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

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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 (HA) is a particularly challenging environment for small endotherms owing to unremitting low O_2 and ambient temperatures. While superior thermogenic capacities have been demonstrated in adults of some HA species, it is unclear if selection has occurred to survive these unique challenges early in development. We used deer mice (*Peromyscus maniculatus*) native to high and low altitude (LA), and a strictly LA species (*Peromyscus leucopus*), raised under common garden conditions, to determine if postnatal onset of endothermy and maturation of brown adipose tissue (BAT) is affected by altitude ancestry. We found that the onset of endothermy corresponds with the maturation and activation of BAT at an equivalent age in LA natives, with 10-day-old pups able to thermoregulate in response to acute cold in both species. However, the onset of endothermy in HA pups was substantially delayed (by approx. 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 HA.

1. 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 remodelling and accelerated development of BAT.

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Table 1. Characteristics of litters born to first-generation (G_1) laboratory-born high (HA) and low altitude (LA) *P. maniculatus* and *P. leucopus* parents. (Dissimilar letters represent significant differences between populations as determined with one-way ANOVA. Data are mean ± s.e.m.)

	P. maniculatus (LA) $n = 7$	*P. maniculatus* (HA) $n = 6$	*P. leucopus* $n = 8$
litter size (no. 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

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]. By 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 owing 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 (*Peromyscus 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 HA. We, therefore, tested two competing hypotheses: (i) adaptation to HA 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; and (ii) adaptation to HA 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. Methods

(a) 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, Colorado (4350 m above sea level (a.s.l.)) and the LA natives (*P.m. nebracensis* and *P. leucopus*) in eastern Nebraska (430 m a.s.l.; [28]). *Peromyscus 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 (approx. 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, rodent chow and water ad libitum). First-generation (G_1) laboratory-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.

(b) Response to acute cold

(i) Thermography

We examined changes in skin surface temperature of pups in response to an acute cold challenge (24°C for 10 min) 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 s^{-1}) using a calibrated thermal camera (FLIR SC660, FLIR systems Inc.). To estimate intrascapular BAT depot (iBAT) and body temperature skin surface temperatures were quantified using ThermaCam Researcher Pro v.2.9 software (FLIR) by drawing a field of view in the intrascapular region (T_{iBAT}) and between hind legs (T_{Back}, [32]). The change in these surface temperatures with acute cold was 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 T_{iBAT}, we fitted a second-order polynomial regression to T_{iBAT} versus time and analysed 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 $_{iBAT}$ within 1°C of initial values (60 s).

(ii) 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, USA). Individual pups were placed in glass chambers (60 ml for P2–8 and 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^{-1} (P2 and P4), 150 ml min^{-1} (P6 and P8) or 175 ml min^{-1} (P10). Excurrent air was dried and drawn though CO_2 (CA-10A) and O_2 (FC-1A) analysers (Sable Systems). Ambient temperature (T_a) was held at 30°C for 10 min to record an initial resting VO_2, then lowered by 0.3°C min^{-1} to a final temperature of 24°C and held for 10 min to determine final rate of O_2 consumption (VO_2). VO_2 was determined at the most stable 30 s during the last 2 min 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 VO_2 and final VO_2 in pups exposed to acute cold (24°C) minus ΔVO_2 of its normothermic (30°C) sibling:

$$\Delta\Delta VO_2 = (\text{final } VO_2 - \text{initial } VO_2)_{\text{acute cold}} - (\text{final } VO_2 - \text{initial } VO_2)_{\text{normothermic}}. \quad (2.1)$$

A positive value indicates a cold-induced increase in VO_2 relative to the normothermic sibling.

(c) Brown adipose tissue maturation

(i) 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 white adipose tissue (WAT) depot was blunt dissected and weighed as a measure of body composition.

(ii) Western blotting

Protein expression of UCP-1 tyrosine hydroxylase (TH) and citrate synthase (CS), were determined by western blotting [34]. Briefly, 20 µg of total BAT protein was separated on 12% SDS PAGE gels and transferred to PVDF membrane. Membranes were probed with 1° antibody (UCP-1; UCP11-A, Alpha Diagnostics International Inc., San Antonio, TX; TH, AB152, EMD Millipore, Temecula, CA; CS, ab129095, Abcam, Woburn, MA, USA) at 1 : 500 followed by horseradish peroxidase-conjugated, goat anti-rabbit 2° antibody at 1 : 10 000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Band density was normalized to total lane protein determined by membrane staining with Coomassie blue.

(iii) Brown adipose tissue histology

Frozen iBAT was coronally sectioned (10 µm) 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 v.4.30 (Laboratory Imaging, Prague). Fluorescent immunohistochemistry was used to identify adrenergic sympathetic neurons (using TH as a marker) and nuclei (4′,6- diamidino-2-phenylindole, DAPI [36]).

(iv) 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, $\Delta\Delta VO_2$), 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 one sample *t*-test within population to determine if Δskin surface temperature was less than 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).

3. Results

(a) Pup growth

Over the first 10 days of postnatal development, pup body mass was significantly different between the three

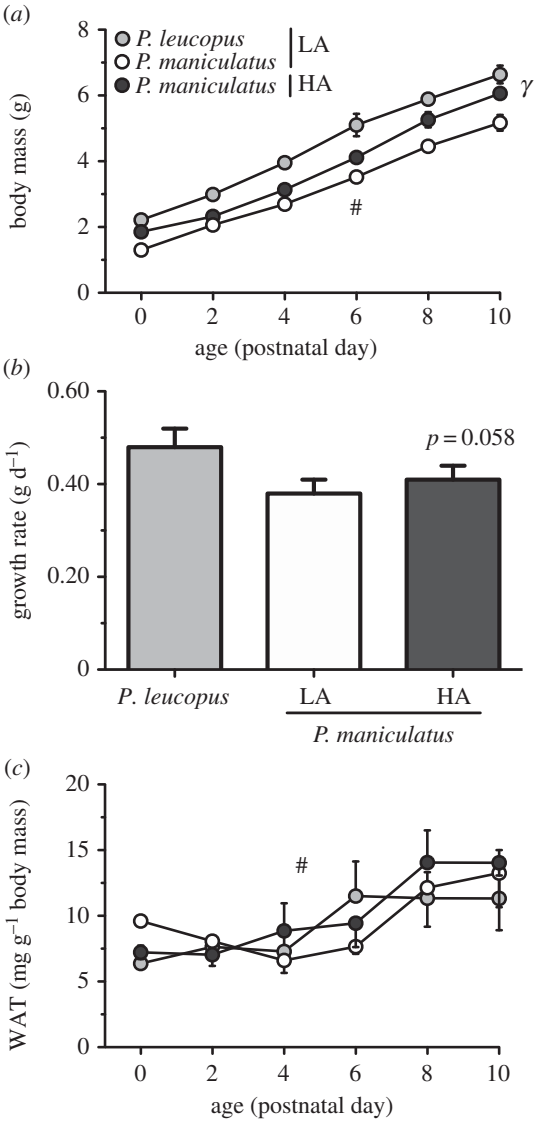

Figure 1. Body mass (*a*), growth rate (*b*), and body composition, as inguinal white adipose tissue (WAT) mass (*c*) during early postnatal development in common garden-raised *Peromyscus maniculatus* of low (LA) and high altitude (HA) ancestry and LA *P. leucopus*. Hash (#), significant main effect of age, gamma (γ) significant main effect of population as determined by two-way RM ANOVA for body mass and two-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.

populations (figure 1*a*; 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 HA 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 1*b*), with *P. leucopus* pups growing slightly faster (0.48 ± 0.04 g d^{-1}) than either of the two *P. maniculatus* populations (LA, 0.38 ± 0.03; HA, 0.41 ± 0.03 g d^{-1}). 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 1*c*). Interestingly, litter size differed significantly with HA mothers having

approximately 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 1).

(b) 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°C and 5.44 ± 0.32°C, respectively. In comparison, HA *P. maniculatus* showed much greater heat loss from T_{Back} (6.87 ± 0.03°C, electronic supplementary material, figures S1, 2*a*) despite having similar starting temperatures (electronic supplementary material, table S1). All mice maintained their surface temperatures more effectively with age, losing significantly less heat with cold exposure (age, 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, T_{Back} $F_{2,69} = 25.203$, $p < 0.001$ and T_{iBAT}, $F_{2,69} = 54.542$, $p < 0.001$; figure 2*a*). *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*a*) 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*, $p = 0.106$; 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. By contrast, at P10 HA pups continued to show persistent heat loss of approximately 2°C for both T_{Back} and T_{iBAT} ($\Delta T_{Back} < 0$°C, $p = 0.018$).

HA pups also showed a developmental latency in thermal endurance compared to LA pups (figure 2*b*). 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 × age, T_{Back}, $F_{10,63} = 7.286$, $p = 0.025$; T_{iBAT}, $F_{10,63} = 2.380$, $p = 0.018$) and was still lower than either LA group (figure 2*b*).

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 × age, $F_{10,62} = 2.891$, $p = 0.005$). LA *P. maniculatus* had a twofold 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 threefold higher than HA pups. HA pups, by contrast showed no increase in cold-induced T_{iBAT} flucuations as they aged (figure 2*e*).

(c) Indirect calorimetry during an acute cold challenge

We measured cold-induced VO_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 VO_2 in response to cold (figure 3*a*). Cold-induced VO_2 increased beyond P6 in all groups, but the timing of this response varied with altitude ancestry (population × age, $F_{8,84} = 3.247$, $p = 0.003$). Both *P. leucopus* and LA *P. maniculatus* showed a significant induction of VO_2 in response to cold at P8 and P10. At P10, cold-induced VO_2 was driven by the combination of an increase in VO_2 in the cold-exposed pups and a decrease in VO_2 in normothermic controls (time × test temperature, *P. leucopus*, $F_{1,6} = 35.693$, $p < 0.0001$; *P. maniculatus*, $F_{1,6} = 13.689$, $p = 0.01$; electronic supplementary material, table S2). By contrast, HA *P. maniculatus* did not show a significant cold-induced VO_2 until P10. In this population, normothermic control pups showed no change in VO_2 (electronic supplementary material, table S2). Cold-induced VO_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 3*b*,*c*).

(d) 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 4*a*). 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 4*b*). Neither iBAT capillary density, area density, nor length density differed between LA (*P. leucopus*) and HA deer mice at any age (figure 4*c*).

To determine if iBAT metabolic phenotype could help explain population differences in thermogenic performance, we assessed protein content of CS (figure 4*d*), and UCP-1 (figure 4*e*). Protein abundance for CS ($p = 0.991$) and UCP-1 ($p = 0.964$) did not differ between the populations. By contrast, at P10 expression of TH, the rate-limiting enzyme in norepinephrine production, was significantly different between the populations (figure 4*f*; $F_{2,17} = 4.634$, $p = 0.025$) with a threefold higher expression in LA pups compared to HA *P. maniculatus* pups. This TH expression is localized to the neurons innervating BAT (electronic supplementary material, figure S2).

4. 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. By 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 threefold 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.

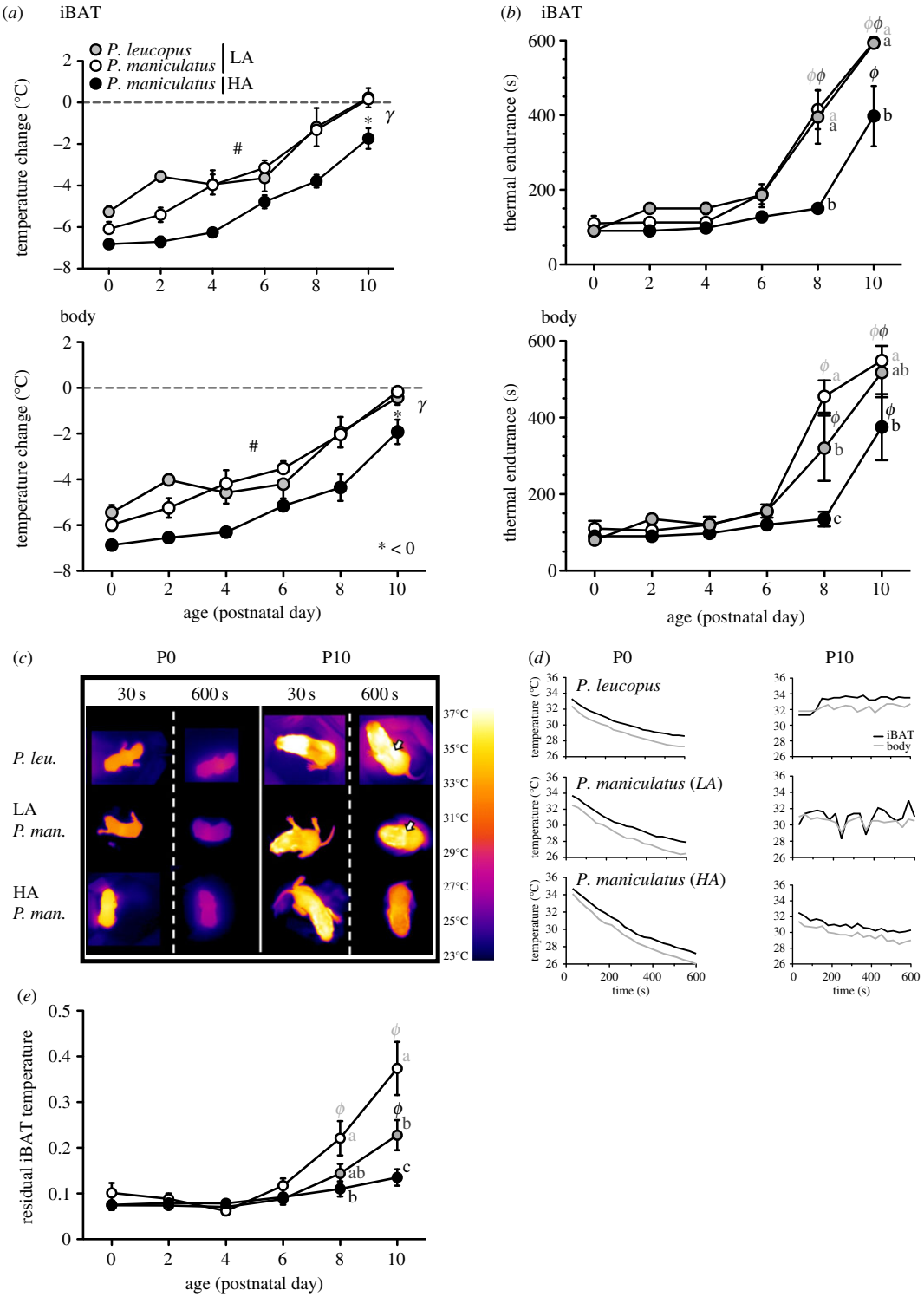

Figure 2. Effect of acute cold exposure (10 min at 24°C) on surface body and intrascapular (iBAT) temperature of common garden-raised *Peromyscus maniculatus* from low (LA) and high altitude (HA) ancestry and LA *P. leucopus* during early postnatal development. (*a*) Temperature change from the start (60 s) to the end (600 s) of exposure. Dashed line represents no heat loss. Asterisk (*), $\Delta T < 0$ (one-sample *t*-test) at P10. (*b*) Thermal endurance, time T_{Back} or T_{iBAT} is maintained within 1°C of start. (*c*,*d*) Representative thermal images and traces. Arrow shows activated iBAT. (*e*) Flucutations of T_{iBAT}. Hash (#), significant main effect of age, gamma (γ), significant main effect of population (two-way ANOVA). When a significant age × population interaction exists, phi (ϕ) denotes a time point is significantly different from postnatal day 2 (P2) within a population. Populations within 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. (Online version in colour.)

(a) Timing of the onset of homeothermic endothermy

We found that the onset of thermogenesis in LA mice occurs between P8 and P10, consistent with previous reports for *P. leucopus* [10]. This species increases metabolic rate with mild, acute cold stress (24°C) at P8 and maintains constant body temperature by P10 [10], in line with our results on both this species and LA deer mice. By 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

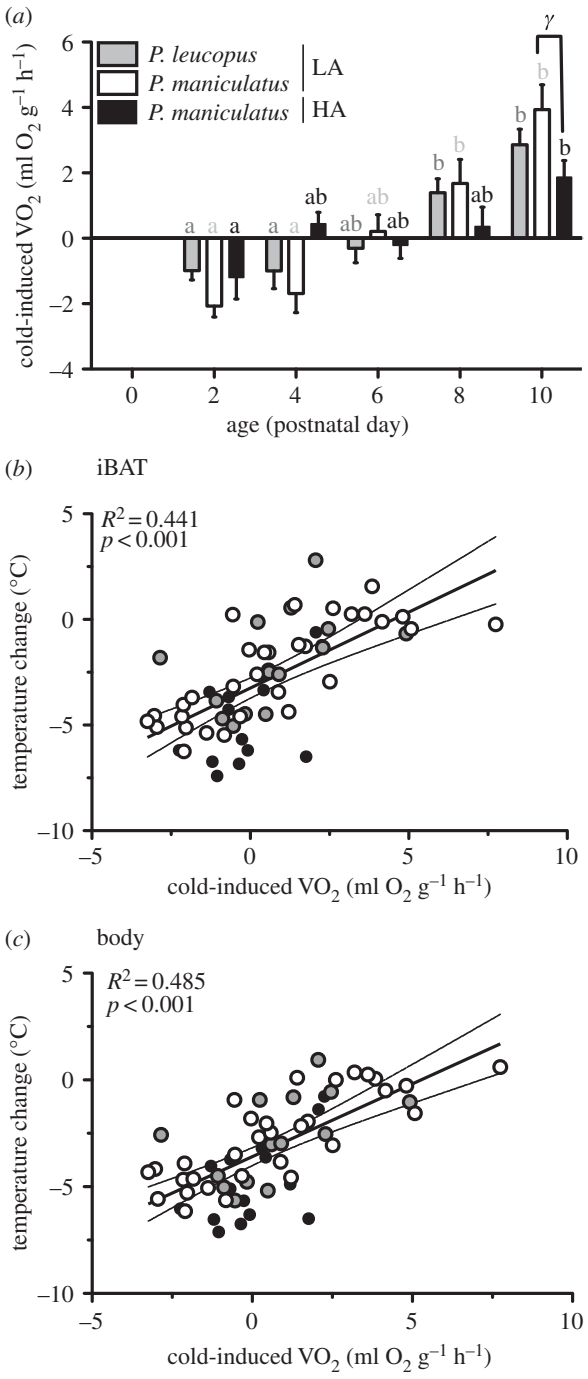

Figure 3. Metabolic response to acute cold (10 min at 24°C) in common garden-raised *Peromyscus maniculatus* from low (LA) and high altitude (HA) ancestry and LA *P. leucopus* during early postnatal development. (*a*) Cold-induced change in oxygen consumption (VO_2) during an acute cold trial relative to a warm control pup litter-mate. Dissimilar letters denote significant difference between age, within population; gamma (γ) denotes significant population difference, within age (Holm's Sidak analysis). Data are mean ± sem. (*b,c*) Linear regression (95% confidence interval) of cold-induced VO_2 and change in body or intrascapular 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).

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 approximately 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 approximately 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.

(b) Brown adipose tissue 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 ($R^2 = 0.96$, electronic supplementary material, figure S3), suggesting that the ability of LA pups to maintain body temperature at P10 is owing 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. UCP-1). Whether this difference in TH

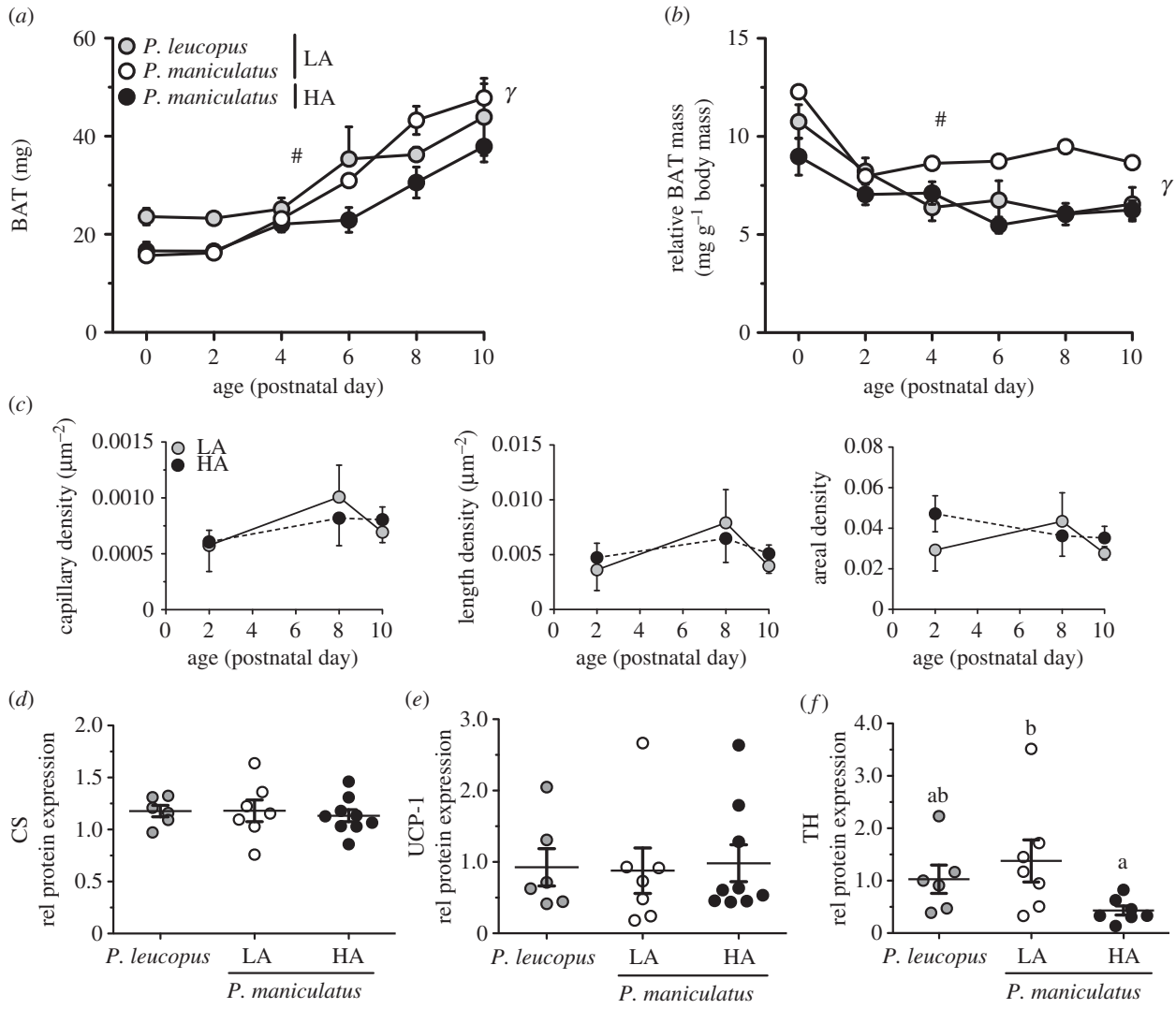

Figure 4. Brown adipose tissue (BAT) maturation during early postnatal (P) development in common garden-raised *Peromyscus maniculatus* from low (LA) and high altitude (HA) ancestry and LA *P. leucopus* (PL). (*a*,*b*) Absolute and mass-specific growth of intrascapular BAT deposit (iBAT). (*c*) Capillarity of iBAT. (*d*–*f*) Protein expression of citrate synthase (CS), uncoupling protein-1 (UCP-1) and tyrosine hydroxylase (TH) in iBAT of P10 pups. Hash (#), significant main effect of age, gamma (γ), significant main effect of population as determined by two-way ANOVA. Bars with dissimilar letters are significantly different as determine by one-way ANOVA. All data are mean ± s.e.m.

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 programme 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 programme of the thermo-effector organs in HA species is an area rife for exploration but outside the scope of this study.

(c) 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 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 owing 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 probably critical for re-warming from bouts of 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.

(d) Conclusion

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 HA favours early onset of 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 HA [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.

Ethics. All procedures were approved by the McMaster University Animal Research Ethics Board in accordance with the Canadian Council for Animal Care guidelines (McMaster Animal Use Protocol no. 16-05-19).

Data accessibility. Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.mt97dv8 [60].

Authors' contributions. C.E.R. and G.B.M. designed the experiment. C.E.R. ran experiments. G.J.T. aided in thermography methodology and data analysis. C.E.R., G.J.T. and G.B.M. edited the manuscript.

Competing interests. We declare we have no competing interests.

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References

1. Crompton AW, Taylor CR, Jagger JA. 1978 Evolution of homeothermy in mammals. *Nature* **272**, 333–336. (doi:10.1038/272333a0)
2. Pembrey MS. 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)
3. Buchanan A, Hill RS. 1947 Temperature regulation in albino rats correlated with determinations of myelin density in the hypothalamus. *Exp. Biol. Med.* **66**, 602–608. (doi:0.3181/00379727-66-16171)
4. Buchanan A, Hill RS. 1949 Myelination of the hypothalamus and its regulation to thermoregulation in the hamster. *Exp. Biol. Med.* **71**, 126–129. (doi:10.3181/00379727-71-17105/)
5. Morrison PR, Ryser FA, Strecker RL. 1954 Growth and the development of temperature regulation in the tundra redback vole. *J. Mammal.* **35**, 376–386. (doi: 10.2307/1378926)
6. Chew RM, Spencer E. 1967 Development of metabolic response to cold in young mice of four species. *Comp. Biochem. Physiol.* **22**, 873–888. (doi:10.1016/0010-406X(67)90778-5)
7. Lagerspetz KYH. 1962 The postnatal development of homoiothermy and cold resistance in mice. *Experientia.* **18**, 282–284. (doi:10.1007/BF02148237)
8. Lagerspetz KYH. 1966 Postnatal development of thermoregulation in laboratory mice. *Helgoländer Wiss. Meeresunters* **14**, 559–571. (doi:10.1007/BF01611645)
9. McManus J. 1971 Early postnatal growth and the development of temperature regulation in the Mongolian gerbil *Meriones unguiculatus*. *J. Mammal.* **52**, 782–792. (doi:10.2307/1378926)
10. Hill RW. 1976 The ontogeny of homeothermy in neonatal *Peromyscus leucopus*. *Physiol. Zool.* **49**, 292–306. (doi:10.1086/physzool.49.3.30155689)
11. Alexander G. 1975 Body temperature control in mammalian young. *Br. Med. Bull.* **31**, 62–68. (doi:10.1093/oxfordjournals.bmb.a071243)
12. Cannon B, Nedergaard J. 2004 Brown adipose tissue: function and physiological significance. *Physiol. Rev.* **84**, 277–359. (doi:10.1152/physrev.00015.2003)
13. Zeltser L. 2015 Developmental influences on circuits programming susceptibility to obesity. *Front. Neuroendocrinol.* **39**, 17–27. (doi:10.1016/j.yfrne.2015.07.002)
14. Bendell JF. 1959 Food as a control of population of white-footed mice, *Peromyscus leucopus noveboracensis* (Fischer). *Can. J. Zool.* **37**, 173–209. (doi:10.1139/z59-021)
15. Howard WE. 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, no. 43*. Ann Arbor, MI: University of Michigan.
16. Hill RW. 1983 Thermal physiology and energetics of *Peromyscus*; ontogeny, body temperature, metabolism, insulation and microclimatology. *J. Mammal.* **64**, 19–37. (doi:10.2307/1380747)
17. Hahn P. 1956 Effect of environmental temperatures on the development of thermoregulatory mechanisms in infant rats. *Nature* **178**, 96–97. (doi:10.1038/178096a0)
18. Cooper K, Ferguson A, Veale W. 1980 Modification of thermoregulatory responses in rabbits reared at elevated environmental temperatures. *J. Physiol.* **303**, 165–172. (doi:10.1113/jphysiol.1980.sp013278)
19. Skála J, Hahn P. 1974 Changes in interscapular brown adipose tissue of the rat during perinatal and

early postnatal development and after cold acclimation VI. Effect of hormones and ambient temperature. *Int. J. Biochem.* **5**, 95–106. (doi:10.1016/0020-711X(74)90050-0)

20. Young JB, Shimano Y. 1998 Effect of rearing temperature on body weight and abdominal fat in male and female rats. *Am. J. Physiol. Regul, Integr. Comp. Physiol.* **274**, 398–405. (doi:10.1152/ajpregu.1998.274.2.R398)
21. Denjean F, Lachuer J, Géloën A, Cohen-Adad F, Moulin C, Barré H, Duchamp C. 1999 Differential regulation of uncoupling protein-1,-2 and -3 gene expression by sympathetic innervation in brown adipose tissue of thermoneutral or cold-exposed rats. *FEBS Lett.* **444**, 181–185. (doi:10.1016/S0014-5793(99)00056-3)
22. Morrison S, Ramamurthy S, Young J. 2000 Reduced rearing temperature augments responses in sympathetic outflow to brown adipose tissue. *J. Neurosci.* **20**, 9264–9271. (doi:10.1523/JNEUROSCI.20-24-09264.2000)
23. Mortola J, Naso L. 1998 Thermogenesis in newborn rats after prenatal or postnatal hypoxia. *J. Appl. Physiol.* **85**, 84–90. (doi:10.1152/jappl.1998.85.1.84)
24. Hayes J. 1989 Altitudinal and seasonal effects on aerobic metabolism of deer mice. *J. Comp. Physiol. B* **159**, 453–459. (doi:10.1007/BF00692417)
25. Beaudry J, McClelland GB. 2010 Thermogenesis in CD-1 mice after combined chronic hypoxia and cold acclimation. *Comp. Biochem. Physiol. B* **157**, 301–309. (doi:10.1016/J.CBPB.2010.07.004)
26. Cadena V, Tattersall GJ. 2014 Body temperature regulation during acclimation to cold and hypoxia in rats. *J. Therm. Biol.* **46**, 56–64. (doi:10.1016/J.JTHERBIO.2014.10.007)
27. McClelland GB, Lyons SA, Robertson CE. 2017 Fuel use in mammals: conserved patterns and evolved strategies for aerobic locomotion and thermogenesis. *Integr. Comp. Biol.* **57**, 231–239. (doi:10.1093/icb/icx075)
28. Cheviron ZA, Bachman G, Connaty A, McClelland GB, Storz JF. 2012 Regulatory changes contribute to the adaptive enhancement of thermogenic capacity in high-altitude deer mice. *Proc. Natl Acad. Sci. USA* **109**, 8635–8640. (doi:10.1073/pnas.1120523109)
29. Cheviron ZA, Bachman G, Storz JF. 2013 Contributions of phenotypic plasticity to differences in thermogenic performance between highland and lowland deer mice. *J. Exp. Biol.* **216**, 1160–1166. (doi:10.1242/jeb.075598)
30. Hayes J, O'Conner C. 1999 Natural selection on thermogenic capacity of high-altitude deer mice. *Evolution* **53**, 1280–1287. (doi:10.2307/2640830)
31. Velotta JP, Ivy CM, Wolf CJ, Scott GR, Cheviron ZA. 2018 Maladaptive phenotypic plasticity in cardiac muscle growth is suppressed in high-altitude deer mice. *Evolution* **72**, 2712–2727. (doi:10.1111/evo.13626)
32. Hodges M, Tattersall G, Harris M, McEvoy S, Richerson D, Deneris E, Johnson R, Chen Z, Richerson G. 2008 Defects in breathing and thermoregulation in mice with near-complete absence of central serotonin neurons. *J. Neurosci.* **28**, 2495–2505. (doi:10.1523/JNEUROSCI.4729-07.2008.)
33. Nakumara K, Morrison S. 2008 A thermosensory pathway that controls body temperature. *Nat. Neurosci.* **11**, 62–71. (doi:10.1038/nn2027)
34. Lui M, Mahalingam S, Patel P, Connaty A, Ivy CM, Cheivron ZA, Storz JF, McClelland GB, Scott GR. 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)
35. 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)
36. Ivy CM, Scott GR. 2017 Control of breathing and ventilatory acclimatization to hypoxia in deer mice native to high altitudes. *Acta. Physiol.* **221**, 266–282. (doi:10.1111/apha.12912)
37. Spicer J, Rundle S. 2006 Plasticity in the timing of physiological development: physiological heterokairy-what is it, how frequent is it, and does it matter? *Comp. Biochem. Physiol. A* **148**, 712–719. (doi:10.1016/j.cbpa.2007.05.027)
38. Pearson OP. 1948 Metabolism of small mammals, with remarks on the lower limit of mammalian size. *Science* **108**, 44. (doi:10.1126/science.108.2793.44)
39. Geiser F, Kenagy G. 1990 Development of thermoregulation and torpor in the golden-mantled ground squirrel *Spermophilus saturates*. *J. Mammal.* **7**, 286–290. (doi:10.2307/1381938)
40. Knight F. 1987 The development of pelage insulation and its relationship to homeothermic ability in an altricial rodent, *Peromyscus leucopus*. *Physiol. Zool.* **60**, 181–190. (doi:10.1086/physzool.60.2.30158642)
41. Oron U, Maltz L, Shefer G, Eilam D. 1998 Histology and enzymatic activity in the postnatal development of limb muscles in rodents. *Physiol. Behav.* **63**, 651–657. (doi:10.1016/S0031-9384(97)00489-7)
42. Baccei M, Bardoni R, Fitzgerald M. 2003 Development of nociceptive synaptic inputs to the neonatal rat dorsal horn: glutamate release by capsaicin and menthol. *J. Physiol.* **549**, 231–242. (doi:10.1113/jphysiol.2003.040451)
43. Takashima Y, Ma L, Mckemy D. 2010 The development of peripheral cold neural circuits based on TRPM8 expression. *J. Neruosci.* **169**, 828–842. (doi:10.1016/j.neuroscience.2010.05.039)
44. Bartness T, Vaughan C, Song C. 2010 Sympathetic and sensory innervation of brown adipose tissue. *Int. J. Obes.* **34**, 36–42. (doi:10.1038/ijo.2010.182)
45. West-Eberhard M. 2005 Developmental plasticity and the origin of species differences. *Proc. Natl Acad. Sci. USA* **102**, 6543–6549. (doi:10.1073/pnas.0501844102)
46. Case T. 1978 On the evolution and adaptive significance of postnatal growth rate in the terrestrial vertebrates. *Q. Rev. Biol.* **53**, 243–282. (doi:10.1086/410622)
47. Farmer C. 2000 Parental care: the key to understanding endothermy and other convergent features in birds and mammals. *Am. Nat.* **155**, 326–334. (doi:10.1086/303323)
48. Farmer C. 2003 Reproduction: the adaptive significance of endothermy. *Am. Nat.* **162**, 826–840. (doi:10.1086/380922)
49. Tattersall G, Leite C, Sanders C, Cadena V, Andrade D, Abe A, Milsom W. 2016 Seasonal reproductive endothermy in tegu lizards. *Sci. Adv.* **2**, e1500951. (doi:10.1126/sciadv.1500951)
50. Farahani R, Kanaan A, Gavrialov O, Brunnert S, Douglas RM, Morcillo P, Haddad GG. 2008 Differential effects of chronic intermittent and chronic constant hypoxia on postnatal growth and development. *Pediatr. Pulm.* **43**, 20–28. (doi:10.1002/ppul.20729)
51. Mortola JP. 1999 How newborn mammals cope with hypoxia. *Resp. Physiol.* **116**, 95–103. (doi:10.1016/S0034-5687(99)00038-9)
52. Mortola JP. 2004 Implications of hypoxic hypometabolism during mammalian ontogenesis. *Resp. Physiol. Neurobiol.* **141**, 345–356. (doi:10.1016/J.RESP.2004.01.011)
53. Halfpenny JC. 1980 Reproductive strategies: intra- and interspecific comparisons within the genus *Peromyscus*. PhD thesis, University of Colorado, Boulder, CO, USA.
54. Bateman N. 1954 The measurements of milk production of mice through preweaning growth of sucking young. *Physiol. Biochem. Zool.* **27**, 163–173. (doi:10.1086/physzool.27.2.30154524)
55. Kaufman DW, Kaufman GA. 1987 Reproduction by *Peromyscus polionotus*: number, size, and survival of offspring. *J. Mammal.* **68**, 275–280. (doi:10.2307/1381466)
56. Gibson AB, Garratt M, Brooks RC. 2015 Experimental evidence that litter size imposes an oxidative challenge to offspring. *J. Exp. Biol.* **218**, 3911–3918. (doi:10.1242/jeb.116327)
57. Hill RW. 1972 The amount of maternal care in *Peromyscus leucopus* and its thermal significance for the young. *J. Mammal.* **53**, 774–790. (doi:10.2307/1379213)
58. Gilbert C, McCafferty D, Giroud S, Ancel A, Blanc S. 2012 Private heat for public warmth: how huddling shapes individual thermogenic responses of rabbit pups. *PLoS ONE* **7**, e33553. (doi:10.1371/journal.pone.0033553)
59. King J, Maas D, Weisman R. 1964 Geographic variation in nest size among species of *Peromyscus*. *Evolution* **18**, 230–234. (doi:10.1111/j.1558-5646.1964.tb01594.x)
60. Robertson CE, Tattersall GJ, McClelland GB. 2019 Data from: Development of homeothermic endothermy is delayed in high-altitude native deer mice (*Peromyscus maniculatus*). Dryad Digital Repository. (https://doi.org/10.5061/dryad.mt97dv8)