Effects of hypoxia at different life stages on locomotory muscle phenotype in deer mice native to high altitudes

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Abstract

Animals native to high altitude must overcome the constraining effects of hypoxia on tissue O_2 supply to support routine metabolism, thermoregulation in the cold, and exercise. Deer mice (*Peromyscus maniculatus*) native to high altitude have evolved an enhanced aerobic capacity in hypoxia, along with increased capillarity and oxidative capacity of locomotory muscle. Here, we examined whether exposure to chronic hypoxia during development or adulthood affects muscle phenotype. Deer mice from a highland population were bred in captivity at sea level, and exposed to normoxia or one of four treatments of hypobaric hypoxia (12 kPa O_2, simulating hypoxia at ~4300 m): adult hypoxia (6-8 weeks), post-natal hypoxia (birth to adulthood), pre-natal hypoxia (before conception to adulthood), and parental hypoxia (in which mice were conceived and raised in normoxia, but their parents were previously exposed to hypoxia). Litter size was similar across treatments, and pups survived the hypoxia exposures and grew to similar body masses at ~6-8 months of age. Hypoxia had no effect on the masses of gastrocnemius and soleus muscles. There was a strong concordance between two distinct histological methods for staining capillaries in the gastrocnemius – alkaline phosphatase activity and binding of *Griffonia simplicifolia* lectin I – each of which showed that capillarity and muscle fibre size were largely unaffected by hypoxia. Maximal activities of several metabolic enzymes (cytochrome oxidase, citrate synthase, isocitrate dehydrogenase, and lactate dehydrogenase) in the gastrocnemius were also largely unaffected by hypoxia. Therefore, the evolved muscle phenotype of high-altitude deer mice is relatively insensitive to hypoxia across life stages.

1. Introduction

High altitude is one of the most challenging terrestrial environments inhabited by endotherms. High-altitude environments are colder than those at sea level, which can elevate the metabolic O_2 demands of thermogenesis (particularly in small endotherms), and they are also hypoxic, which reduces the O_2 available to support those greater O_2 demands. High-altitude natives can somehow overcome this challenge, and can live, reproduce, and sometimes sustain impressive feats of aerobic performance at high altitudes (Hawkes et al., 2013; Hayes, 1989). Growing evidence suggests that the ability of highland natives to thrive in the harsh environment at high altitudes depends on both plasticity (e.g., acclimatization, developmental plasticity, etc.) and evolved changes across the O_2 transport cascade – comprised of ventilation, pulmonary diffusion, circulation, tissue diffusion, and cellular O_2 utilization (Gilbert-Kawai et al., 2014; Ivy and Scott, 2015; Julian et al., 2009; Monge and León-Velarde, 1991; Murray and Horscroft, 2016; Scott, 2011; Storz et al., 2010).

The capacity for O_2 diffusion and mitochondrial O_2 utilization appears to be augmented in the locomotory muscle of several high-altitude natives. Many highland mammals and birds have higher muscle capillarity and oxidative capacity than their lowland counterparts when each is sampled and compared in its native environment (León-Velarde et al., 1993; Mathieu-Costello et al., 1998; Scott et al., 2015; Sheafor, 2003). Differences persist in common garden comparisons in captivity at sea level, suggesting that there is an important genetic (evolved) contribution to the highly capillarized and oxidative phenotype of native highlanders in the wild (Lui et al., 2015; Scott et al., 2009). However, the relative influence on muscle phenotype of environmentally induced plasticity at high altitudes, particularly the influence of hypoxia exposure, is unclear. Counter to the general expectation that tissue O_2 limitation is a primary signal for angiogenesis (Gustafsson, 2011), there are conflicting results about whether the capillarity of locomotory muscle is affected by exposure to chronic hypoxia (e.g., high-altitude acclimatization or hypoxia acclimation in the lab) (Bigard et al., 1991; de Theije et al., 2015; Hoppeler and Vogt, 2001; Lui et al., 2015; Lundby et al., 2004; Mathieu-Costello, 2001; Mathieu-Costello and Agey, 1997). Similar discrepancies have been observed for the effects of high-altitude acclimatization and hypoxia acclimation on muscle oxidative capacity (Beaudry and McClelland, 2010; D'Hulst and Deldicque, 2017; Hoppeler and Vogt, 2001; Jacobs et al., 2016; Mahalingam et al., 2017). However, most previous investigations have examined the

effects of chronic hypoxia on muscle phenotype during adulthood. Although embryonic hypoxia has been shown to increase muscle capillarity in geese (Snyder et al., 1984), it is otherwise poorly understood whether developmental hypoxia has consistent or pronounced effects on muscle phenotype.

The objective of this study was to examine the effects of hypoxia at different life stages on the capillarity and oxidative capacity of locomotory muscle in deer mice (*Peromyscus maniculatus*) native to high altitudes. Deer mice inhabit the broadest altitudinal distribution of any North American mammal, from below sea level in Death Valley California to over 4,300 m elevation in the Rocky Mountains (Hock, 1964; Natarajan et al., 2015; Snyder et al., 1982). High-altitude deer mice maintain high field metabolic rates in the wild, presumably to support the elevated demands of thermogenesis in the cold (Hayes, 1989). Strong directional selection favours a high aerobic capacity (VO_2max) at high altitudes (Hayes and O'Connor, 1999), and highland populations of deer mice have thus evolved an enhanced VO_2max in hypoxia compared to low-altitude mice (Cheviron et al., 2012; Cheviron et al., 2013; Lui et al., 2015; Tate et al., 2017). The underlying mechanism for this evolved increase in VO_2max appears to include several changes in muscle phenotype – including higher capillarity and greater mitochondrial abundance and enzyme activities in the gastrocnemius muscle – and many of these traits are insensitive to hypoxia acclimation in adulthood (Lau et al., 2017; Lui et al., 2015; Mahalingam et al., 2017). However, development at high altitudes is known to affect VO_2max in deer mice (Russell et al., 2008), and environmentally-induced plasticity in response to the full suite of stressors at high altitude is believed to make an important contribution to the skeletal muscle phenotype (particularly metabolic enzyme activities and gene expression) of highland deer mice in the wild (Cheviron et al., 2012; Cheviron et al., 2013; Scott et al., 2015). Here, we sought to determine if this could be underpinned by effects of developmental hypoxia on muscle phenotype.

2. Materials and Methods

2.1. Breeding and hypoxia exposures

Captive breeding populations were established from a wild population of deer mice native to high altitude near the summit of Mount Evans, CO, USA (39°35’18’’N, 105°38’38”W; 4,350 m above sea level) (*P. m. rufinus*). Wild adults were transported to McMaster University (near sea

level) and were bred in captivity to produce lab-raised first-generation progeny. We then established two breeding pairs between adults of these first-generation mice (all of which were from different wild parents), and the two resulting families of progeny (i.e., second generation in the lab) were used in experiments. All mice were held at 24-25°C and a photoperiod of 12 h light: 12 h dark, and were provided with unlimited access to standard rodent chow and water. All animal protocols followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

We used a standardized breeding design to expose second-generation mice to hypoxia, starting at a range of different life stages, with five different treatment groups (Fig. 1). Each breeding pair was first allowed to raise four litters, in order to avoid potential effects of variation in litter size and resource allocation that may arise across the first few litters (Kirkland and Layne, 1989). Each pair then conceived and raised litters 5 and 6 in standard cage conditions of normobaric normoxia until weaning. These progeny from each family were split into two treatment groups, one that remained in normoxia (normoxia control group) and the other that was acclimated to hypobaric hypoxia (barometric pressure of 60 kPa, ~12 kPa O_2; simulating the hypoxia at an elevation of 4,300 m) during adulthood (adult hypoxia group). Litter 7 was also conceived and born in normoxia, but the family was moved to hypobaric hypoxia within 12 h of birth, and the mother and pups remained there together until weaning. After weaning, litter 7 pups continued to be raised in hypobaric hypoxia into adulthood (post-natal hypoxia group). The mother and father continued to be held in hypobaric hypoxia, and were allowed to conceive litter 8, which was born and raised into adulthood in hypobaric hypoxia (pre-natal hypoxia group). After weaning litter 8, breeding pairs were returned to normoxia and were then allowed to conceive and raise litter 9 in normoxia (parental hypoxia group). All litters (and each treatment group) contained a mix of both female and male pups. Exposures to hypobaric hypoxia were conducted using specially designed hypobaric chambers (Lui et al., 2015; McClelland et al., 1998). Cages were cleaned twice a week during hypoxia exposures, which required that mice be returned to normobaria for a brief period (<30 min).

Mice were euthanized (isoflurane overdose followed by cervical dislocation) during adulthood between 6-8 months of age. One gastrocnemius muscle was dissected, weighed, coated in embedding medium, frozen in liquid N_2-cooled isopentane, and stored at -80°C until used for muscle histology. The other gastrocnemius was snap-frozen in liquid N_2 and stored at -

80°C until used for enzyme assays. One soleus muscle was also dissected and weighed. The decision of which muscle, left or right, was used for each measurement was random.

2.2. Muscle histology

We used and compared two histological staining methods to examine the capillarity of the gastrocnemius muscle. Whole muscle was sectioned (10 μm thick) transverse to muscle fiber length in a cryostat maintained at -20°C, and were mounted on slides (SuperFrost Plus; Fisher Scientific, Ottawa, ON, Canada). Alkaline phosphatase activity was stained as we have previously described for deer mouse muscle (Lui et al., 2015; Scott et al., 2015), by incubating sections for 1 h at room temperature in an alkaline (pH 9.3) assay buffer containing 1.0 mM nitroblue tetrazolium, 0.5 mM 5-bromo-4-chloro-3-indoxyl phosphate, 28 mM $NaBO_2$, and 7 mM $MgSO_4$. *Griffonia simplicifolia* lectin I (GSL), which binds to terminal α-galactosyl groups and has been used successfully to detect capillaries in various mouse tissues (Hansen-Smith et al., 1992; Laitinen, 1987), was also used as a marker for capillaries. This was accomplished by first hydrating sections in phosphate buffered saline (PBS; 0.1 M, pH 7.4) and then blocking for 30 min at room temperature in blocking solution (Carbo-Free Blocking Solution, Vector Laboratories, Burlingame, CA, USA). Sections were then incubated for 30 min in Biotinylated GSL (Vector Laboratories) made up at a concentration of 20 μg/ml in PBS containing 1% Triton X-100 and 1.5% bovine serum albumin (PBS/TX/BSA). Sections were rinsed well in PBS, and then incubated for 30 min in ExtrAvidin-Peroxidase diluted 1:50 in PBS/TX/BSA. Sections were again rinsed well in PBS, and finally developed (0.4 mg/ml 3-amino-9-ethyl-carbazole and 0.02% H_2O_2 in 0.05 M sodium acetate buffer; pH 5.0) for ~5 min. Slides were then rinsed in PBS, coated in Aquamount (Fisher Scientific), and cover-slipped.

Images were collected using bright-field microscopy, and several indices of capillarity were measured. ImageJ software (Schneider et al., 2012) was used to quantify capillary density, the average number of capillaries per muscle fibre, and the average transverse area of muscle fibres by an observer that was blind to experimental treatment. We also used NIS Elements Imaging Software (version 4.30, Nikon Instruments, Melville, NY, USA) to measure the number and perimeter of individual capillaries within each image, in order to calculate capillary surface densities. A sufficient number of images was analyzed to account for heterogeneity across the gastrocnemius. This number of images was determined in preliminary measurements, by first

analysing a large excess number of images and then determining the minimum number that is necessary to yield a stable mean value for an individual.

2.3. Enzyme activity assays

We measured the activity of several metabolic enzymes in the gastrocnemius muscle. Whole muscles were powdered under liquid N_2, and small samples were homogenized in 10 volumes of ice-cold homogenization buffer (100 mM KH_2PO_4, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100 at pH 7.2) using a PowerGen 125 homogenizer (Fisher Scientific). Homogenates were then centrifuged at 1000g for 1 min at 4°C. The pellet was discarded and the homogenate was kept on ice until assayed. The activities of cytochrome c oxidase (COX), citrate synthase (CS), isocitrate dehydrogenase (IDH), and lactate dehydrogenase (LDH) were assayed in triplicate at 37°C under the following conditions (in mM at pH 7.2 unless otherwise stated): COX, 0.2 reduced cytochrome c*, 100 KH_2PO_4; CS, 0.5 oxaloacetate*, 0.15 acetyl-coA, 0.15 5,5'-dithiobis-2-nitrobenzoic acid, 100 KH_2PO_4; IDH, 5 isocitrate*, 1.5 NADP, 100 KH_2PO_4; LDH, 5 pyruvate*, 0.3 NADH, 100 KH_2PO_4. Maximal activities (V_{max}) were determined by measuring the change in absorbance over time at 550 nm for COX (ε=28.5 mM^{-1} cm^{-1}), 412 nm for CS (ε=14.15 l mM^{-1} cm^{-1}), and 340 nm for IDH and LDH (ε=6.22 mM^{-1} cm^{-1}), by subtracting the background rate (measured in control reactions without a key substrate, *) from the rates measured in the presence of all substrates. Preliminary experiments verified that substrate concentrations were saturating and were not inhibitory. All substrates for enzyme assays were obtained from Sigma-Aldrich (Oakville, ON, Canada). Cytochrome c was reduced with ascorbate, which was subsequently removed by dialysis.

2.3. Statistics

ANOVA and Bonferroni multiple-comparisons tests were used to evaluate the effects of hypoxia treatment. Two-factor ANOVA was used to test for main effects of treatment and histological staining method on capillarity indices. One-factor ANOVA was otherwise used to test for the main effects of treatment. A significance level of $P<0.05$ was used throughout.

3. Results

3.1. Litter size and body masses

Chronic hypoxia did not appear to affect litter size or adult body mass. Litters 5 and 6 raised in normoxia contained 3 and 5 pups in family 1, and 3 and 4 pups in family 2, respectively. Similar litter sizes were observed in litter 7 exposed to hypoxia from birth to adulthood (family 1, 5 pups; family 2, 4 pups), litter 8 exposed to hypoxia from conception to adulthood (family 1, 3 pups; family 2, 5 pups), and litter 9 in which parents had been hypoxic but conceived and raised pups in normoxia (family 1, 3 pups; family 2, 4 pups). Pups survived the hypoxia treatments, and exhibited similar body masses in adulthood at 6-8 months of age (Table 1).

3.2. Muscle structure and capillarity

Chronic hypoxia had very little effect on the structure and capillarity of locomotory muscles. The mass of the gastrocnemius and soleus muscles were similar across treatment groups (Table 1), as was the size of individual muscle fibres (a measurement that comprises the mean transverse area across the slow oxidative, fast oxidative, and fast glycolytic fibre types in the gastrocnemius) (Table 2). Muscle capillarity was also relatively unaffected by hypoxia (Fig. 2). There were no significant differences in capillary density or capillary surface density across treatment groups (Table 2). There was some modest but significant overall variation in capillary to fibre ratio (significant main effect in ANOVA), driven primarily by higher average values in the adult hypoxia and pre-natal hypoxia groups, but there were no significant pairwise differences between any of the hypoxic treatment groups and the normoxic controls (Table 2). This variation in capillary to fibre ratio was associated with comparable but non-significant variation in muscle fibre size, and therefore appeared to be caused by there being fewer fibres rather than more capillaries per unit area of muscle. Capillarity indices were generally very similar and well correlated between measurements made using alkaline phosphatase activity and *Griffonia simplicifolia* lectin I (GSL) as markers (Table 2, Fig. 3).

3.3. Metabolic enzyme activities in the gastrocnemius muscle

Chronic hypoxia had very little effect on the maximal activity of several metabolic enzymes in the gastrocnemius muscle (Fig. 4). There was a significant treatment effect on the activity of cytochrome c oxidase (the O_2 consuming enzyme of the electron transport system)

detected with ANOVA, driven primarily by higher average values in the adult hypoxia and parental hypoxia groups, but there were no significant pairwise differences between any of the hypoxic treatment groups and the normoxic controls. However, the activities of citrate synthase and isocitrate dehydrogenase (IDH) were unaffected by hypoxia treatment, and the non-significant pattern of variation was inconsistent with the variation in cytochrome c oxidase activity. It is important to note that IDH was assayed using $NADP^+$ as a substrate, so our measurements excluded the NAD^+-dependent form of mitochondrial IDH. Lactate dehydrogenase activity was also unaffected by hypoxia treatment.

4. Discussion

Deer mice native to high altitudes have evolved an enhanced aerobic capacity in hypoxia compared to low-altitude mice (Cheviron et al., 2012; Cheviron et al., 2013; Lui et al., 2015; Tate et al., 2017), for which the underlying mechanism appears to include increases in the capillarity and mitochondrial oxidative capacity of the locomotory muscle (Lui et al., 2015; Mahalingam et al., 2017). Here, we show that the evolved muscle phenotype of high-altitude mice is relatively insensitive to chronic hypoxia across life stages. The structure, capillarity, and maximal activities of several mitochondrial enzymes in locomotory muscle were largely unaltered by hypoxia exposure during pre-natal and post-natal development and during adult life (Tables 1-2 and Fig. 4). Therefore, the underlying mechanisms of evolutionary adaptation to high altitudes may differ from those associated with environmentally-induced plasticity.

4.1. Chronic hypoxia and muscle phenotype

It has recently been suggested that the effects of chronic hypoxia on muscle phenotype depend upon the severity and duration of hypoxia exposure (D'Hulst and Deldicque, 2017), which may explain many of the discrepancies in the literature about whether the capillarity or oxidative capacity of locomotory muscle is affected by chronic hypoxia in humans and other animals (Bigard et al., 1991; Hoppeler and Vogt, 2001; Jacobs et al., 2016; Lui et al., 2015; Lundby et al., 2004; Mahalingam et al., 2017; Mathieu-Costello, 2001; Mathieu-Costello and Agey, 1997). For example, acclimation of adult CD1 mice to hypobaric hypoxia at ~13 kPa (equivalent to the hypoxia at ~4000 m) had no effect on the activities of cytochrome c oxidase or citrate synthase in the gastrocnemius muscle (a muscle with an intermediate mix of fibre types)

(Beaudry and McClelland, 2010), whereas acclimation of C57BL/6J mice to more severe hypoxia at ~8 kPa (equivalent to the hypoxia at ~7500 m) resulted in atrophy of the EDL (predominantly fast fibre types) and soleus (primarily oxidative fibre types) muscles (de Theije et al., 2015). The latter was associated with increased expression of genes involved in HIF signalling (e.g., Glut1, Vegfa), the ubiquitin-proteasome system, and the autophagy-lysosome pathway (de Theije et al., 2015). Nevertheless, across the range of altitudes inhabited by deer mice in the wild, which extends up to at least ~4300 m above sea level, our data suggest that the levels of hypoxia are not severe enough to elicit any change in the capillarity or oxidative capacity of locomotory muscle, even when hypoxia occurs throughout developmental and adult life.

The high muscle capillarity and oxidative capacity in deer mice native to high altitudes (Lui et al., 2015; Mahalingam et al., 2017; Scott et al., 2015) appears to have a genetic basis, and does not appear to be induced in response to living in a hypoxic environment. High-altitude mice have more oxidative fibres in the gastrocnemius muscle than their low-altitude counterparts, as well as a greater mitochondrial abundance within oxidative fibres due to a preferential proliferation of subsarcolemmal mitochondria – the mitochondrial sub-population that is adjacent to the cell membrane and closest to capillaries (Lui et al., 2015; Mahalingam et al., 2017; Scott et al., 2015). These differences are associated with highland mice having higher COX, CS, and IDH activities and lower LDH activity than lowland mice in the gastrocnemius (Lau et al., 2017; Lui et al., 2015). The resulting increase in mitochondrial oxidative capacity could enhance the muscle's capacity for shivering and possible non-shivering thermogenesis in the cold (Mineo et al., 2012). The increase in oxidative capacity could also promote hypoxia resistance, because it would augment the total mitochondrial O_2 flux of the entire muscle and counterbalance the effects of intracellular hypoxia on individual mitochondria – an idea that was proposed by Peter Hochachka and others (Hochachka, 1985; Hochachka et al., 1983). High-altitude deer mice also have a greater capillarity in the gastrocnemius muscle than their low-altitude counterparts, and the difference is greater than would be expected from the population differences in muscle fibre-type composition (Lui et al., 2015; Scott et al., 2015). This could augment the O_2 diffusing capacity from blood to mitochondria, to help offset the potential effects of hypoxaemia and to support the high O_2 demands of thermogenesis and locomotion. Although these various muscle traits are relatively unaffected by exposure to chronic hypoxia, it is possible that (but unknown

whether) plasticity in response to other environmental stressors at high altitude (e.g., cold) contributes to the skeletal muscle phenotype of highland deer mice in the wild (Cheviron et al., 2012; Cheviron et al., 2013; Scott et al., 2015).

4.2. Measurements of muscle capillarity

This study also demonstrated strong concordance between two distinct methods for measuring capillarity in skeletal muscle, alkaline phosphatase activity and binding of *Griffonia simplicifolia* lectin I (GSL) (Figs. 2,3). Previous work has shown that exposing C57BL/6J mice to hypoxia at ~8 kPa for 7-21 weeks reduced the intensity of staining for alkaline phosphatase activity in the gastrocnemius, without changing capillary density or the pattern of GSL staining (Hansen-Smith et al., 1992). We therefore sought to evaluate whether the less severe level of hypoxia used for our exposures (~12 kPa) affected the reliability of the alkaline phosphatase method of staining capillaries in the muscle, and whether a loss in the sensitivity of this method in hypoxia resulted in an underestimation of any potential hypoxia-induced angiogenesis. The strong resemblance in the pattern of capillary staining (Fig. 2) and the high degree of similarity of the measured capillarity indices between methods (Fig. 3; Table 2) suggests that alkaline phosphatase activity is indeed a reliable marker of capillaries in the skeletal muscle of deer mice exposed to hypoxia. Therefore, the lack of any observable variation in muscle capillarity was not a technical artefact (i.e., not resulting from an inability to accurately detect capillaries), but instead reflects a genuine lack of plasticity in response to chronic hypoxia during development and adulthood.

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Table 1

Body and muscle masses.

Treatment	N	Body mass (g)	Gastrocnemius mass (mg g^{-1})	Soleus mass (mg g^{-1})
Normoxia	8	22.4 ± 1.3	4.73 ± 0.22	0.260 ± 0.027
Adult hypoxia	6	25.8 ± 1.7	4.69 ± 0.09	0.287 ± 0.035
Post-natal hypoxia	7	21.8 ± 1.7	4.63 ± 0.28	0.244 ± 0.026
Pre-natal hypoxia	7	21.9 ± 1.7	4.35 ± 0.18	0.271 ± 0.030
Parental hypoxia	7	22.6 ± 1.0	4.48 ± 0.33	0.269 ± 0.027
		ANOVA results		
Treatment effect		$F_{4,30} = 1.04$ $P = 0.403$	$F_{4,30} = 0.426$ $P = 0.788$	$F_{4,30} = 0.275$ $P = 0.892$

Data are reported as means ± s.e.m. Muscle masses are expressed relative to body mass. Statistical results from one-factor ANOVA are shown below data. There were no significant pairwise differences between treatment groups.

Table 2

Capillarity and fibre size in the gastrocnemius muscle.

Treatment	Capillary density (mm^{-2})	Capillaries per fibre	Capillary surface density (μm^{-1})	Mean fibre area (μm^2)
		Alkaline phosphatase activity		
Normoxia	959 ± 31	1.55 ± 0.04	0.0275 ± 0.0008	1633 ± 72
Adult hypoxia	939 ± 72	1.75 ± 0.09	0.0339 ± 0.0034	1886 ± 118
Post-natal hypoxia	855 ± 37	1.53 ± 0.06	0.0298 ± 0.0018	1790 ± 39
Pre-natal hypoxia	969 ± 75	1.76 ± 0.07	0.0297 ± 0.0028	1889 ± 130
Parental hypoxia	927 ± 29	1.60 ± 0.07	0.0271 ± 0.0011	1735 ± 70
		Griffonia simplicifolia lectin I		
Normoxia	980 ± 27	1.58 ± 0.03	0.0261 ± 0.0016	
Adult hypoxia	959 ± 65	1.75 ± 0.09	0.0305 ± 0.0016	
Post-natal hypoxia	924 ± 27	1.57 ± 0.06	0.0315 ± 0.0013	
Pre-natal hypoxia	988 ± 107	1.78 ± 0.06	0.0317 ± 0.0035	
Parental hypoxia	920 ± 30	1.58 ± 0.06	0.0295 ± 0.0019	
		ANOVA results		
Treatment effect	$F_{4,30} = 0.505$	$F_{4,30} = 3.29$	$F_{4,30} = 1.37$	$F_{4,30} = 1.51$
	$P = 0.733$	$P = 0.0238$	$P = 0.269$	$P = 0.226$
Method effect	$F_{1,30} = 2.13$	$F_{1,30} = 0.489$	$F_{1,30} = 0.110$	
	$P = 0.880$	$P = 0.490$	$P = 0.743$	

Data are reported as means ± s.e.m. Capillarity was assessed using two different histological markers of capillaries, alkaline phosphatase activity and binding of *Griffonia simplicifolia* lectin I. The main effects of treatment and histological staining method on capillarity indices were assessed using two-factor ANOVA, and the main effect of treatment on fibre size was assessed using one-factor ANOVA (N as in Table 1). There were no significant pairwise differences between treatment groups.

Figure Legends

Fig. 1. Experimental treatment groups used to evaluate the effects of chronic hypoxia at different life stages in deer mice native to high altitudes. We used two breeding pairs of captive mice that were born of wild parents from Mount Evans, CO, USA at ~4,300 m above sea level. The two resulting families of progeny, the second generation raised in the lab, were used in experiments.

Fig. 2. Analysis of capillarity in the gastrocnemius muscle of deer mice using two histological staining methods. There was strong similarity in the pattern of capillary staining using alkaline phosphatase activity (A,C) and *Griffonia simplicifolia* lectin I (GSL) (B,D) as markers. The muscle from a mouse in the parental hypoxia group is shown, with serial sections stained for each marker in overlapping regions of muscle within (A,B) and outside (C,D) the oxidative core (similar letters represent serial sections of the same muscle fibres between the two markers). Scale bar represents 200 μm.

Fig. 3. Correlation between capillarity indices measured using alkaline phosphatase (AP) activity and *Griffonia simplicifolia* lectin I (GSL) as markers. Solid and dashed black lines represent the linear regression and 95% confidence intervals between the two stains for (A) capillary density (R^2=0.591), (B) capillary to fibre ratio (R^2=0.647), and (C) capillary surface density (R^2=0.317). Dotted grey lines represent equality. Symbols for treatment groups are as follows: normoxic controls, grey triangles; adult hypoxia, white squares; post-natal hypoxia, black triangles; pre-natal hypoxia, white diamonds; parental hypoxia, grey circles.

Fig. 4. Maximal activities of metabolic enzymes in the gastrocnemius muscle. High-altitude deer mice were exposed to one of five treatments: normoxia (N); adult hypoxia (AH); post-natal hypoxia (NH); pre-natal hypoxia (PNH); or parental hypoxia (PH) (see Fig. 1 and Materials and Methods for details). There was a significant overall effect of treatment in one-factor ANOVA on (A) cytochrome c oxidase (COX) activity ($F_{4,30}$ = 4.04, P = 0.010), but not on (B) citrate synthase (CS) activity ($F_{4,30}$ = 0.620, P = 0.076), (C) isocitrate dehydrogenase (IDH) activity ($F_{4,30}$ = 0.965, P = 0.441), or (D) lactate dehydrogenase activity ($F_{4,30}$ = 0.372, P = 0.827). There were no significant pairwise differences between treatment groups for any enzyme. Maximal

enzyme activities are reported as means ± s.e.m. (N as in Table 1) in units of μmol substrate per g tissue per min.

Fig. 1.

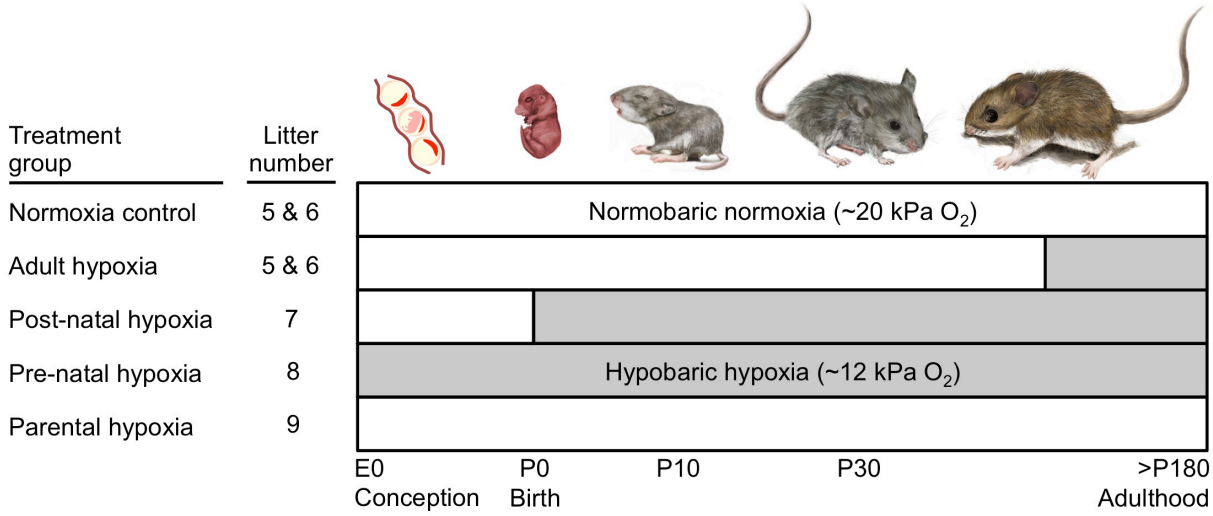

Treatment group
Litter number
Normoxia control
5 & 6
Adult hypoxia
5 & 6
Post-natal hypoxia
7
Pre-natal hypoxia
8
Parental hypoxia
9
Normobaric normoxia (~20 kPa O_2)
Hypobaric hypoxia (~12 kPa O_2)
E0
Conception
P0
Birth
P10
P30
>P180
Adulthood

Fig. 2.

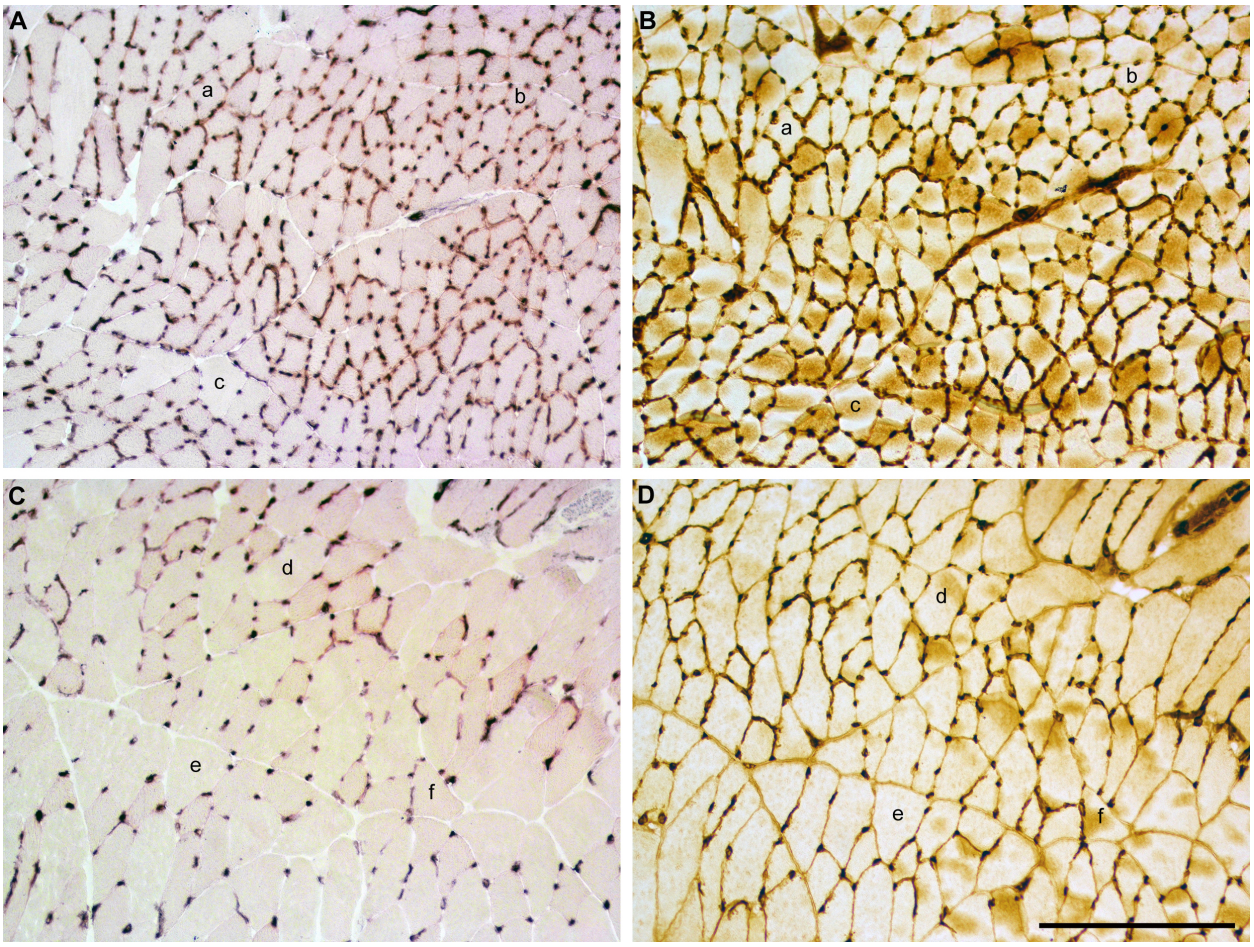


Fig. 3.

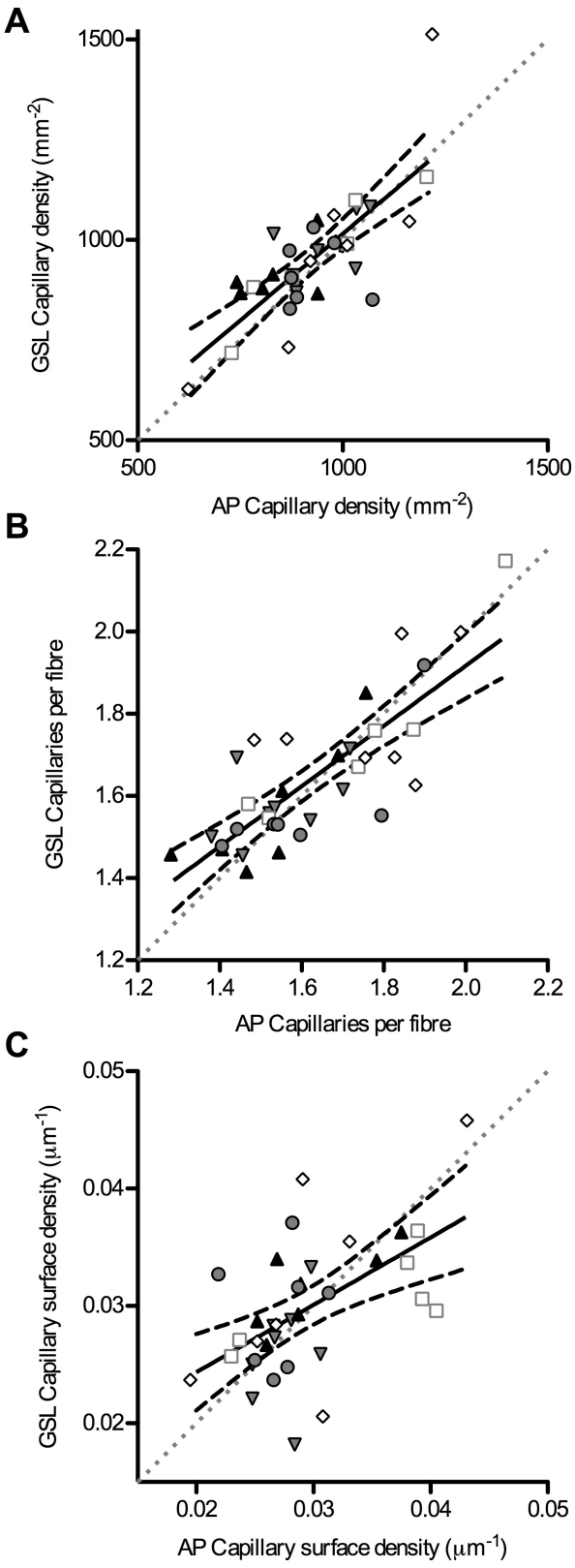
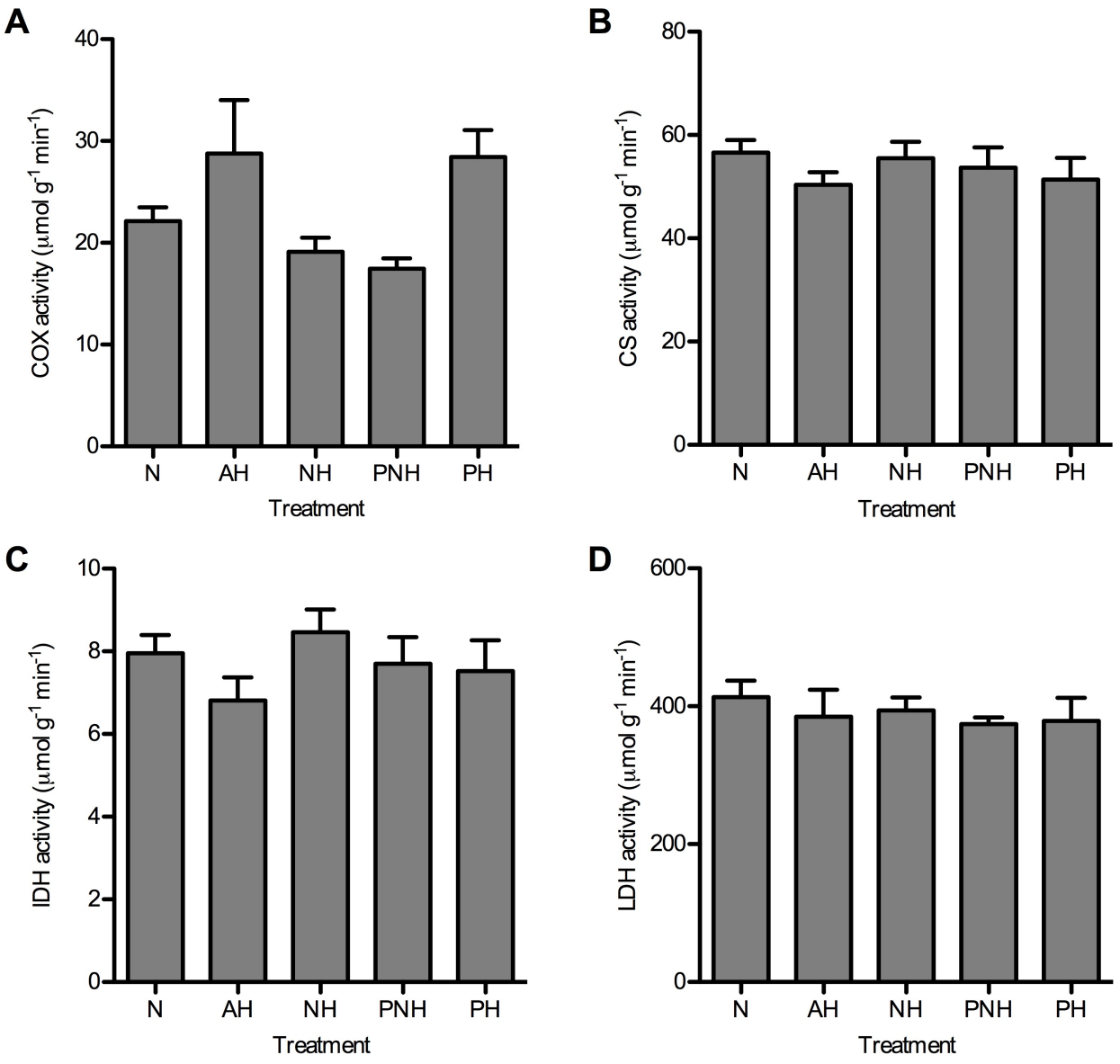

A
1500
1000
500
GSL Capillary density (mm-2)
500
1000
1500
AP Capillary density (mm-2)
B
2.2
2.0
1.8
1.6
1.4
1.2
GSL Capillaries per fibre
1.2
1.4
1.6
1.8
2.0
2.2
AP Capillaries per fibre
C
0.05
0.04
0.03
0.02
GSL Capillary surface density (μm-1)
0.02
0.03
0.04
0.05
AP Capillary surface density (μm-1)

Fig. 4.


A
COX activity (μmol g-1 min-1)
40
30
20
10
0
N
AH
NH
PNH
PH
Treatment
B
CS activity (μmol g-1 min-1)
80
60
40
20
0
N
AH
NH
PNH
PH
Treatment
C
IDH activity (μmol g-1 min-1)
10
8
6
4
2
0
N
AH
NH
PNH
PH
Treatment
D
LDH activity (μmol g-1 min-1)
600
400
200
0
N
AH
NH
PNH
PH
Treatment