Effects of chronic hypoxia on diaphragm function in deer mice native to high altitude

Neal J. Dawson[†,*], Sulayman A. Lyons[†], Danielle A. Henry, and Graham R. Scott

[1]Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON, L8S4K1, Canada

*Correspondence:

Dr. Neal J. Dawson
McMaster University
Department of Biology
1280 Main Street West,
Hamilton, ON, Canada
Phone: (613) 698 6325
E-mail: neal.dawson@gmail.com

[†]Authors contributed equally

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Aim: We examined the effects of chronic hypoxia on diaphragm function in high- and low-altitude populations of *Peromyscus* mice.

Methods: Deer mice (*P. maniculatus*) native to high altitude and congeneric mice native to low altitude (*P. leucopus*) were born and raised in captivity to adulthood, and were acclimated to normoxia or hypobaric hypoxia (12 or 9 kPa, simulating hypoxia at 4300 m and 7000 m) for 6-8 weeks. We then measured indices of mitochondrial respiration capacity, force production, and fatigue resistance in the diaphragm.

Results: Mitochondrial respiratory capacities (assessed using permeabilized fibres with single or multiple inputs to the electron transport system), citrate synthase activity (a marker of mitochondrial volume), twitch force production, and muscle fatigue resistance increased after exposure to chronic hypoxia in both populations. These changes were not well explained by variation in the fibre-type composition of the muscle. However, there were several differences in diaphragm function in high-altitude mice compared to low-altitude mice. Exposure to a deeper level of hypoxia (9 kPa vs 12 kPa) was needed to elicit increases in mitochondrial respiration rates in highlanders. Chronic hypoxia did not increase the emission of reactive oxygen species from permeabilized fibres in highlanders, in contrast to the pronounced increases that occurred in lowlanders. In general, the diaphragm of high-altitude mice had greater capillary length densities, produced less force in response to stimulation, and had shorter relaxation times. The latter was associated with higher activity of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity in the diaphragm of high-altitude mice.

Conclusion: Overall, our work suggests that exposure to chronic hypoxia increases the capacities for mitochondrial respiration, force production, and fatigue resistance of the

diaphragm. However, many of these effects are opposed by evolved changes in diaphragm function in high-altitude natives, such that highlanders in chronic hypoxia maintain similar diaphragm function to lowlanders in sea level conditions.

Introduction

Skeletal muscle is a highly plastic tissue that has a large capacity to remodel in response to patterns of use, environmental changes, and various pathological conditions. The effect of chronic environmental hypoxia on muscle phenotype has received appreciable attention, particularly in studies of locomotory muscle in humans and other animals that spend time at high altitudes[1, 2]. Many (though not all) studies suggest that muscle wasting and/or a loss of oxidative capacity occurs when hypoxia is sufficiently severe and prolonged[3], which can manifest as a loss in mitochondrial volume density or respiratory capacity[4-6]. Chronic hypoxia has also been shown to affect contractile phenotype in some rat studies, favoring a shift from slow- to fast-twitch fiber types[7, 8], increasing twitch force, and decreasing fatigue resistance[9]. These changes in locomotory muscle phenotype could result from intracellular hypoxia and associated oxidative stress, but could also arise from detraining in response to reduced physical activity, and are likely a response to changes in cellular regulators of metabolism[6].

The effects of chronic hypoxia on the diaphragm and on other respiratory muscles have received less attention. Chronic hypoxia may be expected to have distinct effects on the diaphragm than on locomotory muscles, because increases in breathing in response to reduced atmospheric O_2 augment respiratory muscle activity. However, the effects of chronic hypoxia on the contractile function of the diaphragm, which have been explored most extensively in domestic rodents, are somewhat inconsistent. Some studies suggest that chronic hypoxia can augment force production [9, 10], whereas some others suggest that the opposite occurs [11-14]. There are similar inconsistencies in the literature regarding the effects of chronic hypoxia on fatigue resistance and the activities of metabolic enzymes in the diaphragm[10, 11, 13-16]. The reasons for

these discrepancies, and how these findings relate to other non-domesticated species, are poorly understood.

High-altitude natives – who have evolved to cope with the hypoxic environment of montane regions – could provide unique insight into diaphragm function in response to chronic hypoxia, but these organisms have received relatively little attention. North American pika (*Ochotona princeps*) sampled at 3350 m in the wild had higher activities of citrate synthase (CS), β-hydroxyacyl-CoA dehydrogenase, and lactate dehydrogenase (LDH) in the diaphragm compared to the closely related collared pika (*O. collaris*) sampled at 1070 m[17]. This suggests that the metabolic capacity of the diaphragm is elevated in some high-altitude species, but there is otherwise very little known about diaphragm function in high-altitude natives.

The deer mouse, *Peromyscus maniculatus*, inhabits the broadest altitudinal distribution of any North American mammal, from below sea level to more than 4,300 m elevation in the Rocky Mountains[18-20]. High-altitude deer mice sustain high field metabolic rates in the wild, presumably to support the demands of thermogenesis in the colder environment at high altitudes (Hayes, 1989). There is strong directional selection at high altitudes that favours high aerobic capacity (VO_2max) in hypoxia[21], and high-altitude populations show an elevated VO_2max in hypoxia compared to low-altitude populations of deer mice and to low-altitude white-footed mice (*P. leucopus*)[22-25]. High-altitude populations also exhibit a more effective breathing pattern (higher tidal volumes and lower breathing frequencies) than low-altitude populations in normoxia, and unlike lowlanders, highlanders change breathing very little in response to chronic hypoxia[26]. However, the extent to which diaphragm function is altered in highlanders to support an elevated VO_2max, to accommodate a more effective breathing pattern, or to otherwise cope with the hypoxic conditions at high-altitude is unknown. Therefore, we examine here the effects of

chronic hypoxia on diaphragm physiology, considering the underlying determinants of metabolic and contractile function, in high-altitude and low-altitude populations of *Peromyscus* mice.

Results

Effects of chronic hypoxia on oxidative capacity and mitochondrial function

The respiratory capacities of the diaphragm for oxidative phosphorylation (oxphos) increased with chronic hypoxia in the lowland population, but more severe levels of hypoxia were needed to elicit a similar effect of hypoxia exposure in the highland population (Fig. 1a). Respiration rates with pyruvate, malate, and ADP (oxphos *via* complex I; P_{PM}) were 2.0-fold higher after exposure to hypoxia simulating 4300 m (12 kPa O_2) in the lowland population, but a similar level of hypoxia did not increase respiration in highlanders (Figure 1a; Table 1). However, when the high-altitude population was exposed to more severe levels of hypoxia simulating 7000 m (9 kPa O_2), which they appeared to tolerate well and they still exhibited normal activity and behaviour, respiration rates increased by 1.6-fold (Figure 1a; Table 1). Similar effects of hypoxia exposure at 12 kPa O_2 were observed when respiration was measured in the subsequent presence of glutamate to stimulate maximal oxphos *via* complex I (P_{PMG}), succinate to stimulate maximal oxphos *via* complexes I+II (P_{PMGS}), and ascorbate and TMPD to elicit maximal respiration *via* complex IV (P_{Tm}) (Figure 1a; Table 1). There were also similar effects of hypoxia exposure at 9 kPa O_2 in highlanders to those on P_{PM} for P_{PMG}, P_{PMGS}, and P_{Tm} (Figure 1a; Table 1). In contrast, leak state respiration (L_N) was relatively unaffected by hypoxia exposure and did not differ appreciably between populations (Figure 1a; Table 1).

The rate of ROS emission from diaphragm fibres, which was measured simultaneously

with mitochondrial respiration, increased in lowlanders but not in highlanders after hypoxia exposure (Fig. 1b). ROS emission increased ~2-fold after exposure to chronic hypoxia (12 kPa O_2) in lowlanders but did not change in highlanders during leak, P_{PM}, P_{PMG}, or P_{PMGS} (ROS emission cannot be measured in P_{Tm} in the presence of ascorbate and TMPD) (Figure 1b; Table 1). Exposure of highlanders to deeper levels of chronic hypoxia at 9 kPa O_2 was also without any statistically significant effects on ROS emission rate (Figure 1b; Table 1). The increases in ROS emission in lowlanders occurred in parallel to the increases in respiration, because there was no significant variation in rates of ROS emission relative to O_2 consumption in mice exposed to normoxia or 12 kPa hypoxia (Fig. 1c; Table 1). However, highlanders appear to avoid similar increases in ROS emission rates by reducing ROS emission relative to O_2 consumption by roughly half after chronic exposure to 9 kPa hypoxia (Figure 1c; Table 1).

There was variation in the maximal activities of oxidative enzymes that existed in concert with the differences in mitochondrial respiratory capacities (Table 2). The activity of citrate synthase (CS), a citric acid cycle enzyme that is a commonly used marker of mitochondrial abundance, increased after exposure to chronic hypoxia, but did not differ between populations. The activity of cytochrome c oxidase (COX), the terminal oxygen acceptor of the electron transport system, was also increased by exposure to chronic hypoxia. However, this response was driven largely by the lowlanders, and there was a significant overall difference between populations, a nearly significant environment × population interaction (P=0.0525), and no significant effect of hypoxia exposure on COX activity within highlanders (Figure 1).

Chronic hypoxia increased mitochondrial respiration rates relative to CS activity in lowlanders, but not in highlanders, suggesting that mitochondrial quality was altered by hypoxia exposure in the former population (Fig. 1d; Table 1). Oxphos respiration rates relative to CS

activity increased by ~2.0-fold after hypoxia exposure at 12 kPa O_2 in lowlanders but not in highlanders, but this variation was less apparent in the leak state (Fig. 1d; Table 1). Exposure to deeper levels of hypoxia at 9 kPa was without effect on respiration rates relative to CS activity in highlanders (Fig. 1d; Table 1).

The observed variation in the respiratory capacity of diaphragm muscle did not appear to result from variation in fibre-type composition (Fig. 2; Table 3). Exposure to chronic hypoxia decreased the numerical and areal densities of type IIa fibres, in favour of subtle (sometimes non-significant) increases in type IIx and/or IIb fibres. There were no overall population differences in fibre-type composition or fibre size (i.e., no main effect of population in two-factor ANOVA), but the increase in type IIb fibre abundance appeared to be greater in the low-altitude population (based on a significant environment × population interaction). There were no differences in the thickness of the diaphragm between groups, but there was a nearly significant ($p=0.051$) increase (~7-15%) in diaphragm mass in highlanders compared to lowlanders (Table 3).

Despite the relative lack of variation in fibre-type composition between populations, high-altitude mice appeared to have more capillary surface to support gas exchange in the diaphragm (Fig. 2; Table 3). Highlanders had similar capillary densities and capillary to fibre ratios to lowlanders, but ~2.1- to 2.2-fold greater capillary length densities (capillary length per volume of muscle). The distinction between these two measures of capillarity may have arisen from the appreciable difference in capillary morphology, in which staining wrapped the muscle fibers more often in highlanders (such that the capillary was sectioned longitudinally to vessel length) than in lowlanders (in which staining was generally more discrete, indicating that the capillary was sectioned transversely) (Fig. 2), suggesting that vessel tortuosity may be

augmented in the high-altitude population. Chronic hypoxia had no effect on the capillarity of the diaphragm.

Effects of chronic hypoxia on contractile function

Several features of force production by the diaphragm varied between groups, despite the relatively modest variation in fibre-type composition (Fig. 3; Table 4). Exposure to chronic hypoxia increased the peak force and the rate of force development during a muscle twitch by ~1.6 and ~1.5-fold, respectively. However, lowlanders generally produced ~1.7- to 1.8-fold greater peak twitch forces and rates of force development, and they took ~1.2-fold more time to relax after a single twitch. Similar population differences in force production were observed with summation of twitch forces at increasing stimulation frequencies during tests of the force-frequency relationship (Table 4), with lowlanders producing ~1.6-fold higher tetanic force than highlanders when stimulated at 100 Hz.

Chronic hypoxia increased fatigue resistance of the diaphragm (Fig. 4). The initial forces generated in response to stimulation at 70 Hz in the fatigue resistance tests were generally intermediate between the forces measured at 60 and 80 Hz in the force-frequency relationship tests (Table 4); this result shows that force production by the muscle was stable over time and did not decline over the 10 min recovery period between these two tests. However, as muscles were subjected to repeated stimulation over the course of the fatigue resistance test, the proportion of initial contraction force decreased progressively (from ~70-80% of initial contraction force after 1 min of stimulation to only ~20-30% after 20 min). Exposing mice to chronic hypoxia increased the time to 50% of initial force from ~6 to ~10 min, but there were no

significant differences between populations. Similarly, chronic hypoxia increased the proportion of initial force remaining after 5 min of stimulation from ~51% to ~62%, but there were no differences between populations.

We measured Ca^{2+}-dependent ATPase activity in heavy microsomes (which are enriched for sarcoplasmic reticulum) as an index of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), the Ca^{2+}-ATPase involved in the active re-uptake of Ca^{2+} into the sarcoplasmic reticulum from the cytosol. SERCA activity was 1.5- to 1.7-fold greater in highland mice than in lowland mice but was unchanged by exposure to chronic hypoxia (Fig. 5).

Discussion

In this study, we used deer mice (*Peromyscus maniculatus*) native to high-altitude and congeners (*P. leucopus*) native to low-altitude to discern the effects of chronic hypoxia on diaphragm function and to identify potential evolved specializations that are unique to high-altitude natives. Our findings suggest that chronic hypoxia exposure of both lowland and highland mice augments the mitochondrial respiratory capacity, force production, and fatigue resistance of the diaphragm. However, evolved differences in high-altitude mice appeared to oppose many of the environmentally-induced changes in the respiratory capacity and force production of the diaphragm, because highlanders in chronic hypoxia often exhibited similar phenotypes to lowlanders in normoxia. As a result, most aspects of diaphragm function were similar between highlanders in conditions simulating hypoxia at 4300 m and lowlanders in conditions simulating normoxia at sea level.

158 *Effects of Chronic Hypoxia on Diaphragm Function*

159 The respiratory capacity and citrate synthase activity of the diaphragm was augmented
160 after exposure to chronic hypoxia (Fig. 1a; Table 2). Citrate synthase is a commonly used marker
161 of mitochondrial volume, so chronic hypoxia may increase the respiratory capacity of the
162 diaphragm at least in part by inducing mitochondrial growth and biogenesis. The increases in
163 respiratory capacity did not appear to be driven by increases in the abundance of oxidative fibre
164 types in the muscle, and may have even been opposed slightly by the conversion of a small
165 number of IIa fibres into IIx or IIb fibres after exposure to chronic hypoxia (Table 3, Fig. 2).
166 Therefore, adjustments in mitochondrial quantity and/or quality appear to help maintain or
167 increase the respiratory capacity of the diaphragm in *Peromyscus* mice exposed to chronic
168 hypoxia. This contrasts previous studies of domestic C57BL/6J mice, in which chronic hypoxia
169 decreased the volume density and specific respiratory capacity of mitochondria in the
170 diaphragm[16, 27], suggesting that the effects of chronic hypoxia on mitochondrial function may
171 differ appreciably between *Peromyscus* mice and house mice (*Mus musculus*).

172 The contractile force and fatigue resistance of the diaphragm also increased with
173 exposure to chronic hypoxia (Fig. 5). *Peromyscus* mice increase breathing by 1.2- to 1.5-fold in
174 chronic hypoxia at 12 kPa[26], so the increases in force production and fatigue resistance (as well
175 as respiratory capacity) could represent a training effect resulting from increases in muscle
176 activity. This could be partly explained by the apparent shift in some fibres from IIa to IIx/IIb,
177 but in contrast to some previous studies of domestic mice[16], chronic hypoxia did not affect the
178 average size of any muscle fibre types (Table 3). Changes in contractile function after chronic
179 hypoxia do not appear to result from variation in SERCA activity (Fig. 5) or the density of
180 muscle fibres expressing SERCA2[13]. It is possible that chronic hypoxia expanded the relative

volume of myofibrils within diaphragm fibres, but it is unclear how this might be achieved if chronic hypoxia also increases the mitochondrial volume density of the fibres (as discussed in the previous paragraph).

The observed changes in the metabolic and contractile phenotypes of the diaphragm in low-altitude populations after chronic hypoxia (Figs. 1,5) occur in association with changes in breathing. Lowlanders exhibit ventilatory acclimatization to hypoxia (VAH), in which exposure to chronic hypoxia leads to a progressive increase in total ventilation at 12 kPa and it also makes breathing pattern more effective (higher tidal volumes and lower breathing frequencies at a given total ventilation)[26]. VAH appears to be underpinned by growth of the carotid bodies (the chemoreceptor that initiates the hypoxic ventilatory response) in lowland *Permyscus* mice[26], and VAH is generally believed to also result from neuroplasticity in ventilatory control circuits[28]. Therefore, VAH could contribute to the observed effects of chronic hypoxia on diaphragm phenotype in lowlanders by augmenting routine ventilation (and presumably diaphragm activity) and by restructuring the neural networks that control respiratory muscle contraction.

Changes in the Effects of Chronic Hypoxia and Diaphragm Function in High-Altitude Natives

Chronic exposure to deeper levels of hypoxia were needed to increase the respiratory capacity of the diaphragm in high-altitude mice (Fig. 1a). The increases in respiratory capacity observed in lowlanders were not observed in highlanders at 12 kPa O_2, but did occur in highlanders after chronic exposure to 9 kPa O_2 – an O_2 pressure that was well tolerated by the high-altitude mice and was comparable to the level of hypoxia at ~7,000 m elevation. This observation could potentially be explained by population differences in VAH, because highlanders do not exhibit VAH in response to chronic exposure to 12 kPa O_2, unlike the robust

VAH that is exhibited by lowlanders[26]. However, it has yet to be determined if VAH occurs in highlanders after acclimation to 9 kPa O_2, so it remains unclear whether the increased respiratory capacity of their diaphragm in severe hypoxia could be explained by changes in breathing and diaphragm activity. Alternatively, if tissue hypoxia is an important stimulus for increases in respiratory capacity in chronic hypoxia, then this could explain the deeper level of hypoxia that is required to elicit a response in the highlanders. Arterial O_2 saturation is greater in highlanders than in lowlanders at 12 kPa O_2[26], and is presumably associated with parallel differences O_2 supply to the diaphragm and to other tissues. The fact that arterial O_2 saturation in highlanders at 9 kPa is similar to that in lowlanders at 12 kPa may explain why these different environmental conditions led to similar increases in respiratory capacity in each population.

Although the respiratory capacity of the diaphragm increased in chronic hypoxia in both populations, the mechanisms involved appeared to differ in highlanders compared to lowlanders. The increases in respiratory capacity in lowlanders appeared to result at least in part from changes in mitochondrial quality, in which respiratory capacity increased relative to citrate synthase activity (a marker of mitochondrial volume) (Fig. 1). Changes in mitochondrial quality are not necessarily detrimental – increases in mitochondrial quantity and quality can contribute to improvements in locomotory muscle performance and exercise capacity[29] – but those in lowlanders in the present study were correlated with elevated rates of ROS emission. Although ROS are a normal byproduct of mitochondrial metabolism and play important signaling roles in the muscle, disruptions in redox status and oxidative stress have been proposed to underlie some detrimental effects of chronic hypoxia on muscle function[30, 31]. However, the increases in respiratory capacity in highlanders appeared to result solely from changes in mitochondrial abundance, as reflected by similar relative increases in respiratory capacity and CS activity, and

occurred without any change in ROS emission from the fibres (Fig. 1). In fact, exposure to severe hypoxia decreased the ratio of ROS emission relative to O_2 consumption (Fig. 1), a condition that has been shown to distinguish locomotory muscle mitochondria of rats that have been artificially selected for running endurance compared to their more sedentary counterparts[32].

Capillary length density was comparatively higher in the diaphragm of high-altitude mice, which likely increased the area for gas and nutrient exchange between capillaries and muscle fibres (Table 3; Fig. 2). Our findings add to a growing number of studies suggesting that many high-altitude natives have evolved an increased capillarity in skeletal muscles[24, 33-36]. This, along with evolved changes in the distribution of mitochondria closer to capillaries[35, 37], likely improves mitochondrial respiration in high-altitude hypoxia by increasing O_2 diffusing capacity from the blood and increasing the O_2 pressure encountered by the mitochondria.

The diaphragm of high-altitude mice generated less force in response to stimulation, coincident with shorter times to relaxation after contraction and higher SERCA activity, but no difference in fibre-type composition (Figs. 3,5). Because SERCA pumps return Ca^{2+} from the cytosol to the sarcoplasmic reticulum during muscle relaxation, it is possible that the greater SERCA activity in highlanders limited the rise in intracellular [Ca^{2+}] ([$Ca^{2+}]_i$) during stimulation and lowered force generation. Increases in [$Ca^{2+}]_i$ during contraction of skeletal muscle fibres *in vivo* are not typically saturating (e.g., 1-5 μM)[38, 39], so differences in [$Ca^{2+}]_i$ during muscle contraction can affect the force generated by myofibrils. For example, pharmacological inhibition of SERCA has been shown to increase intracellular [Ca^{2+}] and force production during tetanic contraction, but slow the rate of relaxation after tetanus, in flexor brevis muscle fibres from domestic mice[40, 41]. There is some suggestion that increases in haematocrit impair diaphragm function in humans that ascend to high altitude[42]. However, this is unlikely in deer

mice as highlanders have the same haematocrit as lowlanders in normoxia, and lowlanders increase haematocrit more than highlanders after acclimation to hypoxia[24]. Other contributors to force production, such as myofilament force generation or other aspects of Ca^{2+} handling, could also support the observed differences in high-altitude mice[43].

Why would high-altitude mice evolve in such a way that their diaphragm generates less force than that of lowlanders, despite the seeming advantage that diaphragm force might confer for sustaining increases in ventilation at high altitudes? It is unlikely that highlanders have offset the *in vivo* effects of having lower specific force production by evolving a larger diaphragm, because the magnitude of the reduction in force production (Fig. 3) is far greater than the magnitude of the increase in diaphragm mass (Table 3). The reason for this curious reduction in force production is not entirely clear, but it could foreseeably contribute to thermogenesis in the cold environment at high altitudes. Increasing the rate of active reuptake of Ca^{2+} by SERCA could augment Ca^{2+} cycling, overall ATP demand, and thus the heat production by the diaphragm. This would be a different potential mechanism to the heat-generating effects of sarcolipin-induced uncoupling of SERCA pumps[44-47], but both mechanisms could contribute to non-shivering thermogenesis by augmenting Ca^{2+} pumping. Because the diaphragm is not believed to need its full contractile potential during routine conditions, as many fast-contracting fibres are likely only recruited when the diaphragm is accomplishing tasks that require large amounts of force (e.g., sneezing and coughing)[48], the lower force production in highlanders is not expected to restrain their ability to breathe. However, the ideal location of the diaphragm adjacent to many vital organs makes it a potentially valuable source of supplemental heat production. By relinquishing some of the excess force generating capacity of the diaphragm, high-altitude deer mice may have evolved additional means of staying warm in the cold

environment at high altitude.

Materials and Methods

Animals

Adult mice were live trapped in the wild at high altitude on the summit of Mount Evans Colorado (39°35’18’’N, 105°38’38”W; ~4350 m above sea level) (*P. maniculatus rufinus*) and at low altitude on the Great Plains of Nebraska (40°52’12’’N, 96°48’20.3’’W; ~430 m above sea level) (*P. leucopus*), and were transported to McMaster University (elevation 50 m). Mice were bred within each population, and lab-raised progeny were raised to adulthood in captivity in common-garden conditions in normoxia. Adult lab-raised mice (6-12 months of age) from each population were acclimated to (i) standard cage conditions in normobaric normoxia or (ii) hypobaric hypoxia simulating the barometric pressure at an elevation of 4,300 m (barometric pressure of 60 kPa, and O_2 partial pressure of 12 kPa) for 6-8 weeks. For some measurements, high-altitude mice were also acclimated to a more severe level of hypobaric hypoxia that simulated 7,000 m elevation (barometric pressure of 42 kPa, and O_2 pressure of 9 kPa). Specially designed hypobaric chambers were used for hypoxia acclimation, as previously described[24, 37, 49]. Otherwise, mice were held in standard holding conditions (23-25°C, 12:12 light-dark photoperiod) with unlimited access to standard rodent chow and water. After acclimation, mice were euthanized (isoflurane anaesthesia followed by cervical dislocation) and then sampled to measure various aspects of diaphragm function (see below). All procedures were carried out in accordance with guidelines set out by the Canadian Council on Animal Care, and were approved by the McMaster Animal Research Ethics Board.

295

296 *Mitochondrial function in permeabilized muscle fibres*

297 Small samples (~50 mg) of diaphragm muscle were transferred to ice-cold relaxing and
298 preservation buffer (20 mM imidazole, 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 6.56 mM
299 $MgCl_2$, 20 mM taurine, 0.5 mM DTT, 50 mM potassium-methane sulfonate, 5.8 mM Na_2ATP,
300 and 15 mM creatine phosphate, pH 7.1) and were mechanically separated using dissecting
301 probes. Fibres were then chemically permeabilized for 30 min in the same buffer containing 50
302 µg/ml saponin, and rinsed three times for 10 min in respiration buffer (20 mM HEPES, 0.5 mM
303 EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, and 110 mM
304 sucrose, and 1 mg/ml fatty acid-free bovine serum albumin [BSA]; pH 7.1) to wash out
305 endogenous metabolites. Fibres were weighed before respirometry measurements by transferring
306 bundles to respiration solution on a tared analytical balance.

307 *In situ* mitochondrial function was measured in 2 ml of respiration solution in a high-
308 resolution respirometer and fluorometer (Oxygraph-2k with O2k-Fluorescence module;
309 Oroboros Instruments, Innsbruck, Austria) at 37°C under continuous stirring. Fibres (2.1-5.3 mg
310 wet mass) were allowed to rest for 5 min after being transferred to the chamber. Respiration rate
311 was measured from the rate of decline in O_2 concentration in the chamber. Reactive oxygen
312 species (ROS) were measured by the fluorescent detection of resorufin (excitation wavelength of
313 525 nm and AmR filter set, Oroboros Instruments). This was accomplished by adding exogenous
314 superoxide dismutase (22.5 U ml^{-1}; which catalyzes the production of hydrogen peroxide from
315 mitochondrial superoxide), Ampliflu Red (15 µmol l^{-1}), and horseradish peroxidase (3 U ml^{-1};
316 which catalyzes the production of resorufin from hydrogen peroxide and Ampliflu Red) to the

317 respiration buffer. The rate of ROS emission was thus measured as the molar rate of H_2O_2
318 appearance, using exogenous H_2O_2 to calibrate the fluorescent resorufin signal. Respiration and
319 ROS emission rates were first measured after adding malate (2 mM) followed by pyruvate (5
320 mM) to stimulate leak state respiration (L_N). ADP (5 mM) was then added to stimulate ADP-
321 stimulated respiration *via* complex I (P_{PM}), reflecting the mitochondrial capacity for supporting
322 oxidative phosphorylation (oxphos) with pyruvate. Respiration was then measured after each
323 addition of glutamate (10 mM) (P_{PMG}) and succinate (25 mM) (P_{PMGS}) to determine the maximal
324 capacity for supporting oxphos *via* complex I and then complexes I+II (i.e., single and then
325 convergent electron inputs to coenzyme Q), respectively. Cytochrome c (10 µM) was then added
326 to assess the viability of the preparations (increases in respiration are often used as an index of
327 poor outer mitochondrial-membrane integrity, but none of our preparations showed a significant
328 cytochrome c effect of more than 5% above P_{PMGS}). Finally, ascorbate (0.5 mM) followed by
329 N,N,N,N-tetramethyl-p-phenylenediamine (TMPD; 0.5 mM) was used to maximally stimulate
330 complex IV (P_{Tm}). The fibres were then removed from the respirometer, frozen in liquid N_2, and
331 stored at -80°C until assayed for mitochondrial enzyme activities (next section). Respiration and
332 ROS emission rates were measured for at least 3 min in each condition until a steady state was
333 reached. Rates are expressed relative to the wet mass of fibres. The biochemicals used for the
334 measurements described in this section, and elsewhere throughout the Materials and Methods,
335 were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.
336

337 *Mitochondrial enzyme activities*

338 The maximal activities (V_{max}) of citrate synthase (CS) and cytochrome c oxidase (COX)

were assayed at mouse body temperature (37°C) as previously described[24, 50] using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Samples were first homogenized in 10 volumes of ice-cold buffer containing 100 mM KH_2PO_4, 1 mM EGTA, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) at pH 7.2. Homogenates were then centrifuged at 1,000 *g* at 4°C and the supernatant was collected for use in assays. Enzyme activity was assayed in the following conditions: CS, 100 mM KH_2PO_4 (pH 7.2), 0.5 mM oxaloacetate, 0.15 mM acetyl-coA, 0.15 mM 5,5'-dithiobis-2-nitrobenzoic acid; COX, 100 mM KH_2PO_4 (pH 7.2), 0.2 mM reduced cytochrome c (Calzyme Laboratories, CA, USA). V_{max} was measured in triplicate at 412 nm for CS (ε=14.15 l mM^{-1} cm^{-1}) and 550 nm for COX (ε=28.5 mM^{-1} cm^{-1}). Enzyme activities are expressed in units of μmol substrate per g tissue per min. Preliminary experiments determined that all substrate concentrations were saturating.

Diaphragm size, fibre-type composition, and capillarity

Fibre-type composition and capillarity was determined for diaphragm samples that were dissected, coated in mounting medium, and rapidly frozen in 2-methylbutane (cooled to near freezing in liquid N_2). Muscle was sectioned (10 μm) transverse to fibre length in a -20°C cryostat and mounted on slides (Superfrost Plus, Fisher). Enzyme histochemistry was used to stain cryostat sections for myosin-ATPase activity after pre-incubated at pH 4.6 (to identify slow oxidative, type I, fibres), or alkaline phosphatase activity (as a marker of capillaries) using methods we have described previously[35, 36]. Fluorescence immunohistochemistry was used to identify fibre types IIa and IIb. Sections were hydrated in phosphate buffered saline (PBS) and blocked in blocking solution (PBS containing 0.2% Triton X-100, 0.1% w:v sodium azide, 10%

normal goat serum) for 1 h at room temperature. Slides were then incubated overnight with primary antibodies against myosin heavy chain (MHC) IIA (1:10 dilution; SC-71, Developmental Studies Hybridoma Bank [DSHB], University of Iowa) and IIB (1:25 dilution; BA-F3, DSHB). The following day, slides were rinsed well in PBS and then incubated with appropriate secondary antibodies (AlexaFluor 488 at 1:250 dilution and AlexaFluor 594 at 1:500 dilution; Life Technologies, Oregon, USA) for 2 h at room temperature. Slides were then rinsed in PBS and mounted using Vecta Shield (Vector Laboratories Inc., California, USA).

Images were collected using bright-field/fluorescence microscopy and stereological methods were used to make unbiased measurements of several histological variables, as previously described[51, 52], with the assistance of ImageJ software[53]. Type I, IIa, and IIb fibres were identified from images of myosin-ATPase activity, MHC IIA, and MHC IIB, respectively, and the remaining fibres that were not otherwise identified were considered to be type IIx fibres. NIS Elements Imaging Software (version 4.30, Nikon Instruments, Melville, NY, USA) was also used to measure the number, staining area, and perimeter of individual capillaries within each image. Capillary length density was calculated according to Mathieu-Costello[54] as the quotient of capillary areal density (i.e., area of capillary staining relative to total area) and the transverse area of individual capillaries. Because many muscle capillaries are tortuous and are thus sectioned oblique to their length, their apparent measured area is larger than their transverse area. We therefore used the average area of the smallest 10% of identified capillaries to estimate the transverse area of individual capillaries. A sufficient number of images were analyzed to account for heterogeneity, determined in preliminary measurements as the number of images necessary to yield a stable mean value for an individual. We also determined the average thickness of the

diaphragm for each individual mouse by taking the average of several thickness measurements across all images.

Diaphragm masses were obtained in a separate set of mice by dissecting out and weighing diaphragms on a tared analytical balance, and are expressed relative to body mass.

Muscle contractile function

The contractile function of the diaphragm was examined *in vitro* using an isolated muscle test system (Aurora Scientific, Aurora, ON, Canada). Muscle strips were isolated and prepared as previously described[55]. Diaphragms were dissected fresh with ribs attached and placed into ice-cold buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.57 mM $MgSO_4$, 25 mM HEPES and 5.5 mM glucose; pH 7.2). The buffer was continuously bubbled with pure O_2 gas to maintain high O_2 levels (>575 µM or 18.4 mg/L O_2). Strips of muscle were prepared, with the ribs at the distal end and the central tendon at the proximal end, and the remaining muscle tissue was frozen in liquid N_2 and stored at -80°C until assayed for Ca^{2+}-ATPase activity (next section). The rib margin was anchored in place and the central tendon was attached to the force transducer (305C Dual-Mode Muscle Lever, Aurora Scientific) using sutures, and the diaphragm was submerged in the above buffer and maintained at 37°C. The optimal muscle length was determined and set as follows using established procedures[55]. The stimulus voltage was first increased until the peak isometric twitch force was achieved, reflecting full recruitment of fibres in the muscle strip (this maximal stimulation occurred at ~5-15 V for 1 ms, applied using a 701C Electrical Stimulator; Aurora Scientific). The muscle was then subjected to supra-maximal stimulation (10% higher voltage than needed for maximal stimulation) while muscle length was

adjusted with a micro-positioner, until the optimal length was reached that maximized isometric twitch force (optimal length was 8.53 ± 0.21 mm overall, and did not differ across treatment groups; P=0.10). The diaphragm strip was then given 5 min to recover. A single twitch was elicited (supra-maximal stimulation, 1 ms) from which twitch force, time to peak force, and time to 50% relaxation were determined. The force-frequency relationship was then determined by sequentially stimulating the muscle strips at 10, 20, 30, 40, 60, 80 and 100 Hz for 300 ms at each stimulus frequency interspersed by 2 min recovery intervals between each stimulus, and the diaphragm was allowed 10 min to recover. Fatigue resistance was then measured as the decay in force production while the diaphragm was stimulated every second (at 70 Hz for 300 ms) for 20 min[56]. The muscle strip was then removed from the apparatus, the ribs and central tendon were removed, and the wet mass of the muscle tissue was measured. The dry mass was then determined after oven baking the tissue at 60°C for 5 h (the dry mass of muscle strips were 2.12 ± 0.12 mg overall, and did not differ across treatment groups; P=0.5420). Contraction data were recorded and analyzed using the manufacturer's software (Aurora Scientific). Specific forces are expressed in N/cm^2 of muscle cross-sectional area. The latter was approximated as previously described[13], by dividing muscle dry mass by the product of optimal length and muscle density (assumed to be 1.06 g/cm^3).

Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity

We measured Ca^{2+}-dependent ATPase activity in heavy microsomes (which are enriched for sarcoplasmic reticulum) as an index of SERCA activity, using a modification of previously described protocols[57-59] in a SpectraMax Plus 384 spectrophotometer. Samples were homogenized in 10 volumes of ice-cold buffer (100 mM Tris, 250 mM sucrose, 5% glycerol and

1 mM phenylmethylsulfonyl fluoride [PMSF]; pH 7.0) and then centrifuged at 1,000 *g* at 4°C for 2 min. The supernatant was collected and vortexed, then centrifuged at 12,000 *g* at 4°C for 20 min (the preceding centrifugation steps should remove myfibrils, mitochondria, nuclei, and other heavy cellular components[60-62]). The supernatant was again collected and vortexed, then centrifuged at 21,000 *g* at 4°C for 2 h. The resulting pellet (the heavy microsomal fraction that contains sarcoplasmic reticulum) was collected and re-suspended in buffer for use in enzyme assays, and the resulting supernatant (which should include sarcolemmal vesicles, light microsomes, polysomes, and ribosomes[60-62]) was discarded. The rate of change in absorbance at 630 nm (ε=19.6 mM^{-1} cm^{-1}) was measured over 10 min at mouse body temperature (37°C) under the following conditions: 30 mM imidazole, 1 mM ATP, 2.5 µM ruthenium red, 100 mM KCl, 5 mM sodium azide, 200 µM EGTA; pH 6.8. The amount of free inorganic phosphate (P_i) in these conditions was determined by mixing a phosphorus determination solution (0.4 % w:v polyvinal alcohol [89-98 kDa], 75 µM malachite green, and 7.75 mM ammonium heptamolybdate; as described in Chan et al., 1986[58]), 4:1 with the reaction mixture. Malachite green reacts with phosphomolybdate (a product of the reaction of free phosphate and molybdate) and absorbs light at wavelengths 620-650 nm, with peak absorbance at 630 nm. Each sample was assayed in duplicate in the presence and absence of $CaCl_2$, at an appropriate concentration to obtain 80 µM free calcium (using the Maxchelator calculator described by Schoenmakers et al., 1992[63]). SERCA activity was determined by subtracting the values obtained without calcium (background ATPase activity found in the heavy microsomal fraction) from those obtained with 80 µM free calcium (Ca^{2+}-stimulated ATPase activity plus background ATPase activity found in the heavy microsomal fraction), and is expressed in µmol substrate per mg of microsomal protein per min. Preliminary experiments determined that substrate concentrations were saturating.

451

452 *Statistical analysis*

453 Data are presented as means ± SE. Two-factor ANOVA was used to evaluate the main
454 effects of population and environment (normoxia and hypoxia at 12 kPa O_2), and Bonferroni
455 post-tests were used to evaluate pairwise differences between populations within an environment
456 and between environments within each population. For the subset of measurements for which we
457 also collected data from highlanders exposed to more severe levels of hypoxia (9 kPa O_2), we
458 also used one-factor ANOVA and Bonferroni post-tests to evaluate the effects of exposure
459 environment in the highlanders. $P < 0.05$ was considered significant.

460

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470

471 **Conflicting Interests**

472 The authors declare no competing financial interests.

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652

Table 1. Statistical results for permeabilized fibre respiration and ROS emission measurements in the diaphragm of *Peromyscus* mice.

Experimental Results	Population Main Effect Two-Factor ANOVA[1]	Environment Main Effect Two-Factor ANOVA[1]	Interaction Effect Two-Factor ANOVA[1]	Environment Main Effect One-Factor ANOVA[2]
Respiration Rate (Data in Fig. 1A)				
L_N	$F_{1,33}$=2.87, P=0.0997	$F_{1,33}$=0.14, P=0.7118	$F_{1,33}$=4.78, **P=0.0360**	$F_{2,26}$=1.609, P=0.2193
P_{PM}	$F_{1,33}$=2.07, P=0.1592	$F_{1,33}$=10.31, **P=0.0029**	$F_{1,33}$=8.56, **P=0.0062**	$F_{2,26}$=9.531, **P=0.0008**
P_{PMG}	$F_{1,33}$=4.21, **P=0.0483**	$F_{1,33}$=21.17, **P<0.0001**	$F_{1,33}$=13.20, **P=0.0009**	$F_{2,26}$=11.26, **P=0.0003**
P_{PMGS}	$F_{1,33}$=3.63, P=0.0656	$F_{1,33}$=20.18, **P<0.0001**	$F_{1,33}$=8.13, **P=0.0075**	$F_{2,26}$=11.80, **P=0.0002**
P_{Tm}	$F_{1,33}$=5.67, P=**0.0232**	$F_{1,33}$=25.94, **P<0.0001**	$F_{1,33}$=6.07, **P=0.0192**	$F_{2,26}$=18.98, **P<0.0001**
ROS Emission Rate (Data in Fig. 1B)				
L_N	$F_{1,33}$=3.03, P=0.0912	$F_{1,33}$=5.21, **P=0.0291**	$F_{1,33}$=4.42, **P=0.0433**	$F_{2,26}$=0.1194, P=0.8879
P_{PM}	$F_{1,33}$=2.63, P=0.1144	$F_{1,33}$=4.98, **P=0.0325**	$F_{1,33}$=7.98, **P=0.0080**	$F_{2,26}$=0.9385, P=0.4041
P_{PMG}	$F_{1,33}$=4.27, **P=0.0466**	$F_{1,33}$=4.70, **P=0.0374**	$F_{1,33}$=6.49, **P=0.0157**	$F_{2,26}$=1.563, P=0.2285
P_{PMGS}	$F_{1,33}$=4.859, **P=0.0346**	$F_{1,33}$=6.32, **P=0.0170**	$F_{1,33}$=4.095, P=0.0512	$F_{2,26}$=1.151, P=0.3318
ROS Emission/O_2 Consumption Rate (Data in Fig. 1C)				
P_{PM}	$F_{1,33}$=0.3421, P=0.5626	$F_{1,33}$=0.0971, P=0.7573	$F_{1,33}$=0.0036, P=0.9524	$F_{2,26}$=3.477, **P=0.0459**
P_{PMG}	$F_{1,33}$=0.1563, P=0.6952	$F_{1,33}$=0.6931, P=0.4111	$F_{1,33}$=0.0281, P=0.8678	$F_{2,26}$=4.934, **P=0.0153**
P_{PMGS}	$F_{1,33}$=0.0344, P=0.8540	$F_{1,33}$=1.178, P=0.2856	$F_{1,33}$=0.333, P=0.5678	$F_{2,26}$=4.593, **P=0.0195**
Respiration Rate/Citrate Synthase Activity (Data in Fig. 1D)				
L_N	$F_{1,33}$=0.064, P=0.8019	$F_{1,33}$=0.6411, P=0.4290	$F_{1,33}$=14.62, **P=0.0006**	$F_{2,26}$=1.760, P=0.1919
P_{PM}	$F_{1,33}$=5.243, **P=0.0286**	$F_{1,33}$=6.196, **P=0.0180**	$F_{1,33}$=16.68, **P=0.0003**	$F_{2,26}$=0.0698, P=0.3905
P_{PMG}	$F_{1,33}$=8.582, **P=0.0061**	$F_{1,33}$=8.789, **P=0.0056**	$F_{1,33}$=20.59, **P<0.0001**	$F_{2,26}$=0.0398, P=0.5889
P_{PMGS}	$F_{1,33}$=10.13, **P=0.0032**	$F_{1,33}$=10.17, **P=0.0031**	$F_{1,33}$=13.14, **P=0.0010**	$F_{2,26}$=2.775, P=0.0808
P_{Tm}	$F_{1,33}$=8.041, **P=0.0078**	$F_{1,33}$=6.293, **P=0.0172**	$F_{1,33}$=9.538, **P=0.0041**	$F_{2,26}$=0.1463, P=0.8645

[1]Two-factor ANOVA was used to test for significant effects of population and environment in mice exposed to normoxia or hypoxia at 12 kPa O_2. [2]One-factor ANOVA was used to test for significant differences between normoxia, hypoxia at 12 kPa O_2, and severe hypoxia at 9 kPa O_2 within highland mice.

Table 2. Chronic hypoxia increased the citrate synthase activity of the diaphragm in both populations of *Peromyscus* mice, but had population-specific effects on cytochrome c oxidase activity.

Enzyme (μmol/ g tissue/min)	Normoxic Lowlanders	Hypoxic (12 kPa) Lowlanders	Normoxic Highlanders	Hypoxic (12 kPa) Highlanders	Hypoxic (9 kPa) Highlanders	Population Main Effect Two-Factor ANOVA	Environment Main Effect Two-Factor ANOVA	Interaction Effect Two-Factor ANOVA	Environment Main Effect One-Factor ANOVA
Citrate Synthase	53.6 ± 4.5	72.7 ± 5.0†	50.5 ± 5.1	76.7 ± 4.1†	101.6 ± 7.0†,††	$F_{1,33}$=0.01, P=0.9208	$F_{1,33}$=21.35, **P<0.0001**	$F_{1,33}$=0.54, P=0.4675	$F_{2,26}$=41.25, **P<0.0001**
Cytochrome c Oxidase	59.7 ± 3.2	95.98 ± 5.1†	86.7 ± 7.5*	100.1 ± 5.3	102.7 ± 2.4	$F_{1,33}$=7.16, **P=0.0115**	$F_{1,33}$=18.37, **P=0.0001**	$F_{1,33}$=4.05, P=0.0525	$F_{2,26}$=1.644, P=0.2119

Values are given in as the mean ± SEM (n = 8-11). *,†,†† Significant pairwise differences between the highland and lowland populations within the same environment, between normoxia and hypoxia (12 kPa) within the same population, or between moderate (12 kPa) and severe (9 kPa) hypoxia within the highlanders, respectively, in Bonferroni post-tests.

Table 3. Morphometrics of the diaphragm of *Peromyscus* mice.

Muscle Trait	Normoxic Lowlanders	Hypoxic Lowlanders	Normoxic Highlanders	Hypoxic Highlanders	Population Main Effect	Environment Main Effect	Interaction Effect
Diaphragm Mass (mg/g body mass)	3.87 ± 0.13	3.92 ± 0.21	4.45 ± 0.15	4.23 ± 0.23	$F_{1,29}$=4.136, P=0.0512	$F_{1,29}$=1.873, P=0.6683	$F_{1,29}$=0.3599, P=0.5532
Diaphragm Thickness (µm)	406.1 ± 27.6	356.6 ± 26.8	405.5 ± 26.3	405.7 ± 29.7	$F_{1,30}$=0.7280, P=0.4003	$F_{1,30}$=0.7518, P=0.3928	$F_{1,30}$=0.7630, P=0.3893
Numerical Density (%)							
type I fibre	11.3 ± 0.4	9.7 ± 1.0	13.1 ± 1.2	14.0 ± 2.5	$F_{1,20}$=2.66, P=0.1184	$F_{1,20}$=0.03, P=0.8584	$F_{1,20}$=0.44, P=0.5131
type IIa fibre	39.3 ± 6.8	23.2 ± 2.0	34.6 ± 2.4	29.7 ± 2.6	$F_{1,20}$=0.06, P=0.8052	$F_{1,20}$=9.07, **P=0.0069**	$F_{1,20}$=2.54, P=0.1267
type IIx fibre	45.8 ± 7.9	55.5 ± 3.7	43.6 ± 2.4	49.0 ± 3.3	$F_{1,20}$=1.13, P=0.3007	$F_{1,20}$=3.38, P=0.0811	$F_{1,20}$=0.28, P=0.6016
type IIb fibre	3.6 ± 1.1	11.6 ± 3.0	8.7 ± 2.3	7.4 ± 1.2	$F_{1,20}$=0.03, P=0.8562	$F_{1,20}$=2.06, P=0.1664	$F_{1,20}$=3.94, P=0.0609
Areal Density (%)							
type I fibre	15.3 ± 2.2	9.6 ± 0.7	13.9 ± 1.0	16.0 ± 1.7	$F_{1,20}$=1.37, P=0.2553	$F_{1,20}$=0.71, P=0.4091	$F_{1,20}$=3.27, P=0.0858
type IIa fibre	27.2 ± 2.8	16.0 ± 2.1	23.6 ± 1.7	18.0 ± 1.8	$F_{1,20}$=0.15, P=0.6993	$F_{1,20}$=15.47, **P=0.0008**	$F_{1,20}$=1.70, P=0.2074
type IIx fibre	53.8 ± 4.2	59.4 ± 3.8	53.5 ± 1.8	57.2 ± 3.3	$F_{1,20}$=0.15, P=0.6984	$F_{1,20}$=2.07, P=0.1653	$F_{1,20}$=0.10, P=0.7605
type IIb fibre	3.7 ± 1.0	14.9 ± 3.8	9.0 ± 2.1	8.9 ± 1.8	$F_{1,20}$=0.03, P=0.8692	$F_{1,20}$=5.02, P=0.0365	$F_{1,20}$=5.21, **P=0.0335**
Fibre Transverse Area (µm^2)							
type I fibre	1103 ± 184	1501 ± 501	910 ± 82	1219 ± 112	$F_{1,20}$=1.34, P=0.2601	$F_{1,20}$=2.99, P=0.0990	$F_{1,20}$=0.05, P=0.8294
type IIa fibre	706 ± 71	515 ± 72	733 ± 83	749 ± 94	$F_{1,20}$=1.83, P=0.1910	$F_{1,20}$=0.82, P=0.3771	$F_{1,20}$=1.14, P=0.2977
type IIx fibre	1232 ± 203	796 ± 73	1443 ± 165	1439 ± 161	$F_{1,20}$=4.18, P=0.0544	$F_{1,20}$=0.85, P=0.3663	$F_{1,20}$=2.08, P=0.1648

type IIb fibre	1229 ± 212	987 ± 102	1309 ± 226	1475 ± 191	$F_{1,20}$=1.46, P=0.2407	$F_{1,20}$=0.03, P=0.8719	$F_{1,20}$=0.75, P=0.3955
Tissue Capillarity							
capillary density (mm^{-2})	1707 ± 104	1946 ± 203	1483 ± 125	1528 ± 113	$F_{1,31}$=3.72, P=0.0631	$F_{1,31}$=0.72, P=0.4013	$F_{1,31}$=0.72, P=0.4013
capillaries per muscle fibre	1.62 ± 0.08	1.43 ± 0.16	1.59 ± 0.06	1.63 ± 0.14	$F_{1,31}$=0.44, P=0.5124	$F_{1,31}$=0.39, P=0.5370	$F_{1,31}$=0.88, P=0.3563
capillary length density (μm^{-2})	7.4 ± 0.7	6.6 ± 1.0	15.4 ± 1.9	14.8 ± 2.6	$F_{1,31}$=8.60, **P=0.0063**	$F_{1,31}$=0.07, P=0.7904	$F_{1,31}$=0.00, P=0.9590

Values are given in as the mean ± SEM (*n* = 5-9). There were no significant pairwise differences between groups in Bonferroni post-tests.

Table 4. Specific force production (N/cm^2) at different submaximal stimulation frequencies in the diaphragm of *Peromyscus* mice.

Stimulation Frequency	Normoxic Lowlanders	Hypoxic Lowlanders	Normoxic Highlanders	Hypoxic Highlanders	Population Main Effect	Environment Main Effect	Interaction Effect
Force-Frequency Relationship Tests							
10 Hz	11.4 ± 1.8	14.9 ± 1.9	5.4 ± 1.1	9.3 ± 2.0	$F_{1,45}$=10.15, **P=0.0026**	$F_{1,45}$=4.14, **P=0.0478**	$F_{1,45}$=0.01, P=0.9223
20 Hz	12.3 ± 2.0	15.7 ± 2.2	5.6 ± 1.2	10.1 ± 2.4	$F_{1,45}$=8.49, **P=0.0055**	$F_{1,45}$=3.45, P=0.0699	$F_{1,45}$=0.07, P=0.7962
30 Hz	14.7 ± 2.5	17.6 ± 2.7	6.3 ± 1.4	11.6 ± 3.0	$F_{1,45}$=7.53, **P=0.0087**	$F_{1,45}$=2.49, P=0.1214	$F_{1,45}$=0.22, P=0.6440
40 Hz	19.8 ± 3.6	22.8 ± 3.5	8.6 ± 2.0	15.1 ± 3.8	$F_{1,45}$=7.45, **P=0.0090**	$F_{1,45}$=1.91, P=0.1737	$F_{1,45}$=0.25, P=0.6169
60 Hz	29.7 ± 5.0	34.2 ± 4.6	15.5 ± 3.3	23.3 ± 4.8	$F_{1,45}$=7.22, **P=0.0100**	$F_{1,45}$=1.74, P=0.1937	$F_{1,45}$=0.13, P=0.7169
80 Hz	34.8 ± 5.6	43.1 ± 5.5	19.9 ± 4.4	30.4 ± 5.4	$F_{1,45}$=6.38, **P=0.0152**	$F_{1,45}$=2.94, P=0.0933	$F_{1,45}$=0.04, P=0.8467
Fatigue Resistance Tests[1]							
70 Hz	30.9 ± 6.6	37.4 ± 4.9	18.6 ± 4.9	26.1 ± 5.3	$F_{1,34}$=4.46, **P=0.0422**	$F_{1,34}$=1.56, P=0.2202	$F_{1,34}$=0.009, P=0.9268

Values are given in as the mean ± SEM (n = 7-12). [1] Data at 70 Hz are the initial forces generated at the start of the fatigue resistance tests, which were collected after 10 min recovery from the force-frequency relationship tests (see Materials and Methods for details). There were no significant pairwise differences between treatment groups in Bonferroni post-tests.

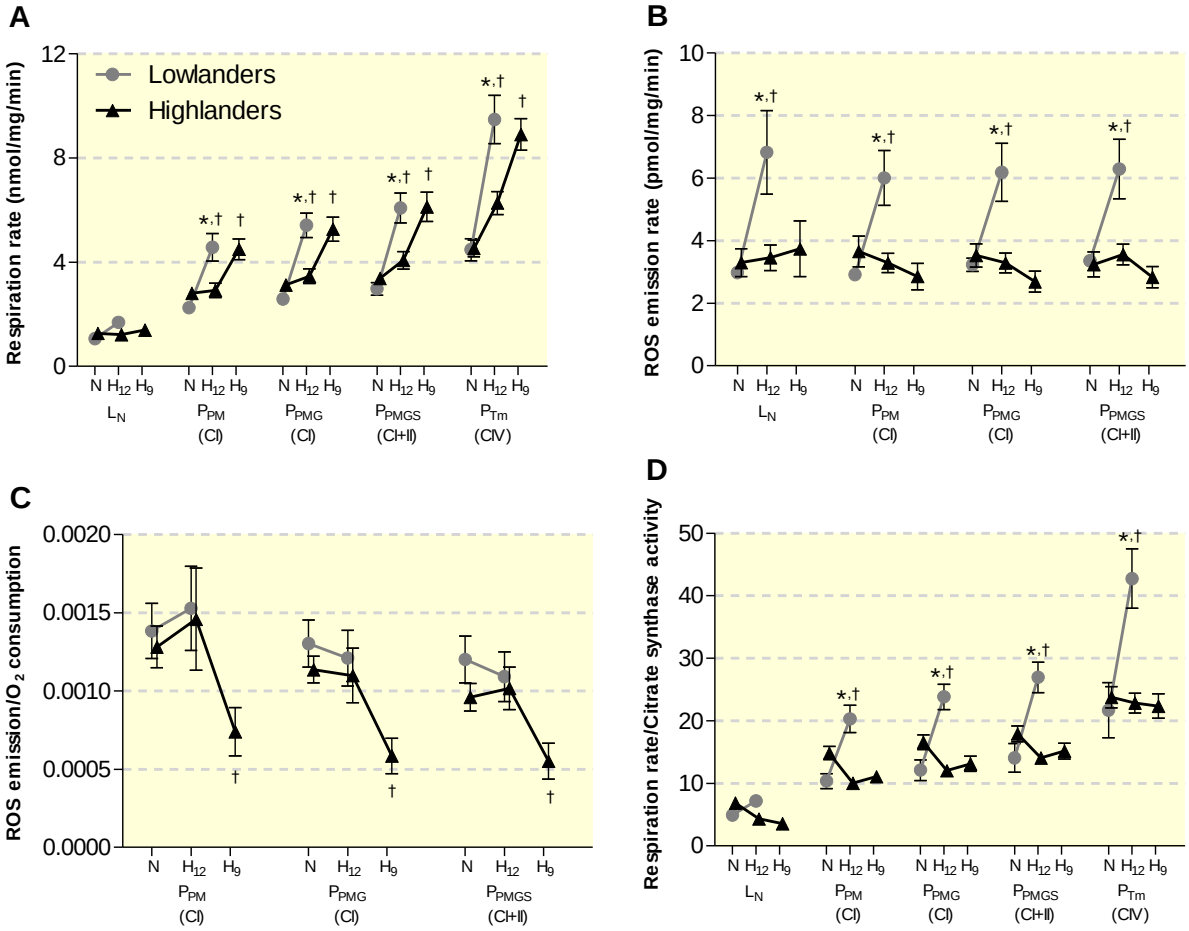

Figure 1. Chronic hypoxia increased the respiration rates (A) of permeabilized diaphragm fibres in both populations of *Peromyscus* mice, but had population-specific effects on ROS emission rates (B) and the ratio of ROS emission to O_2 consumption (C). Respiration and ROS emission rates (expressed per mg mitochondrial protein) were measured in normoxia (20 kPa O_2; N), hypoxia (12 kPa O_2; H_{12}) and more severe hypoxia (9 kPa O_2; H_9) in the leak state (malate and pyruvate - L_N) and during oxidative phosphorylation (P) in the presence of ADP and substrates of complex I (P_{PM}: malate and pyruvate; P_{PMG}: malate, pyruvate, and glutamate), complexes I and II (P_{PMGS}: malate, pyruvate, glutamate, and succinate), and complex IV (P_{Tm}: ascorbate and TMPD). ANOVA results are reported in Table 1. *,† - Significant pairwise differences between the highland and lowland populations within the same environment, or between normoxia and hypoxia within the same population, respectively, in Bonferroni post-tests (n = 8-11).

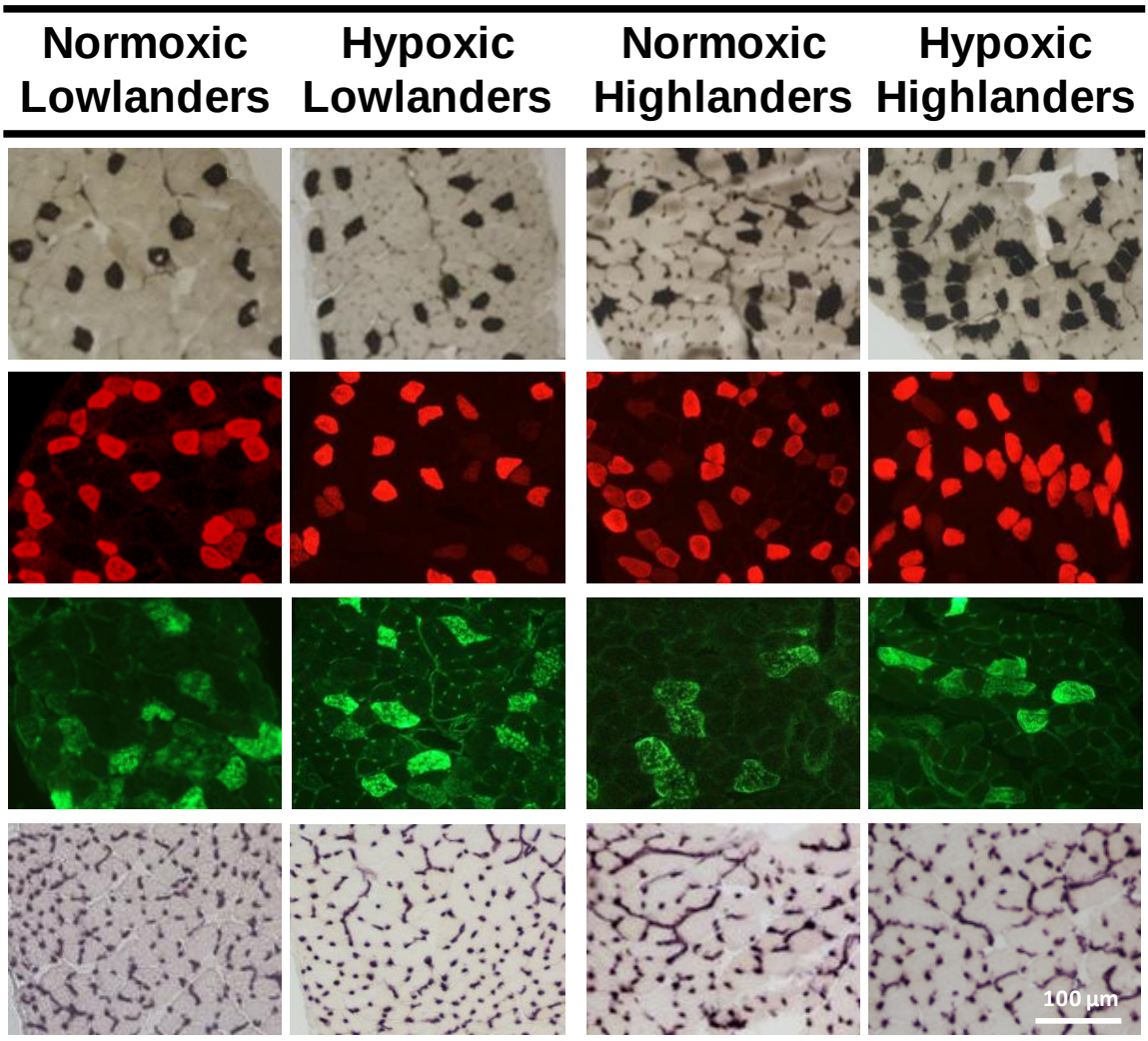

Figure 2. Identification of fibre types and capillaries in the diaphragm muscle. Representative images of each stain is organized sequentially by row in the order (i) myosin-ATPase activity after acidic pre-incubation (a marker of type I fibres), (ii) myosin heavy chain (MHC) IIA (red), (iii) MHC IIB (green), and (iv) alkaline phosphatase activity (a marker of capillaries). All images were acquired at the same magnification.

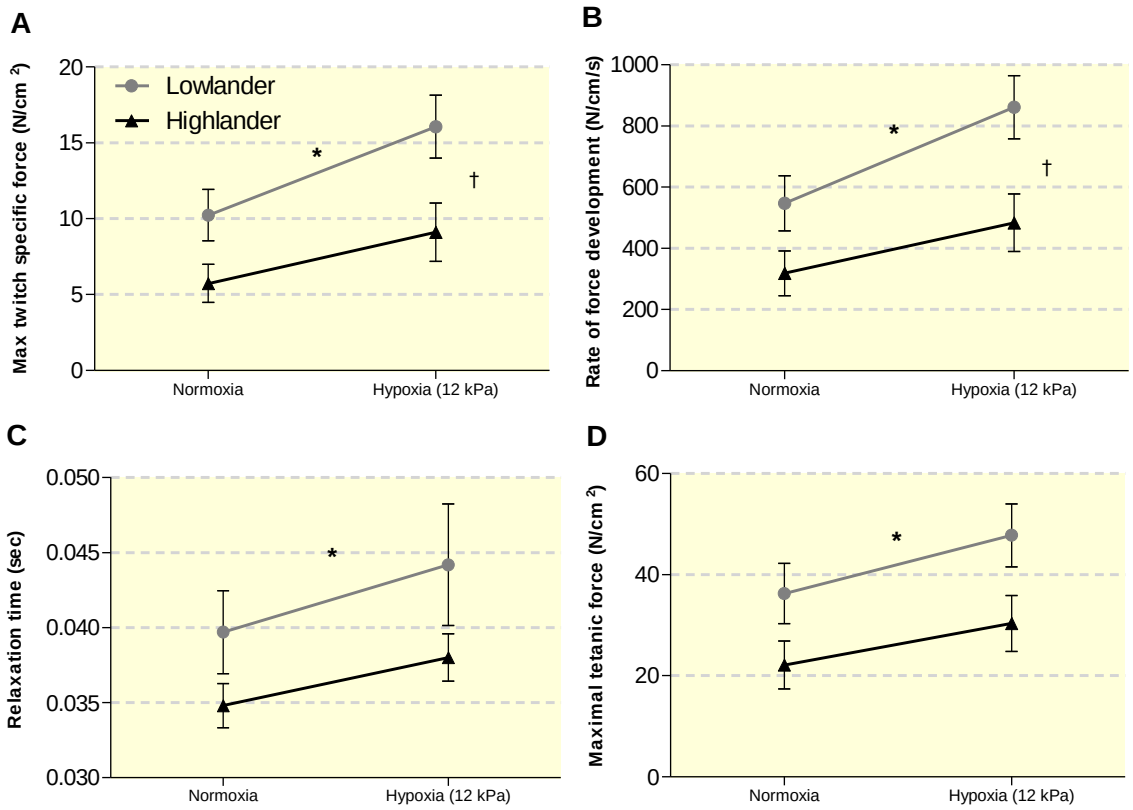

Figure 3. Chronic hypoxia increased force production by the diaphragm in both populations of *Peromyscus* mice (A,D), but the diaphragm of highlanders generally produced less force at a slower rate (B) and relaxed in less time (C). *,† - Significant main effects of population (A, $F_{1,45}$=9.73, P=0.0032; B, $F_{1,45}$=10.46, P=0.0023; C, $F_{1,45}$=4.17, P=0.0474; $F_{1,45}$=7.40, P=0.0093) or environment (A, $F_{1,45}$=6.31, P=0.0156; B, $F_{1,45}$=6.53, P=0.0141; C, $F_{1,45}$=2.01, P=0.1635; D, $F_{1,45}$=2.90, P=0.0957), respectively, in two-factor ANOVA (n = 8-11). There were no significant environment × population interactions (A, $F_{1,45}$=0.45, P=0.5067; B, $F_{1,45}$=0.63, P=0.4319; C, $F_{1,45}$=0.06, P=0.8132; D, $F_{1,45}$=0.08, P=0.7771).

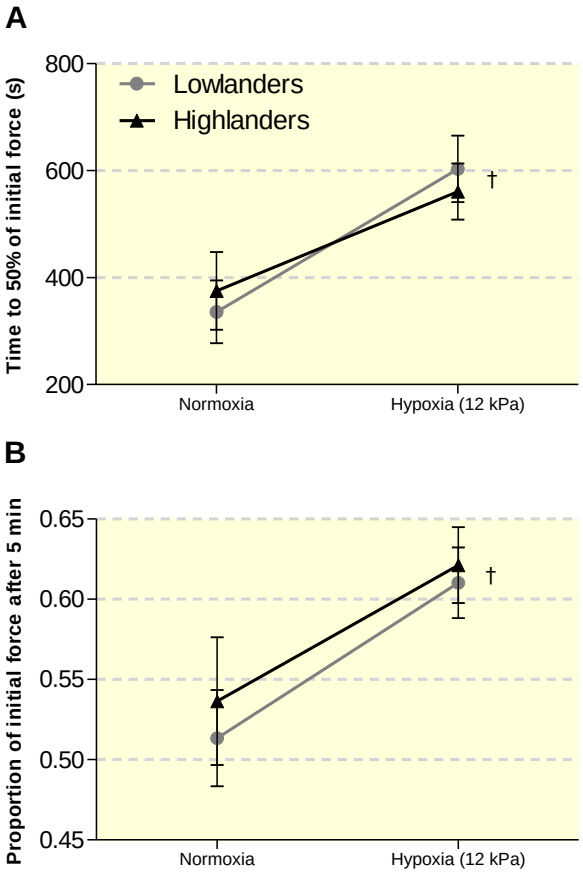

Figure 4. Chronic hypoxia increased fatigue resistance of the diaphragm in both populations of *Peromyscus* mice. Muscles underwent a fatigue protocol in which they were stimulated every second (at 70 Hz for 300 ms) for 20 min (see Materials and Methods). (A) The duration of time the muscles could be stimulated until force production fell to 50% of the initial force. (B) The force production remaining (as a proportion of initial force) after 5 min of stimulation. † - Significant main effect of environment (A, $F_{1,34}$=13.84, P=0.0007; B, $F_{1,34}$=9.86, P=0.0035) in two-factor ANOVA (n = 8-11). There were no significant main effects of population (A, $F_{1,34}$=0.0006, P=0.9809; B, $F_{1,34}$=0.35, P=0.5608) or environment × population interactions (A, $F_{1,34}$=0.45, P=0.5057; B, $F_{1,45}$=0.04, P=0.8359).

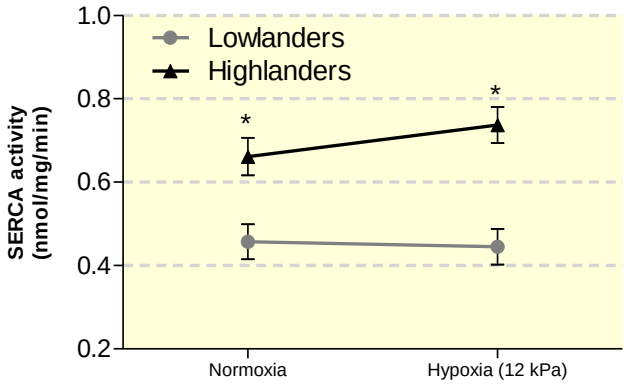

Figure 5. Sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) activity in the diaphragm (assayed in the microsomal fraction and expressed per mg microsomal protein) was greater in highlanders than in lowlanders, but was unaffected by chronic hypoxia. We measured Ca^{2+}-dependent ATPase activity in heavy microsomes (which are enriched for sarcoplasmic reticulum) as an index of SERCA activity (see Materials and Methods for details). There was a significant main effect of population ($F_{1,33}$=32.23, P<0.0001), but not of environment ($F_{1,33}$=0.53, P=0.4707) or environment × population interaction ($F_{1,45}$=1.01, P=0.3205) (n = 8-11). * - Significant pairwise differences between the highland and lowland populations within the same environment in Bonferroni post-tests.