Genetic variation in haemoglobin is associated with evolved changes in breathing in high-altitude deer mice

Catherine M. Ivy[1]*, Oliver H. Wearing[1], Chandrasekhar Natarajan[2], Rena M. Schweizer[3], Natalia Gutiérrez-Pinto[2], Jonathan P. Velotta[3], Shane C. Campbell-Staton[4], Elin E. Petersen[5], Angela Fago[5], Zachary A. Cheviron[3], Jay F. Storz[2], Graham R. Scott[1]

*Corresponding author: Catherine M. Ivy

civy2@uwo.ca

[1]Department of Biology, McMaster University, Hamilton, ON, Canada, L8S 4K1;

[2]School of Biological Sciences, University of Nebraska, Lincoln, NE, USA, 68588;

[3]Divison of Biological Sciences, University of Montana, Missoula, MT, USA, 59812;

[4]Department of Ecology and Evolutionary Biology, University of California, Los Angeles, CA, USA, 90095;

[5]Department of Biology, Aarhus University, Aarhus, Denmark C, 8000

Key words: evolutionary physiology, hypoxia acclimation, pulmonary ventilation, ventilatory acclimatization to hypoxia.

ABSTRACT

Physiological systems often have emergent properties but the effects of genetic variation on physiology are often unknown, which presents a major challenge to understanding the mechanisms of phenotypic evolution. We investigated whether genetic variants in haemoglobin (Hb) that contribute to high-altitude adaptation in deer mice (*Peromyscus maniculatus*) are associated with evolved changes in control of breathing. We created F_2 inter-population hybrids of highland and lowland deer mice to test for phenotypic associations of α- and β-globin variants on a mixed genetic background. Hb genotype had expected effects on Hb-O_2 affinity that were associated with differences in arterial O_2 saturation in hypoxia. However, high-altitude genotypes were also associated with breathing phenotypes that should contribute to enhancing O_2 uptake in hypoxia. Mice with highland α-globin exhibited a more effective breathing pattern, with highland homozygotes breathing deeper but less frequently across a range of inspired O_2, and this difference was comparable to the evolved changes in breathing pattern in deer mouse populations native to high altitude. The ventilatory response to hypoxia was augmented in mice that were homozygous for highland β-globin. The association of globin variants with variation in breathing phenotypes could not be recapitulated by acute manipulations of Hb-O_2 affinity, because treatment with efaproxiral (a synthetic drug that acutely reduces Hb-O_2 affinity) had no effect on breathing in normoxia or hypoxia. Therefore, adaptive variation in haemoglobin may have unexpected effects on physiology in addition to the canonical function of this protein in circulatory O_2 transport.

Summary Statement: High-altitude variants in haemoglobin genes are associated with evolved changes in breathing that likely enhance O_2 uptake in hypoxia in deer mice

INTRODUCTION

High-altitude natives are an exceptional model for understanding the genetic and physiological bases of evolutionary adaptation. Species that are broadly-distributed across altitudes can provide powerful insight into the genetic basis of high-altitude adaptation, because it is possible to examine segregating variation for phenotypes that may contribute to hypoxia tolerance. Recent research has identified many genes that appear to have experienced selection in high-altitude taxa, including genes thought to be involved in O_2 transport, energy metabolism, and hypoxia signalling (Simonson, 2015; Simonson et al., 2012; Storz and Cheviron, 2021). However, in most cases, the specific effects of these genetic variants on physiological function are poorly understood. Identifying these functional effects has the potential to uncover novel and adaptive physiological mechanisms, given the growing appreciation that protein variants can have auxiliary effects that are unrelated to the 'canonical' function of the protein in question (Marden, 2013a).

Evolved changes in haemoglobin have contributed to hypoxia adaptation in many high-altitude mammals and birds (Storz, 2016). Haemoglobin (Hb) is a tetramer containing two α- and two β-chain subunits, and its O_2-binding affinity is an important determinant of O_2 exchange at the lungs and peripheral tissues. Evolved increases in Hb-O_2 affinity have arisen in many high-altitude taxa, and are typically attributable to amino acid replacements in the α- and/or β-chain subunits that increase intrinsic O_2-affinity and/or reduce responsiveness to negative allosteric cofactors (e.g. 2,3-DPG in mammals) (Galen et al., 2015; Jendroszek et al., 2018; Natarajan et

al., 2015a; Natarajan et al., 2016; Natarajan et al., 2018; Projecto-Garcia et al., 2013; Signore et al., 2019; Storz et al., 2010; Tufts et al., 2015; Zhu et al., 2018). An increased Hb-O_2 affinity is generally assumed to safeguard arterial O_2 saturation in hypoxia, but it remains possible that modifications of Hb function contribute to hypoxia tolerance via other physiological mechanisms that are not directly related to circulatory O_2 transport.

Haemoglobin is not generally thought to be directly involved in the regulation of breathing, but some evidence suggests that this might not be the case. On the one hand, pharmacological manipulations that increase Hb-O_2 binding affinity do not generally appear to exert much influence on ventilatory responses to hypoxia in guinea pigs and rats (Birchard and Tenney, 1986; Rivera-Ch et al., 1994), nor do some naturally occurring mutant haemoglobins that have altered O_2 affinity (e.g., Andrew-Minneapolis mutation in the β-globin subunit of humans) (Hebbel et al., 1977). By contrast, affinity-altering mutations in Hbs of mice and sheep are associated with changes in ventilatory sensitivity to O_2 and/or CO_2 (Dawson and Evans, 1966; Izumizaki et al., 2003; Shirasawa et al., 2003). Furthermore, deoxygenated Hb can produce *S*-nitrosothiols that contribute to signalling the ventilatory response to hypoxia in rats (Lipton et al., 2001). These findings together suggest that Hb or its constituent α/β globin monomers may play an underappreciated role in the control of breathing, by a mechanism that is not directly associated with the role of Hb in circulatory O_2 transport.

High-altitude deer mice have evolved an increased Hb-O_2 affinity relative to lowland conspecifics (Snyder et al., 1982; Storz et al., 2010), and this modification of protein function is associated with increased arterial O_2 saturation in hypoxia (Ivy and Scott, 2017; Ivy et al., 2020; Tate et al., 2017; Wearing et al., 2021). Experimental studies have identified the mutations in the α- and β-chain subunits that are responsible for population differences in Hb-O_2 affinity (Jensen

et al., 2016; Natarajan et al., 2013; Natarajan et al., 2015b; Storz et al., 2009; Storz et al., 2010) and population-genetic analyses on sequence variation in the underlying genes provided evidence that the altitudinal patterning of Hb polymorphism is attributable to a history of spatially varying selection that favors different allelic variants in different elevational zones (Storz and Kelly 2008; Storz et al. 2012). High-altitude deer mice have also experienced strong directional selection for increased aerobic capacity for thermogenesis (Hayes and O'Connor, 1999), which has led to evolved increases in maximal rates of O_2 consumption ($\dot{V}O_{2max}$) in hypoxia (Cheviron et al., 2013; Cheviron et al., 2014b; Tate et al., 2017; Tate et al., 2020). Studies of inter-population hybrids of deer mice from high and low altitudes, in which effects of individual genotypes and phenotypes can be assessed on a mixed genetic background, have been used to examine the contribution of Hb-O_2 affinity and other respiratory traits to this evolved increase in $\dot{V}O_{2max}$ (Chappell and Snyder, 1984; Wearing et al., 2021). In addition, high-altitude mice have evolved an enhanced hypoxic ventilatory response and a deeper breathing pattern (larger tidal volumes but lower breathing frequencies at a given level of total ventilation) under routine conditions, both of which should increase alveolar ventilation and be more effective for gas exchange in hypoxia (Ivy and Scott, 2017; Ivy and Scott, 2018; Ivy et al., 2020). However, the potential contribution of genetic variation in Hb to these evolved changes in control of breathing has yet to be examined.

Here we report an investigation of whether high-altitude Hb variants contribute to the evolved changes in control of breathing we have previously reported in high-altitude populations of deer mice (Ivy and Scott, 2017; Ivy and Scott, 2018; Ivy et al., 2020). This was achieved using an F_2 intercross breeding design to isolate the effects of allelic variants of α- and β-globins against an admixed genetic background. We then examined whether the physiological effects of

variation in Hb genes resulted from changes in Hb-O_2 affinity, using efaproxiral (a synthetic drug that acts as a negative allosteric regulator of Hb-O_2 binding) to acutely reduce Hb-O_2 affinity *in vivo* (Donnelly et al., 2006; Khandelwal et al., 1993).

METHODS

Deer mouse populations and breeding designs

Wild adult deer mice were live-trapped at high altitude on the summit of Mount Evans (Clear Creek County, CO, USA at 39°35'18"N, 105°38'38"W; 4,350 m above sea level) (*P. m. rufinus*) and at low altitude on the Great Plains (Nine Mile Prairie, Lancaster County, NE, USA at 40°52'12"N, 96°48'20.3"W; 430 m above sea level) (*P. m. nebrascensis*) and were transported to the University of Montana (elevation 978 m) or to McMaster University (elevation 50 m). The wild mice transported to Montana were used to produce one family of first-generation inter-population hybrids (F_1), created by crossing a highland male and a lowland female. These F_1 hybrids were raised to maturity and were used for full-sibling matings to produce 4 families of second-generation hybrid progeny (F_2). These F_2 hybrids (N=26) were raised to adulthood, a small volume of blood was obtained for genotyping (sampled from the facial vein and then stored at -80°C), and mice were then transported to McMaster for subsequent experiments (see below). Each F_2 hybrid was genotyped (described below) to determine the sequence of its α- and β-globin haplotypes, resulting in the 5 distinct combinations of highland and lowland haplotypes of α- and β-globin that were studied here: N=5 $\alpha^{HH}\beta^{HH}$, N=5 $\alpha^{HH}\beta^{HL}$, N=7 $\alpha^{HH}\beta^{LL}$, N=4 $\alpha^{LL}\beta^{HH}$ and N=5 $\alpha^{LL}\beta^{HL}$. These F_2 hybrid mice were also used for a distinct study on aerobic capacity (Wearing et al., 2021). The wild mice transported to McMaster were bred in captivity to produce first-generation (G_1) progeny within each population. All mice

were held in a standard holding environment (~24-25°C, 12 h:12 h light:dark photoperiod) under normal atmospheric conditions before experiments, and were provided with unlimited access to water and standard mouse chow. All animal protocols were approved by institutional animal research ethics boards.

Adult isoforms of tetrameric haemoglobin from *P. maniculatus* incorporate α-chain subunits that are encoded by two tandem gene duplicates, *HBA-T1* and *HBA-T2* (separated by 5.0 kb on Chromosome 8), and β-chain subunits that are encoded by two other tandem duplicates, *HBB-T1* and *HBB-T2* (separated by 16.2 kb on Chromosome 1) (Hoffmann et al., 2008; Natarajan et al., 2015b). We used a reverse-transcriptase PCR (RT-PCR) approach to obtain sequence data for all four of the adult-expressed α- and β-globin transcripts in the full panel of mice (Natarajan et al., 2015b; Storz et al., 2010). The RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) was used to extract total RNA from red blood cells. We then amplified globin transcripts from 1 μg of extracted RNA using the One-Step RT-PCR system with Platinum *Taq* DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). PCR cycling conditions were as follows: 1 cycle at 50 °C for 30 min, 1 cycle at 95 ˚C for 15 min, 34 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and then a final extension cycle at 72 °C for 3 min. For the α-globin transcripts, we used the same primer pair for *HBA-T1* and *HBA-T2* (forward: CTGATTCTCACAGACTCAGGAAG, reverse: CCAAGAGGTACAGGTGCGAG). For the β-globin transcripts, we used the same RT-PCR primer pair for *HBB-T1* and *HBB-T2* (forward: GACTTGCAACCTCAGAAACAGAC, reverse: GACCAAAGGCCTTCATCATTT). We cloned and sequenced the RT-PCR products using TOPO® TA Cloning Kit (Life Technologies, Carlsbad, CA, USA), and we sequenced at least six clones per gene in order to recover both alleles from the paralogs. Full-length cDNAs of all expressed *HBA* and *HBB* genes were thereby

sequenced at 6-fold coverage, and the haplotype phase of all variable sites was determined experimentally.

Physiological phenotyping of inter-population hybrids with different haemoglobin genotypes

F_2 hybrids were subjected to a series of measurements during adulthood (1-1.5 years old), both before and after exposure to chronic hypoxia. Acute hypoxia responses and haematology were first measured in mice held in normoxia. Four days later, the mice were moved into specifically designed hypobaric chambers that have been previously described (Ivy and Scott, 2017; Lui et al., 2015; McClelland et al., 1998) and were thus acclimated to chronic hypoxia (barometric pressure of 60 kPa, simulating the pressure at an elevation of 4,300 m; O_2 pressure ~12.5 kPa). During this time, mice were temporarily returned to normobaric conditions twice per week for <20 min for cage cleaning. Acute hypoxia responses were measured again after 6-8 weeks in chronic hypoxia. Mice were finally euthanized after a full 8 weeks of chronic hypoxia acclimation with an overdose of isoflurane followed by cervical dislocation, blood was collected for the second set of haematology measurements.

Acute hypoxia responses were measured in unrestrained mice using barometric plethysmography, respirometry, and pulse oximetry techniques that we have used in previous studies (Ivy and Scott, 2017; Ivy and Scott, 2018; Ivy et al., 2020). Mice were placed in a whole-body plethysmography chamber (530 ml) that was supplied with hyperoxic air (30 kPa O_2, balance N_2) at 600 ml min^{-1}. Mice were given 20-40 min to adjust to the chamber until relaxed and stable breathing and metabolism were observed. Mice were then maintained for an additional 20 min at 30 kPa O_2, after which they were exposed to 20-min stepwise reductions in inspired O_2 pressure (PO_2) of 21, 16, 12, 10, and 8 kPa. Dry incurrent gases were mixed using precision flow

meters (Sierra Instruments, Monterey, CA, USA) and a mass flow controller (MFC-4, Sable Systems, Las Vegas, NV, USA), such that the desired PO_2 was delivered to the chamber at a constant flow rate of 600 ml min^{-1}. At the end of this protocol, mice were removed from the chamber and returned to their home cage in the appropriate acclimation condition.

Breathing (total ventilation, breathing frequency, and tidal volume), rates of O_2 consumption (O_2), body temperature (T_b), heart rate, and arterial O_2 saturation were determined during the last 10 min at each PO_2 as follows. Incurrent and excurrent air flows were subsampled at 200 ml min^{-1}; incurrent air was continuously measured for O_2 fraction (FC-10, Sable Systems), and excurrent air was analyzed for water vapour (RH-300, Sable Systems), dried with pre-baked drierite, and analyzed for O_2 and CO_2 fraction (FC-10 and CA-10, able ystems). These data were used to calculate O_2, expressed in volumes at standard temperature and pressure (STP), using established equations (Lighton, 2008). Chamber temperature was continuously recorded with a thermocouple (TC-2000, Sable Systems). Breathing frequency, tidal volume, and total ventilation were measured using whole-body plethysmography as previously described (Ivy and Scott, 2017; Ivy and Scott, 2018) and reported volumes are expressed at body temperature and pressure saturated (T). Air convection requirement (AC) is the quotient of total ventilation and O_2. All the above data was acquired using PowerLab 16/32 and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, USA). T_b was measured using thermosensitive passive transponders (micro LifeChips with Bio-therm technology; Destron Fearing, Dallas, TX, USA), which were implanted subdermally on the left side of the abdomen close to the leg ~2 weeks before normoxic measurements were conducted, along with a hand-held scanner from the same manufacturer. Arterial O_2 saturation and heart rate were measured using MouseOx Plus pulse

oximeter collar sensors and data acquisition system (Starr Life Sciences, Oakmont, PA, USA). This was enabled by removing fur around the neck ~2 days before experiments.

Blood was collected into heparinized capillary tubes for haematology, sampled from the facial vein under light anaesthesia for mice acclimated to normoxia (~130 µl), or by severing the jugular vein for mice that were euthanized and sampled after acclimation to chronic hypoxia (~400 µl). lood Hb content was measured using Drabkin's reagent according to the manufacturer's instructions (i gma-Aldrich, Oakville, ON, Canada). Haematocrit was measured by spinning the blood in the capillary tubes at 12,700 *g* for 5 min. Oxygen dissociation curves were generated at 37 °C for all mice using a Hemox Analyzer (TCS Scientific, New Hope, PA, USA) using 10 µl of whole blood in 5 ml of buffer (50 mmol l^{-1} HEPES, 10 mmol l^{-1} EDTA, 100 mmol l^{-1} KCl, 0.1% bovine serum albumin, and 0.2% antifoaming agent, pH 7.4; TCS Scientific). Hb-O_2 affinity (P_{50}, the PO_2 at which haemoglobin is 50% saturation with O_2) was calculated using Hemox Analytic Software (TCS Scientific). These measurements of blood Hb content, haematocrit, and P_{50} have been previously published (Wearing et al., 2021) but are reported again here to provide insight into the measurements of acute hypoxia responses.

The larger blood samples collected after hypoxia acclimation were also used for measurements of the NO metabolites nitrite, S-nitrosothiols (SNO), and iron-nitrosyl and N-nitrosamine derivatives (FeNO + NNO) in plasma and red blood cells. This was accomplished by reductive chemiluminescence using a Sievers Nitric Oxide Analyzer (NOA model 280i, Boulder, CO, USA) and previously described protocols (Hansen and Jensen, 2010; Yang et al., 2003). Blood was sampled in dim light conditions, spun at 16,000 *g* for 2 min to separate plasma from red blood cells, which were then quickly frozen in liquid N_2 and stored at -80°C. Frozen samples (100 µl) were thawed and immediately incubated at room temperature for 5-10 min in the dark

with a SNO-stabilizing solution (900 µl) described elsewhere (Yang et al., 2003) and then centrifuged (10,000 g for 2 min). The supernatant of each sample was injected into the NOA purge vessel in serial aliquots as is (300 µl, peak A) and after 2 min incubation with sulfanilamide (270 µl, peak B) and sulfanilamide and $HgCl_2$ (270 µl, peak C) to obtain values for nitrite, SNO, and FeNO + NNO from the three peaks, as previously described (Yang et al 2003). These measurements were only conducted for a subset of mice due to an unforeseen technical issue that resulted in the loss of some samples during storage.

Physiological effects of manipulating Hb-O_2 affinity with efaproxiral

Captive G_1 populations of deer mice from high and low altitude, held in standard holding conditions in normoxia, were used to assess the acute effects of manipulating Hb-O_2 binding. Mice were placed in the plethysmography chamber and exposed to normoxic conditions (21 kPa O_2) for 40 min to make baseline measurements. Mice were then removed and given an intraperitoneal injection of either saline or efaproxiral sodium at a volume of 20 ml per kg body mass (Fisher Scientific, Whitby, ON, Canada). Efaproxiral was prepared in sterile saline (0.9% NaCl solution) on the day of experiments and was administered at a dose of 200 mg per kg body mass. Mice were then returned to the chamber, and measurements were made for 50 min in normoxia and 20 min in hypoxia (12 kPa O_2). reathing, O_2, T_b, heart rate, and arterial O_2 saturation were measured in the last 10 min of each exposure as described above. Every individual underwent both saline and efaproxiral injections, conducted in random order and separated by 1 week, and the efaproxiral dose used was determined in preliminary tests to have persistent effects on arterial O_2 saturation for the duration of the experiment.

Statistics

Linear mixed-effects models were used in experiments with F_2 hybrids to test for effects of α and β globin genotype, acclimation environment, and inspired O_2. They were also used in the efaproxiral experiments to test for effects of efaproxiral, mouse population, and inspired PO_2. We initially tested for the random effects of sex and family, but they did not near statistical significance ($P>0.10$) and were therefore removed from the final models reported here. The full results of the linear mixed-effects models are included in the supplementary material (Tables S1-S3), and the salient findings are reported in the Results. Holm-Šidák post-tests were used as appropriate. Statistical analysis was conducted using the lme4 package in R (v. 3.6.0) (Bates et al., 2015) with a significance level of $P < 0.05$. Values are reported as mean ± SEM.

RESULTS

Association of globin genotypes with variation in respiratory physiology in inter-population hybrids

When F_2 hybrids were considered altogether, both acute and chronic hypoxia affected breathing, metabolism, and arterial O_2 saturation (Fig. 1). Adult mice were subjected to acute step-wise decreases in inspired partial pressure of O_2 (PO_2) both before and after chronic hypoxia acclimation (6-8 weeks at ~12 kPa O_2). Total ventilation increased in response to acute hypoxia due to increases in breathing frequency that offset smaller declines in tidal volume. Chronic hypoxia augmented these increases in total ventilation, particularly in response to severe acute hypoxia (acclimation environment×inspired PO_2, $P<0.001$), which arose from significant increases in breathing frequency (environment×PO_2, $P<0.001$). Chronic hypoxia also had significant main effects on several other phenotypes, attenuating declines in arterial O_2 saturation

(SaO_2) (P<0.001) and heart rate (P<0.001) in response to acute stepwise hypoxia, and increasing O_2 consumption rate (P=0.009) and body temperature (P<0.001) (Fig. 1). Chronic hypoxia also increased haematocrit, whole-blood Hb content, and Hb P_{50} (P<0.001 for all environment effects) (Table 1).

Several of these cardiorespiratory and metabolic phenotypes were associated with α-globin and/or β-globin genotype (Tables S1,S2). Below we discuss the statistically significant differences associated with genotype in the Results, but we include the full suite of measurements for each genotype in Supplementary Figures (Fig. S1-S3). There was a strong effect of α-globin genotype (P=0.012), but not β-globin genotype (P=0.711), on arterial O_2 saturation. SaO_2 in severe hypoxia was higher in the highland (H) α-globin genotypes compared to the lowland (L) α-globin genotypes in measurements among normoxia-acclimated mice (Fig. 2A). However, although chronic hypoxia tended to reduce the decline in SaO_2 across genotypes (Fig. 1A), this effect was greater in mice with the α^{LL} genotypes, such that the difference in SaO_2 between genotypes was abolished after hypoxia acclimation (Fig. 2B). This variation in SaO_2 appeared to be associated with variation in Hb-O_2 affinity measured in intact red blood cells (RBC), for which there was also an effect of α-globin genotype (α-globin effect, P<0.001; α-globin×environment, P=0.035) but not β-globin genotype (Table S2). In particular, α^{HH} mice exhibited significantly higher Hb-O_2 affinity (i.e., lower P_{50}) than α^{LL} mice before exposure to chronic hypoxia, but Hb-O_2 affinity decreased in response to chronic hypoxia only in mice with the α^{HH} genotype, such that α-globin genotypes were similar after hypoxia acclimation (Fig. 2C). Measurements of NO metabolites (i.e. nitrite, S-nitrosothiols, iron-nitrosyl and N-nitrosamine derivatives) were made after hypoxia acclimation, and α-globin genotype affected plasma nitrite concentration but had no effect on plasma concentrations of other NO metabolites (Table 2).

There was a strong association of α-globin genotype with variation in breathing pattern, both before and after exposure to chronic hypoxia (Fig. 3). In measurements both before and after hypoxia acclimation, mice with the α^{HH} genotype breathed using significantly deeper breaths (α-globin effect, $P<0.001$) but at a slower frequency (α-globin effect, $P<0.001$) than mice with the α^{LL} genotype, with no significant differences between α-globin genotypes in total ventilation. These differences in breathing pattern persisted across a range of inspired O_2 levels, from hyperoxia at 30 kPa O_2 (when Hb was fully saturated with O_2) to severe hypoxia.

β-globin genotype was associated with variation in the hypoxic ventilatory response, as reflected by a significant interaction between β-globin genotype and inspired PO_2 on total ventilation ($P=0.009$). The association of β-globin genotype with total ventilation was particularly evident among normoxia-acclimated mice that were homozygous for highland α-globin, with no significant associations of β-globin genotype with O_2 consumption rate (Fig. 4, Table S1). Among these normoxia-acclimated mice, those that were homozygous for highland β-globin had higher total ventilation than both heterozygotes and lowland homozygotes at 12 kPa O_2, and higher total ventilation than heterozygotes in more severe levels of acute hypoxia (Fig. 4A). However, these differences between β-globin genotypes disappeared after hypoxia acclimation (Fig. 4B), potentially because the effects of hypoxia acclimation were greater in heterozygotes and lowland homozygotes.

Variation in body temperature (T_b) was associated with both α-globin (α-globin effect, $P=0.007$) and β-globin (β-globin effect, $P=0.037$) genotypes (Fig. S3; Table S1). T_b in normoxia was similar across genotypes, ~36-38 °C on average. Both the magnitude of T_b depression as well as the PO_2 at which T_b depression occurred varied among genotypes, but the magnitude of T_b depression tended be reduced after hypoxia acclimation for all genotypes. However, neither α-

globin nor β-globin genotype had significant associations with O_2 consumption rate before or after hypoxia acclimation (Fig. S3; Table S1).

Effects of acutely manipulating Hb-O_2 affinity on breathing

We next sought to examine whether the association of globin genotype with respiratory phenotypes stems from variation in Hb-O_2 affinity. We used efaproxiral – a synthetic drug that acts as a negative allosteric regulator of Hb-O_2 binding – to reduce Hb-O_2 affinity acutely in captive-bred deer mice from high- and low-altitude populations *in vivo*. This treatment was expected to manifest as a reduction in arterial O_2 saturation in acute hypoxia. Indeed, efaproxiral reduced arterial O_2 saturation in high-altitude deer mice in hypoxia (Fig. 5A) and in low-altitude deer mice in both normoxia and hypoxia (Fig. 5B) compared to saline controls ($P<0.001$ for treatment effect and treatment×PO_2 interaction; Table S3). The magnitude of the effect of efaproxiral on arterial O_2 saturation differed between populations (population×treatment, $P=0.002$; population×treatment×PO_2, $P=0.048$), driven by larger effects in lowlanders than in highlanders. However, efaproxiral had no consistent effects on breathing frequency, tidal volume, or total ventilation in each population (no significant treatment or treatment×PO_2 effects) (Fig. 5). Efaproxiral also had no post-injection effects on oxygen consumption rate, air convection requirement, or body temperature (Table 3). However, efaproxiral did affect heart rate, as reflected by a significant treatment×PO_2 interaction ($P=0.005$) that was driven primarily by increased heart rates in lowlanders after efaproxiral injection (population×treatment, $P=0.012$; Tables 3). Therefore, our treatment was successful in reducing arterial O_2 saturation and leading to potential compensatory changes in heart rate but it had no effect on breathing, in stark contrast to the differences observed between globin genotypes.

DISCUSSION

Evolved changes in Hb-O_2 affinity have contributed to hypoxia adaptation in numerous high-altitude mammals and birds and is often assumed to confer a physiological benefit by safeguarding arterial O_2 saturation in hypoxia (Storz, 2016). However, the possibility that adaptive modifications of Hb function might contribute to other physiological processes that are not directly related to circulatory O_2 transport had been largely unexplored. Here, we show that allelic variants of the α-globin and β-globin genes in high-altitude deer mice are associated with changes in breathing that augment alveolar ventilation in hypoxia. These effects could not be recapitulated by acute changes in arterial O_2 saturation or Hb-O_2 affinity using pharmacological manipulations. Our results suggest that allelic variation in Hb genes are associated with multiple respiratory phenotypes, and may contribute to environmental adaptation via physiological mechanisms that are not commonly ascribed to this protein.

α-globin genotype had a strong association with breathing pattern, in which α^{HH} deer mice breathed deeper but less frequently, a change that likely augments alveolar ventilation without affecting total ventilation. These observed differences in breathing pattern between α-globin genotypes can completely account for previously documented differences that distinguish highland deer mice from lowland conspecifics and a closely related lowland congener (*Peromyscus leucopus*), which are observed in wild deer mice as well as deer mice raised for one or two generations in captivity in normoxia (Ivy and Scott, 2017; Ivy and Scott, 2018; Ivy et al., 2020). This is nicely illustrated in plots of total ventilation against tidal volume (Fig. 6), which are useful for visualizing variation in breathing pattern independent of differences in total ventilation. The pronounced rightward shift in interpopulation hybrids with the α^{HH} genotype compared to the α^{LL} genotype (Fig. 6A), which reflects deeper but less frequent breaths at any

given level of total ventilation, is nearly identical to the rightward shift in highlanders compared to lowlanders in previous measurements of deer mice acclimated to normoxia (Fig. 6B). The first-generation lab-raised mice used for the latter measurements are different from those used for breeding in the current study, but they do represent the physiology of the parents used to generate the F_2 interpopulation hybrids studied here. A similar deepening of breathing pattern has evolved in the high-altitude bar-headed goose (cott et a l., 2007), which also possesses an α-globin variant that contributes to an evolved increase in Hb-O_2 affinity (Natarajan et al., 2018), suggesting that there may be an association between α-globin genotype and breathing pattern in other high-altitude taxa.

β-globin genotype was associated with the hypoxic ventilatory response among normoxia acclimated deer mice, with the $\alpha^{HH}\beta^{HH}$ genotype exhibiting higher total ventilation than $\alpha^{HH}\beta^{HL}$ and $\alpha^{HH}\beta^{LL}$ genotypes at 12 kPa O_2. These findings mirror those in lab-strain mice possessing the Hb re sbyterian β-globin mutation, which is associated with a reduced Hb-O_2 affinity and an attenuated hypoxic ventilatory response (Izumizaki et al., 2003). Differences in β-globin genotype could thus, in addition to α-globin, contribute to the increases in total ventilation in highland deer mice compared to lowland deer mice and *P. leucopus* that we have observed among mice acclimated to normoxia (Ivy and Scott, 2017; Ivy and Scott, 2018; Ivy et al., 2020). However, these associations with β-globin genotype were abolished after hypoxia acclimation, suggesting that β-globin may influence the effects of chronic hypoxia on control of breathing, a process termed ventilatory acclimatization to hypoxia (VAH). If so, β-globin may contribute to the attenuation of VAH that we have previously observed in highland deer mice (Ivy and Scott, 2017; Ivy and Scott, 2018).

The association of globin variants with variation in breathing phenotypes could not be recapitulated by acute manipulations of Hb-O_2 affinity or arterial O_2 saturation. Treatment of deer mice from highland and lowland populations with efaproxiral to reduce Hb-O_2 affinity had no effect on total ventilation or breathing pattern in normoxia or hypoxia. There were differences in the magnitude of the effects of efaproxiral on arterial O_2 saturation and heart rate between populations, possibly because the normally lower Hb-O_2 affinity of lowlanders (Ivy et al., 2020) made them more susceptible to impairments in pulmonary O_2 loading upon further reduction in affinity, but efaproxiral had no effect on breathing in either population. Furthermore, the differences in breathing pattern between α^{HH} and α^{LL} deer mice persisted in hyperoxia (30 kPa O_2) when blood O_2 tension was well above that needed to fully saturate Hb with O_2. However, the effects of efaproxiral on Hb-O_2 affinity are temporary and we only measured the drug's acute effects, and it is possible chronic changes in Hb-O_2 affinity (akin to the differences between adult mice with highland versus lowland globin genotypes) are needed to induce variation in breathing phenotypes. Alternatively, our findings with efaproxiral treatment could also suggest that globin variants regulate breathing via mechanisms that are not directly associated with the role of Hb in circulatory O_2 transport. The globin family is ancient and carrier out various cellular functions beyond O_2 transport (Fago et al., 2004; Kamga et al., 2012). The α/β monomers of Hb are expressed in various non-erythroid cells, including neurons and vascular endothelium (Biagioli et al., 2009; Newton et al., 2006; Richter et al., 2009; Schelshorn et al., 2009; Straub et al., 2012), so it is possible that α and/or β globins expressed in non-erythroid tissues could regulate ventilatory phenotypes.

The association of α-globin genotype with variation in arterial O_2 saturation were contingent upon acclimation environment, consistent with other recent findings (Wearing et al., 2021). Deer mice that were homozygous for highland α-globin (α^{HH}) maintained higher arterial O_2 saturation in hypoxia and had higher Hb-O_2 affinity than lowland homozygous deer mice (α^{LL}) when comparisons were made among normoxia-acclimated deer mice, but these differences were abolished after hypoxia acclimation. This discrepancy could be explained by differences in sensitivity to 2,3-DPG, a key negative allosteric regulator of Hb-O_2 binding in mammalian erythrocytes that can increase in concentration after acclimation to the levels of chronic hypoxia used here (Lenfant et al., 1968; Snyder, 1982). Indeed, previous studies of O_2-binding properties of stripped haemoglobin suggest that 2,3-DPG sensitivity is greater in high-altitude populations of deer mice when measured in the presence of physiologically relevant concentrations of Cl^- (Storz et al., 2010). Therefore, highland homozygotes could have been more sensitive to the increases in red cell 2,3-DPG concentration that may have occurred with hypoxia acclimation, and could have thus exhibited a more pronounced decrease in Hb-O_2 affinity and less pronounced increase in arterial O_2 saturation in hypoxia. This emphasizes the potential advantage of the evolved (genetically-based) reduction in erythrocyte 2,3-DPG levels that has been observed in natural high-altitude populations of deer mice, which arose independent of changes in α-globin genotype (Snyder, 1982; Snyder et al., 1982). This evolved reduction in 2,3-DPG levels counteracts the effects of hypoxia acclimation on this trait, such that 2,3-DPG levels are elevated only slightly in high-altitude populations in the wild (Snyder, 1982). CO_2 and H^+ are also key allosteric regulators of Hb-O_2 binding, and it is possible that differences in alveolar ventilation between genotypes affected blood CO_2/pH and thus influenced our *in vivo*

measurements of arterial O_2 saturation. However, variation in blood CO_2/pH between genotypes would not have persisted in the buffer used for *in vitro* measurements.

The unexpected association between Hb genotype and control of breathing is especially intriguing in light of population genetic evidence for altitude-related selection on the α- and β-globin genes in deer mice (Storz and Kelly, 2008; Storz et al., 2009; Storz et al., 2012). The adaptive relevance of Hb-O_2 affinity is well-established in high-altitude vertebrates, but the present findings force us to consider the possibility that allelic variation in Hb function may affect a broader diversity of physiological processes than previously assumed. Recent studies suggest that Hb functions not just as an O_2 carrier, but also as an O_2 sensor and O_2-responsive transducer of nitric oxide (NO) vasoactivity in the microcirculation, thereby contributing to hypoxic vasodilation that helps match perfusion to tissue O_2 demand (Jensen, 2009; Storz, 2018; Zhang et al., 2015; Zhang et al., 2016). Indeed, our results suggest that α-globin variant in high-altitude deer mice affects the concentrations of NO metabolites in the circulation (Table 2). The results reported here also suggest that the physiological effects of Hb may even transcend circulatory O_2 transport, with direct or indirect effects on control of breathing. The next step is to identify and characterize the causal mechanism underlying the unexpected genotype-phenotype association.

Our findings contribute to a growing awareness that protein polymorphism can sometimes have phenotypic effects that are unrelated to the 'canonical' function of the protein in question. For example, genetic variation in enzymes of central metabolism can affect physiological phenotypes via mechanisms independent of pathway flux due to signalling functions of intermediary metabolites or nonenzymatic 'moonlighting' functions of the enzymes (Marden, 2013a; Marden, 2013b). Such non-canonical effects of haemoglobin variants may be

common, based on previous observations that genetic variation in globins is not always associated with variation in Hb-O_2 binding (Barlow et al., 2017; Cheviron et al., 2014a; Nelson et al., 2019). The realization that there may be physiologically important auxiliary functions still waiting to be discovered in a protein as intensively studied as Hb highlights the importance of maintaining a wide field of vision when investigating causal connections between genotype and phenotype.

ACKNOWLEDGEMENTS

The authors would like to thank the animal care technicians and the members of the Scott and McClelland labs for help and support while completing this project.

COMPETING INTERESTS

No competing interests declared.

AUTHOR CONTRIBUTIONS

G.R.S., J.F.S., and Z.A.C. designed the study. C.N., N.G.-P., J.P.V., R.M.S. and S.C.C.-S. carried out mouse breeding and genetic characterization. C.M.I. and O.H.W. ran and analyzed the *in vivo* experiments. C.M.I., E.E.P., and A.F. carried out blood analyses. C.M.I. and G.R.S. wrote the manuscript, and all authors edited the manuscript.

FUNDING

This research was funded by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to G.R.S (RGPIN-2018-05707). National Sciences

Foundation (NSF) grants to Z.A.C. (IOS-1354934, IOS-1634219, IOS-1755411, and OIA-1736249) and J.F.S. (IOS-1354390 and OIA-1736249), and a National Institutes of Health (NIH) grant to J.F.S. (HL087216). Salary support was provided to C.M.I. by a NSERC Postgraduate Scholarship and an Ontario Graduate Scholarship, to O.H.W. by a NSERC Vanier Canada Graduate Scholarship, to R.M.S. by an NSF Postdoc Research Fellowship in Biology (1612859), to J.P.V. by a NIH National Heart, Lung and Blood Institute Research Service Award Fellowship (1F32HL136124-01), to S.C.C.-S. by a NSF (DBI) Postdoctoral Fellowship in Biology Award (#1612283), and to G.R.S. by the Canada Research Chairs Program.

DATA AVAILABILITY

Physiological data are deposited in Mendeley Data (DOI: 10.17632/mktd4vn3d7.1).

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Figures

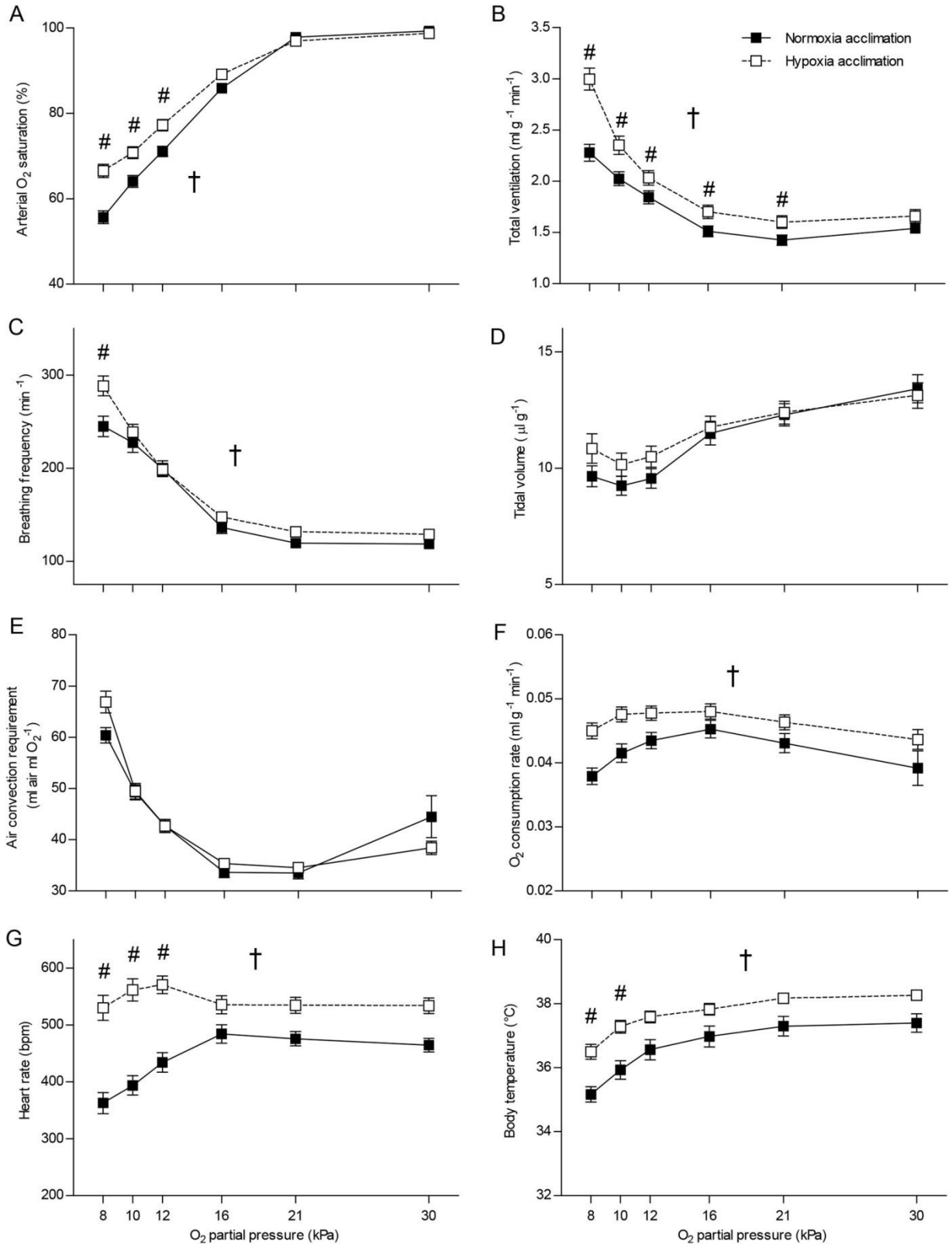

Figure 1. Chronic exposure to hypoxia affected the responses to acute hypoxia in F_2 hybrid deer mice. Values are mean ± SEM (N=26). † represent significant main effects of acclimation environment, # represents a significant pairwise difference between acclimation groups within a PO_2 using Holm-Šidák post-tests.

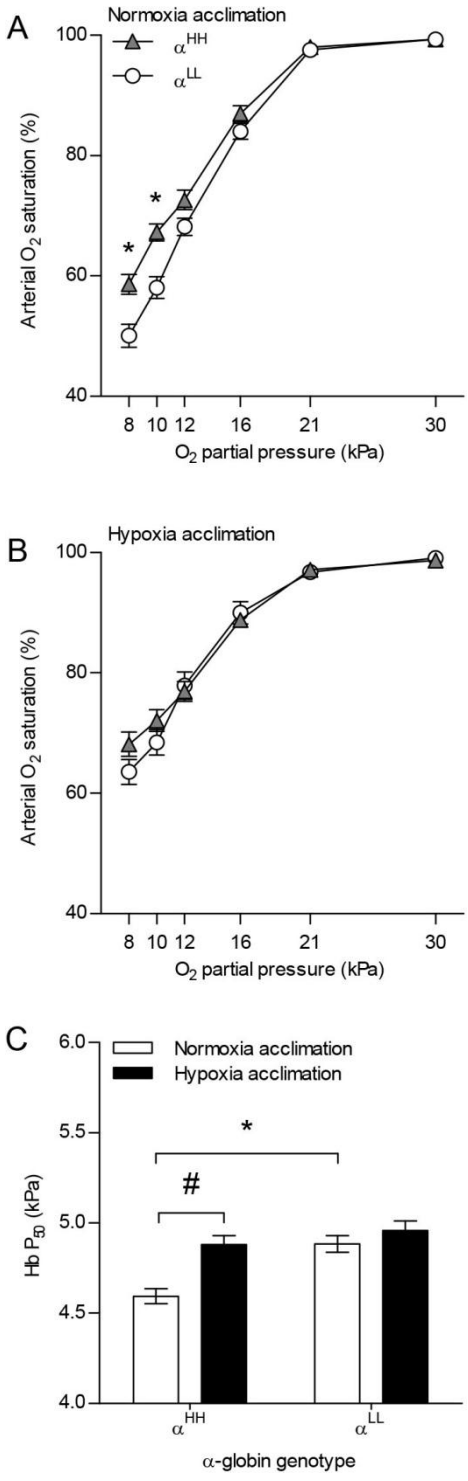

Figure 2. Arterial O_2 saturation during acute hypoxia (A,B) and haemoglobin (Hb) P_{50} (C) were associated with α-globin genotype in F_2 hybrid deer mice before but not after hypoxia acclimation. P_{50} was measured in intact red blood cells (RBC) at pH 7.4 and 37 °C. Different globin genotypes are shown as superscripts with 'L' representing the lowland haplotype and 'H'

representing the highland haplotype. a lues are mean ± EM (α^{HH}, N=17; α^{LL}, N=9). * and # denote significant pairwise differences using Holm-Šidák post-tests between α-globin genotypes within an acclimation environment and between acclimation environments within an α-globin genotype, respectively.

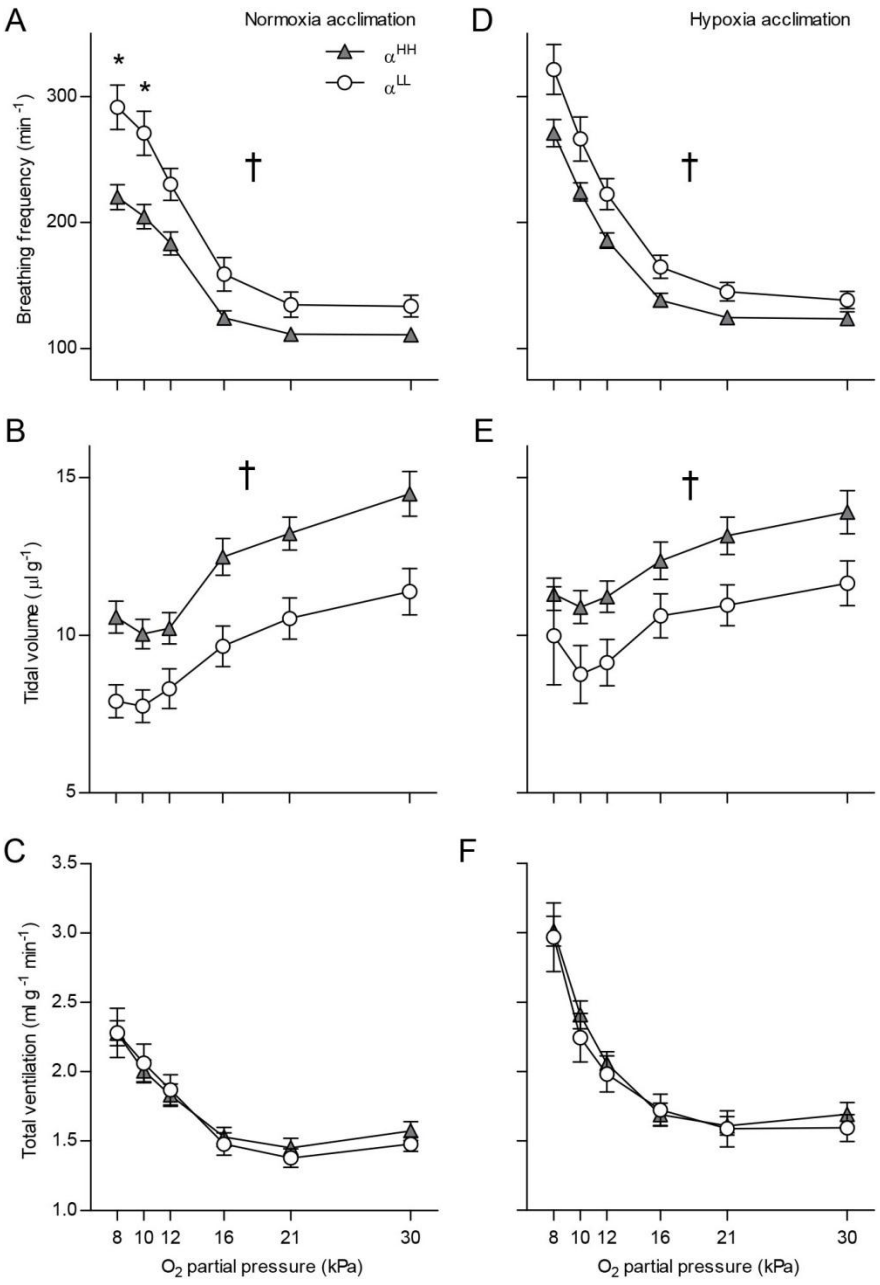

Figure 3. α-globin genotype was associated with variation in breathing pattern in F_2 hybrid deer mice both before (A,B,C) and after (D,E,F) hypoxia acclimation. Genotypes and N as in Fig. 2. a lues are mean ± EM. † represent significant main effects of α-globin genotype, * represent significant pairwise differences between genotypes within a PO_2 using Holm-Šidák post-tests.

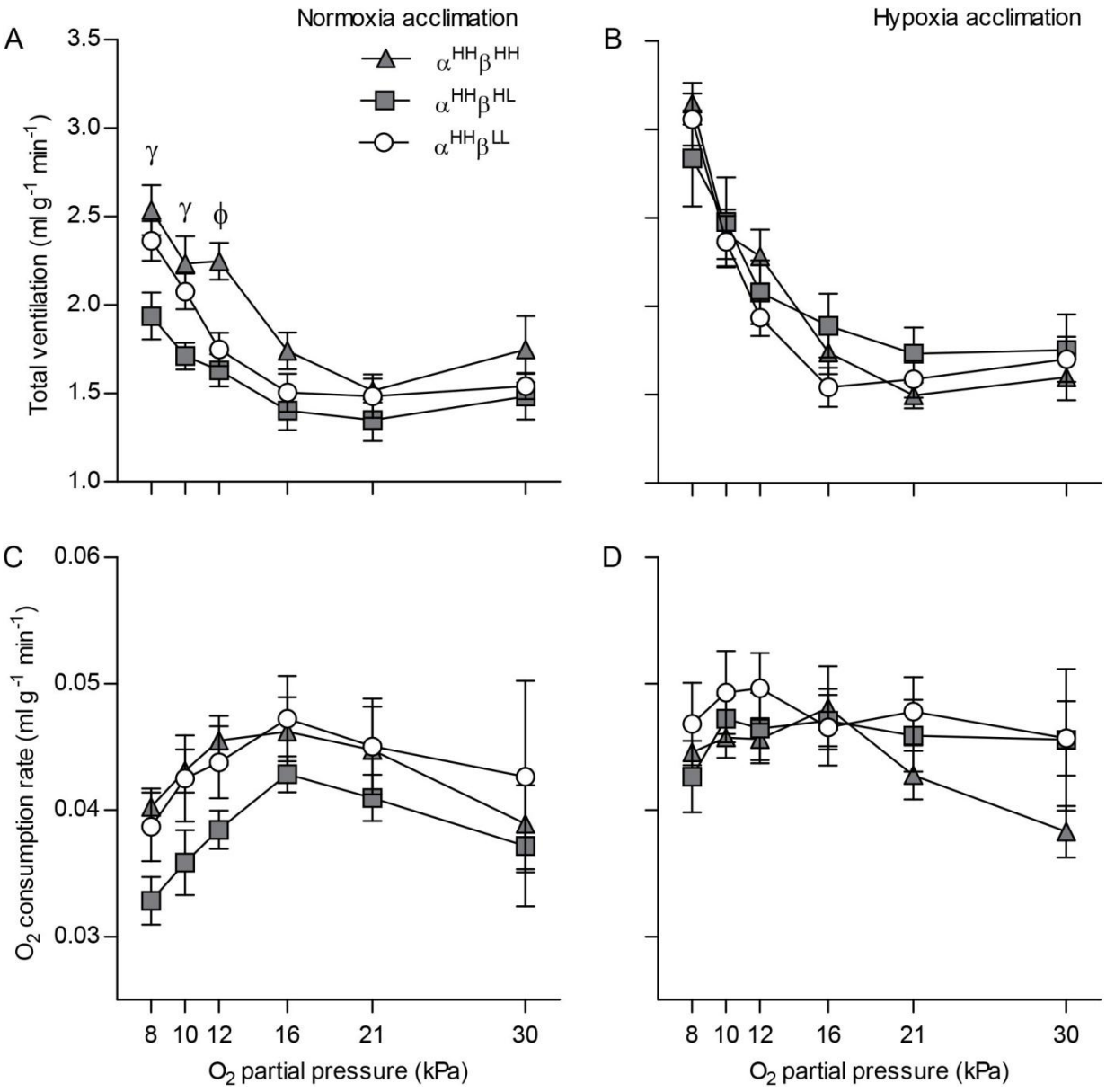

Figure 4. β-globin genotype was associated with variation in the hypoxic ventilatory response in F_2 hybrid deer mice before (A) but not after (B) hypoxia acclimation, without any significant association with O_2 consumption rate (C,D). Genotypes defined in Fig. 2. Values are mean ± EM ($\alpha^{HH}\beta^{HH}$, N=5; $\alpha^{HH}\beta^{HL}$, N=5; $\alpha^{HH}\beta^{LL}$, N=7). γ and ϕ represent significant pairwise differences within a PO_2 between $\alpha^{HH}\beta^{HH}$ and $\alpha^{HH}\beta^{HL}$, or between $\alpha^{HH}\beta^{HH}$ and both $\alpha^{HH}\beta^{HL}$ and $\alpha^{HH}\beta^{LL}$, respectively (Holm-Šidák post-tests).

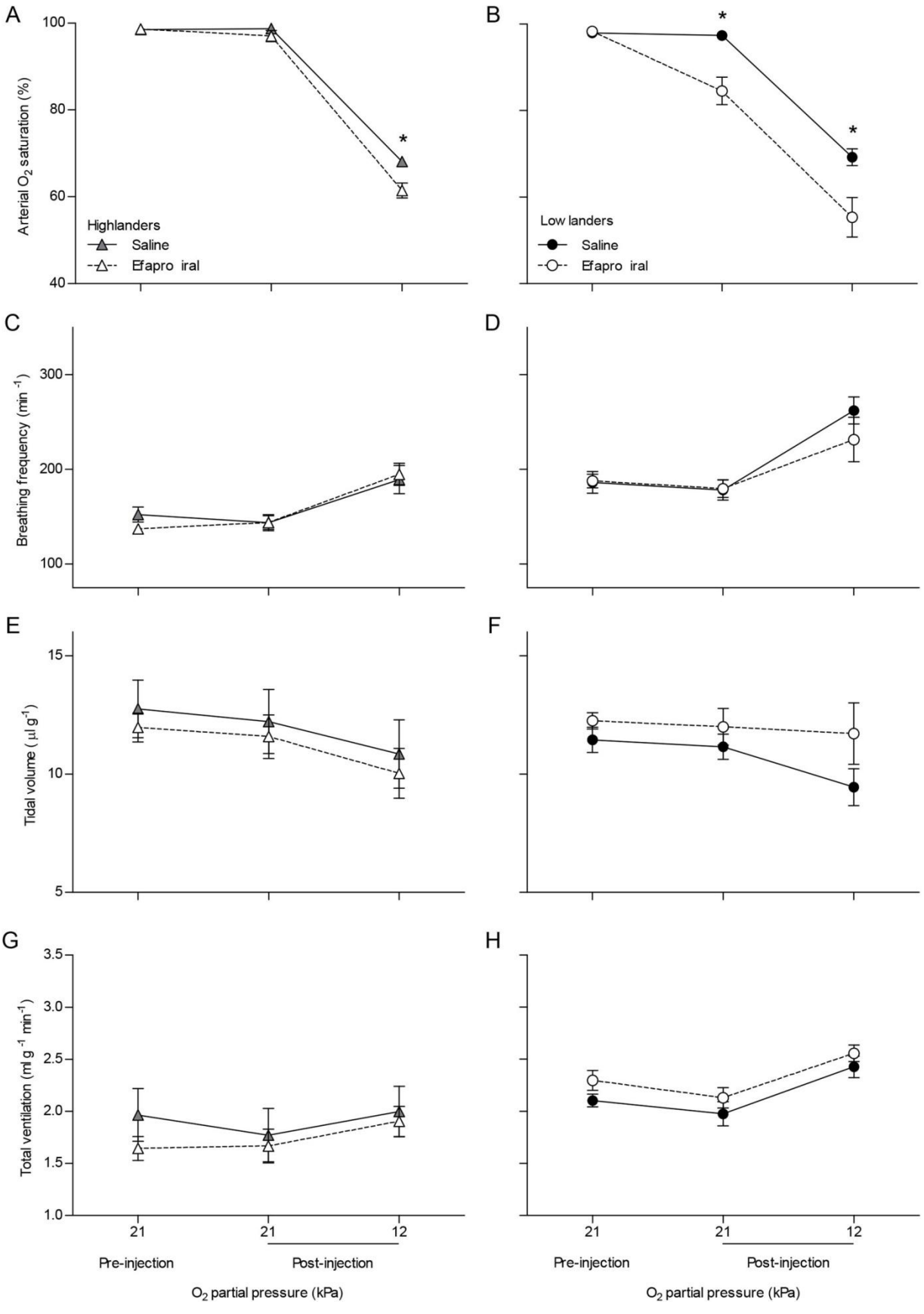

Figure 5. Efaproxiral treatment to reduce haemoglobin-O_2 affinity decreased arterial O_2 saturation but did not influence breathing in highland or lowland populations of deer mice. Values are mean ± SEM (N=6). * represents a significant pairwise differences between saline and efaproxiral (200 mg kg^{-1}) treatments within a PO_2 using Holm-Šidák post-tests.

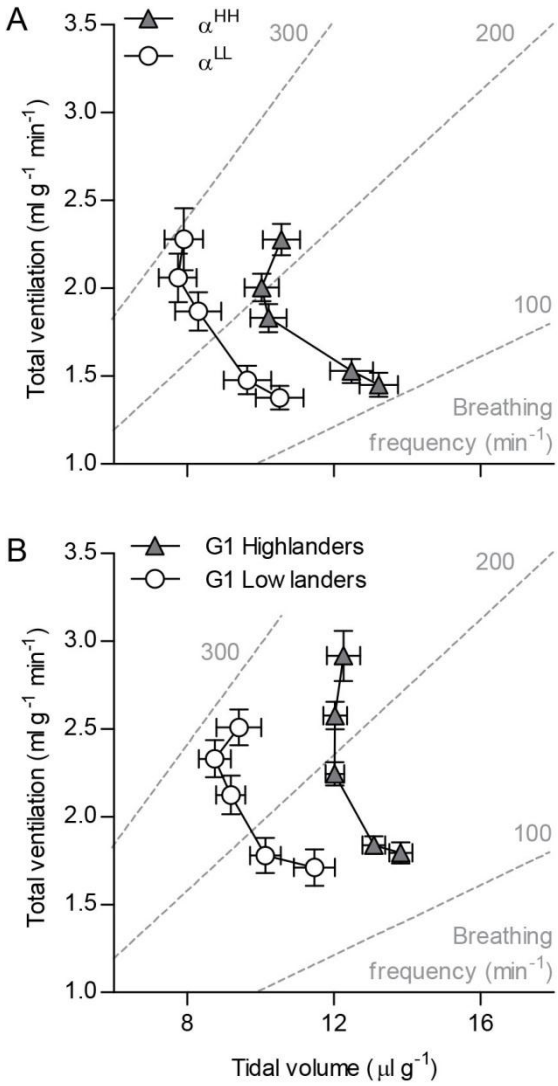

Figure 6. Differences in breathing pattern between α-globin genotypes among the F_2 hybrids studied here (A) were extremely similar to those between high-altitude versus low-altitude populations in comparisons of first-generation (G1) lab-raised deer mice (B). Gray dashed lines represent isopleths of constant breathing frequency. Symbols and error bars represent mean ± SEM (α^{HH}, N=17; α^{LL}, N=9, G1 highlanders, N=30; G1 lowlanders, N=13). Data in (B) are previously published results for adult deer mice acclimated to normoxia (Ivy et al., 2020).

Table 1. Blood responses to chronic hypoxia in F_2 hybrid deer mice.

	Normoxia acclimation	Hypoxia acclimation
Haematocrit (%)	45.70 ± 0.48	59.93 ± 0.87*
Haemoglobin (g dl^{-1})	14.68 ± 0.26	19.03 ± 0.50*
P_{50} (kPa)	4.70 ± 0.04	4.91 ± 0.04*

Values are mean ± SEM (N=26); P_{50}, partial pressure of oxygen where 50% of haemoglobin is saturated, which was measured in intact erythrocytes. * denotes a significant pairwise difference between acclimation groups.

Table 2. Nitric oxide (NO) metabolites measured in plasma and red blood cells after hypoxia acclimation were altered by α-globin genotype in F_2 hybrid deer mice.

	α^{LL}	α^{HH}	P
Plasma			
Nitrite	1.289 ± 0.255 (3)	0.742 ± 0.070* (6)	**0.027**
SNO	0.307 ± 0.045 (3)	0.401 ± 0.061 (6)	0.467
Red blood cells			
Nitrite	0.270 ± 0.151 (5)	0.864 ± 0.231 (8)	0.089
SNO	0.484 ± 0.154 (4)	0.485 ± 0.040 (8)	0.994
FeNO+NNO	0.401 ± 0.186 (5)	0.157 ± 0.054 (8)	0.152

Values are mean ± SEM (N) in units μmol l^{-1}; SNO, S-nitroso compounds (SNO); FeNO+NNO, iron-nitrosyl and N-nitroso compounds. Asterisks indicate a significant difference based on t-test comparisons between genotypes ($P<0.05$).

Table 3. O_2 consumption rate, air convection requirement, heart rate, and body temperature responses for highland and lowland populations of deer mice during manipulation of arterial O_2 saturation using efaproxiral (200 mg kg^{-1}).

		Highlanders		Lowlanders	
	PO_2 (kPa)	Saline	Efaproxiral	Saline	Efaproxiral
		O_2 consumption rate (ml kg^{-1} min^{-1})			
Pre-injection	21	50.7 ± 3.9	37.8 ± 3.4*	61.5 ± 4.2	65.2 ± 5.2
Post-injection	21	43.5 ± 2.9	42.0 ± 3.6	53.7 ± 1.8	58.2 ± 1.8
	12	43.5 ± 0.8	40.9 ± 2.0	52.1 ± 3.4	55.3 ± 3.8
		Air convection requirement (ml air per ml O_2)			
Pre-injection	21	38.2 ± 2.3	35.0 ± 2.5	44.2 ± 2.3	36.0 ± 2.4
Post-injection	21	40.3 ± 4.2	37.0 ± 2.3	40.0 ± 2.8	36.7 ± 1.8
	12	45.9 ± 5.4	47.2 ± 2.3	46.4 ± 2.0	46.9 ± 2.2
		Heart rate (beats min^{-1})			
Pre-injection	21	513 ± 53	583 ± 32	448 ± 42	557 ± 2
Post-injection	21	499 ± 60	540 ± 27	525 ± 36	654 ± 26*
	12	592 ± 36	568 ± 30	597 ± 30	664 ± 39
		Body temperature (°C)			
Pre-injection	21	37.8 ± 0.4	38.7 ± 0.3	38.4 ± 0.4	37.3 ± 0.6
Post-injection	21	36.9 ± 0.8	38.2 ± 0.4	38.0 ± 0.4	37.1 ± 0.5
	12	36.7 ± 0.7	37.7 ± 0.1	37.2 ± 0.4	36.2 ± 0.8

PO_2, partial pressure of O_2. Values are mean ± SEM (N=6). * denotes a significant pairwise difference between saline and efaproxiral injections within a PO_2 and deer mouse population.

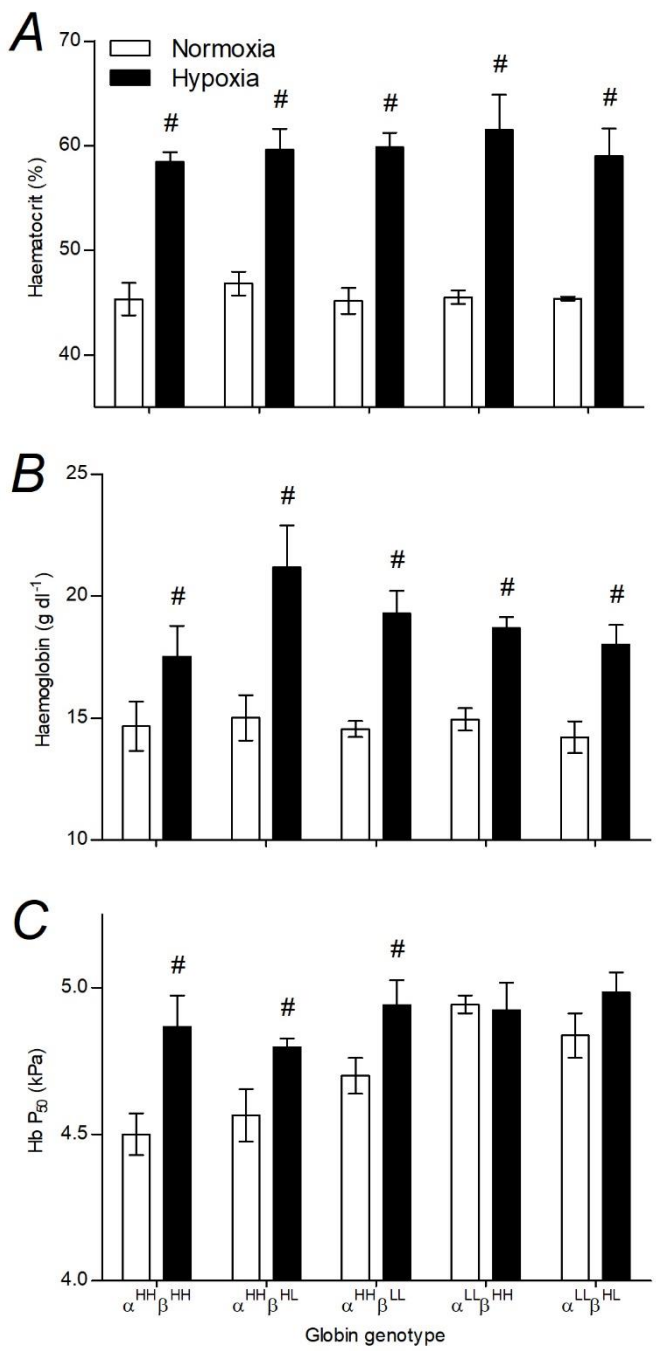

Fig. S1. Haematocrit (A), whole-blood haemoglobin (Hb) content (B), and Hb P_{50} measured in intact erythrocytes (C) increased after hypoxia acclimation in F_2 hybrid deer mice, but only P_{50} was influenced by globin genotype. $\alpha^{HH}\beta^{HH}$ represents mice that are homozygous for the highland α-globin and β-globin genotype (N=5), $\alpha^{HH}\beta^{HL}$ represents mice that are homozygous for the highland α-globin genotype and heterozygous β-globin genotype (N=5), $\alpha^{HH}\beta^{LL}$ represents mice that are homozygous for the highland α-globin genotype and homozygous lowland for the β-globin genotype (N=7), $\alpha^{LL}\beta^{HH}$ represents mice that are homozygous for the lowland α-globin genotype and homozygous for the highland β-globin genotype (N=4), $\alpha^{LL}\beta^{HL}$ represents mice that are homozygous for the lowland α-globin genotype and heterozygous β-globin genotype (N=5). Values are mean ± SEM. # denotes significant pairwise differences using Holm-Šidák post-tests between acclimation environments within a genotype.

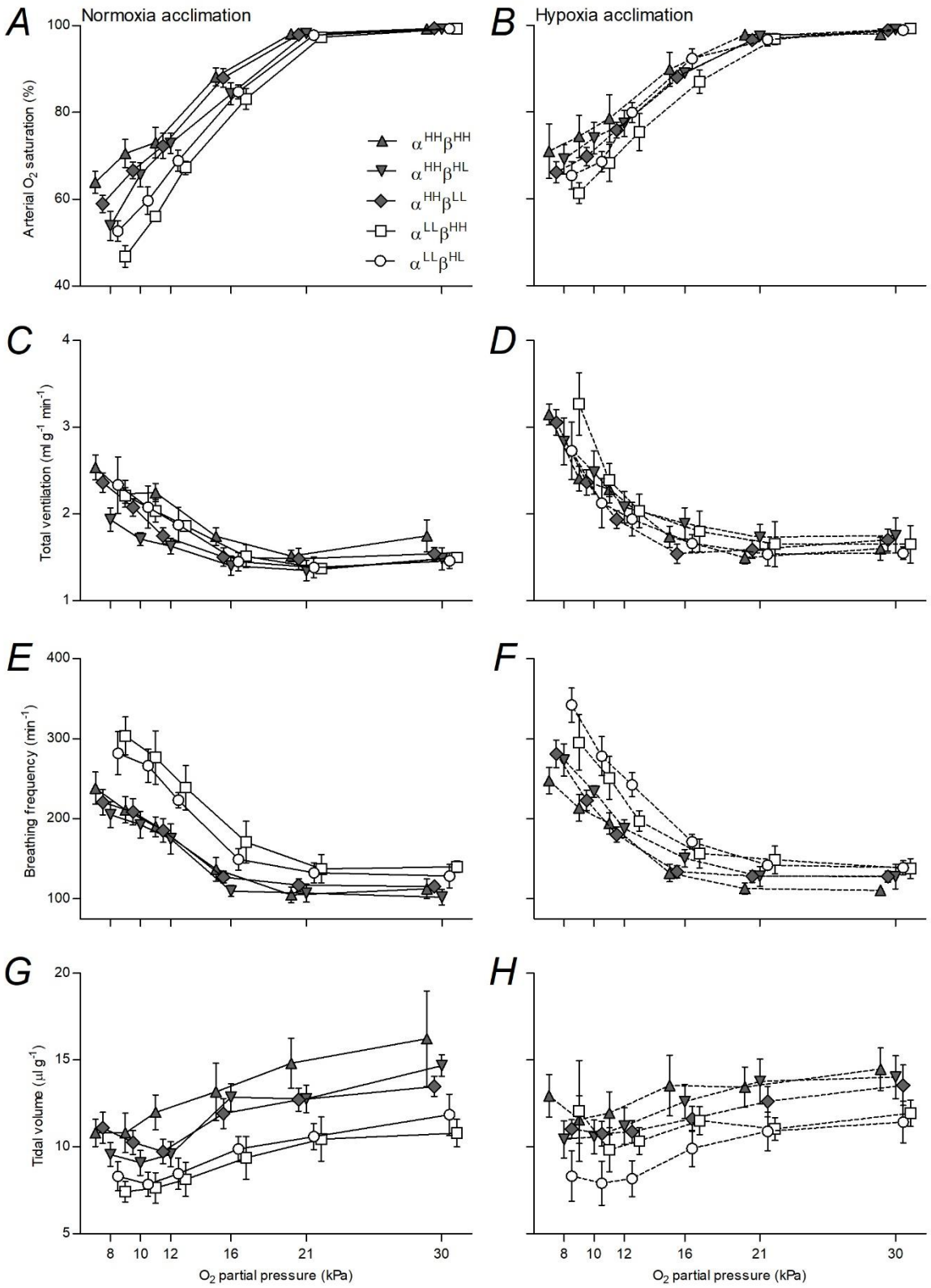

Fig. S2. Arterial O_2 saturation (A,B), total ventilation (C,D), breathing frequency (E,F), and tidal volume (G,H) responses of F_2 hybrid mice before (A,C,E,G) and after (B,D,F,H) hypoxia acclimation in F_2 hybrid mice with different α- and β-globin haplotypes. Values are mean ± SEM (genotypes and N as in Figure S1), symbols at each O_2 partial pressure are offset for clarity..

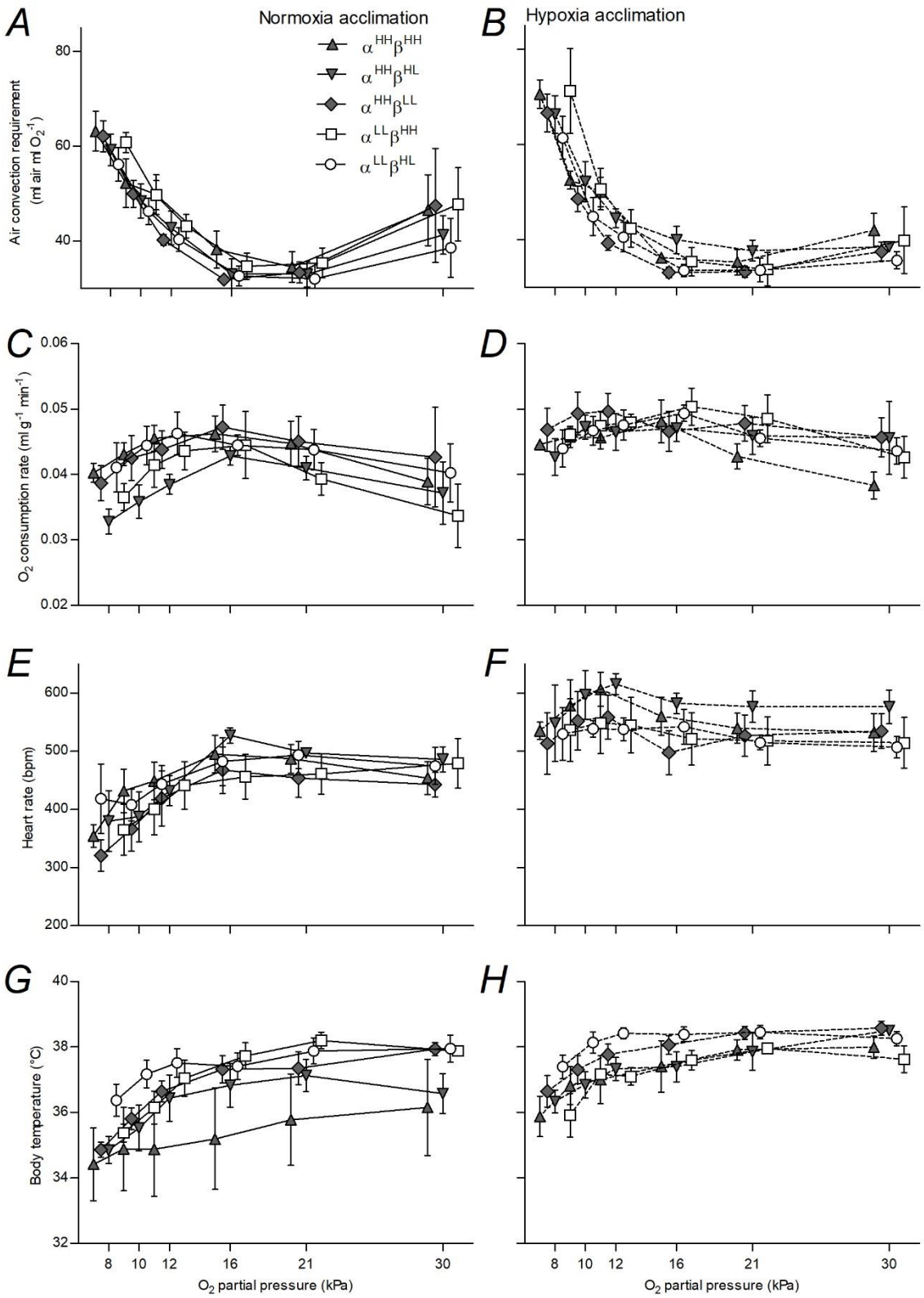

Fig. S3. Air convection requirement (A,B), O_2 consumption rate (C,D), heart rate (E,F), and body temperature (G,H) responses of F_2 hybrid mice before (A,C,E,G) and after (B,D,F,H) hypoxia acclimation in F_2 hybrid mice with different α- and β-globin haplotypes. Values are mean ± SEM (genotypes and N as in Figure S1), symbols at each O_2 partial pressure are offset for clarity..

Table S1. Results of linear mixed-effects models of physiological responses to acute stepwise hypoxia in F_2 hybrid deer mice

		α	β	Env	PO_2	α*Env	β*Env	Env*PO_2	α*PO_2	β*PO_2
Arterial O_2 saturation	F	6.920	0.344	15.41	579.6	0.600	0.510	4.653	n.s.	n.s.
	P	**0.012**	0.711	**<0.001**	**<0.001**	0.553	0.479	**<0.001**	n.s.	n.s.
Total ventilation	F	0.288	1.274	12.33	215.5	0.151	0.223	13.574	n.s.	2.426
	P	0.594	0.289	**<0.001**	**<0.001**	0.700	0.801	**<0.001**	n.s.	**0.009**
Breathing frequency	F	20.61	0.103	4.045	346.7	0.526	1.655	5.197	6.234	n.s.
	P	**<0.001**	0.902	**0.050**	**<0.001**	0.472	0.203	**<0.001**	**<0.001**	n.s.
Tidal volume	F	15.58	1.345	0.836	61.04	0.137	0.147	n.s.	n.s.	n.s.
	P	**0.003**	0.270	0.365	**<0.001**	0.713	0.864	n.s.	n.s.	n.s.
Air convection requirement	F	2.068	47.00	0.141	118.7	0.142	0.387	3.935	n.s.	n.s.
	P	0.157	0.109	0.709	**<0.001**	0.708	0.681	**0.002**	n.s.	n.s.
O_2 consumption rate	F	0.547	0.886	7.495	10.34	0.001	0.036	n.s.	n.s.	n.s.
	P	0.463	0.419	**0.009**	**<0.001**	0.999	0.964	n.s.	n.s.	n.s.
Heart rate	F	1.016	1.524	33.05	10.58	0.661	0.006	13.52	n.s.	n.s.
	P	0.319	0.228	**<0.001**	**<0.001**	0.421	0.994	**<0.001**	n.s.	n.s.
Body temperature	F	7.826	3.582	13.47	76.84	2.100	0.133	1.936	n.s.	n.s.
	P	**0.007**	**0.037**	**<0.001**	**<0.001**	0.154	0.876	0.089	n.s.	n.s.

Env, acclimation environment, PO_2, O_2 partial pressure of inspired air during acute stepwise hypoxia; n.s. not significant and not included in final models (see Methods).

Table S2. Results of linear mixed-effects models of blood responses to chronic hypoxia in F_2 hybrid deer mice

		α	β	Env	α*Env	β*Env
Red cell P_{50}	F	18.22	3.081	13.19	5.037	0.240
	P	**<0.001**	0.063	**<0.001**	**0.035**	0.789
Haematocrit	F	0.018	0.136	223.8	0.211	0.309
	P	0.894	0.874	**<0.001**	0.650	0.737
Haemoglobin	F	0.210	0.049	64.85	0.654	1.063
	P	0.653	0.952	**<0.001**	0.433	0.372

Env, acclimation environment, P_{50}, the partial pressure of O_2 at which haemoglobin is 50% saturation with O_2.

Table S3. Results of linear mixed-effects models of the effects of efaproxiral and inspired PO_2 in highland and lowland populations of deer mice

		Pop	Treat	PO_2	Pop*PO_2	Treat*PO_2	Pop*Treat	Pop*Treat*PO_2
Arterial O_2 saturation	F	4.169	39.15	569.1	4.340	11.41	10.72	3.231
	P	0.068	**<0.001**	**<0.001**	**0.018**	**<0.001**	**0.002**	**0.048**
Breathing frequency	F	17.90	1.113	39.66	n.s	n.s	n.s	n.s
	P	**0.002**	0.296	**<0.001**	n.s	n.s	n.s	n.s
Tidal volume	F	0.046	0.573	6.996	n.s	n.s	7.892	n.s
	P	0.834	0.452	**0.002**	n.s	n.s	**0.007**	n.s
Total ventilation	F	5.607	0.018	11.03	n.s	n.s	7.880	n.s
	P	**0.039**	0.895	**<0.001**	n.s	n.s	**0.007**	n.s
Air convection requirement	F	1.093	0.607	15.15	n.s	n.s	n.s	n.s
	P	0.320	0.439	**<0.001**	n.s	n.s	n.s	n.s
O_2 consumption rate	F	26.98	0.288	4.379	n.s	n.s	7.847	n.s
	P	**<0.001**	0.593	**0.017**	n.s	n.s	**0.007**	n.s
Heart rate	F	2.531	2.538	9.563	n.s	5.765	6.767	n.s
	P	0.143	0.117	**<0.001**	n.s	**0.005**	**0.012**	n.s
Body temperature	F	0.002	1.894	5.683	n.s	n.s	12.48	n.s
	P	0.962	0.174	**0.006**	n.s	n.s	**<0.001**	n.s

Pop, population; Treat, treatment (saline or efaproxiral); PO_2, partial pressure of O_2; n.s., not significant