Evolved reductions in body temperature and the metabolic costs of thermoregulation in deer mice native to high altitude

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Abstract

The evolution of endothermy was instrumental to the diversification of birds and mammals, but the energetic demands of maintaining high body temperature could offset the advantages of endothermy in some environments. We hypothesised that reductions in body temperature help high-altitude natives overcome the metabolic challenges of cold and hypoxia in their native environment. Deer mice (*Peromyscus maniculatus*) from high-altitude and low-altitude populations were bred in captivity to the second generation, and were acclimated as adults to warm normoxia or cold hypoxia. Subcutaneous temperature (T_{sub}, used as a proxy for body temperature) and cardiovascular function were then measured throughout the diel cycle using biotelemetry. Cold hypoxia increased metabolic demands, as reflected by increased food consumption and heart rate (associated with reduced vagal tone). These increased metabolic demands were offset by plastic reductions in T_{sub} (~2˚C) in response to cold hypoxia, and highlanders had lower T_{sub} (~1˚C) than lowlanders in both environmental treatments. Empirical and theoretical evidence suggested that these reductions could together reduce metabolic demands by ~10-30%. Therefore, plastic and evolved reductions in body temperature can help mammals overcome the metabolic challenges at high altitude, and may be a valuable energy-saving strategy in some non-hibernating endotherms in extreme environments.

Keywords

High-altitude adaptation, high-altitude acclimatization, thermoregulation, metabolism, blood pressure, circadian rhythms.

1. Introduction

The evolution of endothermy and the ability to maintain high body temperature has been instrumental to the success and diversification of mammals and birds [1, 2]. By maintaining high body temperature and correspondingly high metabolic rates, endotherms can remain active and support metabolically demanding processes such as locomotion (*e.g.* for hunting, foraging, competing, and/or evading predators) and reproduction across broad ranges of environmental temperature [1, 3-5]. However, heat generation (thermogenesis) necessary for maintaining high body temperature can itself be energetically demanding, thus leading to high food demands and requiring that oxygen (O_2) and metabolic fuels be supplied to thermogenic tissues at high rates [3, 4]. Periods of limited food availability and/or high thermogenic requirements (*e.g.* over winter) can make these demands untenable, such that some endotherms have the ability to temporarily depress body temperature and thermogenesis during times of seasonal hibernation or torpor [6-10]. Whether adaptive reductions in body temperature setpoint may help endotherms reduce metabolic demands in cold environments when they are not hibernating or torpid is less clear. Broad macroevolutionary comparisons suggest that non-hibernating endotherms in cold temperate environments maintain body temperature similar to or even slightly greater than their tropical counterparts [11-13]. However, such broad comparisons do not address the possibility that reductions in body temperature setpoint may have arisen within some distinct lineages as a valuable mechanism for coping with prolonged metabolic challenges.

The extreme environment at high altitude provides examples of animals that have evolved to live in metabolically challenging conditions that are both unavoidable and unremitting. Physiological homeostasis requires that animals balance the supply and demand of O_2 and metabolic fuels for tissues, but this balance can be extremely difficult to maintain at high altitude. Cold temperatures persist year-round at high altitude, raising the demands of aerobic thermogenesis, while low O_2 availability (hypoxia) can limit O_2 supply to support aerobic metabolism [14-17]. High-altitude natives somehow overcome this challenge, successfully supplying O_2 and metabolic fuels at sufficient rates to meet tissue demands. This is achieved in several high-altitude mammals and birds via environmentally-induced plasticity and evolved modifications across the O_2 transport

pathway to augment tissue O_2 supply in cold hypoxia [14-16, 18-21]. However, although many high-altitude endotherms do not hibernate, relatively little attention has been paid to whether evolved reductions in body temperature setpoint might have arisen in high-altitude natives to offset metabolic demands. This could be a particularly valuable energy-saving strategy in high-altitude environments, where hypoxia can constrain the ability to increase metabolic rate.

Deer mice (*Peromyscus maniculatus*) native to high altitude are a powerful model in which to examine the evolution of body temperature regulation [15-17, 22]. The deer mouse is a nocturnal species found across North America [23] and has the largest altitudinal range of any North American mammal [24]. High-altitude populations of deer mice maintain higher field metabolic rates than their low-altitude counterparts [25, 26], likely due to the heightened costs of thermogenesis at high altitudes. High-altitude deer mice also exhibit a high aerobic capacity for thermogenesis during adulthood, achieved through both plastic and evolved changes in physiological pathways of O_2 and metabolic fuel transport [27-36]. Therefore, high-altitude deer mice have a heightened capacity for supplying O_2 and metabolic fuels to tissues, like some other high-altitude taxa. Whether high-altitude deer mice have also reduced the routine demands for O_2 and metabolic fuels is less clear, but such reductions could be highly advantageous when considering that in some instances food availability may also be limited at high altitudes [37]. The ontogenetic development of endothermy is delayed in high-altitude deer mice [38-40], suggesting that metabolic demands of thermogenesis are reduced in early post-natal life stages. Whether they also exhibit strategies to reduce the metabolic demands of thermogenesis and body temperature regulation in later life remains unresolved.

In this study, we test the hypothesis that reductions in body temperature setpoint help high-altitude deer mice reduce metabolic demands and thus cope with the challenges of life at high altitude. Populations of deer mice native to high altitude and low altitude were each bred in captivity. Second-generation mice from both populations were raised to adulthood, and then acclimated to warm normoxia and cold hypoxia in a full-factorial design. We predicted that acclimation to cold hypoxia would reduce body temperature, and that overlaid upon this plastic response, high-altitude populations would have evolved to operate at a lower body temperature than low-altitude populations. We made use of

physiological telemetry devices to measure subcutaneous temperature (T_{sub}) and other physiological variables throughout the diel cycle, in order to avoid the confounding effects of handling, tethering, or anaesthesia [41]. T_{sub} is a strong proxy for core body temperature in small mammals, in which internal temperature gradients are modest [42]. This biotelemetry approach also enabled measurements of cardiovascular function to provide refined insight into the metabolic demands in cold and hypoxic environments.

2. Methods

(a) Animals and environmental treatments

Wild deer mice were live-trapped at high altitude on the summit of Mount Evans (Clear Creed County, CO, USA at 39˚35'18''N, 105˚38'38''W; 4350 m above sea level) and at low altitude in the Great Plains of Nebraska (Buffalo County, NE, USA at 40˚41'58''N, 99˚04'53''W; approx. 660 m above sea level). These wild adults were transported to McMaster University (Hamilton, ON, Canada; 50 m above sea level), and bred within their respective populations for two generations to produce second-generation progeny. These progeny were kept under standard normoxic laboratory conditions (25˚C, ~20 kPa O_2, 12:12-h light-dark photoperiod) until experimentation. In deer mice, embryonic development takes ~24 days, pups can be weaned at ~21 days after birth, females can reproduce by ~2 months of age, and maximum lifespan is ~8 years [39, 43, 44]. At six months of age, mice from each population were assigned to each of two environmental treatments: warm normoxia (25˚C, 20 kPa O_2; 15 lowlanders and 13 highlanders) or cold hypoxia (5˚C, 12 kPa O_2; 12 lowlanders and 15 highlanders). Warm normoxia consisted of standard laboratory conditions. Cold hypoxia was created using previously described hypobaric chambers [31, 45] inside a temperature controlled environmental chamber held at 5˚C. The level of hypoxia used (12 kPa O_2) is roughly equivalent to that at 4350 m above sea level, and 5˚C approximates a temperature that could be encountered within burrows for prolonged periods of the winter [46]. Mice were housed in standard mouse cages (containing 7090 Teklad Sani-Chips® animal bedding; Envigo, Indianapolis, IN, USA) with unlimited access to water and standard rodent chow (Teklad 22/5 Rodent Diet formula 8640; Envigo). Mice in cold hypoxia were briefly (<20 min) returned to normobaria twice per week for cage cleaning and replenishment of food and water.

(b) Surgical instrumentation of physiological telemeters

After 6 weeks of acclimation to warm normoxia or cold hypoxia, deer mice were anaesthetized using isoflurane and surgically instrumented with physiological telemeters (HD-X11, Data Sciences International Inc.) using methods we have previously described [47]. Telemetry implants were positioned subcutaneously on the back in the interscapular space for remote measurement of subcutaneous temperature (T_{sub}) as a proxy for routine body temperature. The left carotid artery was cannulated with the pressure catheter of the telemeter to measure heart rate (f_H) and arterial blood pressure (P_{mean}). Mice were recovered from surgery for 3 days in their respective environmental treatment conditions. A detailed description of the surgical protocol is included in the Supplementary Materials.

(c) Telemetry measurements of routine physiology

Following recovery from surgery, we continuously measured routine T_{sub} and cardiovascular function of freely behaving, unrestrained deer mice. Measurements were made at the same temperature and O_2 levels to which the mice had been acclimated using a temperature and O_2 controlled cabinet (O_2 Control In Vitro Glove Box, Coy Laboratory Products Inc., MI, USA), in which inflowing air and nitrogen were mixed to create warm normobaric normoxia or cold normobaric hypoxia. It was necessary to carry out the telemetric measurements in normobaria (rather than hypobaria) due to the limited pressure calibration range of the blood pressure sensor (670 to 800 mm Hg). Mice were held for 4 d in these conditions to ensure that all telemetry measurements had stabilized at normal values, after which routine physiological measurements were continuously acquired for 48 h using a Matrix 2.0 data acquisition system and Ponemah® software (Data Sciences International), concurrent with measurements of daily food and water consumption. Hourly means of T_{sub}, heart rate (f_H) and mean arterial pressure (P_{mean}) were calculated over the 24-h daily cycle, and the maximum and minimum hourly values were determined.

(d) Pharmacological assessment of cardiovascular control

Following measurements of routine physiology, a subset of mice in warm normoxia (6 lowlanders and 5 highlanders) and cold hypoxia (5 lowlanders and 6 highlanders) were used to determine β_1-adrenergic and vagal tone on the heart. This was achieved by measuring the f_H responses to pharmacological blockade of cardiac β_1-adrenergic receptors (β_1-AR) and muscarinic acetylcholine receptors (mAChR), respectively. Metoprolol (β_1-AR blocker) and atropine (mAChR blocker) were administered on separate consecutive days in random order. Each was given in a series of hourly intraperitoneal (IP) injections of increasing concentration, starting at 2 pm local time (metoprolol – 0.04, 0.4, 4, and 40 mg per kg body mass; atropine – 0.05, 0.5, 5, and 50 mg kg^{-1}; each injected at a volume of 20 ml kg^{-1} in sterile saline). Baseline (*i.e.* pre-injection) f_H was the average between 45 and 15 min before the first injection on each day. The minimum (metoprolol) or maximum (atropine) f_H over a one-minute period was determined 15 to 45 min after each injection. The maximal f_H response (Δf_H) to each blocker was calculated by subtracting post-injection f_H for the dose of blocker eliciting the greatest f_H change from baseline pre-injection f_H. Maximal Δf_H was used as an index of chronotropic tone on the heart.

(e) Organ masses and haematology

Once in vivo physiological measurements were complete, mice were weighed and humanely euthanized by overdose of isoflurane followed by decapitation. Blood was collected, haemoglobin content was measured using Drabkin's reagent (according to instructions from the manufacturer, Sigma-Aldrich), and blood was centrifuged at 12,700g for 5 min to measure haematocrit. Masses of heart ventricles, lungs, liver, and interscapular brown adipose tissue were then measured.

(f) Statistics

Linear mixed models (lme4 package [48] in R Studio, v. 1.4.1103, RStudio Public Benefit Corporation, MA, USA) were used to test for effects of population, environmental treatment, and time of day on routine physiology data collected across the entire 24-h period. Data were first tested for both populations together to evaluate whether populations differed in their response to cold hypoxia, after which the response to cold

hypoxia was tested separately in each population independent of the other. Models were also run to test for effects of population and environmental treatment on maximum and minimum hourly values of routine physiology, food and water consumption, body and organ masses, haematology, and Δf_H for β_1-AR and vagal blockade. Sex and family were included as random factors in all statistical models, but their effects were never significant. Individual was included as a random factor for data with repeated measures across the entire 24-h period. Statistical analyses of organ mass data were carried out on absolute values and included body mass as a covariate, but are presented here relative to body mass as is conventional in the literature. The full results of the linear mixed models are included in electronic supplementary material (tables S1-S7), and the salient findings are reported in the Results. Holm-adjusted Tukey's HSD post-hoc tests were performed to test for pairwise differences between environmental treatments within a population, or between populations within an environmental treatment. Data are presented as mean ± SEM or as box and whisker plots, often along with individual values.

3. Results

(a) Body temperature is reduced by plastic and evolved responses to cold hypoxia

There was significant diel variation in subcutaneous temperature (T_{sub}; time effect, P = 0.013), which was used as a proxy for body temperature, with increasing T_{sub} during the night-time active phase and a rapid reduction in T_{sub} within the first few hours of light during the day-time inactive phase (figure 1a,b). Overlaid upon this diel variation were significant main effects of environmental treatment (P < 0.001) and population (P < 0.001) on $T_{sub.}$ Exposure to cold hypoxia reduced T_{sub} compared to warm normoxic mice, as reflected by strong effects of environmental treatment on T_{sub} within both populations (P < 0.001). Maximum (figure 1c) and minimum (figure 1d) hourly T_{sub} were 1.9-2.4˚C lower on average in cold hypoxia than in warm normoxia (treatment effects, P < 0.001). However, highlanders exhibited lower T_{sub} than lowlanders throughout the diel cycle both in warm normoxia and in cold hypoxia (figure 1a,b), and maximum and minimum T_{sub} were 0.7-1.0˚C lower on average in highlanders (figure 1c,d). Furthermore, in highlanders only, the reduction in T_{sub} during the early morning hours was more rapid in cold hypoxia than in warm normoxia (treatment×time, P = 0.001). Therefore, both plastic responses to cold

hypoxia and evolved changes in the high-altitude population reduced T_{sub} throughout the diel cycle.

Three independent lines of evidence, one empirical and two theoretical, suggested that reductions in body temperature likely help reduce the metabolic demands of thermogenesis. To empirically investigate the effect of changes in body temperature on metabolism in each treatment group, we measured resting O_2 consumption rate ($\dot{V}O_2$) during acute reductions in inspired O_2 (from 21 kPa O_2 to 8 kPa O_2) to reduce body temperature by hypoxic anapyrexia, a well described and pervasive response to severe acute hypoxia across animals [49] (see electronic supplementary material for detailed methods). We then quantified the correlation between relative changes in $\dot{V}O_2$ and T_{sub}. As expected, resting $\dot{V}O_2$ was reduced at lower T_{sub}, and the magnitude of $\dot{V}O_2$ reduction was 5.5-8.1% on average per 1˚C reduction in T_{sub} across treatment groups (electronic supplementary material, figure S1). We also used two theoretical approaches to independently consider the relative effects of changes in body temperature on metabolic rate (see electronic supplementary material). First, we calculated the expected relative effects of temperature on biological rate processes using Q_{10} temperature coefficients of 2 and 3. Indeed, Q_{10} for resting $\dot{V}O_2$ is ~2.1-2.2 in humans and small non-torpid mammals [50, 51]. The expected effect of a 1˚C decrease in body temperature is a 6.7% reduction in $\dot{V}O_2$ for a Q_{10} of 2 and a 10.4% reduction in $\dot{V}O_2$ for a Q_{10} of 3. Second, we used the Scholander-Irving model of thermoregulation [52], in which $\dot{V}O_2$ below the thermoneutral zone is a function of thermal conductance and the difference between body temperature and ambient temperature. In this case, the expected relative effect of a 1˚C decrease in body temperature on $\dot{V}O_2$ was ~3.4%. Based on the magnitude of these relative effects, a plastic ~2˚C reduction in body temperature in response to cold hypoxia combined with an evolved ~1˚C reduction in the high-altitude population could reduce $\dot{V}O_2$ by ~10-30%. These data suggest that reductions in body temperature (measured here as T_{sub}) help reduce metabolic demands at high altitude.

(b) Cold hypoxia is associated with higher heart rates and food consumption, and preservation of mean arterial blood pressure

Heart rates were elevated by ~80-200 beats per minute in cold hypoxia, as reflected by significant main effects of environmental treatment ($P < 0.001$) on f_H across the diel cycle across populations (figure 2a,b). Maximum f_H was 17-20% higher and minimum f_H was 25-30% higher in cold hypoxia compared to warm normoxia in both populations (treatment effects, $P < 0.001$) (figure 2c,d). Similarly, daily food consumption was 37-43% greater in cold hypoxia than in warm normoxia in both populations (treatment effect, $P < 0.001$), with no significant variation in daily water consumption ($P = 0.122$) (table 1). Unlike T_{sub}, there were no significant effects of population on f_H ($P = 0.920$), food consumption ($P = 0.505$), or water consumption ($P = 0.114$).

Mean arterial blood pressure (P_{mean}) was maintained in cold hypoxia relative to warm normoxia in both populations. P_{mean} exhibited diel variation that mirrored the variation in T_{sub} and f_H (time effects, $P < 0.001$), with 6-12 mm Hg higher average pressures during the night-time active phase, but there were no significant effects of environmental treatment ($P = 0.926$) or population ($P = 0.829$) (figure 3a,b). There were 28-37 mm Hg differences in pressure between maximum and minimum P_{mean}, but these metrics were also unaffected by population and environmental treatment (figure 3c,d).

(c) Increased heart rates in cold hypoxia were underlain by changes in vagal tone

Pharmacological assessments of autonomic tone on the heart revealed significant decreases in vagal inhibitory tone in cold hypoxia. Pharmacological blockade of muscarinic acetylcholine receptors with atropine increased f_H, and this index of vagal tone was reduced by 44-63% in cold hypoxia compared to warm normoxia across populations (treatment effect, $P < 0.001$; figure 4a). In contrast, while pharmacological blockade of β_1-adrenergic receptors with metoprolol reduced f_H, this index of β_1-adrenergic tone was not significantly different between cold hypoxia and warm normoxia (treatment effect, $P = 0.172$) (figure 4b). Consistent with the lack of variation in f_H between populations, there were no population differences in vagal tone (population effect, $P = 0.375$) or β_1-adrenergic tone ($P = 0.420$).

(d) Changes in organ masses and haematology in cold hypoxia

Body mass was similar between populations (population effect, P = 0.497) and was unaffected by exposure to cold hypoxia (treatment effect, P = 0.947), but there were several population-specific changes in organ mass and haematology in chronic hypoxia (table 2). As expected, organ mass was strongly affected by body mass ($P \leq 0.001$), so we accounted for body mass as a covariate in the statistical models for all organ mass data. Cold hypoxia increased total ventricle mass by 19-27% (treatment effect, $P < 0.001$), due to increases in mass of the left ventricle and septum (LV + S; $P < 0.001$) and the right ventricle (RV; $P < 0.001$), and cold hypoxia also increased lung mass by 7-24% (P = 0.011). However, these traits were not significantly different between populations. Liver mass increased in cold hypoxia (treatment effect, $P < 0.001$), but the increase was greater in highlanders (21%) than in lowlanders (11%). Indeed, liver mass was 12% greater in highlanders than in lowlanders in cold hypoxia (but not in warm normoxia), which drove the significant main effect of population on liver mass (P = 0.002). Blood haemoglobin content ([Hb]) and haematocrit also increased in cold hypoxia (treatment effects, $P < 0.001$), but these effects were largely driven by higher values in lowlanders than in highlanders. This was evidenced by a significant main effect of population on [Hb] (P = 0.010), and a significant population×treatment interaction for haematocrit Hct (P = 0.012) that was associated with a significant pairwise difference between populations in cold hypoxia. In contrast, the mass of interscapular brown adipose tissue (iBAT) did not differ between environmental treatments (P = 0.107) or populations (P = 0.561).

4. Discussion

High-altitude endotherms that have adapted to cold hypoxic conditions in their native environment can yield appreciable insight into the adaptive evolution of endothermy and body temperature regulation to cope with metabolic challenges. Here, we show that both plastic and evolved reductions in body temperature help reduce metabolic demands in cold hypoxia in deer mice native to high altitude. Chronic exposure to cold hypoxia increased metabolic demands, as reflected by increased heart rate and food consumption, with the former associated with significant reductions in vagal tone on the heart. These metabolic demands were offset by plastic reductions in T_{sub} across the daily cycle in response to cold hypoxia. Furthermore, highlanders had consistently lower T_{sub} than

lowlanders across environmental treatments. Empirical and theoretical evidence suggested that the combined effects of plastic and evolved reductions in body temperature likely helped reduce metabolic demands in cold hypoxia by 10-30%. Therefore, plasticity and further refinement of body temperature by natural selection may help some high-altitude endotherms cope with metabolic challenges in their native environment.

Our findings emphasize the intense metabolic demands that endotherms can experience in high-altitude environments. Previous studies have shown that field metabolic rates are ~57% greater in wild deer mice at high altitude (~3,800 m elevation in the White Mountains) than in those at lower altitudes at nearby locations (1,230 m to 1,830 m elevation) when measured from July to October [25]. Part of this difference may arise from the need to forage over greater distances at high altitude, but colder temperatures above ground (daily average of 8˚C in July, -2˚C in October) and in burrows likely increase the metabolic demands of thermogenesis as well [25, 46]. Our findings here show that the latter effect was likely appreciable, with 21-26% greater f_H (figure 2) and ~37-43% greater food consumption (table 1) in cold hypoxia. Such changes arise during cold exposure because shivering and non-shivering thermogenesis augment blood flow and nutrient supply to skeletal muscles and brown adipose tissues to meet their increased metabolic demands [53-55]. Whole-animal rates of aerobic metabolism and O_2 consumption are thus increased, particularly in smaller endotherms in which their higher surface area to volume ratio makes heat retention more difficult [25, 26, 56-58]. As such, the high demands of thermogenesis at high altitude can amplify the energy and food demands of small endotherms considerably, all while hypoxia may constrain O_2 supply to support increases in aerobic metabolism. Plastic or evolved changes in physiology that help curb these demands should be highly advantageous.

Indeed, our findings suggest that plastic and evolved reductions in body temperature can reduce the metabolic demands of thermogenesis at high altitude. The measurements of T_{sub} here are expected to be slightly lower than core temperature [59] but this difference is often very small in small mammals in which internal temperature gradients are modest [42]. Exposure to cold hypoxia led to plastic reductions in T_{sub} of ~2˚C in both populations (figure 1). Previous research on low-altitude species is unclear as to whether exposure to

hypoxia, cold, or the combination of the two are needed to elicit this plasticity. In bush rats (*Rattus fuscipes*), acclimation to cold alone was shown to reduce body temperature [60]. In house mice, combined exposure to cold and hypoxia was found to reduce body temperature, but in this case exposure to cold or hypoxia alone had no effect [61]. The relative contributions of hypoxia and/or cold to plastic reductions in body temperature in deer mice remain unclear. Nevertheless, the high-altitude population exhibited a further 1˚C reduction in T_{sub} compared to their low-altitude counterparts in both environmental treatments (figure 1). Consistent with the metabolic savings that we estimated from having a lower body temperature, high-altitude deer mice exhibit lower O_2 consumption rates than low-altitude deer mice when compared at an ambient temperature of 0˚C [34]. Energy-saving strategies like body temperature reduction may be particularly advantageous for coping in high-altitude environments, where cold temperatures tend to exaggerate heat loss but hypoxia tends to constrain aerobic metabolism to support heat generation.

Our findings provide a potential example of plasticity-led evolution in a natural population. The plasticity-led evolution hypothesis posits that phenotypic plasticity can often precede and facilitate adaptation to novel environments [22, 62-67]. Specifically, plasticity can induce trait changes that enhance fitness in the initial colonists of a novel environment, after which selection then refines the trait further through genetic changes over time. The high-altitude population studied here likely evolved from a low-altitude ancestor that colonized the Rocky Mountains from the Great Plains [68]. Consistent with the plasticity-led evolution hypothesis, plastic energy-saving reductions in body temperature may have improved fitness in these low-altitude mice that initially colonized higher altitudes. Selection may have then favoured the individuals with the lowest body temperature, thereby leading to further evolved reductions in the high-altitude population. Whether this evolved reduction in body temperature is underpinned by a similar mechanism to that which induces the plastic reduction in body temperature has yet to be determined.

Although plasticity in body temperature is likely adaptive, plasticity of some other traits can be maladaptive in cold hypoxia. For example, chronic hypoxia can induce prolonged sympathoadrenal activation that leads to systemic hypertension, as observed in some previous studies of low-altitude humans [69-78] and rats [79-82], but not in house mice

[47]. Deer mice did not exhibit this pathological response to chronic hypoxia (figure 3), which may have played a role in their ability to colonize high-altitude environments. Increases in blood haemoglobin content and haematocrit are another common response to chronic hypoxia across low-altitude mammals and birds, which in this case were also observed in deer mice (table 2). While such changes may seem beneficial by increasing the O_2 carrying capacity of the blood, this benefit is more than offset by the associated increase in blood viscosity, which augments peripheral vascular resistance, can limit cardiac output and aerobic capacity, can contribute to the pathogenesis of chronic mountain sickness, and can increase the risk of stillbirth and other adverse birth outcomes [15, 83, 84]. However, this plastic response to cold hypoxia is attenuated in high-altitude deer mice, consistent with previous findings [31], suggesting that it was selected against during the process of high-altitude adaptation. Overall, our results and those of many others suggest that phenotypic plasticity is a key determinant of success in high-altitude environments, and that natural selection often reinforces adaptive plasticity and attenuates maladaptive plasticity expressed in response to cold and/or hypoxia [14-16, 19, 20, 22, 31, 67, 84, 85].

Ethics. All animal procedures followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board (Animal Use Protocol 20-01-02).

Data accessibility. Data are available from the Figshare data repository, DOI: 10.6084/m9.figshare.19615761.

Author contributions. O.H.W.: conceptualization, methodology, investigation, formal analysis, writing – original draft, writing – review and editing; G.R.S.: conceptualization, funding acquisition, methodology, supervision, formal analysis, writing – review and editing. Both authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. The authors declare that they have no conflicts of interest.

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Figure legends

Figure 1. Subcutaneous temperatures (T_{sub}), which were measured as a proxy for body temperature, in deer mice from the low-altitude population ('lowlanders') and the high-altitude population ('highlanders'), which were chronically exposed to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2). (a,b) Average hourly T_{sub} over the diel cycle (mean ± SEM) for lowlanders and highlanders in warm normoxia (solid line) and cold hypoxia (dashed line). The shaded background (19:00-07:00) illustrates when lights were off. (c,d) Maximum and minimum values of hourly T_{sub} (box and whisker plots, along with individual data as circles). †, $P < 0.05$ for pairwise differences between environmental treatments within a population; *, $P < 0.05$ for pairwise differences between populations within an environmental treatment; # and ##, $P = 0.068$ and 0.083 for pairwise differences between populations in warm normoxia or cold hypoxia, respectively.

Figure 2. Heart rate (f_H) of deer mice from the low-altitude population ('lowlanders') and the high-altitude population ('highlanders'), which were chronically exposed to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2). (a,b) Average hourly f_H over the diel cycle (mean ± SEM) for lowlanders and highlanders in warm normoxia (solid line) and cold hypoxia (dashed line). (c,d) Maximum and minimum values of hourly f_H (box and whisker plots, along with individual data as circles). See figure 1 for additional details.

Figure 3. Mean arterial pressure (P_{mean}) of deer mice from the low-altitude population ('lowlanders') and the high-altitude population ('highlanders'), which were chronically exposed to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2). (a,b) Average hourly P_{mean} over the diel cycle (mean ± SEM) for lowlanders and highlanders in warm normoxia (solid line) and cold hypoxia (dashed line). (c,d) Maximum and minimum values of hourly P_{mean} (box and whisker plots, along with individual data as circles). See figure 1 for additional details.

688 **Figure 4.** Heart rate responses (Δf_H) to pharmacological blockade of vagal tone (a) and
689 β_1-adrenergic tone (b) in deer mice from the low-altitude population ('lowlanders') and the
690 high-altitude population ('highlanders'), which were chronically exposed to and measured
691 in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2). Box and whisker
692 plots are shown along with individual data as circles. †, $P < 0.05$ for pairwise differences
693 between environmental treatments within a population ($P < 0.05$).
694

Tables

Table 1. Daily food and water consumption.

	Lowlanders		Highlanders	
	Warm Normoxia	Cold Hypoxia	Warm Normoxia	Cold Hypoxia
n	15	12	13	15
Food (mg g^{-1})	199 ± 17	273 ± 21†	185 ± 10	264 ± 15†
Water (mg g^{-1})	360 ± 36	312 ± 18	307 ± 24	268 ± 15

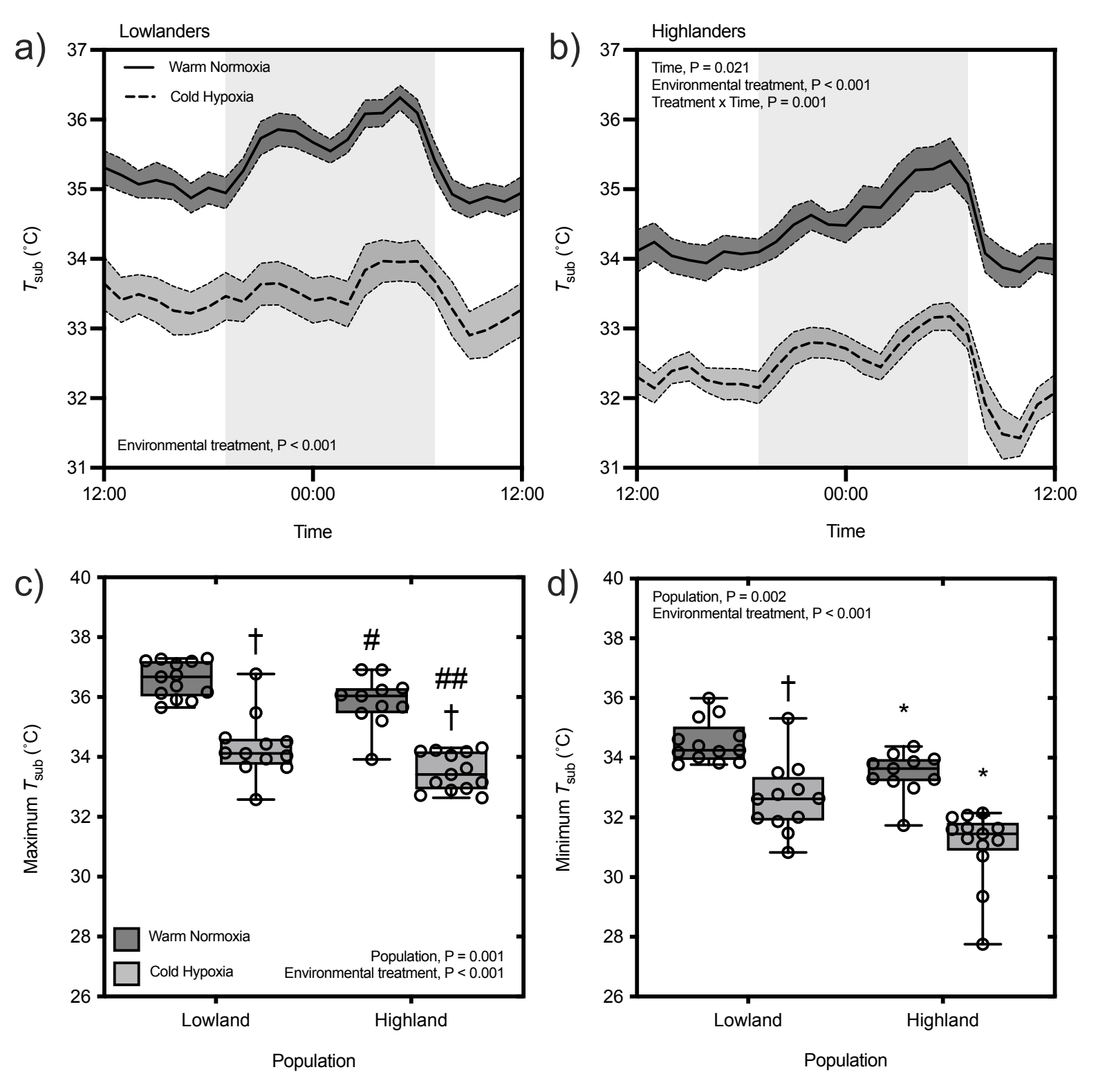
Data are presented as mean ± SEM and are expressed per g body mass. †, $P < 0.05$ for pairwise differences between environmental treatments within a population in Holm-adjusted Tukey's HSD post-tests.

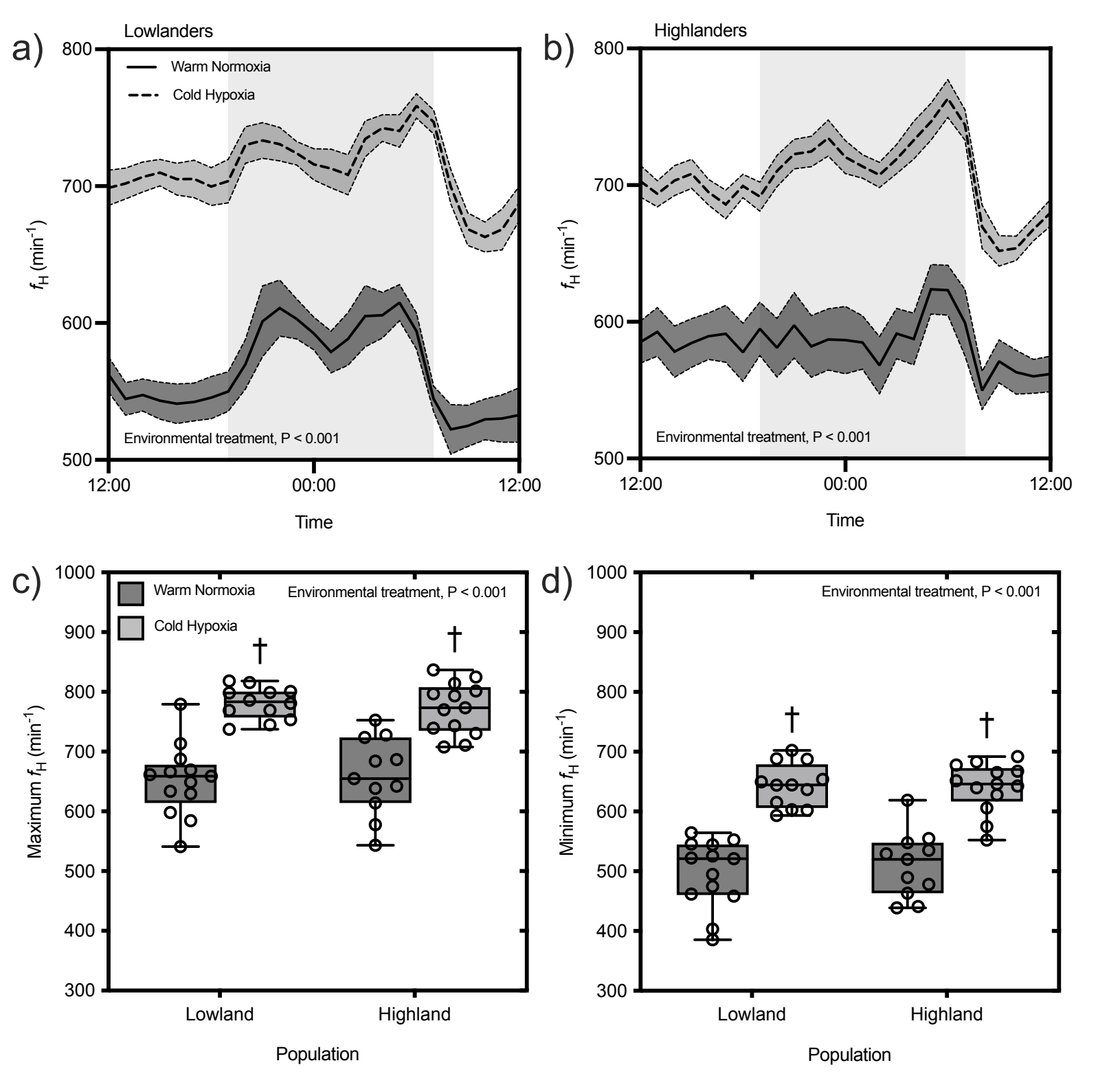
699 **Table 2.** Organ masses and haematology

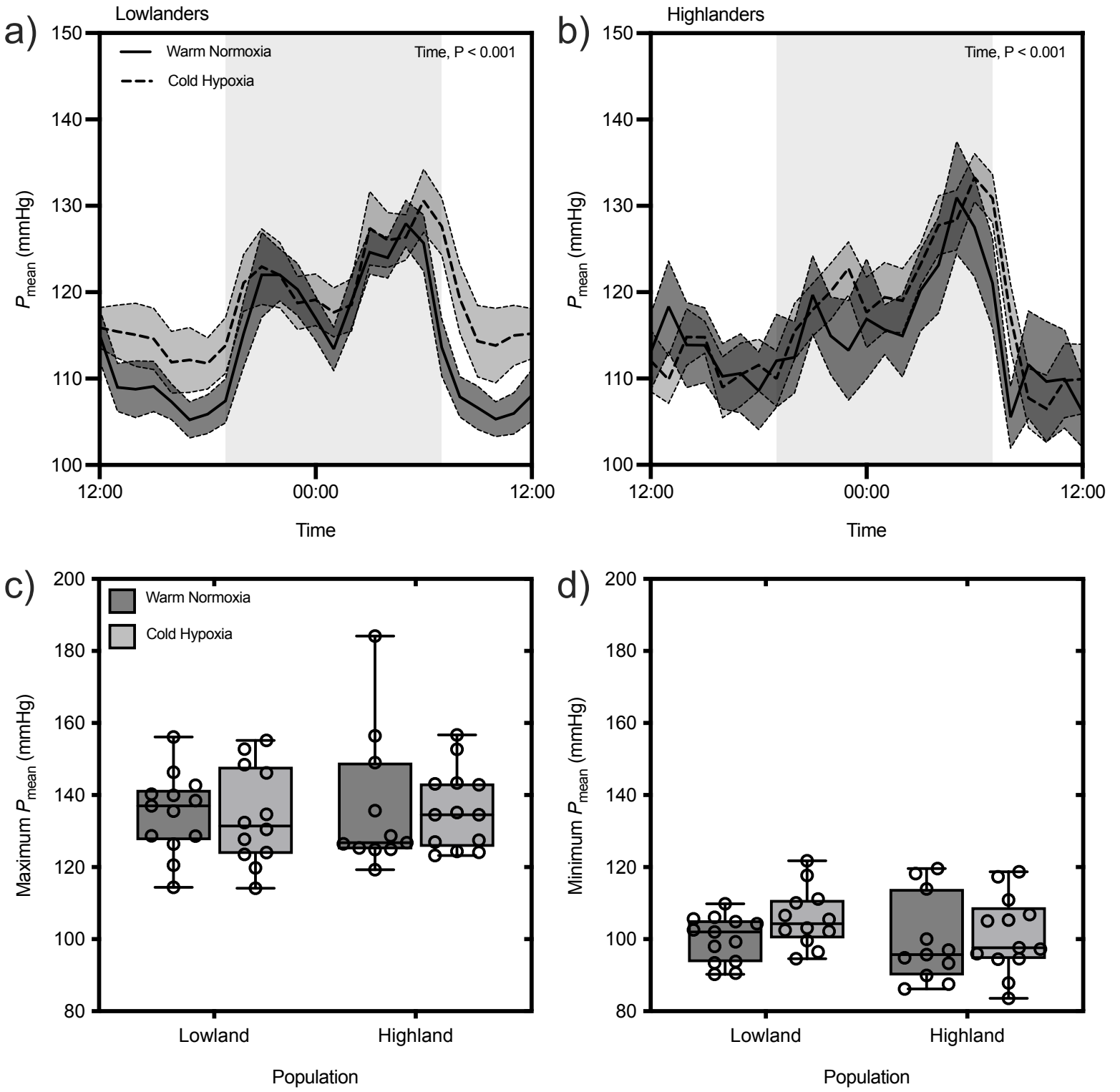
	Lowlanders		Highlanders	
	Warm Normoxia	Cold Hypoxia	Warm Normoxia	Cold Hypoxia
n	14	11	13	13
Animal mass (g)	22.9 ± 1.5	20.8 ± 0.9	19.8 ± 0.6	21.2 ± 0.6
Total ventricle mass (mg g^{-1})	5.65 ± 0.15	7.16 ± 0.27†	5.78 ± 0.15	6.85 ± 0.21†
RV mass (mg g^{-1})	1.33 ± 0.05	1.78 ± 0.09†	1.26 ± 0.05	1.64 ± 0.09†
LV+S mass (mg g^{-1})	4.33 ± 0.15	5.39 ± 0.27†	4.52 ± 0.13	5.22 ± 0.19†
Lung mass (mg g^{-1})	6.79 ± 0.42	8.45 ± 0.50	7.66 ± 0.20	8.20 ± 0.45
Liver mass (mg g^{-1})	40.1 ± 1.4	44.5 ± 1.1†	41.2 ± 1.1	49.9 ± 1.0†*
iBAT mass (mg g^{-1})	5.01 ± 0.31	5.25 ± 0.35	4.54 ± 0.24	5.30 ± 0.51
[Hb] (g dl^{-1})	11.7 ± 0.4	14.3 ± 0.5†	11.2 ± 0.4	12.6 ± 0.4†
Hct (%)	35.7 ± 1.0	45.3 ± 1.7†	35.9 ± 1.2	39.2 ± 0.0†*

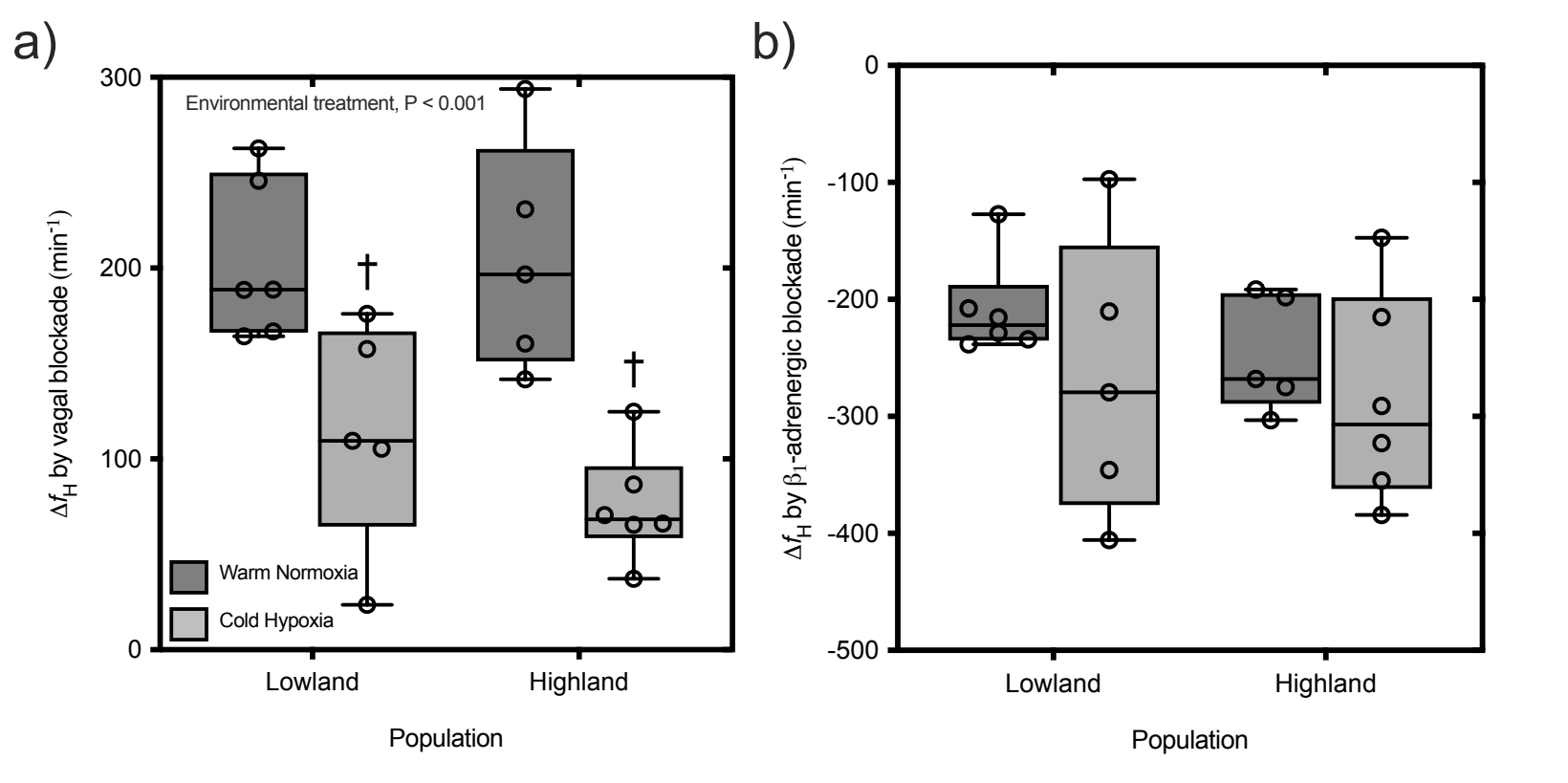
RV, right ventricle; LV+S, left ventricle and septum; iBAT, interscapular brown adipose tissue; [Hb], blood haemoglobin concentration; Hct, haematocrit. Data are presented as mean ± SEM, and organ mass data are expressed per g body mass. †, $P < 0.05$ for pairwise differences between environmental treatments within a population in Holm-adjusted Tukey's HSD post-tests. *, $P < 0.05$ for pairwise differences between populations within an environmental treatment.

700


a)
Lowlanders
Warm Normoxia
Cold Hypoxia
Environmental treatment, P < 0.001
T_{sub} (˚C)
Time
12:00
00:00
12:00
b)
Highlanders
Time, P = 0.021
Environmental treatment, P < 0.001
Treatment x Time, P = 0.001
c)
Maximum T_{sub} (˚C)
Warm Normoxia
Cold Hypoxia
Population, P = 0.001
Environmental treatment, P < 0.001
Lowland
Highland
Population
d)
Minimum T_{sub} (˚C)
Population, P = 0.002
Environmental treatment, P < 0.001


a)
Lowlanders
Warm Normoxia
Cold Hypoxia
Environmental treatment, P < 0.001
f_H (min^{-1})
Time
12:00
00:00
12:00
b)
Highlanders
Environmental treatment, P < 0.001
c)
Warm Normoxia
Cold Hypoxia
Environmental treatment, P < 0.001
Maximum f_H (min^{-1})
Lowland
Highland
Population
d)
Environmental treatment, P < 0.001
Minimum f_H (min^{-1})
Lowland
Highland
Population


a)
Lowlanders
Warm Normoxia
Cold Hypoxia
Time, P < 0.001
P_{mean} (mmHg)
150
140
130
120
110
100
12:00
00:00
12:00
Time
b)
Highlanders
Time, P < 0.001
P_{mean} (mmHg)
150
140
130
120
110
100
12:00
00:00
12:00
Time
c)
Warm Normoxia
Cold Hypoxia
Maximum P_{mean} (mmHg)
200
180
160
140
120
100
80
Lowland
Highland
Population
d)
Minimum P_{mean} (mmHg)
200
180
160
140
120
100
80
Lowland
Highland
Population


a)
Environmental treatment, P < 0.001
300
200
100
0
Δf_H by vagal blockade (min^{-1})
†
†
Warm Normoxia
Cold Hypoxia
Lowland
Highland
Population
b)
0
-100
-200
-300
-400
-500
Δf_H by β_1-adrenergic blockade (min^{-1})
Lowland
Highland
Population

Supplementary Materials to Accompany the Article:

Evolved reductions in body temperature and the metabolic costs of thermoregulation in deer mice native to high altitude

By Oliver H. Wearing and Graham R. Scott

Supplementary Methods

(a) Surgical instrumentation of physiological telemeters

Anaesthesia was induced in an anaesthetic induction chamber using 3% isoflurane balanced with O_2 delivered at 1500 ml min^{-1}. Once a surgical plane of anaesthesia was reached, mice were transferred to a nose cone delivered with isoflurane for maintaining anaesthesia, given a subcutaneous dose of buprenorphine (0.1 mg per kg body weight; dissolved in 0.5 ml sterile 0.9% saline), eye lubricant was applied, and the ventral surface of the neck was shaved and scrubbed using iodine and isopropyl alcohol. The mouse was placed supine on a sterile surgical drape above a heating pad and a surgical plane of anaesthesia was maintained using 1-2% isoflurane delivered to the nose cone. A 15-mm incision was made along the midline of the neck, and the left carotid artery was carefully isolated by blunt dissection. The artery was occlusively cannulated using the fluid-filled pressure catheter of a small-animal radiotelemetry implant capable of measuring blood pressure and temperature (HD-X11, Data Sciences International Inc., MN, USA). The main body of the telemetry implant was tunnelled subcutaneously to a dorsal location in the interscapular space (a location that was determined in preliminary surgeries to be best tolerated by deer mice). The neck incision was sutured closed using an interrupted subcuticular suture (6-0 Vicryl with 10 mm reverse cutting needle, Ethicon Inc., NJ, USA). Mice were then recovered from anaesthesia, placed and housed individually in cages with cellulose bedding (Teklad diamond dry, Envigo, IN, USA) in their respective environmental treatment conditions. Recovering mice were provided with a combined subcutaneous dose of 0.1 mg kg^{-1} buprenorphine and 5 mg kg^{-1} carprofen in 1 ml sterile saline 8 h after surgery, and then another 5 mg kg^{-1} carprofen 12 h and 24 h after that. Unfortunately, 1 warm normoxic lowlander, 1 cold hypoxic lowlander and 2 cold hypoxic highlanders

recovered poorly from surgery and were immediately euthanized. These mice were omitted from the final telemetry dataset.

(b) Physiological responses to acute stepwise hypoxia

We used acute manipulations of inspired O_2 pressure (PO_2) to examine the relationship between subcutaneous temperature (T_{sub}) and O_2 consumption rate ($\dot{V}O_2$) in each treatment group, conducted the day after pharmacological assessments of chronotropic tone on the heart. Each mouse was placed in an open-flow plethysmography chamber (530 ml) and left for 20 to 60 min to become accustomed to the chamber (as reflected by stable resting $\dot{V}O_2$). PO_2 was reduced every 20 min in stepwise increments – 21, 16, 12, 10, 9, and 8 kPa O_2. The desired PO_2 was achieved by mixing compressed O_2 and N_2 using precision flow meters (Sierra Instruments, CA, USA) and a mass flow controller (MFC-4, Sable Systems, NV, USA). Incurrent gas was sub-sampled at 200 ml min^{-1} and used to measure incurrent O_2 with a fuel cell O_2 analyzer (FC-10, Sable Systems). Incurrent flow rate into the chamber (~600 ml min^{-1}) was measured using a precision flow meter (Alicat Scientific, Inc.). Excurrent gas leaving the animal chamber was subsampled at 200 ml min^{-1}, scrubbed of water vapour (Drierite, W.A. Hammond Drierite Co., Ltd., OH, USA), and analyzed for O_2 (FC-10, Sable Systems) and CO_2 (CA-10, Sable Systems). These data were acquired using a PowerLab 16/32 and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, USA). T_{sub} measured by the telemeter implant was acquired continuously (1 kHz sampling rate) using a Matrix 2.0 data acquisition system, and using PhysioTel Connect (ADInstruments, CO, USA) to record the data in Labchart 8 Pro. $\dot{V}O_2$ was determined at rest during the last 10 min at each PO_2 bout, calculated using established formulas [1]. The relationship between changes in $\dot{V}O_2$ and T_{sub} were examined for each of the four experimental groups using linear regressions of relative $\dot{V}O_2$ (quotient of $\dot{V}O_2$ at reduced PO_2 and $\dot{V}O_2$ at 21 kPa O_2) against T_{sub} (see Fig. S1).

(c) Theoretical consideration of the effects of body temperature on metabolic rate

We used two theoretical approaches to calculate the expected relative effects of changes in body temperature (T_b) on metabolic rate. First, we calculated the theoretically

expected effects of a 1˚C change in T_b (ΔT_b) on metabolic rate based on Q_{10} temperature coefficients of 2 and 3, using the following equation for Q_{10}:

$$Q_{10} = \left(\frac{V_1}{V_2}\right)^{\frac{10}{\Delta T_b}}$$

where V_1 is the $\dot{V}O_2$ at the lower temperature and V_2 is the $\dot{V}O_2$ at the higher temperature.

Second, we calculated the expected effects of a 1˚C change in T_b using the Scholander-Irving model of thermoregulation [2]. Stable T_b is maintained when rates of heat loss are balanced by equal rates of metabolic heat production. As a result, $\dot{V}O_2$ at ambient temperatures (T_a) below the thermoneutral zone is a function of the difference between T_b and T_a, and the thermal conductance (C) of the animal (as determined by insulation, etc.):

$$\dot{V}O_2 = C\ (T_b - T_a)$$

Therefore, independent of any changes in C, the effect of a 1˚C change in T_b can be calculated as follows:

$$\left(\frac{V_1}{V_2}\right) = \left(\frac{T_{b1} - T_a}{T_{b2} - T_a}\right)$$

where T_{b1} is the lower T_b and T_{b2} is the higher T_b.

References

1. Lighton J.R.B. 2018 *Measuring Metabolic Rates: A Manual for Scientists*, OUP Oxford.

2. Scholander P.F., Hock R.J., Walters V., Johnson F., Irving L. 1950 Heat regulation in some arctic and tropical mammals and birds. *Biol Bull* **99**(2), 237-258. (doi:10.2307/1538741).

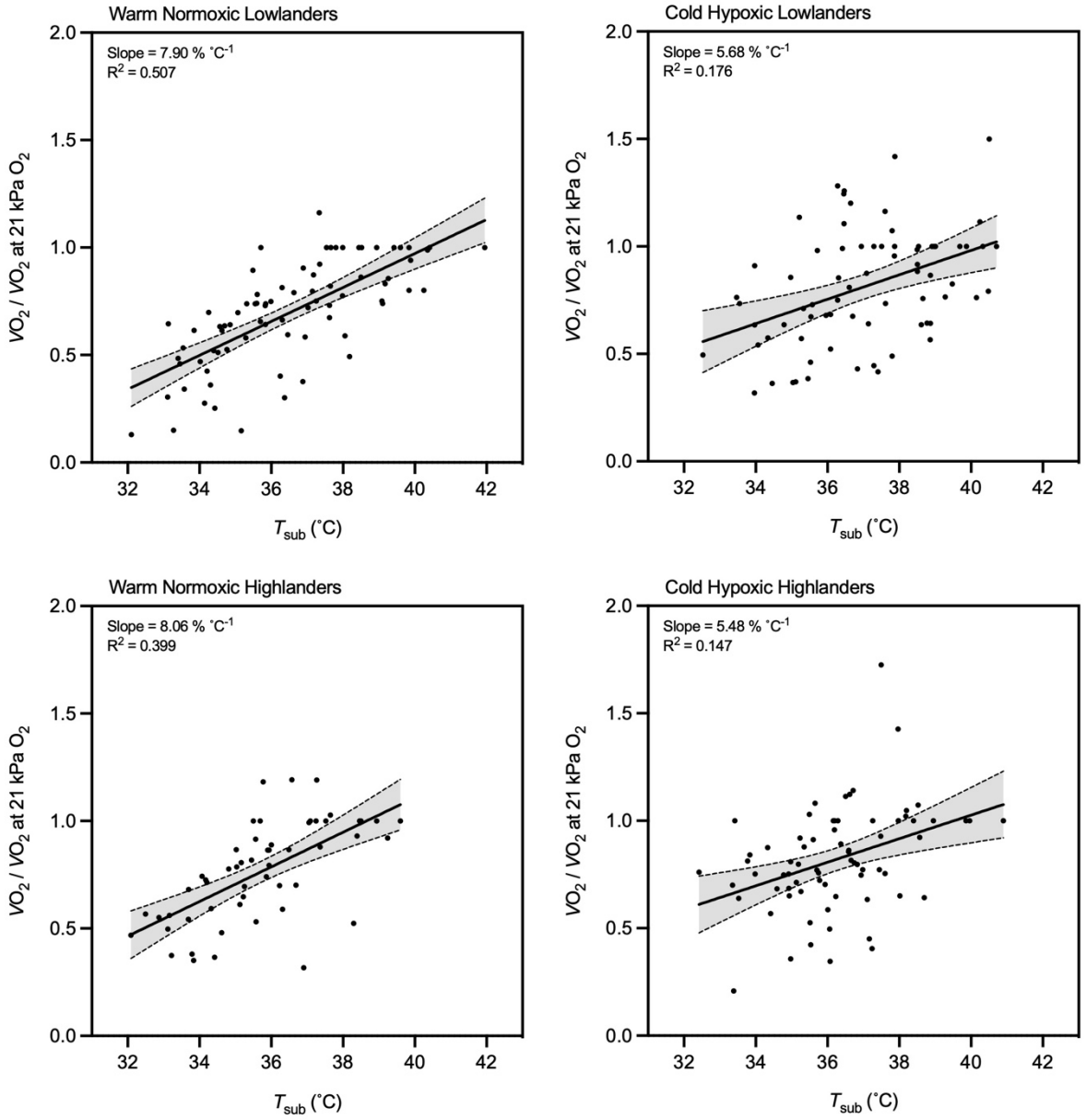


Figure S1. Relationships between subcutaneous temperature (T_{sub}) and resting O_2 consumption rate ($\dot{V}O_2$, relative to $\dot{V}O_2$ at 21 kPa O_2), assessed by using acute hypoxia to cause reductions in T_{sub} without changes in ambient temperature (25˚C) in lowland and highland deer mice chronically exposed to warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2). Circles represent individual measurements, which were made in each animal across several inspired O_2 levels during stepwise exposure to increasingly severe levels of hypoxia (21, 16, 12, 10, 9, and 8 kPa O_2), and solid lines represent linear regressions (dashed lines are 95% confidence intervals of the regression). The number of animals in each group were as follows: warm normoxic lowlanders, n = 13; cold hypoxic lowlanders, n = 12; warm normoxic highlanders, n = 9; cold hypoxic highlanders, n = 12).

Table S1. Results of statistical comparisons using linear mixed models on subcutaneous temperature and cardiovascular function throughout the diel cycle in lowland and highland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Trait	Population (*p*) effect	Environmental treatment (*e*) effect	Time (*t*) effect	*p* x *e* effect	*p* x *t* effect	*e* x *t* effect	*p* x *e* x *t* effect
T_{sub}	**P < 0.001**	**P < 0.001**	**P = 0.013**	P = 0.835	P = 0.318	**P < 0.001**	P = 0.177
f_H	P = 0.920	**P < 0.001**	P = 0.216	P = 0.255	P = 0.347	P = 0.793	P = 0.535
P_{mean}	P = 0.829	P = 0.926	**P < 0.001**	P = 0.538	P = 0.865	**P = 0.031**	P = 0.712

T_{sub}, subcutaneous temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S2. Results of statistical comparisons using linear mixed models on subcutaneous temperature and cardiovascular function throughout the diel cycle in lowland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Trait	Environmental treatment (*e*) effect	Time (*t*) effect	*e* x *t* effect
T_{sub}	**P < 0.001**	P = 0.262	P = 0.064
f_H	**P < 0.001**	P = 0.840	P = 0.546
P_{mean}	P = 0.393	**P < 0.001**	P = 0.193

T_{sub}, subcutaneous temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S3. Results of statistical comparisons using linear mixed models on subcutaneous temperature and cardiovascular function throughout the diel cycle in highland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Trait	Environmental treatment (*e*) effect	Time (*t*) effect	*e* x *t* effect
T_{sub}	**P < 0.001**	**P = 0.021**	**P = 0.001**
f_H	**P < 0.001**	P = 0.109	P = 0.792
P_{mean}	P = 0.652	**P < 0.001**	P = 0.084

T_{sub}, subcutaneous temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S4. Results of statistical comparisons using linear mixed models on maximum and minimum hourly values of subcutaneous temperature and cardiovascular function in lowland and highland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Trait	Population (*p*) effect	Environmental treatment (*e*) effect	*p* x *e* effect
Max. T_{sub}	**P = 0.001**	**P < 0.001**	P = 0.816
Min. T_{sub}	**P = 0.002**	**P < 0.001**	P = 0.368
Max. f_H	P = 0.628	**P < 0.001**	P = 0.553
Min. f_H	P = 0.802	**P < 0.001**	P = 0.619
Max. P_{mean}	P = 0.863	P = 0.689	P = 0.989
Min. P_{mean}	P = 0.399	P = 0.225	P = 0.466

T_{sub}, subcutaneous temperature; f_H, heart rate; P_{mean}, mean arterial blood pressure. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S5. Results of statistical comparisons using linear mixed models on food and water consumption in lowland and highland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Consumption	Population (*p*) effect	Environmental treatment (*e*) effect	*p* x *e* effect
Food	P = 0.505	**P < 0.001**	P = 0.885
Water	P = 0.114	P = 0.122	P = 0.938

Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S6. Results of statistical comparisons using linear mixed models on the response of heart rate (f_H) to pharmacological blockade in lowland and highland deer mice acclimated to and measured in warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Blockade	Population (*p*) effect	Environmental treatment (*e*) effect	*p* x *e* effect
β_1-AR	P = 0.420	P = 0.172	P = 0.770
Vagal	P = 0.375	**P < 0.001**	P = 0.330

β_1-AR, β_1-adrenergic receptor. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.

Table S7. Results of statistical comparisons using linear mixed models on mass data for lowland and highland deer mice acclimated to warm normoxia (25˚C, 21 kPa O_2) or cold hypoxia (5˚C, 12 kPa O_2).

Trait	Body mass effect	Population (p) effect	Environmental treatment (*e*) effect	*p* x *e* effect
Body mass	NA	P = 0.497	P = 0.947	P = 0.408
Ventricles mass	**P < 0.001**	P = 0.882	**P < 0.001**	P = 0.183
RV mass	**P < 0.001**	P = 0.369	**P < 0.001**	P = 0.470
LV+S mass	**P < 0.001**	P = 0.884	**P < 0.001**	P = 0.243
Lungs mass	**P = 0.001**	P = 0.557	**P = 0.011**	P = 0.360
Liver mass	**P < 0.001**	**P = 0.002**	**P < 0.001**	P = 0.240
iBAT mass	**P < 0.001**	P = 0.561	P = 0.107	P = 0.586
[Hb]	NA	**P = 0.010**	**P < 0.001**	P = 0.165
Hct	NA	**P = 0.017**	**P < 0.001**	**P = 0.012**

RV, right ventricle; LV+S, left ventricle and septum; iBAT, interscapular brown adipose tissue; [Hb], blood haemoglobin concentration; Hct, haematocrit. Individual mouse, sex and family were accounted for in the models as random factors. Significant effects (P < 0.05) are shown in bold.