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The Roles of Phenotypic Plasticity and Genotypic Specialization in High Altitude Adaptation

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THE ROLES OF PHENOTYPIC PLASTICITY AND GENOTYPIC SPECIALIZATION IN HIGH ALTITUDE ADAPTATION

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In vertebrates living at high altitude, arterial hypoxemia may be ameliorated by reversible changes in the oxygen-carrying capacity of the blood (regulated by erythropoiesis) and/or changes in blood–oxygen affinity (regulated by allosteric effectors of hemoglobin function). These hematological traits often differ between taxa that are native to different elevational zones, but it is often unknown whether the observed physiological differences reflect fixed, genetically based differences or environmentally induced acclimatization responses (phenotypic plasticity). Here, we report measurements of hematological traits related to blood–O_2 transport in populations of deer mice (*Peromyscus maniculatus*) that are native to high- and low-altitude environments. We conducted a common-garden breeding experiment to assess whether altitude-related physiological differences were attributable to developmental plasticity and/or physiological plasticity during adulthood. Under conditions prevailing in their native habitats, high-altitude deer mice from the Rocky Mountains exhibited a number of pronounced hematological differences relative to low-altitude conspecifics from the Great Plains. However, these differences disappeared after 6 weeks of acclimation to normoxia at low altitude. These results indicate that the naturally occurring hematological differences between highland and lowland mice are environmentally induced and are

largely attributable to physiological plasticity during adulthood. The reciprocal experiment in which highland and lowland natives were subjected to 6 weeks of chronic hypoxia revealed that highland mice may have evolved a blunted erythropoietic response to chronic hypoxia.

As an alternative to plastic responses, genetically based changes in the respiratory properties of hemoglobin can also contribute to hypoxia adaptation in high-altitude vertebrates. Under severe hypoxia, an increase in hemoglobin-O_2 affinity can help preserve an adequate level of tissue oxygenation by enhancing pulmonary O_2 uptake while simultaneously maintaining the pressure gradient that drives O_2 diffusion from capillary blood to the tissue mitochondria. In comparisons between high- and low-altitude species of pikas (*Ochotona princeps* and *O. collaris*, respectively), we demonstrated that the high-altitude species has evolved a derived increase in hemoglobin-O_2 affinity. Using ancestral sequence reconstruction and site-directed mutagenesis, we identified a set of three β-chain substitutions that are responsible for the evolved change in hemoglobin function.

This dissertation is dedicated to my wonderful parents Allan and Eileen Tufts, without your love and support this would not have been possible. You are deeply missed every day and I hope I have made you proud.

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CHAPTER I

PHENOTYPIC PLASTICITY IN BLOOD-OXYGEN TRANSPORT IN HIGHLAND AND LOWLAND DEER MICE*

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ABSTRACT

In vertebrates living at high altitude, arterial hypoxemia may be ameliorated by reversible changes in the oxygen-carrying capacity of the blood (regulated by erythropoiesis) and/or changes in blood–oxygen affinity (regulated by allosteric effectors of hemoglobin function). These hematological traits often differ between taxa that are native to different elevational zones, but it is often unknown whether the observed physiological differences reflect fixed, genetically based differences or environmentally induced acclimatization responses (phenotypic plasticity). Here, we report measurements of hematological traits related to blood–O_2 transport in populations of deer mice (*Peromyscus maniculatus*) that are native to high- and low-altitude environments. We conducted a common-garden breeding experiment to assess whether altitude-related physiological differences were attributable to developmental plasticity and/or physiological plasticity during adulthood. Under conditions prevailing in their native habitats, high-altitude deer mice from the Rocky Mountains exhibited a number of pronounced hematological differences relative to low-altitude conspecifics from the Great Plains: higher hemoglobin concentrations, higher hematocrits, higher erythrocytic concentrations of 2,3-diphosphoglycerate (an allosteric regulator of hemoglobin–oxygen affinity), lower mean corpuscular hemoglobin concentrations and smaller red blood cells. However, these differences disappeared after 6 weeks of acclimation to normoxia at low altitude. The measured traits were also indistinguishable between the F_1 progeny of highland and lowland mice, indicating that there were no persistent differences in phenotype that could be attributed to developmental plasticity. These results indicate that the naturally occurring hematological differences between highland and lowland mice are

environmentally induced and are largely attributable to physiological plasticity during adulthood.

Key Words: physiological plasticity, high altitude, hemoglobin, hematocrit, hypoxia, *Peromyscus maniculatus*, red blood cell

INTRODUCTION

In air-breathing vertebrates living at high altitude, the reduced O_2 tension of inspired air requires compensatory physiological adjustments to ensure an adequate tissue O_2 supply. Some of these adjustments involve reversible changes in O_2-transport by red blood cells, which is a function of the O_2-carrying capacity of the blood (regulated by erythropoiesis) and blood-O_2 affinity (regulated by allosteric effectors of hemoglobin [Hb] function). Mammals that are native to high-altitude environments are typically characterized by a suite of hematological and vascular traits that includes an elevated blood-O_2 affinity, a normal or slightly increased hematocrit (Hct), and increased muscle capillary density (Hall et al. 1936; Chiodi 1962; Bullard et al. 1966; Bullard 1972; Monge and León-Velarde 1991; Weber 1995, 2007; Storz et al. 2010b; Mairbäurl and Weber 2012). In comparisons between species or conspecific populations that are native to different elevational zones, common-garden and/or reciprocal-transplant experiments are required to assess whether observed physiological differences reflect fixed, genetically-based differences or environmentally induced acclimatization responses (phenotypic plasticity). In the latter case, the acclimatization response may involve irreversible changes in phenotype (reflecting developmental plasticity) and/or reversible changes

during adulthood (physiological plasticity or 'phenotypic flexibility'; Piersma and Drent 2003). Regardless of the ontogenetic stage at which the plasticity is manifest, it is generally an open question as to whether a given change in phenotype represents an adaptive acclimatization response that has evolved under the influence of natural selection (Kingsolver and Huey 1998; Huey et al. 1999; Woods and Harrison 2002; Ghalambor et al. 2007).

If physiological differences between highland and lowland natives are attributable to adaptive phenotypic plasticity, then it prompts the question of why traits that are characteristic of hypoxia-tolerant high-altitude species are often not congruent with the typical acclimatization response to hypoxia in lowland species (Monge and León-Velarde 1991; Storz et al. 2010b). Lowland mammals that are not genetically adapted to environmental hypoxia typically respond to chronic O_2 deprivation with an increased erythropoietic activity (resulting in correlated increases in Hb concentration and hematocrit [Hct]) and a decreased blood-O_2 affinity (mediated by an increased red cell concentration of 2,3-diphosphoglycerate [DPG], an allosteric effector that reduces Hb-O_2 affinity; Lenfant et al. 1968 Baumann et al. 1971; Duhm and Gerlach 1971; Mairbäurl et al. 1993; Quatrini et al. 1993). DPG directly reduces Hb-O_2 affinity by preferentially binding and stabilizing deoxygenated Hb, thereby shifting the allosteric equilibrium in favor of the low-affinity 'T-state' quaternary structure. The hypoxia-induced increase in the molar DPG/Hb ratio also indirectly reduces Hb-O_2 affinity by altering the Donnan equilibrium across the red cell membrane, as the increased concentration of nondiffusible anions leads to an associated influx of hydrogen ions, thereby enhancing the Bohr effect (Duhm 1971; Samaja and Winslow 1979).

Within limits, an increased Hb concentration may enhance tissue O_2 delivery under hypoxia because the associated increase in arterial O_2 content can partially compensate for a reduced O_2 saturation. However, results of several empirical and theoretical studies suggest that increasing the Hb concentration of partially saturated blood is not an ideal long-term solution to the problem of chronic hypoxemia because the associated increase in blood viscosity produces an elevated peripheral vascular resistance that can compromise cardiac output (Guyton and Richardson 1961; Bullard 1972; McGrath and Weil 1978; Winslow and Monge 1987; Monge and León-Velarde 1991; Connes et al. 2006; Schuler et al. 2010). Studies of humans at high altitude have suggested that the optimal Hb concentration at rest and at exercise may actually be quite close to the typical sea level value (Winslow 1988; Villafuerte et al. 2004), or perhaps only slightly higher (Reeves and León-Velarde 2004), and it is well documented that excessive polycythemia is a causal factor in the development of chronic mountain sickness (Winslow et al. 1985; Winslow and Monge 1987; Monge and León-Velarde 1991; Rivera-Ch et al. 2007).

The adaptive significance of hypoxia-induced reductions in blood-O_2 affinity depends on the severity of hypoxia as well as the pulmonary O_2 diffusion capacity and numerous other taxon-specific physiological attributes. Under conditions of severe hypoxia, theoretical and experimental results indicate that an increase in red cell DPG concentration and the concomitant decrease in Hb-O_2 affinity will generally have detrimental effects on tissue oxygenation because the reduced arterial O_2 saturation more than offsets any benefit of increased O_2-unloading in the peripheral circulation (Turek et al. 1973; Eaton et al. 1974; Bencowitz et al. 1982; Willford et al. 1982).

Some of the best opportunities for examining plasticity in traits associated with hypoxia tolerance are provided by studies of population-level variation in species that are distributed across steep altitudinal gradients. One such species is the deer mouse (*Peromyscus maniculatus*), which has the broadest altitudinal distribution of any North American mammal (Hock 1964). Since deer mice are continuously distributed from sea level to elevations above 4300 m, this species has proven to be an exemplary subject for research on mechanisms of adaptation and acclimatization to high-altitude hypoxia. An extensive body of work has documented that deer mice native to high-altitude have elevated blood-O_2 affinities relative to lowland conspecifics, and these differences are largely attributable to allelic differences in Hb-O_2 affinity (Snyder 1982; Snyder et al. 1982; Snyder 1985; Chappell and Synder 1984; Chappell et al. 1988; Storz 2007; Storz et al. 2009, 2010a). Additional evidence that allelic variation in Hb function contributes to local adaptation is provided by tests of whole-organism physiological performance involving wild-derived strains that express different Hb variants (Chappell and Synder 1984; Chappell et al. 1988; Hayes and Chappell 1990) and population-genetic analyses of variation in the underlying globin genes (Snyder et al. 1988; Storz 2007; Storz et al. 2007; Storz and Kelly 2008; Storz et al. 2007, 2009, 2012a). In light of this evidence for adaptive, genetically based differences in Hb-O_2 affinity between deer mouse populations that are native to different elevational zones, it is also of interest to assess the role of phenotypic plasticity in modulating aspects of blood-O_2 transport. Here we report measurements of hematological traits related to blood-O_2 transport capacity in populations of deer mice that are native to high- and low-altitude environments. The main objectives were to (i) characterize altitude-related differences in hematological traits, and

(ii) assess what fraction of the observed trait differences are attributable to phenotypic plasticity.

MATERIALS AND METHODS

Animals

In July-August of 2010 and 2011, we collected a total of 118 deer mice from a high-altitude locality in the Southern Rocky Mountains and a low-altitude locality in the Great Plains, 770 km to the East. We collected 58 highland mice from the summit of Mount Evans, Clear Creek Co., Colorado [39°35'18"N, 105°38'38"W; elevation 4350 m a.s.l.] and 60 lowland mice from the prairie grassland of eastern Nebraska (Nine-mile prairie, Lancaster Co. [40°52'12"N, 96°48'20.3"W; elevation 430 m a.s.l.]). All mice were captured using Sherman live traps baited with peanut butter and oats. We drew approximately 200 μl of blood from the maxillary vein of each mouse using a 5 mm Goldenrod lancet (MEDIpoint Inc, Mineola, NY, USA).

A subset of 46 mice (24 highland and 22 lowland) were transferred to a common garden environment at the Animal Research Facility at the University of Nebraska in Lincoln, NE (elevation 300 m) where they were allowed to acclimate for 6 wks. All mice were maintained at a constant temperature (25°C) and on a standard light:dark cycle (12L:12D) for the duration of the experiment. During the 6 wk acclimation period all mice were offered a standard diet *ad libitum* (Harlan Teklad Rodent Chow). After the acclimation period, we again drew blood from each mouse as described above for repeat measurements of the same hematological traits. We also conducted crosses between wild-caught mice from the same collection locality and we reared the resultant F_1 progeny at

the University of Nebraska (Lincoln, NE) and the University of Illinois (Urbana, IL) under the same common-garden conditions described above. A total of 71 F_1 mice (N = 48 and 23 descendants of highland and lowland natives, respectively) were phenotyped after they reached a minimum age of 66 days. All experimental protocols were approved by the Institutional Animal Care and Use Committees at the University of Nebraska (IACUC #522) and the University of Illinois (#10244).

Measurement of hematological traits

We measured Hb concentration in whole blood using a HemoCue Hb 201+ analyzer following the manufacturer's protocol (HemoCue® AB, Ängelholm, Sweden). We measured hematocrit (Hct) as the volume of packed red cells relative to total blood volume in a heparinized capillary tube that was spun at 13,600 x g for 5 min in a ZIPocrit centrifuge (LW Scientific Inc., Lawrenceville, GA, USA). We also calculated mean cell hemoglobin concentration, MCHC (= {Hb concentration}*100/Hct). To measure red cell size, we used a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Gottingen, Germany) to measure the diameter of 10 red cells per sample.

Red cell DPG concentrations were determined spectrophotometrically using a DPG kit following the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA), with the exception that we used sample volumes of 100 µl whole blood rather than 1 mL. We drew blood from a subset of 21 mice (N = 10 and 11 highland and lowland mice, respectively). Blood was collected in chilled heparinized capillary tubes and was immediately deproteinized with 500 µl of cold 0.6 M perchloric acid ($HClO_4$). Samples were centrifuged at 5000 rpm for 10 min, and 400 µl of clear supernatant was then removed and neutralized with 50 µl of 2.5 M potassium carbonate (K_2CO_3). Samples

were kept on ice for at least 60 min and were then centrifuged again at 5000 rpm for 10 min. The supernatant was removed and immediately frozen in liquid nitrogen prior to the spectrophotometric analysis. The concentration of DPG in whole blood was estimated from the coupled reduction of NADH to NAD^+ in the reaction assay at 340 nm (UVIKON 923 B Double Beam UV/VIS Spectrophotometer, Kontron instruments, Milan, Italy). The reaction was completed after 25 min and absorbance did not change noticeably in later readings. Negative controls were run with each analysis and positive controls were performed with human blood.

Statistical Analysis

For measures of Hb concentration in wild-caught mice (phenotyped at the site of capture) and for measures of all hematological traits in the F_1 progeny of wild-caught/lab-acclimated mice, we tested for altitude-related differences in phenotype using standard t-tests. For the subset of mice that were included in the common-garden acclimation experiment, we compared pre- and post-acclimation trait values using a repeated measures two-way ANOVA with native altitude (high vs. low) and time (pre- vs. post-acclimation) included as independent variables. In the analysis of trait variation in the common-garden F_1 mice, we controlled for variation in individual age (66-454 d) by performing an ANCOVA with native altitude as an independent variable and age as a covariate. We did not detect any significant differences between the sexes for any of the hematological traits, so data from males and females were pooled in all analyses. Similarly, we did not detect significant trait variation among sibships within each set of F_1 mice from high- and low-altitude, so data from all families were pooled. Kolmogorov-Smirnov tests (K-S test) did not reveal any significant deviations from normality in any

of the trait-specific data sets. All statistical analyses were conducted using the SAS software package (SAS Institute Inc. 2009) or VassarStats online statistical calculator (vassarstats.net).

RESULTS

Hematological Traits

Under the conditions prevailing in their natural habitats, the highland *P. maniculatus* exhibited a significantly higher Hb concentration than the lowland *P. maniculatus* (mean ± 1 SD, 2.83 ± 0.28 vs. 2.33 ± 0.32 mM, respectively [Hb molecular weight = 64.45 kD]; $t_{116} = 9.079$, $P < 0.0001$; Fig. 1). However, after 6 weeks of acclimation to normoxia in the common-garden laboratory environment, the mean Hb concentration of the highland mice dropped by 8.8% to 2.54 ± 0.21 mM and was statistically indistinguishable from that of the lab-acclimated lowland mice (2.55 ± 0.20 mM; Table 1, Fig. 2A). The F_1 progeny of highland and lowland *P. maniculatus* also had Hb concentrations that were statistically indistinguishable (2.33 ± 0.21 vs. 2.29 ± 0.24 mM, respectively; $t_{69} = 0.739$, $P = 0.489$). Similar to the repeated-measures analysis of Hb concentration (Fig. 2A), an analysis of pre- and post-acclimation measures of Hct, MCHC, red cell size, and red cell DPG concentration for the same subset of highland and lowland mice revealed similar degrees of plasticity (Fig. 2B-E). Highland deer mice exhibited a significantly higher Hct relative to lowland mice (59.67 ± 4.83 vs. 48.94 ± 4.82 %, respectively; Table 1, Fig. 2A), but after 6 wks of acclimation to normoxia, the average Hct of the highland mice dropped by a 17% such that post-acclimation values of the highland and lowland mice were statistically indistinguishable (49.77 ± 3.35 vs. 49.96

± 3.78 %, respectively; Fig. 2B). Relative to the lowland mice, the highland mice had a lower MCHC (4.74 ± 0.28 vs. 5.05 ± 0.45 mM, respectively) and a smaller average red cell size than the lowland mice (5.38 ± 0.12 vs. 5.72 ± 0.41 μm, respectively), but these differences disappeared after acclimation to normoxia (Fig. 2D). The highland mice exhibited a significantly higher red cell DPG concentration (2.16 ± 0.44 vs. 1.47 ± 0.39 mM; Table 1, Fig. 2E), but as with MCHC and red cell size, this difference disappeared after acclimation to normoxia. For each of the hematological traits, the '*in situ*' measurements of wild-caught mice were consistent with data from previous studies of *Peromyscus* mice (Fig. 3).

Similar to the case with Hb concentration, the F_1 progeny of highland and lowland parents did not exhibit significant differences in Hct (49.20 ± 3.21 vs. 50.00 ± 4.26 %; t_{50} = 0.733, P = 0.528), MCHC (4.81 ± 0.34 vs. 4.84 ± 0.44 mM; t_{50} = -0.228, P = 0.841), RBC size (5.57 ± 0.28 vs. 5.45 ± 0.21 μm; t_{42} = 1.380, P = 0.126), or DPG/Hb ratio (2.45 ± 0.51 vs. 2.24 ± 0.61 mM; t_{23} = 0.858, P = 0.458). These results indicate that the naturally occurring hematological differences between highland and lowland mice are environmentally induced and are largely attributable to physiological plasticity during adulthood.

DISCUSSION

Under conditions prevailing in their native habitats, highland deer mice from the summit of Mt. Evans exhibited a number of pronounced hematological differences relative to lowland conspecifics from the Great Plains. In comparison with lowland mice, the highland mice exhibited higher Hb concentrations, higher Hcts, higher DPG/Hb

ratios, lower MCHC values, and smaller red cells. However, these differences disappeared after 6-weeks of acclimation to normoxia at low altitude (Fig. 2). The measured traits were also indistinguishable between the F_1 progeny of highland and lowland mice, indicating that there were no persistent, irreversible differences in phenotype that could be attributed to developmental plasticity or fixed, genetically based differences. Studies of other physiological traits in deer mice have documented similar degrees of plasticity in response to cold and/or hypoxic stress (Hammond et al. 1999, 2001, 2002; Rezende et al. 2004; Chappell et al. 2007; Russell et al. 2008; Rezende et al. 2009; Cheviron et al. 2013).

In the case of Hb concentration and Hct, the native character states of the highland mice were largely congruent with the typical acclimatization response to hypoxia exhibited by lowland mammals that have no known evolutionary history of residence at high-altitude (Monge and León-Velarde 1991). The elevated Hb concentrations and Hcts observed in the highland deer mice are consistent with results of previous studies of *P. maniculatus* (Gough and Kilgore, 1964; Hock, 1964; Sealander, 1964; Dunaway and Lewis, 1965; Thompson et al., 1966; Sawin, 1970; Snyder, 1982; Snyder et al,. 1982; Wyckoff and Frase, 1990; Hammond et al., 1999, 2001; Table S1, Fig. 3). By contrast, most rodent species that are native to high-altitudes appear to have Hb concentrations and Hcts that are substantially lower than those of hypoxia-acclimated laboratory rats or house mice (Hall et al. 1936; Chiodi 1962; Morrison et al. 1963a,b; Bullard et al. 1966) and hematological traits in the majority of high-altitude Andean rodents studied by Morrison et al. (1963a,b) remained unaltered after acclimation to normoxic conditions at sea-level.

In humans, highlanders that are indigenous to the Tibetan and Ethiopian plateaus exhibit low Hb concentrations relative to Andean highlanders that are resident at comparable altitudes (Beall and Reischsman 1984; Winslow et al. 1989; Beall et al. 1990, 1998, 2002; Garruto et al. 2003; Wu et al. 2005; Beall 2006, 2007; Beall et al. 2010; Simonson et al. 2010). The high Hb concentrations of Andean highlanders mirror the expected acclimatization response to hypoxia in lowland natives, whereas the Tibetans and Ethiopians appear to have evolved a blunted erythropoietic response to environmental hypoxia. Andeans also suffer a much higher incidence of chronic mountain sickness (Winslow and Monge 1987). The elevated Hb concentrations of highland deer mice mirror the acclimatization response to hypoxia in lowland species (e.g., Baumann et al. 1971; Duhm and Gerlach 1971), suggesting that the highland deer mice have a hematological profile that is more similar to that of Andean humans than that of Tibetans or Ethiopians. However, additional experiments are required to assess whether highland and lowland mice differ in their acclimation responses to hypoxia, and it is an open question as to whether the elevated Hb concentration of high-altitude deer mice represents an example of adaptive or maladaptive plasticity.

In addition to the potentially adverse effects of increased blood viscosity on cardiac output, an increased Hct can also diminish plasma transport of free fatty acids and other metabolic fuels (McClelland 2004). This is because the fuel transport capacity of blood plasma is determined by the product of plasma flow and fuel concentration, and plasma flow is an inverse function of Hct. In deer mice living at high-altitude, the effects of the elevated Hct on plasma fuel transport may be especially significant because deer

mice rely heavily on fatty acid oxidation to fuel shivering thermogenesis (Cheviron et al. 2012).

The elevated red cell DPG concentrations observed in highland deer mice are mostly consistent with previous reports. Snyder (1982) measured red cell DPG concentrations in deer mice sampled from localities ranging from 2590 m to 4340 m in the White Mountains of eastern California. Although the DPG/Hb ratio was somewhat reduced in the high-altitude sample of mice from 4340 m, there was no monotonic altitudinal trend, as DPG/Hb ratios were highest in mice from intermediate elevations. Similar to the results reported by Snyder (1982), our common-garden experiment revealed a reversal in relative DPG concentrations such that the highland mice actually exhibited a slightly lower baseline DPG/Hb ratio after acclimation to normoxia (Fig. 2E). If the reduced baseline DPG/Hb ratio and elevated Hb-O_2 affinity of highland deer mice are construed as adaptive mechanisms for maintaining an elevated blood-O_2 affinity under hypoxia, then the hypoxia-induced increase in red cell DPG would seem to be counterproductive (Storz et al. 2010b). In considering this seemingly maladaptive acclimatization response, Snyder (1982) suggested that physiological constraints of red cell energy metabolism may prevent evolutionary modifications of intracellular DPG levels since the formation of DPG is stimulated by overall glycolytic activity. However, given that the optimal blood-O_2 affinity is a nonlinear function of ambient PO_2 (Turek et al. 1973; Eaton et al. 1974; Bencowitz et al. 1982; Willford et al. 1982), empirical performance curves are needed to evaluate whether hypoxia-induced reductions in the DPG/Hb ratio are advantageous or disadvantageous at a given altitude. Since changes in the DPG/Hb ratio also affects intracellular pH and Cl^- concentration, and since the adult

Hbs of deer mice and other muroid rodents have ligand-affinities that are more strongly modulated by Cl^- ions than by DPG (Storz et al. 2009, 2010a, 2012b; Runck et al. 2010), the net effects on blood-O_2 affinity are difficult to predict. Moreover, if deer mice have a hyperventilatory response to hypoxia, as in other mammals, the resultant increase in plasma pH (respiratory hypocapnia) may effectively counterbalance the inhibitory effects of DPG on Hb-O_2 affinity (Mairbäurl and Weber 2012).

In contrast to the hematological changes that are typically associated with the acclimatization response to hypoxia in lowland mammals, genetically based changes in Hb structure that increase intrinsic O_2 affinity or that suppress sensitivity to allosteric effectors are generally thought to make more important contributions to hypoxia tolerance in species that are high-altitude natives (Bunn, 1980; Monge and León-Velarde, 1991; Storz, 2007; Weber, 1995, 2007; Storz and Moriyama 2008; Storz et al. 2010b; Mairbäurl and Weber 2012). High-altitude deer mice seem to defy Monge and León-Velarde's (1991) proposed dichotomy between the hypoxia response strategies of highland and lowland natives, as they possess a set of hematological traits that appear to fit both profiles. On the one hand, highland deer mice exhibit elevated Hb concentrations, Hcts, and red cell DPG concentrations relative to lowland mice (Hock 1964; Sawin 1970; Snyder 1982; Snyder et al. 1982), characteristics that seem to fit the profile of a hypoxia-acclimated lowland species. On the other hand, highland deer mice possess structurally distinct Hbs with slightly elevated O_2 affinity (Storz et al. 2009, 2010a), which fits the profile of a genetically adapted, hypoxia-tolerant highland species.

In the case of highly labile hematological traits like Hb concentration, Hct, and red cell DPG concentration that are responsive to minute changes in arterial PO_2 and

acid-base balance, it is probably often the case that altitude-related trait differences between species or between conspecific populations are purely attributable to physiological plasticity, reflecting hypoxia-induced regulatory changes in erythropoietic activity and red cell metabolism. The high degree of plasticity that we have documented for hematological traits in deer mice highlights the importance of using common-garden experiments to assess whether physiological differences between species or conspecific populations represent reversible, constitutively expressed traits or irreversible, fixed differences.

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FIGURES

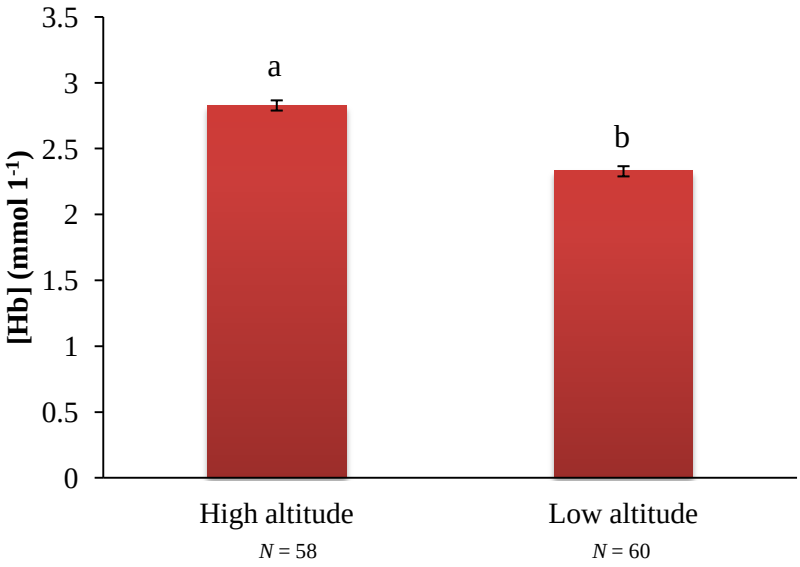

Figure 1. Hemoglobin (Hb) concentrations (mean ± 1 s.e.m.) of highland and lowland *Peromyscus maniculatus* measured at their native altitudes. Significant differences in mean values ($P < 0.05$) are denoted by different letters.

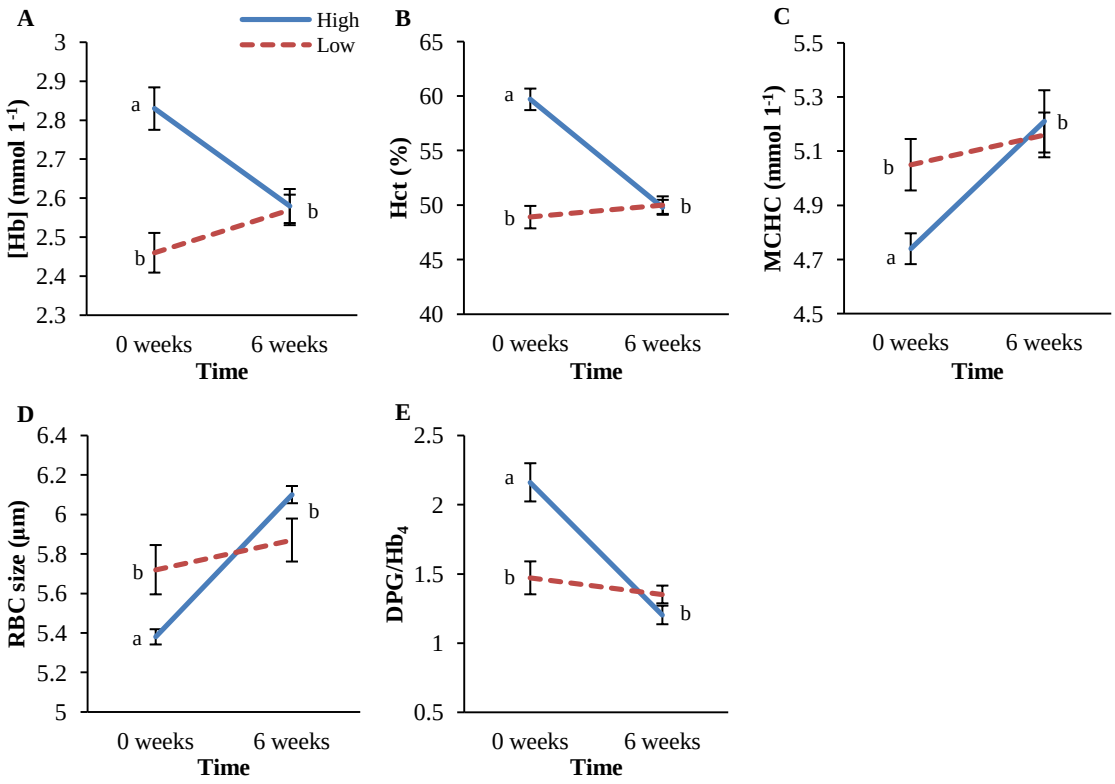

Figure 2. Changes in hematological traits (mean ±1 s.e.m.) in highland and lowland *P. maniculatus* induced by acclimation to normoxia over a 6 week period. (A) Hb concentration, (B) hematocrit (Hct); (C) mean corpuscular Hb concentration (MCHC); (D) red blood cell (RBC) size; and (E) 2,3-diphosphoglycerate (DPG)/Hb_4 ratio. Measures of Hb concentration, Hct, and MCHC are based on 24 highland mice and 22 lowland mice, whereas measures of RBC size and DPG/Hb_4 ratio were based on a subset of 10 highland mice and 11 lowland mice. A repeated-measures ANOVA was used to test for differences in mean values between treatment groups and between pre- and post-acclimation time points. Significant differences in mean values ($P < 0.05$) are denoted by different letters.

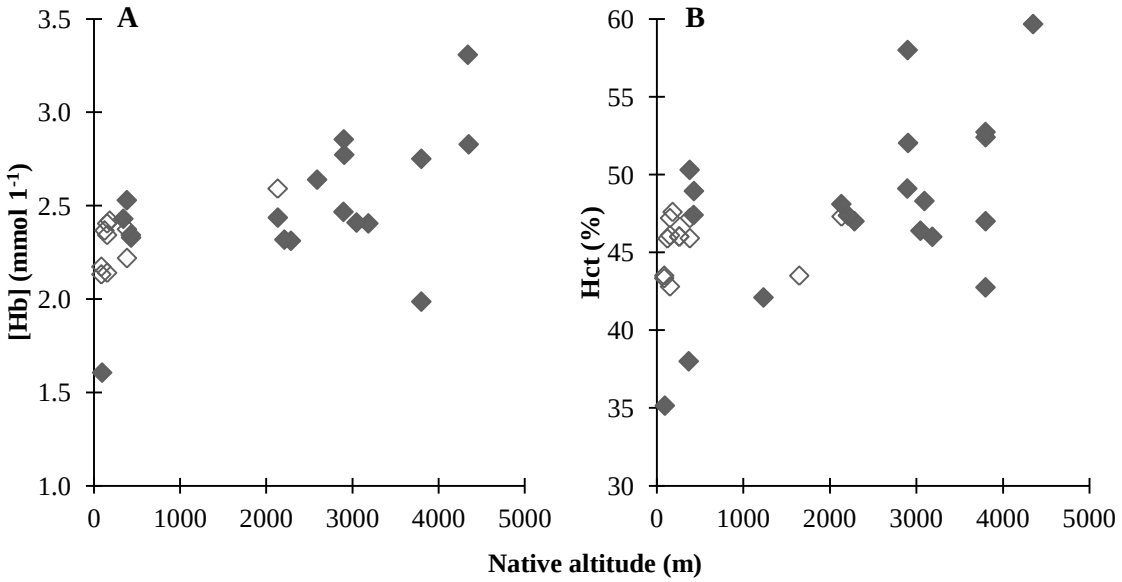

Figure 3. Scatterplot of mean Hb concentration (A) and mean Hct (B) against native altitude for population samples of *P. maniculatus* and other *Peromyscus* species from this and other studies. Filled symbols denote data points for population samples of *P. maniculatus* and open symbols denote data points for other species of *Peromyscus* (*P. boylii*, *P. crinitus*, *P. leucopus*, *P. nuttalli*, and *P. polionotus*). Raw data are provided in supplementary material Table S1.

TABLES

Table 1. Differences in hematological traits between groups of deer mice (high- *versus* low-altitude) and between pre- and post-acclimation time points.

Trait	Factor	d.f.	F	*P*
[Hb]	Altitude	1, 43	14.58	0.0004
	Time	1, 44	2.33	0.1344
	Altitude x Time	1, 44	15.54	0.0003
Hct	Altitude	1, 43	29.99	< 0.0001
	Time	1, 44	29.21	< 0.0001
	Altitude x Time	1, 44	44.24	< 0.0001
MCHC	Altitude	1, 43	2.17	0.1483
	Time	1, 44	8.43	0.0058
	Altitude x Time	1, 44	3.17	0.0821
RBC size	Altitude	1, 18	0.37	0.5490
	Time	1, 19	22.83	0.0001
	Altitude x Time	1, 19	9.82	0.0055
DPG/Hb_4	Altitude	1, 18	7.29	0.0147
	Time	1, 19	27.6	< 0.0001
	Altitude x Time	1, 19	16.69	0.0006

Tests were carried out using a repeated-measures, two-way ANOVA. Hb, hemoglobin; Hct, hematocrit; MCHC, mean corpuscular Hb concentration; RBC, red blood cell; DPG, 2,3-diphosphoglycerate.

Supplementary Table 1. Hematological data for population samples of *P. maniculatus* and other *Peromyscus* species.

Species Name	Altitude (m)	[Hb] g/dl (mM)	Hct (%)	RBC size (µm)	MCHC g/dl (mM)	DPG/Hb	Reference
Peromyscus maniculatus	4350	18.2 (2.8)	59.7	5.4	30.6 (4.7)	2.2	Present study
P. maniculatus	4340	21.3 (3.3)	64.2	-	33.2 (5.2)	1.7	Snyder 1982
P. maniculatus	3801	-	52.4	-	-	-	Hammond et al. 1999
P. maniculatus	3801	-	47.0	-	-	-	Hammond et al. 2001
P. maniculatus	3800	17.7 (2.8)	52.7	-	33.6 (5.2)	1.8	Snyder 1982
P. maniculatus	3800	12.8 (2.0)	42.8	-	30.0 (4.7)	-	Sawin 1970
P. maniculatus	3185	15.5 (2.4)	46.0	-	33.5 (5.2)	-	Thompson et al. 1966
P. maniculatus	3094	-	48.3	-	-	-	Hammond et al. 1999
P. maniculatus	3048†	15.5 (2.4)	46.4	-	33.5 (5.2)	-	Gough and Kilgore 1964
P. maniculatus	2905	17.9 (2.8)	52.0	-	34.4 (5.3)	1.8	Snyder 1982
P. maniculatus	2900	18.4 (2.9)	58.0	5.9	31.7 (4.9)	-	Wyckoff and Frase 1990
P. maniculatus	2896	15.9 (2.5)	49.1	6.4	32.5 (5.0)	-	Sealander 1964
P. maniculatus	2590	17.0 (2.6)	-	-	-	1.7	Snyder 1982
P. maniculatus	2286	14.9 (2.3)	47.0	-	31.6 (4.9)	-	Thompson et al. 1966
P. maniculatus	2210†	14.9 (2.3)	47.4	-	31.6 (4.9)	-	Gough and Kilgore 1964
P. maniculatus	2134	15.7 (2.4)	48.1	6.4	32.8 (5.1)	-	Sealander 1964
P. crinitus	2134	16.7 (2.6)	47.3	6.5	35.2 (5.5)	-	Sealander 1964
P. polionotus	1646	-	43.5	-	-	-	Dunaway and Lewis 1965
P. maniculatus	1234	-	42.1	-	-	-	Hammond et al. 1999
P. maniculatus	430	15.0 (2.3)	48.9	5.7	32.5 (5.1)	1.5	Present study
P. maniculatus	427	15.1 (2.3)	47.4	6.4	31.8 (4.9)	-	Sealander 1962
P. boylii	381	14.3 (2.2)	45.9	6.4	31.0 (4.8)	-	Sealander 1964
P. leucopus	381	15.3 (2.4)	47.2	6.3	32.5 (5.0)	-	Sealander 1964
P. maniculatus	381	16.3 (2.5)	50.3	6.3	32.4 (5.0)	-	Sealander 1964
P. maniculatus	370	-	38.0	-	-	-	Hammond et al. 2001
P. maniculatus	340	15.6 (2.4)	45.9	-	34.1 (5.3)	1.6	Snyder 1982
P. leucopus	259	-	46.0	-	-	-	Dunaway and Lewis 1965
P. nuttalli	259	-	46.0	-	-	-	Dunaway and Lewis 1965
P. leucopus	182	15.6 (2.4)	47.6	6.3	32.8 (5.2)	-	Wyckoff and Frase 1990
P. leucopus	152	15.1 (2.3)	46.1	6.4	32.8 (5.2)	-	Sealander 1964
P. polionotus	152	13.8 (2.1)	42.8	-	32.2 (5.0)	-	Sealander 1964
P. nuttalli	152	15.5 (2.4)	47.2	-	32.7 (5.1)	-	Sealander 1964
P. leucopus	122*	15.3 (2.4)	45.9	6.1	33.2 (5.2)	-	Foreman 1956
P. maniculatus	94	10.4 (1.6)	35.2	-	29.3 (4.6)	-	Sawin 1970
P. gossypinus	84*	13.7 (2.1)	43.5	-	31.6 (4.9)	-	Gough and Kilgore 1964
P. nuttalli	84*	14.0 (2.2)	43.3	-	32.4 (5.0)	-	Gough and Kilgore 1964

† Represents the elevational midpoint of collection localities reported by Gough and Kilgore (1964).
*Estimated altitude based on locality information provided in the text.

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CHAPTER II

HIGHLAND AND LOWLAND DEER MICE EXHIBIT DIFFERENT HEMATOLOGICAL ACCLIMATION RESPONSES TO CHRONIC HYPOXIA

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ABSTRACT

Air-breathing vertebrates living at high altitude face challenges to respiratory gas transport due to the reduced partial pressure of O_2 (P_{O2}). Some species have developed ways of coping with hypoxia by reversibly altering erythropoeisis to enhance the oxygen-carrying capacity of the blood. Here we report a common-garden experiment that was designed to test for differences in acclimation responses to hypoxia in first generation descendents of deer mice (*Peromyscus maniculatus*) that were native to highland and lowland environments. Progeny of wild-caught mice were born and raised in a low altitude common-garden environment to control for developmental plasticity. Experimental animals were acclimated to hypoxia using hypobaric chambers for 6 wks. Hemoglobin (Hb) concentration, hematocrit (Hct), mean cell hemoglobin concentration (MCHC), red blood cell size (RBC size), and red blood cell count (RBC count) were measured before and after hypoxia acclimation. In highland and lowland mice the acclimation response to hypoxia involved significant increases in Hb concentration, Hct, RBC size, and RBC count. MCHC in highland mice increased, but did not change significantly in lowland mice. Differences in the acclimation responses of highland and lowland mice suggest that highland mice may have evolved blunted erythropoietic response to chronic hypoxia.

Key Words: High altitude, hemoglobin, hematocrit, red blood cell, *Peromyscus maniculatus*

INTRODUCTION

In response to acute or chronic hypoxia, mammals typically compensate for the reduced partial pressure of O_2 (P_{O2}) in inspired air by means of reversible or nonreversible changes in hematological traits that increase the O_2 carrying capacity of the blood (Bouverot 1985; Storz et al. 2010b). However, the nature of these acclimatization responses may be quite different in species that have a long evolutionary history of exposure to environmental hypoxia (e.g., high-altitude natives) and those that do not. For example, lowland natives typically respond to acute or chronic exposure to hypoxia by increasing hemoglobin (Hb) concentration and hematocrit (Hct) (Guyton and Richardson 1961; Monge and Whittenbury 1976; Monge and Leon-Velarde 1991; Beall et al. 1998). By contrast, there is some evidence to suggest that highland natives have evolved a blunted erythropoietic response to environmental hypoxia (Monge and Leon-Velarde 1991; Ge et al. 1998; Scott and Milsom 2007; Powell 2008; Storz et al. 2010b). Individuals able to cope with oxygen deficiencies from their hypoxic environment through plastic alterations of certain hematological traits may have a selective advantage over canalized (robustness or the ability to produce the same phenotype regardless of environment) individuals. For example, organisms that are efficient dispersers (i.e. birds or migrating mammals) or live in fine-grained environments may encounter a wide range of environmental conditions and are therefore more likely to evolve phenotypic plasticity because they will be better able to cope with a broad range of environmental variations (van Tienderen 1991; van Tienderen 1997; Storz et al. 2010b). Alternatively, individuals that are poor dispersers (i.e. small mammals) or residents of coarse-grained environments may encounter a limited range of environmental conditions and may have a selective

advantage employing a canalization strategy (Storz et al. 2010b). Therefore, subspecies with different dispersal patterns along a broad elevational distribution often demonstrate a significant amount of phenotypic plasticity (Scheiner 1998; de Jong and Behera 2002; Sultan and Spencer 2002; Storz et al. 2010b). Adaptive phenotypic plasticity may play a crucial role in a species being able to colonize and tolerate extreme environmental conditions and then adapt to these conditions (Ghalambor et al. 2007). For instance, an increase in phenotypic plasticity of hematological traits may be an advantage to organisms that sojourn to high altitude frequently or endure prolonged stays at altitude. Guinea pigs, rabbits, rats and dogs exhibited a plastic response when tested under laboratory conditions by showing an increase in Hb concentration, Hct, and iron metabolism with reduced amounts of oxygen (Meints and Olander 1970). Conversely, animals native to high altitude (i.e. ground squirrels, Yellow-bellied marmots, viscacha, vicuña, llama, Peruvian and Chilean rodents, and some mammals in the Andes) seem to have naturally low Hct values which imply a more adapted (canalized) approach to hypoxic conditions.

Previous studies on deer mice (*Peromyscus maniculatus*) have demonstrated that certain hematological traits in native highland mice exhibit different acclimation responses to normoxia (Tufts et al. 2013). Upon acclimation to low altitude for 6 wks, the high altitude mice showed a decrease in [Hb], Hct, and 2,3-diphosphoglycerate (DPG) levels, but an increase in RBC size and mean cell hemoglobin concentration (MCHC). These results demonstrate that altitudinal variation in hematological traits may be attributable to physiological plasticity during adulthood (Tufts et al. 2013). Our present study reports results of a reciprocal experiment where highland and lowland deer mice

were acclimated to chronic hypoxia to determine how these hematological traits are affected by genetic and physiological differences between these populations. The deer mouse, *Peromyscus maniculatus,* is well-suited to an examination of intraspecific variation because this species has the broadest elevational range of any North American mammal (Hock 1964; Storz 2007). Deer mice have been extensively studied and it is known that residents of high altitude environments exhibit elevated blood-O_2 affinities compared to low altitude individuals mostly due to allelic differences in hemoglobin function (Snyder 1978; Snyder 1981; Snyder 1982; Snyder et al. 1982; Chappell and Snyder 1984; Snyder 1985; Chappell et al. 1988; Storz 2007; Storz et al. 2009; Storz et al. 2010a; Natarajan et al. 2013). The aim of this research was to test for differences between highland and lowland deer mice in the acclimation response to hypoxia. To control for developmental plasticity, we used a common-garden design in which all experimental subjects were first-generation (F_1) descendents of wild-caught/lab-reared mice.

MATERIALS AND METHODS

Study Animals

Peromyscus maniculatus adults were collected from either Mt Evans (Clear Creek County, CO, USA; 39°35’18”N, 105°38’38”W, elevation 4350 m above sea level) or from a prairie grassland in Nebraska (Nine Mile Prairie, Lancaster County, NE, USA; 40°52’12”N, 96°48’20.3”W, elevation 430m above sea level) in 2011. All individuals were acclimated at low altitude conditions at the University of Illinois Urbana-Champaign. F_1 offspring from matings between either high altitude Mt Evans (Colorado)

parents (n = 30) or low altitude Lincoln (Nebraska) parents (n = 20) were used in the subsequent experiments. The mice were shipped to McMaster University and allowed to acclimate for 6 wks in hypobaric chambers to simulate hypoxic conditions. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln (IACUC no. 522) and the University of Illinois, Urbana-Champaign (IACUC no. 10244).

Hematological Measurements

Each individual was sexed and weighed prior to blood collection. All hematological measurements were collected both pre- and post-acclimation to hypoxic conditions for each mouse. Approximately 100 µl of blood was drawn from each individual (n = 50) via the maxillary vein using a 5 mm Goldenrod lancet (MEDIpoint Inc., Mineola, NY, USA). Hemoglobin concentration in whole blood was measured using a HemoCue Hb 201+ analyzer (HemoCue AB, Ängelholm, Sweden). Hematocrit (Hct) was measured as the volume of packed red cells in whole blood and was performed in duplicate using heparinized capillary tubes spun at 13,600 g for 5 min in a ZIPocrit centrifuge (LW Scientific Inc., Lawrenceville, GA, USA). From these measurements we calculated the mean cell Hb concentration (MCHC = Hb concentration*100/Hct). A 10 µl sample of blood was preserved in 990 µl of a 0.85 saline solution and was used to measure mean cell size (diameter of 10 red cells per individual) and RBC count using a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Gottingen, Germany).

Statistical Analysis

For all treatment groups a repeated measures two-way GLIMMIX ANOVA was completed with altitude (normoxia versus hypoxia) and time (pre- versus post-

acclimation) as independent variables. Values for males and females were not significantly different for any hematological traits; therefore both sexes were pooled for each analysis. Unfortunately, the same individuals were not able to be assessed after hypoxia acclimations and therefore statistical analyses were performed by pooling individuals by family groups for highland (n = 6) and lowland (n = 5, 4, or 3 depending on hematological trait) mice. A Kolmogorov-Smirnov (K-S) test was completed for each trait and no significant deviations from normality were detected. Statistical analyses were performed using the SAS software package (SAS Institute Inc., 2009) and an online statistical calculator (physics.csbsju.edu).

RESULTS

Under normoxia, there were no significant differences between highland and lowland mice in Hb concentration (mean ± 1 s.d., 2.37±0.17 versus 2.32±0.13 mmol l^{-1}, respectively (Hb molecular mass 64.45 kDa)), Hct (49.52±2.42 versus 47.72±2.48%, respectively), MCHC (4.78±0.17 versus 4.87±0.13 mmol l^{-1}, respectively), or red cell count (12.83±1.73 versus 10.40±2.48 x 10^6 RBC/mm^3, respectively). However, under hypoxia both highland and lowland mice exhibited a significant increase in Hb concentration (2.84±0.14, P = 0.0018 versus 2.98±0.28, P = 0.0004; respectively) although they were not statistically different from each other at either time point (Table 1; Fig.1A). Both highland and lowland mice also showed a significant increase in Hct after acclimation to hypoxia (P = 0.0084; P = 0.0002, respectively), but highland mice possessed a Hct that was significantly lower than that of lowland mice (56.65±3.58 versus 62.10±6.07%, respectively; P = 0.0495; Table 1; Fig.1B). Highland *P.*

maniculatus showed a significant increase in MCHC after acclimation ($P = 0.0044$) and possessed significantly higher values compared to lowland mice (5.03±0.13 versus 4.83±0.10, respectively; $P = 0.0301$; Table 1; Fig.1C). Under normoxic conditions, highland F_1 mice possessed significantly smaller RBCs than lowland mice ($n = 4$) (5.12±0.05 versus 5.37±0.20 μm, respectively; $P = 0.0168$), both groups showed a significant increase in red cell size after acclimation to hypoxia (highland $P = 0.0229$; lowland $P = 0.0076$). However, after hypoxia acclimation the RBCs of the highland mice remained significantly smaller than those of the lowland mice (5.33±0.13 versus 5.68±0.09 μm, respectively; $P = 0.0018$; Table 1; Fig.1D). Highland ($n = 6$) and lowland ($n = 5$) F_1 mice significantly increased the number of RBC's in parallel under hypoxia (22.37±3.21, $P = 0.0001$ versus 20.43±4.53 x 10^6 RBC/mm^3, $P = 0.0002$; respectively; Table 1; Fig.1E).

DISCUSSION

Under normoxia, the highland and lowland mice exhibited similar hematological profiles. For example, measurements for all traits, except RBC size, were statistically indistinguishable between the two groups. Hb concentrations and RBC counts were statistically indistinguishable between the two groups under both normoxia and hypoxia. However, both groups showed a significant increase in Hb concentration and RBC count that were statistically indistinguishable in response to hypoxia. An increase in Hb concentration is advantageous in hypoxia because it offsets the lower PO_2 and helps to restore arterial oxygen levels (Beall 2001). One possible advantage of a reduced red cell size allows greater production in the overall number of blood cells which in turn would

promote greater circulation of oxygen to respiring tissues. Additionally, smaller red cells would diminish blood viscosity and allow easier flow of blood through constricted capillaries in hypoxia. Studies have shown that mammals with smaller red cells may exhibit some type of biological advantage on the kinetics of O_2 uptake because smaller cells have a greater velocity for O_2 uptake (Holland and Forster 1966). For instance, the rate at which oxygen binds with Hb is a function of the reaction velocity constant and Hb concentration, therefore a high velocity constant and high Hb concentration would allow for lower oxygen diffusion resistance across the membrane and rapid uptake of O_2 (Holland and Forster 1966; Lenfant 1973). Alterations in cell shape and volume may also alter cellular thickness which can affect membrane permeability and consequently oxygen diffusion (Wintrobe 1933; Sirs 1963).

In response to hypoxia, the highland F_1 mice increased Hb concentration (the amount of Hb in whole blood), MCHC (the amount of Hb relative to the packed red cell volume), RBC size (the diameter of RBCs), and RBC count (the number of blood cells). Even though the highland mice possessed smaller RBCs with less Hb, the combined surface area of these cells perhaps permits a more efficient oxygen-carrying capacity. The lowland mice seem to make the same corrections as the highland mice, although they were more drastic in their hematological alterations. For instance, they increased Hct (percentage of RBC's in whole blood) and RBC size to a significantly greater extent than the highland mice. Lowland mice also increased RBC count, but not to the same extent as the highland mice. This might be because they increased the diameter of the blood cells to such an extent that they could not increase RBC count any further. Our findings of these hematological changes in highland and lowland mammals are consistent with other

studies (Wintrobe 1933; Kalabuchov 1937; Gough and Kilgore 1964; Hock 1964; Sealander 1964; Hammond et al. 1999).

Highland F_1 deer mice also seem to react in similar fashion to native highland mice with regard to Hb concentration, Hct, and RBC size in hypoxia (Tufts et al. 2013). Interestingly, the lowland F_1 mice showed lower values than highland F_1 mice and wild-caught mice acclimated to normoxic conditions and higher values after hypoxia exposure in Hb concentration and Hct, suggesting that the lowland mice may have overshot the typical physiological levels for these traits when exposed to chronic hypoxia (data not shown). The highland F_1 deer mice also had virtually the same sized blood cells after hypoxia acclimation as the adult wild-caught native highland mice, which might suggest a genetic predisposition for erythrocytes of this size in hypoxia.

The highland F_1 mice have increased Hb concentration which increases the O_2 carrying capacity, but have maintained a lower Hct and smaller blood cells to avoid the detrimental circulatory consequences of viscous blood. The descendents of lowland deer mice also increased O_2 carrying capacity, but they possessed significantly higher Hct values (high altitude polycythemia), larger erythrocytes, and a significantly lower MCHC, which might increase the cost of pumping blood and impede oxygenation to various tissues because of an increase in blood viscosity and vascular resistance more so than in the highland descendents (Guyton and Richardson 1961; Storz et al. 2010b). The lowland F_1 mice exhibit drastic alterations in the hematological traits measured, these alterations are less intense in the highland descendents suggesting that they possess a more blunted erythropoietic response to chronic hypoxia. This blunted response has also been shown with other mammals including human populations indigenous to high

altitude environments. For example, Tibetan and Ethiopian highlanders at altitudes around 4000 m possess hematological profiles that resemble sea level residents (Beall et al. 1998; Beall 2007). Specifically, Tibetan highlanders were found to have lower levels of Hb concentration compared to acclimated lowland individuals to hypoxia and to Andean highlanders (Beall et al. 1990; Beall 1998; Beall 2001; Beall 2006; Wu and Kayser 2006). Some studies suggest that the blunted response to hypoxia in native highland individuals is a genetically inherited characteristic because acclimatized lowlanders never develop the response and highlanders that spend prolonged periods at low altitude retain the blunted response (Edelmand et al. 1970; Beall et al. 1998; Beall 2000; Beall 2006; Beall 2007). Our results suggest that descendents of highland mice seem to resemble the acclimatization responses observed in Tibetans and Ethiopians at high altitude and that this blunted response may be an important adaptive advantage for surviving in high altitude environments by reducing the stressors engendered by hypoxia.

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FIGURES

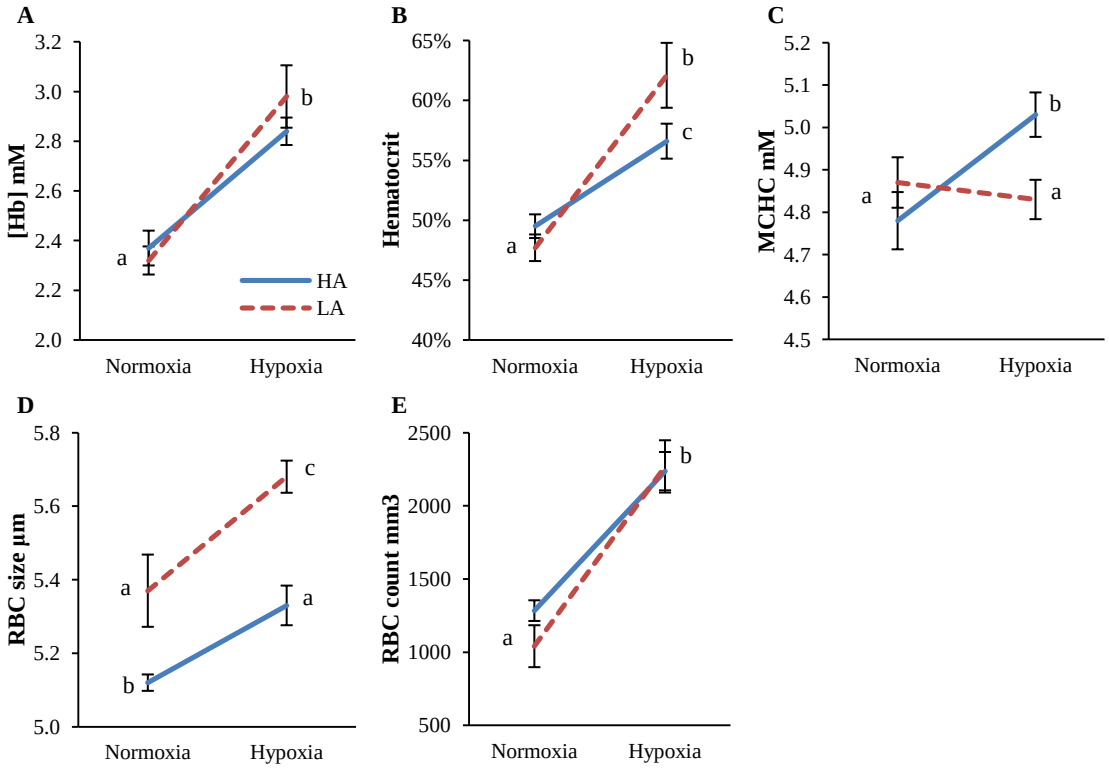

Figure 1. Plasticity in hematological traits (mean ± 1 s.e.m.) from F_1 decedents from either highland or lowland *P. maniculatus* parents subjected to hypoxia for 6 weeks. (A) Hemoglobin concentration, (B) Hematocrit, (C) Mean Cell Hemoglobin Concentration (MCHC), (D) red blood cell (RBC) size, and (E) red blood cell (RBC) count. Sample sizes for highland mice under normoxic and hypoxic conditions were based on six family groups whereas sample sizes for lowland mice under normoxic conditions were based on five family groups ([Hb], Hct, and MCHC), four family groups (RBC size), or three family groups (RBC count), but after acclimation to hypoxia five family groups were used for all measurements except RBC size where four family groups were available. A

repeated-measures ANOVA was used to determine differences in mean values between normoxic and hypoxic conditions, significant differences ($P < 0.05$) are denoted by different letters.

TABLES

Table 1. ANOVA analysis for the differences in various hematological traits between highland and lowland deer mice between normoxia and acclimation to simulated hypoxia.

Trait	**Factor**	**d.f.**	***F***	***P***
[Hb]	Altitude	1, 8	0.29	0.6070
	Time	1, 9	48.96	<0.0001
	Altitude x time	1, 9	1.20	0.3009
Hct	Altitude	1, 8	1.02	0.3428
	Time	1, 9	46.69	<0.0001
	Altitude x time	1, 9	5.30	0.0469
MCHC	Altitude	1, 8	0.94	0.3605
	Time	1, 9	3.43	0.0972
	Altitude x time	1, 9	10.46	0.0103
RBC size	Altitude	1, 8	32.65	0.0004
	Time	1, 8	20.38	0.0020
	Altitude x time	1, 8	0.71	0.4249
RBC count	Altitude	1, 8	4.62	0.0638
	Time	1, 7	104.50	<0.0001
	Altitude x time	1, 7	1.19	0.3123

CHAPTER III

STEPWISE EVOLUTION OF AN INCREASED HEMOGLOBIN-OXYGEN AFFINITY IN A HIGH ALTITUDE PIKA SPECIES (OCHOTONA PRINCEPS)

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ABSTARCT

There are good theoretical and empirical reasons to expect high-altitude animals to have an elevated blood-O_2 affinity relative to closely related lowland taxa. Under severe hypoxia, an increase in blood-O_2 affinity can help preserve an adequate level of tissue oxygenation by enhancing pulmonary O_2 uptake while simultaneously maintaining the pressure gradient that drives O_2 diffusion from the peripheral capillaries to the cells of respiring tissues. Adjustments in blood-O_2 affinity often stem directly from structural changes in hemoglobin (Hb). Such changes can be brought about by amino acid substitutions that increase the intrinsic O_2-affinity of the Hb tetramer and/or mutations that suppress the sensitivity of Hb to the inhibitory effects of allosteric cofactors in red blood cells, such as 2,3-diphosphoglycerate (DPG) in mammals. Here we report an examination of Hb function in high- and low-altitude species of pikas (*Ochotona princeps* and *O. collaris*, respectively). By measuring the oxygenation properties of purified Hbs from each species, we demonstrated that the high-altitude species has evolved a derived increase in hemoglobin-O_2 affinity. The evolved change in protein function was exclusively attributable to an increase in intrinsic O_2-affinity, as the Hbs of both species were equally responsive to the modulating effects of allosteric effectors. Using ancestral sequence reconstruction and site-directed mutagenesis, we identified a set of three β-chain substitutions that are responsible for the evolved change in hemoglobin function.

Key Words: Hypoxia, Mammals, protein evolution, site-directed mutagenesis

INTRODUCTION

A compelling system for investigating mechanisms of biochemical adaptation is provided by the evolution of increased Hb-O_2 affinity in vertebrate species that have adapted to environmental hypoxia. Among mammals, elevated Hb-O_2 affinities have evolved in species that are native to high-altitude environments (Storz 2007; Storz et al. 2009; Storz et al. 2010a) and in fossorial or semi-fossorial species that are adapted to the chronic hypoxia of subterranean burrow systems (Jelkmann et al. 1981; Campbell et al. 2010; Revsbech et al. 2013). Under severe hypoxia, an increased Hb-O_2 affinity can help preserve an adequate level of tissue oxygenation by enhancing pulmonary O_2 uptake while simultaneously maintaining the pressure gradient that drives O_2 diffusion from capillary blood to the tissue mitochondria (Turek et al. 1973; Turek and Kreuzer 1976; Turek et al. 1978a; Turek et al. 1978b; Bencowitz et al. 1982; Mairbaurl 1994; Samaja et al. 2003; Mairbaurl and Weber 2012). Mammalian Hb is a heterotetramer consisting of two α-type subunits (141 amino acids each) and two β-type subunits (146 amino acids each) that each contain a heme group – a porphyrin ring with a ferrous (Fe^{2+}) iron atom capable of reversibly binding a single dioxygen molecule (Dickerson and Geis 1983). Different amino acids (including the polarity of R side chains) instruct the Hb subunits how to fold properly, therefore a single amino acid substitution could drastically affect proper folding of the tertiary and/or quaternary structure of the molecule which may also alter its functional properties (Zuckerkandl and Pauling 1965; Perutz 1983). The Hb tetramer undergoes an oxygenation-linked transition in quaternary structure, whereby the two semi-rigid $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers rotate around each other by 15° during the transition between the deoxy (low affinity [T]) conformation and the oxy (high affinity [R])

conformation (Perutz 1972; Baldwin and Chothia 1979; Shaanan 1980; Fermi and Perutz 1981; Fermi et al. 1984). This oxygenation-linked shift in quaternary structure between the T- and R-states is central to the allosteric function of Hb as an O_2 transport molecule, and it governs the subunit cooperativity of O_2 binding as well as the sensitivity of O_2 binding to allosteric effectors such as Cl^- ions and organic phosphates. Allosteric effectors reduce Hb-O_2 affinity because they preferentially bind to deoxyHb, thereby stabilizing the low-affinity T structure through the formation of additional hydrogen-bonds and salt bridges within and between subunits (Perutz 1970; Perutz 1989; Bettati et al. 1983). Amino acid substitutions that inhibit Cl^- and/or phosphate binding will typically increase Hb-O_2 affinity by shifting the allosteric equilibrium in favor of R-state oxyHb (Weber et al. 2002; Storz et al. 2009; Storz et al. 2010a).

To identify causative mutations and to measure their individual and combined effects, we used a combinatorial protein engineering approach to dissect the mechanistic basis of evolved differences in Hb-O_2 affinity between two sister species of North American pikas that have contrasting altitudinal range limits. Our comparison included the American Pika (*Ochotona princeps*), a predominantly alpine species, and the closely related Collared Pika (*O. collaris*), which has an exclusively lowland distribution in Alaska and western Canada (Hafner and Sullivan 1995; Galbreath et al. 2009; Galbreath et al. 2010). These two Nearctic pika species are thought to have diverged from a common ancestor ~4-8 million years ago, in the early Pliocene or late Miocene (Hafner 1994; Lanier and Olson 2009). We measured the functional properties of native Hbs from *O. princeps* and *O. collaris*, and using ancestral protein resurrection and site-directed mutagenesis, we measured the structural and functional effects of sequential mutations in

all possible pathways connecting the ancestral (low-affinity) Hb and the derived (high-affinity) Hb of the high-altitude species.

MATERIALS AND METHODS

Study organisms and sites

In May-August 2010 and 2011, we collected a total of 13 pikas: seven *Ochotona princeps* individuals from the summit of Mount Evans, Clear Creek Co., Colorado (39°35'18"N, 105°38'38"W; elevation 4350 m above sea level) and six *O. collaris* individuals from Eagle Summit (n = 3; 65°29'36"N, 145°26'01"W; elevation 1131 m above sea level) and Rainbow Ridge, AK (n = 3; 36°18'32"N, 145°40'21"W; elevation 949 m above sea level). All individuals were killed in the field using a .22 caliber rifle or by chest compression when caught with Sherman traps. We collected blood samples via cardiac puncture using a 22-guage needle, bone marrow and liver tissue samples were also collected; all samples were immediately flash frozen in liquid nitrogen. In June-July 2012, *O. dauurica* (n = 3) and *O. pallasi* (n = 1) samples were collected from Mongolia in a similar manner as stated above. In order to have a more comparative study of high and low altitude species, museum tissue samples were obtained from the Museum of Vertebrate Zoology (MVZ; high altitude species *O. princeps* and *O. rufescens*) and the University of Alaska Museum of the North (UAM; low altitude species *O. collaris* and *O. hyperborea*) (Table 1). All animals and procedures were previously approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee (IACUC no. 519; Colorado collecting permit no. 10TR2058; Alaska collecting permit no. 11-057).

Hb characterization and purification

Hemolysates from three *O. princeps* and three *O. collaris* individuals were used to characterize possible Hb isoforms using an isoelectric focusing (IEF) technique (PhastSystem, GE Healthcare Bio-Sciences, Piscataway, NJ). The purification of hemolysates was achieved high performance liquid chromatography (HPLC) by first dialyzing the samples against 20 mM Tris buffer (pH 8.4, sample pI was 7.35) at 4º C for 3 h and then again in a clean 20 mM Tris buffer solution overnight. Subsequently we centrifuged the samples at 14,000 x g to discard cell debris. Next we submitted these hemolysates to an ion-exchange column (HiTrap^{TM} QHP, GE Healthcare Bio-Sciences, Piscataway, NJ) previously equilibrated with 20 mM Tris (pH 8.4) so that all organic phosphates were removed from the Hb samples. To elute Hb bound to the column a gradient of 0 – 0.2 M NaCl was used. We then proceeded to a second dialysis step to desalt the samples against a 10 mM Hepes buffer (pH 7.4) for 3 h and then again overnight. Finally, samples were concentrated (to > 1mM) using 30 K Millipore centrifugal tubes (Millipore, Bedford, MA, USA) at 7,000 x g before storing at -80º C. Heme-oxy concentration (mM) was calculated based on the absorbance peaks in the visible region of the spectrum (577 nm and 540 nm) using known extinction coefficients (Van Assendelft 1970).

Functional analysis

We measured O_2 dissociation curves through a modified O_2 diffusion chamber where shifts in absorption were recorded at stepwise changes of the O_2 tension inside the chamber generated by precision Wösthoff gas-mixing pumps (Weber 1981; Weber 1992; Weber and Fago 2004). The measurements were obtained from a 3 μL thin-layer sample

at 37º C in the presence of 0.1 M Hepes buffer (pH 7.4). In order to characterize the allosteric regulation of Hb-O_2 affinity, we measured O_2 dissociation curves for each sample in the absence of allosteric cofactors (stripped), in the presence of Cl^- ions alone (0.1 M KCl), in the presence of DPG alone (DPG/Hb tetramer ratio = 2.0, [heme] ~0.3 mM), and in the presence of both cofactors.

Values of P_{50} (P_{O2} at 50% saturation of the heme groups) and n_{50} (Hill's cooperativity coefficient at half saturation) were interpolated from the linear portion of Hill plots [log(HbO_2)/[Hb] *vs* logPO_2] based on at least four equilibration steps between 30% and 70% oxygenation. The binding constant and the number of DPG binding sites were measured in one pika from the high and low-altitude populations. Free Cl^- was measured using a model 926S Mark II chloride analyzer (Sherwood Scientific Ltd, Cambridge, UK).

Extractions and amino acid alignments

DNA was extracted from liver tissue using a DNeasy Blood and Tissue kit following manufacturer's protocol (Qiagen, Valencia, CA). Tissue samples from museum vouchers were sequenced using the same methods as wild caught specimens. A non-annotated working draft sequence of *O. princeps* (Genbank accession no. AC235530) along with *O. curzoniae* (Genbank accession no. HBA: EF429202; HBB: DQ839484) were used to design specific primers for each globin gene. We constructed nucleotide sequences for both the α- and β-globin genes using specific primers (HBA-F: 5′ CAGCACCAGCCAATGACCGA 3′; HBA-R: 5′ ACAGGCTGCCACTCAGACTT 3′; HBB-F: 5′ TGTCATCAGTCAGCCTCACC 3′; HBB-R 5′ GGATTCGCACACAGCTCATA 3′). We performed Polymerase Chain Reactions (PCR)

using the Expand High Fidelity PCR system (Roche Applied Science, Indianapolis, IN) (94° C for 2 min; 94° C for 30 s, 55-57° C for 30 s, 72° C for 2 min, and 72° C for 10 min) and samples were screened on a 1% agarose gel. Products of approximately 900 or 1,650 bp were used for sequencing (University of Washington High-Throughput Sequencing Center, Seattle, WA) in triplicate for the α- and β-globin genes, respectfully. Nucleotide consensus sequences were used to predict amino acid sequences for each globin gene, using BioEdit Sequencing Alignment Editor (Ibis Biosciences, Carlsbad, CA). The European rabbit *Oryctolagus cuniculus* (Genbank accession no. M18818) was included as the ancestral outgroup to establish variations within the *Ochotona* genus.

Site-directed mutagenesis

We constructed recombinant Hb (rHb) mutants of *O. princeps*, *O. collaris*, an estimated close ancestor that diverged into these two species, and combinations of single substitution mutants between the ancestral and *O. princeps* forms. The nucleotide sequences of *O. collaris* globin genes were codon-optimized for *Escherichia coli* expression and were then synthesized by Eurofins MWG Operon (Huntsville, Al). The synthetic gene cassette codes consisted of α- and β-globin polypeptides along with a spacer sequence. The globin cassette and *methionine aminopeptidase* were cloned tandemly in the pGM plasmid. Using the OC_pGM plasmid as a template, the *O. princeps* and estimated ancestor expression plasmid were synthesized by site-directed mutagenesis (Fig. 1). We incorporated the amino acid substitution by QuikChange® II XL Site-Directed Mutagenesis kit (Stratagene, LaJolla, CA, USA). The mutagenic primer sequences are shown in Table 2. The mutations in the globin genes were verified by DNA sequencing.

The rHbs were expressed in the JM109 (DE3) *E. coli* strain. The bacterial cells containing the pCOLA-MAP and pGM plasmid were dually selected using Ampicillin and Kanamycin LB plates (Natarajan et al. 201l). Large scale expression for the pika rHbs were conducted in batches of 1.5 – 2 L of TB medium. The cells were grown at 37º C in an orbital shaker at 200 rpm until the absorbance reached 0.6 at 600 nm. The globin genes and MAP genes were induced with 0.2 mM IPTG and the culture was supplemented with hemin (50 μg/ml) and glucose (20 g/L). The bacterial cells were allowed to grow at 28º C for 16 hrs in an orbital shaker with 200 rpm. CO gas was passed through the induced bacterial culture for 15 min and the cells were harvested by centrifugation. The rHbs were purified by HPLC as described previously. To verify the rHb's purity they were separated in 20% SDS-PAGE and also measured for the absorbance spectra at 450 to 600 nm.

RESULTS

Hemoglobin functional properties

From IEF analysis we experimentally confirmed that both pika species express a single tetrameric $\alpha_2\beta_2$ Hb isoform (Fig. 1), consistent with genomic evidence that *O. princeps* possesses single copies of postnatally-expressed α- and β-globin genes. We tested for differences in the oxygenation properties of purified Hbs from the predominantly highland *O. princeps* (*n* = 5 individuals) and the exclusively lowland *O. collaris* (*n* = 5 individuals) species. To gain insights into mechanisms of allosteric regulation, we measured O_2-affinities of purified Hbs (pH 7.40 at 37° C) under four treatments: in the absence of allosteric effectors ('stripped'), in the presence of Cl^- ions

(added as 0.1 M KCl), in the presence of 2,3-diphosphoglycerate (DPG, at two-fold molar excess over tetrameric Hb), and in the simultaneous presence of KCl and DPG. This latter treatment most closely approximates *in vivo* conditions in mammalian red cells.

The experiments revealed that the Hb of *O. princeps* exhibits a uniformly higher O_2-affinity than that of *O. collaris*, as indicated by the fact that O_2-equilibrium curves for *O. princeps* Hb are left-shifted relative to those of *O. collaris* Hb under the same treatment conditions (Fig. 1A, B), and hence, *O. princeps* Hb exhibits lower values of P_{50} (the partial pressure of O_2 at which heme is 50% saturated; Table 3) and n_{50} (cooperativity coefficient; Table 4). The Hb of *O. princeps* exhibited a significantly higher instrinsic O_2 affinity than that of *O. collaris*, as indicated by the lower P_{50} (stripped) values. This difference persisted in the presence of DPG and in the simultaneous presence of both Cl^- and DPG (Fig. 3A). These results indicate that species differences in Hb-O_2 affinity in the presence of allosteric effectors (P_{50} (KCl+DPG)) are exclusively attributable to differences in intrinsic affinity, not differences in the responsiveness to allosteric effectors (Fig. 3B). This is confirmed by dose-response curves for DPG, which demonstrate that Hb-O_2 affinities of both species are allosterically regulated in a similar fashion in spite of the difference in intrinsic affinity (Fig. 2C). Moreover, Hb-O_2 affinities of both species exhibit essentially identical reductions in O_2-affinity in the simultaneous presence of Cl^- ions and DPG (Fig. 2A, B).

Globin gene sequencing

We sequenced the entire α globin and β globin genes in triplicate for all seven pika species in our analysis. The majority of the observed substitutions in the HBA (Fig.

4) and HBB (Fig. 5) chains were isomorphic changes where the ancestral (*Oryctalagus cuniculus*) amino acid was replaced by a similar amino acid with no change in charge or polarity. However, some of the observed amino acid substitutions were non-conservative resulting in a shift in amino acid polarity and/or charge among a few species. A derived substitution of interest was found at site α78(EF8) which consisted of a nonpolar Glycine was substituted to a polar Serine, this mutation was unique to only the high altitude *O. princeps* species (black arrow, Fig. 4). Additionally, we found five amino acid substitutions in the β-chain (black arrows, Fig. 5) that are of interest: β5(A3), β58(E2), β62(E6), β123(H1), and β126(H9). Of the candidate positions in HBB, site 123(H1) and 126(H9) are in a packing contact region (area involving the B, G, H helices and the GH corner to hold the dimer together) between the α_1 and β_1 (or α_2 and β_2) subunits.

A cladogram was inferred using simple parsimony for all seven *Ochotona* spp. and rabbit used in this study; only the five substitutions in the β globin gene were utilized (inferred from Lanier and Olson 2009; Niu et al. 2004; Fig. 6). We postulate that three of the five substitutions occurred exclusively on the lineage leading to *O. princeps* and two on the branch leading to *O. collaris* because these amino acid substitutions were only observed within this species when compared with rabbit and other ancestral *Ochotona* species. Because we found these pronounced differences between our sister species we used the reconstructed cladogram to determine the genotype at the five sites of interest in the β globin gene for the common ancestor between *O. princeps* and *O. collaris*. To identify if the change in Hb-O_2 affinity resulted from an increased affinity in the *O. princeps* lineage or resulted from a decrease of affinity in the *O. collaris* lineage we

constructed recombinant Hb (rHb) mutants and analyzed their functional properties using the aforementioned procedures.

Site-directed mutagenesis functional properties

As a first step in the protein-engineering experiments, we synthesized rHbs representing the wildtype Hbs of *O. princeps* (genotype: β5Ala, β58Ala, β62Thr, β123Thr, β126Val) and *O. collaris* (genotype: β5Gly, β58Pro, β62Ala, β123Ser, β126Ala) and the reconstructed Hb of their common ancestor (Anc; genotype: β5Gly, β58Ala, β62Ala, β123Thr, β126Ala). The triangulated comparison involving the reconstructed ancestral Hb and its modern-day descendants confirmed that the elevated Hb-O_2 affinity of *O. princeps* is a derived character state: P_{50} (KCl+DPG) for the highland *O. princeps* rHb was 21.8 % lower (i.e., O_2 affinity was higher) relative to the ancestor (Fig. 7). Consistent with our experiments on the native Hbs, P_{50} (KCl+DPG) for the highland *O. princeps* rHb was 14.4% lower relative to the lowland *O. collaris* rHb. The reconstructed ancestral β-chain sequence of the two species revealed that three of the substitutions occurred on the branch leading to the high-altitude *O. princeps* (β5Gly→Ala, β62Ala→Thr, and β126Ala→Val), indicating that the derived increase in Hb-O_2 affinity of *O. princeps* is attributable to the independent or joint effects of these three substitutions (Fig. 6). To measure the additive and nonadditive effects of these three substitutions across all possible genetic backgrounds, we used site-directed mutagenesis to synthesize the complete set of mutational intermediates in the 3!=6 possible pathways that connect the inferred ancestral genotype with the derived triple-mutant genotype of *O. princeps* (Fig. 8).

Of the six possible forward trajectories that lead from the ancestor to the *O. princeps* Hb, our experiments revealed that only a single pathway yielded an incremental increase in Hb-O_2 affinity at each successive step: β5Gly→Ala (1st), β62Ala→Thr (2nd), β126Ala→Val (3rd). Each of the possible first steps yielded a significant increase in Hb-O_2 affinity, but two of three possible final steps (β5Gly→Ala and β62Ala→Thr) yielded significant reductions in Hb-O_2 affinity. The β5Gly→Ala substitution significantly increased affinity on the ancestral background, but it significantly decreased affinity on backgrounds in which β62Ala→Thr had already occurred (Fig. 8). This indicates that β5Gly→Ala could have contributed to the derived increase in Hb-O_2 affinity in *O. princeps* if it occurred as the first substitution, but not if it occurred as the final substitution. The β62Ala→Thr substitution significantly increased affinity on the ancestral background, but it significantly decreased affinity on backgrounds in which β5Gly→Ala and β126Ala→Val had already occurred (Fig. 8). As with β5Gly→Ala, the β126Ala→Val substitution could have contributed to the derived increase in Hb-O_2 affinity if it occurred as the first substitution, but not if it occurred as the final substitution. In contrast to the substitutions at sites 5 and 62, the β126Ala→Val substitution always had an affinity-enhancing effect, although this was most pronounced on the ancestral background (the first step) and in the background in which β5Gly→Ala had already occurred (one of two possible second steps; Fig. 8). The β126Ala→Val substitution produced an increased Hb-O_2 affinity on all four possible genotypic backgrounds, although the magnitude of the affinity-enhancing effect varied considerably.

DISCUSSION

Numerous studies have established that when comparing highland mammal species with their lowland congeners there is an increase in Hb-O_2 affinity in the highland species (Hall et al. 1936; Chiodi 1962; Lenfant 1973; Petschow et al. 1977; Monge and Leon-Velarde 1991; Weber and Fago 2004; Storz 2007; Weber 2007; Storz and Moriyama 2008; Storz et al. 2010a; Storz et al. 2010b; Mairbäurl and Weber 2012). Consistent with these previous studies, our results reveal that the high altitude *O. princeps* exhibited a higher Hb-O_2 affinity than *O. collaris* and their recent common ancestor, in the presence and absence of allosteric effectors. This change in O_2 affinity was exclusively attributable to intrinsic effects because both sister species had the same relative sensitivity to allosteric effectors. By analyzing the globin genes of seven *Ochotona* species we were able to conclude that the derived increase in Hb-O_2 affinity of *O. princeps* was attributable to three amino acid substitutions in the β globin gene. Yingzhong and colleagues (2007) sequenced the α and β chains of the Plateau Pika (*O. curzoniae*) and compared the observed substitutions to human Hb genes. They argued that they discovered important substitutions in tight subunit contact sites in the $\alpha_1\beta_1$ contact region and ligand binding sites that they suggested provide an increase in oxygen affinity of *O. curzoniae*. However, most of these sites were identical when compared with other *Ochotona* species and rabbit. Because most of the substitutions Yingzhong et al. (2007) claimed to be important in Hb-O_2 binding affinity of *O. curzoniae* were not unique in highland versus lowland species but between the Order Lagomorpha and humans these substitutions may not, in fact, be responsible for the altitudinal adaptation in the Plateau Pika. This study demonstrates the importance of comparing closely related

species to determine meaningful genotypic differences instead of using phylogenetically disparate species which may yield incorrect conclusions.

If the derived increase in Hb-O_2 affinity in the *O. princeps* lineage evolved under the influence of positive directional selection, then our results suggest that any of the three substitutions could have occurred as the first step in the adaptive walk. The effects of β5Gly→Ala and β62Ala→Thr were opposite in sign when they occurred as second or third steps. This sign epistasis for Hb-O_2 affinity demonstrates that the functional effects of mutations are highly dependent on the temporal order in which they occur. Similar epistatic effects have been documented in other protein-engineering experiments on vertebrate Hbs (Tam et al. 2013; Natarajan et al. 2013; Projecto-Garcia et al. 2013) and sign epistasis for fitness has been documented in microbial experimental evolution studies (Weinreich et al. 2006).

Theoretical questions about the genetics of adaptation have focused on predictions about distributions of effect sites among successive substitutions in an adaptive walk. Our experimental results for pika Hbs demonstrate that each possible pathway through sequence space has a different distribution of phenotypic effect sizes, due to pervasive magnitude as well as sign epistasis between pairs of sites. These results suggest that meaningful estimates of effect-size distributions may only be obtainable in cases where the temporal order of substitutions is known (e.g., in experimental evolution studies).

The evolutionary changes in protein function observed between highland and lowland species of pika and their common ancestor revealed that *O. princeps* evolved an increase in Hb-O_2 affinity. It is likely that the alpine specialist *O. princeps* was able to

adapt to life at high altitude via genetically based changes in Hb which increased the blood-O_2 affinity allowing colonization of their hypoxic environment.

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FIGURES

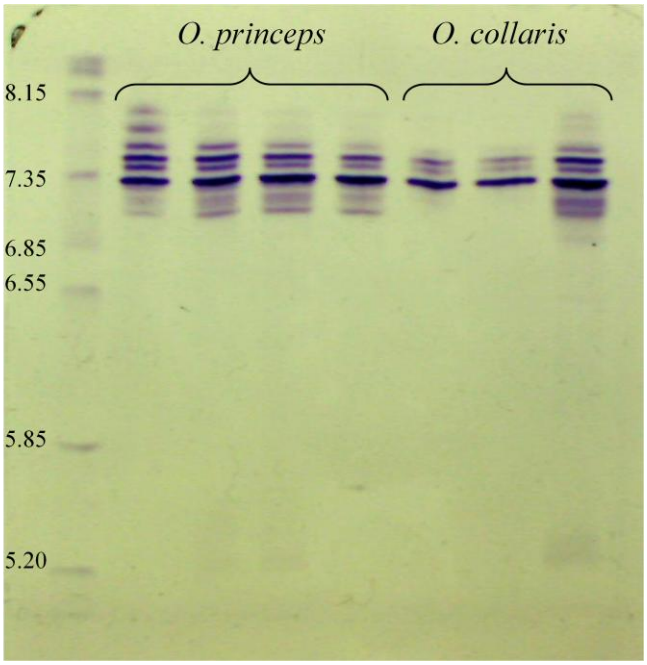


Figure 1. Stained isoelectric focusing (IEF) gel from three *Ochotona princeps* individuals and three *O. collaris* individuals showing a single major tetrameric hemoglobin isoform (dark purple bands around isoelectric point of 7.35) for both species.

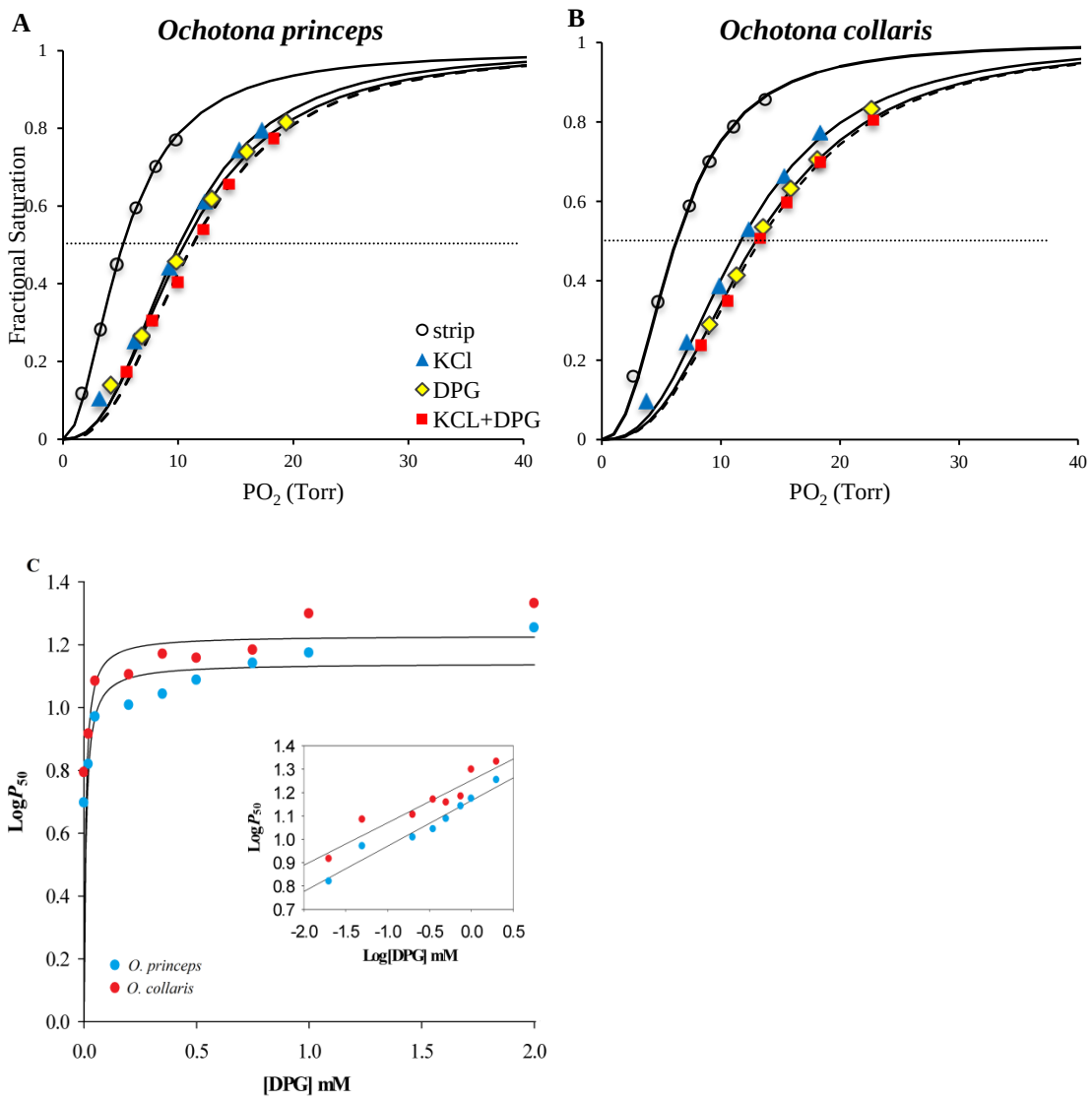


Figure 2. Oxygen dissociation curves at pH 7.40 and 37°C in the presence and absence of allosteric effectors (KCl and DPG) for high altitude *O. princeps* (A) and low altitude *O. collaris* (B). P_{50} (P_{O2} at 50% saturation) is depicted by the horizontal dashed line. Curves for stripped hemolysates (purified Hb without the addition of effectors), in the presence of Cl^- ions (signified as KCl and blue triangle), in the presence of DPG (yellow diamond), and in a combination of both Cl^- and DPG together (red square) are shown. (C) Sensitivity of *O. princeps* (blue circles) and *O. collaris* (red circles) hemolysates to DPG

concentration with log-transformed P_{50} values. The dose-response curves indicate that there is no substantial difference in the sensitivity of *O. princeps* and *O. collaris* Hb to the allosteric effector DPG. The inset figure shows a regression of the logP_{50} vs. log[DPG] and reiterates this finding of similar sensitivities of the two species to DPG.

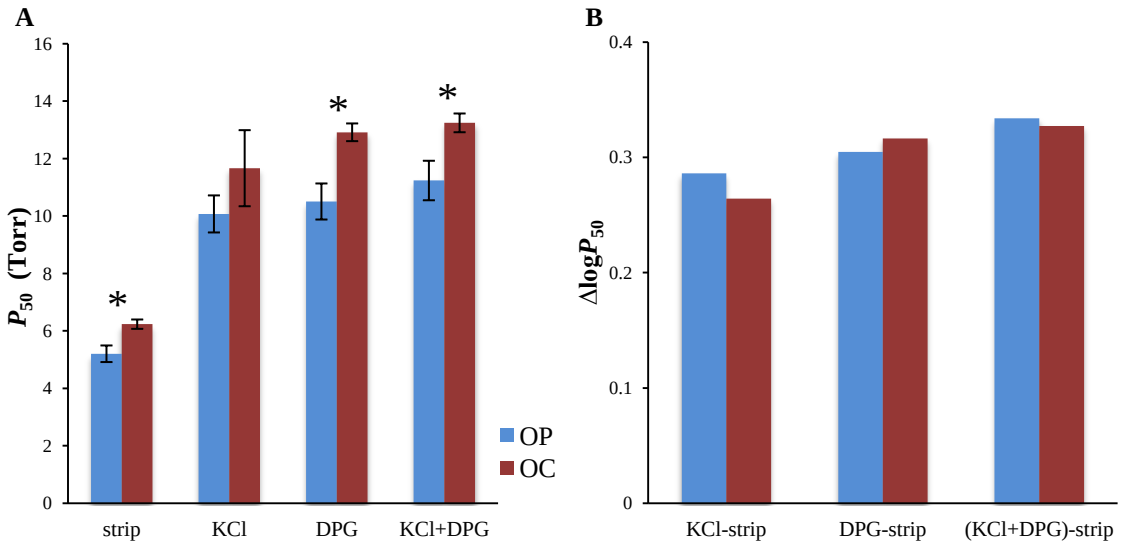
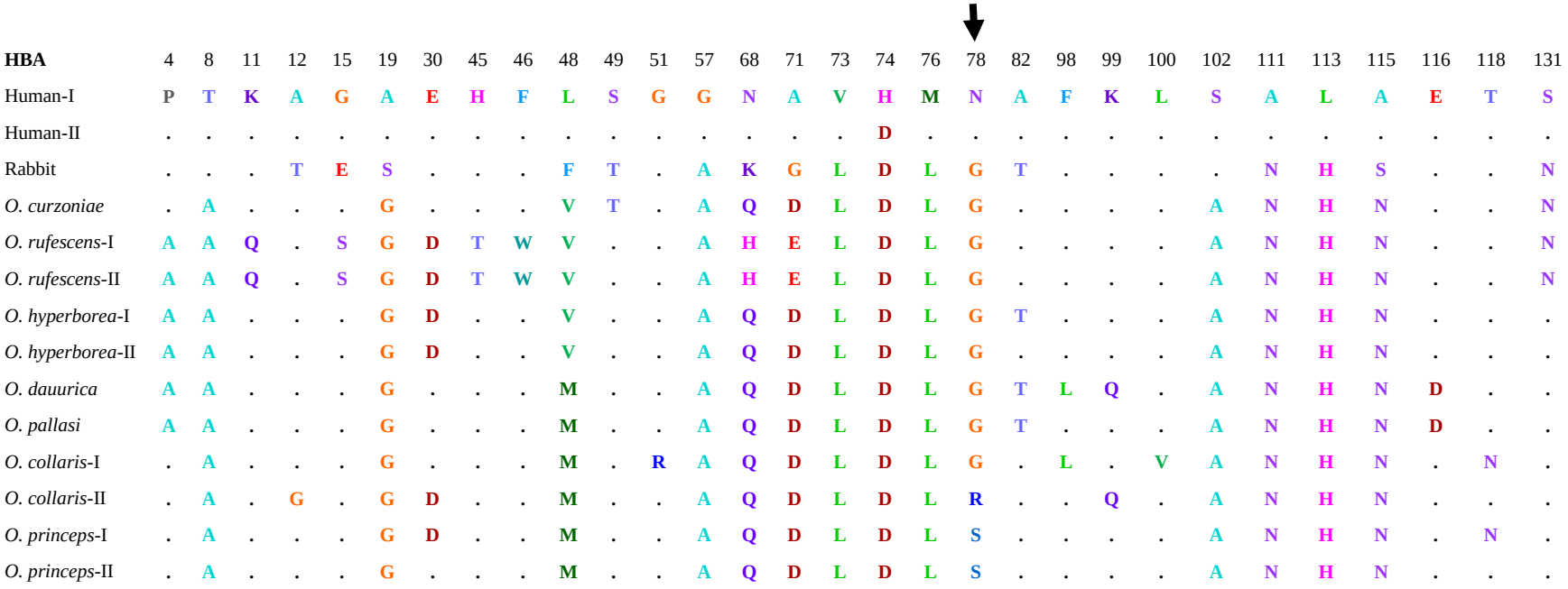


Figure 3. O_2 binding properties of pika Hbs at pH 7.40 and 37°C in the presence and absence of allosteric effectors. (A) P_{50} values for stripped and added allosteric effectors for both high (OP, *O. princeps*) and low (OC, *O. collaris*) altitude pikas. (B) Extent that each effector reduces Hb-O_2 affinity for each species in the presence of a given allosteric effector, expressed as the change in log-transformed P_{50} values. Asterisks denote significant differences between highland and lowland species (t-test); $P < 0.05$, error bars ± SEM.



HBA	4	8	11	12	15	19	30	45	46	48	49	51	57	68	71	73	74	76	78	82	98	99	100	102	111	113	115	116	118	131
Human-I	P	T	K	A	G	A	E	H	F	L	S	G	G	N	A	V	H	M	N	A	F	K	L	S	A	L	A	E	T	S
Human-II	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.
Rabbit	.	.	.	T	E	S	.	.	.	F	T	.	A	K	G	L	D	L	G	T	.	.	.	.	N	H	S	.	.	N
O. curzoniae	.	A	.	.	.	G	.	.	.	V	T	.	A	Q	D	L	D	L	G	.	.	.	.	A	N	H	N	.	.	N
O. rufescens-I	A	A	Q	.	S	G	D	T	W	V	.	.	A	H	E	L	D	L	G	.	.	.	.	A	N	H	N	.	.	N
O. rufescens-II	A	A	Q	.	S	G	D	T	W	V	.	.	A	H	E	L	D	L	G	.	.	.	.	A	N	H	N	.	.	N
O. hyperborea-I	A	A	.	.	.	G	D	.	.	V	.	.	A	Q	D	L	D	L	G	T	.	.	.	A	N	H	N	.	.	.
O. hyperborea-II	A	A	.	.	.	G	D	.	.	V	.	.	A	Q	D	L	D	L	G	.	.	.	.	A	N	H	N	.	.	.
O. dauurica	A	A	.	.	.	G	.	.	.	M	.	.	A	Q	D	L	D	L	G	T	L	Q	.	A	N	H	N	D	.	.
O. pallasi	A	A	.	.	.	G	.	.	.	M	.	.	A	Q	D	L	D	L	G	T	.	.	.	A	N	H	N	D	.	.
O. collaris-I	.	A	.	.	.	G	.	.	.	M	.	R	A	Q	D	L	D	L	G	.	L	.	V	A	N	H	N	.	N	.
O. collaris-II	.	A	.	G	.	G	D	.	.	M	.	.	A	Q	D	L	D	L	R	.	.	Q	.	A	N	H	N	.	.	.
O. princeps-I	.	A	.	.	.	G	D	.	.	M	.	.	A	Q	D	L	D	L	S	.	.	.	.	A	N	H	N	.	N	.
O. princeps-II	.	A	.	.	.	G	.	.	.	M	.	.	A	Q	D	L	D	L	S	.	.	.	.	A	N	H	N	.	.	.

Figure 4. Amino acid sequence alignment of HBA from seven *Ochotona* species aligned with Rabbit (*Ory. cuniculus*) and Human as outgroups. Redundant sequences were removed, only variable sites are displayed, and substitutions that only occurred in a single individual are not shown. The black arrow at residue α78 represents a substitution of Glycine in six *Ochotona* spp. and Rabbit to a Serine in only high altitude *O. princeps* and may play a role in Hb-O_2 affinity.

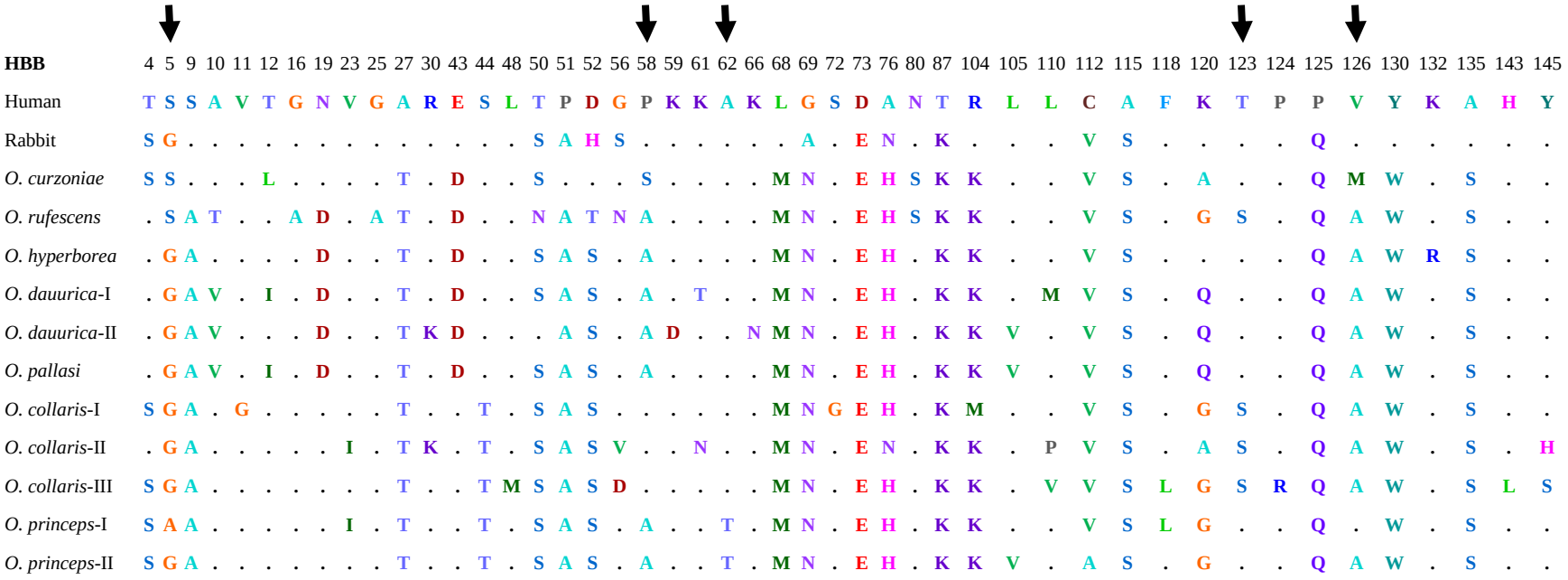


Figure 5. Amino acid sequence alignment of HBB of seven *Ochotona* species aligned with Rabbit (*Ory. cuniculus*) and Human as outgroups. Redundant sequences were removed, only variable sites and substitutions that occurred in more than one individual are shown. The black arrows at residues β5, β58, β62, β123, and β126 represent candidate sites that may be responsible for an increase in Hb-O_2 affinity of high altitude *O. princeps* individuals.

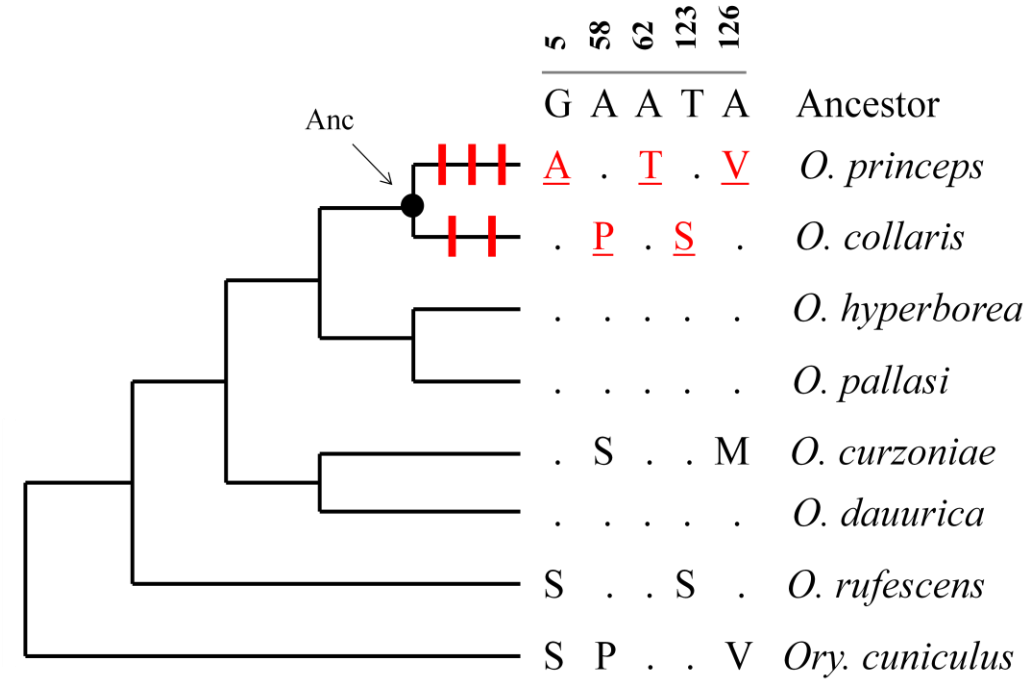


Figure 6. Inferred cladogram of *Ochotona* spp. and Rabbit (*Ory. cuniculus*) as an outgroup, showing the five amino acid substitutions in the β globin gene and estimated genotype for the common ancestor (Anc) between *O. princeps* and *O. collaris*. Protein substitutions that may have an effect on Hb-O_2 affinity are shown in underlined red.

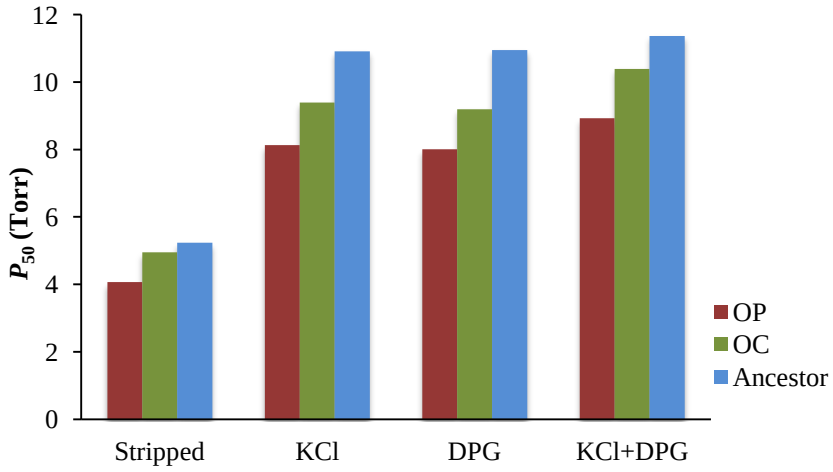


Figure 7. Oxygen binding properties of pika recombinant hemoglobin (rHb) mutants at pH 7.40 and 37°C showing the P_{50} values for stripped and added allosteric effectors for the high altitude (OP, *O. princeps*), low altitude (OC, *O. collaris*), and inferred ancestor pikas.

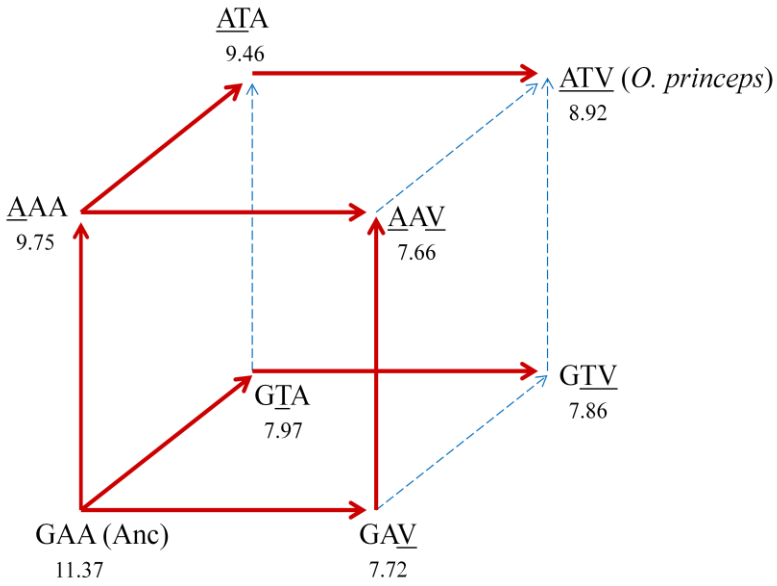


Figure 8. A diagram showing the array of all possible genetic backgrounds and amino acid substitutions in the β globin gene from the inferred ancestor state (Anc) to the high altitude native *Ochotona princeps*. The three letter sequence signifies amino acid substitutions at site β5, β62, and β126, respectively and the underlined letters signify where the substitution(s) occurred. The numbers directly under the three letter sequence are the P_{50} values collected for each recombinant hemoglobin (rHb) mutant. Solid red lines signify an increase in Hb-O_2 affinity while blue dashed lines signify a decrease.

TABLES

Table 1. Species of *Ochotona* used in globin gene alignments, including altitude and location of specimen, whether the sample came from a museum or were collected by us in the field, and the assigned Genbank accession numbers for the α and β genes.

Species	Altitude (m)	Location	Museum/Field	Genbank Accession no.
O. curzoniae	4783	China	Genbank	EF429202; DQ839484
O. princeps	4347	CO	Field caught (NP1195, NP1201-1202)*	KC757706-757708; KC757676-757683
O. princeps	3040	CA	MVZ (202371)	KC757705; KC757674; KC757675
O. rufescens	2533	Iran	MVZ (191920)	KC757684; KC757685; KC757646
O. dauurica	2417	Mongolia	Field caught (NK223524-NK223526)*	KC757688; KC757689; KC757648-757653
O. pallasi	2133	Mongolia	Field caught (NK223869)*	KC757690; KC757654; KC757655
O. collaris	1460	AK	UAM (71425, 100795, 102429)	KC757691-757696; KC757656-757661
O. collaris	1040	AK	Field caught (AF71386-71388; AF67751-67753)ǂ	KC757697-757704; KC757662-757673
O. hyperborea	3.6	Russia	UAM (84368)	KC757686; KC757687; KC757647

*Specimens deposited in the HW Manter lab at UNL
ǂ Specimens deposited at UAM

Table 2. List of specific primers used for site-directed mutagenesis analysis.

Primer name	**Sequences**
OPB G5A SE	GGTGCATCTGAGCGCCGAAGAAAAAGCGG
OPB G5A AS	CCGCTTTTTCTTCGGCGCTCAGATGCACC
OPAB P58A SE	CGGTGATGGGCAACGCGAAAGTGAAAGCG
OPAB P58A AS	CGCTTTCACTTTCGCGTTGCCCATCACCG
OPB A62T SE	TGATGGGCAACGCGAAAGTGAAAACGCATGGCAAAA
OPB A62T AS	TTTTGCCATGCGTTTTCACTTTCGCGTTGCCCATCA
OPB S123T_A126V SE	GGCGGCGAATTTACCCCGCAGGTGCAGGCG
OPB S123T_A126V AS	CGCCTGCACCTGCGGGGTAAATTCGCCGCC
ANB S123T SE	TCTGGGCGGCGAATTTACCCCGCAGGC
ANB S123T AS	GCCTGCGGGGTAAATTCGCCGCCCAGA

Table 3. Mean P50 values (± S.E.M.) for *Ochotona princeps* and *O. collaris* from their native altitudes and recombinant Hb (rHb) mutants for the inferred ancestor, high altitude *O. princeps* (OP), low altitude *O. collaris* (OC), and single amino acid substitution mutants for the ancestor (AN) and *O. princeps* (OP).

P_{50}	stripped	KCl	DPG	KCl+DPG
O. princeps	5.20 ± 0.58	10.07 ± 1.29	10.50 ± 1.25	11.23 ± 1.38
O. collaris	6.23 ± 0.33	11.66 ± 2.65	12.91 ± 0.62	13.24 ± 0.65
OP	4.07	8.12	8.00	8.92
OC	4.95	9.39	9.19	10.39
Ancestor	5.23	10.91	10.95	11.37
AN_AAA	-	9.79	7.55	9.75
AN_GTA	-	7.90	6.75	7.97
AN_GAV	5.66	8.59	7.03	7.72
OP_GTV	4.75	8.61	6.57	7.86
OP_AAV	3.91	7.01	6.68	7.66
OP_ATA	4.39	10.04	8.13	9.46

Table 4. Mean n_{50} values (± S.E.M.) for *Ochotona princeps* and *O. collaris* from their native altitudes and recombinant Hb (rHb) mutants for the inferred ancestor, high altitude *O. princeps* (OP), low altitude *O. collaris* (OC), and single amino acid substitution mutants for the ancestor (AN) and *O. princeps* (OP).

n_{50}	stripped	KCl	DPG	KCl +DPG
O. princeps	2.06 ± 0.15	2.53 ± 0.20	2.41 ± 0.09	2.51 ± 0.16
O. collaris	2.36 ± 0.05	2.54 ± 0.06	2.56 ± 0.22	2.58 ± 0.05
rHb				
Ancestor	2.27	2.62	2.40	2.40
OP	2.23	2.34	2.36	2.40
OC	2.56	2.39	2.39	2.56
AN_AAA	-	1.36	2.85	2.83
AN_GTA	-	2.10	3.83	2.66
AN_GAV	1.22	2.19	2.91	2.58
OP_GTV	2.03	2.10	2.74	2.52
OP_AAV	2.11	2.21	2.23	2.60
OP_ATA	2.00	2.13	2.40	3.04