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RESEARCH ARTICLE

Thermogenesis is supported by high rates of circulatory fatty acid and triglyceride delivery in highland deer mice

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ABSTRACT

Highland native deer mice (*Peromyscus maniculatus*) have greater rates of lipid oxidation during maximal cold challenge in hypoxia (hypoxic cold-induced $\dot{V}_{O_2,max}$) compared with their lowland conspecifics. Lipid oxidation is also increased in deer mice acclimated to simulated high altitude (cold hypoxia), regardless of altitude ancestry. The underlying lipid metabolic pathway traits responsible for sustaining maximal thermogenic demand in deer mice is currently unknown. The objective of this study was to characterize key steps in the lipid oxidation pathway in highland and lowland deer mice acclimated to control (23°C, 21 kPa O_2) or cold hypoxic (5°C, 12 kPa O_2) conditions. We hypothesized that capacities for lipid delivery and tissue uptake will be greater in highlanders and further increase with cold hypoxia acclimation. With the transition from rest to hypoxic cold-induced $\dot{V}_{O_2,max}$, both highland and lowland deer mice showed increased plasma glycerol concentrations and fatty acid availability. Interestingly, acclimation to cold hypoxia led to increased plasma triglyceride concentrations at cold-induced $\dot{V}_{O_2,max}$, but only in highlanders. Highlanders also had significantly greater delivery rates of circulatory free fatty acids and triglycerides due to higher plasma flow rates at cold-induced $\dot{V}_{O_2,max}$. We found no population or acclimation differences in fatty acid translocase (FAT/CD36) abundance in the gastrocnemius or brown adipose tissue, suggesting that fatty acid uptake across membranes is not limiting during thermogenesis. Our data indicate that circulatory lipid delivery plays a major role in supporting the high thermogenic rates observed in highland versus lowland deer mice.

KEY WORDS: *Peromyscus maniculatus*, Cold, Hypoxia, Plasma flow, FAT/CD36, FABP

INTRODUCTION

The constant cold of high altitude places a high demand for aerobic heat production on mammals living in these environments. Small highland native endotherms must also contend with the high rates of heat loss due to their unfavorably large surface area to volume ratios compared with larger species. To counteract the rapid heat loss to the cold high alpine conditions and remain active, these small endotherms must elevate heat production in the face of low environmental oxygen levels. Indeed, field metabolic rates determined for wild highland deer mice (*Peromyscus maniculatus*; Wagner 1845) were higher than those living at low altitude, even during the summer months (Hayes, 1989).

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An elevated cold-induced maximal oxygen consumption in hypoxia (hypoxic cold-induced $\dot{V}_{O_2,max}$, thermogenic capacity) has been associated with increased survival in deer mice living at high altitude (Hayes and O'Connor, 1999). This likely explains why highland deer mice have higher hypoxic cold-induced $\dot{V}_{O_2,max}$ than lowland deer mice (Cheviron et al., 2012). The higher thermogenic capacity observed in highland deer mice is supported by an increased capacity for O_2 delivery to thermo-effector tissues (Tate et al., 2017, 2020). These higher rates of O_2 delivery support higher rates of substrate oxidation, with lipids being the principal fuel for thermogenesis in these mice (Cheviron et al., 2012; Lyons et al., 2021). Indeed, the rates of lipid oxidation during peak thermogenesis in highland deer mice are higher than during exercise (Lau et al., 2017) and the highest observed for mammals (Lyons et al., 2021). However, the underlying mechanisms that allow for these high rates of lipid oxidation to support heat production are currently unclear.

In eutherian mammals such as deer mice, facultative thermogenesis occurs by two main metabolic processes: shivering thermogenesis and non-shivering thermogenesis (NST). Higher hypoxic cold-induced $\dot{V}_{O_2,max}$ in highland versus lowland deer mice are the result of higher rates of both shivering and NST (Robertson and McClelland, 2019; Coulson et al., 2021) and increases in thermogenic capacity are accompanied by increases in lipid oxidation (Lyons et al., 2021). Moreover, only highlanders demonstrate higher rates of NST in response to the chronic cold hypoxia experienced at high altitude (Velotta et al., 2016; Coulson et al., 2021), suggesting that the capacity for substrate oxidation is also affected by chronic cold hypoxia. The prolonged need for heat production can be maintained using the relative large lipid stores in mammals (Weber, 2011), and highland mice tend to have higher body fat compared with lowlanders (Robertson and McClelland, 2021). Indeed, the more limited carbohydrate stores would likely be depleted in only ∼15–20 min, even at metabolic rates for moderate thermogenesis (McClelland et al., 2017). Interestingly, despite their abundance, lipid use is limited during locomotion, and rates of fatty acid oxidation plateau at moderate intensities of aerobic exercise in mammals (Schippers et al., 2014; Lau et al., 2017). Therefore, the high rates of lipid use for thermogenesis compared with exercise would require deer mice to further increase lipid availability to thermo-effector tissues for oxidation.

The pathway for lipid use is a complex multistep process that can be regulated at many pathway steps, from mobilization from storage depots to oxidation in working tissues. Thermo-effector tissues can rely on lipids delivered by the circulation from extracellular supplies, mainly stored as triglycerides in white adipose tissue (WAT) and circulating triglycerides (TGs), or these tissues can use their intracellular supplies, stored as triglyceride-rich lipid droplets in skeletal muscle and brown adipose tissue (BAT). Circulatory delivery of non-esterified fatty acids (NEFAs) depends on plasma NEFA concentration and the rate of plasma flow to working tissues [plasma flow=cardiac output×(1–hematocrit)] (McClelland et al.,

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1994). Uptake into working muscle and BAT is facilitated by membrane transport through the fatty acid translocase (FAT/CD36), and then through the cytosol by fatty acid binding proteins (FABPs) (reviewed in McClelland, 2004). Indeed, the capacity for circulatory and membrane transport has been shown to correlate with organismal and/or tissue oxidative capacity in mice and other mammals (McClelland et al., 1994; Bonen et al., 2000; Bradley et al., 2012; Templeman et al., 2012). Tissue capacity for oxidation of delivered NEFA depends on mitochondrial volume density and the capacity of mitochondria for β-oxidation of fatty acids. In muscle, fatty acids are transported into mitochondria to provide ATP to power shivering, while in BAT, fatty acids activate uncoupling protein 1 (UCP-1) and provide substrate for powering the futile cycling mechanisms involved with non-shivering thermogenesis. Some, or all, of these steps may be a target of selection for increased lipid use to support enhanced rates of thermogenesis in highland deer mice. Different steps may also respond to cold hypoxia acclimation to alter pathway flux at high altitude.

The objective of this study was to examine key steps of the lipid metabolic pathway that may contribute to the higher thermogenic capacity in hypoxia of highland deer mice compared with their lowland counterparts. We compared first generation laboratory born and raised highland and lowland deer mice acclimated to warm normoxic conditions or to cold hypoxic conditions simulating high altitude. We hypothesize that the differences in lipid oxidation rates during hypoxic cold-induced $\dot{V}_{O_2,max}$ between highland and lowland deer mice are due, in part, to differences in the capacity for mobilization, circulatory delivery and uptake of fatty acids into thermo-effector tissues. We further hypothesize that acclimation to cold hypoxic conditions increases the capacity of the lipid pathway to deliver, uptake and oxidize fatty acids in thermo-effector tissues in highland deer mice.

MATERIALS AND METHODS

Animals and acclimation groups

Wild-caught highland native deer mice (*Peromyscus maniculatus rufinus*) were trapped at high altitude at the summit of Mount Evans, CO, USA (4350 m a.s.l.), while lowland native deer mice (*Peromyscus maniculatus nebracensis*) were trapped at low altitude in Nine-mile Prairie, NE, USA (320 m a.s.l.). Wild mice were transferred to McMaster University (90 m a.s.l.) and housed in common laboratory conditions at ∼23°C, a 12 h:12 h light:dark cycle, with food and water *ad libitum*. Mice were bred within their respective population to produce first generation laboratory born and raised mice. Mice used in this study were a mix of both males and females of at least 6 months of age and highland and lowland individuals were randomly assigned to one of two acclimation groups, warm normoxia (23°C and 760 mmHg) or cold hypoxia (5°C and 480 mmHg), using hypobaric chambers (McClelland et al., 1998; Lyons et al., 2021) in a climate-controlled room, simulating an altitude of ∼4300 m. For acclimation to cold hypoxia, deer mice were first housed at 5°C for 24 h at normobaria before being placed in hypobaric chambers. Mice in cold hypoxia were returned to normobaria in cold for a brief period (less than 1 h per week) for cage cleaning and replenishing food and water. All procedures were approved by the McMaster University Animal Research Ethics Board in accordance with guidelines set by the Canadian Council on Animal Care.

Mouse tissue sampling

Tissues were sampled under one of two conditions: at rest or immediately after mice had reached hypoxic cold-induced $\dot{V}_{O_2,max}$. To sample in resting conditions, deer mice were placed into a ∼600 ml respirometry chamber at their acclimation temperature and allowed to settle for ∼30 min undisturbed as outside air was pushed into the chamber at 600 ml min^{-1} using a mass-flow controller (Sable Systems, Las Vegas, NV, USA). After this adjustment period, 5% isoflurane was flowed into the chamber at 600 ml min^{-1} until the mouse was unconscious, then removed from the chamber for cervical dislocation and decapitation. Mice were also sampled immediately after an hypoxic cold-induced $\dot{V}_{O_2,max}$ trial (conditions described in Lyons et al., 2021). In brief, hypoxic cold-induced $\dot{V}_{O_2,max}$ was determined by pushing heliox (12% O_2, 88% He) at 1000 ml min^{-1} using mass flow meters and controllers (Sierra Instruments, Monterey, CA; MFC-4, Sable Systems, NV) through copper coils housed inside a temperature control cabinet and into a respirometry chamber (∼500 ml) cooled to −10°C. Mice were exposed for ∼15 min until it was determined $\dot{V}_{O_2,max}$ was achieved as previously described (Lyons et al., 2021) before being euthanized by introducing 5% isoflurane into the chamber, followed by cervical dislocation and decapitation for blood and tissue collection.

Blood samples were centrifuged at 10,000 ***g*** to separate plasma from erythrocytes and other blood cells. Plasma was quickly frozen in liquid nitrogen and then stored at −80°C until analysis. Other tissues were sampled within 5 min of euthanasia in the following order, right gastrocnemius, right soleus, interscapular BAT (iBAT), right inguinal white adipose tissue (ingWAT), left ingWAT and left gastrocnemius. The right gastrocnemius, right soleus and right ingWAT of mice sampled at rest were set aside for experiments described below. The rest of the tissues were quickly frozen between two aluminium plates cooled in liquid nitrogen and then stored at −80°C until future measurements.

White adipose tissue lipolytic capacity

Lipolytic rate of WAT was assessed *in vitro* using methods reported previously (Price et al., 2008, 2013) with some modifications. Briefly, fresh ingWAT was divided in two equal pieces, both were individually weighed, and then added to separate beakers containing 20 µl mg^{-1} of Krebs Ringer buffer (in mmol l^{-1}: 119.78 NaCl, 15 $NaHCO_3$, 9.99 D-glucose, 4.56 KCl, 2.5 $CaCl_2$ dihydrate, 1.5 sodium phosphate monobasic, 0.70 sodium phosphate dibasic, 0.49 $MgCl_2$ and 4% w/v bovine serum albumin, pH 7.4) kept at 37°C. The tissue was minced with scissors and then the contents of each beaker was transferred to a separate 20 ml glass vial. In one vial norepinephrine (Millipore Sigma, St Louis, MO, USA) was added to a final concentration of 1 µmol l^{-1} (Raclot and Groscolas, 1993), the other vial received no norepinephrine and acted as an unstimulated control. Both control and norepinephrine treated vials were incubated at 37°C in a shaking water bath for 90 min. At the end of the incubation, the solutions were passed through glass microfibre filters (VWR, West Chester, PA, USA) and the filtrate was stored at −80°C for later determination of glycerol concentration. Glycerol concentration was measured using a serum triglyceride determination kit (Sigma-Aldrich) according to the instructions provided by the company. Briefly, 10 µl of each filtrate was mixed with 250 µl of glycerol free reagent and absorbance was determined at 540 nm using a Spectromax Plus 384, 96-well microplate reader (Molecular Devices, Sunnyvale, CA).

Plasma glycerol, triglycerides and fatty acids

Concentrations of plasma glycerol and triglycerides were assessed using a triglyceride quantification assay kit (Sigma-Aldrich). Non-esterified fatty acid concentrations were determined by gas

chromatography as previously described for deer mice (Lyons et al., 2021).

Sample preparation for western blotting and ELISA

The protein expression of FAT/CD36 and FABP were determined using gastrocnemius muscle cytosolic and sarcolemmal fractions prepared as previously described (Templeman et al., 2012), with some minor modifications. Frozen tissue samples were powdered using a liquid N_2 cooled mortar and pestle and ∼70 mg of tissue was homogenized using a glass-on-glass homogenizer in buffer (5 µl mg^{-1} tissue) containing (in mmol l^{-1}): 30 Hepes, 210 sucrose, 2 EGTA, 40 NaCl and protease inhibitor cocktail (0.75× volume). The homogenate was then centrifuged at 600 ***g*** for 10 min at 4°C, and the resulting supernatant was centrifuged at 10,000 ***g*** for 20 min at 4°C. The resultant supernatant was diluted (0.75× volume) with a buffer containing 1.167 mol l^{-1} KCl and 58.3 mmol l^{-1} Na_4PPi at pH 7.4 and centrifuged at 230,000 ***g*** for 2 h at 4°C. The supernatant was collected as the cytosolic fraction and stored at −80°C, while the resulting pellet was resuspended in a buffer containing 10 mmol l^{-1} Tris-HCl and 1 mmol l^{-1} EDTA, at pH 7.4. This suspension was combined with 16% SDS (0.33× volume) and centrifuged at 1100 ***g*** for 20 min at room temperature, with the resulting supernatant collected as the sarcolemmal fraction and stored at −80°C.

Tissue expression of FAT/CD36

Western blotting was used to measure protein abundance of FAT/CD36 in whole tissue homogenates of gastrocnemius muscle and iBAT, as well as the sarcolemmal fraction of the gastrocnemius from deer mice sampled immediately after hypoxic cold-induced $\dot{V}_{O_2,max}$. In brief, frozen gastrocnemius and BAT samples were powdered under liquid N_2 as described above and homogenized in ice-cold RIPA buffer (in mmol l^{-1}: 150 NaCl, 50 Tris-HCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, pH 8.0). Total homogenate protein concentrations were quantified using Bradford assay (Bio-Rad Laboratories Ltd, Mississauga, ON). To avoid interference of SDS in protein quantification, sarcolemmal fraction protein was quantified using BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). A total of 40 µg for gastrocnemius, 20 µg for BAT protein and 30 µg for gastrocnemius sarcolemmal fraction proteins were denatured by heating to 95°C for 5 min in Laemmli sample buffer (Bio-Rad) with β-mercaptoethanol. Proteins were separated on 12% SDS-PAGE gels and then transferred to a PVDF membrane using the Transblot Turbo Transfer System (Bio-Rad). Membranes were incubated overnight at 4°C with 5% skimmed milk in 1× phosphate buffered saline, 0.1% Tween. On the following day, membranes were probed with primary antibody against FAT/CD36 (CD36 polyclonal antibody, Invitrogen, PA-16813) at a dilution of 1:500 for 1 h at room temperature, followed by an incubation with HRP-conjugated secondary antibody (goat anti-rabbit, Invitrogen, cat. #31466) at a dilution of 1:5000 for 1 h at room temperature. Band densities were detected by chemiluminescence and normalized to total lane protein determined using Coomassie Blue. Images were taken and analyzed using a ChemiDoc MP Imaging System (Bio-Rad) and the Image Lab software package (Bio-Rad), respectively (Lui et al., 2015).

Fatty acid binding protein

Concentration of H-FABP in the cytosolic fractions of gastrocnemius muscles was quantified using an enzyme-linked immunosorbent assay (ELIZA), similar to methods described in Templeman et al. (2012). Equal concentrations of total cytosolic protein (0.015 µg) were assayed for each sample, using a commercial H-FABP ELIZA kit for mice (HK413; Hycult Biotech, Uden, Netherlands). H-FABP protein levels are expressed relative to total cytosolic protein content.

Intramuscular triglycerides

Intramuscular triglyceride (IMTG) concentration was determined in the gastrocnemius muscle as described previously (McClelland et al., 1999). Briefly, ∼25–50 mg of powdered frozen muscle was weighed and placed in a glass tube containing 30 µl mg^{-1} of Folch (1:1 chloroform: methanol; Folch et al., 1957) and homogenized using a power homogenizer. Tubes were then shaken for 20 min, followed by a centrifugation at 1811 ***g*** for 10 min at room temperature. The resulting supernatant was then transferred to a new glass tube, while the pellet was washed with 1 ml of Folch and centrifuged again at 1811 ***g***. The supernatants were then combined, followed by the addition of chloroform (0.5× volume), bringing the ratio to 2:1 chloroform: methanol. Next, 26.8 mmol l^{-1} KCl was added (0.25× volume) and the mixture was centrifuged at 2500 rpm for 10 min at room temperature. The aqueous phase (top layer) was discarded, and 2 ml ethanol was added to the organic phase (bottom layer). The mixture was then evaporated completely under N_2 gas at 40°C and resuspended in 250 µl of isopropanol. IMTG concentration was then assessed using the triglyceride quantification assay kit (Sigma-Aldrich).

Enzyme apparent V_{max}

We measured the apparent maximal activities (V_{max}) of β-hydroxyacyl-CoA dehydrogenase (HOAD), citrate synthase (CS) and cytochrome *c* oxidase (COX) in the gastrocnemius. Activities of HOAD and COX were measured on fresh homogenates, while CS activity was measured after homogenates had been frozen and thawed three times. Approximately 30 mg of powdered tissue was homogenized on ice using a glass-on-glass homogenizer in 20 volumes of homogenizing buffer containing 100 mmol l^{-1} potassium phosphate (pH 7.2), 5 mmol l^{-1} EDTA and 0.1% Triton X-100. Assays were performed at 37°C in triplicate and controls for background activities were determined for each assay by omitting substrate. Assay conditions were as follows: COX: 0.1 mmol l^{-1} of reduced cytochrome *c* (omitted in control) in 100 mmol l^{-1} K_2HPO_4 (pH 7.0) at an absorbance of 550 nm. HOAD: 0.1 mmol l^{-1} acetoacetyl-CoA (omitted in control) and 0.28 mmol l^{-1} NADH in 100 mmol l^{-1} triethanolamine·HCl (pH 7.0) at an absorbance of 340 nm. CS: 0.5 mmol l^{-1} oxaloacetate (omitted in control), 0.22 mmol l^{-1} acetyl-CoA, and 0.1 mmol l^{-1} dithiobisnitrobenzoic acid (DTNB) in 40 mmol l^{-1} Tris-HCl (pH 8.0) at an absorbance of 412 nm.

The V_{max} of carnitine palmitoyltransferase (CPT) was measured on isolated mitochondria from freshly dissected gastrocnemius. Isolation of mitochondria from deer mouse muscle has been previously described in Mahalingam et al. (2020). Protein content was measured using a standard Bradford Assay (Bio-Rad). Total CPT activity was measured at 412 nm in the following assay conditions: 5 mmol l^{-1} L-carnitine (omitted in control), 2 mmol l^{-1} palmitoyl-CoA, and 0.2 m DTNB in 100 mmol l^{-1} K_2PO_4 (pH 7.2).

In vitro soleus fatty acid oxidation

The rate of fatty acid oxidation was measured in soleus muscle by tracking the metabolic fate of a radiolabelled fatty acid (Steinberg and Dyck, 2000). In brief, 309.5 mmol l^{-1} $NaHCO_3$ was gassed for 1 h with 100% CO_2 and was then diluted (5.25× volume) with modified Krebs Henseleit buffer (in mmol l^{-1}: 148 NaCl, 5.94 KCl,

Table 1. Body mass and inguinal white adipose tissue (ingWAT) mass of highland and lowland deer mice (*Peromyscus maniculatus*), acclimated to control warm normoxia (23°C, 21 kPa O_2) or cold hypoxia (5°C, 12 kPa O_2) conditions

	Highland		Lowland	
	Warm normoxia	Cold hypoxia	Warm normoxia	Cold hypoxia
Body mass (g)	22.6±1.1*	20.5±1.0*	20.5±2.8	16.2±0.7
IngWAT mass (mg)	245.1±52.0*	194.0±19.1*	113.4±14.6	67.7±8.0
IngWAT g^{-1} body mass ($\times10^3$)	10.1±1.7*	9.6±0.9*	6.7±1.2	4.3±0.6

Values are presented as means±s.e.m. Respective sample sizes for warm normoxia and cold hypoxia were *N*=12 and *N*=9 for highlanders, and *N*=4 and *N*=6 for lowlanders. *Significant differences (*P*<0.05) resulting from Holm–Šídák *post hoc* tests compared with lowland deer mice within an acclimation condition.

3.17 $CaCl_2{\cdot}2H_20$, 1.49 KH_2PO_4 and 1.49 $MgSO_4{\cdot}7\ H_20$, containing 4% w/v fatty acid-free bovine serum albumin (Sigma), 2 mmol l^{-1} pyruvate and 0.5 mmol l^{-1} palmitate, pH 7.4) and gassed (95% O_2, 5% CO_2) at 30°C for 1 h, creating the incubation buffer. The right soleus was removed fresh, weighed, and placed in a 20 ml vial containing 2 ml incubation buffer for 20 min. The soleus was then transferred to second vial containing 2 ml incubation buffer for another 20 min incubation. After the second preincubation, the soleus was transferred to a vial consisting of 0.4 µCi [1-^{14}C]-palmitic acid (Amersham Biosciences, Baie d'Urfé, Quebec), and an open 0.8 ml microcentrifuge tube filled with 400 µl benzethonium hydroxide was placed in the same vial, which was then sealed for 60 min. The soleus was then removed, followed by the addition of 1 ml of 1 mol l^{-1} acetic acid to the vial and immediately capped. The acetic acid added to the vial released $^{14}CO_2$ from the buffer solution, which was collected in the benzethonium hydroxide of the microcentrifuge tube for 60 min. The benzethonium hydroxide was then transferred to a clean scintillation vial with 5 ml scintillation fluid and the counts per minute (CPM) of ^{14}C were measured for 5 min using a Tricarb 2900 TR liquid scintillation analyzer using QuantaSmart 1.31 (Packard Instrument) analysis software.

Statistics

Statistics were performed using the lme4 package (https://CRAN.R-project.org/package=lme4) in R v.4.0.0 (https://www.r-project.org/). We used a 2-way analysis of variance (ANOVA) to assess the main effects of deer mouse population and acclimation environment, and to assess the main effects of population and condition (rest versus $\dot{V}_{O_2,max}$). We also used a 3-way ANOVA to assess the interactions between deer mouse population, acclimation and activity condition, where appropriate. Pairwise Holm–Šídák *post hoc* tests were performed to assess significant interactions. All data are presented as means±s.e.m. A statistical significance value was set at $P<0.05$.

RESULTS

Fat lipolytic capacity and storage

Fat stores were assessed by quantifying the mass of ingWAT relative to body mass. Highland deer mice were heavier (body mass, $F_{1,27}=15.10$, $P<0.001$) and had greater absolute amounts of ingWAT ($F_{1,27}=8.90$, $P=0.01$) compared with lowland deer mice. When expressed relative to body mass, highlanders had 1.8-fold greater ingWAT per g body mass compared with lowland deer mice ($F_{1,27}=8.28$, $P<0.01$). Cold hypoxia acclimation had no effect on body mass ($F_{1,27}=2.67$, $P=0.11$), ingWAT mass ($F_{1,27}=1.28$, $P=0.27$) or relative ingWAT abundance ($F_{1,27}=0.51$, $P=0.56$) in either population (Table 1).

The capacity for WAT to mobilize fatty acids was assessed by measuring the rate of glycerol release of ingWAT *in vitro*. When lipolysis was maximally stimulated by the addition of norepinephrine, there were no significant effects of either population ($F_{1,33}=1.02$, $P=0.32$) or acclimation ($F_{1,33}=0.08$, $P=0.78$) on ingWAT lipolytic capacity (Fig. 1).

Lipid concentrations and circulatory transport rates

Plasma glycerol concentrations were determined as an index of whole-body lipolysis *in vivo*. Plasma glycerol concentrations were approximately 3-fold greater in deer mice after a maximal thermogenic challenge compared with levels in mice at rest ($F_{1,73}=178.35$, $P<0.001$), independent of acclimation condition ($F_{1,73}=3.35$, $P=0.07$). Furthermore, lowland deer mice had significantly greater plasma glycerol concentrations compared with highland deer mice in warm normoxia acclimation conditions (significant population effect; $F_{1,39}=14.68$, $P<0.001$). Cold hypoxia acclimation led to an increase in resting plasma glycerol concentrations in both populations ($F_{1,46}=16.01$, $P<0.001$) and in plasma glycerol concentrations at $\dot{V}_{O_2,max}$ ($F_{1,46}=10.03$, $P=0.004$), but no significant population differences were observed ($F_{1,34}=0.35$, $P=0.56$) (Fig. 2A).

To calculate rates of circulatory transport of plasma lipids we determined the concentrations of plasma NEFA and TGs at rest and immediately after hypoxic cold-induced $\dot{V}_{O_2,max}$. Overall, we found that plasma NEFA concentrations doubled from rest to cold-induced $\dot{V}_{O_2,max}$ ($F_{1,55}=158.04$, $P<0.001$). This occurred in both populations and regardless of acclimation conditions (Fig. 2B). This increase in

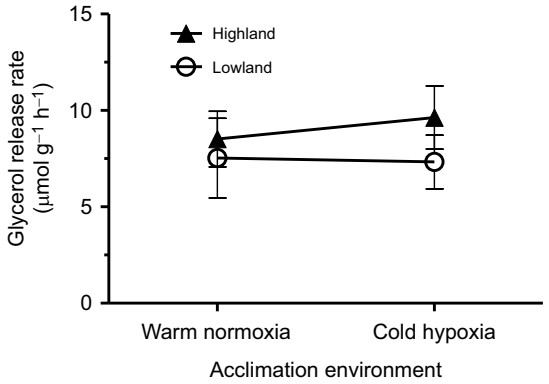

Fig. 1. Rate of glycerol release from inguinal white adipose tissue (ingWAT) in first-generation laboratory born and raised highland and lowland deer mice (*Peromyscus maniculatus*). Deer mice were acclimated to control warm normoxia (23°C, 21 kPa O_2) or cold hypoxia (5°C, 12 kPa O_2) conditions and excised ingWAT was stimulated with 1 µmol l^{-1} norepinephrine and the rate of lipolysis was determined *in vitro* by measuring the rate of glycerol appearance into incubation medium (µmol g^{-1} h^{-1}). Sample sizes for warm normoxia and cold hypoxia were *N*=9 and *N*=9 for highlanders, and *N*=9 and *N*=10 for lowlanders, respectively. Data are reported as means±s.e.m.

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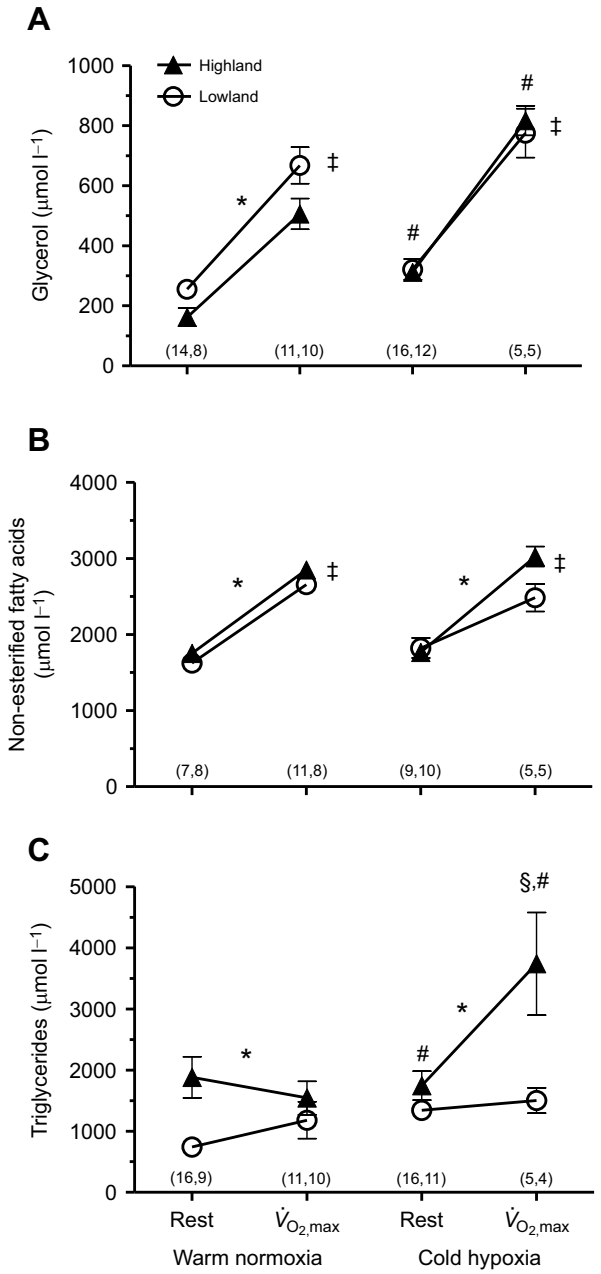

Fig. 2. Plasma concentrations of glycerol and lipids in highland and lowland deer mice. Concentration of glycerol (A), non-esterified fatty acids (NEFAs) (B) and triglycerides (TGs) (C) in first-generation laboratory born and raised highland and lowland deer mice (*Peromyscus maniculatus*), acclimated to control warm normoxia or cold hypoxia conditions, sampled at rest or at cold-induced $\dot{V}_{O_2,max}$. Symbols represent significant differences resulting from Holm–Šídák *post hoc* tests (P<0.05). ‡Significant difference between rest and cold-induced $\dot{V}_{O_2,max}$. *Significant difference between populations. #Significant difference between acclimations. §Cold hypoxia acclimated highland deer mice are significantly different from all other groups. Sample sizes of both deer mouse populations are shown in parentheses underneath symbols within a testing condition (highland, lowland). Data are reported as mean±s.e.m.

total plasma NEFA was the result of significant increases in all the individual fatty acids measured (P<0.05), apart from oleic acid, which did not show a significant increase from resting levels ($F_{1,55}$=3.62, P=0.062) (Table 2). In general, highlanders displayed higher concentrations of total NEFA compared with lowlanders ($F_{1,55}$=9.26, P=0.004) and there was a tendency for NEFAs to increase after cold hypoxia acclimation that approached statistical significance ($F_{1,55}$=3.77, P=0.057).

We also found that plasma TG concentrations were significantly higher in highland compared to lowland deer mice ($F_{1,74}$=12.70, P<0.001). Cold hypoxia acclimation increased circulating TG concentrations in both populations ($F_{1,74}$=4.08, P=0.047). Interestingly, lowland deer mice showed no change in plasma TG with the transition from rest to cold-induced $\dot{V}_{O_2,max}$, but cold hypoxia highland deer mice showed a doubling in plasma TG as energy demand increased (significant population×condition interaction; $F_{1,32}$=6.06, P=0.02) (Fig. 2C).

The rate of circulatory substrate delivery is the product of their concentration and rate of plasma flow (McClelland et al., 1994). Thus, we determined rates of NEFA and TG circulatory delivery by calculating rates of plasma flow using published data for cardiac output and hematocrit during hypoxic cold-induced $\dot{V}_{O_2,max}$ for deer mice in the same acclimation conditions (Tate et al., 2020). Highland deer mice had significantly greater plasma flow rates compared with lowlanders ($F_{1,13}$=13.60, P=0.003) and cold hypoxia acclimation significantly increased plasma flow rates ($F_{1,13}$=13.14, P=0.003) during hypoxic cold-induced $\dot{V}_{O_2,max}$ (Fig. 3A). This led to significantly greater rates of NEFA ($F_{1,13}$=17.53, P=0.001) and TG delivery ($F_{1,13}$=20.23, P <0.001) in highland deer mice compared with lowlanders. Similar to the effect of acclimation on plasma flow, cold hypoxia significantly increased NEFA and TG delivery rates ($F_{1,13}$=14.21, P=0.002, $F_{1,13}$=40.49, P<0.001) during hypoxic cold-induced $\dot{V}_{O_2,max}$ (Fig. 3B,C). There was also a significant population×acclimation interaction, where cold hypoxia acclimation led to a doubling of NEFA delivery rates and tripling of TG delivery rates in highlanders, but not lowlanders ($F_{1,13}$=5.54, P=0.035; $F_{1,13}$=18.00, P<0.001, respectively) (Fig. 3B,C).

Intramuscular triglycerides

The concentrations of IMTG in gastrocnemius muscle were not significantly different between populations ($F_{1,65}$=0.27, P=0.61) and did not change with acclimation ($F_{1,65}$=0.91, P=0.34). Interestingly, there were also no decline in gastrocnemius triglyceride content between rest and cold-induced $\dot{V}_{O_2,max}$ ($F_{1,65}$=2.53, P=0.12) (Fig. 4).

Fatty acid transport proteins

The capacity of thermo-effector tissue for fatty acid uptake was assessed by quantifying the total protein abundance of FAT/CD36 in both iBAT and the gastrocnemius. We found no significant effect of population ($F_{1,16}$=0.12, P=0.734) or acclimation ($F_{1,16}$=0.022, P=0.885) on the abundance of FAT/CD36 in iBAT (Fig. 5).

We also found FAT/CD36 expression in whole tissue homogenates of the gastrocnemius did not show any significant effect of population ($F_{1,19}$=0.042, P=0.841). However, cold hypoxia acclimation led to a significant increased FAT/CD36 protein abundance ($F_{1,19}$=5.674, P=0.028) (Fig. 6A). When sarcolemmal-specific expression of this transporter was assessed in the gastrocnemius at $\dot{V}_{O_2,max}$, we found no significant effect of population ($F_{1,16}$=0.446, P=0.514) or acclimation ($F_{1,16}$=1.162, P=0.297) (Fig. 6B). Furthermore, there were no significant effects of population ($F_{1,17}$=1.18, P=0.29) or acclimation ($F_{1,17}$=0.001, P=0.97) on the abundance of the cytosolic H-FABP in gastrocnemius muscle (Fig. 6C).

Skeletal muscle enzyme activity

To assess any differences in the capacity for aerobic metabolism and for fatty acid oxidation, we determined the apparent V_{max} of specific

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Table 2. Concentrations of individual free fatty acids (μmol l^{-1}) of lowland deer mice (*P. maniculatus*), acclimated to control warm normoxia or cold hypoxia conditions, during rest and cold-induced $\dot{V}_{O_2,max}$

	Highland				Lowland			
	Warm normoxia		Cold hypoxia		Warm normoxia		Cold hypoxia	
	Rest	$\dot{V}_{O_2,max}$	Rest	$\dot{V}_{O_2,max}$	Rest	$\dot{V}_{O_2,max}$	Rest	$\dot{V}_{O_2,max}$
Palmitic acid (16:0)	459±20* (26.2±1.1%)	700±31‡,* (24.6±1.1%)	474±26 (26.8±1.5%)	760.3±37.9‡ (25.1±1.3%)	419±20 (25.8±1.2%)	611±32‡ (23.0±1.2%)	465±35 (25.5±1.9%)	604±39‡ (24.3±1.6%)
Palmitoleic acid (16:1)	135±23* (7.7±1.3%)	178±20‡,* (6.3±0.7%)	150±22 (8.5±1.2%)	191±28 (6.3±0.9%)	181±33 (11.1±2.0%)	253±30‡ (9.5±1.1%)	172±24 (9.4±1.3%)	196±23 (7.9±0.9%)
Stearic acid (18:0)	284±8* (16.2±0.5%)	475±19‡,* (16.7±0.7%)	283±10 (16.0±0.6%)	447±22‡ (14.8±0.7%)	266±13 (16.4±0.8%)	384±21‡ (14.4±0.8%)	277±20 (15.2±1.1%)	392±27‡ (15.8±1.1%)
Elaidic acid (18:1n9t)	223±29* (12.7±1.7%)	351±19‡,* (12.3±0.7%)	195±16 (11.0±0.9%)	401±24‡ (13.3±0.8%)	171±21 (10.5±1.3%)	331±24‡ (12.4±0.9%)	218±22 (11.9±1.2%)	287±33‡ (11.6±1.3%)
Oleic acid (18:1n9c)	110±11 (6.3±0.6%)	106±7 (3.7±0.3%)	121±9 (6.9±0.5%)	110±15 (3.6±0.5%)	148±28 (9.1±1.7%)	117±17 (4.4±0.6%)	148±22 (8.1±1.2%)	98±13 (3.9±0.5%)
Linoleic acid (18:2)	542±23* (30.9±1.3%)	1041±56‡,* (36.5±2.0%)	545±31 (30.8±1.8%)	1118±34‡ (36.9±1.1%)	440±25 (27.1±1.5%)	963±46‡ (36.2±1.7%)	542±57 (29.7±3.1%)	908±70‡ (36.5±2.8%)

Values (μmol l^{-1}) are presented as means±s.e.m. Percentage (%) of specific fatty acid to total NEFA is shown in parentheses underneath. In warm normoxia acclimation, sample sizes for rest and $\dot{V}_{O_2,max}$ were respectively N=7 and N=11 for highland deer mice, and N=8 and N=8 for lowland deer mice, while in cold hypoxia acclimation, sample sizes for rest and $\dot{V}_{O_2,max}$ were respectively N=9 and N=5 for highland deer mice, and N=10 and N=5 for lowland deer mice. Symbols representing significant differences result from Holm–Šídák *post hoc* tests (P<0.05).
‡Significantly different (P<0.05) from resting conditions, within acclimation.
*Significantly different (P<0.05) from lowland deer mice, within acclimation.

Fig. 3. Plasma flow rate and delivery rate of fatty acids and triglycerides at cold-induced $\dot{V}_{O_2,max}$ in hypoxia. Plasma flow rate (A), non-esterified fatty acid (NEFA) delivery rate (B) and triglyceride (TG) delivery rate (C) in highland and lowland deer mice acclimated to control warm normoxia or cold hypoxia conditions, sampled at cold-induced $\dot{V}_{O_2,max}$. Sample sizes for warm normoxia and cold hypoxia were N=7 and N=4 for highlanders, and N=3 and N=3 for lowlanders. Symbols represent significant differences resulting from Holm–Šídák *post hoc* tests (P<0.05). *Significant difference between populations. #Significant difference between acclimations. ‡Cold hypoxia acclimated highlanders are significantly different from all other treatment groups. Data are reported as means±s.e.m. Plasma flow rates were calculated as cardiac output×(1−hematocrit), using previously reported cardiac output and hematocrit for deer mice exposed to the same acclimation conditions (Tate et al., 2017).

enzymes in the gastrocnemius. CPT activity was measured as a marker for fatty acid uptake from the cytosol into the mitochondria. Total CPT activity did not differ between deer mouse populations ($F_{1,28}$=2.47, P=0.13) or with acclimation environments ($F_{1,28}$=1.05, P=0.32) (Fig. 7A). The activity of HOAD, a marker of β-oxidation,

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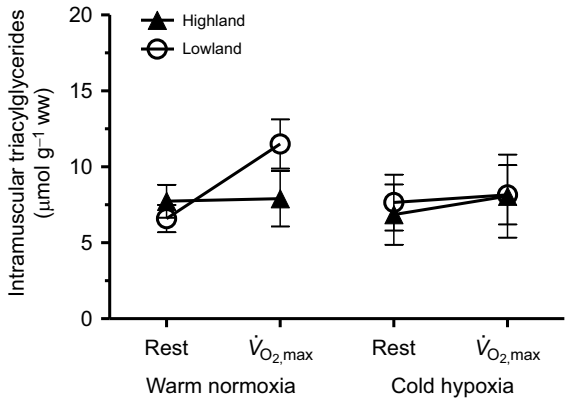

Fig. 4. Intramuscular triglyceride concentration during rest and immediately after cold-induced $\dot{V}_{O_2,max}$. First-generation laboratory born and raised highland and lowland deer mice acclimated to control warm normoxia or cold hypoxia conditions, sampled at rest or immediately after cold-induced $\dot{V}_{O_2,max}$. In warm normoxia acclimation, sample sizes for rest and $\dot{V}_{O_2,max}$ were respectively *N*=16 and *N*=10 for highlanders, and *N*=9 and *N*=9 for lowlanders, while in cold hypoxia acclimation, sample sizes for rest and $\dot{V}_{O_2,max}$ were respectively *N*=9 and *N*=5 for highlanders, and *N*=10 and *N*=5 for lowlanders. Values are presented as μmol of TG g^{-1} of gastrocnemius wet weight (ww). Data are reported as means±s.e.m.

showed no differences between deer mouse populations ($F_{1,30}$ =0.57, *P*=0.46) or acclimation groups ($F_{1,30}$=0.05, *P*=0.83) (Fig. 7B). In contrast, CS activity, a marker for mitochondrial volume, was significantly higher in highland deer mice compared with lowlanders ($F_{1,28}$=5.66, *P*=0.02). However, acclimation had little effect on CS activity ($F_{1,28}$=0.60, *P*=0.45) (Fig. 7C). COX activity, commonly used to assess mitochondrial quality, was significantly higher in cold hypoxia acclimated deer mice ($F_{1,30}$=7.14, *P*=0.01); however, there were no significant population differences ($F_{1,30}$=0.25, *P*=0.62) (Fig. 7D).

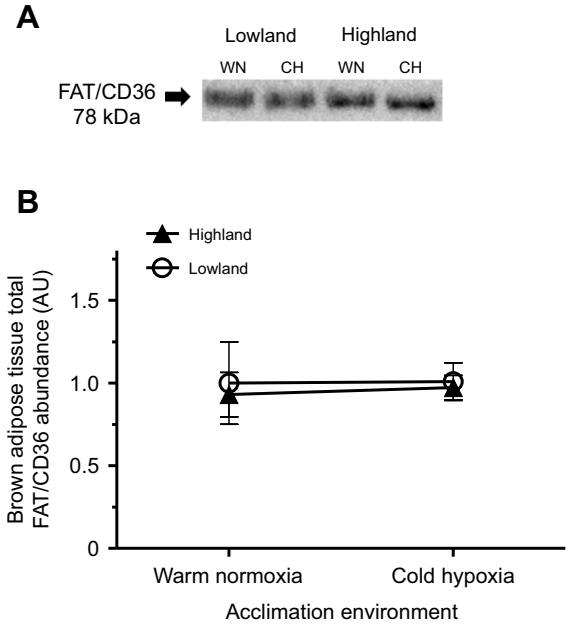

Fig. 5. Relative protein abundance of fatty acid translocase (FAT/CD36) in brown adipose tissue. Representative western blot (A) and plotted protein abundance (B) of FAT/CD36. Sample sizes for warm normoxia (WN) and cold hypoxia (CH) were *N*=5 and *N*=4 for highlanders, and *N*=5 and *N*=6 for lowlanders. Data are reported as means±s.e.m.

Exogenous lipid oxidation

Intact solei were used to measure the rate of exogenous ^{14}C-palmitic acid oxidation. We observed no significant differences between population ($F_{1,29}$=2.63, *P*=0.12) or acclimations ($F_{1,29}$=0.57, *P*=0.46) (Fig. S1).

DISCUSSION

The main objective of this study was to determine if capacities at key steps in the lipid metabolic pathway might explain population differences in maximal lipid oxidation observed at peak thermogenesis in highland and lowland deer mice. Moreover, we assessed whether these steps are modified with acclimation to cold hypoxia simulating the high-altitude environment. Rates of lipid oxidation increased when mice moved from resting to hypoxic cold-induced $\dot{V}_{O_2,max}$ conditions. Both highland and lowland deer mice showed an increase in plasma glycerol and NEFA concentrations after hypoxic cold-induced $\dot{V}_{O_2,max}$, suggesting a significant stimulation of lipolysis and circulatory NEFA availability. Plasma NEFA concentrations were greater in highlanders than lowlanders at both rest and at hypoxic cold-induced $\dot{V}_{O_2,max}$. These population differences may be supported by the greater mass-specific ingWAT depots in highlanders. However, *in vitro* lipolytic capacity of adipocytes from this depot were equivalent in both populations. Furthermore, plasma TGs were also greater in highlanders compared with lowlanders, both at rest and at $\dot{V}_{O_2,max}$, and regardless of acclimation condition. As a result, greater plasma flow rates in highlanders at $\dot{V}_{O_2,max}$ resulted in circulatory NEFA and TG delivery rates that were 3.1- and 7.3-fold greater than in lowlanders, respectively. In contrast, protein abundance of FAT/CD36 in both the gastrocnemius and iBAT did not differ between populations or with acclimation. These data suggest that higher circulatory delivery rates of NEFA and TG to the thermo-effector tissues are primarily responsible for the high rates of lipid oxidation necessary to support the superior hypoxic cold-induced $\dot{V}_{O_2,max}$ observed in highlanders.

Circulatory NEFA and TG availability is dependent on effective convective transport by the cardiovascular system. Circulatory transport of NEFAs is the product of their concentration and plasma flow (McClelland et al., 1994). Highland deer mice have higher plasma flow rates during hypoxic $\dot{V}_{O_2,max}$ than lowlanders (Fig. 3A) as the result of greater cardiac output and a blunted increase in hematocrit in response to cold hypoxia (Tate et al., 2020). During thermogenic $\dot{V}_{O_2,max}$, most of the blood flow is likely directed to BAT and shivering muscle. This high plasma flow would allow highland deer mice to increase NEFA delivery to these thermo-effector tissues. It is possible that acclimation to cold hypoxia changes the relative proportions of blood flow to these tissues. Indeed, when warm acclimated rats are subjected to an acute cold challenge most blood is directed to muscle (Foster and Frydman, 1979), but after cold acclimation there is a redirection of blood flow to BAT (Foster and Frydman, 1979). It is unclear whether the same redistribution of blood flow would occur after cold hypoxia acclimation or with an acute hypoxic cold challenge.

Highland deer mice have larger mass-specific ingWAT depots than lowlanders (Table 1). Given the high energy density of lipids, greater fat stores may be advantageous for sustained periods of heat production (Weber, 2011) and provide additional insulation (Wronska and Kmiec, 2012). However, we have previously found that highland and lowland deer mice have equivalent thermal conductance (Lyons et al., 2021), suggesting that population differences in ingWAT reflect altered energy storage and not insulation. The population differences in WAT depot size were also

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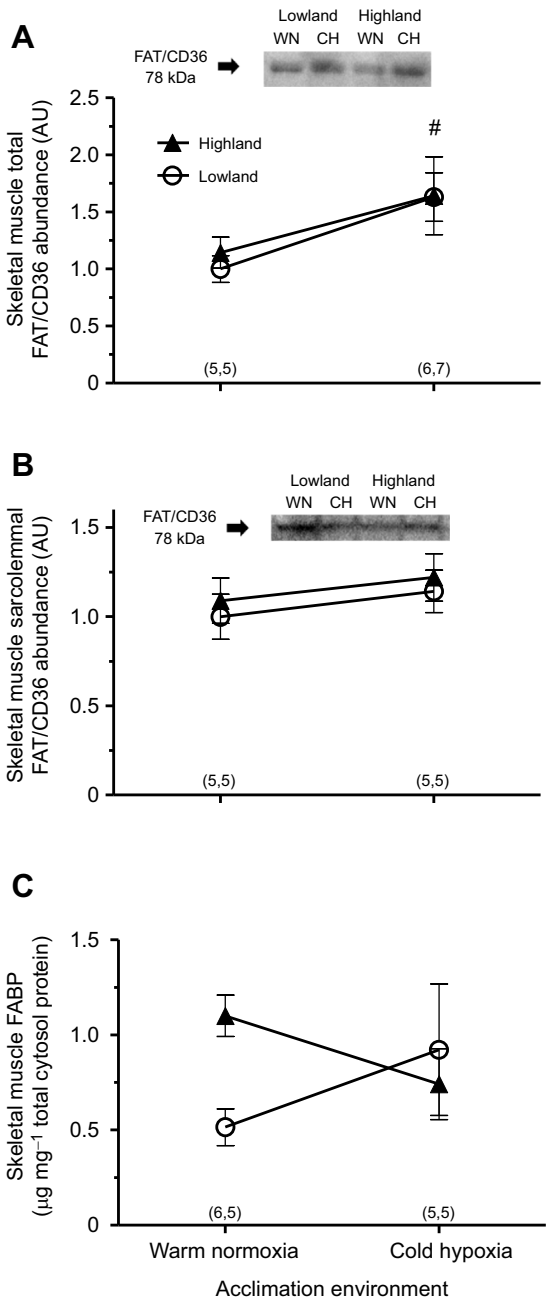

Fig. 6. FAT/CD36 protein expression and fatty acid binding protein (FABP) concentrations in skeletal muscle. (A) FAT/CD36 protein expression in skeletal muscle, (B) sarcolemmal fraction FAT/CD36 protein expression and (C) FABP concentration in the gastrocnemius of highland and lowland deer mice (*Peromyscus maniculatus*), acclimated to control warm normoxia (WN) or cold hypoxia (CH) conditions. Representative western blots are shown (A,B). Symbols represent significant differences resulting from Holm–Šídák *post hoc* tests (P<0.05). #Significant difference between acclimations. Sample sizes of both deer mouse populations are shown in parentheses underneath symbols within an acclimation (highland, lowland). Data are reported as means±s.e.m.

maintained after cold hypoxia acclimation, suggesting that both populations matched food intake to the higher thermogenic demand of the acclimation conditions (Barnett, 1965). This is not the case when cold hypoxia occurred over the early development period, with lowlanders showing a large reduction in fat mass at weaning, while highland pups were able to maintain body composition in these conditions (Robertson and McClelland, 2021). These data suggest that developmental plasticity may have an important influence on the available lipid stores for adult deer mice and may affect thermogenic capacity and/or thermogenic endurance.

Although the size of lipid stores differed between populations, the tissue itself appeared to be phenotypically similar. When the lipolytic capacity of WAT was assessed *in vitro*, we found no differences in the maximal rates of glycerol release in response to norepinephrine stimulation (Fig. 1). However, *in vivo* conditions that stimulate WAT lipolysis and the appearance of NEFAs in the blood may differ between the populations. For example, the release of NEFAs into circulation from WAT is reliant on tissue blood flow and the availability of albumin binding sites (Scow and Chernik, 1970), which may differ between highland and lowland mice. We used circulatory glycerol as a marker for whole-body lipolysis, as glycerol liberated during lipolysis in WAT and skeletal muscle generally appears in the circulation (Weber, 2011), since these tissues lack significant activity of glycerol kinase (Newsholme and Taylor, 1969). Indeed, plasma glycerol showed dynamic changes with the transition from rest to $\dot{V}_{O_2,max}$, which may indicate large increases in lipolysis when maximal aerobic heat production was stimulated (Fig. 2A). However, the highest plasma glycerol concentrations that we measured were similar between highland and lowland deer mice despite significant differences in whole-animal lipid oxidation (Lyons et al., 2021). It is important to note that glycerol turnover and lipid oxidation are not always correlated in mammals (Weber et al., 1993); however, we found that increases in plasma glycerol were associated with increases in plasma NEFAs in deer mice at $\dot{V}_{O_2,max}$, suggesting that the availability of circulatory NEFAs tracks changes in whole-animal lipid oxidation (Fig. 2A,B).

Changes in total plasma NEFA concentrations were likely not the result of differential kinetics in most of the individual fatty acids (Table 2), consistent with results reported for rats undergoing shivering thermogenesis (Vaillancourt et al., 2009). The exception was circulating levels of oleic acid, which remained unchanged as metabolic rate increased (Table 2). This suggests that either mobilization of oleic acid was not induced or release from WAT was matched with an equivalent uptake into heat-producing tissues. Oleic acid is also predominantly found in the sn-2 (middle) position in TGs and is associated with re-esterification. (Karupaiah and Sundram, 2007). Our results may suggest a targeted re-esterification of oleic acid during increased cold exposure in deer mice. An increased availability of NEFA can occur through alterations in the NEFA–TG cycle, where NEFAs are preferentially directed towards oxidation rather than re-esterification (McClelland et al., 2001). This cycle has been observed to change during shivering in rats, where fatty acid re-esterification decreased from 79% to 35% with cold exposure to support the necessary increase in fatty acid oxidation (Vaillancourt et al., 2009). In contrast, hypoxia acclimation has been found to increase the NEFA–TG cycle in lab rats at rest (McClelland et al., 2001), and this accelerated cycling can contribute to thermogenesis by increasing the metabolic rate (Reidy and Weber, 2002). More work needs to be done to better understand how thermogenic demand and oxygen availability influence NEFA–TG cycling in heat production at high altitude.

Along with plasma NEFAs, circulating TGs represent an additional large and often overlooked energy reserve available to support tissue metabolism (Moyes and West, 1995; Magnoni and Weber, 2007; Bartelt et al., 2011). It is possible that circulating NEFAs are not sufficient to sustain the prolonged high rates of lipid oxidation for heat production in mice. However, delivery of NEFAs in the form of TGs could be used by highland deer mice to ensure rapid substrate supply in times of high energy demand. It has been

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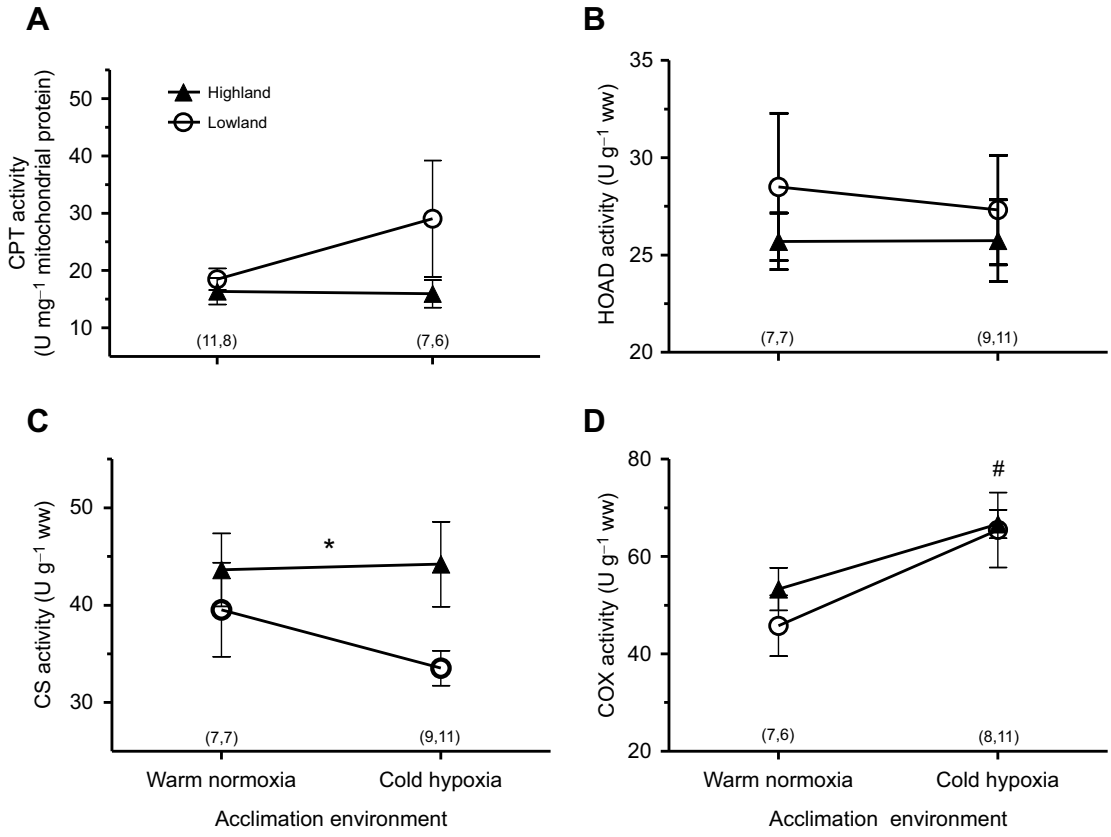

Fig. 7. Apparent maximal enzyme activities (V_{max}) in isolated muscle mitochondria and in the gastrocnemius. V_{max} of carnitine palmitoyl transferase (CPT) in isolated muscle mitochondria (A), and β-hydroxyacyl-CoA dehydrogenase (HOAD) (B), citrate synthase (CS) (C) and cytochrome *c* oxidase (COX) (D) in the gastrocnemius. CPT activity was standardized to mitochondrial protein, and HOAD, CS and COX activities were standardized to g^{-1} wet weight (ww). Symbols represent significant differences resulting from Holm–Šídák *post hoc* tests (P<0.05). *Significant difference between populations. #Significant difference between acclimations. Sample sizes of both deer mouse populations are shown in parentheses underneath symbols within an acclimation (highland, lowland). Data are reported as means±s.e.m.

proposed that circulatory TG may be used by migratory birds to support the extremely high rates of lipid oxidation during prolonged flight (Weber, 2009). The evidence surrounding this phenomenon is currently unclear, with support from research on small migratory passerines (Jenni-Eiermann and Jenni, 1992), but not robins (Gerson and Guglielmo, 2013). Nonetheless, our findings may suggest the high rates of TG circulatory delivery observed in highland deer mice at $\dot{V}_{O_2,max}$ (Fig. 2C, Fig. 3A,C) are used to help support the higher rates of lipid oxidation during thermogenesis compared with lowland deer mice (Lyons et al., 2021). The use of this energy reserve for heat production has been shown to occur in thermoregulating mice where circulatory TGs are cleared by both BAT (Bartelt et al., 2011) and skeletal muscle (Jensen et al., 2008). Mice with elevated lipoprotein lipase show an increase in thermal tolerance during a cold challenge, presumably because of an increased capacity for fat oxidation (Jensen et al., 2008; Bartelt et al., 2011). It is unknown whether lipoprotein lipase abundance or activity is greater in highlanders, enabling them to effectively utilize circulating TGs to assist with fuelling thermogenesis.

We quantified the abundance of FAT/CD36 to determine if capacity for membrane NEFA transport could explain population differences in lipid oxidation at $\dot{V}_{O_2,max}$ (Lyons et al., 2021). While gastrocnemius FAT/CD36 abundance did increase after cold hypoxia acclimation in both populations, there were no differences in FAT/CD36 abundance between the populations (Fig. 6A,B). Furthermore, gastrocnemius FABP content was also the same between highland and lowland deer mice (Fig. 6C). In another hindlimb muscle, the soleus also demonstrated no differences in exogenous lipid oxidation between populations or acclimations (Fig. S1). These results suggest that the capacity for NEFA uptake in shivering muscle may not significantly contribute to population differences observed in whole-animal lipid oxidation during hypoxic cold-induced $\dot{V}_{O_2,max}$ (Lyons et al., 2021). Additionally, we found that IMTG in the gastrocnemius did not change from rest to cold-induced $\dot{V}_{O_2,max}$ (Fig. 4). These data suggest that in deer mice the gastrocnemius primarily relies on circulatory NEFAs to support oxidation during shivering thermogenesis. This higher lipid delivery and greater muscle capillarization (Lui et al., 2015) may be sufficient to support shivering-induced rates of lipid oxidation in the more aerobic gastrocnemii of highland deer mice (Lui et al., 2015; Mahalingam et al., 2017, 2020; Fig. 7C).

At $\dot{V}_{O_2,max}$, NST in BAT has been shown to account for over 50% of total $\dot{V}_{O_2}$ in deer mice (Van Sant and Hammond, 2008). Highland deer mice have been observed to increase NST capacity after cold hypoxia acclimation compared to lowlanders (Coulson et al., 2021). Interestingly, BAT size and mitochondrial density did not explain the increases in NST after cold hypoxia acclimation (Coulson et al., 2021). We found that BAT expression of FAT/CD36 was not affected by cold hypoxia acclimation (Fig. 5), suggesting that BAT capacity for NEFA uptake from the circulation is not part of the plasticity response in NST. However, increased circulatory delivery rates of NEFAs and TGs to BAT may contribute to the increased

lipid oxidation observed at the whole animal level. Additionally, use of intracellular TGs in BAT may increase the upon cold challenge as previously observed in studies in both mice and humans (Blondin et al., 2015, 2017; Labbé et al., 2015).

Conclusions

The current study helps shed light on how the lipid metabolic pathway contributes to the elevated whole-animal lipid oxidation rates observed in highland deer mice during hypoxic cold-induced $\dot{V}_{O_2,max}$. Our results show that highland deer mice have much higher rates of NEFA and TG circulatory delivery compared with lowlanders, and cold hypoxia acclimation further increases these lipid delivery rates. Past work on humans has shown that at high exercise intensities, an increase in circulatory fat availability does not lead to increased muscle fat use (Hargreaves et al., 1991). Although it is unclear if the same is true for exercising deer mice, our data suggests that the limitations of muscle lipid uptake during exercise are circumvented during thermogenesis. This difference in fuel use between these two energetically taxing but distinct activities is probably due to differential recruitment of the metabolically active tissues involved. During thermogenesis BAT accounts for ∼50% of total energy use, but skeletal muscle lipid use remains higher than those observed in deer mice during submaximal exercise (Lau et al., 2017). This suggests that a larger muscle mass may be recruited during shivering compared with locomotion. Collectively, the muscles recruited for shivering use lipids at a high rate, but individually, each shivering muscle may be operating at a metabolic rate that can be supported by circulating lipids, which may explain the high lipid oxidation rates observed in thermoregulating highland deer mice. In the future, determining the destination and uptake rate of circulating NEFAs and TGs into thermo-effector tissues during thermogenesis will help further elucidate how lipids are used during thermogenesis.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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

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Data availability

Data are available from figshare: https://doi.org/10.6084/m9.figshare.19666620.

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