© 2019. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2019) 222, jeb210963. doi:10.1242/jeb.210963

RESEARCH ARTICLE

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

Cayleih E. Robertson* and Grant B. McClelland

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 $\dot{V}_{O_2,max}$). 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 with 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 $\dot{V}_{O_2,max}$ compared with 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.

KEY WORDS: Muscle development, Thermogenesis, Metabolism, Endothermy

INTRODUCTION

At birth, altricial mammals lack the capacity to regulate body temperature through metabolic heat production (Pembrey, 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 (*Peromyscus 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 $\dot{V}_{O_2,max}$), 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 with 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

Department of Biology, McMaster University, 1280 Main St West, Hamilton, ON Canada L8S 4K1.

*Author for correspondence (roberceg@mcmaster.ca)

C.E.R., 0000-0002-6769-2852; G.B.M., 0000-0003-1500-9983

Received 22 July 2019; Accepted 24 September 2019

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.

MATERIALS AND 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*) were trapped at the summit of Mount Evans, CO, USA (4350 m a.s.l.), and the LA natives (*P. maniculatus nebrascensis* 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°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, P2, P4, P6, P8, P10, P14, P21 and P27, one pup per G_1 breeding pair was sampled by an overdose of isoflurane followed by cervical dislocation. The gastrocnemius muscle from one hindlimb was blunt dissected, weighed and freeze-clamped using two liquid N_2-cooled aluminium plates. We used the gastrocnemius 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°C for future molecular analysis. From the other hindlimb, the triceps surae (consisting of gastrocnemius, 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 (10 µm) 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 v.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 randomized and analyzed blind using ImageJ 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 s and eosin for 5 s (Leica Biosystems).

Muscle enzyme activity

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 P27, individual pups were used to determine 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 h to achieve a post-absorptive state prior to BMR trials, after which time they were placed in respirometry chambers (475 ml) maintained within the thermoneutral zone (Hill, 1983) at 28°C 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 three lowest stable (5 min) O_2 traces over 2 h.

NST

Mice were placed in metabolic chambers (475 ml) at 28°C and baseline $\dot{V}_{O_2}$ measurements were obtained after 45 min in normoxia as described above. Noradrenaline (norepinephrine) 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 h 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 min, 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 noradrenaline 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 −5°C and either normoxic (20% O_2) or

Journal of Experimental Biology (2019) 222, jeb210963. doi:10.1242/jeb.210963

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 $\dot{V}_{O_2,max}$ was calculated as the highest stable (10 s) $\dot{V}_{O_2}$ during a 10 min trial (Tate et al., 2017). Rectal body temperature was taken before and after each trial using a rectal probe (RET-4, Physitemp) to ensure that the trial was sufficient to induce hypothermia.

Shivering thermogenesis was estimated in normoxia as $\dot{V}_{O_2,max}$−(NST+BMR) (Wunder and Gettinger, 1996).

Statistics

We used two-way repeated measures (RM) ANOVA to test the effect of population and age on mean pup mass, and one-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 two-way ANOVA to test the effects of population and age on tissue mass, oxidative fiber numerical density, capillary area, enzyme activity and percentage $\dot{V}_{O_2,max}$. Percentage $\dot{V}_{O_2,max}$ 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 $\dot{V}_{O_2}$ ($\dot{V}_{O_2,max}$, NST, BMR), we used two-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–Šidák *post hoc* analysis to perform pairwise comparison between groups. Data are available from figshare (10.6084/m9.figshare.8979842).

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 development and found that *P. leucopus* were larger and grew faster than either population of *P. maniculatus* (age×population $F_{16,176}=12.851$, $P<0.001$; Fig. 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 rate 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 1). Growth patterns of the gastrocnemius muscle also differed between populations (age×population $F_{12,184}=6.684$, $P<0.001$; Fig. 1B). For the first 2 weeks of development, the size of this hindlimb muscle was similar for all three groups. However, after P14, the gastrocnemius 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×population $F_{12,184}=3.516$, $P<0.001$; Fig. 1C). The relative size of the gastrocnemius 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×population $F_{4,47}=3.298$, $P=0.018$;

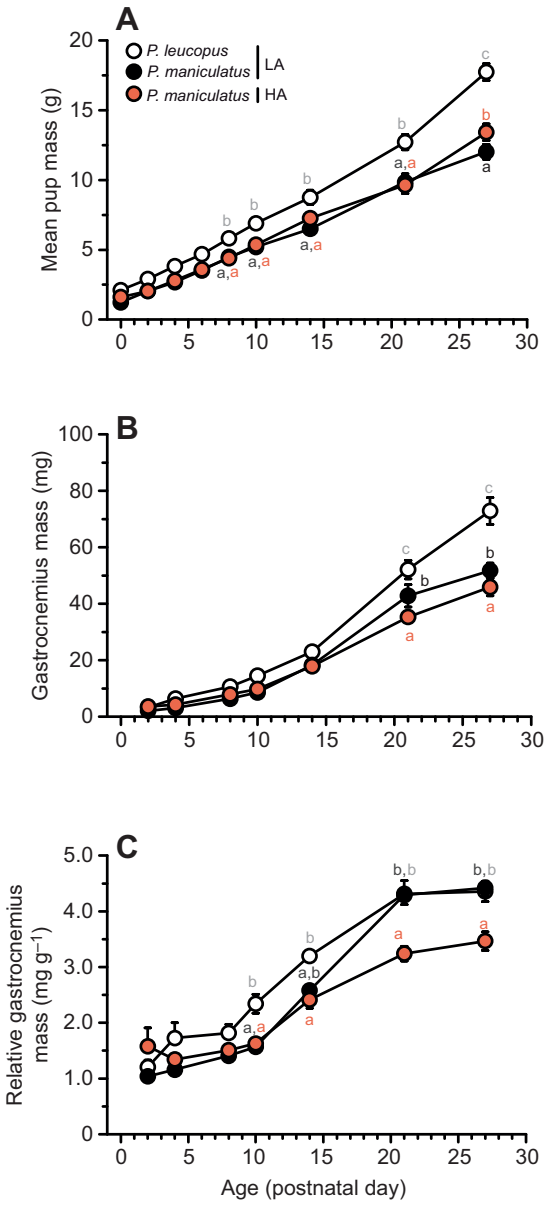

Fig. 1. Postnatal growth of common garden raised *Peromyscus maniculatus* pups of low-altitude (LA, *N*=7) and high-altitude (HA, *N*=8) ancestry and from a LA outgroup species, *Peromyscus leucopus* (*N*=10). Body mass (A), gastrocnemius muscle mass (B) and gastrocnemius mass relative to body mass (C). Dissimilar letters indicate significant population differences within an age as determined by two-way ANOVA or RM ANOVA and Holm–Šidák multiple comparisons. All data are presented as means±s.e.m.

Table 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. maniculatus	
	P. leucopus (*N*=9)	LA (*N*=7)	HA (*N*=8)
Growth rate (g day^{-1})			
Pre-weaning	0.51 ± 0.03^{b}	0.41 ± 0.03^{a}	0.38 ± 0.02^{a}
Post-weaning	0.84 ± 0.07^{c}	0.36 ± 0.08^{a}	0.63 ± 0.04^{b}

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

Journal of Experimental Biology (2019) 222, jeb210963. doi:10.1242/jeb.210963

Fig. 2). At P21 and P27, the muscle fibers of HA pups were generally smaller than those of LA *P. leucopus* (age×population $F_{7,75}$=3.434, P=0.003; Fig. 2B, 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 gastrocnemius enzyme activity. The V_{max} 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. 3A). V_{max} 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. 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×population $F_{4,90}$=4.170, P=0.004; Fig. 3C).

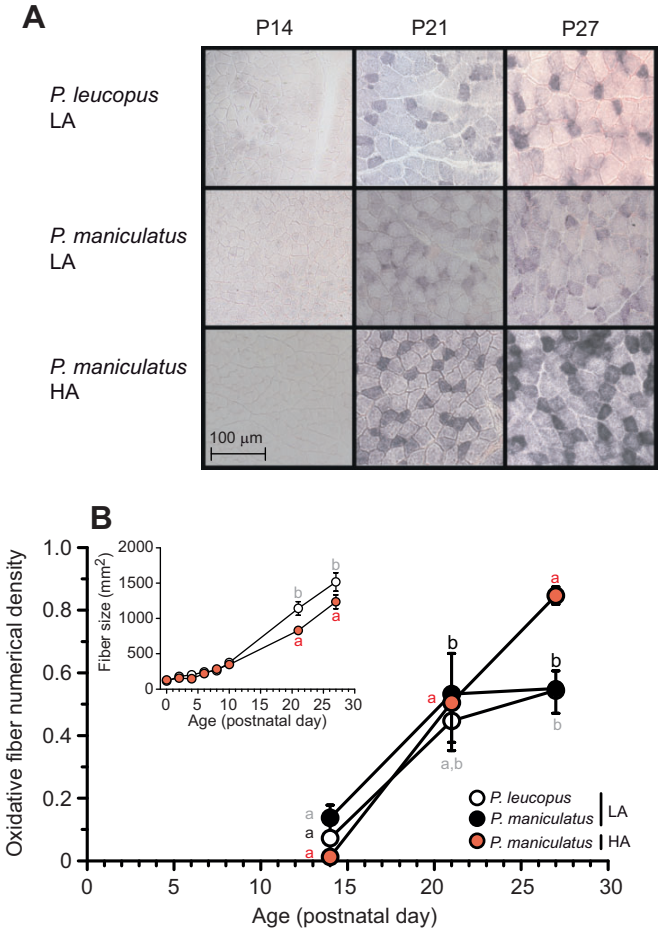

Fig. 2. Maturation of aerobic fiber type of the triceps surae muscle complex in HA and LA *P. maniculatus* and LA *P. leucopus*. (A) Representative images of triceps surae muscle fibers stained for succinate dehydrogenase (SDH) activity to identify aerobic fibers (stained purple) at postnatal day (P)14, P21 and P27. (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 two-way ANOVA and Holm–Šidák 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.

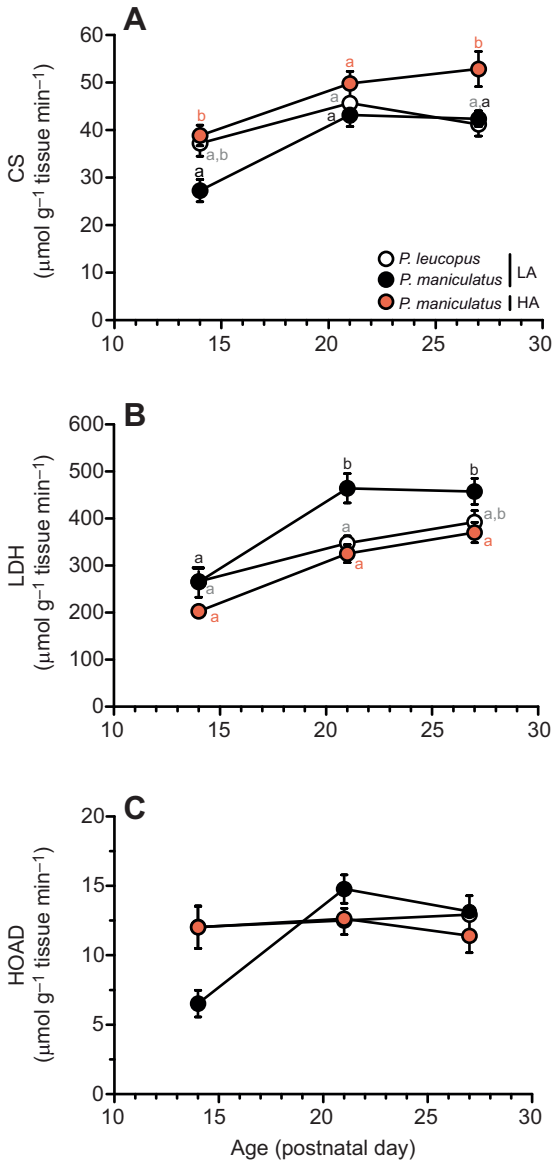

Fig. 3. Muscle enzyme activity in gastrocnemius muscle across postnatal development in HA and LA *P. maniculatus* and LA *P. leucopus*. Apparent V_{max} is shown for (A) citrate synthase (CS), (B) lactate dehydrogenase (LDH) and (C) β-hydroxyacyl-CoA dehydrogenase (HOAD). Dissimilar letters indicate significant population differences within age as determined by two-way ANOVA and Holm–Šidák 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.

Muscle capillarity

In addition to 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×population $F_{4,53}$=3.494, P=0.013; Fig. 4A). Capillary area was larger in HA pups, in part, because the capillaries were more tortuous as capillary

Journal of Experimental Biology (2019) 222, jeb210963. doi:10.1242/jeb.210963

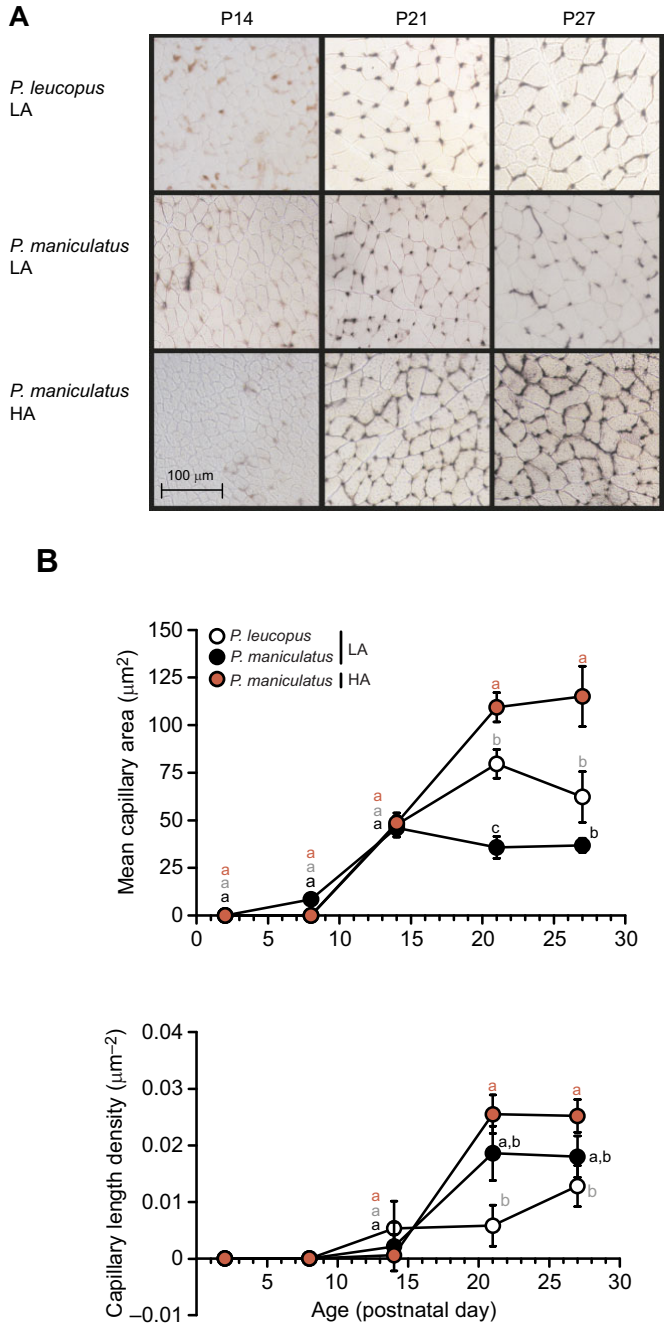

Fig. 4. Maturation of capillarity of the triceps surae muscle complex in HA and LA *P. maniculatus* and LA *P. leucopus*. (A) Representative images of triceps surae muscle fibers stained for alkaline phosphatase (AP) activity to identify capillaries (dark stain) at P14, P21 and P27. (B) Mean capillary area and capillary length density (quotient of areal density and transverse area of the smallest 10% of capillaries). Dissimilar letters indicate significant population differences within age as determined by two-way ANOVA and Holm–Šidák 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.

length density followed an identical trend (age×population $F_{4,51}$=2.577, P=0.048; Fig. 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×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. 5A).

The estimated proportional contribution of shivering thermogenesis to thermogenic capacity changed differentially with age and population (age×population $F_{4,48}$=28.93, P<0.001). For *P. leucopus*, the contribution of shivering thermogenesis remained constant at ∼30% $\dot{V}_{O_2,max}$, while in both LA and HA *P. maniculatus* the contribution of shivering thermogenesis increased with age to ∼55% $\dot{V}_{O_2,max}$ at P27. However, at P14 the contribution of shivering thermogenesis 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. 5B). In fact, in most HA individuals tested, there was no evidence of shivering thermogenesis 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 2). The intrascapular 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×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 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. 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*.

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 with LA conspecifics and 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.

Journal of Experimental Biology (2019) 222, jeb210963. doi:10.1242/jeb.210963

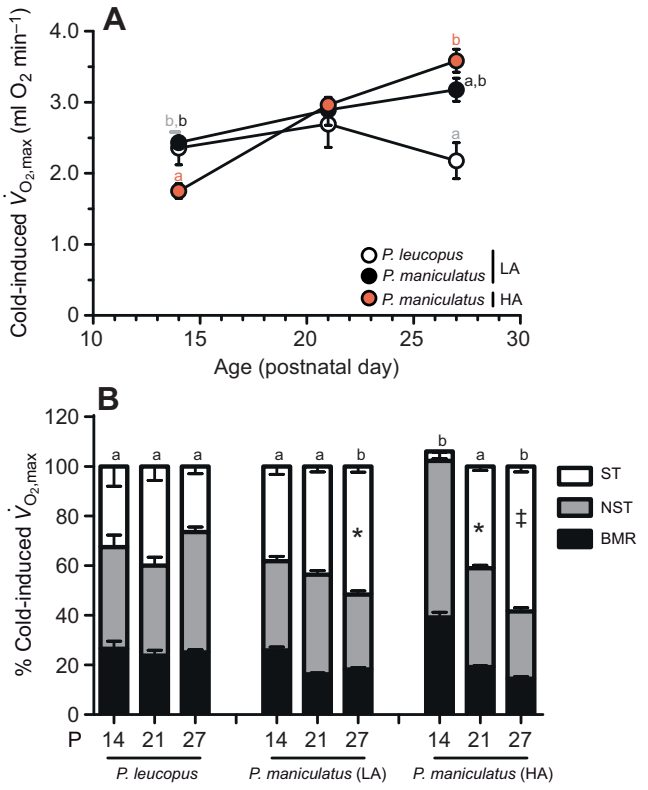

Fig. 5. Thermogenic capacity during postnatal development of HA and LA *P. maniculatus* and LA *P. leucopus*. (A) Estimated marginal mean cold-induced $\dot{V}_{O_2,max}$. (B) Relative contribution (% total) of shivering thermogenesis (ST), non-shivering thermogenesis (NST) and basal metabolic rate (BMR) to cold-induced $\dot{V}_{O_2,max}$. For cold-induced $\dot{V}_{O_2,max}$, dissimilar letters indicate significant population differences within an age as determined by two-way ANCOVA and Tukey's multiple comparisons. For percentage $\dot{V}_{O_2,max}$, dissimilar letters indicate significant population differences of percentage shivering thermogenesis within age as determined by two-way ANOVA and Holm–Šidák multiple comparisons. *Percentage shivering thermogenesis>P14, ‡percentage shivering thermogenesis>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.

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 with 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 1 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 precede 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

Table 2. Estimated marginal mean basal metabolic rate (BMR), noradrenaline-induced non-shivering thermogenesis (NST) and intrascapular brown adipose tissue (iBAT) mass of G_2 HA and LA *P. maniculatus* and LA *P. leucopus*

	P. leucopus (*N*=3–8)			*P. maniculatus*					
				LA (*N*=3–7)			HA (*N*=5–12)		
	P14	P21	P27	P14	P21	P27	P14	P21	P27
BMR (ml O_2 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 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	1.06±0.19	0.92±0.19
Relative iBAT mass (mg 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^{a,b}	7.37±0.63^{b}	6.56±0.63^{a}	9.56±0.55^{a,b}	5.63±0.51^{a,b}

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 two-way ANOVA.

Journal of Experimental Biology (2019) 222, jeb210963. doi:10.1242/jeb.210963

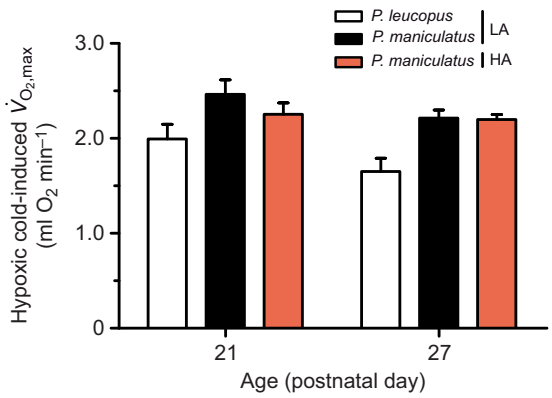

Fig. 6. Estimated marginal mean thermogenic capacity (cold-induced $\dot{V}_{O_2,max}$) in hypoxia (12% O_2) during postnatal development of HA and LA *P. maniculatus* and LA *P. leucopus*. Two-way ANCOVA and Tukey's multiple comparisons test reveal a main effect of both age and population. All data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

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: Arajamma 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 with 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 with 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 (Arajamma 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 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, 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, 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).

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.

Acknowledgements

The authors thank Leanne Zubowski, Debbie Chan, Wafa Khoja and Kwasi Nkansah for their invaluable assistance in data collection. Additionally, we sincerely thank Sulaymon Lyons for his assistance with histological analysis.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.E.R., G.B.M.; Methodology: C.E.R., G.B.M.; Validation: C.E.R.; Formal analysis: C.E.R.; Resources: G.B.M.; Data curation: C.E.R.; Writing - original draft: C.E.R.; Writing - review & editing: C.E.R., G.B.M.; Supervision: G.B.M.; Funding acquisition: G.B.M.

Funding

This work was funded by a Natural Sciences and Engineering Research Council of Canada Discovery Grant, a Natural Sciences and Engineering Research Council of Canada Discovery Accelerator Supplement (G.B.M.) and a Natural Sciences and Engineering Research Council of Canada Graduate Scholarship (C.E.R.).

Data availability

Data are available from the figshare digital repository: doi:10.6084/m9.figshare.8979842

References

Adams, G., McCue, S., Zeng, M. and Baldwin, K. M. (1999). Time course of myosin heavy chain transitions in neonatal rats: importance of innervation and thyroid state. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **276**, 954-981. doi:10.1152/ajpregu.1999.276.4.R954

Agbulut, O., Noirez, P., Beaumont, F. and Butler-Browne, G. (2003). Myosin heavy chain isoforms in postnatal muscle development of mice. *Biol. Cell* **95**, 399-406. doi:10.1016/S0248-4900(03)00087-X

Arajamma, O. and Lagerspetz, K. Y. H. (1978). Postnatal development of shivering in the mouse. *J. Therm. Biol.* **4**, 35-39. doi:10.1016/0306-4565(79)90043-3

Bendell, J. F. (1959). Food as a control of a population of white-footed mice, *Peromyscus leucopus noveboracensis* (Fischer). *Can. J. Zool.* **37**, 173-209. doi:10.1139/z59-021

Brück, K. and Wünnenberg, B. (1965). Untersuchungen über die Bedeutung des multilokulären Fettgewebes für die Thermogenese des neugeborenen Meerschweinchens. *Pflügers Arch.* **283**, 1-16. doi:10.1007/BF00363296

Cheviron, Z. A., Bachman, G., Connaty, A. D., McClelland, G. B. and Storz, J. F. (2012). Regulatory changes contribute to the adaptive enhancement of thermogenic capacity in high-altitude deer mice. *Proc. Nat. Acad. Sci. USA* **109**, 8635-8640. doi:10.1073/pnas.1120523109

Cheviron, Z. A., Connaty, A. D., McClelland, G. B. and Storz, J. F. (2014). Functional genomics of adaptation to hypoxia stress in high-altitude deer mice: transcriptomic plasticity and thermogenic performance. *Evoluion* **68**, 48-62. doi:10.1111/evo.12257

Dawson, N. J., Lyons, S. A., Henry, D. A. and Scott, G. R. (2018). Effects of chronic hypoxia on diaphragm function in deer mice native to high altitude. *Acta Physiol.* **223**, e13030. doi:10.1111/apha.13030

di Prampero, P. E. (1985). Metabolic and circulatory limitations to VO_2max at the whole animal level. *J. Exp. Biol.* **115**, 319-331.

Dubowitz, V. (1963). Enzymatic maturation of skeletal muscle. *Nature* **197**, 1215. doi:10.1038/1971215a0

Geiser, F. (2008). Ontogeny and phylogeny of endothermy and torpor in mammals and birds. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **150**, 176-180. doi:10.1016/j.cbpa.2007.02.041

Glazier, D. S. (1985). Energetics of litter size in five species of *Peromyscus* with generalizations for other mammals. *J. Mammal.* **66**, 629-642. doi:10.2307/1380789

Gokhin, D. S., Ward, S. R., Bremner, S. N. and Lieber, R. L. (2008). Quantitative analysis of neonatal skeletal muscle functional improvement in the mouse. *J. Exp. Biol.* **211**, 837-843. doi:10.1242/jeb.014340

Goldspink, G. and Ward, P. S. (1979). Changes in rodent muscle fibre types during post-natal growth, undernutrition and exercise. *J. Physiol.* **296**, 453-469. doi:10.1113/jphysiol.1979.sp013016

Halfpenny, J. C. (1980). Reproductive strategies: intra- and interspecific comparisons within the genus *Peromyscus*. *PhD thesis*, University of Colorado, Boulder.

Hammond, K. A. and Kristan, D. M. (2000). Responses to lactation and cold exposure by deer mice (*Peromyscus maniculatus*). *Physiol. Biochem. Zool.* **73**, 547-556. doi:10.1086/317757
Hayes, J. P. (1989). Altitudinal and seasonal effects on aerobic metabolism of deer mice. *J. Comp. Physiol. B.* **159**, 453-459. doi:10.1007/BF00692417
Hayes, J. P. and O'Connor, C. S. (1999). Natural selection on thermogenic capacity of high-altitude deer mice. *Evolution* **53**, 1280-1287. doi:10.1111/j.1558-5646.1999.tb04540.x
Hepple, R. T. (2000). Skeletal muscle: microcirculatory adaptation to metabolic demand. *Med. Sci. Sports. Exerc.* **32**, 117-123. doi:10.1097/00005768-200001000-00018
Hepple, R. T., Agey, P. J., Hazelwood, L., Szewczak, J. M., MacMillen, R. E. and Mathieu-Costello, O. (1998). Increased capillarity in leg muscle of finches living at altitude. *J. Appl. Physiol.* **85**, 1871-1876. doi:10.1152/jappl.1998.85.5.1871
Hill, R. W. (1983). Thermal physiology and energetics of *Peromyscus*; ontogeny, body temperature, metabolism, insulation and microclimatology. *J. Mammal.* **64**, 19-37. doi:10.2307/1380747
Hill, R. W. (1976). The ontogeny of homeothermy in neonatal *Peromyscus leucopus*. *Physiol. Zool.* **49**, 292-306. doi:10.1086/physzool.49.3.30155689
Hissa, R. (1964). The postnatal development of homeothermy in the Norwegian lemming (*Lemmus lemmus*). *Experientia* **20**, 326-327. doi:10.1007/BF02171076
Hissa, R. (1968). Postnatal development of thermoregulation in the Norwegian Lemming and the Golden Hamster. *Ann. Zool. Fenn.* **5**, 345-383.
Hissa, R. and Lagerspetz, K. Y. H. (1964). The postnatal development of homoiothermy in the golden hamster. *Ann. Med. Exp. Biol. Fenn.* **42**, 43-45.
Hoppeler, H. and Kayar, S. R. (1988). Capillarity and oxidative capacity of muscles. *Physiology* **3**, 113-116. doi:10.1152/physiologyonline.1988.3.3.113
Howard, W. E. (1949). Dispersal, amount of inbreeding, and longevity in a local population of prairie deer mice on the George Reserve, southern Michigan. *Contributions from the Laboratory of Vertebrate Biology*, 43. University of Michigan, Ann Arbor.
Kaufman, D. W. and Kaufman, G. A. (1987). Reproduction by *Peromyscus polionotus*: number, size, and survival of offspring. *J. Mammal.* **68**, 275-280. doi:10.2307/1381466
Kayser, B., Hoppeler, H., Claassem, H. and Cerretelli, P. (1991). Muscle structure and performance capacity of Himalayan Sherpas. *J. Appl. Physiol.* **70**, 1938-1942. doi:10.1152/jappl.1991.70.5.1938
Lagerspetz, K. Y. H. (1966). Postnatal development of thermoregulation in laboratory mice. *Helogand Wiss Meer.* **14**, 559-571. doi:10.1007/BF01611645
Lau, D. S., Connaty, A. D., Mahalingam, S., Wall, N., Cheviron, Z. A., Storz, J. F., Scott, G. R. and McClelland, G. B. (2017). Acclimation to hypoxia increases carbohydrate use during exercise in high-altitude deer mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **312**, 400-411. doi:10.1152/ajpregu.00365.2016
León-Velarde, F., Sanchez, J., Bigard, A. X., Lesty, C. and Monge-C, C. (1993). High altitude tissue adaptation in Andean coots: capillarity, fibre area, fibre type and enzymatic activities of skeletal muscle. *J. Comp. Physiol. B.* **163**, 52-58. doi:10.1007/BF00309665
Lui, M., Mahalingam, S., Patel, P., Connaty, A., Ivy, C. M., Cheivron, Z. A., Storz, J. F., McClelland, G. B. and Scott, G. R. (2015). High-altitude ancestry and hypoxia acclimation have distance effects on exercise capacity and muscle phenotype in deer mice. *Am. J. Physiol.- Regul. Integr. Comp. Physiol.* **308**, 779-791. doi:10.1152/ajpregu.00362.2014
Madden, C. J. and Morrison, S. F. (2019). Central nervous system circuits that control body temperature. *Neurosci. Lett.* **696**, 225-232. doi:10.1016/j.neulet.2018.11.027
Mahalingam, S., McClelland, G. B. and Scott, G. R. (2017). Evolved changes in the intracellular distribution and physiology of muscle mitochondria in high-altitude native deer mice. *J. Physiol.* **595**, 4785-4801. doi:10.1113/JP274130
Mathieu-Costello, O. (1987). Capillary tortuosity and degree of contraction or extension of skeletal muscles. *Microvasc. Res.* **33**, 98-117. doi:10.1016/0026-2862(87)90010-0
Mathieu-Costello, O., Agey, P. J., Wu, L., Szewczak, J. M. and MacMillen, R. E. (1998). Increased fiber capillarization in flight muscle of finch at altitude. *Respir. Physiol.* **111**, 189-199. doi:10.1016/S0034-5687(97)00119-9
McClelland, G. B. and Scott, G. R. (2019). Evolved mechanisms of aerobic performance and hypoxia resistance in high-altitude natives. *Annu. Rev. Physiol.* **81**, 561-583. doi:10.1146/annurev-physiol-021317-121527
Miller, J. S. (1975). Tactics of energy partitioning in breeding *Peromyscus*. *Can. J. Zool.* **53**, 967-976. doi:10.1139/z75-112
Miller, J. S. (1979). Energetics of lactation in Peromyscus maniculatus. *Can. J. Zool.* **57**, 1015-1019. doi:10.1139/z79-129
Nikel, K. E., Shanishchara, N. K., Ivy, C. M., Dawson, N. J. and Scott, G. R. (2018). Effects of hypoxia at different life stages on locomotory muscle phenotype in deer mice native to high altitudes. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **224**, 98-104. doi:10.1016/j.cbpb.2017.11.009
Oufara, S., Barré, H., Rouanet, J.-L. and Charonnet, J. (1987). Adaptation to extreme ambient temperatures in cold-acclimated gerbils and mice. *Am. J. Physiol.* **253**, R39-R45. doi:10.1152/ajpregu.1987.253.1.R39
Paul, M. J., Tuthill, C., Kauffman, A. S. and Zucker, I. (2010). Pelage insulation, litter size and ambient temperature impact maternal energy intake and offspring development during lactation. *Physiol. Behav.* **100**, 128-134. doi:10.1016/j.physbeh.2010.02.012
Pembrey, M. S. (1895). The effect of variations in external temperature upon output of carbonic acid and the temperature of young animals. *J. Physiol.* **18**, 363-379. doi:10.1113/jphysiol.1895.sp000573
Robertson, C. E., Tattersall, G. J. and McClelland, G. B. (2019). Development of homeothermic endothermy is delayed in high altitude native deer mice *(Peromyscus maniculatus)*. *Proc. R. Soc. B.* **286**, 20190841. doi:10.1098/rspb.2019.0841
Schiaffino, S. and Reggiani, C. (2011). Fiber types in mammalian skeletal muscles. *Physiol. Rev.* **91**, 1447-1531. doi:10.1152/physrev.00031.2010
Scott, G. R., Egginton, S., Richards, J. G. and Milsom, W. K. (2009). Evolution of muscle phenotype for extreme high-altitude flight in the bar-headed goose. *Proc. Roy. Soc. B.* **276**, 3645-3653. doi:10.1098/rspb.2009.0947
Sheafor, B. A. (2003). Metabolic enzyme activities across an altitudinal gradient: an examination of pikas (genus *Ochotona*). *J. Exp. Biol.* **206**, 1241-1249. doi:10.1242/jeb.00226
Sullivan, S. M. and Pittman, R. N. (1987). Relationship between mitochondrial volume density and capillarity in hamster muscles. *Am. J. Physiol. Heart. Circ. Physiol.* **252**, 149-155. doi:10.1152/ajpheart.1987.252.1.H149
Tate, K. B., Ivy, C. M., Velotta, J. P., Storz, J. F., McClelland, G. B., Cheviron, Z. A. and Scott, G. R. (2017). Circulatory mechanisms underlying adaptive increases in thermogenic capacity in high-altitude deer mice. *J. Exp. Biol.* **220**, 3616-3620. doi:10.1242/jeb.164491
Van Wessel, T., de Haan, A., van der Laarse, W. J. and Jaspers, R. T. (2010). The muscle fiber type-fiber size paradox: hypertrophy or oxidative metabolism? *Eur. J. Appl. Physiol.* **110**, 665-694. doi:10.1007/s00421-010-1545-0
Velotta, J. P., Ivy, C. M., Wolf, C. J., Scott, G. R. and Cheviron, Z. A. (2018). Maladaptive phenotypic plasticity in cardiac muscle growth is suppressed in high-altitude deer mice. *Evolution* **72**, 2712-2727. doi:10.1111/evo.13626
White, R. B. Biérinx, A., Gnocchi, V. F. and Zammit, P. S. (2010). Dynamic of muscle fibre growth during postnatal mouse development. *BMC Dev. Biol.* **10**. doi:10.1186/1471-213X-10-21
Wirtz, P., Loermans, H. M. T., Peer, P. G. M. and Reintjes, A. G. M. (1983). Postnatal growth and differentiation of muscle fibres in the mouse I. A histochemical and morphometrical investigation of normal muscle. *J. Anat.* **137**, 109-126.
Withers, P. C. (1977). Measurements of VO2, VCO2, and evaporative water loss with a flow-through mask. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* **42**, 120-123. doi:10.1152/jappl.1977.42.1.120
Wunder, B. A. and Gettinger, R. D. (1996). Effects of body mass and temperature acclimation on the non-shivering thermogenic response of small mammals. In *Adaptations to the Cold: Tenth International Hibernation Symposium* (ed. F. Geiser, A. J. Hulbert and S. C. Nicol), pp. 131-139. Armidale: University of New England Press.