FUEL USE AND METABOLIC ADAPTATIONS TO HIGH ALTITUDE

FUEL USE AND METABOLIC ADAPTATIONS TO HIGH ALTITUDE IN SMALL MAMMALS

By

MARIE-PIERRE SCHIPPERS, B.SC.

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AUTHOR: Marie-Pierre Schippers, B.Sc. (University of Guelph)

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ABSTRACT

Knowledge on fuel use and muscle metabolism in high altitude mammals is very limited. Yet, as the oxidation of carbohydrates offers an oxygen-saving advantage over the oxidation of fatty acids (15-30% more energy produced per oxygen used), one possible adaptation to maintain performance at high altitude is to elevate the use of carbohydrates as a fuel source for energy metabolism. To test this hypothesis, I performed intraspecific and interspecific comparisons of whole-body fuel use and muscle metabolism in closely related high (4000-4500 m) and low altitude (100-300 m) native mice (genus *Phyllotis*), which I collected at different locations in Andean and coastal regions of Peru. My results show a higher proportional use of carbohydrates when oxygen becomes limited in high altitude *Phyllotis* in comparison to their low altitude counterparts. This phenotype does not seem to result from similar phylogenetic history or from a chronic exposure to hypobaric hypoxia during development or adulthood. Accordingly, this thesis provides the first compelling evidence of enhanced carbohydrate utilization as an adaptation to high altitude, a hypothesis proposed nearly 30 years ago. The mechanisms responsible for this shift in fuel use are unknown. There were no strong indications of a greater capacity for carbohydrate oxidation in skeletal and cardiac muscles of high altitude *Phyllotis* mice. Finally, as this thesis provides the first report of whole-body fuel use in mice, a comparison with other mammalian species (rats, dogs and goats) revealed that the current model of mammalian fuel selection, which is thought to be conserved among mammals, does not apply to small mammals. I thus revisited the current model and proposed a new one general to all mammals. This thesis thus provides

significant advancements not only in the field of high altitude physiology but also in the field of mammalian energetics.

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TABLE OF CONTENTS

ABSTRACT ..... **iii**

ACKNOWLEDGEMENTS ..... **v**

TABLE OF CONTENTS ..... **viii**

LIST OF FIGURES ..... **xiii**

LIST OF TABLES ..... **xvi**

LIST OF ABBREVIATIONS ..... **xviii**

DECLARATION OF ACADEMIC ACHIEVEMENT ..... **xx**

CHAPTER 1

GENERAL INTRODUCTION

Maximum oxygen consumption at altitude **2**

Performance at altitude **3**

Mammalian patterns of fuel use **6**

Fuel use at altitude **8**

Muscle tissue responses to high altitude **11**

Criticism of past high altitude adaptation studies **15**

***Phyllotis* mice: a unique system for the study of high altitude adaptation..... 17**

Thesis goals 22

Chapters' summary..... 24

CHAPTER 2

INCREASED USE OF CARBOHYDRATES AT ALTITUDE IN ANDEAN LEAF-EARED MICE (*PHYLLOTIS ANDIUM)*

Abstract 26

Introduction 27

Methods 30

Study design 30
Respirometry 34
Exercise and resting protocols 34
Muscle enzymatic capacities (Vmax) 36
Calculations and Statistical analyses..... 37

Results..... 38

Discussion..... 48

Carbohydrates at altitude..... 48
Performance 50
Muscle enzymatic capacity 51
Limitations and perspectives..... 54

Conclusion..... 55

CHAPTER 3

INCREASED CARBOHYDRATE USE IN HIGH ALTITUDE NATIVE MAMMALS: A 30-YEAR-OLD HYPOTHESIS FINDS SUPPORT IN ANDEAN MICE (*PHYLLOTIS*)

Abstract **57**

Introduction **58**

Methods **60**

Study design 60
Respirometry and *in vivo* measurements 64
Species identification and phylogenetic analyses 64
Muscle enzymatic capacity (Vmax) 65
Statistical analyses 65

Results and Discussion **66**

Aerobic capacity 66
Fuel use 70
Performance 77
Muscle enzymatic capacity 80
Genetic factors, acclimatization and development 83
Summary and perspectives 84

CHAPTER 4

PATTERNS OF FUEL USE DURING LOCOMOTION IN MAMMALS REVISITED: THE IMPORTANCE OF AEROBIC SCOPE

Abstract **86**

Introduction **87**

Materials and methods **90**

Study design 90
Exercise and resting protocols for mice 92
Muscle enzymatic capacity (Vmax) and ELISA 92
Calculations and statistical analyses 93

Results 95

Fuel selection in mammals 95
Acclimation to hypoxia: *in vivo* VO_2 and fuel selection 97
Acclimation to hypoxia: muscle plasticity 101

Discussion 101

Aerobic scope versus VO_2max 104
Effect of acclimation to hypoxia on fuel selection patterns 105
Normoxia versus hypoxia 106
Effect of acclimation to hypoxia on muscle metabolism 107
Conclusion 110

CHAPTER 5

GENERAL DISCUSSION

Increased use of carbohydrates: an adaptation to high altitude 111

Skeletal and cardiac muscle metabolism at high altitude 112

Conserved patterns of mammalian fuel selection 115

Conclusion 118

REFERENCES 119

APPENDIX A 130

APPENDIX B 132

APPENDIX C **133**

APPENDIX D **136**

LIST OF FIGURES

Figure 1.1 Phylogeny of *Phyllotis* species of interest (modified from Steppan 1998) with their respective altitudinal ranges. Branch lengths are not to scale. Altitudinal ranges in bold depict high altitude species..... 20

Figure 2.1 Study design: following capture, low altitude (300 m) and high altitude (4000 m) populations of *P. andium* were transported to the animal facility at sea level. Familiarization to treadmill and data collection commenced 2 and 6 weeks following capture, respectively..... 33

Figure 2.2 Mean + s.e.m. resting respiratory exchange ratio (RER) of the 300 m and 4000 m populations of *P. andium* under (a) normoxic and (b) hypoxic conditions.. 40

Figure 2.3 Mean + s.e.m. percent of total VO_2 used for carbohydrate oxidation during exercise at 75% of VO_2max under a) normoxic and b) hypoxic conditions in the 300 m (open bars) and 4000 m (solid bars) populations of *P. andium*. 42

Figure 2.4 Mean ± s.e.m. VO_2 at each speed of the VO_2max protocol under a) normoxic and b) hypoxic conditions in the 300 m (open circles) and 4000 m (solid circles) populations of *P. andium*..... 45

Figure 2.5 Mean + s.e.m. time to fatigue to treadmill running at 12 m min^{-1} under a) normoxic and b) hypoxic conditions in the 300 m (open bars) and 4000 m (dark bars) populations of *P. andium*. 46

Figure 2.6 Mean + s.e.m. activities (Vmax) of hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), lactate dehydrogenase (LDH), β-hydroxyacyl-CoA dehydrogenase (HOAD), citrate synthase (CS), isocitrate dehydrogenase (IDH), and/or cytochrome c oxidase (COx) in (a) skeletal muscles (gastrocnemius and thigh) and (b) left ventricles of the 300 m (open bars) and 4000 m (dark bars) populations of *P. andium*..... 47

Figure 3.1 Phylogenetic tree of *Phyllotis* species under study, which was used in the independent contrast analysis. Branches are drawn proportional to their actual lengths. 61

Figure 3.2 Estimated marginal mean (± s.e.m.) aerobic capacities (VO_2max) under a) normoxic and b) hypoxic conditions in low altitude (LA; open bars) and high altitude (HA; solid bars) *Phyllotis* species. 67

Figure 3.3 Mean (± s.e.m.) percent of total VO_2 used for carbohydrate oxidation during exercise at 75% of VO_2max under a) normoxic and b) hypoxic conditions in LA (open bars) and HA (solid bars) *Phyllotis* species. 72

Figure 3.4 Mean (± s.e.m.) respiratory exchange ratio (RER) at rest under a) normoxic and b) hypoxic conditions in LA (open bars) and HA (solid bars) *Phyllotis* species. 76

Figure 3.5 Mean (± s.e.m.) time to fatigue to treadmill running at 12 m min^{-1} under a) normoxic and b) hypoxic conditions in LA (open bars) and HA (dark bars) *Phyllotis* species. 78

Figure 3.6 Least-square regressions forced through the origin between independent contrasts of altitude and (a) % CHO at 75% of VO_2max under normoxia (R^2=0.68, *P*=0.087), (b) % CHO at 75% of VO_2max under hypoxia (R^2=0.82, *P*=0.046), (c) resting RER under hypoxia (R^2=0.86, *P*=0.035) ,(d) time to fatigue under normoxia (R^2=0.98, *P*=0.006). 79

Figure 4.1 Percent of oxygen used for CHO oxidation (% VO_2) with relative exercise intensities expressed as (a) % of aerobic capacity (VO_2max) and (b) % of aerobic scope in CD1 mice (this study), rats (McClelland et al. 1998), dogs and goats (Roberts et al. 1996c). 96

Figure 4.2 Mean + s.e.m. activity (Vmax) of hexokinase (HK), phosphofructokinase (PFK), citrate synthase (CS), isocitrate dehydrogenase (IDH), cytochrome c oxidase (COx), β-hydroxyacyl-CoA dehydrogenase (HOAD), pyruvate kinase (PK), and lactate dehydrogenase (LDH) in gastrocnemius of CD1 mice after a chronic exposure to sea level (control) or hypobaric hypoxia (acclimated). 102

Figure 4.3 Mean + s.e.m. activity (Vmax) of hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), lactate dehydrogenase (LDH), citrate synthase (CS), isocitrate dehydrogenase (IDH), cytochrome c oxidase (COx), β-hydroxyacyl-CoA dehydrogenase (HOAD); and mean + s.e.m. of heart type fatty acid binding protein (H-FABP) content in left ventricle of CD1 mice after a chronic exposure to sea level (control) or hypobaric hypoxia (acclimated). 103

Figure 5.1 Mean ± s.e.m. percent of oxygen used for carbohydrate (CHO) oxidation (%VO_2) with relative exercise intensities expressed as (a) % of aerobic capacity (VO_2max) and (b) % of aerobic scope in lowland (open symbols) *Mus muculus* (Chapter 4), *P. amicus*, and *P. limatus*, and highland (filled symbols) *P. andium* and *P. xanthopygus* (Chapter 3). 116

Figure B.1 Protein abundance of pyruvate dehydrogenase complex (PDHtotal) relative to actin in the left ventricles of the 300 m and 4000 m populations of *P. andium*..... 132

Figure C.1 Microarray validation with qPCR using 2 selected genes involved in metabolism (hexokinase 2 (HK2) and isocitrate dehydrogenase 2 (IDH2)) which were differentially expressed between the 300 m and 4000 m populations of *P. andium* by at least 1.45 fold..... 134

Figure C.2 Gene expression relative to values of the 300 m population of *P. andium* of (a) glucose transporter 1 (GLUT1) and (b) glucose transporter 4 (GLUT4) normalized to elongation factor 1-alpha (ef1α) using qPCR 135

LIST OF TABLES

Table 1.1. Climate data and habitats of the sampled populations of *Phyllotis* species investigated in this thesis. 21

Table 2.1 Geographical and altitudinal details of capture sites and description of habitats of the two populations of *P. andium* under study.....31

Table 2.2 : Means ± s.e.m. of body mass and respiratory data of the 300 m and 4000 m populations of *P. andium* under normoxic and hypoxic conditions, together with statistical results of comparisons 39

Table 2.3 Means ± s.e.m. of respiratory exchange ratio (RER) and absolute rates of fuel selection in the 300 m and 4000 m populations of *P. andium* during treadmill running at a target intensity of 75% of VO_2max under normoxic and hypoxic conditions. 43

Table 3.1 Altitudinal ranges and habitats of *Phyllotis* species under study together with geographical and altitudinal details of capture sites. 62

Table 3.2 Estimated marginal means ± s.e.m. of body mass and respiratory data of low (LA) and high altitude (HA) *Phyllotis* species (HA species are in bold) under normoxic and hypoxic conditions 68

Table 3.3 Estimated marginal means of respiratory exchange ratio and absolute rates of fuel selection in LA and HA *Phyllotis* species (HA species are in bold) during treadmill running at a target intensity of 75% of VO_2max under normoxic and hypoxic conditions.....73

Table 3.4 . Mean ± s.e.m. enzyme activities (Vmax) in µmoles min^{-1} g^{-1} wet weight in gastrocnemius and left ventricles of LA and HA *Phyllotis* species.....81

Table 4.1 Mean values of variables used to determine aerobic scope in dogs, goats and rats.. 94

Table 4.2 Resting oxygen consumption (VO_2), resting respiratory exchange ratio (RER), aerobic capacity (VO_2max), speed at VO_2max, and aerobic scope variables of CD1 mice after a chronic exposure to sea level (control) or hypobaric hypoxia (acclimated).....98

Table 4.3 Respiratory exchange ratio (RER), treadmill speeds and carbohydrate (CHO) and lipid oxidation at different relative exercise intensities (%VO_2max or % aerobic scope) under normoxia (21% O_2) and hypoxia (12% O_2) in CD1 mice after a chronic exposure to sea level (control) or hypobaric hypoxia.....100

Table A.1 Means ± s.e.m. of body mass and respiratory data of the low (100 m) and high (3700 m) altitude populations of *P. limatus* under normoxic and hypoxic conditions 130

Table A.2 Means ± s.e.m. of respiratory exchange ratio (RER), percent CHO oxidized, and absolute rates of fuel use in low (100 m) and high (3700 m) altitude populations of *P. limatus* during treadmill running at a target intensity of 75% of VO_2max under normoxic and hypoxic conditions 131

Table C.1 Metabolic genes of interests that were either up-regulated or down-regulated by at least 1.45 fold in left ventricles of HA *P. andium* compared to LA *P. andium*. Data obtained from gene expression profiling experiments using the Mouse Genome 430 2.0 array. 133

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CHO	Carbohydrate
COx	Cytochrome c oxidase
CS	Citrate synthase
ELISA	Enzyme-linked immunosorbent assay
HA	High altitude
HK	Hexokinase
H-FABP	Heat-type fatty acid binding protein
H-LDH	Heart-type lactate dehydrogenase
HOAD	β-hydroxyacyl-CoA dehydrogenase
IDH	Isocitrate dehydrogenase
LA	Low altitude
LDH	Lactate dehydrogenase
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PFK	Phosphofructokinase
PK	Pyruvate kinase
PO_2	Partial pressure of oxygen
RER	Respiratory exchange ratio

UPCH	Universidad Peruana Cayetano Heredia
VCO_2	Rate of carbon dioxide production
VO_2	Rate of oxygen consumption
VO_2max	Maximum oxygen consumption
Vmax	Enzyme maximum activity

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis is organized in a sandwich format approved by McMaster University. It consists of five chapters. Chapter one is a general introduction and summary of the objectives. Chapters two through four represent manuscripts that are in preparation for submission to peer-reviewed scientific journals. Finally, chapter five discusses the major findings of the thesis and indicates the significance and future directions of the research. Appendices A through C presents supplementary data which could not stand as a chapter on its own. Appendix D provides information on two articles published in peer-reviewed journals which are part of this Ph.D. degree but not included as chapters in the present thesis.

Chapter 1: **General introduction**

Chapter 2: **Increased carbohydrate use in the Andean leaf-eared mice (*Phyllotis andium*).**

Authors: Marie-Pierre Schippers, Oswaldo Ramirez, Margarita Arana, and Grant B McClelland

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Chapter 5:		**General discussion**

Appendix A	**Respiratory data and whole body fuel selection in the low (100 m) and high (3700 m) altitude populations of *P. limatus***
Appendix B	**Protein abundance of pyruvate dehydrogenase in the left ventricle of the 300 m and 4000 m populations of *P. andium***
Appendix C	**Differences in gene expression in left ventricle of the 300 m and 4000 m populations of *P. andium***
Appendix D	**Published articles which are part of this Ph.D. degree but not included as chapters in the present thesis**

CHAPTER 1

GENERAL INTRODUCTION

The strategies employed by mammals to circumvent the debilitating effects of low barometric pressure have fascinated physiologists for over 130 years. Paul Bert, the father of modern high altitude physiology and medicine, hypothesized in 1878 that residents of La Paz, Bolivia and other high altitude mammals (llamas, yaks) have gradually adapted to low oxygen levels either by modification of the nature or quantity of hemoglobin (Hb) or by increases in red blood cells (West, 1998). Since then, a plethora of investigations on polycythemia and oxygen transport at high altitude have emerged. It is now recognized that Andean human natives and lowlanders acclimatized to high altitude have increased numbers of red blood cells and that a number of high altitude mammals show modifications in Hb function. Indeed, Andean camelids, such as the guanaco (*Lama guanacoë*), as well as North American high altitude rodents (*Peromyscus maniculatus*) all show high blood oxygen affinities, a trait likely attributable to amino acid substitutions in Hb (Chappell and Snyder, 1984; Piccinini et al., 1990; Poyart et al., 1992; Snyder et al., 1988; Storz, 2007).

Mechanisms of phenotypic plasticity and genetic adaptation in the O_2 cascade in response to hypoxia have been thoroughly studied in humans and in a few other mammalian species (reviewed in Storz et al. 2010). Acclimatization to high altitudes encompasses a series of physiological modifications in the oxygen transport pathway that can be beneficial but also detrimental to long-term survival in hypoxic environments

(Storz et al., 2010). Accordingly, part of the adaptations to high altitude, observed in native residents, appears to be an attenuation of those phenotypes that are maladaptive. Even though different populations and species appeared to have followed different routes of adaptations (Beall, 2007), the end result is successful oxygen delivery and survival in hypoxic environments. In fact, high altitude native mammals usually have similar or even enhanced capacities of O_2 consumption (VO_2max) in comparison to their low altitude closely-related counterparts (Beall, 2007; Chappell and Snyder, 1984; Rezende et al., 2001).

Maximum oxygen consumption at altitude

High altitude native humans (Tibetans and Andeans) at an altitude of ~3900 m usually show maximum oxygen consumption (VO_2max) values similar to lowlanders at sea level and 10-20% higher than acclimatized lowlanders at the same altitude (Beall, 2007). This is because lowlanders suffer a ~20-35% reduction in VO_2max upon exposure to this altitude (Hochachka and Somero, 2002). Acclimatization and long-term residence (over 1 year) gradually attenuates this hypoxia-induced drop in VO_2max, however values usually remain slightly lower than normal sea level ones (Beall, 2007; Marconi et al., 2006). Maintaining adequate levels of VO_2max despite the lower O_2 availability associated with altitude may thus to be important for long-term survival, as it allows for a greater scope of aerobic activity.

In high altitude native rodents, VO_2max has been found to be either higher than low altitude rodents (Chappell and Snyder, 1984; Rezende et al., 2001), or unchanged

when controlling for the acclimatization effects of cold environments (Hayes, 1989a). Moreover, a study controlling for different abiotic and biotic factors influencing VO_2max in rodents along an altitudinal gradient showed that temperature rather than altitude is a better predictor of VO_2max (Rezende et al., 2004). Aerobic capacities in high altitude rodents thus seem to be elevated due to the low temperatures associated with altitude rather than the low oxygen availability per se. Because of allometry (large surface area to volume ratio), small mammals are at disadvantage for heat conservation. Accordingly, it may be essential for small rodents to increase VO_2max at high altitude in order to sustain high rates of thermogenesis for the maintenance of body temperature and thus survival. Indeed, a capture-recapture study in high altitude native North American mice (*Peromyscus maniculatus*) at 3800 m revealed that a high VO_2max at altitude is positively correlated with survival in this population (Hayes and O'Connor, 1999).

Performance at altitude

Because it correlates well with aerobic athletic performance and endurance performance, VO_2max represent a valid measure of physical performance. However, other whole-body measures ought to be considered. If adaptations to high altitude have lead to an enhanced metabolic or work efficiency, such that more ATP is generated per molecule of O_2 or more external work is performed per ATP, a simple VO_2max measurement would not provide an adequate evaluation of performance limits. The whole-body measure of efficiency, often referred as work efficiency, is defined as external work (output) relative to the metabolic cost (input); where external work is

measured by mechanical power output on a cycle ergometer and the metabolic cost by the rate of oxygen consumption (VO_2). A few studies have investigated work efficiency in high altitude mammals. These have mainly focused on high altitude native human populations from the Himalayas and the Andes. It appears that it is still debatable whether these populations have an energetic advantage over lowlanders (Brutsaert, 2008). A few studies have reported higher work efficiencies in high altitude natives (Ge et al., 1994; Haas et al., 1983; Hochachka et al., 1991; Niu et al., 1995), whereas others reported no differences (Brutsaert et al., 2004; Kayser et al., 1994) or even lower efficiency values (Kollias et al., 1968). A number of factors influence work efficiency values: variation in body mass, fitness level, non-steady state dynamics, and exercise intensities above anaerobic threshold, among others. It is likely that some of these factors have influenced a number of studies mentioned above; consequently it still remains unclear whether high altitude natives benefit from a metabolic energetic advantage over lowlanders.

While work efficiency studies of high altitude natives have provided inconsistent data, a few reports on work economy suggest that Himalayan natives have an advantage over low altitude residents (Bastien et al., 2005; Marconi et al., 2005). Comparisons of high altitude Tibetans and low altitude Nepalese reported 8-13% lower VO_2 during walking and running in Tibetans (Marconi et al., 2005).Work economy is defined as the metabolic cost (VO_2) to perform a specific activity. In this measurement, mechanical power output is not assessed. A higher work economy can thus result from elevated metabolic efficiency or economy of locomotion. This measurement can represent an alternative to work efficiency measurements in situations where the latter cannot be easily

quantified. Walking and running represent instances where accurate assessments of work efficiency are challenging because body weight and incline grade must be factored in to provide accurate measures of external work. To my knowledge, no studies have measured walking or running economy in a high altitude native non-human mammal.

Another measure of performance which has received very little attention in the high altitude literature is endurance performance. The only study that has investigated time to fatigue in a high altitude human population reported enhanced endurance performance during treadmill running in Andeans compared to sea level residents in their native environment (reviewed in (Brutsaert, 2008)). The validity of these results however is questionable because no information was provided on the subjects' fitness level. In contrast, another study on deer mice (*Peromyscus maniculatus*) native to altitudes of 1220 m and 3800 m, showed that low altitude mice were actually able to run longer than high altitude mice in their respective native environments (Hock, 1965). In addition, a 90-day acclimatization period at opposite altitudes to their native one revealed that low and high altitude mice have a similar endurance capacity at 3800 m, but that low altitude mice run longer than high altitude mice at 1220 m. It is unclear why these results emerged. As it is widely established that endurance performance is correlated with glycogen depletion (Fitts, 1994), one possible explanation for the reduced endurance ability of high altitude mice compared to low altitude mice at 1220 m is faster glycogen depletion. It has long been hypothesized that high altitude native mammals have evolved a preference for carbohydrates as a fuel source for energy metabolism (Hochachka, 1985). Accordingly, it is possible that high altitude mice fatigued sooner because they were using carbohydrates

at a faster rate, and thus depleted their glycogen stores sooner than the low altitude mice. Unfortunately, little is known on carbohydrate use and glycogen depletion in high altitude mammals, as very few studies have adequately investigated patterns of fuel use in non-human mammals.

Mammalian patterns of fuel use

Mammals mainly dispose of carbohydrates, lipids and proteins as fuel sources for energy metabolism. These fuels differ widely on a number of characteristics such as storage capacity, energy density and speed of conversion to ATP (Weber, 2011). Carbohydrates are generally the fuel of choice for intense activities of short duration. They can produce the highest rates of ATP, but because of their low energy density, only limited quantities are stored. In fact, liver and muscle glycogen, the storage form of carbohydrates, usually account for less than 5% of the total energy reserves of an organism (Weber, 2011). Lipids on the other hand, can be stored in large quantities; they account for over 80% of the body's energy reserves (Weber, 2011). This particular characteristic of lipids is owed to their high energy content per unit mass. However, unlike carbohydrates, lipids show low maximal rates of ATP production. They are thus best suited to fuel low intensity activities of long duration. Lastly, proteins represent a potential source of fuel; however their oxidation is usually kept low to minimize the loss of functional proteins and to avoid metabolic costs of nitrogen excretion. In fact, the contribution of proteins to total ATP synthesis during exercise in mammals is lower than 5% (Carraro et al., 1994; Rennie et al., 1981). Accordingly, models of fuel use of

exercising mammals focus only on carbohydrates and lipids, as the contribution of protein oxidation is negligible.

In mammals, the relative contributions of carbohydrates and lipids to energy metabolism vary with exercise intensity. As the intensity of exercise increases, the proportion of carbohydrates oxidized increases whereas the proportion of lipids decreases concurrently. When exercise intensity is expressed relative to individual aerobic capacities (%VO_2max), a model of fuel selection general to all mammals emerges where the mixture of fuels is predicted by the intensity of exercise (McClelland, 2004; Roberts et al., 1996c; Weber and Haman, 2004). This model has been tested across a range of body sizes (from rats to horses) and aerobic capacities. Same-sized dogs and goats, despite a 2.2 fold difference in VO_2max, used the same mix of fuels at specific relative exercise intensities (%VO_2max) (Roberts et al., 1996c). The pattern also appeared to hold in control and selected lines of mice with inherently different aerobic capacities (Templeman et al., In review). Likewise, modifications of VO_2max arising from acclimation or acclimatization to hypobaric hypoxia in rats and humans also had no effect on the proportion of carbohydrates and lipids oxidized at different relative exercise intensities (Lundby and Van Hall, 2002; McClelland et al., 1998). Given that the data come from many different studies on distantly related species, the generality of the mammalian model of fuel use is quite unusual. It is still unclear why such conserved patterns emerge. Exceptional situations in which fuel use deviates from the predicted model could provide useful information for uncovering the underlying mechanisms. Unusually high fat diets (Jansson and Kaijser, 1982; Phinney et al., 1983) as well as

gender differences (Horton et al., 1998) were found to cause deviations from the model; however there are still no clear explanations for these observed differences. Other potential situations in which a switch in fuel use is expected are worth elucidating. Long-term exposure to the low O_2 levels associated with high altitude represents one of these potential situations.

Fuel use at altitude

Nearly 30 years ago, Hochachka proposed that a greater reliance on carbohydrates would be beneficial at altitude as this fuel provides a greater energy yield per mole of O_2 consumed compared to fatty acids (Hochachka, 1985). Theoretically, the oxygen-saving advantage of oxidizing carbohydrates over fatty acids represents 15-18% more ATP per mole of oxygen used (Brand, 2005). *In vivo* the difference may be even greater, as high as 30% more ATP per O_2 (Daut and Elzinga, 1989), possibly because of the so-called "oxygen-wasting effect" of lipids (Korvald et al., 2000). A number of studies investigating the effects of acute and/or chronic hypoxia exposure on fuel use in men have reported increases in blood glucose utilization at rest and during exercise (Brooks et al., 1991; Cooper et al., 1986; Roberts et al., 1996b) and a decrease in fatty acid uptake in muscle during exercise (Roberts et al., 1996a). However, these studies compared data of individuals exercising at sea level and at high altitude at the same absolute intensities (same work rates) rather than at the same relative intensities in each environment (%VO_2max). A 20-35% reduction in VO_2max is usually experienced by subjects exposed to high altitude hypoxia (Hochachka and Somero, 2002). Consequently,

individuals exercising at altitude at the same absolute work rates as at sea level are actually operating at intensities closer to their VO_2max (higher %VO_2max). Past reports of increased blood glucose utilization may thus be explained by the differences in relative exercise intensities rather than by a metabolic shift in fuel use at altitude. Indeed, a number of studies in which comparisons were made at the same relative intensity of exercise have reported no changes in whole-body rates of carbohydrate and lipid oxidation in men (Lundby and Van Hall, 2002) and rats (McClelland et al., 1998; McClelland et al., 1999) following an acute or chronic exposure to hypobaric hypoxia. It thus appears that acclimatization to high altitude has no effect on fuel selection in lowland mammals. Still further research is needed as a few other reports suggest that acclimatization to high altitudes actually results in a decrease in carbohydrate utilization in women (Braun et al., 2000) and an increase in fatty acid utilization in men (Young et al., 1982) during exercise at the same %VO_2max. Moreover, it may be informative to investigate other mammals as different species may respond to hypoxia in a different manner.

While short-term (hours to months) exposure to hypoxic environments has little effect on patterns of fuel selection, long-term exposure over many generations, on the other hand, could potentially result in modifications of metabolism to increase carbohydrate utilization. The most convincing finding in support of the aforementioned hypothesis is the apparent preference for carbohydrates by the heart of high altitude native human populations (Himalayan and Andean natives). An analysis of heart metabolism using ^{31}P magnetic resonance spectroscopy revealed that Himalayan natives

show a particularly low phosphocreatine / adenosine triphosphate (PCr/ATP) ratio in comparison to North American lowlanders (Hochachka et al., 1996). This low PCr/ATP signature suggests high levels of free ADP, which in turn would satisfy the high Km of ADP-requiring kinases of glycolysis. This finding thus reflects a metabolic organization in the heart of Himalayan natives to preferentially use carbohydrates instead of fatty acids, the latter being the typical fuel used by the heart in a post-absorptive state (Kodde et al., 2007). Similarly, in a separate study on cardiac metabolism of both Andean and Himalayan native individuals, positron emission tomographic measurements revealed elevated rates of glucose uptake in the hearts of both populations of highlanders in comparison to North American lowlanders (Holden et al., 1995). These results suggest that long-term exposure to high altitude could lead to a metabolic re-organization of the heart to preferentially use carbohydrates rather than free fatty acids.

It is still unclear, however, whether this carbohydrate preference occurs at the whole-body level, especially at times when O_2 becomes increasingly limited, such as under hypoxic conditions and during exercise. Hochachka and colleagues (Hochachka et al., 1991) compared respiratory exchange ratios (RER) of Andean natives and lowlanders during an incremental exercise to fatigue and found a higher RER for highlanders at low intensities (below 60% fatigue time). The high RER suggests a greater whole-body use of carbohydrate by high altitude natives, however the experimental approach used in this study raises some concerns. First, RER obtained during an incremental test to fatigue does not provide an accurate estimate of the true RER at each intensity of exercise because stable steady-state VO_2 and VCO_2 are not reached. Secondly, Hochachka and

colleagues (Hochachka et al., 1991) did not compare subjects at the same intensity of exercise relative to VO_2max. The use of fatigue time rather than VO_2max as a measure of relative exercise intensity could have introduced substantial errors. To my knowledge, this study is the only report of whole-body fuel use in a high altitude native mammal. Clearly, accurate assessments of whole-body fuel use in highland mammals are required to test the hypothesis of increased use of carbohydrates at altitude.

Muscle tissue responses to high altitude

Muscle tissue modifications in response to high altitude hypobaric hypoxia have evoked much interest, especially because it was originally hypothesized that long-term exposure to high altitudes positively affects muscle oxidative capacity. Reynafarje (Reynafarje, 1962) first reported an increase in oxidative capacity and myoglobin concentration in sartorius muscle of Andean native men. This study influenced the perception of muscle adaptations to high altitudes among physiologists and contributed to the hypothesis that high altitude residents maintain a high scope for aerobic metabolism in hypoxic environments (Hochachka et al., 1983). It is only several years later that this notion was challenged, as a series of studies from the 1990s reported that skeletal muscles of Andeans and Himalayans were actually characterized by low oxidative capacities (Desplanches et al., 1996; Hochachka, 1992; Kayser et al., 1991). Mitochondrial volume density of the vastus lateralis muscles of Nepalese Sherpas and Andean natives of La Paz was found to be approximately 20% lower than lowlanders (Desplanches et al., 1996; Kayser et al., 1991). Likewise, activities of mitochondrial enzymes such as citrate

synthase (CS) and/or β-hydroxyacyl-CoA dehydrogenase (HOAD), were also found to be similarly reduced (Desplanches et al., 1996; Hochachka, 1992). Interestingly, Tibetans born at low altitude but whose parents were native to high altitude also showed low mitochondrial densities of vastus lateralis as well as low activities of CS and HOAD (Kayser et al., 1996). These results suggest that the trait have a genetic component, although maternal effects during development must also be considered.

Little information is available on muscle metabolism and performance of high altitude native non-human mammals. Hochachka and colleagues (Hochachka et al., 1983) examined the activities of several metabolic enzymes in cardiac muscles of high altitude camelids (llama and alpaca) and high altitude deer (taruca) native to the Andes, and found that oxidative capacities of the heart was enhanced in these species compared to other lowland species. Although this study can be criticized on several aspects, including the fact that comparisons were made on distantly related species (discussed below), it points out that the general agreement that oxidative capacity is reduced in high altitude natives may not apply to cardiac muscle. Moreover, a recent study comparing metabolic enzyme activities of closely related species of pika native to different altitudes demonstrated that citrate synthase (CS) and β–hydroxyacyl CoA dehydrogenase (HOAD) activity of heart was nearly twice as high in the high altitude species compared to the sea-level one (Sheafor, 2003). In contrast, skeletal muscle CS and HOAD activities did not differ between species of different altitudes. Thus, these results suggest that oxidative capacity

of cardiac muscle is elevated in high altitude native mammals whereas the oxidative capacity of skeletal muscle is unchanged or reduced.

Acclimation or acclimatization to high altitudes in lowlanders also seems to induce either decreases or no changes in oxidative capacity of skeletal muscles depending on the magnitude of the hypoxic stress. Chronic exposure to altitudes well above 5000 m were found to induce decreases in mitochondrial density and activities of oxidative enzymes CS and cytochrome c oxidase (COx) (Green et al., 1989; Hoppeler et al., 1990; Howald et al., 1990). However, exposure to a hypoxic stress similar to altitudes of 2500-5000 m (altitudinal range in which highland natives reside), seem to have little effect on oxidative capacities of skeletal muscles of lowland animals. In fact, most studies reported no changes in CS activity following a 1 to 8 weeks acclimation period at these altitudes in skeletal muscles of rats and mice (Beaudry and McClelland, 2010; Kennedy et al., 2001; Le Moine et al., 2011; Perhonen et al., 1996). Likewise, cardiac muscles also show no changes (Kennedy et al., 2001; Templeman et al., 2010) or slight decreases (Cai et al., 2010) in CS activity following a chronic exposure to similar altitudes.

While CS activity is mostly unchanged in skeletal and cardiac muscles following acclimation to high altitudes, HOAD activity, on the other hand, appears to be reduced, especially in cardiac muscles (Daneshrad et al., 2000; Kennedy et al., 2001; Templeman et al., 2010). HOAD is a mitochondrial enzyme involved in the oxidation of fatty acids. Reductions in HOAD may result from decreases in mitochondrial densities or reductions in fatty acid oxidation. Since acclimation to high altitude appears to have little effects on mitochondrial density (insignificant changes in CS activity), these results suggest that

chronic exposure to hypoxia reduces the heart's capacity for fatty acid oxidation. In fact, it is possible that acclimation to high altitudes induces a remodelling of cardiac muscles to enhance glucose oxidation rather than free fatty acid oxidation, as a number of studies have also reported increases in activities of hexokinase (HK) following acclimation to hypoxia (Abdelmalki et al., 1996; Daneshrad et al., 2000). This remodelling of the heart may represent an oxygen-saving strategy likely to be beneficial in situations of low PO_2, as the oxidation of carbohydrates offers more ATP per mole of O_2 than the oxidation of fatty acids (Brand, 2005).

It is widely accepted that aerobic rather than anaerobic pathways are preferred at altitude. The so called lactate paradox, which refers to lower-than-expected accumulations in blood lactate during exercise under hypoxic conditions, has been observed numerous times in high altitude natives and acclimatized lowlanders (reviewed by Hochachka and colleagues (Hochachka et al., 2002)). An increased reliance on anaerobic glycolysis would not be beneficial under chronic hypoxia as it would lead to excess lactate accumulation and fast glycogen depletion, on top of offering a low ATP yield per mole of carbon fuel used. Congruent to this idea, a number of studies have reported reductions in anaerobic capacities (low LDH activities) of skeletal muscles of high altitude mammals (Hochachka, 1992; Hochachka et al., 1983) and of lowland animals acclimatized to hypobaric hypoxia (Le Moine et al., 2011; Rossignol et al., 2003). However, a few studies have also reported elevated LDH activities in muscles of high altitude native pikas and acclimated rats (Daneshrad et al., 2000; Sheafor, 2003). The heart is composed of highly oxidative tissues. Over 90% of cardiac ATP production

is supplied by aerobic pathways (Kodde et al., 2007). Accordingly, the contribution of anaerobic glycolysis to energy production in cardiac muscles is very small. Indeed, rather than producing lactate, the heart is known to be a significant consumer of exogenous lactate produced by muscles and erythrocytes (Kodde et al., 2007). Thus increases in LDH activity in cardiac muscles presumably indicate an enhanced capacity for lactate uptake rather than lactate production. In fact, Sheafor (Sheafor, 2003) also reported that the hearts of high altitude pikas were characterized by high activities of H-LDH (heart type, LDH1), a LDH isozyme which preferentially converts lactate to pyruvate. Furthermore, in a separate study, rats acclimated to hypobaric hypoxia were also found to have a higher relative abundance of LDH1 in cardiac muscle mitochondria compared to control sea level rats (McClelland and Brooks, 2002). In light of this, the observed increases in LDH activity in the heart of high altitude pikas and acclimatized rats can be interpreted as a greater capacity for oxidation of exogenous lactate rather than a high anaerobic capacity.

Criticism of past high altitude adaptation studies

Although most studies on high altitude native mammals have proposed physiological traits to be adaptive, few have followed the current framework for the study of physiological adaptation (Rezende et al., 2005). One major problem of past comparative studies is the use of only two species to draw conclusions on the adaptive significance of a trait (Garland and Adolph, 1994). Since species experience different selective pressures or diverged somehow because of genetic drift, any two species are

likely to show differences in any trait. It is thus imperative that comparative studies use a multi-species comparison approach to study adaptation. Another major problem of past comparative studies of high altitude mammals is that even though three or more species were used, they ignored the phylogenetic relationships of the species. Ignoring the fact that species have a hierarchical relationship violates the assumption of independence, important for most classical statistical analyses (Garland and Carter, 1994). Thus, Garland and Adolph (Garland and Adolph, 1994) and Garland and Carter (Garland and Carter, 1994) stressed that this practice is simply unacceptable and that comparative studies ought to compare at least three species, and use phylogenetically based statistical methods to draw conclusions. Ideally, such comparisons would be performed on species that were reared under common garden conditions for at least two generations to control for the environmental effects during development and ontogeny.

Another shortcoming with the study of high altitude adaptation is that it is very difficult to separate the different environmental factors associated with altitude. For example, low altitude mammals have usually evolved under warmer temperatures than their high altitude counterparts. Careful interpretation of results is thus necessary with regard to inferring that a physiological trait is adaptive to hypoxic environments as other factors (e.g. temperature (Rezende et al., 2004), environmental productivity (Mueller and Diamond, 2001)) could also have contributed to the evolution of the trait.

Phyllotis mice: a unique system for the study of high altitude adaptation

Past studies on metabolic adaptations to high altitudes have focused almost entirely on human populations native to the Andes and Himalayas. Yet, these populations may not be the most powerful models to study high altitude adaptations in mammals for several reasons. First, influx of lowland genes into the highland populations may have been substantial, especially in the last centuries. Indeed, Caucasian admixture in Andean Quechuas is estimated to be around 25% (Salzano and Callegari-Jacques, 1988). Second, compared to other mammals, *Homo sapiens* have remarkably little genetic diversity (Gagneux et al., 1999), reducing the potential for adaptation. Third, humans have colonized highland regions quite recently. It is estimated that colonization of the Andean and Himalayan plateaus date back to about 11,000 and 25,000 years ago, respectively (Aldenderfer, 2003). In contrast, other high altitude mammals are thought to have occupied the highlands for much longer. The presence of llamas, guinea pigs and other rodents in the Andes is estimated to date back to a few million years ago (Dunnum and Salazar-Bravo, 2010; Kramer et al., 1999; Wheeler, 1995). Lastly, human studies are limited to population comparisons within a single species, thus powerful multi-species phylogenetic comparisons cannot be carried out. In light of this, it is evident that more powerful mammalian models are needed to make convincing inferences on the adaptive nature of a number of physiological traits at altitude.

Species of South American wild mice pertaining to the genus *Phyllotis* (Rodentia: Sigmodontinae) offer a powerful model for the study of high altitude adaptations of mammals. This genus comprises several ecologically abundant, closely-

related species that occupy distinct altitudinal ranges in the Andes (Pearson, 1972; Steppan, 1998). Systematic evaluations of the genus *Phyllotis* have provided ample information on the distribution and phylogenetic relationships of species (Pearson, 1958; Pearson, 1972; Steppan, 1998). Moreover, the fossil record suggests that *Phyllotis* may have been at altitude for as long as 2 million years (Kramer et al., 1999), which constitutes ample time for genetic adaptations to occur (Rupert and Hochachka, 2001).

Commonly called leaf-eared mice, *Phyllotis* species occupy mostly rocky and bushy habitats throughout the Andes and surrounding areas from the highlands of Ecuador, all the way down to the southern tip of the continent (Steppan and Ramirez-Baca, In Press). Sigmodontine rodents probably entered South America during the formation of the Panamanian Land Bridge slightly prior to or during the Great American Interchange event (Engel et al., 1998; Webb, 1991), which took place in the Pliocene epoch (5.3-2.6 million years ago (Ma)). They radiated south, possibly along the Andean plateau as it offered a broad and straight corridor (Webb, 1991). By Pleistocene times (~2.5 Ma), sigmodontine rodents were widespread in South America occupying habitats across a wide range of altitudes in the Andes and surrounding coastal lowlands (Palma et al., 2005). The numerous changes in climate and vegetation that occurred in these areas during that time probably facilitated radiation and dispersal from the highlands to lowland areas. In some *Phyllotis*, the split between highland and lowland species, which is estimated to date back to 1.5 Ma, appears to have taken place in the central Andes (Palma et al., 2005). Today the genus comprises of at least 14 species which can be found across

a broad range of elevations, from sea level to 5000 m (Steppan and Ramirez-Baca, In Press) and perhaps even higher (~6000 m, *P. xanthopygus*; (Halloy, 1991)).

Combination of altitudinal distribution and phylogenetic information of several *Phyllotis* species indicate that sister-species from the highlands and lowlands both occur within three separate clades (Figure 1.1). This represents an ideal model system to study adaptations to high altitude as it allows for comparisons of multiple pairs of closely-related species that have evolved at different elevations. This way, the influence of phylogeny on the data is potentially minimal and easily removed. Apart from the different levels of PO_2, lowland and highland habitats differ in a number of factors that could have also shaped species traits. Such factors include temperature, humidity, vegetation, topography, predation, and UV radiation. Table 1.1 provides information on the annual temperature ranges, seasonal average precipitation, relative humidity, and habitat type of sample populations of four *Phyllotis* species which were investigated in this thesis. The Peruvian climate is quite complex, humid (May-Oct.) and dry (Nov.-April) seasons in the coastal lowlands are opposite to the wet (Nov-April) and dry (May-Oct.) seasons of the highlands. In general, the coastal lowlands are desert-like with sparse dry vegetation interspersed by wet riparian areas and lomas (coastal hills with a seasonal mist-fed ecosystem). These habitats are occupied by a number of *Phyllotis* species such as *P. amicus*, *P. limatus and P. andium*. *P. amicus* (altitudinal range: 0-3200 m) is usually found in dryer habitats of sand and rock formations with sparse cover along the coast of northern and central Peru (Steppan and Ramirez-Baca, In Press). Its diet appears to be similar to other *Phyllotis* species (57% leafy vegetation, 17% seeds and 26% insects), but

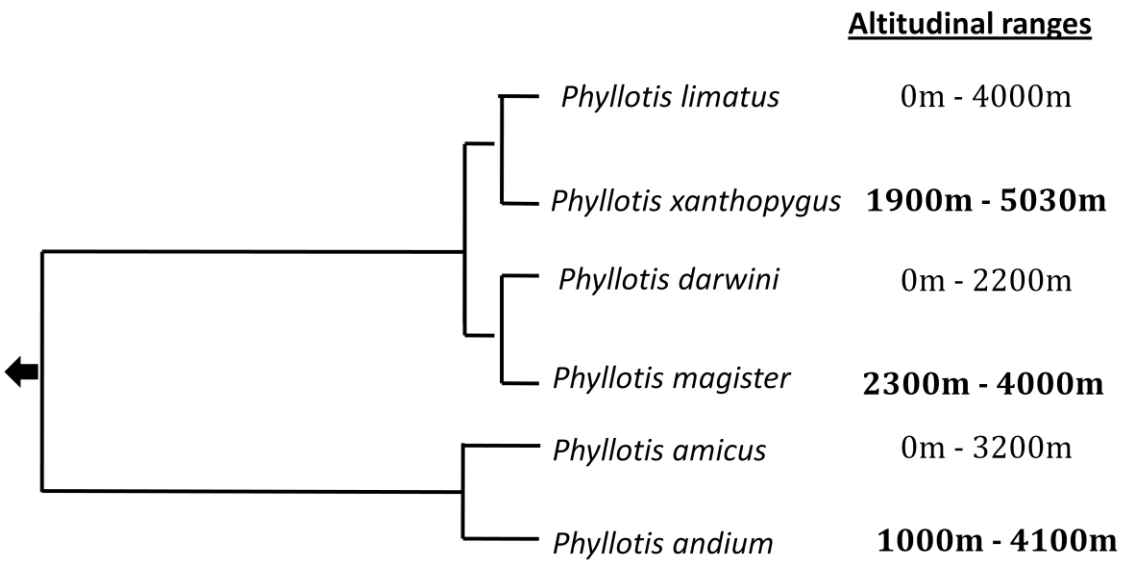

Figure 1.1 Phylogeny of *Phyllotis* species of interest (modified from Steppan 1998) with their respective altitudinal ranges. Branch lengths are not to scale. Altitudinal ranges in bold depict high altitude species.

Table 1.1. Climate data and habitats of the sampled populations of *Phyllotis* species investigated in this thesis.

	P. amicus[1]	*P. andium*[2]	*P. limatus*	*P. xanthopygous*[2]
Altitude (m)	300	4000	100	4500
Temperature range (°C)	13-22	0-17	13-24	0-17
Humidity (%)				
Nov.-April	85	75	63	75
May-Oct.	93	55	81	55
Precipitation (mm)				
Nov.-April	3.5	120	0	120
May-Oct.	22.5	15	0	15
Habitat type				
Nov.-April	Desert[3], sparse dry vegetation	Wet grasslands and shrubs	Dry and riparian vegetation	Wet grasslands and shrubs
May-Oct.	Desert[3], wet vegetation (lomas)	Dry grasslands and shrubs	Dry and riparian vegetation	Dry grasslands and shrub lands

[1] Climate data specific to the microclimate of Lomas de Lachay which is also the site of capture of the low altitude population of *P. andium* (Chapter 2).
[2] Climate data general to Peruvian Highlands
[3] Does not apply to the low altitude population of *P. andium*
References: (Ordoñez and Faustino, 1983); Instituto Geofísico del Perú (www.igp.gob.pe); Servicio Nacional de Meteorología e Hidrología del Perú (SENAMHI; www.senamhi.gob.pe).

contains less seeds (12%) and more insects (41%) (Pizzimenti and De Salle, 1980). *P. limatus* (altitudinal range: 0-4000 m) is also an inhabitant of the Southern arid coast and Pacific slopes of the Andes (Steppan and Ramirez-Baca, In Press). It is commonly found in rocky outcrops, foggy lomas and riparian areas as well as in mixed habitats of cacti and weeds. Lastly, *P. andium* has been reported in a lomas ecosystem in central coastal Peru (Lomas de Lachay) at an altitude of 240-300 m, but it is usually found in the Northern and central highlands at altitudes of 1000 to 4100 m (Steppan and Ramirez-Baca, In Press). Its diet mostly consists of leafy vegetation (74%) with insects (22%) and few seeds (4%) (Pizzimenti and De Salle, 1980). The highlands of central and Southern Peru offer different habitats depending on the altitude and the seasons. Generally, highland *Phyllotis* species such as *P. andium* and *P. xanthopygus* share a number of habitats composed of rock formations, shrubs, grasslands, woody bushes and small forests that are dry from May to October and wet from November to April. *P. xanthopygus* is mostly found in the high Andes in central and southern Peru (altitudinal range 1900-5030 m) (Steppan and Ramirez-Baca, In Press). It is sympatric with *P. andium* in the central highlands and is usually replaced by *P. limatus* at lower elevations (around 2000 m) in the South.

Thesis goals

Owing to its low oxygen levels and temperatures, high altitude environments pose a physiological challenge for maintenance of daily activities and survival in mammals. As such, it is expected that high altitude native mammals show physiological

adaptations to these harsh environments. Limited information is available on the metabolic adaptations to high altitude that occur over evolutionary time. As pointed out in this chapter, most of the advancements have been limited to the O_2 transport pathway. Very little information is available on metabolic adaptations in fuel use and muscle metabolism. In fact, to date, no study has properly examined whole-body fuel use in high altitude mammals (including humans) and little is known on the metabolic organization of their cardiac and skeletal muscle tissues. Moreover, the majority of the high altitude adaptation literature presents inadequate control groups and no mammalian study has used the proper framework to study metabolic adaptations to high altitudes (Garland and Adolph, 1994), which requires the use of several closely related species native to different elevations, from which the influence of their phylogenetic relationships can be removed.

Thus, **(I)** the primary goal of this thesis was to infer on the metabolic adaptations to high altitude in mammals. Since carbohydrates offer an oxygen-saving advantage over fatty acids, I hypothesized that high altitude native mammals have evolved to use more carbohydrates when O_2 becomes limiting. To test this hypothesis, I investigated whole-body fuel use and muscle metabolism in four species of *Phyllotis* mice that are native to different elevations. Specifically, I determined:

1) whole-body fuel use at rest and during exercise under normoxic and hypoxic conditions;
2) performance measures such as aerobic capacity (VO_2max), endurance performance (time to fatigue), and running economy under normoxic and hypoxic conditions;

3) capacity for carbohydrate utilization and fatty acid oxidation in skeletal and cardiac muscles.

Since the effects of acclimation to high altitude on whole body fuel use have only been studied in lowland rats and humans, **(II)** the secondary goal of this thesis was to determine whether acclimation to high altitude also has little effects on whole-body fuel use and muscle metabolism in mice native to low altitude. Specifically, I acclimated CD1 mice (*Mus musculus*) to hypobaric hypoxia or normobaric normoxia for 6 weeks and then determined VO_2max and objectives 1 and 3 described above.

As patterns of fuel selection during exercise are conserved in mammals, **(III)** the third goal of this thesis was to assess whether mice follow the mammalian model of fuel selection. I thus compared patterns of fuel selection of mice (determined as part of goal II) to the ones of rats, goats and dogs published in the scientific literature.

Chapters' summary

Chapter 2 consists of an intraspecific comparison of fuel use and muscle metabolism of high and low altitude populations of *Phyllotis andium*. The results of this study provided the first account of enhanced whole-body use of carbohydrates during submaximal exercise in a high altitude mammal. I followed this study with a more powerful phylogenetic approach to infer on the adaptive nature of that trait. **Chapter 3** consist of an interspecific comparison of whole-body fuel use and muscle metabolism in four species of *Phyllotis* native to high and low altitudes. This study revealed that high

altitude species use more carbohydrates than their low altitude counterparts when oxygen becomes limiting, and that this difference is independent of phylogeny. Thus this chapter provides the first compelling evidence of metabolic adaptation in high altitude mammals.

Chapter 4 reinforces the notion that patterns of whole-body fuel use are unaffected by acclimation to hypobaric hypoxia and also reveals that mice do not follow the current mammalian model of fuel selection. In this chapter, I thus propose a new mammalian model of fuel selection which applies to all mammals. Finally, **chapter 5** provides an evaluation and discussion of the major findings of this thesis, as well as a description of future directions.

CHAPTER 2

INCREASED USE OF CARBOHYDRATES AT ALTITUDE IN ANDEAN LEAF-EARED MICE (*PHYLLOTIS ANDIUM)*

Abstract

For decades, it has been hypothesized that one of the adaptations to maintain performance at high altitude is a preference for carbohydrate oxidation rather than free fatty acid oxidation. The increased use of carbohydrates is thought to offer an oxygen saving advantage over fatty acids, as it theoretically leads to ~15-18% (observed difference 25-30%) more energy per oxygen used. If this represents an important adaptation to high altitude, it should be evident in different populations of the same species that inhabit distinct altitudinal ranges. We thus investigated whole-body fuel selection patterns and biochemical aspects of cardiac and skeletal muscle metabolism in mice native to different altitudes. We captured Andean leaf-eared mice (*Phyllotis andium*) from two geographically separated populations at 300 m and 4000 m in central Peru and measured, after 6 weeks in captivity at sea level, maximum oxygen consumption (VO_2max), time to fatigue, and fuel use at rest and at a submaximal intensity of exercise (75% of VO_2max) under both normoxic and hypoxic (12% O_2) conditions. As hypothesized, the 4000 m population of *P. andium* used proportionately more carbohydrates than the 300 m population when O_2 becomes limited. This preference for carbohydrates was also apparent in the heart, as left ventricles of the high altitude

population were characterized by a higher activity of hexokinase (HK). Skeletal muscles, on the other hand, showed higher activities of citrate synthase (CS), isocitrate dehydrogenase (IDH) and lactate dehydrogenase (LDH), suggesting that skeletal muscles of high altitude mice have an enhanced oxidative capacity and perhaps a greater lactate conversion potential. Our results also show that despite similar VO_2max values between the two populations, the high altitude population was able to run twice as long as the low altitude population. It is possible that the ability of highland mice to use O_2 more efficiently allows for a greater scope of activity in hypoxic environments.

Introduction

Due to its low oxygen levels and temperatures, high altitude represents one of the most hostile environments inhabited by mammals. For over 50 years, physiologists have attempted to uncover the mechanisms that allow high altitude mammals to survive and maintain adequate physical performance in these habitats. While most studies have focused on investigating the O_2 transport pathways, other potential adaptive mechanisms, such as changes in metabolism, have not been extensively researched. For instance, little is known on fuel use at altitude, either in resting or exercising highland animals. Carbohydrates are considered to be the fuel of choice in hypoxic environments (Brooks et al., 1991; Hochachka, 1985) because of its greater yield of ATP per O_2 compared to fatty acids. Theoretically, the difference is about 15-18% (Brand, 2005; Welch et al., 2007), but *in vivo* the observed difference in mammals is closer to 25-30% (Daut and Elzinga, 1989). Yet, only a few studies have investigated carbohydrate utilization in high altitude

native mammals (Hochachka et al., 1996; Hochachka et al., 1991; Holden et al., 1995). These suggest that Andean and Himalayan residents use more carbohydrates than lowlanders, however the validity of these results are uncertain as other factors could be responsible for this difference. In mammals, the proportion of carbohydrates and lipids oxidized during exercise depends on the intensity of exercise relative to the aerobic capacity of the animal (Brooks and Mercier, 1994; Felig and Wahren, 1975; McClelland, 2004; Romijn et al., 1993). As the relative intensity of exercise increases, the contribution of carbohydrates to fuel energy metabolism increases whereas the contribution of lipids decreases concurrently. Past studies have not adequately controlled for the effects of exercise intensity when comparing fuel use at altitude. Thus it is still unknown whether metabolism of high altitude mammals is altered to favour carbohydrate oxidation.

Little is known on the metabolic organization of skeletal and cardiac muscles of high altitude native mammals. A few studies have reported high oxidative capacities in cardiac muscles of high altitude pikas, llamas, alpacas and tarucas (Hochachka et al., 1983; Sheafor, 2003), but little information is available on capacities of enzymes particularly involved in carbohydrate and lipid oxidation. The strongest evidence for altered fuel use in a high altitude native mammal comes from a study using ^{31}P magnetic resonance spectroscopy where Hochachka and colleagues (Hochachka et al., 1996) found a low phosphocreatine / adenosine triphosphate (PCr/ATP) signature in the heart of Himalayan highlanders (Sherpas). These findings suggest a unique metabolic organization of the heart to preferentially use carbohydrates instead of fatty acids. It is thus possible that high altitude mammals have evolved an increased capacity for

carbohydrate oxidation in the heart to accommodate greater *in vivo* rates of carbohydrate utilization. However further research is clearly needed as knowledge on cardiac and skeletal muscle metabolism of high altitude mammals is very limited.

Intraspecific comparisons of low and high altitude populations have proven successful for the study of high altitude adaptation. Highland populations of humans (Tibetans) and North American deer mice (*Peromyscus maniculatus*) have both provided convincing evidence of ongoing natural selection on a number of physiological traits related to the O_2 transport pathway (Beall, 2007; Chappell et al., 1988; Storz, 2007). Yet, these species may only provide limited knowledge on the possible adaptations to high altitude. It appears that gene flow is quite substantial between lowland and highland populations of both humans (Salzano and Callegari-Jacques, 1988) and deer mice (Storz and Dubach, 2004). The influx of lowland genes into the highland populations could be such that only marginal phenotypic differences are likely to emerge. The Andean leaf-eared mouse (*Phyllotis andium*), on the other hand, seems to be an excellent model species for the study of adaptations to high altitudes. Native to the Peruvian Andes, these mice are abundant at elevations ranging from 1000 m and 4100 m, but an isolated population has also been found close to sea level (Steppan and Ramirez-Baca, In Press). Gene flow appears to be quite low between the high altitude populations and this low altitude population (Ramirez O., personal communication). They are thus well-suited to investigate adaptations to high altitude.

To uncover putative metabolic adaptations to high altitudes, we investigated whole-body fuel oxidation, aerobic capacity, endurance performance, and biochemical

aspects of cardiac and skeletal muscle metabolism in high (4000 m) and low (300 m) altitude populations of *P. andium*. As carbohydrate oxidation offers an oxygen-saving advantage over fatty acids, we hypothesized that one of the adaptations to high altitude hypoxia is an increased reliance on carbohydrates as a fuel source for energy metabolism. Our results show that high altitude *P. andium* use proportionally more carbohydrates than its low altitude counterpart when O_2 becomes limited, and that several metabolic adjustments at the muscle level may facilitate physiological function in hypoxic environments.

Methods

Study design

We captured male Andean leaf-eared mice (*Phyllotis andium*) from a low altitude population (300 m, N=10) and a high altitude population (4000 m, N=10) in the province of Lima, Peru using baited Sherman traps (study site location and characteristics outlined in Table 2.1). Upon capture, mice were immediately transported to sea level and kept under identical conditions for a minimum of 6 weeks before experimentation at the animal facility of the Universidad Peruana Cayetano Heredia (UPCH), in Lima, Peru. Animals were housed individually in rodent cages with water and rabbit pellets (Purina) provided *ad libitum*. Photoperiod and temperatures fluctuated with outdoor conditions

Table 2.1 Geographical and altitudinal details of capture sites and description of habitats of the two populations of *P. andium* under study.

Capture sites	Habitat	Altitude (m)
Reserva Nacional Lomas de Lachay 11°21'582''S; 77°22'034''W	Coastal Lomas (wet and dry dwarf forests), rocky areas.	300
Marcahuasi 11°78'928''S; 76°57'558''W	Shrub lands, dry and wet highlands, rocky areas.	4000

(12-13h daylight, 18-23 degrees Celsius). All procedures were approved by the McMaster University Animal Research Ethics Board, the Animal Ethics Committee of the UPCH as well as authorized by the Ministerio de Agricultura and the Instituto Nacional de Recursos Naturales (INRENA) of Peru.

Four weeks prior to the experimental measurements (2 weeks after capture), mice were familiarized to the treadmill-enclosed metabolic chamber for 10 minutes per week at speeds of 4-10 m min^{-1}. The experimental protocol commenced 6 weeks after arrival at sea level. (Figure 2.1). An open flow-through respirometry system (Sable Systems, Las Vegas, NV, USA) with a treadmill-enclosed metabolic chamber or a 600 ml resting chamber were used to measure rates of O_2 consumption (VO_2) and CO_2 production (VCO_2) of individual mice. All mice were subjected to the following *in vivo* experimental measurements under normoxic (normobaric, ~21% O_2) and hypoxic (normobaric but equivalent to 4000 m, ~12% O_2) conditions: (*i*) VO_2max test, followed by measurements of VO_2 and VCO_2 (*ii*) at rest and (*iii*) at an intensity of exercise corresponding to 75% of individual VO_2max, and (*iv*) time to fatigue at a speed of 12 m min^{-1}. These measurements were performed in a random order between noon and 7 pm following a 6-hour fast (except for the VO_2max test) and each mouse was never subjected to more than 1 measurement per day. Subsequently, muscle samples (left ventricle, gastrocnemius, thigh) were harvested, weighed, immediately crushed with pre-cooled aluminum tongs, frozen in liquid nitrogen, and then stored at -80 °C.

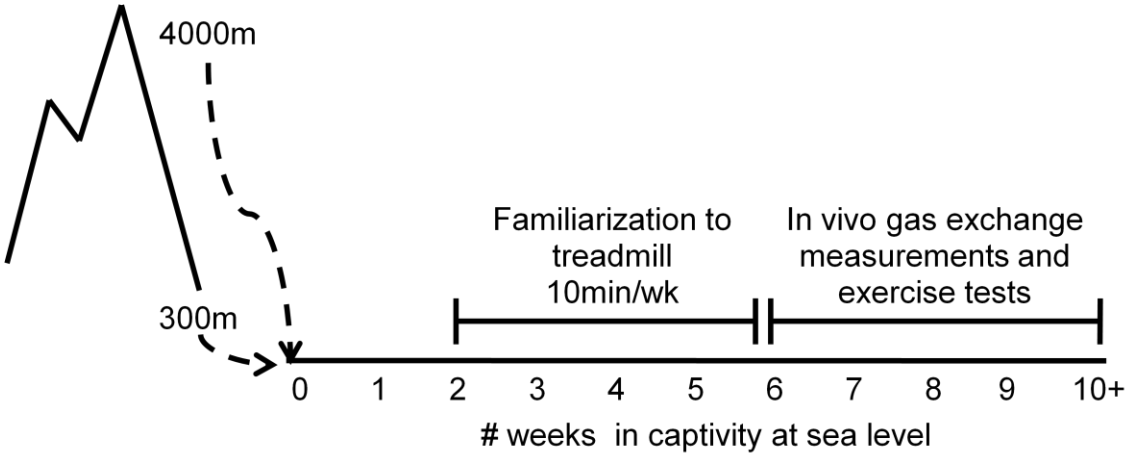

Figure 2.1 Study design: following capture, low altitude (300 m) and high altitude (4000 m) populations of *P. andium* were transported to the animal facility at sea level. Familiarization to treadmill and data collection commenced 2 and 6 weeks following capture, respectively.

Respirometry

Respiratory gases were measured using an open flow-through respirometry system and a rodent treadmill (Columbus Instruments, Columbus, OH, USA) modified to have an enclosed chamber of 21.5 cm long, 5 cm wide and 10 cm high (working section of ~800 ml) equipped with electric stimuli. Incurrent air from outside the building was scrubbed free of water and CO_2 using Drierite, soda lime, and Ascarite. This air was pushed into the metabolic chamber at a flow rate of 2000 ml min^{-1} using a MS5 mass-flow meter and pump (Sable Systems, Las Vegas, NV, USA). A subsample of air from the chamber was obtained at a rate of 200 ml min^{-1} and scrubbed free of water using pre-baked Drierite before entering the O_2 and CO_2 analyzer unit (Foxbox, Sable Systems, Las Vegas, NV, USA). The system was determined to be accurate to within ± 1% for RER (VCO_2 / VO_2) by burning methanol in the chamber. For measurements under hypoxic conditions, we used a compressed gas mixture of 12 % O_2 and balance N_2 as incoming air into the chamber. For resting measurements we used a 600 ml glass jar as a resting chamber with an incoming flow rate of 600 ml min^{-1} and a subsampling rate of 80 ml min^{-1}. Oxygen and carbon dioxide were measured at a rate of 1 sample $second^{-1}$ and recorded on a computer equipped with Expedata data acquisition software (Sable Systems, Las Vegas, NV, USA).

Exercise and resting protocols

Maximum oxygen consumption (VO_2max) was determined by increasing the speed of the treadmill by 3 m min^{-1} every 2 minutes from an initial speed of 4 m min^{-1}.

We determined when the mouse had reached VO_2max according to the following criteria: 1) VO_2 was unchanged as speed increased, 2) mouse could not keep position on the treadmill belt, 3) respiratory exchange ratio (RER) $\geq$ 1. Submaximal exercise intensities (75% of individual VO_2max) were determined from the relationship between VO_2 and speed during the VO_2max test but also by adjusting treadmill speed in real-time to maintain the targeted relative exercise intensity for a period of 20 minutes. We used data from at least 5 minutes of recordings between the 5^{th} and 20^{th} minute of exercise to calculate group means. Recordings which showed important O_2 drift or in which animals refused to run on the treadmill were removed prior to data analysis. Consequently, sample size for each group varied between 7-10 animals. The treadmill had an inclination of 10° for all exercise protocols. Resting rates of VO_2 and VCO_2 were recorded for at least 45 minutes in the resting chamber described above. We used recordings of at least 5 minutes, which corresponded to the lowest VO_2 achieved through the 45 minute period.

We also subjected mice to a time to fatigue test under both normoxic and hypoxic conditions. We allowed mice to rest for 5 min (normoxic condition) or 10 min (hypoxic condition) in the treadmill-enclosed chamber and then gradually increased the treadmill speed (within 2 min) to a final speed of 12 m min^{-1}. Time to fatigue corresponded to the time at which mice showed initial signs of exhaustion (mouse could no longer keep position on the treadmill).

Muscle enzymatic capacities (Vmax)

Muscles were powdered using a liquid N_2-cooled mortar and pestle and homogenized on ice with a glass on glass homogenizer for 1 minute in 20 volumes of homogenizing buffer consisting of 100 mmol l^{-1} potassium phosphate (pH 7.2), 5 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), and 0.1% Triton X-100. All enzymes were measured at 37°C in a Spectromax Plus 384, 96-well microplate reader (Molecular Devices, Sunnyvale, CA, USA). Assays were done in triplicate and control rates (no substrate added) were determined for each assay.

Activities of cytochrome c oxidase (COx), phosphofructokinase (PFK), β-hydroxyacyl-CoA dehydrogenase (HOAD), isocitrate dehydrogenase (IDH), and hexokinase (HK) were measured on fresh homogenates; whereas activities of pyruvate kinase (PK), lactate dehydrogenase (LDH), and citrate synthase (CS) were measured after being frozen and thawed once, twice and three times, respectively. Assay conditions were as follows (in mmol l^{-1}): COx: 100 potassium phosphate (pH 7.0), and 0.1 cytochrome c reduced; PFK: 10 fructose-6-phosphate (F6P) (omitted in control), 4 ATP, 0.28 NADH, 2 AMP, 5 $MgCl_2$, 50 KCl, 5 dithiothreitol (DTT), 1 U aldolase, 50 U triose phosphate isomerase and 8 U α-glycerophosphate dehydrogenase, in 50 triethanolamine (TEA)-HCl (pH 7.6); HOAD: 0.1 acetoacetyl-CoA (omitted in control), 0.28 NADH, and 5 EDTA, in 100 TEA-HCl (pH 7.0); IDH: 1.5 isocitrate (omitted in control), 2 ADP, 0.5 NADP, 1 $MnCl_2$, and 8 MgCl2, in 40 Tris-HCl (pH 7.4); HK: 5 D-glucose (omitted in control), 8 ATP, 8 $MgCl_2$, 0.5 NADP, and 4U glucose-6-phosphate dehydrogenase, in 50 Hepes (pH

7.6); PK: 5 phosphoenol pyruvate (PEP; omitted in control), 5 ADP, 10 $MgCl_2$, 0.15 NADH, 10 fructose-1,6-phosphate, 100 KCl, and 9.25 U lactate dehydrogenase (LDH), in 50 imidazole (pH 7.4); LDH: 1 Pyruvate-Na (omitted in control), and 0.28 NADH, in 40 Tris-HCl (pH 7.4); CS: 0.5 oxaloacetate (omitted in control), 0.22 acetyl-CoA, and 0.1 dithiobisnitrobenzoic acid (DTNB) in 40 Tris-HCl (pH 8.0).

Calculations and Statistical analyses

Rates of O_2 consumption (VO_2) and CO_2 production (VCO_2) were calculated using the equations of Withers (Withers, 1977):

$$VO_2 = [FR \bullet (FiO_2 - FeO_2) - VCO_2 \bullet FiO_2] / (1 - FiO_2) \quad (1)$$

$$VCO_2 = FR \bullet FeCO_2 \quad (2)$$

where FR is the flow rate (ml min^{-1}), Fi is the fractional gas concentration of incurrent air, and Fe is the fractional gas concentration of excurrent air. Carbohydrate and lipid oxidation rates were calculated using the following equation based on Frayn (Frayn, 1983),

$$\text{Carbohydrate oxidation (g min}^{-1}) = 4.55\ VCO_2 - 3.21\ VO_2 \quad (3)$$

$$\text{Lipid oxidation (g min}^{-1}) = 1.67\ VO_2 - 1.67\ VCO_2 \quad (4)$$

assuming that the contribution of protein to overall energy expenditure is negligible during exercise in post-absorptive state (Carraro et al., 1994; Rennie et al., 1981). Values were then converted to µmol of O_2 per minute based on the molar equivalent of gas (1mol O_2 = 22.4 L) and the volume of O_2 consumed for the oxidation of carbohydrates (0.746 L g^{-1}) and lipids (2.03 L g^{-1}).

Comparisons between the low and high altitude populations were performed with t-tests, Mann-Whitney U tests (when data were abnormally distributed and/or showed unequal variances) and two-way analysis of covariance (ANCOVA) with Sidak pairwise comparisons when appropriate, using SPSS (SPSS Inc., Chicago, IL, USA) or the integrated Systat software within Sigmaplot 11.0 (Systat software Inc., San Jose, CA, USA). We also used Pearson correlations to test for positive correlation between exercise intensity and proportion of carbohydrate used. Significance level was set to 0.05. Data are reported as means ± s.e.m, unless otherwise stated. We used one-tail t-tests to test the hypothesis of higher carbohydrate use in the high altitude population.

Results

The low (300 m) and high (4000 m) altitude populations of *P. andium* had comparable body mass (Table 2.2). Moreover, the two populations showed similar aerobic capacities (VO_2max), aerobic scopes and resting metabolic rates (VO_2) under both normoxic and hypoxic conditions (Table 2.2). VO_2max was 16% and 15% lower under hypoxia (compared to normoxic VO_2max) in the low and high altitude populations respectively (two-tail t-tests; 300 m population: t_{18}=3.5, P=0.003; 4000 m population: t_{18}=2.8, P=0.01). At rest, both populations showed similar respiratory exchange ratio (RER) under normoxic conditions (Figure 2.2a; one-tail t-test: t_{18}=0.06, P=0.5), however under hypoxic conditions, the high altitude population had a significantly higher RER than the low altitude population (Figure 2.2b; one-tail t-test: t_{17}=-3.2, P=0.003).

Table 2.2 : Means ± s.e.m. of body mass and respiratory data of the 300 m and 4000 m populations of *P. andium* under normoxic and hypoxic conditions, together with statistical results of comparisons (two-tail t-tests unless otherwise stated).

	Altitude		Statistics
	300 m	*4000 m*	
Body mass (g)	46.7±2.1	45.8±3.4	t_{18}=0.23, *P*=0.8
VO_2max ($ml \cdot min^{-1}$)			
Normoxia	4.57 ±0.13	4.65±0.17	t_{18}=-0.38, *P*=0.7
Hypoxia	3.85±0.09	3.96±0.17	t_{18}=-0.55, *P*=0.6
Aerobic scope ($ml \cdot min^{-1}$)			
Normoxia	3.16±0.12	3.41±0.16	t_{18}=-1.21, *P*=0.2
Hypoxia	2.54±0.12	2.70±0.15	t_{18}=-0.79, *P*=0.4
Resting VO_2 ($ml \cdot min^{-1}$)			
Normoxia	1.41±0.12	1.25±0.09	t_{18}=1.11, *P*=0.3
Hypoxia [a]	1.31±0.09	1.26±0.05	*U*=40, *P*=0.7

[a] Mann-Whitney test

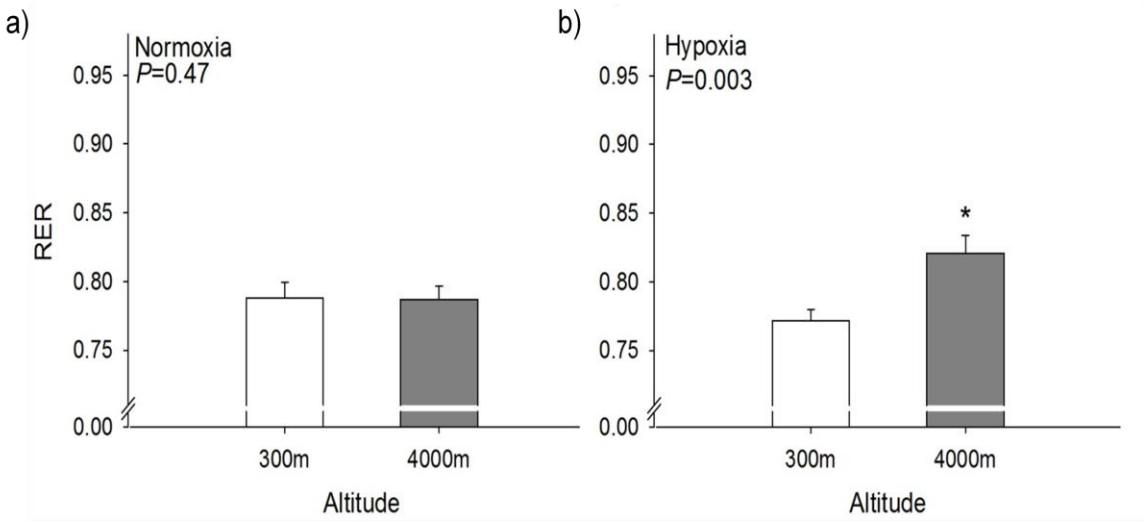

Figure 2.2 Mean + s.e.m. resting respiratory exchange ratio (RER) of the 300 m and 4000 m populations of *P. andium* under (a) normoxic and (b) hypoxic conditions. An asterisk (*) denotes a significant difference between populations, p-values of one-tail t-tests are shown.

When exercised at a target intensity of 75% of their VO_2max under normoxic conditions, the high altitude mice showed a 17% greater reliance on carbohydrates to power locomotion than the low altitude mice, but the difference was not statistically significant (Figure 2.3a; one-tail t-test: t_{17}=-1.6, P=0.062). The power of this particular test (with α=0.05) equalled 0.213, which is below the desired power of 0.8. On an absolute basis, the rate of carbohydrate oxidation was significantly higher in high altitude mice (Table 2.3). The actual exercise intensities relative to VO_2max (% VO_2max) at which mice performed this test were similar to the target intensity (75%) and not different between the two populations (Table 2.3). On the other hand, the actual exercise intensities relative to aerobic scope (% aerobic scope) differed between populations (Table 2.3), but were not correlated to the proportion of carbohydrates used (Pearson correlation: r=0.165, P=0.5).

Under hypoxic conditions, there was a significant difference in the proportion of carbohydrates used between the two populations when exercised at a target intensity of 75% of VO_2max, as the high altitude mice actually used a 46% greater proportion of carbohydrates than the low altitude mice (Figure 2.3b; one-tail t-test: t_{14}=-2.0, P=0.035). On an absolute basis, the high altitude population oxidized carbohydrates and lipids at a higher and lower rate, respectively, than the low altitude population (Table 2.3). The actual exercise intensities relative to VO_2max at which mice performed the exercise test were similar to the target intensities (75%), and both actual relative exercise intensities expressed as % VO_2max and % aerobic scope did not differ between the two populations (Table 2.3).

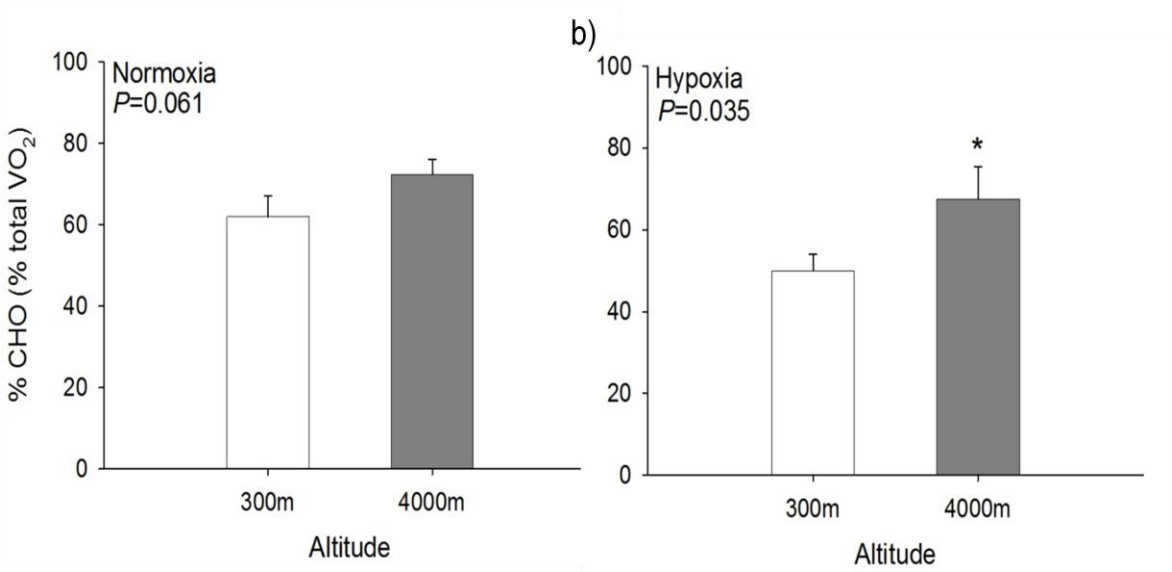

Figure 2.3 Mean + s.e.m. percent of total VO_2 used for carbohydrate oxidation during exercise at 75% of VO_2max under a) normoxic and b) hypoxic conditions in the 300 m (open bars) and 4000 m (solid bars) populations of *P. andium*. An asterisk (*) denotes a significant difference between populations, p-values of one-tail t-tests are shown.

Table 2.3 Means ± s.e.m. of respiratory exchange ratio (RER) and absolute rates of fuel selection in the 300 m and 4000 m populations of *P. andium* during treadmill running at a target intensity of 75% of VO_2max under normoxic and hypoxic conditions, together with statistical results of comparisons (one-tail t-tests) and actual exercise intensities and treadmill speeds. Significant p-values of (P<0.05) are emphasized in bold.

	Altitude		Statistics
	300 m	*4000 m*	
RER			
Normoxia	0.888±0.015	0.919±0.010	t_{17}=-1.6, P=0.062
Hypoxia	0.853±0.012	0.904±0.023	t_{14}=-2.0, ***P*=0.035**
Absolute rate of CHO oxidation (µmol O_2 min^{-1})			
Normoxia	87±7	116±9	t_{17}=-2.7, ***P*=0.008**
Hypoxia	62±5	89±14	t_{14}=-1.8, ***P*=0.045**
Absolute rate of lipid oxidation (µmol O_2 min^{-1})			
Normoxia	54±8	43±5	t_{17}=-1.1, P=0.3
Hypoxia	63±7	40±10	t_{14}=1.8, ***P*=0.047**
Exercise intensity (% VO_2max)			
Normoxia	75.5±0.9	76.1± 0.6	t_{17}=-0.6, P=0.6
Hypoxia	75.9± 2.1	72.1±2.0	t_{14}=1.3, P=0.2
Exercise intensity (% aerobic scope)			
Normoxia	55.4±1.9	68.2±2.1	t_{17}=-4.5, ***P*<0.001**
Hypoxia	58.0±4.4	57.3±3.0	t_{14}=-0.1, P=0.9
Treadmill speed ($m \cdot min^{-1}$)			
Normoxia	5.9±0.5	11.1±0.9	t_{17}=-5.1, ***P*<0.001**
Hypoxia	4.5±0.5	7.3±0.9	t_{14}=-2.6, ***P*=0.023**

Comparisons of total O_2 consumption (VO_2) at each speed of the VO_2max protocol revealed that the high altitude population generally used less oxygen for the same amount of work (Figure 2.4). This trend was significant under normoxic conditions but not under hypoxic conditions (two-way ANCOVA: normoxia: $F_{1,82}$=16.46, P<0.001; hypoxia: $F_{1,70}$=3.26, P=0.075). When subjected to an endurance test at a speed of 12 m min^{-1}, the high altitude population was able to run over twice as long as the low altitude population under both normoxic and hypoxic conditions (Figure 2.5; Mann-Whitney U tests: normoxia: U=16.5, P=0.013; hypoxia: U=13.0, P=0.006).

At the muscle level, the majority of the enzymes investigated had similar activities in the two populations; however the high altitude mice showed higher activities of a number of enzymes involved in carbohydrate metabolism (Figure 2.6). Compared to the low altitude population, the high altitude population had higher activities of mitochondrial enzymes CS (two-tail t-test; t_{18}=-2.8, P=0.012) and IDH (two-tail t-test; t_{18}=-2.1, P=0.048) in the skeletal muscle gastrocnemius and higher activities of LDH in the thigh (Mann-Whitney U tests; U=9.0, P=0.013). Moreover, in the left ventricle, the high altitude population showed higher activities of HK (two-tail t-test; t_{18}=-2.6, P=0.018). Heart weights were similar between the two populations (300 m population: 143 ± 6 mg; 4000 m population: 154 ±13 mg; Mann-Whitney U test: U=44, P=0.7), even when expressed relative to body mass (two-tail t-test: t_{18}=0.05, P=0.9). Similarly, left and right ventricle weights were similar between populations (left ventricle: Mann-Whitney U test: U=45, P=0.7; right ventricle: two-tail t-test: t_{18}=0.08, P=0.9).

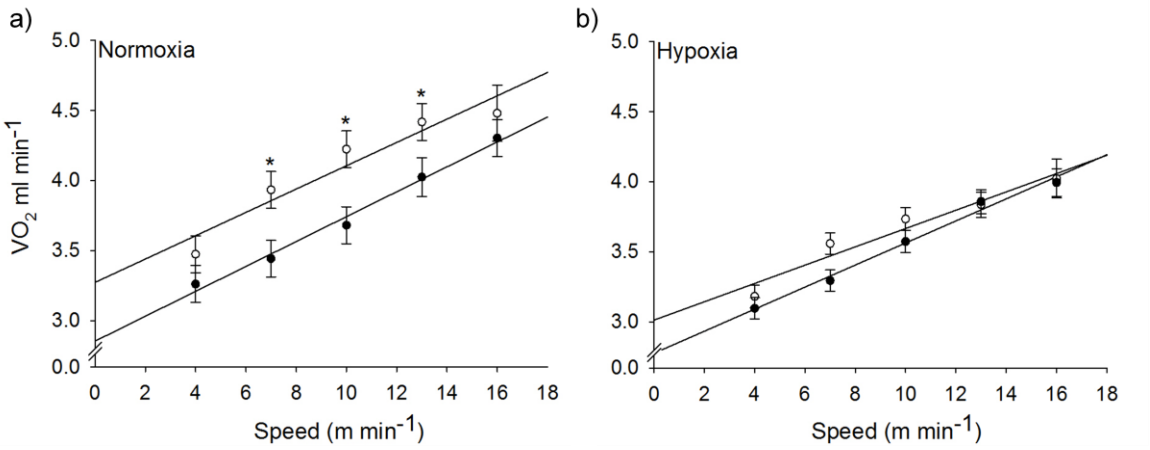

Figure 2.4 Estimated marginal mean VO_2 ± s.e.m. at each speed of the VO_2max protocol under a) normoxic and b) hypoxic conditions in the 300 m (open circles) and 4000 m (solid circles) populations of *P. andium*. An asterisk (*) denotes a significant difference between populations (Sidak pairwise comparisons following a two-way ANCOVA with body mass as covariate, $P<0.05$).

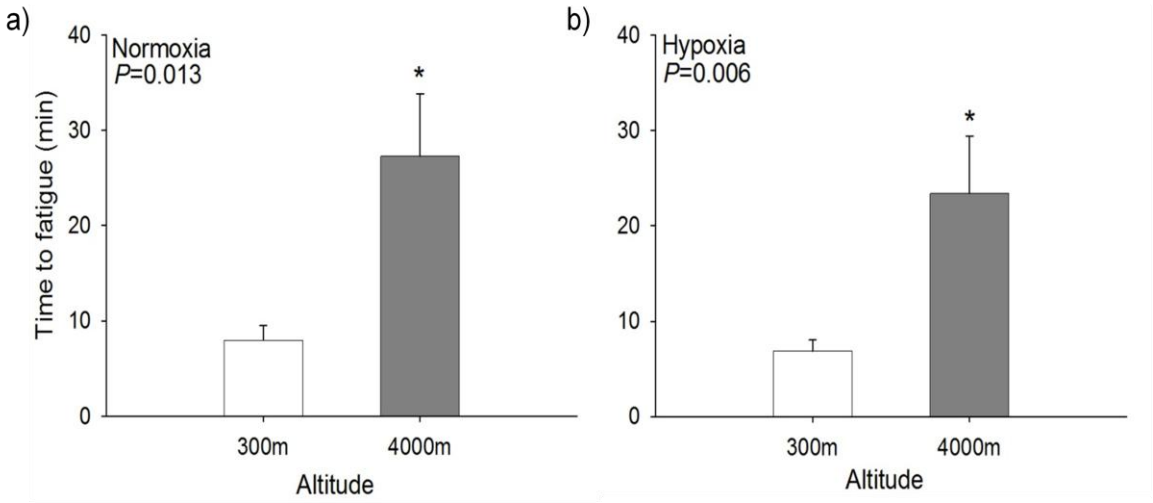

Figure 2.5 Mean + s.e.m. time to fatigue to treadmill running at 12 m min^{-1} under a) normoxic and b) hypoxic conditions in the 300 m (open bars) and 4000 m (dark bars) populations of *P. andium*. An asterisk (*) denotes a significant difference between populations, p-values of Mann-Whitney U tests are shown.

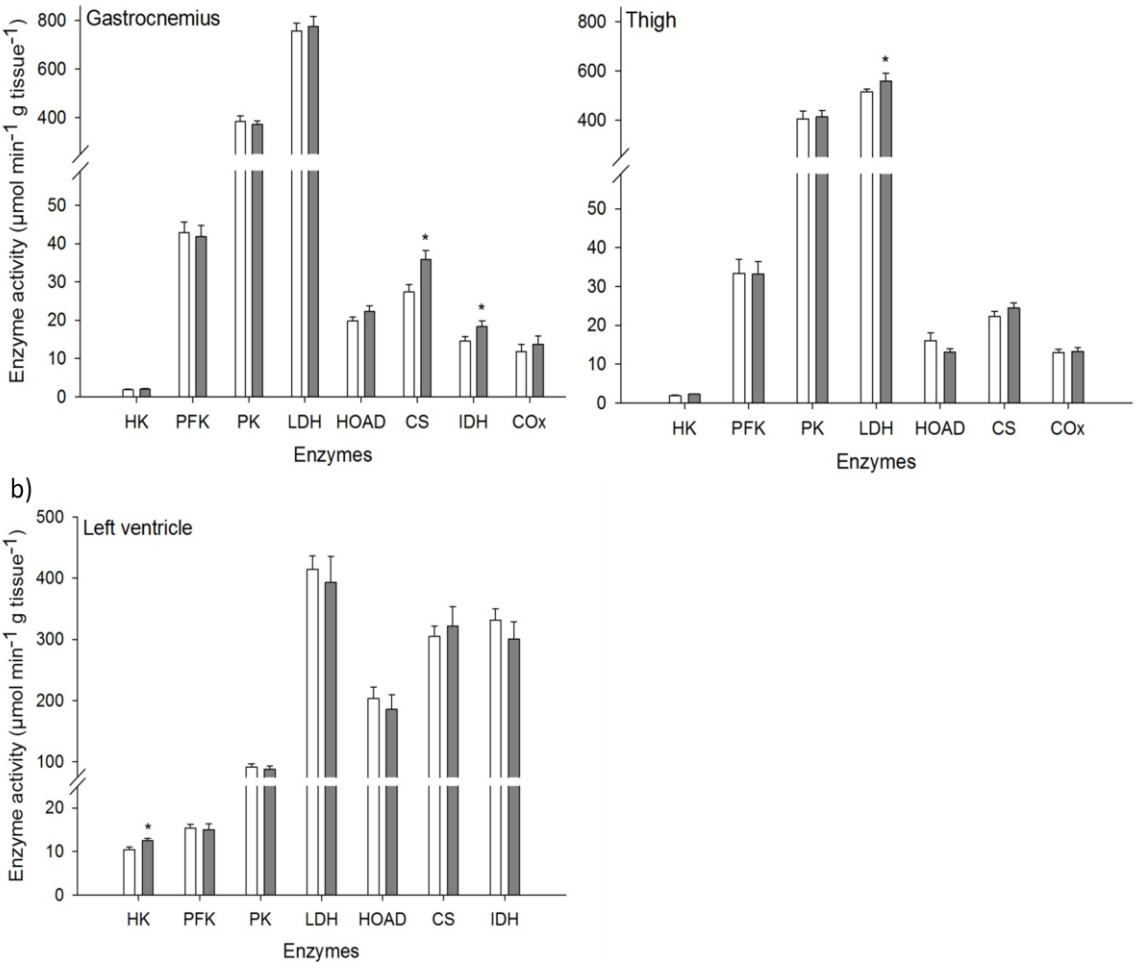

Figure 2.6 Mean + s.e.m. activities (Vmax) of hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), lactate dehydrogenase (LDH), β-hydroxyacyl-CoA dehydrogenase (HOAD), citrate synthase (CS), isocitrate dehydrogenase (IDH), and/or cytochrome c oxidase (COx) in (a) skeletal muscles (gastrocnemius and thigh) and (b) left ventricles of the 300 m (open bars) and 4000 m (dark bars) populations of *P. andium*. Asterisks (*) denote significant differences (two-tail t-tests or Mann-Whitney U tests; $P<0.05$).

Discussion

This study consists of a powerful intraspecific comparison to infer on the metabolic adaptations to high altitude. Compared to its low altitude counterpart, the high altitude population of *P. andium* used proportionally more carbohydrates during exercise, especially under hypoxic conditions. Interestingly, this elevated use of carbohydrates did not compromise endurance performance, as the high altitude population was able to run more than twice as long as the low altitude population. Proper function of metabolic processes in hypoxic environments may have been facilitated by adjustments at the muscle level. In skeletal muscles, greater oxidative capacities may facilitate oxygen utilization in situations of low PO_2, and a high LDH activity in tissues that are mostly glycolytic may accelerate lactate reconversion and allow a prompt recovery following an anaerobic bout of exercise. In cardiac muscles, the higher HK activities suggest enhanced circulatory glucose utilization by the heart, providing a more efficient use of O_2 in this continuously active organ.

Carbohydrates at altitude

Many mammals follow a predictable pattern of fuel use as exercise intensity increases towards an aerobic maximum (McClelland, 2004; Roberts et al., 1996c). However, the oxygen-saving advantage of increasing the proportion of carbohydrate used at any given intensity of exercise is likely beneficial for mammals in situations where O_2 is scarce. At an altitude of 4000 m, every breath of air contains approximately 40% less oxygen than at sea level. This level of hypoxia poses a physiological challenge for most

sea level mammals and usually compromises performance, survival and thus fitness. It is thus expected that any strategy to maximize uptake, delivery, and utilization of oxygen would be used by mammals inhabiting these environments. We believe that the preference for carbohydrates, observed here in high altitude *P. andium* mice when O_2 is limited, represents one such adaptive strategy as it allows for a more efficient utilization of oxygen. Prior studies on fuel use at altitude suggest that this strategy is used by humans permanently residing at high altitudes (Hochachka et al., 1996; Hochachka et al., 1991; Holden et al., 1995). However, these studies were tissue specific or did not adequately take into account the confounding influence of exercise intensity into their experimental design. It is thus questionable whether this elevated carbohydrate utilization exists at the whole-body level and if so, whether it results from differences in relative exercise intensities (exercising at a different % of VO_2max) or from altered fuel selection mechanisms. Since we carefully removed the influence of relative exercise intensity into our comparisons of fuel use during exercise in both populations of *P. andium*, we believe that the greater proportional use of carbohydrates in the high altitude population results from a mechanistic difference in fuel use. This alteration in fuel selection may have arisen as an adaptation to high altitude hypoxia as it allows a more efficient utilization of O_2 during exercise in low O_2 environments. In fact, comparisons of VO_2 at different treadmill speeds (absolute exercise intensity) suggest that high altitude mice use less oxygen for the same amount of work (Figure 2.4). Theoretically, there are several ways in which an animal could increase O_2 to work efficiency, but the increase use of carbohydrates observed here is likely to contribute at least in part to this difference in

VO_2. This oxygen-saving advantage at altitude would clearly be beneficial, as it would allow a greater scope of activity. Energy demanding tasks important for survival such as territorial defence, foraging, predator avoidance and thermogenesis (a capacity especially important at altitude as temperatures plummet with increasing altitude) can thus be adequately performed.

Performance

Although advantageous in low O_2 environments, using carbohydrates at a higher rate can also have its drawbacks. Stored as glycogen, carbohydrates constitute less than 5% of the total energy reserves available in a whole organism (Weber, 2011). It is widely accepted that depletion of this limited resource is correlated with reduced endurance performance (Fitts, 1994). Thus using carbohydrates at a high rate would quickly deplete carbohydrate reserves and consequently shorten time to fatigue. Interestingly, the high altitude population of *P. andium* fatigued later than its low altitude counterpart under both normoxic and hypoxic environments. Under hypoxic conditions, it is not unforeseen that high altitude mice would perform longer than the low altitude mice. The disadvantage of depleting carbohydrates at a higher rate may be offset by their ability to use the limited levels of O_2 efficiently. Thus, in this case, the low altitude mice may be suffering from hypoxia-related cellular disturbances causing fatigue before the high altitude mice exhaust their glycogen stores. On the other hand, under normoxic conditions, the greater endurance performance of the high altitude mice is counterintuitive. Yet, it is possible that by having a slightly higher VO_2max (although not significantly different), the high

altitude mice exercised at a lower % of VO_2max than the low altitude mice during the time to fatigue test, and accordingly were able to run significantly longer. Alternatively, it is also possible that the high altitude mice have enhanced work economy or a greater ability to store glycogen than the low altitude mice. Further research in this area is warranted.

Muscle enzymatic capacity

We measured the activity of several enzymes involved in carbohydrate and lipid oxidation as well as LDH, an indicator of pyruvate - lactate exchange. Enzyme activities or maximum enzymatic flux capacities (Vmax) represent the upper limits of flux at a particular step of a biochemical pathway *in vitro.* Although it does not provide insights on flux *in vivo*, it does offer a measure of the maximum flux rate that can be achieved at a particular step of a metabolic pathway. Since carbohydrates are a more efficient fuel at altitude, we hypothesized that the high altitude population of *P. andium* would have a higher capacity for carbohydrate oxidation than the low altitude population. In the skeletal muscles investigated (gastrocnemius and thigh), the two population showed similar activities of enzymes involved in aerobic glycolysis. Accordingly, there are no indications that the capacity for carbohydrate oxidation is enhanced over lipid oxidation in skeletal muscles. However, the high activities of CS and IDH in the gastronemius suggest that muscle oxidative capacity is elevated in highland mice. The benefits of having high muscle oxidative capacities at altitude are unclear. In fact, previous studies do not show positive associations between altitude and oxidative capacities of skeletal

muscles in high and low altitude native mammals (Hochachka, 1992; Kayser et al., 1991; Sheafor, 2003) and acclimatization or acclimation to high altitudes in lowland animals usually induces either decreases (Green et al., 1989; Hoppeler et al., 1990; Howald et al., 1990) or no changes in activities of oxidative enzymes (Beaudry and McClelland, 2010; Kennedy et al., 2001; Le Moine et al., 2011; Perhonen et al., 1996). Still, it has been suggested that an enhanced oxidative capacity may be useful at altitude as it could prevent the inhibitory effect of intracellular hypoxia on cellular respiration, and thus allowing maintenance of oxidative metabolism in situations of low intracellular PO_2 (Hochachka, 1985). Moreover, a high oxidative capacity may be essential for small mammals inhabiting cold highlands, as the metabolic requirements for thermogenesis are usually greater than at low altitudes.

Skeletal muscles that are considered to be more glycolytic, on the other hand, do not have an enhanced oxidative capacity in high altitude mice, but instead show a greater capacity for anaerobic metabolism. The thigh encompasses a number of skeletal muscles, but as a whole, it is considered to contain more glycolytic fibres than the gastrocnemius (Delp and Duan, 1996). Both populations showed similar activities of all enzymes investigated in the thigh except for LDH, whose activity was higher in the high altitude population. LDH is an equilibrium enzyme catalyzing the pyruvate to lactate exchange, and its activity is usually perceived as an indicator of the anaerobic capacity of a tissue. Since the by-products of anaerobic metabolism can induce a decrease in cell function through pH imbalances (Hochachka and Mommsen, 1983), anaerobic glycolysis is thought to be downregulated in situations of chronic as opposed to acute hypoxia

(Baldwin et al., 1973; Holloszy, 1975; Le Moine et al., 2011). In fact, a number of studies have reported reductions in anaerobic capacity in high altitude mammals (Hochachka et al., 1991; Hochachka et al., 1983; Mensen de Silva and Cazorla, 1973). A high anaerobic capacity in glycolytic fibres is particularly important for burst activities such as predator avoidance. However, there are no clear reasons why high altitude mice would need a greater anaerobic capacity at altitude as burst performance is not affected by oxygen availability (Coudert, 1992), and predatory pressure is probably similar in both low and high altitude environments (Ramirez, O., personal communication). Yet, it is possible that the greater LDH activity in high altitude mice is the result of a higher activity of H-LDH, an isozyme which preferentially converts lactate to pyruvate for oxidation. In glycolytic tissues, a high H-LDH activity would facilitate lactate reconversion after an anaerobic bout and thus accelerate recovery. It is thus possible that the high LDH activity in the high altitude population is an adaptation to hypoxic environments as it could shorten recovery time following a burst activity. In fact, a study comparing different species of pikas across an altitudinal gradient also reported high LDH activities as well as high H-LDH activities in the high altitude native species (Sheafor, 2003), providing further support for the idea that enhanced lactate reconversion capacity is adaptive in high altitude native mammals.

In highly oxidative muscles which are continuously active, such as the heart, a more efficient use of O_2 may be essential in situations of chronic hypoxia. Our results show that the high altitude population of *P. andium* has a higher activity of HK, an enzyme involved in the first step of glycolysis, converting circulatory glucose to glucose-

6-phosphate. High altitude mice may thus have an enhanced capacity for carbohydrate utilization in the heart. This greater capacity for carbohydrate oxidation may have evolved to accommodate greater *in vivo* rates of carbohydrate oxidation, allowing a more efficient use of O_2 due to the oxygen-saving advantage of carbohydrate oxidation over fatty acid oxidation (Brand, 2005). This is consistent with a previous study on high altitude human natives from the Himalayas (Sherpas) who were found to have a low PCr/ATP signature in their heart, a state suggestive of a greater carbohydrate utilization (Hochachka et al., 1996).

Limitations and perspectives

The elevated whole-body use of carbohydrates in the high altitude population of *P. andium* represents a putative adaptation to high altitude hypoxia. In this study, all mice were allowed to acclimatize to similar sea level conditions for a period of 6 weeks before any experimental measurement was taken. This particular amount of time is sufficient to reverse some of the effects of acclimatization to high altitude in sea level natives (Grassi et al., 1996). Moreover, acclimation to high altitude in lowland mice, rats and humans was found to have no effect on whole-body fuel selection when the effect of relative exercise intensity is taken into account (Lundby and Van Hall, 2002; McClelland et al., 1998)(Chapter 4). It is thus unlikely that the elevated use of carbohydrates in highland *P. andium* mice is an effect of acclimatization to high altitudes. The effects of development at altitude, on the other hand, were not accounted for by our experimental protocol. Yet, the same protocol was followed in high (3700 m) and low (sea level) altitude native

populations of *Phyllotis limatus*, a species closely-related to *P. andium*, and no significant differences were found in whole-body use of carbohydrates between the two populations (Appendix A). Development at altitude thus seems to have no effect on the proportion of carbohydrates used. *P. limatus* is abundant in the south of Peru at altitudes ranging from sea level to 4000 m (Steppan and Ramirez-Baca, In Press). Unlike the high and low altitude populations of *P. andium*, the investigated populations of *P. limatus* were found to share haplotypes (data not shown), suggesting a recent gene flow between them. Thus an occupation of habitats at moderate (below 4000 m) rather than high altitudes (above 4000 m) and substantial gene flow between populations may in part explain why the high altitude population of *P. limatus* did not evolve to use more carbohydrates.

Conclusion

This study provides the first evidence of population-level differences in fuel use and muscle metabolism in response to high altitude. By removing the confounding influence of relative exercise intensity on fuel use, this study represents the first compelling intraspecific report of elevated whole body use of carbohydrates in a high altitude native mammal. Our results show that high altitude *P. andium* use proportionally more carbohydrates than its low altitude counterpart when O_2 becomes limited, and that this preference for carbohydrates may be facilitated by a greater capacity for circulatory glucose utilization in the heart and by an enhanced oxidative capacity of some skeletal muscles. Physiological flux rates through metabolic pathways and its regulatory

components need to be investigated to elucidate the mechanisms responsible for this shift in fuel use.

CHAPTER 3

INCREASED CARBOHYDRATE USE IN HIGH ALTITUDE NATIVE MAMMALS: A 30-YEAR-OLD HYPOTHESIS FINDS SUPPORT IN ANDEAN MICE (*PHYLLOTIS*)

Abstract

One of the hypothesized adaptations to maintain performance at high altitude is the preferred use of carbohydrates as a fuel source for energy metabolism because of its oxygen-saving advantage (15-30% more ATP per mole of O_2) over fatty acids. To test this hypothesis, we measured whole-body fuel selection patterns and biochemical aspects of cardiac and skeletal muscle metabolism in four closely related species of Andean mice (*Phyllotis*) native to high (4000-4500 m) and low (100-300 m) altitudes. More specifically, we measured aerobic capacities, time to fatigue and fuel selection at rest and at an intensity of exercise corresponding to 75% of VO_2max under both normoxic and hypoxic (12% O_2) conditions. Taking into account the phylogenetic relationships of the species studied, our results show an increased use of carbohydrates at submaximal relative exercise intensities in the high altitude *Phyllotis* species compared to their low altitude counterparts, providing the first compelling evidence of carbohydrate preference as an adaptation to high altitude. This increased whole-body preference for carbohydrates was not accompanied by an enhanced capacity for carbohydrate oxidation in skeletal muscles, however high oxidative capacities in the heart may be contributing to sustain

high cardiac outputs, thus maintaining oxygen delivery to working skeletal muscles during exercise.

Introduction

Adaptation to high altitude has fascinated physiologists for over 130 years, since Paul Bert (1833-1886) proved that the deleterious effects on animals were the result of low partial pressures of oxygen (West, 1998); but metabolic adaptations to hypoxia have been difficult to demonstrate. The low atmospheric oxygen levels at high altitude constitute a potent and unavoidable physiological stressor from which high altitude mammals are expected to adapt. One of the hypothesized adaptations to maintain performance at altitude is an increased use of carbohydrates as a fuel source for energy metabolism (Brooks et al., 1991; Hochachka, 1985). Using a greater proportion of carbohydrates is thought to be beneficial at altitude as its oxidation offers 15-18% more ATP per O_2 used than the oxidation of fatty acids (Brand, 2005; Welch et al., 2007). This is a stoichiometric difference, the actual difference being possibly greater *in vivo* (~25-30%, (Daut and Elzinga, 1989; Korvald et al., 2000). The study of fuel use in mammals is rather complex because absolute flux and relative proportion (mix) of fuels are influenced by the intensity of exercise performed relative to the aerobic capacity of the subject (Brooks and Mercier, 1994; McClelland, 2004; Roberts et al., 1996c; Weber and Haman, 2004). As the relative intensity of exercise increases (subject exercising at work rates closer to their aerobic capacity) the contribution of carbohydrates toward energy metabolism increases whereas the contribution of fatty acids decreases concurrently. A

few studies have looked at fuel use in high altitude native mammals (Hochachka et al., 1996; Hochachka et al., 1991; Holden et al., 1995). However, these studies have not properly accounted for the effects of relative exercise intensities on fuel use to address the questions of potential metabolic adaptations. Consequently, past reports of increased use of carbohydrates in high altitude native human populations compared to lowland dwellers (Hochachka et al., 1996; Hochachka et al., 1991; Holden et al., 1995) may be explained by differences in relative intensities of exercise rather than by modifications in fuel use regulation per se. Moreover, to infer genetic adaptation one must compare many species and also account for their phylogenetic relationships. In fact, very few studies have used the proper framework to adequately infer on the adaptive nature of a particular physiological trait to high altitudes (Scott et al., 2009a; Scott et al., 2009b; Scott et al., 2011). The majority of the literature on mammalian adaptations to high altitudes is characterized by distantly related species, inadequate control groups, confounding effects of phenotypic plasticity, and / or inappropriate two-species comparisons rather than a multi-species phylogenetic approach (Garland and Adolph, 1994). To properly address this need we performed a multispecies comparison using rodents living in hypoxic environments and compared them at similar relative exercise intensities.

Species of wild mice from the genus *Phyllotis* represent a unique system to study high altitude adaptations as this genus comprises several ecologically abundant, closely-related species that occupy distinct altitudinal ranges in the Andes (Pearson, 1958; Steppan, 1998). In addition, the fossil record suggests the presence of *Phyllotis* species at altitude for as long as 2 million years (Kramer et al., 1999) and accordingly, constitutes ample

time for genetic adaptations to occur (Rupert and Hochachka, 2001), especially since generation time is short in these species.

We thus captured and genotyped four species of *Phyllotis* native to high and low altitudes in the Peruvian Andes (Figure 3.1) and investigated their whole-body fuel use and muscle metabolic capacities. Here we show, using a comparative phylogenetic approach, that high altitude species (*P. andium, P. xanthopygus*) use proportionately more carbohydrates and have higher oxidative capacities of cardiac muscles compared to low altitude species (*P. amicus, P. limatus*). These phenotypes were independent of phylogeny and thus constitute a putative adaptation to high altitude environments. The clear oxygen-saving advantage of using carbohydrates combined with a greater oxidative capacity in the heart suggest a more effective use of oxygen for performing energy demanding tasks at altitude.

Methods

Study design

Males of four different species of leaf-eared mice (genus *Phyllotis*) were captured at different altitudes in Peru (Table 3.1): *Phyllotis amicus* (300 m, N=11); *Phyllotis andium* (4000 m, N=10); *Phyllotis limatus* (100 m, N=12), *Phyllotis xanthopygus* (4500 m, N=10). All mice were captured with Sherman traps and transported to the animal facility of the Universidad Peruana Cayetano Heredia (UPCH), in Lima,

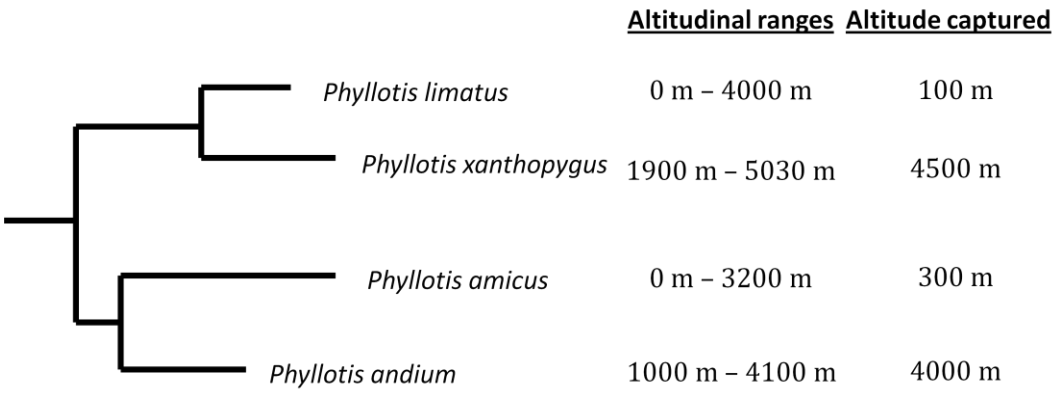

Figure 3.1 Phylogenetic tree of *Phyllotis* species under study, which was used in the independent contrast analysis. Branches are drawn proportional to their actual lengths.

Table 3.1 Altitudinal ranges and habitats of *Phyllotis* species under study together with geographical and altitudinal details of capture sites.

Species	Altitudinal ranges (m)[1]	Habitat[1]	Capture sites	Altitude of capture sites (m)
Phyllotis amicus	0-3200	Desert, rocky areas, sparse vegetation	Reserva Nacional Lomas de Lachay (Lima) 11°21'582''S 77°22'034''W	300
Phyllotis andium	1000-4100	Dry forest, shrub lands, rocky areas and wet highlands	Marcahuasi (Lima) 11°78'928''S 76°57'558''W	4000
Phyllotis limatus	0-4000	Desert, riparian vegetation, rocky areas coastal Lomas	Moro Sama (Tacna) 18°01'227''S 70°50'013''W	100
Phyllotis xanthopygus	1900-5030	Wetlands, *Polylepis* forest, rocky areas, altiplano, grasslands, shrub lands	Añaque (Tacna) 17°25'021''S 69°55'321''W	4500

[1] Steppan and Ramirez In Press

Peru (sea level), where rabbit food pellets and water were provided *ad libitum*. Photoperiod and temperatures were similar to outdoor conditions (~11-13 hours daylight per day, 20-26 degrees Celsius). All procedures were approved by the McMaster University Animal Research Ethics Board, the Animal Ethics Committee of the UPCH as well as authorized by the Ministerio de Agricultura and the Instituto Nacional de Recursos Naturales (INRENA) of Peru.

After at least 6 weeks in captivity at sea level, mice were subjected to different *in vivo* measurements. We first determined (*i*) the aerobic capacity (VO_2max) of all mice and then performed the following measurements in a random order: rates of O_2 consumption (VO_2) and CO_2 production (VCO_2) (*ii*) at rest and (*iii*) at a target exercise intensity corresponding to 75% of individual VO_2max, and (iv) time to fatigue at a speed of 12 m min^{-1}. All measurements were carried out under normoxic (normobaric, ~21% O_2) and hypoxic (normobaric but equivalent to ~4300 m, 12% O_2) conditions in a random order between noon and 7 pm following a 6-hour fast (except for theVO_2max tests, for which the animals were not fasted). Animals were never subjected to more than one experimental procedure per day. Four weeks prior to the experimental measurements, we familiarized all animals to the treadmill-enclosed respirometry chamber by running them at speeds of 4-10 m min^{-1} for 10 minutes once weekly. Following the *in vivo* experimental period and at least 24 hours after the last *in vivo* measurement, we anaesthetized each mouse by placing them in a small container with an isoflurane-soaked cotton ball before performing a cervical dislocation. We quickly extracted left ventricles and skeletal muscles from the hind limbs (gastrocnemius) and immediately weighed and crushed all

tissues with pre-cooled aluminum tongs before storing in liquid nitrogen. Subsequently, we transported all tissues to McMaster University using a cryoshipper and stored them at -80°C until further analyses.

Respirometry and in vivo measurements

We measured VO_2 and VCO_2 using an open flow-through respirometry system and treadmill-enclosed metabolic chamber custom made for mice as described in Chapter 2. We determined VO_2max, time to fatigue, absolute rates of carbohydrate and lipid oxidation at 75% of VO_2max, and resting RER under normoxic and hypoxic conditions as described in Chapter 2.

Species identification and phylogenetic analyses

We sequenced the cytochrome b (cyt b) gene of each individual for species identification and for the determination of genetic variation among species. DNA extraction, amplification and sequencing as well as phylogenetic analyses were performed as previously described (Steppan et al., 2007). Briefly, we extracted complete genomic DNA from a small piece of ear of each mouse using the phenol/chloroform technique, we amplified the entire cyt b gene with primers P484 and P485 described in (Steppan et al., 1999) using polymerase chain reaction (PCR) and sent PCR products for sequencing to Macrogen Corp. (Rockville, MD, USA). With the sequences, we then constructed a phylogenetic tree (Figure 3.1) using the evolutionary model GTR+G (MrModeltest 2.3, (Posada and Crandall, 1998)) and a Bayesian analysis (MrBayes v.3.1.2, (Ronquist and Huelsenbeck, 2003)) with 20000 generations, a sample frequency of 20 and a burnin

fraction of 0.25. The analysis resulted in posterior probabilities of 1 for each node and phylogenetic relationships of species are in accordance with previous reports (Steppan et al., 2007).

Muscle enzymatic capacity (Vmax)

Activities (Vmax) of hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), lactate dehydrogenase (LDH), β-hydroxyacyl CoA dehydrogenase (HOAD), citrate synthase (CS), isocitrate dehydrogenase (IDH), and cytochrome c oxidase (COx,) were measured from the gastrocnemius and the left ventricle of each mouse as described in Chapter 2.

Statistical analyses

Data are reported as means ± s.e.m. unless otherwise stated. We used conventional statistical analyses, assuming independence between species within a node, by doing a two-way analysis of variance or covariance (body mass or exercise intensity as covariates, when applicable) with nodes (node 1 and 2) and altitude (high and low) as factors. Pairwise comparisons were performed with either Sidak *post hoc* tests or t-tests when appropriate. Data abnormally distributed or with unequal variances were log or square root transformed, or otherwise analyzed with a non-parametric test (Mann-Whitney U test). Statistical tests were performed using SPSS (SPSS Inc., Chicago, IL, USA) or Sigmaplot (Systat software Inc., San Jose, CA, USA) with a significance level set to 0.05.

We also performed phylogenetically independent contrast analyses for traits that significantly differed between high and low altitude species. We used the PDAP:PDTREE module (Midford et al., 2005) within Mesquite (Maddison and Maddison, 2006), from which we imported our phylogenetic tree (Figure 3.1), to test for significant positive relationships between independent contrasts of traits and altitudes (low and high, coded with dummy variables, 0 and 1 respectively). Correlations were computed through the origin and we used the one-tailed p-values to determine significance ($P<0.05$).

Results and Discussion

Aerobic capacity

As fuel use is determined by exercise intensity relative to VO_2max in mammals (Brooks and Mercier, 1994; Felig and Wahren, 1975; Romijn et al., 1993), prior to the assessment of whole-body fuel use, VO_2max must be determined. In normoxic conditions, high altitude *Phyllotis* species showed aerobic capacities comparable to their low altitude counterparts. However under hypoxic conditions, VO_2max was significantly reduced by 14-17% in all species except for the high altitude species *P. xanthopygus* (Figure 3.2, Table 3.2). It is widely established that hypoxic conditions equivalent to approximately 4300 m induces a 20-35% decrease in VO_2max in native lowland mammals (Hochachka and Somero, 2002; McClelland et al., 1998). *P. xanthopygus*, a species mostly found in highlands of Peru (up to 5030 m above sea level)

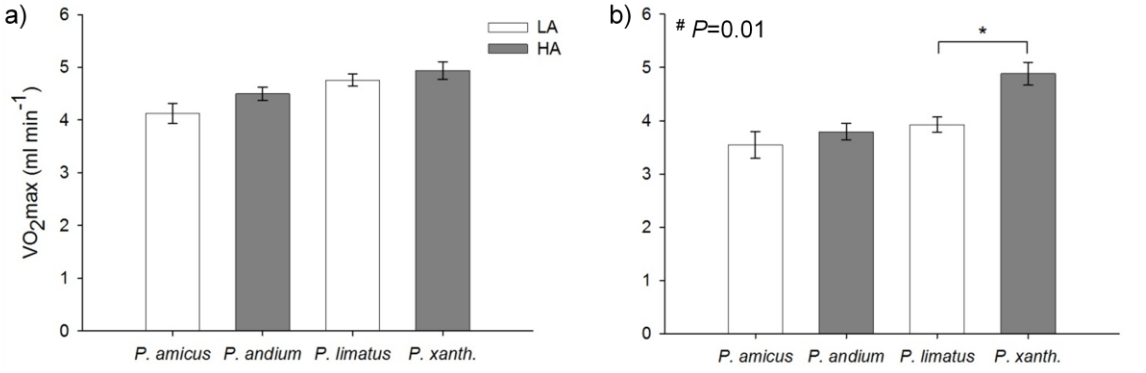

Figure 3.2 Estimated marginal mean (± s.e.m.) aerobic capacities (VO_2max) under a) normoxic and b) hypoxic conditions in low altitude (LA; open bars) and high altitude (HA; solid bars) *Phyllotis* species. An octothorpe indicates ($^{\#}$) a significant effect of altitude (two-way ANCOVA with node and altitude as between-subject factors and body mass as covariate). An asterisk (*) denotes significant pair-wise differences (Sidak *post hoc* test P=0.001).

Table 3.2 Estimated marginal means ± s.e.m. of body mass and respiratory data of low (LA) and high altitude (HA) *Phyllotis* species (HA species are in bold) under normoxic and hypoxic conditions, together with results from a two-way analysis of variance. Significant effects of altitude are emphasized in bold. Asterisks (*) denote significant differences between LA and HA within a node (Sidak *post hoc* $P<0.05$). Original means are shown in parenthesis when different from estimated marginal means.

	Species				Between-subjects factors		
	P. amicus (N=11)	***P. andium*** (N=10)	*P. limatus* (N=12)	***P. xanth.*** (N=10)	Node	Altitude	Node x Altitude
Body mass (g) [c]	26.25±2.07	45.82±2.17*	44.68±1.98	54.82±2.17*	$F_{1,39}$=61.32 P<0 .001	$F_{1,39}$=65.03 ***P*<0.001**	$F_{1,39}$=12.94 P=0.001
VO_2max [a] (ml · min^{-1})							
Normoxia	4.13±0.19 (3.35±0.12)	4.50±0.13 (4.65±0.17)	4.76±0.12 (4.86±0.13)	4.94±0.17 (5.52±0.21)	$F_{1,38}$=9.59 P=0.004	$F_{1,38}$=2.35 P=0.134	$F_{1,38}$=0.58 P=0.452
Hypoxia [b]	3.55±0.25 (2.70±0.09)	3.79±0.16 (3.96±0.17)	3.93±0.14 (4.03±0.16)	4.88±0.22* (5.52±0.25)	$F_{1,38}$=12.01 P=0.001	$F_{1,38}$=7.3 ***P*=0.01**	$F_{1,38}$=2.4 P=0.13
Aerobic scope [a] (ml · min^{-1})							
Normoxia	2.99±0.20 (2.52±0.09)	3.31±0.13 (3.40±0.16)	3.67±0.12 (3.73±0.10)	3.80±0.18 (4.16±0.19)	$F_{1,38}$=10.28 P=0.003	$F_{1,38}$=1.42 P=0.24	$F_{1,38}$=0.467 P=0.50
Hypoxia [b,d]	2.41±0.23 (1.80±0.08)	2.58±0.15 (2.70±0.15)	2.76±0.13 (2.84±0.17)	3.62±0.20* (4.07±0.19)	$F_{1,38}$=11.10 P=0.002	$F_{1,38}$=5.60 ***P*=0.023**	$F_{1,38}$=2.77 P=0.104
Resting VO_2 [a] (ml · min^{-1})							
Normoxia	1.13±0.09 (0.83±0.04)	1.19±0.06 (1.25±0.09)	1.09±0.05 (1.13±0.07)	1.14±0.08 (1.36±0.07)	$F_{1,38}$=0.32 P=0.57	$F_{1,38}$=0.35 P=0.56	$F_{1,38}$=0.01 P=0.93
Hypoxia [c]	1.15±0.08 (0.90±0.04)	1.21±0.05 (1.26±0.04)	1.17±0.05 (1.20±0.04)	1.26±0.07 (1.45±0.09)	$F_{1,38}$=0.66 P=0.42	$F_{1,38}$=2.06 P=0.16	$F_{1,38}$=0.40 P=0.53

[a] body mass used as covariate
[b] means prior to square root transformation
[c] means prior to log transformation
[d] because resting VO_2 was missing for 1 individual *P. andium* and 1 individual *P. xanthopygous*, species mean resting VO_2 were used in the calculation of aerobic scope for these 2 individual mice.

(Steppan and Ramirez-Baca, In Press), is thus exceptional to other mammals in its ability to maintain a normoxic level of VO_2max under hypoxic conditions. Despite the fact that the same phenotype was not observed in the other high altitude species *P. andium*, it is still possible that the preservation of a high VO_2max at high altitude constitutes an adaptation to this hostile environment. Previous reports on aerobic capacities of North American deer mice (*Peromyscus maniculatus*) suggest that, in comparison to the low altitude population, the high altitude population is characterized by a higher VO_2max under hypoxic environments (Chappell et al., 1988; Chappell and Snyder, 1984). A high VO_2max may be beneficial at altitude because 1) it can allow a high level of exercise in spite of the low inspired PO_2 to maintain an acceptable level of performance for energy demanding tasks such as foraging, territorial defence, predator avoidance; and 2) it can help maintain stable body temperature through aerobic thermogenesis at the low ambient temperatures usually experienced at high altitudes. Moreover, field studies suggest that VO_2max is positively correlated with increased fitness in a high altitude population of North American deer mice (Hayes and O'Connor, 1999). It is thus likely that maintaining a high VO_2max represents an adaptation to high altitude environments in small rodents.

Fuel use

Having determined the aerobic capacity of each mouse, we proceeded to determine the proportion of carbohydrates used when mice were running at an intensity corresponding to 75% of their VO_2max (same relative intensity of exercise). The high altitude species used a greater proportion of carbohydrates than the low altitude species

under both normoxic and hypoxic condition (Figure 3.3). As recent evidence suggests that intensity of exercise expressed as a proportion of aerobic scope (in lieu of VO_2max) is a better predictor of the proportion of fuel used (chapter 4), we also used % aerobic scope (Table 3.3) as a covariate into our analyses and found similar results than if the covariate was omitted. In one of the few other studies examining fuel use at altitude, Hochachka and colleagues (Hochachka et al., 1991) reported an increased use of carbohydrates during exercise in high altitude Andean native humans. However their results are difficult to interpret as comparisons were made at work rates close to maximal capacity and without adequate standardization of intensity of exercise relative to VO_2max. Since the high altitude native Andean humans had substantially lower VO_2max compared to lowlanders, it is likely that the slight increase in carbohydrate used observed by Hochachka and colleagues (Hochachka et al., 1991) originated because the high altitude natives were operating at rates closer than their VO_2max rather than because of an inherent change in metabolism. Our results thus constitute the first report of increased carbohydrate use in a high altitude native mammal from which the confounding effects of relative exercise intensities have been adequately taken into account, and thus suggest a true shift in metabolic regulation of fuel use in high altitude native mammals. Moreover, due to our multi-species experimental design our results suggest that this trait is likely to represent an adaptation to high altitude environments. Phylogenetically independent contrasts of proportional use of carbohydrates of all species and altitude showed positive relationships (Figure 3.6a), suggesting that the trait may have evolved independently in the high altitude species.

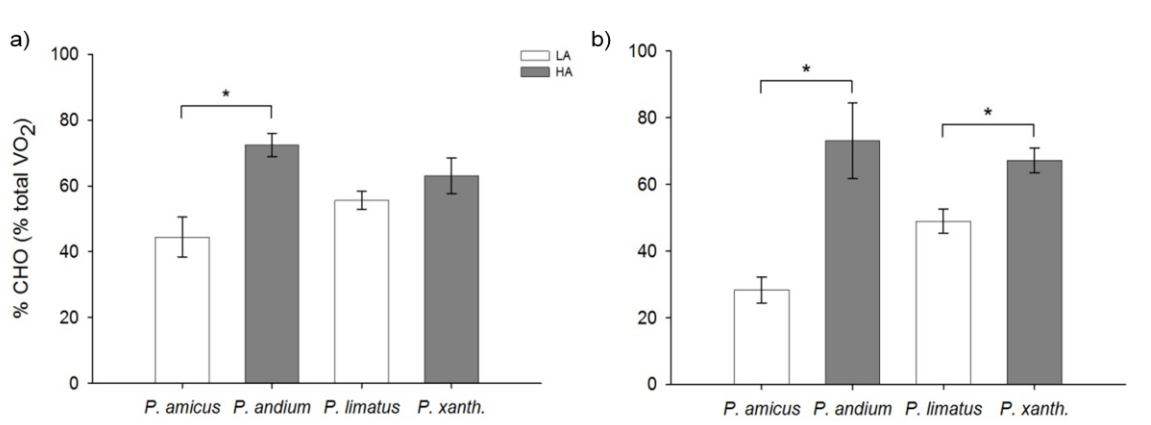

Figure 3.3 Mean (± s.e.m.) percent of total VO_2 used for carbohydrate oxidation during exercise at 75% of VO_2max under a) normoxic and b) hypoxic conditions in LA (open bars) and HA (solid bars) *Phyllotis* species. Asterisks (*) denote significant differences (Sidak *post hoc* test, $P<0.05$).

Table 3.3 Estimated marginal means of respiratory exchange ratio and absolute rates of fuel selection in LA and HA *Phyllotis* species (HA species are in bold) during treadmill running at a target intensity of 75% of VO_2max under normoxic and hypoxic conditions, together with results from a 2-way analysis of variance and actual exercise intensities and treadmill speeds. Significant effects of altitude are emphasized in bold. Asterisks (*) denote significant differences between LA and HA within a node (Sidak *post hoc*, $P<0.05$). Original means are shown in parenthesis when different from estimated marginal means.

	Species				Between-subjects factors		
	P. amicus	***P. andium***	*P. limatus*	***P. xanth.***	Node	Altitude	Node x Altitude
RER							
Normoxia	0.836±0.013	0.919±0.014*	0.869±0.013	0.891±0.015	$F_{1,32}$=0.03 P=0.85	$F_{1,32}$=14.50 ***P*=0.001**	$F_{1,32}$=4.86 P=0.035
Hypoxia	0.789±0.014	0.904±0.015*	0.850±0.013	0.904±0.015*	$F_{1,33}$=4.38 P=0.044	$F_{1,33}$=35.03 ***P*<0.001**	$F_{1,33}$=4.64 P=0.039
Absolute rate of CHO oxidation [a] (µmol O_2 min^{-1})							
Normoxia	84±12 (48±7)	107±8 (116±9)	84±7 (92±6)	87±11 (115±13)	$F_{1,31}$=0.81 P=0.38	$F_{1,31}$=1.43 P=0.24	$F_{1,31}$=1.59 P=0.22
Hypoxia [b]	68±12 (25±4)	80±8 (89±14)	60±7 (67±7)	90±11* (123±11)	$F_{1,32}$=0.38 P=0.54	$F_{1,32}$=4.93 ***P*=0.034**	$F_{1,32}$=0.00 P=0.99
Absolute rate of lipid oxidation [a] (µmol O_2· min^{-1})							
Normoxia	54±10 (60±7)	45±7 (43±5)	75±6 (73±5)	69±10 (64±8)	$F_{1,31}$=5.35 P=0.03	$F_{1,31}$=0.52 P=0.48	$F_{1,31}$=0.07 P=0.79
Hypoxia	47±9 (62±3)	44±6 (40±10)	70±5 (67±4)	71±8 (58±5)	$F_{1,32}$=8.5 P=0.01	$F_{1,32}$=0.01 P=0.89	$F_{1,32}$=0.08 P=0.78

Exercise intensity [c] (% VO_2max)				
Normoxia	77.1±0.5	76.1± 0.6	74.4± 0.8	74.0 ±1.0
Hypoxia	79.3± 0.1	72.1±2.0	77.5±0.6	75.8±0.9
Exercise intensity [c] (% aerobic scope)				
Normoxia	64.6±3.8	68.2±2.1	67.0±1.6	67.2±3.3
Hypoxia	62.5±4.9	57.3±3.0	65.2±1.9	69.9±2.6
Treadmill speed [c] (m min^{-1})				
Normoxia	10.3±1.1	11.1±0.9	14.5±1.0	11.6±0.7
Hypoxia	4.6±0.7	7.3±0.9	10.7±0.7	12±0.9

[a] body mass used as covariate
[b] means prior to square root transformation
[c] original means are displayed

Other lines of evidence of increased carbohydrate use at altitude come from our assessment of fuel use at rest. The respiratory exchange ratio (RER) of high and low altitude *Phyllotis* species was unchanged under normoxia, however under hypoxia, both high altitude species had a higher RER than the low altitude species (Figure 3.4). Since RER gives an idea of the mixture of fuel used, these results suggest that high and low altitude species use a different mixture of fuel at rest in high altitude environments. This difference was also independent of the phylogenetic relationship of species (Figure 3.6b). Even though it is impossible to rule out the potential change in the contribution of proteins in this case, the elevated RER is likely the result of an increase use of carbohydrates and accordingly, these results provide further support to the hypothesis of increased use of carbohydrates as an adaptation to high altitude.

This shift in metabolism toward a greater use of carbohydrates as a fuel source for metabolic functions is likely a way to capitalize on the oxygen-saving advantage of carbohydrates without having to compromise on the aerobic capacity of the animal. Unlike high altitude rodents, a number of high altitude native humans are recognized for their low aerobic capacities (Hochachka et al., 1991; Kayser et al., 1991), thus because they are operating at intensities closer to their VO_2max, they use more carbohydrates at all absolute work rates compared to a lowlander with a high VO_2max. For reasons discussed above (high level of exercise, high thermogenic capacity), maintaining a high VO_2max in small rodents may be essential for survival. Thus, rather than depressing VO_2max in hypoxic environments, selection may have taken place at the level of regulation of fuel use to shift metabolism towards a greater use of carbohydrates.

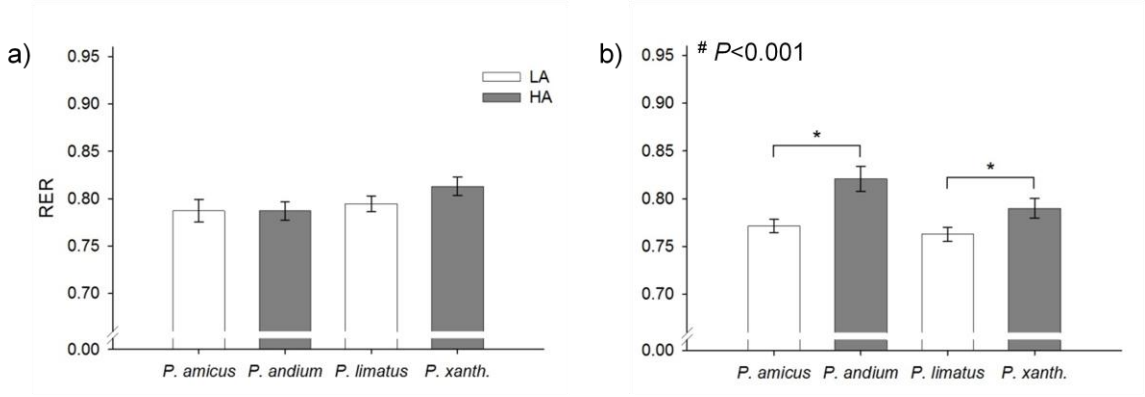

Figure 3.4 Mean (± s.e.m.) respiratory exchange ratio (RER) at rest under a) normoxic and b) hypoxic conditions in LA (open bars) and HA (solid bars) *Phyllotis* species. An octothorpe ($^{\#}$) indicates a significant effect of altitude. Asterisks (*) denote significant differences (Holm-Sidak *post hoc* test, $P<0.05$).

Performance

In high altitude native mice the oxygen-saving strategy of increased use of carbohydrates as fuel for energy metabolism may compromise endurance performance. When mice were subjected to a time to fatigue test on a treadmill at a constant speed of 12 m min^{-1}, the low altitude species actually ran for almost twice as long as the high altitude species under normoxic conditions, whereas no clear pattern emerged under hypoxic conditions (Figure 3.5). By using more carbohydrates at the same relative exercise intensities, high altitude species may deplete their glycogen stores at a higher rate than the low altitude species. It is widely established that time to fatigue is correlated with glycogen depletion (Fitts, 1994). Thus, assuming that both high and low altitude mice have similar glycogen stores, it is possible that high altitude mice fatigued sooner than the low altitude mice because of a faster depletion of glycogen stores. Under hypoxic conditions, both low altitude and high altitude species fatigued quickly, thus the endurance performance of all species seemed to be similarly compromised under hypoxic conditions except that in the high altitude species high rates of glycogen depletion may be responsible for the short time to fatigue whereas in the low altitude species it may be their inability to use O_2 efficiently. In the wild, high altitude mice may not be engaging endurance exercise. Instead, their day to day activities such as predator avoidance, foraging and territorial defence likely consist of moderate or high intensity exercises of short duration. In fact, high altitude deer mice appear to perform most of their daily activities at rates near VO_2max (Hayes, 1989b). Thus, preserving carbohydrate stores may not be as important as using O_2 more efficiently.

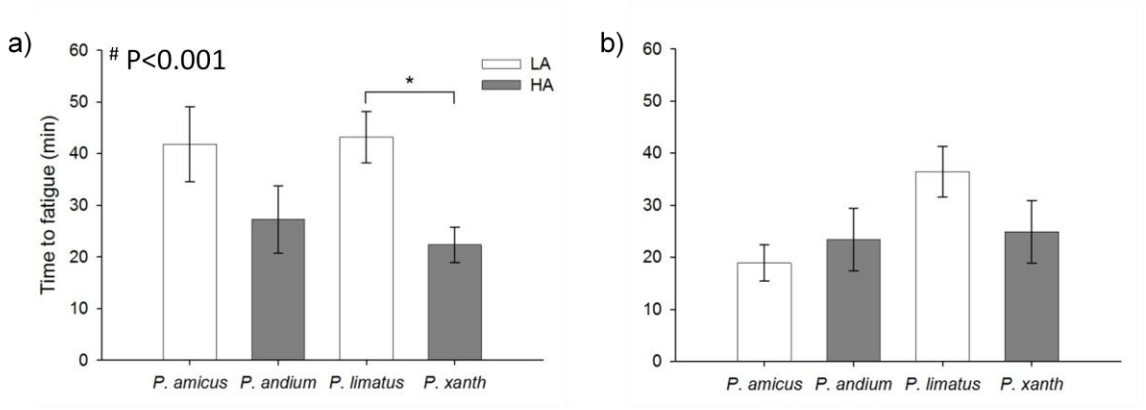

Figure 3.5 Mean (± s.e.m.) time to fatigue to treadmill running at 12 m min^{-1} under a) normoxic and b) hypoxic conditions in LA (open bars) and HA (dark bars) *Phyllotis* species. An octothorpe indicates ($^{\#}$) a significant effect of altitude. An asterisk (*) denote significant differences (Holm-Sidak *post hoc* test, $P<0.05$).

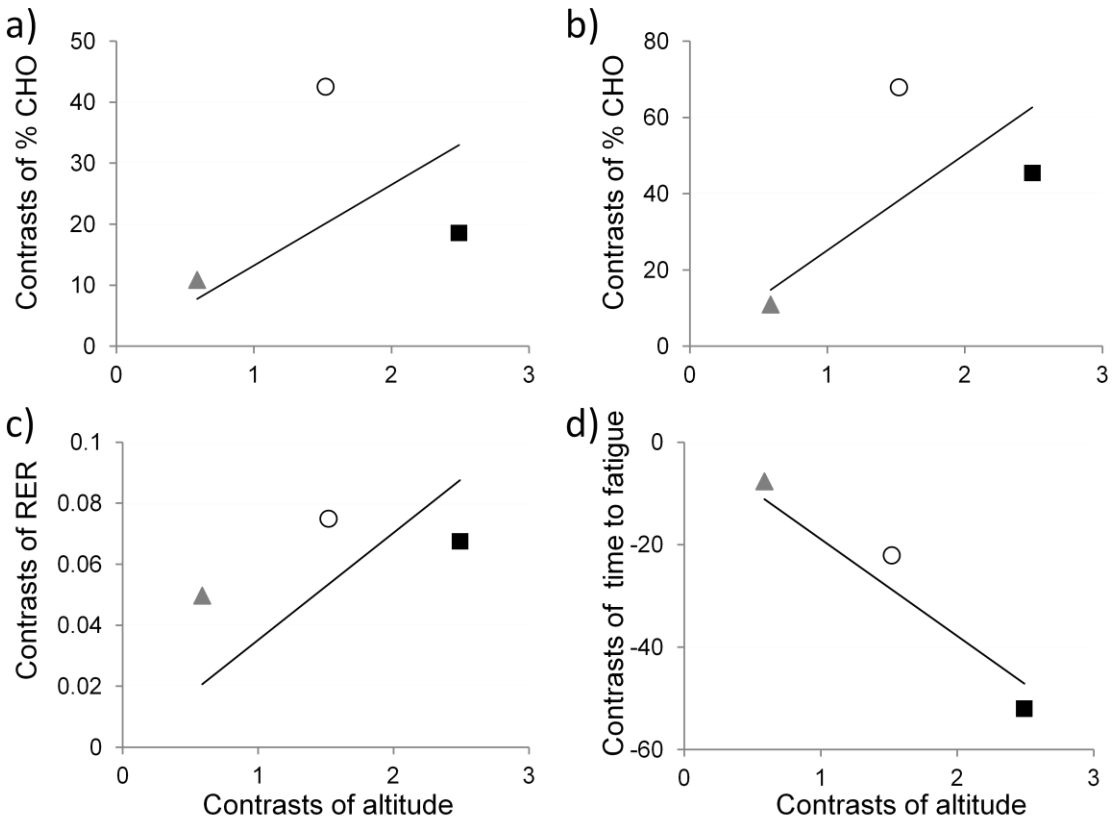

Figure 3.6 Least-square regressions forced through the origin between independent contrasts of altitude and (a) % CHO at 75% of VO_2max under normoxia (R^2=0.68, *P*=0.087), (b) % CHO at 75% of VO_2max under hypoxia (R^2=0.82, *P*=0.046), (c) resting RER under hypoxia (R^2=0.86, *P*=0.035) ,(d) time to fatigue under normoxia (R^2=0.98, *P*=0.006). Grey triangles represent node to node contrast, white circles represent *P. amicus* to *P. andium* contrast, black squares represent *P. limatus* to *P. xanthopygus* contrast.

Muscle enzymatic capacity

To assess whether flux capacity through metabolic pathways is increased at the muscle level to accommodate greater rates of carbohydrate oxidation, we investigated activities (apparent Vmax) of several enzymes involved in carbohydrate and lipid oxidation in the hind limb skeletal muscle gastrocnemius and in the left ventricle. There were no striking differences in enzymatic capacities of gastrocnemius between high and low altitude *Phyllotis* species (Table 3.4), suggesting that skeletal muscles of high altitude native mice do not have a particularly enhanced capacity for carbohydrate oxidation. A similar study comparing species of Pikas (genus *Ochotona*) across an altitudinal gradient also reported little changes in metabolic enzymes in skeletal muscles (Sheafor, 2003). Skeletal muscles are often rested and only require high rates of ATP production sporadically. Consequently, it is not unforeseen that capacity for flux through metabolic pathways is not enhanced in skeletal muscles of high altitude species.

In contrast, for continuously active and oxygen sensitive muscles, such as the heart, higher capacities for carbohydrate oxidation would be beneficial at high altitudes. The heart plays a central role in oxygen delivery, thus proper cardiac function is crucial, especially in low oxygen environments. Our results show that cardiac muscles of high altitude *Phyllotis* species have enhanced oxidative capacities, as activities of citrate synthase (CS) and isocitrate dehydrogenase (IDH) were higher in the high altitude species than in the low altitude species (Table 3.4). A greater oxidative capacity of the heart

Table 3.4 . Mean ± s.e.m. enzyme activities (Vmax) in µmoles min^{-1} g^{-1} wet weight in gastrocnemius (gastroc.) and left ventricles (LV) of LA and HA *Phyllotis* species (HA species are in bold). Significant effects of altitude are emphasized in bold. Asterisks (*) denote significant differences between LA and HA within a node.

	Species				Between-subjects factors		
	P. amicus	***P. andium***	*P. limatus*	***P. xanth.***	Node	Altitude	Node x Altitude
Hexokinase (HK)							
Gastroc.	2.3±0.3	2.0±0.1	2.1±0.2	2.2±0.3	$F_{1,36}$=0.1 P=0.75	$F_{1,36}$=0.1 P=0.73	$F_{1,36}$=0.7 P=0.42
LV	8.7±0.7	12.5±0.6*	7.4±0.4	7.6±0.6	$F_{1,36}$=27.0 P<0.001	$F_{1,36}$= 12.5 ***P=0.001***	$F_{1,36}$=10.4 P=0.003
Phosphofructokinase (PFK)							
Gastroc.	47±3	42±3	44±2	41±3	$F_{1,36}$=0.5 P=0.4	$F_{1,36}$=2.1 P=0.16	$F_{1,36}$=0.1 P=0.74
LV	22.6±1.0	15.0±0.9	11.2±0.5	15.9±0.9	$F_{1,37}$=32.2 P<0.001	$F_{1,37}$=2.3 P=0.14	$F_{1,37}$=43.4 P<0.001
Pyruvate kinase (PK)							
Gastroc.	396±25	372±16	321±13	285±16	$F_{1,36}$=21.3 P<0.001	$F_{1,36}$=3.0 P=0.09	$F_{1,36}$=0.1 P=0.72
LV[a]	121±9	93±5*	98±4	100±4			

Lactate dehydrogenase (LDH)							
Gastroc.	770±32	775±42	828±44	733±57	$F_{1,36}$=0.03 *P*=0.86	$F_{1,36}$=1.0 *P*=0.33	$F_{1,36}$=1.3 *P*=0.27
LV[a]	562±26	448±35*	612±28	708±58			
Hydroxyacyl-CoA dehydrogenase (HOAD)							
Gastroc.	21.5±1.5	22.3±1.5	20.3±0.9	20.6±2.3	$F_{1,36}$=0.9 *P*=0.36	$F_{1,36}$=0.1 *P*=0.73	$F_{1,36}$=0.02 *P*=0.88
LV	256±8	185±24	281±9	292±14	$F_{1,37}$=18.8 *P*<0.001	$F_{1,37}$=3.9 *P*=0.06	$F_{1,37}$=7.3 *P*=0.01
Citrate synthase (CS)							
Gastroc.[a]	38.5±3.3	35.9±2.3	39.6±2.3	42.8±5.3			
LV [b]	273±7	320±13*	371±17	459±29*	$F_{1,37}$=52.1 *P*<0.001	$F_{1,37}$=16.4 ***P*<0.001**	$F_{1,37}$=0.5 *P*=0.50
Isocitrate dehydrogenase (IDH)							
Gastroc.[a]	12.3±1.7	18.4±1.4*	16.3±1.4	17.2±2.5			
LV[a]	223±7	301±28*	301±11	325±12			
Cytochrome c oxidase (COx)							
Gastroc.[b]	16.1±2.2	13.8±2.2	17.3±5.4	15.6±2.5	$F_{1,36}$=0.6 *P*=0.43	$F_{1,36}$=1.5 *P*=0.23	$F_{1,36}$=0.001 *P*=0.97

[a] Mann-Whitney U test or t-tests
[b] Means before log transformation

seems to be a common feature of high altitude native mammals. Previous studies on high altitude pikas (Sheafor, 2003) as well as on high altitude llamas, alpacas and tarucas (Hochachka et al., 1983) also found increases in the activities of several enzymes involved in oxidative pathways. In addition, at least in the high altitude species *P. andium*, heart metabolism appeared to be geared toward a greater uptake of circulatory glucose. We found that activities of hexokinase (HK), an enzyme involved in the first step of glycolysis, were significantly higher in left ventricles of high altitude species *P. andium* compared to its closely-related low altitude counterpart *P. amicus* (Table 3.4). In high altitude native humans (Sherpas), ^{31}P magnetic resonance spectroscopy also revealed a metabolic organization of the heart to preferentially use carbohydrates instead of fatty acids (Hochachka et al., 1996). The oxygen-saving advantage of carbohydrates makes it the fuel of choice in low oxygen environments, and it is thus not surprising that carbohydrate use would be enhanced in an O_2-sensitive tissue in high altitude species.

Genetic factors, acclimatization and development

Phenotypic differences between high and low altitude species may be explained by genetic variations and plasticity during development and adulthood. Here we argue that differences between high and low altitude species of *Phyllotis* are mostly the effect of genetic differences. In the context of acclimatization to high altitudes, some phenotypic changes appear to be reversible, usually within 5 weeks following return to low altitudes (Grassi et al., 1996). Thus our 6-week de-acclimatization period was likely sufficient to remove the effects of acclimatization to high altitude in the high altitude species. In

addition, previous experiments have shown that the proportion of carbohydrates used during exercise does not change with acclimation to high altitude in laboratory mice (*Mus musculus)* (Chapter 4) and rats (*Rattus Norvegicus*) (McClelland et al., 1998). On the other hand, differences arising from the effects of development at different altitudes were not controlled for in this study. Such effects may be irreversible (Russell et al., 2008), and thus common garden experiments are necessary to separate developmental from genetic effects of high altitude. Still, the effects of development on our results, if any, are unlikely to explain most of the variation observed in this study as it is widely accepted, and previously reported in high altitude native mice (*Peromyscus maniculatus)*, that development only partly affects phenotypes (Russell et al., 2008).

Summary and perspectives

The increased whole-body use of carbohydrates and enhanced oxidative capacity of cardiac muscles have likely evolved in high altitude *Phyllotis* species as an adaptation to high altitude environments. Using more carbohydrates represents an oxygen-saving strategy which is likely to allow high altitude *Phyllotis* mice for an enhanced level of activity, thermogenesis, or for allocation of energy into other important physiological processes essential for long-term survival (e.g. immunity, digestion). This preference for carbohydrates in high altitude species was not accompanied by an enhanced capacity for carbohydrate oxidation at the muscle level. Mechanisms of fuel selection in mammals are poorly understood (McClelland, 2004), perhaps in part because of their complexity but perhaps also because they appear to be widely conserved (McClelland, 2004; Roberts et

al., 1996c). The inherent whole-body differences in fuel use between high and low altitude *Phyllotis* species thus provide an exceptional system to elucidate the underlying mechanisms of mammalian fuel selection. Accordingly this study represents a significant discovery in the field of mammalian energetics as it uncovers an ideal system to study the regulatory mechanism of fuel use in mammals. These results also represent significant advancements in the field of high altitude adaptation in mammals as it is the first study to provide compelling evidence for increased carbohydrate use at high altitudes, a hypothesis originally proposed nearly 30 years ago.

CHAPTER 4

PATTERNS OF FUEL USE DURING LOCOMOTION IN MAMMALS REVISITED: THE IMPORTANCE OF AEROBIC SCOPE

Abstract

Fuel selection patterns during exercise are thought to be conserved among mammals when intensity of exercise is expressed relative to VO_2max; however this claim is based on a few mammalian species and has never been tested statistically. We thus investigated fuel use in *Mus musculus* (CD1 mice), a species in which fuel selection patterns have never been determined, and combined this data to previously published data from other mammals (rats, dogs, goats) to statistically evaluate the conserved nature of mammalian fuel selection. Moreover, as the effects of acclimatization to high altitude on fuel use at both the whole-body and muscle levels have not been investigated in lowland native mice, we also subjected a group of CD1 mice to a 6-week exposure to hypobaric hypoxia and investigated whole-body fuel use at 65% and 80% of VO_2max and the capacity for carbohydrate and lipid oxidation in cardiac and skeletal muscles. Our results suggest that exercise intensity expressed relative to aerobic scope, in lieu of VO_2max, is a better predictor of the mix of fuels oxidized during exercise in mammals. We thus revisit the current mammalian model of fuel selection as such: fuel selection patterns during exercise are conserved among mammals when intensity of exercise is expressed relative to aerobic scope. Moreover, we found that acclimation to hypobaric hypoxia does not affect fuel selection in mice but decreases the oxidative capacity of the heart. This

evaluation of the extent of the plasticity of fuel use and muscle metabolism in response to chronic hypoxia in lowland native mice can aid in assessing the adaptive nature of similar traits in high altitude native mammals such as Andean *Phyllotis* mice.

Introduction

Animals power exercise by using the appropriate mix of available fuels based on size of stores, ease of delivery to muscle, and capacity to take up and catabolise different fuels. Early work in humans has shown that the mix of fuels varies with exercise intensity (Edwards et al., 1934). As intensity of exercise increases, the proportion of carbohydrates oxidized increases while the proportion of lipids oxidized decreases. This pattern of fuel selection ignores proteins because their oxidation contributes to less than 5% of the total ATP production during exercise in mammals (Carraro et al., 1994; Rennie et al., 1981). In the last 15 years, comparative examinations of fuel use have shown that a simple model of fuel selection general to all mammals emerges when intensity of exercise is expressed as a fraction of individual aerobic capacities (% VO_2max) (McClelland, 2004; Roberts et al., 1996c; Weber and Haman, 2004). That is, an amalgamation of published means from rats, dogs, goats and humans was used to propose that all mammals oxidize the same mixture of carbohydrates and lipids at any given intensity of exercise relative to VO_2max. Even though it is quite remarkable that such a global pattern emerges in spite of the great phylogenetic distances between species and wide variations in body mass and aerobic capacities, a statistical examination of the conserved nature of this model has not been

performed. Moreover, neither has the current data covered a wide range of mammals or extended the range to small body sizes.

Small mammals such as mice are often used as model species in studies on energetics and fuel use. Yet fuel selection patterns have not been described in a mammal smaller than a rat. Since mass-specific resting metabolic rates increases as body size decreases (Kleiber, 1932), mice generally have a smaller aerobic scope [range of oxidative metabolism from rest to VO_2max] compared to larger mammals of comparable aerobic capacities (Bishop, 1999). These reductions in aerobic scope as body mass decreases could potentially have profound effects on patterns of fuel selection because resting metabolic rates now constitute a higher proportion of VO_2max.

Extending the mammalian fuel use model to small mammals such as mice would also be useful as a hypothesis setting tool for experiments with wild rodents. Several species of wild mice, especially those native to different altitudes (Genus *Peromyscus* and Genus *Phyllotis*), represent unique systems to investigate fuel selection and metabolic adaptations to extreme environments (Hayes and O'Connor, 1999; Nespolo et al., 2003; Russell et al., 2008; Storz, 2007)(Chapter 2 and 3). The study of fuel selection in high altitude mammals is of great interest because of the oxygen-saving advantage of oxidizing carbohydrates over fatty acids (~15-30% more ATP per mole of oxygen (Brand, 2005; Daut and Elzinga, 1989; Welch et al., 2007)). Mammals living at high altitude are thus expected to show an increased reliance on carbohydrates as a fuel source for energy metabolism. This appears to be true in high altitude native *Phyllotis* mice (Chapter 2 and 3) but not in lowland native rats and men following a chronic exposure to hypobaric

hypoxia. In fact, a number of high altitude acclimation studies suggests that the mammalian pattern of fuel use is unaffected by chronic hypoxia (Lundby and Van Hall, 2002; McClelland et al., 1998), whereas others suggest a decrease (Braun et al., 2000; Young et al., 1982) in the relative use of carbohydrates during exercise in acclimated individuals. In light of this, an evaluation of the effects of acclimation to high altitude on fuel use is necessary in lowland mice to assess the extent of the plasticity of the phenotype.

Moreover, little is known on the effects of chronic hypoxia on muscle capacities for carbohydrate and lipid oxidation. In both cardiac and skeletal muscles, oxidative capacity is either unaltered (Beaudry and McClelland, 2010; Kennedy et al., 2001; Le Moine et al., 2011; Perhonen et al., 1996; Templeman et al., 2010), or reduced (Cai et al., 2010) after acclimation to moderate or high altitudes (2500-5000 m), however studies on capacities for carbohydrate or lipid oxidation are scarce. In cardiac muscles, reports of increased hexokinase (HK) activity (Abdelmalki et al., 1996; Daneshrad et al., 2000) and decreased β-hydroxyacyl-CoA dehydrogenase (HOAD) activity (Daneshrad et al., 2000; Kennedy et al., 2001; Templeman et al., 2010) following acclimation to high altitudes suggest a remodelling of the heart to enhance glucose oxidation rather than free fatty acids. Interestingly, one of the proposed adaptations to chronic hypoxia in high altitude native mammals is the preferential use of carbohydrates by the heart (Hochachka et al., 1996; Holden et al., 1995). In the absence of proper evaluations of the effects of acclimation to high altitude on cardiac metabolism, it is unknown whether the phenotype

is unique to high altitude natives or part of the acclimatization response to hypobaric hypoxia common to both lowland and highland mammals.

We thus used CD1 mice (*Mus musculus*) to examine: (1) fuel selection patterns during exercise in a small mammal and (2) the effect of a 6-week acclimation to hypobaric hypoxia on fuel selection patterns and on cardiac and skeletal muscle metabolism. We combined fuel use data of CD1 mice from this study to previously published data of other mammalian quadrupeds (rats: (McClelland et al., 1998); dogs and goats: (Roberts et al., 1996c) to statistically evaluate the conserved nature of mammalian fuel selection patterns. We tested the null hypotheses that 1) small mammals show the same pattern of fuel selection as other mammalian quadrupeds, and 2) chronic hypoxia has little effect on fuel selection patterns and on skeletal and cardiac muscle metabolism.

Materials and methods

Study design

All procedures were approved by the McMaster University Animal Research Ethics Board. Outbred male CD1 mice were obtained at 6 weeks of age (Charles-River, Wilmington, MA, USA) and randomly assigned to a control group (N=12) or a chronic hypoxia acclimated group (N=10). Mice from the acclimated group were placed in a hypobaric chamber kept at a pressure of 0.6 bar (simulating approximately 4300 m of altitude) whereas mice from the control group were housed in the same room but outside the hypobaric chamber (barometric pressures close to sea level value). Animals were kept

in a 12h:12h light:dark cycle with the lights coming on at 7am. The hypobaric chamber was returned to sea level pressures for 30 minutes weekly to provide care for the animals. After an acclimation period of at least 6 weeks, all mice were subjected to the following *in vivo* experimental measurements under normoxic and hypoxic conditions using a respirometry system and metabolic chamber as described in Chapter 2: 1) maximum oxygen consumption (VO_2max) under normoxic (normobaric, ~21% O_2) and hypoxic (normobaric but equivalent to ~4300m, ~12% O_2) conditions (random order) followed by, 2) rates of O_2 consumption (VO_2) and CO_2 production (VCO_2) under normoxic and hypoxic conditions at rest and at target exercise intensities of 65% and 80% of VO_2max. These measurements were performed in a random order between noon and 7pm following a 6-hour fast, except for the VO_2max tests for which mice were not fasted. Each mouse was never subjected to more than 1 measurement per day. During the experimental period, the hypobaric chamber was returned to sea level pressure twice a day for a short period of time (<2 hours), once to remove food and once to perform respirometry measurements. Following the experimental period and at least 24h after the last measurement, mice were quickly anesthetised with isoflurane and immediately killed by cervical dislocation. Cardiac (left ventricles) and skeletal muscles (gastrocnemius) were then quickly extracted, weighed, immediately crushed with pre-cooled aluminum tongs, frozen in liquid nitrogen, and then stored at -80°C for future analysis.

Exercise and resting protocols for mice

Maximum oxygen consumption (VO_2max) was determined by increasing the speed of the treadmill by 3 m min^{-1} every 2 minutes from an initial speed of 7 m min^{-1}. We determined when the mouse had reached VO_2max according to the criteria described in Chapter 2. Submaximal exercise intensities (65% and 80% of individual VO_2max) were determined from the relationship between VO_2 and speed during the VO_2max test but also by adjusting treadmill speed in real-time as described in Chapter 2. We used data from at least 5 minutes of recordings between the 5^{th} and 20^{th} minute of exercise to calculate group means. The treadmill had an inclination of 10° for all exercise protocols. Submaximal exercise at 65% of VO_2max under hypoxia was not possible in most mice because the VO_2 was similar to resting VO_2. For the same reason, exercise at 65% VO_2max under normoxia was not possible for 5 mice from the control group (N=7). Resting VO_2 and VCO_2 were recorded and analyzed as in chapter 2.

Muscle enzymatic capacity (Vmax) and ELISA

Activities (apparent Vmax) of hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), lactate dehydrogenase (LDH), β-hydroxyacyl-CoA dehydrogenase (HOAD), citrate synthase (CS), isocitrate dehydrogenase (IDH), and cytochrome c oxidase (COx,) were measured as described in Chapter 2. Heart-type fatty acid binding protein (H-FABP) content was measured following the recommended protocol of the murine enzyme-linked immunosorbent assay (ELISA) kit by Hycult Biotech (Uden, Netherlands). For this assay, we used a sample size of 10 for each group, but removed an

outlier in the acclimated group to meet the assumption of normal distribution. Interpretation of results are the same with or without the outlier ($P<0.05$).

Calculations and statistical analyses

VO_2, VCO_2 and rates of carbohydrate and lipid oxidation were calculated as described in chapter 2. To reconstruct the overall fuel use pattern in a group of representative quadruped mammals, we used raw data obtained from previously published studies on rats (McClelland et al., 1998), dogs and goats (Roberts et al., 1996c) (Table 4.1). We used individual VO_2max and resting VO_2 values to calculate aerobic scope (VO_2max – resting VO_2) of each animal (Table 4.1) except for the resting VO_2 values of dogs and goats, which were not available for each individual. We thus used previously published mean resting VO_2 values (Taylor et al., 1987) in the calculation of each individual dog and goat. We present regressions of carbohydrate oxidation with relative exercise intensity expressed both as $\%VO_2max$ and as % aerobic scope. Statistical analyses were performed with SPSS (SPSS Inc., Chicago, IL, USA) or Sigmaplot (Systat software Inc., San Jose, CA, USA). We used Pearson correlations, two-tailed t-tests or repeated-measures analyses of variances (ANOVA) with one within subject factor (environment: normoxia and hypoxia) and one between subject factor (group: control and acclimated). We included body mass as a covariate when necessary (resting VO_2, VO_2max, carbohydrate and lipid oxidation) and used Sidak *post hoc* test when applicable. Values are presented as means ± s.e.m.

Table 4.1 Mean values of variables used to determine aerobic scope in dogs, goats and rats. Individual data for each animal was obtained from authors of the studies listed below.

	Dogs	Goats	Rats
N	3	4	12
Body mass (kg)	25±2	30±3	0.307±0.005
Resting VO_2 ($mlO_2\ g^{-1}\ hr^{-1}$)	0.612†	0.504†	1.9±0.1
VO_2max ($mlO_2\ g^{-1}\ hr^{-1}$)	8.8±0.2	4.1±0.1	5.4±0.1
Aerobic scope ($mlO_2\ g^{-1}\ hr^{-1}$)	8.1±0.2	3.6±0.1	3.5±0.1
Study	Roberts et al. 1996		McClelland et al. 1998

† Mean resting VO_2 published in Taylor et al. (1987) was used for each animal.

Results

Fuel selection in mammals

Control CD1 mice fuel selection pattern deviate from the current proposed mammalian model (Figure 4.1a). Mean % carbohydrate oxidation of both exercise intensities studied in mice fell outside the 95% confidence intervals of the polynomial regression generated from dogs and goats. Moreover, the 95% confidence intervals of the linear regression generated from mice do not overlap with the ones from dogs and goats. Interestingly, at low exercise intensities, data from rats also deviate from the current mammalian model of fuel selection. The 95% confidence intervals of the linear regression generated from rats (data not shown) do not overlap with the 95% confidence interval of the polynomial regression from dogs and goats at exercise intensities below 65% of VO_2max.

On the other hand, when exercise intensity is expressed relative to aerobic scope, mice and rats fall within the regression describing the fuel selection pattern of other mammals (Figure 4.1b). Mean % carbohydrate oxidation of both exercise intensities studied in mice and rats fell within the 95% confidence intervals of the polynomial regression of dogs and goats. In addition, nearly all of the area included in the 95% confidence intervals of the linear regressions of mice and rats overlapped with the one from dogs and goats. When all mammals were included in the analyses (mice, rats, dogs and goats), both correlations of % carbohydrate oxidation with exercise intensities

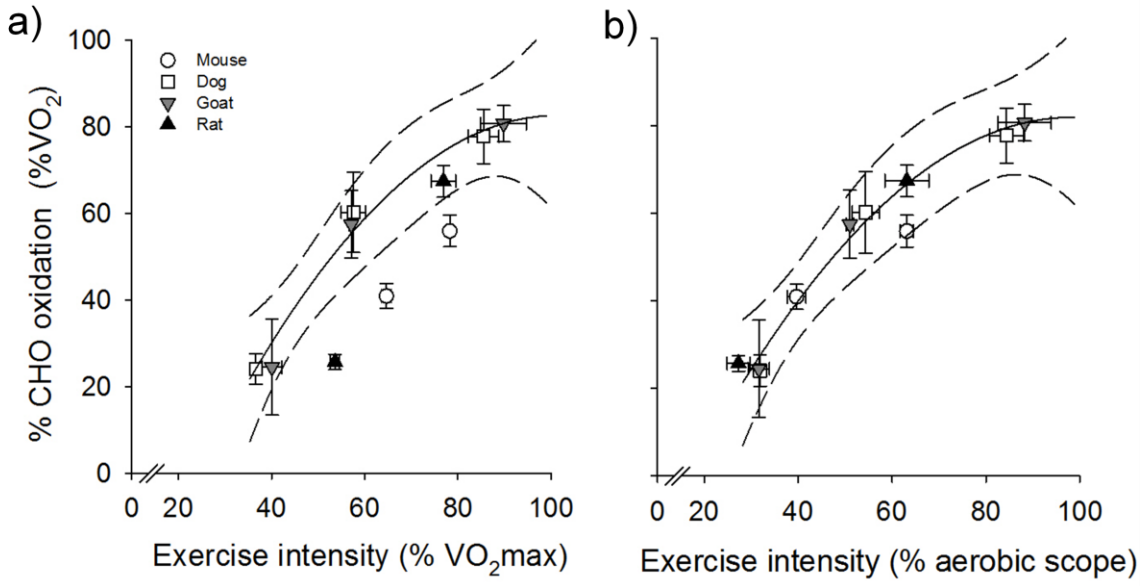

Figure 4.1 Percent of oxygen used for CHO oxidation (% VO_2) with relative exercise intensities expressed as (a) % of aerobic capacity (VO_2max) and (b) % of aerobic scope in CD1 mice (this study), rats (McClelland et al. 1998), dogs and goats (Roberts et al. 1996c). Data shown as mean ± s.e.m. Full lines represent the regression for dogs and goats with its 95% confidence interval (dashed lines).

relative to VO_2max and aerobic scope were significant, however % carbohydrate oxidation was more strongly correlated to exercise intensity relative to aerobic scope (r=0.803, P<0.001) than relative to VO_2max (r=0.725, P<0.001).

Acclimation to hypoxia: in vivo VO_2 and fuel selection

At rest, VO_2 and mass-specific VO_2 did not differ between control and acclimated mice despite lower body mass in acclimated mice (Table 4.2, t_{20}=3.9, P=0.001), however both groups had a lower mass-specific VO_2 under hypoxia (effect of environment, $F_{1,20}$=26.1, P<0.001). There was a significant effect of environment ($F_{1,20}$=6.7, P=0.02) and group ($F_{1,20}$=4.8, P=0.04) in resting RER, however there were no significant differences between control and acclimated mice under normoxia (Sidak *post hoc* test, P=0.14) nor under hypoxia (Sidak *post hoc* test, P=0.13). Likewise, there were no significant differences in the resting RER measured under normoxia and hypoxia within acclimated mice (Sidak *post hoc* test, P=0.11) or control mice (Sidak *post hoc* test, P=0.06).

There were no significant effects of group ($F_{1,19}$=2.8, P=0.11) nor environment ($F_{1,19}$=0.4, P=0.51) in VO_2max, however there was a significant interaction ($F_{1,19}$=11.3, P=0.03). Both control and acclimated mice had a reduced VO_2max under hypoxia (Sidak *post hoc* test, P<0.001), however VO_2max of acclimated mice was only reduced by 17% under hypoxia whereas VO_2max of control mice was reduced by 26%. Consequently, under hypoxia, acclimated mice had a higher VO_2max than control mice (Sidak *post hoc* test, P=0.003). The speed at which VO_2max was reached was 26% and 27% lower under

Table 4.2 Resting oxygen consumption (VO_2), resting respiratory exchange ratio (RER), aerobic capacity (VO_2max), speed at VO_2max, and aerobic scope variables of CD1 mice after a chronic exposure to sea level (control) or hypobaric hypoxia (acclimated). All metabolic tests were done under both normoxia (21% O_2) and hypoxia (12% O_2). Values are means ± s.e.m.

	Control		Acclimated	
	Normoxia	*Hypoxia*	*Normoxia*	*Hypoxia*
N	12		10	
Body mass (g) [a]	38.2±1.0		33.3±0.6[#]	
Resting VO_2 ($mlO_2\ hr^{-1}$) [b]	125±4	103±5	115±8	103±6
Resting VO_2 ($mlO_2\ g^{-1}\ hr^{-1}$)	3.3±0.1	2.7±0.2*	3.4±0.2	3.1±0.2*
Resting RER	0.82±0.01	0.80±0.01	0.83±0.01	0.82±0.01
VO_2max ($mlO_2\ hr^{-1}$) [b]	310±5	225±4*	292±6	241±5*[#]
VO_2max ($mlO_2\ g^{-1}\ hr^{-1}$)	8.07±0.16	5.96±0.15*	8.77±0.16[#]	7.24±0.16*[#]
Speed at VO_2max ($m\ min^{-1}$)	32.5±0.8	24.0±0.9*	35.2±1.1	25.6±1.2*
Aerobic scope ($mlO_2\ g^{-1}\ hr^{-1}$)	4.8±0.2	3.2±0.2*	5.3±0.2	4.1±0.3*[#]

[a] average body mass recorded prior to VO_2max tests
[b] body mass used as covariate
*significantly different from normoxia
[#] significantly different from control

hypoxia in control and acclimated mice, respectively (effect of environment, $F_{1,20}$=107, P<0.001; Sidak *post hoc* tests P<0.001). Acclimated mice reached a slightly higher speed at VO_2max than control mice under both normoxia and hypoxia but the effect was not significant (group main effect, $F_{1,20}$=3.6, P=0.07). Aerobic scope was also significantly reduced under hypoxia in both control and acclimated mice (environment main effect $F_{1,20}$=83, P<0.001) but the aerobic scope of acclimated mice was 28% higher than control mice under hypoxia (Sidak *post hoc* test, P<0.05).

The proportional use of carbohydrates and lipids was unaffected by acclimation to hypobaric hypoxia in CD1 mice (Table 4.3). There were no significant differences in % carbohydrates oxidized between control and acclimated mice at both exercise intensities studied (65% of VO_2max: t_{15}=-0.1, P=0.89; 80% of VO_2max: t_{20}=-1.6, P=0.12) under normoxia. Likewise, under hypoxia, there were no significant changes in % carbohydrates oxidized at 80% of VO_2max (t_{20}=-1.6, P=0.13). At 65% of VO_2max, both control and acclimated mice had similar rates of carbohydrate and lipid oxidation. At 80% of VO_2max, absolute rates of carbohydrate oxidation were higher in acclimated mice under both normoxia and hypoxia, however the difference was significant only when rates were mass-specific (normoxia: t_{20}=-2.4, P=0.03; hypoxia: t_{20}=-2.8, P=0.01). Conversely, there were no significant differences in mass-specific lipid oxidation rates between control and acclimated mice for that intensity. Yet acclimated mice generally showed lower whole-body lipid oxidation rates than control mice, but the difference was significant only at 80% VO_2max under normoxia (t_{20}=2.2, P=0.04).

Table 4.3 Respiratory exchange ratio (RER), treadmill speeds and carbohydrate (CHO) and lipid oxidation at different relative exercise intensities (%VO_2max or % aerobic scope) under normoxia (21% O_2) and hypoxia (12% O_2) in CD1 mice after a chronic exposure to sea level (control) or hypobaric hypoxia. Values are means ± s.e.m.

	Target exercise intensities		
	65% VO_2max	*80% VO_2max*	
	Normoxia	*Normoxia*	*Hypoxia*
N			
Control	7	12	12
Acclimated	10	10	10
Respiratory exchange ratio (RER)			
Control	0.83±0.01	0.87±0.01	0.79±0.01
Acclimated	0.83±0.01	0.9±0.01	0.81±0.01
%CHO oxidation (% of total VO_2)			
Control	41.4±3.2	56.0±3.7	29.3±3.1
Acclimated	42.0±3.0	65.3±4.5	36.6±3.4
Absolute rate of CHO oxidation (µmol O_2 g^{-1} hr^{-1})			
Control	98±8	157±9	62±7
Acclimated	103±8	198±16*	94±10*
Absolute rate of CHO oxidation (µmol O_2 hr^{-1})			
Control	3656±289	6084±462	2357±261
Acclimated	3465±271	6652±551	3088±308
Absolute rate of lipid oxidation (µmol O_2 g^{-1} hr^{-1})			
Control	140±10	125±12	148±7
Acclimated	142±7	104±13	160±7
Absolute rate of lipid oxidation (µmol O_2 hr^{-1})			
Control	5195±333	4722±374	5618±247
Acclimated	4754±231	3458±435*	5277±240
Actual exercise intensities (% VO_2max)			
Control	64.1±1.0	78.2±0.6	78.8±0.6
Acclimated	62.7±0.6	77.2±0.6	78.8±0.6
Actual exercise intensities (% aerobic scope)			
Control	38.5±1.9	63.1±1.4	60.4±1.9
Acclimated	37.8±2.2	61.7±2.1	61.7±2.5
Treadmill speed (m min^{-1})			
Control	5.0±1.2	14.3±0.7	6.0±1.0
Acclimated	5.6±1.0	17.0±1.6	7.9±1.4

*significantly different from control at the same intensity and condition (P<0.05)

Acclimation to hypoxia: muscle plasticity

No changes were found in any of the enzymes investigated in the gastrocnemius of control and acclimated mice (Figure 4.2). However, the activity of enzymes indicative of oxidative capacity (CS, IDH) and of carbohydrate (PK) and lipid (HOAD) oxidation were significantly reduced in the left ventricle (Figure 4.3) of acclimated mice (two-tail t-tests, $P<0.05$). Likewise, H-FABP content was significantly lower in the left ventricle of acclimated mice ($t_{17}=3.4$, $P=0.003$). Left ventricle wet weights were not significantly different between control and acclimated mice (control: 121 ± 4 mg, acclimated: 105 ± 9 mg; $t_{20}=1.8$, $P=0.1$), however acclimated mice showed right ventricular hypertrophy (control: 37 ± 2 mg, acclimated: 45 ± 3 mg; $t_{20}=2.3$, $P=0.03$)

Discussion

In this study we found that, like other mammals, mice increase the use of carbohydrates as exercise intensity increases towards an aerobic maximum. However, the overall pattern of fuel use during exercise is different in mice compared to larger quadrupeds as they rely on carbohydrates to a lesser extent at moderate exercise intensities relative to individual VO_2max. This is likely due to important reductions in aerobic scope as body size decreases, resulting in very low efforts representing high % of VO_2max. We show that when exercise is expressed as a % of aerobic scope rather than aerobic capacity there is a better overall model of mammalian fuel use which extends to the lower range of body size. We thus revisit the mammalian model of fuel selection

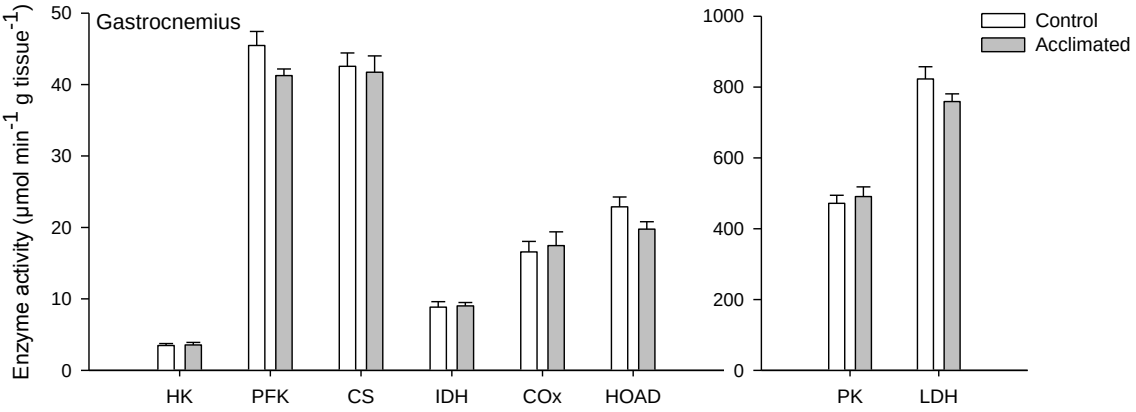

Figure 4.2 Mean + s.e.m. activity (Vmax) of hexokinase (HK), phosphofructokinase (PFK), citrate synthase (CS), isocitrate dehydrogenase (IDH), cytochrome c oxidase (COx), β-hydroxyacyl-CoA dehydrogenase (HOAD), pyruvate kinase (PK), and lactate dehydrogenase (LDH) in gastrocnemius of CD1 mice after a chronic exposure to sea level (control) or hypobaric hypoxia (acclimated).

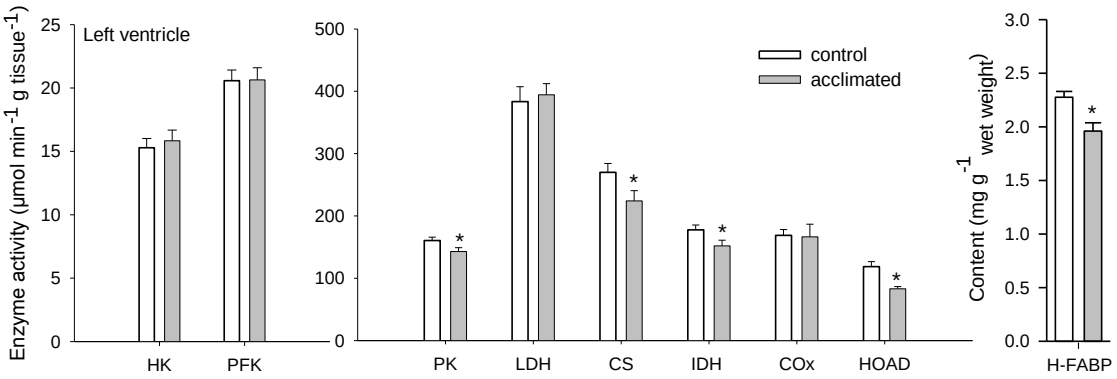

Figure 4.3 Mean + s.e.m. activity (Vmax) of hexokinase (HK), phosphofructokinase (PFK), pyruvate kinase (PK), lactate dehydrogenase (LDH), citrate synthase (CS), isocitrate dehydrogenase (IDH), cytochrome c oxidase (COx), β-hydroxyacyl-CoA dehydrogenase (HOAD); and mean + s.e.m. of heart type fatty acid binding protein (H-FABP) content in left ventricle of CD1 mice after a chronic exposure to sea level (control) or hypobaric hypoxia (acclimated). Asterisks (*) denote significant differences.

previously described by others (McClelland, 2004; Roberts et al., 1996c; Weber and Haman, 2004) as such: exercise intensity expressed as a fraction of aerobic scope (as opposed to VO_2max) determines the relative contribution of carbohydrates and lipids to total energy expenditure in mammals. Moreover, we found that acclimation to hypobaric hypoxia has little effect on whole-body fuel selection pattern, and does not enhance the capacity for carbohydrate oxidation in lowland native mice *Mus musculus*.

Aerobic scope versus VO_2max

When considering aerobic exercise, two definitions are important to understanding relative levels of effort. Aerobic scope expresses the range of oxidative metabolism from rest to maximal exercise, whereas VO_2max is defined as the maximal aerobic capacity of an animal with no consideration given to basal metabolism. Accordingly, our data show that the proportion of carbohydrates and lipids used during exercise is dependent of the maximum amount of oxygen available to fuel locomotion (aerobic scope) as opposed to the maximum amount of oxygen available (VO_2max) to fuel both locomotion and other energy-requiring body functions (resting metabolism). It is not surprising that others (McClelland, 2004; Roberts et al., 1996c; Weber and Haman, 2004) have overlooked the importance of aerobic scope in determining the proportions of fuel used during exercise. Because of allometry, mass-specific resting metabolic rates increase with decreasing body mass (Kleiber, 1932) and accordingly, the fraction of VO_2max corresponding to resting metabolism is much smaller in large mammals than in small mammals. Thus the variation created by differences in mass-specific resting

metabolic rates is negligible in larger mammals but becomes more important as body mass decreases. In fact, we show that like the mice investigated in this study, rats (data from McClelland and colleagues (McClelland et al., 1998)) also deviate from the mammalian pattern of fuel selection at intensities corresponding to a low percentage of their VO_2max. The discrepancy in fuel selection between small and large mammals becomes more pronounced at low exercise intensities (relative to VO_2max) because the fraction of VO_2 corresponding to resting VO_2 becomes increasingly larger as exercise intensity decreases.

Effect of acclimation to hypoxia on fuel selection patterns

Absolute mass-specific rates of carbohydrate oxidation were elevated at 80% of VO_2max in acclimated mice compared to control mice but this increase was scaled with differences in VO_2max. The result is that both groups showed the same proportional use of fuels. Accordingly, our data suggest that acclimation to hypobaric hypoxia does not affect fuel selection patterns in mice at all relative exercise intensities studied under normoxia and hypoxia. This study joins others (Lundby and Van Hall, 2002; McClelland et al., 1998) in supporting the idea that fuel selection patterns are robust to a chronic exposure to high altitude in lowland native mammals, and demonstrates that the preference for carbohydrates observed in high altitude native *Phyllotis* mice is not part of an acclimatization response common to all mammals.

Normoxia versus hypoxia

Both control and acclimated mice suffered reductions in VO_2max under hypoxia; however this reduction was partially mitigated in acclimated mice where VO_2max was reduced to a lesser degree than control mice. This is consistent with the literature on other mammals, including humans, as it is widely established that acute hypoxia (equivalent to ~4200 m) induces a 20-35% decrease in VO_2max and that acclimation to hypoxia substantially lessens this decrease (Brutsaert, 2008; Hochachka and Somero, 2002). The drop in VO_2max, which is largely explained by decreases in O_2 delivery to muscles (Calbet et al., 2003), is probably attenuated in acclimated individuals through acclimation processes aimed at compensating for the deficit in arterial O_2 content.

Acute hypoxia also elicited reductions in resting VO_2 in both control and acclimated mice. This hypoxia induced drop in resting VO_2, also well documented (Gautier, 1996; Mortola, 1993; Robinson and Haymes, 1990; Tattersall and Milsom, 2003), is not compensated by anaerobic metabolism; instead it is the result of a metabolic suppression associated with altered mechanisms of thermoregulation (Mortola, 2004). In fact resting RER was actually slightly lower under hypoxia in both groups of mice, confirming that anaerobic metabolism is not enhanced upon exposure to hypoxia at rest. Similar reports of slight decreases in resting RER under hypoxia in mammals are scarce (Tattersall and Milsom, 2003). A number of studies actually report higher RER values under hypoxia compared to normoxia (Katayama et al., 2007; Liu et al., 2009). Acute hypoxia exposure induces a hyperventilatory response which results in an increase amount of CO_2 exhaled. Thus, under hypoxia, measurements taken prior to reaching

steady states would produce inflated RER values. Tattersall and Milsom (Tattersall and Milsom, 2003) observed high RER values in resting squirrels after 15 minutes of hypoxia exposure, yet after 60 minutes, these returned near normoxic levels or slightly under. We thus believe our RER values to be accurate and that previous reports of elevated RER under hypoxia (Katayama et al., 2007; Liu et al., 2009) are not representative of steady-state values because they were taken within 10 minutes of exposure to hypoxia. Nevertheless, the reasons why a decrease in RER would occur during hypoxia are intriguing. Unlike during exercise, at rest the contribution of protein to total energy metabolism is significant and must thus be taken into account (Carraro et al., 1994; Rennie et al., 1981). Low RER values can thus be interpreted by an increased contribution of proteins, lipids, and or perhaps ketone bodies (Zwemer et al., 2007). While both lipids and proteins are not particularly considered O_2 efficient fuels, ketone bodies on the other hand are (Sato et al., 1995; Veech, 2004). A few studies suggest an increase use of ketones during severe hypoxia exposure (Kirsch and D'Alecy, 1984; Zwemer et al., 2007). It is thus possible that the hypoxia induced decrease in RER observed here is the result of a small increase in ketone metabolism, however further research is warranted.

Effect of acclimation to hypoxia on muscle metabolism

Modifications in muscle phenotype, such as alteration in capacities for flux through metabolic pathways, represent one of the factors potentially affecting whole-body fuel use. We measured the activity (apparent Vmax) of enzymes, which provide insights

on the maximum flux of glycolysis (HK, PFK, PK), pyruvate to lactate (LDH), and β-oxidation (HOAD), as well as aerobic enzymes from the Krebs cycle (CS, IDH) and from the electron transport chain (COx). No changes were found between control and acclimated mice in any of the enzymes investigated in the gastrocnemius, suggesting that the capacity for flux through metabolic pathways is unaffected by chronic hypoxia in that particular skeletal muscle. Generally it is thought that oxidative capacity of skeletal muscle is reduced after chronic exposure to hypoxia (Hoppeler and Vogt, 2001), however this claim is predominantly based on exposures to extreme altitudes (well above 5000 m) (Green et al., 1989; Hoppeler et al., 1990; Howald et al., 1990). Most studies investigating the effects of acclimation to moderate or high altitude (2500-5000m) actually report little changes in skeletal muscle oxidative capacity (no change in Vmax of CS) (Beaudry and McClelland, 2010; Kennedy et al., 2001; Le Moine et al., 2011; Perhonen et al., 1996).

The contribution of cardiac muscle to whole body metabolism is minimal during exercise, and accordingly changes in cardiac muscle fuel use should not significantly affect whole-body fuel selection. However, the effect of chronic hypoxia on cardiac muscle metabolism is of interest here because the heart occupies a central role in oxygen delivery to working muscles during exercise and it is a continuously working organ, especially in situations of low PO_2. Cardiac muscle metabolism is opportunistic; it mainly utilizes fatty acids, but can also use glucose, lactate, ketone bodies and amino acids under certain circumstances (Kodde et al., 2007). We found lower activities of PK, HOAD, CS and IDH in the left ventricle of acclimated mice, suggesting that the capacity for both

carbohydrate and lipid oxidation is reduced in the heart as a result of chronic hypoxia. Decreases in CS and/or HOAD have previously been reported in cardiac muscle in response to chronic hypoxia (Cai et al., 2010; Daneshrad et al., 2000; Kennedy et al., 2001; Templeman et al., 2010) and is consistent with the idea of decreased oxidative capacity of the heart in response to acclimation to high altitudes. However, a lowered capacity for carbohydrate metabolism, suggested by the decrease in PK, is incongruous with a number of high altitude acclimation studies in which it appeared to be either unchanged (Daneshrad et al., 2000) or enhanced (Sivitz et al., 1992) following exposure to chronic hypoxia. Our results thus suggest that acclimation to hypoxia decreases the oxidative capacity of the heart but does not appear to enhance the capacity for carbohydrate utilization.

In this study acclimated mice also exhibited a lower content of H-FABP, a protein which function is to facilitate cellular uptake and transport of fatty acid into the cytoplasm, and potentially influenced by fatty acid oxidation capacity (Schaap et al., 1998). In general, H-FABP content positively correlates with the oxidative capacity of muscles. Accordingly, a low H-FABP content is consistent with the reduced CS and HOAD activity observed in these mice and strengthens the claim of reduced oxidative capacity of the heart in response to hypoxia. Furthermore, our results agree with previous work, where a 20% decrease in myocardial FABP content was observed in rats after a 3-week exposure to hypoxia (Garnier et al., 1993).

Conclusion

This study proposes a new model of fuel selection for exercising mammals, where exercise intensity relative to aerobic scope (in lieu of VO_2max) determines the proportional contribution of carbohydrate and lipid oxidation to total energy expenditure. Unlike the previous model first proposed by Roberts and colleagues (Roberts et al., 1996c) and more recently endorsed by many (McClelland, 2004; Weber, 2011; Weber and Haman, 2004), this pattern promises to hold across a wider range of body size, including mice and potentially smaller mammals. Moreover, this study demonstrates that acclimation to high altitude does not affect whole-body fuel selection patterns and does not induce a metabolic reorganization at the muscle level to enhance the capacity for carbohydrate oxidation. These results are useful in assessing the extent of plasticity of fuel use and muscle metabolism in high altitude native mammals, and thus aid in determining the adaptive nature of the traits investigated in Chapter 2 and 3.

CHAPTER 5

GENERAL DISCUSSION

Increased use of carbohydrates: an adaptation to high altitude

This thesis offers significant advancements in the field of high altitude physiology as it provides the first compelling evidence of elevated carbohydrate utilization in high altitude mammals. This was evident through both intraspecific (Chapter 2) and interspecific (Chapter 3) comparisons of whole body fuel use in *Phyllotis* mice native to high and low altitudes. I believe that the trait evolved as an adaptation to the low PO_2 at high altitude. The benefits associated with using proportionally more carbohydrates than fatty acids are clear: 15- 30% more energy produced per molecule of O_2 consumed (Brand, 2005; Daut and Elzinga, 1989; Korvald et al., 2000; Welch et al., 2007), resulting in a lowered O_2 debt in hypoxic conditions and thus a greater potential to allocate ATP to energy demanding processes or tasks (e.g. immunity, digestion, growth, thermoregulation, reproduction, foraging, predation avoidance). Theoretically, it is possible that this trait would be associated with an increased fitness in low oxygen environments, and given that it is heritable, it could have likely evolved through natural selection. Clearly, empirical evidences are needed to establish its association with fitness and to assess the extent of its heritability.

Unlike most studies on adaptation to high altitude, I carefully evaluated other factors which could also be responsible for the observed phenotypic differences between

the high and low altitude species. These include the phylogenetic relationships of the species and plasticity during development and adulthood. In chapter 3, phylogenetic independent contrast analyses revealed a positive relationship between carbohydrate use and altitude, suggesting that the elevated use of carbohydrates in high altitude *Phyllotis* is independent of phylogeny. Similarly, the trait does not appear to be affected by exposure to high altitude through development and adulthood. The mix of fuel used during exercise was not affected by acclimation to hypobaric hypoxia in *Mus musculus* (Chapter 4) and was similar in high and low altitude native *P. limatus* (Appendix A). Taken together, these results further support the claim that the elevated use of carbohydrates in high altitude native *Phyllotis* mice represents an adaptation to high altitude hypoxia. Future studies should aim at strengthening this claim by investigating fuel use in *Phyllotis* descendents of the populations investigated in this thesis. These would preferably be second generations of the captured mice raised under common garden conditions to remove any environmental effects on the traits under study.

Skeletal and cardiac muscle metabolism at high altitude

Short (6 weeks) and long-term (over many generations) exposures to high altitude seem to have little effect on the capacity for flux through metabolic pathways of skeletal muscles. In the gastrocnemius, activities of enzymes involved in carbohydrate and lipid oxidation were similar among high and low altitude *Phyllotis* species (Chapter 3) and between control and acclimated *Mus musculus* (Chapter 4). Accordingly, the elevated whole-body use of carbohydrates observed in the high altitude *Phyllotis* species

does not seem to be facilitated by an increased capacity for carbohydrate oxidation in skeletal muscles. The mechanisms involved in this shift of whole-body fuel use are unknown. *In vivo* regulation of flux through metabolic pathways is complex and poorly understood, however one likely site of control of carbohydrate oxidation is at the level of pyruvate dehydrogenase (PDH), an enzyme complex responsible for the conversion of pyruvate to acetyl-CoA. This enzyme plays a central role in carbohydrate metabolism, because its activity directly influences whether pyruvate enters anaerobic or aerobic pathways. Regulation of PDH is tightly regulated *in vivo* by reversible phosphorylation and dephosphorylation resulting in the inactivation or activation of PDH (Patel and Korotchkina, 2006). Modifications in the mechanisms involved in the elaborate regulation of the PDH complex could thus affect carbohydrate oxidation rates and change the relative contribution of carbohydrates toward energy metabolism. I attempted to evaluate the degree of activation of PDH at rest in high and low altitude *P. andium* through measurements of protein abundance with western blots. Unfortunately, most commercially available antibodies did not react with the target proteins of *P. andium* mice. I was only able to measure the total abundance of PDH in cardiac muscles, which was not significantly different between the high and low altitude populations (Appendix B, Figure B.1). Future studies should be directed on determining PDH activity in skeletal and cardiac muscles of *Phyllotis* mice during exercise, and perhaps on developing relevant antibodies for these species.

Since previous studies suggested that the heart of high altitude native men is geared towards a greater use of carbohydrates compared to lowlanders (Hochachka et al.,

1996; Holden et al., 1995), I also investigated capacity for carbohydrate and lipid oxidation in cardiac muscles in high and low altitude *Phyllotis* mice. As observed in skeletal muscles, there were no strong indications that high altitude *Phyllotis* mice have evolved a greater capacity for carbohydrate utilization in the heart. However, cardiac muscles of high altitude mice were characterized by high oxidative capacities. This appears to be a common feature of highland mammals, as earlier studies have also reported similar results in Andean camelids, deer (Hochachka et al., 1983) and high altitude pikas (Sheafor, 2003). Conversely, acclimation to high altitude actually decreases the oxidative capacity of cardiac muscles (Chapter 4). The heart is a continuously active organ occupying a central role in oxygen delivery; accordingly, proper cardiac function is essential, especially under hypoxic conditions. High oxidative capacities could contribute to sustain high cardiac outputs, which would ultimately help maintaining adequate O_2 delivery to skeletal muscles. A high oxygen consumption capacity is also particularly important for highland mammals to support the high metabolic requirement of thermogenesis in these cold environments.

Like skeletal muscles, more research is needed on cardiac muscle metabolism of high altitude mammals to assess the relative importance of carbohydrate oxidation *in vivo*. In addition, an integrative approach should be taken to elucidate the mechanisms involved in proper cardiac function at high altitude. One way to tackle the challenge is to look at global differences in gene and protein expression in cardiac muscles of high and low altitude *Phyllotis* mice. Through the use of microarrays, I looked at global differences in gene expression in left ventricles of individuals from the high and low altitude

populations of *P. andium* and found over 20 genes especially important for energy metabolism whose expression was significantly different between populations (Appendix C, Table C.1). Future efforts should be directed towards the validation of the microarrays with real-time PCR (Appendix C, Figure C.1) as well as elucidating the effects of the differential expressions at the protein level.

Conserved patterns of mammalian fuel selection

For over a decade, patterns of fuel selection during exercise were thought to be conserved in mammals when exercise intensity is expressed relative to VO_2max (Roberts et al., 1996c). However, prior to this thesis, this claim had not been tested on small mammals, such as mice; and had never been subjected to any statistical analysis. By combining data of mice (*Mus musculus*) with rats, dogs and goats from previously published studies, I found that exercise intensity relative to aerobic scope (instead of VO_2max) is a better predictor of the proportion of carbohydrates and lipids oxidized (Chapter 4). As part of this thesis, I thus revisited the former claim and proposed that patterns of fuel selection are conserved in mammals when exercise intensity is expressed relative to aerobic scope. Figure 5.1 illustrates patterns of carbohydrate oxidation of all species studied in this thesis. High altitude *Phyllotis* species appear to use a greater proportion of carbohydrates than lowland *Phyllotis* and *Mus muculus* at all exercise intensities, even when they are expressed relative to aerobic scope. This inherent difference between highland and lowland mice represent an exceptional system to

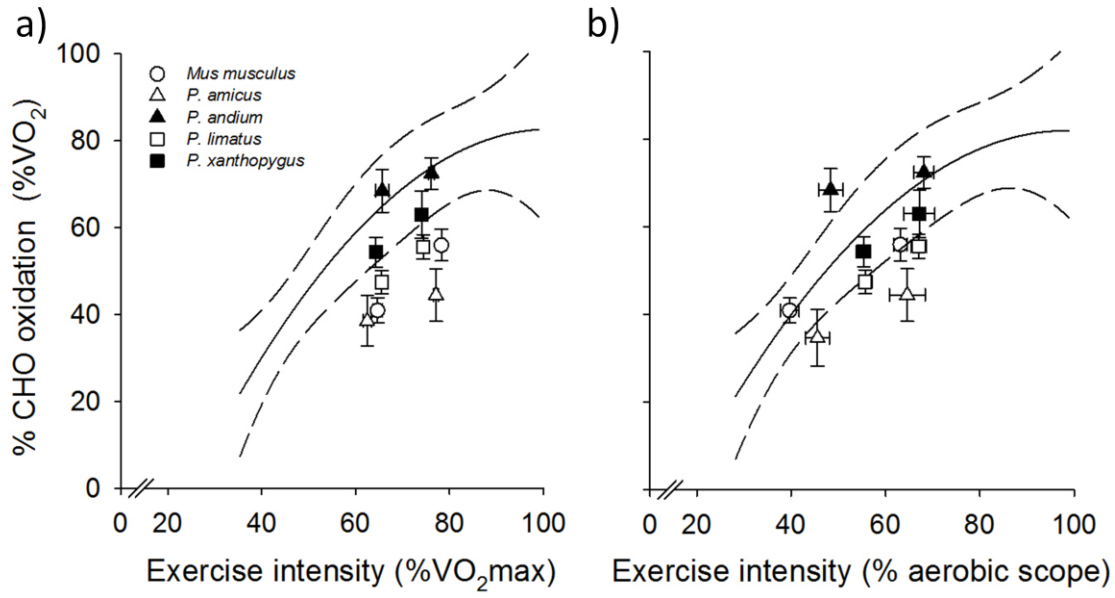

Figure 5.1 Mean ± s.e.m. percent of oxygen used for carbohydrate (CHO) oxidation ($\%VO_2$) with relative exercise intensities expressed as (a) % of aerobic capacity (VO_2max) and (b) % of aerobic scope in lowland (open symbols) *Mus muculus* (Chapter 4), *P. amicus*, and *P. limatus*, and highland (filled symbols) *P. andium* and *P. xanthopygus* (Chapter 3). Full lines represent the regression for dogs and goats (Chapter 4) with its 95% confidence interval (dashed lines).

investigate the underlying mechanisms of mammalian fuel selection, which still remain poorly understood (McClelland, 2004).

It is likely that the general pattern is a reflection of the selective recruitment of different muscles, different fiber types within muscles, and/or different metabolic pathways within fibers (Weber and Haman, 2004). At low exercise intensities slow fibers (type I), specialized for lipid oxidation, are predominantly recruited. As work rates increase, fast (type II) glycolytic fibers are then used to support high rates of ATP production (Laughlin and Armstrong, 1982). Similarly, it is also expected that a selective recruitment of different metabolic pathways occur within muscle fibers. Regulation may take place at several steps along pathways, possibly through calcium dependent signaling or through activation of AMP-activated protein kinase (AMPK) (Jorgensen et al., 2006). The activation of AMPK, which results from decreases in cell energy status (high AMP:ATP), is associated with increased glucose and fatty acid uptake in muscles as well as enhanced fatty acid oxidation. Accordingly, fuel selection during exercise under normoxic and hypoxic conditions may be acutely regulated by AMPK through disturbances in cell energy status. Even though AMPK activity increases in a time and exercise-intensity-dependent manner, it is still unclear whether there is a conserved pattern in its degree of activation with intensities of exercise relative to VO_2max or aerobic scope in normoxic and hypoxic conditions. AMPK activity goes up with increasing absolute or relative exercise intensity under normoxia (Chen et al., 2003; Musi et al., 2001), however humans exercising at the same relative exercise intensity (~72% VO_2max) have a higher muscle AMPK activity and free AMP levels under normoxia than

hypoxia (Wadley et al., 2006). This either suggests that absolute intensity dictates the degree of activation of AMPK acutely or that hypoxia itself results in low AMPK activity because of hypoxia-induced modification in energy status of muscles. Accordingly, metabolic changes triggered by AMPK may affect flux through pathways, but it is unclear whether they take part in regulating the proportional contribution of carbohydrate and lipid oxidation to total energy expenditure at relative exercise intensities.

Conclusion

As pointed out in the introduction (Chapter 1), little was known on whole-body fuel use and muscle metabolism of high altitude mammals. In 1985, Hochachka (Hochachka, 1985) hypothesized that carbohydrates would be the preferred fuel at high altitude because of its oxygen-saving advantage over fatty acids. Nearly three decades later, this thesis provides the first convincing evidence of elevated carbohydrate utilization in a high altitude mammal. This phenotype appears to have evolved as an adaptation to the low PO_2 associated with high altitude as both phylogenetic history, and acclimation to hypobaric hypoxia had little effect on these results. As future studies uncover the mechanisms responsible for the elevated carbohydrate use in high altitude *Phyllotis*, significant advancements would also be made to explain fuel selection in mammals in general. Accordingly, this thesis offers significant advancements not only in the field of high altitude physiology but also in the field of mammalian energetics.

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APPENDIX A

Respiratory data and whole body fuel selection in the low (100 m) and high (3700 m) altitude populations of *P. limatus*

Table A.1 Means ± s.e.m. of body mass and respiratory data of the low (100 m) and high (3700 m) altitude populations of *P. limatus* under normoxic and hypoxic conditions, together with statistical results of comparisons (two-tail t-tests).

	Populations		Statistics
	100 m	*3700 m*	
N	10-12	10-12	
Body mass (g)	44.7±1.7	41.4±2.3	t_{22}=1.2, P=0.3
VO_2max (ml g^{-1} ·hr^{-1})			
Normoxia	6.58±0.17	6.40±0.20	t_{22}=-0.18, P=0.5
Hypoxia	5.41±0.26	5.99±0.21	t_{22}=-1.74, P=0.1
Aerobic scope (ml g^{-1} ·hr^{-1})			
Normoxia	4.99±0.18	4.85±0.16	t_{18}=0.57, P=0.6
Hypoxia	3.65±0.21	4.30±0.20	t_{19}=-2.21, P=0.03
Resting VO_2 (ml g^{-1} hr^{-1})			
Normoxia	1.49±0.07	1.61±0.13	t_{21}=-0.79, P=0.4
Hypoxia	1.58±0.06	1.73±0.10	t_{21}=-1.33, P=0.2
Resting RER			
Normoxia	0.795±0.008	0.776±0.007	t_{21}=-1.61, P=0.1
Hypoxia	0.763±0.008	0.761±0.006	t_{21}=0.20, P=0.8

Table A.2 Means ± s.e.m. of respiratory exchange ratio (RER), percent CHO oxidized, and absolute rates of fuel use in low (100 m) and high (3700 m) altitude populations of *P. limatus* during treadmill running at a target intensity of 75% of VO_2max under normoxic and hypoxic conditions, together with statistical results of comparisons (two-tail t-tests, unless otherwise stated) and actual exercise intensities and treadmill speeds.

	Populations		Statistics
	100 m	*3700 m*	
RER			
Normoxia [a]	0.869±0.008	0.878±0.018	U=47, P=0.9
Hypoxia	0.850±0.010	0.860±0.009	t_{19}=-0.7, P=0.5
% CHO (% total VO_2)			
Normoxia	55.6±2.8	58.6±6.1	
Hypoxia	49.0±3.6	52.4±3.1	
Absolute rate of CHO oxidation (µmol O_2 min^{-1})			
Normoxia	92±6	82±10	
Hypoxia	67±7	72±7	
Absolute rate of CHO oxidation (µmol O_2 g^{-1} hr^{-1})			
Normoxia	119±6	123±10	t_{18}=-0.3, P=0.8
Hypoxia	89±8	106±6	t_{19}=-1.6, P=0.1
Absolute rate of lipid oxidation (µmol O_2 min^{-1})			
Normoxia	73±5	56±8	
Hypoxia	67±4	63±3	
Absolute rate of lipid oxidation (µmol O_2 g^{-1} hr^{-1})			
Normoxia	96±8	92±16	t_{18}=0.2, P=0.8
Hypoxia	92±7	97±8	t_{19}=-0.5, P=0.6
Exercise intensity (% VO_2max)			
Normoxia	74.4±0.8	74.2± 0.9	
Hypoxia	77.5± 0.6	75.6±0.9	
Exercise intensity (% aerobic scope)			
Normoxia	67.0±1.6	60.4±1.8	
Hypoxia	65.2±1.9	64.6±2.2	
Treadmill speed (m min^{-1})			
Normoxia	14.5±1.0	13.8±0.7	
Hypoxia	10.7±0.7	12.7±1.0	

[a] Mann-Whitney U test

APPENDIX B

Protein abundance of pyruvate dehydrogenase in the left ventricle of the 300 m and 4000 m populations of *P. andium*

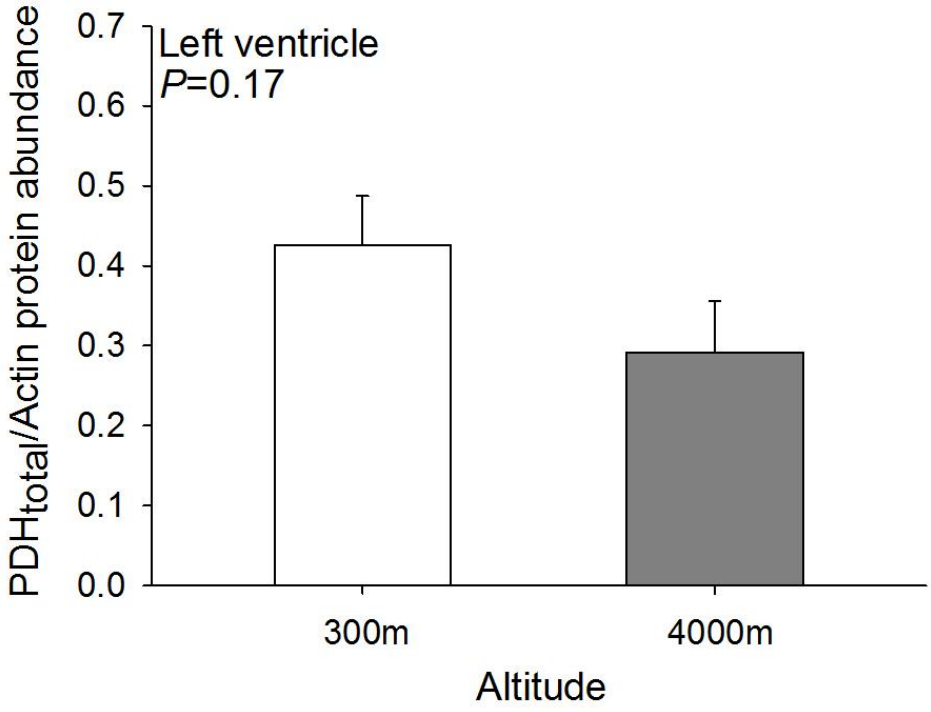

Figure B.1 Protein abundance of pyruvate dehydrogenase complex (PDHtotal) relative to actin in the left ventricles of the 300 m and 4000 m populations of *P. andium.* N= 5 for each population.

APPENDIX C

Differences in gene expression in left ventricle of the 300 m and 4000 m populations of *P. andium*

Table C.1 Metabolic genes of interests that were either up-regulated or down-regulated by at least 1.45 fold in left ventricles of HA *P. andium* compared to LA *P. andium*. Data obtained from gene expression profiling experiments using the Mouse Genome 430 2.0 array. Genes in bold are the selected genes for the validation of these data with qPCR

Gene	Regulation	Fold change
Phosphoenolpyruvate carboxykinase 1, cytosolic (PEPCK)	up	6.8806157
forkhead box K1	up	2.2462761
NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2	up	1.9381446
solute carrier family 25 (mitochondrial carrier, peroxisomal membrane protein), member 17	up	1.604365
ets variant gene 2	up	1.5459765
proline-rich nuclear receptor coactivator 2	up	1.501668
acyl-Coenzyme A oxidase-like	up	1.4747543
solute carrier family 31, member 2	up	1.4646127
isocitrate dehydrogenase 2 (NADP+), mitochondrial	down	1.9231012
phosphatidic acid phosphatase type 2B	down	1.8669349
mitogen activated protein kinase kinase kinase 4	down	1.7051258
phosphofructokinase, liver, B-type	down	1.704957
similar to protein phosphatase 2a, catalytic subunit, beta isoform	down	1.6734873
glutamate oxaloacetate transaminase 2, mitochondrial	down	1.6656879
protein kinase, AMP-activated, gamma 2 non-catalytic subunit	down	1.6555557
translocase of outer mitochondrial membrane 40 homolog-like (yeast)	down	1.6495429
hexokinase 2	down	1.6494145
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	down	1.6450502
mitogen activated protein kinase kinase kinase 1	down	1.6423583
oxoglutarate dehydrogenase (lipoamide)	down	1.6231519
nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	down	1.5698612
protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform	down	1.5172032
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	down	1.4701625

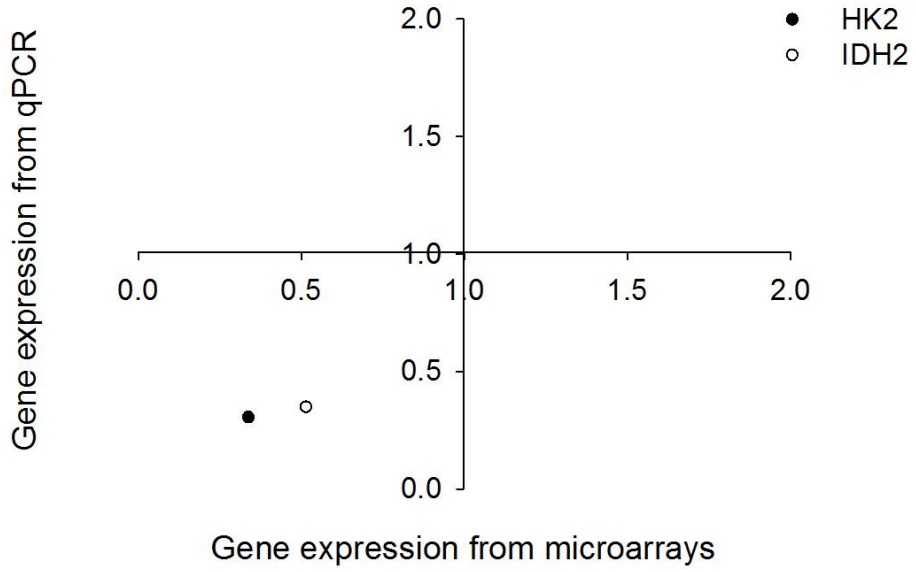

Figure C.1 Microarray validation with qPCR using 2 selected genes involved in metabolism (hexokinase 2 (HK2) and isocitrate dehydrogenase 2 (IDH2)) which were differentially expressed between the 300 m and 4000 m populations of *P. andium* by at least 1.45 fold. Values represent gene expression of left ventricles of the 4000 m population relative to the 300 m population of *P. andium*. Gene expressions from qPCR were normalized to the expression of elongation factor 1 alpha (ef1α) gene.

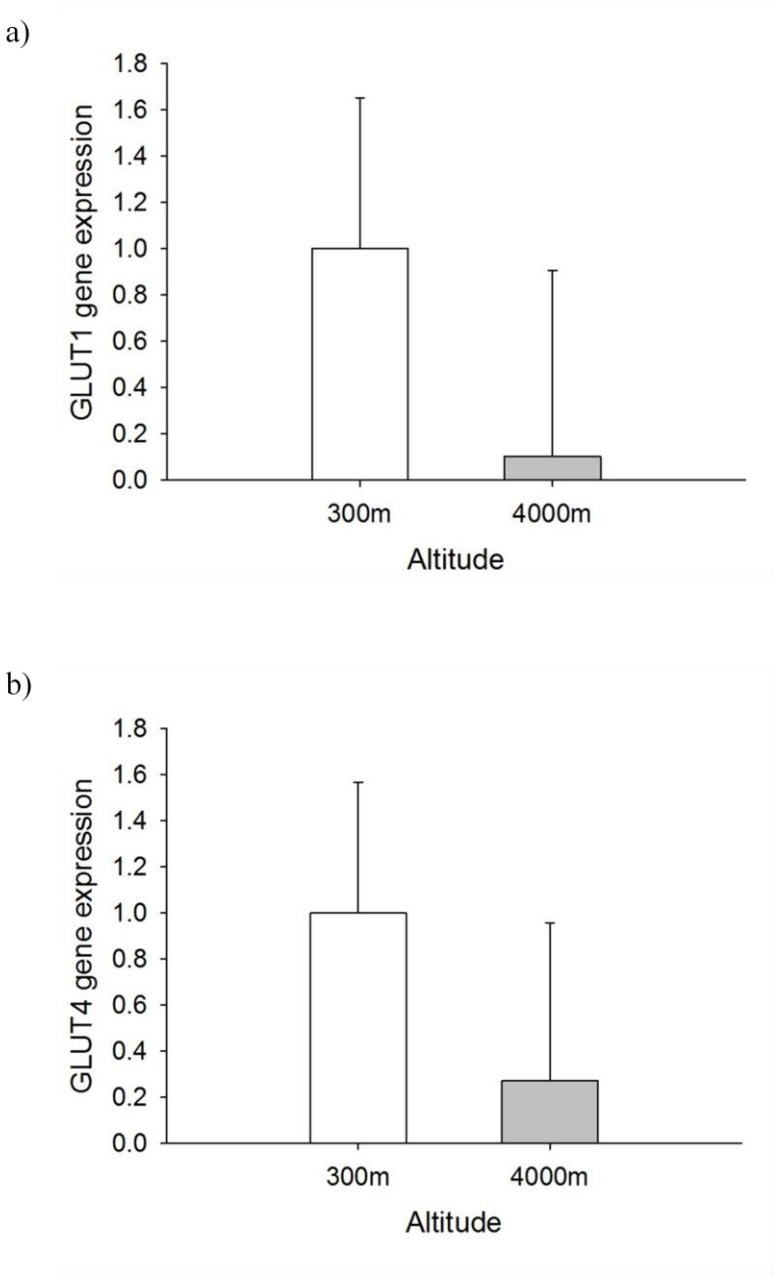

Figure C.2 Gene expression relative to values of the 300 m population of *P. andium* of (a) glucose transporter 1 (GLUT1) and (b) glucose transporter 4 (GLUT4) normalized to elongation factor 1-alpha (ef1α) using qPCR. N=5 for each population. No significant differences were found $P>0.05$.

APPENDIX D

Published articles which are part of this Ph.D. degree but not included as chapters in the present thesis

Article 1:		**Lifetime performance in foraging honeybees: behaviour and physiology.**
	Authors:	Marie-Pierre Schippers, Reuven Dukas, Richard W. Smith, J. Wang, K. Smolen, and Grant B McClelland
	Status:	Published October 2006
	Journal:	Journal of Experimental Biology
	Comments:	This study was conducted by MPS and RD under the supervision of RD and GBM. RWS and KS were responsible for the proteomic analysis and JW for the protein identifications

Schippers, M.-P., Dukas, R., Smith, R.W., Wang, J., Smolen, K., and McClelland, G.B. (2006) Lifetime performance in foraging honeybees: behaviour and physiology. Journal of Experimental Biology. 209:3828-3836.

Article 2:		**Lifetime- and caste-specific flight metabolic rate and muscle biochemistry in honeybees, *Apis mellifera*.**
	Authors:	Marie-Pierre Schippers, Reuven Dukas, and Grant B McClelland
	Status:	Published January 2010
	Journal:	Journal of Comparative Physiology B
	Comments:	This study was conducted by MPS under the supervision of RD and GBM.

Schippers, M.-P., Dukas, R., McClelland, G.B. (2010) Lifetime- and caste-specific flight metabolic rate and muscle biochemistry in honeybees, *Apis mellifera*. Journal of Comparative Physiology B. 180:45-55.