

Repeated elevational transitions in hemoglobin function during the evolution of Andean hummingbirds

Joana Projecto-Garcia^{a,1}, Chandrasekhar Natarajan^{a,1}, Hideaki Moriyama^a, Roy E. Weber^b, Angela Fago^b, Zachary A. Cheviron^{a,c}, Robert Dudley^d, Jimmy A. McGuire^{d,e}, Christopher C. Witt^{f,g,2}, and Jay F. Storz^{a,2,3}

^aSchool of Biological Sciences, University of Nebraska, Lincoln, NE 68588; ^bDepartment of Bioscience, Zoophysiology, Aarhus University, DK-8000 Aarhus, Denmark; ^cDepartment of Animal Biology, School of Integrative Biology, University of Illinois at Urbana-Champaign, Champaign, IL 61801; ^dDepartment of Integrative Biology, University of California, Berkeley, CA 94720-3140; ^eMuseum of Vertebrate Zoology, University of California, Berkeley, CA 94720-3160; and ^fDepartment of Biology and ^gMuseum of Southwestern Biology, University of New Mexico, Albuquerque, NM 87131

Edited by David M. Hillis, University of Texas at Austin, Austin, TX, and approved November 8, 2013 (received for review August 15, 2013)

Animals that sustain high levels of aerobic activity under hypoxic conditions (e.g., birds that fly at high altitude) face the physiological challenge of jointly optimizing blood-O₂ affinity for O₂ loading in the pulmonary circulation and O₂ unloading in the systemic circulation. At high altitude, this challenge is especially acute for small endotherms like hummingbirds that have exceedingly high mass-specific metabolic rates. Here we report an experimental analysis of hemoglobin (Hb) function in South American hummingbirds that revealed a positive correlation between Hb-O₂ affinity and native elevation. Protein engineering experiments and ancestral-state reconstructions revealed that this correlation is attributable to derived increases in Hb-O₂ affinity in highland lineages, as well as derived reductions in Hb-O₂ affinity in lowland lineages. Site-directed mutagenesis experiments demonstrated that repeated evolutionary transitions in biochemical phenotype are mainly attributable to repeated amino acid replacements at two epistatically interacting sites that alter the allosteric regulation of Hb-O₂ affinity. These results demonstrate that repeated changes in biochemical phenotype involve parallelism at the molecular level, and that mutations with indirect, second-order effects on Hb allostery play key roles in biochemical adaptation.

high-altitude adaptation | hypoxia | parallel evolution | protein evolution | epistasis

In air-breathing vertebrates, the optimal Hb-O₂ affinity varies according to the partial pressure of atmospheric O₂ (P_{O₂}) because of the trade-off between the need to maximize arterial O₂ saturation under hypoxia while simultaneously ensuring adequate O₂ unloading in the tissue capillaries (1–8). However, in species that are native to high-altitude environments, it is not known how often and to what extent physiological adaptation to hypoxia is mediated by genetically based modifications of Hb-O₂ affinity (9). Such questions can be resolved by conducting systematic comparative studies of Hb function among species with known phylogenetic relationships and contrasting altitudinal distributions.

In cases where multiple species have adapted independently to high-altitude hypoxia, replicated changes in Hb function may be instructive about the relative accessibility of different design solutions to natural selection. If repeated changes in Hb-O₂ affinity involve parallel amino acid substitutions, then this suggests that adaptive protein evolution may be predisposed to follow particular mutational pathways. If, by contrast, myriad different mutational changes can produce the same functional outcome, then particular design solutions may be selectively accessible from a diverse range of ancestral starting points, and pathways of protein evolution may be highly idiosyncratic.

Among vertebrates, hummingbirds have some of the highest basal metabolic rates and the highest metabolic scopes for activity, and are therefore especially compelling subjects for studies of Hb function and blood-O₂ transport under hypoxia (10–13). We conducted an experimental analysis of Hb function in 10 species of Andean hummingbirds that have dramatically different

altitudinal distributions. The species included in this study fall into three main clades: the Coquettes, the Brilliants, and the Emeralds + Giant Hummingbird (*Patagona gigas*) (Fig. 1A). Each of these three clades contains species that are restricted to low or moderate elevations, as well as independently derived high-elevation species that routinely occur at elevations >4,200 m. We also collected data for one outgroup species from the hermit subfamily (Phaethornithinae), a primarily lowland clade that represents the likely ancestral elevational distribution for hummingbirds (14).

Results and Discussion

Hb Isoform Composition. The Hbs of birds and other jawed vertebrates are heterotetramers, composed of two α -chain and two β -chain subunits (15, 16). During postnatal life, most bird species express two main Hb isoforms in circulating red blood cells: a major isoform, HbA ($\alpha^A_2\beta_2$), with α -chain subunits encoded by the α^A -globin gene, and a minor isoform, HbD ($\alpha^D_2\beta_2$), with α -chain subunits encoded by the α^D -globin gene (17) (Fig. S1). Given that avian HbD has a consistently higher O₂-affinity relative to HbA (17), changes in the intracellular HbA/HbD ratio

Significance

Hummingbirds have exceedingly high oxygen demands because of their elevated rates of aerobic metabolism, and yet they thrive in high-altitude environments in the Andes where oxygen is scarce. Here we report the finding that when hummingbird species colonized new elevational zones, evolutionary changes in the respiratory properties of hemoglobin were repeatedly mediated by the same amino acid replacements. Specifically, ancestral sequence reconstruction and protein engineering experiments revealed that parallel adaptation of hemoglobin function in multiple species is attributable to repeated amino acid replacements at a single pair of interacting sites. This striking parallelism at the molecular level suggests a surprising degree of reproducibility and predictability in adaptive protein evolution.

Author contributions: C.C.W. and J.F.S. designed research; J.P.-G., C.N., H.M., Z.A.C., and C.C.W. performed research; H.M., R.E.W., A.F., J.A.M., C.C.W., and J.F.S. contributed new reagents/analytic tools; J.P.-G., H.M., R.E.W., A.F., Z.A.C., R.D., J.A.M., C.C.W., and J.F.S. analyzed data; and C.C.W. and J.F.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: Complete information for all specimens used in this study is archived on the ARCTOS online database (Table S4). The sequences reported in this paper have been deposited in the GenBank database (accession nos. KF222496, KF222499, KF222501, KF222503, KF222506, KF222510–KF222539).

¹J.P.-G. and C.N. contributed equally to this work.

²C.C.W. and J.F.S. contributed equally to this work.

³To whom correspondence should be addressed. E-mail: jstorz2@unl.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1315456110/-DCSupplemental.

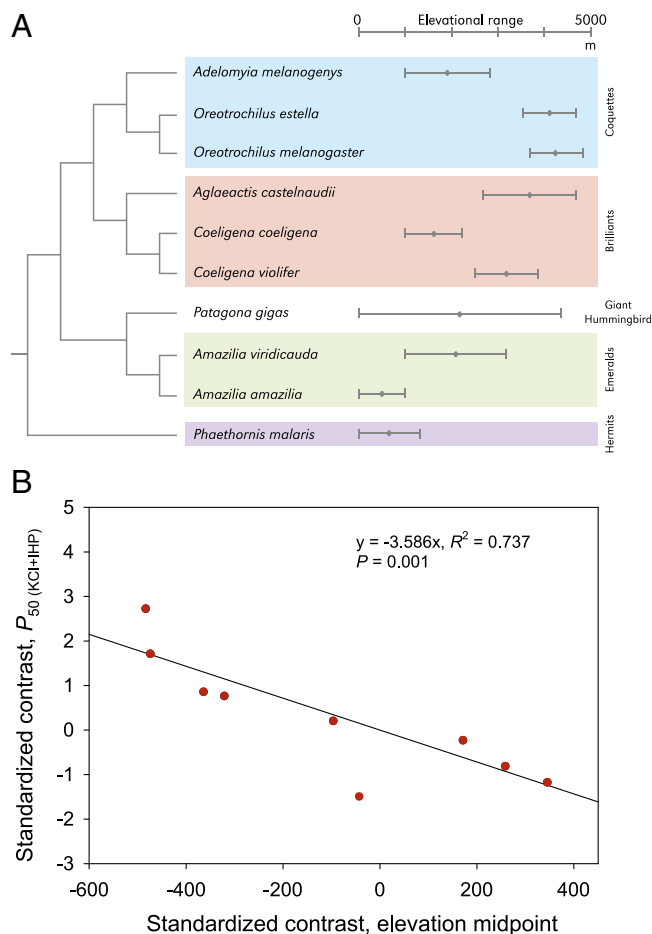


Fig. 1. Phylogenetically independent contrasts reveal a positive association between Hb-O₂ affinity and native elevation in Andean hummingbirds. (A) Phylogenetic relationships (25) and elevational distributions (ranges and midpoints) of 10 hummingbird species included in the analysis of Hb function. (B) Least-squares regression of phylogenetically independent contrasts revealed a significant negative relationship between P₅₀(KCl+IHP) and native elevation (i.e., a positive relationship between Hb-O₂ affinity and elevation). See Table S2 for full results.

could substantially alter blood-O₂ affinity, and it has been suggested that regulatory changes in Hb isoform composition

may contribute to adaptive changes in blood-O₂ transport in high-altitude species (18–20). To test this hypothesis we conducted a proteomic analysis of red cell lysates from each of the 10 hummingbird study species. Results of this analysis revealed that each of the hummingbird species express both HbA and HbD isoforms, and the relative concentration of the minor HbD isoform ranged from 1.6 to 24.2% (mean \pm SD = $13.3 \pm 6.2\%$). However, phylogenetically independent contrasts revealed no clear association between HbA/HbD isoform ratio and native elevation ($R^2 = 0.026$, $P = 0.657$).

Altitudinal Variation in Hb-O₂ Affinity. Evolutionary adjustments in Hb-O₂ affinity can be achieved via changes in intrinsic O₂ affinity or changes in the sensitivity of Hb to the modulating effects of physiological allosteric cofactors, such as Cl⁻ ions and organic phosphates (8, 15). The allosteric regulation of Hb-O₂ affinity involves the oxygenation-linked binding of nonheme ligands that indirectly modulate heme reactivity by shifting the equilibrium between a low-affinity “T-state” and a high-affinity “R-state.” Allosteric cofactor molecules typically reduce Hb-O₂ affinity by preferentially binding and stabilizing deoxygenated Hb, thereby displacing the R↔T equilibrium in favor of the low-affinity T-state conformation (Fig. S2). After isolating and purifying the HbA and HbD isoforms from each hummingbird species, we measured oxygenation properties in the presence and absence of the two main allosteric effectors that regulate Hb-O₂ affinity: inositol hexaphosphate (IHP, a chemical analog of the naturally occurring inositol pentaphosphate in avian red cells, at twofold molar excess over tetrameric Hb) and Cl⁻ ions (added as KCl; 0.1 mol·L⁻¹). Hb-O₂ affinity was indexed by P₅₀, the PO₂ at which Hb is half-saturated. O₂-equilibrium measurements revealed that the major HbA isoforms of the high-altitude hummingbird species were generally characterized by elevated O₂-affinities in the absence of allosteric effectors (“stripped” Hb) and the difference in P₅₀ values between highland and lowland species was amplified in the presence of IHP alone and in the simultaneous presence of both IHP and Cl⁻ ions (Table 1 and Fig. S3).

Regressions based on phylogenetically independent contrasts (PICs) revealed a significantly negative relationship between HbA P₅₀ values and native elevation (i.e., a positive relationship between Hb-O₂ affinity and elevation) (Fig. 1B and Table S1). Similarly, for five species that expressed the HbD isoform at levels sufficient for experimental analysis, regressions based on PICs revealed significantly negative relationships between P₅₀ values and native elevation, both for HbD alone and for the weighted average of HbA and HbD in their naturally occurring relative concentrations (Table S1). From this point onward, we

Table 1. Functional properties of hummingbird HbA isoforms

Species	Stripped		+KCl		+IHP		+KCl+IHP	
	P ₅₀	n ₅₀	P ₅₀	n ₅₀	P ₅₀	n ₅₀	P ₅₀	n ₅₀
<i>Adelomyia melanogenys</i>	2.85 ± 0.01	1.60 ± 0.00	4.60 ± 0.08	1.89 ± 0.08	28.83 ± 1.54	2.16 ± 0.14	32.02 ± 3.84	2.31 ± 0.07
<i>Oreotrochilus estella</i>	2.17 ± 0.12	1.36 ± 0.13	3.39 ± 0.24	1.54 ± 0.13	21.82 ± 1.09	1.98 ± 0.11	20.20 ± 0.28	2.00 ± 0.03
<i>Oreotrochilus melanogaster</i>	2.10 ± 0.06	1.40 ± 0.01	3.86 ± 0.05	1.75 ± 0.05	26.65 ± 0.71	2.25 ± 0.09	19.88 ± 0.27	2.08 ± 0.05
<i>Aglaeactis castelnaudii</i>	2.17 ± 0.06	1.38 ± 0.04	3.23 ± 0.28	1.40 ± 0.02	22.45 ± 0.93	1.51 ± 0.18	17.23 ± 0.66	1.61 ± 0.14
<i>Coeligena coeligena</i>	2.49 ± 0.11	1.48 ± 0.06	4.22 ± 0.16	1.67 ± 0.10	27.83 ± 0.37	1.91 ± 0.08	22.90 ± 3.16	2.19 ± 0.11
<i>Coeligena violifer</i>	2.12 ± 0.04	1.29 ± 0.03	3.74 ± 0.10	1.65 ± 0.08	23.55 ± 0.74	1.96 ± 0.04	19.12 ± 1.27	1.70 ± 0.19
<i>Patagona gigas</i>	2.52 ± 0.06	1.46 ± 0.04	4.14 ± 0.37	1.63 ± 0.13	29.97 ± 1.00	2.28 ± 0.11	25.86 ± 1.66	2.49 ± 0.42
<i>Amazilia viridicauda</i>	2.62 ± 0.03	1.43 ± 0.03	4.47 ± 0.05	1.81 ± 0.05	28.49 ± 1.20	2.13 ± 0.08	24.24 ± 0.87	2.07 ± 0.11
<i>Amazilia amazilia</i>	3.14 ± 0.43	1.38 ± 0.05	5.28 ± 0.25	1.90 ± 0.15	36.77 ± 0.85	2.16 ± 0.08	29.84 ± 0.32	2.42 ± 0.01
<i>Phaethornis malaris</i>	2.83 ± 0.10	1.39 ± 0.12	4.70 ± 0.06	1.83 ± 0.06	37.00 ± 0.44	2.27 ± 0.13	28.13 ± 0.60	2.04 ± 0.11

O₂-affinities (P₅₀, torr) and cooperativity coefficients (*n*₅₀; mean ± SEM) of purified HbA isoforms measured in 0.1 M Hepes buffer at pH 7.40 (± 0.01), 37 °C, in the absence of allosteric effectors (stripped), in the presence of KCl (0.1 M) or IHP (IHP/Hb tetramer ratio = 2.0), and in the presence of both allosteric effectors. [Heme], 0.3 mM. As explained in the text, P₅₀ is an inverse measure of Hb-O₂ affinity. High-altitude species with maximum elevational ranges of >3,000 m are denoted by gray shading.

β85Phe (the N-terminal residue of the F-helix), but the polar -OH side-chain of β83Ser forms additional intermolecular H-bonds that alter the torsion angle of the F-helix, thereby constraining allosteric movement. When nonpolar Gly is replaced by polar, hydrophilic Ser at β83 (as in the predominantly highland *Oreotrochilus*, *A. castelnaudii*, *C. violifer*, *P. gigas*, and *A. viridicauda*) (Fig. 2), the effect on Hb allostery is contingent on the presence of Gly or Ser at β13. Changes in the network of atomic contacts involving β13 and β83 (Table S3) alter the favorability of alternative conformation states for IHP-binding in the central cavity (Fig. 3D), and the resultant changes in the location of IHP-binding account for the observed epistasis for Hb-O₂ affinity in the presence of IHP (Table S2). Our experimental results for the hummingbird rHb mutants are consistent with functional studies of a naturally occurring human Hb mutant, Hb Pyrgos (β83Gly→Asp), which is also characterized by an increased O₂-affinity in the presence of organic phosphates (24).

Parallelism of β-Chain Substitutions Among Species. We sequenced β⁴-globin in 63 hummingbird species and we then used maximum-likelihood and parsimony to map the β13 and β83 replacements onto an independently derived and well-resolved phylogeny (25). This analysis revealed that the substitutions (and, by implication, the associated changes in Hb-O₂ affinity) occurred at least 17 times independently (≥4 and ≥13 transitions between Gly and Ser at β13 and β83, respectively). Maximum-likelihood ancestral-state estimates for native elevation indicated that hummingbird species have shifted upwards and downward during the evolution of the group, in conjunction with repeated substitutions and back-substitutions at β13 and β83 (Fig. 4 and Fig. S5). Hence, the negative correlation between P₅₀ and native elevation (Fig. 1B) is attributable to derived increases in Hb-O₂ affinity in highland lineages, as well as derived reductions in Hb-O₂ affinity in lowland lineages. For example, the common ancestor of the highland genus *Oreotrochilus* (β13Ser-β83Ser) evolved a derived increase in Hb-O₂ affinity relative to the likely ancestral state of the Coquette clade (β13Gly-β83Gly). In contrast, in

the Brilliants the lowland *C. coeligena* (β13Gly-β83Gly) evolved a derived reduction in Hb-O₂ affinity relative to the likely ancestral state for that clade (β13Gly-β83Ser) (Fig. 4). Species' maximum elevation was strongly associated with β13-β83 genotype in a phylogenetic general linear model ($R^2 = 0.53$; $P < 10^{-11}$) (Fig. 4).

Among distantly related species, parallel substitutions at sites β13 and β83 are likely attributable to the repeated fixation of identical-by-state alleles that had independent mutational origins. Among some of the more closely related species, the sorting of ancestral polymorphism may produce the same pattern of parallelism because of the repeated fixation of identical-by-descent alleles in recently diverged lineages (26). Further work is needed to elucidate the mutational origins of the β13 and β83 variants, but it is clear that repeated changes at both sites have contributed to the repeated elevational shifts in Hb function among different lineages. Aside from the variation at sites β13 and β83, no other substitutions in the α¹-, α²-, or β⁴-globin genes exhibited any obvious association with species differences in P₅₀ values for HbA or HbD, although it is likely that particular lineage-specific substitutions (Fig. S4) account for residual variation in Hb-O₂ affinity among species.

Possible Adaptive Significance of Altitudinal Differences in Hb-O₂ Affinity. The evolution of divergent Hb-O₂ affinities between highland and lowland hummingbirds is consistent with theoretical predictions (1–6). At low altitude, a low Hb-O₂ affinity is expected to be physiologically advantageous for hummingbirds and other animals with high mass-specific metabolic rates because O₂ unloading in the peripheral circulation can occur at relatively high PO₂, thereby optimizing tissue oxygenation by increasing the O₂ diffusion gradient between capillary blood and tissue mitochondria. At low altitude, the trade-off with pulmonary O₂ loading is alleviated because arterial O₂ saturation will still be near-maximal. However, under conditions of severe environmental hypoxia at very high altitudes, an increased Hb-O₂ affinity becomes advantageous because tissue O₂ delivery can be

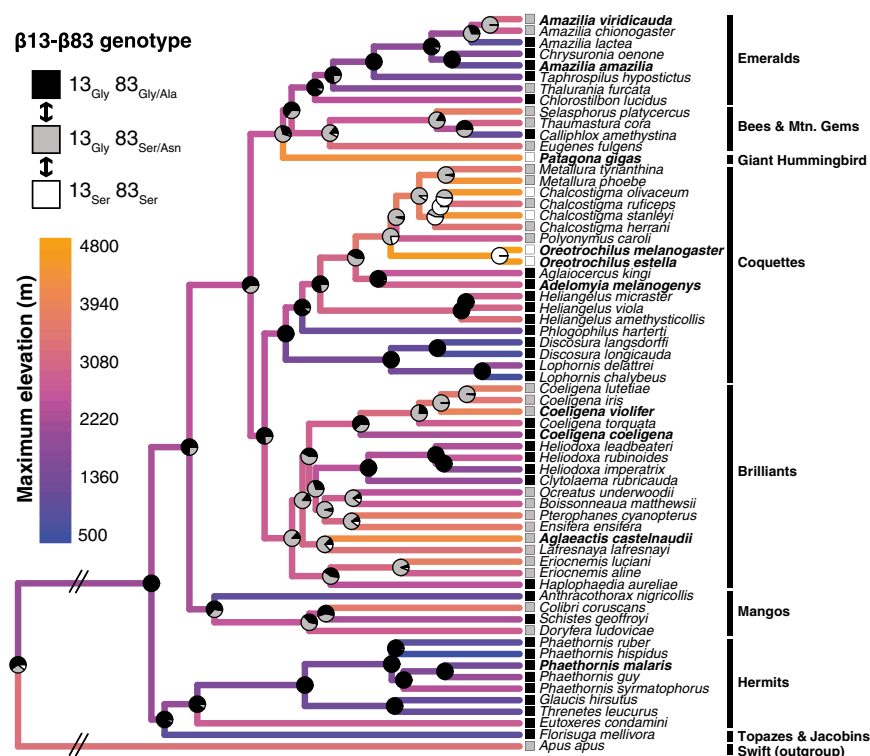


Fig. 4. Ancestral state estimates for β13 and β83 in hummingbirds. Pie diagrams at the nodes indicate the probability of each genotype based on a stepwise, single-rate maximum-likelihood model with two reversible transitions, as indicated in the inset diagram. Terminal branches of the phylogenetic tree are color-coded according to the upper limit of the species' elevational range, and internal branches are color-coded based on maximum-likelihood estimates of the ancestral states. The phylogenetically corrected association between β13-β83 genotype and native elevation was highly significant (see text for details). Parsimony analysis revealed a minimum of 17 changes in genotype across the tree (Fig. S5). β83Asn was observed in a single species, *Doryfera ludoviciae*, and was therefore binned with the β83Ser character state because side-chains of the two residues have the same polarity and the underlying codons are connected by a single mutational step. Similarly, β83Ala was observed in a single species, *Phlogophilus harteri*, and was binned with the β83Gly character state in this analysis. Branch lengths are proportional to relative time, except where indicated. Species names in bold are those that were included in the experimental analysis of Hb function.

preserved more effectively by safeguarding arterial O₂ saturation than by maximizing O₂ unloading from partially desaturated blood (1–6, 8, 9).

Mechanisms of Hb Adaptation and Causes of Parallelism at the Sequence Level. Comparative studies of Hb function in different animal species and experimental studies of naturally occurring or recombinant human Hb mutants have demonstrated that genetically based changes in Hb-O₂ affinity can be produced by numerous possible structural changes (27–29). In Andean hummingbirds, amino acid replacements at β 13 and β 83 contribute to species differences in Hb-O₂ affinity, but it is certainly not because they represent the only possible mutational changes that are capable of producing the observed changes in protein function. Although there may be numerous possible mutations that can produce identical changes in Hb-O₂ affinity, many of those changes are known to have deleterious pleiotropic effects. For example, active site mutations that alter the polarity or hydrophobicity of the distal heme pocket can produce direct changes in the association constant for O₂ binding, but such mutations typically compromise structural stability or increase the susceptibility to heme autoxidation (the spontaneous oxidation of the heme iron from the ferrous Fe²⁺ state to the ferric Fe³⁺ state, which renders Hb functionally inert as an O₂-transport molecule) (29). In contrast, mutations remote from the active site—like those at β 13 and β 83—can potentially produce fine-tuned changes in O₂-affinity with minimal pleiotropic effects through subtle displacements of the allosteric equilibrium (28–30). Within the set of all possible mutations that produce functionally equivalent effects on Hb-O₂ affinity, those that incur a lesser magnitude of deleterious pleiotropy are predicted to have a higher fixation probability, and such mutations may therefore contribute disproportionately to biochemical adaptation (31–33). When such changes are driven by positive directional selection, theory predicts that they are especially likely to evolve in parallel (34).

The parallel β 13 and β 83 substitutions that we have documented in hummingbirds have not been implicated in the adaptation of Hb function in other high-altitude birds or mammals (35–39), although a survey of sequence variation in the globin genes of Andean waterfowl documented a shared β 13Gly/Ser polymorphism in speckled teals (*Anas flavirostris*) and yellow-billed pintails (*Anas georgica*), and in both species the derived Ser variant was present at high frequency in high-altitude populations (40). The phenotypic effects of the β 13Gly/Ser variants in these waterfowl species have not yet been investigated, but the similar altitudinal patterns in Andean ducks and hummingbirds suggest parallel mechanisms of Hb evolution. At β 13 and β 83 in Andean hummingbirds, it may be that recurrent mutation and retention of ancestral polymorphism both contributed to variation in Hb function—variation that was then recruited when selection favored fine-tuned adjustments in blood-O₂ transport (e.g., during elevational range shifts). When closely related species independently adapt to a shared environmental challenge, natural selection may be predisposed to hit upon the same design solution in different lineages if one particularly accessible (and minimally pleiotropic) solution happens to be located within striking distance from the same ancestral starting point.

Methods

Specimen Collection. We preserved blood and tissue samples from voucher specimens of hummingbirds that were collected from numerous Andean localities spanning an elevational range of ~4,500 m (Table S4). Our analysis of Hb function was based on blood samples from 70 hummingbird specimens ($n = 3$ –8 individuals per species). All hummingbirds were live-trapped in mistnets and were bled and killed in accordance with guidelines of the Ornithological Council (41), and protocols approved by the University of New Mexico Institutional Animal Care and Use Committee (Protocol number 08UNM033-TR-100117; Animal Welfare Assurance number A4023-01). All fieldwork was carried out under permits issued by the management

authorities of Peru (76-2006-INRENA-IFFS-DCB, 087-2007-INRENA-IFFS-DCB, and 135-2009-AG-DGFFS-DGEFFS).

For each individual bird, we collected 0.03–0.20 mL of whole blood from the brachial or ulnar vein using heparinized microcapillary tubes. Red blood cells were separated from the plasma fraction by centrifugation, and the packed red cells were then snap-frozen in liquid nitrogen and were stored at –80 °C before use as a source of Hb for experimental studies. We collected liver and pectoral muscle from each specimen as sources of genomic DNA and globin mRNA, respectively. Muscle samples were snap-frozen or preserved using RNAlater and were subsequently stored at –80 °C before RNA isolation. Voucher specimens were preserved along with ancillary data and were deposited in the collections of the Museum of Southwestern Biology of the University of New Mexico and the Centro de Ornitología y Biodiversidad (CORBIDI), Lima, Peru. Complete specimen data are available via the ARCTOS online database (Table S4).

Molecular Cloning and Sequencing. We cloned and sequenced the adult globin genes (α^A , α^D , and β^A -globin) from at least two specimens per species. We used the RNeasy Mini Kit (Qiagen) to isolate RNA, and we used 5' and 3' RACE (Invitrogen Life Technologies) to obtain cDNA sequence for the 5' and 3' UTRs of each adult-expressed globin gene. After designing paralog-specific PCR primers with annealing sites in the 5' and 3' UTRs, complete cDNAs were synthesized for each gene by reverse transcription using the OneStep RT-PCR kit (Qiagen). We cloned gel-purified RT-PCR products into pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen Life Technologies). All new sequences were deposited in GenBank under accession nos. KF222496, KF222499, KF222501, KF222503, KF222506, and KF222510–KF222539.

Characterization of Hb Isoform Composition. We used isoelectric focusing (IEF; PhastSystem, GE Healthcare Bio-Sciences) to characterize Hb isoform composition in red cell lysates from each of the 70 hummingbird specimens. After separating native Hbs by means of IEF, gel bands were excised and digested with trypsin. The resultant peptides were then identified by means of tandem mass spectrometry (MS/MS). Database searches of the resultant MS/MS spectra were performed using Mascot (Matrix Science, v1.9.0), whereby peptide mass fingerprints were used to query a custom database of avian α - and β -chain sequences (17, 42–44), including α^A , α^D , and β^A -globin sequences from each of the surveyed hummingbird species. After separating the HbA and HbD isoforms by native gel IEF and identifying each of the constituent subunits by MS/MS, the relative abundance of the different isoforms in the hemolysates of each individual was quantified densitometrically using ImageJ (45).

Protein Purification and Measurement of Hb-O₂ Equilibria. The HbA and HbD isoforms (isoelectric points = 8.9–9.1 and 6.8–7.3, respectively) were separated and stripped of organic phosphates by means of ion-exchange chromatography. O₂ equilibria of purified Hb solutions [3 μ L thin-layer samples, (heme) 0.3 mM] were measured at 37 °C in the presence of 0.1 M Hepes buffer (pH 7.4). To characterize the allosteric regulation of Hb-O₂ affinity, we measured O₂-equilibrium curves in the absence of allosteric effectors (stripped), in the presence of Cl[–] ions (0.1 M KCl), in the presence of IHP (IHP/Hb tetramer ratio = 2.0), and in the simultaneous presence of both effectors. For details of the purification protocol and the measurement of Hb-O₂ equilibrium curves, see SI Methods.

Vector Construction, Site-Directed Mutagenesis, and Synthesis of rHbs. To produce rHbs for the protein engineering experiments, the α^A - and β^A -globin genes of *A. melanogenys* were synthesized by Genscript after optimizing nucleotide sequences with respect to *Escherichia coli* codon preferences. Gene cassettes for the α^A - and β^A -globin genes and the *methionine aminopeptidase* (MAP) gene were tandemly cloned into the custom pGM expression plasmid described by Natarajan et al. (21). All rHbs were expressed in the JM109 (DE3) *E. coli* strain. See SI Methods for details regarding the site-directed mutagenesis experiments, the expression and purification of the hummingbird rHb mutants, the measurement of rHb oxygenation properties, and the measurement of epistasis.

Ancestral State Estimates. To infer the polarity of character-state changes at β 13 and β 83, we sequenced the β^A -globin gene of 63 hummingbird species with known phylogenetic relationships. Orthologous sequence from the common swift (*Apus apus*) was used as an outgroup. Fifty-six of the 63 nodes in the independently derived phylogeny were resolved with >95% posterior probability (Dataset S1). We estimated ancestral states of the β 13– β 83 genotypes using maximum-likelihood and parsimony with the APE package in R (46). Two of the observed genotypes included rare variants at β 83 (β 13Gly- β 83Asn

and $\beta 13\text{Gly} \rightarrow \beta 83\text{Ala}$) that differed by a single codon change from physiologically similar alternative states ($\beta 83\text{Ser}$ and $\beta 83\text{Gly}$, respectively). We binned each of these singleton changes with the related codon state, resulting in three classes of two-site $\beta 13\text{--}\beta 83$ genotypes. In the maximum-likelihood model, we allowed only the two reversible transitions that each comprised a single nucleotide change. We applied a model with one rate for all transitions because likelihood ratio tests indicated that models with two to four rate parameters were not justified (46) (Fig. 4). For details regarding the phylogenetic topology and phylogenetic comparative methods, see *SI Methods*.

Structural Modeling and Molecular Docking. Homology-models of hummingbird Hb were built by the SWISS-MODEL server in the automated model (47), using *Anas platyrhynchos* Hb (PDB ID code 3EOK) as template. For each of the four rHb mutants, the root-mean-square-deviation was 0.74 Å between model and template and the QMEAN value remained between 0.70 and 0.78 for all models. Molecular docking of IHP in the Hb central cavity was performed

using AutoDock Vina (48). Internal molecular contacts were identified by the Frustratometer program (49).

ACKNOWLEDGMENTS. For assistance in the field, we thank F. Angulo P., E. Bautista O., E. J. Beckman, P. M. Benham, D. Blanco, Centro de Ornitología y Biodiversidad (Lima, Peru), R. W. Dickerman, S. G. DuBay, L. M. Flores, A. B. Johnson, K. G. McCracken, J. A. Otero, A. Quiñonez Z., C. J. Schmitt, D. C. Schmitt, C. G. Schmitt, T. Valqui, W. Vargas C., B. Walker, and N. A. Wright; for assistance in the lab, we thank A. Bang (Aarhus) and A. Rutherford (Lincoln); and we thank K. G. McCracken and two anonymous reviewers for constructive comments on the manuscript. This study was supported by National Institutes of Health Grants R01 HL087216 and HL087216-S1 (to J.F.S.); National Science Foundation Grants IOS-0949931 (to J.F.S.), DEB-0543556 (to J.A.M. and R.D.), and DEB-1146491 (to C.C.W.); the Danish Council for Independent Research, Natural Sciences Grant 10-084565 (to A.F.); the Center for Evolutionary and Theoretical Immunology at the University of New Mexico; and the Faculty of Science and Technology at Aarhus University.

1. Turek Z, Kreuzer F, Hoofd LJ (1973) Advantage or disadvantage of a decrease of blood oxygen affinity for tissue oxygen supply at hypoxia. A theoretical study comparing man and rat. *Pflügers Arch* 342(3):185–197.
2. Turek Z, Kreuzer F (1976) Effect of a shift of the oxygen dissociation curve on myocardial oxygenation at hypoxia. *Adv Exp Med Biol* 75:657–662.
3. Turek Z, Kreuzer F, Ringnald BEM (1978) Blood gases at several levels of oxygenation in rats with a left-shifted blood oxygen dissociation curve. *Pflügers Arch* 376(1):7–13.
4. Turek Z, Kreuzer F, Turek-Maischeider M, Ringnald BEM (1978) Blood O_2 content, cardiac output, and flow to organs at several levels of oxygenation in rats with a left-shifted blood oxygen dissociation curve. *Pflügers Arch* 376(3):201–207.
5. Bencowitz HZ, Wagner PD, West JB (1982) Effect of change in P_{50} on exercise tolerance at high altitude: A theoretical study. *J Appl Physiol* 53(6):1487–1495.
6. Willford DC, Hill EP, Moores WY (1982) Theoretical analysis of optimal P_{50} . *J Appl Physiol* 52(4):1043–1048.
7. Scott GR, Milsom WK (2006) Flying high: a theoretical analysis of the factors limiting exercise performance in birds at altitude. *Respir Physiol Neurobiol* 154(1–2):284–301.
8. Mairbäurl H, Weber RE (2012) Oxygen transport by hemoglobin. *Compr Physiol* 2(2):1463–1489.
9. Storz JF, Scott GR, Cheviron ZA (2010) Phenotypic plasticity and genetic adaptation to high-altitude hypoxia in vertebrates. *J Exp Biol* 213(Pt 24):4125–4136.
10. Suarez RK, Lighton JRB, Brown GS, Mathieu-Costello O (1991) Mitochondrial respiration in hummingbird flight muscles. *Proc Natl Acad Sci USA* 88(11):4870–4873.
11. Suarez RK (1992) Hummingbird flight: Sustaining the highest mass-specific metabolic rates among vertebrates. *Experientia* 48(6):565–570.
12. Suarez RK (1998) Oxygen and the upper limits to animal design and performance. *J Exp Biol* 201(Pt 8):1065–1072.
13. Altshuler DL, Dudley R (2002) The ecological and evolutionary interface of hummingbird flight physiology. *J Exp Biol* 205(Pt 16):2325–2336.
14. McGuire JA, Witt CC, Remsen JV, Dudley R, Altshuler DL (2009) A higher-level taxonomy for hummingbirds. *J Ornithol* 150(1):155–165.
15. Weber RE, Fago A (2004) Functional adaptation and its molecular basis in vertebrate hemoglobins, neuroglobins and cytoglobins. *Respir Physiol Neurobiol* 144(2–3):141–159.
16. Storz JF, Opazo JC, Hoffmann FG (2013) Gene duplication, genome duplication, and the functional diversification of vertebrate globins. *Mol Phylogenet Evol* 66(2):469–478.
17. Grispo MT, et al. (2012) Gene duplication and the evolution of hemoglobin isoform differentiation in birds. *J Biol Chem* 287(45):37647–37658.
18. Hiebl I, Weber RE, Schneegans D, Kesters J, Braunitzer G (1988) Structural adaptations in the major and minor hemoglobin components of adult Ruppell's griffon (*Gyps rueppellii*, Aegyptiinae): A new molecular pattern for hypoxia tolerance. *Biol Chem Hoppe Seyler* 369:217–232.
19. Weber RE, Hiebl I, Braunitzer G (1988) High altitude and hemoglobin function in the vultures *Gyps rueppellii* and *Aegypius monachus*. *Biol Chem Hoppe Seyler* 369(4):233–240.
20. Hoffmann FG, Storz JF (2007) The α D-globin gene originated via duplication of an embryonic α -like globin gene in the ancestor of tetrapod vertebrates. *Mol Biol Evol* 24(9):1982–1990.
21. Natarajan C, et al. (2011) Expression and purification of recombinant hemoglobin in *Escherichia coli*. *PLoS ONE* 6(5):e20176.
22. Weinreich DM, Watson RA, Chao L (2005) Sign epistasis and genetic constraint on evolutionary trajectories. *Evolution* 59(6):1165–1174.
23. Natarajan C, et al. (2013) Epistasis among adaptive mutations in deer mouse hemoglobin. *Science* 340(6138):1324–1327.
24. Wajzman H, et al. (1978) Hémoglobine Pyrgos beta 83 (EF 7) Gly \rightarrow Asp chez un Malien: Identification structurale et propriétés fonctionnelles. *Nouv Rev Fr Hematol* 20(3):403–411.
25. McGuire JA, Witt CC, Altshuler DL, Remsen JV, Jr. (2007) Phylogenetic systematics and biogeography of hummingbirds: Bayesian and maximum likelihood analyses of partitioned data and selection of an appropriate partitioning strategy. *Syst Biol* 56(5):837–856.
26. Clark AG (1997) Neutral behavior of shared polymorphism. *Proc Natl Acad Sci USA* 94(15):7730–7734.
27. Weber RE (2007) High-altitude adaptations in vertebrate hemoglobins. *Respir Physiol Neurobiol* 158(2–3):132–142.
28. Storz JF, Moriyama H (2008) Mechanisms of hemoglobin adaptation to high altitude hypoxia. *High Alt Med Biol* 9(2):148–157.
29. Varnado CL, et al. (2013) Development of recombinant hemoglobin-based oxygen carriers. *Antioxid Redox Signal* 18(17):2314–2328.
30. Mailliet DH, et al. (2008) Interfacial and distal-heme pocket mutations exhibit additive effects on the structure and function of hemoglobin. *Biochemistry* 47(40):10551–10563.
31. Otto SP (2004) Two steps forward, one step back: The pleiotropic effects of favoured alleles. *Proc Biol Sci* 271(1540):705–714.
32. Stern DL, Orgogozo V (2009) Is genetic evolution predictable? *Science* 323(5915):746–751.
33. Streisfeld MA, Rausher MD (2011) Population genetics, pleiotropy, and the preferential fixation of mutations during adaptive evolution. *Evolution* 65(3):629–642.
34. Orr HA (2005) The probability of parallel evolution. *Evolution* 59(1):216–220.
35. Jessen T-H, Weber RE, Fermi G, Tame J, Braunitzer G (1991) Adaptation of bird hemoglobins to high altitudes: Demonstration of molecular mechanism by protein engineering. *Proc Natl Acad Sci USA* 88(15):6519–6522.
36. Weber RE, Jessen T-H, Malte H, Tame J (1993) Mutant hemoglobins ($\alpha^{119}\text{Ala}$ and $\beta^{55}\text{Ser}$): functions related to high-altitude respiration in geese. *J Appl Physiol* (1985) 75(6):2646–2655.
37. Storz JF, et al. (2009) Evolutionary and functional insights into the mechanism underlying high-altitude adaptation of deer mouse hemoglobin. *Proc Natl Acad Sci USA* 106(34):14450–14455.
38. Storz JF, Runck AM, Moriyama H, Weber RE, Fago A (2010) Genetic differences in hemoglobin function between highland and lowland deer mice. *J Exp Biol* 213(Pt 15):2565–2574.
39. Revsbech IG, et al. (2013) Hemoglobin function and allosteric regulation in semi-fossorial rodents (family Sciuridae) with different altitudinal ranges. *J Exp Biol* 216(Pt 22):4264–4271.
40. McCracken KG, et al. (2009) Parallel evolution in the major haemoglobin genes of eight species of Andean waterfowl. *Mol Ecol* 18(19):3992–4005.
41. Fair JM, Paul E (2010) *Guidelines to the Use of Wild Birds in Research*, ed Jones J (Ornithological Council, Washington, DC).
42. Hoffmann FG, Storz JF, Gorr TA, Opazo JC (2010) Lineage-specific patterns of functional diversification in the α - and β -globin gene families of tetrapod vertebrates. *Mol Biol Evol* 27(5):1126–1138.
43. Hoffmann FG, Opazo JC, Storz JF (2011) Differential loss and retention of cytoglobin, myoglobin, and globin-E during the radiation of vertebrates. *Genome Biol Evol* 3:588–600.
44. Hoffmann FG, Opazo JC, Storz JF (2012) Whole-genome duplications spurred the functional diversification of the globin gene superfamily in vertebrates. *Mol Biol Evol* 29(1):303–312.
45. Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with Image J. *Bioinformatics International* 11(7):36–42.
46. Paradis E, Claude J, Strimmer K (2004) APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics* 20(2):289–290.
47. Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics* 22(2):195–201.
48. Trott O, Olson AJ (2010) AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 31(2):455–461.
49. Jenik M, et al. (2012) Protein frustratometer: A tool to localize energetic frustration in protein molecules. *Nucleic Acids Res* 40(Web Server issue):W348–W351.

Supporting Information

Projecto-Garcia et al. 10.1073/pnas.1315456110

SI Methods

Protein Purification. The HbA and HbD isoforms were separated by passing the samples through an ion-exchange chromatography column (HiTrap QHP, 5 × 1 mL, 17–1153-01; GE Healthcare) equilibrated with 20 mM Tris buffer (pH 8.2) and eluted using a linear gradient of 0–0.2 M NaCl. Samples were desalted by overnight dialysis against three changes of 10 mM Hepes buffer (pH 7.6) at 4 °C. Samples were concentrated (to >1 mM heme) using Millipore centrifugal filter units (MW = 30,000; Millipore) at 7,000 × g before freezing at –80 °C. Heme oxy concentration (millimolar) was calculated from the absorbance peaks in the visible region of the spectrum (577 nm and 540 nm) using standard extinction coefficients.

Hb-O₂ Equilibria. O₂-equilibrium curves were measured using a modified O₂ diffusion chamber where changes in absorption (436 nm) of ultrathin (~1.4 μm) layers of Hb solutions were recorded following complete oxygenation (100% saturation) and deoxygenation (0% saturation) of Hb, achieved via equilibration with pure O₂ and N₂, respectively, and complete equilibration to gas mixtures of varying O₂ tension generated by precision Wösthoff gas-mixing pumps, as described previously (1–3). Values of P₅₀ and n₅₀ (Hill's cooperativity coefficient at half-saturation) were interpolated from the linear portion of Hill plots [log ([HbO₂]/[Hb]) vs. log PO₂] based on four to six equilibration steps between 30% and 70% oxygenation. Free Cl[–] concentrations were measured with a model 926S Mark II chloride analyzer (Sherwood Scientific). We used standard concentrations of Cl[–] (0.1 M KCl) and inositol hexaphosphate (IHP; IHP/Hb tetramer ratio = 2.0) (4, 5) that closely approximate intraerythrocytic effector concentrations in vivo (6–8). Predicted P₅₀s of composite hemolysates were calculated as the average value for HbA and HbD, weighted according to the naturally occurring relative concentration of each isoform.

Vector Construction and Site-Directed Mutagenesis. The α⁴- and β⁴-globin genes of *Adelomyia melanogenys* were synthesized by Genscript after optimizing nucleotide sequences with respect to *Escherichia coli* codon preferences. Gene cassettes for the α⁴- and β⁴-globin genes and the *methionine aminopeptidase* (MAP) gene were tandemly cloned into the custom pGM expression plasmid described by Natarajan et al. (9). To maximize efficiency in the posttranslational cleaving of N-terminal methionines from the α- and β-chain polypeptides, an additional copy of the MAP gene was cloned into the pCO-MAP plasmid with a kanamycin resistant gene and was coexpressed with the pGM expression plasmid.

The *A. melanogenys* β⁴-globin was converted into the *Oreotrochilus estella* sequence by engineering two codon changes (β13Gly→Ser and β83Gly→Ser) using site-directed mutagenesis. The same procedure was used to engineer the two possible mutational intermediates between the β-chain Hbs of the two species (β13Ser-β83Gly and β13Gly-β83Ser). The mutagenesis experiments were performed with the QuikChange II XL Site-Directed Mutagenesis kit from Stratagene in accordance with the manufacturer's protocol. The presence of each engineered codon change was verified by DNA sequencing. In addition to the two above-mentioned β-chain substitutions, the major Hb isoforms of *A. melanogenys* and *O. estella* are also distinguished from one another by a single conservative α-chain substitution. We retained the *A. melanogenys* character state at this site (α8Thr) in

all engineered rHbs to control for the effects of substitutions at β13 and β83.

Expression, Purification, and Functional Analysis of Recombinant Hemoglobins. All recombinant hemoglobins (rHbs) were expressed in the JM109 (DE3) *E. coli* strain and the bacterial cells were subject to dual selection in an LB agar plate containing ampicillin and kanamycin to ensure that the transformants receive both the pGM and pCO-MAP plasmids. Large-scale production was conducted in 1- to 1.5-L batches containing TB medium. Cells were grown at 37 °C in an orbital shaker at 200 rpm until absorbance values reached 0.6–0.8 at 600 nm. The cells were induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and were then supplemented with hemin (50 μg/mL) and glucose (20 g/L). The cells were then subsequently grown at 28 °C for 16 h in an orbital shaker at 200 rpm. The bacterial culture was saturated with CO for 15 min and the cells were harvested by centrifugation and stored at –80 °C. Subsequently the cells were resuspended in lysis buffer (3 mL/g of cells, 50 mM Tris base, 1 mM EDTA, 0.5 mM DTT) and lysozyme (1 mg/g cells) was added before sonication. Polyethyleneimine solution (0.5–1%) was added to the crude lysates to precipitate nucleic acids. After centrifugation (15,000 × g for 45 min at 4 °C), the clarified supernatants were dialyzed overnight against three changes of 20 mM Tris buffer (0.5 mM EDTA, 0.5 mM DTT, pH 7.6) at 4 °C. Centrifugation at 14,000 × g was used to pellet cell debris. Recombinant Hbs were then purified in a two-step process using ion-exchange chromatography. In the first step, the sample was passed through a column (HiTrap SPHP, 5 × 5 mL, 17–1152-01) equilibrated with 20 mM Tris buffer (0.5 mM EDTA, 0.5 mM DTT, pH 6.0) and was eluted using a linear gradient of 0–0.5 M NaCl. In the second step the sample was passed through another ion-exchange column (HiTrap QHP, 5 × 5 mL, 17–1153-01; GE Healthcare) equilibrated with 20 mM Tris buffer (0.5 mM EDTA, 0.5 mM DTT, pH 8.5) and was eluted using a linear gradient of 0–0.5 M NaCl. Samples were desalted by overnight dialysis against three changes of 10 mM Hepes buffer (pH 7.6) at 4 °C. If necessary, samples were concentrated to >1 mM heme using Millipore centrifugal filter units (MW = 30,000; Millipore) at 7000 × g before freezing at –80 °C. As a means of quality assessment, absorbance spectra of oxy, deoxy, and CO derivatives were measured at 450–600 nm to confirm that the absorbance maxima of rHb mutants corresponded to those of the native Hbs. O₂-binding equilibria of rHb solutions were measured using the same protocol described above for the native Hb samples, and we used an enzymatic metHb reductase system (10) to maintain heme iron in the ferrous Fe²⁺ state. The measured P₅₀ values for the native Hbs were based on pooled samples from multiple individuals per species, so allelic variation in the α- and β-chain subunits contributes to discrepancies in measured P₅₀ values between the native Hbs, which have a heterogeneous amino acid composition, and the recombinant Hbs, which have an invariant amino acid composition.

Measurement of Epistasis. For the set of four rHb mutants representing each possible two-site combination of amino acid substitutions at β13 and β83, we tested for epistatic deviations from the expectations of an additive model: $\varepsilon = (P_{ii} + P_{jj}) - (P_{ij} + P_{ji})$, where P_{ij} is the measured P₅₀ of the rHb with substitutions i and j at each site. The SE of the measured epistatic deviation, a linear function of P_{ij} , was calculated using the method of error propagation:

$\sigma_\varepsilon = \sqrt{\sigma P_{ii}^2 + \sigma P_{jj}^2 + \sigma P_{ij}^2 + \sigma P_{ji}^2}$, and the 95% confidence interval for ε was computed as $\varepsilon \pm \sigma_\varepsilon \times 1.96$ (11). Epistasis between a given pair of sites was considered to be statistically significant if the 95% confidence interval for ε did not include zero.

Phylogenetically Independent Contrasts. We used a four-gene DNA sequence alignment for 151 species of hummingbirds (12), augmented with 143 additional species and two additional nuclear genes to estimate an ultrametric phylogeny using BEAST (13),

with branch-lengths scaled to relative time (Dataset S1). For the 10 species that were used in the experimental studies of Hb function, we calculated phylogenetically independent contrasts (PICs) of P_{50} values and regressed them against PICs of native elevation. All nodes in the phylogeny of these 10 focal species were resolved with 100% posterior probability. Elevational range data were primarily taken from Parker et al. (14). Results were consistent whether we used the maximum, midpoint, or minimum of the species' elevational range or the actual elevation at which the specimens were collected.

1. Weber RE (1981) Cationic control of oxygen affinity in lugworm erythrocytes. *Nature* 292(5821):386–387.
2. Weber RE (1992) Use of ionic and zwitterionic (Tris/BisTris and HEPES) buffers in studies on hemoglobin function. *J Appl Physiol* (1985) 72(4):1611–1615.
3. Weber RE, et al. (2004) Modulation of red cell glycolysis: Interactions between vertebrate hemoglobins and cytoplasmic domains of band 3 red cell membrane proteins. *Am J Physiol Regul Integr Comp Physiol* 287(2):R454–R464.
4. Imai K (1982) *Allosteric Effects in Haemoglobin* (Cambridge Univ Press, Cambridge, UK).
5. Mairbäurl H, Weber RE (2012) Oxygen transport by hemoglobin. *Compr Physiol* 2(2): 1463–1489.
6. Isaacks RE, et al. (1976) Studies on avian erythrocyte metabolism-IV. relationship between the major phosphorylated metabolic intermediates and oxygen affinity of whole blood in adults and embryos in several galliformes. *Comp Biochem Physiol A* 55(1):29–33.
7. Petschow D, et al. (1977) Causes of high blood O_2 affinity of animals living at high altitude. *J Appl Physiol* 42(2):139–143.
8. Maginniss LA (1985) Red cell organic phosphates and Bohr effects in house sparrow blood. *Respir Physiol* 59(1):93–103.
9. Natarajan C, et al. (2011) Expression and purification of recombinant hemoglobin in *Escherichia coli*. *PLoS ONE* 6(5):e20176.
10. Hayashi A, Suzuki T, Shin M (1973) An enzymic reduction system for metmyoglobin and methemoglobin, and its application to functional studies of oxygen carriers. *Biochim Biophys Acta* 310(2):309–316.
11. Natarajan C, et al. (2013) Epistasis among adaptive mutations in deer mouse hemoglobin. *Science* 340(6138):1324–1327.
12. McGuire JA, Witt CC, Altshuler DL, Remsen JV, Jr. (2007) Phylogenetic systematics and biogeography of hummingbirds: Bayesian and maximum likelihood analyses of partitioned data and selection of an appropriate partitioning strategy. *Syst Biol* 56(5): 837–856.
13. Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 7:214.
14. Parker TA, Stotz DF, Fitzpatrick JW (1996) in *Neotropical Bird Ecology and Conservation*, eds Parker TA, Moskovits DK (Univ Chicago Press, Chicago), pp. 113–436.

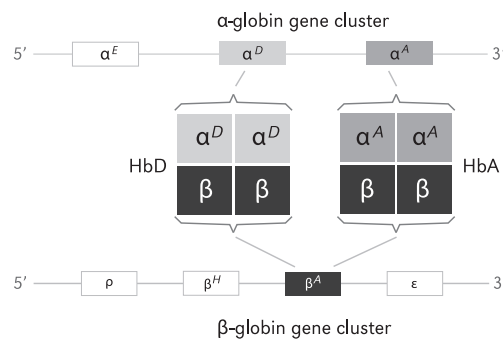


Fig. S1. Postnatally expressed Hb isoforms in avian red blood cells. The major isoform, HbA ($\alpha^A_2\beta_2$), has α -type subunits encoded by the α^A -globin gene, and the minor isoform, HbD ($\alpha^D_2\beta_2$), has α -type subunits encoded by the α^D -globin gene. Both isoforms share identical β -type subunits encoded by the β^A -globin gene. The remaining members of the α - and β -globin gene families (α^E , ρ , β^H , and ϵ -globin) are not expressed at appreciable levels in the definitive erythrocytes of adult birds. Within each gene cluster, the intergenic spacing is not drawn to scale.

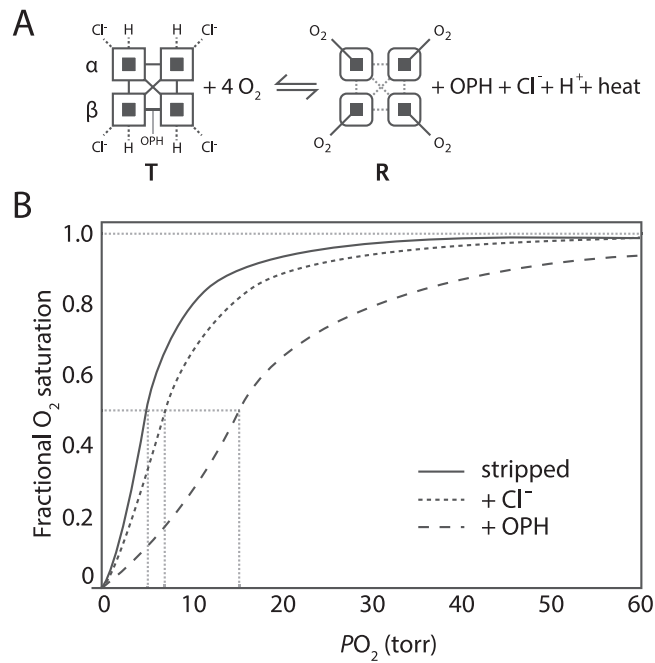


Fig. S2. Diagram illustrating the allosteric regulation of Hb- O_2 affinity. (A) The oxygenation reaction of tetrameric Hb ($\alpha_2\beta_2$) involves an allosteric transition in quaternary structure from the low-affinity T-state to the high-affinity R-state. The oxygenation-induced T \rightarrow R transition entails a breakage of salt bridges and hydrogen bonds within and between subunits (open squares), dissociation of allosterically bound organic phosphates (OPHs), Cl^- ions, and protons, and the release of heat (heme oxygenation is an exothermic reaction). Deoxygenation-linked proton binding occurs at multiple residues in the α - and β -chains, Cl^- binding mainly occurs at the N-terminal α -amino groups of the α - and β -chains in addition to other residues in both chains, and phosphate binding occurs between the β -chains in the central cavity of the Hb tetramer. (B) O_2 -equilibrium curves for purified Hb in the absence of allosteric effectors (stripped) and in the presence of chloride ions (+ Cl^-) and organic phosphates (+OPH). The preferential binding of allosteric effectors to deoxyHb stabilizes the T-state, thereby shifting the allosteric equilibrium in favor of the low-affinity quaternary structure. The O_2 -equilibrium curves are therefore right-shifted (Hb- O_2 affinity is reduced) in the presence of allosteric effectors. Hb- O_2 affinity is indexed by the P_{50} value, the PO_2 at which Hb is half-saturated. The sigmoidal shape of the O_2 -equilibrium curves reflects cooperative O_2 -binding, involving a PO_2 -dependent shift from low- to high-affinity conformations.

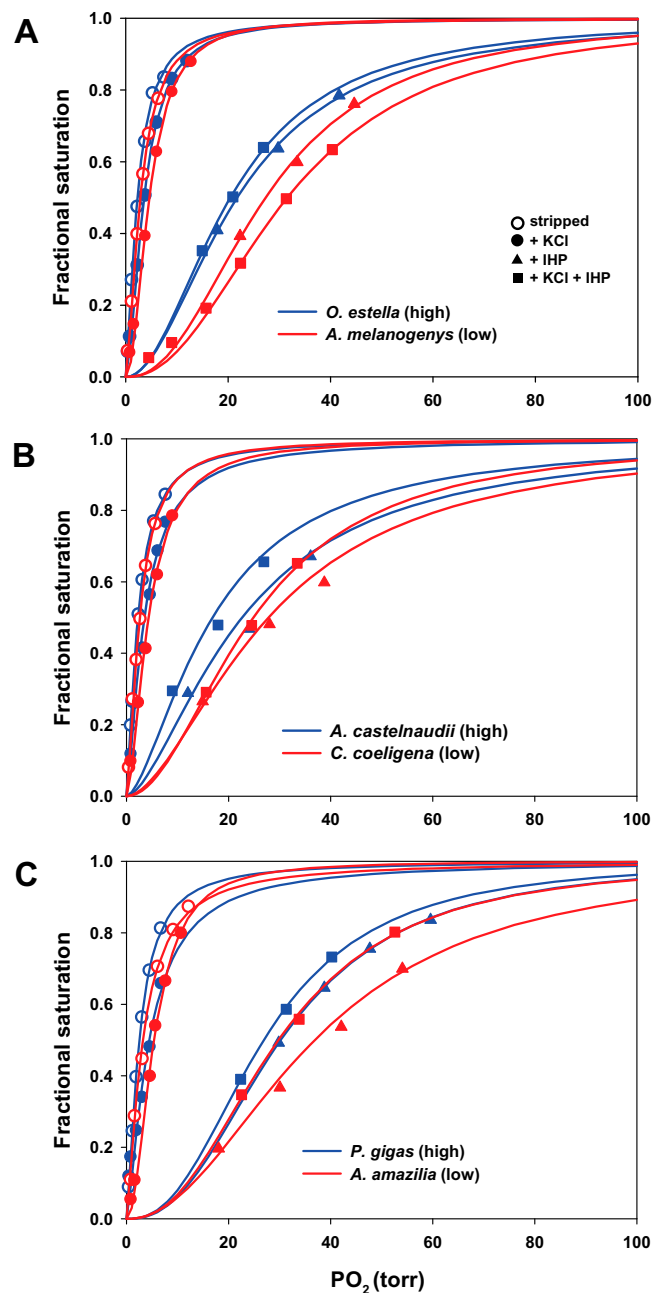


Fig. S3. O₂-equilibrium curves for purified HbA isoforms of high- and low-altitude hummingbird species. (A) Within the Coquettes, the Andean hillstar (*Oreotrochilus estella*), a montane species that occurs at elevations up to ~4,600 m, has a much higher Hb-O₂ affinity (i.e., a left-shifted O₂-equilibrium curve) relative to the speckled hummingbird (*Adelomyia melanogenys*), which is a middle-elevation species (1,000–2,900 m). (B) Within the Brilliants, the white-tufted sunbeam (*Aglæactis castelnaudii*), a montane species that occurs at elevations up to ~4,600 m, has a much higher Hb-O₂ affinity relative to the bronzy Inca (*Coeligena coeligena*), which is native to the subtropical zone (1,000–2,200 m). (C) The giant hummingbird (*Patagona gigas*), which occurs at elevations up to ~4,300 m, has a higher Hb-O₂ affinity than the Amazilia hummingbird (*A. amazilia*), which is generally restricted to sea-level environments. See Table 1 for a summary of data on Hb function for all 10 species.

	α^A -globin										α^D -globin										β^A -globin												
Common swift, <i>Apus apus</i>	8	13	15	22	23	28	69	70	76	83	11	13	15	16	26	30	34	38	55	90	101	111	115	116	118	121	139	13	21	22	44	52	83
<i>Adelomyia melanogenys</i>	T	V	A	E	A	A	A	A	I	L	Q	V	D	K	A	Q	T	Q	I	N	L	T	K	D	T	I	K	G	A	D	S	T	S
<i>Oreotrochilus estella</i>	S	-	-	-	-	T	-	V	-	-	T	-	-	-	-	T	V	T	-	-	-	-	-	-	-	-	-	S	E	Q	-	-	-
<i>Oreotrochilus melanogaster</i>	S	-	-	-	-	T	-	V	-	-	T	-	-	-	-	T	V	T	-	-	-	-	-	-	-	-	-	S	E	Q	N	-	-
<i>Aglaeactis castelnaudii</i>	-	L	G	D	-	T	-	V	-	-	S	I	-	-	-	T	V	-	-	-	-	-	-	-	S	V	-	-	E	Q	-	-	-
<i>Coeligena coeligena</i>	-	L	G	-	-	-	-	-	-	-	S	-	-	-	V	T	V	-	-	D	P	-	E	G	S	V	-	-	E	Q	-	S	G
<i>Coeligena violifer</i>	-	L	G	-	-	-	-	V	M	-	S	I	-	R	-	T	V	-	-	-	-	-	-	-	S	V	-	-	E	Q	-	-	-
<i>Patagona gigas</i>	-	-	-	-	-	T	-	V	-	-	S	-	-	-	-	T	V	T	-	-	-	-	-	-	-	V	E	S	-	-	-	-	-
<i>Amazilia viridicauda</i>	-	I	-	-	D	T	-	V	-	-	T	I	E	-	-	T	V	-	V	-	-	-	-	-	-	V	-	-	-	-	-	-	-
<i>Amazilia amazilia</i>	-	I	-	-	D	T	-	V	-	-	T	I	E	-	-	A	V	-	V	-	-	-	-	-	V	-	-	-	-	E	-	-	G
<i>Phaethornis malaris</i>	-	I	-	-	D	T	P	V	-	F	A	I	-	-	-	T	A	-	V	-	-	S	-	E	-	V	-	-	-	E	-	-	G

Fig. S4. Variable residue positions in a multiple alignment of hummingbird globin sequences. Orthologous sequences from the common swift (*Apus apus*) are included for comparison. High-altitude species with maximum elevational ranges of >3,000 m are denoted by shading. Sequences represent the most common haplotypes for each species. Across all three adult-expressed globin genes (α^A , α^D , and β -globin), 33 of 428 amino acid sites are variable. Of the 33 variable sites, 12 have undergone repeated changes (parallelisms or reversals). Of those 12 sites, only $\beta 13$ and $\beta 83$ have undergone repeated amino acid replacements that are significantly associated with shifts in elevation (see text for details).

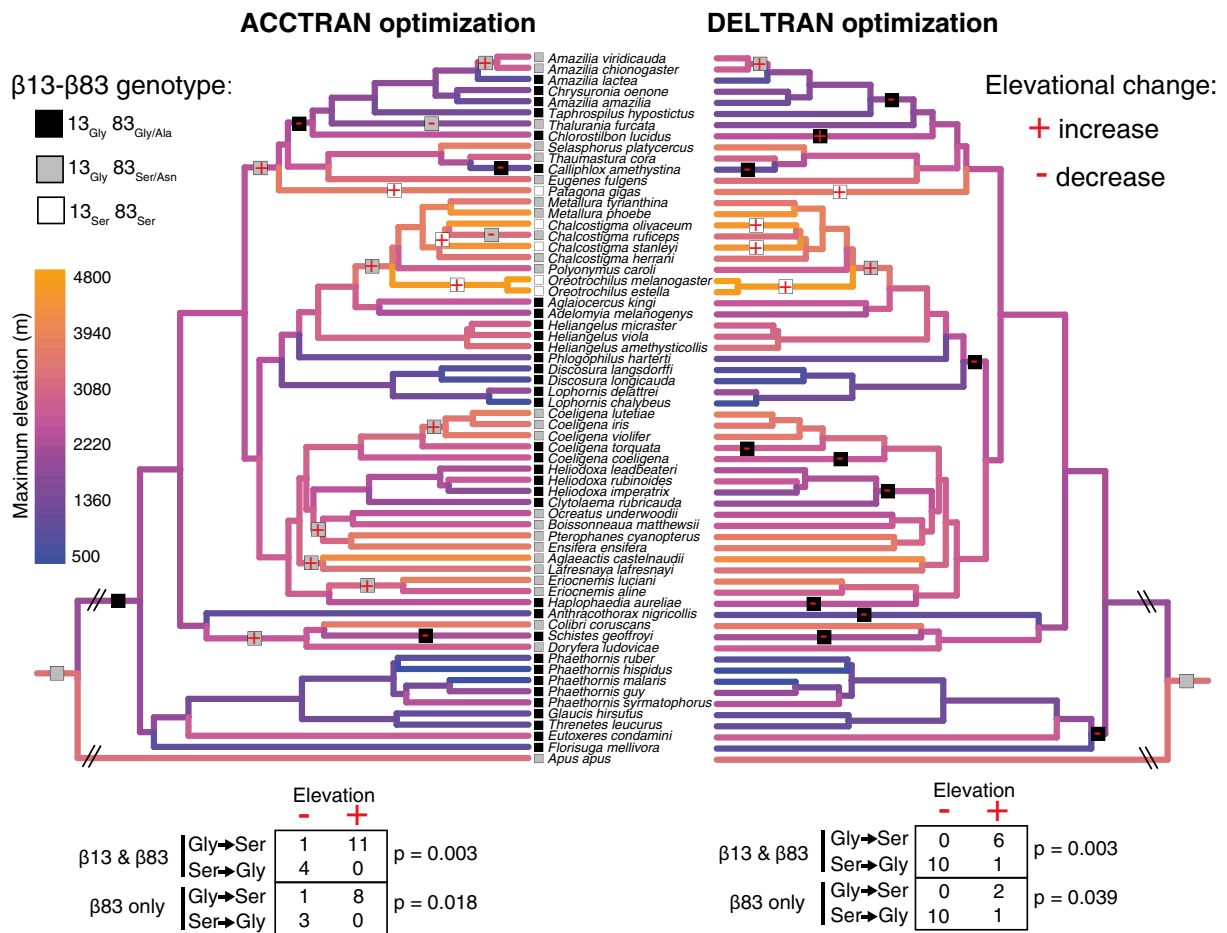


Fig. S5. Parsimony reconstructions reveal repeated substitutions and back-substitutions at $\beta 13$ and $\beta 83$ that are coincident with elevational range shifts during the diversification of Andean hummingbirds. Parsimony reconstructions of 13-83 genotype were performed using accelerated (ACCTTRAN) and delayed (DELTRAN) optimization to maximize reversals and parallel changes, respectively. The minimum number of transitions between Gly and Ser across the phylogeny of these 63 hummingbird species is 17, including 13 changes at $\beta 83$ and 4 changes at $\beta 13$. Regardless of the optimization scheme, Gly→Ser replacements at both sites are associated with upward shifts in elevation (+ symbols) relative to the immediate ancestor, whereas Ser→Gly replacements are associated with downward shifts in elevation (- symbols). In the ACCTTRAN optimized scenario, the change that maps to the common ancestor of all hummingbirds is not associated with any inferred elevation change, which is why only 16 changes were included in the contingency table. Ancestral states for maximum elevation were estimated using maximum-likelihood.

Table S1. Linear regressions of Hb-O₂ affinity (P₅₀, torr) vs. native elevation for South American hummingbirds

X	Y	PIC		Nonphylogenetic	
		R^2	P value	R^2	P value
Midpoint elevation	HbA (stripped)	0.884	5.3×10^{-5}	0.842	1.8×10^{-4}
	HbA (+KCl)	0.538	0.016	0.796	0.001
	HbA (+IHP)	0.430	0.040	0.802	0.001
	HbA (+KCl+IHP)	0.737	0.001	0.651	0.005
	HbD (stripped)	0.877	0.019	0.820	0.034
	HbD (+KCl)	0.767	0.052	0.955	0.004
	HbD (+IHP)	0.890	0.016	0.851	0.026
	HbD (+KCl+IHP)	0.779	0.047	0.800	0.041
	HbA+HbD (stripped)	0.973	0.002	0.970	0.006
	HbA+HbD (+KCl)	0.928	0.008	0.887	0.017
	HbA+HbD (+IHP)	0.995	1.5×10^{-4}	0.995	1.4×10^{-4}
	HbA+HbD (+KCl+IHP)	0.983	9.7×10^{-4}	0.969	0.002
Maximum elevation	HbA (stripped)	0.800	4.7×10^{-4}	0.749	0.001
	HbA (+KCl)	0.497	0.023	0.754	0.001
	HbA (+IHP)	0.384	0.056	0.710	0.002
	HbA (+KCl+IHP)	0.581	0.010	0.512	0.020
	HbD (stripped)	0.978	0.001	0.949	0.005
	HbD (+KCl)	0.967	0.003	0.956	0.004
	HbD (+IHP)	0.987	0.001	0.974	0.002
	HbD (+KCl+IHP)	0.889	0.016	0.926	0.009
	HbA+HbD (stripped)	0.865	0.022	0.793	0.043
	HbA+HbD (+KCl)	0.947	0.005	0.904	0.013
	HbA+HbD (+IHP)	0.893	0.015	0.855	0.024
	HbA+HbD (+KCl+IHP)	0.787	0.045	0.714	0.072

Analysis of the HbA isoform was based on data from all 10 species, whereas the analysis of HbD and the weighted average of both isoforms (HbA+HbD) was based on data from a subset of five species (see main text for details). Coefficients of determination (R^2) and associated P values are given for regressions based on phylogenetically independent contrasts (PICs) and ordinary least-squares regressions that treat values for each species as independent datapoints. Results are shown for PIC and nonphylogenetic regressions using the mid-points and upper limits of the species-typical elevational ranges.

Table S2. Functional properties of recombinant hummingbird HbA mutants

rHb mutant	Stripped		+ IHP		+ KCl + IHP	
	P ₅₀	n ₅₀	P ₅₀	n ₅₀	P ₅₀	n ₅₀
β13Gly-β83Gly	2.74 ± 0.03	1.33 ± 0.04	24.22 ± 0.90	2.03 ± 0.17	19.38 ± 0.41	1.42 ± 0.07
β13Ser-β83Gly	3.21 ± 0.14	1.40 ± 0.03	13.79 ± 0.24	1.52 ± 0.10	10.99 ± 0.06	1.50 ± 0.11
β13Gly-β83Ser	3.08 ± 0.06	1.42 ± 0.02	16.73 ± 0.65	1.52 ± 0.10	11.76 ± 1.09	1.36 ± 0.06
β13Ser-β83Ser	3.21 ± 0.06	1.55 ± 0.07	24.70 ± 0.40	1.98 ± 0.08	16.31 ± 0.90	1.89 ± 0.11

O₂-affinities (P₅₀, torr) and cooperativity coefficients (n₅₀; mean ± SEM) measured in 0.1 M Hepes buffer at pH 7.40, 37 °C. Measurements were conducted in the absence of allosteric effectors (stripped), in the presence of IHP (IHP/Hb tetramer ratio = 2.0), and in the presence of both KCl (0.1 M) and IHP. [Heme], 0.3 mM.

Table S4. Voucher specimens of South American hummingbirds used in the experimental analysis of Hb function

Species	Elevation (m)	NK tissue no.	MSB catalog number and direct Weblink to specimen data
<i>Adelomyia melanogenys</i>	1,395	161266	http://arctos.database.museum/guid/MSB:Bird:27492
<i>Adelomyia melanogenys</i>	1,395	161331	http://arctos.database.museum/guid/MSB:Bird:27552
<i>Adelomyia melanogenys</i>	2,102	163564	http://arctos.database.museum/guid/MSB:Bird:31892
<i>Adelomyia melanogenys</i>	2,111	163645	http://arctos.database.museum/guid/MSB:Bird:31973
<i>Adelomyia melanogenys</i>	2,052	163657	http://arctos.database.museum/guid/MSB:Bird:31985
<i>Adelomyia melanogenys</i>	2,144	163756	http://arctos.database.museum/guid/MSB:Bird:32084
<i>Adelomyia melanogenys</i>	2,147	163838	http://arctos.database.museum/guid/MSB:Bird:32166
<i>Aglaeactis castelnaudii</i>	4,470	159782	http://arctos.database.museum/guid/MSB:Bird:27124
<i>Aglaeactis castelnaudii</i>	4,330	159783	http://arctos.database.museum/guid/MSB:Bird:27125
<i>Aglaeactis castelnaudii</i>	4,030	159798	http://arctos.database.museum/guid/MSB:Bird:27140
<i>Aglaeactis castelnaudii</i>	4,330	159801	http://arctos.database.museum/guid/MSB:Bird:27143
<i>Aglaeactis castelnaudii</i>	4,470	159808	http://arctos.database.museum/guid/MSB:Bird:27149
<i>Aglaeactis castelnaudii</i>	4,300	159809	http://arctos.database.museum/guid/MSB:Bird:27150
<i>Aglaeactis castelnaudii</i>	4,578	169373	http://arctos.database.museum/guid/MSB:Bird:34147
<i>Amazilia amazilia</i>	366	162007	http://arctos.database.museum/guid/MSB:Bird:27595
<i>Amazilia amazilia</i>	366	162009	http://arctos.database.museum/guid/MSB:Bird:27597
<i>Amazilia amazilia</i>	366	162020	http://arctos.database.museum/guid/MSB:Bird:27604
<i>Amazilia amazilia</i>	366	162024	http://arctos.database.museum/guid/MSB:Bird:27608
<i>Amazilia amazilia</i>	366	162026	http://arctos.database.museum/guid/MSB:Bird:31222
<i>Amazilia amazilia</i>	366	162027	http://arctos.database.museum/guid/MSB:Bird:31223
<i>Amazilia amazilia</i>	352	163017	http://arctos.database.museum/guid/MSB:Bird:31453
<i>Amazilia amazilia</i>	132	168989	http://arctos.database.museum/guid/MSB:Bird:33763
<i>Amazilia amazilia</i>	115	169303	http://arctos.database.museum/guid/MSB:Bird:34077
<i>Amazilia viridicauda</i>	3,005	159899	http://arctos.database.museum/guid/MSB:Bird:27227
<i>Amazilia viridicauda</i>	3,005	159900	http://arctos.database.museum/guid/MSB:Bird:27228
<i>Amazilia viridicauda</i>	3,005	159901	http://arctos.database.museum/guid/MSB:Bird:27229
<i>Amazilia viridicauda</i>	2,953	168478	http://arctos.database.museum/guid/MSB:Bird:33259
<i>Amazilia viridicauda</i>	2,953	168480	http://arctos.database.museum/guid/MSB:Bird:33261
<i>Amazilia viridicauda</i>	2,900	168488	http://arctos.database.museum/guid/MSB:Bird:33269
<i>Amazilia viridicauda</i>	2,953	168493	http://arctos.database.museum/guid/MSB:Bird:33274
<i>Coeligena coeligena</i>	2,052	163658	http://arctos.database.museum/guid/MSB:Bird:31986
<i>Coeligena coeligena</i>	2,052	163741	http://arctos.database.museum/guid/MSB:Bird:32069
<i>Coeligena coeligena</i>	2,131	163914	http://arctos.database.museum/guid/MSB:Bird:32242
<i>Coeligena coeligena</i>	2,100	163915	http://arctos.database.museum/guid/MSB:Bird:32243
<i>Coeligena coeligena</i>	2,052	167517	http://arctos.database.museum/guid/MSB:Bird:32345
<i>Coeligena coeligena</i>	2,240	167534	http://arctos.database.museum/guid/MSB:Bird:32362
<i>Coeligena coeligena</i>	2,052	167823	http://arctos.database.museum/guid/MSB:Bird:32651
<i>Coeligena violifer</i>	2,798	163129	http://arctos.database.museum/guid/MSB:Bird:31564
<i>Coeligena violifer</i>	2,778	163210	http://arctos.database.museum/guid/MSB:Bird:31645
<i>Coeligena violifer</i>	2,810	163213	http://arctos.database.museum/guid/MSB:Bird:31648
<i>Coeligena violifer</i>	3,710	163485	http://arctos.database.museum/guid/MSB:Bird:31813
<i>Coeligena violifer</i>	2,858	168451	http://arctos.database.museum/guid/MSB:Bird:33232
<i>Coeligena violifer</i>	3,688	169121	http

Table S4. Cont.

Species	Elevation (m)	NK tissue no.	MSB catalog number and direct Weblink to specimen data
<i>Phaethornis malaris</i>	353	162160	http://arctos.database.museum/guid/MSB:Bird:27711
<i>Phaethornis malaris</i>	350	162199	http://arctos.database.museum/guid/MSB:Bird:27743
<i>Phaethornis malaris</i>	360	162253	http://arctos.database.museum/guid/MSB:Bird:31267
<i>Phaethornis malaris</i>	323	162297	http://arctos.database.museum/guid/MSB:Bird:31273
<i>Phaethornis malaris</i>	365	162333	http://arctos.database.museum/guid/MSB:Bird:27859
<i>Phaethornis malaris</i>	317	162362	http://arctos.database.museum/guid/MSB:Bird:27883

The listed Weblinks to the Museum of Southwestern Biology (MSB) online catalog provide detailed locality information and ancillary data for each of the 70 specimens.

Other Supporting Information Files

[Dataset S1 \(PDF\)](#)

Dataset S1. Phylogeny and trait data for comparative analyses. (A) The phylogeny that was used for the ancestral state estimates of elevation and $\beta 13$ - $\beta 83$ genotype as depicted in Figure 4, in Newick format. Node labels are posterior probability estimates for each clade. Branch lengths are proportional to relative time. (B) The data used for comparative analyses of 63 hummingbird species, including the species' maximum elevation, minimum elevation, and the amino acids at positions $\beta 13$ and $\beta 83$.

A.

```
(apus_apus:42.5,((florisuga_mellivora:21.58251284,(eutoxeres_condamini:19.59179945,((threnetes_leuc
urus:7.593799152,glaucis_hirsutus:7.593799152)1.00:5.458735986,((phaethornis_syrmatophorus:7.0404
04114,(phaethornis_guy:4.536745179,phaethornis_malaris:4.536745179)0.87:2.503658935)1.00:0.7325
615839,(phaethornis_hispidus:7.526412547,phaethornis_ruber:7.526412547)0.94:0.2465531508)1.00:5.
27956944)1.00:6.539264308)1.00:1.990713397)1.00:0.8030054618,(((doryfera_ludovicae:12.78813896,(
schistes_geoffroyi:11.77392866,colibri_coruscans:11.77392866)1.00:1.014210299)1.00:5.76138279,anth
racothorax_nigricollis:18.54952174)1.00:1.5234818,(((haplophaedia_aureliae:11.4814989,(eriocnemis_a
linae:7.215116008,eriocnemis_luciani:7.215116008)1.00:4.266382893)1.00:2.348433693,((lafresnaya_la
fresnaya:11.83844458,aglaeactis_castelnaudii:11.83844458)0.88:1.356892144,(((ensifera_ensifera:10.2
1230199,pterophanes_cyanopterus:10.21230199)1.00:1.625966458,(boissonneaua_matthewsii:10.1139
4209,ocreatus_underwoodii:10.11394209)1.00:1.724326357)0.97:0.5522489052,(clytolaema_rubricauda:
9.184226467,((heliodoxa_imperatrix:4.600810376,heliodoxa_rubinoidea:4.600810376)1.00:0.485043223
3,heliodoxa_leadbeateri:5.085853599)1.00:4.098372868)0.99:3.206290882)1.00:0.4065088099,(coelige
na_coeligena:9.655568467,(coeligena_torquata:6.102444883,(coeligena_violifer:4.772037487,(coeligena
_iris:3.203338608,coeligena_lutetiae:3.203338609)1.00:1.568698878)1.00:1.330407396)1.00:3.5531235
83)1.00:3.141457693)0.73:0.3983105689)1.00:0.6345958663)1.00:1.671283968,(((lophornis_chalybeus:
2.267283906,lophornis_delattrei:2.267283906)1.00:5.543762026,(discosura_longicauda:4.997402833,dis
cosura_langsdorffi:4.997402834)1.00:2.813643099)1.00:6.403304237,(phlogophilus_harterti:13.2108949
2,((heliangelus_amethysticollis:3.530452247,(heliangelus_viola:3.261148903,heliangelus_micraster:3.26
1148903)0.99:0.269303344)1.00:8.587493192,((adelomyia_melanogenys:8.596382441,aglaiocercus_kin
gi:8.596382441)1.00:1.364615777,((oreotrochilus_estella:1.211047725,oreotrochilus_melanogaster:1.211
047726)1.00:6.627644559,(polyonymus_caroli:7.495200005,((chalcostigma_herrani:5.130842394,(chalc
ostigma_stanleyi:4.811536371,(chalcostigma_ruficeps:4.571696761,chalcostigma_olivaceum:4.5716967
61)0.46:0.23983961)0.86:0.3193060232)1.00:0.9657565811,(metallura_phoebae:4.476686487,metallura_
tyrianthina:4.476686487)1.00:1.619912488)1.00:1.398601031)0.94:0.3434922789)1.00:2.122305934)1.0
0:2.15694722)1.00:1.092949481)1.00:1.003455251)1.00:1.286866392)1.00:0.8534145773,(patagona_gi
gas:14.38065922,((eugenes_fulgens:11.524263,((calliphlox_amethystina:3.341953595,thaumastura_cora
:3.341953595)1.00:1.683541112,selasphorus_platycercus:5.025494707)1.00:6.498768296)1.00:2.33638
6548,(chlorostilbon_aureoventris:12.43311726,(thalurania_furcata:11.33366768,(taphrospilus_hypostictu
s:8.855630206,((amazilia_amazilia:4.105048477,chrysoronia_oenone:4.105048477)1.00:1.242588118,(a
mazilia_lactea:2.914837093,(amazilia_chionogaster:1.796389023,amazilia_viridicauda:1.796389023)1.0
0:1.118448069)1.00:2.432799503)1.00:3.507993611)1.00:2.478037477)1.00:1.099449577)1.00:1.42753
2291)1.00:0.520009674)1.00:1.973971915)1.00:3.718372406)1.00:2.312514759)1.00:20.1144817);
```

B.

Species	Maximum elevation (m)	Minimum elevation (m)	$\beta 13$	$\beta 83$
<i>Adelomyia melanogenys</i>	2900	1000	Gly	Gly
<i>Aglaeactis castelnaudii</i>	4200	3100	Gly	Ser
<i>Aglaiocercus kingi</i>	2600	1300	Gly	Gly
<i>Amazilia amazilia</i>	1000	0	Gly	Gly
<i>Amazilia chionogaster</i>	2800	0	Gly	Ser
<i>Amazilia lactea</i>	900	0	Gly	Gly
<i>Amazilia viridicauda</i>	3100	1000	Gly	Ser

<i>Anthracothonax nigricollis</i>	1000	0	Gly	Gly
<i>Apus apus</i>	3300	0	Gly	Ser
<i>Boissonneaua matthewsii</i>	2700	1550	Gly	Ser
<i>Calliphlox amethystina</i>	1050	0	Gly	Gly
<i>Chalcostigma herrani</i>	3400	2700	Gly	Ser
<i>Chalcostigma olivaceum</i>	4500	3150	Ser	Ser
<i>Chalcostigma ruficeps</i>	3300	2250	Gly	Ser
<i>Chalcostigma stanleyi</i>	4400	3350	Ser	Ser
<i>Chlorostilbon aureoventris</i>	2500	0	Gly	Gly
<i>Chrysuronia oenone</i>	1650	0	Gly	Gly
<i>Clytolaema rubricauda</i>	2000	750	Gly	Gly
<i>Coeligena coeligena</i>	2200	1000	Gly	Gly
<i>Coeligena iris</i>	3500	1500	Gly	Ser
<i>Coeligena lutetiae</i>	3750	3000	Gly	Ser
<i>Coeligena torquata</i>	2800	1700	Gly	Gly
<i>Coeligena violifer</i>	3900	2500	Gly	Ser
<i>Colibri coruscans</i>	3600	1300	Gly	Ser
<i>Discosura langsdorffi</i>	800	0	Gly	Gly
<i>Discosura longicauda</i>	700	0	Gly	Gly
<i>Doryfera ludovicae</i>	2800	1200	Gly	Asn
<i>Ensifera ensifera</i>	3600	2200	Gly	Ser
<i>Eriocnemis alinae</i>	2800	2000	Gly	Ser
<i>Eriocnemis luciani</i>	3750	2600	Gly	Ser
<i>Eugenes fulgens</i>	3300	1300	Gly	Ser
<i>Eutoxeres condamini</i>	2750	0	Gly	Gly
<i>Florisuga mellivora</i>	900	0	Gly	Gly
<i>Glaucis hirsutus</i>	1100	0	Gly	Gly
<i>Haplophaedia aureliae</i>	2500	1400	Gly	Gly
<i>Heliangelus amethysticollis</i>	3300	1800	Gly	Gly
<i>Heliangelus micraster</i>	2900	2400	Gly	Gly
<i>Heliangelus viola</i>	3050	2150	Gly	Gly
<i>Heliodoxa imperatrix</i>	1800	900	Gly	Gly
<i>Heliodoxa leadbeateri</i>	2300	1050	Gly	Gly
<i>Heliodoxa rubinoides</i>	2650	1700	Gly	Gly
<i>Lafresnaya lafresnayi</i>	3350	2300	Gly	Ser
<i>Lophornis chalybeus</i>	600	0	Gly	Gly
<i>Lophornis delattrei</i>	2000	0	Gly	Gly
<i>Metallura phoebe</i>	4400	2500	Gly	Ser
<i>Metallura tyrianthina</i>	3500	2400	Gly	Ser
<i>Ocreatus underwoodii</i>	2500	1050	Gly	Ser
<i>Oreotrochilus estella</i>	4600	3400	Ser	Ser
<i>Oreotrochilus melanogaster</i>	4800	3500	Ser	Ser
<i>Patagona gigas</i>	4300	0	Ser	Ser

<i>Phaethornis guy</i>	2000	800	Gly	Gly
<i>Phaethornis hispidus</i>	500	0	Gly	Gly
<i>Phaethornis malaris</i>	1300	0	Gly	Gly
<i>Phaethornis ruber</i>	900	0	Gly	Gly
<i>Phaethornis syrmatophorus</i>	2400	1100	Gly	Gly
<i>Phlogophilus harterti</i>	1200	750	Gly	Ala
<i>Polyonymus caroli</i>	2800	1500	Gly	Ser
<i>Pterophanes cyanopterus</i>	3700	2600	Gly	Ser
<i>Schistes geoffroyi</i>	2250	1100	Gly	Gly
<i>Selasphorus platycercus</i>	3750	1900	Gly	Ser
<i>Taphrospilus hypostictus</i>	1350	750	Gly	Gly
<i>Thalurania furcata</i>	1700	0	Gly	Ser
<i>Thaumastura cora</i>	3000	0	Gly	Ser
<i>Threnetes leucurus</i>	1050	0	Gly	Gly
