

## 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<sub>2</sub> consumption ( $\dot{V}_{O_{2,max}}$ ), during cold exposure] was measured in hypoxia, along with arterial O<sub>2</sub> saturation (Sa<sub>O<sub>2</sub></sub>) 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<sub>O<sub>2</sub></sub> and extracted more O<sub>2</sub> from the blood per heartbeat (O<sub>2</sub> pulse= $\dot{V}_{O_{2,max}}/f_H$ ). Hypoxia acclimation increased  $f_H$ , O<sub>2</sub> 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<sub>2</sub> cascade that augment O<sub>2</sub> circulation and extraction from the blood.

**KEY WORDS:** Evolutionary physiology, High-altitude adaptation, Respiration, O<sub>2</sub> 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<sub>2</sub> consumption for thermogenesis and locomotion while facing a diminished O<sub>2</sub> supply. Growing evidence suggests that high-altitude natives have overcome this challenge through evolved changes in the physiological systems underlying O<sub>2</sub> 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<sub>2</sub> transport cascade – composed of ventilation, pulmonary diffusion, circulation, tissue diffusion and cellular O<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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  $-5^\circ\text{C}$  (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  $1000 \text{ ml min}^{-1}$ , 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  $-5^\circ\text{C}$  (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  $200 \text{ ml min}^{-1}$ , 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  $-80^\circ\text{C}$ . Tissue was sectioned ( $10 \mu\text{m}$ ) perpendicular to the long axis of the heart in a cryostat at  $-20^\circ\text{C}$ . Capillaries were identified by staining for alkaline phosphatase activity for 1 h at room

temperature (assay buffer concentrations in  $\text{mmol l}^{-1}$ : 1.0 nitroblue tetrazolium, 0.5 5-bromo-4-chloro-3-indoxyl phosphate, 28  $\text{NaBO}_2$  and 7  $\text{MgSO}_4$ ; 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

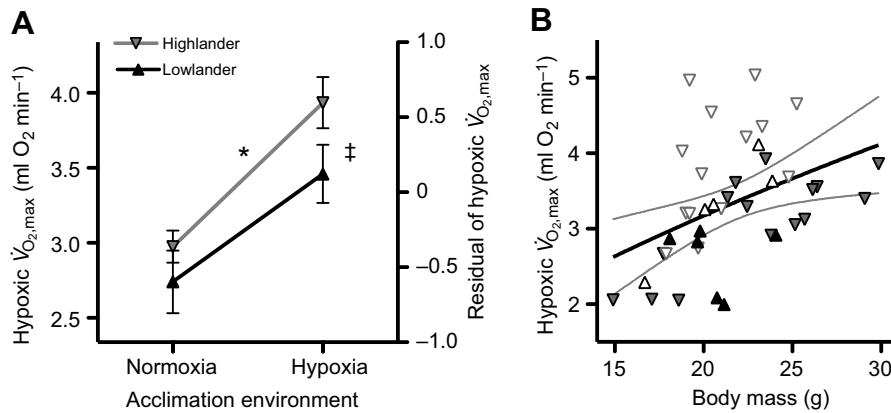
Two-factor ANOVA was generally used to assess the main effects of population altitude and acclimation environment (interactions were also assessed, but were not generally significant and are not reported). Data for  $\dot{V}_{O_2, \max}$  and the amount of  $O_2$  extracted from the blood per heartbeat (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  $\pm$  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,  $22.9 \pm 1.1$  g; hypoxic highlanders,  $21.0 \pm 0.6$  g; normoxic lowlanders,  $20.6 \pm 0.8$  g; hypoxic lowlanders,  $20.9 \pm 1.3$  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



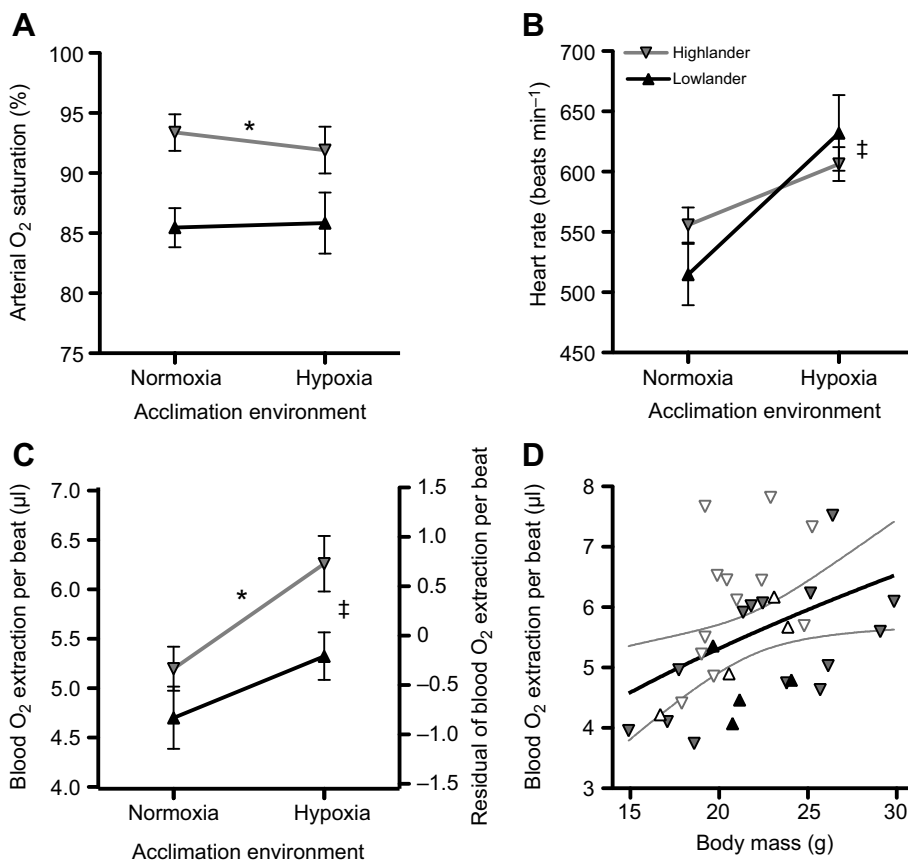
**Fig. 1. Thermogenic capacity, measured in hypoxia as the maximal rate of O<sub>2</sub> consumption ( $\dot{V}_{O_{2,max}}$ ) during acute cold exposure, was enhanced in high-altitude deer mice.** The effects of population altitude and hypoxia acclimation on hypoxic  $\dot{V}_{O_{2,max}}$  were assessed 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 $\pm$ s.e.m.). There were significant main effects of population ( $F_{1,36}=4.42$ ,  $*P=0.043$ ) and hypoxia acclimation ( $F_{1,36}=18.25$ ,  $\dagger P<0.001$ ). (B) Grey lines represent 95% confidence intervals of the allometric regression ( $\nabla$ , normoxic highlanders,  $n=15$ ;  $\nabla$ , hypoxic highlanders,  $n=14$ ;  $\blacktriangle$ , normoxic lowlanders,  $n=6$ ;  $\triangle$ , 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<sub>2</sub> saturation in hypoxia**

Arterial O<sub>2</sub> 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<sub>2</sub> affinities of highlanders (Snyder et al., 1982; Storz et al., 2010a), which would increase SaO<sub>2</sub> at similar conditions of blood O<sub>2</sub> and CO<sub>2</sub> tensions and pH. This observation could also stem from population differences in arterial O<sub>2</sub> tension, arising from differences in pulmonary ventilation or O<sub>2</sub> diffusion. Breathing and pulmonary O<sub>2</sub> 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<sub>2</sub> delivery at hypoxic  $\dot{V}_{O_{2,max}}$  in deer mice.** (A) Arterial O<sub>2</sub> 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 ( $F_{1,30}=15.48$ ,  $\dagger P<0.001$ ) but was similar between populations ( $F_{1,30}=0.129$ ,  $P=0.722$ ). The amount of O<sub>2</sub> extracted from the blood per heartbeat ('O<sub>2</sub> 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<sub>2</sub> 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 ( $F_{1,30}=6.10$ ,  $\dagger P=0.019$ ) (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 ( $\nabla$ , normoxic highlanders,  $n=14$ ;  $\nabla$ , hypoxic highlanders,  $n=12$ ;  $\blacktriangle$ , normoxic lowlanders,  $n=4$ ;  $\triangle$ , hypoxic lowlanders,  $n=4$ ). Data are means $\pm$ 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  $\alpha$ -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.

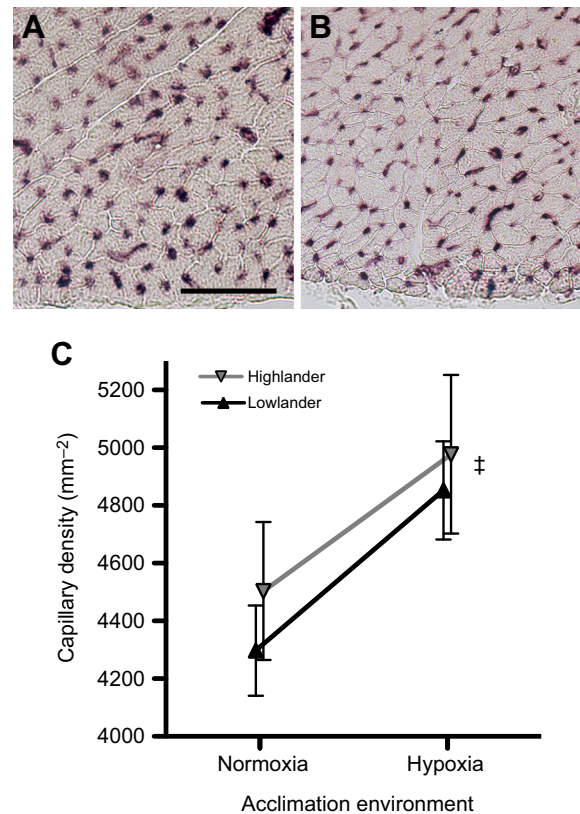
### Differences in cardiac performance appear to underlie differences in thermogenic capacity

Heart rates ( $f_H$ ) during  $\dot{V}_{O_{2,max}}$  in hypoxia were  $\sim 9$ – $23\%$  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  $\sim 25$ – $32\%$  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  $\sim 10$ – $12\%$  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



**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  $\mu$ m). (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  $\pm$  s.e.m., with sample sizes as follows: normoxic highlanders,  $n=9$ ; hypoxic highlanders,  $n=8$ ; normoxic lowlanders,  $n=7$ ; hypoxic lowlanders,  $n=10$ .

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.

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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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