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RESEARCH ARTICLE

Ontogenesis of evolved changes in respiratory physiology in deer mice native to high altitude

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ABSTRACT

High-altitude environments are cold and hypoxic, and many high-altitude natives have evolved changes in respiratory physiology that improve O_2 uptake in hypoxia as adults. Altricial mammals undergo a dramatic metabolic transition from ectothermy to endothermy in early post-natal life, which may influence the ontogenetic development of respiratory traits at high altitude. We examined the developmental changes in respiratory and haematological traits in deer mice (*Peromyscus maniculatus*) native to high altitude, comparing the respiratory responses to progressive hypoxia between highland and lowland deer mice. Among adults, highlanders exhibited higher total ventilation and a more effective breathing pattern (relatively deeper tidal volumes), for mice that were caught and tested at their native altitudes and those lab-raised in normoxia. Lab-raised progeny of each population were also tested at post-natal day (P)7, 14, 21 and 30. Highlanders developed an enhanced hypoxic ventilatory response by P21, concurrent with the full maturation of the carotid bodies, and their more effective breathing pattern arose by P14; these ages correspond to critical benchmarks in the full development of homeothermy in highlanders. However, highlanders exhibited developmental delays in ventilatory sensitivity to hypoxia, hyperplasia of type I cells in the carotid body and increases in blood haemoglobin content compared with lowland mice. Nevertheless, highlanders maintained consistently higher arterial O_2 saturation in hypoxia across development, in association with increases in blood–O_2 affinity that were apparent from birth. We conclude that evolved changes in respiratory physiology in high-altitude deer mice become expressed in association with the post-natal development of endothermy.

KEY WORDS: Hypoxic ventilatory response, Haemoglobin isoforms, Homeothermy, Peripheral O_2 chemoreceptor

INTRODUCTION

High-altitude natives are great models for understanding physiological mechanisms of hypoxia adaptation. High-altitude environments are both cold and hypoxic, which challenges the ability of endotherms to maintain adequate O_2 supply for thermoregulation and exercise. However, many animals are endemic to high altitude, with research suggesting they have overcome the challenges to aerobic metabolism associated with living at high altitude through changes in the O_2 transport pathway (Monge and Leon-Velarde, 1991; Storz et al., 2010a; Tate et al., 2017). The function of the O_2 pathway – composed of pulmonary ventilation (breathing), pulmonary O_2 diffusion, circulatory O_2 delivery, tissue O_2 diffusion and cellular O_2 utilization – is a critical determinant of tissue O_2 supply. As the first step in the O_2 pathway, pulmonary ventilation must occur at appropriate rates and/or using an effective pattern to safeguard O_2 uptake in hypoxic environments (Ivy and Scott, 2015; Storz and Scott, 2019; Storz et al., 2010a).

The control of breathing has evolved to enhance O_2 uptake in many high-altitude taxa. Breathing is stimulated by reductions in arterial O_2 levels in hypoxia, which is sensed by peripheral chemoreceptors in the carotid bodies that initiate the hypoxic chemoreflex and drive the increases in total ventilation (termed the hypoxic ventilatory response, HVR) (Gonzalez et al., 1994; Powell et al., 1998). Several highland taxa exhibit an enhanced HVR compared with their lowland counterparts, including Tibetan humans, plateau pika (*Ochotona curzoniae*) and bar-headed geese (*Anser indicus*) (Beall et al., 1997; Lague et al., 2016; Moore, 2000; Pichon et al., 2009; Scott and Milsom, 2006). These evolved changes have not occurred in some other highland taxa (Beall, 2000; Brutsaert et al., 2005; Ivy et al., 2018; Lague et al., 2017; Schwenke et al., 2007), but in the taxa in which they have arisen, they are likely to be valuable for augmenting O_2 uptake in the hypoxic environment at high altitude to support aerobically demanding activities. However, these previous observations have been made exclusively in adult animals, and we know very little about the ontogenesis of these traits during early development.

Why is it important to study the ontogenetic development of high-altitude phenotypes? Early post-natal life is characterized by many key transitions that facilitate the maturation of an offspring towards independence from its mother. For example, small altricial mammals such as mice are born without the ability to thermoregulate, and thermogenesis and the capacity for homeothermy develop over the first few weeks of post-natal life (Chew and Spencer, 1967; Lagerspetz, 1966). Given this developmental timeline, the unique respiratory phenotypes of high-altitude natives may not be present from birth, but may instead manifest only with the complete development of endothermic homeothermy when the metabolic O_2 costs of life are high. Indeed, the development of several processes important for respiratory O_2 uptake is also occurring during the first few weeks of life in small lowland mammals, such as the maturation of O_2-sensitive cells in the carotid body (Bamford et al., 1999; Carroll and Kim, 2012; Carroll et al., 1993) and the maturation of alveoli in the lungs (Amy et al., 1977). Nevertheless, the timing of these developmental milestones has rarely been studied in high-altitude natives.

The objective of this study was to investigate the post-natal development of the evolved changes in the control of breathing and

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respiratory O_2 uptake in high-altitude deer mice (*Peromyscus maniculatus*). Deer mice are broadly distributed across North America and can be found from sea level to over 4300 m elevation in the Rocky Mountains (Hock, 1964; Natarajan et al., 2015; Snyder et al., 1982). Adults at high altitude sustain high metabolic rates in the wild (Hayes, 1989), and have evolved a higher aerobic capacity ($\dot{V}_{O_2,max}$) in hypoxia compared with low-altitude populations of deer mice as well as with white-footed mice (*Peromyscus leucopus*), a congeneric species that is restricted to low altitudes (Cheviron et al., 2012, 2013, 2014; Lui et al., 2015; Tate et al., 2017). Evolved changes in the O_2 transport pathway have also arisen in high-altitude deer mice, including increases in haemoglobin (Hb)–O_2 affinity and changes in various other traits that influence O_2 supply and utilization by thermogenic tissues in hypoxia (Dawson et al., 2018; Lau et al., 2017; Lui et al., 2015; Mahalingam et al., 2017; Scott et al., 2015; Snyder et al., 1982; Storz et al., 2009, 2010b; Tate et al., 2017). Differences in the control of breathing also exist in high-altitude deer mice relative to low-altitude white-footed mice, as reflected by higher total ventilation and a more effective breathing pattern (deeper but less frequent breaths) in the former in comparisons of adults (Ivy and Scott, 2017a, 2018). We hypothesized that these differences represent evolved changes that are uniquely derived at high altitude, which we tested here by making intraspecific comparisons between high-altitude and low-altitude populations of deer mice. Furthermore, recent research has shown that the ontogenetic development of homeothermy and thermogenesis is delayed in high-altitude deer mice (Robertson and McClelland, 2019; Robertson et al., 2019). Therefore, we also hypothesized that the evolved changes in respiratory physiology in high-altitude deer mice arise in early post-natal life during the developmental transition to endothermy and the acquisition of high aerobic capacity.

MATERIALS AND METHODS

Mouse populations

Deer mice (*Peromyscus maniculatus rufinus* Wagner 1845) 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, 4350 m above sea level), and both deer mice [*Peromyscus maniculatus nebrascensis* (Coues 1877)] and white-footed mice [*Peromyscus leucopus* (Rafinesque 1818); a species that is restricted to low altitudes] were live trapped 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). A subset of wild deer mice were transported to the University of Denver Mount Evans field station (3231 m above sea level) or to the University of Nebraska (430 m above sea level), and were subjected to measurements of acute hypoxia responses (see below) within 1–2 days of capture. Other wild mice were transported to McMaster University (Hamilton, ON, Canada; ∼50 m above sea level) and housed in common laboratory conditions, and were used as parental stock to produce first generation (G1) lab progeny for each mouse population. Breeding pairs were held in individual cages, the male was removed when the female was visibly pregnant, and pups were weaned and moved to separate cages at post-natal day (P)21. Some G1 mice were similarly used as parental stock to produce second generation (G2) progeny for each mouse population. Experiments on captive mice were conducted on several distinct families of G1 deer mice (2 lowland and 5 highland families) during adulthood (at least 6 months of age) and on G2 deer mice during early development on P7, P14, P21 and P30 (6 lowland and 7 highland families). Acute hypoxia responses were measured for high- and low-altitude deer mice only, whereas haematology measurements were made on deer mice and white-footed mice. All captive mice were held in standard holding conditions (24–25°C, 12 h:12 h light:dark photoperiod) with unlimited access to food and water. Animal husbandry and experimentation followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

Acute hypoxia responses

Hypoxia responses were measured in unrestrained mice using barometric plethysmography, respirometry and pulse oximetry techniques that we have used in our previous studies (Ivy and Scott, 2017b, 2018). Mice were placed in a whole-body plethysmography chamber (530 ml) that was supplied with normoxic air (21 kPa O_2, balance N_2) at flow rates appropriate for the size and metabolic rate of the mouse (200 ml min^{-1} for P7, 300 ml min^{-1} for P14, 450 ml min^{-1} for P21 and P30, and 600 ml min^{-1} for adults) at room temperature (∼24°C). Mice were given 20–40 min to adjust to the chamber until relaxed and stable breathing and metabolism were observed. Mice remained at 21 kPa O_2 for an additional 20 min, followed by exposure to acute stepwise reductions in inspired O_2 pressure (P_{O_2}) for 20 min at each step. For wild-caught adults, these stepwise reductions in P_{O_2} were to 12, 10, 8 and 6 kPa O_2. Adult G1 mice and young G2 mice at P14, P21 and P30 were exposed to a slightly less severe protocol, with stepwise reductions in P_{O_2} to 16, 12, 10, 9 and 8 kPa O_2. P7 mice were only exposed to one level of mild hypoxia, at a P_{O_2} of 16 kPa O_2, and as they are not able to thermoregulate at room temperature at this age (Robertson et al., 2019), we helped maintain their normal body temperature over the duration of the experiment by placing a heating pad under the chamber. Incurrent gas composition was set by mixing dry compressed gases using precision flow meters (Sierra Instruments, Monterey, CA, USA) and a mass flow controller (MFC-4, Sable Systems, Las Vegas, NV, USA). Body temperature was measured at the beginning and end of the experiments using a mouse rectal probe (RET-3-ISO, Physitemp).

Breathing and O_2 consumption rate ($\dot{V}_{O_2}$) were determined during the last 10 min at each inspired P_{O_2}. Gas composition was measured continuously in incurrent and excurrent air flows that were subsampled at 150–200 ml min^{-1}. For incurrent air, the subsampled air was dried with prebaked Drierite and then the O_2 fraction was measured using a galvanic fuel cell O_2 analyser (Sable Systems). For excurrent air, water vapour pressure was measured using a thin-film capacitive water vapour analyser (RH-300, Sable Systems), the gas stream was dried with prebaked Drierite, the O_2 fraction was measured as above, and then the CO_2 fraction was measured using an infrared CO_2 analyser (Sable Systems). These data were used to calculate $\dot{V}_{O_2}$, expressed in volumes at standard temperature and pressure (STP) using appropriate equations for dry air as described by Lighton (2008), as well as rates of water loss. Chamber temperature was continuously recorded with a thermocouple (TC-2000, Sable Systems). Breathing frequency and tidal volume were measured from changes in flow across a pneumotachograph in the wall of the plethysmograph chamber, detected using a differential pressure transducer (Validyne DP45, Cancopass, Mississauga, ON, Canada). Tidal volume was calculated using established equations (Drorbaugh and Fenn, 1955; Jacky, 1980) assuming a constant rate of decline in body temperature with declining P_{O_2}, which we have previously shown results in similar tidal volumes to those calculated using direct body temperature measurements at each P_{O_2} (Ivy and Scott, 2017b). Total ventilation was calculated as the

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product of breathing frequency and tidal volume. Total ventilation and tidal volume data are expressed in volumes at body temperature and pressure of saturated air (BTPS). Air convection requirement is the quotient of total ventilation and $\dot{V}_{O_2}$. All of the above data were acquired using a PowerLab 16/32 and Labchart 8 Pro software (ADInstruments, Colorado Springs, CO, USA). Arterial O_2 saturation (Sa_{O_2}) was measured in adult G1 mice, and in young G2 mice at P14, P21 and P30 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.

Immunohistochemistry of the carotid bodies

We examined carotid body development in G2 deer mice at P7, P14, P21 and P30 (*n*=5 highlanders and *n*=5 lowlanders at each age, each of which was from a distinct family) using similar approaches to those we have used previously in adult mice (Ivy and Scott, 2017a). Mice were killed with an overdose of isoflurane followed by decapitation, blood samples (70–300 µl) were taken for haematology (see below), and the bifurcations of the carotid artery were dissected, removed, and fixed in 4% paraformaldehyde for 48 h. The tissue was then cryoprotected in 24% sucrose solution, frozen in embedding medium (Cryomatrix, ThermoFisher Scientific, Waltham, MA, USA) and stored at −80°C. Samples were serially sectioned at 10 µm in a cryostat (Leica CM1860, Wetzlar, Germany) maintained at −20°C, and were then air dried and stored at −80°C. We systematically collected and stained every second section to represent the entirety of each carotid body (Superfrost Plus Fisherbrand, ThermoFisher Scientific). Immunohistochemistry was used to identify type I (glomus) cells (using tyrosine hydroxylase, TH, as a marker), neurons (using neurofilament, NF, and growth-associated protein-43, GAP-43 as concurrent markers) and nuclei (DAPI, 4′,6-diamidino-2-phenylindole, to assist with the identification of individual cells) on one set of carotid body slides; type II cells (using glial fibrillary acidic protein, GFAP, as a marker) and nuclei (DAPI) were identified on the second set of slides. Sections were hydrated in phosphate-buffered saline (PBS; 137 mmol l^{-1} NaCl, 2.68 mmol l^{-1} KCl, 10.0 mmol l^{-1} Na_2HPO_4, 1.76 mmol l^{-1} KH_2PO_4) and treated with blocking solution (PBS containing 0.5% Triton X-100 and 10% normal goat serum) for 1 h. Sections were then incubated overnight in blocking solution containing primary antibodies targeting TH (1:2000 dilution; AB152, Millipore, Billerica, MA, USA), NF (1:100 dilution; MAB1615, Millipore) and GAP-43 (1:2000 dilution; G9264, Sigma-Aldrich, Mississauga, ON, Canada) for the first set of slides, or GFAP (1:1000 dilution; Z033429-2, Agilent Technologies, Santa Clara, CA, USA) for the second set of slides. The following morning, slides were rinsed well in PBS and then incubated for 2 h in blocking solution that contained secondary antibodies (1:400 dilution) against the TH/GFAP primary antibodies (AlexaFluor488, goat anti-rabbit IgG; A11034, Life Technologies, Mississauga, ON, Canada) and (in the first set of slides only) both of the NF and GAP-43 primary antibodies (AlexaFluor594, goat anti-mouse IgG; A11032, Life Technologies). Sections were rinsed well in PBS, and incubated in PBS containing 0.5% Triton X-100 and DAPI (1:100,000 dilution; Sigma-Aldrich) for 25 min. Slides were rinsed thoroughly in PBS and mounted with Vectashield (Vector Laboratories, Brockville, ON, Canada). Sections were imaged using an Olympus microscope with Northern Eclipse software (Elite version 8.0; Empix Imaging, Mississauga, ON, Canada).

The first set of slides was used to measure total projected area in each section of the whole carotid body, neurons and type I cells. The second set of slides was used to measure total projected area of type II cells. All of these area measurements were made using Nikon NIS Elements documentation software (v4.30.02). The number of type I cells in each section was manually counted using ImageJ software (v1.47). Carotid body volume, type I volume, type II volume and neural volume were calculated as the sum of the volumes in each section of the carotid body (the latter was calculated as the product of projection area and section thickness) (Saiki et al., 2006), multiplied by 2 to account for the fact that we only analysed every second slide. Carotid body volume was determined based on the boundaries defined by the neural staining and DAPI clustering. The total number of glomus cells in the carotid body was calculated as the sum of all glomus cells counted across every carotid body section, similarly multiplied by 2.

Haematology and Hb–O_2 affinity

Blood was collected from captive deer mice and white-footed mice to measure haematological traits and Hb–O_2 affinity. Blood Hb content was measured using Drabkin's reagent (Sigma-Aldrich) according to the manufacturer's instructions, and haematocrit was measured by centrifuging blood in a heparinized capillary tube at 12,700 ***g*** for 5 min. Mean corpuscular Hb concentration was calculated as the quotient of blood Hb content and haematocrit. 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 containing 100 mmol l^{-1} Tris, 50 mmol l^{-1} EDTA, 100 mmol l^{-1} KCl, 0.1% bovine serum albumin and 0.2% antifoaming agent at pH 7.4 (TCS Scientific). Oxygen dissociation curves were also generated for adult mice using 100 mmol l^{-1} Hepes (also at pH 7.4) instead of 100 mmol l^{-1} Tris, in order to determine the influence of the assay buffer on P_{50} (the P_{O_2} at which Hb is 50% saturated with O_2; Weber, 1992). Hb–O_2 affinity (P_{50}) was calculated using Hemox Analytic Software (TCS Scientific).

Analysis of Hb isoform composition

We tested for developmental changes in the Hb isoform (isoHb) composition of red blood cells by comparing samples collected at P0/P2 versus P27 from a separate set of G2 highlanders and G2 white-footed mice (*n*=7–8 samples for each developmental time point in each population) from the mice used in the experiments above. Mice were killed with an overdose of isoflurane followed by decapitation, and red blood cell samples (65–90 µl) were collected after whole blood was centrifuged in heparinized capillary tubes (as described above). In the white-footed mice, the samples were from newborns at P0 (*n*=3) and P2 (*n*=5), whereas in highlanders, the samples were all from newborns at P0. We characterized isoHb composition of haemolysates by means of isoelectric focusing (IEF) in combination with tandem mass spectrometry (MS/MS) (Revsbech et al., 2013; Storz et al., 2010b). We electrophoretically separated each isoHb on the basis of net charge using IEF gels with a 5–8 pH gradient (PhastSystem, GE Healthcare Bio-Sciences, Piscataway, NJ, USA). We then used ImageJ (Abràmoff et al., 2004) to obtain densitometric measurements of IEF band intensity to quantify the relative abundance of each identified isoHb. For representative samples of each developmental stage and population, we excised individual bands from the IEF gel to identify the subunit composition of each tetrameric isoHb via MS/MS. For each individual isoHb, we then separated α- and β-type subunits by means of 20% SDS PAGE followed by staining with Coomassie brilliant blue-G.

The α- and β-chain monomers from the SDS gel were digested with trypsin, and the resultant peptides were identified using MS/MS. The peak list of the MS/MS data was generated by Distiller

(Matrix Science, London, UK) using the charge state recognition and de-isotoping with default parameters for quadrupole time-of-flight data. Database searches of the resultant MS/MS spectra were performed using Mascot v1.9.0 (Matrix Science). Specifically, peptide mass fingerprints derived from the MS/MS analysis were used to query a customized reference database of sequences representing the full complement of pre- and post-natally expressed α- and β-type globin genes in deer mice and in white-footed mice (Hoffmann et al., 2008; Natarajan et al., 2015; Storz et al., 2009, 2010b). The following search parameters were used for the MS/MS analysis: no restriction on protein molecular weight or isoelectric point, and methionine oxidation allowed as a variable peptide modification. Mass accuracy settings were 0.15 Da, for peptide mass and 0.12 Da for fragment ion masses. We identified all significant protein hits that matched more than one peptide with P<0.05.

Statistics

For acute hypoxia responses, the main effects of population (lowlander versus highlander) and acute exposure P_{O_2} (repeated measure) and their interactions were evaluated using two-factor ANOVA within wild mice, within G1 adult mice, and within each age of G2 mice. Body mass of wild mice and G1 adult mice was compared between populations using t-tests. For body mass (P7–P30), carotid body traits, Hb–O_2 affinity and other haematological traits, two-factor ANOVA was used to evaluate the main effects of population and age, and their interaction. The full results of these statistical analyses are included in Tables S1–S3, and the salient findings are reported in the Results. Developmental changes in the relative abundance of different Hb isoforms (isoHbs) were tested using two-factor ANOVA. Holm–Šidák *post hoc* tests were used throughout to make pairwise comparisons between highlanders and lowlanders or P0/2 and P27, respectively. Values are

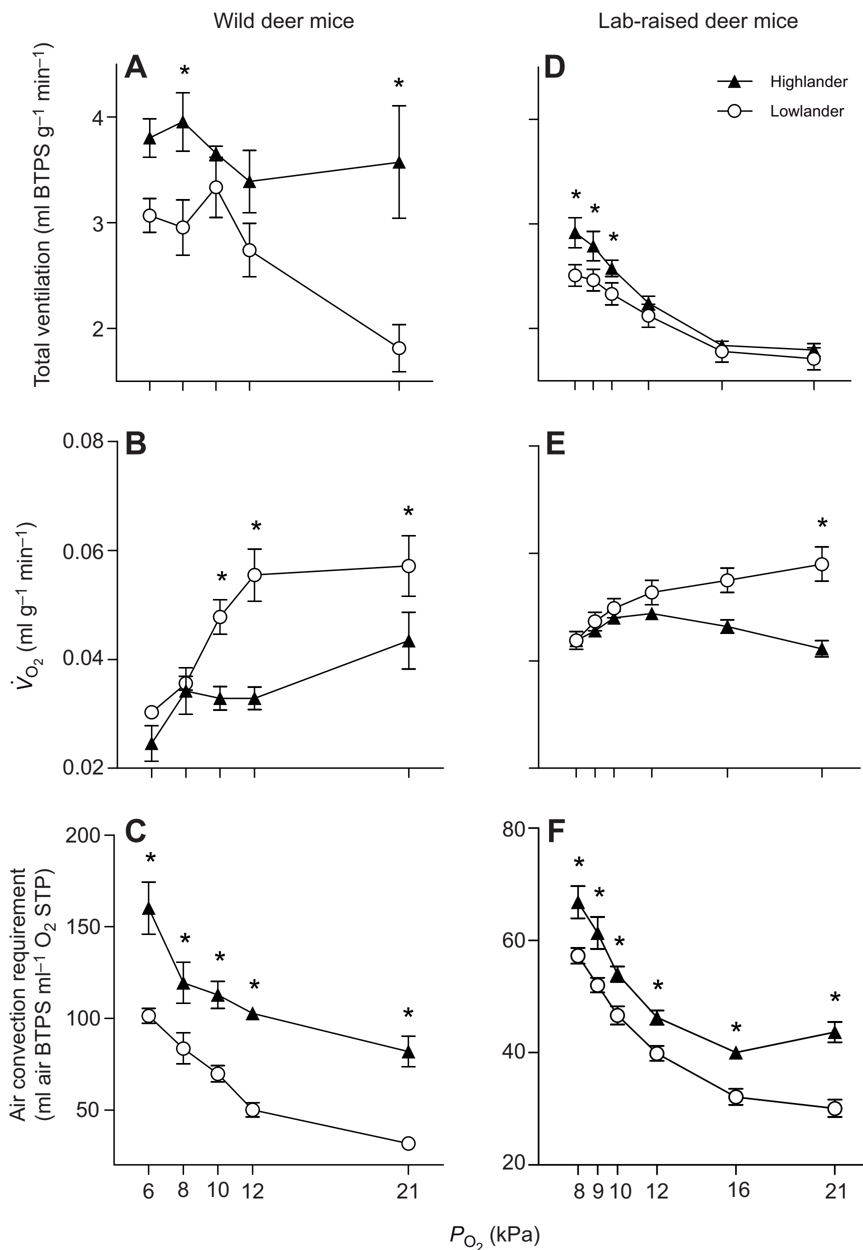

Fig. 1. Wild (left) and lab-raised (right) deer mice from high altitude exhibit differences in breathing and metabolism in hypoxia compared with deer mice from low altitude. (A,D) Total ventilation, (B,E) O_2 consumption rate ($\dot{V}_{O_2}$) and (C,F) air convection requirement versus oxygen partial pressure (P_{O_2}). *Significant pairwise difference between populations within each P_{O_2} using Holm–Šidák *post hoc* tests (n=5 wild lowlanders, n=5 wild highlanders, n=12 lab-raised lowlanders, n=30 lab-raised highlanders). BTPS, body temperature and pressure of saturated air; STP, standard temperature and pressure.

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reported as means±s.e.m. All statistical analysis was conducted with SigmaStat software (v3.5) with a significance level of $P<0.05$.

RESULTS

Ventilatory and metabolic responses to acute hypoxia

There were differences in breathing and metabolism between high- and low-altitude mice in the wild, some of which persisted into the first generation of lab-raised adult mice (Figs 1 and 2; Table S1). Wild highlanders had higher total ventilation compared with wild lowlanders across several inspired P_{O_2} (main effect of population: $P=0.003$; Fig. 1A), but they also had lower $\dot{V}_{O_2}$ ($P=0.008$; Fig. 1B). As a result, air convection requirement was 1.4- to 2.6-fold higher in wild highlanders than in wild lowlanders across the full range of inspired P_{O_2} (population effect: $P<0.001$; Fig. 1C). Differences between populations for adult G1 mice were not as large, but highlanders had higher total ventilation (population effect: $P=0.007$, Fig. 1D) and/or lower $\dot{V}_{O_2}$ (population effect, $P=0.008$; population×P_{O_2} interaction, $P<0.001$; Fig. 1E) than lowlanders, such that air convection requirement was still elevated by 1.2- to 1.5-fold in lab-raised highlanders across the full range of inspired P_{O_2} tested (population effect: $P<0.001$; Fig. 1F). The increases in ventilation in response to acute hypoxia were driven by increasing breathing frequency in both wild and lab-raised mice, and were partially offset by reductions in tidal volume (Fig. 2). In both wild and lab-raised adult mice, highlanders maintained deeper tidal volume and/or lower breathing frequency compared with lowlanders across most levels of inspired P_{O_2} (Fig. 2). These differences were not associated with differences in body mass between highlanders (23.08±0.91 g) and lowlanders (19.06±1.86 g) for wild-caught mice (population effect: $P=0.118$), nor between highlanders (18.87±1.21 g) and lowlanders (22.26±2.06 g) for lab-raised mice (population effect: $P=0.152$).

Population differences in the ventilatory and metabolic responses to hypoxia were established between P14 and P30 (Fig. 3; Table S2). Highland mice did not respond to mild hypoxia at P7, as reflected by a lack of increase in either total ventilation or air convection requirement when exposed to 16 kPa O_2 (Fig. 3A–C). In contrast, at P7 lowland mice maintained total ventilation despite the fall in $\dot{V}_{O_2}$ at 16 kPa O_2, such that air convection requirement increased in response to hypoxia. Both populations had developed ventilatory responsiveness to hypoxia by P14, as reflected by increases in both total ventilation and air convection requirement, and both populations reduced $\dot{V}_{O_2}$ in hypoxia. Lowlanders exhibited higher $\dot{V}_{O_2}$ than highlanders at P14 (population effect: $P<0.001$; Fig. 3E), which persisted at P21 ($P<0.001$; Fig. 3H) and P30 ($P=0.006$; Fig. 3K), similar to the population differences in adults. Highlanders developed higher air convection requirements than lowlanders by P21 ($P=0.032$; Fig. 3I) and this difference persisted at P30 ($P=0.006$; Fig. 3L). The population differences in ventilatory and metabolic traits did not appear to be associated with population differences in body mass, which increased as expected with age (age effect: $P<0.001$) but was generally similar between highlanders

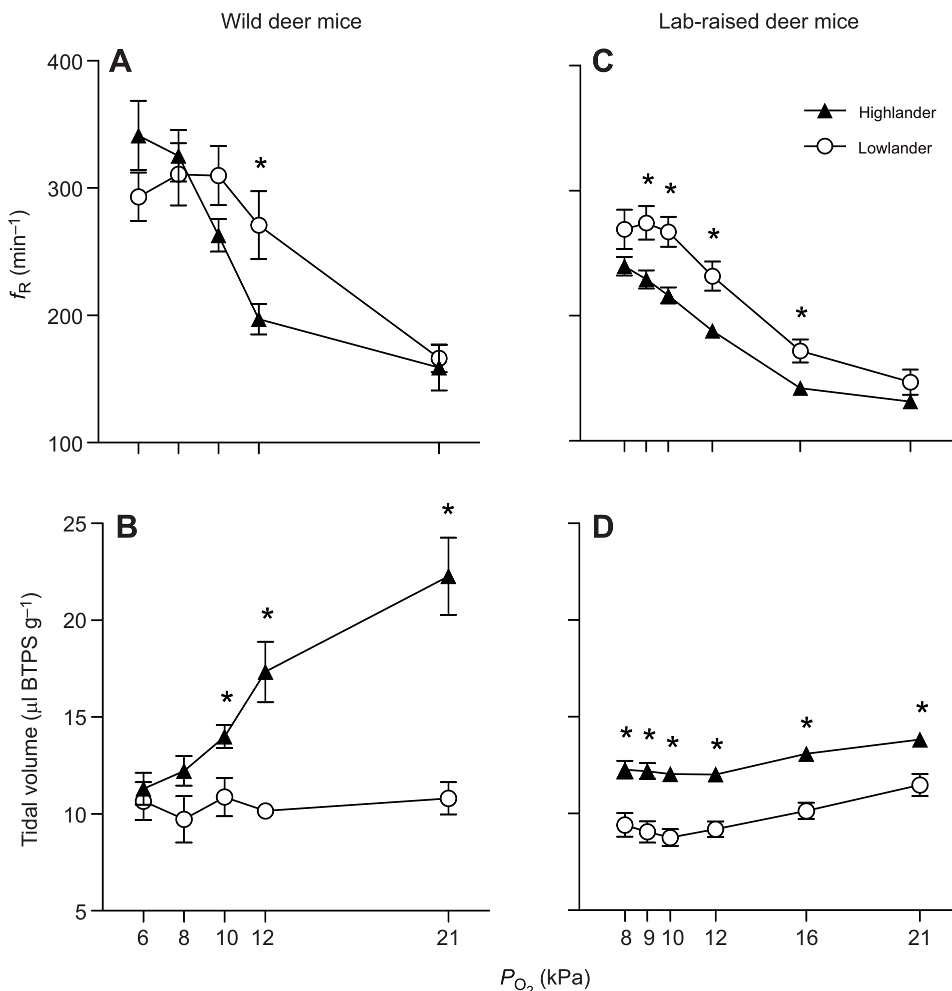

Fig. 2. Wild (left) and lab-raised (right) deer mice from high altitude take deeper but less frequent breaths in hypoxia compared with deer mice from low altitude. (A,C) Breathing frequency (f_R) and (B,D) tidal volume versus P_{O_2}. *Significant pairwise difference between populations within each P_{O_2} using Holm–Šidák *post hoc* tests (*n* as in Fig. 1).

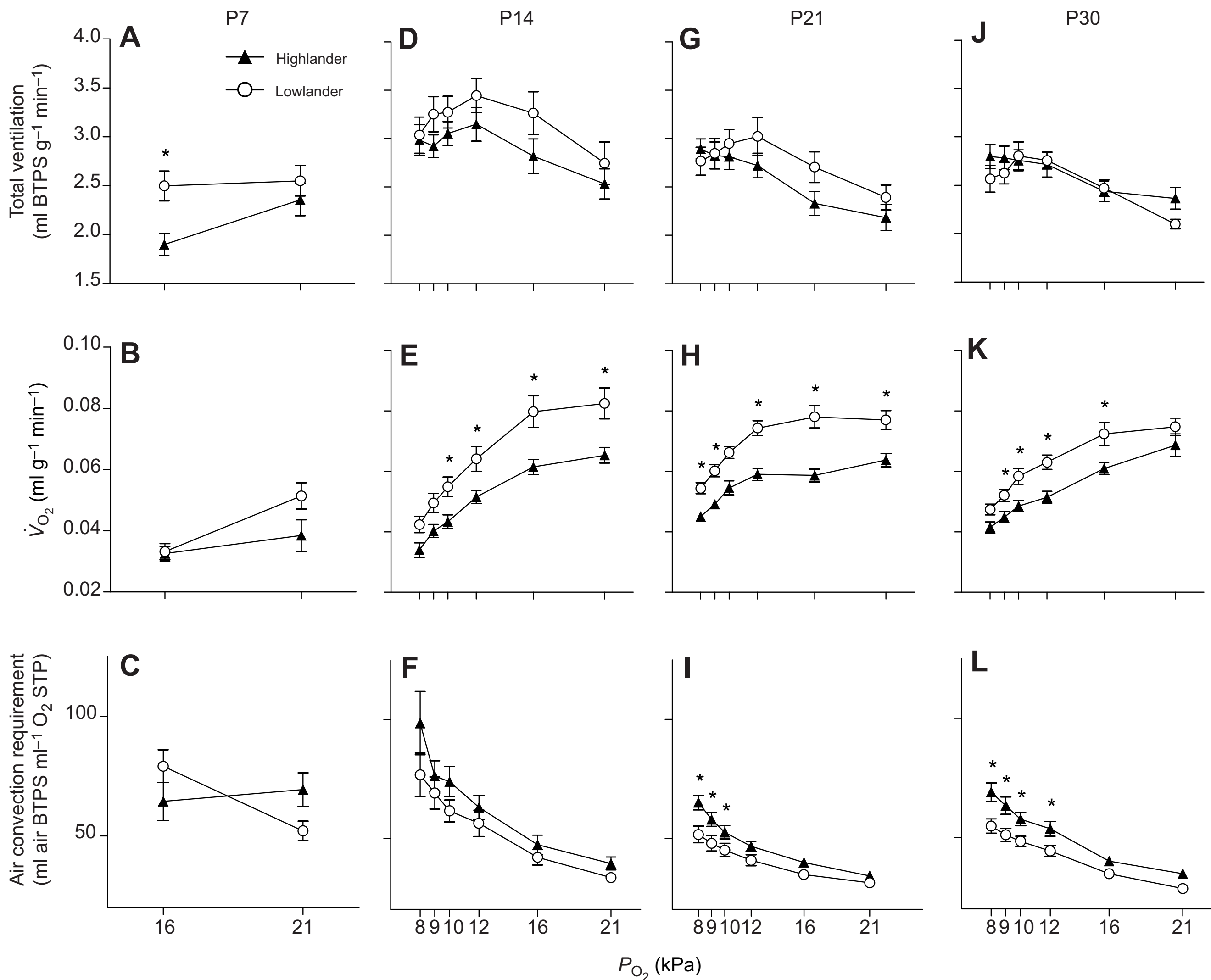

Fig. 3. Highland deer mice are unresponsive to hypoxia at P7 of post-natal development, and begin to express the population differences in ventilation and/or metabolism exhibited by adults at P14, P21 and P30. (A,D,G,J) Total ventilation, (B,E,H,K) $\dot{V}_{O_2}$ and (C,F,I,L) air convection requirement versus P_{O_2}. *Significant pairwise difference between populations within each P_{O_2} using Holm–Šidák *post hoc* tests (*n*=12 P7 lowlanders, *n*=14 P7 highlanders, *n*=11 P14 lowlanders, *n*=14 P14 highlanders, *n*=10 P21 lowlanders, *n*=14 P21 highlanders, *n*=11 P30 lowlanders, *n*=17 P30 highlanders). P, post-natal age (days).

(P7, 4.59±0.18 g; P14, 7.77±0.25 g; P21, 10.22±0.28 g; P30, 13.45±0.54 g) and lowlanders (P7, 4.09±0.13 g; P14, 6.82±0.28 g; P21, 9.65±0.47 g; P30, 12.47±0.43 g).

The differences in breathing pattern between populations that was characteristic of adult mice appeared at P14 of post-natal development (Figs 4 and 5; Table S2). At P7, highlanders had shallower tidal volume than lowlanders (population effect: P=0.005, Fig. 4B). The onset of ventilatory responsiveness to hypoxia by P14 was caused by robust increases in breathing frequency in both populations. However, highlanders exhibited lower breathing frequency than lowlanders at P14 (population effect: P=0.002; Fig. 4C), and this difference persisted at P21 (P<0.001; Fig. 4E) and P30 (P=0.018; Fig. 4G), similar to the population differences observed in adults. Highlanders exhibited deeper tidal volume than lowlanders at P30 (population effect: P=0.007; Fig. 4H), but there was also a trend for highlanders to exhibit deeper tidal volume in the severe hypoxia at earlier ages (Fig. 4D,F). Indeed, when breathing data at 12 kPa O_2 (roughly equivalent to the P_{O_2} at 4300 m elevation) from all captive mice were considered together, by plotting total ventilation as a function of tidal volume, differences in breathing pattern were clearly evident from P14 to adulthood (Fig. 5). There was a progressive reduction in mass-specific rate of total ventilation during development, initially due to a decline in mass-specific tidal volume from P14 to P30 (i.e. changes that parallel the isopleths of constant breathing frequency) followed by a decline in breathing frequency from P30 to adulthood. Nevertheless, highlanders breathed with relatively deeper breaths than lowlanders at any given total ventilation from P14 to adulthood, as reflected by a rightward shift in the relationship between tidal volume and total ventilation (Fig. 5).

Arterial O_2 saturation during acute hypoxia

Highland deer mice maintained higher Sa_{O_2} during hypoxia than lowlanders from P14 to adulthood (Fig. 6; Tables S1 and S2). Sa_{O_2} fell in deer mice of all ages during acute exposure to hypoxia (P_{O_2} effect: P<0.001), but highlanders maintained a higher Sa_{O_2} across a range of reduced inspired P_{O_2} at P14 (population

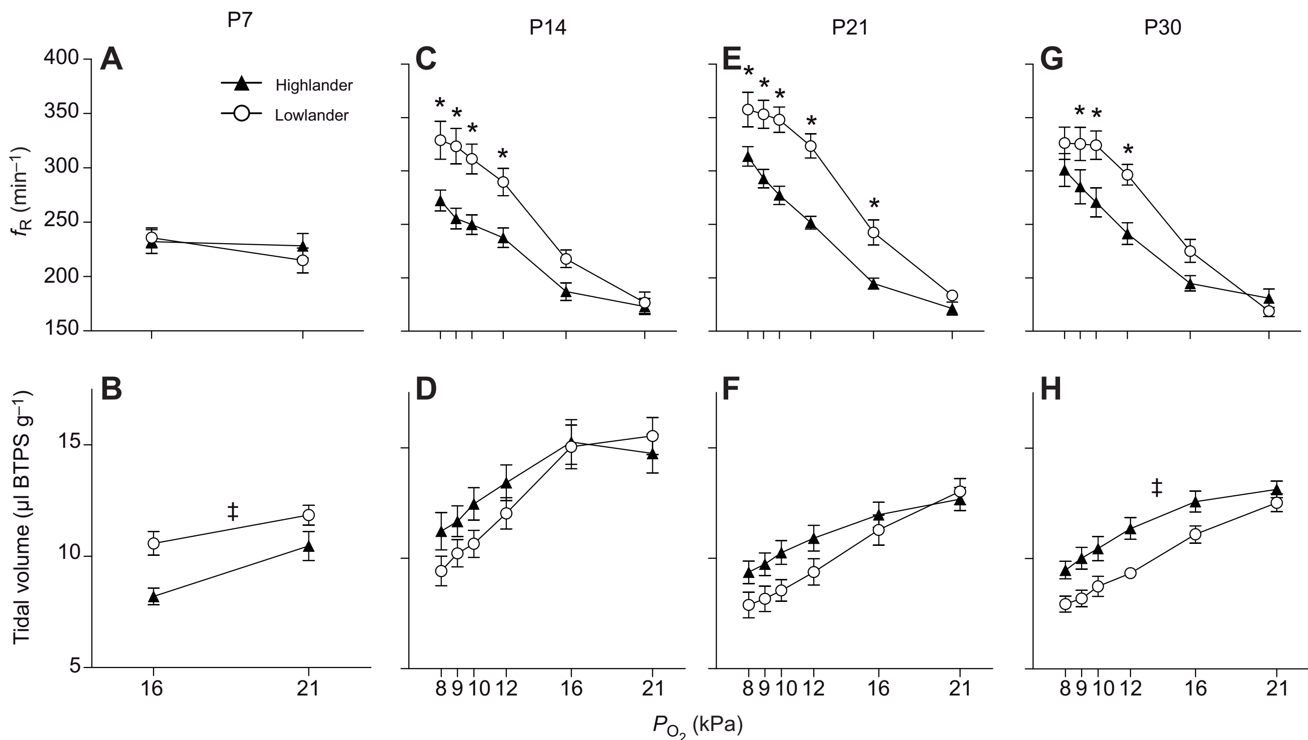

Fig. 4. Breathing pattern during hypoxia diverges between highland and lowland populations of deer mice at P14 and differences persist at P21 and P30. (A,C,E,G) Breathing frequency; and (B,D,F,H) tidal volume versus P_{O_2}. *Significant pairwise difference between populations within each P_{O_2} using Holm–Šidák *post hoc* tests. ‡Significant main effect of population in two-factor ANOVA (*n* as in Fig. 3).

effect: *P*<0.001), P21 (*P*=0.044) and P30 (*P*=0.027), and this difference approached significance in adults (*P*=0.064).

Body temperature during acute hypoxia

Body temperature tended to fall in response to acute hypoxia exposure in all mice (Table 1). Among adult mice caught in the wild, body temperature in normoxia was lower in highlanders than in lowlanders, but lowlanders exhibited a much greater suppression of body temperature in response to hypoxia. This large population difference was not observed in lab-raised mice, in which there were no clear substantial differences in the body temperature response to hypoxia between highlanders and lowlanders. Body temperature in normoxia was similar between populations, and was in the normal range even though ambient temperature (24°C) was probably below the thermal neutral zone for all ages (Hill, 1976).

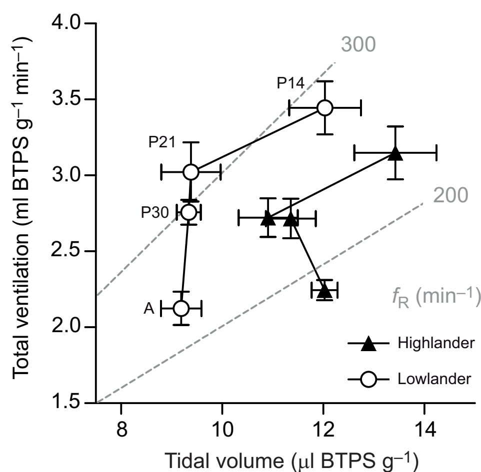

Fig. 5. Highland deer mice consistently maintain deeper tidal volumes at 12 kPa O_2 for any given total ventilation from P14 into adulthood (A). Total ventilation versus tidal volume (*n* as in Figs 1 and 3). Breathing frequency (f_R, min^{-1}) is shown in grey.

Carotid body morphology

Highlanders exhibited a delayed maturation of the carotid body (Fig. 7; Table S3). The total volume of the carotid body was established and remained constant from P7 onwards at a similar volume between populations [highlanders: 27.18(±0.23)×10^5 µm^3, lowlanders: 24.75(±0.14)×10^5 µm^3], as was the volume occupied by neurons [highlanders: 3.24(±0.28)×10^5 µm^3, lowlanders: 2.88(±0.23)×10^5 µm^3]. However, the developmental increases in the total number and volume of type I cells differed between populations, occurring between P7 and P14 in lowland mice but not until between P14 and P21 in highland mice (Fig. 7C,D). Type II cell volume declined from P7 to P14 in both populations, but it returned to initial levels by P21 in lowlanders but not until P30 in highlanders (Fig. 7E). In both populations, an increase in the ratio of type I volume to type II volume occurred in conjunction with the initial proliferation of type I cells (Fig. 7F).

Haematology and Hb–O_2 affinity

We also sought to examine whether differences in haematology might contribute to population differences in blood O_2 content across development. Highlanders exhibited a delayed rise in blood Hb content and haematocrit during development (Table 2; Table S3). Hb content and haematocrit decreased from P0 to P7 and then increased steadily thereafter, but these increases appeared to occur later in highland deer mice than in lowland deer mice and in the strictly lowland native white-footed mice (Table 2).

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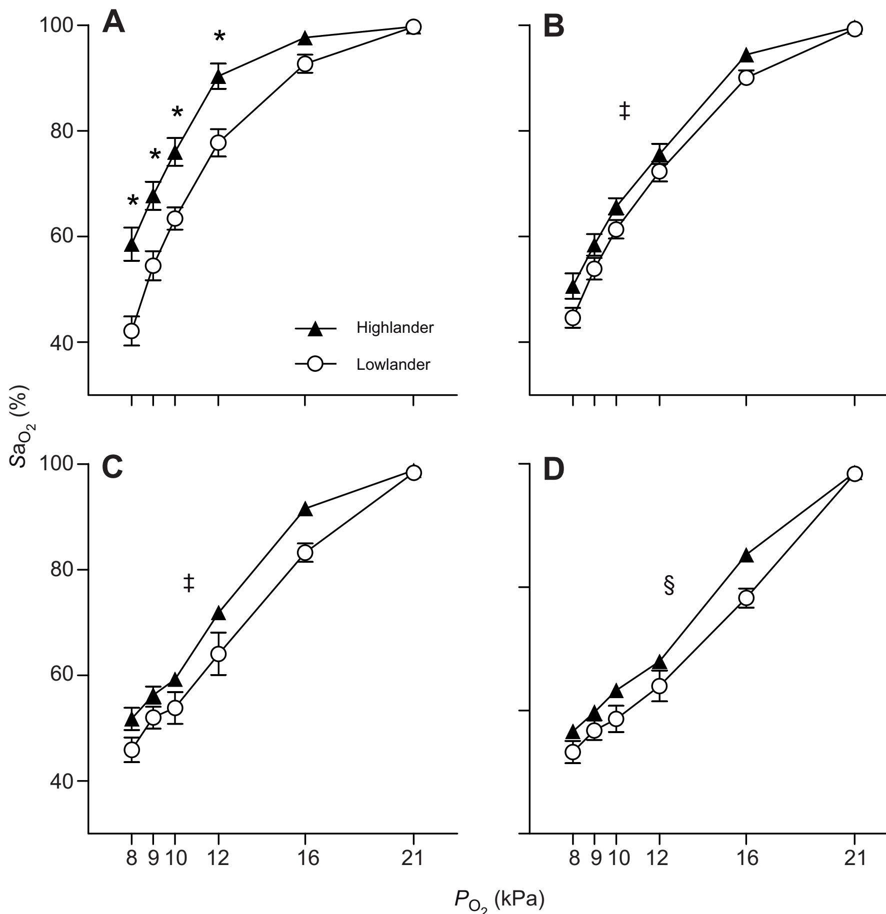

Fig. 6. Highland deer mice maintain higher arterial O_2 saturation (Sa_{O_2}) in hypoxia than lowland deer mice from P14 to adulthood. (A) P14, (B) P21, (C) P30 and (D) adult. ‡Significant main effect of population in two-factor ANOVA; §*P*=0.064 for main effect of population in two-factor ANOVA; *significant pairwise difference between populations within each P_{O_2} using Holm–Šidák *post hoc* tests (*n*=11 P14 lowlanders, *n*=14 P14 highlanders, *n*=10 P21 lowlanders, *n*=14 P21 highlanders, *n*=11 P30 lowlanders, *n*=17 P30 highlanders, *n*=12 lab-raised lowland adults, *n*=30 lab-raised highland adults).

By adulthood, blood Hb content and haematocrit were similar between populations. Mean corpuscular Hb concentration, the quotient of Hb and haematocrit, was unchanged through development and not significantly different between populations (data not shown).

Highlanders had consistently higher Hb–O_2 binding affinity than lowlanders (Fig. 8; Table S3). Red blood cell P_{50} increased between P0 and P7 and then remained stable thereafter, but highlanders maintained lower P_{50} across ages compared with both lowland deer mice and white-footed mice. The combined IEF and MS/MS analyses suggested that the increase in P_{50} from P0 to P7 was unlikely to have resulted from major shifts in red blood cell isoHb composition. IEF of haemolysates from highland mice revealed some subtle shifts in isoHb composition from P0 to P27, as reflected by an increase in the expression of isoHb C, but all ages expressed the same set of four isoHbs (Fig. 8C,D). The consistency across ages was greater in white-footed mice, in which the relative abundance of three expressed isoHbs did not change between P0/2 and P27 (Fig. 8E). The MS/MS analysis revealed that the peptide mass fingerprints of the α- and β-chain subunits comprising each of the electrophoretically distinct isoHb were perfect matches to those expected from translations of nucleotide sequences from the full complement of adult-expressed globin genes (Natarajan et al., 2015; Storz and Kelly, 2008; Storz et al., 2009, 2010b, 2012). We detected no trace of embryonic α- or β-type globins in P0/2 or P27 mice from either population.

Table 1. Body temperature (°C) in normoxia and after acute exposure to stepwise hypoxia in adults and in young mice during early post-natal development

	Highland deer mouse		Lowland deer mouse	
Age	Normoxia	Hypoxia	Normoxia	Hypoxia
Wild adult	35.63±0.33	34.43±0.55	37.81±0.52*	32.40±0.52*
Lab-raised adult	38.16±0.18	36.21±0.25	38.68±0.20	35.79±0.32
P7	34.65±0.11	–	34.79±0.24	–
P14	35.50±0.23	32.38±0.32	36.14±0.24	33.50±0.25*
P21	36.98±0.20	34.74±0.19	37.57±0.12	34.77±0.33
P30	36.70±0.19	35.18±0.16	37.69±0.34*	34.52±0.27*

Hypoxic body temperature is not provided for P7 mice, because body temperature was maintained throughout hypoxia exposure using a heating pad. *Significant pairwise difference at the given time point from highland deer mice (*n* as in Figs 1 and 3). P, post-natal age (days).

DISCUSSION

We have shown that evolved changes in the control and pattern of breathing in high-altitude deer mice arise early after birth during the post-natal development of endothermy. Many high-altitude animals exhibit evolved changes in the control of breathing that make breathing more effective and improve respiratory gas exchange in hypoxia (Beall et al., 1997; Moore, 2000; Pichon et al., 2009; Scott and Milsom, 2006), but the timing of the development of these responses during early life was unresolved. Here, we show that the evolved increases in effective ventilation and the hypoxic ventilatory response that are observed in adult deer mice from high altitude arise between 7 and 21 days after birth, concurrent with the development of homeothermy and increases in thermogenic capacity (Robertson and McClelland, 2019; Robertson et al., 2019). However, the development of ventilatory sensitivity to hypoxia and

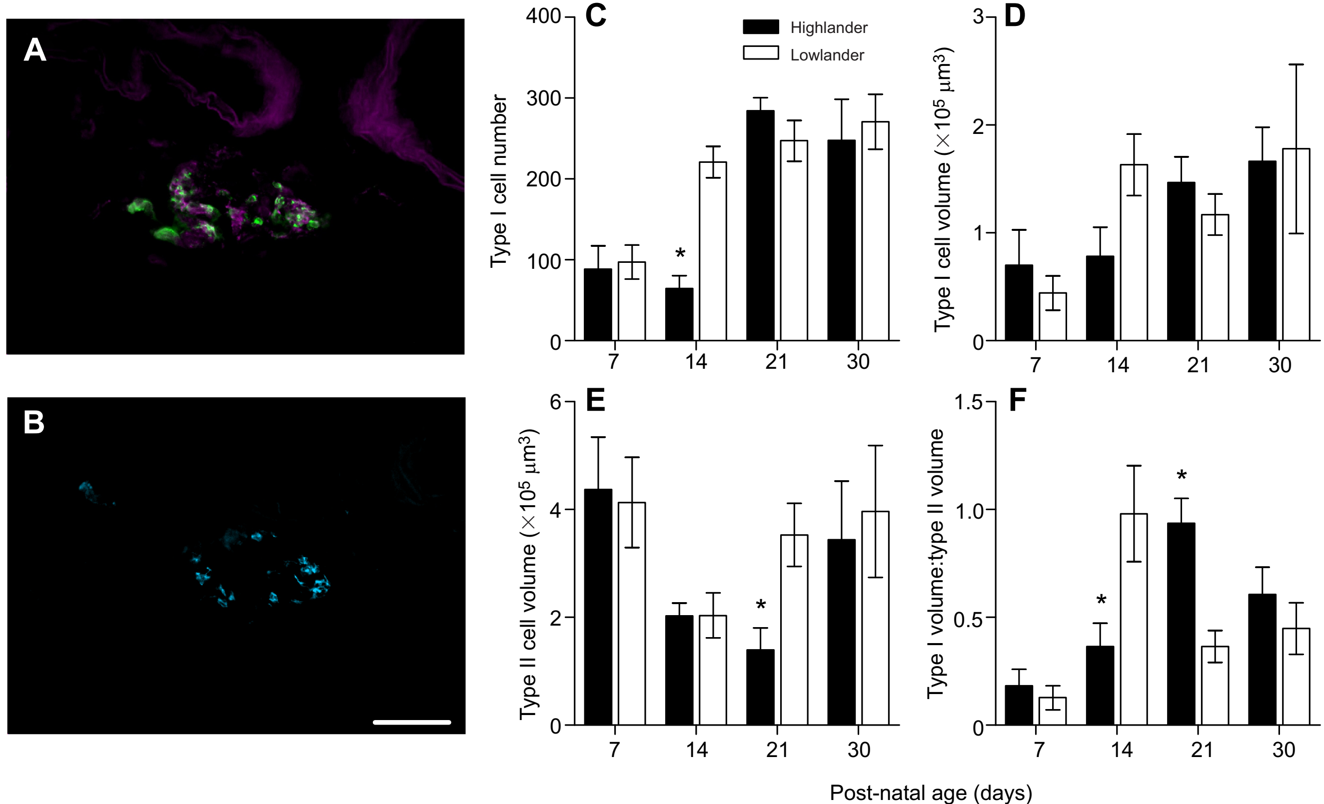

Fig. 7. Carotid body development is delayed in highland deer mice. Fluorescence immunohistochemistry was used to identify (A) type I cells (tyrosine hydroxylase; green) and neurons (neurofilament and growth-associated protein 43; magenta), or (B) type II cells (glial fibrillary acidic protein; blue). Representative images from a lowland mouse at P21. Scale bar: 100 µm. The number (C) and volume (D) of type I cells increased with age, along with developmental changes in the volume of type II cells (E), and the ratio of type I cell volume to type II cell volume (F). *Significant pairwise difference between populations within an age using Holm–Šidák *post hoc* tests (*n*=5 for each group).

the post-natal hyperplasia of O_2-sensitive cells in the carotid bodies are delayed in highlanders compared with lowlanders. The evolved increase in the hypoxic ventilatory response in highlanders does not arise until after this early post-natal phase of carotid body maturation is complete. Therefore, the first few weeks of post-natal life are a dynamic period for high-altitude deer mice, when many of the respiratory and metabolic phenotypes associated with high-altitude adaptation first emerge.

Table 2. Haematology of lab-raised adults and of young mice during early post-natal development

Age	Highland deer mice	Lowland deer mice	Lowland white-footed mice
[Hb] (g dl^{-1})			
P0	15.20±0.69	–	17.39±0.42
P7	11.34±0.47	12.67±0.36	10.99±0.55
P14	12.40±0.33	13.68±0.32	12.68±0.37
P21	12.90±0.27	15.13±0.47*	14.61±0.47*
P30	14.35±0.57	15.32±0.37	14.46±0.56
Adult	16.63±0.54	15.80±0.41	15.14±0.61
Hct (%)			
P0	45.34±1.65	–	53.11±1.05
P7	35.05±1.22	36.88±0.74	35.48±1.26
P14	35.51±1.30	42.45±0.70*	37.43±0.73
P21	39.66±0.74	45.05±1.46*	41.65±1.03
P30	42.18±0.80	45.63±0.74*	45.43±1.01*
Adult	48.59±1.66	43.89±0.85	46.93±2.24

[Hb], haemoglobin concentration; Hct, haematocrit. *Significant difference from highland deer mice (*n* as in Fig. 8A). P, post-natal age (days).

Our findings confirm our other recent findings showing that adult deer mice from high altitude breathe more effectively than their lowland counterparts, with higher total ventilation and preferentially deeper breaths that should increase alveolar ventilation and thus help safeguard Sa_{O_2} in hypoxia (Ivy and Scott, 2017a, 2018). Our previous findings showed that lab-raised highland mice exhibit these differences in comparison with a lowland congener (*P. leucopus*) and that they persist for at least two generations in captivity. However, we had not clearly established in past work whether this trait was unique to highland deer mice or could instead have been a common feature across the *P. maniculatus* species (Ivy and Scott, 2017a, 2018). In this study, we show highland deer mice do indeed exhibit higher total ventilation and preferentially deeper breaths when compared with lowland deer mice. These combined results suggest that the ventilatory phenotype of high-altitude deer mice is a derived trait that uniquely evolved for life at high altitude.

The evolved ventilatory phenotype of adult highlanders was established between 7 and 21 days after birth, concurrent with the development of endothermic homeothermy. Endothermy begins to develop within a few days of birth, and the high aerobic capacity for thermogenesis that is characteristic of adult deer mice from high altitude (Cheviron et al., 2012, 2013; Tate et al., 2017) does not develop until after post-natal day 21 (Robertson and McClelland, 2019; Robertson et al., 2019). This roughly corresponds to the time when juvenile deer mice fully express the enhanced ventilatory

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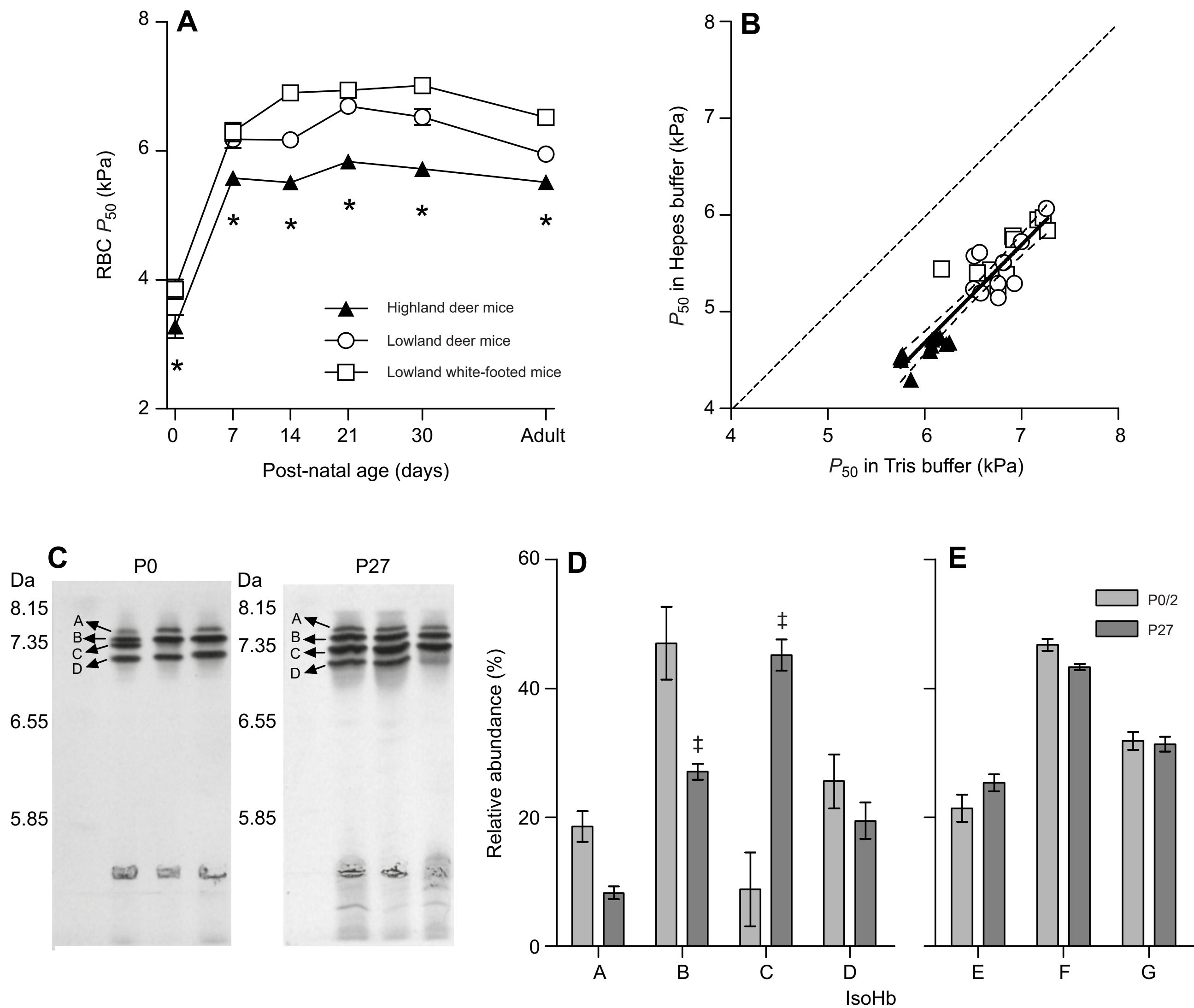

Fig. 8. Highland deer mice exhibit high haemoglobin (Hb)–O_2 binding affinity from birth to adulthood. (A) Red blood cell (RBC) P_{50} increased from P0 to P7, but was consistently lower in highlanders (*n*=5 P0, *n*=12 P7, *n*=14 P14, *n*=14 P21, *n*=17 P30, *n*=10 adults) than in lowland deer mice (*n*=12 P7, *n*=11 P14, *n*=10 P21, *n*=10 P30, *n*=7 adults) and lowland white-footed mice (*n*=4 P0, *n*=11 P7, *n*=12 P14, *n*=11 P21, *n*=11 P30, *n*=10 adults). *Significant pairwise difference between highlanders and lowlanders of both species within an age using Holm–Šidák *post hoc* tests. (B) Tris buffer increased P_{50} values (by ~1.37 kPa) compared with Hepes buffer for RBCs from adult mice, but this effect did not alter the population differences in red blood cell P_{50} (*n*=10 for all groups). (C) Representative isoelectric focusing (IEF) gel showing the different Hb isoforms (isoHb; labelled A–D) expressed in RBCs of highland mice at P0 and P27. (D) IEF analysis revealed variation in the relative abundance of distinct isoHbs with age between P0 (*n*=7) and P27 (*n*=7) in highland mice. (E) IEF analysis revealed highly similar isoHb profiles in samples from lowland white-footed mice at P0/2 (*n*=8) and P27 (*n*=7). ‡Significant pairwise differences in isoHb abundance between age classes of the same species, using Holm–Šidák *post hoc* tests.

sensitivity to O_2 and the breathing pattern that is typical of adults (Figs 3 and 4). These ventilatory phenotypes may be critical to maintaining the high rates of respiratory gas exchange needed to support the O_2 demands of thermogenesis at high altitude.

However, the developmental onset of ventilatory sensitivity to hypoxia appears to be delayed in high-altitude deer mice. Both the hypoxic ventilatory response (as reflected by hypoxia-induced increases in air convection requirement) (Fig. 3) and the post-natal hyperplasia of O_2-sensitive type I cells in the carotid bodies (Fig. 7) occurred later in highland deer mice (P14 and P21, respectively) than in lowland deer mice (P7 and P14, respectively). Carotid body development also occurred later in highlanders than the developmental timing that has previously been reported for domestic lab-strain mice (Kostuk et al., 2011). Furthermore, highlanders exhibited differences in the timing of the developmental changes in the pool of type II cells, which may act as progenitors that differentiate and give rise to new type I cells during carotid body growth (Pardal et al., 2007). These changes in the developmental onset of ventilatory sensitivity to hypoxia may have occurred in conjunction with the development of endothermy, because recent findings suggest that the onset of homeothermy and the expansion of thermogenic capacity are also delayed in highland deer mice compared with both lowland deer mice and white-footed mice (Robertson and McClelland, 2019; Robertson et al., 2019). This may also explain why the post-natal changes in blood Hb content were also delayed in highland deer mice (Table 2).

Highland mice exhibited stronger blood–O_2 affinity from birth to adulthood (Figs 6 and 8). The persistent differences between populations are consistent with previous functional measurements of Hb from adult mice, and can be explained by genetic differences

in the α- and β-chain subunits (Natarajan et al., 2013; Storz et al., 2010b). However, blood–O_2 affinity declined appreciably from birth (P0) to P7 in both mouse populations. These changes did not appear to result from a progressive decrease in the expression of embryonic or fetal globins, as occurs in some other mammals which exhibit significant shifts in red blood cell isoHb composition in the first few weeks of post-natal life to shift blood–O_2 affinity dramatically (Baumann et al., 1972; Blunt et al., 1971; Storz, 2018; Tweeddale, 1973), because both highland deer mice and white-footed mice exhibited adult isoHb composition from birth. The shift in blood–O_2 affinity from P0 to P7 that we observed here may instead result from reductions in the concentration of allosteric modifiers such as 2,3-diphosphoglycerate (DPG) in the red blood cell. Indeed, in mammals that do not express fetal-specific isoHb, fetal red blood cells typically have reduced DPG concentration (with a corresponding reduction in blood P_{50}), to facilitate placental O_2 transfer (Bunn and Kitchen, 1973; Storz, 2018). Blood CO_2 tension can increase at birth and then subside over time (Adamson, 1991), so it is possible that these changes and associated changes in acid–base status between P0 and P7 increase glycolytic DPG production in red blood cells, thereby producing the observed reduction in blood–O_2 affinity. Nevertheless, highlanders maintain consistently greater blood–O_2 affinity, despite the developmental shifts that occur shortly after birth, which probably acts in concert with the unique ventilatory phenotypes of highlanders to help maintain higher arterial O_2 saturation in hypoxia.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.M.I., G.R.S.; Methodology: C.M.I., M.A.G., E.D.S., C.N., J.F.S., G.R.S.; Software: G.R.S.; Formal analysis: C.M.I., M.A.G., E.D.S., C.N.; Investigation: C.M.I., C.N.; Resources: G.R.S.; Data curation: C.M.I., C.E.R.; Writing - original draft: C.M.I., C.E.R., C.N., J.F.S., G.R.S.; Writing - review & editing: C.M.I., M.A.G., E.D.S., C.E.R., C.N., J.F.S., G.B.M., G.R.S.; Visualization: C.M.I., C.E.R., C.N., J.F.S., G.R.S.; Supervision: C.M.I., G.B.M., G.R.S.; Project administration: G.B.M., G.R.S.; Funding acquisition: J.F.S., G.B.M., G.R.S.

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Supplementary information
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