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SHORT COMMUNICATION

Circulatory mechanisms underlying adaptive increases in thermogenic capacity in high-altitude deer mice

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ABSTRACT

We examined the circulatory mechanisms underlying adaptive increases in thermogenic capacity in deer mice (*Peromyscus maniculatus*) native to the cold hypoxic environment at high altitudes. Deer mice from high- and low-altitude populations were born and raised in captivity to adulthood, and then acclimated to normoxia or hypobaric hypoxia (simulating hypoxia at ∼4300 m). Thermogenic capacity [maximal O_2 consumption ($\dot{V}_{O_2,max}$), during cold exposure] was measured in hypoxia, along with arterial O_2 saturation (Sa_{O_2}) and heart rate (f_H). Hypoxia acclimation increased $\dot{V}_{O_2,max}$ by a greater magnitude in highlanders than in lowlanders. Highlanders also had higher Sa_{O_2} and extracted more O_2 from the blood per heartbeat (O_2 pulse=$\dot{V}_{O_2,max}/f_H$). Hypoxia acclimation increased f_H, O_2 pulse and capillary density in the left ventricle of the heart. Our results suggest that adaptive increases in thermogenic capacity involve integrated functional changes across the O_2 cascade that augment O_2 circulation and extraction from the blood.

KEY WORDS: Evolutionary physiology, High-altitude adaptation, Respiration, O_2 transport pathway, Aerobic performance

INTRODUCTION

High-altitude natives are valuable model organisms for understanding how physiological systems evolve. The cold and oxygen-depleted (hypoxic) environment at high altitudes requires that endotherms sustain high rates of O_2 consumption for thermogenesis and locomotion while facing a diminished O_2 supply. Growing evidence suggests that high-altitude natives have overcome this challenge through evolved changes in the physiological systems underlying O_2 transport and utilization (Monge and León-Velarde, 1991; Storz et al., 2010b; Scott, 2011; Ivy and Scott, 2015). Studies of high-altitude natives aimed at understanding the evolution of the O_2 transport cascade – composed of ventilation, pulmonary diffusion, circulation, tissue diffusion and cellular O_2 utilization – are therefore extremely valuable for explaining the physiological mechanisms of evolutionary adaptation.

North American deer mice [*Peromyscus maniculatus* (Wagner 1845)] are an excellent model species for studies of high-altitude adaptation. Their native altitudinal range extends from below sea level in Death Valley, CA, USA, to over 4300 m above sea level in the Rocky Mountains (Hock, 1964; Snyder et al., 1982; Natarajan et al., 2015). High-altitude populations must sustain high metabolic rates in the wild (Hayes, 1989b), and there appears to be strong directional selection on thermogenic capacity [maximal O_2 consumption ($\dot{V}_{O_2,max}$) during cold exposure] to support heat generation in cold alpine environments (Hayes and O'Connor, 1999). In response to this strong selection pressure, high-altitude deer mice have evolved a higher $\dot{V}_{O_2,max}$ in hypoxia than low-altitude populations of deer mice and a congeneric lowland species (white-footed mice, *P. leucopus*) (Cheviron et al., 2012, 2013; Lui et al., 2015). This evidence suggests that highland deer mice have evolved an adaptive increase in thermogenic capacity in hypoxia.

The physiological mechanisms underlying this evolved increase in thermogenic capacity have yet to be fully explained. High-altitude deer mice have evolved a high blood–O_2 affinity compared with their lowland counterparts that contributes to increasing $\dot{V}_{O_2,max}$ in hypoxia (Snyder et al., 1982; Chappell and Snyder, 1984; Storz et al., 2010a; Natarajan et al., 2013), but it is unclear whether this adaptation improves O_2 uptake into the blood *in vivo*. High-altitude deer mice have also evolved a more oxidative and richly vascularized phenotype of the skeletal muscle (used for shivering and locomotion), in association with differential expression of genes involved in aerobic energy metabolism and angiogenesis (Cheviron et al., 2012, 2014; Lui et al., 2015; Scott et al., 2015; Lau et al., 2017; Mahalingam et al., 2017). Development and acclimatization to cold and/or hypoxia are also known to affect $\dot{V}_{O_2,max}$, cardiopulmonary organ sizes and the capacity for non-shivering thermogenesis in deer mice (Hammond et al., 2001, 2002; Chappell and Hammond, 2004; Shirkey and Hammond, 2014; Velotta et al., 2016). However, we know very little about *in vivo* cardiorespiratory function at $\dot{V}_{O_2,max}$ in this species. This study therefore aims to examine the contribution of differences in arterial O_2 saturation and some other aspects of circulatory function to adaptive increases in thermogenic capacity in high-altitude deer mice.

MATERIALS AND METHODS

Animals and acclimation treatments

Captive breeding populations were established from wild deer mouse populations native to high altitude (near the summit of Mount Evans, CO, USA, 39°35′18″N, 105°38′38″W; 4350 m above sea level) (*P. m. rufinus*) and low altitude (Nine Mile Prairie, Lancaster County, NE, USA, 40°52′12″N, 96°48′20.3″W; 430 m above sea level) (*P. m. nebracensis*). Wild adults were transported to McMaster University (near sea level) and housed in common-garden conditions, and were bred within each population to produce laboratory-raised progeny. Mice were raised in standard holding conditions (24–25°C, 12 h:12 h light:dark photoperiod) 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.

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Adult mice were raised to ∼6 months of age, and a randomly selected group of individuals (mix of both sexes) from each population were acclimated to either (1) normobaria in standard normoxic conditions or (2) hypobaric hypoxia (barometric pressure of 60 kPa; equivalent to that at an elevation of ∼4300 m) in specially designed hypobaric chambers that have been described previously (Lui et al., 2015). Cages were cleaned twice a week during acclimations, which required that the hypobaric groups be returned to normobaria for a brief period (<30 min). Mice were subjected to subsequent measurements after 6–8 weeks of acclimation.

Respirometry and pulse oximetry

We measured thermogenic capacity in hypoxia in second-generation (F_2) mice from high-altitude and low-altitude populations. Maximal rates of O_2 consumption ($\dot{V}_{O_2,max}$) were measured during acute cold exposure, using open-flow respirometry in a hypoxic heliox atmosphere (12% O_2, 88% He) at −5°C (Rosenmann and Morrison, 1974; Chappell and Hammond, 2004; Cheviron et al., 2012). Respirometry was carried out in a 0.5 l animal chamber that received a constant incurrent flow rate of 1000 ml min^{-1}, regulated using a mass flow controller (MFC-4, Sable Systems, Las Vegas, NV, USA) and a precision flow control valve that was factory calibrated for heliox (Sierra Instruments, Monterey, CA, USA). The chamber was held inside a freezer, in which the ambient temperature was regulated at or slightly below −5°C (measured with a thermocouple; PT-6, Physitemp, Clifton, NJ, USA), and the incurrent gas flowed through copper coils before entering the chamber. Excurrent gas was subsampled at 200 ml min^{-1}, dried with pre-baked Drierite, and analyzed for O_2 and CO_2 fractions (FoxBox Respirometry System, Sable Systems).

Respirometry experiments were carried out as follows. Baseline O_2 and CO_2 fractions were first measured without an animal in the chamber. Mice were instrumented with a collar sensor to measure heart rate (f_H) and the O_2 saturation of arterial blood (Sa_{O_2}) using a MouseOx Plus pulse oximeter (Starr Life Sciences, Oakmont, PA, USA), and were then transferred to the chamber. The pulse oximetry measurements required that hair be removed from around the neck, which was done 2 days before the experiments using Nair™ hair removal product. Incurrent gas flow rate, chamber temperature, and excurrent O_2 and CO_2 fractions were measured continuously and were acquired using a PowerLab 8/32 and LabChart 8 Pro software (ADInstruments, Colorado Springs, CO, USA). Pulse oximetry measurements were recorded using Starr Life Sciences acquisition software. Rates of O_2 consumption ($\dot{V}_{O_2}$) were calculated using established formulas (Lighton, 2008) and $\dot{V}_{O_2,max}$ was defined as the highest $\dot{V}_{O_2}$ achieved over a 30 s period during the trial, which generally occurred after ∼4–6 min in the chamber, when maximal values of Sa_{O_2} and f_H were also determined. Measurements of core body temperature were made using a rectal probe (RET-3-ISO, Physitemp) immediately after removing the animal from the chamber (after ∼10–12 min in the chamber), and confirmed that all mice were hypothermic at the end of the experiment.

Cardiac histology

Capillarity was measured histologically in the left ventricle of the heart in a separate group of F_1 mice from highland and lowland populations. Mice were euthanized with an overdose of isoflurane followed by cervical dislocation. The ventricles were removed, coated in embedding medium, frozen in liquid N_2-cooled isopentane and stored at −80°C. Tissue was sectioned (10 µm) perpendicular to the long axis of the heart in a cryostat at −20°C. Capillaries were identified by staining for alkaline phosphatase activity for 1 h at room temperature (assay buffer concentrations in mmol l^{-1}: 1.0 nitroblue tetrazolium, 0.5 5-bromo-4-chloro-3-indoxyl phosphate, 28 $NaBO_2$ and 7 $MgSO_4$; pH 9.3). Images were collected systematically using light microscopy, such that there was equal representation of images from across the left ventricle. A blind observer determined the average value of capillary density for each individual.

Statistics

Two-factor ANOVA was generally used to assess the main effects of population altitude and acclimation environment (interactions were also assessed, but were not generally significant and are not reported). Data for $\dot{V}_{O_2,max}$ and the amount of O_2 extracted from the blood per heartbeat (the quotient of $\dot{V}_{O_2}$ and f_H; also called the O_2 pulse) were first corrected for body mass (M_b) before making statistical comparisons. This was accomplished by carrying out least-squares regressions to the equation $Y=aM_b^b$ (using GraphPad Prism software, La Jolla, CA, USA), including all of the data across all groups, and then calculating the residual from the regression for each individual. These residuals were then used in two-factor ANOVA, and are reported graphically on the right *y*-axis. The scale of the left *y*-axis for graphs of our $\dot{V}_{O_2,max}$ and O_2 pulse data shows the sum of the residual and the expected value for an average-sized 21.6-g mouse (i.e. $\dot{V}_{O_2,max}$ or O_2 pulse data corrected to a body mass of 21.6 g). We also performed a supplementary statistical analysis of the effects of body mass, population altitude and acclimation environment on $\dot{V}_{O_2,max}$ and O_2 pulse using linear models (lm) in R (R Core Team, 2016), in which body mass and the variable of interest were log-transformed before making statistical comparisons (the statistical results are extremely similar to those obtained with two-factor ANOVA, and are shown in Table S1). Data are generally reported as means±s.e.m. (except when data points from individual samples are shown). $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Thermogenic capacity in hypoxia is enhanced in high-altitude deer mice

Cold-induced $\dot{V}_{O_2,max}$ (measured in hypoxia) was greatest in high-altitude deer mice, as reflected by a significant population effect in two-factor ANOVA (Fig. 1A). This was particularly apparent after hypoxia acclimation, when $\dot{V}_{O_2,max}$ was 16% higher on average in highlanders than in lowlanders. Because we observed allometric rather than isometric scaling of $\dot{V}_{O_2,max}$ to body mass, we used a residual-based approach to correct for body mass before making these comparisons (Fig. 1B), but we obtained very similar statistical results using linear model statistics (Table S1). Body mass was similar between populations ($F_{1,36}$=1.08, P=0.31) and between acclimation environments ($F_{1,36}$=0.49, P=0.49) (normoxic highlanders, 22.9±1.1 g; hypoxic highlanders, 21.0±0.6 g; normoxic lowlanders, 20.6±0.8 g; hypoxic lowlanders, 20.9±1.3 g).

Our results are consistent with previous findings in deer mice and other high-altitude taxa. The increases in cold- and exercise-induced $\dot{V}_{O_2,max}$ in highland deer mice observed by us and others appear to be greatest in hypoxic conditions, and are not as large in normoxic conditions at sea level, suggesting that highlanders are more resistant to the depressing effects of hypoxia on O_2 transport (Chappell and Snyder, 1984; Hayes, 1989a; Cheviron et al., 2012, 2013; Lui et al., 2015). Similar differences exist in Andean and Tibetan human populations, in which exercise-induced $\dot{V}_{O_2,max}$ is only elevated compared with lowland humans when tested at altitudes above ∼2500 m (Brutsaert, 2016). However, in many of these human studies, it has been hard to distinguish evolved genetic effects from effects of developmental environment and exercise training. Although hypoxia

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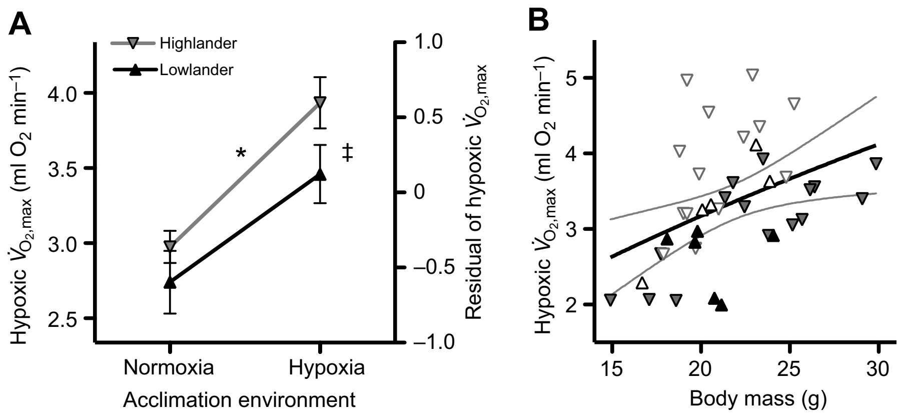

Fig. 1. Thermogenic capacity, measured in hypoxia as the maximal rate of O_2 consumption ($\dot{V}_{O_2,max}$) during acute cold exposure, was enhanced in high-altitude deer mice. The effects of population altitude and hypoxia acclimation on hypoxic $\dot{V}_{O_2,max}$ were assessed by calculating the residuals (A) for an allometric regression of hypoxic $\dot{V}_{O_2,max}$ to body mass (M_b; $\dot{V}_{O_2,max}=0.46M_b^{0.65}$) (B). (A) The left axis shows the hypoxic $\dot{V}_{O_2,max}$ for an average-sized 21.6-g mouse, calculated for each group by adding the residual (shown on the right axis) to the $\dot{V}_{O_2,max}$ predicted at 21.6 g by the regression (means±s.e.m.). There were significant main effects of population ($F_{1,36}$=4.42, *P=0.043) and hypoxia acclimation ($F_{1,36}$=18.25, ‡P<0.001). (B) Grey lines represent 95% confidence intervals of the allometric regression (▽, normoxic highlanders, n=15; ▿, hypoxic highlanders, n=14; ▲, normoxic lowlanders, n=6; △, hypoxic lowlanders, n=5).

exposure during development also has a strong influence on $\dot{V}_{O_2,max}$ in deer mice, directional selection on $\dot{V}_{O_2,max}$ at high altitudes appears to have further increased $\dot{V}_{O_2,max}$ in high-altitude populations (Fig. 1) (Hayes and O'Connor, 1999; Chappell et al., 2007; Russell et al., 2008; Cheviron et al., 2013; Lui et al., 2015).

High-altitude deer mice maintain higher arterial O_2 saturation in hypoxia

Arterial O_2 saturation was ∼6–8% higher in highlanders than in lowlanders at $\dot{V}_{O_2,max}$ in hypoxia (Fig. 2A). This observation likely results at least in part from the greater blood– and haemoglobin–O_2 affinities of highlanders (Snyder et al., 1982; Storz et al., 2010a), which would increase Sa_{O_2} at similar conditions of blood O_2 and CO_2 tensions and pH. This observation could also stem from population differences in arterial O_2 tension, arising from differences in pulmonary ventilation or O_2 diffusion. Breathing and pulmonary O_2 extraction have yet to be examined in deer mice at $\dot{V}_{O_2,max}$, but there appear to be evolved differences in control of breathing by hypoxia under routine conditions in highland deer mice (Ivy and Scott, 2017).

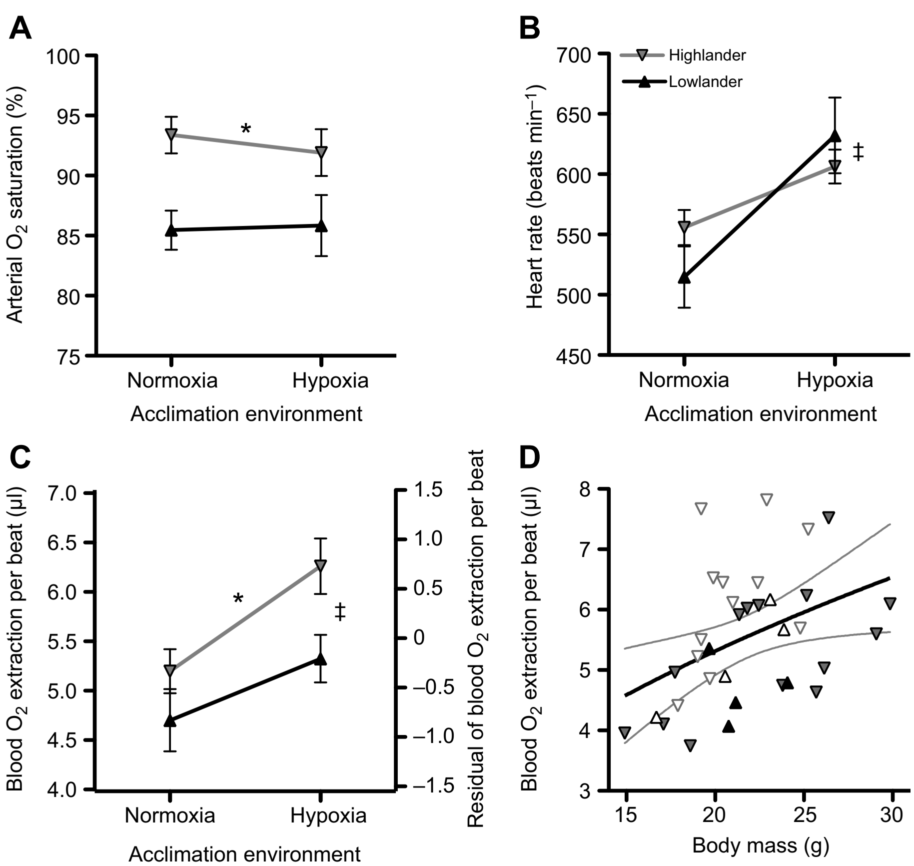

Fig. 2. Population altitude and hypoxia acclimation affect circulatory O_2 delivery at hypoxic $\dot{V}_{O_2,max}$ in deer mice. (A) Arterial O_2 saturation was higher in highland mice ($F_{1,24}$=10.46, *P=0.004) but was unaffected by hypoxia acclimation ($F_{1,24}$=0.063, P=0.803). (B) Heart rate at $\dot{V}_{O_2,max}$ increased after hypoxia acclimation ($F_{1,30}$=15.48, ‡P<0.001) but was similar between populations ($F_{1,30}$=0.129, P=0.722). The amount of O_2 extracted from the blood per heartbeat ('O_2 pulse', quotient of $\dot{V}_{O_2}$ and heart rate), assessed by calculating the residuals (C) for an allometric regression to body mass (M_b; O_2 pulse=$1.15M_b^{0.51}$; D), was greater in highland mice ($F_{1,30}$=4.41, *P=0.044) and increased after hypoxia acclimation ($F_{1,30}$=6.10, ‡P=0.019) (see Fig. 1 and Materials and methods for additional details on this approach). Grey lines in D represent 95% confidence intervals of the allometric regression (▽, normoxic highlanders, n=14; ▿, hypoxic highlanders, n=12; ▲, normoxic lowlanders, n=4; △, hypoxic lowlanders, n=4). Data are means±s.e.m., except in D, where data from individuals are shown.

Sa_{O_2} was unaffected by hypoxia acclimation (Fig. 2A), and was not always associated with clear population differences in $\dot{V}_{O_2,max}$ (Fig. 1). Previous studies using wild-derived strains of deer mice with distinct α-globin haplotypes (on randomized genetic backgrounds) have shown that variation in blood–O_2 affinity affects $\dot{V}_{O_2,max}$, such that mice with higher affinity (typical of highland populations) had the highest $\dot{V}_{O_2,max}$ when acclimated and tested at high altitude (Chappell and Snyder, 1984; Chappell et al., 1988). This relationship is presumed to arise from a positive association between blood–O_2 affinity and Sa_{O_2} in hypoxia, but this has not been tested. Here, the higher Sa_{O_2} in highlanders compared with lowlanders only appears to be associated with increases in $\dot{V}_{O_2,max}$ when mice were acclimated and tested in hypoxia (Fig. 1). However, in normoxia-acclimated mice, highlanders had higher Sa_{O_2} without any clear difference in hypoxic $\dot{V}_{O_2,max}$. This suggests that the influence of Sa_{O_2} on $\dot{V}_{O_2,max}$ may be context dependent, such that the relative benefit of increases in Sa_{O_2} may depend upon interactions with other respiratory traits that change after hypoxia acclimation.

Differences in cardiac performance appear to underlie differences in thermogenic capacity

Heart rates (f_H) during $\dot{V}_{O_2,max}$ in hypoxia were ∼9–23% higher after hypoxia acclimation (Fig. 2B). The amount of O_2 extracted from the blood per heartbeat (‘O_2 pulse’, quotient of $\dot{V}_{O_2}$ and f_H) increased by ∼25–32% after hypoxia acclimation, and was 10–16% greater in the highland population (Fig. 2C, Table S1). The latter observation suggests that cardiac stroke volume (V_S) and/or the absolute O_2 extraction from the blood (Ca_{O_2}–Cv_{O_2}) contributes to the variation in $\dot{V}_{O_2,max}$. This is because all of the above variables are related by the Fick equation, $\dot{V}_{O_2}=f_H \times V_S \times (Ca_{O_2}-Cv_{O_2})$, such that O_2 pulse is equal to the product of stroke volume and blood O_2 extraction. This product must therefore be greater in highlanders and increase with hypoxia acclimation.

The observed difference in cardiac performance was likely associated with variation in O_2 supply to heart tissue. Hypoxia acclimation increased capillary density – a key determinant of O_2 diffusing capacity – by ∼10–12% in the left ventricle (Fig. 3). However, capillary densities were similar between highlanders and lowlanders, so this trait does not underlie population differences in cardiac performance. Nevertheless, it is likely that an interaction between the hypoxia-induced increase in heart capillarity and the population difference in Sa_{O_2} resulted in an improved O_2 supply to cardiac tissue, and may therefore account for the observed differences in cardiac performance and $\dot{V}_{O_2,max}$. High-altitude adaptation and/or hypoxia acclimation could have also improved the heart’s ability to maintain cardiac output during tissue hypoxia. In support of this possibility, some other high-altitude taxa exhibit differences in mitochondrial physiology and metabolic capacity that could improve cardiac function at low O_2 tensions (Sheafor, 2003; Scott et al., 2011; Dawson et al., 2016).

The functional mechanisms of high-altitude adaptation span the O_2 cascade

A key goal of evolutionary physiology is to elucidate the mechanistic basis of adaptive variation in organismal performance (Garland and Carter, 1994; Dalziel et al., 2009). Thermogenesis is a key performance trait that is critical for fitness in endotherms at high altitudes (Hayes and O’Connor, 1999) and can push the respiratory system of many small mammals to its limits (Rosenmann and Morrison, 1974; Chappell and Hammond, 2004). Here, we contribute to the growing evidence suggesting that adaptive increases in thermogenic capacity involve integrated functional changes across the O_2 cascade. $\dot{V}_{O_2,max}$ in hypoxia appears to be enhanced in high-altitude deer mice (Fig. 1) via increases in pulmonary O_2 uptake (Fig. 2A), haemoglobin–O_2 affinity (Snyder et al., 1982; Chappell and Snyder, 1984; Storz et al., 2010a; Natarajan et al., 2013), cardiac performance and/or blood O_2 extraction (Fig. 2C), and the capacity for O_2 diffusion and utilization in skeletal muscle (Cheviron et al., 2012, 2014; Lui et al., 2015; Scott et al., 2015; Mahalingam et al., 2017). Therefore, the concerted evolution of physiological systems underlying O_2 transport and utilization appear to be critical to the process of high-altitude adaptation.

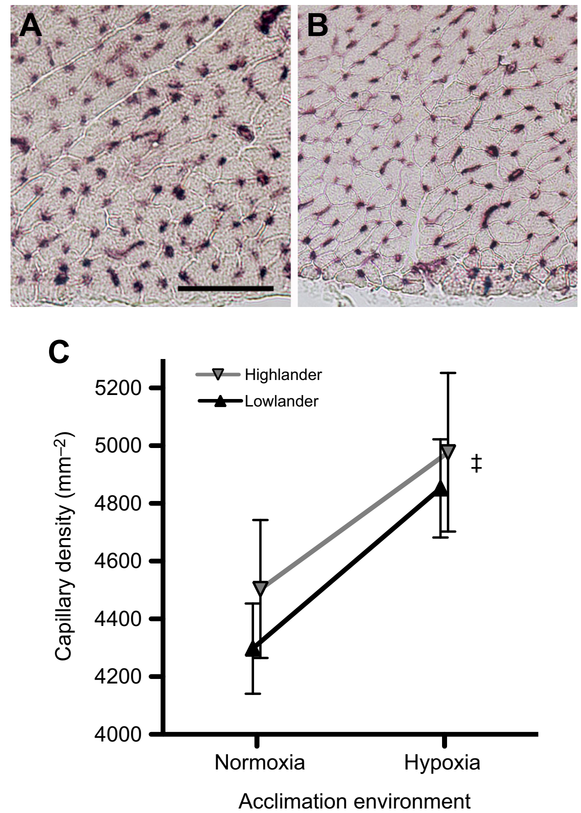

Fig. 3. Capillarity of the heart tissue increased after hypoxia acclimation. (A,B) Representative images of left ventricle tissue near the epicardium, stained for alkaline phosphatase activity to identify capillaries, in normoxia-acclimated deer mice from the low-altitude (A) and high-altitude (B) populations (scale bar, 50 μm). (C) There was a significant main effect of hypoxia acclimation on capillary density ($F_{1,30}$=5.53, ‡P=0.026), but no difference between populations ($F_{1,30}$=0.57, P=0.455). Data are means± s.e.m., with sample sizes as follows: normoxic highlanders, n=9; hypoxic highlanders, n=8; normoxic lowlanders, n=7; hypoxic lowlanders, n=10.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: G.R.S.; Methodology: K.B.T., G.R.S.; Formal analysis: K.B.T.; Investigation: K.B.T., C.M.I., J.P.V., G.R.S.; Writing - original draft: K.B.T., G.R.S.; Writing - review & editing: K.B.T., C.M.I., J.P.V., J.F.S., G.B.M., Z.A.C., G.R.S.; Supervision: J.F.S., G.B.M., Z.A.C., G.R.S.; Funding acquisition: J.F.S., Z.A.C., G.R.S.

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Supplementary information

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Table S1. Statistical results from linear models used to examine the effects of body mass, population altitude, and acclimation environment on VO_2max and O_2 pulse

Source	df	F	Probability > F
VO_2max			
Mass	1	21.6	<0.001
Population altitude	1	4.22	0.048
Acclimation environment	1	27.8	<0.001
Altitude × acclimation	1	0.218	0.644
Residual	35		
O_2 pulse			
Mass	1	13.0	0.001
Population altitude	1	3.87	0.059
Acclimation environment	1	12.2	0.002
Altitude × acclimation	1	0.374	0.546
Residual	29		

df, degrees of freedom; O_2 pulse, the amount of O_2 extracted from the blood per heartbeat (the quotient of VO_2 and f_H); VO_2max, maximal O_2 consumption measured during cold exposure in a hypoxic heliox atmosphere (12% O_2, 88% He).

Body mass (Mb) and the variable of interest were log-transformed before making statistical comparisons, which were carried out using linear models (lm) in R (LogVariable ~ LogMb + Altitude + Acclimation + Altitude×Acclimation).