, J.F. (2012) Regulatory changes contribute to the adaptive enchancement of thermogenic capacity in high-altitude deer mice. *PNAS.* 109, 8635-8640.

Collins, Y., Chouchani, E. T., James, A. M., Menger, K. E., Cochemé, H. M., & Murphy, M. P. (2012). Mitochondrial redox signalling at a glance. *J Cell Sci*, *125*(4), 801-806.

Favier, R., Speilvogel, H., Desplanches, D., Ferretti, G., Kayser, B., and Hopper, H. (1995). Maximal exercise performance in chronic hypoxia and acute normoxia in high altitude natives. J. *Appl. Physiol.* 78: 1868-1874.

Fukuda, R., Zhang, H., Kim, J. W., Shimoda, L., Dang, C. V., & Semenza, G. L. (2007). HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell*, *129*(1), 111-122.

Ghalambor, C. K., McKay, J. K., Carroll, S. P., & Reznick, D. N. (2007). Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Functional ecology*, *21*(3), 394-407.

Hayes, J. P., and O'Conner, C.S. (1999). Natural selection on thermogenic capacity of high-altitude deer mice. *Evolution.* 53, 1280-1287.

Hochachka, P. W. (1992). Muscle enzymatic composition and metabolic regulation in high altitude adapted natives. Int. J. Sports Med. 13, S89-S91.

Hochachka, P. W., Beatty, C. L., Burelle, Y., Trump, M. E., McKenzie, D. C., & Matheson, G. O. (2002). The lactate paradox in human high-altitude physiological performance. *Physiology*, *17*(3), 122-126.

Hochachka, P. W., Gunga, H.C., and Kirsch, K. (1998). Our ancestral physiological phenotype: An adaptation for hypoxia tolerance and for endurance performance. *Proc. Natl. Acad. Sci. 95,* 1915-1920.

Hochachka, P. W., Stanley, C., Merkt, J., & Sumar-Kalinowski, J. (1983). Metabolic

meaning of elevated levels of oxidative enzymes in high altitude adapted animals: an interpretive hypothesis. *Respiration physiology*, *52*(3), 303-313.

Hoffman, D. L., Salter, J. D., & Brookes, P. S. (2007). Response of mitochondrial reactive oxygen species generation to steady-state oxygen tension: implications for hypoxic cell signaling. *American Journal of Physiology-Heart and Circulatory Physiology*, *292*(1), H101-H108.

Hoppeler, H., Kleinert, E., Schlegel, C., Claassen, H., Howald, H., Kayar, S. R., & Cerretelli, P. (1990). II. Morphological adaptations of human skeletal muscle to chronic hypoxia. *International journal of sports medicine*, *11*(S 1), S3-S9.

Hoppeler, H., Vogt, M., Weibel, E. R., and Fluck, M. (2003). Response of skeletal muscle mitochondria to hypoxia. *Experimental Physiology.* 88, 109-119.

Howald, H., Hoppler, H., Claassen, H., Mathiew, O., and Straub, R. (1985). Influence of endurance training on the ultrastructural composition of the difference fibre types in humans. *Pflugers Arch.* 403, 369-376.

Howald, H., Pette, D., Simoneau, J. A., Uber, A., Hoppeler, H., & Cerretelli, P. (1990). III. Effects of chronic hypoxia on muscle enzyme activities. *International journal of sports medicine*, *11*(S 1), S10-S14.

Hurtado, A. (1960). Some clinical aspects of life at high altitudes. *Annals of internal medicine*, *53*(2), 247-258.

Ingwall, J.S. (2002). ATP and the heart. Kluwer, Boston.

Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., ... & Maxwell, P. H. (2001). Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science*, *292*(5516), 468-472.

Jacobs, R. A., Siebenmann, C., Hug, M., Toigo, M., Meinild, A. K., & Lundby, C. (2012). Twenty-eight days at 3454-m altitude diminishes respiratory capacity but enhances efficiency in human skeletal muscle mitochondria. *The FASEB Journal*, *26*(12), 5192-5200.

Kayser, B., Hoppler, H., Claassen, H., and Cerretelli P. (1991) Muscle structure and performance capacity of Himalayan Sherpas. J. Appl. Physiol. 70: 1938-1942.

Kayser, B., Hoppler, H., Desplanches, D., Marconi, C., Broers, B. and Cerretelli P. (1996). Muscle ultrastructure and biochemistry of lowland Tibetans. J. Appl. Physiol. 81: 419-425.

Kinnula, V. L., Huttunen, P., & Hirvonen, J. (1983). Adaptive changes in skeletal muscle mitochondria of the guinea-pig during acclimation to cold. *European Journal of Applied Physiology and Occupational Physiology*, *51*(2), 237-245.

Kocha, K. M., Reilly, K., Porplycia, D. S., McDonald, J., Snider, T., & Moyes, C. D. (2015). Evolution of the oxygen sensitivity of cytochrome c oxidase subunit 4. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *308*(4), R305-R320.

Kolwicz, S. C., & Tian, R. (2014). Fuel Metabolism Plasticity in Pathological Cardiac Hypertrophy and Failure. In *Cardiac Energy Metabolism in Health and Disease* (pp. 169-182). Springer New York.

Kowaltowski, A.J., and Vercesi, A.E. (1999). Mitochondrial damage induced by conditions of oxidative stress. *Free Radical Biology and Medicine.* 26, 463-471.

Lau, D. S., Connaty, A. D., Mahalingam, S., Wall, N., Cheviron, Z. A., Storz, J. F., Scott, G. R. & McClelland, G. B. (2017). Acclimation to hypoxia increases carbohydrate use during exercise in high-altitude deer mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, ajpregu-00365.

Le Moine, C. M., Morash, A. J., & McClelland, G. B. (2011). Changes in HIF-1α protein, pyruvate dehydrogenase phosphorylation, and activity with exercise in acute and chronic hypoxia. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *301*(4), R1098-R1104.

Levett, D. Z., Radford, E. J., Menassa, D. A., Graber, E. F., Morash, A. J., Hoppeler, H., ... & Grocott, M. P. (2012). Acclimatization of skeletal muscle mitochondria to high-altitude hypoxia during an ascent of Everest. *The FASEB journal*, *26*(4), 1431-1441.

MacDougall, J. D., Green, H. J., Sutton, J. R., Coates, G., Cymerman, A., Young, P., & Houston, C. S. (1991). Operation Everest II: structural adaptations in skeletal muscle in response to extreme simulated altitude. *Acta physiologica Scandinavica*, *142*(3), 421-427.

Magalhaes, J., Ferreira, R., Neuparth, M.J., Oliveira, P.J., Marques, F., and Ascensao, A. (2007). Vitamin E prevents hypobaric hypoxia-induced mitochondrial dysfunction in skeletal muscle. *Clinical Science*. 113, 459-466.

Marconi, C., Marzorati, M., and Cerretelli, P. (2006). Work capacity of permanent residents of high altitude. *High altitude medicine and biology*. 7, 105-115.

Martinelli, M., Winterhalder, R., Cerretelli, P., Howald, H., & Hoppeler, H. (1990). Muscle lipofuscin content and satellite cell volume is increased after high altitude exposure in humans. *Experientia*, *46*(7), 672-676.

McClelland, G. B. (2004). Fat to the fire: the regulation of lipid oxidation with exercise and environmental stress. *J Comp Physiol B.* 139, 443-460.

McClelland, G. B., Hochachka, P. W., & Weber, J. M. (1998). Carbohydrate utilization during exercise after high-altitude acclimation: a new perspective. *Proceedings of the National Academy of Sciences*, *95*(17), 10288-10293.

Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. *Biochem J,* 417**,** 1-13.

Murray, A. J., & Horscroft, J. A. (2015). Mitochondrial function at extreme high altitude. *The Journal of Physiology*, 594.5, 1137-1149.

Randle, P. J., Garland, P. B., Hales, C. N., & Newsholme, E. A. (1963). The glucose fatty-acid cycle its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *The Lancet*, *281*(7285), 785-789.

Rennie, M.J., Edwards, R.H.T., Krywawych, S., Davies, C.T.M., Halliday, D., Waterlow, J.C. and Millward, D. J. (1981). Effect of exercise on protein turnover in man. Clin. Sci. 61, 627-639.

Reynafarje, B. (1962). Myoglobin content and enzymatic activity of muscle and altitude adaptation. *Journal of Applied Physiology*, *17*(2), 301-305.

Rosser, B.W. and Hochachka, P. W. (1993). Metabolic capacity of muscle fiers from high-altitude natives. Eur. J. Appl. Physiol. 67, 513-517.

Schippers, M., Ramirez, O., Arana, M., Pinedo-Beral, P., and McClelland, G. B. (2012). *Current Biology*. 22, 2350-2354.

Scott, G. R., Egginton, S., Richards, J. G., & Milsom, W. K. (2009a). Evolution of muscle phenotype for extreme high altitude flight in the bar-headed goose. *Proceedings of the Royal Society of London B: Biological Sciences*, *276*(1673), 3645-3653.

Scott, G. R., Richards, J. G., & Milsom, W. K. (2009b). Control of respiration in flight muscle from the high-altitude bar-headed goose and low-altitude birds. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *297*(4), R1066-R1074.

Semenza, G. L. (2007). Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci. Stke, 2007*(407), cm8

Storz, J.F., Scott, G.R., and Cheviron, Z.A. (2010). Phenotypic plasticity and genetic adaptation to high- altitude hypoxia in vertebrates. *J. Exp. Biol.*213, 4125-4135.

Suarez, R. K. (1992). Ecological implications of metabolic biochemistry. *Experientia* 48, 535-536

Tarnopolsky, M. A., Rennie, C.D., Robertshaw, H.A., Fedak-Tarnopolsky, S.N., Devries, M.C., and Hamadeh, M.J. (2007). Influence of endurance exercise training and sex on intramyocellular lipids and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. *Am J Physiol Regul Integr Comp Physiol.* 292, R1271- R1278.

Taylor, C. R. (1987). Structural and functional limits to oxidative metabolism: insights from scaling. *Annu. Rev. Physiol.* 49, 135-146.Turner, D. L., Hoppeler, H., Hokanson, J. and Weibel, E. R. (1995). Cold acclimation and endurance training in guinea pigs: changes in daily and maximal metabolism. *Respir Physiol.* 101, 183-8.

Valdivia, E. (1958). Total capillary bed in striated muscle of guinea pigs native to the Peruvian mountains. *American Journal of Physiology--Legacy Content, 194*(3), 585-589.

Vock, R., Hoppler, H., Classen H., Wu, D.X., Billeter, R., Webber, J., Taylor, C.R., and Wiebel E.R. (1996). Design of the oxygen and substrate pathways. *J. Exp. Biol.* 199, 1689-1697.

Venediktova, N., Shigaeva, M., Belova, S., Belosludstev, K., Belosludsteva, N., Gorbacheva, O., Lezhnev, E., Lukyanova, L., and Mironova, and Mironova, G. (2013). Oxidative phosphorylation and ion in the mitochondria of two strains of rats varying in their resistance to stress and hypoxia. *Mol Cell Biochem*. 383, 261-269.

Weber, J. (2011). Metabolic fuels: regulating fluxes to select mix. *J. Exp. Biol.* 214, 286-294.

Wijers S.L.J, Schrauwen, P., Saris, W. H.S., and Van Marken Lichtenbelt, W.D. (2008). Human skeletal muscle mitochondrial uncoupling is associated with cold induced adaptive thermogenesis. *Plos One*. 3:e1777.

CHAPTER 2: EVOLVED CHANGES IN THE INTRACELLULAR DISTRIBUTION AND PHYSIOLOGY OF MUSCLE MITOCHONDRIA IN HIGH-ALTITUDE NATIVE DEER MICE

2.1 ABSTRACT

High-altitude natives that have evolved to live in hypoxic environments provide a compelling system to understand how animals can overcome impairments in oxygen availability. We examined whether these include changes in mitochondrial physiology or intracellular distribution that contribute to hypoxia resistance in high-altitude deer mice (*Peromyscus maniculatus*). Mice from populations native to high- and low-altitudes were born and raised in captivity, and as adults were acclimated to normoxia or hypobaric hypoxia (equivalent to 4,300 m elevation). We found that highlanders had higher respiratory capacities in the gastrocnemius (but not soleus) muscle than lowlanders (assessed using permeabilized fibres with single or multiple inputs to the electron transport system), due in large part to higher mitochondrial volume densities in the gastrocnemius. The latter was attributed to an increased abundance of subsarcolemmal (but not intermyofibrillar) mitochondria, such that more mitochondria were situated near the cell membrane and adjacent to capillaries. Hypoxia acclimation had no significant effect on these population differences, but it did increase mitochondrial cristae surface densities of mitochondria in both populations. Hypoxia acclimation also altered the physiology of isolated mitochondria by affecting respiratory capacities and cytochrome c oxidase activities in population-specific manners. Chronic hypoxia decreased the release of reactive oxygen species by isolated mitochondria in both populations. There were subtle differences in O_2 kinetics between populations, with highlanders exhibiting

increased mitochondrial O_2 affinity or catalytic efficiency in some conditions. Our results suggest that evolved changes in mitochondrial physiology in high-altitude natives are distinct from the effects of hypoxia acclimation, and likely provide a better indication of adaptive traits that improve performance and hypoxia resistance at high altitudes.

2.2 INTRODUCTION

Mitochondrial function is essential for aerobic energy production, and limitations in the ability of mitochondria to produce ATP can impact whole-animal performance. Environments with pervasive and unremitting hypoxia, such as at high altitudes, pose a severe challenge to aerobic energy production via oxidative phosphorylation that can affect organismal aerobic metabolism (Buskirk et al., 1967; Lui et al., 2015; Lau et al., 2017; West et al., 1983). Thus, for survival in high-altitude environments, it is clear that the respiratory and circulatory systems play key roles in offsetting the detrimental effects of low environmental oxygen availability on tissue oxygen supply (Gilbert-Kawai et al., 2014; Ivy and Scott, 2015; Storz et al., 2010). However, the importance of changes in mitochondrial physiology for hypoxia resistance, effective locomotion, and thermogenesis at high altitudes is still poorly understood (Murray and Horscroft, 2016).

It has been suggested that increases in tissue oxidative capacity or changes in cellular distribution of mitochondrial volume may offset the reductions in circulatory O_2 supply (Hochacka et al., 1983; Hardy et al., 2009). Although direct measurements of

mitochondrial density and function are extremely rare, studies examining enzyme activities or muscle fibre composition suggest that mammals and birds native to high altitudes increase oxidative capacity in the muscle via either increases in mitochondrial density or by increases in oxidative fibre abundance (Hochacka, 1983; Barrie et al., 1975; Scott et al., 2009; Lui et al., 2015). Interestingly, this does not appear to have occurred in humans native to high altitudes whose muscles have a similar aerobic capacity to lowland natives (Kayser et al., 1991; Kayser et al., 1996; Hoppeler et al., 2003). Oxygen transport could also be enhanced by reducing the capillary to mitochondria diffusion distance through either the redistribution of mitochondria volume to a subsarcolemmal location (Hardy et al., 2009; Murray and Horscroft, 2016), which would act synergistically to the increases in capillarity that have been observed in several highland species (León-Velarde et al., 1993; Mathieu-Costello, 2001; Lui et al., 2015). Bar-headed geese – a species that flies at high altitudes during its migration across the Himalayas (Scott et al., 2015b) – have reduced intracellular diffusion distance by redistributing mitochondria closer to capillaries (Scott et al., 2009), but this has yet to be observed in a high altitude mammal. These data suggest that an increase in muscle aerobic capacity and reduction in O_2 diffusion distances are advantageous at high altitude. What is lacking are data supporting the hypothesis that altered mitochondrial density, distribution and physiology are under selective pressure in a high altitude mammal.

The effect of hypoxia acclimation on phenotypic plasticity has been more extensively studied, but principally in low-altitude natives. There has been disagreement about the extent to which hypoxia acclimation affects tissue oxidative capacity. Many studies on lowland humans have reported that acclimatization to high-altitude conditions in fact decreases muscle aerobic capacity. In some cases this is accompanied by a reduction in muscle fibre size but with the maintenance of capillary density, such that the ratio of capillarity to aerobic capacity increases (Hoppeler et al., 1990; Kayser et al., 1991; Jacobs et al., 2012; Levett et al., 2012). However, many of these observations may have been confounded by decreases in food consumption that can occur with ascent and leads to negative energy balance. In a more recent study when neutral energy balance was maintained, mitochondrial volume density in the muscle was shown to increase with prolonged exposure of lowland humans to high altitude (Jacobs et al., 2016). Comparable studies in non-human mammals are quite limited, but studies in lowland rodents have often suggested that hypoxia acclimation has relatively little effect on the oxidative capacity of muscle (Beaudry and McClelland, 2010; León-Velarde et al., 1993; Lui et al., 2015; Lau et al., 2017; Mathieu-Costello and Agey, 1997). The lack of consensus in the literature has made it difficult to assess whether changes in mitochondrial physiology seen at high altitudes are beneficial or alternatively, maladaptive to aerobic performance.

Studies of high-altitude natives are extremely valuable for disentangling whether the acclimation response in lowlanders (or the various other forms of phenotypic

plasticity) is beneficial or maladaptive at high altitudes, and whether it can be considered to facilitate or impede the process of evolutionary adaptation (Storz et al., 2010). North American deer mice (*Peromyscus maniculatus*) are an excellent model system for addressing this issue, because their native range extends from below sea level in Death Valley, California to ~4300 m above sea level (Hock, 1964; Natarajan et al., 2015; Snyder et al., 1982). Populations from high altitude must sustain high metabolic rates in hypoxia in the wild (Hayes 1989), and evidence suggests there is strong positive selection on aerobic capacity (VO_2max) to support thermogenesis during cold highland winters (Hayes and O'Connor, 1999). Indeed high-altitude deer mice have responded to this strong selection pressure, exhibiting a higher VO_2max in hypoxia than their lowland counterparts (Cheviron et al., 2012; Lui et al., 2015). The underlying mechanism of these evolved differences appears to include a change in the phenotype of locomotory muscle. Highland mice in the wild have higher oxidative enzyme activities, a greater abundance of oxidative fibres, and higher capillarity in the gastrocnemius muscle than lowland mice (Cheviron et al., 2012; Scott et al., 2015a). These population differences persist in first generation (F1) progeny that are raised in captivity, but these evolved differences in muscle aerobic capacity were generally unaffected by hypoxia acclimation (Lui et al., 2015, Lau et al., 2017). However, the mitochondrial physiology that underlies the population differences in muscle phenotype is unknown, awaiting a detailed study of cell structure and mitochondrial function. The objective of this study was to examine the

mechanisms underlying evolved population differences in muscle phenotype in lowland and highland deer mice, and the phenotypic plasticity of muscle in response to hypoxia acclimation. We assessed the respiratory capacity of muscle fibres, the volume density and distribution of mitochondria within fibres, and the functional properties of isolated muscle mitochondria in normoxic or hypoxic acclimated lowland and highland mice.

2.3 METHODS

2.3.1 Experimental animals

Captive breeding populations of high- and low-altitude deer mice were established as previously described (Cheviron et al., 2012; Lui et al., 2015, Lau et al., 2017). High-altitude mice (*P.m. rufinus*) were trapped on the summit of Mt. Evans (Clear Creek County, Colorado; 39°35’18’’N, 105°38’38’’W) at ~4300m above sea level. Low-altitude deer mice (*P.m.nebracensis*) were trapped in Nine-Mile Prairie (Lancaster County, Nebraska; 40°52’12’’N, 96°48’20.3’’W) at ~400 m above sea level. Populations were bred separately in captivity to produce F1 progeny, which were raised to adulthood in normoxia in common laboratory conditions with a constant temperature (25 °C) and light cycle (12L:12D). Mice were provided chow (Teklad Global Rodent Diets, Envigo) and water *ad libitum*. By using F1 generation offspring that were born and raised in a common environment, we control for the effects of developmental and adult phenotypic plasticity. A mix of male and female mice were used in this study and ranged from 16.3 to 32.1 g in body mass, with a similar overlapping range of masses in each population.

All animal care and experimentation followed the guidelines established by the Canadian Council on Animal Care, and were approved by the McMaster University Animal Research Ethics Board.

2.3.2 Experimental design

Adult F1 mice from each population were randomly assigned to one of two acclimation groups for a period of six to ten weeks: 1) normobaric normoxia in standard lab room conditions, or 2) hypobaric hypoxia in hypobaric chambers set to an ambient pressure of 60 kPa (simulating the pressure at an altitude of ~4300 m elevation). Atmospheric pressure was gradually decreased over the first 3 days in the hypoxia group, and kept at that pressure for the remainder of the acclimation period, using previously described methods and custom-made hypobaric chambers (McClelland et al., 1998). The cages were cleaned every 3-4 days, which required that the hypobaric chambers be returned to normobaria for a brief period (< 1 h). Distinct sets of mice were used for (i) respirometry with permeabilized muscle fibres, (ii) transmission electron microscopy, and (iii) respirometry and enzyme assays with isolated mitochondria (see below).

2.3.3 Mitochondrial respiration in permeabilized muscle fibres

Mice were anesthetized using an isoflurane soaked cotton ball and then euthanized by cervical dislocation. The oxidative core of the gastrocnemius muscle and the soleus muscle were removed and placed in ice-cold relaxing and preservation buffer (concentrations in mM: 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56

$MgCl_2 \cdot 6H_2O$, 20 taurine, 15 Na_2Phosphocreatine, 20 imidazole, 0.5 dithiothreitol, 50 methanesulphonate. The muscle fibres were then manually teased apart using dissecting probes (with the assistance of a stereomicroscope) and then permeabilized for 30 min in the same buffer containing saponin (50 µg/ml). Samples then went through three 10 min rinses in respiration solution (in mM: 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose, 1 g l^{-1} fatty acid-free bovine serum albumin; pH 7.1) and were weighed.

Respiration of the muscle fibres was measured using high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) in 2 ml of respiration buffer at a temperature of 37°C. The solution was oxygenated to an oxygen concentration of 450 µM by bubbling compressed oxygen into the chamber. After 5 min, leak respiration was stimulated with 5 mM pyruvate and 2 mM malate (L_N). Oxidative phosphorylation respiration in the presence of 5 mM pyruvate and 2 mM malate was stimulated *via* multiple complexes of the electron transport system with progressive additions of 2 mM ADP ($P_{PM,}$ complex I), 20 mM of glutamate ($P_{PMG,}$ complex I), and 20 mM of succinate ($P_{PMGS,}$ complexes I+II), waiting at least 2 min between each addition until a stable respiration rate could be measured. The chamber was then re-oxygenated and cytochrome oxidase (complex IV) was maximally stimulated with the addition of 1.25 mM ADP, 2 mM ascorbate, and 0.5 mM TMPD (N,N,N′,N′-tetramethyl-p-phenylenediamine) (P_{Tm}).

2.3.4 Transmission electron microscopy

The left hindlimb was removed from the mouse, and the knee and ankle joints were flexed and held at 90° angles. The gastrocnemius muscle was then tied to the bone with suture to hold its length in this position, and the lateral and medial heads of the gastrocnemius were trimmed away so that only the oxidative core remained. The muscle of the oxidative core was again secured firmly with suture on both ends, and was fixed at 4°C for 24 - 48 h in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4. Small muscle blocks were prepared and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h, dehydrated through a graded ethanol series (50%, 70%, 70%, 95%, 95%, 100%, 100%) followed by two changes of 100% propylene oxide (PO), and embedded in Spurr's resin (Spurr, 1969). Ultra-thin sections were cut on a Leica UCT ultramicrotome and placed on copper grids. The sections were post-stained with uranyl acetate and lead citrate. Images were collected using a transmission electron microscope (TEM; JEOL, Peabody, MA, USA).

We made unbiased measurements of mitochondrial volume density and cristae surface density across the oxidative core, using stereological methods that have been previously described (Weibel, 1979; Egginton, 1990; Scott et al., 2009). Mitochondria were classified as subsarcolemmal if they were located between the cell membrane and the outer edges of peripheral myofibrils, and intermyofibrillar if they were instead surrounded by myofibrils on all sides. Sufficient images were analyzed for each sample to

account for heterogeneity, which was determined in preliminary measures as the number of replicate images necessary to yield a stable mean value for the individual mouse. Image locations were selected randomly across the muscle section, and therefore included the representative distribution of fibre types exhibited by the individual.

We also used a semi-quantitative approach to assess the mitochondrial volume densities and intracellular distribution within each fibre type in the muscle. This analysis was important for evaluating whether overall differences in mitochondrial abundance or distribution could be explained by the known differences in muscle fibre-type composition between populations (Lui et al., 2015; Scott et al., 2015a). We first reanalyzed light microscopy images of the oxidative core that were collected as part of a previous study (using the same treatment groups) that reported data for the entire gastrocnemius (Lui et al., 2015) – where full methodologies are described – which allowed us to determine the fibre-type composition within the oxidative core for each population in each treatment group. Numerical densities of oxidative and fast glycolytic fibres were determined from stains of succinate dehydrogenase activity (which identifies oxidative fibres), and numerical densities of slow oxidative fibres were determined by staining for slow myosin immunoreactivity, each using stereological methods that have been well described (Weibel, 1979; Egginton, 1990). The numerical density of fast oxidative fibres was calculated as the difference between the densities of oxidative fibres and slow oxidative fibers. We then used these densities to estimate the proportion of

fibres analyzed by TEM that were of each fibre type, assuming that slow oxidative fibres had the highest mitochondrial volume density, fast oxidative fibres had the next highest, and fast glycolytic fibres had the lowest. For example, if our analyses by light microscopy revealed that a treatment group had 50% slow oxidative fibres on average, then the 50% of fibres with the highest mitochondrial volume density (measured by TEM) were classified as slow oxidative fibres for each individual in that treatment group.

2.3.5 Mitochondrial physiology of isolated mitochondria

Mice were euthanized as described above. The muscles from the entire left hindlimb (including the gastrocnemius and soleus) were removed (~250-400 mg), and immediately transferred to 10 ml of ice-cold isolation buffer (in mM: 100 sucrose, 50 Tris base, 5 $MgCl_2$, 5 EGTA, 100 KCl, 1 ATP). The muscle was minced and then digested for 5 min in the same buffer containing protease (1 mg g^{-1} muscle tissue). The digested muscle was then gently homogenized with six passes of a Potter-Elvehjem Teflon on glass homogenizer (100 rpm). Mitochondria were isolated via differential centrifugation at 4°C. Briefly, homogenates were centrifuged at 700g for 10 min. The resulting supernatant was filtered through cheesecloth and then centrifuged at 1000g for 10 min. The supernatant was centrifuged at 8700g for 10 min and the pellet was resuspended in 10 ml of isolation buffer with bovine serum albumin (BSA, fatty acid-free, at 1% mass:volume) and centrifuged at 8700g. The pellet was resuspended in 10 ml of storage buffer (in mM: 0.5 EGTA, 3 $MgCl_2$, 60 K-methanesulphonate, 20 taurine, 10 KH_2PO_4, 20

Hepes, 110 sucrose, 0.02 vitamin E succinate, 2 pyruvate, 2 malate, pH 7.1) and centrifuged at 8700g. The pellet was finally resuspended in 250-400 µl of storage buffer. Part of this mitochondrial suspension was kept on ice until mitochondrial physiology was measured, and the rest was homogenized in a glass tissue grinder and stored at -80°C for later use in enzyme assays (see below).

The physiology of isolated mitochondria was measured using high-resolution respirometry and fluorometry (Oxygraph-2k with O2k-Fluorescence module, Oroboros Instruments, Innsbruck, Austria). Isolated mitochondria (approximately 40 µg mitochondrial protein) were added to the respirometry chamber with a final volume of 2 ml of respiration buffer (in mM: 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose, 1 g l^{-1} fatty acid-free BSA; pH 7.1) at 37°C. Reactive oxygen species (ROS) was measured by fluorescent detection of resorufin (excitation wavelength of 525 nm and AmR filter set, Oroboros Instruments), which is produced from hydrogen peroxide (H_2O_2) and Ampliflu Red (Sigma-Aldrich) in a reaction catalysed by horseradish peroxidase. Mitochondrial superoxide and H_2O_2 were thus detected together by adding superoxide dismutase (22.5 U ml^{-1}; which catalyzes the production of H_2O_2 from superoxide), horseradish peroxidase (3 U ml^{-1}), and Ampliflu Red (15 µmol l^{-1}) to the chamber. ROS emission was thus measured during the following manipulations as the molar rate of H_2O_2 released from mitochondria, by calibrating the fluorescent resorufin signal with the addition of exogenous H_2O_2. Leak respiration was

stimulated with 5 mM pyruvate and 2 mM malate (L_N). P/O ratios (ATP produced per oxygen atom consumed) were obtained twice with additions of 125 µM of ADP. After each addition of ADP was consumed and the mitochondria reached leak state respiration (L_T), maximal ADP-stimulated respiration (P_{PM}) was induced with 0.6–1 mM ADP. The respiratory control ratio (RCR) was calculated as the ratio of P_{PM}/L_T immediately before and after this saturating addition of ADP. Oxygen levels in the chamber were allowed to reach zero to determine the O_2 kinetics of mitochondrial respiration (see below). The chamber was then re-oxygenated, and the mitochondria were allowed to consume the remaining ADP and reach leak state respiration. The oxygen concentration was again allowed to reach zero to determine oxygen kinetics in the leak state. The chamber was again re-oxygenated and cytochrome oxidase was maximally stimulated with the addition of 1.25 mM ADP, 2 mM ascorbate, and 0.5 mM TMPD (N,N,N′,N′-tetramethyl-p-phenylenediamine).

Respiration (rate of O_2 consumption) and ROS emission rates are expressed per milligram mitochondrial protein. DatLab 2 software (Oroboros Instruments) was used to fit respiration rate (MO_2) data from the transitions into anoxia to the equation: $MO_2=J_{max}\times PO_2/(P_{50}+PO_2)$, where J_{max} is maximal respiration (uninhibited by hypoxia), PO_2 is O_2 tension of the respiration medium, and P_{50} is the PO_2 at which respiration is half of J_{max}. Catalytic efficiency for O_2 was calculated as J_{max}/P_{50}. Delay in response time,

internal zero drift and background O_2 flux of the O_2 sensor were accounted for as previously described (Gnaiger et al., 1995; Gnaiger and Lassnig, 2010).

2.3.6 Enzyme and electron transport chain assays

Apparent V_{max} of mitochondrial complexes I, II, III, IV, and V as well as citrate synthase (CS) in isolated mitochondria were measured at 37°C as previously described (Kirby et al., 2007; Spinazzi et al., 2012) using a Spectromax Plus 384 microplate reader (Molecular Devices). Complexes III and IV were measured after a single freeze/thaw cycle in isotonic buffer (100 mM KCl_2; 25 mM KH_2PO_4; 5 mM $MgCl_2$), complexes I, II and V were measured after a second freeze-thaw cycle in hypotonic buffer (25 mM KH_2PO_4; 5 mM $MgCl_2$), and CS was measured after a third freeze-thaw cycle (in 100 mM Tris, pH 8). Assays were carried out in the following conditions (in mM, unless stated otherwise): Complex I, 0.2 NADH, 0.3 KCN, 0.06 coenzyme Q_{10}, 0.01 rotenone, and 3 mg/ml BSA in 50 KH_2PO_4 (pH 7.5); Complex II, 20 succinate, 0.3 KCN, 0.1 dichlorophenolindophenol (DCPIP), and 0.05 decylubiquinone (DUB) in 25 KH_2PO_4 (pH 7.5); Complex III, 0.075 oxidized cytochrome c, 0.5 KCN, 0.1 EDTA 0.1 decylubiquinol in 25 KH_2PO_4 (pH 7.5); Complex IV, 0.2 reduced cytochrome c, and 0.3 KCN in 50 KH_2PO_4 (pH 7.0); Complex V, 5 ATP, 1 phosphoenolpyruvate (PEP), 0.2 NADH, 1 U/mL pyruvate kinase (PK), 1 U/mL lactate dehydrogenase (LDH), and 0.0005 oligomycin in 50 KH_2PO_4 (pH 7.5); CS, 0.5 acetyl-coA, 0.15 DTNB, 0.5 oxaloacetate, 0.1% vol/vol Triton x-100, in 100 Tris (pH 8.0).

2.3.7 Statistical Analysis

Data are presented as means ± SE. Two-factor ANOVA and Bonferroni multiple-comparisons post-tests were used as appropriate to determine the main effects of population, altitude and acclimation environment, as well as their interaction. A significance level of $P < 0.05$ was used throughout.

2.4 RESULTS

2.4.1 Respiratory Capacities of Permeabilized Skeletal Muscles

The respiratory capacity of permeabilized fibres from the gastrocnemius muscle was elevated in high-altitude deer mice (Figure 1A). ADP stimulated oxphos (oxidative phosphorylation) respiration supported by pyruvate and malate (P_{PM}) was ~40% greater in highland mice than in the lowland mice ($F_{1,26} = 16.88$; $p = 0.0004$), but there was no significant main effect of acclimation ($F_{1,26} = 0.062$; $p = 0.8041$) nor a significant effect of population×acclimation interaction ($F_{1,26} = 0.040$; $p = 0.8426$). Similarly, maximal respiration *via* complex I with the subsequent addition of glutamate (P_{PMG}) ($F_{1,25} = 12.022$; $p = 0.002$) and complexes I+II respiration with the addition of succinate (P_{PMGS}) ($F_{1,26} = 15.354$; $p < 0.001$), were both higher in highland mice compared to lowland mice. There was no main effect of acclimation on P_{PMG} ($F_{1,25} = 0.208$; $p = 0.652$) or P_{PMGS} ($F_{1,26} = 0.204$; $p = 0.656$), and there were no significant interactions (P_{PMG}, $F_{1,25} = 0.0424$, $p = 0.838$; P_{PMGS}, $F_{1,26} = 0.031$, $p = 0.862$). Maximal respiration *via* complex IV stimulated

with ascorbate and TMPD (P_{Tm}) followed a similar pattern, with a significant main effect of population ($F_{1,20} = 5.561$; $p = 0.029$) but with no influence of hypoxia acclimation ($F_{1,20} = 0.902$; $p = 0.354$) or a significant population×acclimation interaction ($F_{1,20} = 1.855$; $p = 0.188$). Leak state respiration in the absence of ATP (L_N) in the gastrocnemius muscle was not significantly different between the populations ($F_{1,26} = 1.635$; $p = 0.212$) and there was no main effect of acclimation ($F_{1,26} = 1.342$; $p = 0.257$) or any significant interaction effect ($F_{1,26} = 0.733$; $p = 0.400$).

In contrast to the gastrocnemius, the respiratory capacity of the soleus muscle was similar in both populations (Figure 1B). There were no significant effects of population ($F_{1,16} = 0.532$; $p = 0.476$), acclimation ($F_{1,16} = 0.004$; $p = 0.949$), or population×acclimation interaction ($F_{1,16} = 1.112$; $p = 0.307$) on P_{PM}. Similarly, there was no main effects of population, acclimation, or their interaction on P_{PMG} (population, $F_{1,16} = 0.340$, $p = 0.568$; acclimation, $F_{1,16} = 0.060$, $p = 0.809$; population×acclimation, $F_{1,16} = 0.575$, $p = 0.459$) or P_{PMGS} (population $F_{1,16} = 0.583$; $p = 0.456$; acclimation $F_{1,16} = 0.175$; $p = 0.681$; interaction $F_{1,16} = 0.611$; $p = 0.446$). P_{Tm} decreased with hypoxia acclimation ($F_{1,16} = 6.818$; $p = 0.019$), by 17% in highlanders and 42% in lowlanders, but there were not significant effects of population ($F_{1,16} = 0.929$; $p = 0.350$) or population×acclimation interaction ($F_{1,16} = 0.815$; $p = 0.380$). L_N was not affected by population and/or treatment (population, $F_{1,16} = 0.027$, $p = 0.871$; acclimation, $F_{1,16} = 2.693$, $p = 0.120$; population×acclimation, $F_{1,16} = 0.760$, $p = 0.396$).

2.4.2 Mitochondrial Abundance and Structure

Highland deer mice had greater mitochondrial volume densities in the gastrocnemius muscle, entirely attributable to a greater abundance of subsarcolemmal mitochondria (Figure 2). Representative electron micrographs from the oxidative core of highland and lowland deer mice are shown in Figure 2A-D, with arrow denoting subsarcolemmal mitochondria and arrowhead denoting intermyofibrillar mitochondria. The total mitochondrial volume density was 25% greater in highland deer mice ($F_{1,16}$ = 18.56, $P < 0.001$), but there were no effects of hypoxia acclimation ($F_{1,16} = 0.049$, $P = 0.827$) or population×acclimation interaction ($F_{1,16} = 0.025$, $p = 0.877$). The population difference in the abundance of subsarcolemmal mitochondria was even larger, with the volume density of subsarcolemmal mitochondria 79% greater in highlanders than in lowlanders (population effect, $F_{1,16} = 54.274$, $P < 0.001$), but there were no population differences in the abundance of intermyofibrillar mitochondria (Fig 2G, $F_{1,16} = 0.01$, $P = 0.923$). Hypoxia acclimation had no significant effects on the abundances of subsarcolemmal ($F_{1,16} = 0.274$; $P = 0.608$) or intermyofibrillar ($F_{1,16} = 0.456$; $P = 0.509$) mitochondria, and the population×acclimation interactions were not significant (subsarcolemmal, $F_{1,16} = 0.274$, $P = 0.608$; intermyofibrillar, $F_{1,16} = 0.456$, $P = 0.509$).

The population differences in mitochondrial abundance cannot be attributed to any differences in fibre-type composition in the gastrocnemius muscle, and occurred in both

slow oxidative and fast oxidative fibre-types. The abundances of slow and fast oxidative fibres far exceeded those of fast glycolytic fibres, and there were no significant main effects of population on muscle fibre-types (Table 1), suggesting that previously observed population differences in fibre-type composition in the gastrocnemius (Lui et al., 2015) were driven largely by regions outside of the oxidative core. Average N_N for each treatment group were used to estimate the mitochondrial volume densities within each fibre type, as described in the methods. In doing so, we found that high-altitude mice generally had greater mitochondrial volume densities in both slow oxidative and fast oxidative fibre-types that were entirely attributable to greater volume densities of subsarcolemmal mitochondria (Table 1). Due to the small number of glycolytic fibres in the oxidative core, we did not evaluate mitochondrial volume densities in this fibre type.

There were no significant population differences in the surface density of mitochondrial cristae, but exposure to chronic hypoxia induced increases of this trait in both populations (Figure 3). Cristae surface density increased by 14% in highland mice and 15% in lowland mice in response to hypoxia acclimation in intermyofibrillar mitochondria ($F_{1,16} = 10.518$; $p=0.005$). A similar pattern was observed in subsarcolemmal mitochondria, with hypoxia acclimation increasing cristae surface density by 11% in highland mice and 7% in lowland mice, but the main effect of acclimation was only marginally significant ($F_{1,16} = 4.353$; $p=0.053$). However, cristae surface density did not differ between populations in either subsarcolemmal (main effect

of population, $F_{1,16} = 0.865$; p=0.366) nor intermyofibrillar ($F_{1,16} = 0.554$; p=0.467) mitochondria, and there were no significant population×acclimation interactions (subsarcolemmal, $F_{1,16} = 0.215$, p = 0.649; intermyofibrillar, $F_{1,16} = 0.00193$, p = 0.966).

2.4.3 Mitochondrial Physiology of Isolated Mitochondria

Isolated mitochondria of highlanders had higher mitochondrial respiration rates than those from lowland deer mice acclimated to normoxia, but rates were similar between the populations after hypoxia acclimation (Figure 4A). P_{PM} was 20% greater in normoxic highland mice than in the corresponding lowland mice ($F_{1,24} = 6.207$; p = 0.020). However, respiration increased by ~32% in response to hypoxia acclimation in lowland mice, whereas highland mice showed the opposite trend, such that there was a significant population×acclimation interaction ($F_{1,24} = 6.503$; p = 0.018) but no clear overall effect of acclimation ($F_{1,24} = 0.552$; p = 0.465). P_{Tm} followed the same pattern, with a significant main effect of population ($F_{1,22} = 11.1$, p = 0.003) in the normoxia group and population×acclimation interaction ($F_{1,22} = 11.05$, p = 0.0031), but no main effect of hypoxia acclimation ($F_{1,22} = 0.307$; p = 0.585). In contrast, L_N did not differ significantly between the populations ($F_{1,24} = 0.821$; p = 0.374) or in response to hypoxia acclimation ($F_{1,24} = 0.833$; p = 0.371), and the population×acclimation interaction was not significant ($F_{1,24} = 2.675$; p = 0.115). Similarly, there were no main effects of population ($F_{1,24} = 0.126$; p = 0.726) or acclimation ($F_{1,24} = 0.0002$; p = 0.989) on leak state

respiration in the presence of ATP (L_T) but there was a significant population×acclimation interaction ($F_{1,24} = 4.44$; $p = 0.046$) (Fig. 4A). Respiratory control ratios (RCR) were always above 5, but the high-altitude population had a higher RCR than the lowland population (Table 2). In contrast, P/O ratios showed no significant effect of population or hypoxia acclimation (Table 2). There were also subtle differences in oxygen kinetics between populations. Mitochondria from highland mice exhibited a significantly greater O_2 affinity (lower P_{50}) during leak respiration. The catalytic efficiency for oxygen (respiration/P_{50}), a measure that best reflects hypoxia sensitivity because it indicates the magnitude of respiration that can be sustained at low PO_2 (Gnaiger et al., 1998), was higher in highlanders during P_{PM} (Table 2).

The maximal activities (V_{max}) of complex III and IV in the electron transport system exhibited similar patterns of variation to those for respiration in isolated mitochondria (Figure 4B). Complex III (CIII) activity was ~51% higher in the highland population compared to the lowland population in normoxia, but population values converged after hypoxia acclimation (population, $F_{1,24} = 8.298$, $p = 0.008$; acclimation, $F_{1,24} = 0.283$, $p= 0.600$; interaction, $F_{1,24} = 0.544$, $p = 0.468$). Similarly, CIV activity was ~52% higher in the highland population compared to the lowland population in normoxia, and they converged after acclimation (population, $F_{1,24} = 19.035$, $p = <0.001$; acclimation, $F_{1,24} = 0.108$, $p = 0.745$; interaction, $F_{1,24} = 11.136$, $p = 0.003$). In contrast, there were no significant differences in activity for complex I (CI) (population, $F_{1,24} = 1.755$, $p = 0.198$;

acclimation, $F_{1,24} = 0.049$, $p = 0.826$; interaction, $F_{1,24} = 1.955$, $p = 0.175$), complex II (CII) (population, $F_{1,24} = 0.053$, $p = 0.820$; acclimation, $F_{1,24} = 0.252$, $p = 0.621$; interaction, $F_{1,24} = 0.351$, $p = 0.559$), complex V (CV) (population, $F_{1,20} = 0.156$, $p = 0.697$; acclimation, $F_{1,20} = 0.142$, $p = 0.710$; interaction, $F_{1,20} = 0.002$, $p = 0.965$), or citrate synthase (CS) (population, $F_{1,24} = 0.319$, $p = 0.577$; acclimation, $F_{1,24} = 0.549$, $p = 0.466$; interaction, $F_{1,24} = 0.482$, $p = 0.494$).

The emission of reactive oxygen species (ROS) from mitochondria was reduced by hypoxia acclimation, but did not differ between populations (Figure 5). The rates of ROS emission reported were measured at ~2.5 kPa O_2, to control for any potential effects of PO_2 on this variable. Mitochondrial ROS emission decreased in both populations after hypoxia acclimation during both P_{PM} ($F_{1,17} = 13.33$, $p = 0.002$) and L_T ($F_{1,23} = 4.469$, $p = 0.0456$). There was no main effect of population on ROS emission during P_{PM} ($F_{1,17} = 0.734$, $p = 0.118$) or L_T respiration ($F_{1,23} = 0.631$, $p = 0.237$), nor were there significant population×acclimation interactions (P_{PM}, $F_{1,17} = 1.43$, $p = 0.248$; L_T, $F_{1,23} = 0.0121$, $p = 0.913$).

2.5 DISCUSSION

The goal of this study was to explore if high-altitude deer mice offset reductions in circulatory O_2 supply during hypoxia with increases in tissue oxidative capacity or changes in mitochondrial distribution, thus contributing to the improvements in aerobic

capacity (VO_2max) in hypoxia exhibited by high-altitude deer mice compared to low-altitude deer mice (Cheviron et al., 2012; Lui et al., 2015). Highland deer mice must sustain high metabolic rates for adequate aerobic locomotion and thermogenesis in the wild (Hayes 1989, Hayes and O'Connor, 1999). We found that skeletal muscles of high-altitude deer mice have higher respiratory capacities and more abundant subsarcolemmal mitochondria compared to low-altitude deer mice. These evolved differences were distinct from the effects of hypoxia acclimation, which increased cristae surface density of mitochondria and reduced reactive oxygen species production in both populations. Evolved increases in oxidative capacity and a redistribution of mitochondria next to the cell membrane and adjacent to capillaries in skeletal muscle may therefore be adaptive for counteracting hypoxia and providing an adequate supply of ATP to support the high demands of exercise and shivering thermogenesis at high altitudes. There is strong positive selection on VO_2max in high-altitude populations, because individuals with a high VO_2max to support thermogenesis in the cold have a distinct survival advantage during harsh winters (Hayes and O'Connor, 1999), so the observed changes in muscle oxidative capacity and mitochondrial distribution likely contribute to improving fitness in the wild.

High-altitude deer mice express a more oxidative phenotype in the gastrocnemius muscle, enhancing its overall capacity for oxygen consumption. Due to the hyperbolic relationship between oxygen tension and mitochondrial respiration rate (Gnaiger, 2001),

having a more oxidative phenotype can help counteract impairments in respiration that can be induced by intracellular hypoxia (Hochachka, 1985). Oxidative capacity of a muscle can be augmented by (i) increasing the abundance of oxidative fibres, (ii) increasing mitochondrial volume density within each muscle fibre, and/or (iii) increasing the respiratory capacity of individual mitochondria. We previously found that gastrocnemius muscles of highland deer mice have more abundant oxidative fibres compared to lowland deer mice across the entire gastrocnemius muscle (Lui et al., 2015), which appears to be attributed largely to the regions outside of the oxidative core (i.e., the lateral and medial heads of the muscle) based on our reanalysis of those data (Table 1). However, muscle fibers in the oxidative core of the gastrocnemius muscle had much higher respiratory capacities (Figure 1) and mitochondrial volume densities are higher in highland mice compared to the lowland mice (Figure 2), independent of any population differences in fibre-type composition. Mitochondrial volume densities were very high in the oxidative fibres of highland deer mice, and much higher than in many lowland mammals. Mitochondrial volume densities vary greatly between fiber-types, muscles, and species. Aerobic muscle fibers generally have mitochondrial volume densities of 3-8% in humans (Alway, 1991) and 1-14% in various other mammals (Mathieu et al., 1981; Desplanches et al., 1990). The mitochondrial abundances we observed here in the oxidative core of the gastrocnemius of high-altitude deer mice (~26%) approach values only seen in the flight muscles of small birds with extremely high metabolic rates, such as

hummingbirds (Suarez et al., 1991).

The higher mitochondrial volume densities found in high-altitude deer mice can be attributed to a greater abundance of subsarcolemmal mitochondria in oxidative muscle fibres, which is likely advantageous for mitochondrial O_2 supply in hypoxic environments. A preferential overabundance of subsarcolemmal mitochondria in the muscle of highland mice situates more mitochondria adjacent to capillaries, and thus reduces the diffusion distance for oxygen. In general, the non-uniform distribution of mitochondria in muscle fibres likely results from reaction-diffusion constraints during aerobic metabolism (Boyle et al., 2003; Hardy et al., 2009). Mathematical modelling has shown that redistributing mitochondria closer to the cell periphery helps muscle fibres overcome diffusion limitations of oxygen delivery (Kinsey et al., 2011; Pathi et al., 2011; 2013). As predicted by these models, increases in muscle fiber size has been associated with a redistribution of mitochondria to the periphery of the cell (Boyle et al., 2003; Hardy et al., 2009; Pathi et al., 2012). Interestingly, the oxidative fibres of high-altitude deer mice are larger in size (Lui et al., 2015), which could provide an additional stimulus (in addition to hypoxia) for the observed subsarcolemmal redistribution of mitochondria. Similar to highland deer mice, oxidative fibres in the pectoralis muscle of the high flying bar-headed goose have a greater proportion of mitochondria in a subsarcolemmal location compared to low altitude geese (Scott et al., 2009; Scott et al., 2015b). In both high-altitude taxa, the mitochondrial redistribution is associated with increases in muscle

capillarity (Scott et al., 2009; Lui et al., 2015), which should together improve O_2 supply in hypoxia and improve performance at high altitudes.

Because subsarcolemmal mitochondria are further from cellular ATPases than intermyofibrillar mitochondria, the diffusion distance could foreseeably be greater for the ATP produced by the more abundant subsarcolemmal mitochondria in highland deer mice. However, recent evidence suggests that the complex and pervasive interconnections within the mitochondrial reticulum of muscle might preclude this possibility (Glancy et al., 2015). According to Glancy et al. (2015) there is a distribution of labour between subsarcolemmal mitochondria, which are specialized for consuming oxygen and generating the proton-motive force, and intermyofibrillar mitochondria, which use the proton-motive force for ATP production. The skeletal muscle of highland deer mice may thus have a greater ability to take up and consume oxygen and generate proton-motive force by virtue of their increased abundance of subsarcolemmal mitochondria, while the ability to produce ATP close to cellular ATPases is maintained because volume densities of intermyofibrillar mitochondria are the same between highland and lowland populations. Otherwise, enhanced shuttling of ATP, such as greater use of the phosphocreatine shuttle (Ventura-Clapier et al., 1998), could help counteract increases in ATP diffusion distance. This may be the case in bar-headed geese, based on observations that creatine sensitivity of mitochondrial respiration is enhanced compared to low-altitude geese, which suggests that the redistribution of mitochondria towards the subsarcolemma

may exist in concert with an active phosphocreatine shuttle to help improve the coupling of ATP supply and demand (Scott et al. 2009b).

Hypoxia acclimation had no effect on the relative proportion of subsarcolemmal mitochondria in either population of deer mice. In contrast, humans after high-altitude acclimatization show a counterintuitive loss of mitochondrial volume density that is greatest in the subsarcolemmal fraction (Murray and Horscroft, 2016). Perhaps the difference in responses between humans and deer mice is the result of experimental design or in the severity of the hypoxia exposures. Brief periods of normoxia, necessary for cage cleaning in the current study, may have potentially influenced the acclimation response in deer mice. However, these brief periods might be expected to have a modest impact relative to the effects of the much longer-term hypoxia acclimation. Alternatively, the hypoxia used to simulate the native high altitude range of deer mice (4300 m) was less than that needed to induce the changes in mitochondrial physiology that have been observed in humans exposed to higher elevations (D'Hulst and Deldicque, 2017).

Hypoxia acclimation had some similar effects on mitochondrial structure in both highland and lowland deer mice, but distinct effects on mitochondrial function between populations. The cristae surface density of mitochondria increased with hypoxia acclimation in both subsarcolemmal and intermyofibrillar mitochondria in both populations. In lowland deer mice, this increased surface density of cristae was associated with increases in both cytochrome c oxidase activity and respiratory capacity of isolated

mitochondria after hypoxia acclimation. In contrast, hypoxia acclimation decreased cytochrome-c oxidase activity in highland deer mice, in concert with a slight but non-significant reduction in pyruvate-stimulated state 3 respiration. Aerobic capacity and mitochondrial function can be influenced by activity level (Hood, 2001; Lira et al., 2010; Weibel et al., 2004), so it is possible that our results were influenced by population differences in activity during hypoxia acclimation. However, both populations were housed in the same conditions and given the same access to food, so any differences in activity would be due to innate (evolved) behavioural differences between populations.

How can the observed effects of hypoxia acclimation on respiration in isolated mitochondria be reconciled with our observations of respiration in permeabilized muscle fibres and of mitochondrial abundance? Muscle fibre respiration was 40-43% higher in highlanders than in lowlanders and did not change with hypoxia acclimation (Figure 1). Mitochondrial abundance was 26-29% higher in highlanders than in lowlanders and was also unchanged by hypoxia acclimation (Figure 2). These observations alone imply that there should also be a modest population difference in the respiratory capacity of isolated mitochondria that is unaltered by hypoxia acclimation, but this was clearly not the case (Figure 4). It is possible that there were differences in mitochondrial function between the tissues used for muscle fibre respiration and TEM (oxidative core of the gastrocnemius muscle) and those used for mitochondrial isolation (all hindlimb muscles). There might have also been changes in mitochondrial physiology during the isolation process,

consistent with some previous studies that have noted qualitatively distinct patterns of variation between isolated mitochondria and permeabilized muscle fibres (Picard et al., 2010; Mathers and Staples, 2015). Although the reasons for this discrepancy are not entirely clear, the combined effects of all determinants of oxidative capacity nevertheless contribute to highland deer mice having a higher oxidative capacity in the gastrocnemius muscle.

Recent work suggests that hypoxia acclimation increases the proportion of carbohydrates oxidized relative to fatty acids when running at 75% of VO_2max in highland but not in lowland deer mice (Lau et al. 2017). Similar differences in fuel selection during exercise have been observed between lowland and highland species of Andean leaf-eared mice (Schippers et al. 2012). Carbohydrate oxidation produces more ATP per molecule of oxygen than the oxidation of fatty acids (Brand, 2005; Welch et al., 2007), and may thus be beneficial for sustaining muscle performance in high-altitude hypoxia. It would be instructive to examine the role of mitochondria in this switch, to determine whether mitochondrial capacities for oxidizing carbohydrates relative to fatty acids might increase in high-altitude mice after hypoxia acclimation, and represent another way in which hypoxia acclimation might have distinct effects on mitochondrial function between populations.

Hypoxia acclimation decreased mitochondrial ROS emission in both populations of deer mice. Some evidence suggests that initial exposure to hypoxic environments

increases the rate of mitochondrial ROS production, because O_2 limitation at COX can render the electron transport system in a more reduced state (Hoppeler et al., 2003). On the one hand, high levels of ROS production could lead to maladaptive increases in oxidative damage to cellular components. On the other hand, increases in ROS production may play an important signalling role in hypoxic environments, and is known to stabilize hypoxia inducible factors (HIF) and thus promote the expression of hypoxia-responsive genes (Chandel et al., 2000). In fact, stabilization of HIF-1α can lead to downstream changes that feedback to reduce ROS production after prolonged hypoxia exposure (Fukuda et al., 2007; Bo et al., 2008; Zhang et al., 2008). It is possible that this contributed to the decrease in mitochondrial ROS emission that we observed here in response to hypoxia acclimation (Figure 5). However, it is unclear whether our observations are caused by a decrease in ROS production by the electron transport system, or by an increase in ROS scavenging by antioxidant systems within the mitochondria (Guzy et al., 2005; Kowaltowski and Vercesi, 1999; Murphy, 2009; Treberg et al., 2010).

2.6 CONCLUSIONS AND PERSPECTIVES

High-altitude natives have evolved exquisite physiological adaptations over millennia that enable them to function under conditions of severe hypoxia. These adaptations provide a strong indication of traits that are especially beneficial for

improving performance and hypoxia resistance at high altitudes. High-altitude adaptation appears to have increased maximal O_2 consumption in hypoxia in highland deer mice (Hayes 1989; Hayes and O'Connor, 1999; Cheviron et al., 2012; Lui et al., 2015). As skeletal muscles are a major determinant of VO_2max, our results suggest that adaptive modifications in the muscle – specifically the respiratory capacity and intracellular distribution of mitochondria – are key to high-altitude adaptation in this species. Given the strong positive selection on VO_2max in high-altitude populations in the wild (Hayes and O'Connor, 1999), the observed changes in muscle phenotype in high-altitude populations likely contribute to improving survival and reproductive fitness. The mechanisms underlying high-altitude adaptation were largely distinct from the effects of hypoxia acclimation, observed here and in many previous studies of high-altitude acclimatization (Murray and Horscroft, 2016), although there were some population differences in the acclimation response. This is one of the first studies to describe adaptations in mitochondrial physiology in non-human mammals native to high altitude, and suggests that natural selection can employ unique solutions to overcome the hypoxic conditions at high altitudes that are beyond those attained by lowlanders after extensive acclimatization.

Here, we used F1 generation mice that were born and raised in normoxia to examine population differences in adults that were acclimated to normoxia or hypoxia, but other life stages and other challenges of high-altitude environments are worth future

consideration. Pre-natal exposure to hypoxia has been shown to affect various aspects of physiology (Hutter & Jaeggi, 2010), including mitochondrial function (Galli et al. 2016), and it would be valuable to investigate how developmental hypoxia impacts mitochondrial physiology in deer mice, and whether the effects of developmental hypoxia differ between high- and low-altitude populations. Furthermore, cold is another key stressor at high altitudes, one that is a significant challenge for small endotherms such as deer mice, and that could lead distinct mechanisms of high-altitude adaptation between small and large animals (e.g., mice versus humans). Cold acclimation is often observed to increase capillarity and aerobic capacity of muscles from lowland mammals (Herpin and Lefaucheur, 1992; Jackson et al., 1987; Mineo et al., 2012; Sillau, 1980; Wickler, 1980), but little is known about this process in high-altitude natives. It will be instructive to examine how cold-induced plasticity (during early development and adulthood) affects mitochondrial physiology in high- and low-altitude populations, alone or in combination with hypoxia, to tease apart the influence of these stressors on muscle aerobic capacity at high-altitudes.

2.8 TABLES AND FIGURES

Table 2.1: Numerical densities and mitochondrial volume densities within each fibre type in the oxidative core of the gastrocnemius muscle of deer mice.

	Acclimation Environment	Lowlanders	Highlanders	Main Effect Variable	F	P
Slow Oxidative Fibres						
N_N (I,m)	Normoxia	0.506±0.035	0.499±0.031	Pop.	0.0004	0.983
	Hypoxia	0.407±0.042	0.412±0.037	Acc.	6.034	0.020
				Pop. × Acc.	0.024	0.878
V_V(mt,I)	Normoxia	26.3±0.63	32.2±1.46*	Pop.	12.608	0.003
	Hypoxia	28.5±0.85	31.9±1.93	Acc.	0.555	0.467
				Pop. × Acc.	0.793	0.386
V_V(ssm,I)	Normoxia	10.7±0.764	15.8±1.06*	Pop.	40.823	<0.001
	Hypoxia	11.9±0.667	17.3±0.709*	Acc.	2.762	0.116
				Pop. × Acc.	0.015	0.905
Vv(imm,I)	Normoxia	15.6±0.695	16.4±1.14	Pop.	0.175	0.681
	Hypoxia	16.5±0.835	14.7±1.93	Acc.	0.081	0.776
				Pop. × Acc.	1.032	0.325
Fast Oxidative Fibres						
N_N (IIa,m)	Normoxia	0.438±0.057	0.429±0.032	Pop.	0.586	0.450
	Hypoxia	0.476±0.044	0.556±0.045	Acc.	3.329	0.079
				Pop. × Acc.	0.917	0.347
V_V(mt,IIa)	Normoxia	15.1±1.769	20.5±1.18*	Pop.	14.765	0.001
	Hypoxia	15.5±1.386	22.1±1.82*	Acc.	0.410	0.531
				Pop. × Acc.	0.159	0.001
V_V(ssm,IIa)	Normoxia	4.07±1.072	9.34±1.01*	Pop.	46.978	<0.001
	Hypoxia	4.30±0.444	11.0±0.825*	Acc.	1.188	0.292
				Pop. × Acc.	0.677	0.423
V_V(imm,IIa)	Normoxia	11.0±1.083	11.1±0.509	Pop.	0.0001	0.991
	Hypoxia	11.2±0.971	11.1±1.42	Acc.	0.002	0.964
				Pop. × Acc.	0.0085	0.928
Glycolytic Fibres						
N_N (IIb,m)	Normoxia	0.057±0.025	0.067±0.015	Pop.	1.647	0.209
	Hypoxia	0.109±0.033	0.040±0.050*	Acc.	0.302	0.587
				Pop. × Acc.	2.837	0.102

N_N (I,m), numerical density of slow oxidative fibres; V_V(mt,I), total mitochondrial volume density (as % fiber volume) of slow oxidative fibres; V_V(ssm,I), subsarcolemmal mitochondrial volume densities of slow oxidative fibres; Vv(imm,I), intermyofibrillar mitochondrial volume densities of slow oxidative fibres; N_N (IIa,m), numerical density of fast oxidative fibres; V_V(mt,IIa), total mitochondrial volume density of fast oxidative fibres; V_V(ssm,IIa), subsarcolemmal mitochondrial volume densities of fast oxidative fibres; V_V(imm,IIa), total mitochondrial volume density of fast oxidative fibres; N_N (IIb,m) numerical density of glycolytic fibres; Pop., Population; Acc., Acclimation; Pop. × Acc., Interaction of Population and Acclimation. For numerical densities, n = 9 individuals for each treatment group, except n = 7 for hypoxia acclimated lowlanders. For mitochondrial volume densities, n = 5 individuals for each treatment group.* Significant pairwise differences between the high and lowland populations within the same acclimation environment.

Table 2.2. Mitochondrial coupling and oxygen kinetics of lowland and highland deer mice acclimated to normoxia or hypoxia.

Variable	Acclimation Environment	Lowlanders	Highlanders	Main Effect Variable	F	P
RCR	Normoxia	6.68±0.647	7.85±0.769	Pop.	9.85	0.005
	Hypoxia	5.87±0.411	8.49±0.452*	Acc.	0.019	0.891
				Pop. × Acc.	1.43	0.244
P/O Ratio	Normoxia	2.8±0.103	2.99±0.061*	Pop	1.44	0.242
	Hypoxia	2.99±0.035ǂ	2.98±0.0759	Acc.	3.19	0.087
				Pop. × Acc.	3.59	0.071
Oxygen Kinetics in P_{PM}						
P_{50}	Normoxia	0.074±0.011	0.058±0.004	Pop.	1.098	0.307
	Hypoxia	0.069±0.005	0.066±0.007	Acc.	0.185	0.672
				Pop. × Acc.	0.474	0.499
Catalytic	Normoxia	8113±626	14429±1661*	Pop.	4.432	0.047
Efficiency	Hypoxia	11517±1700	13125±2866	Acc.	0.241	0.629
				Pop. × Acc.	1.47	0.238
Oxygen Kinetics in L_T						
P_{50}	Normoxia	0.028±0.004	0.019±0.003*	Pop.	5.647	0.028
	Hypoxia	0.022±0.003	0.018±0.001	Acc.	1.401	0.251
				Pop. × Acc.	0.894	0.563
Catalytic	Normoxia	8720±1841	12181±1984	Pop.	1.697	0.208
efficiency	Hypoxia	10202±762	10145±1222	Acc.	0.015	0.905
				Pop. × Acc.	1.81	0.195

RCR, respiratory control ratio (P_{PM}/L_T); P/O Ratio, ATP produced per oxygen atom consumed; P_{PM}, oxphos respiration with pyruvate and malate; L_T , leak respiration in the presence of ATP; $P_{50,}$ PO_2 at 50% inhibition of maximal respiration (expressed in kPa); Catalytic efficiency, the quotient of maximum O_2 flux and P_{50} (expressed in units nmol O_2 mg^{-1} protein min^{-1} kPa^{-1}); Pop., Population; Acc., Acclimation; Pop. × Acc., Interaction of Population and Acclimation. n = 7 individuals for each treatment group for P/O ratios and respiratory control ratios. n = 6 individuals for each treatment group, except n = 5 for normoxia acclimated highland mice for oxygen kinetics. *Significant pairwise differences between the high and lowland populations within the same acclimation environment

Figure 2.1

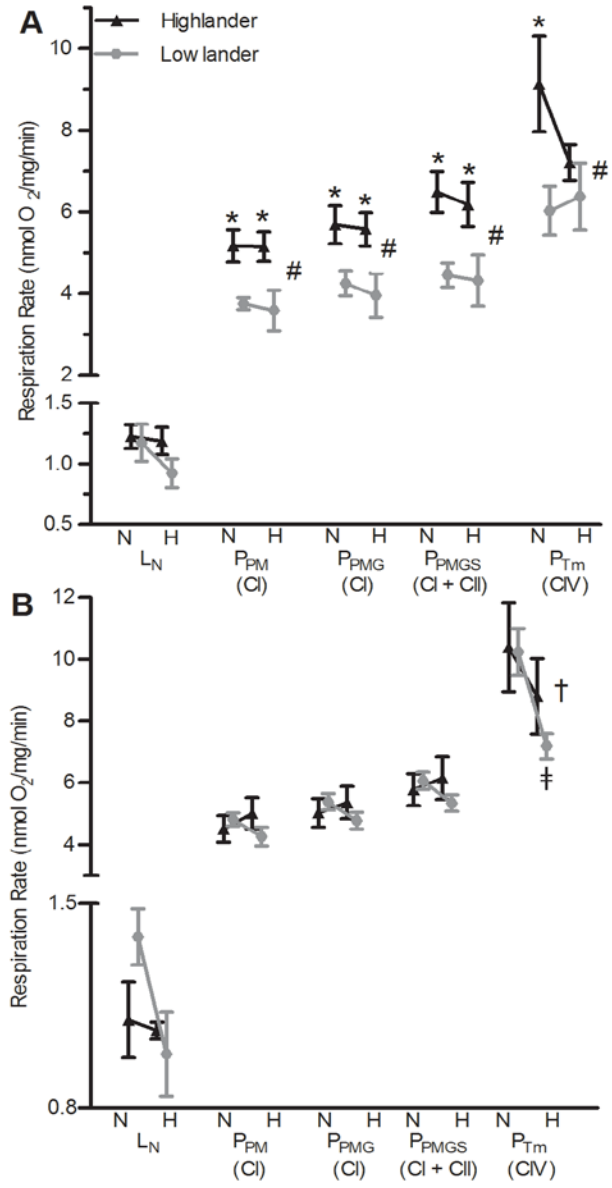
A
Highlander
Low lander
Respiration Rate (nmol O 2/mg/min)
10
8
6
4
2
1.5
1.0
0.5
N H
L N
P PM
(CI)
P PMG
(CI)
P PMGS
(CI + CII)
P Tm
(CIV)
B
Respiration Rate (nmol O2/mg/min)
12
10
8
6
4
1.5
0.8

Figure 2.1: The respiratory capacities of permeabilized muscle fibres from the gastrocnemius (A), but not the soleus (B), were greater in highland deer mice than in lowland deer mice. Leak respiration was measured in the absence of ATP (L_N: malate, pyruvate), and oxidative phosphorylation was measured with substrates of complex I (P_{PM}: malate, pyruvate, ADP; P_{PMG}: malate, pyruvate, glutamate, ADP), complexes I and II (P_{PMGS}: malate, pyruvate, glutamate, succinate, ADP) and maximal complex IV stimulation (P_{Tm}: ADP, ascorbate, TMPD). (A) n = 8 for normoxia acclimated individuals, n = 7 for hypoxia acclimated individuals for L_N, $P_{PM,}$ P_{PMG}, P_{PMGS}; n = 6 individuals for each treatment group for P_{Tm}. (B) n = 5 individuals for each treatment group. N, normoxia acclimation; H, hypoxia acclimation. # Significant main effect of population. † Significant main effect of hypoxia acclimation. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment. ǂ Significant pairwise differences in response to hypoxia acclimation within a population.

Figure 2.2

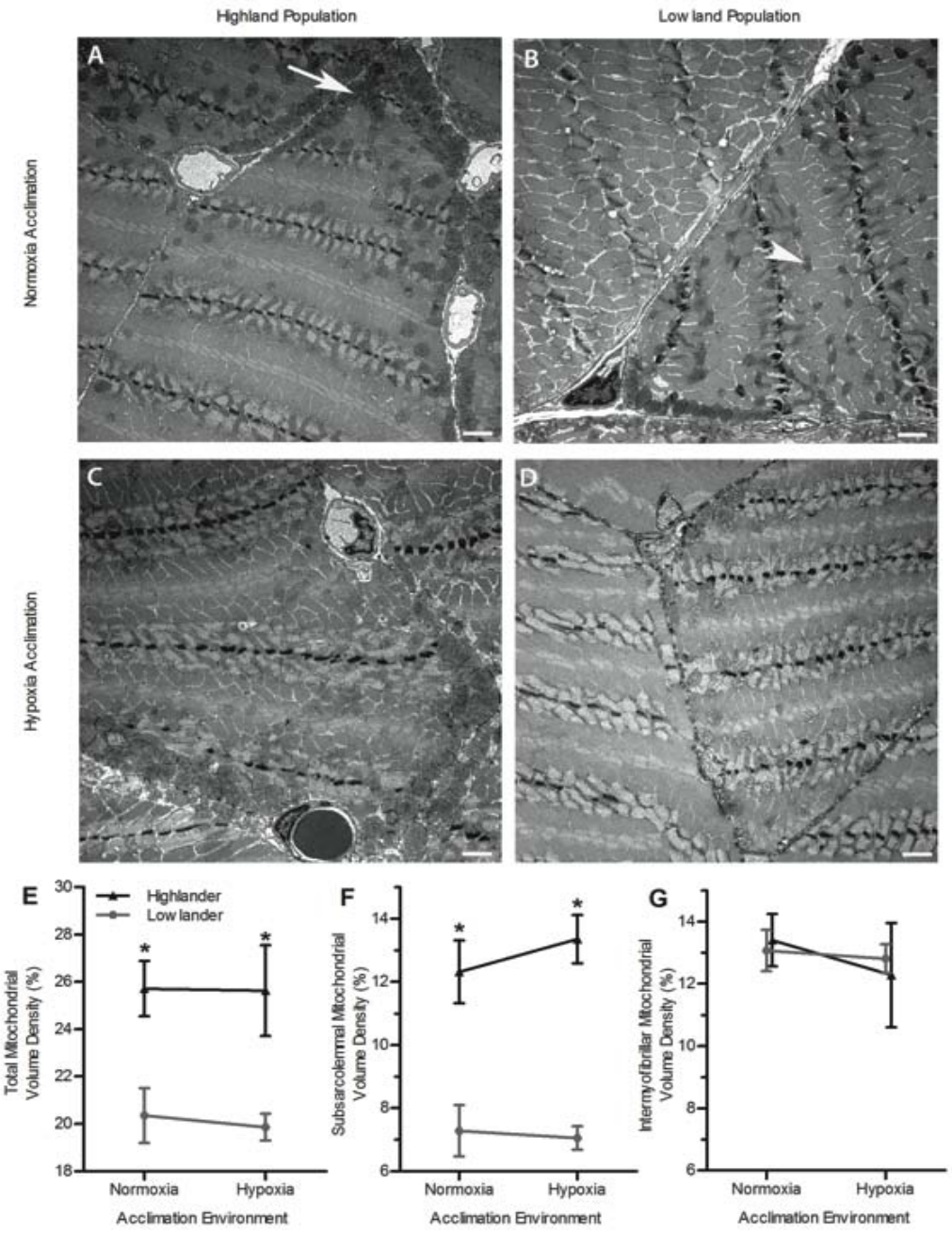
Highland Population
Low land Population
Normoxia Acclimation
Hypoxia Acclimation
A
B
C
D
E
F
G
Highlander
Low lander
Total Mitochondrial Volume Density (%)
Subsarcolemmal Mitochondrial Volume Density (%)
Intermyofibrillar Mitochondrial Volume Density (%)
Normoxia
Hypoxia
Acclimation Environment

Figure 2.2: Highland deer mice had a higher mitochondrial density in the gastrocnemius muscle, due entirely to an increased abundance of subsarcolemmal mitochondria. Representative transmission electron micrographs from the oxidative core of highland (A) and lowland (B) deer mice acclimated to normoxia, and highland (C) and lowland (D) deer mice acclimated to hypoxia. The volume densities of all mitochondria (which includes both subsarcolemmal and intermyofibrillar mitochondria) (E), subsarcolemmal mitochondria (F), and intermyofibrillar mitochondria (G). n = 5 individuals for each treatment group. Data points within an acclimation environment are offset for clarity. Scale bar represents 2 μm. Arrow, subsarcolemmal mitochondria; arrowhead, intermyofibrillar mitochondria. # Significant main effect of population. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment.

Figure 2.3

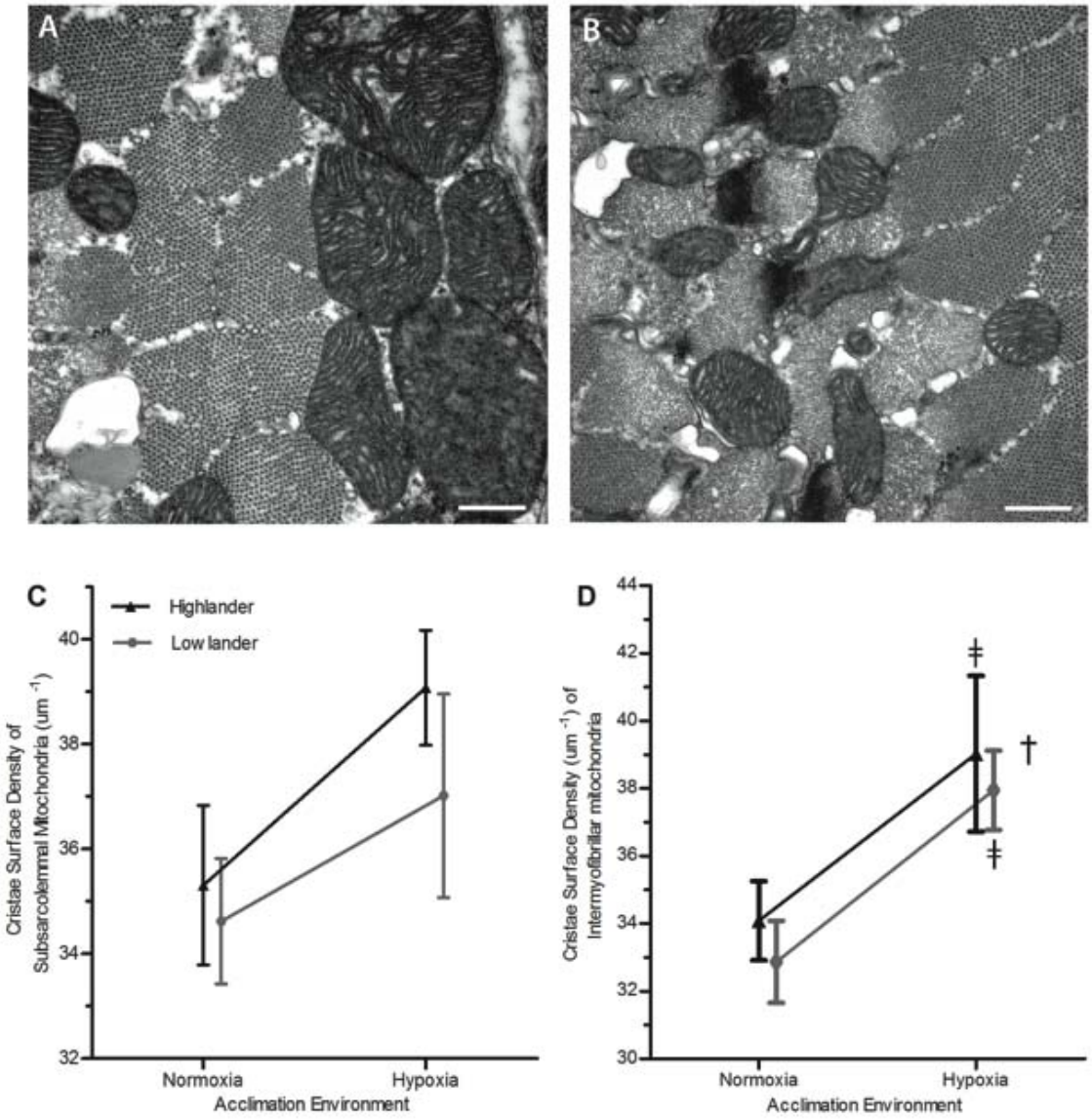
A
B
C
Highlander
Low lander
Cristae Surface Density of Subsarcolemmal Mitochondria (um -1)
40
38
36
34
32
Normoxia
Hypoxia
Acclimation Environment
D
Cristae Surface Density (um -1) of Intermyofibrilar mitochondria
44
42
40
38
36
34
32
30
‡
†
‡
Normoxia
Hypoxia
Acclimation Environment

Figure 2.3: Hypoxia acclimation increased the density of cristae surface in the mitochondria. Representative transmission electron micrographs of subsarcolemmal mitochondria (A) and intermyofibrillar mitochondria (B) from the gastrocnemius muscle of a highland deer mouse. Cristae surface density (μm^2 surface / μm^3 mitochondria) of subsarcolemmal mitochondria (C) and intermyofibrillar mitochondria (D) of highland and lowland deer mice. n = 5 individuals for each treatment group. Data points within an acclimation environment are offset for clarity. † Significant main effect of acclimation. ǂ Significant pairwise differences in response to hypoxia acclimation within a population.

Figure 2.4

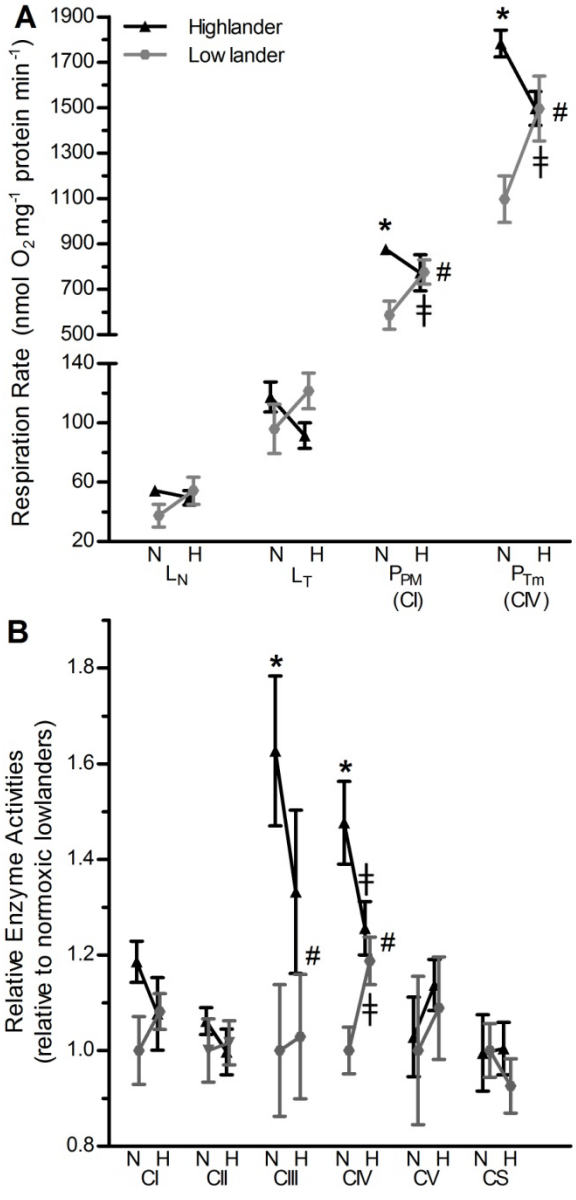
A
Highlander
Low lander
Respiration Rate (nmol O_2 mg^{-1} protein min^{-1})
1900
1700
1500
1300
1100
900
700
500
140
100
60
20
*
#
‡
N H
L_N
L_T
P_{PM} (CI)
P_{Tm} (CIV)
B
Relative Enzyme Activities (relative to normoxic lowlanders)
1.8
1.6
1.4
1.2
1.0
0.8
CI
CII
CIII
CIV
CV
CS

Figure 2.4 Respiration rates (A) and enzyme activities (B) in mitochondria isolated from the hindlimb muscles of deer mice. (A) Leak respiration was measured with pyruvate and malate, both in the absence (L_N) and presence (L_T) of ATP, oxidative phosphorylation was measured with substrates of complex I (P_{PM}: malate, pyruvate, ADP) and with maximal stimulation of complex IV (P_{Tm}: ADP, ascorbate, TMPD). n = 7 individuals for each treatment group, except n = 6 for normoxia acclimated highland and lowland mice for P_{Tm}. (B) Mitochondrial complex activities of lowland mice acclimated to normoxia were set to one to ease comparison, which equalled the following absolute activities for each enzyme (in µmol/mg protein/min): Complex I (CI) = 2.16, Complex II (CII) = 0.82, Complex III (CIII) = 1.87, Complex IV (CIV) = 4.34, Complex V (CV) = 5.19, and citrate synthase (CS) = 5.13. n = 7 individuals for each treatment group for CI, CII, CIII, CIV and CS; n = 6 individuals for each treatment group for CV. Enzyme assays were conducted on the isolated mitochondria that remained after the respiration trials were conducted. N, normoxia acclimation; H, hypoxia acclimation. # Significant main effect of ancestry. † Significant main effect of acclimation. * Significant pairwise differences between the high and lowland populations within the same acclimation environment. ǂ Significant pairwise differences in response to hypoxia acclimation within a population.

Figure 2.5

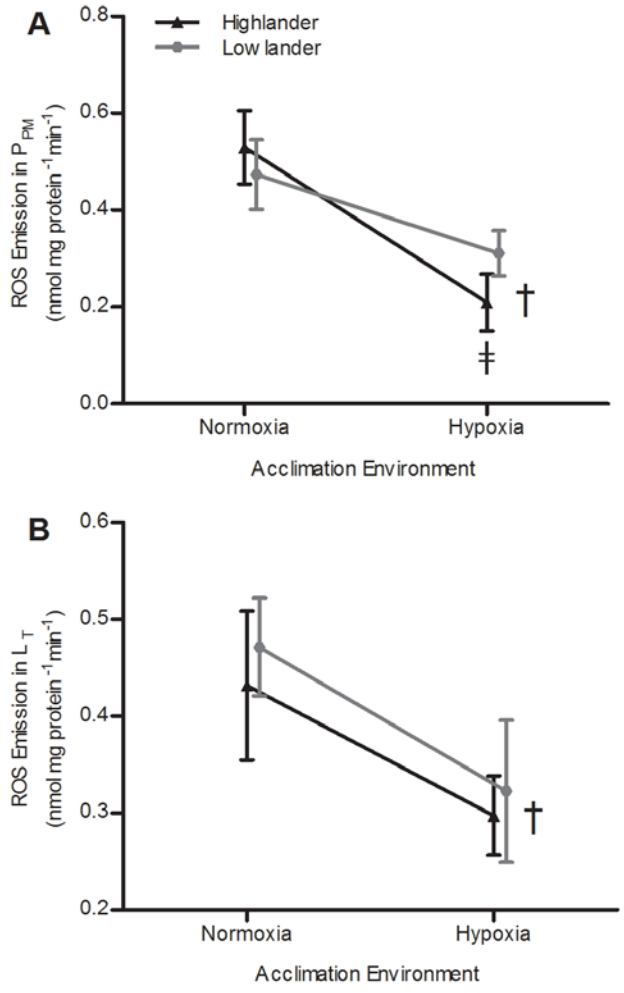
A
Highlander
Low lander
ROS Emission in P_{PM} (nmol mg protein^{-1} min^{-1})
0.8
0.6
0.4
0.2
0.0
†
‡
Normoxia
Hypoxia
Acclimation Environment
B
ROS Emission in L_T (nmol mg protein^{-1} min^{-1})
0.6
0.5
0.4
0.3
0.2
†
Normoxia
Hypoxia
Acclimation Environment

Figure 2.5: The rate of emission of reactive oxygen species (ROS) from mitochondria during oxidative phosphorylation (P_{PM}) (A) and leak (L_T) (B) respiration. ROS emission was coupled to H_2O_2 production using exogenous superoxide dismutase and was measured fluorometrically using Ampliflu Red. Because ROS emission rates may be dependent on O_2 tension (PO_2), the ROS emission rates shown were all measured at PO_2 ~2.5 kPa. (A) n = 5 individuals for each treatment group, except n = 6 for hypoxia acclimated lowland mice. (B) n = 7 individuals for each treatment group, except n = 6 for hypoxia acclimated lowland mice. † Significant main effect of acclimation. ǂ Significant pairwise differences in response to hypoxia acclimation within a population.

2.7 REFERENCES

Alway SE (1991). Is fiber mitochondrial volume density a good indicator of muscle fatigability to isometric exercise? *J Appl Physiol* **70,** 2111-2119.

Barrie E, Heath D, Stella JA & Harris P (1975). Enzyme activities in red and white muscles of guinea-pigs and rabbits indigenous to high altitudes. *Environ Physiol Biochem* **5,** 18–26.

Beaudry JL & McClelland GB (2010). Thermogenesis in CD-1 mice after combined chronic hypoxia and cold acclimation. *Comp Biochem and Physiol B Biochem Mol Biol* ***157,*** 301-309.

Bo H, Wang YH, Li HY, Zhao J, Zhang HY & Tong CQ (2008). Endurance training attenuates the bioenergetics alterations of rat skeletal muscle mitochondria submitted to acute hypoxia: role of ROS and UCP3. *Sheng li xue bao:[Acta physiologica Sinica]* **60,** 767-776.

Boyle KL, Dillaman RM & Kinsey ST (2003). Mitochondrial distribution and glycogen dynamics suggest diffusion constraints in muscle fibers of the blue crab, Callinectes sapidus. *J Exp Zool A Comp Exp Biol* **297,** 1-16.

Brand MD (2005). The efficiency and plasticity of mitochondrial energy transduction. *Biochem Soc Trans* **33,** 897-904.

Buskirk ER, Kollias J, Akers R F, Prokop EK & Reategui EP (1967). Maximal performance at altitude and on return from altitude in conditioned runners. *J Appl Physiol* **23,** 259-266.

Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM & Schumacker PT (2000). Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1α during hypoxia a mechanism of O2 sensing. *J Biol Chem* **275,** 25130-25138.

Cheviron AZ, Bachman GC, Connaty AD, McClelland GB and Storz JF (2012). Regulatory changes contribute to the adaptive enchancement of thermogenic capacity in high-altitude deer mice. *Proc Natl Acad Sci U S A* **109,** 8635-8640.

D'Hulst G and Deldicque L (2017). Human skeletal muscle wasting in hypoxia: a matter of hypoxic dose? *J Appl Physiol* **122**, 406-408.

Desplanches D, Kayar SR, Sempore B, Flandrois R & Hoppeler H (1990). Rat soleus muscle ultrastructure after hindlimb suspension. *J appl physiol* **69,** 504-508.

Egginton S (1990). Numerical and areal density estimates of fibre type composition in a skeletal muscle (rat extensor digitorum longus). *J Anat* **168,** 73-80.

Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV & Semenza GL (2007). HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell* **129,** 111-122.

Galli GL, Crossley J, Elsey RM, Dzialowski EM, Shiels HA, and Crossley DA (2016). Developmental plasticity of mitochondrial function in American alligators, *Alligator mississippiensis*. *Am J Physiol Regul Integr Comp Physiol* **311**, R1164-R1172.

Gilbert-Kawai ET, Milledge JS, Grocott MPW, Martin DS. (2014). King of the mountains: Tibetan and Sherpa physiological adaptations for life at high altitude. *Physiology* **29**, 388-402.

Glancy B, Hartnell LM, Malide D, Yu ZX, Combs CA, Connelly PS, Subramaniam S & Balaban RS. (2015). Mitochondrial reticulum for cellular energy distribution in muscle. *Nature* **523,** 617-620.

Gnaiger E. (2001). Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. *Respir physiol* **128,** 277-297.

Gnaiger E & Lassnig B. (2010). DatLab 2: analysis of oxygen kinetics. *Mitochondr Physiol Network* 02.05, 1-16, http://www.bioblast.at/index.php/ MiPNet02.05_DatLab2_O2Kinetics.

Gnaiger E, Lassnig B, Kuznetsov A, Rieger G & Margreiter R. (1998). Mitochondrial oxygen affinity, respiratory flux control and excess capacity of cytochrome c oxidase. *J Exp Biol* **201**, 1129-1139.

Gnaiger E, Steinlechner-Maran R, Méndez G, Eberl T & Margreiter R. (1995). Control of mitochondrial and cellular respiration by oxygen. *J Bioenerg Biomembr* **27**, 583-596.

Guzy RD, Hoyos B, Robin E, Chen H, Liu L, Mansfield KD, Simon MC, Hammerling U & Schumacker PT (2005). Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. *Cell metab* **1,** 401-408.

Hardy KM, Dillaman RM, Locke BR & Kinsey ST (2009). A skeletal muscle model of extreme hypertrophic growth reveals the influence of diffusion on cellular design. *Am J Physiol Regul Integr Comp Physiol* **296,** R1855-R1867.

Hayes JP (1989). Field and maximal metabolic rates of deer mice (Peromyscus maniculatus) at low and high altitudes. *Physiol Zool* **62,** 732-744.

Hayes JP and O'Connor CS (1999). Natural selection on thermogenic capacity of high-altitude deer mice. *Evolution* **53,** 1280-1287.

Herpin P & Lefaucheur L (1992). Adaptive changes in oxidative metabolism in skeletal muscle of cold-acclimated piglets. *J Therm Biol* **17,** 277-285.

Hochachka PW (1985). Exercise limitations at high altitude: the metabolic problem and search for its solution. In *Circulation, respiration, and metabolism*, ed. Gilles R, pp. 240-249. Springer-Verlag, Berlin.

Hochachka PW, Stanley C, Merkt J, and Sumar-Kalinowski J (1983). Metabolic meaning of elevated levels of oxidative enzymes in high altitude adapted animals: An interpretive hypothesis. *Respir Physiol **52,*** 303-313.

Hock RJ (1964). Physiological responses of deer mice to various native altitudes. In: *The Physiological Effects of High Altitude,* edited by Weihe WH. New York: Macmillan, p. 59–72.

Hood DA (2001). Invited Review: contractile activity-induced mitochondrial biogenesis in skeletal muscle. *J appl physiol* **90,** 1137-1157.

Hoppeler H, Kleinert E, Schlegel C, Claassen H, Howald H, Kayar SR & Cerretelli P (1990). Morphological adaptations of human skeletal muscle to chronic hypoxia. *Int J Sports Med* **11,** S3-S9.

Hoppeler H, Vogt M, Weibel ER & Fluck M (2003). Response of skeletal muscle mitochondria to hypoxia. *Exp Physiol* **88,** 109-119.

Hutter D and Jaeggi E (2010). Causes and mechanisms of intrauterine hypoxia and its impact on the fetal cardiovascular system: a review. *Int J Pediatr* **2010,** 401323.

Ivy CM & Scott GR. (2015). Control of breathing and the circulation in high-altitude mammals and birds. *Comp Biochem Physiol A Mol Integr Physiol* **186**, 66-74.

Jackson CGR, Sillau AH & Banchero N (1987). Fiber composition and capillarity in growing guinea pigs acclimated to cold and cold plus hypoxia. *Exp Biol Med* **185,** 101-106.

Jacobs RA, Lundby AM, Fenk S, Gehrig S, Siebenmann C, Flück D, Kirk N, Hilty MP & Lundby C. (2016). Twenty-eight days of exposure to 3454 m increases mitochondrial volume density in human skeletal muscle. *J Physiol* 594, 1151–1166.

Jacobs RA, Siebenmann C, Hug M, Toigo M, Meinild A-K & Lundby C. (2012). Twenty-eight days at 3454-m altitude diminishes respiratory capacity but enhances efficiency in human skeletal muscle mitochondria. *FASEB J* 26, 5192-5200

Kayser B, Hoppler H, Claassen H & Cerretelli P (1991). Muscle structure and performance capacity of Himalayan Sherpas. *J Appl Physiol* **70,** 1938-1942.

Kayser B, Hoppeler H, Desplanches D, Marconi C, Broers B & Cerretelli P (1996). Muscle ultrastructure and biochemistry of lowland Tibetans. *J Appl Physiol* **81,** 419-425.

Kinsey ST, Locke BR & Dillaman RM (2011). Molecules in motion: influences of diffusion on metabolic structure and function in skeletal muscle. *J exp biol* **214,** 263-274.

Kirby DM, Thorburn DR, Turnbull DM & Taylor RW (2007). Biochemical assays of respiratory chain complex activity. *Mitochondria **80,*** 93-119.

Kowaltowski AJ & Vercesi AE (1999). Mitochondrial damage induced by conditions of oxidative stress. *Free Radic Biol Med* **26,** 463-471.

Lau DS, Connaty AD, Mahalingam S, Wall N, Cheviron ZA, Storz JF, Scott GR & McClelland GB. (2017). Acclimation to hypoxia increases carbohydrate use during exercise 4 in high-altitude deer mice. Am J Physiol Regul Integr Comp Physiol in press

León-Velarde F, Sanchez J, Bigard AX, Brunet A, Lesty C & Monge C (1993). High altitude tissue adaptation in Andean coots: capillarity, fiber area, fiber type and enzymatic activities of skeletal muscle. *J Comp Physiol B* **163,** 52–58.

Levett D, Radford EJ, Menassa DA, Graber EF, Morash AJ, Hoppeler H, Clarke K, Martin DS, Ferguson-Smith AC, Montgomery HE, Grocott MPW, Murray AJ & the Caudwell Xtreme Everest Research Group (2012). Acclimatization of skeletal muscle mitochondria to high-altitude hypoxia during an ascent of Everest. *FASEB J.* **26,** 1431-1441.

Lira VA, Benton CR, Yan Z & Bonen A (2010). PGC-1α regulation by exercise training and its influences on muscle function and insulin sensitivity. *Am J Physiol Endocrinol Metab* **299,** E145-E161.

Lui MA, Mahalingam S, Patel P, Connaty AD, Ivy CM, Cheviron ZA, Storz JF, McClelland, GB & Scott GR (2015). High-altitude ancestry and hypoxia acclimation have distinct effects on exercise capacity and muscle phenotype in deer mice. *Am J Physiol Regul Integr Comp Physiol* **308,** R779-R791.

Mathers KE & Staples JF (2015). Saponin-permeabilization is not a viable alternative to isolated mitochondria for assessing oxidative metabolism in hibernation. *Biol Open* **15,** 858-864.

Mathieu-Costello O (2001). Muscle adaptation to altitude: tissue capillarity and capacity for aerobic metabolism. *High Alt Med Biol* **2,** 413–425.

Mathieu-Costello O & Agey PJ (1997). Chronic hypoxia affects capillary density and geometry in pigeon pectoralis muscle. *Respir Physiol* **109,** 39-52.

Mathieu O, Krauer R, Hoppeler H, Gehr P, Lindstedt SL, Alexander RM, Taylor CR & Weibel ER (1981). Design of the mammalian respiratory system. VII. Scaling mitochondrial volume in skeletal muscle to body mass. *Respir physiol* **44,** 113-128.

McClelland GB, Hochachka PW & Weber JM (1998). Carbohydrate utilization during exercise after high-altitude acclimation: a new perspective. *Proc Natl Acad Sci USA* **95,** 10288-10293.

Mineo PM, Cassell EA, Roberts ME & Schaeffer PJ (2012). Chronic cold acclimation increases thermogenic capacity, non-shivering thermogenesis and muscle citrate synthase activity in both wild-type and brown adipose tissue deficient mice. *Comp Biochem Physiol A Mol Integr Physiol* **161,** 395-400.

Murphy MP (2009). How mitochondria produce reactive oxygen species. *Biochem J* **417,** 1-13.

Murray AJ & Horscroft JA (2016). Mitochondrial function at extreme high altitude. *J Physiol* **594,** 1137-1149.

Natarajan C, Hoffmann FG, Lanier HC, Wolf CJ, Cheviron ZA, Spangler ML, Weber RE, Fago A & Storz JF. (2015). Intraspecific polymorphism, interspecific divergence, and the

origins of function-altering mutations in deer mouse hemoglobin. *Mol Biol Evol* **32,** 978-997.

Pathi B, Kinsey ST, Howdeshell ME, Priester C, McNeill RS & Locke BR (2012). The formation and functional consequences of heterogeneous mitochondrial distributions in skeletal muscle. *J exp biol* **215,** 1871-1883.

Pathi B, Kinsey ST & Locke BR (2011). Influence of reaction and diffusion on spatial organization of mitochondria and effectiveness factors in skeletal muscle cell design. *Biotechnol bioeng* **108,** 1912-1924.

Pathi B, Kinsey ST & Locke BR (2013). Oxygen control of intracellular distribution of mitochondria in muscle fibers. *Biotechnol bioeng* **110,** 2513-2524.

Picard M, Ritchie D, Wright KJ, Romestaing C, Thomas MM, Rowan SL, Taivassalo T & Hepple RT (2010). Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. *Aging Cell*. **9,** 1032-1046.

Schippers MP, Ramirez O, Arana M, Pinedo-Bernal P, and McClelland GB (2012). Increase in carbohydrate utilization in high-altitude Andean mice. *Curr Biol* **22,** 2350-2354.

Scott GR, Egginton S, Richards JG, Milsom WK (2009). Evolution of muscle phenotype for extreme high altitude flight in the bar-headed goose. *Proc R Soc B* **276,** 3645-3653.

Scott GR, Elogio TS, Lui MA, Storz JF & Cheviron ZA (2015a). Adaptive modifications of muscle phenotype in high-altitude deer mice are associated with evolved changes in gene regulation. *Mol Biol Evol* **32,** 1962-1976.

Scott GR, Hawkes LA, Frappell PB, Butler PJ, Bishop CM & Milsom WK (2015b). How bar-headed geese fly over the Himalayas. *Physiology* **30,** 107-115.

Scott GR & Milsom WK (2006). Flying high: a theoretical analysis of the factors limiting exercise performance in birds at altitude. *Respir Physiol Neurobiol* **154,** 284 – 301.

Scott GR, Richards JG & Milsom WK (2009b). Control of respiration in flight muscle from the high-altitude bar-headed goose and low-altitude birds. *Am J Physiol Regul Integr Comp Physiol* **297,** R1066-R1074.

Sillau AH, Aquin L, Lechner A J, Bui MV & Banchero N (1980). Increased capillary supply in skeletal muscle of guinea pigs acclimated to cold. *Respir physiol* **42,** 233-245.

Snyder LRG, Born S & Lechner AJ (1982). Blood oxygen affinity in high- and low-altitude populations of the deer mouse. *Respir Physiol* **48,** 89-105.

Spinazzi M, Casarin A, Pertegato V, Salviati L & Angelini C (2012). Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. *Nat protoc* **7,** 1235-1246.

Spurr AR (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res **26***, 31-43.

Storz JF, Scott GR & Cheviron ZA (2010). Phenotypic plasticity and genetic adaptation to high- altitude hypoxia in vertebrates. *J Exp Biol* **213,** 4125-4135.

Suarez RK, Lighton JR, Brown GS & Mathieu-Costello O (1991). Mitochondrial respiration in hummingbird flight muscles. *Proc Natl Acad Sci USA* **88,** 4870-4873.

Treberg JR, Quinlan CL & Brand MD (2010). Hydrogen peroxide efflux from muscle mitochondria underestimates matrix superoxide production - a correction using glutathione depletion. *FEBS J* **277,** 2766-2778.

Ventura-Clapier R, Kuznetsov A, Veksler V, Boehm E & Anflous K (1998). Functional coupling of creatine kinases in muscles: species and tissue specificity. *Mol cell biochem* **184,** 231-247.

Weibel ER (1979). *Stereological Methods*. Academic Press, Toronto.

Weibel ER, Bacigalupe LD, Schmitt B & Hoppeler H (2004). Allometric scaling of maximal metabolic rate in mammals: muscle aerobic capacity as determinant factor. *Respir Physiol Neurobiol **140,*** 115-132.

Welch KC, Altshuler DL & Suarez RK (2007). Oxygen consumption rates in hovering hummingbirds reflect substrate-dependent differences in P/O ratios: carbohydrate as apremium fuel. *J Exp Biol* **210,** 2146-2153.

West JB, Boyer SJ, Graber, DJ, Hackett PH, Maret KH, Milledge JS, Peters RM, Pizzo, CJ, Samaja M & Sarnquist FH (1983). Maximal exercise at extreme altitudes on Mount Everest. *J Appl Physiol* **55,** 688-698.

Wickler SJ (1980). Maximal thermogenic capacity and body temperatures of white-footed mice (Peromyscus) in summer and winter. *Physiol Zool* **53,** 338-346.

Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, Gonzalez FJ & Semenza GL (2008). Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem* **283,** 10892-10903.

CHAPTER 3: HIGH-ALTITUDE ANCESTRY ALTERS MUSCLE PHENOTYPE AND THE PLASTICITY OF SKELETAL MUSCLE MITOCHONDRIA IN CHRONIC COLD AND/OR HYPOXIA IN DEER MICE

3.1 INTRODUCTION

High altitude environments impose multiple stressors on animals. Two of the major stressors for endothermic animals are hypoxia and cold. Hypoxia at high altitudes makes it hard for lowland species to maintain basal metabolism and the addition of cold increases the oxygen demand to maintain body temperatures. However, many species have adapted to living at high altitudes, despite the low oxygen availability and high oxygen demands. Studying high altitude species will provide insight on how the oxygen transport system of these animals has evolved to overcome the challenges of living in cold hypoxic environments. Animals can enhance oxygen transport at different levels in the oxygen transport cascade (breathing, pulmonary O_2 diffusion, circulatory O_2 delivery, tissue O_2 diffusion, and mitochondrial O_2 utilization (Weibel, 1984; Storz et al., 2010). Mitochondria are the organelles that ultimately use oxygen; however, there has been limited research on how adaptations and/or plasticity of mitochondrial O_2 utilization may enhance performance at high altitudes. Studying the effects of chronic hypoxia, chronic cold and the combination of chronic hypoxia and cold will help tease apart the effects of both stressors and help determine whether the two stressors act synergistically or antagonistically.

Cold exposure in endothermic animals induces both non-shivering thermogenesis and shivering thermogenesis. Non-shivering thermogenesis could occur by mitochondrial uncoupling. It has been suggested that mitochondrial uncoupling can occur in skeletal

muscle via adenine nucleotide translocators (ANT), fatty acids, or changes in mitochondrial membrane composition (Toyomizu et al., 2002; Wijers et al., 2008). Non-shivering thermogenesis may also occur by via futile cycling of the sarco-endoplasmic calcium ATPase (SERCA). Binding of sarcolipin to SERCA leads to uncoupling of SERCA such that ATP is consumed, but calcium is not transported and this futile cycling generates heat (Maurya and Periasamy, 2015). Shivering is a large component of thermogenic capacity in deer mice. At the whole animal level acclimating mice to cold increases maximal aerobic capacity in mammals. Deer mice that were acclimated to cold increased maximal oxygen consumption (VO_{2max}) rates during exercise and thermogenesis. Prior to cold acclimation VO_{2max} during exercise was higher than during thermogenesis, but after cold acclimation VO_{2max} was higher during thermogenesis (Hayes and Chappell, 1986). Similarly, adult white-footed mice that were tested after being acclimatized to winter temperatures had a thermogenic VO_{2max} that was 70% higher than mice that were acclimatized to summer temperatures (Wickler, 1980). An increase in maximal aerobic capacity can be achieved by increasing oxygen delivery or oxidation capacity.

Acclimation of animals to cold has been shown to increase capillarity density, oxidative capacity of skeletal muscle and intracellular lipid droplets presumably to increase the capacity of muscle thermogenesis. The increase in capillary density would help provide more oxygen to mitochondria of shivering muscles. Guinea pigs that were

acclimated to chronic cold (5°C) increased capillary supply to soleus and gastrocnemius muscles (Sillau, 1980). White-footed mice that were acclimatized to winter versus summer temperatures had a higher capillary density (Wickler, 1980). An increase in skeletal muscle oxidative capacity can help provide a continuous supply of ATP aerobically to support thermogenesis or locomotion. Some animals increase oxidative capacity by increasing the amount of oxidative fibres. An increase in the number of oxidative fibres increases the total abundance of mitochondria in the tissue, which leads to an increase in aerobic metabolic capacities. Mice that were acclimated to cold had higher citrate synthase activity in triceps muscles compared to the warm acclimated mice (Mineo et al., 2012). Shivering can occur for a long time, so an increase in lipid droplets may help fuel shivering. Acclimation to cold can also increase the amount of lipid droplets within muscles (Herpin and Lefaucheur, 1992). Lipids should be the fuel of choice if oxygen is not limited during shivering since they provide more ATP per mole of fuel and because there are larger stores of lipids compared to carbohydrates. Whether skeletal muscle mitochondria are involved in non-shivering thermogenesis continues to be debated. It has been observed in human skeletal muscle that cold exposure leads to uncoupling of mitochondria, which results in heat production rather than ATP (Wijers et al., 2008).

Maximal oxygen consumption rate (VO_{2max}) of an animal is lower when measured in hypoxia than when measured in normoxia, but hypoxia acclimation increases aerobic

capacity. High and low altitude deer mice that were acclimated to normoxia had lower maximal oxygen consumption when measured in hypoxia than when measured in normoxia. However, after hypoxia acclimation both high and lowland deer mice increased maximal oxygen consumption rates in hypoxia (Lui et al., 2015). Hypoxia acclimation helps increase aerobic capacity in hypoxia in lowland natives.

Acclimation of lowland natives to hypoxia has resulted in mixed results in tissue oxidative capacity. In humans acclimation to hypoxia has led to muscle atrophy and a maintenance in capillary density, such that capillary to fibre ratio increased (Green et al., 1989; MacDougall et al., 1991). Muscle atrophy could be the result of poor appetite and lethargy (Murray and Horscroft, 2015). There was no change in fibre type distribution, but there were decreases in succinate dehydrogenase, citrate synthase and hexokinase activity (Green et al., 1989). In non-human mammals, acclimation of lowland rodents to hypoxia suggest that there is little effect on the oxidative capacity of muscle (Beaudry and McClelland, 2010; León-Velarde et al., 1993; Lui et al., 2015; Lau et al., 2017; Mathieu-Costello and Agey, 1997). Mitochondria from soleus muscle of mice that were acclimated to hypoxia had lower respiration rates (Gamboa and Andrade, 2012). The response of lowland mammals to hypoxia generally has no effect or a decrease in oxidative capacity of skeletal muscles. However, an increase in oxidative capacity in response to hypoxia acclimation via an increase in mitochondrial respiratory capacity would help counteract

the inhibitory effects of hypoxia since there is a hyperbolic relationship between oxygen tension and mitochondrial respiration rate (Gnaiger, 2001; Hochachka, 1985).

Studying high altitude natives provides some indication of the combined influence of cold and hypoxia. Most studies on high altitude natives and acclimatization studies have been conducted on humans. The human studies suggest that there is a decrease in muscle oxidative capacity matched by a decrease in respiratory capacity of mitochondria (Levett et al., 2012, Jacobs et al., 2012). However, other high altitude animals such as guinea pigs, bar headed geese and deer mice have muscles with higher oxidative capacities (Barrie et al., 1975; Scott, 2009; Cheviron et al., 2012; Cheviron et al., 2014). High altitude natives also had higher capillary densities. Higher muscle oxidative capacities would be beneficial at high altitudes because it can help counteract impairments in respiration induced by intracellular hypoxia (Hochachka, 1985). The effect of cold and hypoxia on skeletal muscle mitochondrial function has not been examined.

In this chapter I tested whether the skeletal muscles of high altitude deer mice have mitochondrial adaptations that help improve performance in cold or hypoxia, and whether acclimation to cold and hypoxia has an antagonistic or synergistic effect on muscle phenotype. I specifically tested the following predictions: 1) skeletal muscles of highland mice will be more oxidative compared to muscles of lowland mice, 2) hypoxia acclimation will increase oxidative capacity in lowland mice, but high altitude deer mice will not have phenotypic plasticity in response to hypoxia, 3) cold acclimation will

increase capillary density, oxidative capacity, lipid oxidation capacity and uncoupling of mitochondria in both high and lowland deer mice since both populations experience fluctuations in temperatures, 4) cold + hypoxia acclimation will increase oxidative capacity to a greater extent than cold or hypoxia alone in lowland mice, but there will be no change in high altitude deer mice.

3.2 METHODS

3.2.1 Experimental Animals

Captive breeding populations of high- and low-altitude deer mice were established as previously described (Cheviron et al., 2012; Lui et al., 2015, Lau et al., 2017). High-altitude mice (*P.m. rufinus*) were trapped on the summit of Mt. Evans (Clear Creek County, Colorado; 39°35’18’’N, 105°38’38’’W) at ~4300m above sea level. Low-altitude deer mice (*P.m.nebracensis*) were trapped in Nine-Mile Prairie (Lancaster County, Nebraska; 40°52’12’’N, 96°48’20.3’’W) at ~400 m above sea level. Populations were bred separately in captivity to produce F1 progeny, which were raised to adulthood in normoxia in common laboratory conditions with a constant temperature (25 °C) and light cycle (12L:12D). Mice were provided chow and water *ad libitum*. All animal care and experimentation followed the guidelines established by the Canadian Council on Animal Care, and were approved by the McMaster University Animal Research Ethics Board.

3.2.2 Experimental Design

Adult F1 mice from each population were randomly assigned to one of four acclimation groups for a period of eighteen to twenty weeks: 1) normobaric normoxia in standard lab room conditions; 2) hypobaric hypoxia in hypobaric chambers set to an ambient pressure of 60 kPa (simulating the pressure at an altitude of ~4300 m elevation); 3) cold normoxia at a constant temperature of 5°C; 4) cold hypobaric hypoxia in hypobaric chambers set to an ambient pressure of 60 kPa and the room was set to a constant temperature of 5°C. For the cold normoxia and cold hypobaric hypoxia groups the mice were moved to a cold room that was at 18°C. The temperature was gradually dropped by 6°C daily until a final temperature of 5°C was reached. For the hypobaric hypoxia and cold hypobaric hypoxia groups the atmospheric pressure was gradually decreased over the first 3 days in the hypoxia group, and kept at that pressure for the remainder of the acclimation period, using previously described methods and custom-made hypobaric chambers (McClelland et al., 1998). The cages were cleaned every 3-4 days, which required that the hypobaric chambers be returned to normobaria for a brief period (< 1 h).

3.2.3 Histology

Mice were anesthetized using an isoflurane and then euthanized by cervical dislocation. One gastrocnemius muscle was coated in embedding medium and frozen in isopentane that was cooled using liquid nitrogen. The frozen samples were stored at -

80°C. 10µm sections were cut transverse to the direction of the muscle fibers using a cryostat set to -20°C. Oxidative muscle fibers (both slow and fast) were identified by staining for succinate dehydrogenase activity. The stain was carried out for 1 hour at room temperature in the following condition: 0.6 mM nitroblue tetrazolium, 2.0 mM KH_2PO_4, 15.4 mM Na_2HPO_4, and 16.7 mM sodium succinate. Capillaries in the gastrocnemius muscle were identified by staining for alkaline phosphatase activity. The stain was carried out for 1 hour at room temperature in the following condition: 1.0 mM nitroblue tetrazolium, 0.5 mM 5-bromo-4-chloro-3-indoxyl phosphate, 28 mM $NaBO_2$, and 7 mM $MgSO_4$ at a pH 9.3. 15 images were collected using a light microscope ensuring that images represented the entire gastrocnemius. Numerical densities and areal densities of oxidative and fast glycolytic fibres were determined from stains of succinate dehydrogenase activity, using stereological methods that have been well described (Weibel, 1979; Egginton, 1990). Measures of capillarity were made using NIS elements program which identifies all the capillaries in an image. Capillary density was calculated by dividing the total number of capillaries in an image by the total area of the image. Capillary surface density was calculated by dividing the sum of the perimeter of the capillaries and dividing by the total area of the image. Capillary to fibre ratios were calculated by dividing the total number of total capillaries by the total number of muscle fibres in an image. The index of tortuosity was calculated by dividing the average area of each independent capillary by the average area of the smallest 10% of all the capillaries.

The smallest capillaries represent those that are sectioned transversely and the larger ones are not included because they are tortuous (Mathieu-Costello, 1987).

3.2.4 Mitochondrial Respiratory Capacities of Isolated Mitochondria

Mice were anesthetized using isoflurane and then euthanized by cervical dislocation. The muscles from the entire left hindlimb (including the gastrocnemius and soleus) were removed (~250-400 mg), and immediately transferred to 10 ml of ice-cold isolation buffer (in mM: 100 sucrose, 50 tris base, 5 $MgCl_2$, 5 EGTA, 100 KCl, 1 ATP). The muscle was minced and then digested for 5 min in the same buffer containing protease (1 mg g^{-1} muscle tissue). The digested muscle was then gently homogenized with six passes of a Potter-Elvehjem Teflon on glass homogenizer (100 rpm). Mitochondria were isolated via differential centrifugation at 4°C. Briefly, homogenates were centrifuged at 700g for 10 min. The resulting supernatant was filtered through cheesecloth and then centrifuged at 1000g for 10 min. The supernatant was centrifuged at 8700g for 10 min and the pellet was resuspended in 10 ml of isolation buffer with bovine serum albumin (BSA, fatty acid-free, at 1% mass:volume) and centrifuged at 8700g. The pellet was resuspended in 10 ml of storage buffer (in mM: 0.5 EGTA, 3 $MgCl_2$, 60 K-methanesulphonate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose, 0.02 vitamin E succinate, 2 pyruvate, 2 malate, pH 7.1) and centrifuged at 8700g. The pellet was finally resuspended in 250-400 µl of storage buffer. Part of this mitochondrial suspension was

kept on ice until mitochondrial physiology was measured, and the rest was homogenized in a glass tissue grinder and stored at -80°C for later use in enzyme assays (see below).

The physiology of isolated mitochondria was measured using high-resolution respirometry and fluorometry (Oxygraph-2k with O2k-Fluorescence module, Oroboros Instruments, Innsbruck, Austria). Isolated mitochondria (approximately 40 µg mitochondrial protein) were added to the respirometry chamber with a final volume of 2 ml of respiration buffer (in mM: 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose, 1 g l^{-1} fatty acid-free BSA; pH 7.1) at 37°C. Leak respiration was stimulated with 5 mM pyruvate and 2 mM malate (L_N). P/O ratios (ATP produced per oxygen atom consumed) were obtained twice with additions of 125 µM of ADP. The respiratory control ratio (RCR) was calculated as the ratio of P_{PM}/L_T immediately before and after this saturating addition of ADP. After each addition of ADP was consumed, mitochondria reached leak state respiration (L_T). Oxidative phosphorylation respiration in the presence of 5 mM pyruvate and 2 mM malate was stimulated *via* multiple complexes of the electron transport system with progressive additions of 2 mM ADP ($P_{PM,}$ complex I), 20 mM of glutamate ($P_{PMG,}$ complex I), 20 mM of succinate ($P_{PMGS,}$ complexes I+II), and 0.5 µM rotenone ($P_{S(Rot),}$ complex II) waiting at least 2 min between each addition until a stable respiration rate could be measured. The chamber was then re-oxygenated and cytochrome c oxidase (complex IV) was maximally stimulated with the addition of 1.25 mM ADP, 2 mM ascorbate, and 0.5 mM TMPD

(N,N,N′,N′-tetramethyl-p-phenylenediamine) (P_{Tm}). The second protocol measured the capacity for fatty acid oxidation. For the second protocol, isolated mitochondria (approximately 40 µg mitochondrial protein) were added to the respirometry chamber with a final volume of 2 ml of respiration buffer. Oxidative phosphorylation respiration was stimulated with 2 mM malate, 40 µM palmitoyl-carnitine and 2 mM ADP followed by 0.2 mM octanoyl-carntine, waiting at least 2 min between each addition until a stable respiration rate could be measured.

3.2.5 Enzyme Assays

3.2.5.1 Gastrocnemius Muscle

Mice were anesthetized using isoflurane and then euthanized by cervical dislocation. One gastrocnemius muscle was frozen in liquid nitrogen and stored in -80°C. The muscle was powdered using a mortar and pestle under liquid nitrogen and then stored in -80°C. This powder was homogenized on ice using a glass on glass homogenizer in 20 volumes of homogenization buffer (concentrations in mM: 100 KH2PO4, 5 ethylenediaminetetraacetic acid, and 0.1% Triton-X-100). Apparent V_{max} of cytochrome oxidase (COX) and citrate synthase (CS) were measured at 37°C as previously described (Cheviron et al., 2012) using a Spectromax Plus 384 microplate reader (Molecular Devices). COX activity was measured shortly after homogenization. CS was measured after three freeze thaw cycles. Assays were conducted under the following conditions (in mM unless stated otherwise): COX, 0.2 reduced cytochrome c in 100 KH_2PO_4 (pH 7.0);

CS, 0.22 acetyl-CoA, 0.15 dithiobisnitrobenzoic acid (DTNB), 0.5 oxaloacetate (omitted for background) in 40 Tris-HCl, (pH 8.0).

3.2.5.2 Isolated Mitochondria

Apparent V_{max} of mitochondrial complex IV and citrate synthase (CS) were measured in isolated mitochondria at 37°C as previously described (Spinazzi et al., 2012) using a Spectromax Plus 384 microplate reader (Molecular Devices). Complex IV was measured after a single freeze/thaw cycle in isotonic buffer (100 mM KCl_2; 25 mM KH_2PO_4; 5 mM $MgCl_2$). CS was measured after a third freeze-thaw cycle (in 100 mM Tris, pH 8). Assays were carried out in the following conditions (in mM, unless stated otherwise): Complex IV, 0.2 reduced cytochrome c, and 0.3 KCN in 50 KH_2PO_4 (pH 7.0); CS, 0.22 acetyl-coA, 0.15 DTNB, 0.5 oxaloacetate, 0.1% vol/vol Triton x-100, in 100 Tris (pH 8.0)

3.3 Statistical Analysis

Data are presented as means ± SE. Two-factor ANOVA and Bonferroni multiple-comparisons post-tests were used as appropriate to determine the main effects of and interactions between population altitude and acclimation environment. A significance level of $P < 0.05$ was used throughout.

3.4 RESULTS

3.4.1 Fibre Type

The gastrocnemius muscle of highland deer mice had a more oxidative fibre composition than that of lowland deer mice (Figure 3.1A,C). The numerical and areal densities were ~15% ($F_{1,46} = 16.017$; $P < 0.001$) and ~25% ($F_{1,46} = 19.703$; $P < 0.001$) higher, respectively, in highland mice compared to lowland mice. There was no main effect of acclimation or interaction effect of acclimation × population in the numerical density (acclimation, $F_{1,46} = 2.266$; $P = 0.093$; acclimation × population, $F_{1,46} = 2.565$; $P = 0.066$) or areal density of oxidative fibres (acclimation, $F_{1,46} = 2.048$; $P = 0.120$; acclimation × population, $F_{1,46} = 2.061$; $P = 0.118$) in two-way ANOVA. Furthermore, there were no pairwise differences detected using Bonferroni post-tests between acclimation environments in the numerical or areal densities of oxidative fibres in highland mice. However, numerical densities of oxidative fibres increased after acclimation to cold ($p = 0.035$) and cold + hypoxia ($p = 0.031$) compared to normoxia acclimation in lowland deer mice. Similarly, lowland mice increased areal density of oxidative fibres after acclimation to cold + hypoxia ($p = 0.041$) and the increase was almost significant after cold acclimation ($p = 0.068$) compared to normoxia acclimated lowland mice. The exact opposite trends were observed in numerical and areal densities of glycolytic fibres (Figure 3.1B,D).

The mass of the gastrocnemius muscle (population, $F_{1,46} = 24.331$; $P < 0.001$;

acclimation, $F_{1,46} = 1.067$; $P = 0.372$; acclimation × population, $F_{1,46} = 2.247$; $P = 0.095$) (Figure 3.2A) and the transverse area of the gastrocnemius muscle (population, $F_{1,46} = 7.613$; $P = 0.008$; acclimation, $F_{1,46} = 1.005$; $P = 0.399$; acclimation × population, $F_{1,46} = 1.107$; $P = 0.356$) (Figure 3.2B) were similar or higher in lowland mice. Both the mass and the transverse area of the gastrocnemius muscle did not differ with acclimation in either population when tested using Bonferroni post-tests between acclimation environments. The total number of oxidative fibers in the gastrocnemius muscle did not differ between the two populations or with acclimation (population, $F_{1,46} = 1.605$; $P = 0.212$; acclimation, $F_{1,46} = 1.181$; $P = 0.327$; acclimation × population, $F_{1,46} = 1.939$; $P = 0.137$) (Figure 3.2C). The total number of glycolytic fibers in the gastrocnemius muscle was higher in the lowland deer mice but there was no change with acclimation (population, $F_{1,46} = 24.462$; $P < 0.001$; acclimation, $F_{1,46} = 1.528$; $P = 0.220$; acclimation × population, $F_{1,46} = 0.733$; $P = 0.538$) (Figure 3.2D). The oxidative fibres of highland mice and lowland mice acclimated to normoxia or hypoxia were the same size, but the highland mice had larger oxidative fibres than lowland mice after cold and cold + hypoxia acclimation, but there was no main effect of acclimation (population, $F_{1,49} = 15.909$; $P < 0.001$; acclimation, $F_{1,49} = 0.578$; $P = 0.632$; acclimation × population, $F_{1,49} = 4.561$; $P = 0.007$) (Figure 3.2E). In lowland mice there were no pairwise differences detected using Bonferroni post-tests between acclimation environments in the size of oxidative fibres with acclimation. In highland mice the size of the oxidative fibres were similar between

all the acclimations, except cold acclimated mice had significantly larger oxidative fibres compared to hypoxia acclimated mice. The size of glycolytic fibres did not differ between the populations or with acclimation (population, $F_{1,48} = 0.812$; $P = 0.372$; acclimation, $F_{1,48} = 0.381$; $P = 0.767$; acclimation × population, $F_{1,48} = 2.016$; $P = 0.124$) (Figure 3.2F).

3.4.2 Capillarity

The capillarity of the gastrocnemius muscle of highland mice is greater than in lowland mice (Figure 3.3). Capillarity of the gastrocnemius muscle was quantified using capillary surface density (population, $F_{1,43} = 22.956$; $P < 0.001$; acclimation, $F_{1,43} = 0.328$; $P = 0.805$; acclimation × population, $F_{1,43} = 2.869$; $P = 0.047$), capillary density (population, $F_{1,43} = 28.673$; $P < 0.001$; acclimation, $F_{1,43} = 1.407$; $P = 0.254$; acclimation × population, $F_{1,43} = 2.550$; $P = 0.068$), capillary per muscle fibre (population, $F_{1,37} = 54.966$; $P < 0.001$; acclimation, $F_{1,37} = 1.888$; $P = 0.148$; acclimation × population, $F_{1,37} = 2.274$; $P = 0.096$), and using an index of tortuosity (population, $F_{1,38} = 40.816$; $P < 0.001$; acclimation, $F_{1,40} = 1.703$; $P = 0.183$; acclimation × population, $F_{1,40} = 1.878$; $P = 0.150$). All four indices of capillarity were higher in the high altitude mice. There was very little change in the measures of capillarity in response to acclimation. In the lowland mice there were hardly any differences in the measures of capillarity with acclimation, except there was a significant increase in capillary density after acclimation to cold in comparison to normoxia ($p = 0.043$). Similarly highland mice did not change measures of capillarity in response to acclimation. Highland mice had a higher capillary to fibre ratio

after cold acclimation compared to hypoxia and cold + hypoxia acclimations and the index of tortuosity differed between cold and hypoxia acclimations.

3.4.3 Tissue and Mitochondrial Oxidative Enzyme Activity

The gastrocnemius tissue of highland was more oxidative indicated by COX activity being ~31% higher (population, $F_{1,54} = 13.258$; $P < 0.001$; acclimation, $F_{1,54} = 12.647$; $P < 0.001$; acclimation × population, $F_{1,54} = 2.655$; $P = 0.058$) and CS activity being ~42% higher (population, $F_{1,55} = 25.955$; $P < 0.001$; acclimation, $F_{1,55} = 8.012$; $P < 0.001$; acclimation × population, $F_{1,55} = 2.097$; $P = 0.111$) (Figure 3.4A,B). Pairwise differences were detected using Bonferroni post-tests between acclimation environments. COX activity was higher in highland population acclimated to hypoxia or cold. CS activity was higher in highland mice acclimated to hypoxia and cold + hypoxia. Hypoxia acclimation did not change COX activity or CS activity in comparison to normoxia acclimated mice in either population. In highland mice, COX and CS activity increased significantly in comparison to normoxia acclimation after acclimation to cold and cold + hypoxia. In lowland mice, COX activity increased after cold + hypoxia acclimation compared to normoxia.

Highland mice had higher COX activity per mg of mitochondrial protein compared to lowland mice in all acclimation environments, except after hypoxia acclimation (population, $F_{1,50} = 24.828$; $P < 0.001$; acclimation, $F_{1,50} = 3.896$; $P = 0.014$; acclimation × population, $F_{1,50} = 2.899$; $P = 0.044$) (Figure 3.4C,D). Acclimation did

cause slight changes in COX activity per mg of mitochondrial protein, but only the decrease in activity after acclimation to cold + hypoxia in highland was significant using Bonferroni post-tests between acclimation environments significant. The mitochondria of highland mice had higher CS activity per mg of mitochondrial protein in normoxia and cold acclimations. (population, $F_{1,52} = 15.274$; $P < 0.001$; acclimation, $F_{1,52} = 5.074$; $P = 0.004$; acclimation × population, $F_{1,52} = 5.367$; $P = 0.003$). Pairwise differences were detected using Bonferroni post-tests between acclimation environments. In highland mice, acclimation to hypoxia, cold, or cold + hypoxia did not alter CS activity per mg of mitochondrial protein in comparison to normoxia acclimated highland deer mice, but cold acclimated had mice higher CS activity compared to hypoxia acclimated mice. Lowland mice acclimated to hypoxia, cold, or cold + hypoxia had similar activity per mg of mitochondrial protein, which was significantly higher than normoxia acclimated lowland mice.

3.4.4 Mitochondrial Respiration

3.4.4.1 Mitochondrial Efficiency

The mitochondria of highland mice acclimated to normoxia are of better quality compared to lowland deer mice, but they are similar in all other acclimations. Highland mice have mitochondria that are more coupled in normoxia compared to lowland mice indicated by higher RCR values and there was a significant main effect of acclimation (population, $F_{1,53} = 6.337$; $P = 0.015$; acclimation, $F_{1,53} = 15.829$; $P < 0.001$; acclimation

× population, $F_{1,53} = 1.455$; $P = 0.237$) (Figure 3.5A). Hypoxia acclimation did not change RCR values compared to normoxia acclimation in high and lowland deer mice. Highland mice had significantly lower RCR values after acclimation to cold and cold + hypoxia compared to both hypoxia and normoxia acclimation. Lowland deer mice showed a similar trend but RCR values were only significantly lower after cold and cold + hypoxia in comparison to hypoxia acclimation. The second measure of mitochondrial quality was P/O ratios which is an indication of how efficiently mitochondria use oxygen. Highland mice have mitochondria that are more efficient at producing ATP for given amount of oxygen in normoxia and there was a significant effect of acclimation in both populations (population, $F_{1,51} = 4.980$; $P = 0.030$; acclimation, $F_{1,51} = 7.043$; $P < 0.001$; acclimation × population, $F_{1,51} = 1.450$; $P = 0.239$) (Figure 3.5B). Acclimation to hypoxia and hypoxia + cold did not change P/O ratios in either population compared to normoxia acclimation. The highland mice had lower P/O ratios after acclimation to cold in comparison to normoxia and hypoxia acclimations. P/O ratios did not change as much in the lowland deer mice, but the P/O ratios after cold acclimation were lower compared to lowland mice acclimated to hypoxia. Leak state respiration rates in the presence ATP (L_T) were similar between populations, except highland mice had higher rates after cold acclimations compared to cold acclimated lowland mice. There was a main effect of population and a main effect of acclimation in leak state respiration rates (population, $F_{1,55} = 6.302$; $P = 0.015$; acclimation, $F_{1,55} = 22.415$; $P < 0.001$; acclimation × population, $F_{1,55} = 4.371$; $P =$

0.008) (Figure 3.5C). Leak state respiration rates did not differ between normoxia and hypoxia acclimation in either population. Leak state respiration rates were significantly higher in cold and cold + hypoxia compared to normoxia and hypoxia acclimation in both populations.

3.4.4.2 Mitochondrial capacity to oxidize carbohydrates and fatty acids

The ability to oxidize carbohydrates and fatty acids was measured indirectly by stimulating mitochondrial respiration with pyruvate (P_{PM} = malate + pyruvate + ADP), palmitoyl carnitine and octanoyl-carnitine (P_{MPal} = malate + palmitoyl-carnitine + ADP; $P_{MPalOct}$ = malate + palmitoyl-carnitine + octanoyl-carnitine + ADP) (Figure 3.6). Pyruvate stimulated respiration was higher overall in highland deer mice, but acclimation had different effects on respiration rates between populations (population, $F_{1,54} = 25.890$; $P < 0.001$; acclimation, $F_{1,54} = 3.541$; $P = 0.020$; acclimation × population, $F_{1,54} = 14.487$; $P < 0.001$). Highland mice had significantly lower respiration rates after acclimation to hypoxia compared to all other acclimations. Lowland mice had similar respiration rates after hypoxia, cold, and cold + hypoxia acclimation which was significantly higher than respiration rates of lowland mice acclimated to normoxia (Figure 3.6A). There was no difference in mitochondrial respiration rates stimulated by palmitoyl-carnitine compared to respiration rates stimulated by octanoyl-carnitine (Figure 3.6B,C). Highland mice had higher palmitoyl-carnitine stimulated respiration rates in normoxia and there was a main

effect of acclimation (population, $F_{1,51} = 4.965$; $P = 0.030$; acclimation, $F_{1,51} = 10.039$; $P < 0.001$; acclimation × population, $F_{1,51} = 1.973$; $P = 0.130$). Mice acclimated to hypoxia, cold and cold + hypoxia had similar respiration rates to mice acclimated to normoxia. Both high and lowland mice had higher fatty acid stimulated respiration rates after acclimation to cold in comparison to hypoxia acclimation (Figure 3.6B).

3.4.4.3 Mitochondrial respiratory capacities

Mitochondrial respiratory capacities were higher in highland mice compared to lowland deer mice. Maximum Complex I (CI) respiration was stimulated using malate + pyruvate + ADP + glutamate (P_{PMG}). Maximum CI respiration rates were higher in highland mice and there was a significant effect of acclimation that differed between populations (population, $F_{1,54} = 32.388$; $P < 0.001$; acclimation, $F_{1,54} = 3.578$; $P = 0.020$; acclimation × population, $F_{1,54} = 12.829$; $P < 0.001$) (Figure 3.7A). In highland mice, maximum CI respiration rates were similar in mice acclimated to normoxia, cold and cold + hypoxia, but respiration rates decreased after hypoxia acclimation. In lowland mice maximum CI respiration rates were similar in mice acclimated to hypoxia, cold, cold + hypoxia, which was significantly higher than mice acclimated to normoxia.

Maximum Complex II (CII) respiration was also higher in highland deer mice and there was a significant effect of acclimation that differed between populations (population, $F_{1,52} = 37.917$; $P < 0.001$; acclimation, $F_{1,52} = 7.002$; $P < 0.001$; acclimation × population, $F_{1,52} = 4.155$; $P = 0.010$) (Figure 3.7B). Maximum CII respiration was

stimulated using malate + pyruvate + ADP + glutamate + succinate + rotenone (P_{Srot}). In highland mice cold acclimation decreased maximum CII respiration rates compared to all other acclimations. Lowland mice acclimated to hypoxia significantly increased CII respiration rates compared to all other acclimations.

Maximum CI+II respiration rates were also higher in the highland mice (population, $F_{1,54} = 47.439$; $P < 0.001$; acclimation, $F_{1,54} = 1.911$; $P = 0.139$; acclimation × population, $F_{1,54} = 13.306$; $P < 0.001$) (Figure 3.7C). Maximum CI+II respiratory capacity was stimulated using malate + pyruvate + ADP + glutamate + succinate (P_{PMGS}). In highland mice maximum CI+II respiration rates were similar in mice acclimated to normoxia, cold and cold + hypoxia, but respiration rates decreased after hypoxia acclimation. In lowland mice maximum CI+II respiration rates were similar in mice acclimated to hypoxia, cold, cold + hypoxia, which was higher than mice acclimated to normoxia. The results were similar when complex IV was maximally stimulated artificially using ADP, ascorbate and TMPD (P_{Tm}) (population, $F_{1,51} = 18.388$; $P < 0.001$; acclimation, $F_{1,51} = 9.021$; $P < 0.001$; acclimation × population, $F_{1,51} = 8.817$; $P < 0.001$) (Figure 3.7D). Generally maximal respiratory capacities were higher in the highland mice compared to lowland mice in all the acclimations, except hypoxia acclimation where respiratory capacities converged.

3.5 DISCUSSION

At high altitudes, increased aerobic capacity is under directional selection in deer mice (Hayes and O'Connor, 1999). Our results suggest that high-altitude adaptation has resulted in F1 generation highland deer mice having a more oxidative muscle phenotype indicated by both high and lowland populations having the same number of oxidative fibres in the gastrocnemius muscle, but highlanders having a reduced abundance of glycolytic fibres, highland mice having higher capillarity and higher activity of oxidative enzymes. Measures of mitochondrial efficiency and respiratory capacities were also higher in highland mice. Cold and/or hypoxia acclimations had effects on muscle metabolism that were population specific; furthermore, these stressors had a complex interaction that was not entirely synergistic or antagonistic. These were F1 generation that were born and raised in common garden conditions; therefore, differences between the two populations are likely due to genetic differences between the two populations.

3.5.1 Fibre Type Distribution

The gastrocnemius muscle of highland mice was more oxidative. Highland deer mice had higher numerical and areal densities of oxidative fibres compared to lowland deer mice in normoxia and hypoxia, but these values converged after cold and cold + hypoxia acclimation due to a shift in fibre type distribution in the lowland mice. In lowland mice the increase in abundance of oxidative fibres was matched by increased CS or COX activity with cold or cold + hypoxia acclimation. Highland mice also had

increased CS and COX activity after cold and cold + hypoxia acclimation which suggests that there may have been a switch from fast oxidative to slow oxidative fibres, an increase in mitochondrial content within oxidative fibres or an increase in mitochondrial respiratory capacities, since the abundance of oxidative fibers was constant. The higher areal and numerical densities of oxidative fibres in highland deer are consistent with previous findings in wild deer mice (Scott et al., 2015), F1 generation deer mice (Lui et al., 2015) and high altitude birds (Scott et al., 2009). Highland mice had gastrocnemius muscles that were smaller than lowland mice. The numerical densities and the mass of the gastrocnemius muscle were used to calculate the total number of oxidative and glycolytic fibres. The total number of oxidative fibres in the gastrocnemius muscle did not differ between the two populations. However, the total number of glycolytic fibres was greater in lowland mice. Therefore, the proportion of oxidative fibres was higher in the highland mice compared to the lowland mice.

Mammals living in cold environments need to thermoregulate and an increase in oxidative fibres in cold environments can enhance shivering and non-shivering thermogenesis (Mineo et al., 2012; Sander et al., 2008). Fibre type distribution of highland mice was fixed, whereas lowland mice had the plasticity to increase the proportion of oxidative fibres after acclimation to cold and cold + hypoxia. These findings suggest that lowland deer mice maintain phenotypic plasticity in response to cold, but not in response to hypoxia. These findings also support Banchero's (1987)

argument that hypoxia alone does not stimulate changes in muscle structure; he suggested that changes occurred when hypoxia was coupled with cold or exercise. Cold acclimations have in fact increased the abundance of oxidative fibres in other mammals (Herpin and Lefaucheur, 1992; Behrens and Himms-Hagen, 1977). Furthermore, guinea pigs that were acclimated to hypoxia, cold, and cold + hypoxia, only increased the proportion of oxidative fibres in response to cold or cold + hypoxia acclimation (Jackson et al., 1987). Similarly, hypoxia acclimation in humans did not increase the abundance of oxidative fibers (Green et al., 1989).

3.5.2 Capillarity of the Gastrocnemius Muscle

Capillarity of the gastrocnemius muscle was higher in the highland mice. High altitude birds also have more capillaries compared to low altitude birds (Leon-Velarde et al., 1993; Scott et al., 2009). In both highland and lowland mice capillary surface density, capillary density, capillary to fiber ratio, and the index of tortuosity did not change in response to hypoxia acclimation. However in humans, acclimation and acclimatization to hypoxia usually results in muscle atrophy and no change in capillary density in skeletal muscles (Green et al., 1989; Hoppeler, 1990). In highland mice cold acclimation increased capillary to fiber ratio and cold + hypoxia acclimation increased the index of tortuosity. Indices of capillarity did not change much with acclimation in lowland mice, except that capillary density increased with cold acclimation. An increase in capillarity in hypoxic environments would help counteract hypoxia by increasing oxygen delivery to

muscles. In cold environments an increase in capillarity may be required in order to support the increase in oxidative fibers or to support heat generation by mitochondria that are uncoupled. In lowland mice the increase in capillary density likely supports the increase in numerical density of oxidative fibers in response to cold acclimation. Guinea pigs that were acclimated to cold also increased capillarity in the gastrocnemius and soleus muscles (Sillau et al., 1980). In highland mice the increase in capillary to fiber ratio and index of tortuosity can help counteract the increased uncoupling of mitochondria and less efficient use of oxygen to produce ATP. It has been previously shown that F1 highland mice acclimated to normoxia and hypoxia have enhanced capillarity to a greater extent than expected from having a greater abundance of oxidative fibers, which suggests that it is an adaptation that increases oxygen supply to counteract hypoxia (Lui et al., 2015). The higher capillarity of highland mice is most likely due to genetic adaptations to living at high altitudes, since lowland animals are not able to attain the same level of capillarity even with acclimation.

3.5.3 Mitochondrial Efficiency

The mitochondria of highland mice were better coupled and had higher P/O ratios than mitochondria of lowland deer mice. Coupled mitochondria are more efficient at producing ATP. RCR values were higher in highland mice after normoxia acclimation, but these values were not significantly different after hypoxia acclimation. Hypoxia acclimation did not significantly alter the coupling of mitochondria in either population.

Ensuring that mitochondria are coupled can be beneficial in a hypoxic environment where ATP production may be inhibited. Mitochondria that are better coupled with lower leak respiration rates are able to reduce the energy required to maintain the membrane potential of mitochondria (Gnaiger et al., 2000). RCR values and leak control ratios improved in response to 28 days of high altitude acclimatization in lowland native humans (Jacobs et al., 2012), but RCR and leak control ratios did not change with 9-11 days of acclimatization to high altitude (Jacobs et al., 2013). Cold and cold + hypoxia acclimations decreased mitochondrial coupling significantly in highland mice and lowland mice showed a similar trend. Similarly, leak state respiration rates increased after acclimation to cold and cold + hypoxia in both populations. Cold and cold + hypoxia acclimation decreased mitochondrial efficiency. The mitochondria of highland deer mice were more efficient at producing ATP per molecule of oxygen. P/O ratios were higher in highland mice after normoxia acclimation, but these values were not significantly different after hypoxia acclimation. The ability to maintain or slightly improve P/O ratios in a hypoxic environment can be crucial in order to use oxygen efficiently. P/O ratios were significantly lower in highland mice after cold acclimation, but P/O ratios in response to cold + hypoxia acclimation were similar to normoxic values. The decrease in P/O ratios in cold acclimated mice, along with decreased RCR values and increased leak state respiration rates most likely contribute to generation of heat in a cold environment, in order to maintain body temperatures. In human skeletal muscles it has been reported

that cold induced uncoupling of mitochondria is associated with adaptive thermogenesis (Sander et al., 2008). Cold acclimation decreases mitochondrial efficiency; therefore, improved mitochondrial efficiency of highland native deer mice is likely at adaptation to hypoxia at high altitudes.

3.5.4 Mitochondrial Fuel Selection

At the level of the mitochondria highland deer mice did not increase their capacity to oxidize carbohydrates and both populations did not decrease their capacity to oxidize fatty acids in response to hypoxia acclimation. In order to benefit from fuel selection, mice acclimated to hypoxia should use a greater proportion of carbohydrates relative to fatty acids to take advantage of the more efficient use of oxygen by carbohydrates in ATP production (Brand, 2005; Welch et al., 2007). Highland mice actually decreased both carbohydrate and fatty acid oxidation capacities. Lowland mice did increase carbohydrate oxidation capacity, but there was no change in fatty acid oxidation capacity. At the whole animal level highland deer mice that were acclimated to hypoxia increase the proportion of carbohydrates utilized when running at 75% of VO_{2max} (Lau et al. 2017). This suggests that highland mice increase carbohydrate use without changing mitochondrial fuel oxidation capacity. In cold environments it would be advantageous to increase the ability to oxidize fatty acids to support shivering thermogenesis, but both high and lowland animals did not significantly increase fatty acid oxidation capacities after cold or cold + hypoxic acclimations. Wild highland deer mice are able to oxidize lipids at a faster rate

compared to lowland deer mice (Cheviron et al., 2012). Furthermore, cold acclimations of lowland native mammals usually increase lipid oxidation capacity in mammals (Shabalina et al., 2010; Begin-Heick and Heick, 1977). The increase in lipid oxidation capacity may be due to increased rate of transport or availability of fatty acids upstream of mitochondria, which may explain the lack of change in fatty acid oxidation capacity in isolated mitochondria with acclimation.

3.5.5 Mitochondrial Respiratory Capacities

Mitochondrial respiratory capacities were higher in highland mice acclimated to normoxia, cold and cold + hypoxia. Highland mice significantly decreased mitochondrial respiratory capacities of CI, CI+CII and CIV in response to hypoxia acclimation, whereas, lowland mice significantly increased respiratory capacities. In highland mice cold and cold + hypoxia acclimation did not alter maximal respiratory capacities of CI, CI+CII or CIV. The reduction in respiratory capacity induced by hypoxia acclimation was recovered by cold acclimation in highland mice acclimated to both cold and hypoxia. In lowland mice, maximal respiratory capacities in CI, CI+CII and CIV were similar in hypoxia, cold and cold + hypoxia acclimated mice, which was significantly higher than respiratory capacities in normoxia acclimated mice. Interestingly, although both cold and hypoxia alone increased respiratory capacities, the combined stressor did not increase respiratory capacities to a greater extent. The changes in respiratory capacities of the mitochondria correspond with COX and CS activity within the mitochondria. In skeletal

muscles of humans acclimated to hypoxia or acclimatized to high altitude it resulted in a decrease in tissue respiratory capacities (Hoppeler et al., 1990; Jacobs et al., 2012; Jacobs et al., 2013), except in a recent study that maintained neutral energy balance (Jacobs et al., 2016).

An increase in respiratory capacity of individual mitochondria may increase the oxidative capacity of the tissue, which is advantageous in environments that are cold or hypoxic. In a hypoxic environment an increase in respiratory capacity of individual mitochondrion is beneficial since there is a hyperbolic relationship between oxygen tension and mitochondrial respiration rate (Gnaiger, 2001); having mitochondria with higher respiratory capacities can help counteract impairments in respiration induced by intracellular hypoxia (Hochachka, 1985). Cold environments increase ATP requirements to maintain body temperatures; mitochondria with higher respiratory capacities are able to produce ATP at higher rates. The increase in respiratory capacity of mitochondria in response to hypoxia, cold and cold + hypoxia acclimations in lowland mice would help improve performance of these mice. The decrease in respiratory capacities in highland mice in response to hypoxia acclimation was initially surprising, but consistent with findings in the first data chapter. These results suggest that the increase in mitochondrial respiratory capacity is advantageous in hypoxic or cold environments, since lowland mice do increase respiratory capacities in response to hypoxia acclimation. However, high altitude mice have mitochondria that are able to increase respiratory capacities beyond

that of lowland mice, which is apparent in normoxia, cold, and hypoxia + cold acclimated highland mice. The increased respiratory capacity of highland is likely an adaptive response to both hypoxia and cold at high altitudes.

3.6 CONCLUSION

High altitude environments increase the oxidative capacity of skeletal muscles. F1 generation high altitude deer mice have a more oxidative phenotype compared to lowland mice that is maintained even after hypoxia, cold and cold + hypoxia acclimation. Lowland deer mice increase the oxidative capacity of skeletal muscle via increases in mitochondrial respiratory capacity and/or increases in oxidative fibers in response to hypoxia or cold acclimations. The greater oxidative phenotype of highland mice likely explains the better whole animal performance of highland mice.

3.7 TABLES AND FIGURES

Figure 3.1

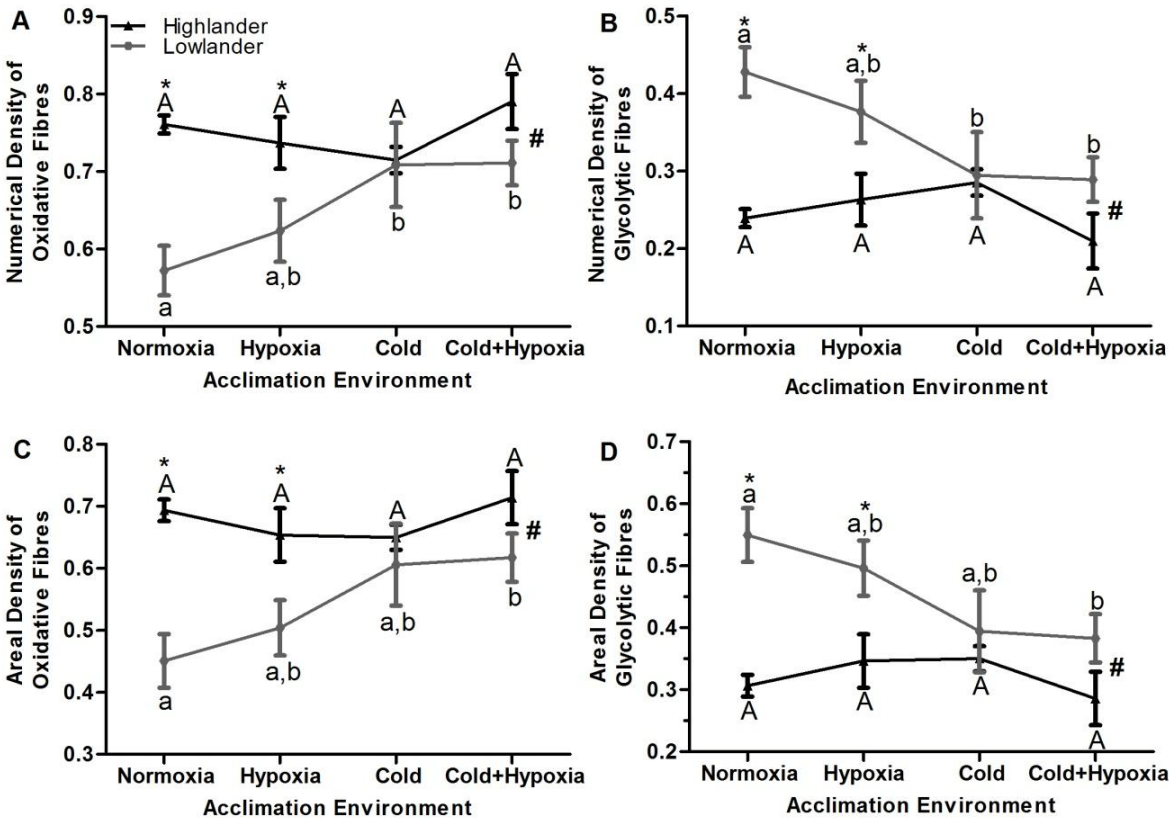

Figure 3.1: Highland mice had a more oxidative phenotype in the gastrocnemius muscle. The numerical density (A,B) and areal density (C,D) of oxidative and glycolytic fibres in the gastrocnemius muscle of high and lowland mice acclimated to normoxia, hypoxia, cold, and cold+hypoxia. # Significant main effect of population. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment. Different uppercase letters represent significant pairwise differences in response to acclimation within highland mice. Different lowercase letters represent significant pairwise differences in response to acclimation within lowland mice.

Figure 3.2

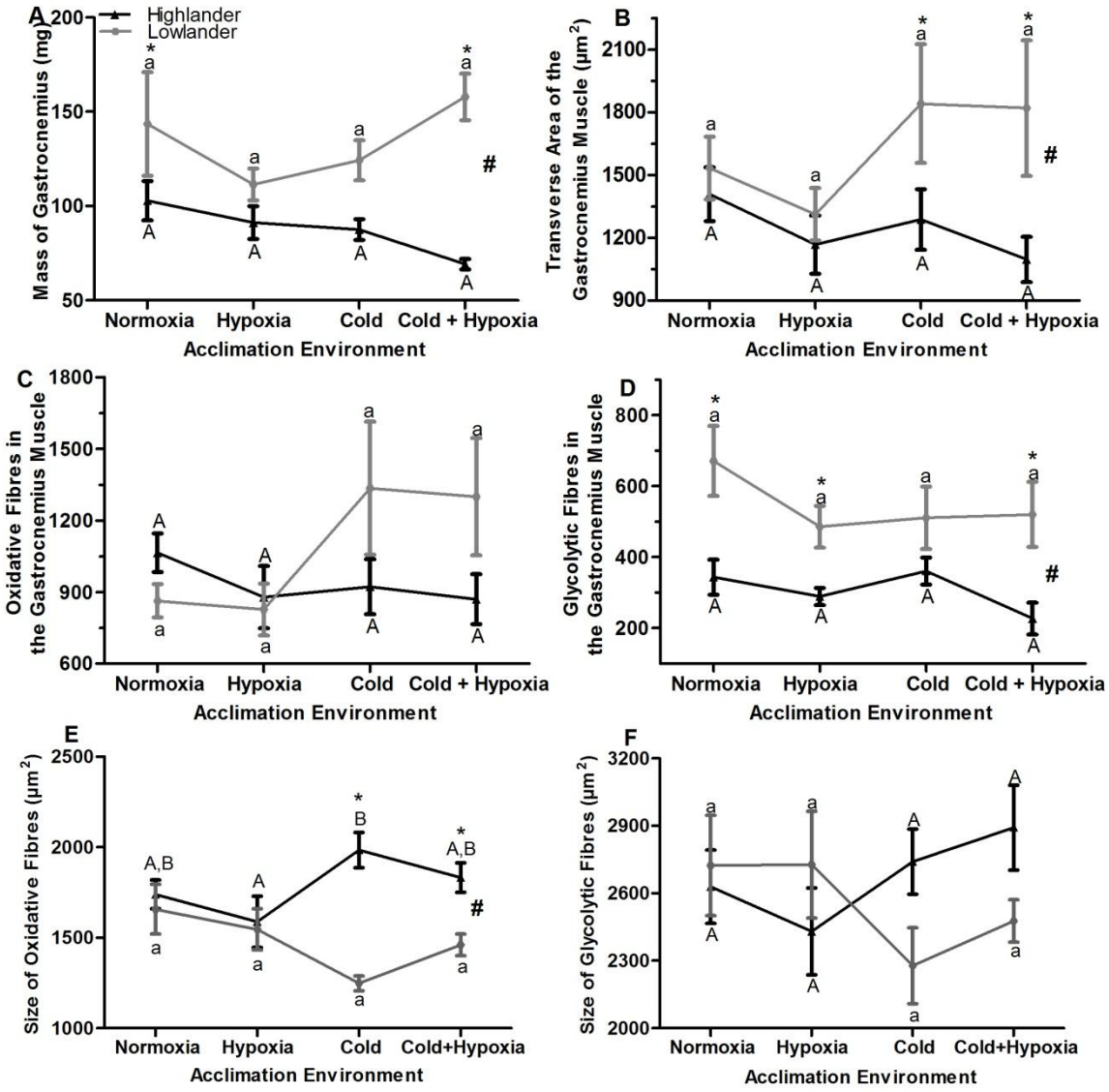
A
Highlander
Lowlander
Mass of Gastrocnemius (mg)
200
150
100
50
Normoxia
Hypoxia
Cold
Cold + Hypoxia
Acclimation Environment
B
Transverse Area of the Gastrocnemius Muscle (μm²)
2100
1800
1500
1200
900
C
Oxidative Fibres in the Gastrocnemius Muscle
1800
1500
1200
900
600
D
Glycolytic Fibres in the Gastrocnemius Muscle
800
600
400
200
E
Size of Oxidative Fibres (μm²)
2500
2000
1500
1000
Cold+Hypoxia
F
Size of Glycolytic Fibres (μm²)
3200
2900
2600
2300
2000

Figure 3.2 Muscle mass (A), total transverse area (B), total number of oxidative fibres (C), total number of glycolytic fibres (D), average size of individual oxidative fibres (E) and average size of individual glycolytic fibres (F) in the gastrocnemius muscle of high and lowland deer mice acclimated to to normoxia, hypoxia, cold, and cold+hypoxia. # Significant main effect of population. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment. Different uppercase letters represent significant pairwise differences in response to acclimation within highland mice. Different lowercase letters represent significant pairwise differences in response to acclimation within lowland mice.

Figure 3.3

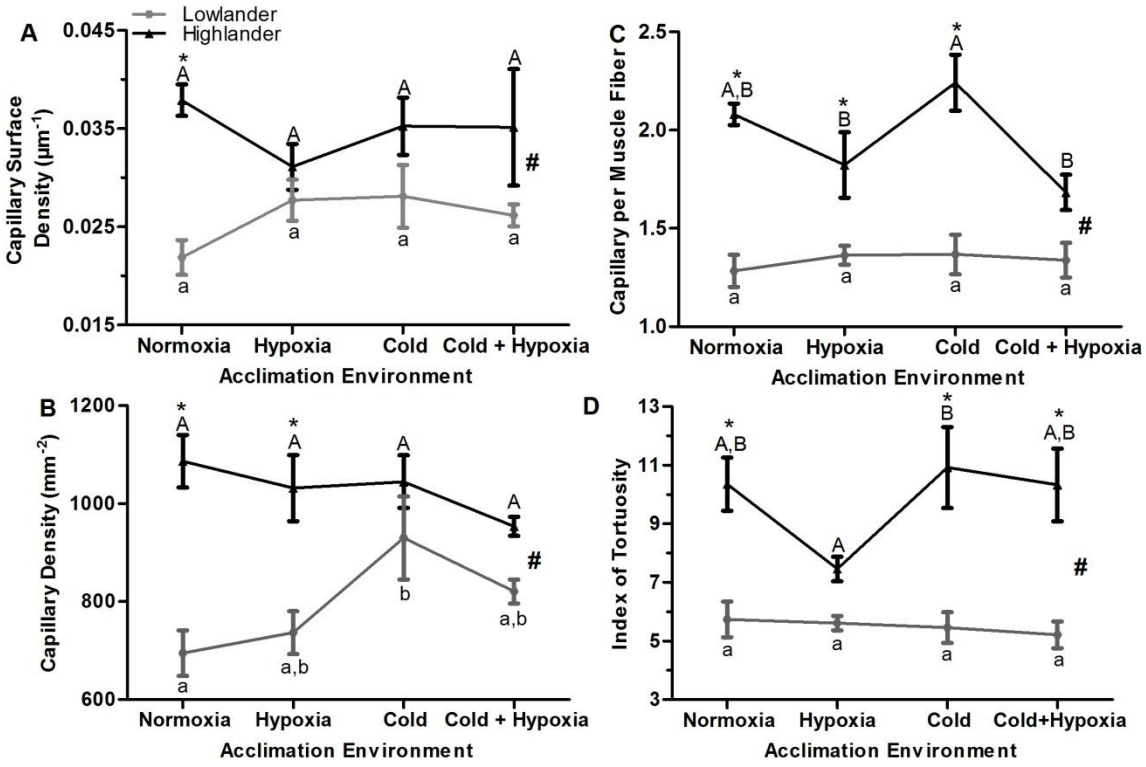
A
Lowlander
Highlander
Capillary Surface Density (μm-1)
0.045
0.035
0.025
0.015
Normoxia
Hypoxia
Cold
Cold + Hypoxia
Acclimation Environment
B
Capillary Density (mm-2)
1200
1000
800
600
C
Capillary per Muscle Fiber
2.5
2.0
1.5
1.0
D
Index of Tortuosity
13
11
9
7
5
3
Cold+Hypoxia

Figure 3.3: Capillarity and tortuosity of capillaries in the gastrocnemius muscle were higher in the highland mice. Four indices of capillarity were quantified: capillary surface density (A), capillary density (B), capillary per muscle fiber (C) and index of tortuosity in high and lowland deer mice acclimated to normoxia, hypoxia, cold and cold + hypoxia. # Significant main effect of population. † Significant main effect of acclimation. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment. Different uppercase letters represent significant pairwise differences in response to acclimation within highland mice. Different lowercase letters represent significant pairwise differences in response to acclimation within lowland mice.

Figure 3.4

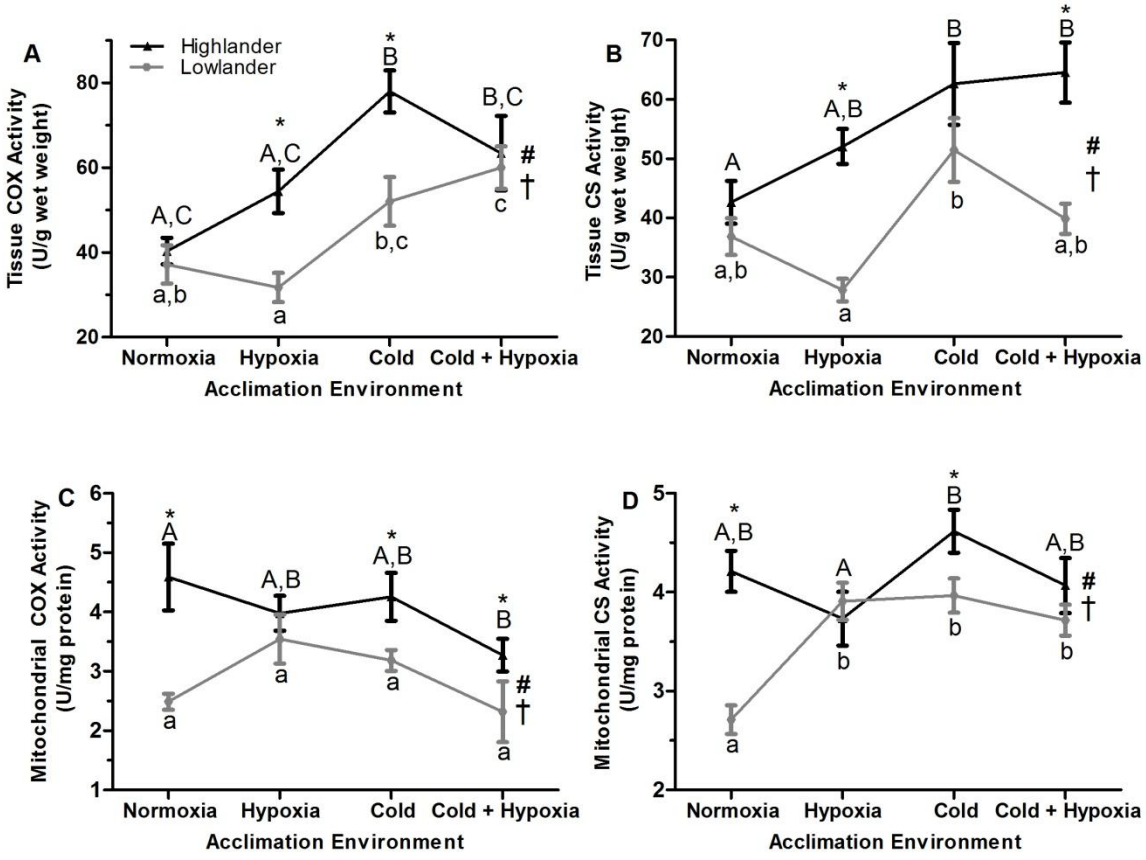
A
Highlander
Lowlander
Tissue COX Activity (U/g wet weight)
Normoxia
Hypoxia
Cold
Cold + Hypoxia
Acclimation Environment
B
Tissue CS Activity (U/g wet weight)
C
Mitochondrial COX Activity (U/mg protein)
D
Mitochondrial CS Activity (U/mg protein)

Figure 3.4: Maximal COX and CS activities (Vmax) (U = µmol/min) in the gastrocnemius tissue (A,B) and isolated mitochondria of hindlimb muscles (C,D) in high and lowland deer mice acclimated to normoxia, hypoxia, cold and cold + hypoxia. # Significant main effect of population. † Significant main effect of acclimation. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment. Different uppercase letters represent significant pairwise differences in response to acclimation within highland mice. Different lowercase letters represent significant pairwise differences in response to acclimation within lowland mice.

Figure 3.5

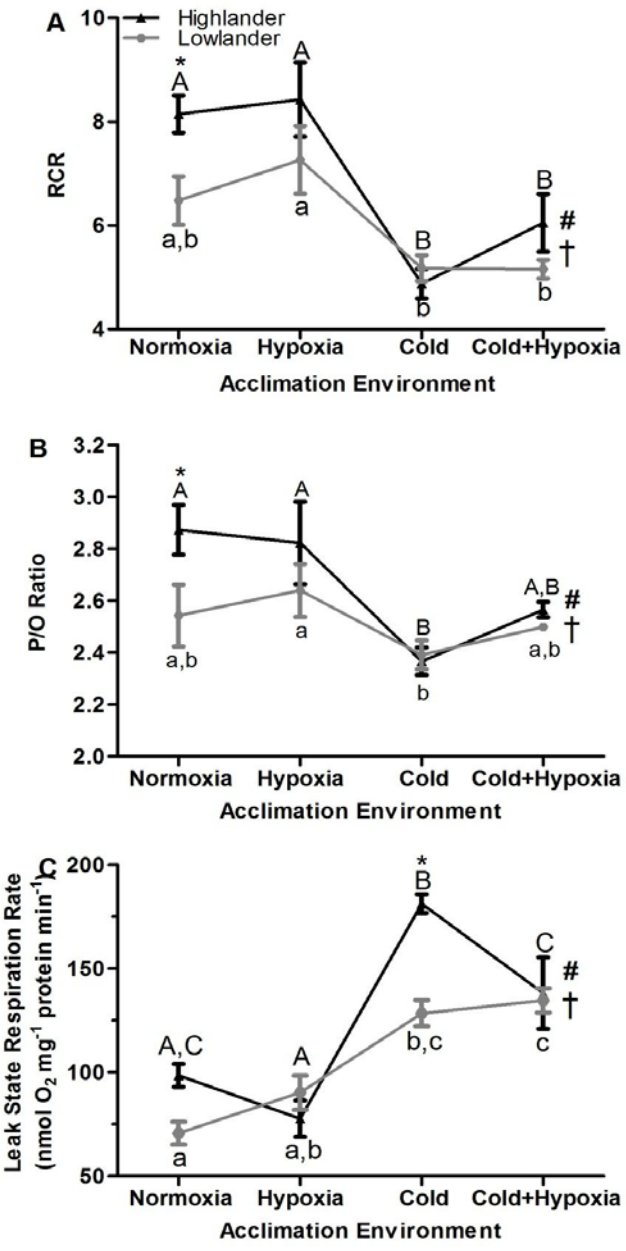
A
Highlander
Lowlander
RCR
10
8
6
4
*
A
A
a,b
a
B
b
B
b
#
†
Normoxia
Hypoxia
Cold
Cold+Hypoxia
Acclimation Environment
B
P/O Ratio
3.2
3.0
2.8
2.6
2.4
2.2
2.0
*
A
A
a,b
a
B
b
A,B
a,b
#
†
Normoxia
Hypoxia
Cold
Cold+Hypoxia
Acclimation Environment
C
Leak State Respiration Rate
(nmol O2 mg-1 protein min-1)
200
150
100
50
A,C
a
A
a,b
*
B
b,c
C
c
#
†
Normoxia
Hypoxia
Cold
Cold+Hypoxia
Acclimation Environment

Figure 3.5: Respiratory control ratio (RCR) (A), ATP produced per oxygen atom (P/O ratio) (B) and leak state respiration rates in the presence of ATP (L_T) (C) in isolated mitochondria from the hindlimb muscles of high and lowland deer mice acclimated to normoxia, hypoxia, cold and cold + hypoxia. In graph C leak respiration was measured with pyruvate and malate in the presence of ATP. # Significant main effect of population. † Significant main effect of acclimation. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment. Different uppercase letters represent significant pairwise differences in response to acclimation within highland mice. Different lowercase letters represent significant pairwise differences in response to acclimation within lowland mice.

Figure 3.6

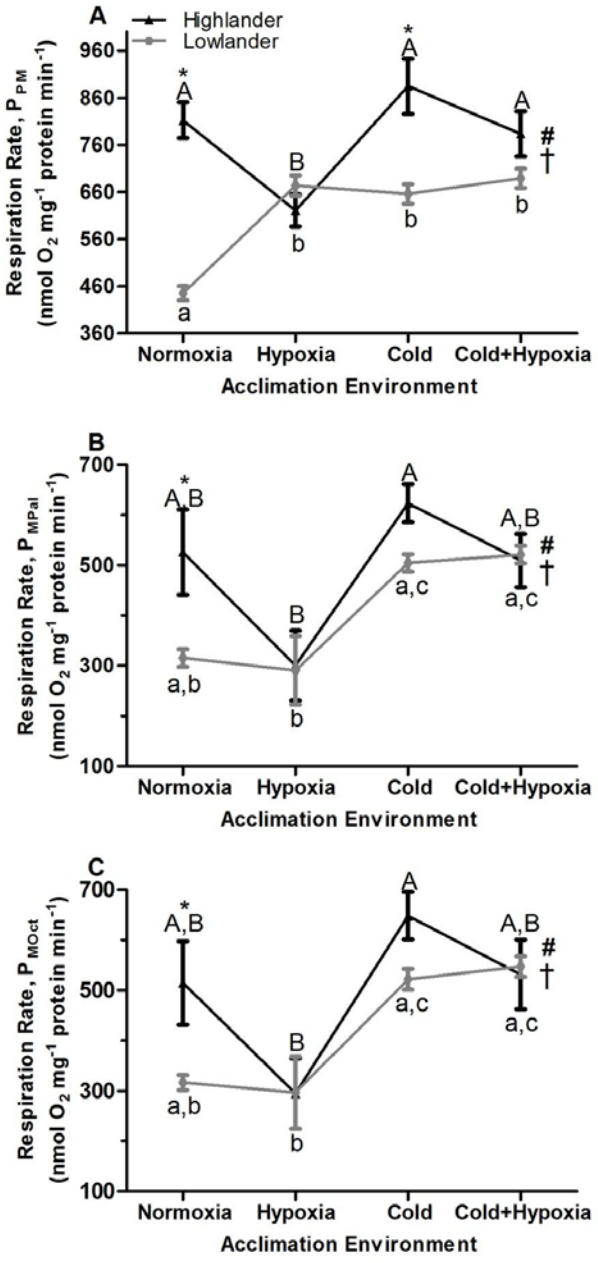
A
Highlander
Lowlander
Respiration Rate, P_{PM} (nmol O_2 mg^{-1} protein min^{-1})
960
860
760
660
560
460
360
Normoxia
Hypoxia
Cold
Cold+Hypoxia
Acclimation Environment
B
Respiration Rate, P_{MPal} (nmol O_2 mg^{-1} protein min^{-1})
700
500
300
100
Normoxia
Hypoxia
Cold
Cold+Hypoxia
Acclimation Environment
C
Respiration Rate, P_{MOct} (nmol O_2 mg^{-1} protein min^{-1})
700
500
300
100
Normoxia
Hypoxia
Cold
Cold+Hypoxia
Acclimation Environment

Figure 3.6: Highland mice had higher capacity to oxidize carbohydrates and fatty acids. Oxidative phosphorylation respiration rates were measured for carbohydrate oxidation capacity (P_{PM} = malate + pyruvate + ADP) (A) and fatty acid oxidation capacity (P_{MPal} = malate + palmitoyl-carnitine + ADP (B) $P_{MPalOct}$ = malate + palmitoyl-carnitine + octanoyl-carnitine + ADP) (C) in mitochondria isolated from the hindlimb muscles of high and lowland deer mice acclimated to normoxia, hypoxia, cold and cold + hypoxia. # Significant main effect of population. † Significant main effect of acclimation. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment. Different uppercase letters represent significant pairwise differences in response to acclimation within highland mice. Different lowercase letters represent significant pairwise differences in response to acclimation within lowland mice.

Figure 3.7

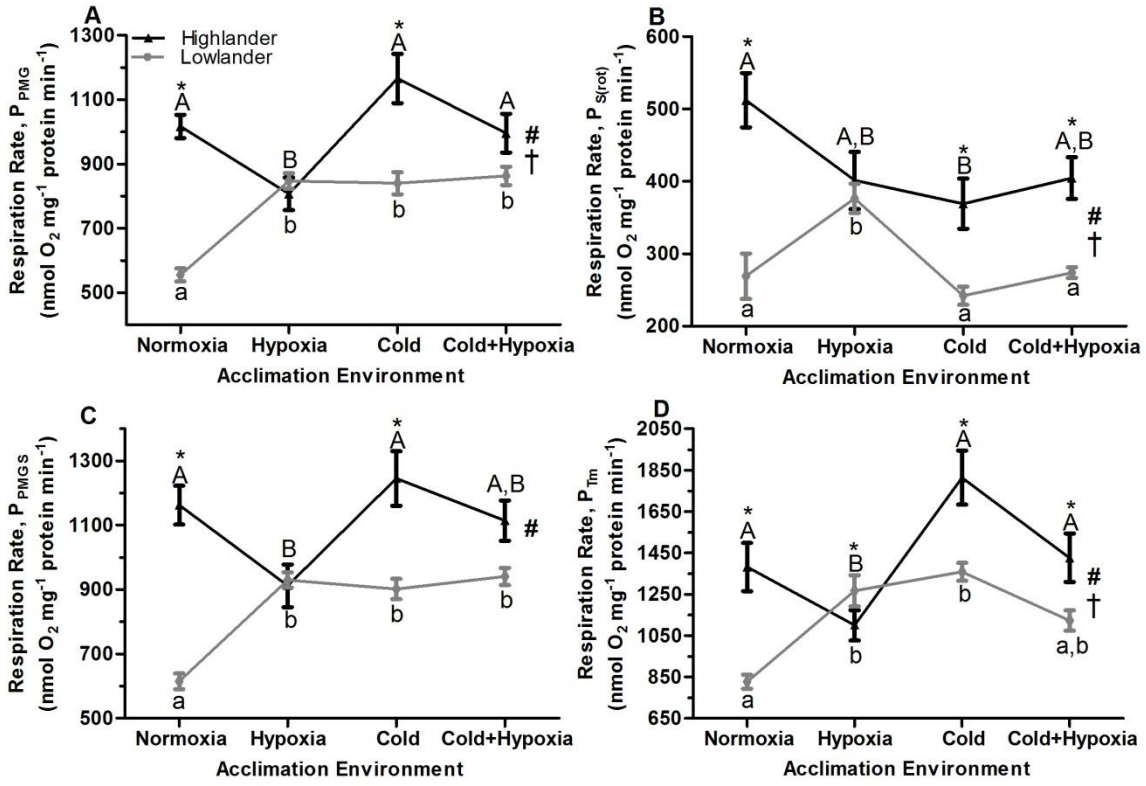
A
Highlander
Lowlander
Respiration Rate, P_{PMG} (nmol O_2 mg^{-1} protein min^{-1})
1300
1100
900
700
500
Normoxia
Hypoxia
Cold
Cold+Hypoxia
Acclimation Environment
B
Respiration Rate, $P_{S(rot)}$ (nmol O_2 mg^{-1} protein min^{-1})
600
500
400
300
200
C
Respiration Rate, P_{PMGS} (nmol O_2 mg^{-1} protein min^{-1})
D
Respiration Rate, P_{Tm} (nmol O_2 mg^{-1} protein min^{-1})
2050
1850
1650
1450
1250
1050
850
650

Figure 3.7: Mitochondrial respiratory capacities of complex I (A), complex II (B), complex I+II (C), and complex IV (D) were all higher in the highland mice compared to lowland mice. Maximum oxidative phosphorylation respiration rates of complex I (P_{PMG} = malate + pyruvate + ADP + glutamate), CII ($P_{S(Rot)}$ = malate + pyruvate + ADP + glutamate + succinate + rotenone), complex I + II (P_{PMGS} = malate + pyruvate + ADP + glutamate + succinate) and complex IV (P_{Tm} = ADP + ascorbate + TMPD) were measured in isolated mitochondria from the hindlimb muscles of high and lowland deer mice acclimated to normoxia, hypoxia, cold and cold + hypoxia. # Significant main effect of population. ✝ Significant main effect of acclimation. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment. Different uppercase letters represent significant pairwise differences in response to acclimation within highland mice. Different lowercase letters represent significant pairwise differences in response to acclimation within lowland mice.

3.8 REFERENCES

Barrie, E., Heath, D., Stella, J. A., & Harris, P. (1974). Enzyme activities in red and white muscles of guinea-pigs and rabbits indigenous to high altitude. *Environmental physiology & biochemistry*, *5*(1), 18-26.

Brand, M. D. (2005). The efficiency and plasticity of mitochondrial energy transduction. *Biochemical Society Transactions*, *33*(5), 897-904.

Cheviron, Z. A., Connaty, A. D., McClelland, G. B., & Storz, J. F. (2014). Functional genomics of adaptation to hypoxic cold-stress in high-altitude deer mice: transcriptomic plasticity and thermogenic performance. *Evolution*, *68*(1), 48-62.

Cheviron, Z. A., Bachman, G. C., Connaty, A. D., McClelland, G. B., & Storz, J. F. (2012). Regulatory changes contribute to the adaptive enhancement of thermogenic capacity in high-altitude deer mice. *Proceedings of the national academy of sciences*, *109*(22), 8635-8640.

Gamboa, J. L., & Andrade, F. H. (2012). Muscle endurance and mitochondrial function after chronic normobaric hypoxia: contrast of respiratory and limb muscles. *Pflügers Archiv-European Journal of Physiology*, *463*(2), 327-338.

Gnaiger, E., Méndez, G., & Hand, S. C. (2000). High phosphorylation efficiency and

depression of uncoupled respiration in mitochondria under hypoxia. *Proceedings of the National Academy of Sciences*, *97*(20), 11080-11085.

Green, H. J., Sutton, J. R., Cymerman, A., Young, P. M., & Houston, C. S. (1989). Operation Everest II: adaptations in human skeletal muscle. *Journal of Applied Physiology*, *66*(5), 2454-2461.

Hayes, J. P., & Chappell, M. A. (1986). Effects of cold acclimation on maximum oxygen consumption during cold exposure and treadmill exercise in deer mice, Peromyscus maniculatus. *Physiological zoology*, *59*(4), 473-481.

Herpin, P., & Lefaucheur, L. (1992). Adaptative changes in oxidative metabolism in skeletal muscle of cold-acclimated piglets. *Journal of thermal biology*, *17*(4-5), 277-285.

Hoppeler, H., Kleinert, E., Schlegel, C., Claassen, H., Howald, H., Kayar, S. R., & Cerretelli, P. (1990). II. Morphological adaptations of human skeletal muscle to chronic hypoxia. *International journal of sports medicine*, *11*(S 1), S3-S9.

Hochachka, P. W., Gunga, H.C., and Kirsch, K. (1998). Our ancestral physiological phenotype: An adaptation for hypoxia tolerance and for endurance performance. *Proc. Natl. Acad. Sci.* 95, 1915-1920.

Jackson, C. G. R., Sillau, A. H., & Banchero, N. (1987). Fiber composition and capillarity

in growing guinea pigs acclimated to cold and cold plus hypoxia. *Experimental Biology and Medicine*, *185*(1), 101-106.

Jacobs, R. A., Boushel, R., Wright-Paradis, C., Calbet, J. A., Robach, P., Gnaiger, E., & Lundby, C. (2013). Mitochondrial function in human skeletal muscle following high-altitude exposure. *Experimental physiology*, *98*(1), 245-255.

Jacobs, R. A., Siebenmann, C., Hug, M., Toigo, M., Meinild, A. K., & Lundby, C. (2012). Twenty-eight days at 3454-m altitude diminishes respiratory capacity but enhances efficiency in human skeletal muscle mitochondria. *The FASEB Journal*, *26*(12), 5192-5200.

Kayser, B., Hoppler, H., Desplanches, D., Marconi, C., Broers, B. and Cerretelli P. (1996). Muscle ultrastructure and biochemistry of lowland Tibetans. J. Appl. Physiol. 81: 419-425.

Levett, D. Z., Radford, E. J., Menassa, D. A., Graber, E. F., Morash, A. J., Hoppeler, H., ... & Grocott, M. P. (2012). Acclimatization of skeletal muscle mitochondria to high-altitude hypoxia during an ascent of Everest. *The FASEB journal*, *26*(4), 1431-1441.

Lau, D. S., Connaty, A. D., Mahalingam, S., Wall, N., Cheviron, Z. A., Storz, J. F., ... & McClelland, G. B. (2017). Acclimation to hypoxia increases carbohydrate use during exercise in high-altitude deer mice. *American Journal of Physiology-Regulatory,*

Integrative and Comparative Physiology, ajpregu-00365.

Lui, M. A., Mahalingam, S., Patel, P., Connaty, A. D., Ivy, C. M., Cheviron, Z. A., ... & Scott, G. R. (2015). High-altitude ancestry and hypoxia acclimation have distinct effects on exercise capacity and muscle phenotype in deer mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *308*(9), R779-R791.

MacDougall, J. D., Green, H. J., Sutton, J. R., Coates, G., Cymerman, A., Young, P., & Houston, C. S. (1991). Operation Everest II: structural adaptations in skeletal muscle in response to extreme simulated altitude. *Acta physiologica Scandinavica*, *142*(3), 421-427.

Mathieu-Costello, O. (1987). Capillary tortuosity and degree of contraction or extension of skeletal muscles. *Microvascular research*, *33*(1), 98-117.

Maurya, S. K., & Periasamy, M. (2015). Sarcolipin is a novel regulator of muscle metabolism and obesity. *Pharmacological research*, *102*, 270-275.

Mineo, P. M., Cassell, E. A., Roberts, M. E., & Schaeffer, P. J. (2012). Chronic cold acclimation increases thermogenic capacity, non-shivering thermogenesis and muscle citrate synthase activity in both wild-type and brown adipose tissue deficient mice. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *161*(4), 395-400.

Reynafarje, B. (1962). Myoglobin content and enzymatic activity of muscle and altitude adaptation. *Journal of Applied Physiology*, *17*(2), 301-305.

Scott, G. R., Egginton, S., Richards, J. G., & Milsom, W. K. (2009). Evolution of muscle phenotype for extreme high altitude flight in the bar-headed goose. *Proceedings of the Royal Society of London B: Biological Sciences*, *276*(1673), 3645-3653.

Scott, G. R., Elogio, T. S., Lui, M. A., Storz, J. F., & Cheviron, Z. A. (2015). Adaptive modifications of muscle phenotype in high-altitude deer mice are associated with evolved changes in gene regulation. *Molecular biology and evolution*, msv076.

Shabalina, I. G., Hoeks, J., Kramarova, T. V., Schrauwen, P., Cannon, B., & Nedergaard, J. (2010). Cold tolerance of UCP1-ablated mice: a skeletal muscle mitochondria switch toward lipid oxidation with marked UCP3 up-regulation not associated with increased basal, fatty acid-or ROS-induced uncoupling or enhanced GDP effects. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1797*(6), 968-980.

Sillau, A. H., Aquin, L., Lechner, A. J., Bui, M. V., & Banchero, N. (1980). Increased capillary supply in skeletal muscle of guinea pigs acclimated to cold. *Respiration physiology*, *42*(3), 233-245.

Storz, J.F., Scott, G.R., and Cheviron, Z.A. (2010). Phenotypic plasticity and genetic adaptation to high- altitude hypoxia in vertebrates. *J. Exp. Biol.*213, 4125-4135.

Toyomizu, M., Ueda, M., Sato, S., Seki, Y., Sato, K., & Akiba, Y. (2002). Cold-induced mitochondrial uncoupling and expression of chicken UCP and ANT mRNA in chicken skeletal muscle. *FEBS letters*, *529*(2-3), 313-318.

Weibel, E.R. (1979). *Stereological Methods*. Academic Press, Toronto.

Welch, K. C., Altshuler, D. L., & Suarez, R. K. (2007). Oxygen consumption rates in hovering hummingbirds reflect substrate-dependent differences in P/O ratios: carbohydrate as apremium fuel'. *Journal of Experimental Biology*, *210*(12), 2146-2153.

Wickler, S. J. (1980). Maximal thermogenic capacity and body temperatures of white-footed mice (Peromyscus) in summer and winter. *Physiological Zoology*, *53*(3), 338-346.

Wijers, S. L., Schrauwen, P., Saris, W. H., & van Marken Lichtenbelt, W. D. (2008). Human skeletal muscle mitochondrial uncoupling is associated with cold induced adaptive thermogenesis. *PloS one*, *3*(3), e1777.

CHAPTER 4: HIGH ALTITUDE ANCESTRY AND HYPOXIA ACCLIMATION AFFECT CARDIAC MITOCHONDRIAL PHYSIOLOGY IN DEER MICE

4.1 INTRODUCTION

Previous research on mitochondrial function in highland deer mice has focused on skeletal muscle (chapters 2 and 3). However, skeletal muscles may not be the most sensitive to reductions in oxygen availability. The heart is an obligate aerobic organ that requires continuous supply of oxygen in order to meet its energy demands. Mass specific ATP requirements of a human heart at rest are similar to those of skeletal muscles during intense exercise (Ingwall, 2002). When the heart is stressed and works harder the ATP demands increase many fold. The heart has high ATP turnover rates; therefore, ATP production needs to be closely coupled to ATP demands for the heart to function normally (Ingwall, 2002). In order to sustain ATP supply at such high rates the heart is able to metabolize lipids, carbohydrates, lactate, ketone bodies and amino acids, but the main fuel source is lipids (Ingwall, 2002). Since the heart requires a continuous supply of oxygen to produce ATP aerobically, it is very sensitive to hypoxia. It is unclear whether there are changes in mitochondrial respiratory capacities that help counteract the effects of hypoxia in the heart.

One strategy to maintain aerobic metabolism in an environment that has reduced oxygen availability is to maximize ATP production for a mole of oxygen. Oxidation of glucose or glycogen as a fuel source produces 15% more ATP per mole of oxygen compared to oxidation of fatty acids (Brand, 2005; Hochachka et al., 1991; Korvald et al., 2000). This suggests that high altitude animals may increase carbohydrate use relative to

fatty acids to economize oxygen use. The hypothesis was also supported at the whole animal level in a study by Schippers et al. (2012), in which two species of Andean mice (genus: Phyllotis) native to high altitude utilized a greater proportion of carbohydrates during exercise compared to low altitude species under both normoxia and hypoxia. Similarly, high altitude deer mice that were acclimated to hypoxia increased carbohydrate oxidation and the relative reliance on carbohydrates while running at 75% of their maximal oxygen consumption (Lau et al., 2017). The metabolism of high altitude populations is shifted towards aerobic carbohydrate oxidation to produce energy and to use oxygen more efficiently.

A second strategy to counteract reduced oxygen availability is to increase aerobic and oxidative capacity of the tissue. An increase in aerobic capacity of the tissue is not the same as a change in efficiency of oxygen use. Increasing capacity for oxidative phosphorylation of carbohydrates rather than anaerobic glycolysis improves the yield of ATP per carbon molecule since oxidative phosphorylation produces more ATP per molecule of glucose compared to anaerobic glycolysis. Hochachka et al., (1983) studied heart enzymes from the high altitude Alpaca, Llama and Taruca and all three animals had higher mitochondrial densities in their cardiomyocytes compared to lowland animals. The enzyme activities of high altitude populations also have lower anaerobic glycolytic capacities and the glycolytic pathway results in more oxidation of carbohydrates rather than fermentation. These high altitude natives have high ratios of citrate synthase

activities to lactate dehydrogenase activities, which is indicative of an increased relative oxidative capacity compared to anaerobic metabolic capacities (Hochacka et al., 1983). High altitude native humans also have lower blood lactate concentrations compared to lowlanders after VO_2 max tests (Hochachka et al., 2002); lactate is a product of a mismatch between pyruvate production and oxidation. This suggests that humans native to high altitude use more carbohydrates via oxidative phosphorylation than anaerobic glycolysis. Animals native to high altitude also increase oxygen supply to the heart; they have higher capillary densities in their left ventricle (Jurgens et al., 1988; Scott et al., 2011). These studies suggest that cardiac tissue of highland natives are more aerobic compared to lowland mammals.

Lowland mammals acclimated to hypoxia conserve oxygen by using more carbohydrates and increase aerobic capacity of the heart. Rats that were exposed to systemic hypoxia via cobalt chloride treatment or iso-volumic hemodilution reduced their expression of genes involved in fatty acid oxidation in their heart and increased their reliance on glucose during hypoxia (Razeegi et al., 2001). Similarly, CD1 mice acclimated to chronic hypoxia significantly decreased fatty acid oxidation capacity (Templeman et al., 2010). Animals acclimated to hypoxia also increased oxygen supply to the heart; rats that were exposed to hypobaric hypoxia increased capillary density in the heart (Turek and Kreuzer, 1972). An increase in ATP produced per mole of oxygen and an increase aerobic capacity is advantageous when oxygen availability is limited.

The heart is able to oxidize lactate and increasing lactate oxidation capacities may be beneficial at high altitudes in order to use all of the carbon atoms from glucose and in return supplement ATP production in a hypoxic environment. Lactate is produced by a reaction catalyzed by lactate dehydrogenase (LDH) in which pyruvate and NADH are converted to lactate and NAD^+. As a near-equilibrium enzyme, LDH also catalyzes the reverse reaction and converts pyruvate to lactate. LDH is composed of H (heart type) and M (muscle type) subunits that differ in function. The H subunit has a higher affinity for lactate and is inhibited by high pyruvate concentrations, so it favours lactate oxidation to pyruvate. The M subunit has a higher affinity for pyruvate and is inhibited by high lactate concentrations; therefore, it favours pyruvate reduction to lactate (Malmqvist et al., 1991; Quistroff et al., 2011). LDH in the heart is primarily composed of H-LDH (Krieg et al., 1967). High altitude native humans have lower blood lactate concentrations after exercise (Hochachka et al., 2002, West, 1986), which may be supported by an increased oxidation of lactate. Heart tissue of pikas native to high altitude have higher heart type LDH activities compared to low altitude pikas (Sheafor, 2003). Rats that were acclimated to long-term hypobaric hypoxia increased LDH protein content in heart mitochondria (McClelland and Brooks, 2001). How lactate moves between tissues and within a cell is explained by the lactate shuttle hypothesis (Brooks et al., 1999), which has an intercellular component and an intracellular component. The cell-cell lactate shuttle has been confirmed over the years (Bangsho et al., 1991; Bergman et al., 1999; Mazzio et al.,

1986) and posits that lactate released from one cell could serve as a carbon source in other cells; for example, the heart can take up lactate circulating in the blood and convert it to pyruvate. The current intracellular lactate hypothesis posits that cytosolic lactate is transported into the mitochondrial intermembrane space via a monocarboxylate transporter where it is converted to pyruvate and NADH by mitochondrial LDH (Brooks et al., 1999; Kane, 2014). The pyruvate is then oxidized to acetyl CoA in the mitochondrial matrix and enters the Kreb's cycle, ultimately producing ATP via oxidative phosphorylation. Although there is compelling evidence that lactate can be oxidized by mitochondria (Elustondo et al., 2013; Passaraella et al., 2014; Brooks et al., 1999), some studies were not able to observe lactate oxidation by mitochondria (Yoshida et al., 2007; Sahlin et al., 2002; Rassmusen et al., 2002). Lactate oxidation has been observed in isolated subsarcolemmal mitochondria (Elustondo et al., 2013). It has been argued that the lack of lactate oxidation by mitochondria in studies may be due to mitochondria not being intact or using isolation/respiratory media that inhibit lactate oxidation (Passaraella et al., 2014). Removing lactate from the cytosol and increasing intracellular lactate shuttling will help keep cytosolic lactate levels low. This allows cytosolic LDH to continue to produce lactate from pyruvate, generating NAD^+ to support glycolysis while keeping ratios of NAD^+ to NADH homeostatic. Lactate is a smaller molecule and more easily diffusible compared to NAD^+. Lactate import into the mitochondria would be faster than than NAD^+ efflux; therefore lactate production and import is a more effective way to

maintain NAD^+ to NADH ratios. High altitude natives would be able to make use of the additional fuel source by increasing the activity intracellular lactate shuttle.

In this chapter I tested the hypothesis that the hearts of high altitude deer mice have adaptations that help counteract the effects of hypoxia. Specifically, I tested the following predictions: 1) the heart tissue of highland mice will have higher oxidative capacities compared to the lowland mice, 2) hypoxia acclimation will increase the oxidative capacity of the left ventricle in both the high and low altitude populations, 3) the mitochondria of highland mice will increase their capacity to oxidize carbohydrates relative to fatty acids in response to hypoxia acclimation, 4) highland mice will use the intracellular lactate shuttle to a greater extent to support ATP production in comparison to the low altitude population, 5) hypoxia acclimation will increase the use of the lactate as a fuel source in both populations

4.2 METHODS

4.2.1 Experimental Animals

Captive breeding populations of high- and low-altitude deer mice were established as previously described (Cheviron et al., 2012; Lui et al., 2015, Lau et al., 2017). High-altitude mice (*P.m. rufinus*) were trapped on the summit of Mt. Evans (Clear Creek County, Colorado; 39°35’18’’N, 105°38’38’’W) at ~4300m above sea level. Low-altitude deer mice (*P.m.nebracensis*) were trapped in Nine-Mile Prairie (Lancaster County, Nebraska; 40°52’12’’N, 96°48’20.3’’W) at ~400 m above sea level. Populations were

bred separately in captivity to produce F1 progeny, which were raised to adulthood in normoxia in common laboratory conditions with a constant temperature (25 °C) and light cycle (12L:12D). Mice were provided chow (Teklad Global Rodent Diets, Envigo) and water *ad libitum*. All animal care and experimentation followed the guidelines established by the Canadian Council on Animal Care, and were approved by the McMaster University Animal Research Ethics Board.

4.2.2 Experimental Design

Adult F1 mice from each population were randomly assigned to one of two acclimation groups for a period of six to ten weeks: 1) normobaric normoxia in standard lab room conditions, or 2) hypobaric hypoxia in hypobaric chambers set to an ambient pressure of 60 kPa (simulating the pressure at an altitude of ~4300 m elevation). Atmospheric pressure was gradually decreased over the first 3 days in the hypoxia group, and kept at that pressure for the remainder of the acclimation period, using previously described methods and custom-made hypobaric chambers (Lui et al., 2015; Lau et al., 2017; McClelland et al., 1998). The cages were cleaned every 3-4 days, which required that the hypobaric chambers be returned to normobaria for a brief period (< 1 h).

4.2.3 Mitochondrial Respiration in Permeabilized Muscle Fibres

Mice were anesthetized using isoflurane and then euthanized by cervical dislocation. The heart was removed and the left ventricle was cut into small ~5mg pieces and placed in ice-cold relaxing and preservation buffer (concentrations in mM: 2.77

CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2 \cdot 6H_2O$, 20 taurine, 15 Na_2Phosphocreatine, 20 imidazole, 0.5 dithiothreitol, 50 methanesulphonate. The muscle fibres were then manually teased apart using dissecting probes (with the assistance of a stereomicroscope) and then permeabilized for 30 min in the same buffer containing saponin (50 µg/ml). Samples then went through three 10 min rinses in respiration solution (in mM: 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose, 1 g l^{-1} fatty acid-free bovine serum albumin; pH 7.1) and were weighed. Three different pieces of left ventricle were teased and permeabilized.

Respiration of the muscle fibres was measured using high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) in 2 ml of respiration buffer at a temperature of 37°C. Three different mitochondrial respiration protocols were used to measure respiration rates using different combinations of substrates. For all three protocols the solution was oxygenated to an oxygen concentration of 450 µM by bubbling compressed oxygen into the chamber. For the first protocol, 5 minutes after adding the fibres, leak respiration was stimulated without adenylates (L_N) by adding 5 mM pyruvate and 2 mM malate. Oxidative phosphorylation respiration was stimulated *via* multiple complexes of the electron transport system with progressive additions of 2 mM ADP ($P_{PM,}$ complex I), 20 mM of glutamate ($P_{PMG,}$ complex I), and 20 mM of succinate ($P_{PMGS,}$ complexes I+II), waiting at least 2 min between each addition until a stable respiration rate could be measured. The chamber was then re-oxygenated and cytochrome c oxidase

(complex IV) was maximally stimulated with the addition of 1.25 mM ADP, 2 mM ascorbate, and 0.5 mM TMPD (N,N,N′,N′-tetramethyl-p-phenylenediamine) (P_{Tm}). The second protocol measured the capacity for fatty acid oxidation. For the second protocol, 5 minutes after the addition of the fibres, oxidative phosphorylation respiration was stimulated with 2 mM malate, 40 µM palmitoyl-carnitine and 2 mM ADP, followed by 0.2 mM octanoyl-carnitine, waiting at least 2 min between each addition until a stable respiration rate could be measured. The third protocol measured lactate oxidation capacity; 5 minutes after the addition of the fibres, oxidative phosphorylation respiration was stimulated with progressive additions of 1.25 mM ADP, 0.2 mM malate and 2 mM NAD+, 30 mM L-lactate, and 5 µM Cinnamate, waiting at least 2 min between each addition until a stable respiration rate could be measured (Elustondo et al., 2013). A concentration of 5 µM cinnimate was used to inhibit pyruvate transporters on the inner mitochondrial membrane, higher concentrations raise concern about whether the lactate transporter may be inhibited (Elustondo et al., 2013).

4.2.4 Tissue and Mitochondrial Enzyme Assays

Enzyme activities in left ventricle tissue were assayed in samples that were frozen in liquid nitrogen and stored in -80°C. The tissue was powdered using a mortar and pestle under liquid nitrogen and then stored in -80°C. This powder was homogenized on ice using a glass on glass homogenizer in 20 volumes of homogenization buffer (concentrations in mM: 100 KH_2PO_4, 5 ethylenediaminetetraacetic acid, and 0.1% Triton-

X-100). The apparent V_{max} of citrate synthase (CS), hexokinase (HK), ß-hydroxyacyl-CoA dehydrogenase (HOAD) and lactate dehydrogenase (LDH) in the left ventricle tissue were measured at 37°C as previously described for deer mice (Cheviron et al., 2012, Lui et al, 2015; Lau et al., 2017) using a Spectromax Plus 384 microplate reader (Molecular Devices). Hexokinase activity, followed by HOAD activity, was measured shortly after homogenization. LDH activity was measured after two freeze thaw cycles and CS was measured after three freeze thaw cycles. Assays were conducted under the following conditions (in mM unless stated otherwise): HK, 50 hepes, 8 $MgCl_2$, 0.5 NADP, 8 ATP, 4 units of glucose-6-PDH, 5 glucose (omitted for background) (pH= 7.6); HOAD, 100 TEA-HCl, 5 EDTA, 0.28 NADH, 0.1 acetyl Co-A (omitted for background) (pH = 7.0); LDH, 40 Tris-HCl, 0.28 NADH, 2.4 pyruvate-Na (omitted for background) (pH = 7.4); CS, 40 Tris-HCl, 0.22 acetyl-CoA, 0.1 dithiobisnitrobenzoic acid (DTNB), 0.5 oxaloacetate (omitted for background) (pH 8.0).

Enzyme activities were also assayed in mitochondria that were isolated from left ventricle tissue. Mice were anesthetized using an isoflurane soaked cotton ball and then euthanized by cervical dislocation. The heart was removed, and the left ventricle dissected and immediately transferred to 10 ml of ice-cold isolation buffer (in mM: 100 sucrose, 50 Tris base, 5 $MgCl_2$, 5 EGTA, 100 KCl, 1 ATP). The muscle was minced and then digested for 5 min in the same buffer containing protease (1 mg g^{-1} muscle tissue). The digested muscle was then gently homogenized with six passes of a Potter-Elvehjem

Teflon on glass homogenizer (100 rpm). Mitochondria were isolated via differential centrifugation at 4°C. Homogenates were centrifuged at 700g for 10 min. The resulting supernatant was filtered through cheesecloth and then centrifuged at 1000g for 10 min. The supernatant was centrifuged at 8700g for 10 min and the pellet was resuspended in 10 ml of isolation buffer with bovine serum albumin (BSA, fatty acid-free, at 1% mass:volume) and centrifuged at 8700g. The pellet was resuspended in 10 ml of storage buffer (in mM: 0.5 EGTA, 3 $MgCl_2$, 60 K-methanesulphonate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose, 0.02 vitamin E succinate, 2 malate, pH 7.1) and centrifuged at 8700g. The pellet was finally resuspended in 250-400 µl of storage buffer. Part of this mitochondrial suspension was kept on ice until enzyme assays were conducted. Mitochondrial suspensions were not homogenized so that mitochondria were intact. Apparent V_{max} of CPT1 and LDH in isolated mitochondria were measured at 37°C using a Spectromax Plus 384 microplate reader (Molecular Devices). CPT1 was measured in mitochondrial isolation buffer, 0.1 mM (DTNB), 0.1 mM palmitoyl-CoA, 5 mM L-carnitine (omitted for background), with and without 50 µM malonyl-CoA. (Morash et al., 2008). LDH activity was determined in intact and burst isolated mitochondria. Intact mitohcondria were assayed in isotonic storage buffer, with 0.28 mM NADH, 2.4 mM pyruvate-Na (omitted for background). LDH activity was negligible when mitochondria were kept intact. The isolated mitochondria were burst by freeze thawing in liquid nitrogen and LDH activity within the mitochondria was measured in 40 mM Tris-HCl,

0.28 mM NADH, 2.4 mM pyruvate-Na (omitted for background) (pH = 7.4) (Passarella et al., 2014).

4.2.5 Statistical Analysis

Data are presented as means ± SE. Two-factor ANOVA and Bonferroni multiple-comparisons post-tests were used as appropriate to determine the main effects of and interactions between population altitude and acclimation environment. A significance level of $P < 0.05$ was used throughout.

4.3 RESULTS

The respiratory capacity of mitochondria in the left ventricle in the highland mice was greater than lowland mice in normoxia, but similar after hypoxia acclimation (Figure 4.1). Oxidative phosphorylation (OXPHOS) respiration, when stimulated by palmitoyl-carnitine (P_{MPal}), was ~67% higher in the high-altitude population compared to the low-altitude population acclimated to normoxia, but respiration rates were similar after hypoxia acclimation. ($F_{1,20} = 5.60$; $p = 0.028$). There was no main effect of acclimation ($F_{1,20} = 8.77\times10^{-6}$; $p = 0.998$), but there was a significant effect of population × acclimation interaction ($F_{1,20} = 6.980$; $p = 0.016$). Similarly, OXPHOS respiration stimulated by pyruvate and malate (P_{PM}) ($F_{1,22} = 6.652$; $p = 0.017$) and maximal complex I respiration stimulated by subsequent addition of glutamate (P_{PMG}) ($F_{1,22} = 4.323$; $p = 0.049$) were higher in the high altitude population compared to the low altitude population in normoxia acclimated mice, but respiration rates were similar after hypoxia acclimation. There was no main effect of acclimation on P_{PM} ($F_{1,22} = 0.385$; $p = 0.542$) or P_{PMG} ($F_{1,22} = 1.206$; $p = 0.284$), and there were no significant interaction (P_{PM}, $F_{1,22} = 0.630$, $p = 0.436$; P_{PMG}, $F_{1,22} = 1.083$, $p = 0.309$). Maximal complex I+II respiration stimulated by the addition of succinate was significantly higher in the high altitude population in normoxia, but these rates converged after hypoxia acclimation (P_{PMGS}) ($F_{1,22} = 4.606$; $p = 0.043$). Respiration rates significantly increased by ~26% in response to hypoxia acclimation in the low altitude mice, but there was no main effect of acclimation since highland mice did

not change respiration rates in response to hypoxia ($F_{1,22} = 3.621$; $p = 0.070$) and there was no effect of population×acclimation ($F_{1,22} = 1.513$; $p = 0.232$). There was no difference in maximal complex IV activity stimulated by TMPD and ascorbate (P_{TM}) (population, $F_{1,16} = 1.983$, $p = 0.178$; acclimation $F_{1,16} = 0.336$, $p = 0.570$; interaction of population×acclimation, $F_{1,16} = 1.206$, $p = 0.288$).

In order to examine the relative capacities for oxidizing carbohydrates and fatty acids, oxphos respiration rates stimulated by malate and pyruvate were divided by respiration rates stimulated by palmitoyl-carnitine (Figure 4.2). In response to hypoxia acclimation the highland population and lowland population had different responses as indicated by a significant interaction effect between population and acclimation ($F_{1,22} = 8.141$; $p = 0.009$). The highland population significantly increased their capacity to oxidize carbohydrates relative to fatty acids by 25% in response to hypoxia acclimation. The lowland population did not have a significant change in their ability to oxidize carbohydrates compared fatty acids in response to hypoxia acclimation. There was no main effect of population ($F_{1,22} = 1.257$; $p = 0.617$) or acclimation ($F_{1,22} = 1.085$; $p = 0.309$).

Citrate synthase activity did not differ between the high and low altitude populations which is indicative of similar mitochondrial volume densities (population, $F_{1,35} = 0.751$, $p = 0.392$; acclimation, $F_{1,35} = 0.845$, $p= 0.364$; interaction, $F_{1,35} = 0.162$, $p= 0.690$) (Figure 4.3A). In response to hypoxia acclimation hexokinase (HK) activity

increased in the highland deer mice by ~30% and by ~32% the lowland deer mice ($F_{1,32}$ = 9.213, p = 0.005). HK activity did not differ significantly differ between high and low altitude deer mice ($F_{1,32}$ = 2.020, p = 0.165) and there was no interaction effect between population×acclimation ($F_{1,32}$ = 0.014, p = 0.908) (Figure 4.3B). Highland deer mice showed a trend towards a decrease in HOAD activity while the lowland population showed a trend towards an increase in response to acclimation, which resulted in a significant interaction effect of population×acclimation ($F_{1,33}$ = 6.513, p = 0.016). HOAD activity did not differ between the two populations ($F_{1,33}$ = 0.279, p = 0.601) and there was no main effect of acclimation ($F_{1,33}$ = 0.009, p = 0.924) (Figure 4.3C). Similarly, mitochondrial CPT1 activity significantly decreased by ~79% in the highland mice, but increased significantly by ~75% in the lowland mice in response to hypoxia acclimation, which resulted in a significant interaction effect of population×acclimation ($F_{1,12}$ = 17.38, p = 0.001). Mitochondrial CPT1 activity did not differ between populations ($F_{1,12}$ = 1.101, p = 0.315) or in response to acclimation ($F_{1,12}$ = 0.046, p = 0.834) (Figure 4.3D).

Lactate respiration ($P_{MNad+Lac}$) was 37% higher in the high altitude population than in the lowland population in normoxia, but respiration rates were similar after hypoxia acclimation ($F_{1,28}$ = 8.495, p = 0.007) (Figure 4.4). There was no main effect of acclimation ($F_{1,28}$ = 1.573, p = 0.220), nor an interaction of population×acclimation ($F_{1,28}$ = 1.849, p = 0.184). Pairwise comparison showed that the respiration rates were significantly different between the populations in normoxia, but converged after hypoxia

acclimation. OXPHOS respiration on malate and NAD+ (P_{MNad+}) did not differ between the populations or in response to acclimation (population, $F_{1,28} = 1.778$, $p = 0.193$; acclimation $F_{1,28} = 0.755$, $p = 0.392$; interaction of population×acclimation $F_{1,28} = 2.863$, $p = 0.102$). After the addition of cinnimate (Cin) to block lactate entry into the mitochondria, respiration rates were similar to that of P_{MNad+} and there were no significant differences between the populations (population, $F_{1,28} = 2.950$, $p = 0.097$; acclimation $F_{1,28} = 0.957$, $p = 0.336$; interaction of population×acclimation $F_{1,28} = 1.695$, $p = 0.204$) (Figure 4.4).

Tissue lactate dehydrogenase (LDH) activity was ~32% greater in the highland deer mice compared to the lowland deer mice ($F_{1,35} = 10.741$, $p = 0.002$) (Figure 4.5A). The highland population had a significantly higher tissue LDH activity compared to the lowland population in both acclimation environments. There was no main effect of acclimation environment ($F_{1,35} = 0.314$, $p = 0.579$) and there was no interaction effect of population×acclimation ($F_{1,35} = 0.095$, $p = 0.760$). LDH activity within the mitochondria was ~47% greater in the highland deer mice compared to the lowland deer mice ($F_{1,26} = 6.103$, $p = 0.02$) (Figure 4.5B). However, there was no main effect of population ($F_{1,26} = 0.225$, $p = 0.640$), nor interaction of population×acclimation ($F_{1,26} = 0.001$, $p = 0.974$)

4.4 DISCUSSION

Mitochondrial adaptations and phenotypic plasticity in highland mice help counteract the effects of hypoxia. Respiratory capacity of the left ventricle is higher in highland deer mice compared to lowland deer mice. Unlike the highland mice, the lowland mice increased respiratory capacity in the left ventricle after hypoxia acclimation. The differences were due to mitochondrial respiratory capacities, not mitochondrial content. The highland population increased their capacity for carbohydrate oxidation relative to fatty acid oxidation capacity in response to hypoxia acclimation. Highland deer mice also have a greater capacity to oxidize lactate via the intracellular lactate shuttle compared to the lowland mice.

The enhanced aerobic capacity of highland mice in the left ventricle compared to lowland mice allows for greater oxygen consumption. Having a more oxidative phenotype can help counteract the effects of hypoxia. It has been suggested that the reduced maximum respiratory capacity of a tissue due to hypoxia can be overcome by increasing the oxidative capacity of the tissue allowing greater oxygen flux to the tissue in order to offset the inhibitory effects of hypoxia (Hochachka, 1985). This increased oxidative capacity of muscles has been observed in a few animals native to high altitude (Dawson et al., 2016; Hochachka, 1985; Lui et al., 2015; Lau et al., 2017; Scott et al., 2009). The oxidative phenotype of a muscle can be increased via an increase in oxidative fibre, increasing volume mitochondrial density within fibres, or by increasing respiratory

capacities of individual mitochondria. The left ventricle is only composed of cardiac fibres and citrate synthase activities, a marker of mitochondrial content was similar between the populations; therefore, the increased oxidative phenotype can be attributed to differences in mitochondrial quality. The mitochondria of the highland mice have higher respiratory capacities, which may help the heart continue to function normally when intracellular oxygen is low.

The respiratory capacity of the left ventricle in response to hypoxia appears to be a fixed trait in the highland population, but phenotypic plasticity was maintained in the lowland population. The respiratory capacities did not differ significantly after hypoxia acclimation in the highland population. The lowland population significantly increased respiration rates in response to hypoxia acclimation when mitochondrial complexes one and two were maximally stimulated. Since both populations of mice are born and raised at approximately sea level, differences in respiratory capacity likely have a genetic basis.

The relative increased capacity for carbohydrate oxidation compared to fatty acids in the left ventricle tissue of highland population after hypoxia acclimation may increase the efficiency of oxygen use in ATP production at the whole animal level. The hearts of humans that are native to high altitude show a preference for glucose metabolism compared to humans that live at low altitudes (Holden et al., 1995). These findings similar to the increased proportion of carbohydrates oxidized when running at 75% of VO2max in high altitude deer mice that were acclimated to hypoxia (Lau et al. 2017).

Similarly, Phyllotis mice from high altitude also use a greater proportion of carbohydrates during submaximal exercise compared to their low altitude counterparts (Schippers et al., 2012). The left ventricle of lowland deer mice did not show a change in relative fuel oxidation capacity of carbohydrates and fatty acids after hypoxia acclimation. When lowland deer mice were acclimated to hypoxia and ran at 75% of VO2max there was no change in the proportion of carbohydrates used to support exercise (Lau et al. 2017). The lack of ability to increase the proportion of carbohydrates after hypoxia acclimation in lowland animals has been reported in rats (McClelland et al., 1998, 1999) and humans (Braun et al., 1999; Lundby et al., 2002). These findings suggest that the ability to increase the proportion of carbohydrate use in response to hypoxia acclimation may be an adaption to living at high altitudes.

The switch in relative capacity for oxidizing carbohydrates and fatty acids at the tissue level in the left ventricle of highland mice was supported by changes in enzyme activities. The increase in carbohydrate oxidation capacity in the high altitude mice after hypoxia acclimation was supported by a significant increase in hexokinase activity. An increase in hexokinase activity is indicative of an increased capacity for glucose oxidation. Overexpression of hexokinase in cardiomyocytes of mice led to higher ATP content in hypoxia, but there was no difference in normoxic conditions (Ye et al., 2005). In an oxygen-limited environment maximizing ATP produced per molecule of oxygen by increasing glucose oxidation capacity relative to fatty acids improves ATP production

significantly (Brand, 2005; Welch et al., 2007). The increase in relative use of carbohydrates was also due to decreased fatty acid oxidation capacity after hypoxia acclimation in the highland mice. The left ventricle tissue of highland mice showed a trend towards a decrease in respiration capacity when stimulated with palmitoyl-carnitine and in HOAD activity. The highland mice also significantly decreased carnitine palmitoyltransferase 1 (CPT1) activity in isolated mitochondria. CPT1 helps control the entry of long-chain fatty acids into the mitochondria (McGarry et al. 1983). Lowland mice also increased hexokinase activity in response to hypoxia acclimation and this was matched by a trend towards an increase in mitochondrial oxidation capacity of carbohydrates. However, left ventricle tissue of highland mice showed a trend towards an increase in respiration capacity when stimulated with palmitoyl-carnitine and in HOAD activity. Therefore, lowland mice increased oxidation capacity of both carbohydrates and fatty acids, such that there was no change in relative capacity for oxidizing carbohydrates compared to fatty acids in response to hypoxia acclimation.The increased capacity to oxidize carbohydrates relative to fatty acid in the highland mice after hypoxia acclimation at the tissue level was supported by increased enzymatic oxidation capacity of glycolysis and decreased enzymatic oxidation and transport capacity of fatty acids.

The left ventricles of highland mice have higher lactate dehydrogenase (LDH) activities. Similarly, pikas and torrent ducks that are native to high altitude have higher LDH activities in the left ventricle compared to their low altitude counterparts (Brandon,

2003; Dawson et al., 2016). In pikas the increased LDH activity was due to increased heart type LDH activity (Brandon, 2003). Rats that were acclimated to hypobaric hypoxia increased expression of LDH in the heart due to increased heart type LDH expression in the mitochondrial fraction (McClelland and Brooks, 2000). An increase in LDH activity in the heart in response to living at high altitudes or in hypoxia most likely supports lactate oxidation rather than lactate production due to an increase in heart type LDH. There are five different isoenzymes of LDH that are composed of four subunits each. The subunits are either heart type (H) or muscle type (M) and they differ in enzymatic activity. The H subunits favour conversion of lactate to pyruvate; whereas, the M subunits favour the conversion of pyruvate to lactate. The main function of LDH is determined by the subunits that it is composed of, if LDH is composed of four H subunits it favours lactate oxidation to pyruvate whereas LDH composed of four M subunits favours the revere reaction. An increase in overall LDH activity in the left ventricle can either increase lactate production or increase lactate oxidation depending on the predominant isozyme of LDH that is present. The previous findings suggest that an increase in LDH activity in the left ventricle in response to hypoxia exposure results in an increase in heart type LDH resulting in oxidation of lactate as an alternative carbon source (Brandon, 2003; Dawson et al., 2016; McClelland and Brooks, 2000).

Our observation that highland mice have a higher capacity for mitochondrial lactate oxidation suggests that they might have a greater capacity for using the

intracellular lactate shuttle. According to the intracellular lactate shuttle hypothesis cytosolic lactate is transported into the intermembrane space of the mitochondria where it is converted to pyruvate before entering the mitochondrial matrix. Although existence of an intracellular lactate shuttle has not been universally accepted, many studies have shown that mitochondria are able to oxidize lactate using permeabilized muscle fibres (Passarella et al., 2014; Jacobs et al., 2013; Elustando et al., 2013). Our study lends support to the intracellular lactate shuttle hypothesis and the existence of LDH within the mitochondrial intermembrane space. The muscle fibres were permeabilized using saponin, which preferentially reacts with cholesterol leading to perforation of the sarcolemma and loss of cytosolic metabolites, cofactors, enzymes and co-enzymes. Therefore, there should not be any cytosolic LDH in the permeabilized fibre preparations. Addition of 5uM cinnimate inhibits the mitochondrial pyruvate transporter located on the inner mitochondrial membrane and this reduced respiration rates to pre-lactate rates, which suggests that lactate is converted to pyruvate in the intermembrane space and that it is pyruvate that enters the mitochondrial matrix. We also tested whether LDH is located within the mitochondria by isolating mitochondria from the LV and measuring LDH activity in both intact and non-intact mitochondria. There was no LDH activity in the intact mitochondria, but LDH activity was detected after bursting the mitochondria. These results lend strong support for the existence of LDH in the intermembrane space.

The ability of mitochondria to take up lactate rather than conversion of lactate to

pyruvate in the cytosol can be beneficial in hypoxic conditions. The uptake of lactate by mitochondria may help balance lactate production via glycolysis and oxidation of lactate for ATP production without having a build up of lactate. Furthermore, it permits the cytosolic muscle type LDH to regenerate NAD^+ for glycolysis, while keeping homeostatic NAD^+ to NADH ratios in the cytosol (Sun et al., 2012). Lactate is also more readily diffusible into the mitochondria compared to NAD^+ efflux during increased glycolytic flux; therefore, lactate production and import may be a more effective way of maintaining NAD+/NADH ratios (Kane, 2014). In addition, removal of lactate from the cytosol may allow for increased uptake of lactate from the blood without accumulating lactate in the heart or inhibiting glycolysis due to accumulation of pyruvate if lactate is converted to pyruvate in the cytosol. The increased ability to oxidize lactate as an alternative fuel source may explain the reduced blood lactate in high altitude natives (Hochachka et al., 2002).

Highland mice have adaptations to mitochondrial physiology that enhance performance at high altitudes. Furthermore, most of the mitochondrial variables that were measured were fixed in the highland mice with very little change in response to hypoxia acclimation. Whereas, the mitochondria of the lowland mice seem have a more plastic phenotype and are able to increase respiratory capacity. Since the heart is continuously working and hypoxia sensitive it is important for high altitude animals to have ways to deal with hypoxia. The increased respiratory capacity in the highland mice that was

matched by lowland mice after hypoxia acclimation suggests that genetic assimilation has occurred in the highland population. The acclimation response of highland mice was unique from the response of lowland mice. The increase in relative oxidation capacity of carbohydrates compared to fatty acids in response to hypoxia acclimation in the highland mice would also help deal with hypoxia. Overall, the results suggest that the heart tissue of highland would perform better in hypoxia.

4.5 TABLES AND FIGURES

Figure 4.1

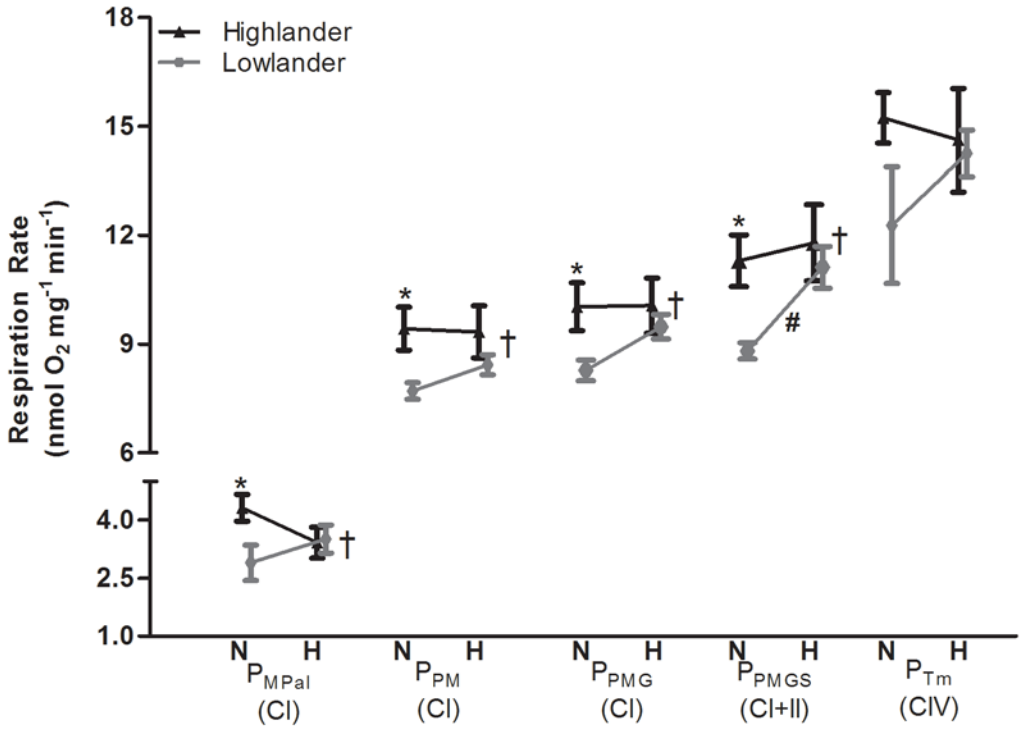

Figure 4.1: The left ventricle respiratory capacities of permeabilized muscle were greater in high-altitude deer mice than in low-altitude deer mice. N = normoxia acclimation environment, H = hypoxia acclimation environment, oxidative phosphorylation respiration was measured with substrates of complex I (CI) (P_{MPal} = malate + palmitoyl-carnitine + ADP; P_{PM} = malate + pyruvate + ADP; P_{PMG} = malate + pyruvate + ADP + glutamate), complexes I and II (CI+II) (P_{PMGS} = malate + pyruvate + ADP + glutamate + succinate) and maximum complex IV (CIV) respiration (P_{Tm} = ADP + ascorbate + TMPD). † Significant main effect of population. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment. # Significant pairwise differences in response to hypoxia acclimation within a population.

Figure 4.2

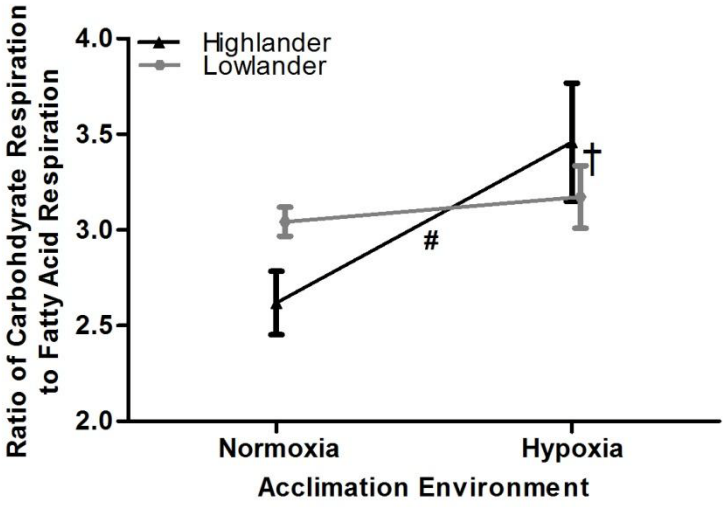
Ratio of Carbohdyrate Respiration to Fatty Acid Respiration
4.0
3.5
3.0
2.5
2.0
Highlander
Lowlander
#
†
Normoxia
Hypoxia
Acclimation Environment

Figure 4.2: High altitude deer mice increase capacity to oxidize carbohydrates relative to fatty acids in response to hypoxia acclimation, as reflected by the ratio of P_{PM} (P_{Mpal} = malate + pyruvate + ADP) to P_{MPal} (P_{PM} = malate + palmitoyl-carnitine + ADP) in permeabilized left ventricle muscle. ✝ Significant main effect of population. # Significant pairwise differences between the highland and lowland populations within the same acclimation environment.

Figure 4.3

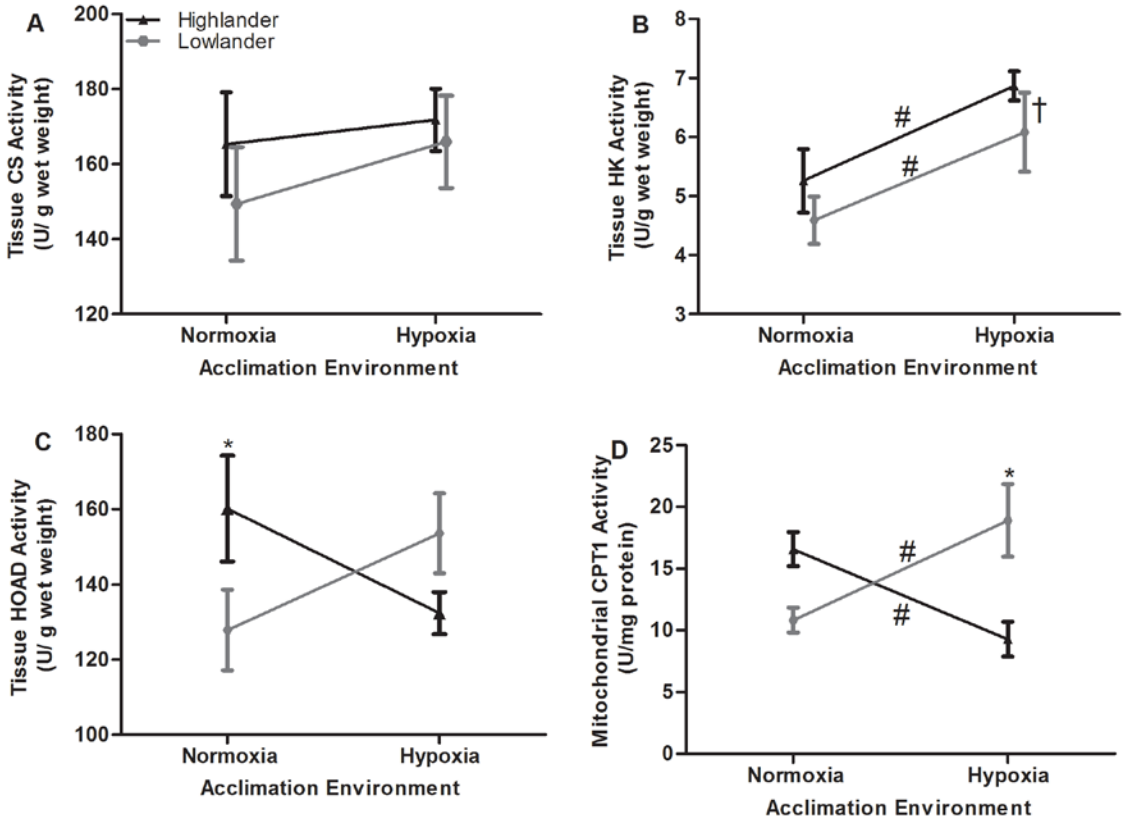
A
Highlander
Lowlander
Tissue CS Activity (U/ g wet weight)
200
180
160
140
120
Normoxia
Hypoxia
Acclimation Environment
B
Tissue HK Activity (U/g wet weight)
8
7
6
5
4
3
#
#
†
Normoxia
Hypoxia
Acclimation Environment
C
Tissue HOAD Activity (U/ g wet weight)
180
160
140
120
100
*
Normoxia
Hypoxia
Acclimation Environment
D
Mitochondrial CPT1 Activity (U/mg protein)
25
20
15
10
5
0
#
#
*
Normoxia
Hypoxia
Acclimation Environment

Figure 4.3: Maximal enzyme activities (V_{max} in U = μmol/min) in the left ventricle tissue (A, citrate synthase; B, hexokinase; C, HOAD) or in mitochondria isolated from left ventricle tissue (D, CPT1) in high and low altitude deer mice acclimated to normoxia or hypoxia.† Significant main effect of population. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment. # Significant pairwise differences in response to hypoxia acclimation within a population.

Figure 4.4

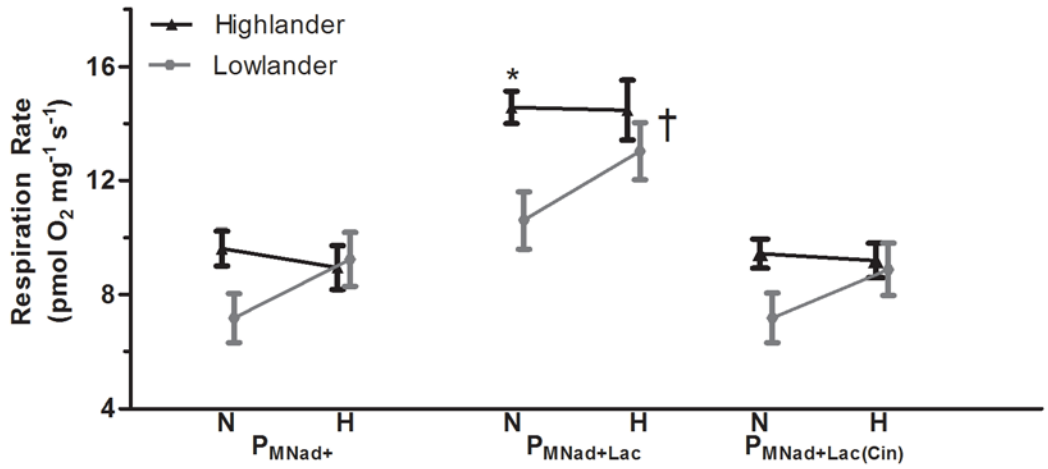
Highlander
Lowlander
Respiration Rate
(pmol O_2 mg^{-1} s^{-1})
16
12
8
4
*
†
N
H
P_{MNad+}
N
H
$P_{MNad+Lac}$
N
H
$P_{MNad+Lac(Cin)}$

Figure 4.4: The left ventricle respiratory capacities of permeabilized muscle fibres stimulated with lactate were greater in high-altitude deer mice than in low-altitude deer mice. N = normoxia acclimation environment, H = hypoxia acclimation environment, oxidative phosphorylation respiration was measured with substrates of complex I (P_{MNAD+} = malate + NAD^+; $P_{MNAD+Lac}$ = malate + NAD^+ + lactate; $P_{MNAD+Lac(Cin)}$ = malate + NAD^+ + lactate + 5 µM cinnimate. ✝ Significant main effect of population. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment.

Figure 4.5

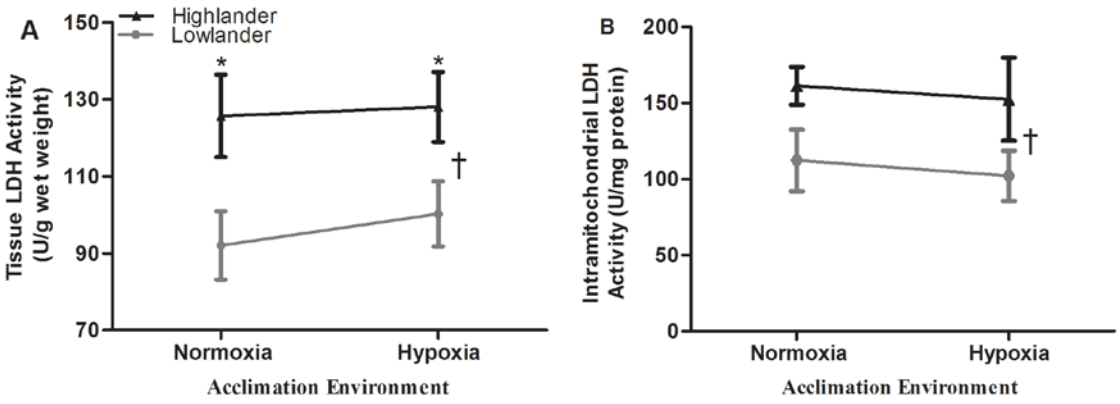
A
Highlander
Lowlander
Tissue LDH Activity (U/g wet weight)
150
130
110
90
70
*
*
†
Normoxia
Hypoxia
Acclimation Environment
B
Intramitochondrial LDH Activity (U/mg protein)
200
150
100
50
0
†
Normoxia
Hypoxia
Acclimation Environment

Figure 4.5: Maximal LDH activities (Vmax) in the tissue (A) and in isolated mitochondria (B) in the left ventricle of high and low altitude deer mice acclimated to normoxia or hypoxia.† Significant main effect of population. * Significant pairwise differences between the highland and lowland populations within the same acclimation environment.

4.6 REFERENCES

Bangsbo, J., Gollnick, P. D., Graham, T. E., & Saltin, B. (1991). Substrates for muscle glycogen synthesis in recovery from intense exercise in man. *The Journal of physiology*, *434*(1), 423-440.

Bergman, B. C., Wolfel, E. E., Butterfield, G. E., Lopaschuk, G. D., Casazza, G. A., Horning, M. A., & Brooks, G. A. (1999). Active muscle and whole body lactate kinetics after endurance training in men. *Journal of applied physiology*, *87*(5), 1684-1696.

Brand, M. D. (2005). The efficiency and plasticity of mitochondrial energy transduction. *Biochemical Society Transactions*, *33*(5), 897-904.

Braun, B., Mawson, J. T., Muza, S. R., Dominick, S. B., Brooks, G. A., Horning, M. A., Rock, P.B., Moore, L.G., Mazzeo, R.S., Ezeji-Okoye, S.C., & Butterfield, G. E. (2000). Women at altitude: carbohydrate utilization during exercise at 4,300 m. *Journal of Applied Physiology*, *88*(1), 246-256.

Brooks, G. A., Dubouchaud, H., Brown, M., Sicurello, J. P., & Butz, C. E. (1999). Role of mitochondrial lactate dehydrogenase and lactate oxidation in the intracellular lactate shuttle. *Proceedings of the National Academy of Sciences*, *96*(3), 1129-1134.

Elustondo, P. A., White, A. E., Hughes, M. E., Brebner, K., Pavlov, E., & Kane, D. A.

(2013). Physical and functional association of lactate dehydrogenase (LDH) with skeletal muscle mitochondria. *Journal of Biological Chemistry*, *288*(35), 25309-25317.

Hochachka, P. W., Beatty, C. L., Burelle, Y., Trump, M. E., McKenzie, D. C., & Matheson, G. O. (2002). The lactate paradox in human high-altitude physiological performance. *Physiology*, *17*(3), 122-126.

Hochachka, P. W., Stanley, C., Matheson, G. O., McKenzie, D. C., Allen, P. S., & Parkhouse, W. S. (1991). Metabolic and work efficiencies during exercise in Andean natives. *Journal of Applied Physiology*, *70*(4), 1720-1730.

Hochachka, P. W., Stanley, C., Merkt, J., & Sumar-Kalinowski, J. (1983). Metabolic meaning of elevated levels of oxidative enzymes in high altitude adapted animals: an interpretive hypothesis. *Respiration physiology*, *52*(3), 303-313.

Holden, J. E., Stone, C. K., Clark, C. M., Brown, W. D., Nickles, R. J., Stanley, C., Hochachka, P. W. Enhanced cardiac metabolism of plasma glucose in high-altitude natives: adaptation against chronic hypoxia. *Journal of Applied Physiology* 79: 222–228, 1995.

Ingwall, J.S. (2002). ATP and the heart. Kluwer, Boston.

Lau, D. S., Connaty, A. D., Mahalingam, S., Wall, N., Cheviron, Z. A., Storz, J. F., Scott

G.R. & McClelland, G. B. (2017). Acclimation to hypoxia increases carbohydrate use during exercise in high-altitude deer mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, ajpregu-00365.

Jürgens, K. D., Pietschmann, M., Yamaguchi, K., & Kleinschmidt, T. (1988). Oxygen binding properties, capillary densities and heart weights in high altitude camelids. *Journal of Comparative Physiology B*, *158*(4), 469-477.

Korvald, C., Elvenes, O. P., & Myrmel, T. (2000). Myocardial substrate metabolism influences left ventricular energetics in vivo. *American Journal of Physiology-Heart and Circulatory Physiology*, *278*(4), H1345-H1351.

Lundby, C. E., & Van Hall, G. (2002). Substrate utilization in sea level residents during exercise in acute hypoxia and after 4 weeks of acclimatization to 4100 m. *Acta Physiologica*, *176*(3), 195-201.

Mazzeo, R. S., Brooks, G. A., Schoeller, D. A., & Budinger, T. F. (1986). Disposal of blood [1-13C] lactate in humans during rest and exercise. *Journal of applied physiology*, *60*(1), 232-241.

McClelland, G. B., Hochachka, P. W., & Weber, J. M. (1998). Carbohydrate utilization during exercise after high-altitude acclimation: a new perspective. *Proceedings of the*

National Academy of Sciences, *95*(17), 10288-10293.

McClelland, G. B., Hochachka, P. W., & Weber, J. M. (1999). Effect of high-altitude acclimation on NEFA turnover and lipid utilization during exercise in rats. *American Journal of Physiology-Endocrinology And Metabolism*, *277*(6), E1095-E1102.

Morash, A. J., Kajimura, M., & McClelland, G. B. (2008). Intertissue regulation of carnitine palmitoyltransferase I (CPTI): mitochondrial membrane properties and gene expression in rainbow trout (Oncorhynchus mykiss). *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1778*(6), 1382-1389.

Quistorff, B., & Grunnet, N. (2011). The isoenzyme pattern of LDH does not play a physiological role; except perhaps during fast transitions in energy metabolism. *Aging (Albany NY)*, *3*(5), 457-460.

Razeghi, P., Young, M. E., Abbasi, S., & Taegtmeyer, H. (2001). Hypoxia in vivo decreases peroxisome proliferator-activated receptor α-regulated gene expression in rat heart. *Biochemical and biophysical research communications*, *287*(1), 5-10.

Schippers, M. P., Ramirez, O., Arana, M., Pinedo-Bernal, P., & McClelland, G. B. (2012). Increase in carbohydrate utilization in high-altitude Andean mice. *Current Biology*, *22*(24), 2350-2354.

Scott, G. R., Schulte, P. M., Egginton, S., Scott, A. L., Richards, J. G., & Milsom, W. K. (2011). Molecular evolution of cytochrome c oxidase underlies high-altitude adaptation in the bar-headed goose. *Molecular biology and evolution*, *28*(1), 351-363.

Templeman, N. M., Beaudry, J. L., Le Moine, C. M., & McClelland, G. B. (2010). Chronic hypoxia-and cold-induced changes in cardiac enzyme and gene expression in CD-1 mice. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1800*(12), 1248-1255.

Turek, Z., Grandtner, M., & Kreuzer, F. (1972). Cardiac hypertrophy, capillary and muscle fiber density, muscle fiber diameter, capillary radius and diffusion distance in the myocardium of growing rats adapted to a simulated altitude of 3500 m. *Pflügers Archiv*, *335*(1), 19-28.

Welch, K. C., Altshuler, D. L., & Suarez, R. K. (2007). Oxygen consumption rates in hovering hummingbirds reflect substrate-dependent differences in P/O ratios: carbohydrate as apremium fuel'. *Journal of Experimental Biology*, *210*(12), 2146-2153.

West, J. B. (1986). Lactate during exercise at extreme altitude. *Federation proceedings*, 45 (13), 2953-2957.

Ye, G., Donthi, R. V., Metreveli, N. S., & Epstein, P. N. (2005). Overexpression of hexokinase protects hypoxic and diabetic cardiomyocytes by increasing ATP

generation. *Cardiovascular toxicology*, *5*(3), 293-300.

CHAPTER 5: SUMMARY

5.1 GENERAL SUMMARY AND CONCLUSIONS

Genotype and environment determine an animal's phenotypic characteristics. These characteristics can be broadly categorized into biochemical, physiological and morphological traits. These traits determine an animal's performance, which natural selection can act on (Garland & Carter, 1994). High altitude environments are ideal for investigating evolutionary physiology. Aerobic capacity of wild caught high altitude deer mice is higher than the aerobic capacity of wild caught low altitude deer mice (Cheviron et al., 2012) and thermogenic capacity of highland mice is also under strong directional selection (Hayes and O'Connor, 1999). Changes in performance of an animal can be attributed to phenotypic plasticity or genetic adaptations (Garland & Carter, 1994). However, there hasn't been much research on the underlying characteristics at the level of the muscles that contribute to these differences in performance between high and lowland deer mice.

Aerobic capacity can be increased via enhancements along the oxygen cascade or through fuel selection (Favier et al., 1995; Hochachka, 1998; Beall, 2006; Beal, 2007; Schippers et al., 2012). I wanted to test whether the higher aerobic capacity of highland mice can be explained by enhanced aerobic capacity at the level of the tissue, specifically changes in mitochondrial abundance and physiology. Aerobic capacity of a tissue can be increased at high altitudes by increasing capillarity, increasing mitochondrial abundance, increasing the abundance of oxidative fibres, increasing mitochondrial volume density

within each muscle fibre, increasing the respiratory capacity of individual mitochondria, redistributing mitochondria closer to the sarcolemma and/or increasing the proportion of carbohydrate used during exercise.

Studying first generation (F1) mice born and raised at low altitude allows us to tease apart developmental and adult phenotypic plasticity from genetic adaptations. Aerobic capacity of F1 high altitude deer mice is higher than the aerobic capacity of F1 generation low altitude deer mice (Lui et al., Cheviron et al., 2013). Differences observed in F1 generation mice that were born and raised in common garden conditions strongly suggests that there are genetic differences due to ancestry. The gastrocnemius muscle of F1 highland deer mice had a greater abundance of mitochondria, more subsarcolemmal mitochondria, more capillaries, more efficient mitochondria indicated by higher RCR values and P/O ratios, higher mitochondrial respiratory capacities, and a greater capacity to oxidize both carbohydrates and fatty acids. The left ventricle of F1 highland deer mice also had mitochondria with higher respiratory capacities and greater capacity to oxidize both carbohydrates and fatty acids. These differences contribute to the muscles of highland mice having a higher aerobic capacity, which likely factors into highland deer mice having a higher aerobic capacity. However, one of the drawbacks of using F1 generation mice is the potential effect of epigenetics (Allis et al., 2007). Some of the differences observed between the F1 high and lowland deer mice may be explained by the fact that the genetic programming of the parents of highland mice were influenced by

being born and raised at high altitudes. Using second generation mice would have removed this confounding factor, and I could have been more certain that the differences were in fact entirely due to genetic differences between the two populations.

Acclimating the lowland and highland deer mice to hypoxia, cold or cold + hypoxia provides insight on adult phenotypic plasticity. However, this model system is unique in that I was able to look at the interplay of high altitude ancestry on phenotypic plasticity. In the left ventricle the respiratory capacity of mitochondria was higher in normoxia acclimated highland mice, but respiratory capacities converged after hypoxia acclimation. This was mainly because the highland mice had a fixed phenotype that did not change with acclimation to hypoxia, whereas the lowland mice increased respiratory capacities. This suggests that genetic assimilation likely occurred in the highland mice. In skeletal muscles hypoxia acclimation did not change mitochondrial abundance or efficiency in either high or lowland deer mice, but there was a significant change in mitochondrial respiratory capacities. Interestingly, the respiratory capacities both high and low altitude populations converged after hypoxia acclimation. The response was population specific; highland mice decreased respiratory capacities and lowland deer mice increased respiratory capacities. In response to cold and cold + hypoxia, mitochondrial respiratory capacities were not different from normoxia in highland mice, but respiratory capacities increased in lowland deer mice. Acclimation to cold in addition to hypoxia reversed the decrease in respiratory capacities in the highland deer mice. This

suggests that there is a complex interaction between cold and hypoxia that helps restore mitochondrial function to what was observed in normoxia acclimated highland deer mice. Lowland deer mice increased mitochondrial capacities, in response to cold and cold + hypoxia acclimations. After cold + hypoxia acclimation almost all measures of mitochondrial efficiency and mitochondrial capacity were similar between high and lowland deer mice. This suggests that genetic assimilation occurred in high altitude deer mice in response to the combined stress of hypoxia and cold. This is one of the few studies in which high and lowland animals were acclimated to cold, hypoxia and cold + hypoxia and it clearly demonstrated that there are distinct responses that are population specific.

Increased lactate oxidation capacity in heart mitochondria of normoxia acclimated highland mice and convergence of lactate oxidation capacity between the high and low altitude deer mice after hypoxia acclimation was an exciting novel finding. This was one of the first studies that looked at mitochondrial lactate oxidation capacity in cardiac tissue. A few studies have demonstrated that skeletal muscle mitochondria are also able to oxidize lactate (Elustado et al., 2013; Jacobs et al., 2013; Passaraella et al., 2014). The lactate paradox has two perplexing aspects: 1) lower blood lactate in VO_{2max} tests in highland natives compared to lowlanders 2) lower blood lactate in VO_{2max} tests in hypoxia-acclimated lowlanders compared to unacclimated lowlanders (Hochachka et al., 2002). If highland ancestry and hypoxia acclimation can also increase mitochondrial

lactate oxidation in skeletal muscle in addition to cardiac muscle it may help reduce blood lactate levels, shedding some insight on how blood lactate levels may be reduced.

At the whole animal level, the highland mice have higher aerobic capacities compared to lowland mice during running VO_{2max} when tested in hypoxia after the mice were acclimated to normoxia or hypoxia (Lui et al., 2012). After cold acclimation, the highland mice still have higher mitochondrial respiratory capacities so I would expect highland mice to still have a higher running $VO_{2max.}$ After acclimation to cold and hypoxia the high and lowland mice had mitochondria that are more similar functionally, but highland mice still had greater CS activity in the gastrocnemius tissue indicative of higher mitochondrial content. In skeletal muscle of lowland deer mice normoxia acclimated mice had the lowest oxidative capacity, followed by hypoxia acclimated mice, followed by cold acclimated mice, and acclimation to cold and hypoxia increased the oxidative capacity of the lowland mice to the greatest extent. If an increase in oxidative capacity determines performance of an animal at high altitudes then VO_{2max} of lowland deer mice should also increase with acclimation. Cold induced maximal oxygen consumption in hypoxia is higher in highland mice, but similar between high and lowland deer mice after cold + hypoxia acclimation (Wall et al., unpublished). Although cold induced maximal oxygen consumption is not exactly the same as VO_{2max} during running, the aerobic capacity of the populations may converge due to the increase in oxidative capacity of muscle tissue in lowland mice.

In conclusion, high altitude deer mice have mitochondria that are adapted to life at high altitudes that contribute to their muscles being more oxidative compared to lowland counterparts.

5.2 REFERENCES

Allis, C. D., Jenuwein, T., Reinberg, D., & Caparros, M. L. (Eds.). (2007). Epigenetics.

Beall, C.M. (2006). Andean, Tibetan, and Ethiopian patterns of adaptation to high-altitude hypoxia. *Integrative and Comparative Physiology*. 46,18-24.

Beall, C.M. (2007). Two routes to functional adaptations: Tibetan and high-altitude natives. *PNAS.* 104,8655-8660.

Cheviron, Z. A., Bachman, G. C., Connaty, A. D., McClelland, G. B., & Storz, J. F. (2012).

Regulatory changes contribute to the adaptive enhancement of thermogenic capacity in high-altitude deer mice. *Proceedings of the national academy of sciences*, *109*(22), 8635-8640.

Cheviron, Z. A., Bachman, G. C., & Storz, J. F. (2013). Contributions of phenotypic plasticity to differences in thermogenic performance between highland and lowland deer mice. *Journal of Experimental Biology*, *216*(7), 1160-1166.

Elustondo, P. A., White, A. E., Hughes, M. E., Brebner, K., Pavlov, E., & Kane, D. A. (2013). Physical and functional association of lactate dehydrogenase (LDH) with skeletal muscle mitochondria. *Journal of Biological Chemistry*, *288*(35), 25309-25317.

Favier, R., Speilvogel, H., Desplanches, D., Ferretti, G., Kayser, B., and Hopper, H. (1995). Maximal exercise performance in chronic hypoxia and acute normoxia in high altitude natives. *J. Appl. Physiol.,* 78: 1868-1874.

Garland Jr, T., & Carter, P. A. (1994). Evolutionary physiology. *Annual Review of Physiology*, *56*(1), 579-621.

Hayes, J. P., & O'Connor, C. S. (1999). Natural selection on thermogenic capacity of high-altitude deer mice. *Evolution*, 1280-1287.

Hochachka, P. W., Beatty, C. L., Burelle, Y., Trump, M. E., McKenzie, D. C., & Matheson, G. O. (2002). The lactate paradox in human high-altitude physiological performance. *Physiology*, *17*(3), 122-126.

Hochachka, P. W., Gunga, H.C., and Kirsch, K. (1998). Our ancestral physiological phenotype: An adaptation for hypoxia tolerance and for endurance performance. *Proc. Natl. Acad. Sci.*, 95, 1915-1920.

Jacobs, R. A., Meinild, A. K., Nordsborg, N. B., & Lundby, C. (2013). Lactate oxidation in human skeletal muscle mitochondria. *American Journal of Physiology-Endocrinology and Metabolism*, *304*(7), E686-E694.

Lui, M. A., Mahalingam, S., Patel, P., Connaty, A. D., Ivy, C. M., Cheviron, Z. A., ... &

Scott, G. R. (2015). High-altitude ancestry and hypoxia acclimation have distinct effects on exercise capacity and muscle phenotype in deer mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *308*(9), R779-R791.

Passarella, S., Paventi, G., & Pizzuto, R. (2014). The mitochondrial L-lactate dehydrogenase affair. *Frontiers in neuroscience*, *8*, 407.

Schippers, M. P., Ramirez, O., Arana, M., Pinedo-Bernal, P., & McClelland, G. B. (2012). Increase in carbohydrate utilization in high-altitude Andean mice. *Current Biology*, *22*(24), 2350-2354.