Journal of Comparative Physiology B
https://doi.org/10.1007/s00360-021-01423-4

ORIGINAL PAPER

Phenotypic plasticity to chronic cold exposure in two species of *Peromyscus* from different environments

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Received: 15 July 2021 / Revised: 17 November 2021 / Accepted: 24 November 2021

Abstract

Effective thermoregulation is important for mammals, particularly those that remain winter-active. Adjustments in thermoregulatory capacity in response to chronic cold can improve capacities for metabolic heat production (cold-induced maximal oxygen consumption, $\dot{V}O_2max$), minimize rates of heat loss (thermal conductance), or both. This can be challenging for animals living in chronically colder habitats where necessary resources (i.e., food, O_2) for metabolic heat production are limited. Here we used lowland native white-footed mice (*Peromyscus leucopus*) and highland deer mice (*P. maniculatus*) native to 4300 m, to test the hypothesis that small winter-active mammals have evolved distinct cold acclimation responses to tailor their thermal physiology based on the energetic demands of their environment. We found that both species increased their $\dot{V}O_2max$ after cold acclimation, associated with increases in brown adipose tissue mass and expression of uncoupling protein 1. They also broadened their thermoneutral zone to include lower ambient temperatures. This was accompanied by an increase in basal metabolic rate but only in white-footed mice, and neither species adjusted thermal conductance. Unique to highland deer mice was a mild hypothermia as ambient temperatures decreased, which reduced the gradient for heat loss, possibly to save energy in the chronically cold high alpine. These results highlight that thermal acclimation involves coordinated plasticity of numerous traits and suggest that small, winter-active mammals may adjust different aspects of their physiology in response to changing temperatures to best suit their energetic and thermoregulatory needs.

Keywords Brown adipose tissue · Thermogenesis · Thermoneutral zone · Mice · Uncoupling protein · Metabolism

Introduction

Effective thermogenesis is an important trait in endotherms as it enables them to defend an elevated body temperature (T_b) when faced with fluctuating or extreme ambient temperatures (T_a) (Tattersall et al. 2012). The thermoneutral zone is a range of T_a where endotherms can maintain normal T_b without adjustments in energy expenditure. However, when T_a falls below the thermoneutral zone, thermoregulation relies on an animal's ability to induce metabolic heat production to counteract heat loss to the colder surroundings (Tattersall et al. 2012). This can be very energetically expensive for small mammals living in cold climates due to higher surface area-to-volume ratios and a more limited ability to increase insulation that accelerate rates of heat loss compared to larger species (Tattersall et al. 2012; Scholander et al. 1950). The ability to effectively defend T_b also becomes more challenging when the necessary resources (i.e., food or O_2) for metabolic heat production are limiting and when T_a is low, as during winter at higher latitudes (Wickler 1980). In species that remain winter-active, seasonal adjustments in physiology allow these mammals to survive by either improving capacities for elevated rates of heat production, by minimizing rates of heat loss, or by adjustments to both (Fristoe et al. 2015).

Maximal thermogenic oxygen consumption (cold-induced $\dot{V}O_2max$) is an important aspect of an endotherm's thermal biology as it sets the upper limit to aerobic heat production. This thermogenic capacity is supported primarily by high rates of lipid oxidation (Lyons et al. 2021). Despite the metabolic cost associated with elevated aerobic capacity, many small winter-active endotherms increase $\dot{V}O_2max$ on a seasonal basis (e.g., Wickler 1980), which permits them

Communicated by G. Heldmaier.

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Published online: 06 January 2022

to remain active in the cold (Sears et al. 2006). In more controlled lab settings, it is well documented that cold acclimation also increases $\dot{V}O_2max$ in captive populations of *Peromyscus* mice (Hayes and Chappell, 1986; Rezende et al. 2004, 2009; Van Sant and Hammond, 2008; Tate et al. 2017, 2020) and in other small endotherms (e.g., Heldmaier et al. 1982). A shortened photoperiod also elicits increases in $\dot{V}O_2max$ (e.g., Heldmaier et al. 1981), demonstrating that this trait is highly plastic in response to changing environmental conditions.

However, $\dot{V}O_2max$ is not the only trait that affects an animal's thermal performance, and other physiological traits can also vary in response to changing environmental conditions. For example, both basal metabolic rate (BMR) and thermal conductance (C_{min}) can be altered in response to chronic cold exposure (Fristoe et al. 2015) to improve heat production and retention, respectively. According to the classic Scholander–Irving model of heat transfer (Scholander et al. 1950), within an environment the quotient of BMR and C_{min} affects the critical temperature difference between T_b and T_a (ΔT_m) that an endotherm can maintain at the lower end of their thermoneutral zone (Naya et al. 2013). These properties are argued to be part of a coordinated system for heat transfer between an endotherm and its surrounding environment and provides a model to predict mass-independent thermal adaptation (Naya et al. 2013; Fristoe et al. 2015). For example, a higher BMR to C_{min} ratio would reduce the ambient temperature at the lower end of the thermoneutral zone beyond which an animal must increase aerobic heat production to maintain T_b (i.e., the lower critical temperature, T_{lc}; Fristoe et al. 2015). How BMR, C_{min} and T_{lc} change in response to cold acclimation in mammals from different thermal environments is unclear but can lend insight into variation in thermal strategies. Few studies have taken a holistic view of thermal performance and variation in cold acclimation responses. Instead, many studies have focused primarily on changes in maximal thermogenic capacity (e.g., Hayes and Chappell, 1986).

The effect of cold acclimation on BMR cannot be easily predicted in endotherms. For example, results from studies using lab-born and -reared deer mice (DM, *Peromyscus maniculatus*) have been equivocal, showing either an increase (Mason 1974) or no change in BMR with cold acclimation (Van Sant and Hammond 2008; Russell and Chappell 2007). BMR is also thought to be directly associated with cold-induced $\dot{V}O_2max$, and mice artificially selected for high $\dot{V}O_2max$ showed a correspondingly higher BMR (Wone et al. 2011). This relationship between BMR and $\dot{V}O_2max$ has also been documented across seasons in wild-caught DM (Hayes 1989). The link between BMR and $\dot{V}O_2max$ is likely driven by the high maintenance costs of tissues that support high metabolic rates, such as brown adipose tissue (BAT) (Rezende et al. 2009; Wone et al. 2011). Indeed, cold acclimation and seasonal acclimatization in rodents have been shown to increase BAT mass, the expression of uncoupling protein 1 (UCP-1, the protein regulating induced heat production), and mitochondrial density (Jacobsson et al. 1994; Milner and Trayhurn 1989; Klaus et al. 1988). Whether these changes lead to an increase in BMR may depend on relative changes in BAT mass or UCP-1 content. The former would increase basal costs of maintaining a bigger tissue, while the latter may result in no change in BMR if not accompanied by tissue hypertrophy. Therefore, whether a link between $\dot{V}O_2max$ and BMR exists in an individual depends on the mechanistic basis for that animal's cold acclimation response. The relationship between BMR and $\dot{V}O_2max$ is an important aspect of an organism's physiology as their quotient represents the fractional aerobic scope.

Endotherms might also lower C_{min} in response to cold acclimation, primarily through increases in insulation. This strategy is limited for small species as significant increases in fur thickness may impede locomotion (Gordon 2012). Alternatively, heat retention can be improved by reducing the gradient between T_b and T_a through a lowering of T_b. An assumption of the Scholander–Irving model is that T_b is constant across the thermoneutral zone, but when measured, T_b has been shown to be quite labile in mice, varying both diurnally and with changes in T_a (Beaudry and McClelland 2010; Gordon 2012). Hypothermia might be a useful strategy in environments with limited food or oxygen availability, as it reduces the energy requirements necessary for thermoregulation as T_a declines. Small endotherms native to different thermal environments may have evolved distinct strategies by altering different physiological traits when acclimating to cold conditions.

To understand how phenotypic plasticity of different thermal traits may interact to drive the cold acclimation response, we examined two species of *Peromyscus* mice native to environments with vastly different thermal regimes. We used white-footed mice (WFM, *P. leucopus*) from the Great Plains where summer temperatures are likely often within their thermoneutral zone, but winter temperatures fall far below their T_{lc}. We also examined mice from a highland population of deer mice (DM; *P. maniculatus*) native to the Rocky Mountains, where they experience cooler temperatures year-round, despite living at the same latitude as WFM. The low oxygen availability in the high alpine may impair effective thermogenesis by reducing the overall scope for aerobic activities (Hammond et al. 2002). As a result, there has been directional selection for high aerobic capacity in highland DM, which was positively associated with winter survival (Hayes and O'Connor 1999). Wild-caught highland DM also exhibit significantly higher $\dot{V}O_2max$ in hypoxia than DM from a lowland population native to the same latitude, and the strictly low-altitude WFM (Cheviron

et al. 2012). Previous studies have shown that environmentally induced plasticity of $\dot{V}O_2max$ contributes to the high thermogenic capacity in wild highland DM (Cheviron et al. 2013; Coulson et al. 2021; Robertson and McClelland 2021; Tate et al. 2020). However, variation in BMR, C_{min} and ΔT_m can also play important roles in adjusting effective thermoregulation to match environmental conditions. It is unclear if plasticity in some or all these traits underlie the ability of mice to adjust thermoregulation in response to cold acclimation; however, it is possible that some strategies may be more favored than others, depending on the environment.

Thermoregulation represents an extreme, high energetic cost for endotherms, and winter-active mammals must balance the energetic demands of thermoregulation with energy supply. We hypothesized that small winter-active mammals have evolved distinct cold-acclimation responses to tailor their thermal physiology based on the energetic demands of their environment. We predicted that in both highland DM and WFM, cold acclimation increases cold-induced $\dot{V}O_2max$, in part due to changes in BAT phenotype. However, any changes in BMR, C_{min}, T_{lc}, and ΔT_m will reflect differences in their ancestral thermal history. Specifically, we hypothesized that highland DM, native to a chronically cold and hypoxic ecosystem, have prioritized reducing energy expenditure and maximizing aerobic scope, relying on increasing heat retention. In contrast, we hypothesized that WFM, who need only cope with cold temperatures seasonally, will maximize heat production of BAT (increasing BAT size), leading to increases in BMR. To test these predictions, we used second-generation lab-born and raised highland DM and WFM and determined changes in BMR, C_{min}, ΔT_m, T_{lc} and cold-induced $\dot{V}O_2max$ with cold acclimation. Due to the inherent limitations of two species comparisons (Garland and Adolph 1994), we examined responses to cold acclimation in DM and WFM separately.

Materials and methods

Animals and acclimation design

We used second-generation laboratory-born and raised individuals kept in common conditions at sea level, from DM (*P. maniculatus rufinus*) derived from wild populations native to high altitude (Clear Creek County, Colorado, 39°35′18′′N, 105°38′38′′W, 4350 m a.s.l.) and WFM (*P. leucopus*) from Eastern Nebraska (Nine-Mile Prairie, Lancaster County, Nebraska, 40°52′12′′N, 96°48′20.3′′W, 430 m a.s.l.) as previously described (Cheviron et al. 2012). Mice from both species were split into two groups of ten individuals, ensuring an equal distribution of sexes and families, and acclimated for at least six weeks in one of two treatment groups, controls (25 °C) or cold (5 °C). All procedures were approved by the McMaster University Animal Research Ethics Board in accordance with guidelines from the Canadian Council on Animal Care.

Four to five days before the start of acclimations, each mouse was briefly anaesthetized using 2% isoflurane to have a thermo-sensitive passive transponder RFID chip (LifeChips with BioThermo Technology, Destron Fearing) implanted subcutaneously on the lateral side of the abdomen where the abdomen meets the hind limb. These chips allowed for non-invasive determination of body temperature and identification while mice moved freely in their home cages and during the metabolic rate measurements (see below). After a minimum of five days after implantation of the transponder chips, the cold acclimation group was moved to a temperature-controlled room. Room temperature was gradually decreased over the first four days, from 25 °C to the final temperature of 5 °C. Once the final temperature was reached, the mice were acclimated for a period of six weeks before testing. Both cold-acclimated and control mice were kept in a constant photoperiod of 12:12-h light–dark cycle, and were given standard rodent chow and water ad libitum. After the six-week acclimation period, experiments were conducted within a three-week period. Animals remained in their acclimation condition until testing was complete.

Indirect calorimetry

Metabolic rates as $\dot{V}O_2$ and carbon dioxide production $\dot{V}CO_2$ were determined over a range of T_a using a flow-through respirometry system (Sable Systems, NV), as previously described for mice (Beaudry and McClelland 2010; Coulson et al. 2021; Robertson and McClelland 2019). After a 4-h fast, mice were placed in a metabolic chamber (volume ~ 250 ml) housed in an insulated Peltier cabinet (Sable Systems). Outside air was scrubbed of H_2O and CO_2 using Drierite (W.A. Hammond Company, Xenia, OH), Soda Lime and Ascarite (Fisher Scientific, Pittsburg, PA) and flowed into the chamber at a rate of 500 ml min^{-1} using a mass flow controller. A subsample of the excurrent air was dried with pre-baked Drierite (White et al. 2006) and passed through O_2 and CO_2 analyzers at a rate of approximately 150 ml min^{-1} using a subsampling pump. Mice were allowed to become accustomed to the metabolic chamber for 20 min at 30 °C, after which time, temperature was decreased by 2 °C every 25 min to a final temperature of 20 °C. This temperature range is consistent with previous studies that found a T_{lc} of 25.5–27.5 °C in wild-caught DM and WFM (Tomasi 1985; Deavers and Hudson 1981). Metabolic rates at each temperature were calculated using Eq. 3b in Withers (1977) and by averaging the lowest 2 min of $\dot{V}O_2$ when the mouse was inactive but awake (verified using a webcam). Body temperature was monitored at 5-min intervals by reading the

imbedded RFID chips using a receiver placed underneath the metabolic chamber. Experiments typically occurred between the hours of 12 pm–6 pm.

Minimum thermal conductance (C_{min}) was calculated for each mouse according to the equation from Scholander et al. (1950),

$$C_{\min} = \frac{\dot{V}o_2}{(T_b - T_a)} \quad (1)$$

Cold-induced $\dot{V}O_2$max was determined for each mouse as previously described (Tate et al. 2017; Robertson and McClelland 2019) on a separate day from BMR determinations. For these measurements, mice were not fasted, and outside air was replaced with a mixture of 20% O_2 and 80% Helium (Heliox) to increase thermal conductance so $\dot{V}O_2$max could be induced at a higher temperature than with air to avoid cold injury (Rosenmann and Morrison 1974). Heliox was flowed through the metabolic chamber at 1300 ml min^{-1} and the excurrent air was subsampled for O_2 and CO_2 determinations as described above. Mice were placed in a temperature cabinet at 0 °C and the cabinet was cooled to −5 °C over a period of 10 min, and measurements continued until $\dot{V}O_2$max was observed. Cold-induced $\dot{V}O_2$max was defined as the highest 1 min of $\dot{V}O_2$ during the trial. Rectal temperatures were taken immediately before and after the $\dot{V}O_2$max trial to ensure mice were hypothermic and for accurate comparisons with temperature readings from the implanted chips.

Tissue mass

Mice were euthanized by isoflurane overdose followed by cervical dislocation, as previously described (Lau et al. 2017). Lungs, kidneys, liver, interscapular brown adipose tissue (iBAT), and one of the gastrocnemius muscles were quickly dissected and wet mass determined. The entire gastrointestinal tract was removed and cleaned of its contents before being weighed. The white adipose tissue was also sampled from the inguinal region (ingWAT). Tissues were then dried (one half of the liver and BAT) in an oven at 200 °C and were considered fully desiccated once the mass of the tissue stabilized within ± 0.5 mg following repeated measurements over the span of 24 h.

Western blot

Approximately one half of the iBAT depot was quickly frozen after mice were sacrificed for determination of UCP-1 and citrate synthase (CS) protein content by western blot as previously described (Coulson et al. 2021; Robertson et al. 2019). BAT samples were powdered using a liquid N_2-cooled mortar and pestle, and ~ 20 mg of powder was homogenized in ice-cold RIPA buffer containing (in mM) 150 NaCl, 1.0% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, in 50 Tris–HCl, pH 8.0. Proteins were then denatured at 95 °C for 5 min in Laemmli sample buffer containing 750 mM β-mercaptoethanol (BioRad, Mississauga, ON, Canada). Total protein concentrations were determined using a Bradford Assay (Bio-Rad). Then 20 µg of total protein was loaded into each lane of a pre-cast 12% sodium dodecyl sulfate–polyacrylamide gels (Bio-Rad) and separated for 45 min at 120 V followed by 15 min at 150 V using a Mini-Protein Tetra System (Bio-Rad). Proteins were then transferred from the gel to polyvinylidene difluoride membranes (PVDF) for 7 min at 25 V using a Trans Blot Turbo Transfer System (Bio Rad). Membranes were blocked overnight at 4 °C using 5% skim milk powder in phosphate-buffered saline Tween buffer solution (PBST; 1.5 mM $NaH_2PO_4 \cdot H_2O$, 8.1 mM Na_2HPO_4, 145.5 mM NaCl, 0.05% Tween 20 at pH 7.4). Membranes were then incubated for 1 h at room temperature with primary antibody (UCP1, UCP11-A, Alpha Diagnostics International inc., San Antonio, TX, USA; CS, ab129095, Abcam, Woburn, MA, USA) at a dilution of 1:500 using antibody-diluting agent (1% bovine serum albumen (BSA) suspended in PBST). Membranes were then rinsed 3 times with PBST for 10 min each, and then incubated at room temperature for 1 h with donkey anti-rabbit horseradish peroxidase conjugated, secondary antibody at a dilution of 1:10,000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibody binding was quantified using enhanced chemiluminescence substrates (ECL Clarity solution; Bio Rad) and band intensity was detected using a ChemiDoc MP Imaging System (Bio Rad). To each gel, a common protein sample from 1 group (cold-acclimated DM) was included to account for any variation in membrane protein transfer efficiency. Expression levels for all samples were determined relative to this common sample. In addition, each sample was normalized to the amount of loaded protein determined by quantifying all bands revealed by Coomassie blue staining (Sanchez et al. 1992). Band quantification was performed using Image Lab Software (Bio Rad).

Statistics

The T_{lc} was determined for each individual mouse using a continuous two-phase regression model to determine the inflection point where $\dot{V}O_2$ begins to increase with a decrease in T_a, as described previously (Nickerson et al. 1989; Campbell and Hochachka 2000). The effect of cold acclimation on metabolic variables, tissue masses and relative UCP-1 expression was analyzed within each species using *t* tests (GraphPad Prism version 9.1.0 for Mac, and in R (https://www.r-project.org/). Statistical outliers were detected using the Grubb's test and removed. These data points included 1 liver, 1 iBAT, 1 WAT wet mass, and 1

BAT and 1 intestine dry mass in control WFM. 1 liver wet weight and 1 liver dry weight value were removed for cold-acclimated WFM. Changes in T_b as T_a declined were determined using a mixed effects analysis, with T_a and acclimation as main effects and individual mouse as a random effect. Regression and correlation analysis across all individual mice were used to compare residual variation in BMR and $\dot{V}O_2$max to tissue mass, independent from body mass as determined by allometric regression (GraphPad Prism version 9.1.0 for Mac, and SPSS, IBM).

Results

Basal metabolic rate (BMR), thermal conductance and lower critical temperature

Body mass did not significantly change for either species with cold acclimation ($t_{12}=1.14$, $p=0.278$ for WFM and $t_{14}=0.673$, $p=0.512$ for DM; Table 1). With cold acclimation, WFM showed a significant 24% increase in BMR ($t_{17}=2.967$, $p=0.008$), but thermal conductance ($t_{13}=0.330$, $p=0.747$) did not change (Fig. 1A and C). In contrast, highland DM show no change in either BMR ($t_{14}=0.845$, $p=0.412$) or conductance ($t_{11}=0.385$, $p=0.707$) in response to cold acclimation (Fig. 1B and D). This resulted in a significant increase in BMR/ C_{min} ratio in WFM with cold acclimation (9.1 ± 0.6 versus 12.3 ± 0.8; $t_{13}=3.38$, $p=0.005$) but not in highland DM (9.9 ± 0.9 versus 10.3 ± 0.4; $t_{11}=0.402$, $p=0.695$).

The lower critical temperature (T_{lc}) of the thermoneutral zone was determined by monitoring $\dot{V}O_2$ with decreasing T_a. We found that with cold acclimation the T_{lc} decreased significantly by ~2 °C in WFM from 27.0 ± 0.3 °C to 25.1 ± 0.7 °C ($t_{16}=2.662$, $p=0.017$; Fig. 2B). Similarly, highland DM showed a decline in T_{lc} with cold acclimation by ~2.5 °C from 27.0 ± 0.4 °C to 24.5 ± 0.6 °C ($t_{11}=-3.359$, $p=0.006$; Fig. 2C). We monitored body temperature during the respirometry trials (Fig. 2A), which allowed us to determine ΔT_m, the difference between T_b and T_a at the T_{lc}. Although with cold acclimation, WFM showed a decrease in T_b by 2.3 °C as T_a was reduced from 30 °C to their T_{lc}, they experience a significant increase in ΔT_m from 9.5 ± 0.4 °C to 11.8 ± 0.9 °C ($t_{13}=2.509$, $p=0.026$; Fig. 2D). Cold-acclimated highland DM also experienced a drop in T_b of ~2.6 °C as T_a declined from 30 °C ($p<0.001$; Fig. 2A). This resulted in a ΔT_m that was similar in cold-acclimated mice compared to controls ($t_7=1.40$, $p=0.206$; Fig. 2E). In fact, by

Table 1 Body mass (g) and wet and dry tissue masses (mg) of various tissues in white-footed mice (*P. leucopus*) and highland deer mice (*P. maniculatus*) after acclimation to control warm or cold conditions

	White-footed mice		Highland deer mice		Residual BMR vs residual tissue mass	
	Control	Cold	Control	Cold	r^2	P
Body mass	30.8 ± 4.1	29.8 ± 2.6	19.3 ± 1.2	18.5 ± 1.2	–	–
Liver						
Wet	466 ± 36	492 ± 36	361 ± 31	415 ± 41	0.018	0.547
Dry	166 ± 22	153 ± 10	110 ± 9	133 ± 15	0.00001	0.962
Muscle						
Wet	148 ± 11	139 ± 8	98 ± 7	71 ± 9*	0.012	0.610
Dry	39 ± 3	36 ± 2	26 ± 2	19 ± 2*	0.009	0.658
ingWAT						
Wet	924 ± 238	509 ± 143	312 ± 78	430 ± 87	0.022	0.487
Dry	429 ± 43	341 ± 111	205 ± 50	300 ± 81	0.024	0.468
Lungs						
Wet	183 ± 7.8	207 ± 8.6	156 ± 6.6	152 ± 11	0.036	0.377
Dry	43 ± 2	45 ± 2	35 ± 1	34 ± 2	0.068	0.217
Intestine						
Wet	2093 ± 173	2590 ± 145*	1604 ± 101	2088 ± 155*	0.096	0.075
Dry	414 ± 21	433 ± 24*	334 ± 23	435 ± 32*	0.151	0.067
Kidney						
Wet	298 ± 15	383 ± 40	234 ± 14	250 ± 17	0.434	0.003
Dry	79 ± 4	98 ± 11	61 ± 2	65 ± 3	0.476	< 0.001

The regression coefficients (r^2) are presented for residual variation in basal metabolic rate (BMR) versus residual variation in tissue mass from allosteric regressions with body mass and across all mice.

Muscle, gastrocnemius; ingWAT, inguinal white adipose tissue. Values are means ± s.e.m.

*Significantly different from controls within a species

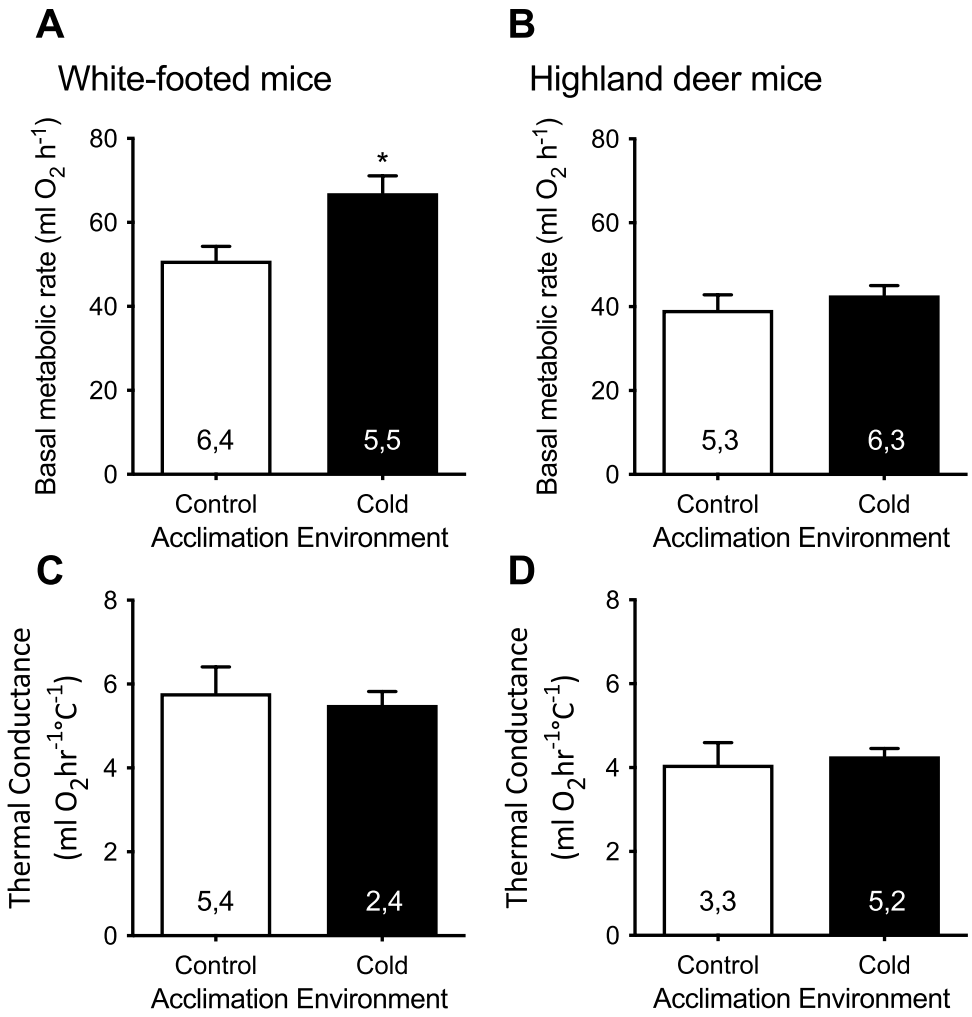

Fig. 1 Basal metabolic rate (BMR) and thermal conductance (C_{min}) was determined in white-footed mice (**A** and **C**) and in a highland native population of deer mice (**B** and **D**) both lab-born and raised to the second generation, either kept in control conditions (23 °C) or with acclimation to cold (5 °C). Groups sizes are reported in parentheses as number of males and females (M,F). *Denotes a significant difference ($p < 0.05$) between control and cold-acclimated groups within a species

dropping their T_b highland DM reduced the T_b–T_a difference at 20 °C from a potential 17 °C (if T_b was maintained at 37 °C) to 13.7 ± 0.5 °C.

Tissue mass

BMR reflects the maintenance costs of tissue metabolism, and changes in tissue mass may influence responses of BMR to chronic cold exposure. We found that cold acclimation led to significant increases in intestinal mass (wet and dry mass) in both WFM and highland DM ($t_{11} = 2.207$, $p = 0.050$ for WFM and $t_8 = 2.620$, $p = 0.030$ for DM; Table 1). All other tissues were unaffected by acclimation (Table 2), except for the gastrocnemius muscle, which decreased in mass in highland DM with cold acclimation compared to controls ($t_9 = -2.279$, $p = 0.050$). Using data from all individual mice from both species and acclimations, we found that there was a significant correlation between residual variation in kidney dry mass and residual variation in BMR relative to body mass ($F_{1,23} = 20.90$, $p = 0.0001$, $r^2 = 0.476$, Pearson $r = 0.69$), likely driven by cold-acclimated WFM. Residual variation in BMR and residual variation in intestinal dry mass show a relationship that approached statistical significance ($F_{1,21} = 2.22$, $P = 0.067$, $r^2 = 0.151$, Pearson $r = 0.309$). Masses of the other tissues measured failed to show a significant relationship with BMR (Table 1).

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

We determined $\dot{V}O_2$max as a measure of maximal thermogenic capacity. We found that $\dot{V}O_2$max was significantly influenced by cold acclimation in both species, with increases of ~ 40–47% ($t_{14} = 4.285$, $p < 0.001$ for WFM and $t_{14} = 6.519$, $p < 0.001$ for DM; Fig. 3A and B). Since we also measured $\dot{V}CO_2$, we calculated the respiratory exchange ratio ($\dot{V}CO_2/\dot{V}O_2$) at $\dot{V}O_2$max. Values close to 0.71 confirm the high reliance on lipids to power heat production (Lyons et al. 2021). In highland DM, cold acclimation resulted in a significant decrease in RER from 0.789 ± 0.007 to 0.739 ± 0.003 ($t_9 = -10.378$, $p < 0.001$), suggesting a greater reliance on lipids as the primary fuel after chronic cold (Fig. 3D). Lowland WFM showed no change in RER with

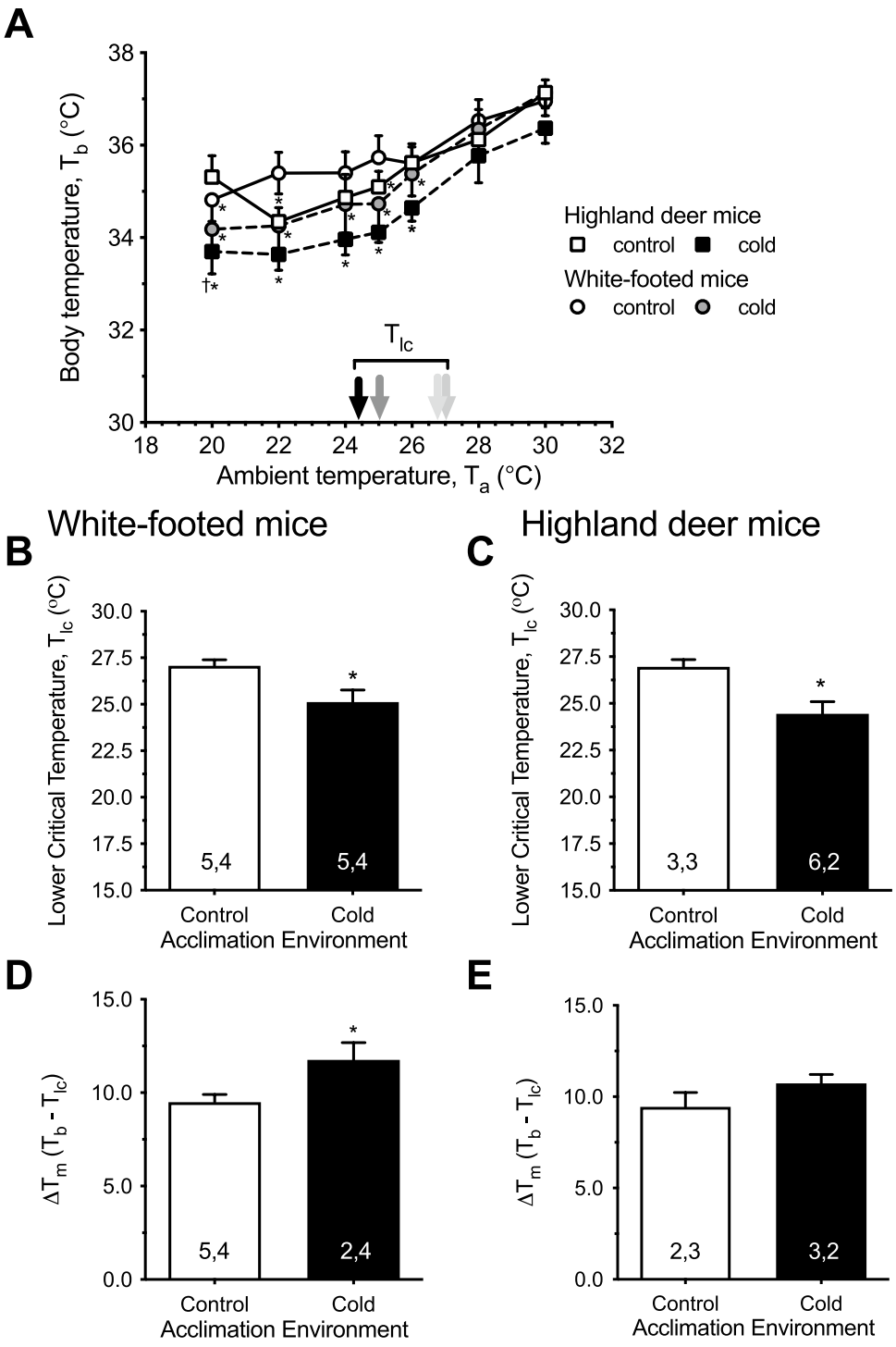

Fig. 2 Body temperature (T_b) was monitored across ambient temperatures (T_a) from 20 °C to 30 °C in white-footed mice and highland deer mice held in control condition or cold conditions (**A**). All groups showed significant reductions in T_b compared to $T_a = 30$ °C as T_a declined (*). Arrows indicate lower critical temperatures (T_{lc}) for each group. †significantly different from control mice within a species. Lower critical temperature was reduced with cold acclimation in both species (**B** and **C**). The critical temperature difference (ΔT_m) between body temperature (T_b) and T_{lc} showed a significant increase with cold acclimation in white-footed mice (**D**) but not deer mice (**E**). Groups sizes are reported in parentheses as number of males and females (M,F). *In **B**–**E** denotes a significant difference ($p < 0.05$) between control and cold-acclimated groups within a species

cold acclimation ($t_{13} = -0.824$, $p = 0.415$). When fuel oxidation rates were determined using indirect calorimetry (Frayn 1983), the relative use of lipids increased from 71 ± 2% to 88 ± 1% of total $\dot{V}O_2$ in highland DM with cold acclimation compared to the controls. $\dot{V}O_2$max and BMR values were compared for each individual mouse as fractional aerobic scope ($\dot{V}O_2$ max /BMR), and cold acclimation resulted in no change in fractional scope in WFM ($t_{17} = 1.522$, $p = 0.147$). This was the result of an increase in both $\dot{V}O_2$max and BMR in response to chronic cold (Fig. 3E). In contrast, highland DM showed an increase in aerobic scope with cold acclimation because BMR did not change but $\dot{V}O_2$max was elevated compared with controls ($t_{11} = 3.344$, $p = 0.006$; Fig. 3F).

Table 2 Statistical summary for wet and dry masses of various tissues in white-footed mice (*P. leucopus*) and highland deer mice (*P. maniculatus*)

		White-footed mice		Highland deer mice	
		t	*P*	*t*	*P*
Liver	Wet	$t_{10}=0.506$	0.624	$t_9=1.058$	0.318
	Dry	$t_8=-0.511$	0.623	$t_9=1.238$	0.247
Muscle	Wet	$t_{11}=-0.651$	0.529	$t_9=-2.364$	0.044
	Dry	$t_{10}=-0.835$	0.423	$t_9=-2.279$	0.050
ingWAT	Wet	$t_8=-1.489$	0.173	$t_9=1.015$	0.337
	Dry	$t_8=-0.743$	0.480	$t_8=1.001$	0.346
Lungs	Wet	$t_{12}=1.997$	0.069	$t_8=-0.290$	0.779
	Dry	$t_{11}=0.483$	0.638	$t_6=-0.314$	0.764
Intestine	Wet	$t_{11}=2.207$	0.050	$t_8=2.620$	0.030
	Dry	$t_{10}=2.785$	0.020	$t_9=2.570$	0.031
Kidney	Wet	$t_8=1.957$	0.088	$t_9=0.715$	0.493
	Dry	$t_8=1.165$	0.139	$t_9=1.137$	0.286

Muscle, gastrocnemius; ingWAT, inguinal white adipose tissue

Values are the result of t tests for each tissue comparing cold-acclimated and control mice within a species

iBAT mass

As increases in overall thermogenic capacity likely involve changes in thermo-effector tissue phenotype, we determined whether the size of iBAT was influenced by cold acclimation. WFM showed growth in iBAT by 34% with cold acclimation, an increase that approached statistical significance ($t_{10}=2.112$, $p=0.066$; Fig. 4A). The iBAT mass across all mice used in this study showed an allometric relationship with body mass as iBAT mass $= 3.136 \times$ body mass$^{0.874}$. Thus, when expressed relative to body mass, WFM showed an increase in relative iBAT mass with cold acclimation from 2.83 ± 0.21 mg g$^{-0.874}$ to 3.49 ± 0.29 mg g$^{-0.874}$, but this increase did not to reach statistical significance ($P=0.094$). Highland DM showed a strong response to cold acclimation with a 37% increase in absolute iBAT mass ($t_8=2.396$, $p=0.043$; Fig. 4B). This was also true for relative iBAT mass, which increased from 2.46 ± 0.25 mg g$^{-0.874}$ to 3.63 ± 0.39 mg g$^{-0.874}$ ($P=0.036$).

UCP-1 and CS expression in iBAT

The capacity for iBAT to produce heat upon activation can be augmented through increases in mitochondrial volume and/ or expression of UCP-1, key to determining rates of heat production by this tissue (Cannon and Nedergaard 2004). We used protein expression of CS as an index of iBAT mitochondrial volume density and found CS expression did not change with cold acclimation ($t_{12}=1.071$, $p=0.305$; Table 3). However, expression of UCP-1 was responsive to acclimation and increased significantly ($t_{12}=4.509$, $p<0.001$; Table 3) leading to 290% greater UCP-1/CS in cold-acclimated WFM compared to controls ($t_8=4.511$, $p=0.002$; Fig. 4C). Similarly, in highland DM, a 210% increase in UCP-1/CS ($t_8=4.158$, $p=0.004$; Fig. 4D) is driven by an increase in UCP-1 expression ($t_8=2.56$, $p=0.034$; Table 3). Moreover, examining data from across all mice showed a positive correlation between UCP1/CS and the residual variation in cold-induced $\dot{V}O_2$max relative to body mass ($F_{1,22}=13.82$, $p=0.001$, $r^2=0.39$).

Discussion

The goal of this study was to examine variation in thermoregulatory mechanisms and phenotypic plasticity to cold acclimation in *Peromyscus* mice from different thermal habitats. Our results support the hypothesis that these species have both common and distinct thermal strategies in response to chronic cold exposure. We found that both WFM and highland native DM broadened their thermoneutral zone with cold acclimation by reducing the lower critical temperature. In WFM, this was also associated with an increase in BMR. In contrast, highland DM showed no change in BMR with cold acclimation. Instead, these mice lowered their T_b to a greater extent as environmental temperatures declined, reducing the T_a to T_b thermal gradient. Both species also demonstrated an increase in cold-induced $\dot{V}O_2$max after cold acclimation, likely the result of increases in iBAT mass and in the expression of UCP-1 protein in this thermo-effector tissue. Differences in acclimation responses in BMR resulted in highland DM having a greater fractional aerobic scope. Our results highlight that thermal acclimation involves coordinated plasticity of numerous traits and suggests that small, winter-active mammals may adjust different aspects of their physiology in response to changing temperatures to best suit their energetic and thermoregulatory needs. In particular, a shallow hypothermia may be an important aspect of thermal acclimation strategy in stressful environments.

BMR, C_{min} and T_{lc}

Changes in BMR and C_{min} can affect thermoregulatory capacity in rodents by modifying heat production and retention, respectively. Indeed, an analysis of 127 temperate rodent species spanning multiple climates found that mean annual temperature was a strong predictor of C_{min} and BMR, with species from colder environments having higher BMR and lower C_{min} than those in warmer climes (Naya et al. 2013). Based on thermal environment alone, this analysis would predict that highland DM should show an increase in BMR and decrease in C_{min} with cold acclimation as a mechanism to increase thermoregulatory capacity to survive the

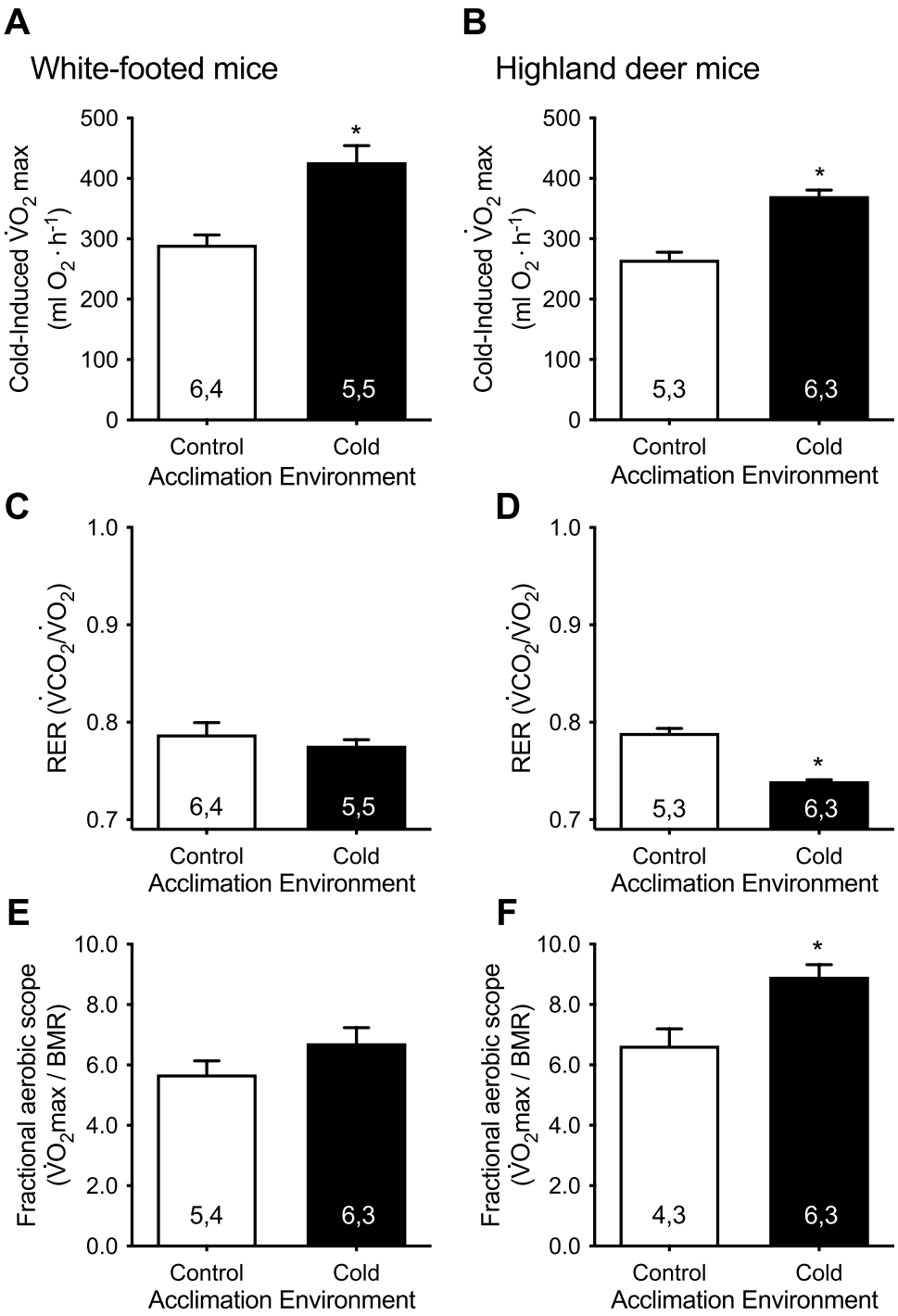

Fig. 3 Cold-induced maximal oxygen consumption ($\dot{V}O_2$max) was determined in white-footed mice (**A**) and in a highland native population of deer mice (**B**), both lab-born and -raised to the second generation, in control conditions (23 °C) or after acclimation to cold (5 °C). Respiratory exchange ratios (RER = ($\dot{V}CO_2/\dot{V}O_2$) were calculated at $\dot{V}O_2$max (C and D), and the fractional aerobic scope, as the quotient of $\dot{V}O_2$max and basal metabolic rate (BMR), for white-footed mice (**E**) and deer mice (**F**). Groups sizes are reported in parentheses as number of males and females (M,F). *Denotes a significant difference ($p < 0.05$) between control and cold-acclimated groups within a species

continuous cold of the high alpine, relative to the seasonal cold experienced by WFM. However, cold is not the only stressor at high altitude, they also tend to be more arid, and rainfall also predicts both BMR and C_{min} (Naya et al. 2013). Nevertheless, highland DM are unlikely to ever experience temperatures within their thermoneutral zone when outside of their burrows, and cold is a persistent stressor throughout the year. The daily energy expenditure of wild deer mice is also ~ 60% higher in highlanders compared to lowlanders (Hayes 1989). In addition, highland native mice must cope with unremitting hypobaric hypoxia. Taken together, these data suggest that highland DM may not be able to afford an increase in BMR. Indeed, we found second-generation lab-born and -raised highland DM did not alter either BMR or C_{min} in response to cold acclimation. A thermal strategy that involves maintenance of BMR and seasonal flexibility in thermal conductance would be beneficial in environments that require sustained thermogenesis but do not support

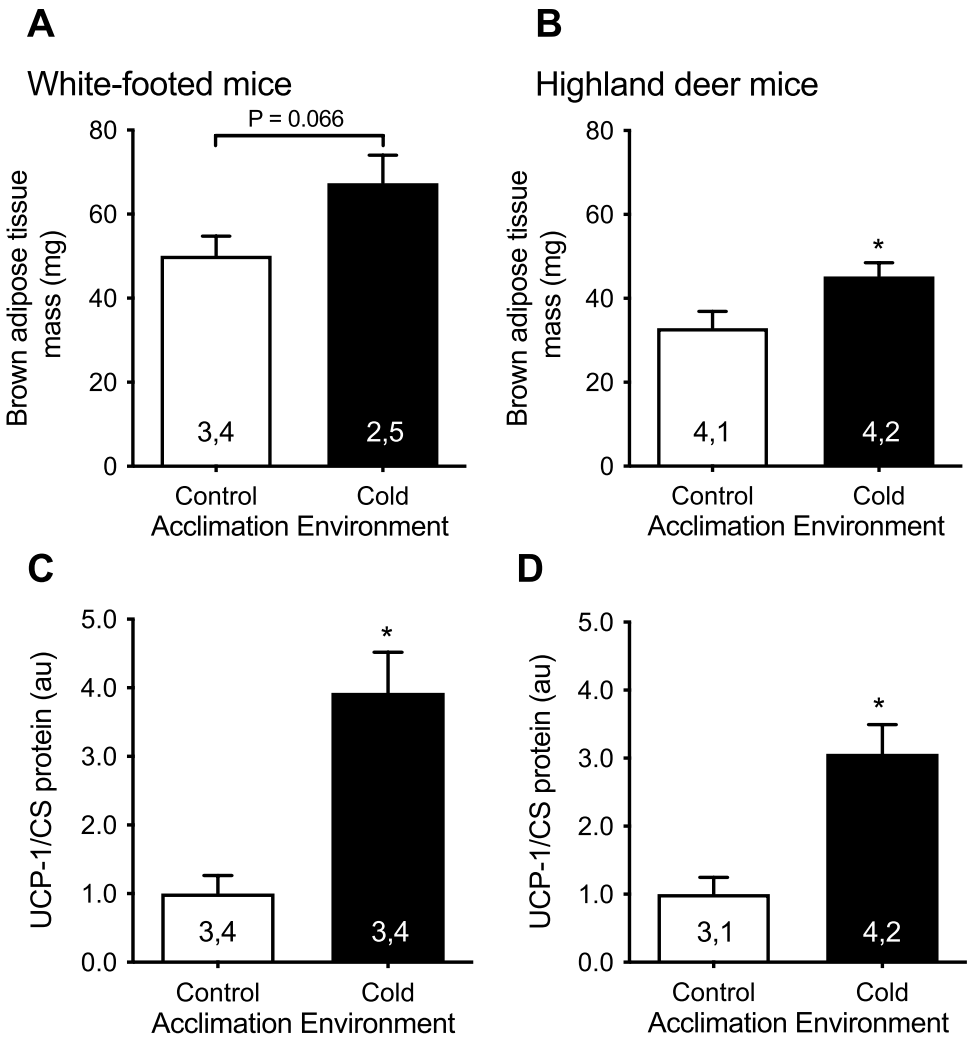

Fig. 4 Brown adipose tissue (BAT) mass was determined in white-footed mice (**A**) and in a highland native population of deer mice (**B**), both lab-born and -raised to the second generation, in control conditions (23 °C) or after acclimation to cold (5 °C). BAT mass varied allometrically with body mass (M) across all individuals as $BAT = 3.136 \times M^{0.874}$. Protein expression for UCP-1/CS relative to control mice in white-footed mice (**C**) and highland deer mice (**D**). Groups sizes are reported in parentheses as number of males and females (M,F). *Denotes a significant difference ($p < 0.05$) between control and cold-acclimated groups within a species

high-quality diets (Naya et al. 2013), as is the case with high altitude. In the highland DM, BMR did not change with cold acclimation despite significant increases in intestinal mass (Table 1), which may allow for enhanced nutrient extraction, especially if high-altitude native mice exploit nutrient-poor diets in the winter.

Table 3 Protein expression of uncoupling protein (UCP)-1 and citrate synthase (CS) in brown adipose tissue of white-footed mice (*P. leucopus*) and highland deer mice (*P. maniculatus*) acclimated to the cold relative to control conditions

	White-footed mice		Highland deer mice	
	Control	Cold	Control	Cold
UCP-1	1.00 ± 0.30	3.24 ± 0.40*	1.00 ± 0.32	1.88 ± 0.19*
CS	1.00 ± 0.12	0.85 ± 0.08	1.00 ± 0.14	0.73 ± 0.05

Values are means ± s.e.m. See Fig. 4 for sample sizes. *Significantly different from controls within a species

In contrast to highland DM, the WFM did increase BMR with cold acclimation, but C_{min} was unaffected by this exposure. This higher BMR was associated with an increase in the relative size of both the intestine and iBAT mass, and likely reflects increased maintenance costs of these larger tissues. An increase in the size of the GI tract is a common response to chronic cold in small mammals, reflecting increases in food consumption with increased thermogenic demands (e.g., Heroux and Gridgeman 1958; Hammond and Wunder 1995; Chi and Wang 2010), and wild WFM are known to adjust the mass of their intestine seasonally (Derting and Hornung 2003).

Few studies have examined changes in T_{lc} with cold acclimation in mice or other small mammals. In a study of OF1 strain mice (*Mus musculus*) Oufara et al. (1987) found little change in T_{lc} after 4 weeks at 4 °C. In contrast, cold-acclimated gerbils (*Gerbillurus campestris*) showed a decrease in T_{lc} that was paralleled by increased BMR and a lowering of defended T_b at low T_a (Oufara et al. 1987). Here we show that cold acclimation had a significant effect on T_{lc}

which decreased by ~ 2.5 °C in highland DM and ~ 2.0 °C in WFM (Fig. 2B,C). To our knowledge, this is the first study that demonstrates the effect of cold acclimation on the breadth of the thermoneutral zone in *Peromyscus* mice. The classic Scholander–Irving model predicts this can occur by either an increase in BMR or a decrease in C_{min} or a combination of changes, because it allows for T_b regulation at lower T_a within the thermoneutral zone (Fristoe et al. 2015). This appears to be the strategy employed by WFM, where cold acclimation led to an increase in BMR, with an associated broadening of ΔT_m (Fig. 2D). However, the adaptive significance of this broadening of the thermoneutral zone is unclear. The annual temperature experienced by wild WFM from the Great Plains involves seasonal fluctuations in and out of their thermoneutral zone. This means that as WFM move from summer to winter, they exist in two dramatically different physiologically states. The reduced T_{lc} should only occur during winter months and could mean that cold-acclimated WFM can easily maintain a more stable T_b within at slightly lower range of T_a. However, during the winter months, wild WFM are known to move their nests to underground burrows (e.g., Wolf and Durr 1986). The ambient temperature of these underground burrows is high and stable (Hayward 1965), reducing the need for WFM to maintain a stable T_b themselves. Outside the burrows, they would likely never experience temperatures warm enough to take advantage of their lowered T_{lc}. It is therefore possible that the lowered T_{lc} in WFM is merely an indirect consequence of an increase in BMR.

While the ambient temperatures at high altitude in the Rocky Mountains do fluctuate seasonally, they are always below the T_{lc} of highland DM. Therefore, unlike the WFM, the highland DM are unlikely to ever experience temperatures within their thermoneutral zone when outside of their burrows. Highland DM showed no change in either BMR or C_{min} with cold acclimation but did show a decrease in T_{lc}. This was a result of reduced T_b to T_a gradients for heat loss as T_a declined. This mechanism is not accounted for in the classic Scholander–Irving model, which assumes that T_b remains constant within the thermoneutral zone. However, T_b can be highly variable in small mammals (Boyles et al. 2019). For example, mark-recapture studies have shown that average T_b in wild bush rats during the winter was 0.4 °C lower than in the summer. Moreover, after cold acclimation, they reduced T_b by 0.9 °C compared to warm-acclimated controls (Glanville and Seebacher 2010a, b). Djungarian hamsters also undergo a regulated hypothermia (by over 1 °C) in response to cold stress (Heldmaier et al. 1985). Reducing the gradient for heat loss reduces the need to increase BMR, possibly a valuable thermogenic strategy when temperatures decline in resource poor environments.

$\dot{V}O_2$max, and aerobic scope

Increased thermogenic capacity is a common response to cold acclimation in small mammals (Merritt and Zegers 2002). Consistent with previous studies on *Peromyscus* (Chappell and Hammond 2004; Tate et al. 2020), we show that cold acclimation increased cold-induced $\dot{V}O_2$max in both WFM and highland DM. Thermogenic capacity is the sum of BMR, shivering thermogenesis, and non-shivering thermogenesis (Wunder and Gettinger 1996), and cold acclimation is often associated with increases in the capacity for non-shivering thermogenesis (Heldmaier et al. 1982; Li et al. 2001; Li and Wang 2005; Boratyński et al. 2016), effectively increasing thermogenic capacity. Since non-shivering thermogenesis occurs primarily in BAT, the capacity for heat production reflects increases in BAT mass and/or mitochondrial and UCP-1 content (Himms-Hagen 1986). Indeed, we found cold acclimation led to increased iBAT mass and UCP-1 protein content, but not in CS content used as an index of mitochondrial volume density. This resulted in a larger tissue with greater UCP-1 content per unit mitochondria providing great capacity for non-shivering thermogenesis. Consistent with this conclusion was a positive relationship between UCP/CS and residual variation in cold-induced $\dot{V}O_2$max relative to body mass, across species and acclimations ($r^2 = 0.39$, $p = 0.001$). Interestingly, we have previously shown that the BAT phenotype of highland DM is resistant to chronic hypoxia exposure (Coulson et al. 2021), which impedes the cold-induced remodeling of BAT in lowland lab mice (Beaudry and McClelland 2010), suggesting similar changes to those observed here occur at high altitude.

One advantage of increasing cold-induced $\dot{V}O_2$max is to allow for a great aerobic reserve ($\dot{V}O_2$max − field metabolic rate), which is important when thermogenic costs of an environment are high. However, changes in fractional aerobic scope ($\dot{V}O_2$max/BMR) can impact any potential gains in aerobic reserve. An increase in aerobic scope seen in highland DM would provide a greater range for aerobic activities and potentially reduce overall field metabolic rate (which includes BMR and all other aerobic activities). This would be beneficial at high altitude, where DM have been shown to often operate near their hypoxic $\dot{V}O_2$max (Hayes 1989). How do highland DM increase cold-induced $\dot{V}O_2$max without a corresponding increase in BMR? We found that during cold acclimation the mass of the gastrocnemius muscle decreased in highland DM but not WFM. While this was the only skeletal muscle measured in this study, it could indicate an overall decrease in the muscle mass of highland DM, a phenomenon that has been previously reported in rats (Heroux and Gridgeman 1958). A decrease in lean body mass would lower BMR and could theoretically offset the costs

of increasing intestine and iBAT size. Relative increases and decreases in the masses of different metabolically active tissues could explain why some previous reports have found increases in BMR with cold acclimation and others have not.

In contrast to highland DM, we found that aerobic scope remained constant with cold acclimation in WFM due to increases in both cold-induced $\dot{V}O_2$max and BMR. The acclimation response to cold in WFM likely reflects the need to support an increased $\dot{V}O_2$max, as organs adjust to augment heat production, driving an increase in BMR. This is a costly strategy as a higher BMR would increase daily energy expenditure, even within the thermoneutral zone. However, a high cold-induced $\dot{V}O_2$max allows *Peromyscus* mice to spend more time foraging for food in the cold (Sears et al. 2006). Our data suggest that the thermal acclimation response of WFM is geared toward rapidly increasing heat production rather than modifying heat retention and likely reflects a need to move between seasons that vary within or below their thermoneutral zone.

Conclusion

Here we have shown that two species of *Peromyscus* mice native to the same latitude and geographically adjacent, but experiencing vastly different thermal regimes, use distinct strategies to acclimate to cold. Both highland DM and WFM increase $\dot{V}O_2$max via plasticity in the iBAT. This increase in maximal aerobic capacity requires an increase in nutrient intake, which is supported by remodeling of the digestive tract and supporting tissues. In WFM, changes in $\dot{V}O_2$max are coupled with a higher BMR, which likely increases daily energy expenditure, but allows them to guard a high, stable T_b. By contrast, highland DM may always need a high $\dot{V}O_2$max, which they can further increase while maintaining a constant BMR. This allows highlanders to increase aerobic scope in the coldest part of the year. Our data suggest that highland DM may occasionally use shallow hypothermia to save energy when thermogenic demands are highest. The use of moderate heterothermy, outside the scope of torpor or hibernation, as an energy saving strategy is poorly understood. Our data highlight the importance of considering how seasonal temperatures change relative to an animal's thermoneutral zone to truly understand appropriate acclimation strategies.

Funding This research was funded by a Natural Science and Engineering Research Council of Canada (NSERC) Discovery Grant to G.B.M. G.B.M. was also a recipient of an NSERC Discovery Accelerator grant. C.E.R. was the recipient of a NSERC postgraduate scholarship.

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