

**THE REGULATORY ROLE OF MITOCHONDRIAL SUBSTRATE KINETICS  
TO SUPPORT LIPID OXIDATION IN SHIVERING MUSCLE IN DEER MICE**

MSc. Thesis – C.E. Baragar; McMaster University – Department of Biology

**FUELLING THE FIRE: MITOCHONDRIAL FUEL SELECTION FOR  
SUSTAINING SHIVERING THERMOGENESIS IN THE HIGH-ALTITUDE  
DEER MOUSE, *PEROMYSCUS MANICULATUS***

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MOUSE, *PEROMYSCUS MANICULATUS*

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## **LAY ABSTRACT**

Some animals can survive extremely harsh climates, such as high altitude. High altitude is characterized by unremitting cold and thin air, and these challenges can constrain aerobic activities in mammals. The North American deer mouse can thrive at high altitude by actively generating large amounts of body heat in a process known as thermogenesis. The deer mouse relies primarily on fats as fuel to support thermogenesis, but the cellular mechanisms that regulate the use of lipids to power thermogenesis remain unclear. To address this question, I induced shivering in deer mice from both high- and low-altitude populations that I exposed to simulated high- or low-altitude conditions. I then examined the effects of these treatments on the ability of shivering muscle to consume oxygen and fuel for thermogenesis. My thesis contributes to the current understanding of how mammals manage their energy supply to survive in a challenging environment.

## ABSTRACT

High altitude is characterized by chronically low ambient temperatures and oxygen. To survive, highland native deer mice (*Peromyscus maniculatus*) are capable of high rates of prolonged thermogenesis due to elevated aerobic capacity ( $\dot{V}O_{2\max}$ ) in hypoxia. Deer mice primarily use fats to fuel their high metabolic rates for heat production. Carnitine palmitoyl-transferase 1 (CPT-1) is a rate-limiting step in mitochondrial fat oxidation, and a reduction in CPT-I sensitivity for its substrate L-carnitine is associated with a reduction in muscle fat use during high intensity exercise in mammals. Sensitivity of mitochondrial metabolism to ADP also changes with exercise. It is currently unknown whether similar mechanisms underpin regulation of fuel use during shivering, but I predicted that sensitivities to ADP and L-carnitine would be greater in highlanders than lowlanders and increase with acclimation. To address this question, I examined mitochondrial sensitivity to substrates involved in the fat oxidation pathway in low- and high- altitude deer mice born and raised in common laboratory conditions. Mice were also acclimated to high altitude condition of cold hypoxia to examine if the plasticity of these traits were affected by altitude ancestry. Consistent with previous findings, both high and lowland mice increased their cold-induced  $\dot{V}O_{2\max}$  following cold hypoxia acclimation and rely primarily on lipids to fuel thermogenesis. High- and low-altitude deer mice responded differently to chronic cold hypoxia with highlanders showing a ~7-fold greater ADP sensitivity than lowlanders following acclimation. In contrast to the expected outcome, highlander deer mice tended to have a reduced sensitivity to L-carnitine compared to lowlanders that approached statistical significance.

Neither sensitivity to palmitoylcarnitine sensitivity nor mitochondrial expression of FAT/CD36, thought to aid in mitochondrial fat delivery, showed differences between population or changes with acclimation, indicating that limitations to lipid oxidation during shivering likely occur at, or upstream of, CPT-I in the deer mouse.

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How do you begin an ending? At the beginning, of course. When I first started talking to Grant about doing a masters project, he sent along a paper from Petrick and Holloway that I could not, for the life of me, comprehend. Today, I know this paper upside-down and backwards. To me, that's progress.

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## Table of Contents

<b>LAY ABSTRACT</b> .....	<b>IV</b>
<b>ABSTRACT</b> .....	<b>V</b>
<b>ACKNOWLEDGEMENTS</b> .....	<b>VII</b>
<b>LIST OF FIGURES AND TABLES</b> .....	<b>XII</b>
<b>LIST OF ABBREVIATIONS AND SYMBOLS</b> .....	<b>XVI</b>
<b>DECLARATION OF ACADEMIC ACHIEVEMENT</b> .....	<b>XVIII</b>
<b>CHAPTER 1: GENERAL INTRODUCTION</b> .....	<b>1</b>
<b>1.1 Endothermy</b> .....	<b>1</b>
<b>1.2 High altitude</b> .....	<b>2</b>
1.2.1 Coping with hypoxia.....	2
1.2.2 Mitochondrial function at high altitude.....	5
<b>1.3 Thermogenesis</b> .....	<b>7</b>
1.3.1 Non-shivering Thermogenesis .....	8
1.3.2 Shivering Thermogenesis .....	10
<b>1.4 Fuel use in skeletal muscle</b> .....	<b>11</b>
1.4.1 Fuel use during exercise .....	11
1.4.2 Fuel use during shivering.....	12
1.4.2.1 The lipid oxidation pathway .....	13
1.4.2.2 Mitochondrial ADP transport .....	14
1.4.2.3 Mitochondrial lipid transport .....	16
1.4.2.4 Fuel interactions .....	16
1.4.3 Lipid use in shivering skeletal muscle .....	20
<b>1.5 Thesis Aims</b> .....	<b>21</b>
<b>1.6 References</b> .....	<b>23</b>
<b>CHAPTER 2: Fuelling the fire: Mitochondrial fuel selection for sustaining shivering thermogenesis in the high-altitude deer mouse, <i>Peromyscus maniculatus</i></b> .....	<b>33</b>
<b>2.1 Abstract</b> .....	<b>33</b>
<b>2.2 Introduction</b> .....	<b>34</b>
<b>2.3 Methods</b> .....	<b>40</b>

2.3.1 Animals and acclimations.....	40
2.3.2 Acute cold exposure and tissue sampling .....	41
2.3.3 Permeabilized gastrocnemius fiber respiration.....	43
2.3.4 Mitochondrial Isolation.....	45
2.3.5 Mitochondrial expression of FAT/CD36 .....	46
2.3.6 Statistics .....	47
<b>2.4 Results.....</b>	<b>48</b>
2.4.1 Figures and Tables.....	48
2.4.2 Body Mass and Cold-induced $\dot{V}O_2$ .....	57
2.4.3 ADP-supported respiration.....	57
2.4.4 Lipid-supported respiration.....	58
2.3.5 Mitochondrial FAT/CD36 Protein Abundance .....	58
<b>2.5 Discussion.....</b>	<b>59</b>
2.5.1 High-altitude show an altered ADP sensitivity response following cold hypoxia acclimation .....	60
2.5.2 High lipid oxidation rates during shivering in highland mice are not associated with increases in CPT-I substrate sensitivity.....	61
2.5.3 Summary and Conclusions.....	64
<b>2.6 References.....</b>	<b>66</b>
<b>CHAPTER 3: DISCUSSION.....</b>	<b>71</b>
<b>3.1 Mitochondrial respiratory kinetics at submaximal substrate concentrations as a measure of mitochondrial function.....</b>	<b>72</b>
<b>3.2 High- and low-altitude populations of deer mice show different ADP sensitivity responses in chronic cold hypoxia.....</b>	<b>73</b>
<b>3.3 Mitochondrial lipid transport.....</b>	<b>76</b>
3.3.1 High altitude mice do not increase sensitivity to P-CoA in chronic cold hypoxia.....	76
3.3.2 The regulation of lipid oxidation during shivering in high altitude mice is not associated with increases in CPT-I sensitivity to L-carnitine.....	77
<b>3.4 Lipid transport into the mitochondrial matrix .....</b>	<b>79</b>
<b>3.5 Summary and Conclusion.....</b>	<b>81</b>
<b>3.6 References.....</b>	<b>83</b>
<b>Appendix.....</b>	<b>89</b>



## LIST OF FIGURES AND TABLES

**Figure 1** Oxygen consumption ( $\dot{V}O_2$ , in mL g<sup>-1</sup> min<sup>-1</sup>) (A) and RER (B) of second and third generation highland and lowland deer mice (*Peromyscus maniculatus*) that were acclimated to thermal neutral (TN; 30°C, 21 kPa O<sub>2</sub>, 12:12 L:D) or cold hypoxia (CH; 4°C, 12 kPa O<sub>2</sub>, 8:16 L:D) conditions and then exposed to acute cold hypoxia (heliox).  $\dot{V}O_2$  (A) increased with CH acclimation (main effect of acclimation,  $F_{1,37} = 59.25$   $p < 0.0001$ ). The respiratory exchange ratios (RER; C) showed no significant effect of population ( $F_{1,37} = 1.55$ ,  $p > 0.05$ ) or acclimation ( $F_{1,37} = 0.80$ ,  $p > 0.05$ ). Asterisks indicate significant pairwise difference in highland deer mice within an acclimation environment. Data are means  $\pm$  S.E.M.,  $n = 6-11$ .

**Figure 2.** Mitochondrial respiration (nmol O<sub>2</sub> mg wet tissue<sup>-1</sup> min<sup>-1</sup>) in permeabilized fibers from the gastrocnemius muscle of high- (HA) and low-altitude (LA) native deer mice acclimated to either cold hypoxia (CH) or thermoneutral (TN) conditions. Leak<sub>PM</sub> was measured in the absence of ADP (in the presence of blebbistatin, pyruvate and malate). Complex I and II (CII<sub>PMGS</sub>)-stimulated respiration were measured immediately following the ADP kinetic protocol. CI<sub>PMG</sub> was maximally stimulated by pyruvate, malate, and glutamate, and CII<sub>PMGS</sub> respiration was maximally stimulated by, subsequently adding succinate. Highland native deer mice appear to increase their complex I respiration (CI<sub>PMG</sub>), compared to lowlanders (near-significant main effect of population;  $F_{1,25} = 3.88$ ;  $p = 0.06$ )  $n = 5-10$ . Bars represent the means  $\pm$  S.E.M.

**Figure 3.** Effect of cold hypoxia acclimation (CH) on ADP-supported mitochondrial respiration in post-shivering muscle fibers from low-altitude (LA) and high-altitude (HA) deer mice. The fibers were sampled immediately (1-2 mins) after shivering in cold hypoxia (heliox). Mitochondrial sensitivity to ADP in CH mice (**A**) and TN mice (**B**). In (**C**), there is a significant interaction effect of population and acclimation in mitochondrial sensitivity to ADP ( $K_m$ ), although no changes in maximum mitochondrial respiration were detected (**D**),  $n = 4-10$ . Symbols represent the means  $\pm S.E.M$ , significant differences # represent a  $p < 0.05$ .

**Figure 4.** Effect of cold hypoxia acclimation on palmitoyl Co-A (P-CoA)-supported mitochondrial respiration in shivering low-altitude (LA) and high-altitude (HA) deer mice. CPT-I sensitivity to P-CoA in CH mice (**A**) and TN mice (**B**).  $K_m$ s do not vary across acclimation groups and populations (**C**), although HA mice acclimated to cold hypoxia appear to increase mitochondrial respiration stimulated by P-CoA (**D**),  $n = 3-7$ . Symbols represent the means  $\pm S.E.M$ .

**Figure 5.** Effect of cold hypoxia acclimation on palmitoylcarnitine (PC)-supported mitochondrial respiration in shivering low-altitude (LA) and high-altitude (HA) deer mice. Mitochondrial sensitivity to PC in CH mice (**A**) and TN mice (**B**). No significant differences were identified in either mitochondrial sensitivity (**C**) or maximum palmitoylcarnitine-supported respiration (**D**),  $n = 3-6$ . Symbols represent the means  $\pm S.E.M$ .

**Figure 6.** Effect of cold acclimation on L-carnitine-supported mitochondrial respiration in shivering low-altitude (LA) and high-altitude (HA) deer mice. CPT-I sensitivity to L-carnitine in CH mice (**A**) and TN mice (**B**). Mitochondrial sensitivity (**C**) shows a population effect that approaches statistical significance ( $p = 0.0562$ ), and mitochondrial respiration supported by L-carnitine showed a trend to increase with acclimation (**C**),  $n = 4-9$ . Symbols represent the means  $\pm$  *S.E.M.*

**Figure 7.** Relative protein abundance of fatty acid translocase (FAT/CD36) in isolated mitochondria from the gastrocnemius. Plotted relative protein abundance of FAT/CD36 (**A**) and representative western blots (**B**) of mitochondrial FAT/CD36 (above) and SERCA2 (below),  $n = 5-7$ . Data represent the means  $\pm$  *S.E.M.*

**Table 1.** Summary of mitochondrial substrate sensitivities ( $K_m$ ) from Michaelis-Menten relationships from the gastrocnemius muscle of shivering deer mice. Second and third generation lab-born mice from high and low altitude populations of *P. maniculatus* were acclimated to either cold hypoxia (CH) or thermal neutral normoxia (TN) conditions and were then exposed to cold hypoxia (heliox) for 15 minutes. The muscle was sampled immediately (1-2 minutes) after shivering. Data represent the mean  $\pm$  standard error of the mean.

**Table 2.** Summary of mitochondrial substrate sensitivities ( $K_m$ ) from Michaelis-Menten relationships from the gastrocnemius muscle of shivering deer mice. Second and third generation lab-born mice from high and low altitude populations of *P. maniculatus* were acclimated to either cold hypoxia (CH) or thermal neutral normoxia (TN) conditions and were then exposed to cold hypoxia (heliox) for 15 minutes. The muscle was sampled immediately (1-2 minutes) after shivering. Data represent the mean  $\pm$  standard error of the mean.

## LIST OF ABBREVIATIONS AND SYMBOLS

ADP – Adenosine diphosphate  
ANOVA – Analysis of variance  
ANT – Adenine nucleotide transporter  
ATP – Adenosine triphosphate  
BAT – Brown adipose tissue  
iBAT – Interscapular brown adipose tissue  
BIOPS – Biopsy permeabilization buffer  
CAT – Carnitine acetyltransferase  
CACT – Carnitine-acylcarnitine translocase  
CH – Cold Hypoxia  
CI<sub>PMG</sub> – Mitochondrial complex I-stimulated respiration, supported by pyruvate, malate, and glutamate  
CII<sub>PMGS</sub> – Mitochondrial complex I and II-stimulated respiration, supported by pyruvate, malate, glutamate, and succinate  
CoA – Coenzyme A  
COX – Cytochrome c oxidase  
CPT-I – Carnitine palmitoyl-transferase I  
CPT-II – Carnitine palmitoyl-transferase II  
CS – Citrate synthase  
CT – Computerized tomography  
ETS – Electron transport system  
FA – Fatty acyl  
FFA – Free fatty acid  
HA – High altitude native population  
HOAD – 3-hydroxyacyl-CoA dehydrogenase  
JO<sub>2</sub> – O<sub>2</sub> flux  
K<sub>m</sub> – Michaelis-Menten constant  
LA – Low altitude native population  
Leak<sub>PM</sub> – Leak mitochondrial respiration, supported by pyruvate and malate  
M-CoA – Malonyl CoA  
Mir05 – Mitochondrial respiration medium  
MR – Metabolic rate  
NE – Norepinephrine  
NST – Non-shivering thermogenesis  
O<sub>2</sub> – Oxygen  
OXPHOS – Oxidative phosphorylation  
PC – Palmitoylcarnitine  
P-CoA – Palmitoyl Co-A  
PDH – Pyruvate dehydrogenase  
ROS – Reactive oxygen species  
SEM – Standard error of the mean  
SERCA – Sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPase 2

$T_a$  - Ambient temperature

$T_b$  - Body temperature

TN – Thermal neutral normoxia

TNZ – Thermal neutral zone

UCP1 – Uncoupling protein 1

VDAC – Voltage-dependent anion channel

$V_{max}$  – Maximum rate of substrate-to-product conversion

$\dot{V}CO_2$  – Whole-animal maximum rate of  $CO_2$  production

$\dot{V}O_{2max}$  – Whole-animal maximum rate of  $O_2$  consumption

$\dot{V}O_2$  – Whole-animal rate of  $O_2$  consumption

$^{\circ}C$  – Degrees Celsius

[ ] – Concentration symbol

## **DECLARATION OF ACADEMIC ACHIEVEMENT**

This thesis is organized in sandwich format, as recommended, and approved by members of my supervisory committee and approved by McMaster university. It consists of three chapters. Chapter 1 is a general introduction to the background material of this subject. Chapter 2 is in manuscript-style format, summarizing the background information, methods, findings, and a brief discussion of the findings. Chapter 3 is an in-depth examination of the major findings of this thesis and how these results contribute to the current knowledge base in this field.

### CHAPTER 1: GENERAL INTRODUCTION

### CHAPTER 2: FUELLING THE FIRE: FUELLING THE FIRE: MITOCHONDRIAL FUEL SELECTION FOR SUSTAINING SHIVERING THERMOGENESIS IN THE HIGH-ALTITUDE DEER MOUSE, PEROMYSCUS MANICULATUS

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Comments: The majority of data in this study was collected by C.E.B, under the supervision of G.B.M. S.A.L. taught dissections and respirometry techniques, and assistance with enzyme assays was given by D.S.

### CHAPTER 3: GENERAL DISCUSSION

## CHAPTER 1: GENERAL INTRODUCTION

### 1.1 Endothermy

Endothermy, or the physiological generation of heat through metabolism, is a defining feature of mammals. The major advantages to maintaining a relatively high and stable body temperature ( $T_b$ ) hinge upon the effects of temperature on biochemical reactions: as temperature increases, so does reaction rate (Bennet and Reuben, 1979; Tattersall *et al.*, 2012). Thus, as endothermy and high metabolic rates (MR) evolved, so did the capacity for sustaining prolonged aerobic activities, such as locomotion or thermogenesis (Clarke and Pörtner, 2010; Tattersall *et al.*, 2012).

However, maintaining an elevated  $T_b$  is energetically costly (Schubert *et al.*, 2010); for instance, the caloric demands of maintaining basal MR can be more than five times greater in endotherms than in ectotherms of a similar size (Karasov and Diamond, 1985). The already steep energetic demands of endothermy increase when a mammal must increase MR to compensate for low ambient temperatures ( $T_a$ ). The thermoneutral zone (TNZ) is a range of  $T_a$  throughout which an animal can regulate its  $T_b$  without increasing MR (Tattersall *et al.*, 2012). However, when  $T_a$  drops below the TNZ, homeothermic endotherms must increase their metabolism to defend  $T_b$  against increased heat loss to the cold environment (Tattersall *et al.*, 2012). Defending  $T_b$  is particularly challenging for small endotherms, such as rodents. Relative to larger species, they have a high surface area that interfaces with their ambient environment. Consequently, they experience greater rates of heat loss in the cold (Scholander *et al.*, 1950).

## **1.2 High altitude**

For endotherms, high altitude environments present a combination of physiological challenges, primarily, persistent low temperatures and hypoxia. The main physiological barrier to aerobic performance at high altitude is the reduction in aerobic scope due to the reduced oxygen availability (Hochachka, 1985). Since the aerobic exigencies of daily life remain as high or higher at altitude (Hayes and O'Connor, 1999), reducing oxygen demand by suppressing metabolism for extended periods of time is not a viable solution for animals that remain active year-round. Furthermore, snow cover, low temperatures and high windspeeds make high altitude one of the harshest environments inhabited by land animals. The deer mouse (*Peromyscus maniculatus*) can be found from below sea level (−80 m a.s.l.) to the high Sierras (4,300 m a.s.l.), granting them the broadest altitudinal distribution of any North American mammal (Hock, 1964; Snyder *et al.*, 1982). Highland and lowland populations of deer mice are genetically distinct from one another (Storz *et al.*, 2012), and high-altitude populations have evolved unique genetic variations of hypoxia resistance mechanisms, such as *EPAS1* and the genes that encode haemoglobin (Schweizer *et al.*, 2019; Storz *et al.*, 2012). For these reasons, the deer mouse is an ideal candidate for high altitude research.

### **1.2.1 Coping with hypoxia**

Despite these chronic environmental challenges, some endotherms have adapted to high altitude by maintaining non-limiting concentrations of O<sub>2</sub> at the mitochondria (Hochachka, 1985). Research on high altitude native humans demonstrates that there is no one adaptational combination that enables high altitude survival. For example, Tibetans and Andeans have adapted to high altitude (> 4,000m) for thousands of years

(Aldenerfer, 2000). Both populations maintain a similar aerobic scope and submaximal O<sub>2</sub> consumption at high altitude as lowland populations at sea level (Marconi *et al.*, 2006; Beall *et al.*, 2007), but through different adaptations. For instance, Tibetans “breathe deeper,” but maintain a lower haemoglobin concentration in their blood, whereas Andeans do the opposite, overall resulting in a similar level of oxygen saturation in the blood (reviewed by Beall, 2007).

Many high-altitude species evolved fixed genetic enhancements at one or more steps along the O<sub>2</sub> transport cascade that improve O<sub>2</sub> delivery to working tissues (Velotta *et al.*, 2018; McClelland and Scott, 2019; Storz and Cheviron, 2021). The bar-headed goose is capable of extreme high-altitude flight, and it models many evolved traits that enhance high altitude performance, including deeper breathing, increased haemoglobin O<sub>2</sub> affinity, and redistribution of flight muscle mitochondria closer to capillaries (Scott *et al.*, 2009a; Scott *et al.*, 2009b; Natarajan *et al.*, 2018). High fliers evolved larger lungs and deeper breathing, and this is also the case for high altitude humans and rodents (Callison *et al.*, 2022). Compared to lowlanders, highlander deer mice are also better at extracting O<sub>2</sub> from the bloodstream by increasing their heart rate and their cardiac output (Tate *et al.*, 2017). Highlander deer mice have evolved a greater haemoglobin O<sub>2</sub> affinity (Storz *et al.*, 2012; Wearing *et al.*, 2021). High altitude deer mice have consistently higher activities of fatty acid oxidation and glycolytic enzymes (*e.g.*, COX, HOAD, CS), as well as greater capillarity and oxidative fiber density in their gastrocnemius muscle (Lui *et al.*, 2015; Lau *et al.*, 2017). Compared to lowlanders, some high-altitude species have strategically recruited mitochondria closer to the capillaries to increase their rate of

O<sub>2</sub> diffusion into working tissues, as seen in the bar-headed goose by redistributing mitochondria (Scott *et al.*, 2009b) or in the deer mouse by increasing subsarcolemmal content (Mahalingam *et al.*, 2017). Notably, cold hypoxia acclimation recruits oxidative fibres and increases capillarity in *P. leucopus* but produces no effect in highlanders (Mahalingam *et al.*, 2020). This finding tells us that the effects of chronic cold and hypoxia interact, adding a layer of complexity to the high-altitude adaptive profile.

$\dot{V}O_2\text{max}$  declines with PO<sub>2</sub>. One strategy for improving aerobic performance in hypoxia is to increase the maximal rate of oxygen consumption, and this trend is particularly seen in rodents (Lui *et al.*, 2015; Schippers *et al.*, 2012). However, some high-altitude species buck the trend; for instance, high altitude *P. andium* do not increase their  $\dot{V}O_2\text{max}$  (Schippers *et al.*, 2012; McClelland and Scott, 2019). Furthermore, it is unclear whether increasing  $\dot{V}O_2\text{max}$  in chronic hypoxia is associated with reproductive success in humans (Moore, 2017; McClelland and Scott, 2019), suggesting that enhancing  $\dot{V}O_2\text{max}$  is not essential to high altitude success.

High altitude hypoxia adaptations are superimposed with a unique suite of plastic responses to hypoxia in highland endotherms (McClelland and Scott, 2019). In some cases, high altitude natives can induce a more dramatic shift in a trait than lowlanders in chronic hypoxia; for instance, hypoxia acclimation increases the rate of carbohydrate use in highlanders, but not lowlanders, during exercise (Lui *et al.*, 2015). Additionally, highlanders circumvent many of the maladaptive responses to chronic hypoxia that are seen in lowlanders, including minimizing the hypoxic pulmonary hypertension response (Beall, 2007; Groves *et al.*, 1993; Ge *et al.*, 1998) or blunting the rise in haemoglobin

concentration that occurs in lowland species upon chronic hypoxia to prevent an increase in blood viscosity (Black *et al.*, 1980; Beall *et al.*, 1984; Horscroft *et al.*, 2017).

### **1.2.2 Mitochondrial function at high altitude**

As the site of adenosine triphosphate (ATP) production and the majority of oxygen consumption (Murray and Horscroft, 2016), the mitochondria are often dubbed “the powerhouse of the cell” and play a critical role in mediating aerobic metabolism and energy homeostasis (Brand and Murphy, 1987; Brand, 2005). The machinery for making ATP – the electron transport system (ETS) – resides within the inner mitochondrial membrane, and the dense ultrastructure of this membrane (the cristae) lends the mitochondria its ability to generate large amounts of ATP through oxidative phosphorylation (OXPHOS; Soubannier and McBride, 2009). Oxidative phosphorylation, the process by which animals convert fuel and oxygen into ATP, is a central component of animal physiology. Being the cellular system with the greatest metabolite delivery, OXPHOS also mediates many cellular signalling systems. (Wilson, 2017).

Many mitochondrial studies incorporate measurements of maximum OXPHOS capacity, either through measuring the activities of mitochondrial enzymes, comparing mitochondrial volume within a tissue, or by directly measuring mitochondrial respiration (Hood *et al.*, 2018; Scott *et al.*, 2018). Metrics of mitochondrial quality can be measured using high resolution respirometry, by using permeabilized fibres or isolated mitochondria. While isolated mitochondria are the preferred method for when cytosolic factors or diffusion limitations may interfere with respiration measurements, differential centrifugation to isolate the mitochondria may introduce confounding factors (*e.g.*,

potential mitochondrial damage or biased selection of mitochondria (*e.g.*, swollen mitochondria). In contrast, permeabilized fibers preserve the functional and structural integrity of the mitochondria and are likely a better reflection of mitochondrial conditions *in situ* (reviewed by Kuznetsov *et al.*, 2008).

Species native to high altitude would benefit from increasing their mitochondrial O<sub>2</sub> consumption to support aerobic performance (*e.g.*, during exercise or thermogenesis), and this is reflected at the mitochondrial level. Highland deer have a greater OXPHOS capacity, which is supported by greater subsarcolemmal mitochondrial volume than lowlanders (Mahalingam *et al.*, 2017), and increased O<sub>2</sub> affinity ( $P_{50}$ ) in SS mitochondria (Dawson and Scott, 2022). Highland deer mice have a higher density of oxidative fibres in their skeletal muscles than congenics from a strictly lowland species (*P. maniculatus*) when acclimated to hypoxia (Lui *et al.*, 2015). Cold acclimation has increased mitochondrial volume density in other rodents (Buser *et al.*, 1982; Bruton *et al.*, 2010), although this has not been shown in deer mice.

Qualitatively, mitochondria respond to high altitude adaptation quite differently across species. For instance, highland deer mice and *P. leucopus* increased Leak respiration and decreased mitochondrial coupling efficiency (Mahalingam *et al.*, 2020), which contrasts starkly with the adaptational strategy of Tibetan Sherpas, which favours O<sub>2</sub> economy (Horscroft *et al.*, 2017). Although, Mahalingam *et al.* (2020) have speculated that the “loosening” of mitochondrial membrane integrity may be a sign of adaptive non-shivering thermogenesis in skeletal muscle. In isolated mitochondria from highlander deer mice, acclimation to cold or cold hypoxia yielded no increases in

OXPHOS capacity, which contrasts with *P. leucopus*. Warm hypoxia decreased OXPHOS capacity isolated muscle mitochondria from highlander deer mice (Mahalingam *et al.*, 2020), but does not affect OXPHOS capacity in permeabilized muscle fibres from the same species (Mahalingam *et al.*, 2017).

Recent evidence has emerged that submaximal mitochondrial respiration may be a more reliable marker of *in vivo* physiology, because *in situ*, mitochondria are rarely performing at maximum capacity (Petrick and Holloway, 2019; Petrick and Holloway, 2020). Chance and Williams (1955) were among the first to perform Michaelis-Menten titrations on isolated mitochondria, and the technique has since advanced through incorporating permeabilized fibres and the use of myosin-II inhibitors (Petrick and Holloway, 2020). It is possible that implementing submaximal respiration metrics could improve the understanding of the dynamic relationships between ancestry, plasticity, and environment at high altitude.

### **1.3 Thermogenesis**

Thermoregulation by thermogenesis, or heat production by thermo-effector tissues, is an energy consuming process that allows endotherms to compensate for heat lost to the external environment. In mammals, thermogenesis is a by-product of inefficient mitochondrial ATP production or ATP utilization, and mainly occurs via two mechanisms: (1) non-shivering thermogenesis (NST), by way of brown adipose tissue (BAT), or (2) shivering thermogenesis by the skeletal muscles. NST produces heat via futile cycling of protons across the mitochondrial membrane by uncoupling protein 1 (UCP1; Cannon and Nedergaard, *et al.*, 2004), and shivering generates heat by increasing

MR through involuntary muscle contractions that do not generate locomotory work. As highly aerobic processes, both shivering and non-shivering thermogenesis are difficult to perform at high altitude, an environment that compounds the chronic effects of reduced O<sub>2</sub> availability and low temperature. Particularly for small endotherms, which experience a relatively high surface area over which heat loss occurs, thermoregulation adds significant costs to daily energy expenditure. Thus, the high aerobic capacity of highlanders like deer mice presumably evolved to enhance thermogenesis (Cheviron *et al.*, 2014, Lui *et al.*, 2015, Scott *et al.*, 2015). Highlanders such as deer mice also have an enhanced plasticity in response to chronic cold and hypoxia, which has been shown to increase thermogenic capacity (Van Sant and Hammond, 2008; Cheviron *et al.*, 2012; Tate *et al.*, 2020; Coulson *et al.*, 2021). As will be reviewed in these next sections, chronic cold exposure enhances thermogenic capacity by stimulating the remodeling of both BAT and shivering mechanisms across several rodent species.

### **1.3.1 Non-shivering Thermogenesis**

NST is initiated by the stimulation of  $\beta_3$ -adrenergic receptors by norepinephrine (NE), leading to a signalling cascade that simultaneously increases UCP1 transcription and inhibits apoptosis in brown adipocytes (reviewed by Cannon and Nedergaard, 2004). NST upregulation and BAT development as an acclimatization response to the cold is critical to survival for small winter-active animals (Himms-Hagen, 1985; Cannon and Nedergaard, 2004; Velotta *et al.*, 2016), and has been shown to account for over 50% of thermogenic capacity in deer mice (Van Sant and Hammond, 2008). Experiments from UCP1 ablated mice reveal that UCP1 drives the thermogenic power of BAT, and its contributions cannot be matched by the combined efforts of UCP2 and UCP3

physiologically (Golozoubova *et al.*, 2001; Nedergaard *et al.*, 2001; Golozoubova *et al.*, 2006). As such, UCP1 is considered the mediator of adaptive non-shivering thermogenesis (Nedergaard *et al.*, 2001).

NST plasticity in response to cold acclimation/acclimatization is well documented in rodents, (Beaudry and McClelland, 2010; Cannon and Nedergaard, 2004; Golousova *et al.*, 2006; Van Sant and Hammond, 2008) with a particular focus on high altitude species, such as *Peromyscus* sp. (Chappell, 1984; Chappell and Hammond, 2004; Rezende *et al.*, 2004;). As shown in these works, BAT recruitment can take many forms, including but not limited to increasing UCP1 content, BAT mass, mitochondrial content, mitochondrial membrane leakiness, and BAT sensitivity to NE, all with the goal of enhancing the oxidizing capacity of BAT.

Recent insights from high altitude species tease apart the differences between the plastic and ancestral underpinnings of NST performance. Indeed, Velotta *et al.* (2016) found that, when testing wild populations in their native habitat, high altitude deer mice have an enhanced capacity for NST, compared to their lowland conspecifics and to lab reared F1 highlanders, indicating an environmental influence behind the evolved higher thermogenic capacity in highlanders (Cheviron *et al.*, 2012, 2014). This enhanced BAT function in wild highlanders was associated with upregulated transcript expression in a suite of genes that remodel BAT O<sub>2</sub> delivery to enhance NST, including genes for angiogenesis, brown adipocyte proliferation, and reduced apoptosis of BAT (Velotta *et al.*, 2016). Population differences were also found at the mitochondrial level for both wild and lab reared F1 high altitude mice, including increased UCP1 transcription and

expression (Velotta *et al.*, 2016). Mitochondria demonstrate an enhanced phenotypic plasticity response in highlanders, where there was an increase in UCP1-supported mitochondrial respiration in isolated iBAT mitochondria following cold hypoxia acclimation (Coulson *et al.*, 2021).

### **1.3.2 Shivering Thermogenesis**

Skeletal muscle is the largest fraction of body mass in mammals (Rowland *et al.*, 2014). It is important to note that skeletal muscle contractions contribute to both exercise and shivering, and acclimation/acclimatization or adaptations to enhance one performance trait may induce changes in the other; for instance, it may be possible to ‘train’ muscles by shivering frequently in a cold environment, thereby improving shivering (Cannon and Nedergaard, 2004). Furthermore, Hayes and Chappell (1986) found that cold acclimation increased running  $\dot{V}O_2\text{max}$  (9%) in addition to cold-induced  $\dot{V}O_2\text{max}$  (31%) in deer mice. The imbalance in cold acclimation enhancements between these two performance traits is likely caused by a strong selective pressure on thermogenic capacity in smaller mammals, the inclusion of NST in  $\dot{V}O_2\text{max}$  measurements, and regulation differences in the metabolic pathways that uphold both performance traits (McClelland and Scott, 2019). Shivering of the skeletal muscles can contribute considerably (up to ~ 30-50%) to thermogenesis (Van Sant and Hammond, 2008), although the influences of cold and hypoxia on shivering in high altitude mammals are mixed. For instance, Nespolo *et al.* (1999) determined that shivering thermogenesis in *P. xanthopygus* increased by 200% following a moderate cold acclimation, whereas cold acclimation (5°C) in deer mice did not affect shivering output (Van Sant and Hammond, 2008).

The heat generated by shivering is produced by the major ATPases of the cell, primarily, Na<sup>+</sup>/K<sup>+</sup> ATPase, myosin ATPase, and the sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA; Pant *et al.*, 2016). fMRI and PET/CT scans in humans have unearthed the principal anatomical components of the signalling pathway that stimulates shivering, beginning with temperature sensors in the skin and integrating input from the brain, spine, thermo-effector organs and core temperature sensors (although it remains unclear whether core temperature is generates feed-back signals through slight fluctuations, or if cold defense mechanisms are activated before core temperature can drop; reviewed by Haman and Blondin, 2017). Furthermore, it is the change in the temperature of the skin, rather than the absolute temperature, that stimulates signalling pathways from the brain to thermo-effector organs (Ran *et al.*, 2016).

Skeletal muscle may also be capable of NST, whereby sarcolipin uncouples Ca<sup>2+</sup> transport from SERCA ATP hydrolysis (Pant *et al.*, 2016). Whether it is considered adaptive thermogenesis remains controversial (Block *et al.*, 1994; Campbell and Dicke, 2018), but it is suggested that sarcolipin supported NST significantly affects thermogenic performance (Rowland *et al.* 2015). There is also some evidence of functional UCP recruitment to skeletal muscle mitochondria following cold acclimation in penguins (Talbot *et al.*, 2004), but this has yet to be confirmed in the deer mouse.

## **1.4 Fuel use in skeletal muscle**

### **1.4.1 Fuel use during exercise**

Fuel selection during aerobic exercise is influenced by intensity, work rate, and species (Brooks and Mercier, 1994; Schippers *et al.*, 2014; reviewed by Weber, 2011). As work rate increases, lipid oxidation can no longer support metabolic exercise, and cellular

metabolism undergoes a coordinated shift to carbohydrate use (Brooks and Mercier, 1994; Weber, 2011). Carbohydrate oxidation generates more ATP per mole O<sub>2</sub> (Brand, 2005), making carbohydrates the most O<sub>2</sub>-economical choice of fuel. Roberts *et al.* (1996) posited that it is advantageous to increase  $\dot{V}O_{2\max}$  to blunt the effects of increasing work rate on exercise intensity (in %  $\dot{V}O_{2\max}$ ), allowing carbohydrate metabolism to power higher absolute work rates. High altitude species may take advantage of the conservative O<sub>2</sub> consumption rate in carbohydrates, independent of changes to aerobic scope (McClelland *et al.*, 2017). For example, Schippers *et al.* (2012) found that wild high-altitude populations of Andean mice evolved a greater reliance on carbohydrates during treadmill exercise in hypoxia compared to lowlanders. Similarly, hypoxia-acclimated deer mice of high-altitude ancestry demonstrated an enhanced ability to oxidize carbohydrates during exercise in hypoxia, despite showing no difference in fuel selection from lowlanders prior to acclimation (Lau *et al.*, 2017). Together, these findings point towards convergent evolution of enhanced carbohydrate metabolism among high altitude species (McClelland *et al.*, 2017).

#### **1.4.2 Fuel use during shivering**

When recovery opportunities are limited, many organisms rely on lipids to power sustained aerobic activities (Vallaincourt *et al.*, 2009; Cheviron *et al.*, 2012; Lyons *et al.*, 2021; Lyons and McClelland, 2022). Lipids generate more ATP per gram than carbohydrates, and in mammals, lipids make up ~80% of endogenous lipid stores (Weber, 2011). While there are trade-offs to consuming lipids at high altitude, which require more O<sub>2</sub> per mole for oxidation than do carbohydrates (Weber, 2011), sustaining thermogenesis is presumably selected for over O<sub>2</sub> economy in this capacity.

#### **1.4.2.1 The lipid oxidation pathway**

To summarize the lipid oxidation pathway, free fatty acids (FFAs) are generated from storage as triacylglycerols in adipose tissue and transported to the adipocyte membrane. If they are not recycled back into triacylglycerols, they are transported into the circulatory system by way of fatty acid translocase, FAT/CD36. FFAs are bound and mobilized through the plasma by way of albumin; upon reaching the myocyte, albumin passes FFAs off to FAT/CD36 and FFAs enter the cell. FFAs are then transported through the cytosol by way of fatty acid binding proteins. At the outer mitochondrial membrane, FFAs are converted into fatty acyl-Coenzyme As (fatty acyl-CoAs). To enter the mitochondria, FA-CoAs are converted into FA-carnitines via carnitine palmitoyl-transferase I (CPT-I) and then transported across the outer membrane by carnitine acyltransferase; once translocated from the cytoplasm into the mitochondria by carnitine acylcarnitine transferase (CACT) in a 1:1 exchange with L-carnitine (Stephens *et al.*, 2007), acylcarnitines are transported into the matrix and converted back into fatty acyl-Co-As by CPT-II (McClelland, 2004). Once inside, the  $\beta$ -oxidation spiral yields one acetyl Co-A per turn (reviewed in McClelland, 2004; Stephens, 2007; Weber, 2011; Spriet, 2014).

To uphold increases in energy demand, metabolic pathways for lipid and carbohydrate oxidation must be upregulated in parallel to meet these demands, and aerobic metabolism is therefore constrained by the rate of substrate delivery to the mitochondria (McClelland and Scott, 2019). Similar signals from feedforward and feedback systems within a cell yield increases in both carbohydrate and lipid oxidation, and, therefore, cannot account for intensity-based changes in fuel consumption (Petrick

and Holloway, 2020). Furthermore, exercise research has indicated that metabolic control is not contingent on mitochondrial OXPHOS capacity (Phillips *et al.*, 1996; Green *et al.*, 1992). All other factors remaining the same, each metabolite in a pathway can act as a regulator upon that pathway (Wilson, 2017). This next section will explore substrate-based regulatory control at mitochondrial level, and how those control mechanisms can support aerobic activities, *e.g.*, exercise.

#### **1.4.2.2 Mitochondrial ADP transport**

The proton motive force generated by the ETS is used to transform ADP into ATP via ATP synthase. Positioned at such a key step in aerobic metabolic pathways, ADP can exert a tremendous amount of regulatory control over these pathways (Klingenberg, 2008). For instance, free ADP (ADP not bound to other proteins) oversees the regulation of the phosphorylation potential within cells, and, therefore, extends control over post-translational modifications within the cell that are tied to MR (Brand and Murphy, 1997; Meyer and Foley, 1996). In most cells, [ATP] and [Pi] are much greater than [ADP], so changes in [ADP] are much more influential on phosphorylation regulation (Wilson, 2017).

ADP enters the mitochondria by diffusing through voltage-dependent anion channel (VDAC) and crosses the intermembrane space where it passes through adenine nucleotide transporter (ANT), an antiporter that exchanges ATP for ADP in a 1:1 manner (Aliev *et al.*, 2011). ADP transport into the mitochondria is highly regulated, and the mitochondrial sensitivity to ADP has been of interest since as early as 1955 (Chance and Williams, 1955). ADP sensitivity has been shown to increase with OXPHOS capacity (Dudley *et al.*, 1987), and independently from increases in mitochondrial volume (Green

*et al.*, 1992; Phillips *et al.*, 1996). In contrast, mitochondrial sensitivity to ADP is attenuated during exercise in mice (Barbeau *et al.*, 2018; Miotto and Holloway, 2016), potentially to maintain cytosolic concentrations of ADP, but can be improved with exercise training (Ludzki *et al.*, 2015).

There are many potential sources of ADP transport regulation. ATP synthase, or complex V of the ETS, harnesses the proton motive force created by the electron transport chain to create ATP from ADP and inorganic phosphate (P<sub>i</sub>). ATP synthase shows some signs of regulatory control over mitochondrial ADP consumption; for example, fatiguing contractions in mouse gastrocnemius muscle revealed that several proteins in the ETS undergo post-translational modifications (PTMs) (Kramer *et al.*, 2018; as summarized by Petrick and Holloway, 2019b). In these mice, ATP synthase was S-glutathionylated at two locations, although whether the net outcome of these PTMs is inhibitory or stimulatory remains ambiguous (Kramer *et al.*, 2018).

ANT has been characterized by several forms of PTMs (Yan & Sohal, 1998; Feng *et al.*, 2008; Queiroga *et al.*, 2010; Mielke *et al.*, 2014). Perhaps the most compelling is the acetylation of lysine 23, which shows an apparent inhibitory effect on ANT1, but is attenuated following acute bouts of exercise in humans (Mielke *et al.*, 2014). ANT1 protein content has been shown to increase following exercise training in mice (Ludzki *et al.*, 2015), although it cannot solely account for increases in ADP sensitivity that arise through exercise training (Ludzki *et al.*, 2015).

Palmitoylcarnitine has been shown to affect the ultrastructure of the mitochondria, by expanding the intermembrane space and clustering the folds of the cristae (Toleikis *et*

*al.*, 2020). Toleikis *et al.* (2020) determined that the presence of palmitoylcarnitine increased mitochondrial ADP sensitivity in saponin-skinned rat heart, indicating that increasing the intermembrane space may have a stimulatory effect on ADP transport.

#### **1.4.2.3 Mitochondrial lipid transport**

CPT-I is essential for fatty acyl-CoA transport into the mitochondria (as reviewed in McClelland, 2004), and L-carnitine is its substrate. L-carnitine is not synthesized in skeletal muscle; it must be acquired from the liver, where it is made endogenously, or from diet, and subsequently transported to skeletal muscle via blood circulation (Evans and Fornasini, 2003). The regulation of CPT-I activity is not well understood, although intensity-based exercise studies have yielded some insight. CPT-I has a known and somewhat controversial allosteric inhibitor, Malonyl-Coenzyme A (M-CoA). M-CoA content has been shown to decrease with exercise intensity in rodents, but decreases in M-CoA have been shown not to influence increases in fatty acid uptake and oxidation at low and moderate exercise intensities (Odland *et al.*, 1998; van Loon *et al.*, 2001). Additionally, CPT-I can be phosphorylated, potentially by kinases that are known to increase with exercise intensity (Miotto *et al.*, 2017), although this mechanism and its potential to regulate CPT-I activity is not well understood.

#### **1.4.2.4 Fuel interactions**

ADP plays an important role in mitigating mitochondrial ROS production by reducing the membrane potential, and consequently, the rate of superoxide production, while increasing the rate of substrate oxidation (Ludzki *et al.*, 2015). In turn, ROS production is a suspected candidate for mediating ADP sensitivity during exercise, for ROS production has been associated with acetylation and glutathionylation at several

residues of ANT (Barbeau *et al.*, 2018; Willis *et al.*, 2018; Petrick and Holloway, 2020). ROS production is also thought to increase with exercise duration, which may have implications in sustaining glycolytic flux by increasing cytosolic [ADP] (Pearson *et al.*, 2014).

Palmitoyl-CoA (P-CoA) was first identified as an inhibitor of ATP sensitivity by Pande *et al.* (1971), who discovered that mitochondrial respiration was reduced in the presence of P-CoA at submaximal, but not maximal, ADP concentrations in rat cardiac mitochondria. P-CoA and other long-chain fatty acids have since been identified as competitive inhibitors of ADP-stimulated respiration *via* inhibition of ANT (Morel *et al.*, 1974; Ho and Pande, 1974), and have also been shown to attenuate the ability of ADP to mitigate mitochondrial ROS production (Ludzki *et al.*, 2015). However, ANT inhibition by P-CoA is inhibited following exercise training in humans, producing an overall enhancement of ADP sensitivity (Ludzki *et al.*, 2015).

Current understanding of the physiological role of L-carnitine stems largely from [CB1]research that was motivated by the nutrition and exercise industries (Stephens *et al.*, 2007). Fritz and colleagues' initial discovery that mitochondrial membranes are impermeable to long-chain fatty acyl-CoA, but not to long-chain fatty acylcarnitines, established that L-carnitines are essential for translocating large fatty acids into skeletal muscle mitochondria (Fritz *et al.*, 1955; Fritz and Yue, 1963; Fritz and Marquis, 1965; Stephens *et al.*, 2007), which is now understood to be the primary role for L-carnitine. A secondary but equally influential role for L-carnitine is its ability to consume acetyl-CoA, generating acetylcarnitine and consequently managing the size of the free acetyl-CoA

pool within the matrix (Virmani *et al.*, 2015; Virmani and Cirulli, 2022). Acetyl-CoA is either generated from pyruvate dehydrogenase (PDH) or from  $\beta$ -oxidation, and it typically accumulates when an increased power output from skeletal muscle generates a rise in cytosolic ADP, consequently stimulating glycolytic enzymes, including PDH (Howlett *et al.*, 1998; Spriet, 2014). As with many products of the TCA cycle, acetyl-CoA inhibits PDH, and when PDH outpaces the mitochondrial ability to consume acetyl-CoA, carnitine acyl transferase (CAT) can combine L-carnitine and acetyl-CoA to reduce the size of the acetyl-CoA pool and restore glycolytic flux (Randle *et al.*, 1963; Virmani *et al.*, 2015; Virmani and Cirulli, 2022).

The primary roles of L-carnitine – being to (i) effect long-chain fatty acyl translocation into the mitochondria, and (ii) to modulate the acetyl-CoA : CoA-SH ratio by buffering acetyl-CoA and acetylcarnitine – confer natural points of regulation for fuel metabolism that have been explored since the 1960s, especially in the context of declining fat oxidation at high exercise output (Brooks and Mercier, 1994; as reviewed by Stephens *et al.*, 2007). CPT-I, which sits on the outer mitochondrial membrane, is considered a rate-limiting step to fatty acid oxidation, as this enzyme catalyzes the reversible esterification that creates acylcarnitines (McGarry and Brown, 1997; McClelland, 2004). The underlying reasons for limiting fat oxidation remain underexplored.

During high intensity exercise, PDH activity increases, which corresponds to a decline in lipid oxidation and muscle L-carnitine availability (Romjin *et al.*, 1995; van Loon *et al.*, 2001). Experiments on systemic carnitine deficiency revealed that removing

85% of L-carnitine stored in skeletal muscle was associated with a 75% drop in palmitic acid oxidation (reviewed by Stephens *et al.*, 2007). In rats and horses, L-carnitine feeding improved intramuscular carnitine pools (Rivero *et al.*, 2002; Bacurau *et al.*, 2003) and fatigue resistance (Bacurau *et al.*, 2003). In contrast, dietary or intravenous supplementation of L-carnitine were not shown bolster carnitine pools or improve lipid oxidation during long-term exercise in humans (Brass *et al.*, 1994; Brass, 2000; Stephens *et al.*, 2006a), but muscle infusion of L-carnitine does alter muscle fuel metabolism and improve glycogen stores (Stephens *et al.*, 2006b), suggesting that in humans, L-carnitine transport into the muscles may be limiting (Stephens *et al.*, 2007). Together, these findings have generated a prominent theory that as L-carnitine buffers the accumulation of acetyl-CoA in skeletal muscle during high a work output, the free L-carnitine pool is diminished, consequently constraining fat oxidation in the mitochondria (Stephens *et al.*, 2007; Stephens *et al.*, 2019).

In the locomotory muscle of exercising lab mice, CPT-I sensitivity to L-carnitine was shown to decrease in an intensity-dependent manner (Petrick and Holloway, 2019). Furthermore, the apparent L-carnitine  $K_m$ s in these mice remained lower than the reported concentrations of free L-carnitine in lab mice, indicating that L-carnitine availability was non-limiting during exercise and at rest (ter Veld *et al.*, 2009; Constantin-Teodosiu *et al.*, 1991). Based on these findings, CPT-I sensitivity to L-carnitine is a promising mechanism of regulation of fatty acid oxidation during exercise. Whether L-carnitine sensitivity regulates lipid oxidation in shivering muscle remains unknown.

### 1.4.3 Lipid use in shivering skeletal muscle

Previous research on lipid oxidation in skeletal muscles has largely focused on the decline lipid consumption during exercise that occurs above 60-70%  $\dot{V}O_2\text{max}$  in favour of aerobic or anaerobic carbohydrate use (Hargreaves *et al.*, 1991; Brooks and Mercier, 1994; Romjin *et al.*, 1995; Weber, 2011; Schippers *et al.*, 2014). In the deer mouse, maximum cold-induced  $\dot{V}O_2$  surpasses exercising  $\dot{V}O_2\text{max}$  (Lyons *et al.*, 2021). Respiratory exchange ratios (RER) have revealed that lipid oxidation is 3-fold greater at 0°C than at 30°C (Lyons *et al.*, 2021), this is likely supported in highlanders by upregulated transcripts and activities for fat oxidation enzymes in the gastrocnemius (Cheviron *et al.*, 2012), as well as elevated plasma free fatty acids (FFA) and increased plasma flow rate at cold-induced  $\dot{V}O_2\text{max}$  to increase fat delivery following cold hypoxia acclimation (Tate *et al.*, 2017; Lyons and McClelland, 2022). Furthermore, the gastrocnemius muscle in highlander deer mice demonstrate enhanced  $^{13}\text{C}$ -labeled palmitate oxidation following cold hypoxia acclimation (Lyons *et al.*, 2021). Until recently, the barriers to high rates of lipid oxidation were thought to be a lack of substrate availability for CPT-I (Stephens *et al.*, 2018). However, lipid oxidation during high intensity exercise enters a refractory period even when lipid availability is artificially increased (Hargreaves *et al.*, 1991; Romjin *et al.*, 1995). Interestingly, fats can only be used aerobically (McClelland, 2004), and the indications of high lipid oxidation in gastrocnemius muscle contrast with what has been observed above 60-70%  $\dot{V}O_2\text{max}$  during exercise. FAT/CD36 has been shown to translocate to the mitochondrial membrane during exercise in an intensity-dependent manner (Campbell *et al.*, 2004; Holloway *et al.*, 2006). Furthermore, recent findings from Petrick and Holloway (2019)

determined that CPT-I sensitivity to L-carnitine decreases during high intensity exercise. Whether this mechanism underpins the ability of shivering muscle to maintain high rates of lipid oxidation in skeletal muscle warrants further investigation.

### **1.5 Thesis Aims**

In this study, we used second generation lab-born and raised deer mice from high low altitude ancestry. We acclimated these mice for at least six weeks to either thermoneutral conditions (30°C in normoxia) or simulated high-altitude conditions of cold hypoxia (4°C in 12 kPa O<sub>2</sub>). Intense shivering was induced with an acute cold exposure in hypoxia, and permeabilized fibres from oxidative core of the gastrocnemius muscle were prepared immediately post-shivering to examine mitochondrial function using high-resolution respirometry. We tested the hypothesis that, compared to lowland conspecifics, deer mice native to high altitude would demonstrate altered mitochondrial respiration kinetics that promote their high rates of whole-animal lipid oxidation observed during thermogenesis (Lyons *et al.*, 2021). We predicted highlander mitochondria would show greater sensitivity to ADP and lipid oxidation substrates to promote the use of lipids during intense shivering.

In chronic cold hypoxia, highland deer mice also demonstrate enhanced thermogenesis in response to cold hypoxia acclimation by dramatically increasing their cold-induced  $\dot{V}O_2\text{max}$  (Lyons *et al.*, 2021) and by upregulating genes that promote thermogenic endurance (Cheviron *et al.*, 2013), compared to lowlanders. At the mitochondrial level, highlanders evolved altered plasticity from lowlanders by upregulating CS and COX independently from changes in oxidative fiber abundance

(Mahalingam *et al.*, 2020<sub>[GM3]</sub>). Thus, we further predict, that highland deer mice will show an increase in mitochondrial sensitivity to ADP and L-carnitine following cold hypoxia acclimation, while lowlanders will show no change.

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## **CHAPTER 2: Fuelling the fire: Mitochondrial fuel selection for sustaining shivering thermogenesis in the high-altitude deer mouse, *Peromyscus maniculatus*.**

### **2.1 Abstract**

High altitude is characterized by chronically low ambient temperatures and oxygen. To survive, highland native deer mice (*Peromyscus maniculatus*) are capable of high rates of prolonged thermogenesis due to elevated aerobic capacity ( $\dot{V}O_2\text{max}$ ) in hypoxia. Deer mice primarily use fats to fuel their high metabolic rates for heat production. Carnitine palmitoyl-transferase 1 (CPT-1) is a rate-limiting step in mitochondrial fat oxidation, and a reduction in CPT-I sensitivity for its substrate L-carnitine is associated with a reduction in muscle fat use during high intensity exercise in mammals. Sensitivity of mitochondrial metabolism to ADP also changes with exercise. It is currently unknown whether similar mechanisms underpin regulation of fuel use during shivering, but we predicted that sensitivities to ADP and L-carnitine would be greater in highlanders than lowlanders and increase with acclimation. To address this question, we examined mitochondrial sensitivity to substrates involved in the fat oxidation pathway in low- and high- altitude deer mice born and raised in common lab conditions. Mice were also acclimated to the high-altitude condition of cold hypoxia to examine if the plasticity of these traits were affected by altitude ancestry. Consistent with previous findings, both high and lowland mice showed an increase of their cold-induced  $\dot{V}O_2\text{max}$  following cold hypoxia acclimation and rely primarily on lipids to fuel thermogenesis. High- and low-altitude deer mice responded differently to chronic cold acclimation resulting with highlanders showing a ~7-fold greater ADP sensitivity than lowlanders following acclimation. In contrast to the expected outcome, highlander deer mice tended to have a

reduced sensitivity to L-carnitine compared to lowlanders that approached statistical significance. Neither sensitivity to palmitoylcarnitine sensitivity nor mitochondrial expression of FAT/CD36, thought to aid in mitochondrial fat delivery, showed differences between population or changes with acclimation, indicating that limitations to lipid oxidation during shivering likely occur at, or upstream of, CPT-I in the deer mouse.

## **2.2 Introduction**

In mammals, exercise and shivering are both carried out by contracting skeletal muscle, but the metabolic pathways that initiate these two activities are very different (McClelland, 2004, Weber, 2011; Spriet *et al.*, 2014; Lyons *et al.*, 2021; Lyons and McClelland, 2022). As the ultimate site of oxygen and fuel consumption, the mitochondria define the upper limits of aerobic performance within skeletal muscle, and there is a strong relationship between the type of fuel oxidized by the mitochondria and the capacity for work performed by the muscles (Hochachka, 1985; Brooks and Mercier, 1994; Weber, 2011). For instance, as exercise intensity increases to ~60-70% of maximal exercise-induced oxygen consumption ( $\dot{V}O_{2\max}$ ) in mammals, fuel metabolism switches from depending primarily on lipids to carbohydrates (Brooks and Mercier, 1994; Romjin *et al.*, 1995; Weber, 2011). This shift in fuel use is caused by the comparatively low rate of maximal rate ATP production supported by lipids (McClelland, 2004; Weber, 2011), which constrains exercise intensity. However, lipid oxidation yields more ATP per gram than carbohydrates because lipids are more chemically reduced than other fuels (Weber, 2011). This makes lipids ideal for supporting sustained aerobic activity, such as thermogenesis.

Much of the current understanding of mitochondrial function comes from examining the maximal respiratory capacities of the mitochondrial complexes (Kuznetsov *et al.*, 2008; Gnaiger, 2012). In addition to the classic metrics of maximal mitochondrial respiration, mitochondrial kinetics at submaximal respiration is increasingly recognized as important for understanding *in vivo* constraints on ATP production (Ludzki *et al.*, 2015; Holloway, 2017). For instance, ADP sensitivity is known to decline during moderate intensity exercise in mice and in humans (Ludzki *et al.*, 2015; Miotto *et al.*, 2016; Petrick and Holloway, 2019). Furthermore, mitochondrial enzyme sensitivities have been shown to change in an intensity-dependent manner which correspond to changes in whole-animal performance (Petrick and Holloway, 2019). While enzyme sensitivity continues to be examined in exercising muscle, to our knowledge, this subject has yet to be explored in shivering muscle. Shivering capacity is known to vary greatly across and within mammalian species (Nespolo *et al.*, 1999; Van Sant and Hammond, 2008; Robertson and McClelland, 2019; Lyons *et al.*, 2021), and it is likely that submaximal mitochondrial respiration, in addition to maximal respiration, contributes to this variation.

Thermogenesis, or endogenous heat production, is a complex performance trait that allows homeothermic endotherms such as mammals to maintain a stable body temperature in face of ambient temperatures below their thermal neutral zone (TNZ). Thermogenesis in small eutherian mammals occurs through two different mechanisms: (i) non-shivering thermogenesis (NST), where heat production is driven by futile proton cycling across the inner mitochondrial membranes in brown adipose tissue (BAT), mediated by uncoupling protein (UCP)-1 (Cannon and Nedergaard, 2004); or (ii) shivering thermogenesis (ST) in

skeletal muscles, whereby mitochondrial ATP production powers involuntary muscle contractions that generate no locomotory work (Hemingway, 1963). Mammalian species challenges can be dramatic for small mammals like rodents, because of their large surface area to volume ratio, and because they are likely operating near their metabolic ceiling in the wild at high altitude, based on estimates of their daily energy expenditure (Hayes, 1989b). Over the last few decades, plasticity of non-shivering thermogenesis involving BAT in response to chronic cold and combined cold hypoxia has garnered much interest (reviewed by Cannon and Nedergaard, 2004; Velotta *et al.*, 2016; Coulson *et al.*, 2021). While NST is a major contributor to thermogenesis, shivering can contribute between 40-50% of thermogenic output (Van Sant and Hammond, 2008). The capacity for shivering thermogenesis has been shown to increase by 2-fold following cold acclimation in rodents (*e.g.*, *P. xanthopygus*, Nespolo *et al.*, 1999). Due to the importance of ST to overall heat production, the underlying metabolic mechanisms responsible for shivering thermogenesis warrant further investigation.

As the site of oxygen consumption and aerobic ATP production, mitochondrial performance is central to whole-animal aerobic performance. Previous research on mitochondrial physiology in high-altitude species has focused on adaptive and plastic responses that enhance muscle O<sub>2</sub> consumption, although the mechanism of change varies considerably across species. For instance, studies examining enzyme activities and muscle phenotypes in high altitude deer mice have revealed fixed genetic traits that support aerobic performance in hypoxia, such as elevated oxidative fiber content, oxidative enzyme activities and enhanced pulmonary O<sub>2</sub> extraction and O<sub>2</sub> delivery (Lui *et al.*, 2015;

Lau *et al.*, 2017; Tate *et al.*, 2020). High altitude natives such as deer mice can also develop altered plastic responses to the cold and hypoxia compared to lowlanders. Following hypoxia acclimation, high altitude deer mice upregulate carbohydrate metabolism pathways, including upregulating hexokinase activity and the capacity for circulatory glucose uptake (Lau *et al.*, 2017). Additionally, compared to lowlanders, high altitude deer mice increase their hematocrit and haemoglobin concentrations in high altitude simulated conditions (Tufts *et al.*, 2013), and highlanders display an altered thermogenic endurance response to hypoxia acclimation (Cheviron *et al.*, 2013).

The deer mouse is a well-established model for high altitude hypoxia, as this species inhabits the broadest altitudinal distribution in North America. For high altitude species such as the deer mouse, natural selection might act on mitochondrial volume density, respiratory capacity, coupling efficiency, reducing muscle leak respiration and/or increasing leak respiration in brown adipose tissue to improve aerobic performance. In terms of myocytes structure, these mice have a greater density of subsarcolemmal (SS) mitochondria, which are located close to the cell membrane and near capillaries. This increase in SS mitos explain the overall increase in total mitochondrial volume density (Mahalingam *et al.*, 2017). Functionally, mitochondria expressed in skeletal muscle of highlanders evolved an increase in OXPHOS capacity, respiratory control ratio, and O<sub>2</sub> sensitivity during leak respiration (Mahalingam *et al.*, 2017). Moreover, it is the subsarcolemmal mitochondria that appear to drive many of these changes (Dawson and Scott, 2022). In chronic cold hypoxia, highlanders demonstrate an altered plastic response in their mitochondria, which increases oxidative enzyme activities of COX and CS in the

gastrocnemius muscle independently from changes in oxidative fibre abundance, unlike in native lowland congeners (*P. leucopus*; Mahalingam *et al.*, 2020). CS is a known marker of mitochondrial abundance, and these results suggest highlanders increase their mitochondrial abundance in response to cold hypoxia. However, many of these traits do not change with hypoxia acclimation alone and may be fixed at a higher level in highlanders (Mahalingam *et al.*, 2017).

Previous work has focused on identifying changes in the oxygen transport that improve aerobic function in highland deer mice, but less is known about how high altitude adapted animals power thermogenesis for prolonged periods. [MG12] Of what we know in the deer mouse, high altitude natives have fixed genetic traits that enhance their capacity for oxidizing lipids in muscle mitochondria (Cheviron *et al.*, 2012; Lau *et al.*, 2017). Whole-animal lipid oxidation in deer mice is threefold greater in moderate cold exposure compared to thermal neutral conditions, and this finding is mirrored by <sup>13</sup>C-labeled palmitate oxidation, with high altitude mice demonstrating enhanced <sup>13</sup>C- palmitate oxidation following cold hypoxia acclimation (Lyons *et al.*, 2021). Furthermore, elevated plasma free fatty acids (FFA) in these mice combine with increased plasma flow rate at  $\dot{V}O_2\text{max}$  to increase fat delivery in cold hypoxia acclimated mice (Tate *et al.*, 2017; Lyons and McClelland, 2022). However, the mechanism responsible for enhanced mitochondrial lipid used *in vivo* during shivering by high altitude deer mice is unclear.

Much of the current understanding of patterns of fuel use comes from studies on how muscle metabolism is powered during aerobic exercise. At low exercise intensities in mammals, lipid oxidation predominates but is known to decrease as exercise intensity

increases (Brooks and Mercier, 1994; Weber and Haman, 2004; Romjin *et al.*, 1995; Schippers *et al.*, 2014), even when lipid availability is artificially elevated (Hargreaves *et al.*, 1991). The mechanisms underlying this shift remain unclear, but the primary limitations to lipid consumption in the mitochondria are presumed to be cell transport limitation and a reduction of substrate availability. Specifically, limitation in fatty acid availability for the carnitine palmitoyl transferase-I (CPT-I) reaction, which catalyzes a key step in the transport of fats into the mitochondria (Stephens, 2018). However, recent work from Petrick and Holloway (2019) suggests that sensitivity of mitochondria to substrates impacts fuel use *in vivo* by regulating muscle fuel use relative to exercise intensity. They suggest that it is a reduced sensitivity to L-carnitine with the transition from low to high intensity exercise that helps explain the declining reliance on fat oxidation as muscles metabolism increases. Whether a similar shift in mitochondrial substrate sensitivity occurs in skeletal muscle with intense shivering is unknown. However, the high reliance on lipids to power both moderate and intense shivering in deer mice (Lyons *et al.*, 2021, Lyons and McClelland, 2022) suggests distinct regulation of mitochondrial metabolism compared with during aerobic exercise. Whether population differences in lipid oxidation for thermogenesis between lowland and highland deer mice or changes that occur with acclimation to cold hypoxia involve modifications of mitochondrial kinetics is also unclear.

To address this knowledge gap, we used second generation lab-born and raised deer mice from high and low altitude ancestry. We acclimated these mice for at least six weeks to either thermoneutral conditions (30°C in normoxia) or simulated high-altitude conditions of cold hypoxia (5°C in 480 mmHg). Intense shivering was induced with an

acute cold exposure in hypoxia, and permeabilized fibres from oxidative core of the gastrocnemius muscle were prepared immediately post-shivering to examine mitochondrial function using high-resolution respirometry. We tested the hypothesis that, compared to lowland conspecifics, deer mice native to high altitude would demonstrate altered mitochondrial respiration kinetics that promote their high rates of whole-animal lipid oxidation observed during thermogenesis (Lyons *et al.*, 2021). We predicted highlander mitochondria would show greater sensitivity to ADP and lipid oxidation substrates to promote the use of lipids during intense shivering.

Highland deer mice also demonstrate enhanced thermogenesis response to chronic cold hypoxia by dramatically increasing their cold-induced  $\dot{V}O_2\text{max}$  (Lyons *et al.*, 2021) and by upregulating genes that promote thermogenic endurance (Cheviron *et al.*, 2013), compared to lowlanders. At the mitochondrial level, highlanders evolved altered plasticity by upregulating CS and COX independently from changes in oxidative fiber abundance (Mahalingam *et al.*, 2020). Thus, we further predict, that highland deer mice will show an increase in mitochondrial sensitivity to ADP and L-carnitine following cold hypoxia acclimation, while lowlanders will show no change.

## 2.3 Methods

### 2.3.1 Animals and acclimations

Wild highland native deer mice (*Peromyscus maniculatus rufinus*) were trapped at the summit of Mount Blue Sky, CO, USA (4350 m *a.s.l.*) and lowland native deer mice (*Peromyscus maniculatus nebracensis*) in Kearney, NE, USA (600m *a.s.l.*). Mice were transferred to McMaster University (90 m *a.s.l.*) and housed in common garden conditions of ~23°C, 12 h:12 h L:D cycle, with food and water available *ad libitum*. The

deer mice were bred within their respective populations to produce second and third generation (G1 and G2) lowland and highland deer mice. This study used both male and female mice of at least 6 months of age, which were randomly assigned to one of two acclimation groups: thermal neutral normoxic conditions (TN; 30°C and 760 mmHg; 12:12 L:D) using a rodent incubator (Powers Scientific), or cold hypoxia (CH; 5°C and 480 mmHg; 12:12 L:D) using hypobaric chambers (McClelland *et al.*, 1998; Lui *et al.*, 2015) kept in a cold room, as previously described (Lyons and McClelland, 2022). Mice in the cold hypoxia acclimation group were kept for 24h at 5°C in normobaria before being placed in the hypobaric chambers. Mice were briefly removed (<1h per week) from their acclimations for cage cleaning and to replenish food and water. All procedures were approved by the McMaster University Animal Research Ethics Board in accordance with guidelines set by the Canadian Council on Animal Care.

### **2.3.2 Acute cold exposure and tissue sampling**

The gastrocnemius muscles of lowland and highland mice were sampled immediately after steady-state shivering at their maximal cold-induced oxygen consumption ( $\dot{V}O_2\text{max}$ ) in hypoxia, as described previously (Lyons *et al.*, 2021). Briefly, hypoxic cold-induced  $\dot{V}O_2$  was determined by pushing dry, CO<sub>2</sub>-free heliox (12% O<sub>2</sub>, 88% He; Rosenmann & Morrison, 1974) at 1050 ml min<sup>-1</sup> using mass flow meters and controllers (Sierra Instruments, Monterey, CA; MFC-4, Sable Systems, NV) through copper coils housed inside a temperature control cabinet and into a respirometry chamber (~500 ml) cooled to 5°C for TN mice, and -5°C for CH mice. Mass-specific  $\dot{V}O_2$  was determined using the following equation (Chappell *et al.*, 2007; Lighton, 2008):

$$\dot{V}O_2 = FR_i \frac{(F_iO_2 - F_eO_2) \times F_eO_2(F_eCO_2 - F_iCO_2)}{(1 - F_eO_2)}$$

Where  $FR_i$  is the flow rate. Relative to the chamber,  $F_i$  and  $F_e$  denote fractional incurrent and excurrent gas concentrations, respectively. To reduce variance the log values of body mass and  $\dot{V}O_2$  were taken prior to covariate analysis. After allowing the cabinet to cool to  $-5^\circ\text{C}$  for cold hypoxic mice or to  $5^\circ\text{C}$  for thermal neutral mice, The excurrent air was subsampled at a rate of  $\sim 200 \text{ mL min}^{-1}$ , dried with pre-baked Drierite and then passed through  $\text{CO}_2$  and  $\text{O}_2$  gas analyzers (Sable Systems). The mice were exposed to acute cold hypoxia for 15 minutes, and  $\dot{V}O_{2\text{max}}$  was defined as the greatest  $\dot{V}O_2$  over a 5-10 second period. Mass-specific  $\dot{V}CO_2$  was calculated using the following equation (Lighton, 2008):

$$\dot{V}CO_2 = FR_i \frac{(F_eCO_2 - F_iCO_2) \times F_eCO_2(F_iO_2 - F_eO_2)}{1 - F_eCO_2}$$

Whole animal rates of substrate oxidation were assumed to have minimal contributions from protein oxidation, based on the observation that protein oxidation contributes only  $\sim 5\%$  to fuelling exercise (Bulow, 1988; Gessaman and Nagy, 1988; Lyons *et al.*, 2021). The following equation was used to calculate the relative contributions of  $\text{CO}_2$  production and  $\text{O}_2$  consumption (the respiratory exchange ratio, RER; Frayn, 1983):

$$\text{RER} = \frac{\dot{V}\text{CO}_2}{\dot{V}\text{O}_2}$$

Once the acute cold hypoxia trial was complete, mice were then euthanized by introducing 5% isoflurane into the chamber, followed by cervical dislocation. The right gastrocnemius, and then muscles from the entire left hindlimb, were sampled within 5 minutes of euthanasia. The right gastroc was quickly transferred into ice-cold BIOPS containing (in mM), 50 MES, 7.23 K<sub>2</sub>EGTA, 2.77 CaK<sub>2</sub>EGTA, 20 imidazole, 0.5 dithiothreitol, 20 taurine, 5.77 ATP, 15 PCr, and 6.56 MgCl<sub>2</sub>·H<sub>2</sub>O; (pH 7.1) until permeabilization was initiated. Muscles from the left hindlimb (~250-400 mg) were immediately transferred to 10 mL ice-cold mitochondrial isolation buffer containing (in mM), 100 sucrose, 50 Tris base, 5 MgCl<sub>2</sub>, 5 EGTA, 100 KCl, 1 ATP.

### **2.3.3 Permeabilized gastrocnemius fiber respiration**

Under a stereomicroscope, the oxidative core of the gastrocnemius muscle was trimmed of fat and connective tissue before being teased apart into fibre bundles with dissecting probes in ice-cold BIOPS, as previously described for deer mice (Mahalingam *et al.*, 2017). Fibre bundles were then shaken on ice for 30 minutes in BIOPS at 50  $\mu\text{g mL}^{-1}$ . Samples then went through 3  $\times$  10 min washes on ice in Mir05 buffer containing (in mM), 0.5 EGTA, 3 MgCl<sub>2</sub>, 60 potassium lactobionate, 20 taurine, 10 KH<sub>2</sub>PO<sub>4</sub>, 20 Hepes, 110 sucrose, 1 g L<sup>-1</sup> fatty acid-free BSA (pH 7.1). Samples were kept on ice until experiments began.

We used high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) to measure mitochondrial respiration in permeabilized fibres. The

fibres were added to 2 ml of oxygenated Mir05 respiration buffer ( $\sim 450 \mu\text{M O}_2$ ) and held at  $37^\circ\text{C}$ , with constant stirring at 750 rpm. At no time during the respirometry was oxygen concentration allowed to drop below  $200 \mu\text{M O}_2$ . To prevent muscle contraction,  $5 \mu\text{M}$  blebbistatin (a myosin II inhibitor) was added to each fibre bundle. Following respirometry measurements,  $20 \mu\text{M}$  cytochrome c was added to the chamber and an increase in respiration of  $<10\%$  was used as an indication that mitochondrial membrane integrity was not disrupted.

Respirometry measurements were adapted from Petrick and Holloway (2019). Briefly, rates of respiration ( $\text{JO}_2$ ) were determined with titration of ADP concentrations ( $25, 75, 325, 575, 875, 2625 \mu\text{M}$ ) in the presence of  $5 \text{ mM}$  pyruvate and  $2 \text{ mM}$  malate ( $n = 11/\text{group}$ ). Following each ADP titration protocol,  $1.25 \mu\text{M}$  ADP,  $5 \text{ mM}$  pyruvate,  $20 \text{ mM}$  glutamate and  $20 \text{ mM}$  succinate was added to determine OXPHOS capacity of complexes I and II of the ETS. Palmitoyl-CoA (P-CoA) sensitivity was determined in the presence of  $2 \text{ mM}$  malate,  $2 \text{ mM}$  ADP and  $1 \text{ mM}$  L-carnitine by titrating  $2, 6, 14, 30,$  and  $70 \mu\text{M}$  PCo-A ( $n = 4-6/\text{group}$ ). Palmitoylcarnitine (PC) sensitivity was determined in the presence of  $2 \text{ mM}$  malate and  $2 \text{ mM}$  ADP, and PC titrations were performed ( $0.5, 1.5, 2.5, 4.5, 8.5, 16.5, 36.5 \mu\text{M}$ ). Finally, L-carnitine sensitivity was determined in the presence of  $2 \text{ mM}$  ADP,  $2 \text{ mM}$  malate,  $40 \mu\text{M}$  PCo-A by titrating L-carnitine ( $0.5, 1.5, 3.5, 8.5, 13.5, 23.5, 48.5, 98.5, 173.5, 248.5, 348.5, 848.5, 1358.5, 1858.5 \mu\text{M}$ ).

Calculated predictions of mitochondrial enzyme flux were determined using the kinetic properties depicted by the mitochondrial respiration data. L-carnitine, PCo-A and

PC-supported mitochondrial affinities, respectively, were determined using the Michaelis-Menten equation:

$$V = \frac{V_{\max} \times [S]}{K_m + [S]}$$

Whereby  $V$  denotes the enzyme reaction velocity,  $V_{\max}$ , the maximum enzyme reaction velocity,  $[S]$ , the substrate concentration, and  $K_m$ , the enzyme's substrate sensitivity.

#### **2.3.4 Mitochondrial Isolation**

Left hindlimb skeletal muscles were sampled and minced with scissors in 10 mL of isolation buffer and digested in the same buffer with protease ( $1 \text{ mg} \cdot \text{g}^{-1}$  muscle). The digested muscle was then homogenized with a Potter-Elvehjem Teflon mortar and pestle for six passes at 100 rpm. The homogenate was centrifuged at  $700 \times g$  at  $4^\circ\text{C}$  for 10 minutes. The supernatant was then filtered through a cheesecloth into a new centrifuge tube and then centrifuged at  $1000 \times g$  at  $4^\circ\text{C}$  for 10 minutes. The supernatant was transferred into a new tube and spun at  $8700 \times g$  for 10 minutes, the supernatant was discarded, and the remaining pellet was resuspended in 10 mL isolation buffer with BSA ( $1 \text{ mg mL}^{-1}$ ) and spun at  $8700 \times g$  for 10 minutes. This process was repeated two more times, resuspending the pellet in 10 mL isolation buffer between spins. Finally, the pellet was resuspended in  $100\mu\text{L}$  isolation buffer. The isolated mitochondria were divided into aliquots and frozen for western blot analysis (see below).

### **2.3.5 Mitochondrial expression of FAT/CD36**

Western blotting was performed on isolated mitochondria from muscles after shivering to quantify FAT/CD36 protein abundance. To test for any contamination of mitochondrial fractions by cytosolic proteins, SERCA2 was also quantified, with whole tissue homogenates pooled from 5 TN deer mouse acting as a positive control. Whole tissue homogenates were processed as follows: frozen gastrocnemius was powdered liquid N<sub>2</sub> cooled mortar and pestle and subsequently homogenized in RIPA buffer (in mmol l<sup>-1</sup>: 150 NaCl, 50 Tris-HCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, pH 8.0) at a dilution of 25 µl mg<sup>-1</sup> tissue and homogenized using a glass-on-glass homogenizer. Protein concentrations of tissue homogenates and isolated mitochondria were quantified using Bradford assay (Bio-Rad Laboratories Ltd, Mississauga, ON). A total of 35 µg for whole-gastrocnemius homogenate and 2.5µg for isolated mitochondria proteins were denatured by heating to 95°C for 5 min in 4× Laemmli sample buffer (Bio-Rad) with 10% β-mercaptoethanol. Proteins were separated on 12% SDS-PAGE gels and then transferred to a PVDF membrane using the Transblot Turbo Transfer System (Bio-Rad). Membranes were incubated overnight at 4°C with 5% skimmed milk in 1× phosphate buffered saline, 0.1% Tween. On the following day, membranes were probed with primary antibody against FAT/CD36 (CD36 polyclonal antibody, Invitrogen, PA-16813) or against SERCA2 (Serca2-ATPase Monoclonal Antibody, 2A7-A1, ThermoFisher Scientific) at a dilution of 1:2000 and at a dilution of 1:1000, respectively, for 1 h at room temperature. Membranes probed for FAT/CD36 were then incubated with HRP-conjugated secondary antibody (goat anti-rabbit, Invitrogen, cat. #31466) at a dilution of 1:5000 for 1h at room temperature. Membranes probed for SERCA2 were

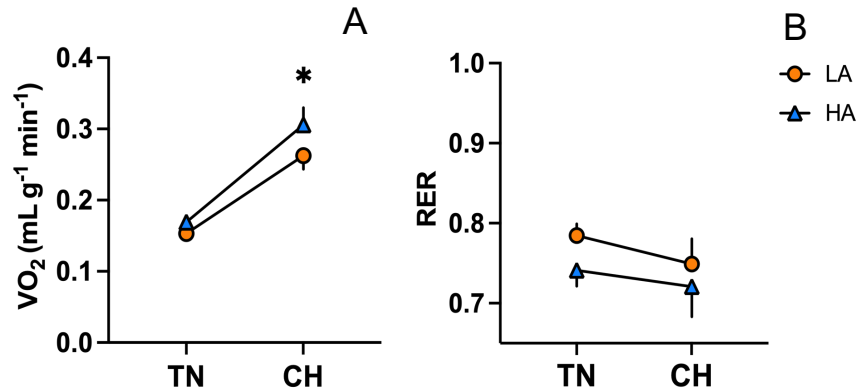
then incubated with HRP-conjugated secondary antibody (goat anti-rabbit, Invitrogen, cat. #31466) at a dilution of 1:5000 for 1 h at room temperature. Band densities were detected by chemiluminescence and normalized to selected lane protein determined by staining the membranes with Coomassie Blue. ChemiDoc MP Imaging System (Bio-Rad) was used to image the membranes, and the Image Lab software package (Bio-Rad) was used to analyze the band densities (Lyons and McClelland, 2022).

### **2.3.6 Statistics**

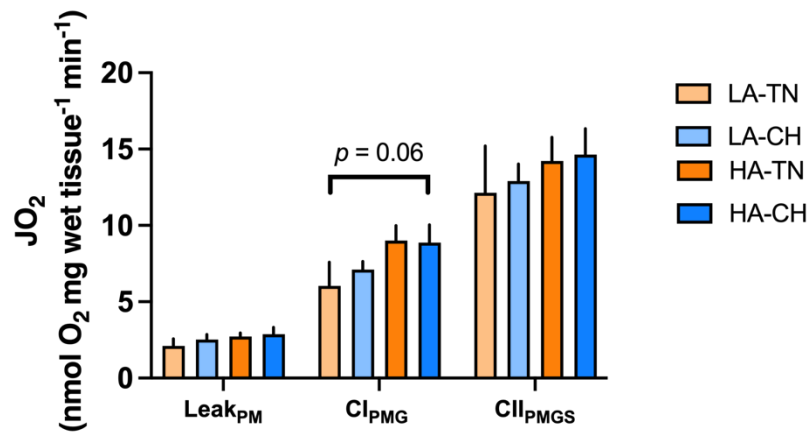
Statistical analysis of data was completed using GraphPad Prism software (GraphPad Prism version 10.0.0 for Mac, GraphPad Software, Boston, Massachusetts USA). Two-way analysis of variance (ANOVA) tests were conducted using body mass as a covariate where appropriate. ADP, Pi, L-carnitine, and PCo-A titrations were analyzed using non-constrained Michaelis-Menten Kinetics using GraphPad Prism Software. The significance level was  $p < 0.05$ . Both population and acclimation were used as main effects within the tests. Outliers within the data were determined using a Grubbs' Test on the GraphPad Outlier Calculator, whereby any values with  $p < 0.05$  were considered outliers and were removed from the data set. Covariate analyses were performed in R 4.3.1 using the car package.

## 2.4 Results

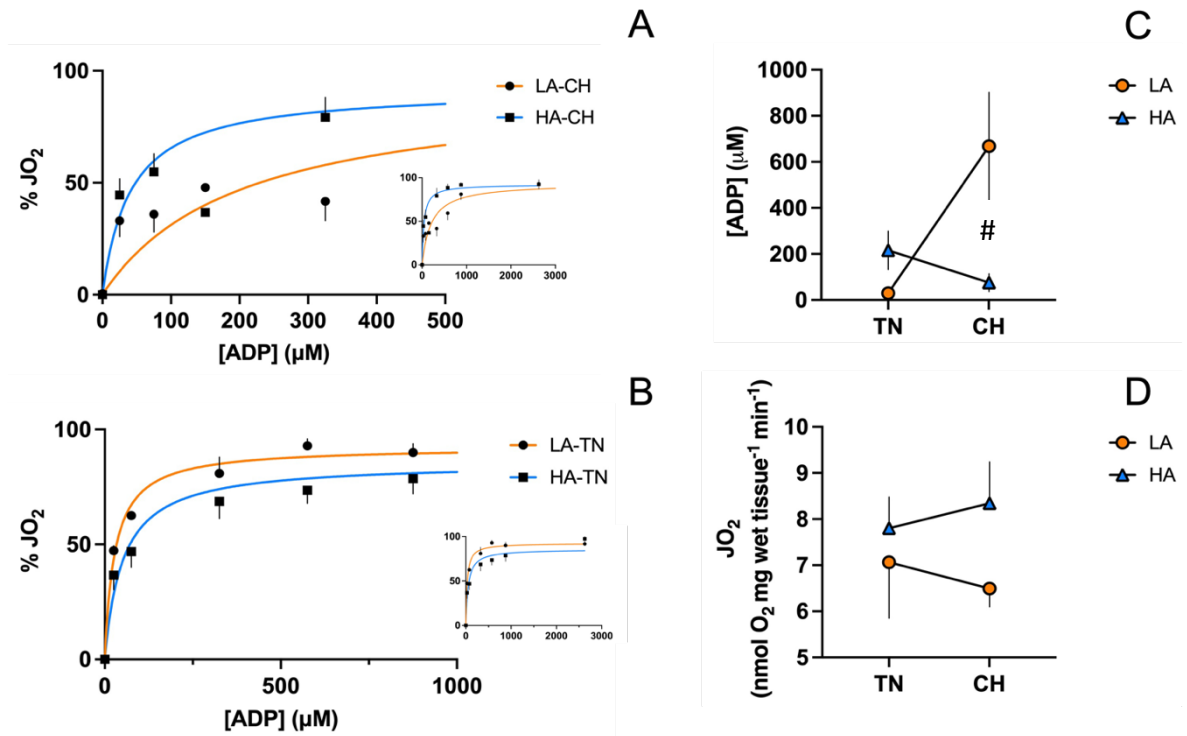
### 2.4.1 Figures and Tables



**Figure. 1** Oxygen consumption ( $\dot{V}O_2$ , in mL g<sup>-1</sup> min<sup>-1</sup>) (A) and RER (respiratory exchange ratio;  $\dot{V}CO_2/\dot{V}O_2$ ) (B) of second and third generation highland and lowland deer mice (*Peromyscus maniculatus*) that were acclimated to thermal neutral (TN; 30°C, 21 kPa O<sub>2</sub>, 12:12 L:D) or cold hypoxia (CH; 4°C, 12 kPa O<sub>2</sub>, 8:16 L:D) conditions and then exposed to acute cold hypoxia (12% O<sub>2</sub>, 88% He).  $\dot{V}O_2$  (A) increased with CH acclimation (main effect of acclimation,  $F_{1,37} = 59.25$   $p < 0.0001$ ). The respiratory exchange ratios (RER; C) showed no significant effect of population ( $F_{1,37} = 1.55$ ,  $p > 0.05$ ) or acclimation ( $F_{1,37} = 0.80$ ,  $p > 0.05$ ). Asterisks indicate significant pairwise difference in highland deer mice between acclimation environments. Data are means  $\pm$  S.E.M.,  $n = 8-12$ .

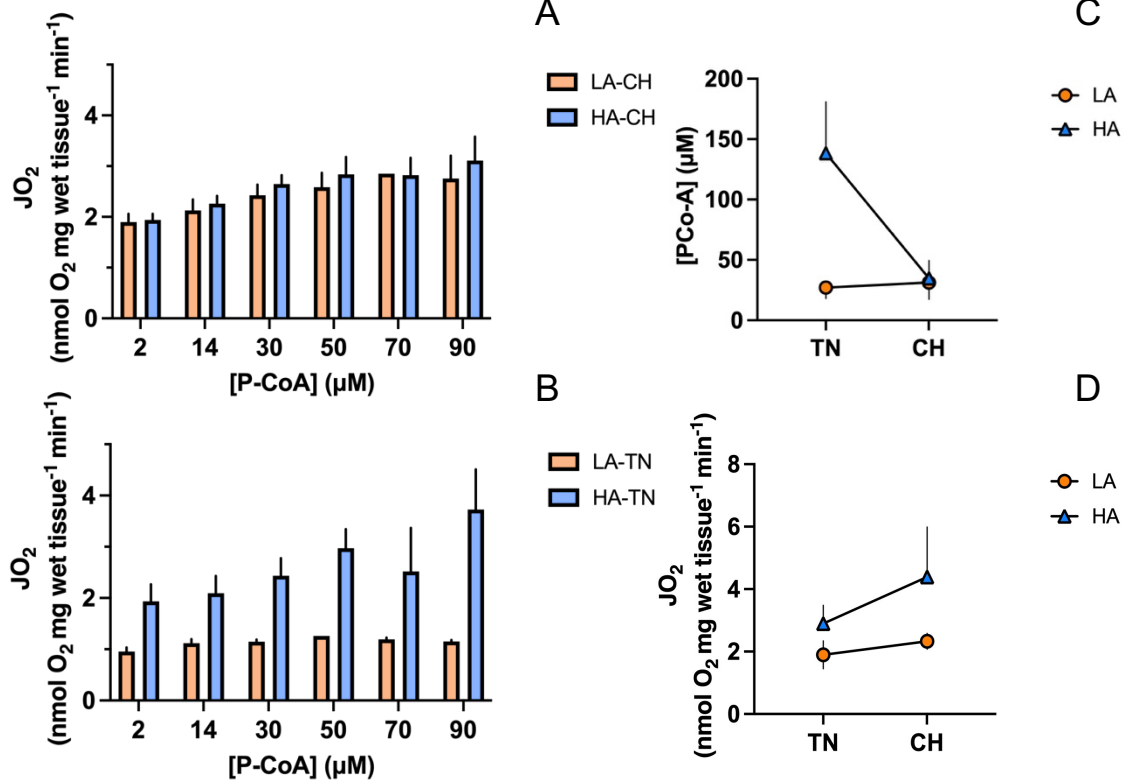


**Figure 2.** Mitochondrial respiration ( $\text{nmol O}_2 \text{ mg wet tissue}^{-1} \text{ min}^{-1}$ ) in permeabilized fibers from the gastrocnemius muscle of high-altitude (HA) and low-altitude (LA) native deer mice acclimated to either cold hypoxia (CH) or thermoneutral (TN) conditions.  $\text{Leak}_{\text{PM}}$  was measured in the absence of ADP (in the presence of blebbistatin, pyruvate and malate). Complex I and II ( $\text{CII}_{\text{PMGS}}$ )-stimulated respiration were measured immediately following the ADP kinetic protocol.  $\text{CI}_{\text{PMG}}$  was maximally stimulated by pyruvate, malate, and glutamate, and  $\text{CII}_{\text{PMGS}}$  respiration was maximally stimulated by, subsequently adding succinate. Highland native deer mice show a trend to increase their complex I respiration ( $\text{CI}_{\text{PMG}}$ ), compared to lowlanders (near-significant main effect of population;  $F_{1,25}=3.88$ ;  $p = 0.06$ )  $n = 5-10$ . Bars represent the means  $\pm$  *S.E.M.*

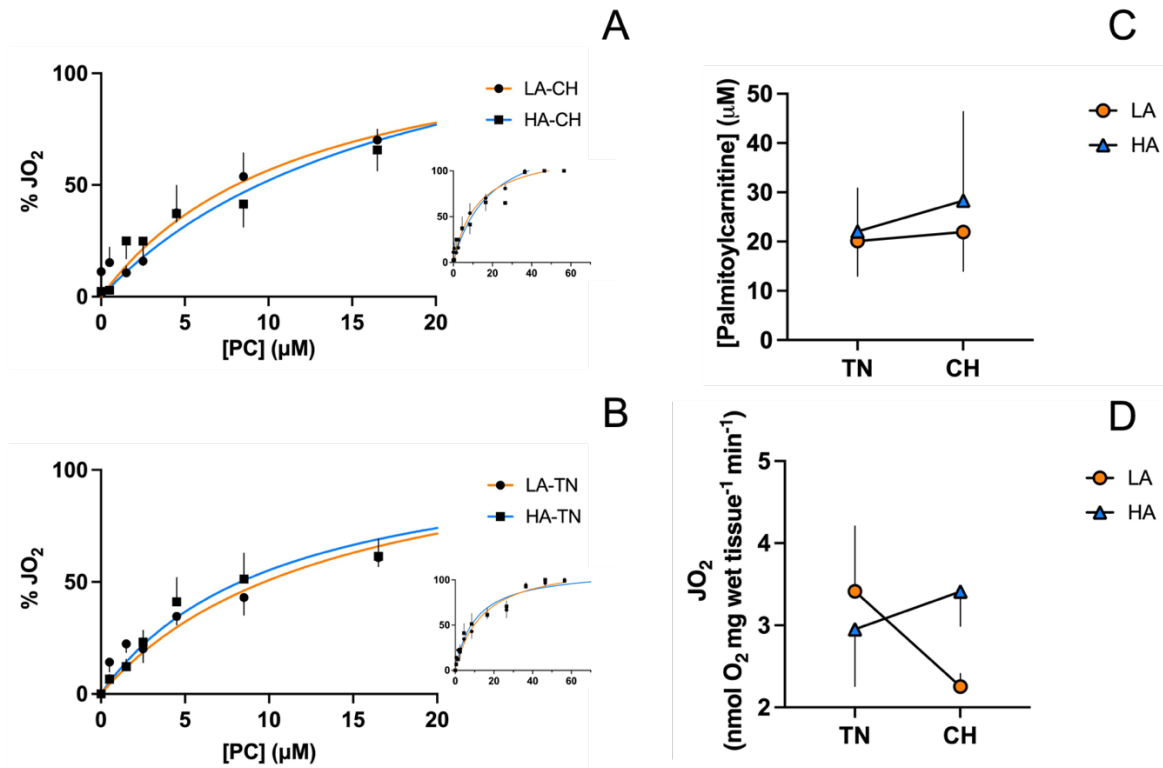


**Figure 3.** Effect of cold hypoxia acclimation (CH) on ADP-supported mitochondrial respiration in post-shivering muscle fibers from low-altitude (LA) and high-altitude (HA) deer mice. The fibers were sampled immediately (1-2 mins) after shivering in hypoxia. Mitochondrial sensitivity to ADP in CH mice (A) and TN mice (B). In (C), there is a significant interaction effect ( $F_{1,23} = 5.56$ ,  $p = 0.027$ ) of population and acclimation on apparent ADP  $K_m$  although no changes in maximum mitochondrial respiration were detected (D; Table 2),  $n = 4-10$ . Symbols represent the means  $\pm$  *S.E.M.*, significant differences # represent a  $p < 0.05$ .

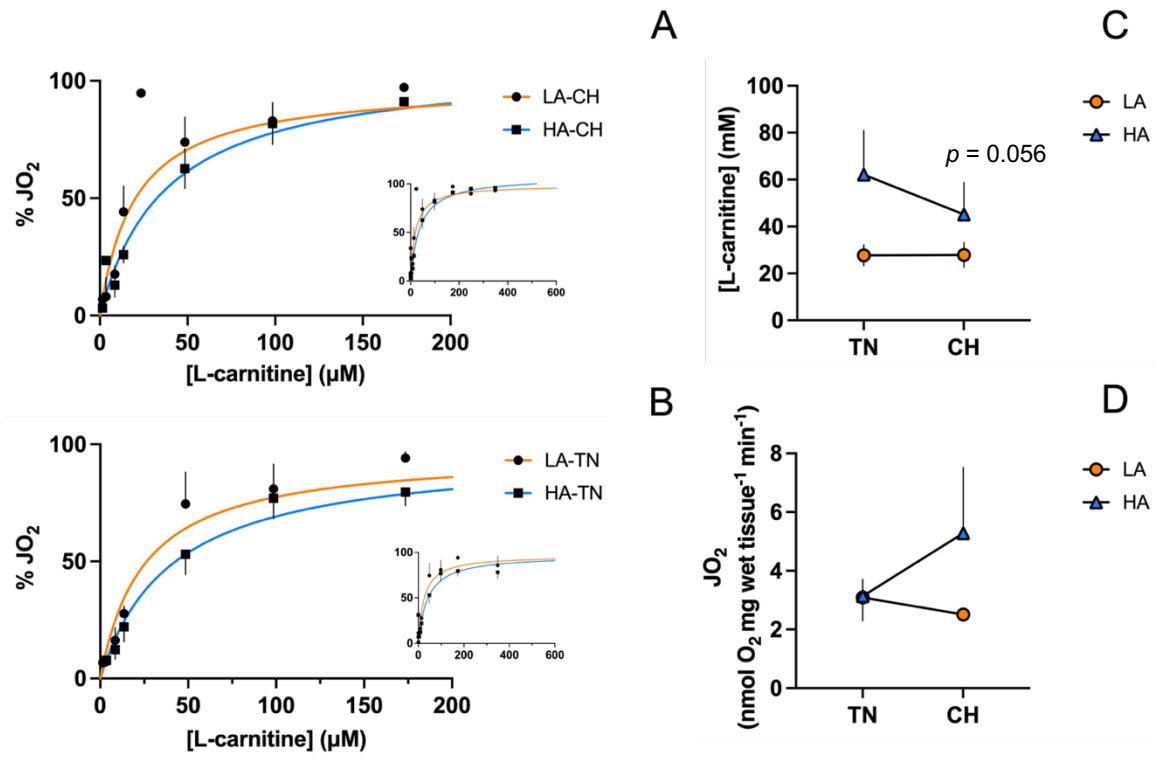
[CB15]



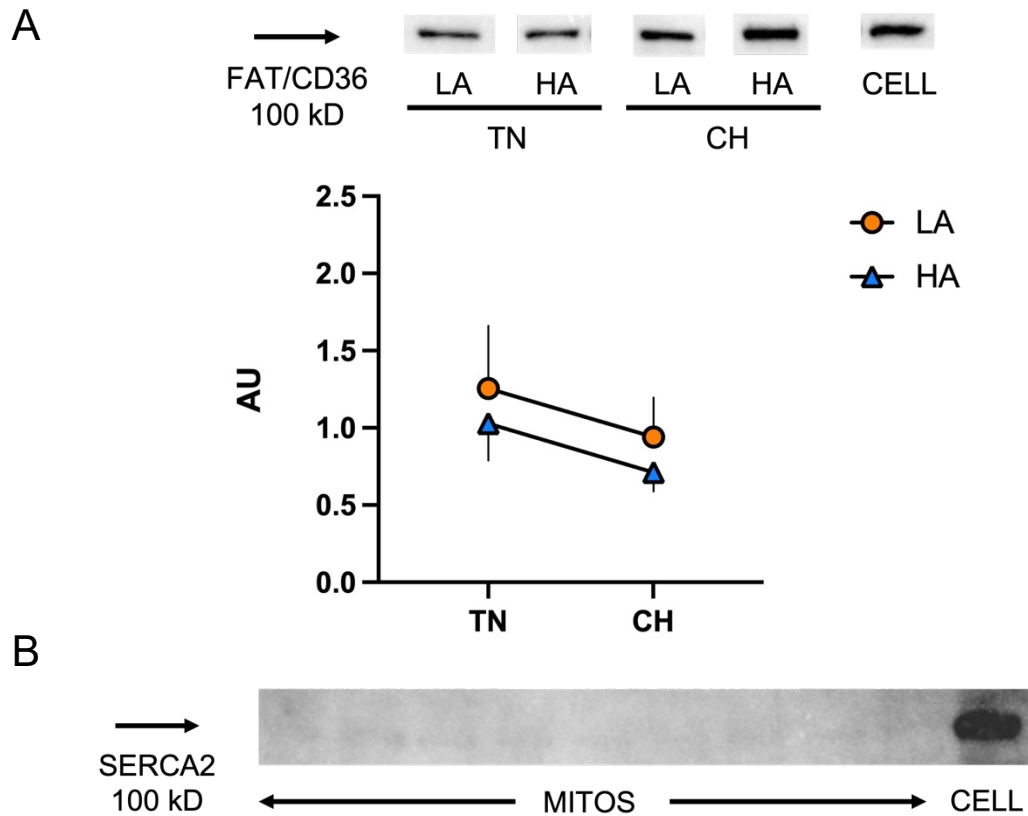
**Figure 4.** Effect of cold hypoxia acclimation on palmitoyl Co-A (P-CoA)-supported mitochondrial respiration in shivering low-altitude (LA) and high-altitude (HA) deer mice. CPT-I sensitivity to P-CoA in CH mice (**A**) and TN mice (**B**). No significant differences were identified in either mitochondrial PCo-A sensitivity (**C**) or maximum P-CoA-supported respiration (**D**),  $n = 3-7$ . Symbols represent the means  $\pm$  *S.E.M.*



**Figure 5.** Effect of cold hypoxia acclimation on palmitoylcarnitine (PC)-supported mitochondrial respiration in shivering low-altitude (LA) and high-altitude (HA) deer mice. Mitochondrial sensitivity to PC in CH mice (**A**) and TN mice (**B**). No significant differences were identified in either mitochondrial palmitoylcarnitine sensitivity (**C**) or maximum palmitoylcarnitine-supported respiration (**D**),  $n = 3-6$ . Symbols represent the means  $\pm$  *S.E.M.*



**Figure 6.** Effect of cold hypoxia acclimation on L-carnitine-supported mitochondrial respiration in shivering low-altitude (LA) and high-altitude (HA) deer mice. CPT-I sensitivity to L-carnitine in CH mice (**A**) and TN mice (**B**). Mitochondrial sensitivity (**C**) shows a population effect that approaches statistical significance ( $F_{1,21} = 4.08$ ;  $p = 0.056$ ),  $n = 4-9$ . Symbols represent the means  $\pm$  *S.E.M.*



**Figure 7.** Relative protein abundance of fatty acid translocase (FAT/CD36) in isolated mitochondria from the gastrocnemius of high altitude (HA) and low altitude (LA) mice acclimated to cold hypoxia (CH) or thermal neutral (TN) conditions. Relative protein abundance of FAT/CD36 (A) and representative western blots (B) of mitochondrial FAT/CD36 (above) and sarcoplasmic endoplasmic reticulum-2 (SERCA2; below),  $n = 5-7$ . Data represent the means  $\pm$  *S.E.M.*

**Table 1.** Summary of mitochondrial substrate sensitivities (apparent  $K_m$ , in  $\mu M$ ) from Michaelis-Menten relationships from the gastrocnemius muscle of shivering deer mice. Second and third generation lab-born mice from high (HA) and low (LA) altitude populations of *P. maniculatus* were acclimated to either cold hypoxia (CH) or thermal neutral normoxia (TN) conditions and were then exposed to cold hypoxia (heliox) for 15 minutes. The muscle was sampled immediately (1-2 minutes) after shivering. Data represent the mean  $\pm$  standard error of the mean.

Substrate	TN			CH			F-statistic	P-value		
	LA		HA		LA				HA	
	n	Mean $\pm$ SEM	n	Mean $\pm$ SEM	n	Mean $\pm$ SEM			n	Mean $\pm$ SEM
<b>ADP</b>	4	7.05 $\pm$ 3.52	10	216.07 $\pm$ 83.53	8	668.45 $\pm$ 233.84	5	75.46 $\pm$ 38.44	<b>Population</b> F (1,23) 1.51 <b>Acclimation</b> F (1,23) 2.27 <b>Interaction</b> F (1,23) 5.56	0.231 0.145 0.027
<b>PCo-A</b>	3	27.09 $\pm$ 9.07	7	138.37 $\pm$ 42.60	4	31.31 $\pm$ 13.84	6	34.76 $\pm$ 14.89	<b>Population</b> F (1,16) 2.75 <b>Acclimation</b> F (1,16) 2.06 <b>Interaction</b> F (1,16) 2.43	0.117 0.170 0.139
<b>PC</b>	3	20.12 $\pm$ 7.12	6	22.05 $\pm$ 8.78	6	21.92 $\pm$ 7.93	4	28.31 $\pm$ 18.09	<b>Population</b> F (1,15) 0.13 <b>Acclimation</b> F (1,15) 0.12 <b>Interaction</b> F (1,15) 0.03	0.719 0.728 0.847
<b>L-carnitine</b>	5	27.72 $\pm$ 4.43	7	62.10 $\pm$ 18.83	9	27.87 $\pm$ 5.22	4	45.13 $\pm$ 13.65	<b>Population</b> F (1,21) 4.08 <b>Acclimation</b> F (1,21) 0.43 <b>Interaction</b> F (1,21) 0.44	0.056 0.517 0.510

**Table 2.** Summary of mitochondrial substrate consumption rates ( $V_{\max}$ , in  $\text{nmol O}_2 \text{ mg}^{-1} \text{ min}^{-1}$ ) from Michaelis-Menten relationships from the gastrocnemius muscle of shivering deer mice. Second and third generation lab-born mice from high (HA) and low (LA) altitude populations of *P. maniculatus* were acclimated to either cold hypoxia (CH) or thermal neutral normoxia (TN) conditions and were then exposed to cold hypoxia (heliox) for 15 minutes. The muscle was sampled immediately (1-2 minutes) after shivering. Data represent the mean  $\pm$  standard error of the

mean.	TN		CH		F-statistic	P-value		
	LA	HA	LA	HA				
Substrate	n	Mean $\pm$ SEM	n	Mean $\pm$ SEM	n	Mean $\pm$ SEM	F-statistic	P-value
ADP	5	7.07 $\pm$ 1.21	10	7.80 $\pm$ 0.68	9	6.49 $\pm$ 0.40	5	8.34 $\pm$ 0.89
								Population F (1,25) 2.79 0.107
								Acclimation F (1,25) 0.0004 0.985
								Interaction F (1,25) 0.52 0.477
PCo-A	5	1.89 $\pm$ 0.45	6	2.89 $\pm$ 0.59	6	2.33 $\pm$ 0.25	6	4.39 $\pm$ 1.60
								Population F (1,19) 2.72 0.115
								Acclimation F (1,19) 1.08 0.311
								Interaction F (1,19) 0.32 0.575
PC	3	3.41 $\pm$ 0.79	6	2.95 $\pm$ 0.70	7	2.25 $\pm$ 0.16	4	3.41 $\pm$ 0.42
								Population F (1,16) 0.40 0.534
								Acclimation F (1,16) 0.40 0.532
								Interaction F (1,16) 2.18 0.159
L-carnitine	5	3.09 $\pm$ 0.79	7	3.14 $\pm$ 0.57	9	2.51 $\pm$ 0.16	4	5.29 $\pm$ 2.23
								Population F (1,21) 2.71 0.114
								Acclimation F (1,21) 0.83 0.371
								Interaction F (1,21) 2.53 0.126

### 2.4.2 Body Mass and Cold-induced $\dot{V}O_2$

Neither environment nor population significantly affected body mass between high and low altitude deer mice ( $p > 0.05$ ). To achieve high rates of shivering prior to mitochondrial respiration experiments, we performed acute cold hypoxia-induced  $\dot{V}O_2$  trials on the mice, during which they were exposed for 15 minutes to 12% O<sub>2</sub>, 88% He at 5°C or -5°C for TN and CH treatment groups, respectively. We found that compared to thermoneutral mice,  $\dot{V}O_2$  increased 1.7-fold in cold hypoxia acclimated mice from both populations (Fig. 1), but no population effect ( $F_{1,36} = 2.99, p = 0.09$ ) or interaction effect ( $F_{1,36} = 0.63, p = 0.43$ ) was found.

### 2.4.3 ADP-supported respiration

The mitochondrial respiratory capacity in permeabilized fibres from the gastrocnemius muscle showed no differences overall across population and acclimation. Leak respiration (Leak<sub>PM</sub>; Fig. 2) yielded no effects of population ( $F_{1,25} = 1.51, p = 0.23$ ), acclimation ( $F_{1,25} = 0.57, p = 0.5$ ), as was the case with respiration from complex I (CI<sub>PMG</sub>; population,  $F_{1,25} = 3.88, p = 0.60$ ; acclimation,  $F_{1,25} = 0.15, p = 0.70$ ) and with complex I + II (CII<sub>PMGS</sub>; population,  $F_{1,25} = 0.94, p = 0.34$ ; acclimation,  $F_{1,25} = 0.08, p = 0.76$ ) (Fig. 2). There were no interaction effects for Leak<sub>PM</sub> ( $F_{1,25} = 0.11, p = 0.74$ ), CI<sub>PMG</sub> ( $F_{1,25} = 0.24, p = 0.63$ ) or CII<sub>PMGS</sub> ( $F_{1,25} = 0.01, p = 0.92$ ).

We aimed to characterize whether altitude ancestry or acclimation environment influences mitochondrial sensitivity to ADP, as seen in Fig. 3. There was an interaction effect of population and acclimation for ADP sensitivity, shown in Fig. 3C (Table 1A;  $p < 0.05$ ), with LA mice increasing apparent  $K_m$  by ~100-fold and HA mice decreasing  $K_m$  by ~65% following CH acclimation, although there are no significant effects of

population or acclimation (Table 1A;  $p > 0.05$ ). ADP-supported mitochondrial  $V_{\max}$  (Fig. 3D) does not change significantly with population or acclimation (Table 1B;  $p > 0.05$ ).

#### **2.4.4 Lipid-supported respiration**

We next examined whether acclimation or ancestry influenced CPT-I supported respiration in permeabilized fibres. CPT-I sensitivity to P-CoA (Fig. 4) shows no significant effect of population, despite mean HA  $K_m$  reporting as ~4-fold greater than in lowlanders for the TN acclimation as seen in Fig. 4C (Table 1A;  $p > 0.05$ ). Maximal P-CoA -supported respiration ( $V_{\max}$ ; Fig. 4D) similarly showed no change across population, acclimation, or any interaction effects (Table 1B;  $p > 0.05$ ). Mitochondrial respiratory sensitivity to PC (Fig. 5) did not show any significant differences in  $K_m$  (Fig. 5C, Table 1A,  $p > 0.05$ ) or  $V_{\max}$  (Fig 5D, Table 1B,  $p > 0.05$ ) across population or acclimation. Finally, we performed titrations using L-carnitine and found that population differences in CPT-I sensitivity to L-carnitine approach significant (Fig. 6C,  $p = 0.056$ ), although no acclimation or population effects were seen (Table 1A,  $p > 0.05$ ). L-carnitine-supported  $V_{\max}$  (Fig. 6D) did not show any population, acclimation, or interaction effects (Table 1B,  $p > 0.05$ ).

#### **2.3.5 Mitochondrial FAT/CD36 Protein Abundance**

We detected the presence of the lipid transporter FAT/CD36 in isolated mitochondria from the entire hindlimb (Fig. 7A). We detected no effect of population ( $F_{1,20}=1.44$ ,  $p = 0.24$ ) or acclimation ( $F_{1,20}=0.74$ ,  $p = 0.40$ ) across treatment groups for FAT/CD36. We used SERCA2 as a positive control for mitochondrial purity and detected no presence of SERCA2 in the mitochondrial preparations (Fig. 7B).

## 2.5 Discussion

The aim of this study was to determine if mitochondrial respiration kinetics of shivering muscle could help explain population differences and acclimation effects on whole-animal lipid oxidation to support thermogenesis. We confirmed that deer mice consume largely lipids to support peak rates of thermogenesis (as indicated by RERs in Fig.1), consistent with previous results for this species (Cheviron *et al.*, 2012; Lyons *et al.*, 2021). Acclimation to cold hypoxia led to an increase in cold-induced  $\dot{V}O_{2\max}$  (Fig. 1). Cold hypoxia acclimation also induced an altered ADP sensitivity response between high- and low-altitude native mice, whereby ADP sensitivity was greater in highlanders than lowlanders, which supports the secondary prediction of this study (Fig. 3). High- and low- altitude mice did not show a change in L-carnitine sensitivity following cold hypoxia acclimation, and L-carnitine sensitivity appears to be fixed at a lower level in highlanders, in contrast with what was predicted ( $p = 0.056$ ; Fig. 6). There were no differences across acclimation or population in palmitoylcarnitine sensitivity or FAT/CD36 abundance. Additionally, the observed changes in mitochondrial substrate kinetics were not associated with significant corresponding changes in  $V_{\max}$ . These results highlight that mitochondrial kinetics determined at submaximal substrate concentrations can provide valuable insights into understanding of mitochondrial fuel selection that cannot be determined when the mitochondria are maximally respiring. While these results cannot confirm that CPT-I is an important control point for lipid oxidation, they do indicate that the rate-limiting steps to fatty acid oxidation likely occur at or upstream of CPT-I along the fat oxidation pathway.

### **2.5.1 High-altitude show an altered ADP sensitivity response following cold hypoxia acclimation**

Altitude ancestry influences the acclimation response leading to altered ADP sensitivity, as cold hypoxia acclimation caused ADP  $K_m$  to decrease in highlanders, and increase in lowlanders (Fig. 3C), resulting in an interaction effect of environment and population (Table 1). In contrast to the response in highlanders, previous work in exercising humans and lab rodents have shown that mitochondrial ADP sensitivity is reduced in skeletal muscle following high intensity exercise (Ludzki *et al.*, 2015; Miotto *et al.*, 2019). However, ADP sensitivity is greater in oxidative fibres, as seen when comparing permeabilized fibres of rodents from the heart (apparent ADP  $K_m \sim 310$ ; Kuznetsov *et al.*, 1996; Beaudoin *et al.*, 2014) and the gastrocnemius (apparent ADP  $K_m \sim 700 - 1050$ ; Petrick and Holloway, 2019; Miotto *et al.*, 2019). Additionally, exercise training in rats led to improved ADP sensitivity in saponin-skinned red gastrocnemius, but not in the soleus, and this difference was associated with a 40% greater  $V_{max}$  in the gastrocnemius compared to the soleus (Burrelle and Hochachka, 1985). The red core of the gastrocnemius in deer mice has a greater proportion of oxidative fibers (Lui *et al.*, 2015), and the apparent ADP  $K_m$  in cold hypoxic highlanders is comparable to rat permeabilized heart (Beaudoin *et al.*, 2014). Thus, it is possible that the increase in ADP sensitivity is supported by the more oxidative fiber type in highlanders.

Reductions in ADP sensitivity have also been linked to increases in stress associated with reactive oxygen species (ROS; Smith *et al.*, 2014; Ludzki *et al.*, 2015; Holloway *et al.*, 2018; Miotto *et al.*, 2019), potentially to induce a hypoxia signalling cascade (Smith *et al.*, 2017; Miotto and Holloway, 2019; Dawson and Scott, 2022).

Isolated intermyofibrillar and subsarcolemmal mitochondria from altitude deer mice show reduced ROS emissions after chronic hypoxia exposures, and it was suggested that these mice are more effective at minimizing oxidative stress, either by minimizing ROS production or enhancing antioxidant pathways (Dawson and Scott, 2022). Perhaps high-altitude deer mice can maintain higher rates of ADP sensitivity in cold hypoxia because they are less impacted by the negative effects of ROS.

The apparent ADP  $K_m$  has also been shown to decrease in the presence of fatty acids in skinned rat cardiac fibres, with a proposed mechanism being that the presence of fatty acyl-carnitines (i.e., palmitoylcarnitine) change the mitochondrial ultrastructure, whereby acylcarnitines could induce conformational changes in the adenine nucleotide transporter (ANT; Toleikis *et al.*, 2020). This conformational change in ANT from the M-state to the C-state is thought to reduce the apparent  $K_m$  by increasing the size of the intermembrane space and reducing the size of the matrix (Toleikis *et al.*, 2020). It has also been suggested that the capacity for acylcarnitine oxidation relative to pyruvate oxidation is increased in highlanders compared to lowlanders (Dawson and Scott, 2022). It is possible that the enhanced ADP sensitivity in highlanders is linked to their increased capacity to support fatty acid oxidation.

### **2.5.2 High lipid oxidation rates during shivering in highland mice are not associated with increases in CPT-I substrate sensitivity**

CPT-I function is regulated by many factors, and consequently is a major regulatory control point in the fat oxidation pathway (Zammit *et al.*, 1997; McGarry *et al.*, 1997). It was previously shown that CPT-I sensitivity to L-carnitine decreases in an intensity-dependent manner during exercise in lab mice, and this was suggested as a

mechanism for the shift towards carbohydrate oxidation in the transition from moderate to high-intensity exercise (Petrick and Holloway, 2019). We therefore predicted that CPT-I sensitivity to L-carnitine would not decrease in shivering deer mice, since shivering deer mice maintain high rates of lipid oxidation during shivering even at cold-induced  $\dot{V}O_{2\max}$  (Lyons *et al.*, 2021; Lyons and McClelland, 2022). In contrast to our prediction, high-altitude mice showed a trend to reduce their sensitivity to L-carnitine (increasing their apparent  $K_m$ ) during shivering (a nearly significant population effect,  $p = 0.056$ ; Fig. 6). Perhaps the  $\sim 2$ -fold greater  $V_{\max}$  following cold hypoxia acclimation in high altitude mice compensates for lower sensitivity to L-carnitine, but this acclimation effect also did not reach statistical significance ( $p = 0.371$ ).

L-carnitine serves many roles in the cell. The primary role of L-carnitine is to mediate lipid as the substrate for CPT-I, as was discovered in 1955 (Fritz, 1955). Carnitine acetyltransferase (CAT) transforms free carnitine and acetyl-CoA to make acetyl-carnitine, so L-carnitine plays a secondary role as a buffer to modulate the size of the acetyl-CoA pool in the mitochondria (Virmani *et al.*, 2015; Martinez-Rayes and Chandel, 2020; Virmani and Cirulli, 2022). During carbohydrate oxidation, acetyl-CoA is produced from pyruvate oxidation catalyzed by the enzyme pyruvate dehydrogenase (PDH). Acetyl-CoA is also produced by beta-oxidation of fatty acids, and both pathways converge with oxaloacetate via the citrate synthase reaction to participate in the tricarboxylic acid (TCA) cycle (Martinez-Rayes and Chandel, 2020). During exercise at higher power outputs, free cytosolic ADP increases (Howlett *et al.*, 1998; Spriet, 2014), stimulating the glycolytic pathway and leading to an accumulation of acetyl-CoA.

Acetyl-CoA accumulation inhibits PDH, and free L-carnitine reacts with and buffers acetyl-CoA to relieve the inhibition on carbohydrate oxidation (Randle *et al.*, 1963; Virmani *et al.*, 2015; Virmani and Cirulli, 2022). Highlander deer mice potentially reduce CPT-I sensitivity to L-carnitine to preserve free L-carnitine so that it is available to balance the acetyl-CoA:CoA ratio and avoid inhibition of PDH. Alternatively, the systems for producing and consuming acetyl-CoA are better matched in highland deer mice, lessening the consumption of free carnitine to buffer acetyl-CoA. Substrate concentration and  $K_m$  are usually matched *in vivo* to maximize regulatory control (Schnell and Maini, 2000), so an increase in the apparent  $K_m$  of L-carnitine might be accompanied by an increase in free L-carnitine availability.

CPT-I sensitivity to L-carnitine does not appear to limit fat burning for shivering thermogenesis in deer mice, and furthermore, mice from all groups appear to equally recruit FAT/CD36 to mitochondria with shivering (Fig. 7). Therefore, the underlying mechanisms of why highlanders achieve high rates of lipid oxidation likely occur at steps that are upstream of CPT-I along the fat oxidation pathway. For instance, cold hypoxia-acclimated deer mice increase total cell FAT/CD36 to their cell membranes, and highlander deer mice have increased rates of free fatty acid delivery in the plasma, especially when acclimated to cold hypoxia (Lyons and McClelland, 2022). There is some evidence from data on isolated mitochondria that highlanders increase the capacity of CPT-II following cold hypoxia acclimation (Fig. 5D; Lyons and McClelland, 2022), although it is unknown whether there are also changes in CPT-I activity in these mice.

Furthermore, the data suggest that the rate of transport through FAT/CD36 is equivalent across treatment groups in this study (Fig. 8).

High and low altitude mice do not increase sensitivity to P-CoA following cold hypoxia acclimation (Fig. 4C, D). This absence of statistical significance is likely due to low sample size. An accumulation of cytosolic P-CoA is known to hinder ADP transport by competitively binding to ANT (Ho and Pande, 1974), and this effect has been linked to a reduction in ROS attenuation (Mielke *et al.*, 2014; Ludzki *et al.*, 2015). In highlanders, it is possible that the potential inhibitory effects of P-CoA on ADP transport are minimized by the increased ADP sensitivity observed following cold hypoxia acclimation.

### **2.5.3 Summary and Conclusions**

Deer mice native to high altitude rely on high rates of lipid oxidation to sustain thermogenesis in the chronic cold of the high alpine. Recent findings show that these patterns of lipid metabolism are supported by elevated rates of circulatory delivery of free fatty acids and triglycerides to heat generative tissues in highlanders (Lyons *et al.*, 2021; Lyons and McClelland, 2022). This study helps shed light on the mitochondrial mechanisms of regulatory control on lipid metabolism at submaximal rates of lipid oxidation. Notably, we determined that high altitude mice acclimated to cold hypoxia have a greater mitochondrial sensitivity to ADP than do lowlanders. These findings contrast with findings from some exercise studies, where there is an intensity-related decrease in ADP sensitivity (Ludzki *et al.*, 2015; Miotto *et al.*, 2019), but are consistent with some studies that report an increase in ADP sensitivity in association with increased muscle oxidative capacity (Kuznetsov *et al.*, 1996; Beaudoin *et al.*, 2014; Petrick and

Holloway, 2019; Miotto *et al.*, 2019). Furthermore, we found that in high altitude mice, CPT-I sensitivity to L-carnitine showed a trend to be reduced by cold hypoxia acclimation, contrary to the initial prediction of this study. Taken together, these results suggest that high altitude deer mice show an enhanced plasticity of some but not all aspects of mitochondrial respiration kinetics during shivering. However, to gain a full appreciation for the role of ancestry and plasticity play in the mitochondrial sensitivity of shivering deer mice, this study should be expanded to also compare respiratory kinetic from muscles of non-shivering mice in thermoneutral condition. Measuring the concentration of free L-carnitine in shivering would help to further uncover the roles of L-carnitine as a regulator of lipid oxidation. These next steps will provide a deeper understanding of the impressive rates of lipid consumption that supports the survival of high-altitude deer mice.

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### CHAPTER 3: DISCUSSION

This thesis presents novel research on mitochondrial kinetics in skeletal muscle of a high-altitude adapted species, the deer mouse (*Peromyscus maniculatus*) after shivering thermogenesis in hypoxia. Specifically, this research investigated the ability of these mice to maintain high rates of lipid oxidation during high intensity shivering, and whether population differences between low- and high-altitude deer mice in mitochondrial function are due to fixed traitson evolved phenotypic plasticity to the cold hypoxia of high altitude.

I [GM16] acclimated high- and low-altitude deer mice to either cold hypoxic or thermal neutral normoxic conditions. At the end of the acclimation mice were forced to shiver in 12% O<sub>2</sub>, 88% He at -5°C or 5°C, for TN and CH mice, respectively (Fig. 1A). To maintain a stable T<sub>b</sub> when T<sub>a</sub> falls below the TNZ, homeothermic endotherms increase their metabolic rate to match endogenous heat production with heat lost to the external environment (Tattersall *et al.*, 2012; Clarke and Pörtner, 2010). I found that there was a strong environment effect on  $\dot{V}O_2$  ( $p < 0.0001$  [GM17]), whereby cold hypoxia increased  $\dot{V}O_2$  by ~2-fold in both populations of deer mice, which indicates a greater capacity for matching the metabolic demands of thermoregulation This finding is consistent with previous work that found a strong acclimation response of  $\dot{V}O_2$  to cold hypoxia in deer mice (Lyons *et al.*, 2021; Tate *et al.*, 2020). Furthermore, both populations of mice had an RER between 0.7 and 0.8, indicating that they rely primarily on lipid oxidation while shivering (Fig. 1B), which confirms the findings from Lyons *et al.* (2021) and Cheviron *et al.* (2012). [GM18] The aims of this research were thus to determine whether the high rates of whole-animal lipid oxidation that are observed in shivering deer mice are

supported by mitochondrial kinetics during submaximal respiration. The present experiments reveal that (i) altitude ancestry influences the way mitochondrial ADP sensitivity responds to cold hypoxia acclimation in deer mice. Specifically, high altitude mice have a greater sensitivity to ADP than lowlanders in cold hypoxia. (ii) Lipid oxidation in these mice is not associated with changes in CPT-I sensitivity to L-carnitine; in fact, L-carnitine sensitivity appears to be lower in highlanders than in lowlanders, in contrast to the initial prediction of this study.

### **3.1 Mitochondrial respiratory kinetics at submaximal substrate concentrations as a measure of mitochondrial function**

As the site of O<sub>2</sub> consumption and ATP production, the mitochondria are essential to aerobic life. The mitochondria play many important roles in the cell, including mediation of cell death, gene expression, and immune response, (Martinez-Rayes and Chandel, 2020), but the initial discovery that mitochondrial function and respiratory capacity correlate directly to whole-animal aerobic capacity, by Hollozy (1967), have made mitochondrial oxidative capacity the central focus of aerobic performance research. More recently, examining kinetics of submaximal mitochondrial respiration has been acknowledged to better reflect mitochondria function *in vivo* (Petrick and Holloway, 2020).

Substrate availability [GM19] regulates mitochondrial function (Petrick and Holloway, 2020). Aerobic activities (*e.g.*, exercise) are fuelled by a combination of carbohydrates and lipids (Brooks and Mercier, 1994; Weber, 2011), and membrane transport of these substrates is considered a key rate-limiting mechanism influencing oxidative phosphorylation (Petrick and Holloway, 2019b). During low- to moderate-

intensity exercise, lipids are the primary fuel sources in mammals, but their contribution to ATP production declines as intensity increases (Brooks and Mercier, 1994; Weber, 2011). Many studies on exercise metabolism have examined mitochondrial sensitivity to lipids [GM20] to gain insight on intensity- and training-dependent mitochondrial fuel selection and regulation (Ludzki *et al.*, 2015; Miotto *et al.*, 2016; Petrick and Holloway, 2019a). However, very little [GM21] is known about how these forms of regulation are different in shivering deer mice, which maintain high rates of lipid oxidation during high intensity shivering (in contrast to patterns of fuel use during exercise) (Cheviron *et al.*, 2012; McClelland *et al.*, 2017; Lyons *et al.*, 2021; Lyons and McClelland, 2022; Fig. 1). In this study, I examined the influence of high-altitude acclimation and ancestry on the mitochondrial regulation of fat oxidation via mitochondrial membrane substrate transport in shivering deer mice, namely CPT-I and ANT, through their sensitivities to their substrates, ADP, P-CoA, and L-carnitine.

### **3.2 High- and low-altitude populations of deer mice show different ADP sensitivity responses in chronic cold hypoxia.**

Altitude ancestry influences the acclimation response to cold hypoxia for ADP sensitivity in deer mice. There was a significant [GM22] interaction effect of acclimation and population on ADP sensitivity (Fig. 3C), whereby ADP  $K_m$  was ~7-fold greater in low-altitude mice following chronic cold hypoxia exposure.

Much of the current understanding of ADP kinetics in contracting muscle comes from exercise research. The effects of exercise on mitochondrial ADP sensitivity are varied, depending on the intensity, duration, training history, species, and the presence of creatine. For instance, high-intensity and moderate exercise has been shown to increase

and decrease ADP sensitivity (Perry *et al.*, 2012; Ydfors *et al.*, 2016; Petrick and Holloway, 2019), and chronic exercise training has been shown to reduce sensitivity (Guerro *et al.*, 2005; Ludzki *et al.*, 2015).

One explanation for why highlanders and lowlanders show different plastic responses in their mitochondrial sensitivity to ADP during shivering could be that highlanders have a more oxidative phenotype in their muscles. Based on calculations of free ADP at a given  $\dot{V}O_2$ , ADP sensitivity has been shown to increase with oxidative capacity, as seen in rodents and humans following exercise training (Green *et al.*, 1992; Phillips *et al.*, 1996; Dudley *et al.*, 1987). ADP sensitivity is also greater in more oxidative tissue fiber types; for example, literature from rodent studies indicates that permeabilized fibers from heart muscle are ~ 2 to 3- fold more sensitive to ADP than from permeabilized gastrocnemius fibers (ADP  $K_m$  of ~300  $\mu$ M in the heart, vs. ~1050  $\mu$ M in the gastrocnemius; Kuznetsov *et al.*, 1996; Beaudoin *et al.*, 2014; Petrick and Holloway, 2019; Miotto *et al.*, 2019).

Highlander deer mice acclimated to thermoneutral normoxia conditions have a comparable sensitivity to those of rat heart permeabilized fibres (ADP  $K_m$  = 214  $\mu$ M), and their ADP sensitivity increases nearly 3-fold following cold hypoxia acclimation (Table 1). Many traits involving aerobic performance are fixed at a higher level in high-altitude deer mice, compared to lowlanders. For instance, highlanders demonstrate a greater capillarity, area of aerobic fibers, mitochondrial enzyme activities, (Lui *et al.*, 2015; Lau *et al.*, 2017), mitochondrial volume density, and oxidative capacity in the gastrocnemius muscle of highlanders (Mahalingam *et al.*, 2017), and chronic hypoxia did

not alter the expression of these traits. High altitude mice also constitutively upregulate transcription of genes involved in lipid oxidation and mitochondrial respiration (Cheviron *et al.*, 2012, 2014), compared to lowlanders.

These fixed genetic traits in highlander deer mice are superimposed upon an enhanced plasticity in several aerobic performance traits in hypoxia. For instance, in chronic hypoxia, highlanders can increase their carbohydrate use during exercise, unlike lowlanders (Lau *et al.*, 2017). Furthermore, de-acclimation experiments from wild highlanders reveal that highlanders have an enhanced ability to increase thermogenic endurance; when tested *in situ* versus when de-acclimated from conditions in the wild, wild highlanders also reveal that transcriptomic plasticity underpins their more aerobic phenotype (Cheviron *et al.*, 2014). Thus, the fixed and plastic adaptations that contribute to the highly oxidative profile of highlander mice probably underlie the acclimation response to ADP sensitivity in these mice.

Another explanation for why ADP sensitivity is attenuated during exercise is due to the damage accrued from redox stress (Smith *et al.*, 2013; Ludzki *et al.*, 2015; Holloway *et al.*, 2018; Miotto *et al.*, 2019). Mitochondrial ROS production is linked to a rise in membrane potential caused when ADP is not available to interact with ATP synthase (Ludzki *et al.*, 2015). The underlying causes of increased mitochondrial-specific ROS production in contracting muscle are possibly linked to a decline in ADP sensitivity, as is observed during exercise (Miotto *et al.*, 2019). ROS emissions during exercise have been associated with mitochondrial biogenesis and the induction of oxidative gene transcription in mice (Miotto *et al.*, 2019). High altitude deer mice show reduced ROS

emissions in chronic hypoxia, and it was suggested that these mice are more effective at maintaining redox homeostasis, either by minimizing ROS production or enhancing antioxidant pathways (Dawson and Scott, 2022). Perhaps high-altitude deer mice can sustain greater rates of ADP-mediated electron flux through their electron transport system because the enhanced aerobic capacity within their skeletal muscle (Mahalingam *et al.*, 2017; Mahalingam *et al.*, 2020) is accompanied by a better capacity for managing ROS production.

Additionally, the transport proteins that are responsible for bringing ADP into the mitochondria could be a source of variation for ADP sensitivity between high- and low-altitude populations of mice. The voltage-dependent anion channel (VDAC), ANT and ATP synthase are all known to be modified by glutathionylation and/or acetylation (Kerner *et al.*, 2012; Mielke *et al.*, 2014; Kramer *et al.*, 2018), and determining whether there are differences in their post-translational modifications or their protein content could be an interesting next step in understanding ADP transport in deer mice.

### **3.3 Mitochondrial lipid transport**

#### **3.3.1 High altitude mice do not increase sensitivity to P-CoA in chronic cold hypoxia**

Palmitoyl-CoA and other long-chain fatty acids can moderate fatty acid oxidation through their ability to inhibit ADP transport (Ho and Pande, 1974; Morel *et al.*, 1974; Ludzki *et al.*, 2015). In this study, I found that P-CoA  $V_{\max}$  was not affected by environment or population (Table 2), although in highlanders,  $V_{\max}$  increased nearly 2-fold following cold hypoxia acclimation. Furthermore, high altitude mice do not increase sensitivity to PCo-A (apparent  $K_m = \sim 35 \mu\text{M}$ <sub>[GM23]</sub>; Table 1) following cold hypoxia acclimation, although their apparent  $K_m$  is lower than the reported values in

permeabilized mouse and rat skeletal muscle, and rat heart (40-120  $\mu\text{M}$ ; Smith *et al.*, 2012; Beaudoin *et al.*, 2014; Barbeau *et al.*, 2018). Having a relatively greater sensitivity to P-CoA may be beneficial to highlanders by reducing free PCo-A concentrations to minimize the inhibitory effect of P-CoA on ADP transport through ADP (Ho and Pande, 1974). Additionally, the reduced availability of ADP for ATP synthase has been associated with increased ROS production (Miotto *et al.*, 2019). Elevated cytosolic PCo-A levels have been linked to a reduced capacity to lower ROS production (Mielke *et al.*, 2014; Ludzki *et al.*, 2015); thus, reducing P-CoA content by increasing sensitivity to P-CoA could also reduce ROS exposure.

### **3.3.2 The regulation of lipid oxidation during shivering in high altitude mice is not associated with increases in CPT-I sensitivity to L-carnitine**

Recent evidence from exercising mice demonstrates that CPT-I sensitivity to L-carnitine decreases in an intensity-dependent manner (Petrick and Holloway, 2019a), proposing that CPT-I serves as a control point through sensitivity to L-carnitine, in addition to L-carnitine availability. We therefore predicted that shivering deer mice would either maintain or increase CPT-I sensitivity to L-carnitine to maintain the high rates of lipid oxidation observed at the whole animal level in this species (Lyons *et al.*, 2021; Lyons and McClelland, 2022; Fig. 1B). Contrary to the prediction, there was a near-significant population effect ( $p = 0.056$ ) of high-altitude mice reducing CPT-I sensitivity to L-carnitine (increasing their apparent  $K_m$ ) during shivering (Fig. 6). Additionally, the  $K_m$  values of high-altitude deer mice are roughly equal to or slightly greater than those of sedentary mice and lab mice exercising at a moderate and high intensity (Petrick and Holloway, 2019; Table 2). Although  $V_{\text{max}}$  was  $\sim 2$ -fold greater in

cold hypoxia acclimated high altitude mice compared to thermoneutral normoxia conditions (Table 2), this effect did not reach statistical significance.

L-carnitine is primarily known for its role as the substrate for CPT-I, the rate-limiting fatty acid oxidation enzyme, but L-carnitine plays an additional role in maintaining the acetyl-CoA pool when acetyl-CoA begins to accumulate faster than it can be consumed by the mitochondria (Stephens *et al.*, 2007; Spriet *et al.*, 2014). This accumulation usually occurs when flux through glycolytic pathways is high, *e.g.*, during exercise. Acetyl-CoA can inhibit pyruvate dehydrogenase (PDH) when it accumulates. Thus, free L-carnitine can converge with acetyl-CoA to form acetyl-carnitine via carnitine acyltransferase (CAT), and restore PDH flux (Stephens *et al.*, 2007; Spriet *et al.*, 2014; Stephens *et al.*, 2018). A potential explanation for why highland deer mice have an reduced L-carnitine sensitivity is that acetyl-CoA production and consumption is evenly matched, which would reduce the need for L-carnitine to buffer acetyl-CoA, and consequently increase the free L-carnitine pool.<sup>[GM24]</sup> This theory has also been proposed to explain why acyl-carnitine pools<sup>[GM25]</sup> are not diminished at low exercise intensities (Sahlin, 1990; Constantin-Teodosiu *et al.*, 1991).

In addition to their role in fatty acid oxidation, high levels of acyl-CoAs and acyl-carnitines are also known to inhibit several enzymes, including PDH and ANT (Morel *et al.*, 1974; Ho and Pande, 1974; Virmani and Cirulli, 2022). To optimize regulatory control, Schnell and Maini (2000) posited that the apparent  $K_m$  and cellular concentration for a given substrate are “in the neighbourhood” of each other and that changes in concentration would be mirrored in the  $K_m$ . Based on this theory, the trends of apparent

$K_m$ s seen in cold hypoxic highlanders imply that these mice maintain larger pools of L-carnitine than of PCo-A or palmitoylcarnitine (Table 1). This could benefit highlanders by allowing them to exert more refined regulatory control over lipid oxidation and glycolytic flux.

In humans, L-carnitine availability drops dramatically following exercise (by ~70%) (van Loon *et al.*, 2001). In contrast, in lab mice exercising at a moderate intensity, free L-carnitine availability drops moderately from ~250 to ~175  $\mu$ M (Primassin *et al.*, 2008; ter Veld *et al.*, 2009). However, the apparent  $K_m$  in mice at rest (~23  $\mu$ M) and exercising at a high intensity (~35  $\mu$ M) remains well below the levels of available L-carnitine (Petrick and Holloway, 2019), suggesting that the intensity-dependent decline in CPT-I sensitivity attenuates lipid oxidation in lab mice. The levels of L-carnitine availability in deer mice are unknown, but identifying how free L-carnitine availability is affected by shivering would provide valuable insight into the regulatory role of L-carnitine in lipid oxidation.

### **3.4 Lipid transport into the mitochondrial matrix**

We found that FAT/CD36 was present in deer mouse mitochondria during shivering, and that there were no differences in content across treatment groups and populations (Fig. 7) FAT/CD36, which is typically associated with fatty acid transport across cell membranes, can also be found in mitochondrial membranes (Bonen *et al.*, 2004; Campbell *et al.*, 2004; Bezaire *et al.*, 2006; Holloway *et al.*, 2006). In mitochondria, FAT/CD36 is thought to increase the rate of fat uptake by translocating acylcarnitines from CPT-I to CACT (Bezaire *et al.*, 2006). Furthermore, some research

on isolated mitochondria in humans has shown that FAT/CD36 was recruited to mitochondria to aid exercise-induced increases in lipid oxidation (Holloway *et al.*, 2006). While mitochondrial FAT/CD36 content does not change with population or environment in shivering deer mice, Lyons and McClelland (2022) found that tissue FAT/CD36 expression increased in gastrocnemius after deer mice were acclimated to cold hypoxia. Based on this evidence, it seems that changes in mitochondrial FAT/CD36 do not contribute to population differences in lipid oxidation between populations.

I found that mitochondrial sensitivity to palmitoylcarnitine was unchanged across all groups, and while the two populations appear to respond differently to acclimation regarding maximum palmitoylcarnitine-stimulated respiration, their responses were not significantly different (Table 2). The apparent  $K_m$  reported for palmitoylcarnitine in this study is similar to the reported sensitivity in resting and exercising lab mice ( $K_m \sim 20\text{-}23 \mu\text{M}$ ; Petrick and Holloway, 2019), but lower than in permeabilized fibers from rat heart ( $K_m \sim 55$ ; Toleikis *et al.*, 2020). Palmitoylcarnitine has been shown to affect the ultrastructure of the mitochondria by producing similar effects to the drug carboxyatractyloside, which increases the intermembrane space by constraining the folds of the cristae. Specifically, palmitoylcarnitine is thought to induce a conformational change in ANT from the M-state to the C-state, which creates a more open, or “orthodox,” structure of the intermembrane space (Toleikis *et al.*, 2020). Deer mice have been shown to increase cristae surface density following hypoxia acclimation (Mahalingam *et al.*, 2017). However, considering the lack of effect of environment or population on palmitoylcarnitine affinity, palmitoylcarnitine is probably not responsible

for any changes to mitochondrial ultrastructure. Furthermore, based on this evidence it appears that the points of selection for enhancing lipid oxidation during shivering occur at CPT-I or further upstream along this pathway in the deer mouse.

### **3.5 Summary and Conclusion**

High altitude deer mice can maintain high rates of lipid oxidation to support the metabolic demand of shivering at a high intensity. Current research has shown that these highland mice acclimated to cold hypoxia display the highest mass-specific lipid oxidation rates of any mammal during cold-induced  $\dot{V}O_2$  trials (Lyons *et al.*, 2021). Furthermore, these highlanders have greater rates of circulatory lipid transport, and can increase their plasma triglyceride content following cold hypoxia acclimation (Lyons and McClelland, 2022). At the mitochondrial level, research on permeabilized fibres shows that highlander deer mice had greater mitochondrial respiratory capacities and a greater subsarcolemmal mitochondrial volume density (Mahalingam *et al.*, 2017). This study aimed to determine if these adaptations in the high-altitude deer mouse are overlaid by regulatory mechanisms that occur during submaximal mitochondrial respiration.

We found that that high altitude mice acclimated to cold hypoxia increase their mitochondrial sensitivity to ADP, which contrasts with findings from exercise studies. ANT is a promising point of investigation for understanding how ADP sensitivity may fluctuate across populations and acclimation treatments, since ANT has been shown to interact with several factors, such as ROS production and the presence of long-chain fatty acids, such as P-CoA (Ludzki *et al.*, 2015; Miotto *et al.*, 2019), and is susceptible to post-translational modifications (Yan & Sohal, 1998; Feng *et al.*, 2008; Queiroga *et al.*, 2010;

Mielke *et al.*, 2014). We also found that in highland mice, CPT-I appears to reduce sensitivity to L-carnitine with cold hypoxia acclimation. This finding contrasts with the initial prediction of this study, and a plausible explanation is that highland deer mouse mitochondria produce and consume acetyl-coA at a more evenly matched rate, reducing the need to use L-carnitine as an acetyl-CoA buffer. The physiological reasons for variation in submaximal CPT-I performance could stem from differences in L-carnitine availability (Stephens *et al.*, 2007; Stephens, 2018), although it is currently unknown whether glycolytic and lipid oxidation pathways deplete L-carnitine pools during shivering. Regardless, these results suggest that high altitude ancestry in deer mice is capable of altered plasticity at submaximal levels of mitochondrial respiration during shivering. The natural next step to this study is to examine submaximal mitochondrial function and lipid consumption in non-shivering deer mice. Furthermore, identifying the concentrations of L-carnitine, palmitoylcarnitine and P-CoA in skeletal muscle from these mice would increase our understanding of how L-carnitine mediates the pools of P-CoA and L-carnitine to balance lipid and carbohydrate oxidation pathways. These future studies will expand on the story of the deer mouse and its incredible story of survival in an extreme environment – high altitude.

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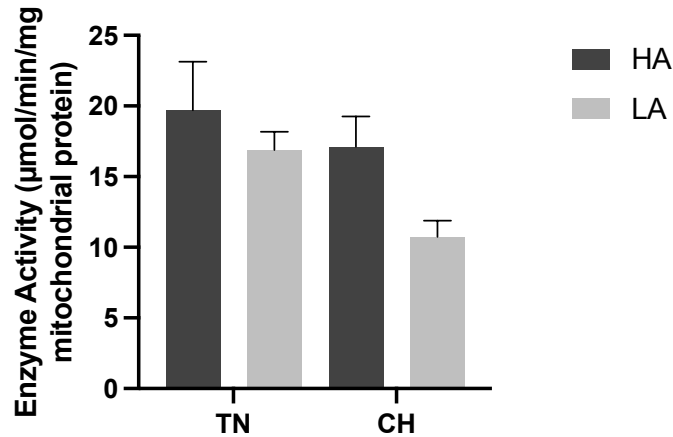
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## **Appendix**

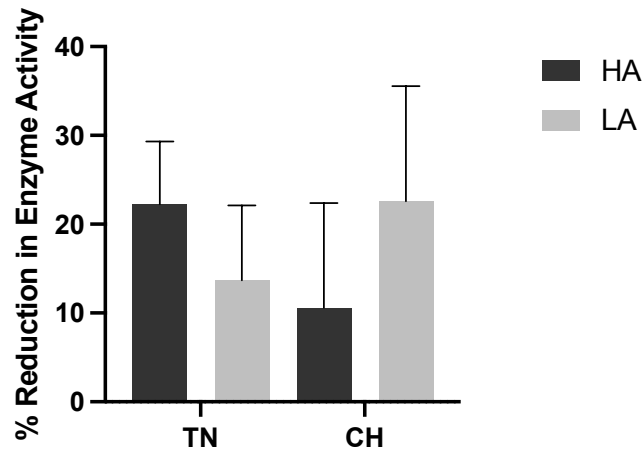
The following results were collected in collaboration with Darian Steele, and these figures were created with his assistance.

### *Methods for measuring CPT-I activity and L-carnitine titrations in isolated mitochondria*

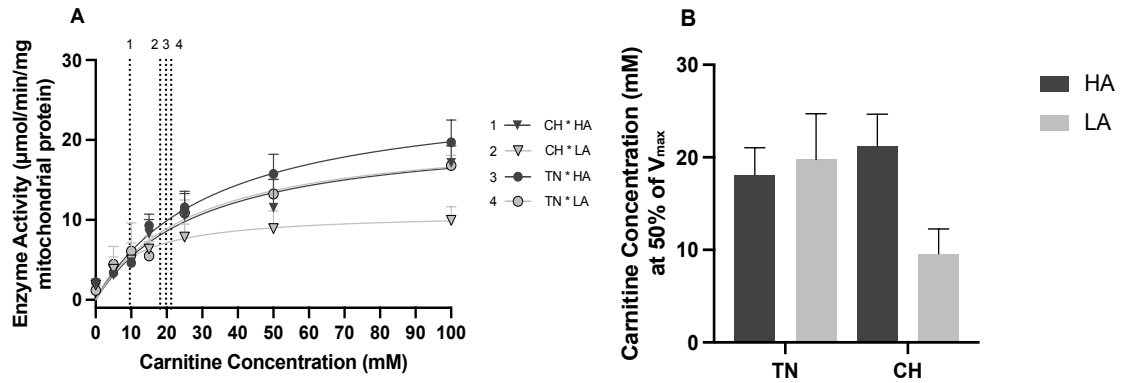
The apparent  $V_{max}$  of CPT-I was determined using intact isolated mitochondria that were diluted in ddH<sub>2</sub>O. Prior to determining CPT-I activity, total mitochondrial protein was quantified using a Bradford assay (Bio-Rad, Mississauga, ON). CPTI  $V_{max}$  was measured at 37°C in Mir05 with 0.2mM DTNB, 2mM palmitoyl-CoA and 5mM L-carnitine (Lyons and McClelland, 2022). Enzyme activity was also measured using a titration of L-carnitine (0.01, 0.05, 0.1, 0.5, 1.0, 10, and 100mM). All enzyme assays were run in 96 well format at 412 using a Spectromax Plus 384 microplate reader (Molecular Devices).



**Figure A1.** Apparent  $V_{\max}$  of carnitine palmitoyl-transferase I (CPT I) in isolated mitochondria from the left hindlimb muscles of cold hypoxia (CH)- and thermoneutral (TN)-acclimated high altitude (HA) and low altitude (LA) deer mice,  $n = 3-5$ . Activity was presented as  $\mu\text{mol mg}^{-1} \text{min}^{-1}$  mitochondrial protein. There was no interaction effect between environment and population ( $F_{1,13} = 0.71$ ,  $p = 0.41$ ), nor a significant effect of acclimation ( $F_{1,13} = 3.44$ ,  $p = 0.09$ ). The population effect approached significance ( $F_{1,13} = 3.90$ ,  $p = 0.07$ ). Data are presented as mean  $\pm$  SEM.



**Figure A2.** Percentage reduction of carnitine palmitoyl-transferase I (CPT-I) activity in the presence 1.4 mM of known CPT I allosteric inhibitor malonyl-CoA in isolated mitochondria from the hindlimb muscles of cold hypoxia (CH) and thermoneutral (TN) acclimated high altitude (HA) and low altitude (LA) deer mice,  $n = 3-5$ . Based on % reduction in enzyme activity, the inhibitory effect of malonyl-CoA did not produce significant effect of environment ( $F_{1,12} = 0.211$ ,  $p = 0.65$ ), population ( $F_{1,12} = 0.21$ ,  $p = 0.87$ ) or interaction between environment and population ( $F_{1,12} = 0.60$ ,  $p = 0.45$ ). Data are presented as mean  $\pm$  SEM.



**Figure A3.** Carnitine palmitoyl-transferase I (CPT I) enzyme activity ( $\mu\text{mol}/\text{min}/\text{mg}$ ) in isolated mitochondria from the hindlimb muscles of cold hypoxia (CH) and thermoneutral (TN) acclimated high altitude (HA) and low altitude (LA) deer mice. **A** Enzyme activity at increasing L-carnitine concentrations (mM). **B**  $K_m$ , or L-carnitine concentration at 50% of  $V_{\text{max}}$  (mM),  $n = 3-5$ . There was no significant effect of environment ( $F_{1,8} = 1.152, p = 0.31$ ), population ( $F_{1,8} = 0.71, p = 0.422$ ) or interaction between the two ( $F_{1,8} = 3.14, p = 0.31$ ) on the apparent  $K_m$  of L-carnitine. Data are presented as mean  $\pm$  SEM.