Coordinated Changes Across the O₂ Transport Pathway Underlie Adaptive Increases in Thermogenic Capacity in High-Altitude Deer Mice

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Abstract

Animals native to the hypoxic and cold environment at high altitude provide an excellent opportunity to elucidate the integrative mechanisms underlying the adaptive evolution and plasticity of complex traits. The capacity for aerobic thermogenesis can be a critical determinant of survival for small mammals at high altitude, but the physiological mechanisms underlying the evolution of this performance trait remain unresolved. We examined this issue by comparing high-altitude deer mice (*Peromyscus maniculatus*) to low-altitude deer mice and white-footed mice (*P. leucopus*). Mice were bred in captivity and adults were acclimated to each of four treatments: warm (25°C) normoxia; warm hypoxia (12 kPa O₂); cold (5°C) normoxia; or cold hypoxia. Acclimation to hypoxia and/or cold increased thermogenic capacity in deer mice, but hypoxia acclimation led to much greater increases in thermogenic capacity in highlanders than in lowlanders. The high thermogenic capacity of highlanders was associated with increases in pulmonary O₂ extraction, arterial O₂ saturation, cardiac output, and arterial-venous O₂ difference. Mechanisms underlying the evolution of enhanced thermogenic capacity in highlanders were partially distinct from those underlying the ancestral acclimation responses of lowlanders. Environmental adaptation has thus enhanced phenotypic plasticity and expanded the physiological toolkit for coping with the challenges at high altitude.
1. Introduction

Explaining the mechanistic basis for adaptive variation in organismal performance is a central and long-standing goal of evolutionary physiology [1, 2]. Organismal performance traits are complex phenotypes supported by the coordinated function of various organ systems. The capacity for sustained aerobic exercise, for example, is supported by systems for partitioning O2 and metabolic fuels to the mitochondria in active locomotory muscles [3, 4]. Studies of the O2 transport pathway, the conceptual steps involved in O2 transport from the environment to O2 utilization by mitochondria (ventilation, pulmonary diffusion, circulation, tissue diffusion, and mitochondrial O2 utilization), have been particularly useful for understanding the systems-level mechanisms underlying the evolution of aerobic exercise performance. Some comparative studies have suggested that variation in aerobic exercise capacity – reflected by the maximal rate of O2 consumption (\(\dot{V}_{\text{O}_2\text{max}}\)) that can be achieved during exercise – is underpinned by matching variation in the capacity of multiple steps in the O2 pathway [5, 6]. Such observations led to the concept of symmorphosis, which proposed that structural design is optimized to match but not exceed functional demands, such that each step in the O2 pathway has an equivalent capacity to support O2 flux [5, 6]. However, results from artificial selection experiments [7-9] and theoretical modelling of the respiratory system [10, 11] have suggested that evolved changes in exercise \(\dot{V}_{\text{O}_2\text{max}}\) do not require matched changes across the O2 pathway, and can arise from changes in just a single step. The truth may lie somewhere between these two extremes, but there have been relatively few comparative studies of the evolution of \(\dot{V}_{\text{O}_2\text{max}}\) that have fully investigated the function of the O2 transport pathway.

Thermogenic capacity is another vital organismal performance trait that can push the limits of aerobic metabolism. Often measured as the \(\dot{V}_{\text{O}_2\text{max}}\) during acute cold exposure, values of thermogenic \(\dot{V}_{\text{O}_2\text{max}}\) can equal or exceed values of \(\dot{V}_{\text{O}_2\text{max}}\) during exercise in small mammals [12-14]. As a result, thermogenic capacity requires high rates of O2 flux through the O2 transport pathway, but unlike aerobic exercise, aerobic thermogenesis involves activity of both muscles (shivering thermogenesis) and adipose tissues (non-shivering thermogenesis). Thermogenic capacity can influence survival, fitness, and the ability of small mammals to stay active in the cold [15, 16]. However, the systems-level mechanisms underlying the evolution of thermogenic capacity are poorly understood.
Thermogenic capacity is also a plastic trait that can increase in response to chronic cold-exposure via metabolic adjustments in thermogenic tissues [17-21], so studies of this performance trait can provide general insight into the plasticity of complex phenotypes. Theory and empirical evidence show that phenotypic plasticity can facilitate the survival and reproductive success of initial colonizers of a novel environment, and plasticity can then evolve by various forms of genetic accommodation and move the colonizing population closer to the fitness optimum [22-25]. However, most previous studies of the evolution of plasticity in complex traits have not considered the tight functional integration that can exist between the component mechanisms that underlie such traits [26]. Studies of thermogenic capacity and its underlying physiological mechanisms have the potential to shed light on this issue, and to better understand the role of phenotypic plasticity in environmental adaptation.

Animals native to high-altitude environments provide an excellent opportunity to elucidate the mechanisms underlying the plasticity and adaptive evolution of thermogenic capacity. The cold and oxygen-depleted (hypoxic; low partial pressure of O$_2$, P$_{O_2}$) environment at high altitude requires that endotherms maintain high rates of O$_2$ transport and utilization for thermogenesis while facing a diminished O$_2$ availability. Growing evidence suggests that high-altitude natives overcome this challenge through evolved changes in various cardiorespiratory and metabolic phenotypes [4, 27, 28]. However, with the exception of high-altitude humans, who do not suffer the same thermoregulatory challenges as do small mammals in the cold, there have been few comparative studies that have fully investigated the function of the O$_2$ pathway at $\dot{V}_{O_2,max}$ in high-altitude natives [28, 29].

Deer mice (Peromyscus maniculatus) native to high elevation are a powerful model for understanding the physiological bases of phenotypic plasticity and local adaptation. This species can be found across a wide altitudinal range, from below sea level in Death Valley, CA, USA to over 4300 m above sea level in the Rocky Mountains [30, 31]. At high altitude, free-ranging deer mice sustain high metabolic rates [32] and there is evidence that increased thermogenic $\dot{V}_{O_2,max}$ improves survival [15], presumably as a result of the high demands for heat generation in cold alpine environments. Various studies suggest that high-altitude deer mice have responded to this strong selection pressure by evolving increases in $\dot{V}_{O_2,max}$ in hypoxia, based on comparisons to low-altitude populations of deer mice and white-footed mice (P. leucopus; a congener that is restricted to low altitudes) [33-35]. Highland deer mice have a particularly strong advantage in
hypoxic environments, because thermogenic $\dot{V}_{O_2\text{max}}$ (measured in hypoxia) increases by a much greater magnitude after hypoxia acclimation in highlanders than in lowlanders [36]. The functional changes in the $O_2$ transport pathway that underlie this evolved increase in thermogenic $\dot{V}_{O_2\text{max}}$ have yet to be fully explained. Furthermore, cold acclimation is known to increase thermogenic $\dot{V}_{O_2\text{max}}$ and cardiopulmonary organ sizes in deer mice [12, 20, 37], but it is unknown whether the acclimation response to cold or to the combination of cold and hypoxia has evolved in high-altitude populations. This study therefore aims to examine how acclimation to hypoxia and cold – alone or in combination – affects thermogenic $\dot{V}_{O_2\text{max}}$ in hypoxia, to determine whether high-altitude deer mice have evolved heightened acclimation responses, and to elucidate the functional changes in the $O_2$ pathway that contribute to enhancements of thermogenic $\dot{V}_{O_2\text{max}}$.

2. Methods

(a) Acclimation treatments

Captive breeding populations were established from wild deer mouse populations native to high altitude (near the summit of Mount Evans, CO, USA; 4350 m above sea level) and from wild populations of both deer mouse and white-footed mouse from low altitude (Nine Mile Prairie, Lancaster County, NE, USA; 430 m above sea level), as described previously [36, 38] and detailed in the Supplementary Methods (electronic supplementary material). First generation adult mice from each population were acclimated to each of four acclimation environments: (1) warm (25°C) normobaric normoxia (barometric pressure ~100 kPa, $P_{O_2}$ ~20 kPa); (2) warm (25°C) hypobaric hypoxia (barometric pressure of 60 kPa, $P_{O_2}$ of 12.5 kPa); (3) cold (5°C) normobaric normoxia; and (4) cold (5°C) hypobaric hypoxia. Routine husbandry and the use of hypobaric chambers to create hypoxia have been described previously [35, 39], and cold conditions were maintained in large environmental chambers with temperature control.

(b) Cardiorespiratory measurements at thermogenic $\dot{V}_{O_2\text{max}}$ in hypoxia

Thermogenic $\dot{V}_{O_2\text{max}}$ was measured after 6-8 weeks of acclimation using open-flow respirometry during exposure to a cold (-5°C) hypoxic heliox gas mixture (12% O$_2$, 88% He). We employed methods we have described previously [36, 38] and are detailed in the
Supplementary Methods. Concurrent measurements of breathing were made by plethysmography, and measurements of arterial O\textsubscript{2} saturation and heart rate were made using a MouseOx Plus pulse oximeter (Starr Life Sciences, PA, USA).

We subsequently cannulated a subset of these mice to sample and measure the O\textsubscript{2} content of mixed venous blood at thermogenic $\dot{V}_{O_2}^{\text{max}}$. These measurements were made on highland deer mice and lowland white-footed mice across all four acclimation environments, but could only be made on lowland deer mice in warm normoxia and cold hypoxia. After at least three days recovery in the appropriate acclimation environment from the initial $\dot{V}_{O_2}^{\text{max}}$ measurement, mice were surgically implanted with a central venous cannula using standard surgical procedures under sterile conditions (see Supplementary Methods, electronic supplementary material). This was achieved by occlusively cannulating the jugular vein using a microrenathane catheter, advancing the catheter into the central venous cavity, and externalizing it through the skin at the nape of the neck. Mice were recovered at room temperature for 4-6 h and then at their appropriate acclimation environment for at least 3 days. A second thermogenic $\dot{V}_{O_2}^{\text{max}}$ trial was then conducted under the same exposure conditions as the first, and a 50 µl sample of central venous blood was collected at $\dot{V}_{O_2}^{\text{max}}$ and immediately analyzed for total O\textsubscript{2} content at 37°C using the Tucker method [40]. A second venous blood sample was then collected for measurement of haemoglobin content using Drabkin’s Reagent (Sigma-Aldrich, Oakville, ON, Canada). The $\dot{V}_{O_2}^{\text{max}}$ values from the second $\dot{V}_{O_2}^{\text{max}}$ trial did not differ statistically from those measured in the first, as described in the Supplementary Methods (electronic supplementary material), where the standard equations used to calculate venous O\textsubscript{2} saturation, cardiac output, stroke volume, and pulmonary O\textsubscript{2} extraction can also be found.

(c) Statistical analyses

Linear mixed-effects models were used to test for effects of mouse population, acclimation $P_{O_2}$, and acclimation temperature, and were performed using the lme4 package [41] in R [42]. We first included data from across all four acclimation environments to test for the fixed effects of population, $P_{O_2}$, and temperature as well as all possible interactions between these three factors, which allowed us to evaluate whether populations differed in their interactive response to hypoxia and cold. However, this approach did not allow us to examine the potential
population differences in the acclimation responses to hypoxia or cold on their own, which required two additional series of tests. We tested for the fixed effects of population, acclimation $P_{O_2}$, and the interaction between them by only including data for the warm acclimation environments, which allowed us to evaluate potential population differences in the response to chronic hypoxia in the absence of cold. We tested for the fixed effects of population, acclimation temperature, and the interaction between them by only including data among the normoxic acclimation environments, which allowed us to evaluate potential population differences in the response to chronic cold in the absence of hypoxia. Each series of tests was initially run including all of the potential interactions between the fixed factors, including body mass as a covariate and the random effects of sex and family. If any of the interactions or if the effects of body mass, sex, or family did not approach significance ($P \geq 0.1$), we carried out a second final test in which these particular effects were removed. Only highlanders and lowland white-footed mice were included in models for the subset of cardiovascular measurements that were not also made in lowland deer mice. The full results of the linear mixed-effects models are included in the electronic supplementary material (tables S1-S6), and the salient findings are reported in the Results. Tukey post-hoc tests were performed using the multcomp package in R [43]. These statistical analyses were carried out on absolute values of traits that were not corrected for body mass (because effects of body mass were accounted for in statistical models), but some data presented here are expressed relative to body mass as is conventional in the literature ($V_{O_2\text{max}}$, ventilatory and cardiac volumes). We also calculated Pearson correlations between $V_{O_2\text{max}}$ and its potential determinants using GraphPad Prism software (version 8.4; La Jolla, CA, USA), for which the P-values are reported in the Results and additional details are in electronic supplementary material table S7. $P < 0.05$ was considered statistically significant.

3. Results

(a) Thermogenic $V_{O_2\text{max}}$ in hypoxia

Thermogenic $V_{O_2\text{max}}$ in hypoxia was strongly affected by acclimation environment (figure 1), as reflected by highly significant effects of acclimation $P_{O_2}$ and temperature in linear mixed-effects models (table 1), but highland deer mice exhibited an especially pronounced acclimation response to hypoxia. As expected, body mass differed between species, as white-
footed mice (26.7 ± 1.4 g in warm normoxia) were generally larger than both highland (19.6 ± 0.8 g) and lowland (22.2 ± 1.1 g) deer mice (P<0.001) (electronic supplementary material, table S8). Body mass had statistically significant effects on \( \dot{V}_{O_2 \text{max}} \) in linear mixed-effects models and it varied across treatment groups (electronic supplementary material, tables S1-S6, S8), driven primarily by modest declines after acclimation to warm hypoxia in the lowland populations (P=0.009 for the main effect of acclimation \( P_{O_2} \)). After taking effects of body mass into account, hypoxia acclimation at warm temperature had a strong main effect on \( \dot{V}_{O_2 \text{max}} \) (P<0.001) that was driven entirely by deer mice (\( \dot{V}_{O_2 \text{max}} \) did not differ between warm hypoxia and warm normoxia for white-footed mice). However, the effects of hypoxia acclimation were much greater in highlanders compared to lowlanders (P<0.001 for the interaction between population and acclimation \( P_{O_2} \), and \( \dot{V}_{O_2 \text{max}} \) was 1.3- to 1.7-fold higher in highlanders than in lowlanders of both species in comparisons between populations acclimated to warm hypoxia. By contrast, cold acclimation had a strong main effect on \( \dot{V}_{O_2 \text{max}} \) (P<0.001) that was similar in magnitude across populations (non-significant population×temperature interaction). Acclimation to the combination of hypoxia and cold increased \( \dot{V}_{O_2 \text{max}} \) in all populations (~1.4- to 1.7-fold compared to warm normoxic mice), but the relative importance of each environmental parameter on the acclimation response differed between populations, as reflected by a significant population×\( P_{O_2} \)×temperature interaction (P=0.045). In lowlanders of both species, the response to cold hypoxia appeared to be slightly greater than the sum of the individual responses to hypoxia or cold alone. For example, in lowland deer mice, the sum of the magnitude of the responses to warm hypoxia and cold normoxia (~0.04 ml min\(^{-1}\) g\(^{-1}\) in each case) was slightly less than the magnitude of the response to cold hypoxia (~0.09 ml min\(^{-1}\) g\(^{-1}\)), when each response magnitude was calculated as the average absolute difference from warm normoxia. Similarly, in white-footed mice, the sum of the responses to warm hypoxia and cold normoxia (~0 and ~0.04, respectively) was slightly less than the response to cold hypoxia (~0.05 ml min\(^{-1}\) g\(^{-1}\)). This was not the case in highlanders, however, in which the response to warm hypoxia was just as large as the response to cold hypoxia.

(b) Breathing and pulmonary O\(_2\) uptake at \( \dot{V}_{O_2 \text{max}} \)
Breathing was measured by plethysmography to examine whether it contributed to some of the variation in \( \dot{V}_{O_2\text{max}} \) across treatment groups (figure 2, table 1). Breathing frequency at \( \dot{V}_{O_2\text{max}} \) appeared to vary across groups, largely because highlanders had higher breathing frequencies than lowlanders after acclimation to warm hypoxia (P=0.007 for population×\( P_{O_2} \) interaction) or cold normoxia (P=0.015 for population effect). Tidal volumes at \( \dot{V}_{O_2\text{max}} \) varied little across acclimation environments in both populations of deer mice, such that the patterns of variation in total ventilation appeared to be very similar to the variation in breathing frequency for these populations. However, hypoxia acclimation tended to reduce tidal volume at \( \dot{V}_{O_2\text{max}} \) in white-footed mice, which is likely responsible for the significant population×\( P_{O_2} \) interaction for this trait (P=0.014), and there was also a significant population×\( P_{O_2} \) interaction for total ventilation (P=0.001). Nevertheless, total ventilation was significantly correlated with \( \dot{V}_{O_2\text{max}} \) across all groups (P=0.004).

Pulmonary O\(_2\) extraction at \( \dot{V}_{O_2\text{max}} \) also appeared to vary across treatment groups (figure 3, table 1). Pulmonary O\(_2\) extraction tended to increase in cold and/or hypoxic acclimation environments compared to warm normoxic controls, as reflected by significant main effects of acclimation \( P_{O_2} \) (P=0.024) and temperature (P=0.040). There was a significant main effect of population overall (P=0.002), driven largely by higher values of pulmonary O\(_2\) extraction in highlanders that were greatest after hypoxia acclimation. Pulmonary O\(_2\) uptake was strongly correlated with \( \dot{V}_{O_2\text{max}} \) across all groups (P<0.0001).

(c) Circulatory O\(_2\) transport at \( \dot{V}_{O_2\text{max}} \)

We observed significant variation in arterial O\(_2\) saturation (measured by pulse oximetry) and the content of haemoglobin in the blood across treatment groups (table 1). Arterial O\(_2\) saturation varied little in response to acclimation environment but saturation was consistently ~7% to 12% higher in highlanders than in lowlanders of both species (P<0.001 for population effect) (figure 4A), and there was a significant correlation between arterial O\(_2\) saturation and \( \dot{V}_{O_2\text{max}} \) across all groups (P=0.0004). Blood haemoglobin content increased in response to hypoxic but not cold acclimation environments, as reflected by main effects of acclimation \( P_{O_2} \) (P=0.024) but not temperature (P=0.574) (table 2). However, blood haemoglobin content tended
to be highest in white-footed mice (P<0.001 for population effect) and the response to hypoxia
acclimation was generally similar between highland and lowland deer mice (non-significant
population×$P_{O_2}$ interactions). As a result, blood haemoglobin content was not correlated to
$\dot{V}_{O_2}\text{max}$ across groups (P=0.465).

There was also some variation across treatment groups in venous $O_2$ saturation at $\dot{V}_{O_2}\text{max}$
(figures 4B, table 1), which was calculated from measurements of blood haemoglobin content and
the $O_2$ content of mixed venous blood sampled from cannulated mice (electronic supplementary
material, table S9). In contrast to arterial $O_2$ saturation, linear mixed-effects models comparing
highland deer mice and lowland white-footed mice did not detect any significant population
effects on venous $O_2$ saturation. However, there was a significant main effect of cold acclimation
on venous $O_2$ saturation (P=0.004), driven largely by a lower value in white-footed mice
compared to highland deer mice in cold normoxia. Tissue $O_2$ extraction – calculated as the
difference between average arterial and venous $O_2$ saturations – was generally higher in
highlanders (~77-83%) than in lowlanders (~71-74%), due primarily to the higher arterial $O_2$
saturations in highlanders (figure 4A). The apparent drop in venous $O_2$ saturation in cold-
acclimated white-footed mice did not appear to increase tissue $O_2$ extraction (~72% in this
group), because it was associated with a non-significant decline in arterial $O_2$ saturation
compared to other acclimation environments. There were some differences across acclimation
groups in blood acid-base status and lactate concentration at $\dot{V}_{O_2}\text{max}$ – cold acclimation groups
appeared to have increased venous pH and bicarbonate concentration and decreased plasma
lactate compared to warm acclimation groups – but there were no appreciable or consistent
differences between populations (electronic supplementary material, figures S1,S2).

Differences in cardiac output (calculated using the Fick equation) appeared to contribute
to the variation in $\dot{V}_{O_2}\text{max}$ across treatment groups (figure 5, table 1), based on the strong
correlation between cardiac output and $\dot{V}_{O_2}\text{max}$ across all groups (P<0.0001). Highland mice
generally had higher cardiac output at $\dot{V}_{O_2}\text{max}$ than lowland white-footed mice, as reflected by a
significant population effect across all environments (P=0.029) that appeared to be largely due to
variation in stroke volume that neared significance (P=0.067 for population effect across all
environments). The significant main effects of both acclimation $P_{O_2}$ and temperature (P<0.001
each) suggested that cardiac output at $V_{O_2}\text{max}$ increased in mice from cold and/or hypoxic
acclimation environments compared to warm normoxic controls, but the magnitude of the changes suggested that these effects were driven much more by highland deer mice than lowland white-footed mice. The relative influence of changes in stroke volume versus heart rate on cardiac output between acclimation environments also differed across populations. For stroke volume, there was a significant population×\(P_O_2\) interaction across all environments (P=0.020) and there appeared to be greater increases in stroke volume in highlanders in response to hypoxic and/or cold acclimation environments, reaching values that were 1.6- to 1.8-fold greater on average than lowlanders of both species in cold hypoxia. For heart rate, there was a significant population×temperature interaction (P<0.001), in large part because cold acclimation environments tended to reduce heart rates at \(\dot{V}_{O_2\text{max}}\) in highlanders but not in lowlanders (as compared to heart rates of mice from the warm normoxic environment). As such, the increase in cardiac output at \(\dot{V}_{O_2\text{max}}\) in response to cold-hypoxia acclimation was driven primarily by increases in heart rate in lowland white-footed mice, but increases in stroke volume were a larger contributor in highland deer mice.

4. Discussion

Deer mice at high altitudes sustain high metabolic rates to support thermogenesis [32] and appear to be subject to strong directional selection for increased thermogenic capacity in hypoxia [15]. Here, we show that adaptive increases in thermogenic \(\dot{V}_{O_2\text{max}}\) in hypoxia arise from evolved changes and plasticity in response to the major stressors at high altitude, hypoxia and cold. High-altitude mice exhibited an exaggerated increase in \(\dot{V}_{O_2\text{max}}\) in response to chronic hypoxia, which appeared to completely dominate the response to concurrent hypoxia and cold. The variation in thermogenic \(\dot{V}_{O_2\text{max}}\) appeared to be explained by evolved and environmentally induced variation across the \(O_2\) transport pathway, including breathing, pulmonary \(O_2\) extraction, arterial \(O_2\) saturation, cardiac output, and tissue \(O_2\) extraction. Therefore, both evolutionary adaptation and phenotypic plasticity contribute to coordinated changes in the function of the \(O_2\) pathway that lead to adaptive increases in thermogenic capacity in deer mice at high altitudes.

(a) Combined effects of hypoxia and cold on thermogenic capacity
The effects of chronic hypoxia and/or cold on thermogenic capacity suggest that phenotypic plasticity can improve the ability of small mammals to cope with the cold environment at high altitude. Cold acclimation is well known to increase thermogenic \(\dot{V}_{O_2}\text{max}\) as well as the capacity for non-shivering thermogenesis (NST) in deer mice and other small mammals [17, 21, 44-48]. Hypoxia acclimation does not generally increase \(\dot{V}_{O_2}\text{max}\) in humans [49]; however, it has been shown to increase \(\dot{V}_{O_2}\text{max}\) in rodents during thermogenesis or exercise when measured in hypoxia, but not necessarily when measured in normoxia, suggesting that the responses of rodents to chronic hypoxia act primarily to reduce the depressive effects of hypoxia on \(\dot{V}_{O_2}\text{max}\) [35, 36, 50]. Little was previously known about how \(\dot{V}_{O_2}\text{max}\) changes after chronic exposure to hypoxia and cold in combination, but prior studies in house mice suggest that these stressors have opposing (but additive) effects on the capacity for NST in normoxia, such that cold hypoxic mice have similar NST capacity to warm normoxic mice [17]. Our results in lowland deer mice suggest that hypoxia and cold as acclimation treatments have additive or more than additive effects that increase thermogenic \(\dot{V}_{O_2}\text{max}\) in hypoxia when they occur in combination, possibly because cold acclimation tends to increase \(\dot{V}_{O_2}\text{max}\) in normoxia while hypoxia acclimation makes \(\dot{V}_{O_2}\text{max}\) less sensitive to reductions in environmental \(P_{O_2}\).

Plastic changes across the \(O_2\) transport pathway appeared to underlie the increases in thermogenic capacity in response to chronic hypoxia and/or cold. Previous studies in rats also found that cold acclimation increased \(O_2\) consumption at cold temperatures via increases in cardiac output, with no change in the arterial-venous difference in \(O_2\) saturation or \(O_2\) content, and the increased cardiac output largely served to increase blood flow to multiple depots of brown adipose tissue throughout the body [46]. Hypoxia acclimation has also been found to increase exercise \(\dot{V}_{O_2}\text{max}\) in hypoxia (but not in normoxia) in rats, in association with decreases in arterial \(CO_2\) tension (which could reflect an increase in alveolar ventilation) and with increases in arterial \(P_{O_2}\) and \(O_2\) saturation, blood haemoglobin content, and tissue \(O_2\) extraction [50]. However, cardiac output and heart rate at \(\dot{V}_{O_2}\text{max}\) were lower after hypoxia acclimation in this particular study [50], and subsequent arterial pacing studies suggested that these reductions in cardiac output constrained the plastic increases in \(\dot{V}_{O_2}\text{max}\) [51]. Similarly, heart rate at \(\dot{V}_{O_2}\text{max}\) was reduced after acclimation to warm hypoxia in lowland deer mice, but this was not observed in other populations, and cardiac output was highest in the cold hypoxic groups of all populations.
Therefore, responses to chronic cold may over-ride effects of chronic hypoxia that could otherwise constrain cardiac output and $\dot{V}_{O_2\text{max}}$ in lowland mice during acclimation to high-altitude conditions.

(b) High-altitude deer mice have evolved an enhanced hypoxia acclimation response

Our findings here suggest that directional selection for high thermogenic capacity at high altitude [15] has increased $\dot{V}_{O_2\text{max}}$ in highland mice by amplifying the plastic response to chronic hypoxia, consistent with our previous findings [36]. These findings are consistent with a scenario where, upon colonization of the high-altitude environment, directional selection on $V_{O_2\text{max}}$ increased the magnitude of adaptive phenotypic plasticity in this trait, and thus shifted the population mean closer to the fitness optimum [22]. Our results contribute to growing evidence suggesting that high-altitude natives of various taxa have evolved to become more resistant to the depressive effects of hypoxia on $\dot{V}_{O_2\text{max}}$ than their low-altitude counterparts [52].

It is intriguing to consider why highland and lowland deer mouse populations exhibited similar plasticity of $\dot{V}_{O_2\text{max}}$ in response to the combination of cold and hypoxia. Although this may call into question the adaptive significance of the enhanced plasticity in response to warm hypoxia in highlanders, the population difference in the interaction between cold and hypoxia (i.e., significant population $\times P_O_2 \times$ temperature interaction) suggests that it may still have adaptive significance. The strong response of highlanders to hypoxia alone appeared to dominate the acclimation response to hypoxia and cold in combination, in stark contrast to the responses of lowland mice. We speculate that this strong hypoxia response of highlanders may allow them to respond more strongly than lowlanders if they are exposed to colder temperatures than those used for cold acclimations here ($5^\circ\text{C}$). Indeed, $5^\circ\text{C}$ may underestimate the intensity of cold exposure at high altitude in the wild, because the high peaks of the Rocky Mountains are snow covered for much of the year. Future studies of plasticity in response to hypoxia at colder temperatures are needed to explore this possibility.

Strong increases in some of the systems-level determinants of O$_2$ transport likely contributed to the evolved increase in $\dot{V}_{O_2\text{max}}$ in response to hypoxia acclimation in high-altitude deer mice. Increases in breathing frequency at $\dot{V}_{O_2\text{max}}$ after acclimation to warm hypoxia were much greater in highlanders than in lowlanders, which would be expected to help increase
thermogenic $\dot{V}_{O_2}^{max}$ if it augmented alveolar ventilation. However, highlanders tended to have
relatively low tidal volumes at $\dot{V}_{O_2}^{max}$, and as a result, variation in total ventilation was not
clearly associated with the increased thermogenic $\dot{V}_{O_2}^{max}$ in highlanders after acclimation to
warm hypoxia. Highlanders may have also relied upon more pronounced increases in pulmonary
O$_2$ extraction after acclimation to warm hypoxia to augment O$_2$ uptake into the blood. Cardiac
output exhibited a particularly strong increase of ~1.4-fold in highlanders after acclimation to
warm hypoxia compared to warm normoxic controls. This is in stark contrast to low-altitude
mice, in which cardiac output at $\dot{V}_{O_2}^{max}$ changed very little as a result of hypoxia acclimation,
and to previous studies in rats, in which cardiac output at $\dot{V}_{O_2}^{max}$ decreased after exposure to
chronic hypoxia [50].

Recent theoretical evidence suggests that the evolution of plasticity in complex traits
depends upon the level of functional integration between the multiple component mechanisms
underlying those traits [26]. For thermogenic capacity, the integration between its underlying
component mechanisms (i.e., steps in the O$_2$ transport pathway) is extensive. For example,
increases in arterial O$_2$ saturation may be of little benefit to aerobic capacity if they are not
combined with increases in tissue O$_2$ extraction [53]. Our findings here suggest that this
integration may contribute to the enhanced plasticity of thermogenic $\dot{V}_{O_2}^{max}$ in chronic hypoxia
in highlanders. The effect of hypoxia acclimation on $\dot{V}_{O_2}^{max}$ (which increased ~1.7-fold in warm
hypoxia compared to warm normoxia) was greater in magnitude than the effects of hypoxia
acclimation on any of its systems-level determinants from across the O$_2$ transport pathway.
Therefore, no single component can explain the evolved increase in the plasticity of $\dot{V}_{O_2}^{max}$ in
highlanders, but it is instead explained by the interactive effects of changes in plasticity and/or
mean trait value for each of these components. For example, the effects of increased plasticity in
cardiac output combined with increased mean values of arterial O$_2$ saturation and tissue O$_2$
extraction (neither of which were plastic themselves) could together be responsible for
amplifying the increase in O$_2$ transport to thermogenic tissues and $\dot{V}_{O_2}^{max}$ after hypoxia
acclimation.

(c) Coordinated changes across the O$_2$ transport pathway augment thermogenic capacity in
high-altitude deer mice
Our results suggest that high-altitude deer mice have evolved functional changes across the O₂ pathway to support thermogenic performance in hypoxia. Several plastic physiological processes – breathing frequency, pulmonary O₂ extraction, cardiac output, and stroke volume – were often higher in highlanders than in lowlanders, particularly after hypoxia acclimation. Some other physiological processes exhibited very little plasticity – namely, arterial O₂ saturation and the arterial-venous difference in O₂ saturation – but were consistently greater in highlanders than in lowlanders. These latter changes may be at least partly explained by the evolved increases in haemoglobin-O₂ affinity [31, 54, 55] and in the capillarity and oxidative capacity of skeletal muscle [35, 56-59] in high-altitude deer mice. Therefore, evolutionary adaptation to high altitude has amplified some of the mechanisms that contribute to plasticity in lowlanders, but it has also expanded the physiological toolkit for increasing thermogenic $\dot{V}_{O_2,\text{max}}$ under hypoxic conditions. Our results also suggest that the evolution of thermogenic $\dot{V}_{O_2,\text{max}}$ in high-altitude deer mice has occurred through similar mechanisms to the increases in exercise $\dot{V}_{O_2,\text{max}}$ in some human populations native to high altitude. For example, the augmented exercise $\dot{V}_{O_2,\text{max}}$ in high-altitude humans in hypoxia is associated with higher pulmonary O₂ diffusing capacity and cardiac output [4, 28, 29, 52]. However, in many human studies, it has been difficult to disentangle the genetic and environmental components of variation in high-altitude phenotypes [52, 60]. Our results here suggest that both plastic and evolved changes in the O₂ pathway of high-altitude deer mice have contributed to the success of these animals in harsh alpine environments.

**Ethics.** All procedures followed guidelines set out by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board (Animal Use Protocol 16-01-02).

**Data accessibility.** Data are available from the Dryad Digital Repository:

https://doi:10.5061/dryad.rjdfn2z7d [61].

**Author contributions.** Z.A.C., J.F.S., G.B.M., and G.R.S. designed the study. K.B.T., O.H.W., and C.M.I. ran the experiments and analyzed the data. K.B.T., O.H.W., and G.R.S. wrote the manuscript, and all authors edited the manuscript.
Competing interests. The authors declare no competing interests.

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increases in thermogenic capacity in high-altitude deer mice. *Dryad Digital Repository.*
([https://doi.org/10.5061/dryad.rjdfn2z7d](https://doi.org/10.5061/dryad.rjdfn2z7d)).
Figure Legends

**Figure 1.** Thermogenic capacity, measured in hypoxia as the maximal rate of O₂ consumption ($\dot{V}_{O_2\text{max}}$) during acute cold exposure, was found to vary across populations and acclimation treatments in statistical tests using linear mixed-effects models (table 1). **••** Significant pairwise difference in highland deer mice compared to lowland white-footed mice or both lowland populations, respectively, within an acclimation environment (P<0.05). Data are means ± s.e.m., with N for each group indicated within each bar.

**Figure 2.** Breathing at $\dot{V}_{O_2\text{max}}$ was found to vary across populations and acclimation treatments. See figure 1 for symbol definitions and statistical details.

**Figure 3.** Pulmonary O₂ extraction at $\dot{V}_{O_2\text{max}}$ was found to vary across populations and acclimation treatments. See figure 1 for symbol definitions and statistical details.

**Figure 4.** Arterial O₂ saturation at $\dot{V}_{O_2\text{max}}$ was augmented in high-altitude mice. See figure 1 for symbol definitions and statistical details.

**Figure 5.** Cardiac output at $\dot{V}_{O_2\text{max}}$ was found to vary across populations and acclimation treatments. See figure 1 for symbol definitions and statistical details.
Figure 1

Thermogenic $\dot{V}_{O_2, max}$ in hypoxia (ml min$^{-1}$ g$^{-1}$).

- Highland deer mice
- Lowland deer mice
- Lowland white-footed mice

Acclimation environment:
- Warm normoxia
- Warm hypoxia
- Cold normoxia
- Cold hypoxia

**Statistical significance**
Figure 2

A. Total ventilation (ml min\(^{-1}\) g\(^{-1}\))

- Highland deer mice
- Lowland deer mice
- Lowland white-footed mice

B. Tidal volume (µl g\(^{-1}\))

C. Total ventilation (ml min\(^{-1}\) g\(^{-1}\))

Legend:
- Warm normoxia
- Warm hypoxia
- Cold normoxia
- Cold hypoxia

Note: * and ** indicate statistical significance.
Figure 3

- **Highland deer mice**
- **Lowland deer mice**
- **Lowland white-footed mice**

**Acclimation environment**

- **Warm normoxia**
- **Warm hypoxia**
- **Cold normoxia**
- **Cold hypoxia**

**Pulmonary O₂ extraction (%)**

*Note: Data points marked with an asterisk (*) indicate significant differences.*
Figure 4

A

Arterial $O_2$ saturation (%)

<table>
<thead>
<tr>
<th>Acclimation environment</th>
<th>Highland deer mice</th>
<th>Lowland deer mice</th>
<th>Lowland white-footed mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm normoxia</td>
<td>14</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Warm hypoxia</td>
<td>6</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Cold normoxia</td>
<td>14</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Cold hypoxia</td>
<td>6</td>
<td>6</td>
<td>13</td>
</tr>
</tbody>
</table>

B

Venous $O_2$ saturation (%)

<table>
<thead>
<tr>
<th>Acclimation environment</th>
<th>Warm normoxia</th>
<th>Warm hypoxia</th>
<th>Cold normoxia</th>
<th>Cold hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9</td>
<td>4</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5

(A) Cardiac output (ml min\(^{-1}\) g\(^{-1}\))

(B) Heart rate (min\(^{-1}\))

(C) Stroke volume (µl g\(^{-1}\))

Acclimation environment:
- Warm normoxia
- Warm hypoxia
- Cold normoxia
- Cold hypoxia
Table 1. Summary of the results of linear mixed-effects models that were used to test for effects of population and acclimation environment.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Only warm environments</th>
<th>Only normoxic environments</th>
<th>All environments</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{O_2 \text{max}}$</td>
<td>$P_{O_2}$, Pop×$P_{O_2}$</td>
<td>T</td>
<td>$P_{O_2}$, T, Pop×$P_{O_2}$, Pop×$P_{O_2}$×T</td>
</tr>
<tr>
<td>$\dot{V}_1$</td>
<td>Pop, Pop×$P_{O_2}$</td>
<td>-</td>
<td>T, Pop×$P_{O_2}$</td>
</tr>
<tr>
<td>$V_T$</td>
<td>Pop, $P_{O_2}$, Pop×$P_{O_2}$</td>
<td>-</td>
<td>Pop, $P_{O_2}$, Pop×$P_{O_2}$</td>
</tr>
<tr>
<td>$f_R$</td>
<td>Pop, $P_{O_2}$, Pop×$P_{O_2}$</td>
<td>Pop, T</td>
<td>Pop, $P_{O_2}$, T</td>
</tr>
<tr>
<td>$E_{L_{O_2}}$</td>
<td>Pop, $P_{O_2}$</td>
<td>T</td>
<td>Pop, $P_{O_2}$, T</td>
</tr>
<tr>
<td>$S_{aO_2}$</td>
<td>Pop</td>
<td>Pop</td>
<td>Pop, $P_{O_2}$</td>
</tr>
<tr>
<td>$\dot{Q}$</td>
<td>$P_{O_2}$</td>
<td>Pop, T</td>
<td>Pop, $P_{O_2}$, T</td>
</tr>
<tr>
<td>$V_S$</td>
<td>-</td>
<td>Pop, T</td>
<td>T, Pop×$P_{O_2}$</td>
</tr>
<tr>
<td>$f_H$</td>
<td>-</td>
<td>-</td>
<td>$P_{O_2}$, Pop×T, $P_{O_2}$×T</td>
</tr>
<tr>
<td>[Hb]</td>
<td>Pop, $P_{O_2}$</td>
<td>Pop</td>
<td>Pop, $P_{O_2}$</td>
</tr>
<tr>
<td>$S_{vO_2}$</td>
<td>-</td>
<td>T</td>
<td>$P_{O_2}$, T</td>
</tr>
</tbody>
</table>

$V_{O_2 \text{max}}$, maximal rate of O$_2$ consumption; $\dot{V}_1$, total ventilation; $V_T$, tidal volume; $f_R$, breathing frequency; $E_{L_{O_2}}$, pulmonary O$_2$ extraction; $S_{aO_2}$, arterial O$_2$ saturation; $\dot{Q}$, cardiac output; $V_S$, stroke volume; $f_H$, heart rate; [Hb], blood haemoglobin content; $S_{vO_2}$, mixed venous O$_2$ saturation; $P_{O_2}$, partial pressure of O$_2$. Significant main effects and interactions between population (Pop) and acclimation $P_{O_2}$ and/or temperature (T) are shown for models including data from all acclimation environments, only data from warm environments, and only data from normoxic environments. See Methods for a full description and electronic supplementary material (tables S1-S6) for full results.
Table 2. Blood haemoglobin content (g dl⁻¹) of mice in each acclimation environment.

<table>
<thead>
<tr>
<th>Acclimation environment</th>
<th>Highland deer mice</th>
<th>Lowland deer mice</th>
<th>Lowland white-footed mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm (25°C) normoxia</td>
<td>15.7 ± 0.5 (13)</td>
<td>16.9 ± 0.9 (9)</td>
<td>17.5 ± 0.7 (14)</td>
</tr>
<tr>
<td>Warm (25°C) hypoxia</td>
<td>17.9 ± 1.0 (10)</td>
<td>18.1 ± 0.4 (6)</td>
<td>20.5 ± 0.6 (14)</td>
</tr>
<tr>
<td>Cold (5°C) normoxia</td>
<td>16.2 ± 0.5 (24)</td>
<td>14.7 ± 1.2 (6)</td>
<td>18.2 ± 0.5 (17)</td>
</tr>
<tr>
<td>Cold (5°C) hypoxia</td>
<td>17.2 ± 1.5 (11)</td>
<td>19.9 ± 1.0 (9)</td>
<td>21.3 ± 1.0 (12)</td>
</tr>
</tbody>
</table>

Data are means ± s.e.m. (N)