© 2021. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2021) 224, jeb242279. doi:10.1242/jeb.242279

RESEARCH ARTICLE

Plasticity of non-shivering thermogenesis and brown adipose tissue in high-altitude deer mice

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ABSTRACT

High altitude environments challenge small mammals with persistent low ambient temperatures that require high rates of aerobic heat production in face of low O_2 availability. An important component of thermogenic capacity in rodents is non-shivering thermogenesis (NST) mediated by uncoupled mitochondrial respiration in brown adipose tissue (BAT). NST is plastic, and capacity for heat production increases with cold acclimation. However, in lowland native rodents, hypoxia inhibits NST in BAT. We hypothesize that highland deer mice (*Peromyscus maniculatus*) overcome the hypoxic inhibition of NST through changes in BAT mitochondrial function. We tested this hypothesis using lab born and raised highland and lowland deer mice, and a lowland congeneric (*Peromyscus leucopus*), acclimated to either warm normoxia (25°C, 760 mmHg) or cold hypoxia (5°C, 430 mmHg). We determined the effects of acclimation and ancestry on whole-animal rates of NST, the mass of interscapular BAT (iBAT), and uncoupling protein (UCP)-1 protein expression. To identify changes in mitochondrial function, we conducted high-resolution respirometry on isolated iBAT mitochondria using substrates and inhibitors targeted to UCP-1. We found that rates of NST increased with cold hypoxia acclimation but only in highland deer mice. There was no effect of cold hypoxia acclimation on iBAT mass in any group, but highland deer mice showed increases in UCP-1 expression and UCP-1-stimulated mitochondrial respiration in response to these stressors. Our results suggest that highland deer mice have evolved to increase the capacity for NST in response to chronic cold hypoxia, driven in part by changes in iBAT mitochondrial function.

KEY WORDS: Mitochondria, Acclimation, Uncoupling protein-1, *Peromyscus maniculatus*, *Peromyscus leucopus*

INTRODUCTION

The generation of metabolic heat (thermogenesis) is an important mechanism used by endotherms to maintain a high body temperature in face of varying environmental temperatures. In small mammals, non-shivering thermogenesis (NST) is a significant component of whole-animal thermogenic capacity (Cannon and Nedergaard, 2004; Janský, 1973; Himms-Hagen, 1985; Heldmaier, 1971; Heldmaier et al., 1989). In fact, NST can contribute more than 50% of cold-induced $\dot{V}_{O_2,max}$ (thermogenic capacity) in some rodents (Van Sant and Hammond, 2008). The major site of NST in eutherian mammals is brown adipose tissue (BAT), where energy from uncoupled mitochondrial respiration, mediated by uncoupling protein 1 (UCP-1) is almost entirely released as heat (Cannon and Nedergaard, 2004). Heat production by NST occurs as the result of activation of UCP-1 by free fatty acids released in response to sympathetic activation of BAT upon cold exposure (Cannon and Nedergaard, 2004; Fedorenko et al., 2012).

Thermogenic capacity is a plastic trait that is influenced by multiple environmental factors. Prolonged exposure to cold is known to increase overall thermogenic capacity in rodents (Cannon and Nedergaard, 2011), which helps in the preparation for seasonal variation in ambient temperatures (e.g. Böckler and Heldmaier, 1983; Klaus et al., 1988). A high thermogenic capacity is especially important for those species that are winter active as it correlates with increased activity levels in the cold (Sears et al., 2006). In rodents, seasonal variation in thermogenic capacity is primarily driven by changes in NST, through remodeling of BAT (Klaus et al., 1988; Van Sant and Hammond, 2008). Studies on wild white-footed mice and bank voles show that NST capacity increases in preparation for winter (Klaus et al., 1988; Wickler, 1980). This plasticity of NST has also been observed in cold-acclimated lab mice (e.g. Beaudry and McClelland, 2010; Lacy et al., 1978; Oufara et al., 1987). The mechanisms that underlie increases in the capacity for NST are thought to involve either changes in: (i) the size of BAT depots, (ii) brown adipocyte phenotype, and/or (iii) mitochondrial function. For example, cold acclimation in rodents has been shown to increase BAT mass by both hypertrophy (Klingenspor et al., 1996) and hyperplasia (Bukowiecki et al., 1982), but also by increasing tissue oxidative capacity through greater mitochondrial abundance and/or the mitochondrial expression of UCP-1 (Beaudry and McClelland, 2010; Klingenspor et al., 1996; Smith and Roberts, 1964). The respiratory capacity of isolated BAT mitochondria may also be a target of plasticity, through increases in UCP-1-induced respiration and capacity for substrate catabolism (e.g. β-oxidation of fatty acids), enhancing brown adipocyte heat production (Ballinger et al., 2016; Heim et al., 2017; Yu et al., 2002).

High altitude regions are generally cooler year-round than those of low altitude at the same latitude (Hayes, 1989). Since low oxygen availability is known to restrict aerobic metabolism, the high alpine climate presents a significant thermoregulatory challenge for small mammals inhabiting these regions. Indeed, deer mice [*Peromyscus maniculatus* (Wagner 1945)] native to high altitude have higher daily energy requirements than their low altitude conspecifics, presumably because of the high demand for thermogenesis (Hayes, 1989). This high aerobic demand has likely contributed to directional selection for enhanced aerobic capacity seen in high altitude deer mice (Hayes and O'Connor, 1999). As a result, high altitude deer mice have evolved a higher thermogenic capacity compared with both their low altitude conspecifics and a strictly low altitude congeneric

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Received 20 January 2021; Accepted 12 April 2021

[white-footed mice, *Peromyscus leucopus* (Rafinesque 1818)] (Cheviron et al., 2012, 2014). This higher capacity for aerobic thermogenesis in highland deer mice is associated with constitutively greater aerobic capacity of skeletal muscle (Cheviron et al., 2014; Lui et al., 2015; Scott et al., 2015), presumably to enhance the capacity for shivering. However, NST is a major component of thermogenesis in deer mice (Van Sant and Hammond, 2008), suggesting that BAT function also contributes to the greater thermogenic capacity in highlanders. Indeed, Velotta et al. (2016) found that wild highland deer mice had higher NST than lowland conspecifics when measured at their native altitudes. These population differences in NST may represent genetically fixed traits or may be the product of phenotypic plasticity induced by chronic exposure to cold hypoxia in the high alpine. Comparisons of NST and interscapular BAT (iBAT) transcriptomics between wild deer mice from low and high altitudes, and their first-generation laboratory born and raised descendants, suggest phenotypic plasticity (developmental and/or adult) plays an important role in the evolved higher thermogenic capacity seen at high altitude (Velotta et al., 2016).

In low altitude natives, hypoxia has antagonistic effects on cold-induced changes in NST and BAT remodeling (Beaudry and McClelland, 2010). This response is distinct from the effect of either cold or hypoxia acclimation alone (Beaudry and McClelland, 2010). While cold acclimation has a stimulatory effect on BAT growth and metabolic remodeling, hypoxia acclimation tends to have an inhibitory effect. Although the effects of hypoxia acclimation on NST have not been extensively studied, it has been shown to increase cytochrome *c* oxidase activity and stimulate UCP-1 mRNA expression in BAT but decreases the capacity for NST in CD-1 strain lab mice (Beaudry and McClelland, 2010). In contrast, when combined with cold, hypoxia led to a blunting of the cold-induced increases in NST (Beaudry and McClelland, 2010). Whether these antagonistic influences of combined cold hypoxia on NST and BAT function persist in highland natives in response to the combined stressors experienced in their native environment is presently unknown.

In this study, we used first generation lab born and raised lowland and highland deer mice (*P. maniculatus*), and the strictly lowland white-footed mice (*P. leucopus*) acclimated to warm normoxia control conditions and to cold hypoxia simulating high altitude. We tested the hypothesis that deer mice native to high altitude have evolved a robust response to cold hypoxia acclimation that results in an increase in NST due to BAT remodeling. We predicted that cold hypoxia acclimation will lead to higher rates of NST, greater BAT mass, and an augmented capacity for brown adipocytes and their mitochondria to increase heat production in highlanders. In contrast, we predict cold hypoxia will have antagonistic effects in lowlanders resulting in no net change in NST, BAT mass or its capacity for heat production.

MATERIALS AND METHODS

Animal model

Captive breeding populations were established from wild-caught mice, as previously described (Cheviron et al., 2012; Lau et al., 2017; Tate et al., 2017). Lowland deer mice (*P. maniculatus nebrascensis*) and white-footed mice (*P. leucopus*) were captured at Nine-mile Prairie, NE, USA (320 m a.s.l.). Highland deer mice (*P. maniculatus rufinus)* were captured at the summit of Mount Evans, CO, USA at 4348 m a.s.l. Wild-caught mice were transported to McMaster University in Hamilton, ON, Canada (~90 m a.s.l.) and housed in common laboratory conditions for breeding within their respective populations to the first generation. Mice were held under standard temperature (25°C) at sea level with a 12 h:12 h light:dark cycle and had access to standard mouse chow and water *ad libitum*.

Acclimation conditions

Mice of all three groups, which included adult mice from a mix of different families and sexes, were exposed to two acclimation conditions for 6–8 weeks: warm normoxia (WN, control conditions) and cold hypoxia (CH). Mice in the WN acclimation were held at atmospheric pressure of ~101 kPa and at a room temperature of 25°C, while CH mice were held in hypobaric chambers (McClelland et al., 1998) at 60 kPa housed in a 5°C cold room, simulating conditions at an altitude of 4300 m a.s.l. The cages were cleaned once per week, which required the CH mice to be temporarily exposed to normobaric conditions (<1 h per week). All experiments and protocols approved by the McMaster University Animal Research Ethics Board in accordance with the Canadian Council for Animal Care.

Non-shivering thermogenesis

To assess rates of NST, mice were first removed from their cages and immediately placed in metabolic chambers (475 ml) maintained within their thermoneutral zone at 28°C, using a temperature control cabinet (Sable Systems, Las Vegas, NV, USA). Mice were allowed to adjust to the chambers for 45 min while metabolic rate was monitored by flow-through respirometry in normoxia, as previously described (Robertson and McClelland, 2019). Briefly, air dried and stripped of CO_2 (Drierite, W. A. Hammond, Xenia, OH; soda lime and Ascarite, Fisher Scientific, Pittsburgh, PA) was pushed through the chamber at a rate of 1000 ml min^{-1}, and a subsample of the excurrent air was dried before being drawn through CO_2 and O_2 analyzers (Sable Systems). Mice were then removed from the metabolic chamber and injected subcutaneously with a standardized dose of norepinephrine (NE) based on body mass (Wunder and Gettinger, 1996) in a total of 500 µl saline. We found in preliminary experiments that any increase in metabolism due to injection of saline alone returned to baseline within 10 min. Thus, we quantified NST as the 5 min of the maximal NE-induced $\dot{V}_{O_2}$ while animals were not active (verified using a webcam) and after the effect of saline was complete. We also calculated the increase in $\dot{V}_{O_2}$ above basal metabolic rate (BMR) as NST–BMR (Robertson and McClelland, 2019). BMR for each group was estimated from their average mass using the allometric relationship of body mass and BMR from a separate set of 179 individuals across all three populations in our captive breeding colony, as the lowest $\dot{V}_{O_2}$ in the post-absorptive state, awake and in thermoneutral conditions during 3 h of measurement (N. Wall and G.B.M., unpublished data). To calculate $\dot{V}_{O_2}$ we used the following equations from Withers (1977):

$$\dot{V}_{CO_2} = \dot{V}_{I_{stp}}(F_{E_{CO_2}} - F_{I_{CO_2}}), \quad (1)$$

$$\dot{V}_{O_2} = \frac{\dot{V}_{I_{stp}}(F_{I_{O_2}} - F_{E_{O_2}}) - \dot{V}_{CO_2} \times F_{I_{O_2}}}{(1 - F_{I_{O_2}})}, \quad (2)$$

where $\dot{V}_{CO_2}$ is production of CO_2, $\dot{V}_I$ is the flow rate of incurrent air [at standard temperature and pressure (stp)] into the metabolic chamber, and F is the fractional concentration of O_2 or CO_2 in the incurrent (I) or excurrent (E) air.

Western blotting

Mice were euthanized by an overdose of anesthetic (isoflurane), followed by cervical dislocation to sample iBAT, a major BAT depot in mice (de Jong et al., 2015), which was stored at −80°C before analysis. Western blotting was used to quantify the protein expression of UCP-1, as previously described (Robertson et al., 2019). Unless described otherwise, all reagents were purchased

from Millipore Sigma (St. Louis, MO). Frozen iBAT samples were powdered under liquid N_2 and 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. Total homogenate protein was quantified via Bradford assay (Bradford, 1976) and 15 µg per sample was denatured by heating to 95°C for 5 min in Laemmli sample buffer (Bio-Rad, Mississauga, ON, Canada) with β-mercaptoethanol. Proteins were separated on 12% SDS PAGE gels and then transferred to PVDF membrane. Membranes were probed with primary antibody against UCP-1 (UCP-11-A, Alpha Diagnostics International Inc., San Antonio, TX) at a dilution of 1:500, followed by HRP-conjugated secondary antibody at 1:10,000 (goat anti-rabbit, Santa Cruz Biotechnology, Santa Cruz, CA). Band density was normalized to total lane protein determined by membrane staining with Coomassie Blue, using the Image Lab software package (Bio-Rad). Owing to low availability of *P. leucopus* in our captive colony, these measurements were only carried out on lowland and highland *P. maniculatus*.

Mitochondrial isolation

BAT mitochondria were isolated as described previously (Cannon and Nedergaard, 2008; McFarlane et al., 2017; Muleme et al., 2006). For each mitochondrial preparation, iBAT was blunt dissected from one to two individual mice of the same family, cleaned of white adipose tissue and muscle, pooled, and immediately placed in ice-cold homogenization buffer (HB: 250 mmol l^{-1}sucrose, 1 mmol l^{-1} EGTA, 10 mmol l^{-1} Hepes, pH 7.4). Tissue was minced and then mechanically homogenized in a glass mortar with a Teflon pestle (6 strokes at 100 rpm) in ice-cold HB. The homogenate was filtered through two layers of cheesecloth and the filtrate was centrifuged at 4°C for 10 min at 8700 ***g***. The pellet was resuspended in HB and centrifuged again at 800 ***g*** for 10 min. The supernatant was transferred to a clean tube and was centrifuged again at 8700 ***g*** for 10 min. The pellet was resuspended in HB and centrifuged at 8700 ***g*** for 10 min twice more. The final pellet was then resuspended in ∼300 µl of HB and kept on ice until further use. Total mitochondrial protein content was quantified by Bradford assay in triplicate.

Mitochondrial respiration

Respiration of isolated iBAT mitochondria was determined by high-resolution respirometry using Clark-type polarographic oxygen electrodes (Oxygraph-2k; Oroboros, Innsbruck Austria) in a total volume of 2 ml respiration buffer [110 mmol l^{-1} sucrose, 60 mmol l^{-1} K-lactobionate, 20 mmol l^{-1} Hepes, 20 mmol l^{-1} taurine, 10 mmol l^{-1} KH_2PO_4, 3 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} EGTA and 0.1% (w/v) fatty-acid free BSA, pH 7.1]. Calibration was performed using air-saturated buffer and an oxygen depleted buffer (using a yeast suspension). Measurements were made with constant stirring at 750 rpm and at 37°C. Background respiration through complex I, in the absence of exogenous fatty acid stimulation, was determined using 50 µg of isolated BAT mitochondria in the presence of 1 mmol l^{-1} pyruvate and 1 mmol l^{-1} malate. UCP-1-stimulated uncoupled respiration was induced by the addition of 1 mmol l^{-1} malate and 1 mmol l^{-1} octanoyl-carnitine (8:0) or 25 µmol l^{-1} palmitoyl-carnitine (16:0) [free fatty acids (FFAs) of different chain lengths], at a concentration determined in preliminary experiments. The addition of 2 mmol l^{-1} GDP was used to inhibit UCP-1-mediated respiration (Shabalina et al., 2004), and thus GDP-sensitive respiration (accounting for any residual respiration after GDP addition) is reported as respiration directly attributed to UCP-1 stimulation. Respiration due to oxidative phosphorylation was determined by adding 1.25 mmol l^{-1} ADP, which was then inhibited with the addition of 1.25 µmol l^{-1} oligomycin. All respiration measurements were conducted in triplicate, except for measurements with the addition of FFAs, which were measured once per mitochondrial preparation. Isolated mitochondria not used for respiration were stored at −80°C for enzyme assays.

Enzyme assays

Following one round of freeze–thaw, isolated iBAT mitochondria were assayed to determine the apparent V_{max} of citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (HOAD). Each assay was performed in triplicate, and an assay without added substrate was performed to correct for any background activity. Assay conditions were as follows for CS: 0.5 mmol l^{-1} oxaloacetate, 0.15 mmol l^{-1} acetyl-CoA, 0.05% Triton X-100 and 0.15 mmol l^{-1} 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.04 mmol l^{-1} Tris-HCl (pH 8.0). For HOAD: 0.15 mmol l^{-1} acetoacetyl-CoA, 0.15 mmol l^{-1} NADH in 0.1 mmol l^{-1} triethanolamine (pH 7.0). Assays were carried out in 96-well format at 37°C using a Spectromax Plus 384 microplate reader (Molecular Devices).

Statistical analysis

Data were analyzed by a two-way analysis of variance (ANOVA), an analysis of covariance (ANCOVA) with body mass as a covariate and population and acclimation as main effects, or a linear mixed model with mass as a covariate and family and/or sex as random variables, in R (https://www.r-project.org/). Multiple comparisons were performed using Tukey HSD or Holm–Šídák *post hoc* tests. Data are presented as means±s.e.m.

RESULTS

Non-shivering thermogenesis (NST)

We assessed the capacity for NST and the phenotypic plasticity of this important performance variable in lowland deer mice and white-footed mice, compared to highland deer mice with and without acclimation to CH conditions. We defined NST as peak rates of $\dot{V}_{O_2}$ after injection of norepinephrine and found it to be similar between all groups of mice acclimated to control WN conditions (P>0.05). Acclimation to CH led to an increase in NST but only in highlanders, where it was 1.3-fold higher than in warm normoxic conditions (P=0.03). Rates of NST above predicted BMR were also similar across groups kept in WN. There was an effect of CH but only in highlanders (Fig. 1), where NST-BMR increased by 1.7-fold from 1.15±0.21 ml min^{-1} to 1.92±0.26 ml min^{-1}, a rate significantly higher than that observed in *P. leucopus* (1.35±0.25 ml min^{-1}) under the same conditions (P=0.04).

iBAT mass and UCP-1 protein expression

To investigate the underlying traits responsible for changes in NST with acclimation, we assessed iBAT mass and expression of UCP-1. Changes in iBAT mass and/or UCP-1 expression have both been shown to correlate with changes in NST in other studies (McDevitt and Andrews, 1997; Beaudry and McClelland, 2010). Since iBAT mass varies as a function of body mass in other mammals (Aleksiuk and Frohlinger, 1971; Król and Speakman, 2019; Smith and Roberts, 1964), we first determined this relationship for deer mice and white-footed mice. We found that iBAT mass varied directly with body mass across populations and with acclimation conditions (Fig. 2A). After accounting for any differences in body mass, we found that iBAT mass tended to be lower in white-footed mice than lowland deer mice, but this difference did not reach statistical significance with multiple comparisons test (P=0.06). However, there was no significant effect of CH acclimation on iBAT mass in any of the

Journal of Experimental Biology (2021) 224, jeb242279. doi:10.1242/jeb.242279

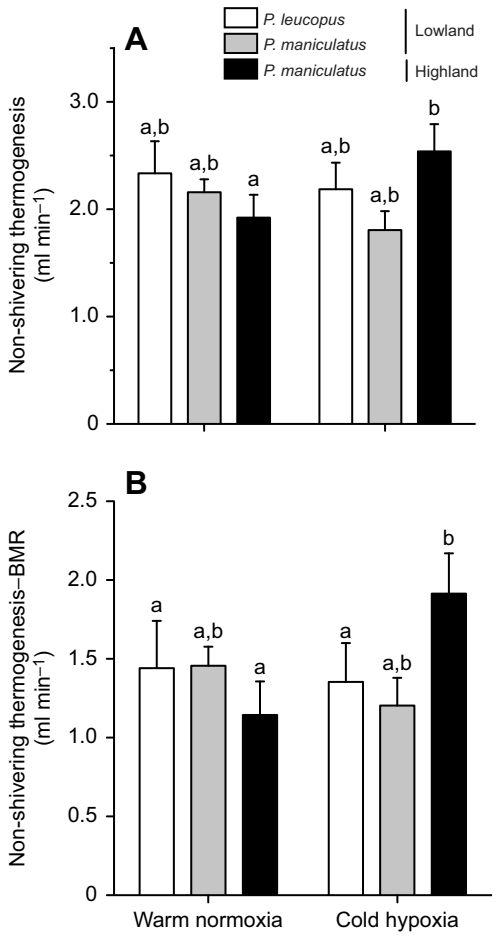

Fig. 1. Norepinephrine-induced non-shivering thermogenesis (NST) and NST-basal metabolic rate of lowland native white-footed mice (*Peromyscus leucopus*) and deer mice (*Peromyscus maniculatus*) compared with highland native deer mice acclimated to 6-8 weeks of either warm normoxia (control conditions) or cold hypoxia. (A) Norepinephrine-induced NST. (B) Norepinephrine-induced NST–BMR (predicted from average body mass for each group based on BMR=5.229×$M_b^{0.689}$). Sample sizes (mass and predicted BMR) for warm normoxia and cold hypoxia, respectively, were N=9 (20.7±1.5 g, 0.70 ml min^{-1}) and N=4 (16.6±0.7 g, 0.60 ml min^{-1}) for lowland deer mice; N=6 (29.3±1.7, 0.89 ml min^{-1}) and N=6 (26.5±1.9 g, 0.83 ml min^{-1}) for white-footed mice; and N=6 (24.0±3.6 g, 0.78 ml min^{-1}) and N=5 (17.4±0.8 g, 0.62 ml min^{-1}) for highland deer mice. Using mass as a covariate, NST showed a significant population×acclimation interaction ($F_{2,28}$=4.676, P=0.018). Similarly, for NST–BMR when mass was used as a covariate, the effect of acclimation approached significance ($F_{1,28}$=3.77, P=0.062), and there was a significant population×acclimation interaction ($F_{2,28}$=5.32, P=0.011). Bars that do not share a letter are significantly different from each other as determined by multiple comparisons (Tukey). Values presented as means±s.e.m.

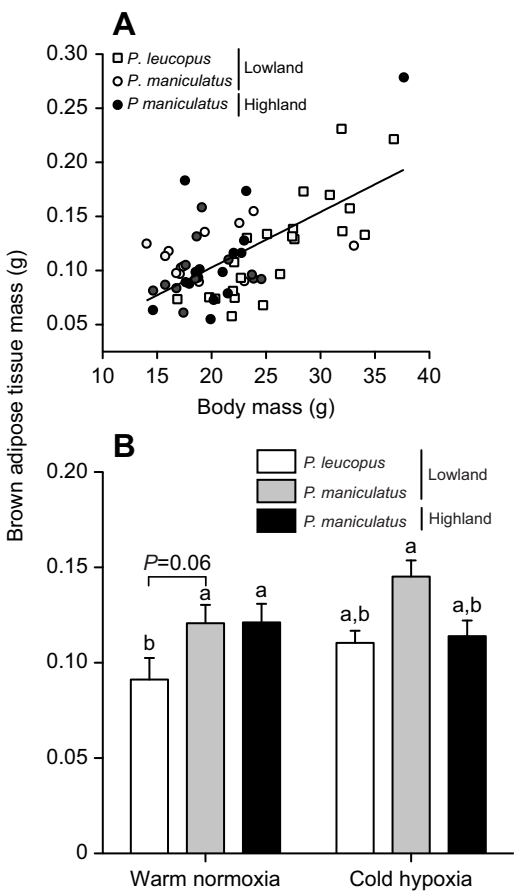

Fig. 2. Mass of interscapular brown adipose tissue (iBAT) in lowland *P. leucopus*, and in lowland and highland *P. maniculatus*. (A) Mass (M_b) of iBAT varied with body mass as BAT_{mass}=0.0052×$M_b^{0.997}$. (B) Mass of iBAT in lowland and highland native mice, all acclimated to 6–8 weeks of either warm normoxia (control conditions) or cold hypoxia. Samples sizes (and average body mass) for warm normoxia and cold hypoxia, respectively, were N=9 (20.7±1.8 g) and N=4 (17.3±1.9 g) for lowland deer mice; N=10 (26.2±1.8 g) and N=12 (26.2 ±1.4 g) for white-footed mice; and N=16 (21.0±1.3 g) and N=12 (19.3±1.0 g) for highland deer mice. Using mass as a covariate, there was a significant effect of population ($F_{2,55}$=4.802, P=0.012) but not of acclimation ($F_{1,55}$=2.589, P=0.113) nor a significant interaction ($F_{2,55}$=2.53, P=0.089). Bars that do not share a letter were significantly different from each other as determined by multiple comparisons (Tukey). Values in B are presented as means±s.e.m.

experimental groups. In fact, all mice maintained consistent iBAT mass across the two acclimation conditions (Fig. 2B).

In contrast, CH acclimation led to an increased expression of UCP-1 protein (Fig. 3), but only in highland deer mice. Compared with highlanders kept in WN conditions, those acclimated to CH showed a 1.9-fold increase in UCP-1 expression (P<0.01), to levels seen in CH-acclimated lowlanders.

Isolated mitochondria respiration and enzyme activities

To understand if the metabolic properties of mitochondria might contribute to differences in NST, we isolated mitochondria from iBAT and determined basal and UCP-1-stimulated rates of respiration. When we assessed respiration using pyruvate and malate (in the absence of exogenous ADP or FFAs), WN acclimated lowland deer mice showed higher rates of this background respiration than both white-footed mice (P=0.03) and highland deer mice (P=0.01). In general, lowland deer mice also had higher rates of background respiration than all other groups after CH acclimation (Table 1). The exception was white-footed mice, which showed an increase in background respiration with CH acclimation (P<0.001) to rates that were equivalent to both lowland and highland deer mice. When respiration was further stimulated using exogenous FFAs, which are known to activate UCP-1, rates increased in all WN acclimated mice, but respiration was higher in lowland deer mice compared with either white-footed mice (P=0.006) or highland deer mice (P=0.016). In contrast, only highland deer mice showed an increase in FFA-simulated respiration after CH acclimation, although the increase was only marginally significant compared with levels in the control

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(*P*=0.054). Nevertheless, all CH acclimated deer mice and white footed mice had similar FFA-stimulated respiration rates. FFA-stimulated respiration was strongly inhibited by the addition of GDP, by 4- to 5-fold in all groups. Respiration that was directly attributed to UCP-1 (i.e. GDP-sensitive palmitoyl-carnitine respiration) was compared between WN acclimated mice and showed no significant difference between populations. However, both highland deer mice (*P*=0.062) and white-footed mice (*P*=0.07) showed a strong trend to increase respiration, by 1.6-fold and 1.8-fold, respectively, after CH acclimation (Fig. 4). Indeed, after CH acclimation, highland mice had higher rates of respiration than white-footed mice in warm normoxia (*P*=0.018). Regardless, all groups showed similar rates of mitochondrial respiration after CH acclimation. We also found that acyl chain length had little impact on FFA-stimulated respiration, with similar rates of UCP-1-dependent respiration with octanoyl-carnitine to those with palmitoyl-carnitine. When oxidative phosphorylation was induced by the addition of ADP, rates of respiration were relatively low, and only ∼20–40% the rate of UCP-1-stimulated respiration (Table 1).

Differences in mitochondrial respiration were not related to mitochondrial-specific activities (apparent V_{max}) of CS or HOAD. In fact, CS activity tended to decline with CH acclimation (significant acclimation effect), likely driven by reductions in activity in the two LA species. In contrast, mitochondrial HOAD activity showed little change in activity with acclimation (Fig. 5).

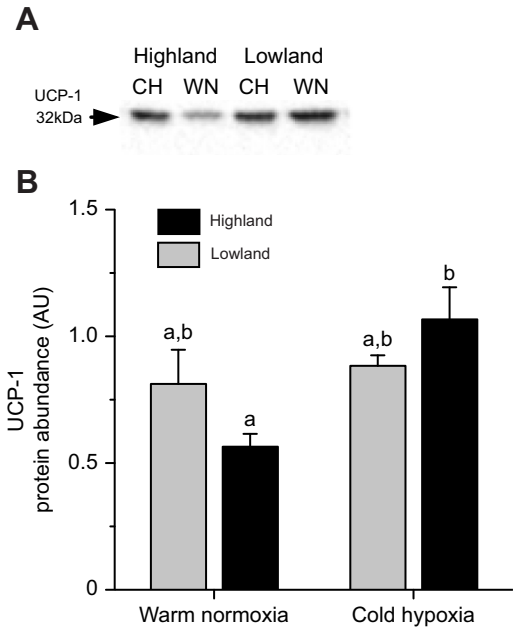

Fig. 3. Relative protein expression of uncoupling protein (UCP)-1 in brown adipose tissue of lowland compared with highland native deer mice acclimated to 6-8 weeks of either warm normoxia (control conditions) or cold hypoxia. Representative western blot (A) and plotted protein abundance (B) of UCP-1. Sample sizes for warm normoxia and cold hypoxia, respectively, were *N*=5 and *N*=6 for lowland deer mice, and *N*=5 and *N*=4 for highland deer mice. There was a significant effect of acclimation ($F_{1,16}$=8.435, *P*=0.010) and a significant population×acclimation interaction ($F_{1,16}$=5.525, *P*=0.032). Bars that do not share a letter were significantly different from each other as determined by multiple comparisons (Tukey). Values presented as means±s.e.m.

DISCUSSION

The main objective of this study was to determine if highland native deer mice have evolved an enhanced plasticity in NST with acclimation to cold hypoxia (CH) conditions that simulate their native altitude. Our data support this hypothesis, with highland mice showing a significant increase in NST after acclimation. In contrast, lowland conspecifics and congenerics showed no change in NST when acclimated to the same conditions. Variation in NST with CH acclimation was not driven by changes in iBAT mass, which remained consistent across experimental groups and acclimation conditions. However, acclimation to CH in highland deer mice led to an increase in the expression of UCP-1 in iBAT and in UCP-1-stimulated respiration of isolated mitochondria, changes not seen in lowlanders.

We found that high altitude native mice increased NST by 32% with acclimation to conditions that simulate an elevation of 4300 m, while low altitude native mice showed little change in this trait in response to the same conditions. Indeed, the response to CH acclimation was even greater for NST-BMR, which increased by 67% from WN in highlanders. This suggests that environment-induced plasticity plays an important role in elevated NST seen in wild highland deer mice measured in their native environment, when compared with lowland mice measured at low altitude (Velotta et al., 2016). This population difference likely reflects the influence of the persistent low temperatures experienced by the highland mice, even in summer (Hayes, 1989; Velotta et al., 2016).

Table 1. Respiration rates (in nmol O_2 min^{-1} mg^{-1} protein) of isolated mitochondria from brown adipose tissue of first generation lab born and raised lowland native deer mice (*P. maniculatus*) and white-footed mice (*P. leucopus*) compared with highland native deer mice, all acclimated to 6-8 weeks of either warm normoxia (control conditions) or cold hypoxia

	P. maniculatus (highland)		*P. maniculatus* (lowland)		*P. leucopus* (lowland)	
	Warm normoxia	Cold hypoxia	Warm normoxia	Cold hypoxia	Warm normoxia	Cold hypoxia
Pyruvate+malate	115±27^{a}	201±19^{a,b}	250±15^{b}	301±32^{b}	114±36^{a}	299±31^{b}
Palmitoyl-carnitine	271±34^{a}	413±9^{b}	437±13^{b,c}	491±25^{b}	228±23^{a}	378±53^{a,b}
Octanoyl-carnitine	227±25^{a}	374±51^{a,b}	362±60^{a,b}	543±65^{b}	274±41^{a}	324±38^{a,b}
+GDP	61±12^{b,c}	76±13^{b}	131±17^{a}	119±26^{a,b}	61±6^{c}	74±11^{b}
ADP	103±15^{a}	103±10^{a}	167±21^{b}	NA	77±13^{a}	67±10^{a}
+Oligomycin	58±10^{a}	71±12^{a}	116±16^{b}	NA	64±9^{a,b}	64±10^{a}

There was a significant effect of population ($F_{2,39}$=9.223, *P*<0.001) and acclimation ($F_{1,39}$=21.716, *P*<0.001) on basal respiration with pyruvate+malate as substrates. When UCP-1 was stimulated using palmitoyl-carnitine there were significant effects of both population ($F_{2,39}$=8.145, *P*=0.001) and acclimation ($F_{1,39}$=15.844, *P*<0.001). Respiration using octanoyl-carnitine also showed a significant effect of both population ($F_{2,29}$=4.255, *P*=0.024) and acclimation ($F_{1,29}$=10.814, *P*=0.003). When UCP-1 was inhibited with the addition of GDP, respiration showed a significant effect of population ($F_{2,39}$=9.373, *P*<0.001). Oxidative phosphorylation with the addition of ADP showed a significant effect of population ($F_{2,34}$=14.721, *P*<0.001). Respiration after the inhibition of oxidative phosphorylation using oligomycin showed a significant effect of population ($F_{2,32}$=7.798, *P*=0.002). Means that do not share a letter were significantly different from each other within a substrate, as determined by multiple comparisons. See Fig. 4 for details on sample sizes. Values presented as means±s.e.m.

Journal of Experimental Biology (2021) 224, jeb242279. doi:10.1242/jeb.242279

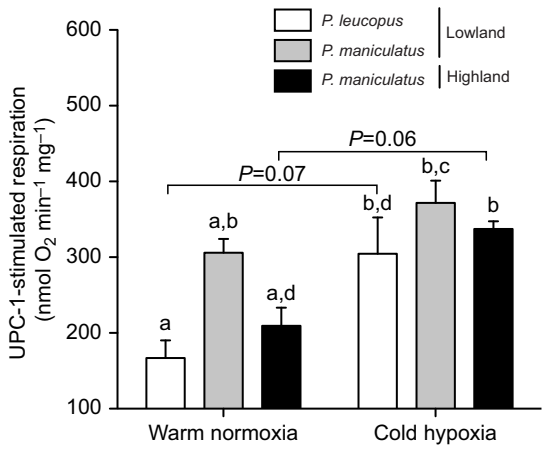

Fig. 4. UCP-1-stimulated respiration in isolated mitochondria of interscapular brown adipose tissue of *P. leucopus* and *P. maniculatus*. UCP-1-stimulated respiration, measured as palmitoyl-carnitine-stimulated respiration that was GDP sensitive (in nmol O_2 min^{-1} mg^{-1}) in mitochondria isolated from interscapular brown adipose tissue from mice acclimated to 6–8 weeks of either warm normoxia (control conditions) or cold hypoxia. Sample sizes (each sample represents BAT pooled from *N*=2 mice of the same family) for warm normoxia and cold hypoxia, respectively, were *N*=8 and *N*=6 for lowland deer mice, *N*=5 and *N*=10 for white-footed mice, and *N*=8 and *N*=8 for highland deer mice. There was a significant effect of acclimation ($F_{1,39}$=17.17, *P*<0.001) and an effect of population that approached statistical significance ($F_{2,39}$=2.99, *P*=0.062). Bars that do not share a letter were significantly different from each other as determined by multiple comparisons (Tukey). Values presented as means±s.e.m.

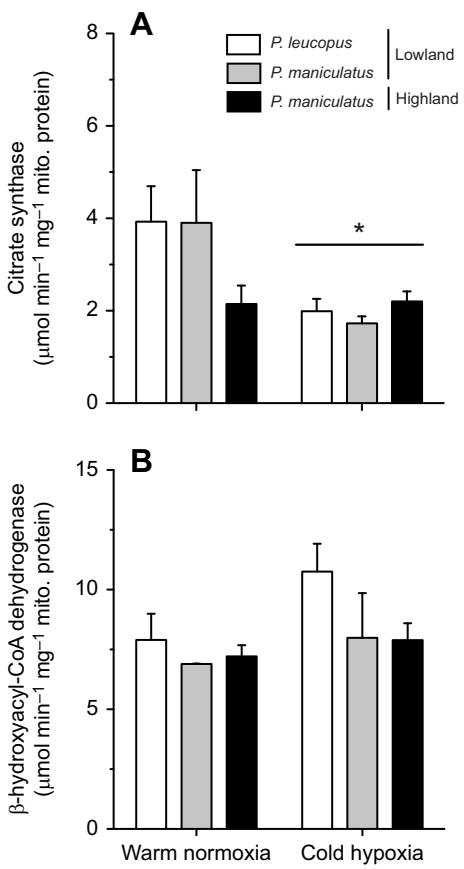

Fig. 5. Apparent V_{max} of citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (HOAD) of isolated mitochondria from *P. leucopus* and *P. maniculatus*. Enzyme activity of (A) CS and (B) HOAD in isolated mitochondria. Sample sizes for warm normoxia and cold hypoxia, respectively, were *N*=3 and *N*=3 for lowland deer mice, *N*=9 and *N*=5 for white-footed mice, and *N*=7 and *N*=10 for highland deer mice. For HOAD there was an effect of acclimation that approached statistical significance ($F_{1,28}$=3.726, *P*=0.064). For CS there was a significant effect of acclimation (*$F_{1,28}$=3.298, *P*=0.008), and a nearly significant population×acclimation interaction ($F_{2,28}$=2.838, *P*=0.076). Values presented as means±s.e.m.

Indeed, descendants of lowland and highland deer mice born and raised in common laboratory conditions had equivalent NST capacities prior to acclimation (Fig. 1; Velotta et al., 2016). It is well recognized that cold acclimation increases NST capacity by ∼40% in lowland mammals, including laboratory mice and deer mice (Beaudry and McClelland, 2010; Oufara et al., 1987; Van Sant and Hammond, 2008). However, the addition of chronic hypoxia exposure can blunt the effect of cold on thermogenic capacity and NST (Beaudry and McClelland, 2010). Our data show that highland deer mice have overcome the antagonistic effect that hypoxia has on the plasticity of BAT-based heat production, with a distinct response to the combination of stressors experienced at high elevations. Here, we show that iBAT also undergoes phenotypic plasticity in response to CH with increased rates of NST that would enable highland mice to cope with the cold conditions at altitude.

Increases in NST capacity seen in highland mice with CH were not associated with an increase in iBAT mass, in contrast to changes in the size of this tissue that other species experience with seasonal changes in temperature (e.g. *Apodemus flavicollis*, Klaus et al., 1988). Acclimation to cold alone is known to induce BAT growth in rodents (Bukowiecki et al., 1982; Nedergaard et al., 1980; Smith and Roberts, 1964), but when combined with hypoxia we found any potential growth was inhibited in deer mice and white-footed mice (Fig. 2). This is consistent with data from laboratory mice, where acclimation to equivalent CH conditions did not influence absolute iBAT mass (Beaudry and McClelland, 2010). Limiting BAT growth may be beneficial in hypoxic environments to minimize the metabolic costs associated with maintaining a large and metabolically active tissue. Instead, what may underlie the CH-stimulated increase in NST are changes in the capacity of brown adipocytes for heat production. Consistent with this idea, wild highland deer mice show 1.5-fold higher *Ucp1* gene expression in iBAT compared with lab-reared descendants (Velotta et al., 2016). Furthermore, we found that lab-reared highland deer mice increased UCP-1 protein expression and had greater UCP-1-stimulated mitochondrial respiration after CH acclimation. The benefit of increased UCP-1 expression is that it provides a higher capacity for uncoupled respiratory capacity, without affecting basal proton leak (Shabalina et al., 2010). Interestingly, basal respiration, presumably to support proton leak independent of UCP-1 stimulation, tended to be lower in highland deer mice compared with lowland deer mice (Table 1). Therefore, iBAT thermogenic capacity is increased to support higher NST rates, with limited effect on energy expenditure when mice are not actively thermoregulating. These changes, along with increased expression of genes involved in BAT vascularization (Velotta et al., 2016) and an enhanced systemic blood flow (Tate et al., 2017, 2020), contribute to the increased capacity for NST seen in CH acclimated highlanders.

The environmentally induced plasticity of NST at high altitude likely contributes to the high overall thermogenic capacity seen in highland deer mice (Cheviron et al., 2012, 2014). NST is known to contribute >50% of total thermogenic capacity in rodents (e.g. *Phyllotis xanthopugus*, Nespolo et al., 1999; *P. maniculatus*, Van Sant and Hammond, 2008). The rates of NST for WN acclimated deer

mice and white- footed mice contributed 35–57% of previously reported thermogenic capacity determined in hypoxia (Tate et al., 2020). After CH acclimation, lowland mice generally showed a decline in the contribution of NST to total thermogenesis, but highland deer mice showed an increase in the relative contribution of NST. The adaptive significance of increased NST reliance with CH acclimation in highland deer mice is presently unclear. While NST may be a more efficient method of heat production than shivering, highland deer mice have a more oxidative muscle phenotype relative to lowlanders (Lui et al., 2015), presumably for high rates of shivering. However, CH acclimation does not influence the aerobic phenotype of gastrocnemius muscle in highland deer mice (Mahalingam et al., 2020), suggesting that the capacity for shivering remains unchanged. While high alpine environments are colder than low altitude sites of the same latitude, they still experience seasonal and daily temperature variation. Thus, the evolved plasticity of NST in response to CH may be essential for further increases in thermogenic capacity during the coldest parts of the year at high altitude.

Similarly to other rodent species, our lowland native *Peromyscus* spp. showed no increase in NST with CH (e.g. Beaudry and McClelland, 2010), suggesting this may be a conserved response for lowland native mice. The mechanistic underpinnings of the antagonistic effect of hypoxia on cold in lowlanders is unclear, although there are several possibilities. The hypothalamus integrates ambient and body temperature information, activating both shivering and NST when required. Hypoxia tends to lower the body temperature setpoint in the hypothalamus of rodents, making them less responsive to cold temperatures (Tattersall and Milsom, 2009). Hypoxia has also been shown to inhibit adrenergic signaling in lowland rats (Voelkel et al., 1981), which is a requisite for BAT recruitment (Cannon and Nedergaard, 2004). Together, these data may explain the resistance of NST and iBAT phenotype to CH acclimation in lowland natives if BAT activation is generally inhibited. Previous studies on adrenergic signaling in hypoxia have focused primarily on cardiac function (León-Velarde et al., 2001; Voelkel et al., 1981) and the effects of hypoxia on adrenergic signaling in BAT during hypoxia have not been explored. Moreover, active BAT requires appropriate O_2 and fuel delivery (Bartelt et al., 2011; Heim and Hull, 1966). Highland deer mice maintain higher arterial O_2 saturation than lowlanders in hypoxia (Tate et al., 2017), suggesting that highlanders can deliver sufficient O_2 to activated BAT in hypoxia while lowlanders may not. Together, these traits likely allow for a robust environmentally induced increase in NST and thermogenic capacity necessary to survive the harsh high alpine conditions.

Conclusions

Our results suggest that evolved plasticity of thermogenic capacity is important for survival in adult deer mice at high altitude, consistent with previous reports (Cheviron et al., 2014; Tate et al., 2020; Velotta et al., 2016). Here, we show that highland deer mice have an enhanced plasticity to cold hypoxia that increases the capacity for NST. Skeletal muscles of highland deer mice show constitutively higher aerobic capacities compared with lowland mice, and this aerobic phenotype is resistant to hypoxia (Lui et al., 2015; Mahalingam et al., 2017) and CH (Mahalingam et al., 2020) acclimation. This suggests that plasticity of whole animal thermogenic capacity in highland mice is principally the result of changes in NST rather than shivering. Our data also suggest that it is an evolved plasticity of iBAT function that contributes to enhanced rates of NST-based heat production at high altitude.

In this study, we have found that highland deer mice increase NST capacity with CH acclimation by functionally remodeling iBAT mitochondria. However, the convergence in iBAT phenotype between mice from different altitudes after CH acclimation suggests that iBAT phenotype cannot completely explain changes in whole-animal thermogenesis. Instead, differences may lie at the integrated whole-tissue or whole-animal level. A greater capacity for tissue O_2 delivery and blood O_2 carrying capacity of highland deer mice in hypoxia may underly some of these mechanisms. The phenotypic plasticity of BAT and NST observed in high altitude deer mice contributes to high thermogenic capacity, which allows them to be active in high altitude conditions and invade an ecological niche inaccessible to other mice. Future areas of study to determine the proximate mechanism behind this enhanced plasticity include comparisons of thermogenic function in intact brown adipocytes between acclimations, fuel provisioning to BAT mitochondria *in vivo* and contributions to NST from other depots of BAT and other tissues, such as beige fat.

Acknowledgements

The authors thank N. Dawson for technical assistance with enzyme assays and G. Scott for use of his Oroboros Oxygraph-2k.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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

Funding

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

Data availability

Data are available from figshare: https://doi.org/10.6084/m9.figshare.14357297.v1

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