Evolution and developmental plasticity of lung structure in high-altitude deer mice

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Abstract

Hypoxia at high altitudes can constrain the ability of endotherms to maintain sufficient rates of pulmonary O_2 transport to support exercise and thermogenesis. Hypoxia can also impede lung development during early post-natal life in some mammals, and could thus accentuate constraints on O_2 transport at high altitude. We examined how these challenges are overcome in deer mice (*Peromyscus maniculatus*) native to high altitude. Lung structure was examined in highland and lowland populations of deer mice and lowland populations of white-footed mice (*P. leucopus*; a congener restricted to low altitude) that were bred in captivity. Among mice that were born and raised to adulthood in normoxia, highland deer mice had higher alveolar surface density and more densely packed alveoli. The increased alveolar surface density in highlanders became fully apparent at juvenile life stages at post-natal day 30 (P30), after the early developmental period of intense alveolus formation before P21. Alveolar surface density was maintained in highlanders that were conceived, born, and raised in hypoxia (~12 kPa O_2), suggesting that lung development was not impaired by post-natal hypoxia as it is in many other lowland mammals. However, developmental hypoxia increased lung volume and thus augmented total alveolar surface area from P14. Overall, our findings suggest that evolutionary adaptation and developmental plasticity lead to changes in lung morphology that should improve pulmonary O_2 uptake in deer mice native to high altitude.

Introduction

Developmental plasticity is the process whereby the conditions experienced in early development adjust an organism's phenotype, and these effects can sometimes be irreversible and persist into later adult life (Burggren 2019; Burggren and Reyna 2011; Moczek et al. 2011). The phenotypic responses to environmental stressors during early life can differ from those during adulthood, and responses can even differ as a function of the stage of development at which stressors are experienced (*e.g*., prenatal *versus* postnatal development) (Burggren and Reyna 2011; Carroll 2003). Developmental plasticity is known to shape a range of physiological phenotypes, including the systems responsible for supporting metabolism and O_2 transport (Bavis 2005; Burggren and Reyna 2011; Carroll 2003; Frappell and Mortola 1994; Vulesevic and Perry 2006; Wood et al. 2017). However, in many cases, the persistence and life stage-specificity of developmental plasticity remains poorly understood.

Hypoxia (low O_2) exposure during early development can lead to plastic changes in lung structure, but the responses to hypoxia during postnatal development appear to depend on when hypoxia occurs. In placental mammals, the overall branching pattern of the bronchial tree is established during pre-natal development such that the lungs are functional at birth (Metzger et al. 2008), but there are species differences in the developmental timeline for the formation of most alveoli (the primary gas exchange units of the lungs). Septation (the partitioning of saccules into alveoli) occurs during post-natal development in many species (*e.g.,* humans, mice, rats, many marsupials, etc.) but it occurs during pre-natal development in some others (*e.g*., guinea pig, sheep) (Burri 1984; Frappell and MacFarlane 2006; Massaro and Massaro 2002). In the former species that undergo alveolarization during early post-natal life, this period represents a critical window for lung development, when hypoxia exposure impedes lung development by impairing septation (Ambalavanan et al. 2008; Blanco et al. 1991). Hypoxia exposure at later stages of post-natal development after this window has the opposite effect, enhancing lung volume and alveolar surface area (Bartlett and Remmers 1971; Burri and Weibel 1971). Furthermore, in species that undergo alveolarization before birth, post-natal hypoxia does not disrupt alveolar dimensions or lung volume (Hsia et al. 2005; Tenney and Remmers 1966). However, less is known about how life stage-specific effects might combine if hypoxia occurs throughout early development and persists into later life.

High-altitude environments are both hypoxic and cold, and can thus constrain the ability of endotherms to maintain sufficient rates of tissue O_2 supply to support the high metabolic demands of exercise and thermogenesis (Frappell et al. 2007; McClelland and Scott 2019; Storz and Scott 2019). Detrimental effects of hypoxia on early post-natal lung development could be debilitating at high altitude by further constraining tissue O_2 supply. And yet many populations or species that are native to high altitude have larger lungs and/or alveolar surface density contributing to greater pulmonary O_2 extraction compared to their low-altitude counterparts (Brutsaert et al. 2004; Brutsaert et al. 1999; Lechner 1977; Maina et al. 2017; Mortola et al. 1992; Pearson and Pearson 1976). However, in many previous studies, it has often been difficult to distinguish whether the distinct features of high-altitude natives arise from the effects of developing in a hypoxic environment or from evolved (genetically-based) adaptations to high altitude (Brutsaert 2016; Moore 2017). In high-altitude taxa that undergo alveolarization during early post-natal life, the effects of hypoxia exposure on lung development during and after this critical window are poorly understood.

In the current study, we examine these issues in high-altitude populations of the deer mouse (*Peromyscus maniculatus*). Deer mice are broadly distributed across North America and have the largest elevational range of any North American mammal, from near sea level to over 4300 m (Natarajan et al. 2015; Snyder et al. 1982). High-altitude populations of deer mice maintain high field metabolic rates (Hayes 1989b) and are under strong directional selection for a high aerobic capacity ($\dot{V}O_2max$) for thermogenesis in the wild (Hayes and O'Connor 1999). As a result, high-altitude deer mice have evolved increased $\dot{V}O_2max$ in hypoxia compared to both low-altitude populations of deer mice and white-footed mice (*P. leucopus*), a closely related species that is restricted to low altitudes (Cheviron et al. 2012; Cheviron et al. 2013; Lui et al. 2015; Tate et al. 2017; Tate et al. 2020). This increase in $\dot{V}O_2max$ is associated with evolved increases in pulmonary O_2 extraction and arterial O_2 saturation (Tate et al. 2017; Tate et al. 2020). Previous work by others has shown that acclimation to high-altitude hypoxia increases lung volume and diffusing capacity in deer mice (Dane et al. 2018), but it remains unknown whether there are evolved changes in lung structure in high-altitude populations. Here, we compare high-altitude deer mice to low-altitude mice during adulthood and early development in normoxia, and also examine the effects of hypoxia exposure during post-natal development in high-altitude mice. We thus test whether evolutionary adaptation to high altitude and/or

developmental plasticity lead to changes in lung morphology that could enhance pulmonary O_2 uptake in the hypoxic environment at high altitude.

Materials and Methods

Deer mouse populations and breeding design

Captive breeding populations were established from wild populations of deer mice native to high altitude near the summit of Mount Evans, CO, USA (39°35'18"N, 105°38'38"W; 4,350 m above sea level), and from wild populations of both deer mice and white-footed mice native to 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). Wild adults were transported to McMaster University (~50 m above sea level) and housed in common laboratory conditions in ambient (normoxic) air, and were used as parental stock to produce first-generation lab progeny for each mouse population. Breeding pairs were held individually in standard mouse cages, and the male was removed when the female was visibly pregnant. Pups were weaned and moved to separate cages at post-natal day (P) 21, and were then raised to adulthood (at least 6 months of age). First-generation mice from both deer mouse populations were similarly used as parental stock to produce second-generation progeny. Both first- and second-generation mice were used for the comparisons described below. Mice were generally held at 24-25 °C and a photoperiod of 12 h light: 12 h dark, and were provided with unlimited access to standard rodent chow 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 (AUP #16-01-02).

Population comparisons of adult mice

First-generation mice were used to make comparisons between populations in adulthood. Mice from all three populations were chronically exposed to 1) normobaric normoxia at an O_2 partial pressure (PO_2) of ~20 kPa (i.e., standard holding conditions) or to 2) hypobaric hypoxia at a PO_2 of ~12.5 kPa (barometric pressure of 60 kPa, simulating the pressure at ~4,300m). High-altitude mice were also exposed to 3) hypobaric hypoxia at a PO_2 of ~9 kPa (barometric pressure of 43 kPa, simulating the pressure at ~7,000m). The mice used for these treatments came from 7 families of high-altitude deer mice (n=1-2 per family per treatment), 2 families of low-altitude

deer mice (n=2-3 per family per treatment), and 8 families of white-footed mice (n=1-2 per family per treatment). We studied adult mice across a range of ages beyond 6 months, with an overall average and standard deviation of 16 and 5 months. Specially designed hypobaric chambers were used for exposure to chronic hypoxia, as previously described (Lui et al. 2015; McClelland et al. 1998). Mice in hypobaric hypoxia were temporarily returned to normobaric conditions twice per week for less than 20 min for cage cleaning. Mice were euthanized with an over-dose of isofluorane followed by decapitation after 6-8 weeks of chronic exposure, and were then processed for lung histology (see below). Before euthanasia, mice were used as part of previous studies for *in vivo* measurements of breathing in resting, unrestrained, and un-instrumented conditions along with haematology (Ivy et al. 2020).

Population comparisons during post-natal development

Second-generation deer mice were used to make comparisons between high- *versus* low-altitude populations of deer mice across early post-natal and juvenile development (7 high-altitude families and 7 low-altitude families). We only examined deer mice in this comparison in order to focus on evolved intraspecific differences that likely resulted from high-altitude adaptation, without the additional sources of variation that differentiate deer mice from white-footed mice (large differences in body mass, etc.). Mice were raised in standard laboratory conditions in normoxia, and were euthanized as described above and processed for lung histology at P7, P14, P21 (before weaning), and P30. Before euthanasia, mice were used for *in vivo* measurements of breathing and haematology as described above and reported previously (Ivy et al. 2020).

Effects of developmental hypoxia in high-altitude mice

Second-generation mice from the high-altitude population were used to examine the effects of hypoxia exposure across early post-natal and juvenile development (4 separate families from those used for population comparisons). This was achieved using a standardized breeding design to expose mice from each family to both normoxia or hypoxia. Each breeding pair was first allowed to raise 4 litters that were not used in this study, in order to avoid potential effects of variation in litter size and resource allocation that may arise across the first few litters (Kirkland and Layne 1989). Each breeding pair then conceived and raised litter 5 in standard

cage conditions of normobaric normoxia (normoxia control group). After weaning, the pups of litter 5 remained in normoxia but the mother and father were moved to hypobaric hypoxia at a PO_2 of ~12.5 kPa (as described above). Parents were then allowed to conceive litter 6 in hypobaric hypoxia, and the resulting pups were born, weaned, and raised in hypobaric hypoxia (developmental hypoxia group). Mice were euthanized as described above and processed for lung histology at P7, P14, and P30. Before euthanasia, mice were used for *in vivo* measurements of breathing and haematology as described above and reported elsewhere (Ivy and Scott 2020).

Lung histology

After euthanasia, lungs were inflated with 10% formalin at a constant pressure of 30 cm H_2O, and lung volume was measured as previously described (Scherle 1970). Lungs were then fixed in 10% formalin for 72 h and stored in 70% ethanol until paraffin embedding. Embedded lungs were sectioned using a microtome at a thickness of 5 μm and were mounted on Superfrost Plus microscope slides (Fisher Scientific; Mississauga, ON, Canada). Sections were taken at each of 3-4 different locations along the rostrocaudal axis of both the left and right lungs. Sections were then stained for hematoxylin and eosin as follows. Sections were deparaffinized with two washes of xylene for 10 min each, then incubated in 2 changes of 100% ethanol and one of 95% ethanol for 5 min each. Sections were then washed in distilled water for 5 min, stained with Gills II haematoxylin for 2 min, washed in water for 1 min, and stained with eosin for 45 s. Sections were then rinsed in water and dehydrated using one wash of 95% ethanol and two washes of 100% ethanol for 5 min each. Sections were cleared with two changes of xylene for 10 min each, then coverslipped with Permount (Fisher Scientific).

Stained sections were imaged for analysis using an upright brightfield microscope. Images were taken at 200X magnification from 3 different regions within each section, yielding 12-18 images for analysis per individual. Stereological methods were then used to make unbiased morphometric measurements of alveolar surface density (surface area per volume of lung parenchyma) and the total alveolar surface area of the animal, as previously recommended (Mühlfeld et al. 2013). Alveolar density was quantified as the number of distinct alveoli per imaged area of lung parenchyma.

Statistical analyses

We used linear mixed-effects models to test for the effects of environment, population, and/or age using the lme4 package (Bates et al. 2015) in R (version 4.0.3; R Core Team 2020). For population comparisons of adult mice, we tested for the main effects of population and environment (normoxia *versus* hypoxia at a PO_2 of 12.5 kPa) and their interaction. For population comparisons during post-natal development, we tested for the main and interactive effects of population and age. For the effects of developmental hypoxia in high-altitude mice, we tested for the main and interactive effects of developmental environment and age. We used a backwards model selection approach, in which all initial models also included sex and family as random factors, as well as body mass as a covariate. Age was also included as a random factor for adult comparisons. When random factors or covariates had P values above 0.1, they were removed by stepwise backward deletion (starting with the term with the highest P value) and the model was re-run until all random factors and covariates in the model had P values below 0.1. When main effects or interactions were significant, we performed Tukey post-hoc comparisons between populations within each environment/age (in the experiments seeking to compare populations during adulthood or development) or between environments within each age (in the experiment on the effects of developmental hypoxia). Data are reported as means ± SEM, along with individual values for the key data shown in figures. $P<0.05$ was considered to be significant.

Results

Population comparisons of adult mice

The key population difference we observed was that high-altitude deer mice had relatively high alveolar surface density of the lungs as compared to their low-altitude counterparts (Fig. 1; Tables 1,2). Our comparisons of adult mice were influenced by a significant main effect of population on body mass ($P = 0.002$), driven primarily by larger body masses in white-footed mice (Table 1). These population differences in body mass were associated with expected differences in absolute lung volume (Table 2), but there was no significant effect of population on lung volumes expressed relative to body mass (population effect, $P = 0.553$; Fig. 1A). In contrast, there was a significant main effect of population on alveolar surface density ($P < 0.001$), driven by 10-13% higher values in high-altitude mice than in both low-altitude taxa on average (Fig. 1B), and pairwise comparisons between highland deer mice and white-footed mice

were significant in both normoxia and hypoxia. However, due to subtle non-significant variation in lung volume, there was no significant effect of population on the total alveolar surface area of the entire lungs expressed relative to body mass (Fig. 1C).

Hypoxia acclimation had modest effects on lung volume in adult mice (Fig. 1; Tables 1,2). There was a significant ($P = 0.043$) main effect of acclimation environment on absolute lung volume, and although the main effect on mass-specific lung volume was not significant ($P = 0.097$), mass-specific lung volumes were 11-17% larger on average in all populations after acclimation to 12 kPa O_2. This variation in lung volume appeared to drive the overall increases in total alveolar surface area after acclimation to 12 kPa O_2, because there was no effect of environment on alveolar surface density ($P = 0.332$). Indeed, there were main effects of environment on both absolute ($P = 0.015$) and mass-specific ($P = 0.041$) values of total alveolar surface area.

Age had a significant effect on many lung variables among adult mice (Table 2). Lung volumes and total alveolar surface densities in particular tended to increase with age. Although age was accounted for as a random effect in our statistical models, it is possible that this additional source of variation influenced some of the comparisons described above. Whereas the ages of the high-altitude deer mice (mean ± S.D. of 15 ± 4 months) and low-altitude white-footed mice (15 ± 5 months) studied were generally similar, the low-altitude deer mice examined were somewhat older (23 ± 6 months). We therefore sought to carry out a more detailed comparison between high-altitude and low-altitude populations of deer mice at specific controlled ages during development.

Population comparisons during post-natal development

The increased alveolar surface density in high-altitude deer mice became fully apparent at juvenile life stages (Figs. 2,3, Tables 3,4). Development from post-natal day (P) 7 to P30 was associated with 3.0- to 3.2-fold increases in body mass (main effect of age, $P < 0.001$), in association with only 2.1- to 2.7-fold increases in absolute lung volume ($P < 0.001$; Table 3), such that mass-specific lung volume declined at P30 to 0.7- to 0.8-fold of the values at P7 ($P < 0.001$; Fig. 2A). There was also appreciable alveolarization that occurred across post-natal development (Fig. 3). Development from P7 to P30 was associated with 1.7- to 1.8-fold increases in alveolar density ($P < 0.0001$; Table 3) and 1.3- to 1.5-fold increases in alveolar

surface density ($P < 0.001$; Fig. 2B). As a result, absolute values of total alveolar surface area increased with age ($P < 0.001$; Table 3) in near proportion to the increases in body mass, and mass-specific total alveolar surface area changed little with age (Fig. 2C). There were no significant population effects or population×age interactions on the developmental growth of body mass or lung volume, but there were significant population effects on several alveolar measurements (Table 4). Alveolar surface density was greater in highlanders than in lowlanders at P30 (Fig. 2B), which appeared to drive the significant main effect of population on this trait ($P = 0.014$). There were also significant population effects on alveolar density ($P = 0.008$) and total alveolar surface area ($P = 0.002$) that were driven by higher values in highlanders (Table 3). These findings applied across sexes and families, as neither of these variables had any significant effects on lung traits (Table 4).

Effects of developmental hypoxia in high-altitude mice

Hypoxia during peri-natal development appeared to increase lung volume and thus expand total alveolar surface area (Figs. 4,5, Tables 5,6). Litter size ranged from 4 to 9 pups across families and was unaffected by hypoxia exposure (normoxia controls, mean ± S.D. of 6.3 ± 2.2; developmental hypoxia 6.3 ± 2.6; $P = 0.999$ in two-tailed t-test). There was no effect of developmental hypoxia on the increases in body mass with age (Table 5), but there were significant environment effects on both absolute lung volume ($P = 0.016$; Table 5) and mass-specific lung volume ($P = 0.012$; Fig. 4A). Hypoxia had the greatest effect on lung volume at P14. Developmental hypoxia did not appear to impede alveolarization during post-natal development (Fig. 5). Both alveolar surface density and alveolar density increased with age in both treatment groups (age effects, $P < 0.001$ for both) such that there was no significant effect of developmental hypoxia on these traits (Fig. 4B, Tables 5,6). As a result, the increases in lung volume with developmental hypoxia led to increases in total alveolar surface area for both the absolute (environment effect, $P = 0.021$) and mass-specific ($P = 0.001$) values of this trait, and the effects of developmental hypoxia were greatest at P14 and P30 (Fig. 4C, Table 5). There was significant inter-family variation for many of these traits, which was accounted for as a random effect in our statistical models, but there were no significant effects of sex (Table 6).

Discussion

Deer mice at high altitudes must sustain high metabolic rates (Hayes 1989a), and evolutionary adaptation to high altitude has increased their aerobic capacity in hypoxia (Hayes and O'Connor 1999; Tate et al. 2017; Tate et al. 2020). Our findings here suggest that changes in lung structure may partially underlie these performance traits. Deer mice native to high altitude had higher alveolar surface density and more densely packed alveoli, which arose at the juvenile life stage and persisted into adulthood (Figs. 1-3). Developmental hypoxia increased lung volume, without impeding alveolarization, such that hypoxia led to an associated increase in total alveolar surface area (Figs. 4,5). These results suggest that both evolved and plastic increases in the surface area for pulmonary O_2 diffusion may contribute to increasing aerobic capacity in deer mice at high altitude.

Deer mice were generally smaller than white-footed mice and there were no intraspecific differences in body mass between high- and low-altitude populations. Bergmann's rule predicts that taxa in the colder environments at high latitudes and altitudes will tend to be larger, which reduces surface area to volume ratio and may thus facilitate heat retention (Blackburn et al. 1999; Gutiérrez-Pinto et al. 2014). The interspecific differences in body mass between deer mice and white-footed mice are inconsistent with Bergmann's rule, because the range of deer mice extends far further north than that of white-footed mice (Bedford and Hoekstra 2015). The lack of any intraspecific differences in body mass between high- and low-altitude populations is also inconsistent with Bergmann's rule, consistent with findings in some other endothermic species in which body mass does not increase and may even decrease with elevation (Gutiérrez-Pinto et al. 2014). It is likely that the variation (or lack thereof) in body mass among deer mice and white-footed mice is shaped by ecological pressures other than the potential drive to reduce heat loss by reducing surface area to volume ratio.

Our finding that highland mice have increased alveolar surface density is consistent with findings in other small mammals sampled at high altitude in the wild (Lechner 1977; Pearson and Pearson 1976). However, the population differences in lung structure seen here were based on observations from mice raised for two generations in captivity in common conditions, suggesting that they have an evolved genetic basis. The full realization of these differences in lung structure at the juvenile life stage is consistent with the developmental trajectory of aerobic capacity. Mice are poikilothermic at birth, and highland deer mice do not exhibit higher aerobic capacity than their lowland counterparts until after weaning at P21 and by P27 (Robertson and

McClelland 2019). Similarly, the evolved differences in lung structure observed here arise sometime between P21 and P30 (Fig. 2). Therefore, the period after weaning when mice become independent and then become juveniles is a key phase of development when many of the evolved differences in respiratory physiology and metabolism arise in high-altitude deer mice (Ivy et al. 2020; Robertson and McClelland 2019). It remains to be determined whether evolved differences in the pulmonary vasculature or the fine structure of the blood-gas barrier, as exists in some other high-altitude taxa (Maina et al. 2017), might also arise along a similar developmental timeline in high-altitude deer mice.

High-altitude deer mice are resistant to the detrimental effects of hypoxia on the formation of alveoli during early post-natal development. The inhibitory effect of hypoxia on lung growth in some placental mammals is restricted to a short critical period after birth when alveoli form by septation (Burri 1984; Massaro and Massaro 2002). In mice and rats, this critical period occurs within the first 14 days after birth, when hypoxia exposure has been shown to impair alveolar formation (Ambalavanan et al. 2008; Blanco et al. 1991). Hypoxia had no such effect in high-altitude deer mice, in which hypoxia exposure until P14 had no significant effect on alveolar surface density and it increased alveolar density (Fig. 4, Table 5). Continued exposure to hypoxia from P14 to P30 also had no effect on alveolar surface density (Fig. 4), nor did chronic hypoxia in adulthood (Fig. 1). However, hypoxia exposure during early development (Fig. 4) and adulthood (Fig. 1) appeared to increase lung volume and thus increased total alveolar surface area (likely in association with greater total numbers of alveoli per animal). It is possible that longer-term exposure to hypoxia, throughout early peri-natal development and into adulthood, could have even more pronounced changes in lung structure. Indeed, several previous studies have found that hypoxia can increase lung volume and total alveolar surface area when exposure starts after the critical period of alveolar formation (Bartlett and Remmers 1971; Blanco et al. 1991; Burri and Weibel 1971; Dane et al. 2018).

Although both evolutionary adaptation and hypoxia-induced plasticity appear to enhance the surface area for pulmonary O_2 diffusion, they appear to act through distinct mechanisms. High-altitude adaptation appears to have increased alveolar packing to increase the amount of alveolar surface per volume, whereas hypoxia exposure appears to increase lung volume. Whether high-altitude adaptation has led to evolved changes in developmental plasticity of lung structure remains to be determined. Hypoxia acclimation during adulthood appeared to have

similar effects on lung structure in highland and lowland mice, but the plastic response to developmental hypoxia has yet to be compared between highland and lowland mice. This information will be pertinent to understanding how climate change might impact deer mouse populations across elevational gradients, as warming temperatures drives some lowland *Peromyscus* mice to higher altitudes (Moritz et al. 2008; Rowe et al. 2015). Nevertheless, the evolution and plasticity of lung structure may be critical for augmenting the O_2 diffusing capacity of the lungs and helping high-altitude deer mice maintain high metabolic rates to cope with the hypoxic and cold environment at high altitude.

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Availability of data and material

The datasets generated and analyzed during the current study are available at https://doi.org/10.6084/m9.figshare.13549367.v1.

Conflict of interest

The authors declare no conflicts of interest.

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Figure Legends

Figure 1. Lung volume (A), alveolar surface density (B), and total alveolar surface area (C) in adult mice acclimated to either normoxia or hypoxia. All mice were acclimated to 12 kPa O_2, but only highlanders were acclimated to 9 kPa O_2. Bars indicate mean ± SEM and symbols represent individual values, and the number of males and females in each group are indicated within each bar of panel A (N_{males}, $N_{females}$). Letters indicate significant pairwise differences between populations within a given environment, where bars sharing the same letter are not significantly different ($P<0.05$).

Figure 2. Lung volume (A), alveolar surface density (B), and total alveolar surface area (C) in deer mice during early post-natal and juvenile development in normoxia. Bars indicate mean ± SEM and symbols represent individual values, and the number of males and females in each group are indicated within each bar of panel A (N_{males}, $N_{females}$). * Significant pairwise difference between populations within a given age ($P<0.05$).

Figure 3. Representative images of lung parenchyma at post-natal day (P) 7 (A,B), P14 (C,D), P21 (E,F), and P30 (G,H) for deer mouse populations from low altitude (A,C,E,G) and high altitude (B,D,F,H) that were born and raised in captivity in normoxia. Scale bar represents 200 μm.

Figure 4. Lung volume (A), alveolar surface density (B), and total alveolar surface area (C) in high-altitude deer mice during early post-natal and juvenile development in normoxia or hypoxia. Bars indicate mean ± SEM and symbols represent individual values, and the number of males and females in each group are indicated within each bar of panel A (N_{males}, $N_{females}$). * and †, Significant ($P<0.05$) or nearly significant ($P = 0.0556$) pairwise difference between environments within a given age, respectively.

Figure 5. Representative images of lung parenchyma at post-natal day (P) 7 (A,B), P14 (C,D), and P30 (E,F) for high-altitude deer mice conceived, born, and raised in normoxia (A,C,E) or hypoxia (B,D,F). Scale bar represents 200 μm.

Table 1: Body mass and absolute lung volumes and total alveolar surface area in comparisons of adult mice.

Trait	Acclimation environment	Highland deer mice	Lowland deer mice	Lowland white-footed mice
Body mass (g)	Normoxia	19.4 ± 1.7^{a}	22.1 ± 2.4^{ab}	25.6 ± 2.2^{b}
	Hypoxia (12 kPa O_2)	20.7 ± 1.1	21.0 ± 0.6	25.1 ± 1.3
	Hypoxia (9 kPa O_2)	21.1 ± 1.0	NA	NA
Lung volume (µl)	Normoxia	681 ± 49^{a}	897 ± 77^{ab}	958 ± 84^{b}
	Hypoxia (12 kPa O_2)	884 ± 59^{a}	978 ± 17^{a}	1128 ± 89^{b}
	Hypoxia (9 kPa O_2)	908 ± 76	NA	NA
Total alveolar surface area (cm^2)	Normoxia	725 ± 50	860 ± 63	866 ± 57
	Hypoxia (12 kPa O_2)	942 ± 82	949 ± 36	1021 ± 74
	Hypoxia (9 kPa O_2)	925 ± 66	NA	NA

Values are represented as mean ± SEM (N as per Fig. 1). Letters indicate significant pairwise differences between populations in a given environment, where values that share the same letter are not significantly different ($P<0.05$).

Table 2: Results of linear mixed-effects models for comparisons of adult mice.

Trait	Pop	Env	Pop× Env	M_b	Age	Sex
M_b	$F_{2,47}$=6.92	$F_{2,47}$=0.53	$F_{2,47}$=0.42	-	ns	$F_{1,47}$=6.82
	P=0.002	P=0.590	P=0.660			**P=0.012**
V_L (µl)	$F_{2,47}$=8.21	$F_{2,47}$=3.38	$F_{2,47}$=0.06	ns	$F_{1,47}$=25.5	ns
	P<0.001	**P=0.043**	P=0.938		**P<0.001**	
V_L (µl/g)	$F_{2,46}$=0.60	$F_{2,46}$=2.46	$F_{2,46}$=0.21	-	$F_{1,46}$=12.6	$F_{1,46}$=4.99
	P=0.553	P=0.097	P=0.809		**P<0.001**	**P=0.030**
S_V[alv,par] (μm^{-1})	$F_{2,47}$=10.9	$F_{2,47}$=1.13	$F_{2,47}$=0.26	ns	$F_{1,47}$=8.84	ns
	P<0.001	P=0.332	P=0.768		**P=0.005**	
S_L[alv] (cm^2)	$F_{2,47}$=1.62	$F_{2,47}$=4.58	$F_{2,47}$=0.01	ns	$F_{1,47}$=12.1	ns
	P=0.201	**P=0.015**	P=0.994		**P=0.001**	
S_L[alv] (cm^2/g)	$F_{2,46}$=1.26	$F_{2,46}$=3.43	$F_{2,46}$=0.05	-	$F_{1,46}$=5.14	$F_{1,46}$=3.82
	P=0.294	**P=0.041**	P=0.952		**P=0.028**	**P=0.057**

Traits: M_b, body mass; V_L, lung volume; S_V[alv,par], alveolar surface density; S_L[alv], total alveolar surface area of the lungs. Predictor variables: Pop, population; Env, environment (normoxia or hypoxia). Covariate: M_b. Random factors: Age; Sex; Family. ns, not significant and excluded from final models (family was never significant and excluded from all final models).

Table 3: Body mass, absolute lung volume and total alveolar surface area, and alveolar density in population comparisons during post-natal development.

Trait	Age	Highland deer mice	Lowland deer mice
Body mass (g)	P7	4.53 ± 0.35	3.92 ± 0.20
	P14	7.62 ± 0.41	6.81 ± 0.39
	P21	9.96 ± 0.48	9.31 ± 0.52
	P30	13.55 ± 0.93	12.55 ± 0.59
Lung volume (µl)	P7	295 ± 19	238 ± 16
	P14	398 ± 25	367 ± 41
	P21	518 ± 40	442 ± 37
	P30	622 ± 19	635 ± 38
Total alveolar surface area (cm^2)	P7	121 ± 7	95 ± 7
	P14	200 ± 17	175 ± 18
	P21	289 ± 23	242 ± 17
	P30	378 ± 12	335 ± 19
Alveolar density (alveoli/mm^2)	P7	218 ± 17	207 ± 16
	P14	359 ± 10*	284 ± 23
	P21	379 ± 30	335 ± 22
	P30	387 ± 22	358 ± 13

P, postnatal age in days. Values are represented as mean ± SEM (N as per Fig. 2). *, Significant pairwise difference between populations at a given age ($P < 0.05$).

Table 4: Results of linear mixed-effects models for population comparisons during post-natal development.

Trait	Pop	Age	Pop× Age	M_b
M_b	$F_{1,36}$=3.61	$F_{3,36}$=82.8	$F_{3,36}$=0.05	-
	P=0.066	**P<0.001**	P=0.986	
V_L (µl)	$F_{1,35}$=2.78	$F_{3,35}$=60.8	$F_{3,35}$=1.46	$F_{1,35}$=13.7
	P=0.104	**P<0.001**	P=0.242	**P<0.001**
V_L (µl/g)	$F_{1,36}$=0.01	$F_{3,36}$=9.25	$F_{3,36}$=1.53	-
	P=0.912	**P<0.001**	P=0.223	
S_V[alv,par]	$F_{1,36}$=6.68	$F_{3,36}$=26.9	$F_{3,36}$=1.41	ns
	P=0.014	**P<0.001**	P=0.254	
S_L[alv] (cm^2)	$F_{1,35}$=11.1	$F_{3,35}$=94.5	$F_{3,35}$=0.26	$F_{1,35}$=9.14
	P=0.002	**P<0.001**	P=0.851	**P=0.004**
S_L[alv] (cm^2/g)	$F_{1,36}$=3.19	$F_{3,36}$=1.13	$F_{3,36}$=0.18	-
	P=0.082	P=0.351	P=0.997	
N_A[alv,par]	$F_{1,36}$=8.01	$F_{3,36}$=24.3	$F_{3,36}$=0.85	ns
	P=0.008	**P<0.001**	P=0.476	

Traits: M_b, body mass; V_L, lung volume; S_V[alv,par], alveolar surface density; S_L[alv], total alveolar surface area of the lungs; N_A[alv,par], alveolar density. Predictor variables: Pop, population; Age (categorical). Covariate: M_b, body mass. Random factors: Sex; Family. ns, not significant and excluded from final models (neither sex nor family were ever significant and were excluded from all final models).

Table 5: Body mass, absolute lung volume and total alveolar surface area, and alveolar density in comparisons to examine the effects of developmental hypoxia in high-altitude deer mice.

Trait	Age	Normoxia controls	Developmental hypoxia
Body mass (g)	P7	4.28 ± 0.51	4.19 ± 0.47
	P14	7.15 ± 0.60	6.61 ± 0.35
	P30	14.20 ± 1.29	14.02 ± 0.31
Lung volume (μl)	P7	268 ± 21	277 ± 28
	P14	413 ± 22	480 ± 16*
	P30	644 ± 36	733 ± 40*
Total alveolar surface area (cm^2)	P7	121 ± 13	117 ± 14
	P14	229 ± 14	271 ± 7†
	P30	418 ± 16	478 ± 36*
Alveolar density (alveoli/mm^2)	P7	257 ± 37	474 ± 23
	P14	288 ± 43	403 ± 29
	P30	434 ± 28	456 ± 34

P, postnatal age in days. Values are represented as mean ± SEM (N as per Fig. 4). * and †, Significant ($P<0.05$) and nearly significant ($P = 0.053$) pairwise differences between populations at a given age.

Table 6: Results of linear mixed-effects models for the effects of developmental hypoxia in high-altitude deer mice.

Trait	Env	Age	Env× Age	M_b	Family
M_b	$F_{1,30}$=0.37	$F_{2,30}$=185	$F_{2,30}$=0.06	-	$F_{3,30}$=15.1
	P=0.548	**P<0.001**	P=0.938		**P<0.001**
V_L (µl)	$F_{1,29}$=6.57	$F_{2,29}$=163	$F_{2,29}$=1.62	$F_{1,29}$=3.46	$F_{3,29}$=5.50
	P=0.016	**P<0.001**	P=0.214	P=0.073	**P=0.004**
V_L (µl/g)	$F_{1,30}$=7.07	$F_{2,30}$=16.3	$F_{2,30}$=1.10	-	$F_{3,30}$=6.05
	P=0.012	**P<0.001**	P=0.346		**P=0.002**
S_V[alv,par]	$F_{1,30}$=0.09	$F_{2,30}$=114	$F_{2,30}$=1.15	ns	$F_{3,30}$=3.78
	P=0.769	**P<0.001**	P=0.329		**P=0.021**
S_L[alv] (cm^2)	$F_{1,30}$=5.92	$F_{2,30}$=217	$F_{2,30}$=2.03	ns	$F_{3,30}$=5.77
	P=0.021	**P<0.001**	P=0.148		**P=0.003**
S_L[alv] (cm^2/g)	$F_{1,30}$=12.8	$F_{2,30}$=14.9	$F_{2,30}$=5.33	-	$F_{3,30}$=4.69
	P=0.001	**P<0.001**	**P=0.010**		**P=0.008**
N_A[alv,par]	$F_{1,29}$=0.29	$F_{2,29}$=57.4	$F_{2,29}$=0.80	$F_{1,29}$=5.54	$F_{3,29}$=10.3
	P=0.592	**P<0.001**	P=0.460	**P=0.026**	**P<0.001**

Traits: M_b, body mass; V_L, lung volume; S_V[alv,par], alveolar surface density; S_L[alv], total alveolar surface area of the lungs; N_A[alv,par], alveolar density. Predictor variables: Env, environment (normoxia or hypoxia); Age (categorical). Covariate: M_b, body mass. Random factors: Sex; Family. ns, not significant and excluded from final models (sex was never significant and was excluded from all final models).

Figure 1

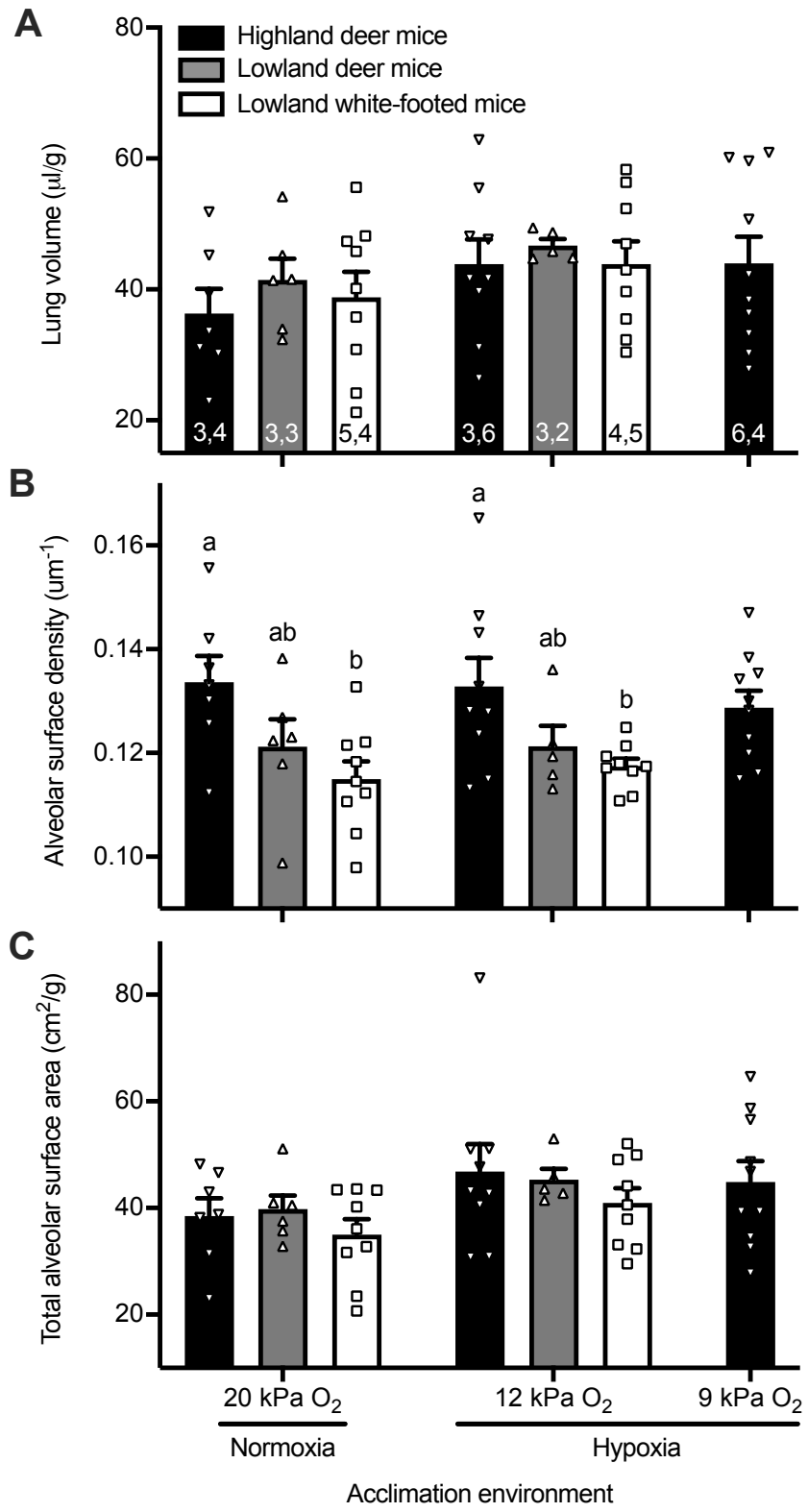
A
Highland deer mice
Lowland deer mice
Lowland white-footed mice
Lung volume (μl/g)
80
60
40
20
3,4
3,3
5,4
3,6
3,2
4,5
6,4
B
Alveolar surface density (um-1)
0.16
0.14
0.12
0.10
a
ab
b
a
ab
b
C
Total alveolar surface area (cm2/g)
80
60
40
20
20 kPa O2
12 kPa O2
9 kPa O2
Normoxia
Hypoxia
Acclimation environment

Figure 2

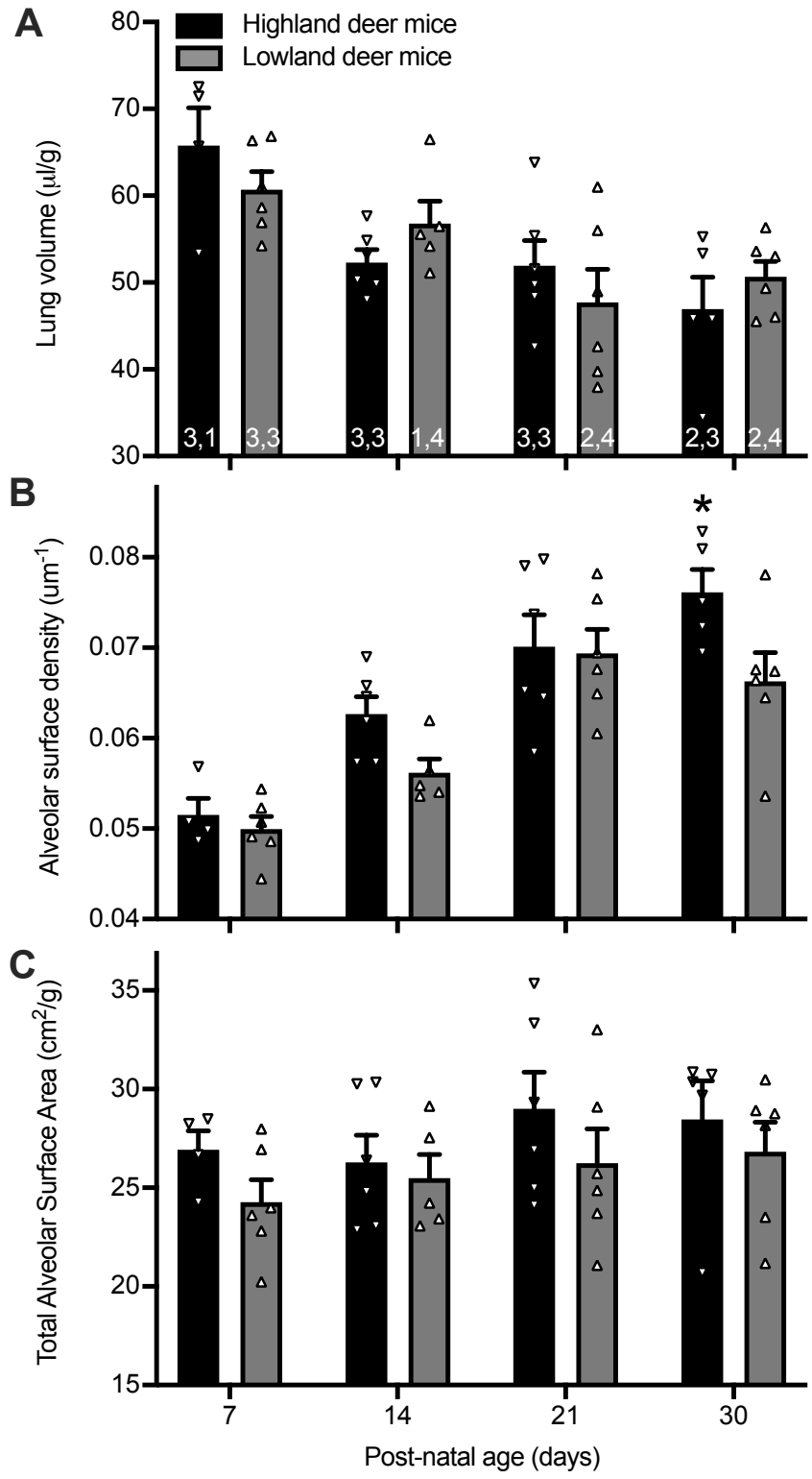
A
Highland deer mice
Lowland deer mice
Lung volume (μl/g)
80
70
60
50
40
30
3,1
3,3
3,3
1,4
3,3
2,4
2,3
2,4
B
Alveolar surface density (um-1)
0.08
0.07
0.06
0.05
0.04
*
C
Total Alveolar Surface Area (cm2/g)
35
30
25
20
15
7
14
21
30
Post-natal age (days)

Figure 3

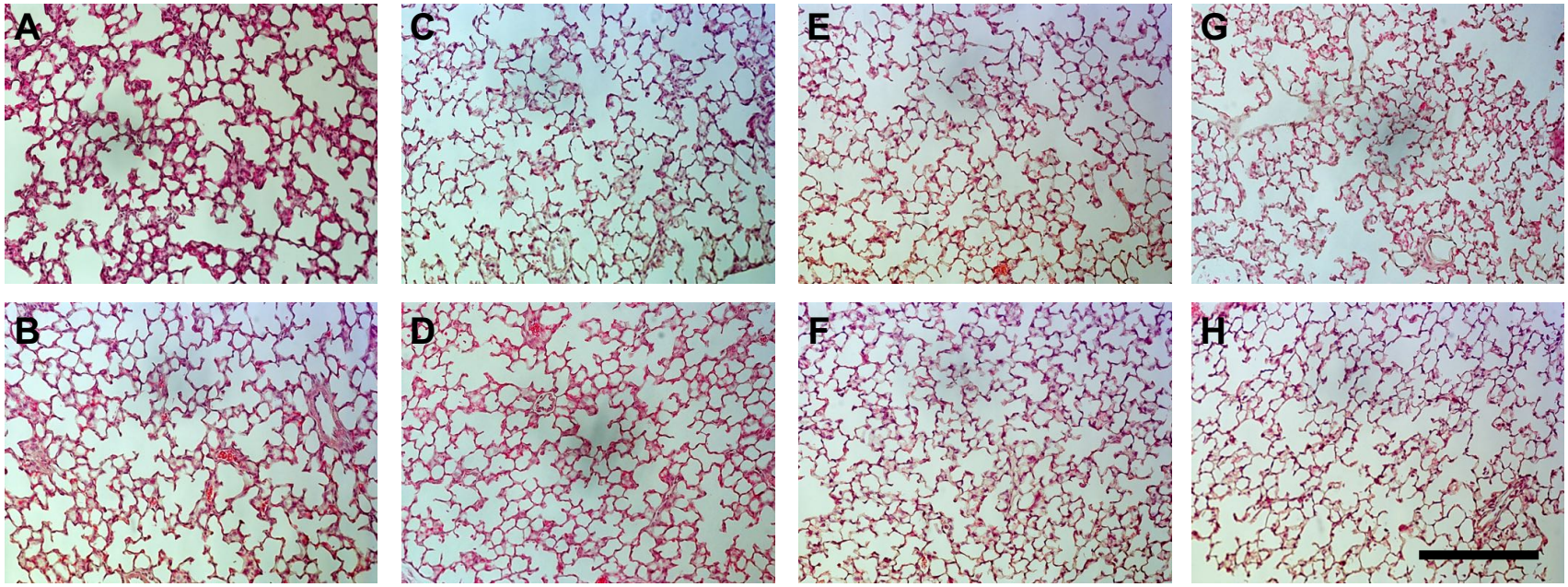

Figure 4

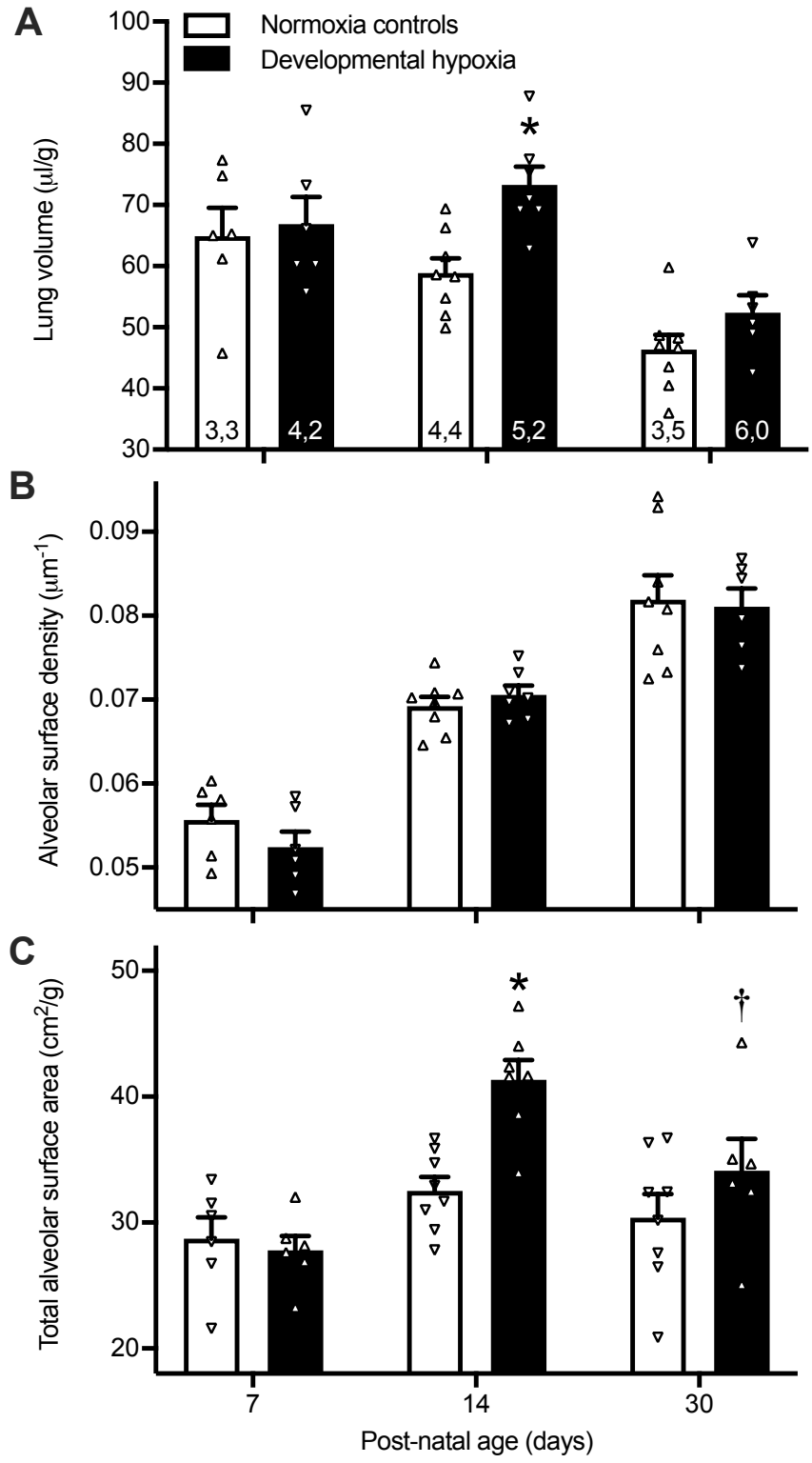
A
Normoxia controls
Developmental hypoxia
Lung volume (µl/g)
100
90
80
70
60
50
40
30
3,3
4,2
4,4
5,2
3,5
6,0
*
B
Alveolar surface density (µm-1)
0.09
0.08
0.07
0.06
0.05
C
Total alveolar surface area (cm2/g)
50
40
30
20
*
†
7
14
30
Post-natal age (days)

Figure 5