DEVELOPMENTAL AND MATERNAL THERMOREGULATORY ADAPTATIONS TO HIGH ALTITUDE IN THE NORTH AMERICAN DEER MOUSE, *PEROMYSCUS MANICULATUS*

DEVELOPMENTAL AND MATERNAL THERMOREGULATORY ADAPTATIONS TO HIGH ALTITUDE IN THE NORTH AMERICAN DEER MOUSE, *PEROMYSCUS MANICULATUS*

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LAY ABSTRACT

Cold temperatures and less available oxygen make mountain ranges challenging places for small mammals to live. We know very little about how young high-altitude animals overcome these challenges. I have shown that young deer mice adapted to high altitude receive substantially less parental care than their low altitude counterparts, yet they are able to grow at the same rate. They also do to not attempt to burn fuel and oxygen to produce heat until they much older than low altitude mice. Suppressing energetically costly heat production may allow them to save energy, and ultimately survive in their extreme environment.

ABSTRACT

Altricial mammals develop the capacity to independently thermoregulate during the first few weeks of postnatal development. This shift in performance is driven by the maturation of the two primary thermo-effector organs, brown adipose tissue (BAT) and skeletal muscle which are non-functional at birth. In wild rodent populations this is also a time of high mortality (50-95%), making the physiological systems that develop during this period potential targets for natural selection. High altitude is a particularly challenging environments for small endotherms due to unremitting low O_2 availability and consistently low ambient temperatures. As a result, some high-altitude taxa have evolved a superior *adult* thermogenic capacity. However, it is unclear if selection has occurred to survive these unique challenges early in development. The goal of my thesis is to address this gap in knowledge and assess developmental thermoregulatory adaptations in an altricial high altitude endotherm. I used deer mice (*P. maniculatus*) native to high and low altitude population, and a strictly low altitude species (*P. leucopus*), all raised under common garden conditions to assess how high altitude adaptation has altered the onset of endothermy, developmental plasticity of thermogenic capacity and maternal care. I have shown that selection has fundamentally delayed the maturation of the BAT and skeletal muscle in high altitude natives and has altered their capacity for developmental plasticity. High altitude pups are able to maintain growth rates and body composition under cold hypoxic conditions despite receiving less maternal care than their low altitude counterparts. These data suggest that high altitude adapted mice have evolved to suppress energetically costly thermogenesis during development.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance

a.s.l: Above sea level

B-3-AdR: Beta adrenergic receptor- 3

BAT: Brown adipose tissue

BMR: Basal metabolic rate

BSA: Bovine serum albumin

CAD: Capillary areal density

CLD: Capillary length density

CH: Combined cold hypoxia

CO_2: Carbon dioxide

CS: Citrate synthase

CT: micro-computed tomography

FDG: [^{18}F] fluorodeoxyglucose

DAPI: 4',6- diamidino-2-phenylindole

G: Generation reared in lab

GA: Gastrocnemius muscle

HA: High altitude

H&E: Hemotoxylin and eosin

HOAD: β-hydroxy acyl-CoA dehydrogenase

LA: Low altitude

LDH : Lactate dehydrogenase

NE: Norepinephrine

mmHg: barometric pressure in milometers of mercury

NST: Non-shivering thermogenesis

O_2: Oxygen

P: Postnatal day

PBST: Phosphate-buffered saline

PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PPAR: peroxisome proliferator-activated receptor

PO_2: partial pressure of oxygen

PVDF: polyvinylidene difluoride

RM: Repeated measures

RT: Room temperature

s.e.m.: Standard error of the mean

SDH: Succinate dehydrogenase

SDS PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

ST: Shivering thermogenesis

SUV: Standardized uptake values

T_a: Ambient temperature

T_b: Body temperature

T_{BACK}: Skin surface temperature between hindlimbs

Ti_{BAT}: Interscapular brown adipose tissue temperature

TCA cycle: Tricarboxylic acid

TH: Tyrosine hydroxylase

TNZ: Thermo neutral zone

UCP-1: Uncoupling protein 1

$\dot{V}O_2$: Rate of oxygen consumption

$\dot{V}O_2max$: Maximal rate of O_2 consumption

WAT: White adipose tissue

ingWAT: inguinal WAT depot

ZT: Zeitgeber time

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis is organized in a sandwich format and consists of six chapters. Chapter 1 is an overview of background material and hypotheses tested. Chapter 2 through 5 are manuscripts that are published, submitted or ready to be submitted for publication in a peer reviewed scientific journal. Chapter 2 is referred to as Robertson et al. 2019, Chapter 3 is referred to as Robertson and McClelland, 2019. Chapter 6 summarizes the major findings of this thesis and places these findings in the context of current literature.

CHAPTER 1 **GENERAL INTRODUCTION**

CHAPTER 2 **DEVELOPMENT OF HOMEOTHERMIC ENDOTHERMY IS DELAYED IN HIGH ALTITUDE NATIVE DEER MICE (PEROMYSCUS MANICULATUS)**

Authors: Cayleih E. Robertson, Glenn J. Tattersall and Grant B. McClelland

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CHAPTER 3 **DEVELOPMENTAL DELAY IN SHIVERING LIMITS THEROMGENIC CAPACITY OF JUVENILE HIGH-ALTITUDE DEER MICE (PEROMYSCUS MANICULATUS)**

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CHAPTER 4 **DEVELOPMENTAL PLASTICITY OF BROWN ADIPOSE TISSUE-DRIVEN NON-SHIVERING THERMOGENESIS TO COLD IS LOST WITH HIGH ALTITUDE ADAPTATION**

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CHAPTER 5 **MATERNAL ADAPTATIONS TO HIGH ALTITUDE**

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CHAPTER 6 **GENERAL DISCUSSION**

APPENDIX A. **SUPPLEMETNAL MATERIALS**

CHAPTER 1 GENERAL INTRODUCTION

One of the principle aims of comparative physiology is to understand the evolution of complex physiological systems. As a result, understanding how genotypes and environments interact to produce adaptive phenotypes in extreme environments is of great interest to this field. However, isolating the effect of a given environmental variable on an adaptive trait presents a variety of challenges. An animal's phenotype is the product of genotype × environment interactions which can manifest in a variety of ways throughout the organism's lifetime. First, the phenotype may be influenced by the animal's rearing environment. Every organism has a genetically pre-programmed developmental trajectory along which ontogeny normally proceeds. This represents the timing and order of developmental milestones that occur in a consistent environment and reflects the organisms' genotype. This developmental program may be altered in response to environmental perturbations experienced during certain critical windows of development (Burggren and Reyna, 2011). One possible outcome of an altered developmental trajectory is irreversible developmental plasticity, which is defined as the ability of a single genotype to produce separate adult phenotypes by altering developmental trajectory in response to environmental stimuli early in life (Moczeck et al., 2011). Additionally, rearing environment may encompass both the abiotic factors acting directly on the developing organism but also include the influences of maternal effects. Maternal effects encompass any non-genetic influence of the mother on offspring phenotype (Wolf and Wade, 2009). Mammalian mothers, in particular, can affect the

phenotype of their offspring at several different critical windows, but focus has been primarily at two perinatal periods. Prenatally maternal nutrition status, stress level and PO_2, can all impact fetal growth and development (reviewed in Murphy et al., 2006). Postnatally, quality of maternal care can program altered offspring physiology (Weaver et al., 2004).

In recent years it has become increasingly clear that developmental plasticity must be considered as a potential driver of both individual fitness and evolutionary change as it allows young organisms to better match their phenotype to their environment. (West-Eberhard, 2005). This may be particularly important in altricial species who experience extremely high mortality rates during the first few weeks of life (e.g. 49-96% *Peromysucus* mice; Hill, 1983). Therefore, any change in phenotype in response to an environmental cue, which improved survival would likely become a target of selection. The adult phenotype may also be influenced by phenotypic flexibility in response to the adult environment. These two plastic responses can also interact, where an organism's prior experience with a stressor during development may influence their capacity for adult phenotypic flexibility to that stressor (**Figure 1.1**, Chappell et al., 2007; Russell et al., 2008). In stressful environments the interactions between developmental plasticity and reversable adult acclimation response should be important drivers of fitness. However, these interactions are poorly understood and rarely studied (Beaman et al., 2016).

High altitude (HA) environments are particularly valuable for these kinds of studies as the two major selective pressures, low partial pressure of oxygen (PO_2) and low ambient

temperature, are well known. Additionally, changes in these environmental variables typically occur over a small spatial range (Körner, 2007). As a result, there is a great deal of both genetic and phenotypic variation between high and low altitude (LA) conspecifics across numerous taxa (Keller et al., 2013). Physiological adaptations of adult HA natives have been extensively studied and are beginning to be well characterized (McClelland and Scott, 2019). However, very little is understood about how HA adapted species survive in high alpine environments during sensitive stages of development (Ivy and Scott, 2015). Altricial HA species may be particularly prone to developmental adaptations as many of the physiological systems that adults use do not mature until well after birth. The **goal** of this thesis is to address this gap in knowledge in relation to the thermogenesis of HA adapted populations of the North American deer mouse (*Peromyscus maniculatus*).

1.1 LITERATURE REVIEW

To understand the importance of thermogenesis during early development it is essential to review what is known regarding thermogenesis in adult mammals, the major thermo-effector organs involved, endothermy during development in altricial species and the ontogeny of those same thermo-effector tissues.

Mammalian Thermogenesis and Thermo-effector Organs:

To maintain homeostatic body temperature (T_b) in the face of cold exposure mammals engage in aerobic thermogenesis, which requires a drastic increase in O_2 consumption from basal metabolic rate (BMR) to maintain heat production to match heat

loss (Scholander et al., 1950). Mammalian thermogenesis is centrally regulated by the preoptic area of the anterior hypothalamus which integrates signals from central and peripheral thermo-sensors and activates the thermo-effector organs (for review see Madden and Morrison, 2019). Behavioural thermoregulation is also an important aspect of the response to cold, particularly in small mammals; however, for the purpose of this review I will focus on the thermo-effector organs capable of producing metabolic heat. This process involves metabolic heat production via two distinct mechanisms, shivering (ST) and non-shivering thermogenesis (NST).

Shivering Thermogenesis: Shivering, or the repetitive, involuntary and uncoordinated contraction of skeletal muscle, produces heat through the hydrolysis of ATP with little useful work performed (Hemingway, 1963). Shivering intensity is determined by the physical and metabolic properties of the muscles involved (Taylor-Burt et al., 2015). There are two distinct forms of shivering in humans, continuous low intensity shivering, which is performed by Type I, slow-oxidative muscle fibers, and short, intense bouts of shivering which involves the recruitment of Type II fibers (Haman et al., 2004). In rodents ST is responsible for the initial response to acute, severe cold exposure. However, as adult mammals acclimate to cold ST decreases (Hemingway, 1963). During chronic cold exposure NST is also recruited and is thought to inhibit further ST unless conditions become severe (Cannon and Nedergaard, 2004). ST can also be suppressed during hypoxia, which inhibits neural recruitment of skeletal muscle in the cold (Gautier et al., 1987).

Non-shivering thermogenesis. In mammals the primary site of NST is brown adipose tissue (BAT; Foster and Frydman, 1978). This highly vascularized tissue is unique to the placental mammals (Jastroch et al., 2018) and is characterized by small multilocular adipocytes with a high mitochondrial density (Cannon and Nedergaard, 2004). These mitochondria express uncoupling protein-1 (UCP-1), which uncouples the oxidation of substrates from ATP production by dissipating the proton gradient established by the electron transport chain across the inner mitochondrial membrane (Cannon and Nedergaard, 2004). BAT is recruited and UCP-1 activated upon cold stimulation by sympathetic neurons that release norepinephrine (NE; Bartness et al., 2010). This response is mediated primarily by *β*-3 adrenergic receptors (AdR), though BAT also expresses β-1, α-1 and α-2 receptors. Activation of β-3 AdR initiates lipolysis of triglyceride stores within brown adipocytes and the resulting free fatty acids activate UCP-1 and further power thermogenesis by providing fuel for *β*- oxidation in the mitochondria (Cannon and Nedergaard, 2004). Adrenergic stimulation of BAT also dramatically increases glucose uptake of the tissue (Shimizu et al., 1993). The largest and best studied depot of BAT is found in the interscapular region (iBAT) in rodents, however, there are numerous other depots (13 in *P. maniculatus*) found throughout the body (Rauch and Hayward, 1969; de Jong et al., 2015).

Role of Adult phenotypic flexibility

The two primary thermo-effector organs are irreversibly linked by common central control and crosstalk of various endocrine factors. Severe cold stress maximally

recruits both ST and NST (Wunder and Gettinger, 1996). Therefore, an increase in thermogenic capacity likely involves the coordination of both tissues. In the wild, mammals experience seasonal and diurnal fluctuations in temperature and must adjust their capacity for metabolic heat production accordingly (e.g., Lynch, 1973; van den Berg, 2018). BAT is particularly phenotypically plastic in small mammals and is thought to be the primary driver of these changes (van Sant and Hammond, 2008). In many species chronic cold exposure has been found to induce BAT hyperplasia, increase UCP-1 expression, and vascularization of the tissue (see Wang and Seale, 2016 for review). Interestingly, hypoxia directly inhibits the activation of BAT (Madden and Morrison, 2005) and when combined with cold, limits plasticity of this tissue in LA natives (Mortola and Naso, 1997; Beaudry and McClelland, 2010; Cadena and Tattersall, 2014). Some of the mechanisms which drive environmentally induced plasticity of BAT have been uncovered. For example, chronic NE stimulation upregulates peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which in turn regulates mitochondrial biogenesis and UCP-1 expression (Puigserver et al., 1998; Puigserver, 2005)

Ontogeny of Thermogenesis

Small altricial mammals, such as deer mice, are born without the capacity to maintain T_b (Pembry, 1895). During this time pups must rely entirely on parental care to maintain T_b. This early phase of development is followed by the maturation of thermoregulation including first the onset of NST followed by ST as well as an increase

in insulation (fur, fat, etc.). At this intermediary stage between poikilothermy and full homeothermic endothermy, animals can maintain constant T_b but only within a narrow range of ambient temperatures (Lagerspetz, 1966, Hill, 1976). Finally, pups develop adult-like homeothermy, which usually coincides with the timing of forays away from the nest (Chew and Spencer, 1967). In general, rodents develop their full thermoregulatory capacity within 1 month of birth.

Development of BAT: In most rodents BAT is present at birth, though the cells are small and lipid poor (Barnard et al., 1970). In laboratory mice *Ucp-1* is expressed in the fetus BAT, though the tissue may require the large NE stimulation at birth to begin maturing (Xue et al., 2007). This tissue matures rapidly after birth, which is likely due to the initial reliance of neonatal rodents on NST as thermogenesis develops.

Development of Skeletal Muscle: Skeletal muscle also develops postnatally, as neither final fiber size nor fiber type is established at birth (Dubowitz, 1963). In mice, rats and hamsters it takes several weeks for adult muscle phenotype to be established (e.g., Goldspink and Ward, 1979; Adams et al., 1999, Gokhin et al., 2008, Agbulut et al., 2003). In contrast to BAT, which develops rapidly, the slow postnatal maturation of skeletal muscle results in a protracted development of ST relative to NST (Arajamma and Lagerspetz, 1979). For example, young *Peromyscus leucopus* pups can increase metabolic rate in the face of a mild cold exposure by postnatal day (P)8, however, this species does not visibly shiver until P13 (Hill, 1976).

The timing of the onset of endothermy could theoretically be adjusted in several ways. An evolved shift in the timing of physiological events is known as physiological heterochrony (Gould, 1977; Spicer, 2006). For example, the Norwegian lemming, a cold-adapted subarctic species can thermoregulate earlier after birth than most other altricial rodents studied (e.g., mice, rats, or golden hamsters; Lagerspetz, 1966). This contrasts with physiological heterokairy, where the maturation of a system is altered in response to environmental factors (Spicer and Burggren, 2003, Spicer and Rundle, 2007). This is one form of developmental plasticity. For example, maturation of BAT can be accelerated in response to low ambient temperature in LA rodents. Changes in tissue mass, UCP-1, and innervation of BAT have all been reported in cold reared rodent pups (Skala and Hahn, 1974; Denjean et al., 1999; Morrison et al., 2000). In contrast, both prenatal and postnatal hypoxia exposure can inhibit BAT growth and maturation (Mortola and Naso, 1998). Given that cold and hypoxia may have antagonistic effects on BAT function and development it is unclear how HA-adapted altricial mammals respond to their rearing environment.

Thermogenesis at high altitude

For small obligate endotherms, whose large surface area to volume ratio promotes rapid heat loss to the environment, the stressors found at HA (low PO_2 and low temperature) are particularly challenging. The O_2-limited nature of the HA environment limits the capacity for aerobically demanding activities such as thermogenesis and necessitates an important energetic trade-off that is not fully understood (Beaudry and

McClelland, 2010; Cadena and Tattersall, 2014). As a result of these combined stressors, small mammals who inhabit high alpine environments typically operate close to their maximum aerobic capacity ($\dot{V}O_2max$; Hayes, 1989). Despite these thermoregulatory challenges, it is small altricial rodents and lagomorphs that have the broadest altitudinal ranges of any mammals (e.g., *Peromyscus maniculatus*, Hock, 1964; *Phyllotis xanthopygus,* Kramer et al., 1999; *Ochotona curzoniae*, Ci et al., 2008).

HA populations of the North American deer mouse (*P. maniculatus*) has evolved an enhanced adult thermogenic capacity (Cheviron et al., 2012). A higher cold-induced $\dot{V}O_2max$ increases activity levels in the cold (Sears et al., 2006). This trait is of particular ecological and physiological relevance as HA deer mice do not hibernate and, therefore, must remain active year-round (Hayes, 1989). Mark and recapture studies have shown that individuals with a high adult cold induced $\dot{V}O_2max$ are more likely to survive during cold conditions (Hayes and O'Connor, 1999). This quantifiable effect on fitness makes *Peromyscus* thermogenic capacity an ideal performance measure to study genotype × environment interactions for an adaptive trait.

This increase in thermogenic capacity is driven by numerous physiological adaptations, including the two key thermo-effector organs, BAT and skeletal muscle. HA native deer mice, like many other HA adapted endotherms, have a highly vascularized and aerobic adult skeletal muscle phenotype (León -Velarde et al., 1993, Hepple et al., 1998, Mathieu Costello et al., 1998, Kayser et al., 1991, Sheafor, 2003, Scott et al., 2009; Lui et al., 2015; Lau et al., 2017; Mahalingam et al., 2017). These specialized aerobic

muscles also have an elevated capacity for lipid oxidation (Cheviron et al., 2012, 2014, Lau et al., 2017), presumably to support sustained rates of shivering. When tested in the wild, HA mice also have a higher capacity for NST under hypoxia than their LA conspecifics (Velotta et al., 2016). However, it is unclear if this difference is a result of genetic adaption to HA or adult phenotypic flexibility.

Seasonal variation in thermogenic capacity does occur in HA mammals (e.g., Hayes, 1989; Wang et al., 2006). This is particularly interesting given that hypoxia blunts the flexibility of NST in LA natives (Beaudry and McClelland, 2010). Adult phenotypic flexibility of thermogenic capacity has also been demonstrated experimentally in HA deer mice (Van Sant and Hammond, 2008, Cheviron et al., 2013; Tate et al., 2017). For example, when HA and LA wild caught mice were de-acclimated in sea level laboratory conditions for 6 weeks, thermogenic capacity dropped in both populations, though population differences in $\dot{V}O_2max$ persisted (Cheviron et al., 2013). Re-acclimating laboratory reared populations of HA native deer mice as adults to either cold or hypoxia increases their thermogenic capacity (Van Sant and Hammond, 2008; Tate et al., 2017). This complex phenotype is therefore driven by both evolved genetic adaptations and adult phenotypic flexibility. However, first generation (G_1) laboratory-reared progeny of wild HA deer mice have a lower thermogenic capacity than their wild-caught parents. Adult phenotypic flexibility is not enough to explain this difference in thermogenic capacity, suggesting that developmental environment also plays an important role in shaping this adaptive phenotype.

1.2 AIMS AND OBJECTIVES

It has been estimated that in the wild 49-96% of *Peromyscus* die within the first 5 weeks of life (See Hill, 1983 for review). Therefore, the physiological systems that are developing during this critical period are likely under intense natural selection. Altricial species that inhabit extreme environments such as HA are likely particularly sensitive during this period. However, almost nothing is known about how developmental environment shapes important physiological processes such as thermoregulation in extreme environments. The **primary objective** of this thesis is to understand how young, HA adapted altricial endotherms overcome the thermoregulatory challenges of their environment and determine the relative importance of developmental environment on thermogenesis. To address these objectives, I used a common-garden experimental design to comprehensively assess genotype × environment interactions at various ontogenetic stages in HA and LA deer mice. For this study I used the second-generation (G2) lab-reared descendants of two wild caught populations of *P. maniculatus*. The HA population (*P.m. rufinus*) was trapped on the summit of Mount Evans, Colorado (4350 m a.s.l.), while the LA population (*P.m. nebrascensis*) were trapped at Nine Mile Prairie (430 m a.s.l.) in Eastern, Nebraska. These two populations are genetically distinct from one another, and it is thought that the HA population evolved from LA ancestors (Natarajan et al., 2015). Additionally, I have also used the closely related, but strictly low-altitude white-footed mouse (*P. leucopus*) as a LA outgroup. The white-footed mouse lives sympatrically with *P. maniculatus* but is found exclusively at LA (Bedford and Hoekstra,

2015). *P. leucopus* and *P. maniculatus* diverged between 5.5-11.7 million years ago (Velotta et al., 2018). Using this experimental design, I have conducted a series of studies outlined in the following 4 chapters, to test the over arching ***hypothesis*** that small, altricial mammals adapted to high altitude (HA) have evolved developmental adaptations in response to the intense thermoregulatory challenges of that environment.

Chapter 2. Ontogeny of homeothermic endothermy and BAT: The goal of this study was to assess if underlying genetic adaptations have altered the developmental onset of homeothermic endothermy and BAT maturation in HA adapted pups. Using G2 pups, aged 0-10 days raised under common garden conditions, I tested two competing hypotheses:

Hypothesis 1. Adaptation to HA favours earlier onset of independent endothermy to cope with the intense thermoregulatory demands in the high alpine.

Hypothesis 2. Adaptation to HA delays the onset of independent endothermy to preserve limited energy resources as a response to lower O_2 availability.

Chapter 3. Ontogeny of Shivering thermogenesis and maturation of skeletal muscle: The goal of this study was to assess if underlying genetic adaptations have altered the maturation of skeletal muscle and the onset of shivering thermogenesis. I measured maturation of the hindlimb muscles and quantified the developmental onset of shivering thermogenesis in G2 pups from birth to 1 month old, raised under common garden conditions.

Hypothesis. Adaptation to HA accelerates the maturation of skeletal muscle allowing juveniles to induce shivering thermogenesis in the cold.

Chapter 4. Developmental plasticity to cold: The goal of this study was to determine the capacity of HA adapted pups for developmental plasticity to cold. I used a common garden experimental design to isolate the effects of genotype, developmental environment, and adult environment. I exposed G2 pups to either control, prenatal cold (14^{o}C), or postnatal cold (14^{o}C) and measured BAT phenotype and the metabolic response to cold during development, as adults and following a subsequent adult cold acclimation (5^{o}C). I also tested the closely related but exclusively low altitude white footed mouse (*Peromyscus leucopus*).

Hypothesis: HA adapted mice use developmental plasticity to cold, driven by BAT, to cope with the extreme thermoregulatory challenges of their environment.

Chapter 5. Maternal adaptations: The goal of this study was to assess how HA adapted female mice, who inhabit a cold-hypoxic environment that already pushes energy expenditure close to its maximum, and who consistently have larger litters than their low-altitude counterparts, balance energy allocation. G1 dams reared their litters under control (20^{o}C, 760mmHg) and simulated HA (5^{o}C, 430mmHg) conditions. I measured maternal energy intake, energy output and quality of care during lactation. Furthermore, I measured short- and long-term consequences of maternal energy allocation on offspring body mass, body composition, and thermogenic capacity.

Hypothesis: There is a fundamental trade-off at high altitude between the energetic demands of lactation and thermoregulatory demands of pups that has altered maternal care.

1.3 FIGURES

Figure 1.1

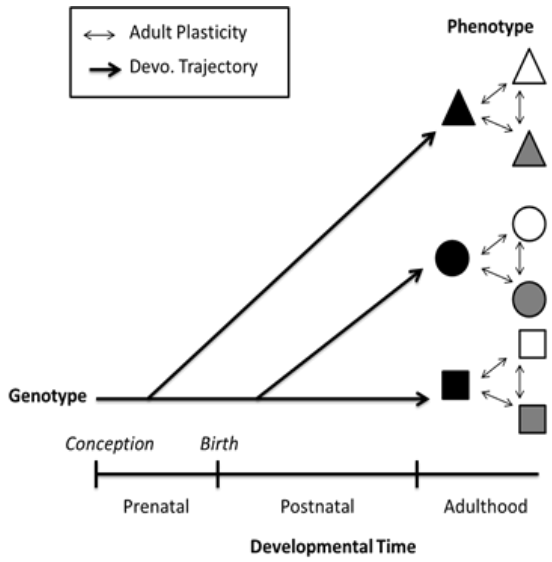

Figure 1.1 One genotype produces many phenotypes. Developmental trajectory is determined by genetic developmental program. It can be permanently altered by environmental or maternal factors during critical stages of development resulting in alternate adult phenotypes (*developmental plasticity*; ▲, ■, ●). Environmental stressors experienced during adulthood can also reversibly impact phenotype (*adult phenotypic flexibility*). Thus, gene × environment interactions occur throughout an organism's life.

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CHAPTER 2 Development of homeothermic endothermy is delayed in high altitude native deer mice (*Peromyscus maniculatus)*

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2.1 ABSTRACT

Altricial mammals begin to independently thermoregulate during the first few weeks of postnatal development. In wild rodent populations this is also a time of high mortality (50-95%), making the physiological systems that mature during this period potential targets for selection. High altitude is a particularly challenging environment for small endotherms due to unremitting low O_2 and ambient temperatures. While superior thermogenic capacities have been demonstrated in adults of some high-altitude species, it is unclear if selection has occurred to survive these unique challenges early in development. We used deer mice (*P. maniculatus*) native to high and low altitude, and a strictly low altitude species (*P. leucopus*), raised under common garden conditions, to determine if postnatal onset of endothermy and maturation of BAT is affected by altitude ancestry. We found that the onset of endothermy corresponds with the maturation and activation of brown adipose tissue (BAT) at an equivalent age in low altitude natives, with 10-day old pups able to thermoregulate in response to acute cold in both species. However, the onset of endothermy in high altitude pups was substantially delayed (by ~2 days), possibly driven by delayed sympathetic regulation of BAT. We suggest that this delay may be part of an evolved cost-saving measure to allow pups to maintain growth rates under the O_2-limited conditions at high altitude.

2.2 INTRODUCTION

Homeothermy is a defining trait for most endothermic placental mammals, and the capacity for sustained metabolic heat production is considered one of the hallmarks of mammalian evolution (1). Despite the importance of this trait small altricial mammals, such as mice are born without the physiological ability to regulate body temperature (2). Instead, the capacity to independently thermoregulate develops postnatally, with the transition from poikilothermy to homeothermic endothermy occurring sometime within the first weeks after birth (3-10). This dynamic period of development is characterized by rapid maturation of the thermo-effector organ brown adipose tissue (BAT) and the neural circuitry that senses ambient temperature (11-13).

Mortality can be extremely high (50-95%) during the early postnatal period in wild rodent populations (14-16) suggesting that the developmental window when thermoregulatory systems are maturing is under intense natural selection. Altricial species living in thermally challenging environments may have evolved specific physiological adaptations to survive this critical period. Studies using laboratory rodent models demonstrate that BAT maturation and age of onset of endothermy respond to altered rearing conditions. Rearing young rodents in cold typically causes the onset of endothermy to shift to earlier points in development (17,18). These performance shifts have been associated with phenotypic remodeling and accelerated development of BAT. For example, tissue mass, mitochondrial content, uncoupling protein-1 (UCP-1) expression and innervation of BAT have all been reported to increase (16, 19-22). In contrast, postnatal hypoxia leads to an inhibition of both BAT growth and maturation

(23). However, it is unclear how the development of these systems may have adapted to chronic exposure to these conditions in altricial species living in extreme environments.

Unremitting low O_2 and ambient temperatures at high altitude (HA) are particularly challenging for small mammals due to the high aerobic demand of thermogenesis (24). HA environments require both the efficient use of limited O_2 and adequate generation of heat, an energetic trade-off that is not fully understood (25-27). These two abiotic stressors are considered the primary selective pressures for animals living in the high alpine. Indeed, some HA rodents (e.g., deer mice) have evolved a greater adult thermogenic capacity than their low altitude (LA) conspecifics (28,29). This trait has been shown to be under strong directional selection at HA to improve winter survivability and therefore fitness (30). However, it is unclear how alpine natives have evolved to survive these unique challenges early in development; but the maturation of BAT-based heat production is a likely target of selection.

To address this gap, we used LA and HA North American deer mice (*Peromyscus maniculatus*), and a related but strictly LA species (*P. leucopus*) to determine if the postnatal maturation of BAT and the development of homeothermic endothermy is influenced by altitude ancestry. There are two possible directions in which the timing of the onset of endothermy could shift in response to the stressors at high altitude. We therefore tested two competing hypotheses:1. Adaptation to high altitude favours earlier onset of independent endothermy to cope with the intense thermoregulatory demands in the high alpine. If so, the BAT of HA pups should mature faster and the ability to

thermoregulate occur at a younger age than LA pups. 2. Adaptation to high altitude delays the onset of independent endothermy to preserve limited energy resources as a response to lower O_2 availability. If so, the BAT of HA pups should mature more slowly and the ability to thermoregulate should lag behind LA pups.

2.3 METHODS

Experimental Animals

Mice were from a captive breeding colony established using separate stocks of wild-caught deer mice and white-footed mice (*P. leucopus*). The HA natives, (*P.m. rufinus*) were trapped at the summit of Mount Evans, CO (4350 m a.s.l.) and the LA natives (*P.m. nebrascensis and P. leucopus*) in Eastern Nebraska (430 m a.s.l.; 28). *P. leucopus* are closely related to, and live sympatrically with, *P. maniculatus* but are found exclusively at LA (31). Wild mice were transported to McMaster University (~90 m a.s.l.) and bred within their respective populations under common garden conditions (24^{o}C, 760mmHg, 14h:10h light:dark cycle, rodent chow and water *ad libitum*). First generation (G_1) lab-born mice were mated to produce G_2 offspring. All pups used in this study were from G_2. All procedures described were approved by the McMaster University Animal Research Ethics Board.

Response to Acute Cold

Thermography

We examined changes in skin surface temperature of pups in response to an acute cold challenge (24^{o}C for 10 minutes) at postnatal day (P) 0, 2, 4, 6, 8 and 10. All pups from a litter (for litter size see Table 1) were removed from insulated nests and placed in separate plastic weigh boats to prevent huddling. Thermal images were captured (0.2 frames/second) using a calibrated thermal camera (FLIR SC660, FLIR systems Inc.). Skin surface temperatures were quantified using ThermaCam Researcher Pro v2.9 software (FLIR) by drawing a field of view in the interscapular region (T_{iBAT}) and between hind legs (T_{Back}, 32). The change in these surface temperatures with acute cold were determined as $T_{10\ min} - T_{1\ min}$. When BAT is recruited it displays a characteristic pattern of temperature fluctuation (33). To quantify fluctuation in TiBAT, we fitted a second order polynomial regression to T_{iBAT} versus time and analyzed the absolute residuals as an indirect measure of iBAT activation. Furthermore, to assess thermal endurance we determined the time pups could maintain constant T_{Back} or T_{iBAT} within 1^{o}C of initial values (60 s).

Indirect Calorimetry

Cold-induced metabolic rate was determined in individual pups at P2, 4, 6, 8 and 10 using open flow respirometry (Sable Systems, Las Vegas, NV). Individual pups were placed in glass chambers (60 ml for P2-8, 100 mL for P10) inside a temperature-controlled cabinet (Sable Systems). Dry, CO_2-free air was pulled through the chambers at 100 mL/min (P2 and P4), 150 mL/min (P6 and P8), or 175 mL/min (P10). Excurrent air was dried and drawn though CO_2 (CA-10A) and O_2 (FC-1A) analyzers (Sable Systems).

Ambient temperature (T_a) was held at 30°C for 10 minutes to record an initial resting $\dot{V}O_2$, then lowered by 0.3°C/min to a final temperature of 24°C and held for 10 min to determine a final $\dot{V}O_2$. $\dot{V}O_2$ was determined at the most stable 30s during the last 2 minutes at 30 and 24°C. A second pup from the same litter was held at a constant 30°C (trial order randomized) to account for any influence of maternal separation or handling stress. Cold-induced metabolic rate was calculated as the difference (Δ) between initial $\dot{V}O_2$ and final $\dot{V}O_2$ in pups exposed to acute cold (24°C) minus $\Delta\dot{V}O_2$ of its normothermic (30°C) sibling:

$$\Delta\Delta\dot{V}O_2 = (\text{Final } \dot{V}O_2 - \text{Initial } \dot{V}O_2)_{\text{acute cold}} - (\text{Final } \dot{V}O_2 - \text{Initial } \dot{V}O_2)_{\text{normothermic}} \quad (1)$$

A positive value indicates a cold-induced increase in $\dot{V}O_2$ relative to the normothermic sibling.

Brown Adipose Tissue Maturation

Tissue Sampling

Two littermates from each family were euthanized with an overdose of isoflurane followed by cervical dislocation at P0, 2, 4, 6, 8, and 10. For one pup the iBAT depot was blunt dissected, weighed, flash frozen and stored at -80°C. For the other pup, iBAT was frozen in embedding medium (Cryomatrix, Thermo Scientific) for histological analysis. The inguinal WAT depot was blunt dissected and weighed as a measure of body composition.

Western Blotting

Protein expression of uncoupling protein-1(UCP-1) tyrosine hydroxylase (TH) and citrate synthase (CS), were determined by western blotting (34). Briefly, 20μg of total BAT protein was separated on 12% SDS PAGE gels and transferred to PVDF membrane. Membranes were probed with 1^{o} antibody (UCP-1; UCP11-A, Alpha Diagnostics International Inc., San Antonio, TX; TH, AB152, EMD Millipore, Temecula, CA; CS, ab129095, Abcam, Woburn, MA) at 1:500 followed by HRP-conjugated, goat anti-rabbit 2^{o} antibody at 1:10000 (Santa Cruz Biotechnology, Santa Cruz, CA). Band density was normalized to total lane protein determined by membrane staining with Coomassie blue.

BAT Histology

Frozen iBAT was coronally sectioned (10μm) at -30°C using a Cryostat CM1860 (Leica Biosystems, Nossloch) and stained for alkaline phosphatase (AP) activity to identify capillaries as previously described (34, 35). Capillary density (total capillary number/ surface area), capillary areal density (total capillary surface area/ BAT surface area), and capillary length density (tortuosity) were quantified using the NIS-Elements Imaging Software version 4.30 (Laboratory Imaging, Prague). Fluorescent immunohistochemistry was used to identify adrenergic sympathetic neurons (using tyrosine hydroxylase, TH, as a marker) and nuclei (4',6- diamidino-2-phenylindole, DAPI, 36).

Statistical Analysis

To avoid pseudo-replication, a single replicate represents the mean of all individuals tested from the same family and sample sizes represent the number of families within a group. A two-way repeated measures (RM) ANOVA was used to assess the effects of population and age on pup mass. When we had incomplete representation from each family at each age (tissue growth, Δskin surface temperature, $\Delta\Delta\dot{V}O_2$), we used two-way ANOVA to determine the main effects of population and age. One-way ANOVA was used to determine the effect of population on litter parameters, and protein expression. In the case of significant interactions between main effects, a Holm's Sidak *post hoc* analysis was used. We used a 1 sample t-test within population to determine if Δskin surface temperature was <0 at P10, to confirm that pups were homeothermic at this age. All analyses were performed using Sigma Stat (SysStat Software Inc., San Jose, CA, USA) or R software (R Foundation, Vienna, Austria).

2.4 RESULTS

Pup growth

Over the first 10 days of postnatal development, pup body mass was significantly different between the three populations (**Figure 2.1A;** Population, $F_{2,105}$ =13.027, P < 0.001; Age, $F_{5,102}$ =419.044, P <0.001). Pups of LA *P. leucopus* were the largest at birth (2.22 ± 0.08 g), 1.7 times larger than the LA *P. maniculatus* pups who were the smallest of the three groups (1.30 ± 0.09 g), and high-altitude native pups were intermediate in size (1.86 ± 0.05 g). These population differences were generally maintained throughout

development. However, there was a significant interaction between population and age ($F_{10,97}$=1.986, P=0.044) and population differences in growth rates approached statistical significance ($F_{2,21}$ =3.268, P=0.058, **Figure 2.1B**), with *P. leucopus* pups growing slightly faster (0.48 ± 0.04 g/day) than either of the two *P. maniculatus* populations (LA, 0.38 ± 0.03; HA, 0.41 ± 0.03 g/day). WAT, used as an index of body composition, was similar between all three groups (P=0.549) and increased with age ($F_{5,104}$ =6.589, P<0.001, **Figure 2.1C**). Interestingly, litter size differed significantly with HA mothers having ~1.6 times as many pups as either LA species ($F_{2,19}$ =7.366, P=0.004). Thus, total litter mass provisioned by HA mothers was larger than their LA counterparts, both at birth ($F_{2,18}$ =9.716, P=0.001) and at P10 ($F_{2,18}$ =7.563, P=0.004) by 46-85%, respectively (**Table 2.1**).

Heat loss and thermal endurance in response to acute cold

Using video thermography we assessed changes in body skin surface (T_{Back}) and iBAT (T_{iBAT}) temperature during acute cold, providing an indication of thermoregulatory ability and iBAT recruitment. For newborn pups, regardless of population, T_{Back} rapidly declined during acute cold. T_{Back} of LA *P. maniculatus* and *P. leucopus* dropped by 5.98 ± 0.34 ^{o}C and 5.44 ± 0.32 ^{o}C, respectively. In comparison, HA *P. maniculatus* showed much greater heat loss from T_{Back} (6.87 ± 0.03 ^{o}C, **Figure S1, 2.2A**) despite having similar starting temperatures (**Table S1**). All mice maintained their surface temperatures more effectively with age, losing significantly less heat with cold exposure (Age for T_{Back}, $F_{5,86}$ =54.542, P<0.001 and T_{iBAT}, $F_{5,86}$ =57.988, P<0.001). However, altitude ancestry

differences in heat loss were maintained (Population for T_{Back} $F_{2,69}$ =25.203, P<0.001 and T_{iBAT}, $F_{2,69}$ =54.542, P<0.001; **Figure 2.2A**). *Post hoc* analysis revealed that HA pups had a greater decline in surface temperatures across all ages compared to both *P. leucopus* (P< 0.001) and LA *P. maniculatus* (P<0.001; **Figure 2.2A**) who were not different from each other (P=0.144). By P10 the two LA populations could maintain stable T_{Back} in response to acute cold (ΔT_{Back} was not different from 0°C; *P. leucopus*, P=0.106 and LA *P. maniculatus*, P=0.211). At this age the dual lobes of the iBAT were clearly visible in thermal images of LA pups suggesting that the tissue was active and producing heat (**Figure 2.2D**). In contrast, at P10 HA pups continued to show persistent heat loss of ~2°C for T_{Back} (ΔT_{Back} < 0°C, P=0.018).

HA pups also showed a developmental latency in thermal endurance compared to LA pups (**Figure 2.2B**). Thermal endurance was low from P0 to P6 in all groups. LA pups showed a significant increase in thermal endurance with age and at P10 could maintain homeostatic T_{Back} for the entire cold trial. In HA pups endurance was only improved at P10 (Population x Age for $T_{Back,}$ $F_{10,63}$ =7.286, P=0.025 and T_{iBAT}, $F_{10,63}$=2.380, P=0.018) and was still lower than either LA group (**Figure 2.2B**).

The capacity in LA pups by P10 to maintain stable T_{Back} during acute cold coincided with increased fluctuations in T_{iBAT}. Early in development, T_{iBAT} fluctuations with cold were very minimal but increased significantly as pups aged ($F_{2,62}$ =16.464, P <0.001). However, this pattern was only seen in pups from LA ancestry (Population x Age, $F_{10,62}$ =2.891, P= 0.005). LA *P. maniculatus* had a two-fold increase in cold-

induced T_{iBAT} flucutations relative to HA *P. maniculatus* and *P. leucopus* at P8. At P10 all three populations were significantly different, where cold-induced T_{iBAT} fluctuations in *P. leucopus* and LA *P. maniculatus* were two and three-fold higher than HA pups. HA pups, in contrast showed no increase in cold-induced T_{iBAT} fluctuations as they aged. (**Figure 2.2F**).

Indirect calorimetry during an acute cold challenge

We measured cold-induced $\dot{V}O_2$ to determine when pups were able to induce a metabolic response to cold. We found that young pups (P2-P6) were unable to increase $\dot{V}O_2$ in response to cold (**Figure 2.3A**). Cold-induced $\dot{V}O_2$ increased beyond P6 in all groups, but the timing of this response varied with altitude ancestry (Population x Age, $F_{8,84}$ =3.247, P=0.003). Both *P. leucopus* and LA *P. maniculatus* showed a significant induction of $\dot{V}O_2$ in response to cold at P8 and P10. At P10 cold-induced $\dot{V}O_2$ was driven by the combination of an increase in $\dot{V}O_2$ in the cold-exposed pups and a decrease in $\dot{V}O_2$ in of normothermic controls (Time x Test Temperature, *P. leucopus*, $F_{1,6}$ =35.693, P<0.001; *P. maniculatus*, $F_{1,6}$ =13.689, P=0.01; **Table S2**). In contrast, HA *P. maniculatus* did not show a significant cold-induced $\dot{V}O_2$ until P10. In this population, normothermic control pups showed no change in $\dot{V}O_2$ (**Table S2**). Cold-induced $\dot{V}O_2$ was positively correlated with ΔT_{iBAT} (R^2 =0.441, P<0.001) and ΔT_{Back} (R^2=0.485, P<0.001; **Figure 2.3B, C**).

Maturation of brown adipose tissue

We found that the iBAT deposit grew continuously over the first 10 days of development in all three groups (Age, $F_{5,104}$ =30.88, P<0.001), though it was smaller in HA pups (Population, $F_{2,104}$=7.586, P<0.001; **Figure 2.4A**). When expressed relative to body mass iBAT was largest at birth but remained at a constant proportion of body mass at all other ages (Age, $F_{5,104}$ =16.258, P<0.001), suggesting iBAT mass tracked overall growth. Relative iBAT mass was highest in LA *P. maniculatus* compared to the two other groups (Population, $F_{2,104}$ =32.932, P<0.001) (**Figure 2.4B**). Neither iBAT capillary density, area density, nor length density differed between LA (*P. leucopus*) and HA deer mice at any age (**Figure 2.4C**).

To determine if iBAT metabolic phenotype could help explain population differences in thermogenic performance, we assessed protein content of CS (**Figure 2.4D**), and UCP-1 (**Figure 2.4E**). Protein abundance for CS (P=0.991) and UCP1 (P=0.964) did not differ between the populations. In contrast, at P10 the expression of TH, the rate-limiting enzyme in norepinephrine production, was significantly different between the populations (**Figure 2.4F**, $F_{2,17}$ =4.634, P=0.025) with a 3-fold higher expression in LA pups compared to HA *P. maniculatus* pups. This TH expression is localized to the neurons innervating BAT (**Figure S2**).

2. 5 DISCUSSION

The main objective of this study was to determine if the postnatal onset of homeothermic endothermy is influenced by altitude ancestry in deer mice. We show that

P. maniculatus pups of HA ancestry delay the onset of endothermy compared to LA pups. Over the first 10 postnatal days HA pups did not guard skin surface temperature in the face of an acute cold challenge. In contrast, by P8 pups of LA *P. maniculatus* and *P. leucopus* could increase aerobic metabolism, and by P10 could maintain homeostatic body temperatures in response to cold. At P10 BAT was phenotypically similar regardless of altitude ancestry, except for a 3-fold lower expression of TH seen in HA pups, suggesting neurotransmitter synthesis and neural activation of this tissue is delayed in this population. To our knowledge this is the first study to demonstrate that the developmental trajectory of thermogenesis is altered as an adaptation to HA.

Timing of the onset of homeothermic endothermy

We found that the onset of thermogenesis in LA mice occurs between P8-P10, consistent with previous reports for *P. leucopus* (10). This species increases metabolic rate with mild, acute cold stress (24^{o}C) at P8 and maintains constant body temperature by P10 (10), in line with our results on both this species and LA deer mice. In contrast, we found deer mice of HA ancestry did not respond metabolically to acute cold until P10 and could not maintain body temperature. These data suggest that developmental milestones of endothermy are highly conserved in LA native mice, and that the delay in thermogenesis observed in HA natives is a derived trait that represents a fundamental shift in the developmental trajectory of this population. This may be an example of physiological heterochrony, an evolved change in timing of the development of a physiological process (37).

Homeothermy is determined not only by metabolic heat production but also by an animal's ability to retain heat, which in turn is linked to body size (38). Neonates born less than 4 g typically cannot maintain body temperature and must first reach a critical body mass before they can successfully retain heat (6,39). The predicted critical mass for LA *P. maniculatus* is ~4.8-5.4 g (6). We found that despite the greatest disparity in mass, the two LA species had almost identical development of thermal endurance and thermal capacity. Additionally, by the time LA pups could successfully thermoregulate (P10) all three groups reached the critical mass predicted for effective thermoregulation (6). The HA native pups were of an intermediate size, with similar proportions of WAT compared to LA native pups, yet they showed delays in all aspects of thermoregulatory capacity by at least 2 days, suggesting a fundamental shift in their physiology that is independent of body size.

Another critical factor for heat retention is insulation. Neonatal *Peromyscus* have sparse but visible hairs by P4 and insulation continues to increase linearly with age (40). Lack of pelage at P8 may explain why cold-induced metabolic rate was insufficient to maintain skin surface temperature of LA pups. We did not measure pelage, and it is possible that population differences in fur density may contribute to the greater heat loss observed in HA pups. However, we also found a direct correlation between the cold-induced metabolic rate and change in body temperature. Our data suggest that metabolic heat production accounts for ~ 48.5% of the change in body temperature in cold-exposed pups and that neither body size nor insulation can fully account for the delay in the

thermoregulatory ability of HA natives. Instead our data point to a direct effect of HA adaptation on the thermoregulatory systems.

BAT maturation and recruitment

It is likely that metabolic heat production in the first 10 days of postnatal development is driven exclusively by the maturation of non-shivering thermogenesis in BAT as skeletal muscles of neonatal mice are immature at this stage (6,16, 41). Maturation of iBAT appears fixed in deer mice regardless of altitude ancestry, increasing in size at similar rates, with similar vascularization. By P10 all pups expressed similar levels of the mitochondrial marker CS and the functional thermogenic protein UCP-1. However, T_{iBAT} is tightly correlated to T_{Back} throughout development (R^2=0.96, **Figure S3**), suggesting that the ability of LA pups to maintain body temperature at P10 is due to the activation of BAT. How then is the cold-induced activation of BAT delayed in HA native pups?

In altricial rodents the neuronal circuits that sense cold are established sometime during the first two weeks of postnatal development (e.g., *Rattus,* 42; *Mus,* 43). We observed a fluctuating pattern of iBAT during acute cold, indicative of active centrally mediated regulation, which increases with age in LA but not HA pups at the onset of thermogenesis (P8-P10). Lower expression of the enzyme TH in the neurons innervating iBAT of HA native pups suggests that neurotransmitter synthesis and neural activation of this tissue is delayed in this population. Any delay in the innervation of BAT would inhibit cold-induced recruitment of this tissue (32, 44). This may explain why HA pups

fail to recruit their iBAT at P10, despite the presence of functional thermogenic machinery (e.g., UCP1). Whether this difference in TH expression is specific to the neurons innervating BAT or part of a more global delay in the development of autonomic pathways in HA pups remains to be seen.

The common garden nature of our study allowed us to uncover genetically based differences in developmental milestones as a function of altitude ancestry. The differences in timing of the onset of endothermy and recruitment of BAT suggest a shift in the developmental program of these traits as a result of selective pressures at HA. However, it is important to consider the influence that rearing environment may play in shaping thermogenic phenotypes in the wild (45). The primary abiotic stressors at HA, low PO_2 and temperature, are known to alter the development of BAT in laboratory rodents (22, 23). For example, rat pups reared in cold increase innervation of BAT by 70% (22). It is possible that HA pups may require this chronic environmental stimulus and may not exhibit the same developmental delays in their native environment. The mechanistic changes responsible for the altered developmental program of the thermo-effector organs in HA species is an area rife for exploration but outside the scope of this study.

Adaptive benefits of delayed development of homeothermic endothermy

Maintaining an elevated and constant body temperature is critical for the growth and maturation of young mammals. In fact, it has been theorized that endothermy evolved specifically to facilitate increased postnatal growth rates (46-49). Why then

would the onset of endothermy be delayed in HA environments, which are characterized by low ambient temperatures year-round?

At HA, low PO_2 may limit aerobic ATP production, causing resources to be allocated to maintenance metabolism (basal metabolic rate, thermal regulation and physical work) instead of growth or reproduction. This is beneficial in the short term but has detrimental effects on fitness if sustained. Reduced growth rates are observed in many vertebrates when development occurs in chronic hypoxia (e.g., 23, 50). Young mammals typically respond to acute hypoxia by suppressing thermoregulation to reduce metabolic demand and conserve energy (51, 52). This acute response may have been canalized at HA to allow pups to allocate their limited ATP resources towards growth.

The trade-off between growth and thermoregulation may be critically important given the large litter sizes of HA deer mice, which are consistent with wild HA populations (53). Postnatal growth in mice is limited by milk availability (54) and pups from larger litters have less access to milk and therefore slower growth rates (55). However, despite large litter sizes, we observed that HA pups maintained similar growth rates to LA pups. While mothers in our study were fed *ad libitum*, larger litter sizes should still increase intra-litter competition for milk (56). If HA pups are nutrient limited due to increased competition from litter mates, then suppressing energetically costly thermogenesis may help them recoup some of their energetic costs in favour of maintaining growth rates even in normoxia. This may also allow the preservation of BAT for adulthood, when it is likely critical for re-warming from torpor (30,31).

While larger litters may impose an energetic cost, there is a thermoregulatory benefit to having many siblings. We performed all measurements on individual pups, isolated from both their parents and litter mates. This was necessary to assess the maturation of the physiological systems responsible for heat production. However, *Peromyscus* pups huddle when their mothers are absent (57). The decreased surface area relative to the Volume of the whole litter allows pups to retain heat for several hours in the absence of an exogenous heat source, even at low ambient temperature (58). Additionally, mothers of various wild LA *Peromyscus* species from Northern latitudes alter their nests to further increase insulation (59). Our data suggest that HA pups rely more heavily on parental care and/or huddling with nestlings allowing for equivalent postnatal growth rates to LA pups. How HA mothers balance their own metabolic needs while caring for larger, less capable litters than their LA conspecifics, under harsh alpine conditions, is an important area for future exploration.

Conclusions

We have provided direct evidence that HA adaptation delays the transition from poikilothermy to homeothermic endothermy. This may be driven by a delay in the sympathetic regulation of BAT. These findings reject the hypothesis that adaptation to high altitude favours earlier onset of independent endothermy. Instead, we suggest this delay is a cost-saving adaptation that allows HA pups to maintain growth rates under the O_2-limited conditions. This seemingly paradoxical result is at odds with the elevated thermogenic capacity seen in adult HA mice (28, 29), which improves overwinter

survival and is under directional selection at high elevations (31). Our findings stress the importance of understanding how the same selective pressures can act differently depending on life history stage to truly understand the evolution of an adaptive physiological trait.

2.6 ACKNOWLEDGEMENTS

We would like to thank Vicky Lai for her analysis of BAT histology.

2.7 FIGURES AND TABLES

Table 2.1. Characteristics of litters born to first-generation (G_1) laboratory-born high (HA) and low altitude (LA) *P. maniculatus* and *P. leucopus* parents.

	***P. maniculatus* (LA)** **N=7**	***P. maniculatus* (HA)** **N=6**	***P. leucopus*** **N= 8**
Litter Size (# pups)	4.86 ± 0.55^a	7.67 ± 0.42^b	4.56 ± 0.67^a
Litter Mass at birth (g)	6.44 ± 0.98^a	14.10 ± 1.01^b	9.90 ± 1.37^c
Litter Mass P10 (g)	24.81 ± 2.43^a	45.92 ± 4.57^b	31.42 ± 4.00^b

Dissimilar letters represent significant differences between populations as determined with One-Way ANOVA. Data are mean ± s.e.m.

Figure 2.1

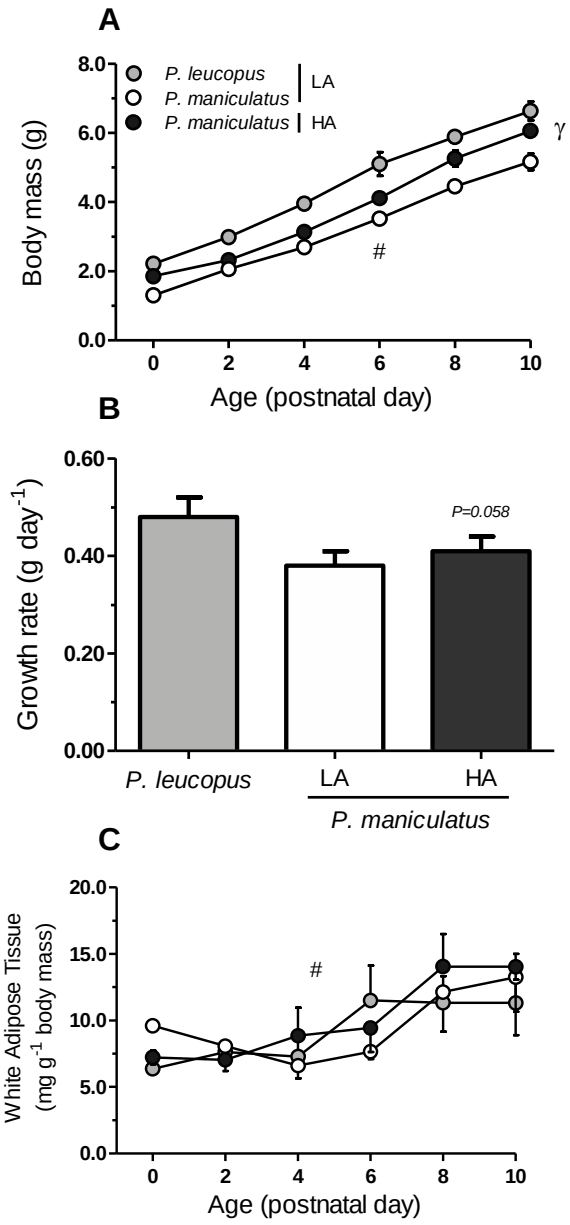
A
P. leucopus
P. maniculatus | LA
P. maniculatus | HA
Body mass (g)
Age (postnatal day)
γ
#
B
Growth rate (g day-1)
P=0.058
P. leucopus
LA
HA
P. maniculatus
C
White Adipose Tissue (mg g-1 body mass)
Age (postnatal day)
#

Figure 2.1. Body mass (A) and body composition, as inguinal white adipose tissue (WAT) mass (B) during early postnatal development in common garden-raised *Peromyscus maniculatus* of low (LA) and high altitude (HA) ancestry and LA *P. leucopus*. $^{\#}$ significant main effect of age, $^{\gamma}$ significant main effect of population as determined by 2-Way RM ANOVA for body mass and 2-Way ANOVA for WAT. Sample sizes (N) for WAT of *P. leucopus*, LA and HA *P.maniculatus* by age are as follows P0 (5,6,6), P2(5,7,8), P4 (5,7,9), P6 (5,7,6), P8 (5,6,10) and P10 (7,7,11) All data are mean ± s.e.m.

Figure 2.2

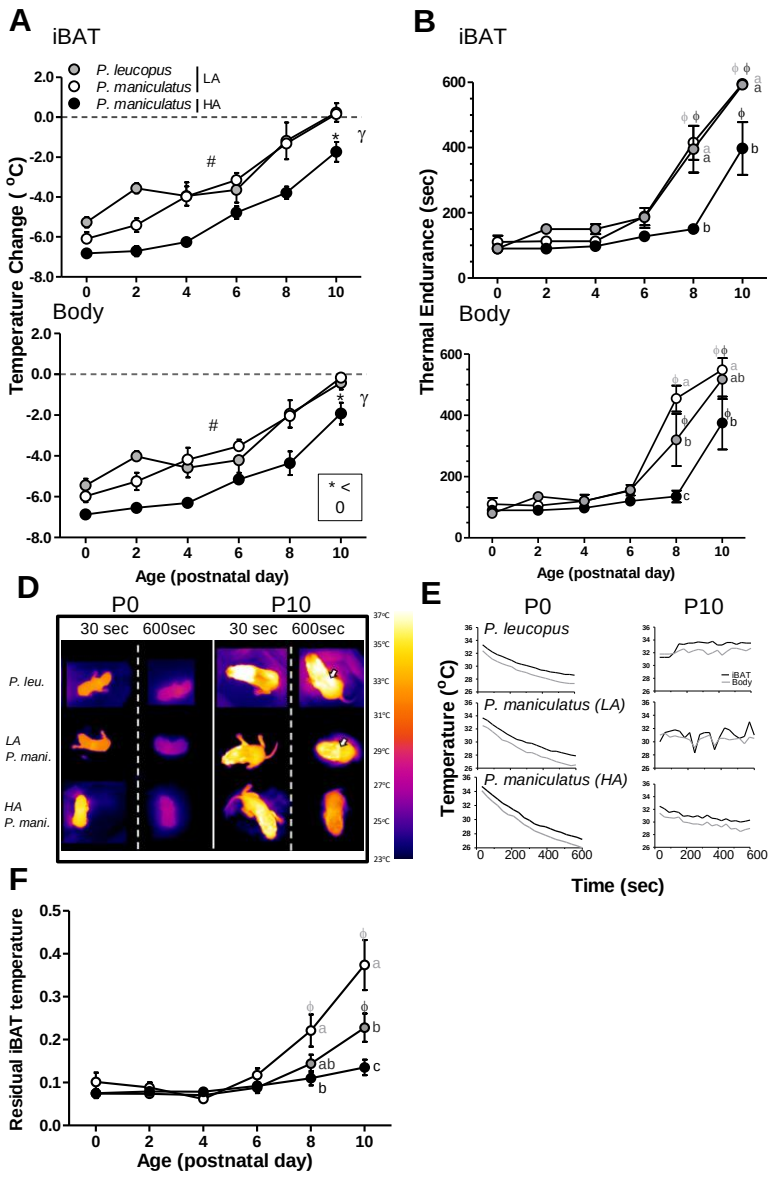
A
iBAT
P. leucopus
P. maniculatus | LA
P. maniculatus | HA
Temperature Change (°C)
Body
Age (postnatal day)
B
iBAT
Thermal Endurance (sec)
Body
Age (postnatal day)
D
P0
P10
30 sec
600sec
P. leu.
LA P. mani.
HA P. mani.
E
P0
P10
P. leucopus
P. maniculatus (LA)
P. maniculatus (HA)
Temperature (°C)
iBAT
Body
Time (sec)
F
Residual iBAT temperature
Age (postnatal day)

Figure 2.2. Effect of acute cold exposure (10 min at 24^{o}C) on surface body and interscapular (iBAT) temperature of common garden-raised *Peromyscus maniculatus* from low (LA) and high altitude (HA) ancestry and LA *P. leucopus* during early postnatal development. (A) Temperature change from the start (60s) to the end (600s) of exposure. Dashed line represents no heat loss. * $\Delta T < 0$ (one-sample t-test) at P10. (B) Thermal endurance as time T_{Back} or T_{iBAT} is maintained within 1^{o}C of starting T. (D, E) Representative thermal images at P0 and P10 taken at the beginning and end of an acute cold trial. Arrow shows activated iBAT (F) Abolute residuals of regressions of T_{iBAT} over time during an acute cold trial. $^{\#}$ Significant main effect of age, $^{\gamma}$ significant main effect of population (2-Way ANOVA). When a significant age x population interaction exists, $^{\phi}$ denotes a time point is significantly different from postnatal day 2 (P2) within population. Populations within an age with dissimilar letters are significantly different as determined by Holm's-Sidak analysis. N of *P. leucopus*, LA and HA *P. maniculatus* by age are as follows P0 (3,3,4), P2 (4,4,4), P4 (6,7,4), P6 (5,7,4), P8 (6,6,4) and P10 (5,7,4). Data are mean ±s.e.m.

Figure 2.3.

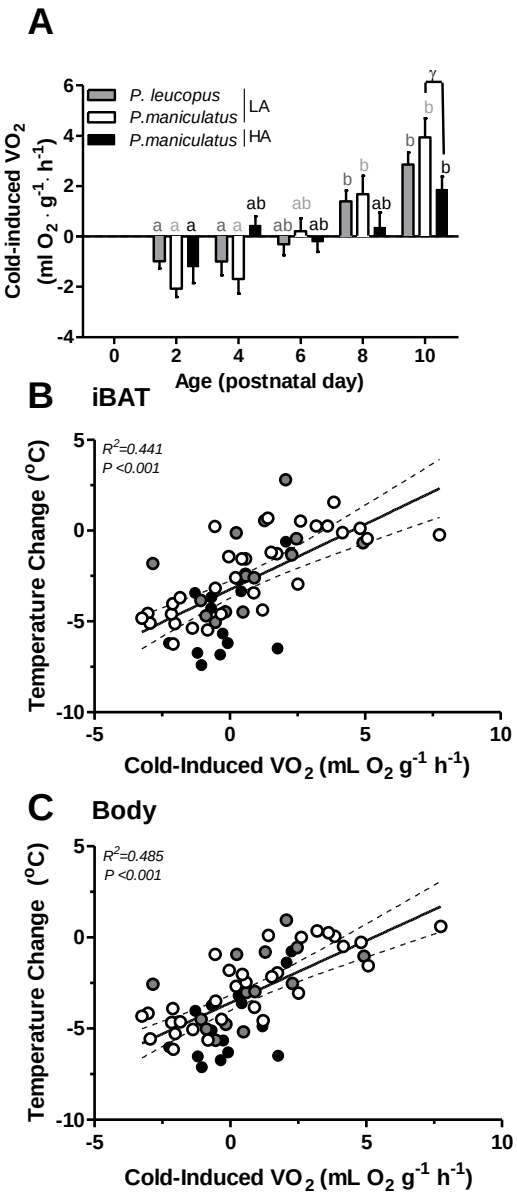
A
P. leucopus
P.maniculatus LA
P.maniculatus HA
Cold-induced VO2 (ml O2 · g-1 · h-1)
Age (postnatal day)
B iBAT
R2=0.441
P <0.001
Temperature Change (°C)
Cold-Induced VO2 (mL O2 g-1 h-1)
C Body
R2=0.485
P <0.001
Temperature Change (°C)
Cold-Induced VO2 (mL O2 g-1 h-1)

Figure 2.3. Metabolic response to acute cold (10 min at 24ºC) in common garden-raised *Peromyscus maniculatus* from low (LA) and high altitude (HA) ancestry and LA *P. leucopus* during early postnatal development. (A) Cold-induced change in oxygen consumption ($\dot{V}O_2$) during an acute cold trial relative to a warm control pup litter-mate. Dissimilar letters denote significant difference between age, within population; $^{\gamma}$ denotes significant population difference, within age (Holm's Sidak analysis). Data are mean ± sem. (B, C) Linear regression (95% CI) of cold induced $\dot{V}O_2$ and change in body or interscapular brown adipose tissue (iBAT) surface temperatures. Sample sizes (N) of *P. leucopus*, LA and HA *P. maniculatus* by age: P2(5,6,9), P4 (4,7,8), P6 (7,7,7), P8 (7,6,5) and P10 (7,7,7).

Figure 2.4.

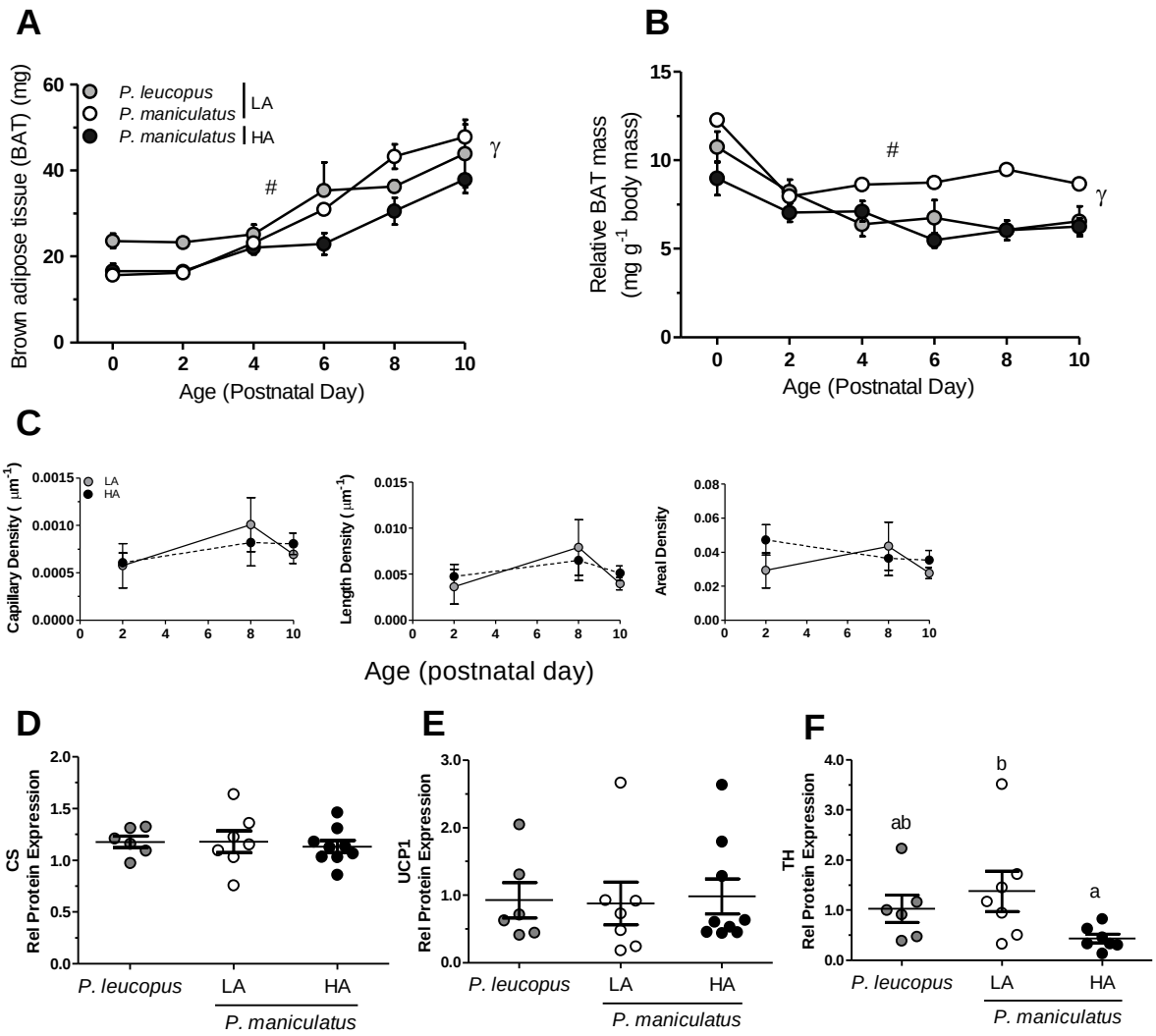
A
Brown adipose tissue (BAT) (mg)
P. leucopus
P. maniculatus
P. maniculatus
LA
HA
#
γ
Age (Postnatal Day)
B
Relative BAT mass (mg g-1 body mass)
#
γ
Age (Postnatal Day)
C
Capillary Density (μm-1)
Length Density (μm-1)
Areal Density
LA
HA
Age (postnatal day)
D
CS
Rel Protein Expression
P. leucopus
LA
HA
P. maniculatus
E
UCP1
Rel Protein Expression
P. leucopus
LA
HA
P. maniculatus
F
TH
Rel Protein Expression
ab
b
a
P. leucopus
LA
HA
P. maniculatus

Figure 2.4. Brown adipose tissue (BAT) maturation during early postnatal (P) development in common garden-raised *Peromyscus maniculatus* from low (LA) and high altitude (HA) ancestry and LA *P. leucopus* (PL). (A, B) Absolute and mass-specific growth of interscapular BAT deposit (iBAT). (C) Capillary density, length and area density of iBAT. (D-F) Protein expression of citrate synthase (CS), uncoupling protein-1(UCP-1) and tyrosine hydroxylase (TH) in iBAT of P10 pups. $^{\#}$ Significant main effect of age, $^{\gamma}$ significant main effect of population as determined by 2-Way ANOVA. Bars with dissimilar letters are significantly different as determine by 1-Way ANOVA. All data are mean ± s.e.m.

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CHAPTER 3 Developmental delay in shivering limits thermogenic capacity in juvenile high-altitude deer mice (*Peromyscus maniculatus*)

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3.1 ABSTRACT

Many endotherms native to cold and hypoxic high altitude (HA) environments have evolved a highly vascularized and aerobic skeletal muscle. This specialized muscle phenotype contributes via shivering to an enhanced capacity for aerobic thermogenesis (cold-induced $\dot{V}O_2max$). However, it is unclear how selection at HA for shivering thermogenesis acts early in the development of small altricial mammals, which are born with immature skeletal muscles and without the capacity for homeothermic endothermy. We have previously shown that postnatal maturation of brown adipose tissue and non-shivering thermogenesis is delayed in HA native deer mouse pups (*Peromyscus maniculatus*). To assess whether HA adaptation has also altered the developmental program of skeletal muscle and shivering thermogenesis, we used laboratory-reared descendants of deer mice native to low altitude (LA, 430 m a.s.l) and HA (4350 m a.s.l.) and a LA congeneric outgroup (*P. leucopus*). We found that LA juveniles were able to shiver robustly at 2 weeks after birth. However, HA juveniles were unlikely able to shiver at this point, resulting in a 30% lower capacity for thermoregulation compared to lowlanders. It was only at 27 days after birth that HA juveniles had established the aerobic muscle phenotype characteristic of HA adults and a superior cold-induced $\dot{V}O_2max$ compared to LA mice of the same age. The capacity for shivering may be

delayed in HA mice to allow energy to be allocated to other important processes such as growth.

3.2 INTRODUCTION

At birth altricial mammals lack the capacity to regulate body temperature through metabolic heat production (Pembry, 1895). Instead, they develop homeothermic endothermy over the early postnatal period as the primary thermo-effector organs and their corresponding regulatory systems mature. This dynamic life stage is characterized first by the rapid maturation of brown adipose tissue (BAT) and non-shivering thermogenesis (NST; Lagerspetz, 1966). In contrast, the skeletal muscles of altricial mammals are extremely immature at birth (Dubowitz, 1963). In these species, it takes several weeks before muscle phenotype is fully established (e.g., Goldspink and Ward, 1979; Adams et al., 1999, Gokhin et al., 2008, Agbulut et al., 2003). The relatively slow postnatal maturation of skeletal muscle corresponds with a gradual onset of the capacity to engage in shivering (Arajamma and Lagerspetz, 1978). For example, shivering was not observed in the white-footed mouse (*Peromyscus leucopus*) until approximately 2-weeks after birth (Hill, 1976).

In adult endotherms, a well-developed capacity for shivering thermogenesis is especially important in the unremitting low ambient temperatures characteristic of high-altitude (HA) ecosystems. This environment is particularly challenging for small obligate endotherms as their high rates of heat loss increase the demand for aerobic heat production in the face of low O_2 availability. As a result of these combined selective

pressures many birds and mammals native to high alpine regions have evolved highly vascularized and aerobic adult skeletal muscle phenotypes (León -Velarde et al., 1993, Hepple et al., 1998, Mathieu Costello et al., 1998, Kayser et al., 1991, Sheafor, 2003, Scott et al., 2009; Lui et al., 2015; Lau et al., 2017). This high muscle aerobic capacity is accompanied by an elevated capacity for lipid oxidation in adult HA native deer mice (*P. maniculatus*, Cheviron et al., 2012, 2014, Lau et al., 2017), presumably to support high rates of shivering. These underlying adaptations have allowed HA deer mice to evolve a high thermogenic capacity (cold-induced $\dot{V}O_2max$), which contributes directly to fitness by improving survival (Hayes and O'Connor, 1999).

Given that the skeletal muscle of altricial mammals is immature at birth, the aerobic muscle fiber type, metabolic capacity, and capillarity characteristic of this adaptive HA muscle phenotype, may be absent early in postnatal development of HA deer mice. It is unclear at what age HA native deer mice develop this phenotype which is so critical for adult performance. The early postnatal period, during which muscle phenotype is established, is characterized by extremely high mortality rates (50-95%) in wild rodent populations (e.g., Bendell, 1959; Howard, 1949). Thus, the physiological systems that develop during this period are likely subject to intense natural selection (Hill, 1983). It is unknown how natural selection in extreme environments, such as HA, may have altered the postnatal maturation of skeletal muscle and/ or the capacity for shivering thermogenesis to allow juveniles to cope with the constant cold and hypoxia at high elevations. Given the importance of muscle phenotype for adult thermoregulation, the

timing of the maturation of this system likely has major performance consequences for the thermoregulatory ability of juveniles at HA. Additionally, we have previously demonstrated that in the first 10 postnatal days, activation and regulation of NST in BAT is delayed in HA native deer mouse pups compared to lowlanders (Robertson et al., 2019). As a result, HA pups are unable to adequately thermoregulate during this early developmental window, inconsistent with their superior thermogenic capacity as adults (Cheviron et al., 2014). Given the delay in BAT maturation, HA juveniles may rely more heavily on skeletal muscle-based thermogenesis. Therefore, natural selection may have accelerated the development of this tissue at high elevations.

To assess whether HA adaptation has altered the developmental program of skeletal muscle and shivering thermogenesis, we used low-altitude (LA) and HA North American deer mice (*P. maniculatus*) and the closely related but strictly LA white-footed mice (*P. leucopus*) born and raised in common garden conditions at LA. We tested the hypothesis that adaptation to HA accelerates the maturation of skeletal muscle allowing juveniles to induce shivering thermogenesis in the cold. If so, we predict that HA pups will utilize shivering earlier in development than their LA counterparts. We further predict that the thermoregulatory capacity of HA juveniles will only exceed that of LA mice once they have developed a more aerobic muscle phenotype.

3.3 METHODS

Experimental Design

Juvenile mice used in this study were the second generation (G_2) laboratory-born progeny of a captive breeding colony of HA and LA native deer mice, *P. maniculatus*, and white-footed mice, *P. leucopus* (Rafinesque 1818). In both 2013 and 2014 the HA wild-caught breeding stock, *P. maniculatus rufinus* (Merriam, 1890) were trapped at the summit of Mount Evans, CO, USA (4350 m a.s.l.) and the LA natives, *P. maniculatus nebrascensis* (Coues, 1877) and *P. leucopus*, were trapped at Nine Mile Prairie, NE, USA (430 m a.s.l.) as previously described (Cheviron et al, 2012). Wild-caught mice were transported to McMaster University, Canada (~90 m a.s.l.) and bred within their respective populations under common garden conditions (24^{o}C, 760 mmHg, 14 h:10 h light:dark cycle, with rodent chow and water *ad libitum*). First generation (G_1) mice were mated within their respective populations to produce the G_2 offspring used in this study (Robertson et al., 2019). All pups were weaned at postnatal day 21 (P21) and housed with their same-sex littermates post weaning. Given that *P. leucopus* are closely related to *P. maniculatus* but are found exclusively at LA we used this species as a LA outgroup (Velotta et al., 2018). All procedures were approved by the McMaster University Animal Research Ethics Board.

Skeletal Muscle Maturation

Tissue Sampling

At postnatal days P0, 2, 4, 6, 8, 10, 14, 21 and 27 one pup per G1 breeding pair was sampled by an overdose of isoflurane followed by cervical dislocation. The

gastrocnemius (GA) muscle from one hindlimb was blunt dissected, weighed and freeze-clamped using two liquid N_2-cooled aluminum plates. We used the GA as a representative muscle as previous studies have shown it is involved in shivering thermogenesis (Oufara et al., 1987) and that phenotypic differences between LA and HA adult mice correlate strongly with whole animal thermogenic capacity (Cheviron et al., 2012, 2014). Muscle tissue was stored at -80^{o}C for future molecular analysis. From the other hindlimb the triceps surae (consisting of GA, plantaris and soleus) was frozen in embedding medium (Cryomatrix, Thermo Scientific) for histological analysis.

Muscle Histology

We assessed developmental changes in the numerical density of aerobic muscle fibers and capillarity as previously described for adult deer mice (Lui et al., 2015; Mahalingam et al., 2017). Briefly, frozen triceps surae was sectioned transversely (10μm) at -20°C using a cryostat (Leica Biosystems CM1860). Capillaries were identified by staining for alkaline phosphatase activity. Capillary area was quantified as previously described (Mathieu-Costello, 1987) using NIS-Elements Imaging Software version 4.30 (Laboratory Imaging, Prague, Czechia). Briefly, capillary areal density (CAD) was determined as the ratio of capillary area (μm^2) to transverse muscle area (μm^2). As it has previously been suggested that skeletal muscle capillaries of HA deer mice are more tortuous than those of LA deer mice (Lui et al., 2015; Scott et al., 2015) we used capillary length density (CLD) as a measure of tortuosity. CLD is the quotient of areal density and the transverse area of the smallest 10% of capillaries (Dawson et al., 2018). Aerobic

muscle fibers were identified by staining using succinate dehydrogenase (SDH) activity (Lui et al., 2015). The numerical density of oxidative fibers was determined as the number of oxidative fibers divided by the total number of fibers. Images were analyzed using Image J software. Muscle fiber size was determined in LA *P. leucopus* and HA *P. maniculatus* pups using hematoxylin and eosin (H&E) staining. Briefly, slides were fixed in 95% ethanol then incubated in Gills II hematoxylin for 30 seconds and eosin for 5 seconds (Leica Biosystems).

Muscle Enzyme Activitiy

The apparent V_{max} of citrate synthase (CS), lactate dehydrogenase (LDH) and β-hydroxyacyl-CoA dehydrogenase (HOAD) was assayed to assess capacity for the TCA cycle, anaerobic glycolysis and fatty acid oxidation, respectively, using assay conditions previously described (Cheviron et al., 2012; Lui et al., 2015; Lau et al., 2017).

Respirometry

At P14, P21 and _27 individual pups were used to determine either basal metabolic rate (BMR), NST or maximum thermogenic capacity (cold-induced $\dot{V}O_2$ max) by indirect calorimetry as described below.

BMR

Juveniles were removed from their nests and fasted for 4 hours to achieve a post-absorptive state prior to BMR trials, after which time they were placed in respirometry

chambers (475 ml) maintained at within the thermoneutral zone (Hill, 1983) at 28ºC using a Peltier Cabinet (Sable Systems, Las Vegas, NV, USA). Outside air was dried and stripped of CO_2 using Ascarite and soda lime before flowing through the 475 mL chambers at a rate of 1000 mL min^{-1} using a mass flow controller (Sable Systems). Excurrent air was subsampled, dried with magnesium perchlorate and passed through CO_2 (CA-10A) and O_2 (FC-1A) analyzers (Sable Systems) for determination of oxygen consumption ($\dot{V}O_2$). BMR was calculated with equation 3b from Withers (1977) using the average of the 3 lowest stable (5 min) O_2 traces over 2h.

NST

Mice were placed in metabolic chambers (475 ml) at 28ºC and baseline $\dot{V}O_2$ measurements were obtained after 45 minutes in normoxia as described above. Norepinephrine (NE) was then injected subcutaneously at a standardized dose (Wunder and Gettinger, 1996) in a total of 500 µl of saline, and mice were returned to the respirometry chamber for 1 hour or until $\dot{V}O_2$ returned to baseline. We determined in a preliminary study that any change in $\dot{V}O_2$ due to subcutaneous injection of saline alone returned to baseline $\dot{V}O_2$ after ~10 minutes, twice as fast as with intraperitoneal injections. Thus, we determined maximal NST (NST_{max}) as the highest stable (5 min) $\dot{V}O_2$ with subcutaneous NE injection after this time point. NST was then calculated as NST_{max} – BMR.

Thermogenic Capacity (Cold-induced $\dot{V}O_2$ max): To determine maximal cold-induced $\dot{V}O_2$ juveniles were placed in respirometry chambers at -5ºC and either normoxic (20%

O_2) or hypoxic (12% O_2) heliox (O_2 with He) was flowed through the chambers at a rate of 1000 ml min^{-1}. Subsampled air was dried before analysis for changes in O_2 and CO_2 (Fox Box Respiratory System, Sable Systems). Data acquisition was performed using LabChart software (ADI instruments) and cold-induced $\dot{V}O_2max$ was calculated as the highest stable (10 seconds) $\dot{V}O_2$ during a 10-minute trial (Tate et al., 2017). Rectal body temperature was taken before and after each using a rectal probe (RET-4, Physitemp) trial ensure that the trial was sufficient to induce hypothermia.

Shivering thermogenesis (ST) was estimated in normoxia as $\dot{V}O_2max$ – (NST + BMR) (Wunder and Gettinger, 1996).

Statistics

We used 2-Way repeated measures (RM) ANOVA, to test the effect of population and age on mean pup mass and 1-Way ANOVA to test the effect of population on growth rate. For these data we used family as a replicate, using the mean of all offspring from a G_1 breeding pair as a single measurement. We used 2-Way ANOVA to test the effects of population and age on tissue mass, oxidative fiber numerical density, capillary area, enzyme activity and $\%\dot{V}O_2$ max. Percent $\dot{V}O_2$ max data were arcsine square root transformed and values of 0% were assigned a value of 1/4X, where X=$\dot{V}O_2$ max, prior to analysis. For measures of $\dot{V}O_2$ ($\dot{V}O_2max$, NST, BMR) we used 2-Way ANCOVA to test the effects of population and age with body mass as a covariate. When significant interactions ($P<0.05$) were found between population and age we used Holm-Sidak post

hoc analysis to perform pairwise comparison between groups. Data are available at 10.6084/m9.figshare.8979842.

3.4 RESULTS

Pup and Muscle Growth

We tracked the growth of deer mouse (*P. maniculatus*) and white-footed mouse (*P. leucopus*) pups over the first 30 days of postnatal (P) development and found that *P. leucopus* were larger and grew faster than either population of *P. maniculatus* (Age x Population $F_{16,176}$=12.851, P< 0.001, **Fig. 3.1A**). While *P. maniculatus* pups were being provisioned by their mothers, their size and growth rates were similar regardless of altitude ancestry; post-weaning (P21) growth rates accelerated in HA and LA *P. leucopus* pups (Population $F_{2,22}$=14.487, P<0.001), resulting in a significant size differential between all three groups by P27 (Fig. 1, **Table 3.1**). Growth patterns of the GA muscle also differed between populations (Age x Population $F_{12,184}$=6.684, P< 0.001, **Fig. 3.1B**). For the first 2 weeks of development, the size of this hindlimb muscle was similar for all three groups. However, after P14 the GA of HA pups diverged and was smaller than that of either LA group. This altitude difference in muscle size was further exacerbated when expressed relative to body mass, with HA pups showing disproportionately small muscles at P21 and P27(Age x Population $F_{12,184}$=3.516, P< 0.001, **Fig. 3.1C**). The relative size of the GA muscle was identical in LA pups after P14, despite the large interspecies difference in body size.

Maturation of Muscle Metabolic Capacity

At birth, muscle fibers of the hindlimb were small and undifferentiated in all groups. By P14, all pups showed a small number of oxidative fibers in these muscles as determined by SDH staining. However, the numerical density of oxidative fibers rapidly increased from P14 to P21 in all groups. Between P21 and P27, there was a further increase in oxidative fiber numerical density but only in HA juveniles (Age x Population $F_{4,47}$=3.298, P= 0.018, **Fig. 3.2**). At P21 and P27 the muscle fibers of HA pups were generally smaller than those of LA *P. leucopus* (Age x Population $F_{7,75}$=3.434, P= 0.003, **Fig. 3.2, inset**), which is characteristic of a higher proportion of Type I and Type IIa fibers relative to the larger Type IIb fibers.

This change in aerobic capacity of the muscle was reflected in GA enzyme activity. The Vmax of CS, a marker of mitochondrial density, increased similarly in all groups from P14 to P21 (Age $F_{2,90}$ = 8.173, P <0.001) and was higher in HA pups at P27 relative to both LA populations (Population $F_{2,90}$ = 14.451, P< 0.001, **Fig. 3.3A**). Vmax of LDH also increased with age (Age $F_{2,92}$ = 11.820, P <0.001) but was lower in HA pups than in LA *P. maniculatus* (Population $F_{2,92}$ = 36.337, P< 0.001, **Fig. 3.3B**). Interestingly, HOAD activity was generally consistent between populations and across ages. It was slightly lower in LA *P. maniculatus* at P14 relative to that of HA pups but increased by P21 and remained consistent from this point onwards (Age x Population $F_{4,90}$=4.170, P= 0.004, **Fig. 3.3C**).

Muscle Capillarity

Along with immature muscle fibers, the skeletal muscle of LA and HA pups was poorly vascularized at birth. We were unable to detect capillaries in the hindlimb muscles using alkaline phosphatase staining at P2 and found negligible vascularization at P8 regardless of altitude ancestry. By P14, the capillary area was similar between all groups. This level remained constant in LA pups for the remainder of development but increased further by P21 in HA pups such that capillary area in the hindlimb was 2 to 3-fold higher than that of LA pups for the remainder of development (Age x Population $F_{4,53}$=3.494, P= 0.013, **Fig. 3.4A**). Capillary area was larger in HA pups, in part, because the capillaries were more tortuous as capillary length density followed an identical trend (Age x Population $F_{4, 51}$ = 2.577, P=0.048, **Fig. 3.4B**).

Thermogenesis

We tracked the whole-animal thermogenic performance of deer mouse (*P. maniculatus*) and white-footed mouse (*P. leucopus*) pups over the age range where muscle phenotype diverged (P14 – P27). We found that mass-corrected thermogenic capacity (cold-induced $\dot{V}O_2$ max) did not change across the second 2-weeks of postnatal development in LA pups of either species. However, HA pups showed a different pattern with age (Age x Population $F_{4,47}$= 10.756, P < 0.001). At P14, the thermogenic capacity of HA pups was lower than that of either LA group but increased continuously over the next 2 weeks. By P21, the thermogenic capacity of HA pups was equivalent to that measured in LA native mice and exceeded that of LA mice by P27 (**Fig. 3.5A**).

The proportional contribution of shivering thermogenesis (ST) to thermogenic capacity changed differentially with age and population (Age x Population $F_{4,48}$ =28.93, P< 0.001). For *P. leucopus*, the contribution of ST remained constant at ~ 30% $\dot{V}O_2$max, while in both LA and HA *P. maniculatus* the contribution of ST increased with age to ~55% $\dot{V}O_2$max at P27. However, at P14 the contribution of ST in HA pups was dramatically lower than that in either LA *P. maniculatus* or *P. leucopus* (*post hoc* P<0.001), accounting for only 3.8% of $\dot{V}O_2$max in this population (**Fig. 3.5B**). In fact, in most HA individuals tested there was no ST measured at P14, with combined BMR and NST meeting or exceeding cold-induced $\dot{V}O_2$max. Neither absolute NST nor BMR changed with age or population (**Table 3.2**). The interscapular BAT (iBAT) grew between P14 and weaning (P21) and then showed a post weaning decline in size (P27) in all populations (Age $F_{2,52}$= 11.050, P<0.001; data not shown). However, this change was not proportional to body size (Age x Population $F_{4,52}$=2.760, P=0.036). At P14 LA *P. maniculatus* had a higher proportion of iBAT than either *P. leucopus* or HA *P. maniculatus*. However, from P21 onwards there was no difference between the *P. maniculatus* populations regardless of altitude ancestry (**Table 3.2**).

Finally, we measured cold-induced $\dot{V}O_2$ under hypoxia (12% O_2) to determine the extent to which thermogenic capacity during postnatal development was limited by O_2 availability. Generally, both HA and LA *P. maniculatus* had a greater hypoxic $\dot{V}O_2$ max than LA *P. leucopus* (Population $F_{2,25}$=16.065, P<0.001; **Fig. 3.6**). Hypoxic $\dot{V}O_2$ max decreased slightly with age (Age $F_{1,25}$= 46.085, P<0.001), which was driven by

statistically non-significant decreases in both LA populations (*P. maniculatus* P=0.089, *P. leucopus* P=0.17) but not HA *P. maniculatus*.

3.5 DISCUSSION

The main objective of this study was to determine whether altitude ancestry influences the postnatal maturation of skeletal muscle and the onset of shivering thermogenesis in deer mice. In contrast to our predictions, we found that the postnatal maturation of the lower hindlimb muscles and the onset of shivering thermogenesis were not accelerated in HA deer mice. In fact, HA mice showed a substantial delay in the developmental onset of shivering and a lower overall capacity for thermogenesis early in postnatal development, compared to LA conspecifics and to LA *P. leucopus*. It was not until P21 that HA deer mice developed the capacity to shiver, and not until P27 that their skeletal muscle matured to establish the specialized aerobic phenotype characteristic of HA adults. Thermogenic capacity in HA juveniles surpassed that of lowlanders only after this more aerobic muscle phenotype was fully developed, and the capacity for shivering increased. To our knowledge this is the first study to show that the postnatal maturation of skeletal muscle and of shivering thermogenesis is altered with adaptation to HA.

Maturation of Skeletal Muscle Aerobic Phenotype

Skeletal muscles of altricial mammals, such as mice, fully mature and assume their adult phenotype sometime in the first weeks after birth (Dubowitz, 1963). However, the timing of key steps in muscle postnatal development is poorly understood in all but a few species (White et al., 2010, Schiafino and Reggiani, 2011), and to our knowledge has never been

studied in relation to adaptive variation in wild populations. We found that at birth, regardless of altitude ancestry, the lower hindlimb muscles of deer mice were small and underdeveloped, consistent with data from laboratory mice (Wirtz et al., 1983). Muscles in both LA and HA mice grew proportionately with increases in body size over the first 10 days of development after which muscle growth accelerated. Postnatal muscle growth in mice is generally attributed to hypertrophy of muscle fibers (Gokhin et al., 2008; White et al., 2010) and we found that muscle fiber size increased significantly in both LA and HA mice over the first 27 days after birth. However, later in development (P21-P27), HA juveniles had smaller muscle fibers compared to LA mice. This may be indicative of a higher proportion of oxidative fibers which tend to be smaller than glycolytic fibers, an adaptation thought to minimize O_2 diffusion distance from the capillaries to the mitochondria (van Wessel et al, 2010). Overall, these smaller fibers likely contributed to smaller gastrocnemius muscles in HA juveniles after P21, even after considering any differences in body mass between experimental groups. These smaller muscle fibers and overall muscle size are also seen in adult HA deer mice (Lui et al., 2015).

We found that many of the traits that are characteristic of a specialized HA muscle phenotype were established in HA mice over the first month of postnatal development, but interestingly not all traits matured at a similar pace. For example, muscle capillarity diverged between HA and LA pups at P21, suggesting that HA mice have an increased capacity for O_2 delivery to muscle by this age. Evidence for a fiber-type difference at this age is supported by the higher capacity for pyruvate to lactate exchange in LA pups (LDH

activity) relative to HA pups over this same time period (P21-P27). However, differences in muscle aerobic fiber numerical density (SDH staining) were not apparent until one week later at P27. This contrasts with adult skeletal muscle where capillarity tends to vary in close association with muscle fiber aerobic capacity (e.g., Sullivan and Pittman, 1987; Hoppeler and Kayar, 1988). Another important mitochondrial protein, the citric acid cycle enzyme CS also showed greater activity in HA mice by P27. CS is often used as an indirect marker of mitochondrial Volume and these data suggest that it may take at least 4 weeks of postnatal development to establish the higher mitochondrial Volume density seen in adult HA deer mice (Mahalingam et al., 2017). In skeletal muscle of adults there is a tight relationship between mitochondrial Volume density and muscle fiber capillarity, which allows maximal O_2 extraction from blood (Hepple, 2000). This developmental pattern, where increases in capillarity precedes increases in mitochondria, may be a mechanism to ensure that muscle cells have an adequate O_2 supply prior to increasing aerobic capacity. This may prevent a mismatch between O_2 supply and demand in the maturing muscle of HA mice.

Interestingly, there was no change in the capacity for lipid oxidation (indexed by HOAD activity) across development in HA juveniles, despite the importance of lipid metabolism in adults of this population (Cheviron et al., 2012). This may represent a temporal mismatch in the development of mitochondrial quantity versus quality and indicates that not all aspects of the HA adult muscle phenotype are established by P27. Taken together these data serve as an important reminder that maturation of adult muscle phenotype is a

combination of many independently developed functional parameters (e.g., mitochondrial Volume, capillarity, myosin heavy chain isoform) that eventually preferentially cluster together in the mature animal (Schiaffino and Reggiani, 2011). The underlying mechanisms responsible for this developmental program are unclear but the regulatory gene networks that drive the dynamic changes during ontogeny of skeletal muscle in HA mice are likely complex and this is an important area for future study.

It is notable that the developmental patterns for the traits we measured were similar in muscle of both LA *P. maniculatus* and *P. leucopus*. This suggests that the developmental trajectory seen in HA muscle is a derived phenotype that has evolved at higher elevations. Given our common garden experimental design, we attribute the observed differences in LA and HA skeletal muscle to genetically based changes in the developmental program. It is also possible that evolved differences in environmentally induced plasticity influence the development of skeletal muscle in the wild. However, adult deer mouse skeletal muscle is generally resistant to hypoxia-induced phenotypic flexibility (Lui et al., 2015; Lau et al., 2017; Mahalingam et al., 2017), and rearing deer mice in chronic hypoxia had little effect on adult muscle phenotype (Nikel et al., 2018). Thus, the pattern of muscle maturation we observed likely reflects the developmental trajectory of wild deer mice. What then are the functional and fitness consequences of mismatched developmental timing of important muscle traits on whole-animal thermal performance in juvenile mice?

Development of Thermogenic Capacity

Like other altricial mammals, the development of homeothermic endothermy in young deer mice first involves the maturation of BAT and its regulation early in the postnatal period (P0 - P10; Robertson et al., 2019). Any further increase in thermogenic capacity after this point may involve either increases in the capacity for NST and/or the developmental onset of shivering. In altricial mammals there is a gradual postnatal onset of shivering, which occurs at irregular bouts in response to cold, and with a much lower amplitude compared with that in adults (direct quantification using electromyography in guinea pig, Brück and Wünnenberg, 1965; golden hamster, Hissa and Lagerspetz, 1964, Norwegian lemming, Hissa, 1968; Egyptian fruit bat, Hissa, 1964; and laboratory mouse; Araajamaa and Lagerspetz, 1978). We found that LA deer mice were able to shiver at P14, with shivering thermogenesis contributing ~35% to cold-induced $\dot{V}O_2$ max. Surprisingly, at this same age stimulation of NST in HA pups could account for 100% of cold-induced $\dot{V}O_2$ max, suggesting an inability to shiver. Lack of shivering likely led to the 30% lower thermogenic capacity of HA mice compared to LA mice at P14. Importantly, regardless of altitude ancestry, at P14 all measures of muscle phenotype were similar. Yet LA juveniles could shiver robustly in response to cold while HA juveniles could not. This implies that the whole-animal performance differences at this age are not due to differences in muscle phenotype. It has been suggested that shivering occurs later in ontogeny relative to NST because of the slow development of the muscles compared to BAT (Lagerspetz, 1966). However, the gradual onset of shivering in altricial mammals is also caused by the postnatal development of the neural pathways that regulate heat production (Arajamaa and Lagerspetz, 1978). Thus, it is possible that HA

mice have delayed maturation of the regulatory systems that govern the shivering reflex. This is supported by our previous study, where delayed activation of BAT in HA pups over the first 10 days of postnatal development appeared to be driven by a delay in the sympathetic innervation of the tissue (Robertson et al., 2019). Given that BAT in the HA mice appears to be functional at P14, the shared neural circuitry of the shivering and non-shivering pathways upstream of the motor neurons is likely intact (Madden and Morrison, 2019). Therefore, the delay in shivering may be due to underdeveloped efferent activation of muscle in response to cold.

Between ages P14 and P27, $\dot{V}O_2$max steadily increased in HA pups, while LA pups showed no significant change in thermogenic capacity over the same period. During this time, absolute rates of NST did not change, therefore, the resulting increase in thermogenic capacity must be due to the onset of shivering in HA pups. Despite the delay in the onset of shivering thermogenesis, the capacity for shivering increased continuously from 2 to 4 weeks of postnatal development in HA deer mouse pups and their $\dot{V}O_2$max surpassed that of LA pups by P27, consistent with population divergence in muscle phenotype. While the specialized aerobic phenotype of HA muscle is not required for shivering thermogenesis, once established it likely drives the improved thermogenic performance of HA relative to LA mice.

It is notable that cold-induced $\dot{V}O_2$ max was only higher in 27-day old HA juveniles when tested under normoxic conditions. In contrast, when tested under hypoxia, thermogenic capacity was severely limited and did not differ between LA and HA mice.

As previously discussed, HA mice at this age do not yet have the high capacity for lipid oxidation that is seen in adults. In adult mice, this gastrocnemius muscle metabolic profile directly correlates with thermal performance under hypoxia (Cheviron et al., 2012; Cheviron et al., 2014) and could explain the lower performance of juveniles under hypoxia at this age. Additionally, thermogenesis is limited not just by muscle performance but also by O_2 availability (di Prampero, 1985). Many HA natives, including deer mice, have a suite of respiratory adaptations (hemoglobin affinity, breathing pattern, etc…) that increase their capacity for O_2 delivery (McClelland and Scott, 2019). Many of these traits are also established postnatally and could lag behind skeletal muscle development, limiting thermogenesis under hypoxia. Importantly, these findings emphasize that even at this more mature age, HA juveniles are likely not proficient thermoregulators in their native environment.

Adaptive Benefits of Delaying Shivering?

If an elevated cold-induced $\dot{V}O_2$ max is a critical adaptation for adult HA deer mice, why then do they delay the development of shivering thermogenesis and overall thermogenic capacity, particularly under hypoxia? The answer may lie in the high energetic cost of thermoregulation at HA. For example, field metabolic rate data suggest that adult HA deer mice routinely operate much closer to their $\dot{V}O_2$max than LA deer mice in the wild (Hayes, 1989). During the postnatal period pups can avoid this energetic cost by relying on their mothers as an external heat source (Hill, 1983). It is notable that HA thermogenic capacity only begins to surpass that of LA pups after weaning at P21. This

is also the point where many of the phenotypic traits of the skeletal muscle diverge. HA pups may not require the full development of thermogenic capacity before this point if they rely more heavily on maternal care than their LA conspecifics. However, female HA *P. maniculatus* give birth to larger litters of pups than either LA *P. maniculatus* or *P. leucopus*, both in the wild and in captivity (Robertson et al., 2019; Halfpenny, 1980). Large litter size increases intra-litter competition for food, limiting growth rate in rodents (Kaufman and Kaufman, 1987). Wild and captive bred LA *Peromyscus* mothers compensate for large litter sizes by increasing food intake (Miller, 1975; Miller, 1979; Glazier, 1985) allowing postnatal growth rates of their pups to be maintained. This response is exacerbated in the cold where rodent mothers must further increase food intake to meet their own energetic needs (Hammond and Kristan, 2000; Paul et al., 2010). To provision their large litters in cold conditions, female HA deer mice may spend substantially more time foraging for food than their LA counterparts, leaving their pups unattended. HA pups would therefore experience longer or more frequent bouts of cold. Thus, HA pups may have evolved to prioritize the maintenance of postnatal growth rates under these conditions by limiting their capacity for energetically costly thermogenesis over the nursing period. We see evidence for this in the similar pre-weaning growth rates of HA pups and their LA conspecifics, despite the HA mice having greater intra-litter competition for milk. Instead of attempting to maintain body temperature when their mothers are absent from the nest, juveniles may save energy by allowing body temperature to drop. Whether this is accomplished actively, by purposefully entering a torpor-like state, or passively as a result of an immature thermoregulatory system remains

to be seen (Geiser, 2008). This growth versus thermoregulation trade-off likely manifests throughout postnatal development at HA as we have also found that the maturation of BAT-based NST is delayed in HA deer mice (Robertson et al., 2019).

3.6 CONCLUSIONS

We have shown for the first time that the aerobic muscle phenotype, characteristic of many HA native endotherms, is established over the first month after birth in HA deer mice. Additionally, we have shown that juvenile HA deer mice have a limited capacity to independently thermoregulate early in postnatal development because of a delayed ability to engage in shivering thermogenesis relative to their LA conspecifics. This delay is likely a result of the regulatory systems that activate shivering, rather than the maturation of the skeletal muscle itself. Previously, we have shown that the earlier postnatal onset of NST is also delayed in HA deer mice because of a similar latency in BAT activation (Robertson et al., 2019). Taken together these findings suggest that HA mice repress thermogenesis after birth until weaning. While these findings may seem paradoxical given the superior thermogenic capacity of adult mice native to high elevations, it is likely that this is a critical adaptation that allows pups and juveniles to preserve energy and maintain high postnatal growth rates in this extreme environment.

3.7 ACKNOWLEDGEMENTS

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3.8 FIGURES AND TABLES

Table 3.1. Postnatal growth rates of second generation (G_2) laboratory-born high-altitude (HA) and low-altitude (LA) *Peromyscus maniculatus* and LA *Peromyscus leucopus*.

	P. leucopus **(9)**	***P. maniculatus*** **LA (7)**	***P. maniculatus*** **HA (8)**
Pre-Weaning Growth Rate ($g \cdot day^{-1}$)	0.51 ± 0.03^{b}	0.41 ± 0.03^{a}	0.38 ± 0.02^{a}
Post-Weaning Growth Rate ($g \cdot day^{-1}$)	0.84 ± 0.07^{c}	0.36 ± 0.08^{a}	0.63 ± 0.04^{b}

Dissimilar letters represent significant differences between populations as determined with One-Way ANOVA. Data are presented as means ± s.e.m. Sample sizes for each group are in parentheses.

Table 3.2. Estimated marginal mean basal metabolic rate (BMR), norepinephrine-induced non-shivering thermogenesis (NST) and interscapular brown adipose tissue (iBAT) mass of G2 HA and LA *P. maniculatus* and LA *P. leucopus*.

	P. leucopus **(3-8)**			***P. maniculatus*** **(LA) (3-7)**			***P. maniculatus*** **(HA) (5-12)**		
Age (postnatal day)	P14	P21	P27	P14	P21	P27	P14	P21	P27
BMR ($ml\ O_2 \cdot min^{-1}$)	0.58 ±0.12	0.55 ±0.10	0.53 ±0.17	0.55 ±0.03	0.43 ±0.04	0.53 ±0.11	0.53 ±0.02	0.53 ±0.08	0.44 ±0.03
NST ($ml\ O_2 \cdot min^{-1}$)	0.76 ±0.19	1.00 ±0.09	1.19 ±0.47	0.77 ±0.10	1.01 ±0.11	0.92 ±0.29	0.83 ±0.12	1.0 6±0.19	0.92 ±0.19
Relative iBAT mass ($mg \cdot g^{-1}$)	5.97 ±0.89[a]	7.36 ±0.58[a]	4.67 ±0.55[a]	9.92 ±0.58[b]	9.10 ±0.58[ab]	7.37 ±0.63[b]	6.5 6±0.63[a]	9.56 ±0.55[ab]	5.63 ±0.51[ab]

Data (means ± s.e.m.) are for the second 2 weeks of postnatal development (P, postnatal day). Dissimilar letters indicate significant differences between populations within age as determined by 2-Way ANOVA. Sample sizes for each group are in parentheses.

Figure 3.1

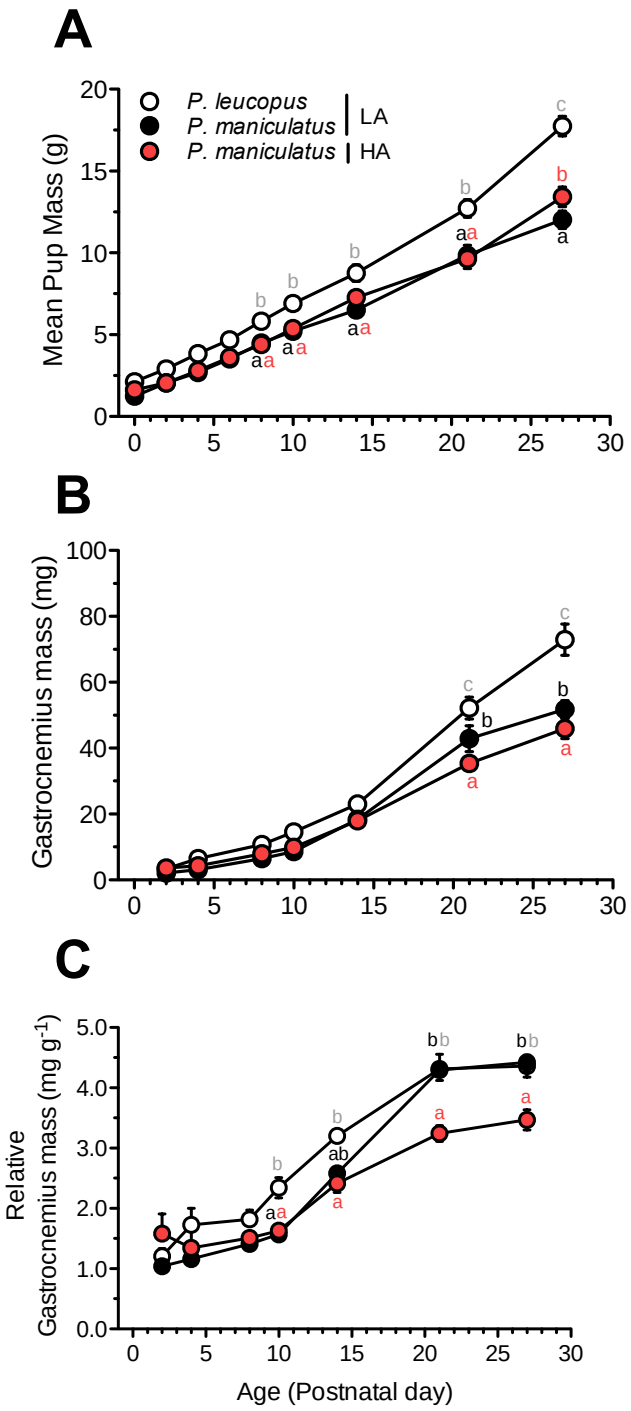
A
Mean Pup Mass (g)
P. leucopus
P. maniculatus | LA
P. maniculatus | HA
B
Gastrocnemius mass (mg)
C
Relative Gastrocnemius mass (mg g-1)
Age (Postnatal day)

Fig. 3.1. Postnatal growth of common garden raised *Peromyscus maniculatus* pups of low-altitude (LA, N=7) and high-altitude (HA, N=8) ancestry and from a LA outgroup species, *Peromyscus leucopus* (N=10). Body mass (A), gastrocnemius muscle mass (B), and gastrocnemius mass relative to body mass (C). Groups with dissimilar letters indicate significant population differences within an age as determined by 2-Way ANOVA or RM ANOVA and Holm's Sidak multiple comparisons. All data are presented as means ± s.e.m.

Figure 3.2

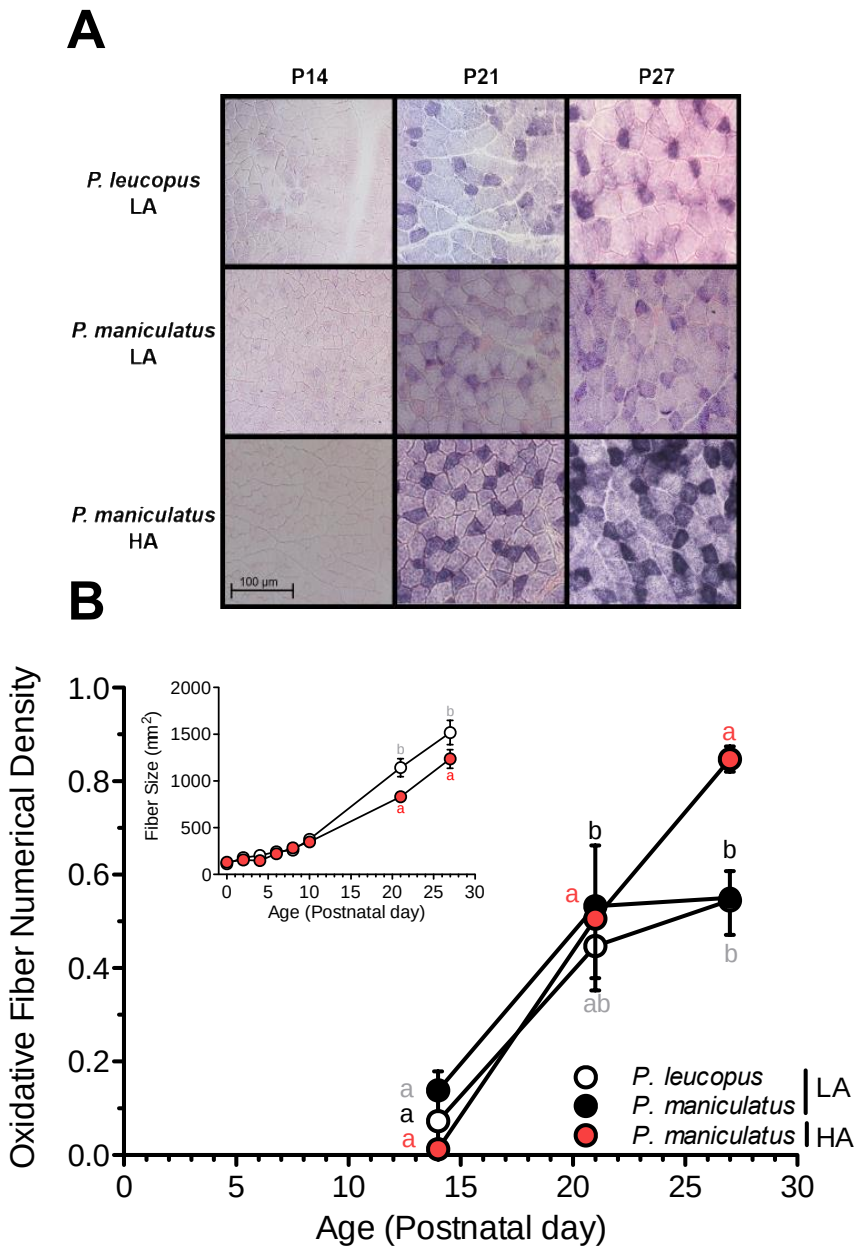
A
P14
P21
P27
P. leucopus
LA
P. maniculatus
LA
P. maniculatus
HA
100 µm
B
Oxidative Fiber Numerical Density
Age (Postnatal day)
Fiber Size (mm²)
Age (Postnatal day)
P. leucopus
P. maniculatus
P. maniculatus
LA
HA

Fig. 3.2. Maturation of aerobic fiber type of the triceps surae muscle complex in HA and LA *P. maniculatus* and LA *P. leucopus*. (A). Representative images of triceps surae muscle fibers stained for succinate dehydrogenase (SDH) activity to identify aerobic fibers (stained purple) at postnatal day (P)14, P21 and P27 days after birth. (B). Histological analysis of numerical density of aerobic fibers (SDH staining). Inset depicts mean fiber size across age in LA and HA mice as determined by H&E staining. Dissimilar letters indicate significant population differences within an age as determined by 2-Way ANOVA and Holm- Sidak multiple comparisons. Sample sizes (*N*) of *P. leucopus* and LA and HA *P. maniculatus* by age: P14, *N*=3, 6, 8, P21, *N*=7, 7, 8 and P27, *N*=10, 8, 8. All data are presented as means ± s.e.m.

Figure 3.3

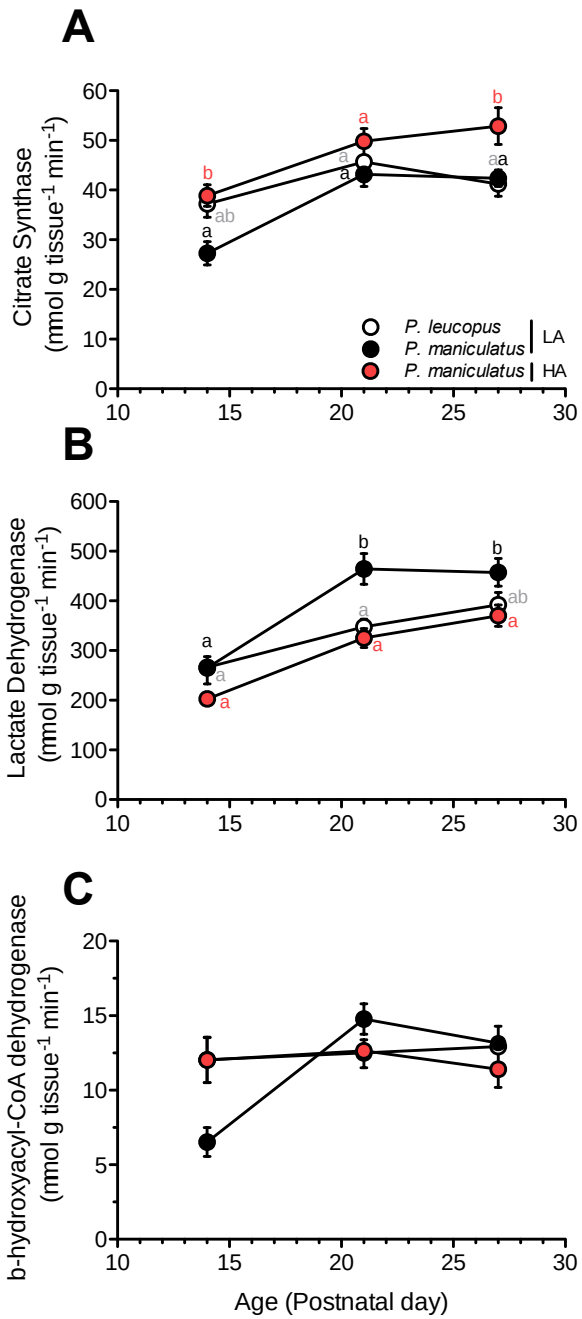
A
Citrate Synthase
(mmol g tissue-1 min-1)
P. leucopus
P. maniculatus
LA
P. maniculatus
HA
B
Lactate Dehydrogenase
(mmol g tissue-1 min-1)
C
b-hydroxyacyl-CoA dehydrogenase
(mmol g tissue-1 min-1)
Age (Postnatal day)

Fig. 3.3. Muscle enzyme activity in gastrocnemius muscle across postnatal development in HA and LA *P. maniculatus* and LA *P. leucopus*. Apparent Vmax is shown for (A) citrate synthase (CS), (B) lactate dehydrogenase (LDH) and (C) β-hydroxyacyl-CoA dehydrogenase (HOAD). Dissimilar letters indicate significant population differences within age as determined by 2-Way ANOVA and Holm-Sidak multiple comparisons. Sample sizes (*N*) of *P. leucopus* and LA and HA *P. maniculatus* by age: P14, *N*=9, 10, 10, P21, *N*=12, 11, 12 and P27, *N*=15, 10, 12. All data are presented as means ± s.e.m.

Figure 3.4

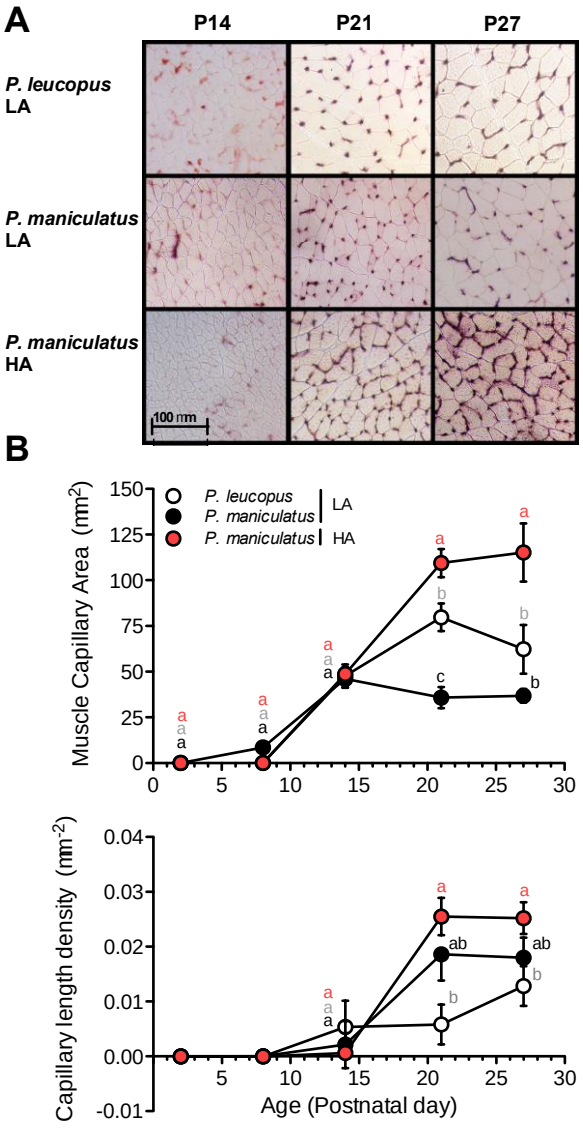
A
P14
P21
P27
P. leucopus
LA
P. maniculatus
LA
P. maniculatus
HA
100 mm
B
P. leucopus
P. maniculatus
P. maniculatus
LA
HA
Muscle Capillary Area (mm2)
Capillary length density (mm-2)
Age (Postnatal day)

Fig.3.4. Maturation of capillarity of the triceps surae muscle complex in HA and LA *P. maniculatus* and LA *P. leucopus*. (A). Representative images of triceps surae muscle fibers stained for alkaline phosphatase (AP) activity to identify capillaries (dark stain) at P14, P21 and P27. (B). Mean capillary area and capillary length density (quotient of areal density and the transverse area of smallest 10% of capillaries). Dissimilar letters indicate significant population differences within age as determined by 2-Way ANOVA and Holm-Sidak multiple comparisons. Sample sizes (*N*) of *P. leucopus* and LA and HA *P. maniculatus* by age: P14, *N*=5, 5, 7, P21, *N*=7, 4, 9 and P27, *N*=7, 7, 11. All data are means± s.e.m.

Figure 3.5

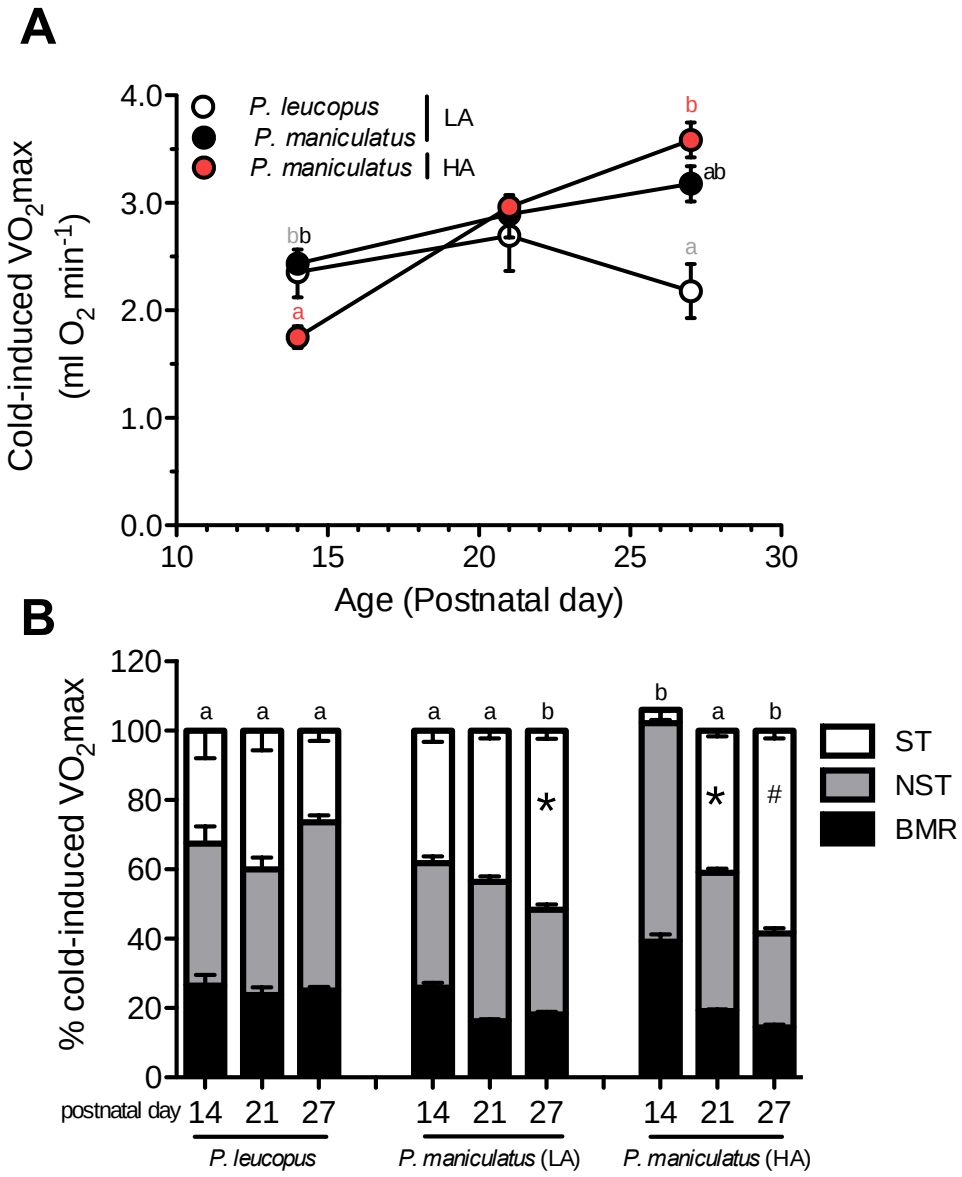
A
Cold-induced VO2max (ml O2 min-1)
4.0
3.0
2.0
1.0
0.0
10
15
20
25
30
Age (Postnatal day)
P. leucopus
P. maniculatus
P. maniculatus
LA
HA
b
ab
bb
a
a
B
% cold-induced VO2max
120
100
80
60
40
20
0
a a a a a b b a b
* * #
ST
NST
BMR
postnatal day 14 21 27 14 21 27 14 21 27
P. leucopus
P. maniculatus (LA)
P. maniculatus (HA)

Fig. 3. 5. Thermogenic capacity during postnatal development of HA and LA *P. maniculatus* and LA *P. leucopus*. (A). Estimated marginal mean of cold-induced $\dot{V}O_2max$. (B). Relative contributions (% total) of shivering thermogenesis (ST), non-shivering thermogenesis (NST) and basal metabolic rate (BMR) to cold-induced $\dot{V}O_2max$. For cold-induced $\dot{V}O_2max$, dissimilar letters indicate significant population differences within an age as determined by 2-Way ANCOVA and Tukey's multiple comparisons. For percentage $\dot{V}O_2max$ dissimilar letters indicate significant population differences of percentage ST within age as determined by 2-Way ANOVA and Holm-Sidak multiple comparisons. * Percentage ST > P14, # percentage ST > P21 within a population. Sample sizes (*N*) of *P. leucopus* and LA and HA *P. maniculatus* by age: P14, *N*=4, 7, 5, P21, *N*=3, 8, 10 and P27, *N*=6, 7, 7. All data are means ± s.e.m.

Figure 3.6

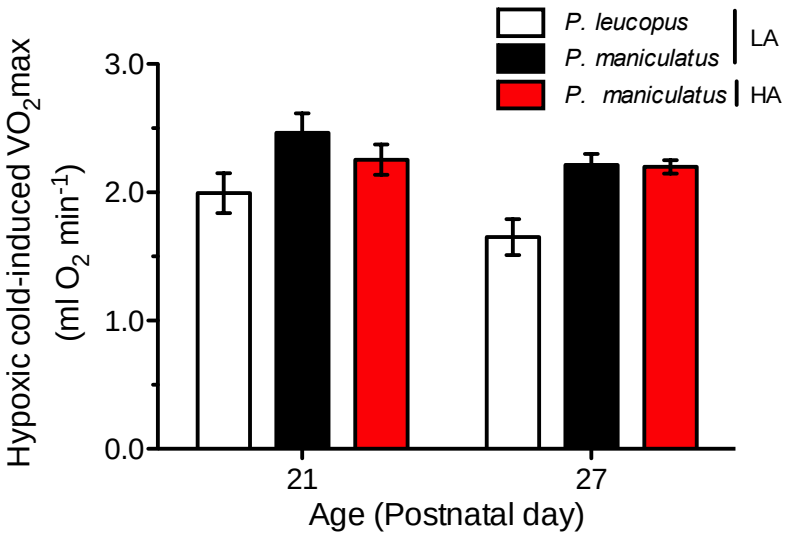
P. leucopus
P. maniculatus
P. maniculatus
LA
HA
Hypoxic cold-induced VO2max
(ml O2 min-1)
3.0
2.0
1.0
0.0
21
27
Age (Postnatal day)

Fig. 3. 6. Estimated marginal mean thermogenic capacity (cold-induced $\dot{V}O_2max$) in hypoxia (12% O_2) during postnatal development of HA and LA *P. maniculatus* and LA *P. leucopus*. 2-Way ANCOVA and Tukey's multiple comparisons test reveal a main effect of both age and population. All data are means ± s.e.m.

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CHAPTER 4 Developmental plasticity of brown adipose tissue-driven non-shivering thermogenesis to cold is lost with high altitude adaptation

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4.1 ABSTRACT

An adaptive physiological trait is the result of gene by environment interactions leading to evolved fixed traits or phenotypic flexibility. Rearing environment can influence physiological phenotypes by altering developmental trajectories, adult phenotype or the adult acclimation response. High-altitude (HA) deer mice have evolved an enhanced adult thermogenic capacity to survive the low oxygen and temperatures characteristic of their environment. To understand the influence of developmental plasticity on thermogenic capacity we used lab-reared descendants of *Peromyscus* mice adapted to low-altitude (LA, 400m a.s.l.) and HA (4350m a.s.l.). Pups were cold exposed (14°C) either prenatally (conception-birth) or postnatally (birth to 27 days old). We tested the hypothesis that developmental plasticity to cold in HA adapted mice, driven by brown adipose tissue (BAT), allows them to cope with the extreme thermoregulatory challenges of their environment. We found that cold stimulated BAT growth and accelerated developmental onset of endothermy in LA pups. Early postnatal cold exposure permanently remodeled BAT, resulting in increased BAT activity and thermogenic capacity as adults. However, developmental plasticity to cold was only seen in LA natives suggesting that it is lost with adaptation to HA. To test the effect of rearing temperature on the adult cold acclimation response we further acclimated a subset of mice to 6 weeks at 5°C once they reached maturity. Early postnatal cold exposure limited the adult acclimation response in LA and

HA mice. Prenatal cold had a similar effect, though only in lowlanders. Our findings suggest a complex interaction between the effects of developmental and adult environment drives thermogenesis in the wild, and this interaction is strongly influenced by altitude ancestry.

4.2 INTRODUCTION

An animal's adult physiological phenotype is the result of gene by environment interactions leading to evolved fixed traits and phenotypic flexibility (e.g., Schlichting and Pigliucci, 1995; Price et al., 2003). In recent years it has become increasingly clear that rearing environment also shapes adult phenotype through developmental plasticity (Mozcek, et al., 2011; Mueller et al., 2015; Beamen et al., 2016; Wells, 2019). This phenomenon is a potential driver of both individual fitness and evolutionary change as it may allow young organisms to better match their phenotype to their environment (West-Eberhard, 2005). These two plastic responses can also interact, where an organism's prior experience with a stressor during development may influence their capacity to respond to that same stressor later in life (e.g., Chappell et al., 2007; Russell et al., 2008; Beaman et al., 2016). Thus, adult phenotype may be influenced by environmental stressors acting at any life stage. In addition to having a persistent effect on adult phenotype, environmental stressors experienced during critical windows of development can alter the timing and order of developmental milestones, influencing the physiology of the juvenile as well as the adult (Burggren and Reyna, 2011). As a result, understanding the role that developmental environment plays in shaping adaptive physiological traits can be remarkably complex.

Animals adapted to high altitude (HA) are an excellent model for studying complex genotype by environment interactions. Alpine environments are characterized by two main selective pressures, unremitting low ambient temperatures and low environmental PO_2, resulting in numerous physiological differences between closely related high and low altitude (LA) adapted populations and species (Keller et al., 2013, McClelland and Scott, 2019). Importantly, many of the developmental and adult responses to acute hypoxia, particularly cardio-respiratory changes, of LA taxa are maladaptive in the face of chronic hypoxia (Bavis, 2005; Moore, 2004; Storz et al., 2010). As a result, phenotypic flexibility to this environmental stressor has been altered or blunted in many HA adapted species (e.g., Beal, 2007; Frisancho, 1975; Ge et al., 1998; Velotta et al., 2018). However, cold is a relevant environmental stressor regardless of altitude. The aim of this study is to determine if HA adaptation has altered the developmental response to a cold rearing environment.

The chronic cold of HA is uniquely challenging for small obligate endotherms because aerobic heat production is limited by the low O_2 availability (Hayes, 1989). To cope with this combination of stressors, small rodents that inhabit this ecosystem have evolved an elevated cold-induced $\dot{V}O_2max$ (thermogenic capacity; Hayes and O'Connor, 1999; Cheviron et al., 2012). This trait directly increases fitness by improving survival (Hayes and O'Connor, 1999) and also increases the animal's ability to remain active in the cold (Sears et al., 2006). HA native deer mice (*Peromyscus maniculatus*) tested in the wild have been shown to have either a superior thermogenic capacity as adults (Cheviron et al.,

2013) or a greater adult acclimation response to cold (Chappell et al., 2007) compared to those reared at LA, indicating that this trait is driven in part by some aspect of their developmental environment.

One potential physiological mechanism driving the plasticity of whole animal thermogenic performance is brown adipose tissue (BAT). BAT is the primary site of non-shivering thermogenesis (NST) in placental mammals and is a major contributor to overall adult thermogenic capacity in deer mice (van Sant and Hammond, 2008). BAT functions by uncoupling aerobic respiration from ATP production through uncoupling protein-1 (UCP-1; Cannon and Nedergaard, 2004). However, in altricial species, such as deer mice, the capacity of BAT to engage in thermogenesis only develops after birth. In LA natives, early life cold exposure can accelerate the maturation of BAT (Mouroux et al., 1990) and the onset of endothermy (Hahn, 1956; Jensen et al., 1980). For some altricial species, postnatal cold exposure is even required for normal BAT development (Jurić-Lekić et al., 2018; Nagaya et al., 2019). Early cold exposure can also permanently remodel BAT in laboratory rodents, resulting in an adult phenotype that is more responsive to cold (Cooper et al., 1980; Bertin et al., 1990; Mouroux et al., 1990; Morrison et al., 2000; Young et al., 2002). These studies highlight the influence of early-life environmental experience on BAT phenotype and whole-animal capacity for metabolic heat production. BAT and NST are also subject to phenotypic flexibility in adult rodents (Cannon and Nedergaard, 2004) including *Peromyscus* (e.g., Lynch, 1973; van Sant and Hammond, 2008) in response to cold. Given the inherent plasticity of BAT

it is the likely proximate mechanism of both developmental and adult plasticity of thermogenesis in LA adapted species. However, these complex interactions have not been tested in the context of HA adaptation.

We have previously shown that the postnatal maturation of brown adipose tissue (BAT) and the onset of BAT-based non shivering thermogenesis (NST) is delayed in HA native deer mouse pups compared to LA natives raised under common garden conditions (Robertson et al., 2019). This may be an example of physiological heterochrony, or an evolved difference in the timing developmental milestones (Spicer, 2006). However, it is equally possible that in their native environment, HA pups rely on an environmental stimulus to promote BAT maturation.

Using a common garden model and breeding to the second generation in captivity to isolate the effects of genotype, developmental environment and adult environment, we have asked three questions about the effect of early cold exposure on BAT of HA native deer mice. Does early cold exposure alter the developmental timing of BAT maturation? Does early cold exposure change the developmental trajectory of BAT development and altered adult BAT phenotype? Does early cold exposure alter phenotypic flexibility of adult mice to cold? To address these questions, we assessed the response to early cold exposure in HA native deer mice and in the closely related but exclusively LA white footed mouse (*P. leucopus*). LA was the ancestral condition in *Peromyscus,* therefore assessing the capacity for developmental plasticity in response to cold in this extant species provides some insight into the state of that ancestral population (Velotta et al.,

2018). Using this design, we tested the **hypothesis** that HA adapted mice use developmental plasticity to cold, driven by BAT function, to cope with the extreme thermoregulatory challenges of their native environment.

4.3 METHODS

4.3.1 Experimental Animals

All mice used in this study were from the second generation (G2) laboratory-born progeny of a captive breeding colony of HA native deer mice (*Peromyscus maniculatus*) and LA native white-footed mice (*P. leucopus*). The wild-caught, HA breeding stock (*P. m. rufinus*) were captured at the summit of Mount Evans, CO (4350 m a.s.l.) and the LA natives (*P. leucopus*) from Nine Mile Prairie, NE (430 m a.s.l.) as previously described (Cheviron et al, 2012). Wild-caught mice were transported to McMaster University, Canada (~90 m a.s.l.) and bred within their respective populations under common garden conditions (24ºC, 760mmHg, 14h:10h light cycle, rodent chow and water *ad libitum*). First generation (G1) lab-born mice were mated within their respective populations to produce the G2 offspring used in this study (see Robertson et al., 2019). All pups were weaned at postnatal day (P) 21 and housed with their same-sex littermates post weaning. All procedures were approved by the McMaster University Animal Research Ethics Board.

4.3.2 Experimental Design

Developmental Plasticity: In a preliminary study we found no effect of litter order from an individual breeding pair on adult thermogenic capacity (N=78). However, there was a significant effect of family on this performance measure. Therefore, to control for the effect of family, 3 different litters (litter number 4-6) from 14 unique G1 breeding pairs (7HA, 7LA) were used in this experiment. The litters, along with both parents, underwent 3 treatments (**Figure 4.1**): 1) Control (24^{o}C); 2) Prenatal Cold (14^{o}C, fertilization to birth); and 3) Postnatal Cold (14^{o}C, birth to P30). After the designated treatment period all pups were raised to adulthood (P90) under common garden control conditions (24^{o}C). Due to the sequential nature of our experimental design we wanted to ensure that previous exposure of parents to cold did not influence any measure of offspring phenotype. To do this we also tested a fourth litter from each breeding pair after the completion of the experiment. These pups were not statistically different from controls for any of the measurements (data not shown).

Adult Phenotypic Flexibility: Once they had reached adulthood two individuals (1male and 1 female) from each developmental treatment, and from a subset of families (3 HA, and 3 LA families), were further acclimated to cold for 6 weeks (5^{o}C) to test the effect of developmental temperature on the adult acclimation response to cold.

4.3.3 Onset of homeothermic endothermy (juveniles):

We have previously shown that the metabolic response to acute cold is not active in *Peromyscus* pups until P8 (LA) or P10 (HA) when raised under control conditions. To assess if cold exposure accelerates the onset of homeothermic endothermy the metabolic

response to acute cold was measured at P6, 8 and 10 as previously described (Robertson et al., 2019). Briefly, single pups were placed in glass respirometry chambers (60 ml for P6-8, 100 mL for P10) inside a temperature-controlled cabinet (Sable Systems, NV). Dry, CO_2 -free air was pulled through the chambers at a flow rate of 150 mL/min for P6 and P8, and 175 mL/min for P10. The excurrent air was dried and then drawn though CO_2 and O_2 analyzers (Sable Systems) for determination of $\dot{V}O_2$. Ambient temperature (T_a) was maintained at 30^{o}C within the respirometry chamber for 10 minutes and an initial resting metabolic rate was recorded. T_a was then lowered at a rate of 0.3^{o}C/min to a final temperature of 24^{o}C and held at that temperature to determine metabolic rate for 10 min. $\dot{V}O_2$ was calculated from the most stable 30 s recording during the last 2 minutes at each temperature (30 and 24^{o}C). To account for any potential influence of maternal separation or handling stress on $\dot{V}O_2$, a second pup from the same litter was placed in a separate chamber at constant temperature of 30^{o}C for the entire 30-minute period and $\dot{V}O_2$ was determined as described above (trial order randomized at each postnatal age). Pups were weighed at the beginning and end of each trial to account for water loss and the average of these two weights was used to calculate mass specific $\dot{V}O_2$. To determine the direct effect of acute cold on $\dot{V}O_2$ and to account for any temperature-independent effects on $\dot{V}O_2$, the delta-delta change in metabolic rate was calculated as the difference between initial $\dot{V}O_2$ and final $\dot{V}O_2$ in pups exposed to acute cold (24^{o}C) minus the difference in initial and final $\dot{V}O_2$ of its normothermic sibling held at a constant 30^{o}C, according to the equation:

$$\Delta\Delta\dot{V}O_2 = (\text{Final } \dot{V}O_2 - \text{Initial } \dot{V}O_2)_{\text{acute cold}} - (\text{Final } \dot{V}O_2 - \text{Initial } \dot{V}O_2)_{\text{normothermic}} \quad (1)$$

where a positive value indicates a cold-induced increase in $\dot{V}O_2$ relative to a normothermic sibling.

4.3.4 Adult Thermogenic Capacity

Adult mice were placed in flow through glass respirometry chambers (~500ml) held in a Peltier cabinet (Sable Systems) at -5^{o}C for 10 minutes. Normoxic (20% O_2) or hypoxic (12% O_2) heliox gas (O_2 with He) was flowed through the chambers at a rate of 1000 ml/min (Tate et al., 2017; Robertson and McClelland, 2019). Excurrent air was subsampled, dried and passed through O_2 and CO_2 analyzers (FoxBox Respirometry System, Sable Systems). Data acquisition was performed using LabChart software (ADI instruments). Cold-induced $\dot{V}O_2$ max was defined as the lowest stable 10 s of the O_2 trace (Tate et al., 2017). After mice reach $\dot{V}O_2$ max, both $\dot{V}O_2$ and body temperature fall, entering a state of hypothermia (Hayes and Chappell, 1986). Body temperature was taken before and after each trial, using a rectal thermometer (RET-3 ISO, Physitemp, NJ) to ensure that the acute cold exposure was sufficient to elicit hypothermia and therefore ensure that $\dot{V}O_2$max was reached. Two adult mice from each litter (one male, one female, when possible) were measured. Mice were allowed 48 hours between normoxic and hypoxic trials (order randomized). Cold-induced $\dot{V}O_2$max was measured in the same individuals before and after adult cold acclimation.

4.3.5 Adult BAT activation

BAT activation in adult mice was quantified following norepinephrine (NE; Sigma) injection using combined small animal positron emission tomography (PET, Philip Mosaic, Andover, MA) and micro-computed tomography (CT, Xspect System, Gamma Medica-Ideas, NorthRidge, CA) imaging (Crane et al., 2014). Two days prior to imaging, hair was chemically removed from the tail (Nair, Carter-Wallace) so the tail vein could be visualized. An intraperitoneal injection of a standard dose (Wunder and Gettinger, 1996) of NE was administered 15 min prior to an injection of [^{18}F] fluorodeoxyglucose (FDG) (~10 MBq, Hamilton Health Sciences and McMaster University) injected into the tail vein. Mice underwent PET 15 minutes following injection, immediately followed by CT for 5 minutes. To calibrate for Hounsfield units a water-filled tube was CT-scanned alongside each mouse. All imaging took place under anesthesia (isoflurane) to ensure that mice remained immobile during scans. Cold acclimated mice were allowed to acclimate to room temperature (24^{o}C) for at least 1 h prior to the start of the procedure.

Images were analyzed using AMIDE software. The interscapular BAT (iBAT) depot was identified using the combined PET/CT scan. A cylindrical region of interest (650 mm^3) was drawn between the shoulder blades and BAT activity (FDG- tissue uptake) and the mean standard uptake values (SUV) of the voxels in this region were determined (Kinahan and Fletcher, 2011).

4.3.6 BAT maturation

Tissue Sampling One individual from each litter was euthanized with an overdose of isoflurane followed by cervical dislocation at postnatal day P6, P30 and P90. All mice

acclimated to cold as adults were also sampled. The interscapular brown adipose tissue depot (iBAT) was blunt dissected, quickly cleaned of white adipose tissue (WAT) under a dissecting microscope, flash frozen and stored at -80^{o}C for future molecular analysis.

Western Blot Analysis The protein expression of uncoupling protein (UCP)-1 and citrate synthase (CS), were determined by western blotting as previously described (Robertson et al., 2019). Briefly, BAT was powered using a liquid N_2-cooled mortar and pestle and 20 mg was homogenized in ice-cold RIPA buffer (150mM NaCl, 1.0% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, in 50mM Tris-HCl, pH 8.0). Total protein concentration was determined using a Bradford Assay (Bio-Rad, Mississauga, ON, Canada). Denatured protein (20 μg) was separated in pre-cast 12% sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad) in a Mini-Protein Tetra System (Bio-Rad; 120 V for 45 min, 150 V for 15 min) and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio Rad) using a Trans Blot Turbo Transfer System (Bio Rad; 25 V for 7 min). PVDF membranes were incubated overnight at 4^{o}C in blocking buffer (5% fat-free milk in PBST; 1.5 mM $NaH_2PO_4^{-}$• H_2O, 8.1 Na_2HPO_4, 145.5 NaCl, 0.05% Tween 20, pH 7.4) and then probed for 1 h at room temperature (RT) with primary antibody for UCP1 (UCP11-A, Alpha Diagnostics International Inc., San Antonio, TX) or CS (ab129095, Abcam, Woburn, MA) each diluted 1:500 in antibody diluting agent (1% bovine serum albumin (BSA) suspended in PBST) followed by 1 h at RT with donkey anti-rabbit horseradish peroxidase conjugated, secondary antibody (1:10000, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed with PBST (1x 15 min and 3 x 5min) following

incubation with each antibody. Membranes were then developed using ECL Clarity solution (Bio Rad) and band intensity was quantified by chemiluminescence using a ChemiDoc MP Imaging System and Image Lab software (Bio Rad). A common sample was included on each gel to control for transfer efficiency, and all samples are expressed relative to this standard. Each sample was normalized to total lane protein determined by membrane staining with Coomassie blue.

4.3.7 Statistics and Analysis

Using family as a replicate a 1-way repeated measures (RM) ANOVA and 2-way RM-ANOVA was used to test the effect of developmental treatment on birth weight and growth. We used 1-Way ANOVA to test the effect of developmental treatment on BAT mass (P6, adult), WAT mass (P6, adult), BAT protein expression (P6, adult) and BAT glucose uptake (adult). We used 2-way ANOVA to test the effect of developmental treatment and age on pup cold-induced $\dot{V}O_2$. We used 1-Way ANCOVA with body mass as a covariate to test the effect of developmental treatment on adult cold-induced $\dot{V}O_2max$. We used 1-Way ANOVA, within developmental treatment to test the effect of adult cold acclimation on BAT mass and BAT protein expression. To test the effect of adult acclimation on BAT glucose uptake we used RM-ANOVA within developmental treatment, as these measures were performed on the same individuals before and after cold exposure. To test the effect of adult acclimation on cold-induced metabolic rate we used a multi-level linear model, with restricted maximum likelihood estimation within developmental treatment with body mass and time as fixed factors and individual as a

random effect. This was to account for body mass (the covariate) varying over time (Bolker et al., 2009). All statistical tests were performed within population and all analysis was performed using SigmaStat and R software.

4.4 RESULTS

4.4.1 Birth Weight and Growth Rate

At birth, LA *P. leucopus* pups were larger if their mothers were exposed to cold during pregnancy compared to when pregnancy occurred in warm conditions (Developmental treatment; $F_{2,10} = 9.800$, P=0.004). However, this effect did not persist after birth and there was no further influence of developmental cold exposure on body mass at any age in this species (Developmental treatment x Age; $F_{22, 165} = 0.632$, P= 0.892; Figure 4.2 A).

In contrast, HA pups were the same size at birth regardless of pregnancy temperature (Developmental treatment; $F_{2,8}$= 0.0291, P=0.971). While HA mice remained similar in size throughout most of postnatal development, developmental cold exposure influenced adult (P90) body mass (Developmental treatment x Age; $F_{22, 88}$=2.518, P= 0.001). As adults, HA mice whose mothers had been cold exposed during pregnancy were slightly larger than controls, while those who had been raised in cold were slightly smaller (Figure 4.2 B).

We have previously reported that HA deer mice give birth to more pups per litter than LA *P. leucopus* (Robertson et al., 2019), a result confirmed in this study (Population; $F_{1,27}$ =

10.174, P=0.004). Litter size was not affected by developmental cold exposure in either population (Developmental treatment $F_{2,27}$ = 0.496, P=0.614).

4.4.2 Early Effects of Developmental Cold

BAT Maturation

While there was no effect of developmental temperature on overall body mass of LA pups, body composition was affected by both prenatal and postnatal cold exposure. At P6 the iBAT of cold-reared LA pups was 30% larger than that of those reared in warm conditions (Developmental treatment; $F_{2,26}$ =4.314, P=0.024) even when corrected for body mass (Developmental treatment; $F_{2,26}$ =7.498, P=0.003). These pups were also leaner than controls, having only ~54% of the inguinal WAT of control pups (Developmental treatment; $F_{2,26}$ = 5.092, P=0.014). Pups whose mothers had experienced cold during pregnancy were also leaner than controls with ~62% of the inguinal WAT but they had similar BAT mass (Figure 4.3 A, B). There was also no effect at P6 of either developmental cold exposure on BAT UCP-1 or CS protein expression (Figure 3C) suggesting neither mitochondrial quantity nor quality changed.

Cold-exposed HA pups were also leaner than controls at P6, however this effect was only seen in those whose mothers that had experienced cold during pregnancy (Developmental treatment; $F_{2,27}$ = 7.316, P=0.003) (Figure 4.3 D). Pups who experienced prenatal cold had ~60% less inguinal WAT than controls. However, there was no effect of either developmental cold exposure on BAT mass (Developmental treatment; $F_{2,26}$ = 1.824,

P=0.181) (Figure 4.3 E). Similar to LA pups, at P6 neither mitochondrial nor UCP-1 content of iBAT was affected by either prenatal or postnatal cold exposure in HA pups (Figure 4.3 F).

Onset of Endothermy

In line with our previous findings (Robertson et al., 2019), control LA pups were not able to mount a robust metabolic response to acute cold until they were at least 8 days old (Age; $F_{2,18}$ = 12.655, P<0.001). Both prenatal and postnatal cold exposure shifted the onset of this response to an earlier age in LA pups (Developmental treatment x Age; $F_{4, 48}$=2.326, P= 0.07). At P6 both prenatal and postnatal cold-exposed pups were able to robustly increase metabolic rate in response to acute cold to the same extent as control 8-day old pups. Postnatal cold exposed pups were able to further increase cold-induced metabolic rate at P10 (Age; $F_{2,17}$ = 10.935, P<0.001) while prenatal cold exposed pups did not further increase cold-induced $\dot{V}O_2$ after P6 (Age; $F_{2,13}$= 0.555, P=0.587; Figure 4.4 A).

We have previously shown that the onset of endothermy is delayed in HA pups relative to lowlanders. As such, they are not able to mount a metabolic response to cold until they are 10-days old (Robertson et al., 2019). We found similar results in control HA pups in this study (Age; $F_{2,41}$=5.361, P=0.009). However, this developmental trajectory remained fixed regardless of developmental temperature as neither prenatal nor postnatal cold exposure shifted the onset of endothermy earlier in this population (Developmental treatment x Age; $F_{4, 41}$=0.499, P= 0.736) (Figure 4.4 B).

4.4.3 Persistent Effects of Developmental Cold

Adult BAT phenotype

Developmental temperature had no persistent effect on adult BAT mass in LA *P. leucopus* despite early differences in BAT growth (Developmental treatment; $F_{2,14}$ = 2.273, P=0.140) (Figure 4.5 A). Additionally, neither mitochondrial marker CS (Developmental treatment; $F_{2,12}$ = 0.012, P=0.988) nor UCP-1 (Developmental treatment; $F_{2,12}$ = 0.138, P=0.872) content of adult BAT differed between the treatment groups. The ratio of UCP-1 per CS therefore remained constant (Figure 4.5 B).

There was also no effect of developmental cold exposure on absolute adult BAT mass in HA mice (Developmental treatment; $F_{2,13}$ = 2.361, P=0.133). However, mice whose mothers had been cold exposed during pregnancy had slightly more BAT relative to body mass than controls (Developmental treatment; $F_{2,13}$ = 3.074, P=0.081) (Figure 4.5 C). Despite these subtle differences in proportion of BAT there was no persistent effect of developmental temperature on adult BAT mitochondria (Developmental treatment; $F_{2,12}$ = 2.056, P=0.179) or UCP-1 (Developmental treatment; $F_{2,12}$ = 1.206, P=0.333) content (Figure 4.5 D).

NE-induced Glucose Uptake

NE-stimulated FDG uptake into iBAT was higher than controls only in adult LA mice who had been reared in cold for the first 30 days for postnatal development (Developmental treatment; $F_{2,8}$ = 5.127, P=0.037) (Figure 5.6 A). There was no such

effect of developmental temperature on FDG uptake in BAT of HA mice (Developmental treatment; $F_{2,15} = 0.921$, P=0.420) (Figure 5.6 B).

Cold-induced $\dot{V}O_2max$

Along with an elevated BAT glucose uptake, there was a persistent effect of postnatal cold exposure on adult thermogenic capacity. Cold-reared LA mice had a higher cold-induced $\dot{V}O_2max$ than controls and mice exposed to cold prenatally (Developmental treatment; $F_{2,37} = 4.956$, P=0.012) (Figure 7A). However, this effect was only present when tested under normoxia and the cold-induced hypoxic (12% O_2) $\dot{V}O_2max$ was similar among all LA groups (Developmental treatment; $F_{2,37} = 0.571$, P=0.600) (Figure 7B).

Surprisingly, HA mice showed no effect of early life cold exposure (prenatal or postnatal postnatal) on cold-induced $\dot{V}O_2max$. This was true when tested under both normoxic (Developmental treatment; $F_{2,29} = 0.523$, P=0.598) (Figure 7C) and hypoxic (Developmental treatment; $F_{2,29} = 1.463$, P=0.248) conditions (Figure 7D).

4.4.4 Effects of Developmental Cold on Adult Cold Acclimation Response

BAT phenotype

To determine if early life cold exposure influenced adult phenotypic plasticity, we tested a subset of adult mice from each development treatment before and after a subsequent cold acclimation. When acclimated to cold as adults (5^{o}C, 6 weeks) BAT mass relative to body mass showed a small but significant decreased in control LA mice (Adult Cold; $F_{1,10}$

= 7.213, P=0.023). However, no such change was seen in mice who had been exposed to cold prenatally (Adult Cold; $F_{1,8}$ = 0.250, P=0.631) or postnatally (Adult Cold; $F_{1,10}$ = 0.0726, P=0.793) (Figure 4.8 A). In contrast, UCP-1 protein expression increased ~3 fold in response to adult cold acclimation with no change in CS in all LA mice regardless of developmental temperature (UCP-1/ CS; Adult Cold; $F_{1,33}$ = 13.348, P<0.001) (Figure 8B).

In contrast to LA mice, relative BAT mass increased with adult cold acclimation in control (Adult Cold; $F_{1,9}$ = 13.360, P=0.005) and postnatal cold HA mice (Adult Cold; $F_{1,10}$ = 17.384, P=0.002). However, HA mice whose mothers had been cold exposed during pregnancy did not alter relative BAT mass with adult cold acclimation (Adult Cold; $F_{1,9}$ = 0.167, P=0.900) (Figure 4.8 D). A similar pattern was seen with UCP-1 expression, which increased dramatically in response to adult cold (~6-7-fold) in control and cold-reared HA mice but did not change in those exposed to cold prenatally. Similar to LA mice, mitochondrial content of BAT did not change in any experimental group in response to adult cold acclimation (UCP-1/CS; Adult Cold x Developmental Cold $F_{3, 31}$ = 3.466, P=0.028) (Figure 4.8 E).

NE-induced BAT glucose uptake

In LA mice there was no change in BAT FDG uptake after adult cold acclimation, regardless of developmental cold exposure (Figure 4.8 C).

In contrast, in HA mice there was an overall increase in BAT FDG uptake after adult cold acclimation (Adult Cold; $F_{1,30} = 7.949$, P=0.008), but this effect was only significant in mice whose mothers had been cold-exposed during pregnancy ($F_{1,5} = 6.667$, P=0.049) (Figure 4.8F).

Cold-induced $\dot{V}O_2$ max

LA mice raised in control conditions increased their cold-induced $\dot{V}O_2$max by ~30% after adult cold acclimation ($F_{1,4} = 80.874$, P<0.001). However, prenatal ($F_{1,4} = 2.976$, P=0.159) and postnatal ($F_{1,4} = 1.375$, P=0.306) resulted in no change in $\dot{V}O_2$max with cold acclimation (Figure 4.9 A). In contrast, all mice increased hypoxic $\dot{V}O_2$max following adult cold acclimation regardless of developmental treatment (Control: $F_{1,4} = 22.549$, P=0.009; Prenatal: $F_{1,4} = 15.148$, P=0.018; Postnatal: $F_{1,4} = 45.972$, P=0.003) (Figure 4.9B).

In HA mice both control ($F_{1,4} = 14.366$, P=0.019) and prenatal cold-exposed ($F_{1,4} = 24.301$, P=0.008) increased cold-induced $\dot{V}O_2$max after adult cold acclimation. However, mice who had been cold-reared failed to show this increase ($F_{1,4} = 1.427$, P=0.298) (Figure 4.9 C). For HA mice, hypoxic cold-induced $\dot{V}O_2$max increased in controls following adult cold acclimation ($F_{1,4} = 7.336$, P=0.054), but this post acclimation increase was not seen in mice that had prior cold experience either prenatally ($F_{1,4} = 2.752$, P=0.172) or postnatally ($F_{1,4} = 0.036$, P=0.859) (Figure 9D).

4.5 DISCUSSION

The goal of this study was to assess the importance of developmental plasticity on neonatal and adult thermoregulation in two closely related LA and HA adapted *Peromyscus* species. Here, I identified two temperature-sensitive critical windows in the development of thermoregulation in neonatal *Peromyscus* mice. Cold exposure during either of these windows resulted in an altered developmental trajectory and adult phenotype. However, the magnitude of developmental plasticity and its consequences for whole-animal thermoregulatory performance both during ontogeny and into adulthood varied drastically between high altitude adapted *P. maniculatus* and the low altitude native *P. leucopus*. To our knowledge this is the first study to demonstrate that the capacity for developmental plasticity may have diverged between HA and LA adapted species.

4.5.1 Prenatal Cold Exposure

The first critical window I identified was the prenatal period. Compared to warm exposed controls, LA native *P. leucopus* mothers who were cold exposed throughout their pregnancy produced larger, leaner pups. These pups attempted to thermoregulate earlier in development and demonstrated a limited capacity for further phenotypic flexibility in response to cold as adults. It is important to note that pups in this study are likely not directly experiencing cold stress while *in utero* as they are buffered from variations in ambient temperature by their mother's own thermoregulatory ability (Wells, 2019). In fact, pregnant mammalian mothers will carefully guard against fluctuations in body

temperature even in species that are normally heterothermic (Oelkrug et al., 2015). As a result, the impacts of prenatal cold exposure on offspring phenotype reported here are likely due to indirect effects of ambient temperature on the mother. Maternal cold exposure during pregnancy has been shown to alter offspring phenotype other LA mammals such as rats (e.g., Dahlöf et al., 1978; Tazumi et al., 2005; Lian et al., 2018) and sheep (e.g., Symonds et al., 1992; Clarke et al., 1997). The ability of mothers to transfer information about their environment to their offspring is thought to be mediated primarily through elevated levels of maternal glucocorticoids (for review see Braun et al., 2013; Moisiadis and Matthews, 2014), a mechanism that appears to be conserved across vertebrate taxa (Thayer et al., 2018) .

Whether or not this altered developmental trajectory and blunted adult phenotypic flexibility is adaptive is unclear (Beamen et al., 2016). A higher cold-induced metabolic rate that occurs early in lowlanders during postnatal development could benefit pups during mild cold exposure. However, the increased energetic requirement of thermoregulation could deplete lipid reserves. A reduced capacity for adult phenotypic flexibility may have more dire consequences as the ability to seasonally increase thermogenic capacity could impede overwintering survival (Lynch, 1973).

Maternal stress during pregnancy can result in offspring phenotypes that are maladaptive, in the case of increased propensity for metabolic diseases (e.g., Hales and Barker, 1992). Alternatively, maternal stress can result in adaptive changes as seen with increased stress in pregnant red squirrels in response to real or perceived increases in population density.

These stressed mothers give birth to larger and faster growing kits, who as a result have an increased chance of survival due to their more robust body size (Dantzer et al., 2013). However, it is often difficult to tease apart the costs and benefits of these altered phenotypes (Jones, 2005). There is some evidence that cold stress in pregnant ewes can alter the development of BAT in their lambs (Clarke et al., 1997; Budge et al., 2000). Interestingly, despite an elevated cold-induced metabolic rate early in postnatal development (P6) I saw no change in the BAT size or phenotype in those pups whose mothers had been cold exposed during pregnancy. Therefore, the underlying physiological mechanism driving this change in early cold induced metabolic rate is also unclear.

These responses in LA native mice contrast the response to prenatal cold exposure in HA adapted mice. HA pups born to mothers who were cold exposed during pregnancy were also leaner during development than controls. However, unlike lowlanders there was no change in developmental onset or magnitude of cold-induced metabolic rate, suggesting that this developmental pattern may be resistant to maternal stress. However, once these mice reached adulthood the persistent effects of their prenatal stressor became apparent. Prenatal cold- exposed HA mice were larger than controls as adults and had slightly higher proportional iBAT mass. Unlike the response seen in lowlanders, HA mice were able to robustly increase cold-induced $\dot{V}O_2max$ by 30% in the face of adult cold acclimation. However, the phenotypic flexibility of iBAT mass and BAT thermogenic machinery responded very differently in prenatal cold versus control siblings

demonstrating developmental plasticity alters the mechanistic underpinnings of adult cold acclimation.

There are two possible explanations for this divergent response to prenatal cold in HA and LA adapted *Peromyscus.* HA adaptation may have directly altered the sensitivity to maternal stress in the fetus. For example, if mothers are chronically stressed expression of 11β hydroxysteroid dehydrogenases which guard the fetus against high levels of maternal glucocorticoids (Benediktsson et al., 1997) can be altered in both the placenta and the brain of the fetus (Jensen Peňa et al., 2012). Conversely, HA adaptation may have lowered the sensitivity of the mothers to cold stress. While 14^{o}C is well below the thermal neutral zone of *P. maniculatus*, it is within the upper range of ambient temperatures that a HA female would experience in the wild. HA mice also have an attenuated capacity to secrete catecholamines in response to stress (Scott et al., 2019). This may also hold true for glucocorticoids as well, which would dampen the signal received by the developing fetus. Regardless of the proximate mechanisms involved, the response to prenatal cold has diverged between these two populations.

4.5.2 Postnatal Cold Exposure

Unlike prenatal cold exposure, postnatal cold has a well documented influence on the development of thermogenesis in rodents (e.g., Skala and Hahn, 1974; Denjean et al., 1999; Morrison et al., 2000). Consistent with these results I found that cold-reared LA *P. leucopus* showed increased BAT mass during development, and an earlier postnatal metabolic response to acute cold. As adults these mice had a higher cold-induced

$\dot{V}O_2max$ that was likely driven, in part, by elevated BAT activity, as measured by NE-stimulated BAT glucose uptake. These results also corroborate previous reports from this species that show that postnatal cold stimulates BAT growth (Hill, 1983). Given the consistency in this response across Rodentia it is likely that this developmental plasticity represents a conserved, ancestral response. This may be a mechanism to allow LA pups born near the end of the breeding season (in early autumn) to quickly prepare for the coming winter. However, like prenatal cold, postnatal cold exposure produces an adult phenotype with limited capacity for further adult flexibility in response to cold.

Unlike lowlanders, the development of homeothermic endothermy in HA native pups appears to be unresponsive to rearing at 14^{o}C. This suggests that the delay in the onset of endothermy previously reported in high altitude pups (Robertson et al., 2019; Robertson and McClelland, 2019) is likely also occurring in the wild. I have previously suggested that this is a cost saving mechanism, that evolved to protect valuable lipid stores and to promote growth. Support for this hypothesis can be seen in the postnatal cold exposed LA pups, who, despite similar birth and growth rates, have only ~50% of the inguinal WAT stores as their control siblings. No such fat loss is seen in HA pups.

It is likely that the ancestral *P. maniculatus* that initially colonized HA had a similar sensitivity to low rearing temperatures as the LA *P. leucopus* used in this study. However, in the face of the chronic cold and hypoxic conditions of HA, the loss of that plasticity may represent a critical adaptation for HA taxa. These data are supported by work comparing wild caught and first-generation laboratory born LA and HA *P. maniculatus*

(Velotta et al., 2016). In this study there was a strong effect of rearing environment on NST in lowlanders but no effect on highlanders. Alternatively, high altitude pups may be buffered from low ambient temperatures during postnatal development by increased maternal care. This could occur due to altered nest building (King et al., 1964) or an increase in the amount of time high-altitude mothers spend in the nest with their pups.

Despite the lack of change in the onset of homeothermic endothermy in highlanders there were effects of rearing temperature on this group as adults, suggesting that some form of developmental plasticity has been maintained. As adults, HA mice that were reared at 14^{o}C were slightly smaller than controls, and unlike their prenatal cold exposed siblings, showed a limited capacity for further phenotypic flexibility in response to cold. These findings further emphasize that there is a complex interaction between developmental temperature and the capacity for further adult phenotypic flexibility in both LA and HA adapted species.

4.5.3 Interactions between developmental and adult rearing temperature

Control adult animals of both species reacted to cold exposure in very similar ways. A 6 week acclimation to 5^{o}C caused an increase in normoxic and hypoxic cold-induced $\dot{V}O_2max$ as well as a 3-to -5-fold increase in UCP-1 expression. These data suggest that remodelling of iBAT contributes to the increase in overall thermogenic capacity. In HA adapted animals this also involved an increase in BAT mass. Interestingly, there was no change in BAT glucose uptake, suggesting that unlike in laboratory rodents, adult cold acclimation did not alter levels of glucose transporters (Olichon-Berthe et al., 1992;

Shimizu et al., 1993). This may be a strategy for overwintering. Normally small mammals (<100 g) survive winter by conserving energy rather than increasing thermogenic capacity (Lovegrove, 2005). However, *Peromyscus* are active year round, and therefore cannot avoid actively thermoregulating. A high cold-induced $\dot{V}O_2max$ allows increase foraging (Sears et al., 2006) and improves adult survivability (Hayes and O'Connor, 1999). It is therefore surprising that both prenatal and postnatal cold exposure limits adult phenotypic flexibility of whole animal thermogenic capacity (albeit in highlanders the former only occurs in hypoxic cold induced $\dot{V}O_2max$). There is some evidence that exposure to a developmental environmental condition that increases adult $\dot{V}O_2max$ (running or thermogenic) can limit further increases in that trait (Chappell et al., 2007; Russell et al., 2008) in *Peromyscus*. However, these studies use a combination of wild caught and lab-reared mice under a mixture of cold, hypoxic and combined cold/hypoxic conditions that make it difficult to isolate the effects of any one environmental factor. It has been suggested that developmental plasticity will limit adult acclimation capacity if phenotype and environment are well matched. This theoretically reduces the cost of mounting an adult acclimation response (Beaman et al., 2016).

In this study, developmental cold (prenatal or postnatal) only attenuates increases in normoxic $\dot{V}O_2max$ of LA mice. Hypoxic $\dot{V}O_2max$ by contrast, increases with adult acclimation and was not influenced by early life cold exposures. This suggests that these mice are capable of phenotypic flexibility, that is only apparent when O_2 is limiting. Interestingly, hypoxia acclimation alone also increases $\dot{V}O_2max$ in LA and HA *P.*

maniculatus due to a suite of underlying cardio-respiratory changes (Tate et al., 2017). It is possible that cold may act to increase hypoxic thermogenic capacity through similar mechanisms. Interestingly in both pre and postnatal cold exposed LA mice there was a robust change in UCP-1 content of the BAT following adult cold acclimation. Why then is there no change in normoxic $\dot{V}O_2max$? It is important to remember that cold-induced $\dot{V}O_2max$ is the result of both shivering and non-shivering thermogenesis. Any increase in one factor could be masked by a decrease in the other. Perhaps prenatal or postnatal cold limits the capacity for shivering thermogenesis resulting in little change in overall thermogenic capacity.

A similar mechanism could be at work in HA mice. However, in this case the response depends on the timing of the initial cold stress. In postnatal cold exposed mice, there was no change in either hypoxic or normoxic cold-induced $\dot{V}O_2max$ despite increases in both BAT mass and UCP-1 content. It is possible that these mice may have a limited capacity to activate BAT. The BAT of wild caught mice has much lower expression levels of β3 adrenergic receptor mRNA compared to their laboratory reared controls, suggesting that rearing environment may shape the way that BAT is regulated/ activated by cold (Velotta et al., 2016). In our study prenatal cold exposed highlanders increase normoxic cold induced $\dot{V}O_2max$ and norepinephrine-stimulated FDG uptake without changing BAT mass or UCP-1 content. This is further evidence that suggests that BAT may be differentially regulated in these mice. While this may initially appear counterintuitive, BAT is a highly metabolically active tissue that when stimulated consumes high levels of

lipid, carbohydrates and O_2, all precious resources for a high-altitude mouse. The capacity for adult phenotypic flexibility to cold is clearly conserved in high altitude mice, given that naïve controls responded identically to their lowland congenerics. Our data suggest that the early life experiences of HA mice may act to fine-tune this response in a way that is better suited for the combined cold/hypoxia of high alpine regions.

4.6 CONCLUSIONS

When animals first colonized HA their existing capacity for phenotypic flexibility may have produced phenotypes that were adaptive, maladaptive or neutral (Velotta and Cheviron, 2018). Our data suggest that the delayed developmental trajectory of thermogenesis in HA mice that we have previously reported (Robertson et al., 2019) is not sensitive to cold in the 2 developmental windows tested. While we have found evidence of some developmental plasticity to cold in HA deer mice, it is limited when compared to lowlanders, and only apparent in the face of subsequent adult cold challenges. This suggests that ancestral plasticity exhibited by lowlanders may have been maladaptive in the chronically cold and hypoxic HA environment particularly during postnatal development, and was subsequently selected against. This blunting of the response to cold may allow HA pups to conserving energy during development, by limiting energetically costly thermogenesis. Finally, our data suggest that there may be an important role of maternal care and/ or physiology in highlanders, that buffers pups against perturbations in ambient temperature both *in utero* and during the sensitive early postnatal period.

4.7 FIGURES

Figure 4.1

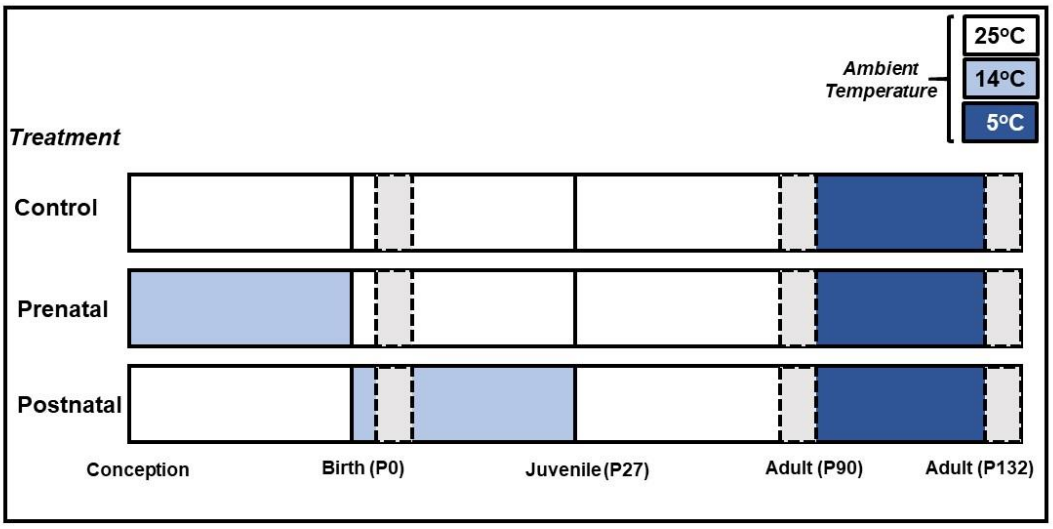

Figure 4.1 Experimental Design. Second generation lab born high altitude *Peromyscus maniculatus* and low altitude *P. leucopus* were acclimated to cold (14ºC) during prenatal or postnatal development. Dashed box represents measurements of cold induced metabolic rate at postnatal day (P) 6-10, P90 and P132, after 6 weeks of adult acclimation to cold (5ºC).

Figure 4.2

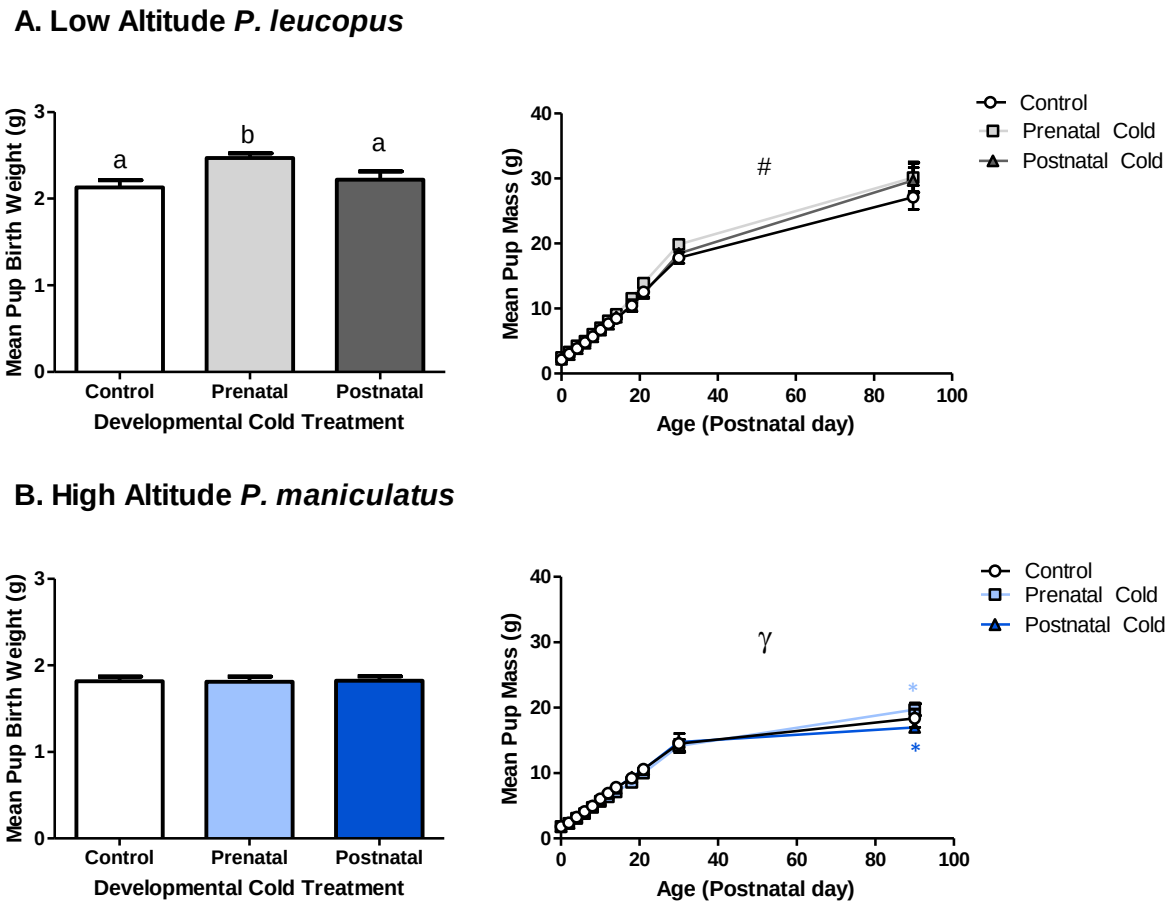
A. Low Altitude P. leucopus
Mean Pup Birth Weight (g)
a
b
a
Control
Prenatal
Postnatal
Developmental Cold Treatment
Mean Pup Mass (g)
#
Age (Postnatal day)
Control
Prenatal Cold
Postnatal Cold
B. High Altitude P. maniculatus
Mean Pup Birth Weight (g)
Control
Prenatal
Postnatal
Developmental Cold Treatment
Mean Pup Mass (g)
γ
*
*
Age (Postnatal day)
Control
Prenatal Cold
Postnatal Cold

Figure 4.2. Effect of prenatal and postnatal cold (14ºC) exposure on birth weight and growth rate of second-generation lab-born low altitude native *P. leucopus* (A) and high-altitude native *P. maniculatus* (B). For birth weight bars with dissimilar letters are significantly different as determined by 1-Way RM ANOVA. # indicates an overall main effect of age across developmental treatments as determined by 2-Way RM ANOVA. γ represents a significant interaction between developmental treatment and age, and * indicates a significant difference from control within age. To avoid pseudo replication family (litters with same parents) was considered as a single biological replicate. Therefore, within a developmental treatment all masses of pups from a single breeding pair were averaged. All data are presented as mean ± sem.

Figure 4.3

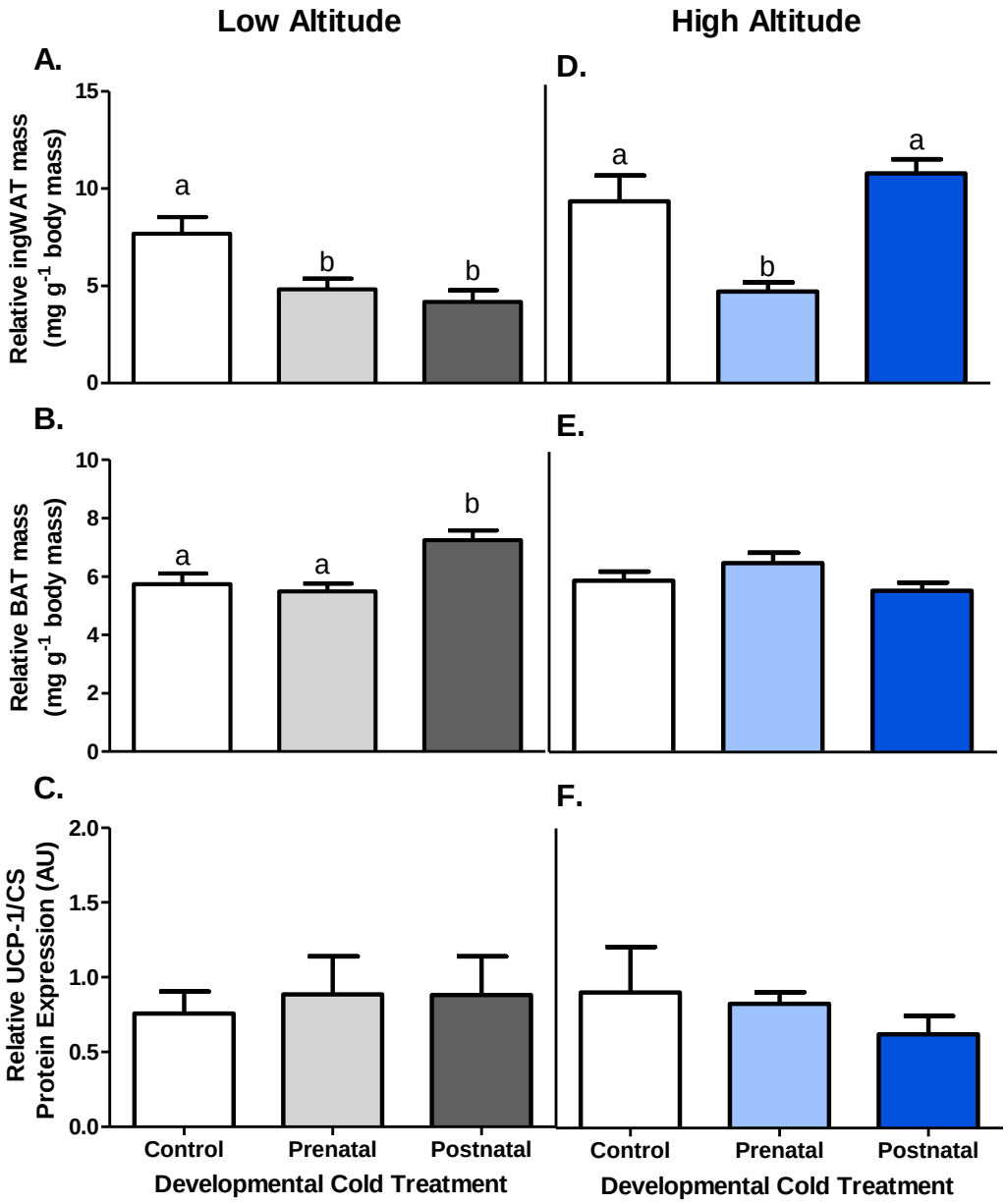
Low Altitude
High Altitude
A.
D.
Relative ingWAT mass (mg g-1 body mass)
15
10
5
0
a
b
b
a
b
a
B.
E.
Relative BAT mass (mg g-1 body mass)
10
8
6
4
2
0
a
a
b
C.
F.
Relative UCP-1/CS Protein Expression (AU)
2.0
1.5
1.0
0.5
0.0
Control
Prenatal
Postnatal
Developmental Cold Treatment
Control
Prenatal
Postnatal
Developmental Cold Treatment

Figure 4.3. Effect of prenatal and postnatal cold (14ºC) exposure on white (WAT) and brown adipose tissue (BAT) phenotype of 6-day old, second generation lab-born low altitude native *P. leucopus* (A-C) and high-altitude native *P. maniculatus* (D-F). Within a population bars with dissimilar letters are significantly different as determined by 1-Way ANOVA. All data are presented as mean ± sem.

Figure 4.4

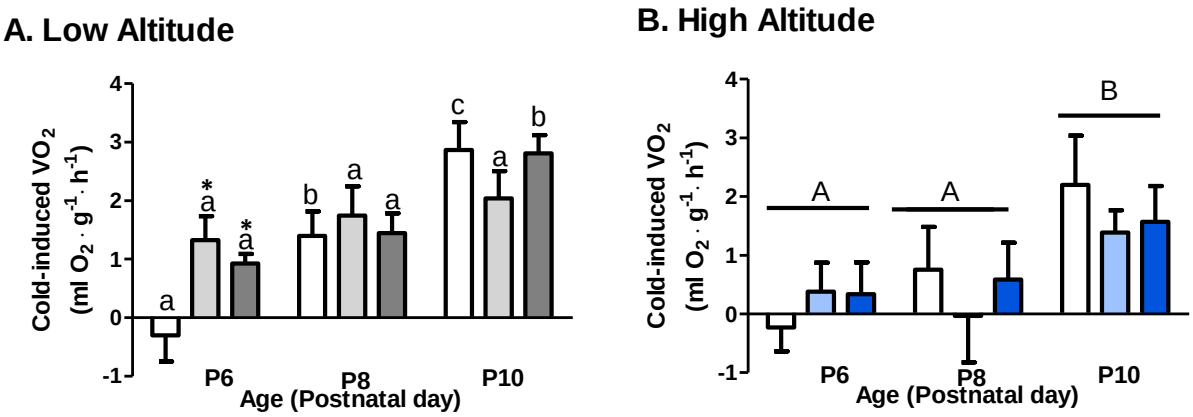
A. Low Altitude
B. High Altitude
Cold-induced VO2 (ml O2 · g-1 · h-1)
Age (Postnatal day)
P6
P8
P10
a
a*
a*
b
a
a
c
a
b
A
A
B

Figure 4.4 Effect of prenatal and postnatal cold (14ºC) exposure on the developmental onset of endothermy in second generation lab-born low altitude native *P. leucopus* (A) and high-altitude native *P. maniculatus* (B). Within population upper case letters represent an overall effect of age. Lower case letters represent effect of age within developmental treatment and * indicates a significant difference from control within age as determined by 2-Way ANOVA. All data are presented as mean ± sem.

Figure 4.5

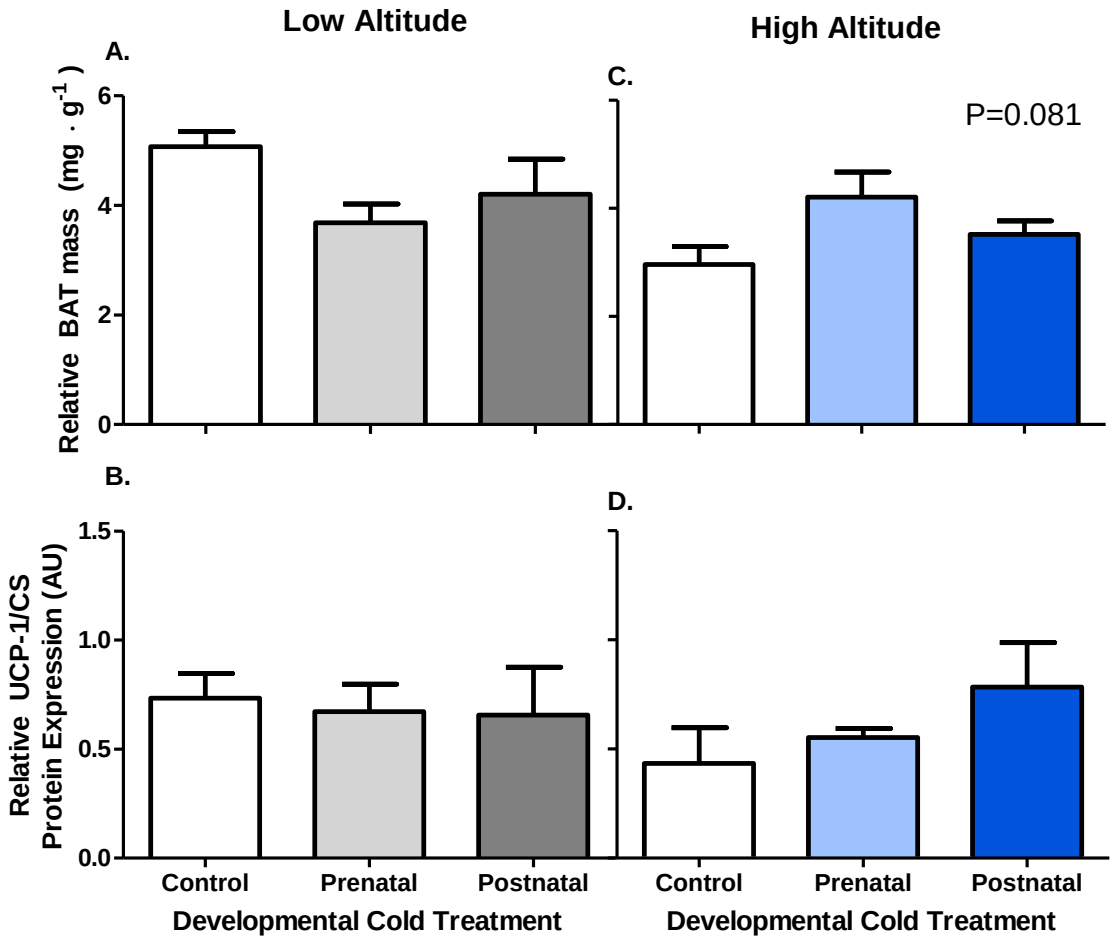
Low Altitude
High Altitude
A.
C.
P=0.081
Relative BAT mass (mg · g-1)
6
4
2
0
B.
D.
Relative UCP-1/CS Protein Expression (AU)
1.5
1.0
0.5
0.0
Control
Prenatal
Postnatal
Control
Prenatal
Postnatal
Developmental Cold Treatment
Developmental Cold Treatment

Figure 4.5 Effect of prenatal and postnatal cold (14ºC) exposure on adult brown adipose tissue (BAT) phenotype of second-generation lab-born low altitude native *P. leucopus* (A) and high-altitude native *P. maniculatus* (B). All data are presented as mean ± sem.

Figure 4.6

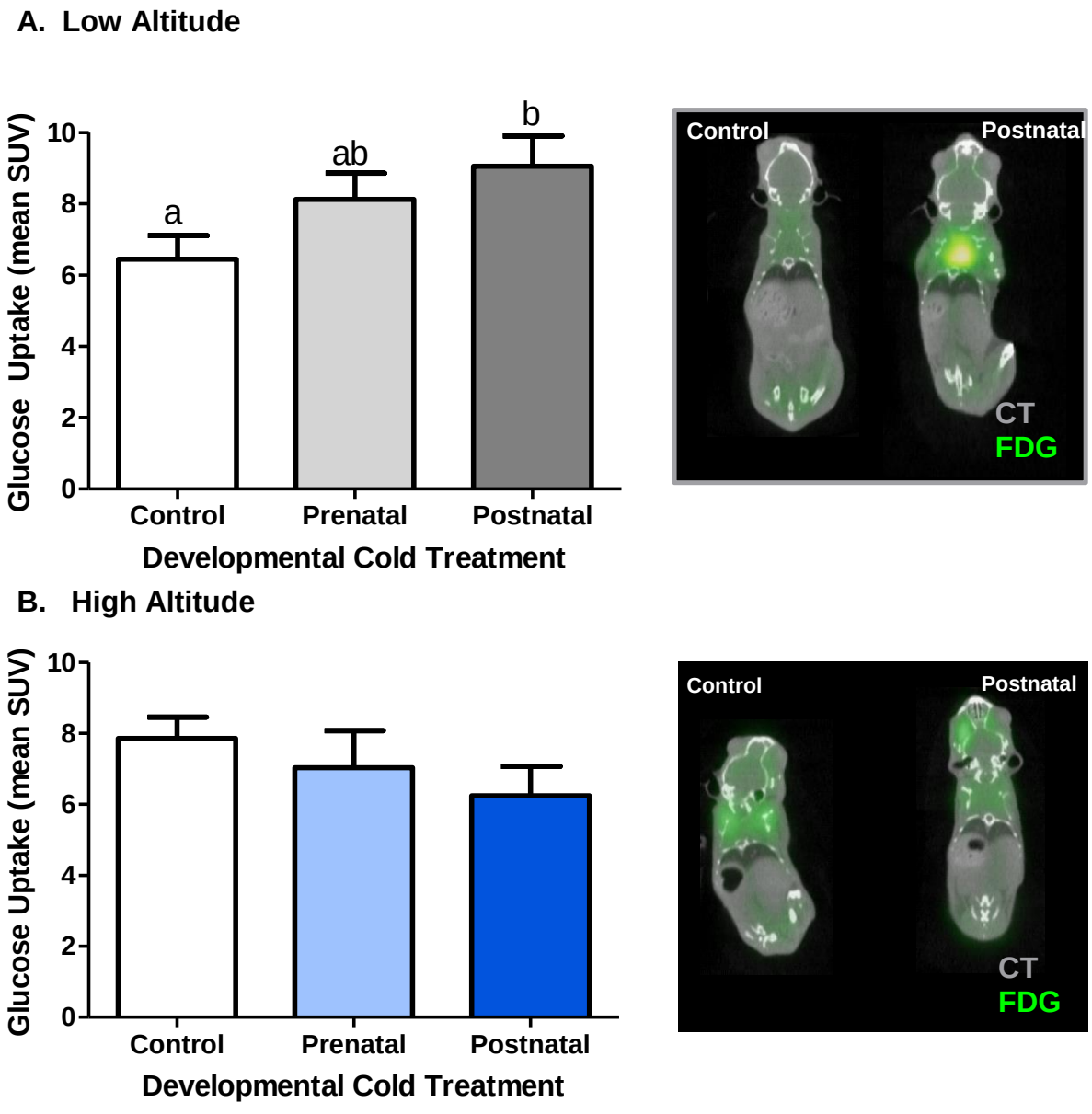
A. Low Altitude
Glucose Uptake (mean SUV)
10
8
6
4
2
0
a
ab
b
Control
Prenatal
Postnatal
Developmental Cold Treatment
Control
Postnatal
CT
FDG
B. High Altitude
Glucose Uptake (mean SUV)
10
8
6
4
2
0
Control
Prenatal
Postnatal
Developmental Cold Treatment
Control
Postnatal
CT
FDG

Figure 4.6 Effect of prenatal and postnatal cold (14ºC) exposure on norepinephrine induced F^{18}deoxyglucose uptake of adult brown adipose tissue (BAT) in second-generation lab-born low altitude native *P. leucopus* (A) and high-altitude native *P. maniculatus* (B). Bars with dissimilar letters are significantly different as determined by 1 Way ANOVA. All data are presented as mean ± sem.

Figure 4.7

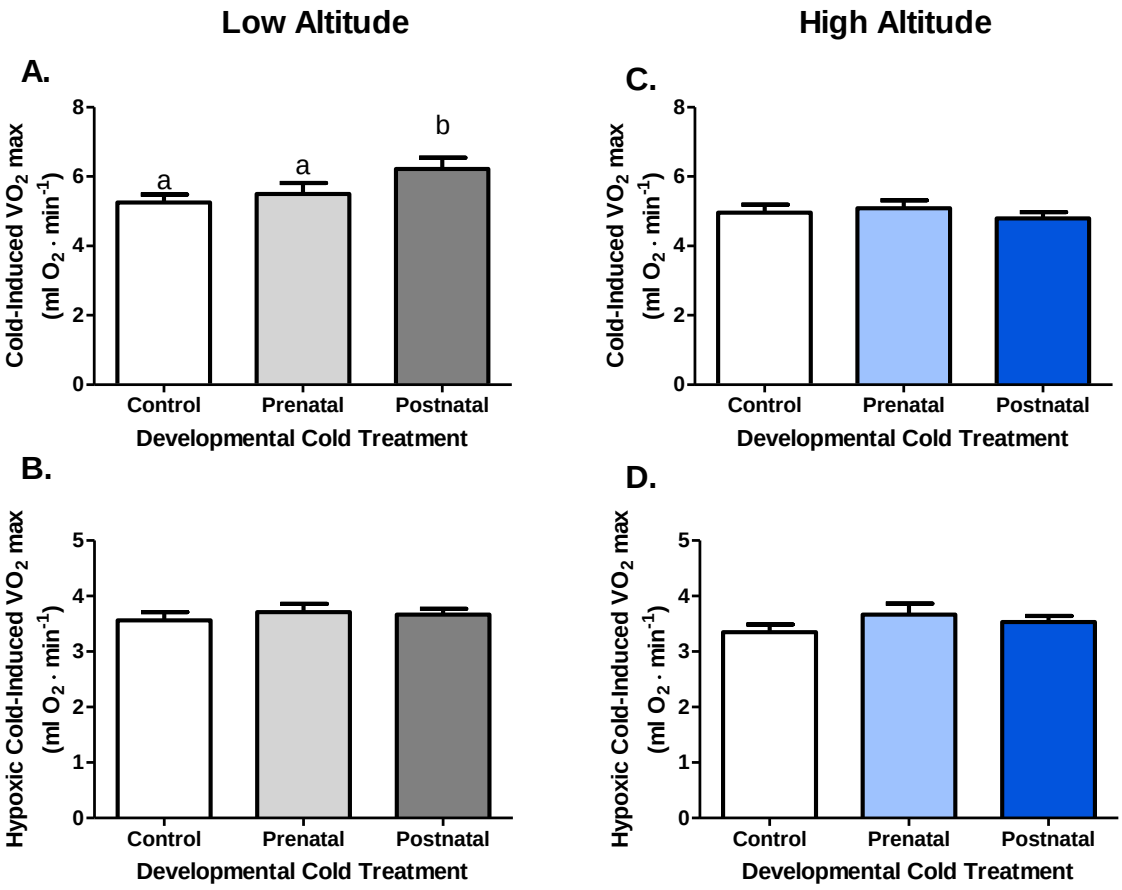
Low Altitude
High Altitude
A.
B.
C.
D.
Cold-Induced VO2 max (ml O2 · min-1)
Hypoxic Cold-Induced VO2 max (ml O2 · min-1)
a
a
b
Control
Prenatal
Postnatal
Developmental Cold Treatment

Figure 4.7 Effect of prenatal and postnatal cold (14ºC) exposure on normoxic (21% O_2) and hypoxic (12% O_2) thermogenic capacity of second-generation lab-born low altitude native *P. leucopus* (A, B) and high-altitude native *P. maniculatus* (C, D). Bars with dissimilar letters are significantly different as determined by 1 Way ANCOVA, with body mass as a covariate. Data are presented as mean ± sem of estimated marginal means of cold-induced $\dot{V}O_2$ max.

Figure 4.8

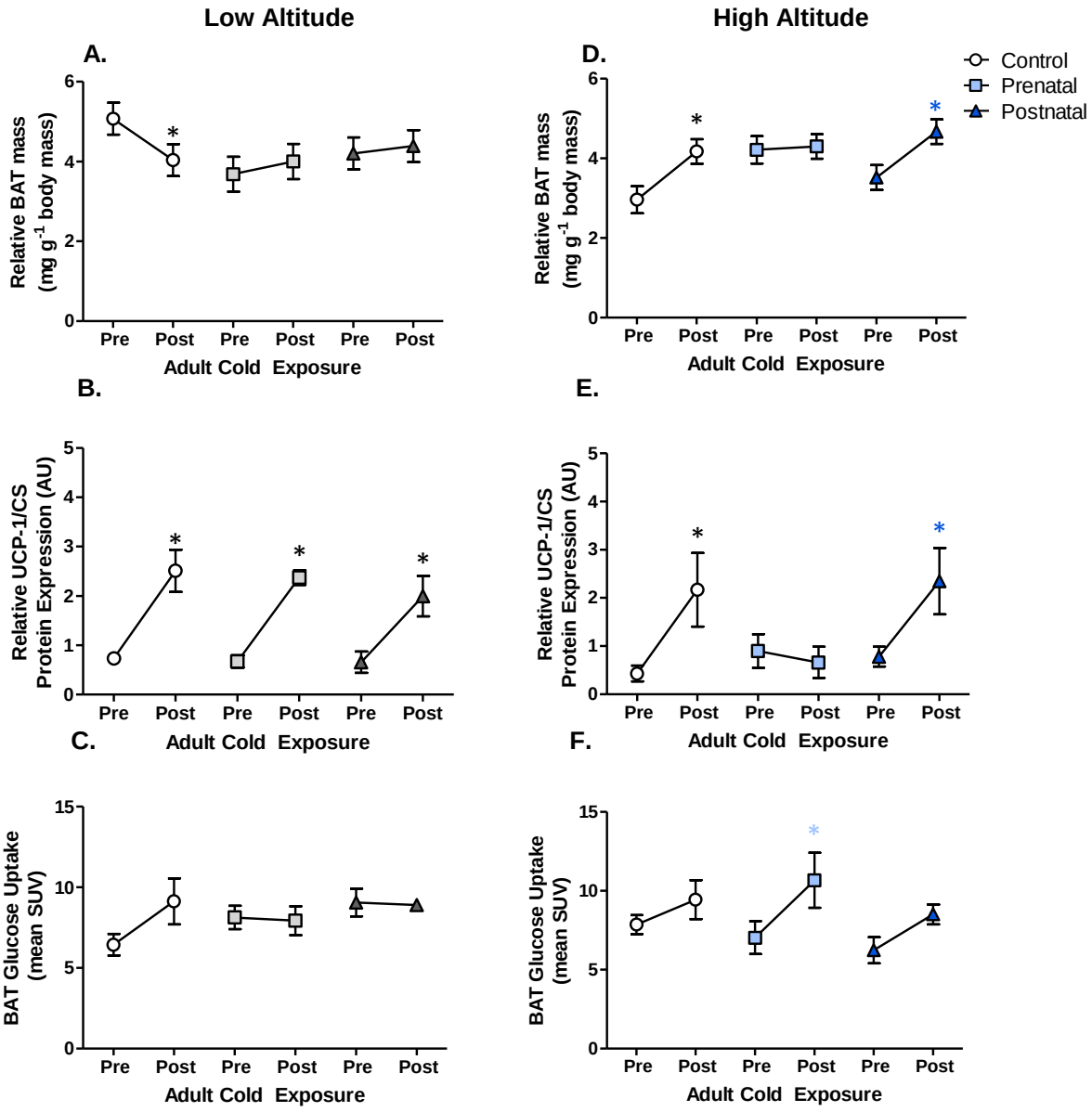
Low Altitude
High Altitude
A.
D.
B.
E.
C.
F.
Control
Prenatal
Postnatal
Relative BAT mass (mg g-1 body mass)
Relative UCP-1/CS Protein Expression (AU)
BAT Glucose Uptake (mean SUV)
Pre
Post
Adult Cold Exposure

Figure 4.8 Effect of prenatal and postnatal cold (14ºC) exposure on adult acclimation response of brown adipose tissue (BAT) to cold (6 weeks, 5ºC) in second-generation lab-born low altitude native *P. leucopus* (A-C) and high-altitude native *P. maniculatus* (D-F). * indicates significant effect of adult acclimation within developmental treatment as determined by1 Way ANOVA (UCP-1 content, BAT mass) or 1-Way RM ANOVA (glucose uptake). Data are presented as mean ± sem.

Figure 4.9

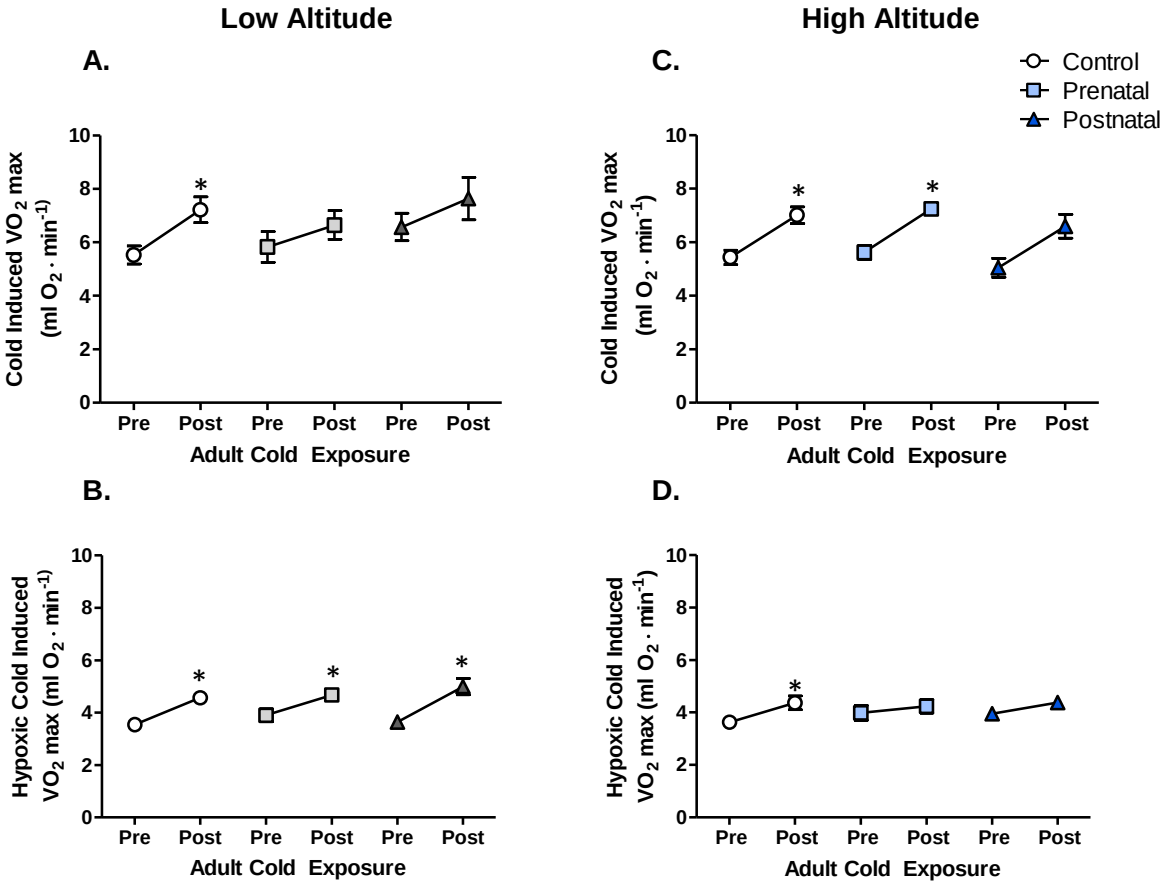
Low Altitude
High Altitude
A.
B.
C.
D.
Control
Prenatal
Postnatal
Cold Induced VO2 max (ml O2 · min-1)
Hypoxic Cold Induced VO2 max (ml O2 · min-1)
Pre
Post
Adult Cold Exposure
0
2
4
6
8
10
*

Figure 4.9 Effect of prenatal and postnatal cold (14ºC) exposure on adult acclimation response to cold of thermogenic capacity in second generation lab-born low altitude native *P. leucopus* (A, B) and high-altitude native *P. maniculatus* (C, D). * indicates significant effect of adult acclimation within developmental treatment as determined by multi-level model. Data are presented as mean ± sem of estimated marginal means of cold-induced $\dot{V}O_2max$.

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CHAPTER 5 Maternal adaptation to high altitude

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5.1 ABSTRACT

Low oxygen and temperatures at high altitude (HA) are particularly challenging for small mammals due to the high energetic costs of aerobic thermogenesis. Altricial mammals cannot maintain body temperature at birth and rely on maternal care while these thermoregulatory systems develop. The development of thermoregulation is delayed in HA deer mouse pups suggesting a greater need for maternal care. Thus, we tested the hypothesis that HA adaptation alters maternal care, using lab-reared descendants of deer mice native to low altitude (LA; 400 m a.s.l.) and HA (4350 m a.s.l.). Mothers reared their litters under control (20^{o}C, 760mmHg) and simulated HA (5^{o}C, 430mmHg) conditions. We characterized maternal energetic demands and quality of care. We found that the metabolic cost of lactation at HA is exceptionally high. The resting metabolic rate of lactating HA females was 70% of $\dot{V}O_2max$ while the cost for LA females was even greater (85%). Surprisingly, HA mothers spent considerably less time caring for their pups under both rearing conditions. Both populations altered their maternal behavior in response to simulated HA cold hypoxia, however, LA pups were significantly developmentally delayed and had reduced thermogenic capacity as adults. In contrast, HA pups developed normally and had increased thermogenic capacity as adults. Our data suggest an energetic maternal/offspring conflict at HA that manifests as a trade-off in the quality/efficiency of care.

5.2 INTRODUCTION

High altitude (HA) regions are characterized by low ambient temperatures and hypobaric hypoxia. As a result of this combination of environmental stressors, the daily energy expenditure of small obligate endotherms who live at HA is much higher than lowlanders of the same species. These animals routinely operate close to their maximum aerobic capacity ($\dot{V}O_2max$) (Hayes, 1989). This high cost of living limits aerobic reserve ($\dot{V}O_2max$- field metabolic rate), leaving very little scope for additional aerobically demanding activities. Surprisingly, across numerous mountain ranges small rodents and lagomorphs have the highest altitudinal ranges amongst mammals (Hock, 1964; Kramer et al., 1999, Ci et al., 2008). These species inhabit HA regions year-round and undergo their entire reproductive cycle under these cold and hypoxic conditions. This environment poses significant physiological challenges, especially during life stages when energetic demands are exceptionally high.

For a female mammal, lactation represents the most energetically demanding period of her adult life (Miller, 1979; Prentice and Prentice, 1998; Speakman, 2008). In mice, milk output during lactation accounts for ~50% of the energy derived from food (Johnson et al., 2001). The primary driver of a lactating mother's energy requirement is litter size, with more pups requiring more milk production (Miller, 1979). We and others have reported that in species of rodents with broad altitudinal distributions, females from higher elevation populations give birth to larger litters than their low altitude (LA) counterparts (e.g., *Phyllotis xanthopygus*, Sassi et al., 2018; *Peromyscus maniculatus*¸ Halfpenny, 1980; Robertson et al., 2019). It is currently unknown how these HA females

successfully provision these large litters in an environment that is already exceptionally energetically demanding.

At LA rodent mothers typically respond to the demand of an increased litter size by increasing food intake (Miller, 1975; Miller, 1979; Glazier, 1985). Moreover, food intake increases even further when lactating mothers are cold exposed (Hammond and Kristan, 2000; Johnson and Speakman, 2001; Paul et al., 2010; Zhao, 2011), suggesting that HA mothers with their large litters and chronically cold environment require much more food than lowlanders experiencing warmer temperatures.

The hypoxia characteristic of HA puts an additional constraint on lactation. Milk production is inhibited at real or simulated altitudes > 3000 m above sea level (a.s.l.) in several LA natives (e.g., Moore and Price, 1948; Weihe, 1965; Bruder et al., 2008). This inhibition of milk production results in severe growth restrictions in their offspring. When this hypoxia is combined with cold, LA native rodents are unable to produce enough milk for their pups, even when supplemented with high fat diets. As a result, offspring mortality is extremely high (Weihe, 1965). Females from mammalian species native to HA must somehow overcome this limitation.

In HA adapted populations of the North American deer mouse (*Peromyscus maniculatus*) the cost of lactation is likely exacerbated by the fact that young pups may require increased maternal care. In *Peromyscus* mice, and other altricial species, mothers act not only as a source of food but also as an external source of heat until pups are able to independently thermoregulate (Hill, 1972). We have previously shown that in deer mice,

HA adapted pups develop the capacity to thermoregulate more slowly than their LA conspecifics (Robertson et al., 2019; Robertson and McClelland, 2019). For example, in contrast to lowland mice at 10 days old, highland mice are unable to maintain body temperature even in the face of very mild cold stress (10 minutes at 24^{o}C). This delay in the development of thermogenesis continues until they are weaned at 21 days old after which they quickly surpass lowlanders in their overall thermogenic capacity (Robertson and McClelland, 2019). During the nursing period, HA pups may therefore rely more heavily on external maternal heat. When HA are raised in the cold there is no effect on pup physiology suggesting that mothers may buffer pups from low ambient temperature (See Chapter 4). This creates a fundamental trade-off for females between an increased need to forage to support the high energetic requirements of lactation in the cold and time spent caring for and incubating their pups.

Time spent nursing (and therefore incubating) pups is a behavioral trait that is incredibly variable in laboratory rodents (Champange, 2003). This variation has also been identified in wild populations of LA *P. maniculatus.* However, in wild mice this trait is flexible, with mothers spending more time caring for pups when population density is low and thus competition for food is reduced (Stewart and McAdam, 2014). Despite the seemingly overwhelming limits to lactation for HA female mammals, to our knowledge, maternal care has never been studied in a HA adapted population. We hypothesized that there is a fundamental trade-off at HA between the energetic demands of lactation and thermoregulatory demands of pups that has altered maternal care. To test this hypothesis,

we examined maternal energy output and behavior in LA and HA adapted populations of *P. maniculatus*.

5.3 METHODS

5.3.1 Animals

Dams used in this study were the first generation (G_1) laboratory-born progeny of wild-caught HA and LA native deer mice (*P. maniculatus*). Wild HA mice (*P.m. rufinus*) and LA mice (*P.m. nebrascensis*) were trapped at the summit of Mount Evans, CO (4350 m a.s.l.) and from Nine Mile Prairie, NE (430 m a.s.l.; Cheviron et al, 2012), respectively. Wild mice were transported to McMaster University, Canada (~90 m a.s.l.) and bred within their respective populations, under common garden conditions (24^{o}C, 760 mmHg, 14 h:10 h light:dark cycle, with rodent chow and water *ad libitum*). Once they reached adulthood (90 days old) 10 G_1 females from each population were mated to unrelated G_1 males with the same altitude ancestry. Once pregnancy was confirmed, males were removed until the litter was weaned at postpartum day (P) 21. After weaning, pups were housed with their same-sex littermates until adulthood. Breeding success in captivity is highly variable so we allowed breeding pairs to have 3 subsequent litters for females to establish their own maternal care phenotype before starting the experiment with litter number 4. All procedures were approved by the McMaster University Animal Research Ethics Board.

5.3.2 Experimental Design

Once the dams gave birth to their fourth litter, they were given 24 hours with their pups undisturbed. On P1 mothers and pups were weighed and placed in a clean cage. Food was weighed and the cage was moved to an observation room with the same light cycle, temperature and oxygen level as the breeding room described above. All measures were repeated on the next litter, raised under HA simulating cold hypoxia. Once the litter was born, mothers and pups were moved into a 5^{o}C. The next day (P1) mothers, pups and food were weighed and placed in a clean cage in hypobaric chambers at 430 mmHg (equivalent to 12% O_2 at sea level). These conditions mimic the summit of Mount Evans, CO during the summer months. We quantified maternal behaviour when between P2 to P8. Cages were not changed until after the final maternal care observation so that mothers were not disturbed. After final maternal care observation on P8 we performed a maternal provisioning test (see below). Maternal resting metabolic rate (RMR) was measured during peak lactation at P12 and P14 (Król and Speakman, 2003). At P21 mothers and all pups were weighed, and pup body composition was determined for 1 male and 1 female from each litter. After weaning at P21, pups were returned to control conditions and raised until adulthood (P90), when thermogenic capacity was measured (detailed below). If females did not successfully raise an experimental litter to weaning, they were allowed 1 additional attempt in that experimental treatment before being removed from the study.

5.3.4 Maternal Behaviour

Comprehensive protocols for scoring maternal behaviour have been previously developed for lab rodents (Champange, 2003) and validated in wild *P. maniculatus* (Stewart and

McAdam, 2014). We performed maternal behavior observations starting at 12:00 pm on pup P2 until 8:00 am on P8. Five observation periods of 1 h each were performed during a 24-hour cycle, 3 equally spaced during the light phase (8:00 am, 12:00 pm and 5: 00 pm) and 2 during the dark phase under red light (10:30 pm, 2:30 am). During each 1-hour observation period 21 observations were made, once every 3 minutes starting at time 0. Maternal behavior was first scored as nursing (either attached pups visible or incubating pups in nest) or not nursing (not in contact with pups). Further behaviors were noted that were not mutually exclusive (eating, drinking, grooming, active, nest building, carrying pups). Each mother's scores for each behavior were averaged for every timepoint across all 7 days.

5.3.5 Provisioning test

On P8, mothers were separated from pups for 4 hours, during that time mothers were fed *ad libitum* but pups became post-absorptive. Pups remained in the home cage, while mothers were removed to a new cage. At the end of this separation, mothers and pups were weighed. Each pup was given a distinctive mark with permanent marker so they could be identified. Mothers were returned to the home cage with pups and filmed for 3 h. At the end of the 3 h pups and mothers were re-weighed. Time spent nursing was assessed from the videos. Provisioning rate was calculated as: litter weight gain · 1h nursing^{-1} (Hager and Johnstone, 2005; 2007; Stewart and McAdam, 2014). We used the ratio of litter weight gain to maternal weight loss over the time spent nursing as a measure of provisioning efficiency.

5.3.6 Maternal Resting Metabolic Rate

We used flow through respirometry to analyze maternal resting metabolic rate under warm/normoxic or cold/hypoxic conditions. Trials were performed on P12 and P14, which should correspond to peak lactation (Król and Speakman, 2003). Mothers were removed from cages and placed in 475 mL plexiglass metabolic rate chambers. Chambers were placed inside a Peltier cabinet (Sable Systems, NV) to maintain ambient temperature at either 28^{o}C or 5^{o}C. Incurrent air was either outside air or 12% O_2 balanced with nitrogen, dried and stripped of CO_2 before flowing through the chambers at a rate of 1000 mL/ min. Air flow was controlled using a mass flow controller (Sable Systems). Excurrent air was subsampled at a rate of 150 mL/min, dried with magnesium perchlorate and passed through O_2 and CO_2 analyzers (Sable Systems). Trials lasted 2h and resting metabolic rate (RMR) was determined as the average of the 3 lowest stable 5 min $\dot{V}O_2$ traces after 45 minutes. Data acquisition and analysis were performed using Expedata software (Sable Systems).

5.3.7 Pup Body Composition

We used a quantitative magnetic resonance imager (EchoMRI 4-in1, EchoMRI, Houston, Texas) to determine lean mass (g) and fat mass (g) of pups at P21 and adulthood (P90). Body composition was determined as: $100 \cdot \text{fat mass} \cdot \text{body mass}^{-1}$.

5.3.8 Offspring thermogenic capacity

To assess the long-term effects of maternal care phenotype on offspring phenotype we measured thermogenic capacity as cold-induced $\dot{V}O_2max$ under normoxic (21% O_2) and hypoxic conditions (12% O_2; Robertson and McClelland, 2019) of offspring once they reached adulthood (P90). Briefly, adult mice were placed in sealed glass respirometry chambers (475 ml) at -5^{o}C. Heliox air (21% or 12% O_2 with He) was flowed through the chambers at a rate of 1000 ml·min^{-1}. Subsampled excurrent air was dried before being flowed through O_2 and CO_2 analyzers (Fox Box Respiratory System, Sable Systems). Data acquisition was performed using LabChart software (ADI instruments) and cold-induced $\dot{V}O_2max$ was calculated as the highest stable (10 seconds) $\dot{V}O_2$ (Tate et al., 2017). Rectal body temperature was taken before and after each trial to ensure that the trial was sufficient to induce hypothermia.

5.3.9 Statistical Analysis

We used 2-Way analysis of variance (ANOVA) to test the effects of altitude ancestry (population) and environment on litter size, maternal weekly energy intake, maternal resting metabolic rate, Δ litter mass per nursing bout, provisioning rate, provisioning efficiency, maternal behaviour and pup body composition at weaning. We used 2- Way ANOVA to test the effects of time and ancestry on daily food intake. We used a multivariate analysis of variance (MANOVA) to test the effects of ancestry, environment and time on maternal body mass. To deal with a significant treatment x population interaction we then used 2- Way repeated measures (RM) ANOVA within population to test the effect of time and environmental conditions on maternal body mass and pup mass.

We used a chi squared test to compare the proportions of pups in a litter who lost weight during a nursing bout. We used a linear regression to test the effects of litter size on Δ pup mass per nursing bout. We used 2 -Way ANOVA within sex to test the sex specific effects of altitude ancestry and rearing environment on adult body mass, body composition. We used 2-Way Analysis of covariance (ANCOVA) with body mass as a covariate to test the sex specific effects of altitude ancestry and rearing environment on offspring adult thermogenic capacity (cold-induced $\dot{V}O_2$ max). We used Tukey post hoc analysis whenever there were significant interactions.

5.4 RESULTS

Maternal Energy Intake

We tracked food intake over the initial lactation period (P2- P8). All mothers increased food consumption over this period (Postnatal Day, $F_{6,77}$ =5.035, P <0.001). It was not surprising that HA mothers consumed more food than LA mothers during lactation (Population, $F_{1,77}$ = 26.924, P<0.00) (**Figure 5.1A**). This increased food consumption in HA mothers could be mostly accounted for by their increased litter size, as food intake per pup was more consistent between the populations (Population, $F_{1,77}$ =3.337, P=0.072) (**Figure 5.1B**). We tracked weekly, rather than daily, food intake in cold hypoxia, to avoid opening the hypobaric chambers. There was no increase in food consumption in cold hypoxia in either population (Treatment, $F_{1,20}$=0.089, P=0.768) (**Figure 5.1C**). Once again, HA mothers ate more than LA (Population, $F_{1,20}$ =4.523, P=0.046), but consumed the same amount of food per pup (Population, $F_{1,19}$ =0.416, P=0.505) (**Figure 5.1D**).

Maternal Energy Output

HA mothers were heavier throughout the lactation period than LA mothers, regardless of environmental conditions (Population, $F_{1,72}$=43.474, P < 0.001). The initial response to cold hypoxia also differed between the populations (Population x Treatment, $F_{1,1}$=5.562, P=0.021). When pups are 8 days old, cold hypoxia exposed HA mothers were heavier than controls while LA mothers are lighter (**Figure 5.2A**). As previously reported (Robertson et al, 2019; Robertson and McClelland, in revision) HA mothers in this study gave birth to more pups per litter than LA mothers (Population, $F_{1,19}$=6.539, P=0.019). Pup number was consistent between control and cold hypoxia exposed litters (Treatment, $F_{1,19}$=1.536, P=0.230) (**Figure 5.2B**).

The RMR of lactating females was similar between the populations (Population, $F_{1,21}$ =0.364, P= 0.553), regardless of exposure conditions. Under control conditions the RMR of lactating females was 1.5-1.8-fold higher than virgin females. Under cold hypoxia conditions the RMR of LA and HA mothers increased substantially, by ~36-52% (Treatment, $F_{1,21}$=20.627, P<0.001) (**Figure 5.2C**).

We used weight gain after a single nursing bout to assess maternal provisioning rate. During a single nursing bout, litters as a whole gained similar amounts of weight per hour (Population $F_{1,17}$=1.293, P=0.271). We compared litter weight gain to maternal weight loss over the same nursing bout to calculate provisioning efficiency. Under cold and hypoxia litters gained less weight during a single nursing bout for every g their mothers lost regardless of population (Treatment, $F_{1,17}$=5.438, P=0.032; Population $F_{1,17}$=0.586,

P=0.454) (**Figure 5.2D**). During a single nursing bout roughly 20-30% of pups in a litter lost weight during the nursing bout (**Figure 5.2E**). This proportion did not differ significantly between any of the groups tested ($_X^2$ = 1.089, P=0.780). When assessing individual pups weight changes over a single nursing bout, taking into account pups who lost weight there was no effect of population ($F_{1,110}$ =0.274, P=0.602) or environment ($F_{1,110}$ =0.366, P=0.546) on the mean pup weight change. There was also no correlation of this factor with litter size (R^2= 0.006, P=0.653).

Maternal Behaviour

Peromyscus are nocturnal so not surprisingly, both LA (Time; $F_{4,45}$=19.453, P<0.001) and HA (Time; $F_{4,45}$=21.105, P<0.001) spent ~95% of their time during the day in their nests, nursing their pups (**Figure 5.3A**). During the night HA mothers spent 30% less time in contact with/nursing pups (Population; $F_{1,11}$= 6.172, P=0.030) and 20% more time (Population; $F_{1,11}$= 3.405; P=0.092) being active (running, climbing etc. **Figure 5.3B**). Overall, cold hypoxia exposure caused mothers from both populations to increase the time spent in the nest nursing their pups (LA Treatment, $F_{1,45}$ =5.685, P=0.021; HA Treatment, $F_{1, 45}$ = 4.454, P=0.040). However, the population differences during nightly time spent nursing were maintained (Population, $F_{1,7}$=18.400, P=0.004). Interestingly, under cold hypoxia HA mothers spent a similar time nursing pups as LA mothers under warm-normoxic control conditions. Under cold hypoxia, 3 of 7 HA mothers continuously abandoned their litters resulting in 100% pup mortality. After 2 subsequent breeding attempts, they were removed from the trial. 1 LA mother died during cold hypoxia

exposure. All other LA mothers raised their pups to weaning without abandonment under cold hypoxia.

Postnatal Offspring Phenotype

Under common garden control conditions, LA and HA pups were the same size throughout the nursing period (Population; $F_{1,30}$= 1.547, P=0.218). Postnatal cold/hypoxia caused growth restrictions in both LA (Age x Treatment; $F_{2,8}$=25.660, P<0.001) and HA (Age x Treatment; $F_{2,6}$=6.420, P=0.032) pups. However, this effect was much more severe in LA pups who grew to only 65% of their control siblings by weaning in cold hypoxia, compared to HA pups who grew to 88% (**Figure 5.4A**). LA pups also showed a substantial decrease in % body fat by weaning, dropping from ~23% body fat to ~18.5%. This effect was not seen in HA pups (Population x Treatment; $F_{1,34}$ = 5.196, P=0.029) who maintained an average body composition of ~23% body fat in cold hypoxia (**Figure 5.4B**).

Offspring Adult Phenotype

We found persistent effects of nursing environment on adult phenotype in both LA and HA mice. However, males and females responded very differently. We therefore examined the sexes separately.

As adults, there was no difference in body mass of LA and HA male mice regardless of developmental treatment (Population, $F_{1,12}$ =0.908, P=0.359; Treatment, $F_{1,12}$=0.432, P=0.523). This was also true of control females, however LA females raised in CH were

~30% smaller than controls (Population x Treatment, $F_{1,13} = 3.738$, P=0.075) (**Figure 5.5A**).

LA mice were generally leaner than HA mice ($F_{1,23} = 12.406$, P=0.002). This is driven partially by female LA mice who are slightly leaner than males (Population x Sex, $F_{1,23} =4.523$, P=0.044). There was no effect of rearing environment on body composition of female mice, with LA females having ~ 8% body fat relative to ~16% body fat in HA females regardless of treatment (Population, $F_{1,12} =9.924$, P=0.008; Treatment, $F_{1,12}=0.007$, P=0.934). In males, LA who had been raised in CH increased fat mass to have a similar body composition as HA males (Population x Treatment, $F_{1,11}=6.641$, P=0.026) (**Figure 5.5B**).

Cold hypoxia exposure had different effects on adult thermogenic capacity of male LA and HA mice (Normoxic Population x Treatment $F_{1,11} =4.706$, P=0.023; Hypoxic Population x Treatment $F_{1,11} = 2.294$, P=0.158). There was no effect of development exposure on LA male thermogenic capacity. This was true of both normoxic ($F_{1,4}=0.090$, P=0.779) and hypoxic ($F_{1,4}=0.588$, P=0.4861) cold-induced $\dot{V}O_2$ max. In contrast, early cold hypoxia exposure increased adult thermogenic capacity on HA mice when tested under either normoxia ($F_{1,6}=39.58$, P<0.001) or hypoxia ($F_{1,6}=5.246$, P=0.062). The population differences in response to early cold hypoxia exposure were much more pronounced in female mice (Normoxic Population x Treatment $F_{1,12} =11.045$, P=0.006; Hypoxic Population x Treatment $F_{1,12} =12.33$, P=0.004). Early cold hypoxia exposure had a similar effect on HA female mice, who had higher cold-induced $\dot{V}O_2$ max than controls

under normoxia ($F_{1,6}$=8.140, P=0.029) and hypoxia ($F_{1,6}$=8.893, P=0.025). LA female mice responded very differently to early cold hypoxia. Both normoxic ($F_{1,5}$=17.533, P=0.009) and hypoxic ($F_{1,5}$=23.155, P=0.005) cold-induced $\dot{V}O_2$ substantially decreased in those mice who had been exposed to cold hypoxia as pups (**Figure 5.5 C, D**).

5.5 DISCUSSION

Lactation is the most energetically costly life stage for an adult female mammal (Speakman and McQueenie, 1996). The energy she derives from food can be allocated towards her own maintenance costs or her nursing offspring in the form of milk or care behaviour. This dichotomy of energy provision creates a maternal-offspring conflict (Trivers, 1974), which is especially challenging for mothers with larger litters (Rogowitz, 1996). We tested the hypothesis that there is a fundamental trade-off at HA between the energetic and thermoregulatory demands of pups that has altered maternal care. The goal of this study was to assess how HA adapted mice, who inhabit a cold hypoxic environment that already pushes energy expenditure close to its maximum (Hayes 1989), and who consistently have larger litters than their LA counterparts, manage this trade-off. Here, we have shown substantial differences in the maternal behaviour of HA and LA deer mice, that are strongly influenced by both altitude ancestry and environment. This variation has consequences on offspring phenotype that persist into adulthood.

5.5.1 Altitude ancestry alters maternal care

Our data suggest that under common garden conditions HA and LA females allocate their energy reserves differently during lactation. We found that under warm normoxic

conditions, lactating HA mothers consume more food than LA mothers. This difference in food consumption can partially be explained by the greater number of pups HA nurse. Indeed, increasing food consumption due to increased litter size is a common response in many species including *P. maniculatus* (Miller, 1975). However, this explanation may be overly simplistic. Resting metabolic rate of HA and LA females during lactation was the same, as was provisioning rate and provisioning efficiency. In our study a single nursing bout early in lactation (postnatal day 8) caused litters to gain 0.2-0.31g·h^{-1} regardless of altitude ancestry. This finding is identical to the rates in wild LA *P. maniculatus gracilis* (0.315 g·h^{-1}, Stewart and McAdam, 2014) and suggests that when HA mothers are nursing, they are producing similar quantities of milk as their LA counterparts. It is possible that HA mothers are producing more nutrient dense milk. Indeed, the milk of wild house mice (*Mus musculus*) bred in the cold (3^{o}C) for 10 generations has a higher fat and protein content (Barnett and Dickson, 1984). However, studies on HA adapted humans, yaks and dairy cows found no association between milk content and elevation (Bartl et al., 2009; Qiao et al., 2013; Barsila et al., 2014; Quinn et al., 2016). While we have not measured milk energetic content directly, the similar RMR values suggest that the metabolic cost of milk production is also similar between LA and HA mice (Derting and Austin, 1988). We again see this reflected in similar provisioning efficiencies, measured as the ratio of maternal weight loss to litter gain after nursing. Maternal weight loss during a nursing bout is thought to be reflective of milk output (Hager and Johnstone, 2005; 2007).

Despite similarities in milk output when nursing, HA mothers spend substantially less time nursing their pups than LA mothers (37 vs 65% of nocturnal activity). Instead, HA mothers spend more than half of their time during the night outside of the nest, away from their pups. A full 30% of their time is spent engaging in aerobic exercise (running, climbing, jumping etc.) compared to a mere 11% of time LA mothers engage in these activities. HA mothers also maintain heavier body mass during lactation than LA females. Altogether these data suggest that in common garden conditions HA mothers invest less of their total energy in their offspring during lactation than LA mothers, despite consuming similar amounts of food per pup.

5.5.2 Flexibility of maternal care in response to simulated high altitude

In simulated HA conditions we found no change in food intake in either population. This result was surprising as others have found that cold exposure alone increases food intake in both lactating (Hammond and Kristan, 2000) and non-lactating (Hammond et al., 2001) *P. maniculatus*. In fact, it has been suggested that food intake during lactation is limited by a mother's ability to dissipate metabolically produced heat (the heat dissipation limit (HDL) hypothesis, see Speakman and Król, 2010 for review). Therefore, in cold environments lactating females can typically consume more food than would otherwise be possible (Johnson and Speakman, 2001). However, hypoxia is a known anorexic agent, and decreases food intake in lactating rats (Bruder et al., 2008). In non-lactating, adult *P. maniculatus* the increase in food intake seen in the cold is somewhat, but not completely ablated at 3800 m a.s.l. (Hammond et al., 2001). Our study conditions mimic

4350 m a.s.l., a more severe form of hypobaric hypoxia. It is possible that this level of hypoxia acts completely antagonistically to the effects of cold on food intake resulting in the lack of change reported here for both populations.

While lactating in cold and hypoxia the maternal RMRs increased substantially (by 36-52%) but remained similar between the two populations. However, these values represent a remarkable 70 and 85% of reported hypoxic cold-induced $\dot{V}O_2max$ (Cheviron et al., 2013) of HA and LA mice, respectively. Remarkably, these high resting metabolic rates are sustained without a corresponding increase in food intake. How then are lactating females able to cope with the energetic demands of offspring care when they have so little aerobic reserve ($\dot{V}O_2max$ – field metabolic rate; Hayes, 1989)?

In many species, simulated or natural hypobaric hypoxia limits milk output (e.g., Walton and Uruski, 1946; Moore and Price, 1948; Weihe, 1965; Bruder et al., 2008). However, we saw no change in litter mass gained during a single nursing bout under cold and hypoxia. We did, however, see a decrease in provisioning efficiency. These data suggest that mothers are not saving energy by limiting milk output in cold hypoxia, but each bout of nursing is more costly for the mothers in terms of mass lost. This finding provides direct evidence for a maternal- offspring energetic conflict in simulated HA (Trivers, 1974). This energetic conflict is particularly notable because both populations increased the amount of time spent nursing in cold hypoxia. In fact, HA mothers in cold and hypoxia altered their behavior to match that of LA mothers under control conditions.

HA mothers may have compensated for the increase in energetically costly nursing bouts in cold hypoxia by decreasing nocturnal activity levels substantially (26% decrease). LA mothers also decreased activity levels but to a much lesser extent (6% decrease). This is due to LA mother already spending much of their time inactive (11% of time vs 37% for HA) and, therefore, having very little scope for further reducing activity levels to save energy. This likely explains the significant weight loss in LA but not HA mothers. HA mothers appear to have energy available in control conditions for either activity or maintenance costs that is then shifted to nursing in HA conditions. What is the source of this energy reserve and how are HA mothers able to provision larger litters of pups without increasing milk output? The answer may lie in the physiology and behavior of the pups themselves.

HA mothers routinely have litters with more pups than teats (6) meaning that they cannot feasibly provision all offspring simultaneously. It is well known that increased litter size increases intra-litter competition for food (Seitz, 1954), and that pups from larger litters tend to be smaller (Kaufman and Kaufman, 1987; Rogowitz, 1996). Despite this well documented phenomenon, HA pups were the same size or larger than their LA counterparts, especially when raised under simulated HA. During a single nursing bout, we did not find any evidence that more pups from HA litters were failing to gain weight, suggesting that their own metabolic costs are low. One possible way that HA pups could reduce their metabolic rate is by not actively thermoregulating when they experience bouts of cold in the absence of their mothers (Hill, 1972).

During the same period where maternal care was monitored in this study (postpartum day 2-8) we have previously shown that LA pups will activate their brown adipose tissue (BAT) and elevate their metabolic rate in response to an acute cold exposure. In contrast, HA pups show a significant reduction in body temperature and do not activate BAT or increase metabolic rate at these ages (Robertson et al., 2019). In fact, we have shown that HA pups have a consistently blunted metabolic response to cold throughout the nursing period (Robertson and McClelland, in revision). We have suggested that this is a cost saving strategy that allows HA pups to allocate energy towards growth rather than thermogenesis. At least one other small, altricial mammalian species, the dessert hamster (*Phodopus roborovskii*) has been shown to use this "precocial torpor" strategy early in postnatal development to save energy in the cold (Geiser et al., 2019). HA mothers may be able to successfully provision their pups, while providing less milk and care than lowlanders due to the lowered metabolic needs of their offspring. What influence do these differing strategies have on offspring phenotype?

5.5.3 Effects on offspring phenotype

Rearing environment can have profound effects on offspring physiology through adaptive or maladaptive developmental plasticity. In species with maternal care, rearing environment is the result of both the abiotic stressors of the surrounding environment and the mother's ability to buffer her offspring against them (Well, 2019). We have found that when raised under simulated HA conditions LA and HA deer mice show evidence of maladaptive and adaptive developmental plasticity, respectively. Despite increasing care,

LA mothers are unable to buffer their pups against adverse effects of experiencing simulated HA during postnatal development. However, these effects are much more pronounced in female offspring. As adults, cold hypoxia raised LA female mice are smaller than controls, with a substantially lower thermogenic capacity. Neonatal hypoxia also differentially effects the breathing response in males and females; however, in this case it is females who appear less effected (Bavis, 2005). It is unclear if LA males are less sensitive to postnatal cold hypoxia or if they simply recover more effectively when returned to control conditions. In contrast, raising HA mice at simulated HA better prepares them to face the challenges of that environment as adults. These mice have higher cold-induced $\dot{V}O_2max$ as adults when tested under either normoxic or hypoxic conditions than their warm normoxic raised siblings. An elevated cold-induced $\dot{V}O_2$ max confers a fitness benefit at high altitude, improving survival (Hayes and O'Connor, 1999). In contrast, cold alone had no effect on adult thermogenic capacity in HA mice (Chapter 4). It is important to note that pups in an insulated nest can be somewhat buffered against cold ambient temperatures. However, they cannot escape the unremitting low O_2 availability of HA. These findings suggest that this adaptive developmental plasticity has evolved in response to the specific rearing conditions found at HA, though the proximate and ultimate mechanisms driving this response are unclear (West-Eberhard, 2005).

5.6 CONCLUSIONS

We have shown that the metabolic costs of lactation at HA are incredibly high and require lactating mothers to adjust energy allocation dramatically. LA adapted females are not able to do so effectively, resulting in negative short- and long-term consequences for the

growth and physiology of their offspring. In contrast, HA mothers compensate for the increased energetic demands of HA, in part, by decreasing extraneous aerobic activity. This is likely aided by their pups, who suppress energetically costly thermoregulation during the nursing period (Robertson et al., 2019; Robertson and McClelland, 2019). This may be an example of maternal- offspring coadaptation (Wolf and Brodie, 1998), which occurs when selection favours the coevolution of a combination of parental and offspring traits that ultimately improve offspring fitness. These changes in maternal and offspring physiology and behavior were likely driven by an increased maternal- offspring conflict for the limited resources at HA.

5.7 FIGURES

Figure 5.1

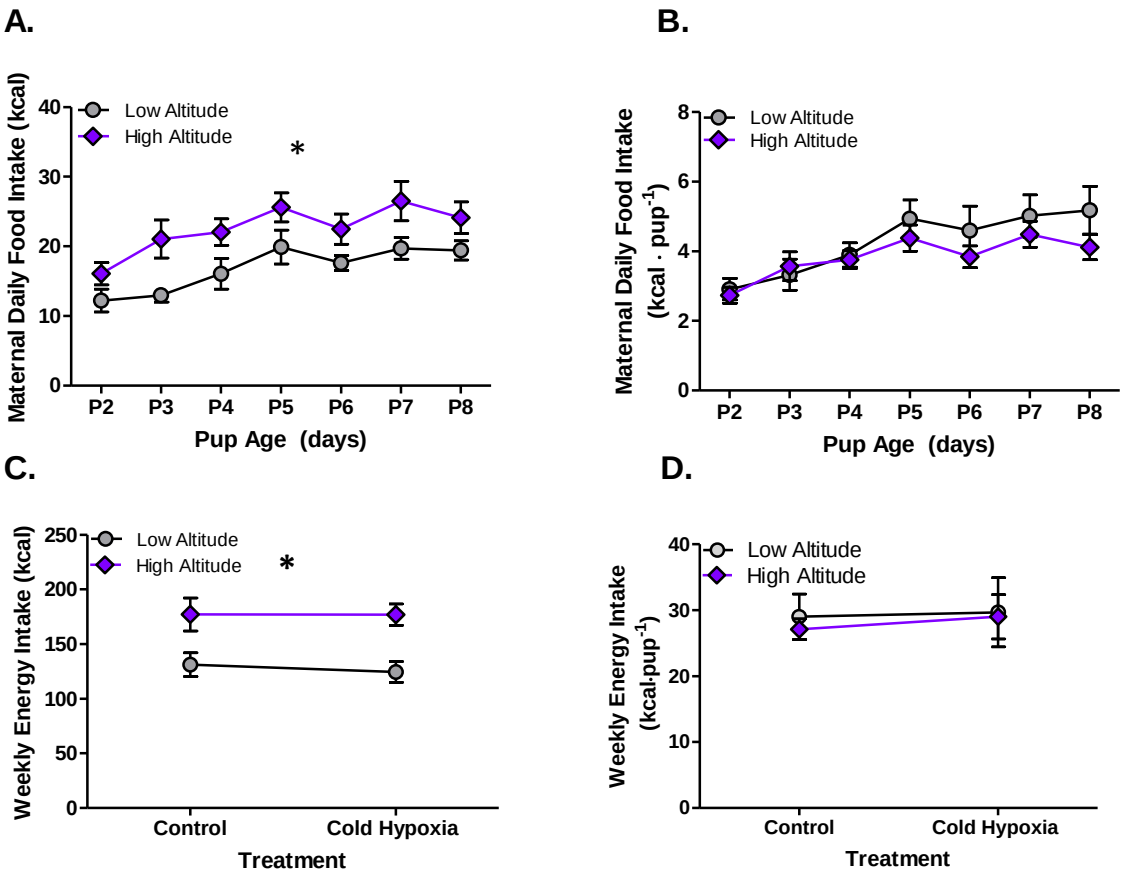

Figure 5.1 Energy intake of during postpartum (P) days 2-8 of low (LA) and high (HA) altitude native *Peromyscus maniculatus* mothers, under control and simulated high altitude (cold hypoxia, CH) conditions. * indicates significant main effect of altitude ancestry as determined by 2- Way ANOVA. Data are mean ± s.e.m.

Figure 5.2

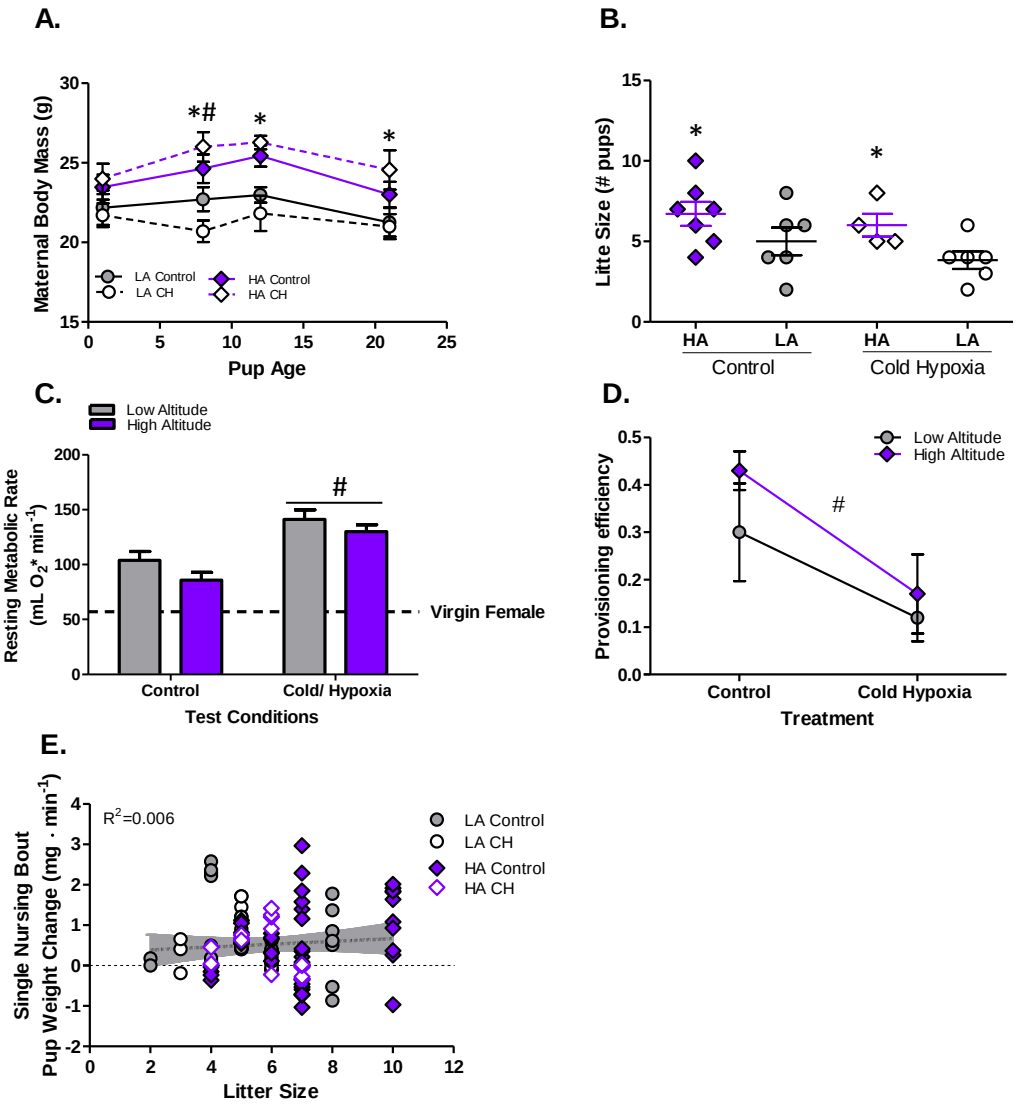
A.
Maternal Body Mass (g)
Pup Age
LA Control
LA CH
HA Control
HA CH
B.
Litte Size (# pups)
HA
LA
HA
LA
Control
Cold Hypoxia
C.
Low Altitude
High Altitude
Resting Metabolic Rate
(mL O2* min-1)
Virgin Female
Control
Cold/ Hypoxia
Test Conditions
D.
Low Altitude
High Altitude
Provisioning efficiency
Control
Cold Hypoxia
Treatment
E.
R2=0.006
LA Control
LA CH
HA Control
HA CH
Single Nursing Bout
Pup Weight Change (mg · min-1)
Litter Size

Figure 5.2 Energy output of nursing low (LA) and high (HA) altitude native *Peromyscus maniculatus* mothers, under control and simulated high altitude (cold hypoxia, CH) conditions. A). Maternal body mass throughout the nursing period. B). Litter size. C). Resting metabolic rate (RMR) of lactating females measured at peak lactation (postpartum day 12-14). Dashed line represents mean RMR of virgin females. D). Provisioning efficiency, ratio of provisioning rate (litter mass gain in $g \cdot h^{-1}$) to rate maternal weight loss ($g \cdot h^{-1}$). E) Change (Δ) pup mass per nursing bout. Dashed line at 0 represents no net change. Solid line represents the linear line of best fit, with 95% confidence intervals (grey). * indicates effect of altitude ancestry population effect, # indicates effect of environment as determined by 2- Way ANOVA. Data (A-D) are mean ± s.e.m.

Figure 5.3

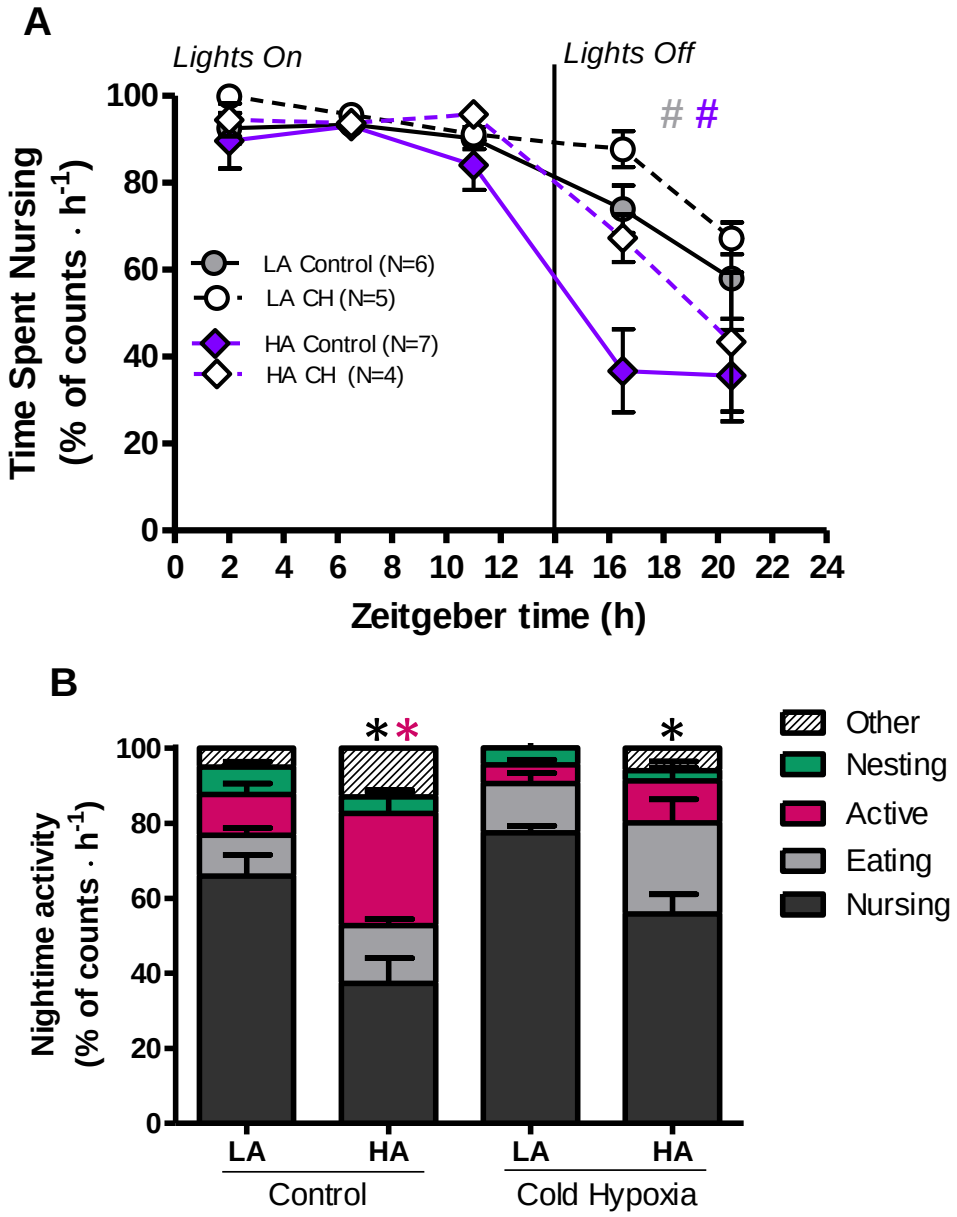
A
Lights On
Lights Off
# #
Time Spent Nursing
(% of counts · h-1)
100
80
60
40
20
0
LA Control (N=6)
LA CH (N=5)
HA Control (N=7)
HA CH (N=4)
0 2 4 6 8 10 12 14 16 18 20 22 24
Zeitgeber time (h)
B
Nightime activity
(% of counts · h-1)
* *
*
Other
Nesting
Active
Eating
Nursing
LA
HA
LA
HA
Control
Cold Hypoxia

Figure 5.3 Effect of simulated high altitude (cold hypoxia, CH) on maternal behaviour of low (LA) and high (HA) altitude deer mice (*Peromyscus maniculatus)* during days 2-8 of lactation. A). Daily pattern of time spent nursing. Zeitgeber time (ZT) 0 is 6:00 am EST. The solid vertical line indicates ZT hour 14, when lights were turned off. # indicates a main effect of treatment on behaviour. B). Proportional time spent of nighttime activities. * indicates a significant population difference within treatment ($P<0.1$). All data are presented as mean ± s.e.m. An N=1 represents all data from a single mother over 7 repeated day/night cycle.

Figure 5.4

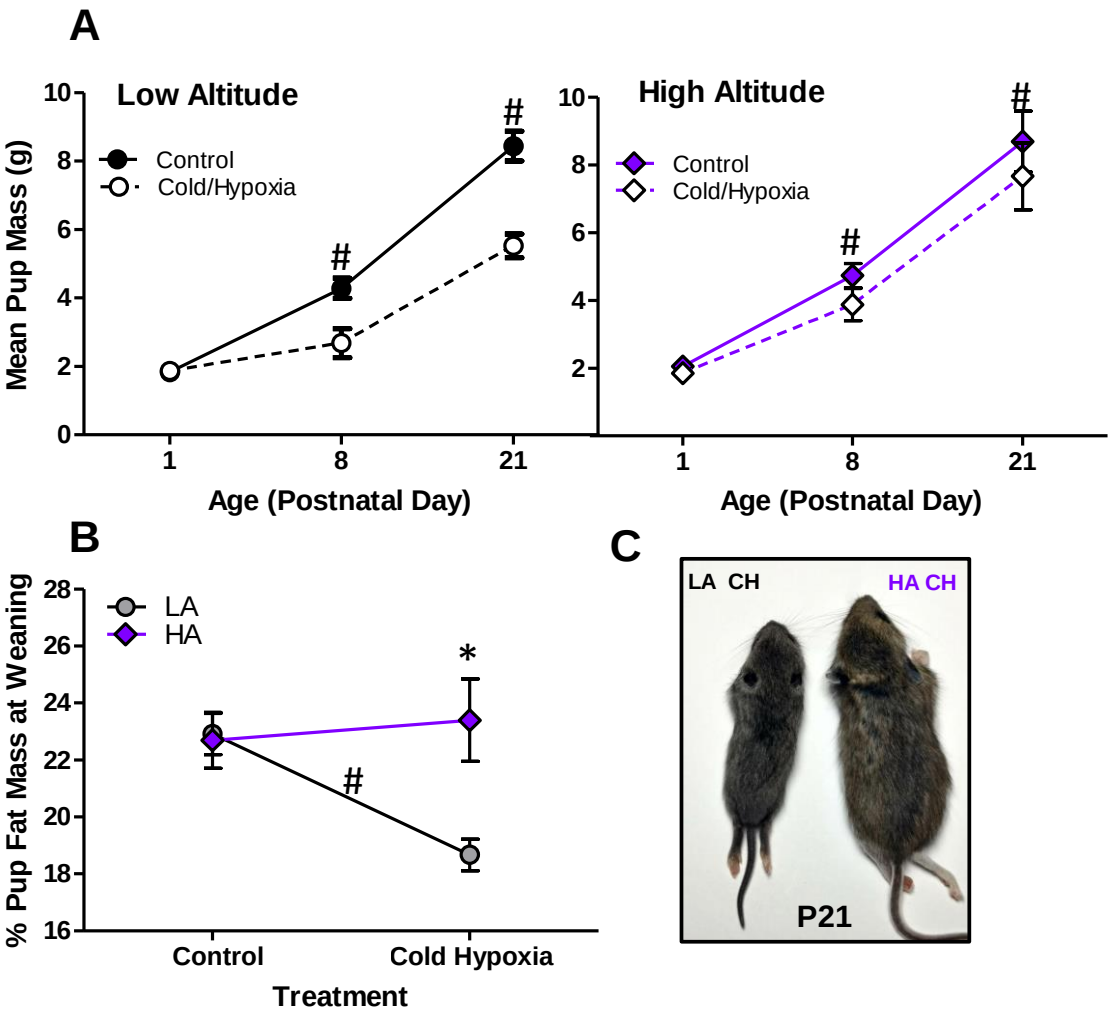
A
Low Altitude
Control
Cold/Hypoxia
Mean Pup Mass (g)
Age (Postnatal Day)
High Altitude
Control
Cold/Hypoxia
Age (Postnatal Day)
B
LA
HA
% Pup Fat Mass at Weaning
Control
Cold Hypoxia
Treatment
C
LA CH
HA CH
P21

Figure 5.4 Effect of simulated high altitude on postnatal growth during nursing of low (LA) and high (HA) altitude native *Peromyscus maniculatus* pups. A). Growth until weaning. B). Body composition (% fat mass) at weaning C). Representative images of LA and HA cold/hypoxia reared pups at weaning (P21). # indicates significant effect of treatment within population, * indicates significant effect of population within treatment. All data are mean ±s.e.m.

Figure 5.5

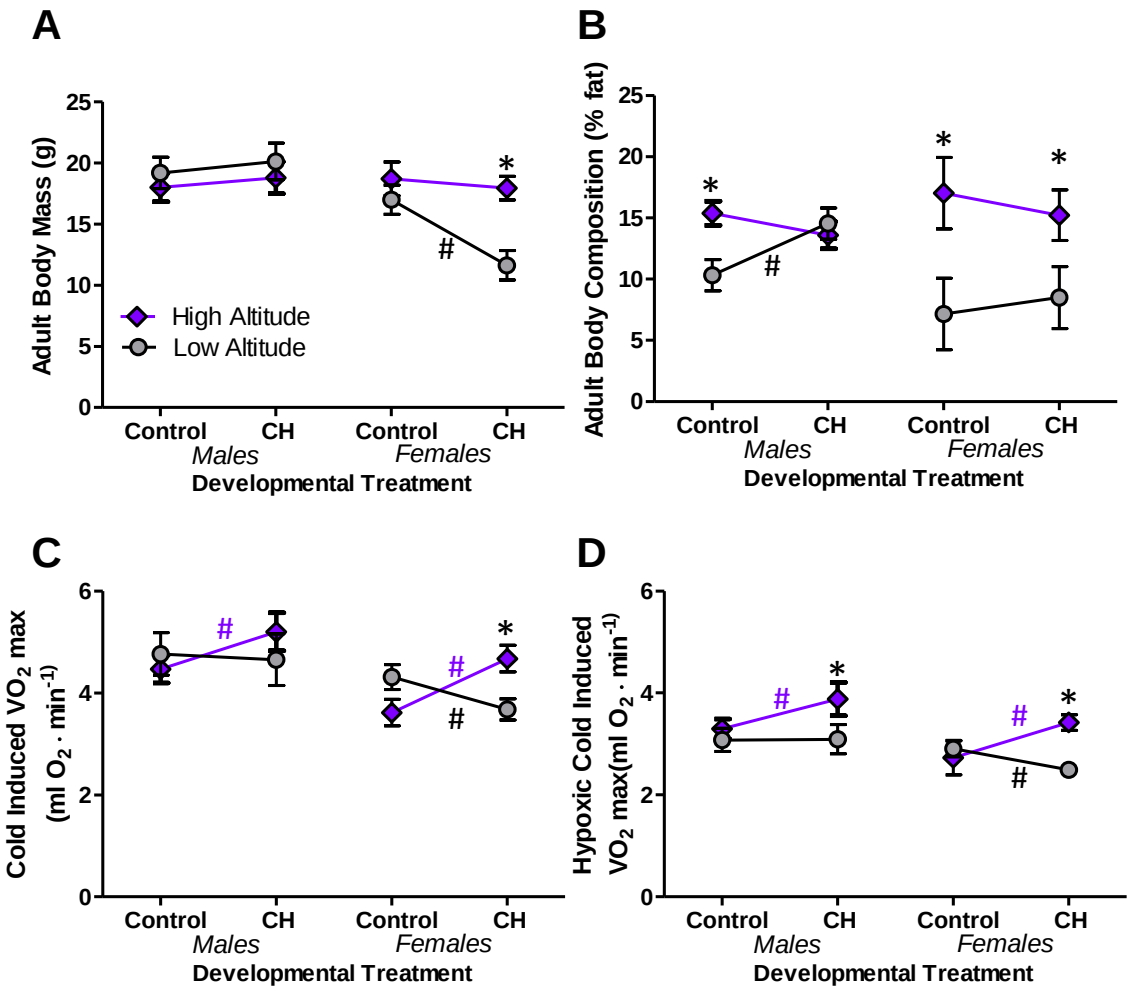
A
Adult Body Mass (g)
25
20
15
10
5
0
High Altitude
Low Altitude
Control
CH
Control
CH
Males
Females
Developmental Treatment
B
Adult Body Composition (% fat)
Developmental Treatment
C
Cold Induced VO2 max
(ml O2 · min-1)
Developmental Treatment
D
Hypoxic Cold Induced
VO2 max(ml O2 · min-1)
Developmental Treatment

Figure 5.5 Effect of developmental environment on adult phenotype of low and high altitude native *Peromyscus maniculatus* raised in control or high altitude simulating cold hypoxia (CH). A). body mass, B). body composition (% fat mass), C). thermogenic capacity (cold-induced $\dot{V}O_2$max) in normoxia, D). cold-induced $\dot{V}O_2$max in hypoxia. **#** indicates a significant effect of developmental treatment within altitude ancestry. * indicates a significant effect of altitude ancestry within treatment as determined by 2-Way ANOVA or 2-Way ANCOVA. Data are presented as mean body mass and body composition or estimated marginal mean of $\dot{V}O_2$ max corrected for body size ± s.e.m.

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CHAPTER 6 GENERAL DISCUSSION

The broad goal of my dissertation was to elucidate the role that developmental environment plays in shaping adaptive physiological traits. Specifically, I tested the hypothesis that a small, altricial mammal adapted to high altitude (HA) has evolved developmental adaptations in response to the intense thermoregulatory challenges of that environment. Through the experiments outlined in chapters 2-5 of this thesis, I have provided evidence in support of this hypothesis in HA populations of the North American deer mouse (*Peromyscus maniculatus*). I have identified evidence of both physiological heterochrony and an altered capacity for developmental plasticity in this species. Briefly, I have demonstrated a delay in the postnatal onset of both non-shivering (Chapter 2) and shivering thermogenesis (Chapter 3). Unlike low altitude (LA) adapted species, this developmental pattern is unaffected by rearing temperature (Chapter 4). Finally, HA pups show adaptive developmental plasticity when raised under simulated HA despite receiving less maternal care than their LA conspecifics (Chapter 5). The results of my thesis provide compelling evidence that HA pups have evolved to delay the developmental onset of thermogenesis as a cost-saving mechanism. However, is supressing thermoregulation in favour of growth a feasible strategy to employ at HA? If so, what are the potential proximate mechanisms responsible for this phenomenon?

6.1 The growth/thermoregulation trade-off: A feasible form of HA adaptation?

Any energetic trade-off between growth and thermoregulation is driven by the high energetic costs of metabolic heat production (e.g., Newkirk et al., 1995). In fact, one of the hallmarks of endothermy is a 3-10-fold higher metabolic rate compared to similar

sized ectotherms (e.g., Bennett and Reuben, 1979; Else and Hulbert, 1981). Cold rearing temperatures cause growth restrictions in many young LA mammals due to the increased energetic demands of thermoregulation, despite mothers increasing milk production (e.g., Hill, 1972; Hackländer et al., 2002; Zhao, 2011; Gilbert et al., 2012; Zhao et al., 2013). Small mammals are particularly prone to a cold-induced energy imbalance when they produce metabolic heat before they are large enough, or well insulated enough (with fur, fat, etc.), to successfully retain that heat when ambient temperatures drops below a mild cold stress (Pearson, 1948; Chew and Spencer, 1967; Hill, 1976; Knight, 1987; Hill, 2017). I showed that common garden raised *Peromyscus* increased metabolic rate in response to cold at least 2 days before they could maintain body temperature (T_b) in that same cold challenge (Robertson et al., 2019).

Specifically, thermoregulation threatens energy balance by depleting lipid stores. As adults, both HA and LA *Peromyscus* are thought to fuel thermogenesis exclusively through lipid oxidation (Cheviron et al., 2012). Additionally, HA mice have numerous physiological adaptations which promote higher rates of lipid oxidation during adult thermogenesis compared to lowlanders (Cheviron et al., 2012; 2014; Lau et al., 2017). These high lipid oxidation rates benefit adult mice in the chronically cold conditions of HA, as lipids are an energy dense fuel source which is ideal for endurance activities (McClelland et al., 2017). Indirect calorimetry in the postnatal period indicated that lipid metabolism also predominately fuels the metabolic response to cold in *Peromyscus* neonates (respiratory exchange ratio ~0.7). Pre and postnatal cold exposed LA *P.*

leucopus developed a metabolic response to cold earlier in ontogeny than controls. They also showed a significant decline in lipid reserves (inguinal white adipose tissue, ingWAT), likely as a result of this shift (Chapter 4). These results provide evidence that thermogenesis early in development can cause an energy imbalance in young *Peromyscus* mice by depleting fat stores.

The energetic costs of thermoregulation are greater at HA than at LA, even during the summer months (Hayes, 1989). Under these conditions, the depletion of lipid stores by thermogenesis early in development could be detrimental to survival. In fact, juvenile rats raised under simulated HA are unable to consume enough food to offset their increased metabolic costs and end up starving to death (Weihe, 1965). Increasing the fat content of milk by feeding dams high fat diets, helps alleviate growth restriction at HA, but mortality rates are still high (Weihe, 1965). I saw a similar, though less severe effect of simulated HA on growth in young LA *P. maniculatus* (Chapter 5). LA mothers were unable to offset the metabolic costs of cold hypoxia. As a result, LA pups raised in these HA simulating conditions were significantly smaller and leaner than their control siblings. However, HA native pups were able to maintain growth rates and body composition at weaning in these conditions, suggesting they are not suffering the same energy imbalance as their LA counterparts. Instead, HA pups may overcome the growth/thermoregulation trade-off by supressing thermogenesis during development to maintain growth rates and preserve valuable lipid stores.

HA pups may be taking advantage of this strategy even under relatively warm conditions. For example, HA pups consistently have more siblings to compete against for limited milk resources (Halfpenny, 1980; Sassi et al., 2017; Robertson et al., 2019). I determined in Chapter 5 that HA *P. maniculatus* mothers do not compensate for this increase in offspring number by increasing milk output. Therefore, any given HA pup is receiving less milk than a corresponding LA individual of the same age. This should limit growth, even under warm normoxic conditions (Seitz, 1954; Kaufman and Kaufman, 1987). However, HA pups consistently grow at similar rates to their LA conspecifics. Is delaying the developmental onset of thermogenesis enough to recoup the cost of increased intra-litter competition?

If we use the ingWAT depot as a marker of onboard fat stores, we can estimate the cost-savings of supressing thermoregulation at different stages of development. For example, at 8 days old, common garden raised LA pups increase metabolic rate in response to acute exposure to room temperature (24ºC), while HA pups do not (Robertson et al., 2019). We can therefore estimate the lipid oxidation rate of 8-day old LA pups, should nest temperature drop to 24ºC, using the following formula (Frayn, 1983):

(1) Lipid Oxidation ($g \cdot h^{-1}$) = (1.67*($\dot{V}O_2$ ($ml \cdot h^{-1}$) $\cdot 1000^{-1}$)) - (1.67*(VCO_2($ml \cdot h^{-1}$) $\cdot 1000^{-1}$))

LA pups in mild cold will increase lipid oxidation rates to 0.01 $g \cdot h^{-1}$. At this rate it would take only 5 h to completely deplete the equivalent of their ingWAT depot at 24ºC. Notably, it is not uncommon for wild, LA *Peromysucus* mothers to leave their nests for

several hours when resource availability is low, resulting in growth restricted pups (Hill, 1972). Young HA pups do not incur this cost, instead allowing their T_b to drop with ambient temperature. Therefore, the 2-day delay in the maturation of the metabolic response to mild cold does result in significant cost savings for developing highlanders (Robertson et al., 2019).

In a more extreme example, at P14 LA *Peromyscus* have a high thermogenic capacity, using both shivering and non-shivering thermogenesis (Robertson and McClelland, in revision. For LA *P. maniculatus* at this age it would only take ~1.2 h operating at their cold-induced $\dot{V}O_2max$ to deplete their ingWAT. At this age HA pups also metabolically respond to cold. However, they do not robustly shiver and as a result thermogenic capacity is significantly lower than their LA conspecifics (Robertson and McClelland, 2019). It would therefore take 14-day old HA pups, operating at their $\dot{V}O_2max$, ~4 h to deplete the equivalent of their ingWAT depot. This is far less realistic scenario than the previous example, as even wild adult HA *P. maniculatus* can only sustain >90% of $\dot{V}O_2$ max for ~ 25 minutes (Cheviron et al., 2013). However, using estimated rates of lipid oxidation illustrates the potential cost savings of HA pups during both mild and intense cold exposures if they limit thermogenesis during development.

Young HA pups may be able allow T_b to drop in order to save energy as, like many neonatal rodents, *Peromyscus* are remarkably resistant to deep hypothermia (Hill, 2017). For example, *Peromyscus* pups (P3-10) survived repeated 3h bouts at T_b of 1-2^oC without any adverse consequences on later predator avoidance or parental care received (Hill,

2017). The cost of rewarming from a bout of hypothermia is substantial, however, use of an external heat source dramatically reduces the cost of re-warming (Geiser et al., 2002; Currie et al., 2015) especially in neonates (Wacker et al., 2017). It is therefore feasible that HA pups drop T_b when their mothers are away from the nest, and then passively re-warm once she returns, without incurring a major energetic cost.

Based on the results of the studies presented here it is unclear if HA pups use a regulated hypometabolism to supress metabolic rate in response to cold or if T_b drops passively and subsequently metabolic rates are low as a result of a Q_{10} effect. Adult *P. maniculatus* are known users of daily torpor (Sheafor and Snyder, 1996). However, the use of torpor during ontogeny is poorly understood in most altricial species (Geiser, 2008). There is some limited evidence of other altricial neonates actively supressing metabolism during development to save energy in the cold. However, this use of torpor only occurs after endothermy has already developed (Geiser et al., 2019). Simultaneous measurements of T_b and $\dot{V}O_2$, throughout ontogeny, are needed to determine if HA pups use torpor to actively supress metabolism.

Large litter sizes may provide an additional thermoregulatory benefit for HA pups. Together a litter has a reduced surface area to Volume ratio compared to isolated pups, and thus, lower rates of heat loss (Hill, 1976). Huddling allows altricial neonates to save energy by retaining heat passively, without activating brown adipose tissue (BAT; Gilbert et al., 2012). It is possible that the larger litter sizes reported here and elsewhere for HA

mice (Robertson et al., 2019; Halfpenny, 1980; Sassi et al., 2017) may permit pups to retain some heat during prolonged maternal absences.

Taken together, it is conceivable that a developmental delay in thermogenesis evolved at HA to save energy during the postnatal period. While the energetic trade-off between growth and thermoregulation may be the ultimate mechanism driving suppression of thermogenesis, what are the possible underlying regulatory mechanisms at work?

6.2 Turning off the furnace: Development of BAT and muscle

I have shown that the phenotype of both BAT and skeletal muscle differs between HA and LA deer mouse pups across postnatal development (Robertson et al., 2019; Robertson and McClelland, in revision). In adult deer mice, adult ancestry influences the pattern of gene expression in both tissues (Cheviron et al., 2012; 2014; Scott et al., 2015; Velotta et al., 2016). In collaboration with researchers at the University of Montana, we have recently analyzed the transcriptome of BAT and skeletal muscle across development. The goal of this work is to begin to understand the tissue level regulatory mechanisms underlying whole animal performance. We find unique developmental transcriptional profiles of these two thermo-effector tissues based on altitude ancestry. Additionally, we identified several groups of gene transcripts whose expression is strongly correlated with whole animal cold-induced metabolic rate (Velotta et al., in prep).

In BAT, one of the modules, which was positively correlated with cold-induced metabolic rate, was enriched for genes involved in “axon guidance” (Velotta et al., in prep). One of those genes, which is down regulated in BAT of HA pups, is *calsyntenin 3β*, which codes

for a newly discovered BAT specific protein that aids in the secretion of neural growth factor S100B (Zeng et al., 2019). S100B promotes the sympathetic innervation of BAT, and *calsyntenin 3β* knock-out mice have fewer sympathetic neurons in their BAT, are unable to regulate T_b and have more white adipose tissue compared to controls (Zeng et al., 2019). I have previously shown that HA pups also have less sympathetic innervation of their BAT (Robertson et al., 2019), which could be explained by impaired secretion of S100B. Together these data suggest that the delay in the postnatal onset of NST may be partially due to decreased sympathetic regulation of BAT in HA pups. Plastic changes in BAT are thought to be driven by chronic norepinephrine (NE) stimulation in response to cold (Cannon and Nedergaard, 2014). If HA pups do indeed have fewer NE releasing neurons during development, this could also explain their lack of developmental plasticity in response to cold (Chapter 4).

In addition to decreased expression of genes associated with neural development, many metabolism-associated genes were downregulated in the BAT of HA pups. There was a similar pattern of differential gene expression in the skeletal muscle of HA and LA mice. In this tissue, we found that at P14, genes associated with oxidative phosphorylation, glycolysis and the TCA cycle were all significantly down regulated in HA pups (Velotta et al., in prep). The global downregulation of genes involved in multiple metabolic pathways points to the involvement of metabolic master regulators. Indeed, one of the genes we identified, that was downregulated in HA skeletal muscle at P14 was *peroxisome proliferator-activated receptor α* (PPARα; Isseman and Green, 1990).

PPARα is a transcription factor that increases the expression of many of the genes involved in lipid metabolism, including those involved in fatty acid synthesis, transport and uptake as well as β-oxidation (Yoon, 2009). Downregulation of *PPARα* provides mechanistic support for the hypothesis that suppression of lipid metabolism is an important cost saving strategy in these animals.

We have only begun to explore the mechanistic underpinnings of the developmental thermoregulatory delays identified here. There are many avenues for future studies to further elucidate these mechanisms. For example, using a bioinformatics approach we can test if the genes that are differentially expressed in the BAT and muscle of HA and LA pups are enriched with promoter regions for specific transcription factors. We can also test if these genes, or those that code for their transcription factors, show signatures of natural selection in HA populations. Additionally, we can look for differential patterns of methylation and other epigenetic markers.

6.3 Adaptive Significance and Conclusions

"One reason to focus on the physiology of juveniles and nestlings is that for most individuals it is the *only* physiology ever experienced"- Hill, R.W. 1983

For a small, HA mammal there are two major life stages, where mortality rates are likely very high. Any trait that improves fitness during these potential bottlenecks would be an important target of selection. The first stage is the postnatal period - mortality rates during the first weeks of development have not been estimated at HA but in LA *Peromyscus* they range from 49-96% (Howard, 1949; Bendell, 1959). The suppression of energetically

costly thermoregulation during postnatal development is one way by which HA pups may survive this critical life-history stage, and successfully reach maturation.

For those individuals who survive through to adulthood the second major challenge is their first winter. Overwinter survival in adult HA deer mice have been estimated to be only ~18% (Wilde et al., 2019). We and others have identified numerous cardio-respiratory and metabolic adaptations in adult HA *P. maniculatus* (McClelland and Scott, 2019 for review). However, here I have provided evidence that HA deer mice have also evolved an altered capacity for developmental plasticity in response to both the prenatal and postnatal environment. This adaptive response improves overall adult thermogenic capacity, a trait that has been shown to directly increase fitness at HA (Hayes and O'Connor, 1999).

Peromyscus mice are thought to be the most widely distributed mammals in North America (Bedford and Hoekstra, 2015). Wilfred Osgood once wrote of these mice "It is probable that a line, or several lines, could be drawn from Labrador to Alaska and thence to southern Mexico throughout which not a single square mile is not inhabited." (Osgood, 1909). However, of all the members of the *Peromyscus* genus only *P. maniculatus* has successfully colonized elevations > 4000 m a.s.l. (Hock, 1964; Baker, 1968; Velotta et al., 2018). Analysis of mitochondrial *cytochrome b* sequences have revealed the HA adapted populations of *P. maniculatus*, from separate mountain ranges, are more closely related to each other than their closest geographical LA counterparts (Natarajan et al., 2015). The

developmental changes I have reported here may help explain why these mice has been so successful in high alpine environments where others have failed to establish.

Here I have provided evidence that supressing thermogenesis is a feasible mechanism by which HA pups can maintain growth rates and preserve valuable fat stores in their cold, hypoxic environment. Decreased sympathetic innervation of BAT and a coordinated down regulation of genes involved in metabolism, in both BAT and skeletal muscle, likely underly changes in whole animal thermogenic performance during development and into adulthood. These changes in thermogenic performance have the potential to improve fitness throughout the lifespan of HA mice and may be critical components of HA adaptation in the North American deer mouse. Overall, for comparative physiologists it is important to remember that when we study adult organisms, we can easily overlook that their greatest physiological challenges may have already occurred.

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APPENDIX A

Supplementary Materials for Chapter 2

Table S1. Initial (60 sec at 24^{o}C) skin surface temperature of second-generation (G_2) laboratory-born high altitude (HA) and low altitude (LA) native *P. maniculatus* and *P. leucopus* pups at postnatal (P) days 0-10. All data are mean ± sem.

Age	HA *P. maniculatus*	LA *P. maniculatus*	LA *P. leucopus*
P0	33.22 ± 0.29	32.42 ± 0.41	32.43 ± 0.05
P2	32.69 ± 0.42	32.15 ± 0.50	33.31 ± 0.35
P4	33.44 ± 0.30	33.77 ± 0.19	34.07 ± 0.24
P6	33.48 ± 0.32	32.75 ± 0.16	33.79 ± 0.35
P8	32.54 ± 0.32	32.53 ± 0.06	32.58 ± 0.39
P10	31.17 ± 0.20	30.89 ± 0.07	31.83 ± 0.29

Table S2. Metabolic rate (oxygen consumption, VO_2) of second-generation (G_2) laboratory-born high altitude (HA) and low altitude (LA) native *P. maniculatus* and *P. leucopus* pups at postnatal (P) days 2-10 measured in control warm (30°C) or acute cold (24°C) conditions. All pups were held for at 30 °C at the beginning of the metabolic rate trials and these initial values are means of both temperature groups. All data are mean ± sem.

	Pup VO_2 (mL O_2 g^{-1} h^{-1})				
	P2	P4	P6	P8	P10
P. leucopus					
Initial	5.58±0.41	4.65±0.44	5.04±0.42	5.86±0.25	6.19±0.19
Control	5.99±0.66	5.43±0.83	4.93±0.67	5.10±0.49	4.43±0.43
Cold	5.17±0.52	3.95±0.67	5.03±0.85	6.36±0.53**	6.81±0.32*
P. maniculatus *(LA)*					
Initial	4.07±0.57	5.48±0.48	5.28±0.58	5.07±0.71	6.01±1.32
Control	5.32±0.57	5.61±0.43	6.23±0.84	5.21±0.93	5.30±0.22
Cold	3.70±0.89**	5.16±0.66	5.22±1.12	5.59±1.02*	8.17±0.67 **
P. maniculatus *(HA)*					
Initial	3.38±0.43	4.58±0.41	4.79±0.51	4.98±0.56	5.07±0.35
Control	3.85±0.70	5.14±0.34	5.41±0.60	5.58±0.57	5.31±0.43
Cold	3.71±0.67	4.83±0.69	6.02±0.96	5.71±1.04	7.36±0.35**

** Significant interaction between time in chamber (initial vs final) and test temperature (control vs cold) with 2-Way RM ANOVA ($P<0.05$). * $P=0.07$.

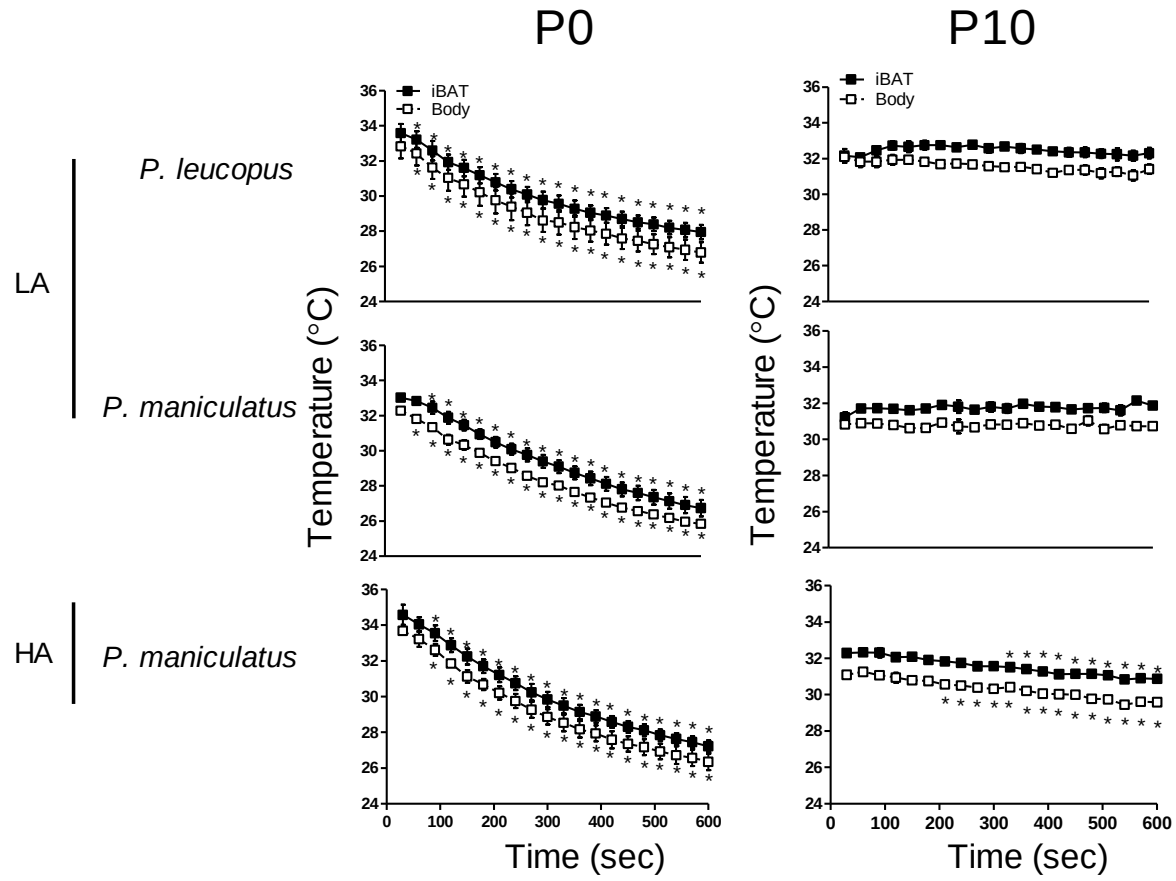

Figure S1. Surface body and intrascapular brown adipose tissue (iBAT) temperatures of newborn (P0) and 10-day postnatal (P10) aged *Peromyscus maniculatus* from low (LA, N for P0 and P10 were 3 and 7 litters, respectively) and high altitude (HA, N for P0 and P10 were 4 and 4 litters, respectively) ancestry and low altitude *P. leucopus* (N for P0 and P10 were 3 and 4 litters, respectively) exposed to acute cold (23^{o}C) for 10 minutes. * denotes a significant decrease in temperature compared to the 30 sec time point, as determined by 1-Way RM-ANOVA of temperature over time and Holms-Sidak analysis. All data are mean ± s.e.m.

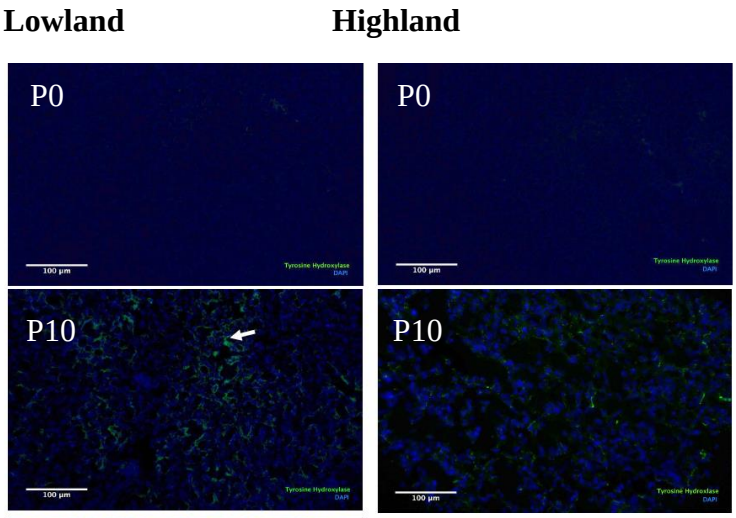

Figure S2. Immunohistochemistry with tyrosine hydroxylase-specific antibodies (green) at postnatal days P0 and P10 in iBAT of common-garden raised *Peromyscus* pups from low (LA) and high (HA) altitude ancestry.

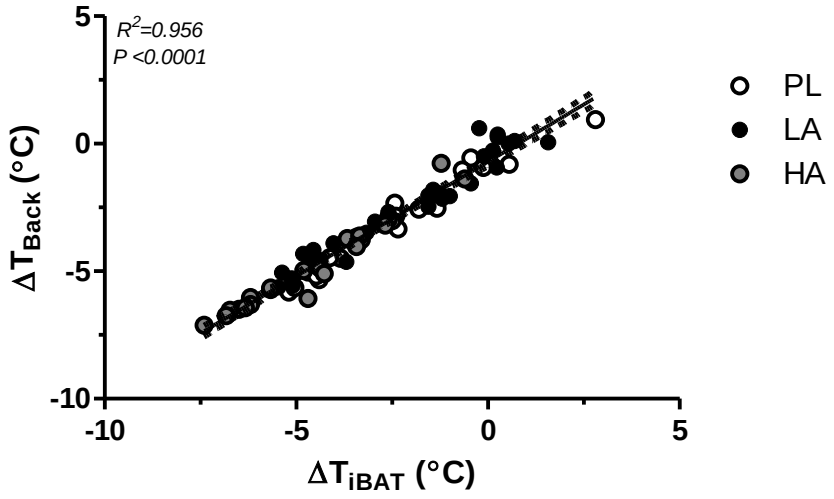

Figure S3 Linear regression (95% CI) of change in body and intrascapular brown adipose tissue (iBAT) surface temperatures (determined by thermography) with acute cold in *Peromyscus leucopus* (PL), LA and HA *P. maniculatus*.