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RESEARCH ARTICLE

Adaptive increases in respiratory capacity and O_2 affinity of subsarcolemmal mitochondria from skeletal muscle of high-altitude deer mice

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Abstract

Aerobic energy demands have led to the evolution of complex mitochondrial reticula in highly oxidative muscles, but the extent to which metabolic challenges can be met with adaptive changes in physiology of specific mitochondrial fractions remains unresolved. We examined mitochondrial mechanisms supporting adaptive increases in aerobic performance in deer mice (*Peromyscus maniculatus*) adapted to the hypoxic environment at high altitude. High-altitude and low-altitude mice were born and raised in captivity, and exposed as adults to normoxia or hypobaric hypoxia (12 kPa O_2 for 6–8 weeks). Subsarcolemmal and intermyofibrillar mitochondria were isolated from the gastrocnemius, and a comprehensive substrate titration protocol was used to examine mitochondrial physiology and O_2 kinetics by high-resolution respirometry and fluorometry. High-altitude mice had greater yield, respiratory capacity for oxidative phosphorylation, and O_2 affinity (lower P_{50}) of subsarcolemmal mitochondria compared to low-altitude mice across environments, but there were no species difference in these traits in intermyofibrillar mitochondria. High-altitude mice also had greater capacities of complex II relative to complexes I + II and higher succinate dehydrogenase activities in both mitochondrial fractions. Exposure to chronic hypoxia reduced reactive oxygen species (ROS) emission in high-altitude mice but not in low-altitude mice. Our findings suggest that functional changes in subsarcolemmal mitochondria contribute to improving aerobic performance in hypoxia in high-altitude deer mice. Therefore, physiological variation in specific mitochondrial fractions can help overcome the metabolic challenges of life at high altitude.

KEYWORDS

bioenergetics, biological evolution, high-altitude hypoxia, muscle mitochondria, reactive oxygen species

Abbreviations: COX, cytochrome c oxidase; CS, citrate synthase; IMM, intermyofibrillar mitochondria; OXPHOS, oxidative phosphorylation; PO_2, partial pressure of O_2; P_{50}, PO_2 at 50% inhibition of mitochondrial respiration; ROS, reactive oxygen species; SDH, succinate dehydrogenase; SSM, subsarcolemmal mitochondria.

1 | INTRODUCTION

The high aerobic energy demands of muscle function have led to the evolution of complex mitochondrial reticula in highly oxidative striated muscles.[1,2] Mitochondria are located in multiple distinct locations throughout muscle cells, including locations near the sarcolemma and nearby capillaries ("subsarcolemmal mitochondria" or "paravascular mitochondria") as well as locations more closely associated with myofibrils ("intermyofibrillar mitochondria") near the I-band and elsewhere.[3,4] The structural organization of the mitochondrial reticulum connecting these mitochondria has important consequences for cellular energy distribution,[5] and the electrical connections throughout the reticulum enable mitochondria in different locations of the cell to carry out distinct functions.[3,6] As a result, changes in metabolic demands on the muscle, such as in response to exercise training or evolutionary adaptation to extreme environments, can be associated with changes in the abundance of distinct mitochondrial fractions (e.g., increased volume density of subsarcolemmal mitochondria).[7–9] Previous findings have also demonstrated that subsarcolemmal and intermyofibrillar mitochondria can have different respiratory properties and different functional responses to exercise training.[10–12] Nevertheless, the extent to which metabolic challenges can be met with adaptive changes in the physiology of specific mitochondrial fractions remains poorly understood.

Mitochondrial O_2 kinetics is a relatively unexplored area of mitochondrial physiology that could have important implications for muscle metabolism. Although mitochondrial respiration is insensitive to O_2 across a broad range of high O_2 levels, elevated rates of metabolic ATP demand and mitochondrial O_2 consumption can reduce cellular O_2 pressure to levels that have the potential to limit mitochondrial respiration.[13,14] Indeed, recent work suggests that mitochondrial O_2 affinity (calculated as P_{50}, the partial pressure of O_2 at 50% inhibition of respiration by hypoxia) can have an impact on maximal O_2 consumption during exercise,[15,16] and affinity increases in association with maximal O_2 consumption after exercise training.[17] Mitochondrial O_2 affinity might be expected to have an even greater influence in hypoxic environments where cellular O_2 pressure can be reduced further, and evidence from fish suggests that mitochondrial O_2 affinity is increased in taxa that are more tolerant of environmental hypoxia.[18,19] In contrast, studies of endotherms have yielded equivocal conclusions on whether increases in mitochondrial O_2 affinity are a common adaptation to hypoxic environments.[8,9] It is largely unknown whether O_2 affinity differs between subsarcolemmal and intermyofibrillar mitochondria, or whether the O_2 affinity of mitochondrial fractions exhibits distinct responses to changes in metabolic demands.

High-altitude natives provide an excellent opportunity to examine how adaptive variation in mitochondrial physiology contributes to aerobic performance. Cold temperatures at high altitude can demand high rates of aerobic metabolism to support thermogenesis, particularly in small mammals by virtue of their high surface area to volume ratio,[20] while hypoxia at high altitude can constrain O_2 supply to support mitochondrial respiration. Many high-altitude natives appear to have evolved solutions to help overcome these challenges, and thus avoid potential imbalances between mitochondrial O_2 supply and demand.[21,22] Recent evidence suggests that evolved changes in the physiology and abundance of muscle mitochondria are involved in these solutions,[23–26] but the role of distinct mitochondrial fractions remains unresolved. Some high-altitude birds and small mammals have greater abundance of subsarcolemmal mitochondria in skeletal muscles compared to low-altitude taxa,[8,9] but this is not true of Tibetan humans.[24] Besides mitochondrial abundance, it is unknown whether the physiology of subsarcolemmal mitochondria is distinctly altered in high-altitude natives to help improve respiration during hypoxia.

Deer mice (*Peromyscus maniculatus*) native to high altitude are a powerful model for understanding the mitochondrial mechanisms underlying aerobic performance. Deer mice are found across a broad altitudinal range, from near sea level to over 4300 m elevation in the Rocky Mountains,[27,28] and high-altitude populations must maintain high field metabolic rates to support heat generation in cold montane habitats.[20] High-altitude populations also exhibit high aerobic capacity (VO_2max) in hypoxia (both during thermogenesis and intense exercise), and this has been shown to be an evolved trait in comparisons to both low-altitude populations of deer mice and white-footed mice (*P. leucopus*), a closely related species that is restricted to low altitudes.[29–33] This evolved increase in VO_2max is associated with several changes in the phenotype of the gastrocnemius—a large hind limb muscle involved in shivering and locomotion—including increases in capillarity, density of oxidative fiber types, mitochondrial volume density, and respiratory capacity of permeabilized muscle fibers, along with the differential expression of genes regulating metabolism and capillary growth (detected using RNA-Seq).[8,31,34–36] The increased mitochondrial volume density in high-altitude deer mice is entirely caused by a greater abundance of subsarcolemmal mitochondria,[8] but the physiology of the separate subsarcolemmal and intermyofibrillar fractions of muscle mitochondria had yet to be assessed. This study therefore examined the physiology of subsarcolemmal and intermyofibrillar mitochondria in high-altitude populations of the deer mouse and in the low-altitude white-footed mouse. Mice were acclimated to normoxia and hypoxia to

consider effects of the environment on the physiology of each mitochondrial fraction. We examined mitochondrial respiration, O_2 kinetics, and reactive oxygen species (ROS) emission using a comprehensive substrate titration protocol to assess distinct fuel pathways (carbohydrate vs. lipid substrates), distinct points 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 mitochondrial physiology has evolved in high-altitude deer mice to help improve aerobic performance in hypoxia, predicting that high-altitude deer mice will exhibit greater mitochondrial respiratory capacity and O_2 affinity than low-altitude mice.

2 | MATERIALS AND METHODS

2.1 | Animals and environmental exposures

Adult deer mice (*P. maniculatus*) from a population native to high altitude were caught on the summit of Mount Evans Colorado (39°35′18″N, 105°38′38″W; ~4350 m above sea level). Adult white-footed mice (*P. leucopus*) were caught at low altitude on the Great Plains of Nebraska (40°52′12″N, 96°48′20.3″W; ~430 m above sea level). Mice were then transported to McMaster University (elevation 50 m) where they were bred in captivity. First-generation lab-bred offspring from three distinct highland families and four distinct lowland families were used for experiments. These first-generation mice were raised to adulthood (6–12 months of age) at ~25°C with a 12:12 light–dark photoperiod and were housed in standard mouse cages (containing 7090 Teklad Sani-Chips® animal bedding; Envigo, Indianapolis, IN, USA) with unlimited access to water and standard rodent chow (Teklad 22/5 Rodent Diet formula 8640; Envigo). Adults were then acclimated for 6–8 weeks to normobaric normoxia (standard lab conditions) or to hypobaric hypoxia simulating an elevation of 4300 m (partial pressure of O_2 ~12 kPa, barometric pressure ~60 kPa). The total number of mice used for experiments was eight normoxic highlanders (five males, three females), eight hypoxic highlanders (three males, five females), six normoxic lowlanders (five males, one female), and seven hypoxic lowlanders (five males, two females). However, sample loss precluded one set of measurements, so the number of mice used for each measurement is clearly indicated in each figure and table. Hypobaric hypoxia was achieved using specially designed hypobaric chambers that have been described previously.[37,38] After acclimation, mice were euthanized (isoflurane overdose followed by cervical dislocation), body mass was immediately measured, both gastrocnemii muscles were harvested and weighed, and these muscles were used to isolate mitochondria. All animal procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care and were approved by McMaster Animal Research Ethics Board.

2.2 | Mitochondrial isolation

The gastrocnemii were transferred immediately after harvesting 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, pH 7.4), the composition of which has been used for mitochondrial isolations in previous research with mammals and birds.[8,9,34,39,40] The muscle was minced and was then gently homogenized with six passes of a Potter–Elvehjem Teflon on glass homogenizer (100 r.p.m.). Separate mitochondrial fractions were then isolated via differential centrifugation at 4°C as follows. Homogenates were centrifuged at 1000 *g* for 10 min. The resulting supernatant (which would contain primarily subsarcolemmal mitochondria, SSM) was separated from the pellet and retained, and the pellet was resuspended in 10 ml of isolation buffer containing nagarse protease (1 mg per g of original muscle tissue) and left on ice to digest for 5 min. The digest was filtered through cheesecloth and centrifuged at 1000 *g* for 10 min to remove cellular debris, and the supernatant (which would contain primarily intermyofibrillar mitochondria, IMM) was retained. Both of these SSM and IMM fractions were then centrifuged at 8700 *g* for 10 min, the pellets were resuspended in 10 ml of fresh isolation buffer containing bovine serum albumin (BSA, fatty acid-free, at 1% mass:volume), and centrifuged again at 8700 *g*. These pellets were then resuspended in 10 ml of storage buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM potassium methanesulfonate, 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 again at 8700 *g*. The pellets were finally resuspended in a small volume of storage buffer (500 μl for IMM fraction; 175–350 μl for SSM fraction depending on the perceived yield). Part of this mitochondrial isolate was kept on ice until mitochondrial physiology was measured, and the rest was stored at −80°C for later use in enzyme assays.

2.3 | Mitochondrial physiology measurements

Mitochondrial physiology was measured at 37°C using high-resolution respirometry and fluorometry in a Oxygraph-2k with O2k-Fluorescence module (Oroboros

Instruments, Innsbruck, Austria). Isolates of SSM and IMM fractions were each added to a separate respirometry chamber (using ~50 and ~30 μg mitochondrial protein, respectively) that contained 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 fatty acid-free BSA; pH 7.1). This respiration buffer (MiR05) and the modified storage buffer above are well-established media for the prolonged stability of mitochondria in vitro.[41,42] Respiration rate was measured as the rate of decline in O_2 concentration in the chamber, which contained a final volume of 2 ml. Reactive oxygen species (ROS) emission rates were measured by fluorescence detection of resorufin (excitation wavelength of 525 nm, AmR filter set, Oroboros Instruments), which is produced from hydrogen peroxide (H_2O_2) and Ampliflu Red (10 μM; Sigma-Aldrich, Oakville, Ontario, Canada) in a reaction catalyzed by horseradish peroxidase (3 U/ml) and superoxide dismutase (SOD; 22.5 U/ml). Calibration of the fluorescent resorufin signal was done with the addition of exogenous H_2O_2.

Mitochondrial respiration and ROS emission of SSM and IMM fractions were measured in each of two separate protocols. The first protocol was used to assess mitochondrial respiratory capacities for carbohydrate oxidation and O_2 kinetics. Malate (2 mM) and pyruvate (5 mM) were added to stimulate leak state respiration without adenylates ($L_{N,PM}$). Oxidative phosphorylation (OXPHOS) was then stimulated with the addition of 5 mM ADP, reflecting mitochondrial respiratory capacity for pyruvate oxidation (P_{PM}). Glutamate (10 mM) was then added to stimulate OXPHOS capacity via complex I (P_{PMG}). Succinate (25 mM) was next added to determine OXPHOS capacity via complexes I + II (P_{PMGS}). Mitochondria were then allowed to consume all the O_2 in the chamber to determine the O_2 kinetics of mitochondrial respiration. The chamber was then reoxygenated and exogenous cytochrome c (10 μM) was added to assess the integrity of the outer mitochondrial membrane, and the effect on respiration was always modest (~5% on average and always <10%). Finally, ascorbate (0.5 mM) followed by N,N,N,N-tetramethyl-p-phenylenediamine (TMPD; 0.5 mM) was used to measure the respiratory capacity of complex IV (P_{Tm}). ROS emission rates are not reported after the addition of cytochrome c, ascorbate, and TMPD, because they are strongly redox-active.

The second protocol was used to assess mitochondrial respiratory capacities for fatty acid oxidation. Malate (2 mM) and palmitoyl-carnitine (50 μM) were added to stimulate leak state respiration without adenylates ($L_{N,\ PcM}$), then OXPHOS was stimulated with 5 mM ADP (P_{PcM}). Octanoyl-carnitine (0.5 mM) was added to determine the mitochondrial respiratory capacity for oxidizing multiple acyl-carnitines (P_{PcOcM}). This was followed by sequential additions of glutamate (P_{PcOcMG}), pyruvate ($P_{PcOcMGP}$), and succinate ($P_{PcOcMGPS}$) at the concentrations used in the first protocol. In both the first and second protocols, mitochondrial respiration and ROS emission rates are expressed per mg mitochondrial protein, which was measured using the Bradford assay (following instructions of the manufacturer; Bio-Rad Laboratories, Montreal, Canada).

Mitochondrial O_2 kinetics were examined using DatLab 2 software (Oroboros Instruments, Innsbruck, Austria). The relationship between mitochondrial respiration rate (VO_2) and partial pressure of O_2 (PO_2) during the entry into anoxia was fitted to the equation $VO_2 = V_{max} \times PO_2/(P_{50} + PO_2)$, where V_{max} is the mitochondrial respiration uninhibited by hypoxia and P_{50} is the PO_2 at which respiration is 50% of V_{max}. The response time delay of the oxygen sensor, sensor drift, and background O_2 flux of the O_2 sensor were accounted for as previously recommended.[43,44]

2.4 | Enzyme assays

The maximal activities of citrate synthase (CS), cytochrome c oxidase (COX; complex IV), and succinate dehydrogenase (SDH; complex II) were measured in mitochondrial isolates using assay protocols that have been previously described.[45,46] Isolates were first homogenized in 10 volumes of ice-cold buffer (100 mM KH_2PO_4, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.2), then centrifuged at 1000 *g* at 4°C, and the supernatant was collected for use in assays. Enzyme activity was assayed at 37°C using a SpectraMax Plus 384 spectrophotometer and Softmax Pro software (Molecular Devices, Sunnyvale, CA, USA) in the following conditions: CS, 100 mM KH_2PO_4, 0.5 mM oxaloacetate, 0.15 mM acetyl-coA, 0.15 mM 5,5′-dithiobis-2-nitrobenzoic acid, pH 8.0; COX, 100 mM KH_2PO_4, 0.2 mM reduced cytochrome c, pH 7.2; SDH, 20 mM succinate, 0.05 mM decylubiquinone, 0.05 mM dichlorophenolindophenol, 0.3 mM KCN, pH 7.5. Activities were measured in triplicate at 412 nm for CS (extinction coefficient [ε], 14.15 mM^{-1} cm^{-1}), 550 nm for COX (ε, 28.5 mM^{-1} cm^{-1}), and 600 nm for SDH (ε, 21.9 mM^{-1} cm^{-1}), and are expressed in units of μmol substrate per mg mitochondrial protein per min. Preliminary experiments determined that all substrate concentrations were appropriate for eliciting maximal enzyme activity.

2.5 | Statistical analysis

Two-factor ANOVA was used to evaluate the effects of species, acclimation environment, and their interaction. The full set of *p*-values for all two-factor ANOVAs are reported in tables, and some key *p*-values are also reported in the text. When main effects or interactions were significant, we carried out Holm–Šídák post hoc tests for pairwise differences between species within an environment and/or between environments within each species, and we report significant pairwise differences using symbols on associated figures and tables. $p < .05$ was considered statistically significant. Statistical tests were performed using Prism software (version 9.3; GraphPad Software, San Diego, CA).

3 | RESULTS

The yield of subsarcolemmal mitochondria (SSM) in isolates from the gastrocnemius muscle was much greater in high-altitude deer mice ("highlanders") than in low-altitude white-footed mice ("lowlanders") (Figure 1, Table 1). This was reflected by a significant overall difference in SSM yield between highlanders and lowlanders (main effect of species in two-factor ANOVA, $p < .001$), but SSM yield was unaffected by acclimation environment (main effect of environment, $p = .640$). However, the yield of intermyofibrillar mitochondria (IMM) was similar between highlanders and lowlanders (species effect, $p = .160$) and was unaffected by acclimation environment (environment effect, $p = .811$). Highlanders also had smaller gastrocnemius muscles than lowlanders (species effect, $p = .044$) after accounting for the species differences in body mass (Table 2).

The respiratory capacity of subsarcolemmal mitochondria was greater in highlanders than in lowlanders (Figure 2, Table 1). This was reflected by the significant overall difference in the respiratory capacity of SSM between highlanders and lowlanders (species effect, $p < .001$), and the effects of hypoxia acclimation also differed between species (species × environment, $p = .036$). Indeed, hypoxia acclimation appeared to increase SSM respiratory capacity in highlanders but decrease it in lowlanders, which resulted in values that were 68% higher in highlanders after hypoxia acclimation. In contrast, highlanders and lowlanders had similar IMM respiratory capacities (species effect, $p = .967$), and hypoxia led to an overall decline in respiration that was similar in magnitude between species (environment effect, $p = .049$).

Substrate control ratios were calculated from respiration rates measured using different combinations of mitochondrial substrates (Tables 3 and 4), and the observed

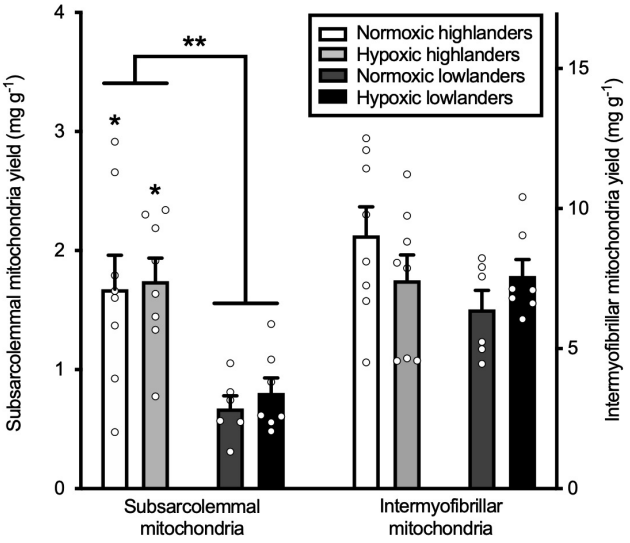

FIGURE 1 The yield of subsarcolemmal mitochondria in isolates from the gastrocnemius muscle is greater in highlanders than in lowlanders. Yield (mg of mitochondrial protein per g muscle mass) is expressed with bars representing means + SE and circles representing individual values. *Significant ($p < .05$) pairwise differences between highlanders and lowlanders within the same acclimation environment in Holm–Šídák post hoc tests. **Significant ($p < .05$) main effect of species in two-factor ANOVA (full ANOVA results are shown in Table 1).

variation was suggestive of further differences in mitochondrial physiology between highlanders and lowlanders (Figure 3, Table 1). When OXPHOS respiration supported by pyruvate oxidation (P_{PM}) or by maximal stimulation of complex I (P_{PMG}) was expressed relative to total OXPHOS capacity via complexes I + II (P_{PMGS}), highlanders had lower values than lowlanders overall in both SSM and IMM (species effects, $p \leq .001$). The species differences in this trait were greatest after hypoxia acclimation, due to differences between species in the effects of hypoxia acclimation (species × environment for P_{PMG}/P_{PMGS}, $p = .037$ and .050, respectively). These results suggest that highlanders may have an increased OXPHOS capacity for complex II relative to complexes I + II combined. However, the capacity for acyl-carnitine oxidation relative to total OXPHOS capacity ($P_{PcOcM}/P_{PcOcMGS}$) was much more similar overall between species in both SSM and IMM (species effects, $p = .126$ and .203), although the unique effect of hypoxia acclimation in highlanders persisted (species × environment, $p = .040$ and .050). The respiratory capacity of complex IV (P_{Tm}) relative to P_{PMGS}, a measure of the excess capacity of cytochrome c oxidase, was similar between species and environments in both SSM and IMM.

We next sought to determine whether the apparent O_2 affinity of mitochondria differed between highlanders and lowlanders (Figure 4, Table 1). The effect of hypoxia on

TABLE 1 *p*-values from two-factor ANOVA analyses of data shown in figures

	Species main effect	Environment main effect	Species × environment
Subsarcolemmal mitochondria			
Yield	<0.001	0.640	0.883
Respiratory capacity	0.002	0.868	0.036
P_{PM}/P_{PMGS}	0.001	0.115	0.194
P_{PMG}/P_{PMGS}	<0.001	0.035	0.037
$P_{PcOcM}/P_{PcOcMGPS}$	0.126	0.107	0.040
P_{Tm}/P_{PMGS}	0.497	0.753	0.887
P_{50}	0.010	0.464	0.423
CS activity	0.346	0.293	0.358
COX activity	0.203	0.148	0.913
SDH activity	0.108	0.179	0.084
ROS-P_{PMGS}	0.711	0.025	0.188
ROS/O_2-P_{PMGS}	0.555	0.008	0.058
Intermyofibrillar mitochondria			
Yield	0.160	0.811	0.116
Respiratory capacity	0.967	0.049	0.910
P_{PM}/P_{PMGS}	0.001	0.354	0.111
P_{PMG}/P_{PMGS}	0.001	0.145	0.050
$P_{PcOcM}/P_{PcOcMGPS}$	0.203	0.237	0.145
P_{Tm}/P_{PMGS}	0.145	0.250	0.783
P_{50}	0.300	0.990	0.897
CS activity	0.189	0.008	0.591
COX activity	0.005	0.703	0.264
SDH activity	0.002	0.320	0.701
ROS-P_{PMGS}	0.292	0.014	0.146
ROS/O_2-P_{PMGS}	0.497	0.199	0.385

Note: See associated figures for symbol abbreviations.

TABLE 2 Body mass and gastrocnemius mass

	Body mass	Gastrocnemius mass
Normoxic highlanders	20.5 ± 0.9 (8)*	7.68 ± 0.61 (8)*
Hypoxic highlanders	17.9 ± 0.8 (8)*	7.94 ± 0.15 (8)
Normoxic lowlanders	34.4 ± 2.3 (6)	9.20 ± 0.47 (6)
Hypoxic lowlanders	27.9 ± 1.4 (7)	8.28 ± 0.38 (7)
Species effect	$p < .001$	$p = .044$
Environment effect	$p = .012$	$p = .456$
Species × environment	$p = .505$	$p = .191$

Note: Body mass (g) and gastrocnemius mass (mg per g body mass) are reported as mean ± SE (N). *p*-values reported are for the main effects and interactions from two-factor ANOVA, and * denotes significant pairwise differences ($p < .05$) between highlanders and lowlanders within the same acclimation environment in Holm–Šídák post hoc tests.

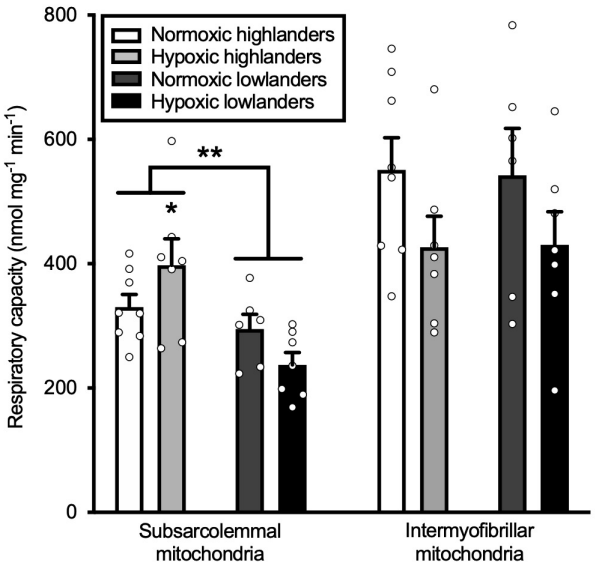

FIGURE 2 The respiratory capacity of subsarcolemmal mitochondria is greater in highlanders than in lowlanders. The respiratory capacity (nmol O_2 per mg of mitochondrial protein per min) of each individual was the greatest value of respiration achieved during oxidative phosphorylation (*P*) using substrates of complexes I and II (i.e., whichever of the P_{PMGS} or $P_{PcOcMGPS}$ states in Table 2 was greater), and is expressed with bars representing means + SE and circles representing individual values. *Significant ($p < .05$) pairwise differences between highlanders and lowlanders within the same acclimation environment in Holm–Šídák post hoc tests. **Significant ($p < .05$) main effect of species in two-factor ANOVA (full ANOVA results are shown in Table 1).

mitochondrial respiration rate (VO_2) at low partial pressures of O_2 (PO_2) is well described by a hyperbolic curve with the equation $VO_2 = V_{max} \times PO_2/(P_{50} + PO_2)$. P_{50} (i.e., the partial pressure of O_2 at 50% inhibition of respiration by hypoxia) is a measure of O_2 affinity, and a lower value of P_{50} reflects an increased O_2 affinity (V_{max} is the mitochondrial respiration uninhibited by hypoxia). We measured P_{50} during OXPHOS respiration via complexes I + II (P_{PMGS}). SSM P_{50} was generally lower in highlanders than in lowlanders (species effect, $p = .010$), and the pairwise difference between species was significant after hypoxia acclimation (Figure 4A). The combined effects of reduced P_{50} and increased respiratory capacity in SSM (and thus a greater V_{max}) in highlanders lead to an appreciable change in the O_2 kinetics of respiration at low O_2 pressures (Figure 4B). In contrast, IMM P_{50} was similar between species and environments, with no significant effects in ANOVA, although P_{50} was much lower on average (i.e., O_2 affinity was higher) than the P_{50} of SSM (Figure 4A).

TABLE 3 Respiration of mitochondria isolated from the gastrocnemius muscle

	Normoxic highlanders	Hypoxic highlanders	Normoxic lowlanders	Hypoxic lowlanders
Subsarcolemmal mitochondria				
$L_{N,PM}$	11.8 ± 2.0 (8)	11.4 ± 2.2 (8)	13.7 ± 3.7 (6)	11.2 ± 2.0 (7)
P_{PM}	103 ± 17 (8)	76 ± 21 (8)	154 ± 39 (6)	132 ± 24 (7)
P_{PMG}	145 ± 23 (8)	102 ± 23 (8)	185 ± 44 (6)	165 ± 25 (7)
P_{PMGS}	254 ± 33 (8)	330 ± 19 (8)*	251 ± 41 (6)	227 ± 22 (7)
P_{Tm}	835 ± 126 (8)	1118 ± 125 (8)*	816 ± 122 (6)	668 ± 97 (7)
$L_{N,PcM}$	32.8 ± 5.8 (8)	36.1 ± 10.7 (7)	40.5 ± 14.5 (6)	35.0 ± 1.6 (7)
P_{PcM}	132 ± 17 (8)	92 ± 21 (7)	104 ± 25 (6)	92 ± 6 (7)
P_{PcOcM}	129 ± 17 (8)	90 ± 22 (7)	102 ± 22 (6)	91 ± 6 (7)
P_{PcOcMG}	200 ± 22 (8)	147 ± 39 (7)	174 ± 26 (6)	165 ± 22 (7)
$P_{PcOcMGP}$	197 ± 22 (8)	142 ± 36 (7)	166 ± 24 (6)	156 ± 21 (7)
$P_{PcOcMGPS}$	305 ± 22 (8)	340 ± 64 (7)*	249 ± 15 (6)	227 ± 22 (7)
Intermyofibrillar mitochondria				
$L_{N,PM}$	33.1 ± 5.4 (8)	18.4 ± 2.6 (8)†	24.0 ± 3.7 (6)	19.6 ± 1.6 (7)
P_{PM}	286 ± 50 (8)	174 ± 48 (8)	334 ± 53 (6)	280 ± 41 (7)
P_{PMG}	380 ± 57 (8)	234 ± 55 (8)	423 ± 61 (6)	345 ± 44 (7)
P_{PMGS}	534 ± 56 (8)	415 ± 44 (8)	542 ± 75 (6)	431 ± 52 (7)
P_{Tm}	1215 ± 68 (8)	1119 ± 58 (8)	1165 ± 155 (6)	976 ± 85 (7)
$L_{N,PcM}$	40.3 ± 5.5 (8)	26.8 ± 6.1 (7)	36.2 ± 3.1 (6)	35.6 ± 4.7 (7)
P_{PcM}	191 ± 22 (8)	101 ± 27 (7)†	161 ± 9 (6)	138 ± 21 (7)
P_{PcOcM}	186 ± 23 (8)	100 ± 26 (7)†	161 ± 10 (6)	135 ± 21 (7)
P_{PcOcMG}	329 ± 43 (8)	192 ± 50 (7)	337 ± 52 (6)	279 ± 36 (7)
$P_{PcOcMGP}$	318 ± 41 (8)	184 ± 47 (7)	317 ± 47 (6)	263 ± 33 (7)
$P_{PcOcMGPS}$	468 ± 53 (8)	329 ± 54 (7)	438 ± 60 (6)	349 ± 44 (7)

Note: Respiration measured at each of the respiration states shown is reported as mean ± SE (N) in units nmol O_2 per mg mitochondrial protein per min. L_N and P, leak respiration (in the absence of ADP) and respiration during oxidative phosphorylation, respectively, with additional subscripts representing substrates (P, pyruvate; M, malate; G, glutamate; S, succinate; Tm, TMPD; Pc, palmitoyl-carnitine; Oc, octanoyl-carnitine). *,†Significant pairwise differences ($p < .05$) in Holm–Šídák post hoc tests between highlanders and lowlanders within the same acclimation environment, or between acclimation environments (normoxia vs. hypoxia) within a species, respectively (full two-factor ANOVA results are shown in Table 4).

The observed variation in mitochondrial respiratory capacities and O_2 kinetics led us to examine whether there were differences in maximal activities of mitochondrial enzymes between highlanders and lowlanders (Figure 5, Table 1). Neither citrate synthase (CS) nor cytochrome c oxidase (COX) activities differed between species and environments in subsarcolemmal mitochondria (i.e., no significant effects in ANOVA). However, there was an overall effect of hypoxia acclimation (environment effect, $p = .008$) that reduced CS activity in intermyofibrillar mitochondria across both species, in parallel with the significant effect of hypoxia acclimation on respiratory capacity (Figure 2). Furthermore, COX activity in IMM was greater overall in highlanders than in lowlanders (species effect, $p = .005$). Succinate dehydrogenase (SDH) activity tended to be greater in mitochondria from highlanders than in mitochondria from lowlanders, as reflected by a significant pairwise difference between species in hypoxia in SSM, and significant species effect ($p = .002$) and pairwise differences between species in IMM.

We also sought to examine whether mitochondrial ROS emission differed between highlanders and lowlanders. In both SSM and IMM, hypoxia acclimation led to a pronounced reduction in the ROS emission rate measured at total OXPHOS capacity via complexes I + II (P_{PMGS}) in highlanders (Figure 6, Table 1), which drove the significant environment effect in ANOVA ($p = .025$), and similar reductions were observed in most other respiration states during leak and OXPHOS respiration (Tables 5 and 6). In the SSM, this coincided with similar variation in the ratio of ROS emission to O_2 consumption (Figure 6; environment effect, $p = .008$; species × environment, $p = .058$).

TABLE 4 *p*-values from two-factor ANOVA analyses of mitochondrial respiration data in Table 3

	Species main effect	Environment main effect	Species × environment
Subsarcolemmal mitochondria			
$L_{N,PM}$	0.732	0.560	0.670
P_{PM}	0.043	0.347	0.933
P_{PMG}	0.080	0.272	0.679
P_{PMGS}	0.082	0.385	0.102
P_{Tm}	0.075	0.529	0.102
$L_{N,PcM}$	0.719	0.903	0.626
P_{PcM}	0.447	0.160	0.447
P_{PcOcM}	0.470	0.172	0.434
P_{PcOcMG}	0.892	0.285	0.454
$P_{PcOcMGP}$	0.768	0.246	0.423
$P_{PcOcMGPS}$	0.030	0.861	0.444
Intermyofibrillar mitochondria			
$L_{N,PM}$	0.316	0.018	0.186
P_{PM}	0.124	0.101	0.554
P_{PMG}	0.184	0.057	0.554
P_{PMGS}	0.831	0.053	0.949
P_{Tm}	0.296	0.128	0.614
$L_{N,PcM}$	0.655	0.186	0.222
P_{PcM}	0.894	0.017	0.143
P_{PcOcM}	0.835	0.017	0.182
P_{PcOcMG}	0.306	0.041	0.395
$P_{PcOcMGP}$	0.363	0.036	0.352
$P_{PcOcMGPS}$	0.926	0.043	0.644

Note: See Table 3 for symbol abbreviations.

However, there were no significant effects of hypoxia acclimation on ROS emission rate or ROS/O_2 in lowlanders in either the SSM or IMM.

4 | DISCUSSION

High-altitude populations of deer mice have evolved an increased aerobic capacity (VO_2max) in hypoxia compared to their low-altitude counterparts,[29–33] in association with multiple changes in the metabolic phenotype of skeletal muscle.[8,31,34–36] Our findings here show that functional differences in subsarcolemmal mitochondria from skeletal muscle may also contribute to improving aerobic performance in hypoxia in high-altitude deer mice. Subsarcolemmal mitochondria from high-altitude mice had greater respiratory capacity and O_2 affinity than those from low-altitude mice, leading to an appreciable change in O_2 kinetics that increased respiration at low O_2 pressures (Figures 2 and 4). These differences were specific to subsarcolemmal mitochondria, as these traits were similar between species in intermyofibrillar mitochondria. High-altitude mice also reduced mitochondrial ROS emission in chronic hypoxia, in contrast to low-altitude mice in which ROS emission was unaltered by chronic hypoxia (Figure 6). Therefore, our results suggest that adaptive changes in the physiology of specific mitochondrial fractions can help overcome the metabolic challenges of life in the harsh environment at high altitude.

4.1 | Differences in mitochondrial physiology in high-altitude mice

The effects of both greater yield and greater OXPHOS capacity of subsarcolemmal mitochondria should combine to augment muscle respiratory capacity in high-altitude deer mice (Figures 1 and 2). Indeed, previous studies have shown that the respiratory capacity of permeabilized fibers from gastrocnemius muscle is ≥40% higher in high-altitude deer mice than in conspecifics from low altitude.[8] This difference in highlanders is associated with an increased abundance of oxidative fiber types and an increased volume density of subsarcolemmal mitochondria in the muscle.[8,31] Similar differences have been observed in the high-flying bar-headed goose in comparison to closely related low-altitude species.[9,47] However, in high-altitude deer mice, differences in fiber-type composition and mitochondrial volume density cannot entirely explain their greater muscle respiratory capacity.[8,31] Our results here show that this discrepancy is likely accounted for by differences in the respiratory capacity per volume of subsarcolemmal mitochondria. It also expands upon the findings of previous comparisons between high-altitude deer mice and low-altitude white-footed mice.[34] That work showed that highlanders generally had greater respiratory capacity than lowlanders in measurements on mitochondria isolated from the entire hind limb musculature (without separating separate fractions).[34] Our results here suggest that these differences were driven primarily by differences in the subsarcolemmal fraction.

Multiple potential mechanisms could account for the greater O_2 affinity of subsarcolemmal mitochondria in highlanders compared to lowlanders (Figure 4). Mitochondrial O_2 affinity depends strongly on the relative catalytic activity of cytochrome c oxidase (COX), with higher P_{50} observed in states with greater relative activation of the electron transport system (e.g., leak vs. OXPHOS, OXPHOS via complex I vs. complexes I + II).[8,14,17] This mechanism could explain the observed

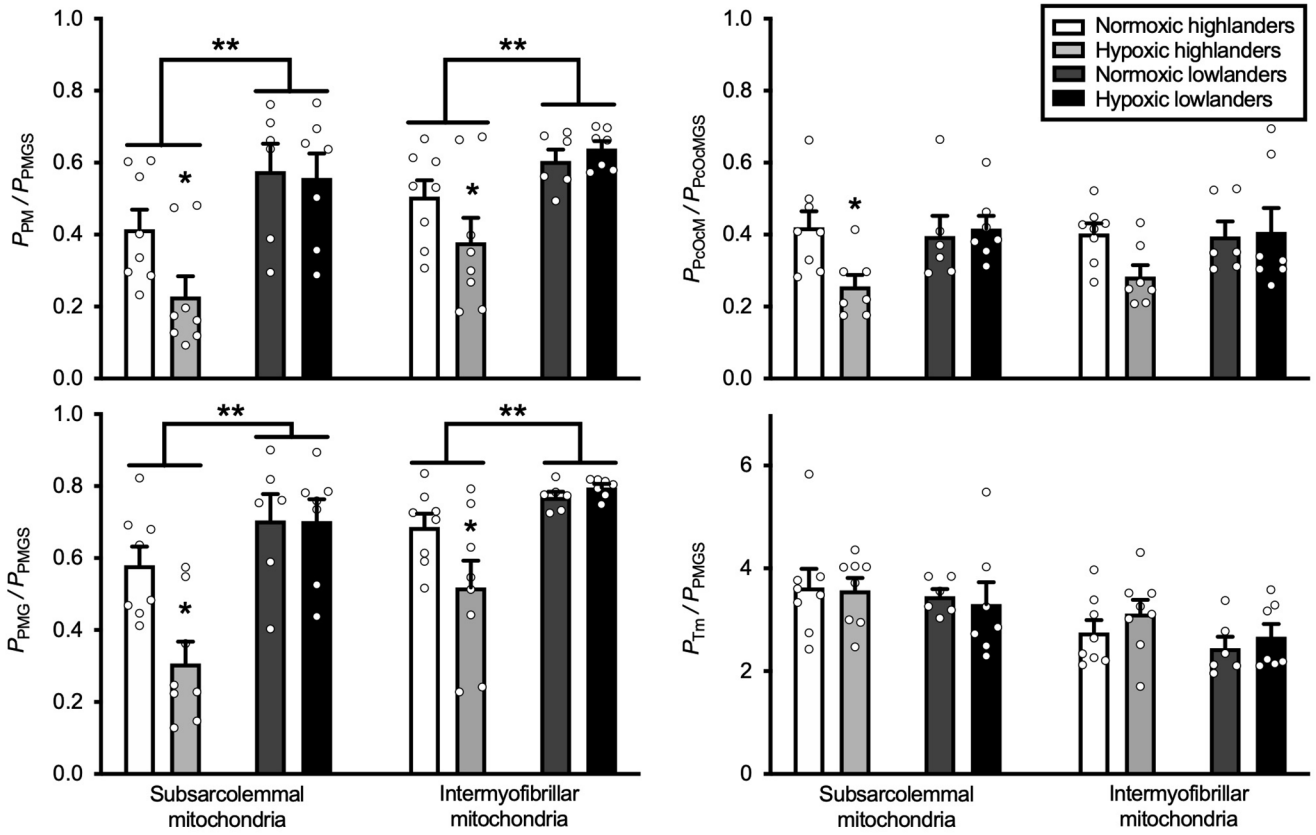

FIGURE 3 Substrate control of mitochondrial respiration during oxidative phosphorylation (OXPHOS, *P*) differed between highlanders and lowlanders. Substrate control ratios were calculated relative to maximal OXPHOS respiration via complexes I and II during each mitochondrial experiment (P_{PMGS} or $P_{PcOcMGS}$ as appropriate) for OXPHOS respiration measurements using each of four substrate combinations: maximal oxidation of pyruvate (P_{PM}); maximal oxidation of acyl-carnitines (P_{PcOcM}); maximal respiration via complex I (P_{PMG}); and respiratory capacity of cytochrome c oxidase (complex IV; P_{Tm}). 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; S, succinate; Tm, TMPD and ascorbate. Bars represent means + SE and circles representing individual values. *Significant ($p < .05$) pairwise differences between highlanders and lowlanders within the same acclimation environment in Holm–Šídák post hoc tests. **Significant ($p < .05$) main effect of species in two-factor ANOVA (full ANOVA results are shown in Table 1).

variation in O_2 affinity if highlanders had greater excess capacity of COX than lowlanders, such that highlanders supported OXPHOS respiration with lower relative COX activity. This was clearly not the case, because highlanders had similar COX activities (Figure 5) and similar COX excess capacities (P_{Tm}/P_{PMGS} in Figure 3) in subsarcolemmal mitochondria compared to lowlanders. It is instead possible that mitochondrial O_2 affinity is increased in highlanders due to a greater O_2 affinity of the COX enzyme, as observed in some hypoxia-tolerant fish.[18] If so, this raises the question of whether the species differences in O_2 affinity result from functional changes in COX subunit(s) that are uniquely expressed in the subsarcolemmal fraction, because the O_2 affinity of intermyofibrillar mitochondria was similar between species. For example, COX subunit 4 has two alternate isoforms, COX4-1 and COX4-2, which differ in expression between normoxia and hypoxia and result in different O_2 affinities of the COX enzyme.[48–50] However, it remains unclear whether high-altitude deer mice have evolved sequence differences in any COX subunit isoforms that might alter O_2 affinity.

Species differences in substrate control ratios suggest that other changes in mitochondrial function have arisen in high-altitude deer mice (Figure 3). The much greater difference between highlanders and lowlanders for P_{PM}/P_{PMGS} than for $P_{PcOcM}/P_{PcOcMGS}$ suggests that the capacity for acyl-carnitine oxidation relative to pyruvate oxidation may be greater in highlanders than in lowlanders. This is consistent with recent findings that high-altitude deer mice have greater rates of lipid oxidation during maximal thermogenesis than their low-altitude counterparts, in association with greater whole-body lipid stores and greater β-hydroxyacyl-CoA dehydrogenase activity in the gastrocnemius.[51–53] The lower OXPHOS capacity via complex I relative to complexes I + II (P_{PMG}/P_{PMGS}) in highlanders than in lowlanders suggests that OXPHOS capacity via complex II is increased in

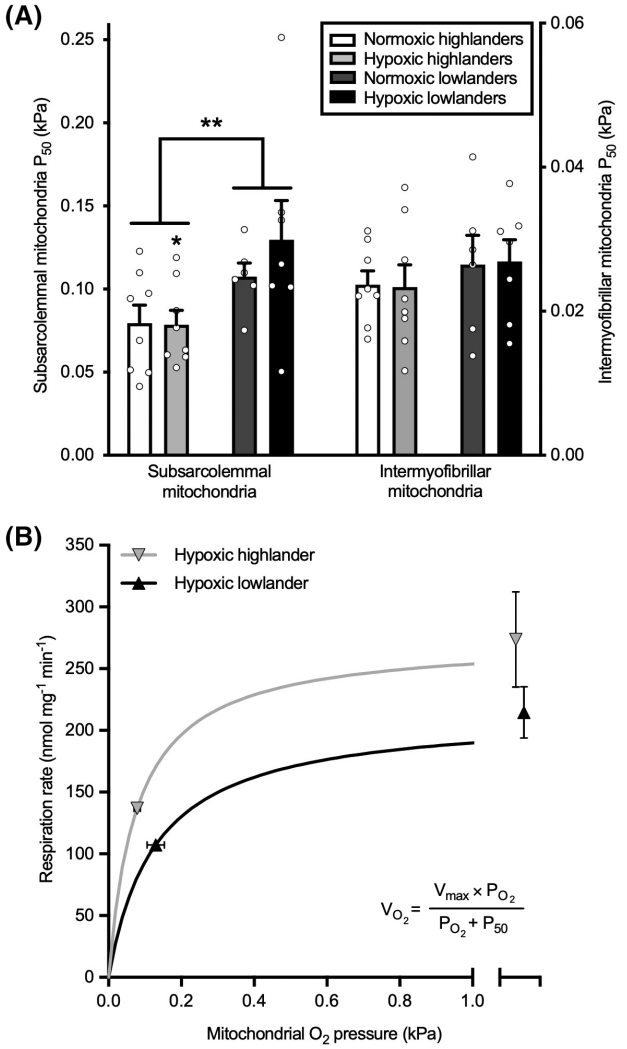

FIGURE 4 The apparent O_2 affinity of respiration by subsarcolemmal mitochondria is greater in highlanders than in lowlanders. (A) Mitochondrial O_2 affinity was measured during oxidative phosphorylation via complexes I and II (the P_{PMGS} state in Table 2) and is expressed as the P_{50} (the partial pressure of O_2 at 50% inhibition of respiration by hypoxia), such that lower P_{50} reflects increased affinity. Bars represent means + SE and circles represent individual values. *Significant ($p < .05$) pairwise differences between highlanders and lowlanders within the same acclimation environment in Holm–Šídák post hoc tests. **Significant ($p < .05$) main effect of species in two-factor ANOVA (full ANOVA results are shown in Table 1). (B) Hyperbolic relationship between respiration rate of subsarcolemmal mitochondria (V_{O_2}) and partial pressure of O_2 (P_{O_2}) for hypoxia-acclimated mice. P_{50} and the fitted respiration rate uninhibited by hypoxia (V_{max}) are shown as triangles representing means ± SE, from which hyperbolic curves described by the equation shown are plotted using the mean values of P_{50} and V_{max}.

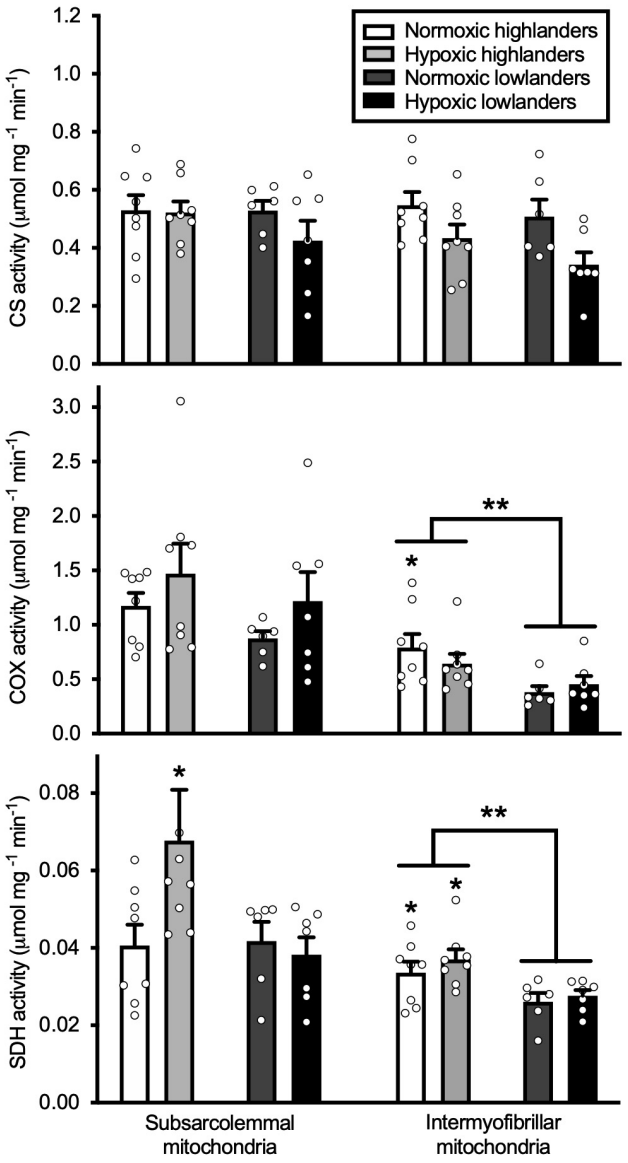

FIGURE 5 The activity of enzymes involved in oxidative phosphorylation differed between highlanders and lowlanders. Maximal activities (µmol of substrate per mg of mitochondrial protein per min) of citrate synthase (CS), cytochrome c oxidase (COX; complex IV), and succinate dehydrogenase (SDH; complex II) were assayed in mitochondrial isolates. Bars represent means + SE and circles represent individual values (one individual data point at 0.157 for SDH in subsarcolemmal mitochondria from hypoxic highlanders is beyond the limit of the y-axis scale). *Significant ($p < .05$) pairwise differences between highlanders and lowlanders within the same acclimation environment in Holm–Šídák post hoc tests. **Significant ($p < .05$) main effect of species in two-factor ANOVA (full ANOVA results are shown in Table 1).

highlanders, which may be at least partly due to increased activities of succinate dehydrogenase (Figure 5). Consistent with this finding, several high-altitude waterfowl from the Andes have greater succinate dehydrogenase activity in the flight muscle than their low-altitude relatives.[45] However, these observations are in stark contrast to the effects of high-altitude acclimatization at 5260 m in lowland humans, in which OXPHOS capacity via complex I (P_{PMG}) increased relative to OXPHOS capacity via complex II (OXPHOS with

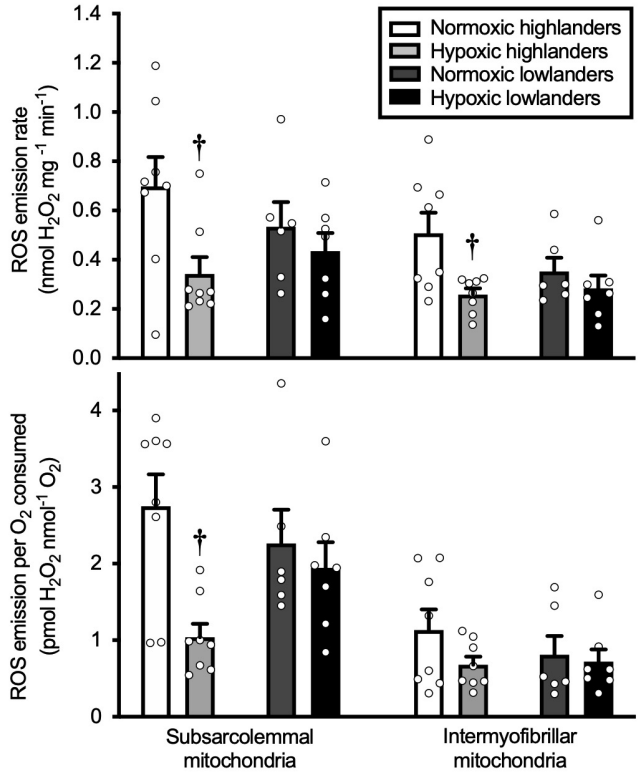

FIGURE 6 Rates of emission of reactive oxygen species (ROS) from mitochondria were reduced in highlanders but not in lowlanders after hypoxia acclimation. ROS emission was coupled to H_2O_2 production using exogenous superoxide dismutase and was measured fluorometrically using Ampliflu Red, and the data reported here were measured during oxidative phosphorylation via complexes I and II (the P_{PMGS} state in Table 2) as absolute emission rates (nmol H_2O_2 per mg of mitochondrial protein per min) or emission relative to O_2 consumption (pmol H_2O_2 per nmol O_2). Bars represent means + SE and circles represent individual values. †Significant ($p < .05$) pairwise differences between acclimation environments (normoxia vs. hypoxia) within a species in Holm–Šídák post hoc tests (full two-factor ANOVA results are shown in Table 1).

succinate and rotenone).[54] The latter response in humans was associated with the increased demands for NADH oxidation resulting from protein catabolism,[54] which may underlie the common observation that chronic hypoxia exposure induces muscle atrophy.[55,56] Chronic exposure to the levels of hypoxia used here (equivalent to those at ~4300 m elevation) does not reduce fiber size in deer mice or white-footed mice,[31,34,36] potentially due to taxonomic differences or differences in the magnitude of hypoxia,[57] which may explain the discrepancy between studies.

The species differences in mitochondrial ROS emission indicate that high-altitude deer mice may exhibit unique mechanisms for maintaining ROS homeostasis in chronic hypoxia (Figure 6). Similar responses to chronic hypoxia have been observed in several hypoxia-tolerant ectotherms, including freshwater turtle,[58] epaulette shark,[59] and mummichog killifish,[60] suggesting that reductions in mitochondrial ROS emission represent a common response to chronic hypoxia in hypoxia-tolerant taxa. Mitochondrial ROS emission represents the difference between ROS production by the electron transport system and ROS consumption by mitochondrial antioxidant pathways, either of which could be adjusted to reduce ROS emission in chronic hypoxia.[61] The former could be achieved by reductions in proton motive force, which could reduce superoxide production by reducing the accumulation of electrons in mitochondrial electron carriers. The latter could be achieved by increases in matrix NADPH availability to support the consumption of H_2O_2 (itself produced from superoxide via superoxide dismutase) through thioredoxin- and glutathione-dependent antioxidant pathways. H_2O_2 consumption by the glutathione-dependent pathway may be a particularly important determinant of some differences in mitochondrial ROS emission between species, based on comparisons of skeletal muscle and heart mitochondria between mice and naked mole rats.[62] Whatever the mechanism, reductions in mitochondrial ROS emission could help protect against oxidative stress, as mitochondrial ROS production was long considered a harmful byproduct of the electron transport system. However, ROS are now known to have many important roles in cell signaling,[63,64] so reductions in mitochondrial ROS emission could play an important signaling function in chronic hypoxia.

4.2 | Differences between subsarcolemmal versus intermyofibrillar mitochondria

The greater OXPHOS capacity we observed in intermyofibrillar mitochondria (IMM) than in subsarcolemmal mitochondria (SSM) (Figure 2) is consistent with previous studies.[11,12] In lowlanders, respiratory capacities were 84% and 82% greater in IMM than in SSM in normoxia and hypoxia, respectively. This difference appeared to be lower in highlanders, in which IMM respiratory capacities were 67% greater in normoxia and only 7% greater in hypoxia. In mitochondria isolated from the quadriceps of rats, differences in OXPHOS respiration between IMM and SSM were greater when supported via complex I (glutamate) than complex II (succinate and rotenone), in association with higher activities of succinate dehydrogenase in SSM, suggesting that there is greater reliance on complex I in IMM.[11] Our findings are largely consistent with this possibility, as IMM tended to have higher ratios of OXPHOS respiration via complex I (P_{PMG}) relative to complexes I + II combined (P_{PMGS}) (Figure 3) and lower SDH activities (Figure 5) than SSM.

The divergent effects of chronic hypoxia on IMM versus SSM build upon previous findings showing that

TABLE 5 Reactive oxygen species (ROS) emission from mitochondria isolated from the gastrocnemius muscle

	Normoxic highlanders	Hypoxic highlanders	Normoxic lowlanders	Hypoxic lowlanders
Subsarcolemmal mitochondria				
$L_{N,PM}$	0.638 ± 0.120 (8)	0.336 ± 0.046 (8)†	0.528 ± 0.059 (6)	0.378 ± 0.056 (7)
P_{PM}	0.662 ± 0.124 (8)	0.390 ± 0.055 (8)	0.543 ± 0.086 (6)	0.444 ± 0.078 (7)
P_{PMG}	0.661 ± 0.115 (8)	0.380 ± 0.054 (8)	0.515 ± 0.093 (6)	0.427 ± 0.080 (7)
$L_{N,PcM}$	0.641 ± 0.100 (8)*	0.382 ± 0.048 (7)†	0.355 ± 0.029 (6)	0.264 ± 0.024 (7)
P_{PcM}	0.672 ± 0.135 (8)*	0.405 ± 0.034 (7)	0.340 ± 0.025 (6)	0.299 ± 0.032 (7)
P_{PcOcM}	0.673 ± 0.150 (8)*	0.394 ± 0.035 (7)	0.320 ± 0.024 (6)	0.295 ± 0.032 (7)
P_{PcOcMG}	0.710 ± 0.170 (8)*	0.375 ± 0.029 (7)†	0.332 ± 0.024 (6)	0.302 ± 0.034 (7)
$P_{PcOcMGP}$	0.666 ± 0.137 (8)*	0.370 ± 0.026 (7)†	0.343 ± 0.031 (6)	0.289 ± 0.023 (7)
$P_{PcOcMGPS}$	0.667 ± 0.127 (8)*	0.363 ± 0.027 (7)†	0.342 ± 0.033 (6)	0.263 ± 0.017 (7)
Intermyofibrillar mitochondria				
$L_{N,PM}$	0.582 ± 0.105 (8)	0.240 ± 0.024 (8)†	0.356 ± 0.052 (6)	0.281 ± 0.049 (7)
P_{PM}	0.530 ± 0.090 (8)	0.294 ± 0.023 (8)†	0.377 ± 0.055 (6)	0.301 ± 0.051 (7)
P_{PMG}	0.515 ± 0.091 (8)	0.293 ± 0.026 (8)†	0.342 ± 0.047 (6)	0.302 ± 0.056 (7)
$L_{N,PcM}$	0.360 ± 0.064 (8)*	0.179 ± 0.023 (7)†	0.188 ± 0.038 (6)	0.149 ± 0.025 (7)
P_{PcM}	0.365 ± 0.070 (8)*	0.210 ± 0.027 (7)†	0.163 ± 0.036 (6)	0.150 ± 0.023 (7)
P_{PcOcM}	0.358 ± 0.067 (8)*	0.208 ± 0.027 (7)†	0.151 ± 0.037 (6)	0.148 ± 0.024 (7)
P_{PcOcMG}	0.357 ± 0.066 (8)*	0.202 ± 0.027 (7)†	0.156 ± 0.032 (6)	0.149 ± 0.018 (7)
$P_{PcOcMGP}$	0.357 ± 0.064 (8)*	0.209 ± 0.028 (7)†	0.146 ± 0.033 (6)	0.142 ± 0.021 (7)
$P_{PcOcMGPS}$	0.353 ± 0.062 (8)*	0.204 ± 0.027 (7)†	0.151 ± 0.033 (6)	0.133 ± 0.020 (7)

Note: ROS emission measured at each of the respiration states shown are reported as mean ± SE (N) in units nmol H_2O_2 per mg mitochondrial protein per min. See Table 3 for symbol abbreviations for each respiration state. *,† Significant pairwise differences ($p < .05$) in Holm–Šídák post hoc tests between highlanders and lowlanders within the same acclimation environment, or between acclimation environments (normoxia vs. hypoxia) within a species, respectively (full two-factor ANOVA results are shown in Table 6).

plastic responses to changes in metabolic demands can differ between mitochondrial fractions. For example, in mitochondria isolated from the gastrocnemius of rats, endurance training and hind limb immobilization lead to pronounced changes in OXPHOS respiration in SSM, while IMM were unaltered by the same treatments.[12] We found that while chronic hypoxia reduced OXPHOS capacity in IMM in both species, it appeared to increase OXPHOS capacity in SMM in highlanders (Figure 2). Therefore, effects of chronic hypoxia on muscle metabolism assessed in permeabilized muscle fibers and tissue samples, such as those observed in recent studies of high-altitude acclimatization in humans,[23,54,65] might be underlain by distinct functional changes in each mitochondrial fraction. However, mice in the current study were housed at relatively warm temperatures and did not have access to running wheels, and it would be instructive to consider how chronic exposure to hypoxia, cold temperatures, and/or altered activity levels interact to affect SSM and IMM.

Mitochondrial O_2 affinity was greater on average in IMM than in SSM (Figure 4). Our values of P_{50} are comparable to previous measurements of P_{50} in human muscle mitochondria respiring with substrates of complexes I + II.[17] In fact, compared to the findings of this previous study, which did not separate mitochondrial fractions, our SSM P_{50} measurements are slightly higher and our IMM P_{50} measurements are slightly lower.[17] The lower P_{50} in IMM did not appear to result from a lower relative activity of cytochrome c oxidase, because IMM had lower COX excess capacities (Figure 3) and lower COX activities (Figure 5) than SSM. Other differences between mitochondrial fractions may account for the differences in O_2 affinity, such as differential expression of COX subunit isoforms or emergent differences in the control of oxidative phosphorylation at low O_2.[3,49,50]

4.3 | The role of mitochondria in high-altitude adaptation

Our findings lead us to conclude that evolved changes in mitochondrial physiology, specific to subsarcolemmal mitochondria of skeletal muscle, contribute to high-altitude adaptation in deer mice. The important implications of

TABLE 6 *p*-values from two-factor ANOVA analyses of mitochondrial ROS emission data in Table 5

	Species main effect	Environment main effect	Species × environment
Subsarcolemmal mitochondria			
$L_{N,PM}$	0.677	0.010	0.355
P_{PM}	0.724	0.054	0.356
P_{PMG}	0.589	0.050	0.292
$L_{N,PcM}$	0.005	0.013	0.210
P_{PcM}	0.014	0.074	0.184
P_{PcOcM}	0.020	0.106	0.175
P_{PcOcMG}	0.037	0.086	0.150
$P_{PcOcMGP}$	0.022	0.045	0.156
$P_{PcOcMGPS}$	0.011	0.020	0.154
Intermyofibrillar mitochondria			
$L_{N,PM}$	0.186	0.005	0.062
P_{PM}	0.250	0.019	0.204
P_{PMG}	0.200	0.047	0.158
$L_{N,PcM}$	0.028	0.017	0.110
P_{PcM}	0.009	0.078	0.135
P_{PcOcM}	0.006	0.100	0.112
P_{PcOcMG}	0.006	0.072	0.098
$P_{PcOcMGP}$	0.003	0.082	0.101
$P_{PcOcMGPS}$	0.003	0.054	0.126

Note: See Tables 3 and 5 for symbol abbreviations.

our findings are emphasized by recent research in humans on the contribution of mitochondrial capacities and O_2 kinetics to aerobic performance. This work has suggested that the total mitochondrial respiratory capacity of the muscle likely exceeds the capacity for O_2 transport from the circulation in vivo.[66] This excess capacity for mitochondrial respiration should lead to submaximal activation of the ETS in vivo, and a correspondingly lower mitochondrial P_{50} than during maximal OXPHOS capacity in vitro.[15,17] This reduction in P_{50} in vivo allows for a lower mitochondrial PO_2 and may thus facilitate O_2 diffusion from the blood.[15] Therefore, the increases in respiratory capacity (Figure 2) and O_2 affinity (Figure 4) of subsarcolemmal mitochondria observed here likely make important contributions to the evolved increase in VO_2max in highlanders. Highlanders have increased capacity for supplying O_2 to the muscle in hypoxia, by virtue of increases in arterial O_2 saturation, cardiac output, and muscle capillarity,[31–33,35] so their increased mitochondrial respiratory capacity should be important for maintaining mitochondrial excess capacity. When combined with an increased inherent mitochondrial O_2 affinity, highlanders should be capable of maintaining lower mitochondrial P_{50} and PO_2 in vivo, and thus amplify O_2 extraction from the blood. This is supported by the observation that O_2 extraction measured at VO_2max in hypoxia is greater in highlanders than in lowlanders.[33]

When considered in light of findings in other taxa, our results suggest that high-altitude adaptation has led to distinct changes in mitochondrial physiology in different species. Mitochondrial abundance and respiratory capacity in muscle are greater in high-altitude deer mice compared to their low-altitude counterparts, which bears similarity to previously observed differences between the high-flying bar-headed goose and low-altitude waterfowl. However, this is contrasted by Tibetan humans, which exhibit lower mitochondrial abundance and OXPHOS capacity in the muscle than lowland humans.[23,24] Tibetans also have reduced mitochondrial capacity for lipid oxidation,[23] in contrast to high-altitude deer mice (Table 3, Figure 3)[34] and to several high-altitude waterfowl from the Andes as compared to their lowland relatives.[45] Some of these discrepancies could be explained by differences in metabolic demands at high altitude, which are anticipated to be greater in deer mice and bar-headed geese than in humans. Deer mice maintain high-field metabolic rates at high altitude,[20] likely due to the costs of thermogenesis to cope with cold, which is expected to be substantial by virtue of their small body size and high surface area to volume ratio. Similarly, bar-headed geese maintain high metabolic activity while flying at high altitude during their migration across the Himalayas.[67] Discrepancies between high-altitude taxa may also be explained by differences in the O_2 transport cascade, because the influence of changes in mitochondrial OXPHOS capacity and O_2 affinity on muscle metabolism likely depend on the rates of mitochondrial O_2 supply.[16] Future work aimed at appreciating these relationships will help shed further insight into the contribution of variation in mitochondrial physiology to aerobic performance and environmental adaptation.

AUTHOR CONTRIBUTIONS

Neal J. Dawson and Graham R. Scott designed research; Neal J. Dawson performed research; Neal J. Dawson and Graham R. Scott analyzed the data and wrote the paper.

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DISCLOSURES

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the Figshare repository at https://doi.org/10.6084/m9.figshare.19714663.v1.

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