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Chronic cold exposure induces mitochondrial plasticity in deer mice native to high altitudes

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Key points

- Small mammals native to high altitude must sustain high rates of thermogenesis to cope with cold. Skeletal muscle is a key site of shivering and non-shivering thermogenesis, but the importance of mitochondrial plasticity in cold hypoxic environments remains unresolved.
- We examined high-altitude deer mice, which have evolved a high capacity for aerobic thermogenesis, to determine the mechanisms of mitochondrial plasticity during chronic exposure to cold and hypoxia, alone and in combination.
- Cold exposure in normoxia or hypoxia increased mitochondrial leak respiration and decreased phosphorylation efficiency and OXPHOS coupling efficiency, which may serve to augment non-shivering thermogenesis. Cold also increased muscle oxidative capacity, but reduced the capacity for mitochondrial respiration via complex II relative to complexes I and II combined.
- High-altitude mice had a more oxidative muscle phenotype than low-altitude mice.
- Therefore, both plasticity and evolved changes in muscle mitochondria contribute to thermogenesis at high altitude.

Abstract Small mammals native to high altitude must sustain high rates of thermogenesis to cope with cold and hypoxic environments. Skeletal muscle is a key site of shivering and non-shivering thermogenesis, but the importance of mitochondrial plasticity in small mammals at high altitude remains unresolved. High-altitude deer mice (*Peromyscus maniculatus*) and low-altitude white-footed mice (*P. leucopus*) were born and raised in captivity, and chronically exposed as adults to warm (25°C) normoxia, warm hypoxia (12 kPa O_2), cold (5°C) normoxia, or cold hypoxia. We then measured oxidative enzyme activities, oxidative fibre density and capillarity in the gastrocnemius, and used a comprehensive substrate titration protocol to examine the function of muscle mitochondria by high-resolution respirometry. Exposure to cold in both normoxia or hypoxia increased the activities of citrate synthase and cytochrome oxidase. In lowlanders, this

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was associated with increases in capillary density and the proportional abundance of oxidative muscle fibres, but in highlanders, these traits were unchanged at high levels across environments. Environment had some distinct effects on mitochondrial OXPHOS capacity between species, but the capacity of complex II relative to the combined capacity of complexes I and II was consistently reduced in both cold environments. Both cold environments also increased leak respiration and decreased phosphorylation efficiency and OXPHOS coupling efficiency in both species, which may serve to augment non-shivering thermogenesis. These cold-induced changes in mitochondrial function were overlaid upon the generally more oxidative phenotype of highlanders. Therefore, both plasticity and evolved changes in muscle mitochondria contribute to thermogenesis at high altitudes.

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Introduction

Heat production by thermo-effector tissues (often termed 'adaptive thermogenesis') is critical to endothermy and is a key component of energy expenditure (Nowack *et al.* 2017; Periasamy *et al.* 2017; Fuller-Jackson & Henry, 2018). Skeletal muscle plays a key role in thermogenesis through the process of shivering, which is supported by a broad range of postural and locomotory muscles in the limbs and trunk (Hemingway, 1963; Günther *et al.* 1983; Pearson *et al.* 2005). The brown adipose tissue of placental mammals supports non-shivering thermogenesis, which involves the regulated uncoupling of oxidative phosphorylation by uncoupling protein (UCP) 1, and is particularly important in small mammals (Cannon & Nedergaard, 2004; Gaudry *et al.* 2018; Jastroch *et al.* 2018). Skeletal muscle probably also supports non-shivering thermogenesis in both mammals and birds (Duchamp *et al.* 1991; Duchamp & Barre, 1993; Block, 1994; Nowack *et al.* 2017), but the mechanisms remain controversial (Rowland *et al.* 2015; Campbell & Dicke, 2018). The potentially important role of the protein sarcolipin in uncoupling Ca^{2+} transport from ATP hydrolysis by SERCA (sarcoplasmic reticulum Ca^{2+}-ATPase) pumps has gained interest in the biomedical research community (Bal *et al.* 2012; Betz & Enerbäck, 2018), but a regulated uncoupling of oxidative phosphorylation in muscle mitochondria may also play a role (Grav & Blix, 1979; Talbot *et al.* 2004; Teulier *et al.* 2016). In both cases, increases in flux through pathways of mitochondrial energy metabolism – either to support ATP synthesis or to counteract proton leak – is an important source of heat production.

Chronic cold exposure leads to increases in thermogenic capacity that are key to maintaining thermoregulatory homeostasis (Foster & Frydman, 1979; Rezende *et al.* 2004; Tate *et al.* 2020), and muscle plasticity appears to play an important role in this process. Cold acclimation is well known to increase the volume of mitochondria in skeletal muscle (Buser *et al.* 1982; Bal *et al.* 2016, 2017*a*,*b*), which may arise via Ca^{2+}-stimulated mitochondrial biogenesis (Bruton *et al.* 2010; Maurya *et al.* 2018). Some evidence also suggests that cold acclimation can lead to plastic changes in the physiology of mitochondria in skeletal muscle, but the exact nature of these changes in mitochondrial 'quality' have varied across studies. For example, the range of mitochondrial responses to cold acclimation have included increases in leak respiration and uncoupling (Monemdjou *et al.* 2000; Mollica *et al.* 2005), increases in respiratory capacity under phosphorylating conditions (Zaninovich *et al.* 2003; Arruda *et al.* 2008), increases in relative capacity for lipid oxidation (Shabalina *et al.* 2010), or no response at all (Mineo *et al.* 2012). Furthermore, past studies have largely employed only a few mitochondrial substrates, so they have been unable to provide a comprehensive indication of how the entire electron transport system is adjusted during chronic cold exposure. More research is needed to fully appreciate the role of mitochondrial plasticity in skeletal muscle in cold environments.

Small mammals native to high altitude provide an excellent opportunity to understand the mechanisms of mitochondrial plasticity that support thermoregulation in the cold. The cold environment at high altitude requires that small mammals maintain high rates of mitochondrial metabolism to support thermogenesis (Hayes, 1989). The thin air at high altitude also has a low partial pressure of O_2 (hypoxia), which could constrain O_2 supply to support mitochondrial respiration. However, the effects of combined exposure to cold and hypoxia on muscle and mitochondrial plasticity are poorly understood. A previous study of domestic mice found that neither cold nor hypoxia, alone or in combination, affected the maximal activities of citrate synthase or cytochrome *c* oxidase (common mitochondrial markers) in the gastrocnemius muscle (Beaudry & McClelland, 2010), but we otherwise know very little about how mitochondrial quantity or quality change during acclimation to these challenges.

Studies of mitochondrial plasticity in high-altitude natives have the potential to shed light on this issue, and to lead to a better understanding of the general role of mitochondrial plasticity in challenging environments.

Deer mice (*Peromyscus maniculatus*) native to high altitude are a powerful model for understanding the mitochondrial mechanisms of phenotypic plasticity and local adaptation. Deer mice can be found across a wide elevational range, from sea level to over 4300 m above sea level in the Rocky Mountains (Snyder *et al.* 1982; Natarajan *et al.* 2015). High-altitude populations of this species maintain high metabolic rates in the wild (Hayes, 1989) and there is evidence for strong directional selection for increased thermogenic capacity (cold-induced $\dot{V}_{O_2\,max}$) (Hayes & O'Connor, 1999), presumably as a result of the high demands for heat generation in cold alpine environments. High-altitude deer mice appear to have responded to this strong selection pressure with evolved increases in thermogenic capacity in hypoxia, based on comparisons to low-altitude populations of deer mice and white-footed mice (*P. leucopus*; a congener that is restricted to low altitudes) (Cheviron *et al.* 2012, 2013). Furthermore, chronic exposure to cold and/or hypoxia increases thermogenic capacity in high-altitude deer mice and their low-altitude counterparts (Chappell & Hammond, 2004; Rezende *et al.* 2004; Tate *et al.* 2017, 2020). The changes in muscle and mitochondrial physiology that underlie this variation in thermogenic capacity have yet to be fully explained, but our previous work suggests that evolved increases in the oxidative capacity of several skeletal muscles may be involved (Dawson *et al.* 2018; Scott *et al.* 2018). In the gastrocnemius muscle in particular, high-altitude deer mice have evolved increased oxidative capacity as a result of increases in abundance of oxidative fibre types, mitochondrial volume density and mitochondrial respiratory capacity (Lui *et al.* 2015; Scott *et al.* 2015; Mahalingam *et al.* 2017; Robertson & McClelland, 2019). Hypoxia acclimation had relatively few effects on these traits in either high- or low-altitude populations of deer mice, but the effects of acclimation to cold or cold hypoxia are unknown. This study therefore aims to examine how acclimation to hypoxia and cold – alone or in combination – affects muscle phenotype and mitochondrial physiology in high-altitude populations of deer mice and in a closely related but exclusively low-altitude species, the white-footed mouse. We use a comprehensive substrate titration protocol to examine multiple aspects of mitochondrial function, including distinct fuel pathways (carbohydrate *versus* lipid substrates), distinct branches of electron entry into the electron transport system (complex I or II substrates), and integrated mitochondrial function with a fully reconstituted tricarboxylic acid cycle (substrates of both complex I and II). We hypothesized that cold environments would induce mitochondrial plasticity to increase oxidative capacity, potentially to augment heat production, and that responses to cold would occur more strongly in highlanders than in lowlanders.

Methods

Ethical approval

All procedures were approved by the McMaster University Animal Research Ethics Board, and followed principles set out by the Canadian Council on Animal Care as well as *The Journal of Physiology* (Grundy, 2015). All steps were taken to minimize pain and suffering during all procedures.

Animals and acclimation treatments

Captive breeding populations were established from wild populations of deer mouse native to high altitude (*P. m. rufinus*; from near the summit of Mount Evans, CO, USA, 39°35′18″N, 105°38′38″W; 4350 m above sea level) and from wild populations of the exclusively low-altitude white-footed mouse (*P. leucopus*; from Nine Mile Prairie, Lancaster County, NE, USA, 40°52′12″N, 96°48′20.3″W; 430 m above sea level). Wild mice were transported to McMaster University in Hamilton, ON, Canada (<100 m above sea level), housed in common laboratory conditions, and were used as a parental stock to produce first-generation lab progeny for each mouse population. Progeny of each population were raised to at least ∼6 months of age and were then used in experiments. Mice were held in standard holding conditions (∼25°C, 12 h:12 h light:dark photoperiod) and were provided with unlimited access to water and standard mouse chow.

First generation adult mice from each population were chronically exposed to each of four acclimation environments for 18–20 weeks: (1) warm (25°C) normobaric normoxia (barometric pressure ∼100 kPa, P_{O_2} ∼20 kPa), the standard holding condition; (2) warm (25°C) hypobaric hypoxia (barometric pressure of 60 kPa, P_{O_2} of 12.5 kPa); (3) cold (5°C) normobaric normoxia; and (4) cold (5°C) hypobaric hypoxia. No mice died during these acclimation treatments. Hypobaric conditions were achieved using specially designed hypobaric chambers that have been described previously (McClelland *et al.* 1998; Lui *et al.* 2015; Ivy & Scott, 2017). Cold conditions were maintained in environmental chambers with temperature control, which were sufficiently large to house the hypobaric chambers for acclimations to cold hypoxia. All mice experienced a 12 h:12 h light:dark photoperiod and were provided with an *ad libitum* supply of mouse chow and water during chronic exposures. Cage cleaning was conducted twice per week, which required that mice in hypobaric conditions be returned to normobaria for a brief period (< 20 min). At the end

of chronic exposures, mice were killed by anaesthetic overdose (inhalation of >5% isoflurane vapour) followed by cervical dislocation, body mass and gastrocnemius muscle mass were measured, and muscles were sampled for a variety of additional measurements described below. We could not make all of these measurements on all mice, so *n* (which represents the number of mice used for each measurement) is clearly indicated in each figure and table.

Histology

Histology and stereology were used to measure the abundance of oxidative fibres and capillaries in the gastrocnemius muscle. The entire gastrocnemius was coated in embedding medium, frozen in liquid N_2-cooled isopentane, and stored at −80°C. Tissue was sectioned (10 μm) transverse to muscle fibre length in a cryostat maintained at −20°C. Oxidative muscle fibres (both slow and fast) were identified by staining for succinate dehydrogenase activity for 1 h at room temperature (assay buffer concentrations in mM: 1.0 nitroblue tetrazolium, 2.0 KH_2PO_4, 15.4 Na_2HPO_4, 16.7 sodium succinate). Capillaries were identified by staining for alkaline phosphatase activity for 1 h at room temperature (assay buffer concentrations in mM: 1.0 nitroblue tetrazolium, 0.5 5-bromo-4-chloro-3-indoxyl phosphate, 28 $NaBO_2$, 7 $MgSO_4$; pH 9.3). Images were collected systematically across the entire section of the gastrocnemius using an upright brightfield microscope. Stereological methods were used to make unbiased measurements of several variables (areal and numerical densities of oxidative fibres, average transverse area of oxidative and glycolytic fibres, capillary density, capillary to fibre ratio), as previously described (Weibel, 1979; Egginton, 1990), during which the observer was blind to experimental conditions. Sufficient images were analysed for each sample to account for heterogeneity, determined by the number of replicates necessary to yield a stable mean value (at least 15 images were collected and analysed).

Enzyme assays

The maximal activities of citrate synthase (CS) and cytochrome *c* oxidase (COX) were measured in the gastrocnemius, as the activities of these enzymes have been shown to be reliable markers of oxidative capacity and mitochondrial content in skeletal muscle (Reichmann *et al.* 1985; Larsen *et al.* 2012). The entire gastrocnemius was frozen in liquid N_2 and was powdered by grinding the tissue in a liquid N_2-cooled mortar and pestle, and the powdered tissue was stored at −80°C. Tissue powder (≥20 mg) was homogenized on ice using a Dounce glass tissue grinder in 20 volumes of homogenization buffer (100 mM KH_2PO_4, 5 mM ethylenediaminetetraacetic acid, and 0.1% Triton-X-100; pH 7.2). COX activity was assayed shortly after homogenization, and CS was measured later after storing the homogenate at −80°C. Activity was assayed at 37°C using a Spectromax Plus 384 microplate reader (Molecular Devices, San Jose, CA, USA) by measuring the change in absorbance over time at 550 nm (COX) or 412 nm (CS). Assays were conducted in triplicate under the following conditions: COX, 0.2 mM reduced cytochrome *c*, 100 mM KH_2PO_4 (pH 8.0); CS, 0.23 mM acetyl-coA, 0.5 mM oxaloacetate, 0.15 mM DTNB (dithiobisnitrobenzoic) acid, 40 mM Tris (pH 8.0). Enzyme activities were determined by subtracting the background rate (measured in control reactions that contained no cytochrome *c* or oxaloacetate, respectively) from the rates measured in the presence of all substrates, and are reported relative to muscle tissue mass (in grams). Preliminary experiments verified that substrate concentrations were saturating. We used the same procedure to measure enzyme activities in isolated mitochondria (see below), except that these activities are reported per milligram mitochondrial protein.

Mitochondrial isolation and respirometry

Mitochondria were isolated from the muscles from one entire hindlimb (including the gastrocnemius) by differential centrifugation. Muscles were removed (∼250–400 mg), immediately transferred to 10 ml of ice-cold isolation buffer (100 mM sucrose, 50 mM Tris, 5 mM $MgCl_2$, 5 mM EGTA, 100 mM KCl, 1 mM ATP), and minced. The tissue was digested for 5 min by adding nagarse protease (1 mg per g muscle tissue), which digests the myofibrils and liberates intermyofibrillar mitochondria, such that the subsequent isolation procedure yields a mix both subsarcolemmal and intermyofibrillar mitochondrial subpopulations. This digested muscle was then gently homogenized with six passes of a Potter-Elvehjem homogenizer (100 rpm). Homogenate was filtered through cheesecloth and then centrifuged at 1000 *g* for 10 min at 4°C. The resulting supernatant was centrifuged at 8700 *g* for 10 min at 4°C (the same for all further centrifugation). The pellet was resuspended in 10 ml of isolation buffer containing bovine serum albumin (BSA, fatty acid-free, at 1% mass per volume) and centrifuged. The pellet was resuspended in 10 ml of storage buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM potassium methanesulphonate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 0.02 mM vitamin E succinate, 2 mM pyruvate, 2 mM malate; pH 7.1) and centrifuged. Finally, the pellet was resuspended in 250–400 μl of storage buffer. Part of this mitochondrial suspension was kept on ice until mitochondrial physiology was measured, and the rest was

Table 1. Statistical results of two-way ANOVA

	Environment		Species		Environment × species	
	F	*P*	*F*	*P*	*F*	*P*
Muscle CS	7.63	<0.001	31.1	<0.001	1.73	0.172
Muscle COX	7.56	<0.001	20.2	<0.001	0.924	0.435
M_g	0.575	0.633	40.6	<0.001	0.920	0.434
M_b	4.39	0.006	19.5	<0.001	0.672	0.571
A_A[ox,m]	2.05	0.120	19.7	<0.001	2.06	0.118
N_N[ox,m]	2.27	0.093	16.0	<0.001	2.57	0.066
A[ox]	0.301	0.824	10.1	0.003	2.13	0.109
A[gly]	0.183	0.908	<0.001	0.986	0.412	0.745
N_A[cap,m]	0.936	0.431	39.5	<0.001	1.52	0.222
N_N[cap,f]	1.02	0.391	59.8	<0.001	3.85	0.015
P_{PM}	7.66	<0.001	42.3	<0.001	13.4	<0.001
P_{PMG}	9.35	<0.001	48.0	<0.001	11.8	<0.001
P_{PMGS}	5.61	0.002	51.5	<0.001	10.8	<0.001
$P_{S(Rot)}$	3.38	0.025	45.2	<0.001	3.47	0.023
P_{Tm}	11.9	<0.001	22.1	<0.001	7.10	<0.001
P_{PcM}	10.1	<0.001	4.84	0.033	1.89	0.144
P_{PcOcM}	10.3	<0.001	3.74	0.059	1.57	0.208
Mito CS	5.07	0.004	15.3	<0.001	5.37	0.003
Mito COX	3.16	0.032	19.0	<0.001	1.66	0.188
P_{PMGS}/CS	1.44	0.241	13.5	<0.001	1.50	0.227
P_{PM}/P_{PMGS}	0.135	0.939	3.17	0.081	0.076	0.973
P_{PMG}/P_{PMGS}	2.65	0.059	1.91	0.173	0.434	0.730
$P_{S(Rot)}/P_{PMGS}$	17.0	<0.001	5.52	0.023	1.00	0.399
P_{PcOcM}/P_{PMGS}	6.46	<0.001	1.87	0.178	0.473	0.703
$L_{N,PM}$	5.93	0.001	4.11	0.048	3.85	0.014
$L_{T,PM}$	31.7	<0.001	6.91	0.011	6.15	0.001
$L_{N,PcM}$	6.91	0.001	3.02	0.089	6.91	<0.001
P/O	7.04	<0.001	4.98	0.030	1.45	0.239
$1 - L_{T,PM}/P_{PM}$	19.5	<0.001	4.28	0.044	1.73	0.172

Abbreviations: $A_{[ox]}$ and $A_{[gly]}$, average transverse area of oxidative and glycolytic fibres, respectively; A_A[ox,m] and N_N[ox,m], areal density and numerical density of oxidative fibres, respectively; L_N, leak respiration in the absence of ATP; L_T, leak respiration in the presence of ATP; M_b, body mass; M_g, gastrocnemius mass; muscle CS and muscle COX, maximal enzyme activity of citrate synthase and cytochrome *c* oxidase, respectively, in gastrocnemius muscle; mito CS and mito COX, maximal enzyme activity of CS and COX, respectively, in isolated mitochondria; N_A[cap,m], capillary density; N_N[cap,f], capillary to fibre ratio; *P*, respiration during oxidative phosphorylation with subscripts representing substrates or inhibitors (P, pyruvate; M, malate; G, glutamate; S, succinate; Rot, rotenone; Tm, TMPD; Pc, palmitoyl-carnitine; Oc, octanoyl-carnitine); P/O, phosphorylation efficiency, the ratio of ATP production to atomic oxygen consumption; $1 - L_{T,PM}/P_{PM}$, OXPHOS coupling efficiency.

homogenized in a glass tissue grinder and stored at −80°C for later use in enzyme assays as described above.

The physiology of isolated mitochondria was measured using two protocols of high-resolution respirometry. The first protocol was aimed at assessing mitochondrial respiratory capacities for carbohydrate oxidation. Isolated mitochondria (approximately 40 µg mitochondrial protein) were added to respiration buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g l^{-1} fatty acid-free BSA; pH 7.1) within the respirometry chamber of an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria), which was maintained at 37°C and set to a final volume of 2 ml. Leak respiration in the absence of adenylates was stimulated with 5 mM pyruvate and 2 mM malate ($L_{N,PM}$). P/O ratios (ATP per oxygen atom) were then determined by adding submaximal ADP (125 µmol l^{-1}) and then allowing all ADP to be consumed to reach leak respiration in the presence of ATP ($L_{T,PM}$). This ADP titration was performed twice, and the average P/O ratio is reported. The full capacity for oxidative phosphorylation via multiple complexes of the electron transport system was then stimulated with progressive additions of maximal ADP at 2 mM ($P_{PM,}$ complex I), 20 mM of glutamate ($P_{PMG,}$ complex I), 20 mM of succinate ($P_{PMGS,}$ complexes I+II), and 0.5 µM rotenone ($P_{S(Rot),}$ complex II), waiting at least 2 min between each addition until a stable

respiration rate could be measured. The chamber was then re-oxygenated and cytochrome oxidase (complex IV) was maximally stimulated with the addition of 1.25 mM ADP, 2 mM ascorbate, and 0.5 mM TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine) (P_{Tm}).

The second protocol was aimed at assessing mitochondrial respiratory capacities for fatty acid oxidation. Isolated mitochondria were added to the respirometry chamber as above. Leak respiration was stimulated with 2 mM malate and 40 μM palmitoyl-carnitine ($L_{N,PcM}$). Oxidative phosphorylation was then stimulated with 2 mM ADP ($P_{PcM,}$ complex I) followed by 0.2 mM octanoyl-carnitine ($P_{PcOcM,}$ complex I). Respiration rate (rate of O_2 consumption) is always expressed per milligram mitochondrial protein, which was measured using the Bradford assay (following instructions of the manufacturer; Bio-Rad Laboratories, Montreal, Canada).

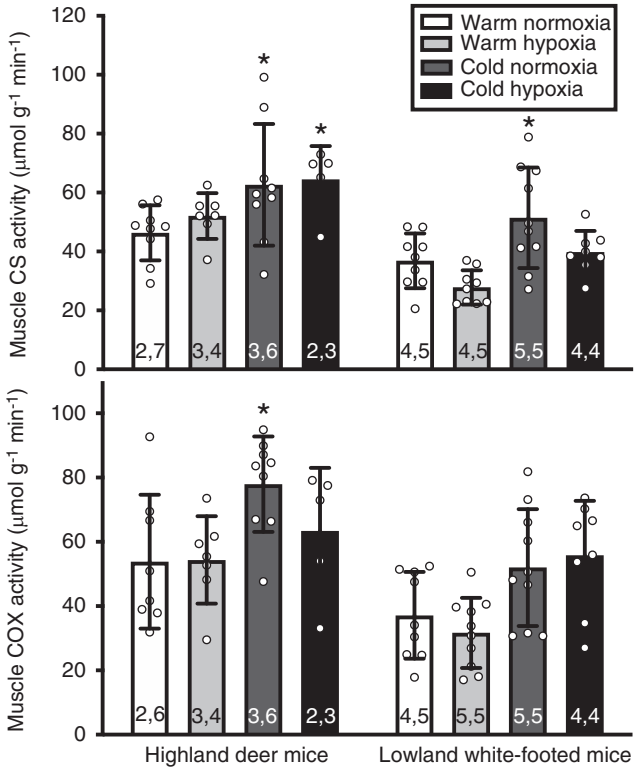

Figure 1. Chronic cold exposure affects citrate synthase (CS) and/or cytochrome *c* oxidase (COX) activity in the gastrocnemius muscle
Mice from each species were bred in captivity and acclimated during adulthood to each of four treatments: warm (25°C) normoxia; warm hypoxia (12 kPa O_2); cold (5°C) normoxia; or cold hypoxia. Bars are means ± SD, with the number of males and females in each group indicated within each bar (N_{males}, $N_{females}$), and circles represent individual values.*Significant ($P < 0.05$) pairwise differences from warm normoxic controls within a species using Holm-Sidak *post hoc* tests (two-factor ANOVA results are shown in Table 1).

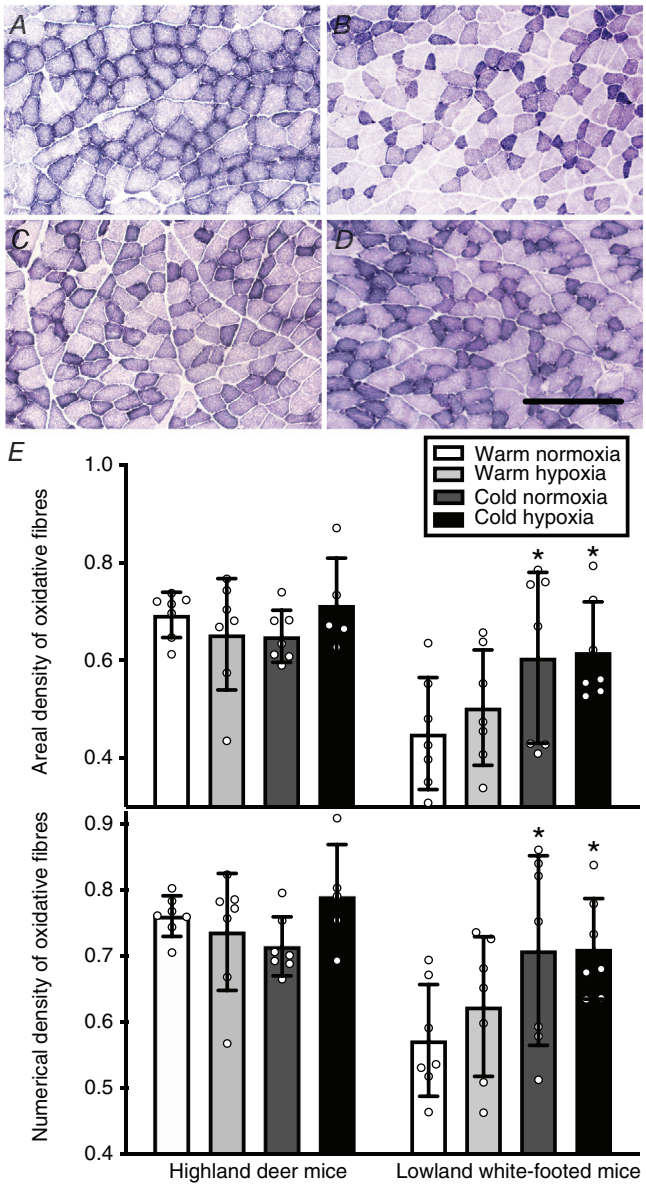

Figure 2. Chronic cold exposure increased the abundance of oxidative fibres in the gastrocnemius muscle of lowlanders, but not of highlanders
Mice from each species were bred in captivity and acclimated during adulthood to each of four treatments: warm (25°C) normoxia; warm hypoxia (12 kPa O_2); cold (5°C) normoxia; or cold hypoxia. *A–D*, histological staining of succinate dehydrogenase activity to identify oxidative fibres in highlanders (*A*, *C*) and lowlanders (*B*, *D*) held in warm normoxia (*A*, *B*) or cold hypoxia (*C*, *D*). Scale bar represents 200 μm. *E*, areal density of oxidative fibres (A_A[ox,m], the area of oxidative fibres relative to the total transverse area of the muscle) and numerical density of oxidative fibres (N_N[ox,m], number of oxidative fibres relative to the total number of fibres) are shown as bars as means ± SD (*N* as in Table 2), and circles represent individual values.*Significant ($P < 0.05$) pairwise differences from warm normoxic controls within a species using Holm-Sidak *post hoc* tests (two-factor ANOVA results are shown in Table 1). [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2. Gastrocnemius muscle phenotypes

	Acclimation environment			
	Warm (25°C) normoxia	Warm (25°C) hypoxia	Cold (5°C) normoxia	Cold (5°C) hypoxia
Highland deer mice				
M_g	99.7 ± 26.9 (4, 9)	91.7 ± 20.2 (8, 7)	92.1 ± 29.0 (5, 8)	84.5 ± 25.4 (2, 7)
M_b	20.3 ± 3.6 (4, 9)	20.6 ± 3.4 (8, 7)	23.7 ± 3.9 (5, 8)	21.8 ± 4.1 (2, 7)
A[ox]	1752 ± 240 (3, 4)	1660 ± 374 (5, 2)	1987 ± 299 (4, 3)	1648 ± 435 (1, 4)
A[gly]	2629 ± 500 (3, 4)	2518 ± 526 (5, 2)	2748 ± 441 (4, 3)	2688 ± 562 (1, 4)
N_A[cap,m]	1067 ± 152 (3, 4)	1033 ± 206 (5, 2)	1045 ± 142 (4, 3)	1019 ± 97 (1, 4)
N_N[cap,f]	2.10 ± 0.15 (3, 4)	1.86 ± 0.43 (5, 2)	2.16 ± 0.38 (4, 3)	1.67 ± 0.33 (1, 4)*
Lowland white-footed mice				
M_g	137.3 ± 48.6 (9, 8)	122.3 ± 31.8 (6, 10)	138.6 ± 44.1 (5, 7)	146.7 ± 41.6 (6, 6)
M_b	24.2 ± 5.0 (9, 8)	22.8 ± 3.9 (6, 10)	26.6 ± 5.2 (5, 7)	27.2 ± 3.8 (6, 6)
A[ox]	1570 ± 321 (3, 4)	1546 ± 302 (3, 4)	1361 ± 312 (4, 3)	1503 ± 131 (5, 2)
A[gly]	2850 ± 886 (3, 4)	2695 ± 721 (3, 4)	2607 ± 950 (4, 3)	2444 ± 319 (5, 2)
N_A[cap,m]	684 ± 139 (3, 4)	746 ± 130 (3, 4)	878 ± 183 (4, 3)	832 ± 87 (5, 2)
N_N[cap,f]	1.28 ± 0.22 (3, 4)	1.41 ± 0.25 (3, 4)	1.32 ± 0.14 (4, 3)	1.45 ± 0.16 (5, 2)

Abbreviations: A[ox] and A[gly], average transverse area (μm^2) of oxidative and glycolytic fibres, respectively; M_b, body mass (g); M_g, gastrocnemius mass (mg); N_A[cap,m], capillary density (mm^2); N_N[cap,f], capillary to fibre ratio. Data are means ± SD (N_{males}, $N_{females}$). *Significant ($P < 0.05$) pairwise difference from warm normoxic controls within a species using Holm-Sidak *post hoc* tests (two-factor ANOVA results are shown in Table 1).

Statistics

Two-factor ANOVA was used to assess the effects of acclimation environment, species, and their interaction. When effects of environment or environment × species interaction were significant, we carried out Holm-Sidak *post hoc* tests to test for pairwise differences from warm normoxic controls within each species. Statistical tests were carried out using GraphPad Prism software (version 8.4; La Jolla, CA, USA). Data are reported as individual values and/or means ± SD. $P < 0.05$ was considered to be significant.

Results

We examined the effects of chronic exposure to hypoxia and/or cold on muscle phenotype and mitochondrial physiology in both high-altitude deer mice and low-altitude white-footed mice. There were significant main effects of species for most measured traits (Table 1), consistent with our previous findings showing that high-altitude deer mice have evolved increased oxidative capacity in the gastrocnemius muscle, as a result of increases in abundance of oxidative muscle fibre-types, mitochondrial volume density, and mitochondrial respiratory capacity (Lui *et al.* 2015; Scott *et al.* 2015, 2018; Mahalingam *et al.* 2017; Robertson & McClelland, 2019). Here, we focus on the effects of acclimation environment to examine whether mitochondrial plasticity in skeletal muscle contributes to thermogenesis and aerobic performance in high-altitude environments.

Muscle phenotype

The oxidative capacity of the gastrocnemius muscle was strongly affected by chronic exposure to cold environments (Fig. 1; Table 1). There was a significant main effect of acclimation environment on citrate synthase activity ($P < 0.001$), driven by increases in cold and cold hypoxia in highlanders and increases in cold in lowlanders. Acclimation environment also had a strong effect on cytochrome *c* oxidase activity ($P < 0.001$), particularly in highlanders in the cold. However, there was no significant effect of acclimation environment on gastrocnemius mass ($P = 0.633$) (Table 2).

The contribution of changes in the abundance of oxidative fibres to variation in oxidative capacity appeared to differ between species (Fig. 2; Table 1). The overall effect of acclimation environment was not significant for either the areal density ($P = 0.120$) or the numerical density of oxidative fibres ($P = 0.093$). However, there were significant main effects of species for both traits ($P < 0.001$) and only lowlanders increased oxidative fibre densities in response to cold and cold hypoxia (Fig. 2*E*). These effects of cold environments in lowlanders were not associated with any changes in the transverse area of oxidative or glycolytic fibres, nor was there a significant main effect of acclimation environment on fibre sizes ($P = 0.824$ and 0.908), but highlanders tended to have

larger oxidative fibres (species effect, $P = 0.003$) but not glycolytic fibres ($P = 0.986$) (Table 2). The variation in abundance of oxidative fibres was associated with variation in capillary density (Table 2), as reflected by a strong positive correlation between capillary density and the areal density of oxidative fibres (Fig. 3), and a significant species × environment interaction for this trait ($P = 0.015$). These results suggest that increases in the abundance of oxidative fibres in cold environments probably contributed to the variation in CS and COX activities in lowlanders, but not in highlanders.

Mitochondrial physiology

Respiratory capacities of mitochondria isolated from hindlimb muscle were affected by acclimation environment, but the effects appeared to differ between species (Fig. 4; Tables 1 and 3). In highlanders, cold environments had little effect on respiratory capacities during oxidative phosphorylation but acclimation to warm hypoxia reduced respiratory capacity, when measured using substrates of complex I (malate, pyruvate, glutamate, acyl-carnitines) or II (succinate) (Table 3), or using substrates of complexes I and II combined (Fig. 4). In lowlanders, by contrast, all three hypoxic and/or cold environments increased respiratory capacity measured using substrates of complex I (excluding malate and acyl-carnitines in warm hypoxia) or complexes I+II. As a result, there were significant species × environment interactions for respiratory capacity using several substrate combinations (Table 1). However, when respiratory capacity (complexes I+II) was expressed relative to the citrate synthase activity of isolated mitochondria (Fig. 4), the effects of environment ($P = 0.241$) and species × environment interaction ($P = 0.227$) were no longer significant, but the species differences remained (species effect, $P < 0.001$). Nevertheless, these data suggest that cold-induced increases in mitochondrial OXPHOS capacity may

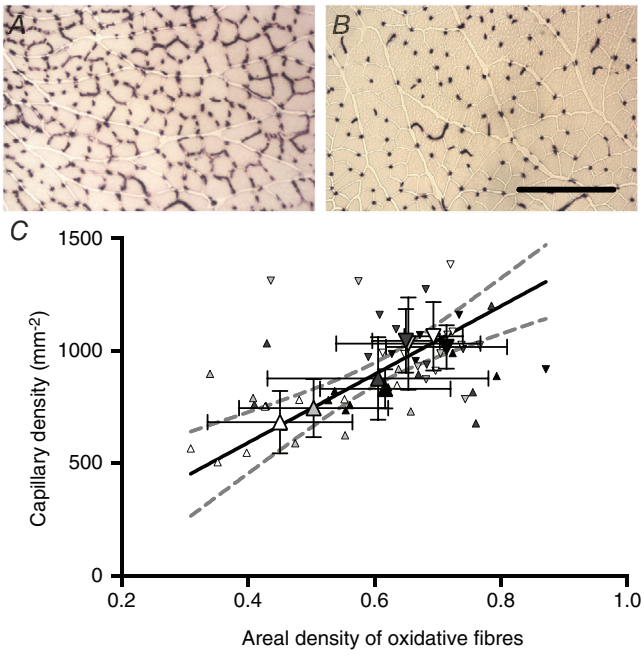

Figure 3. Capillarity varied with the abundance of oxidative fibres in the gastrocnemius muscle
A and *B*, histological staining of alkaline phosphatase activity to identify capillaries in highlanders (*A*) and lowlanders (*B*) held in warm normoxia. Scale bar represents 200 μm. *C*, there was a strong correlation ($P = 0.0008$) between capillary density and the areal density of oxidative fibres in the gastrocnemius. Large symbols are means ± SD and small symbols are individual values (highlanders, downward triangles; lowlanders, upward triangles; dashed lines are 95% confidence interval of linear regression; *N* as in Table 2). [Colour figure can be viewed at wileyonlinelibrary.com]

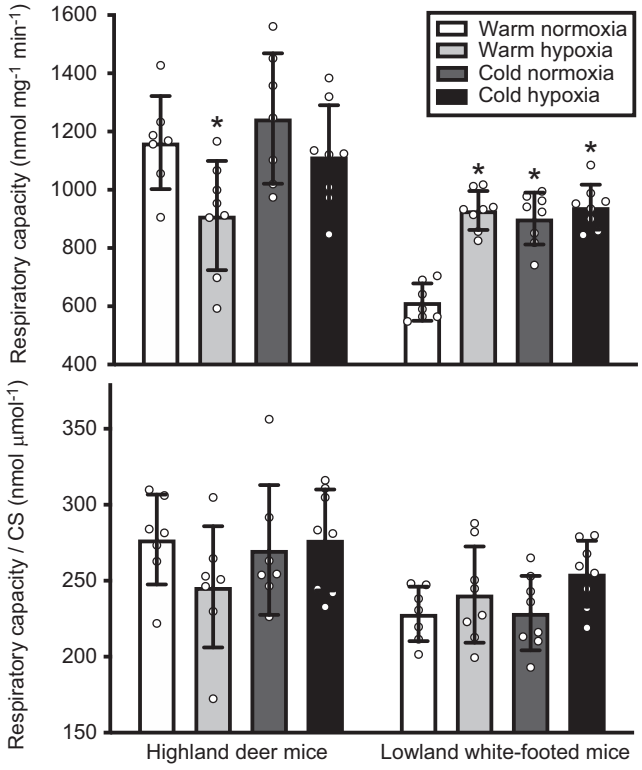

Figure 4. Chronic cold and/or hypoxia exposure affected respiration during oxidative phosphorylation (OXPHOS, *P*) of isolated mitochondria from hindlimb muscle
Mice from each species were bred in captivity and acclimated during adulthood to each of four treatments: warm (25°C) normoxia; warm hypoxia (12 kPa O_2); cold (5°C) normoxia; or cold hypoxia. Respiratory capacity during OXPHOS (P_{PMGS}) measured with substrates of complexes I and II (pyruvate, malate, glutamate, succinate), expressed per milligram mitochondrial protein or relative to the activity of citrate synthase (CS) assayed in isolated mitochondria. Bars are means ± SD and circles represent individual values (*N* as in Table 3).*Significant ($P < 0.05$) pairwise differences from warm normoxic controls within a species using Holm-Sidak *post hoc* tests (two-factor ANOVA results are shown in Table 1).

Table 3. Physiology of isolated muscle mitochondria

	Acclimation environment			
Respiration condition	Warm (25°C) normoxia	Warm (25°C) hypoxia	Cold (5°C) normoxia	Cold (5°C) hypoxia
Highland deer mice				
$L_{N,PM}$	61.5 ± 11.4 (3, 4)	51.3 ± 10.8 (5, 3)	81.8 ± 7.8 (2, 5)*	65.8 ± 13.1 (2, 6)
$L_{N,PcM}$	61.7 ± 3.7 (3, 4)	58.0 ± 27.7 (4, 3)	87.3 ± 14.2 (2, 5)*	55.7 ± 9.5 (2, 6)
P_{PM}	813 ± 101 (3, 4)	621 ± 97 (5, 3)*	885 ± 155 (2, 5)	784 ± 136 (2, 6)
P_{PMG}	1016 ± 96 (3, 4)	807 ± 143 (5, 3)*	1165 ± 202 (2, 5)	995 ± 171 (2, 6)
$P_{S(Rot)}$	512 ± 92 (3, 3)	401 ± 104 (4, 3)*	369 ± 92 (2, 5)*	405 ± 81 (2, 6)*
P_{Tm}	1382 ± 310 (3, 4)	1101 ± 207 (5, 3)*	1814 ± 348 (2, 5)*	1426 ± 310 (2, 5)
P_{PcM}	526 ± 224 (3, 4)	301 ± 185 (4, 3)*	624 ± 101 (2, 5)	510 ± 150 (2, 6)
P_{PcOcM}	515 ± 220 (3, 4)	301 ± 190 (4, 3)*	648 ± 125 (2, 5)	532 ± 195 (2, 6)
CS activity	4.21 ± 0.55 (3, 4)	3.73 ± 0.72 (4, 3)	4.62 ± 0.58 (2, 5)	4.07 ± 0.79 (2, 6)
COX activity	4.59 ± 1.38 (3, 3)	3.98 ± 0.78 (4, 3)	4.26 ± 1.07 (2, 5)	3.27 ± 0.78 (2, 6)
Lowland white-footed mice				
$L_{N,PM}$	45.2 ± 12.7 (5, 2)	62.2 ± 23.4 (2, 6)*	63.5 ± 4.6 (3, 5)*	61.5 ± 14.4 (3, 5)*
$L_{N,PcM}$	39.7 ± 11.3 (4, 2)	80.3 ± 25.8 (2, 5)*	58.8 ± 10.4 (3, 4)	54.0 ± 12.6 (3, 5)
P_{PM}	446 ± 40 (5, 2)	674 ± 62 (2, 6)*	656 ± 59 (3, 5)*	690 ± 59 (3, 5)*
P_{PMG}	555 ± 53 (5, 2)	847 ± 68 (2, 6)*	840 ± 97 (3, 5)*	862 ± 82 (3, 5)*
$P_{S(Rot)}$	269 ± 83 (5, 2)	350 ± 90 (2, 6)	242 ± 36 (3, 5)	274 ± 21 (3, 5)
P_{Tm}	827 ± 82 (4, 2)	1267 ± 212 (2, 6)*	1359 ± 124 (3, 5)*	1123 ± 141 (3, 5)*
P_{PcM}	316 ± 43 (4, 2)	291 ± 182 (2, 5)	505 ± 46 (3, 4)*	521 ± 51 (3, 5)*
P_{PcOcM}	317 ± 37 (4, 2)	297 ± 189 (2, 5)	522 ± 55 (3, 4)*	548 ± 57 (3, 5)*
CS activity	2.71 ± 0.39 (5, 2)	3.91 ± 0.53 (2, 6)*	3.97 ± 0.49 (3, 5)*	3.72 ± 0.44 (3, 5)*
COX activity	2.49 ± 0.36 (5, 2)	3.54 ± 1.17 (2, 6)	3.18 ± 0.50 (3, 5)	2.32 ± 1.45 (3, 5)

L_N and P, respiration during leak (without ATP present) or oxidative phosphorylation (units: nmol O_2 mg^{-1} mitochondrial protein min^{-1}), with subscripts representing the following substrates (or inhibitors): P, pyruvate; M, malate; G, glutamate; S, succinate; Rot, rotenone; Tm, TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine); Pc, palmitoyl-carnitine; Oc, octanoyl-carnitine. Maximal enzyme activities for citrate synthase (CS) and cytochrome *c* oxidase (COX; units: μmol substrate mg^{-1} mitochondrial protein min^{-1}). Data are means ± SD (N_{males}, $N_{females}$).*Significant ($P < 0.05$) pairwise differences from warm normoxic controls within a species using Holm-Sidak *post hoc* tests (two-factor ANOVA results are shown in Table 1).

contribute to variation in muscle oxidative capacity in lowlanders, but not in highlanders.

Changes in substrate control ratios were suggestive of further variation in mitochondrial physiology across acclimation environments (Fig. 5). Substrate control ratios were calculated as the respiration supported by a particular substrate combination relative to the maximal respiration supported by substrates of complexes I and II combined (i.e. OXPHOS capacity in Fig. 4). These ratios can provide evidence for qualitative adjustments in mitochondrial function, in which an increase in substrate control ratio reflects an increase in the relative influence of particular substrates over mitochondrial respiration (Gnaiger, 2009). We were specifically interested in examining the relative capacities for respiration via complex I (P_{PMG}/P_{PMGS}) and complex II ($P_{S(Rot)}/P_{PMGS}$), as the two sites of electron entry to the electron transport system, as well as the relative capacities for oxidation of pyruvate (P_{PM}/P_{PMGS}) and acyl-carnitines (P_{PcOcM}/P_{PMGS}), as key mitochondrial substrates of carbohydrate and fatty acid oxidation, respectively. The most substantial change in substrate control occurred for the relative capacity of complex II ($P_{S(Rot)}/P_{PMGS}$) (environment effect, $P < 0.001$), as reflected by pronounced decreases in relative complex II capacity in cold acclimation environments in highlanders and lowlanders. Variation in other substrate control ratios was less pronounced. There were no significant environment ($P = 0.059$) or species ($P = 0.173$) effects on the relative capacity of complex I (P_{PMG}/P_{PMGS}). There was also no significant variation in the relative capacity for pyruvate oxidation (P_{PM}/P_{PMGS}) (environment effect, $P = 0.939$; species effect, $P = 0.081$). However, there was a significant environment effect on the relative capacity for acyl-carnitine oxidation (P_{PcOcM}/P_{PMGS}) ($P < 0.001$), which appeared to be driven by decreases in warm hypoxia, but the pairwise differences between warm hypoxia and warm normoxia were not significant in white-footed mice ($P = 0.061$) nor in deer mice ($P = 0.520$).

In addition to the above effects on respiratory capacity, cold acclimation environments also had a strong effect on leak respiration and phosphorylation efficiency of mitochondria isolated from hindlimb muscle (Fig. 6; Table 1). There was a significant main effect of acclimation environment on leak respiration measured in the

presence of ATP ($L_{T,PM}$, $P < 0.001$), primarily as a result of significant increases in leak respiration in both cold and cold hypoxic acclimation environments for both species (Fig. 6). However, there were significant species differences in the response acclimation environment, as reflected by a significant species × environment interaction ($P = 0.001$) that appeared to result from a much stronger increase in leak respiration in highlanders in cold normoxia and an increase in lowlanders in warm hypoxia (Fig. 6). Similarly, there were significant main effects of acclimation environment on leak respiration measured in the absence of ATP ($L_{N,PM}$ and $L_{N,PcM}$, $P = 0.001$ for both) along with significant species × environment interactions ($L_{N,PM}$, $P = 0.014$; $L_{N,PcM}$, $P < 0.001$) that were driven by increases in cold and/or hypoxia (Table 3). There was also a significant main effect of acclimation environment on phosphorylation efficiency (P/O ratio; $P < 0.001$) and OXPHOS coupling efficiency ($1 - L_{T,PM}/P_{PM}$; $P < 0.001$), each of which decreased in cold and cold hypoxic acclimation environments in one or both species. However, there was a significant effect of species for both phosphorylation efficiency ($P = 0.030$) and OXPHOS coupling efficiency ($P = 0.044$), which appeared to be caused by lower values in lowlanders in warm environments.

Discussion

Deer mice at high altitudes maintain high metabolic rates to support thermogenesis (Hayes, 1989) and are subject to strong directional selection for increased thermogenic capacity (Hayes & O'Connor, 1999). Here, we show that chronic exposure to cold environments induces mitochondrial plasticity in skeletal muscle that may contribute to thermogenesis (Fig. 7). Chronic exposure to cold or cold hypoxic environments increased oxidative capacity of the gastrocnemius muscle, as reflected by

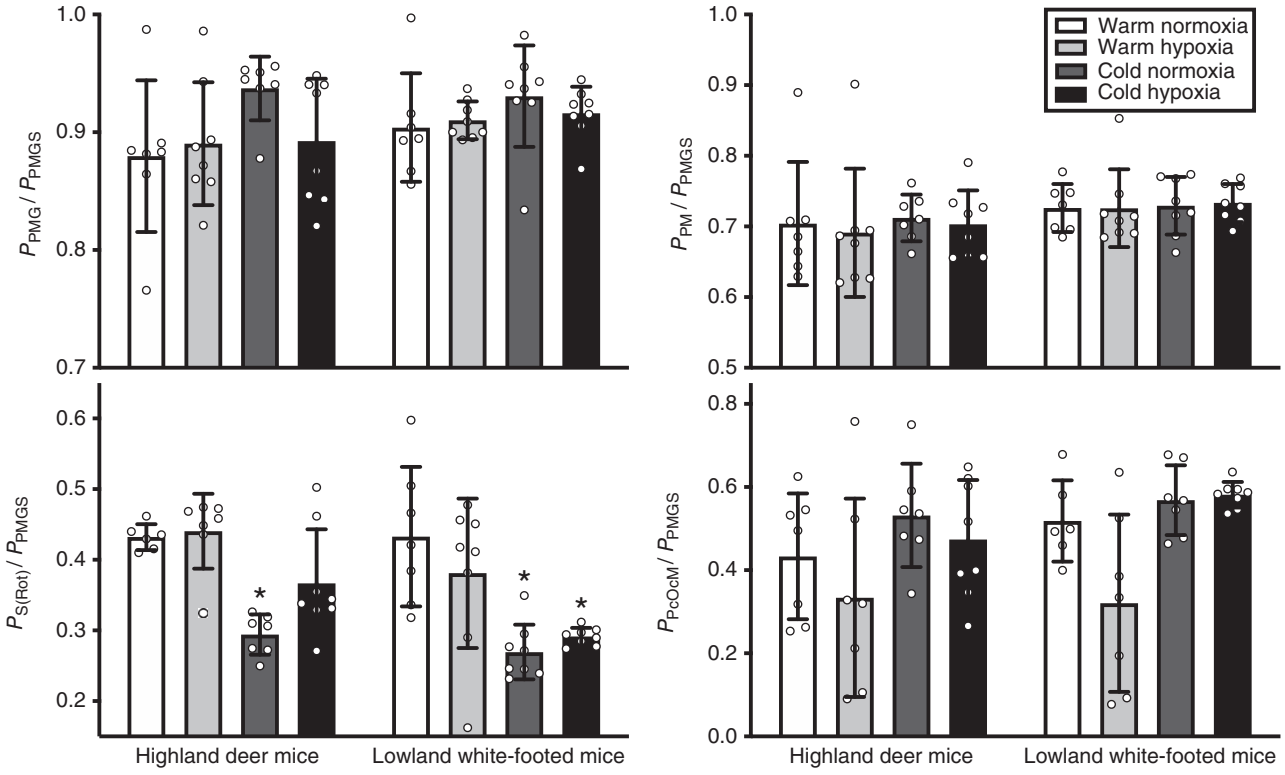

Figure 5. Chronic cold exposure altered substrate control of respiration during oxidative phosphorylation (OXPHOS, *P*) for isolated mitochondria from hindlimb muscle
Mice from each species were bred in captivity and acclimated during adulthood to each of four treatments: warm (25°C) normoxia; warm hypoxia (12 kPa O_2); cold (5°C) normoxia;or cold hypoxia. Substrate control ratios were calculated relative to maximal OXPHOS respiration via complexes I+II (P_{PMGS}) for OXPHOS respiration measurements using each of four substrate combinations: maximal respiration via complex I (P_{PMG}); maximal respiration via complex II ($P_{S(Rot)}$); maximal oxidation of pyruvate (P_{PM}); and maximal oxidation of acyl-carnitines (P_{PcOcM}). Subscripts represent the following substrates (or inhibitors) that were used to elicit each respiratory state: G, glutamate; M, malate; P, pyruvate; Pc, palmitoyl-carnitine; Oc, octanoyl-carnitine; Rot, rotenone; S, succinate. Bars are means ± SD and circles represent individual values (*N* as in Table 3).*Significant ($P < 0.05$) pairwise differences from warm normoxic controls within a species using Holm-Sidak *post hoc* tests (two-factor ANOVA results are shown in Table 1).

CS and COX activities, which appeared to occur more strongly in high-altitude deer mice than in low-altitude white-footed mice (Fig. 1). Both species made adjustments in mitochondrial function in response to cold exposure, including relative reductions in complex II capacity for OXPHOS (Fig. 5), increases in leak respiration, and decreases in phosphorylation efficiency and OXPHOS coupling efficiency (Fig. 6). Some of the latter changes occurred more strongly in highlanders than in lowlanders, and may serve to uncouple OXPHOS to augment heat production. These plastic changes in response to cold exposure were overlaid upon the generally more oxidative phenotype of highlanders. Therefore, both evolutionary adaptation and phenotypic plasticity of muscle mitochondria appear to contribute to improving thermogenesis in deer mice at high altitudes.

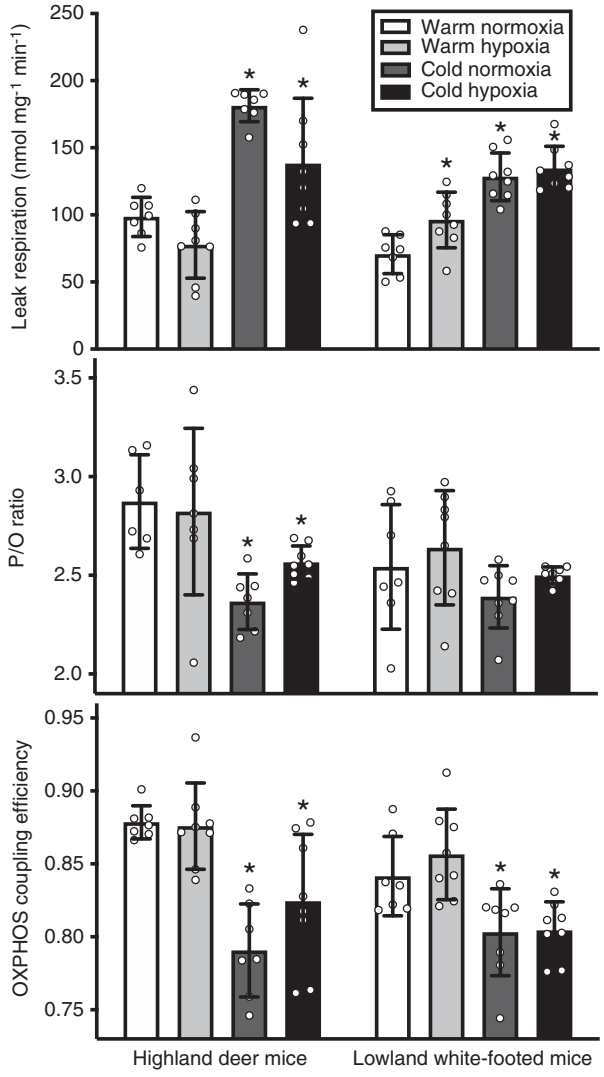

Figure 6. Chronic cold exposure increased leak respiration and reduced phosphorylation efficiency (P/O ratio) and OXPHOS coupling efficiency for isolated mitochondria from hindlimb muscle
Mice from each species were bred in captivity and acclimated during adulthood to each of four treatments: warm (25°C) normoxia; warm hypoxia (12 kPa O_2); cold (5°C) normoxia; or cold hypoxia. Leak respiration was measured in the presence of ATP with pyruvate and malate as substrates ($L_{T,PM}$). OXPHOS coupling efficiency was calculated as $1 - L_{T,PM}/P_{PM}$ (P_{PM}, maximal respiratory capacity for OXPHOS with pyruvate and malate as substrates; Table 3). Bars are means ± SD and circles represent individual values (*N* as in Table 3).*Significant ($P < 0.05$) pairwise differences from warm normoxic controls within a species using Holm-Sidak *post hoc* tests (two-factor ANOVA results are shown in Table 1).

Mitochondrial plasticity in response to cold and hypoxia

Chronic cold exposure induced mitochondrial uncoupling in skeletal muscle, as reflected by increases in leak respiration along with decreases in phosphorylation efficiency and OXPHOS coupling efficiency (Figs 6 and 7), patterns that are consistent with some previous findings in domestic mice and rats (Monemdjou *et al.* 2000; Mollica *et al.* 2005). Cold-induced mitochondrial uncoupling has long been considered to be an important potential source of heat production, derived from the increased metabolic flux required to sustain higher rates of leak respiration. In fact, an early suggestion that skeletal muscle can support non-shivering thermogenesis came from observations of cold-induced increases in leak respiration and decreases in phosphorylation efficiency in mitochondria isolated from fur seal muscle (Grav & Blix, 1979). Our results here suggest that cold-induced mitochondrial uncoupling also occurs during concurrent exposure to hypoxia, a noteworthy result considering that hypoxia could be expected to place a premium on metabolic efficiency to cope with O_2 limitation.

Chronic cold exposure also increased mitochondrial respiratory capacity in white-footed mice (Fig. 4), consistent with some previous studies of mice, rats and rabbits (Zaninovich *et al.* 2003; Arruda *et al.* 2008; Shabalina *et al.* 2010), but not others (Monemdjou *et al.* 2000; Mollica *et al.* 2005; Mineo *et al.* 2012). In the latter studies, the two using mitochondria isolated from hindlimb muscle examined respiratory capacity with electron entry via complex II (succinate and rotenone) (Monemdjou *et al.* 2000; Mollica *et al.* 2005). However, our results suggest that cold exposure leads to a preferential decline in respiratory capacity via complex II relative to complexes I+II ($P_{S(Rot)}/P_{PMGS}$) (Fig. 5). Therefore, at least some of the discrepancy in the literature may have arisen from differences in the choice of substrates used to support mitochondrial respiration. This emphasizes the importance of using a comprehensive substrate titration protocol for examining mitochondrial function, like the one used here, in order to fully appreciate mitochondrial plasticity. Nevertheless, our finding that high-altitude deer

mice did not increase mitochondrial respiratory capacity in cold environments (Fig. 4) reveals a clear species difference in the cold response (Fig. 7).

The reason for the preferential decline in respiratory capacity via complex II relative to complexes I+II is not entirely clear, but it could reflect changes in mitochondrial composition. Exercise training leads to a greater upregulation of complex I than complex II in the skeletal muscle of humans, largely as a result of increases in the content of mitochondrial supercomplexes assembled from complexes I, III and IV (Greggio *et al.* 2017), so it is possible that increased shivering activity with cold exposure had similar effects and thus altered the stoichiometry of complex II relative to other complexes. Cold exposure could have also altered fuel use, thus changing the relative use of NADH and leading to adjustments in the relative abundance of complex I *versus* complex II (Acín-Pérez *et al.* 2014). Future work examining mitochondrial supercomplexes and the relative protein abundance of each individual complex could provide insight into this issue.

Chronic exposure to hypoxia alone had relatively few effects on mitochondrial physiology. Hypoxia had no effect on mitochondrial coupling or phosphorylation efficiency, unlike the strong effects of cold (Fig. 6). Furthermore, the effects of chronic hypoxia on mitochondrial respiratory capacity were inconsistent between highlanders and lowlanders, a result that is consistent with our previous comparisons between high-and low-altitude populations of deer mice (Mahalingam *et al.* 2017). Specifically, chronic hypoxia increased OXPHOS capacity in white-footed mice, but not in high-altitude deer mice. These divergent responses to hypoxia were eliminated after normalizing mitochondrial respiration to citrate synthase activity as a TCA cycle marker (Fig. 4), suggesting that coordinated regulation of the electron transport system and TCA cycle is involved in the adjustments in OXPHOS capacity.

There are few previous studies of chronic hypoxia on mitochondrial physiology, but measurements of mitochondrial respiration in permeabilized muscle fibres from low-altitude humans suggest that high-altitude acclimatization has little effect on OXPHOS capacity (Horscroft *et al.* 2017; Chicco *et al.* 2018). However, the increased OXPHOS capacity in white-footed mice was not associated with a change in the capacity for oxidation of acyl-carnitines in chronic hypoxia (Table 3), and the substrate control ratio for acyl-carnitine oxidation appeared to decline (Fig. 5). This contrasts with previous findings in humans using permeabilized fibres from the vastus lateralis muscle, in which chronic hypoxia increased the respiratory capacity for lipid oxidation without affecting OXPHOS capacity (Chicco *et al.* 2018). The mitochondrial capacity for lipid oxidation is not expected to limit submaximal rates of lipid oxidation *in vivo*. Indeed, chronic hypoxia does not alter relative fuel use in low-altitude mice running at 75% of exercise $\dot{V}_{O_2\,max}$ (Lau *et al.* 2017), nor does chronic hypoxia affect relative fuel use in lowland humans or in other lowland taxa (McClelland *et al.* 1998; Lundby & Van Hall, 2002). Mitochondrial capacity for lipid oxidation may play a more important role during the high rates of lipid oxidation that are observed in mice during maximal aerobic thermogenesis (Cheviron *et al.* 2012).

Mitochondrial physiology in high-altitude natives

The results here confirm our previous findings that high-altitude deer mice have evolved a high oxidative capacity in skeletal muscle through several mechanisms (Scott *et al.* 2018). Highlanders have increased oxidative enzyme activities (CS, COX, hydroxyacyl-CoA dehydrogenase) and increased relative abundance of oxidative fibre-types in the gastrocnemius muscle compared to lowland deer mice (Lui *et al.* 2015;

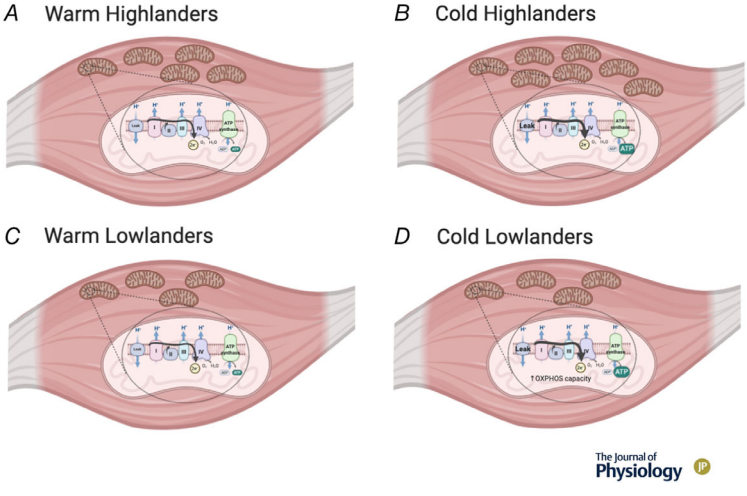

Figure 7. Summary of mitochondrial responses to chronic cold exposure in the gastrocnemius of *Peromyscus* mice
Cold exposure increases mitochondrial leak, thus reducing phosphorylation and/or OXPHOS coupling efficiency, and decreases the capacity for mitochondrial respiration via complex II relative to complexes I and II combined (shown as a thickened arrow along the path of electron flow from complex I to III). High-altitude deer mice (highlanders; *A* and *B*) appear to respond to cold with increases in mitochondrial volume (depicted by the increased number of mitochondria), whereas low-altitude white-footed mice (lowlanders; *C* and *D*) respond to cold with increases in mitochondrial OXPHOS capacity and oxidative fibre density (latter not shown). Exposure to hypoxia had only modest effects or no effects on these phenotypes. Figure created with BioRender.com. [Colour figure can be viewed at wileyonlinelibrary.com]

Scott *et al.* 2015; Lau *et al.* 2017), similar to our comparison to lowland white-footed mice here. These evolved phenotypes arise in early juvenile development concurrent with the ontogenesis of a high thermogenic capacity (Robertson & McClelland, 2019). Highlanders also have increased mitochondrial volume density within muscle fibres, by a magnitude that is comparable to the increased CS activity, and which results entirely from an increased abundance of subsarcolemmal mitochondria (Mahalingam *et al.* 2017). These phenotypes do not appear to change in response to chronic hypoxia exposure, either during adulthood or in early development (Lui *et al.* 2015; Mahalingam *et al.* 2017; Nikel *et al.* 2017). However, our results here suggest that chronic exposure to cold environments does increase CS and/or COX activities in highlanders, such that they still appear to maintain higher activities than lowlanders (Fig. 1), which probably contributes to their high capacity for lipid-fuelled thermogenesis in the wild (Cheviron *et al.* 2012). These cold-induced increases in CS and COX in highlanders did not arise from changes in the abundance of oxidative fibres (Fig. 2) or in the oxidative capacity of isolated mitochondria (Fig. 4, Table 3), suggesting that they may instead result solely from mitochondrial biogenesis within particular fibre types. In lowland white-footed mice, by contrast, cold-induced changes in muscle enzyme activities were associated with changes in the abundance of oxidative fibres and in mitochondrial oxidative capacity. It is possible that these species differences in the cold response were associated with differences in locomotor activity or in the use of shivering or non-shivering thermogenesis during cold exposure, but these traits have yet to be measured. Nevertheless, in considering the importance of skeletal muscle for shivering and non-shivering thermogenesis, the high oxidative capacity of skeletal muscle probably evolved in high-altitude deer mice as a result of selection for increased thermogenic capacity in the cold and hypoxic alpine environment.

Conclusions

Our results support growing evidence for an important role of mitochondrial plasticity in the mechanisms used by animals to cope in challenging environments. Both species adjusted mitochondrial function in response to chronic cold exposure, including relative reductions in complex II capacity for OXPHOS, increases in leak respiration, and decreases in phosphorylation efficiency and OXPHOS coupling efficiency (Fig. 7). Cold exposure had no effect on the maximal respiratory capacity of mitochondria in highland deer mice, which already had a high capacity in warm conditions, but cold exposure did increase mitochondrial respiratory capacity in white-footed mice (Fig. 7). These results suggest that several mechanisms of mitochondrial plasticity may play a key role in adaptive thermogenesis in skeletal muscle, and may contribute to the strong increases in thermogenic capacity that these species exhibit in response to cold exposure (Chappell & Hammond, 2004; Rezende *et al.* 2004; Tate *et al.* 2017, 2020).

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Additional information

Data availability statement

The data that support the findings of this study are available in the published figures or in the Supporting information for this article.

Competing interests

The authors declare that they have no competing interests.

Author contributions

Experiments were performed at McMaster University. G.R.S., G.B.M., Z.A.C. and J.F.S. conceived and funded the work, and all authors were involved in study design. S.M. performed the experiments. S.M. and G.R.S. analysed the data and drafted the article. All authors were involved in data interpretation and critical revision of the article. All authors qualify for authorship, approved the final version of the article, and agree to be accountable for all aspects of the work. All those who qualify for authorship are listed as authors.

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Keywords

evolutionary physiology, high-altitude adaptation, lipid oxidation, mitochondrial metabolism, substrate control ratio

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document
Table S1. Individual data to accompany the summary data for body and muscle masses that is provided in Table 2.
Table S2. Individual data to accompany the summary data for muscle histological phenotypes that is provided in Table 2.
Table S3. Individual data to accompany the summary data for isolated mitochondria phenotypes that is provided in Table 3.