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Chandrasekhar Natarajan
University of Nebraska-Lincoln, chandrasekhar.natarajan@unl.edu

Federico G. Hoffmann
University of Nebraska - Lincoln, fhoffmann2@unl.edu

Roy E. Weber
Aarhus University, roy.weber@bios.au.dk

Angela Fago
Aarhus University, Denmark, angela.fago@biology.au.dk

Christopher C. Witt
University of New Mexico, cwitt@unm.edu

See next page for additional authors

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Natarajan, Chandrasekhar; Hoffmann, Federico G.; Weber, Roy E.; Fago, Angela; Witt, Christopher C.; and Storz, Jay F., "Predictable convergence in hemoglobin function has unpredictable molecular underpinnings" (2016). *Jay F. Storz Publications*. 65.
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Authors
Chandrasekhar Natarajan, Federico G. Hoffmann, Roy E. Weber, Angela Fago, Christopher C. Witt, and Jay F. Storz

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EVOLUTION

Predictable convergence in hemoglobin function has unpredictable molecular underpinnings

Chandrasekhar Natarajan,[1] Federico G. Hoffmann,[2] Roy E. Weber,[3] Angela Fago,[3] Christopher C. Witt,[4] Jay F. Storz[1]*

To investigate the predictability of genetic adaptation, we examined the molecular basis of convergence in hemoglobin function in comparisons involving 56 avian taxa that have contrasting altitudinal range limits. Convergent increases in hemoglobin-oxygen affinity were pervasive among high-altitude taxa, but few such changes were attributable to parallel amino acid substitutions at key residues. Thus, predictable changes in biochemical phenotype do not have a predictable molecular basis. Experiments involving resurrected ancestral proteins revealed that historical substitutions have context-dependent effects, indicating that possible adaptive solutions are contingent on prior history. Mutations that produce an adaptive change in one species may represent precluded possibilities in other species because of differences in genetic background.

A fundamental question in evolutionary genetics concerns the extent to which adaptive convergence in phenotype is caused by convergent or parallel changes at the molecular sequence level. This question has important implications for understanding the inherent repeatability (and, hence, predictability) of molecular adaptation. One especially powerful approach for addressing this question involves the examination of phylogenetically replicated changes in protein function that can be traced to specific amino acid replacements. If adaptive changes in protein function can only be produced by a small number of possible mutations at a small number of key sites—representing "forced moves" in genotype space—then evolutionary outcomes may be highly predictable. Alternatively, if adaptive changes can be produced by numerous possible mutations—involving different structural or functional mechanisms, but achieving equally serviceable results—then evolutionary outcomes may be more idiosyncratic and unpredictable (*1*–*4*). The probability of replicated substitution at the same site in different species may be further reduced by context-dependent mutational effects (epistasis), because a given mutation will only contribute to adaptive convergence if it retains a beneficial effect across divergent genetic backgrounds (*4*).

To assess the pervasiveness of parallel molecular evolution and to investigate its causes, we examined replicated changes in the oxygenation properties of hemoglobin (Hb) in multiple bird

[1]School of Biological Sciences, University of Nebraska, Lincoln, NE 68588, USA. [2]Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology and Institute for Genomics, Biocomputing, and Biotechnology, Mississippi State University, Mississippi State, MS 39762, USA. [3]Zoophysiology, Department of Bioscience, Aarhus University, DK-8000 Aarhus, Denmark. [4]Department of Biology and Museum of Southwestern Biology, University of New Mexico, Albuquerque, NM 87131, USA.
***Corresponding author. E-mail: jstorz2@unl.edu**

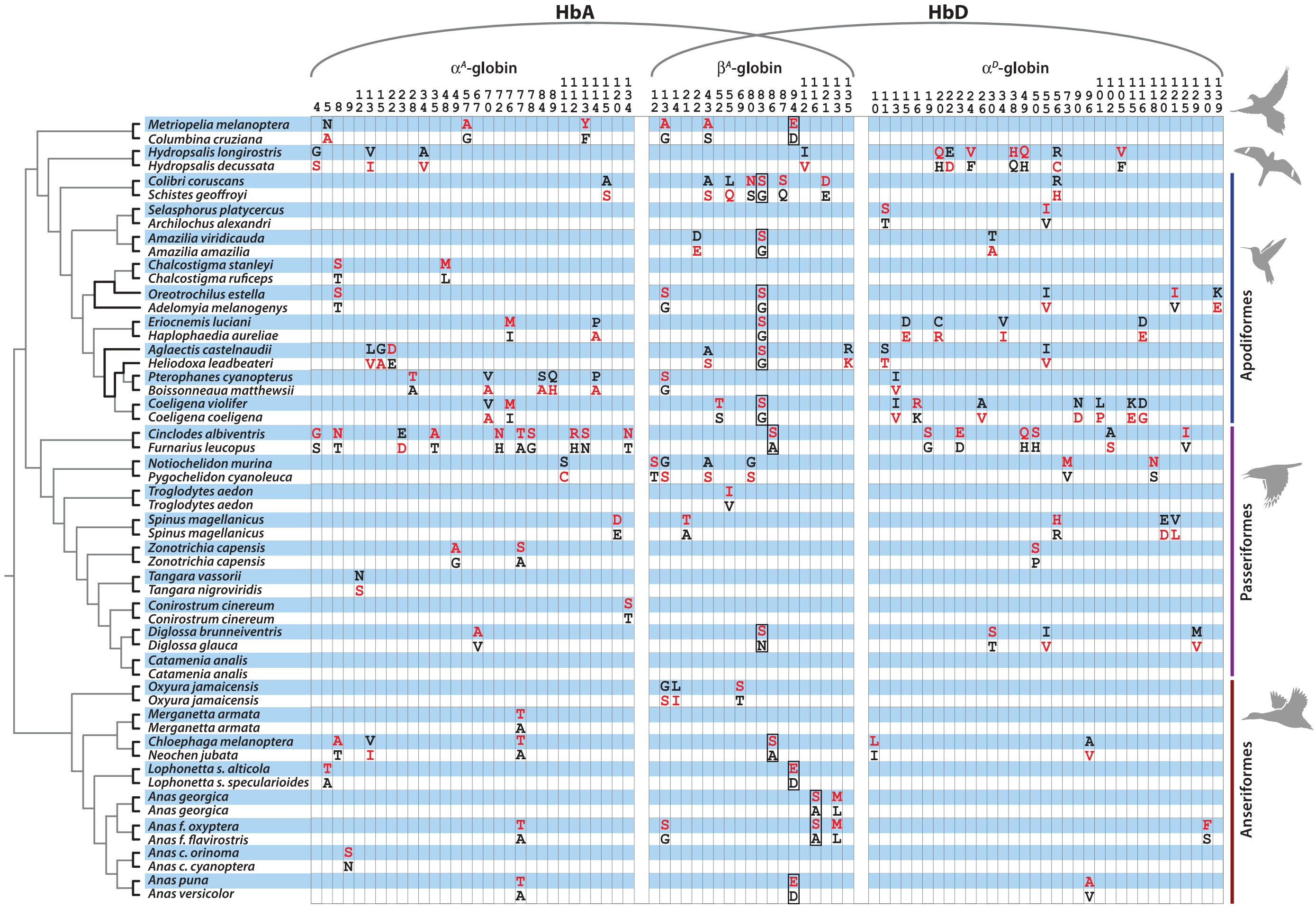

Fig. 1. Amino acid differences that distinguish the Hbs of each pair of high- and low-altitude taxa. Derived (nonancestral) amino acids are shown in red lettering, and rows corresponding to high-altitude taxa are shaded in blue. Subunits of the major HbA isoform are encoded by the α^A- and β^A-globin genes, whereas those of the minor HbD isoform are encoded by the α^D- and β^A-globin genes. Phylogenetically replicated β-chain replacements that contribute to convergent increases in Hb-O_2 affinity (N/G83S, A86S, D94E, and A116S) are outlined. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

species that have independently colonized high-altitude environments. Specifically, we tested whether high-altitude taxa have convergently evolved derived increases in Hb-O_2 affinity, and we assessed the extent to which such changes are attributable to parallel amino acid substitutions. We performed comparisons of Hb function in 56 avian taxa making up 28 pairs of high- and low-altitude lineages (table S1). The comparisons involved pairs of species or conspecific populations that are native to different altitudes.

Under severe hypoxia, an increased Hb-O_2 affinity can help sustain tissue O_2 delivery by safeguarding arterial O_2 saturation while simultaneously maintaining the pressure gradient for O_2 diffusion from capillary blood to the tissue mitochondria, so altitude-related changes in Hb function likely have adaptive relevance (*5*, *6*). Evolutionary increases in Hb-O_2 affinity can be caused by amino acid mutations that increase intrinsic O_2 affinity and/or mutations that suppress the sensitivity of Hb to the inhibitory effects of allosteric effectors such as Cl^- ions and organic phosphates (*5*, *7*).

In a highly influential paper on biophysical mechanisms of protein evolution, Perutz (*7*) predicted that adaptive changes in functional properties of vertebrate Hb are typically attributable to "a few replacements at key positions." According to Perutz, amino acid substitutions that can be expected to make especially important contributions to evolutionary changes in Hb-O_2 affinity involve heme-protein contacts (affecting intrinsic heme reactivity), intersubunit contacts (affecting the oxygenation-linked, allosteric transition in quaternary structure), and binding sites for allosteric effectors (*7*). If Perutz is correct that adaptive modifications of Hb function are typically attributable to a small number of substitutions at key positions, then it follows that the same mutations will be preferentially fixed in different species that have independently evolved Hbs with similar functional properties. For example, in high-altitude vertebrates that have convergently evolved elevated Hb-O_2 affinities, Perutz's hypothesis predicts that parallel amino acid substitutions should be pervasive.

Most bird species express two tetrameric ($\alpha_2\beta_2$) Hb isoforms in adult red blood cells: (i) the major hemoglobin A (HbA) isoform, which incorporates α-chain products of the α^A-globin gene, and (ii) the minor HbD isoform, which incorporates products of the closely linked α^D-globin gene. Both isoforms share the same β-chain subunits. By cloning and sequencing the adult-expressed globin genes, we identified all amino acid differences that distinguish the Hbs of each pair of high- and low-altitude taxa. The comparative sequence data revealed phylogenetically replicated replacements at numerous sites in the α^A-, α^D-, and β^A-globins (Fig. 1 and figs. S1 and S2).

After identifying the complete set of Hb substitutions that distinguish each pair of high- and low-altitude taxa, we experimentally assessed how many of the replicated amino acid replacements actually contributed to convergent changes in Hb function. To characterize the functional mechanisms that are responsible for evolved changes in Hb-O_2 affinity, we measured P_{50} (the O_2 partial pressure at which Hb is 50% saturated) of purified Hbs in the presence and absence of Cl^- ions and the organic phosphate inositol hexaphosphate (IHP) (*8*). We focus on measures of P_{50} in the

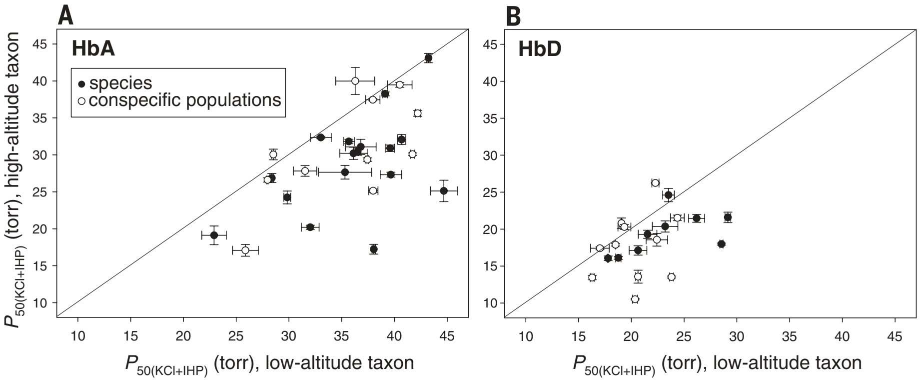

Fig. 2. Convergent increases in Hb-O_2 affinity in high-altitude Andean birds. (**A**) Plot of $P_{50(KCl+IHP)}$ (± 1 SE) for HbA in 28 matched pairs of high- and low-altitude taxa. Data points that fall below the diagonal line (*x* = *y*) denote cases in which the high-altitude member of a given taxon pair possesses a higher Hb-O_2 affinity (lower P_{50}). Comparisons involve replicated pairs of taxa, so all data points are phylogenetically independent. (**B**) Plot of $P_{50(KCl+IHP)}$ (± 1 SE) for the minor HbD isoform in a subset of the same taxon pairs in which both members of the pair express HbD. $P_{50(KCl+IHP)}$, O_2 partial pressure at which Hb is 50% saturated in the presence of chloride and inositol hexaphosphate.

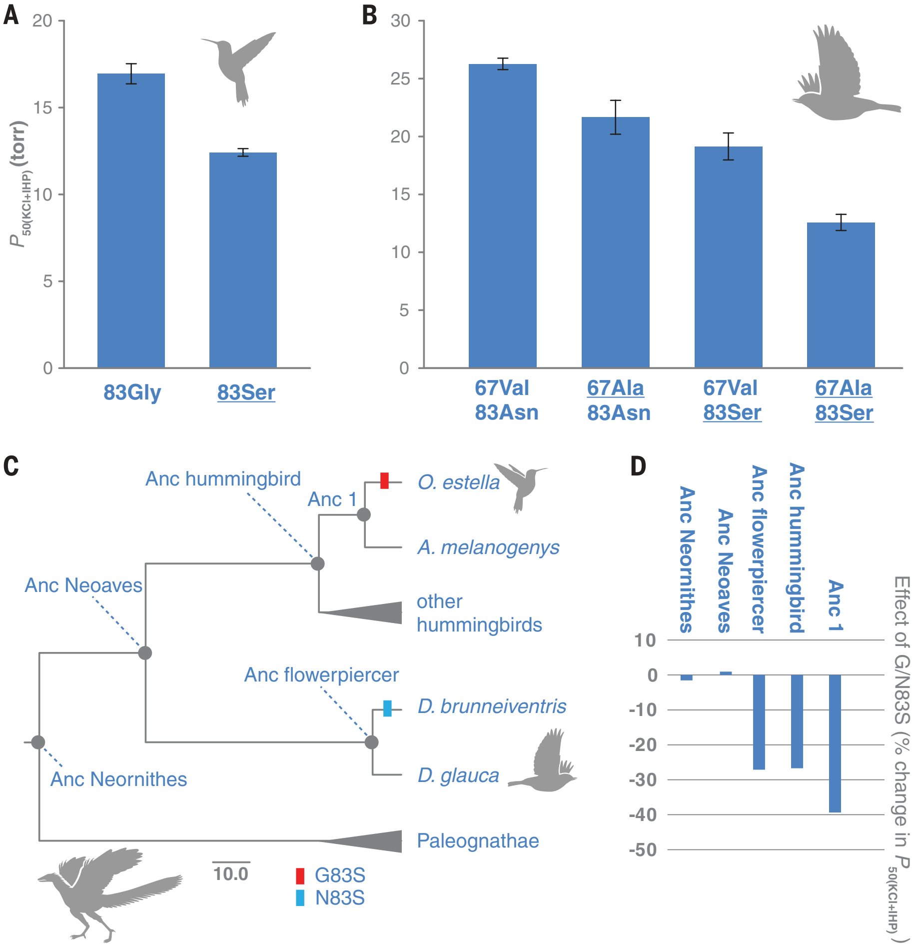

Fig. 3. Phenotypic effects of substitutions at β83 are conditional on genetic background. (**A**) The engineered G83S mutation produced a significant reduction in $P_{50(KCl+IHP)}$ (increase in Hb-O_2 affinity) in the reconstructed Hb of the hummingbird ancestor. (**B**) The engineered A67V and N83S mutations produced additive reductions in $P_{50(KCl+IHP)}$ in the reconstructed Hb of the flowerpiercer ancestor. Underlining indicates derived (nonancestral) amino acids. (**C**) Diagrammatic tree with time-scaled branch lengths showing internal nodes that we targeted for ancestral protein resurrection. Scale bar, 10 million years. (**D**) N/G83S mutations produced significant increases in Hb-O_2 affinity (expressed as reductions in $P_{50(KCl+IHP)}$) in the ancestors of hummingbirds and flowerpiercers. Substitutions at the same site produced no detectable effects in Anc Neoaves or Anc Neornithes.

presence of Cl^- and IHP, because this experimental treatment is most relevant to in vivo conditions in avian red blood cells.

HbD exhibited uniformly higher O_2 affinities than HbA in all examined taxa (table S2), consistent with results of previous studies (*9*–*13*). This consistent pattern of isoform differentiation suggests that up-regulating the expression of HbD could provide a ready means of increasing blood O_2 affinity. However, our results demonstrate that this regulatory mechanism does not play a general role in hypoxia adaptation, because there was no consistent trend of increased HbD expression among high-altitude taxa (Wilcoxon signed-rank test, $Z = -0.775$, $P = 0.441$, $n = 26$; table S3 and fig. S3).

Phylogenetically independent comparisons involving all 28 taxon pairs revealed that highland natives have generally evolved an increased Hb-O_2 affinity relative to that of their lowland counterparts, a pattern that is consistent for both HbA (Wilcoxon's signed-rank test, $Z = -4.236$, $P < 0.0001$, $n = 28$; Fig. 2A and table S2) and HbD ($Z = -2.875$, $P = 0.0041$, $n = 20$; Fig. 2B and table S2). In all pairwise comparisons in which the high-altitude taxa exhibited significantly higher Hb-O_2 affinities ($n = 22$ taxon pairs for HbA and 15 taxon pairs for HbD), the measured differences were almost entirely attributable to differences in intrinsic O_2 affinity, rather than differences in sensitivity to Cl^- or IHP (table S4). Thus, genetically based increases in Hb-O_2 affinity were not generally associated with a diminution of allosteric regulatory capacity (i.e., O_2 affinity could still be modulated by erythrocytic changes in anion concentrations), in contrast to the case with some high-altitude mammals (*5*, *14*, *15*).

Results of experiments based on both native Hb variants and engineered, recombinant Hb mutants revealed that only a subset of replicated replacements actually contributed to convergent increases in Hb-O_2 affinity in high-altitude taxa (table S5). These include replicated replacements at just four β-chain sites: N/G83S, A86S, D94E, and A116S. β116 is an $\alpha_1\beta_1$ intersubunit contact, and β94 plays a key role in allosteric proton binding; neither of the other replicated replacements—and few of the affinity-enhancing nonreplicated replacements—involved heme contacts, intersubunit contacts, or canonical binding sites for allosteric effectors.

Our experiments revealed a striking pattern of convergence in the oxygenation properties of Hb in high-altitude natives (Fig. 2, A and B), and, in several cases, convergent increases in Hb-O_2 affinity were caused by parallel substitutions at key residues that mediate protein allostery (e.g., D94E in the β-chains of high-altitude ground doves and waterfowl; Fig. 1 and table S5). However, in the majority of cases, convergent increases in Hb-O_2 affinity were attributable to nonreplicated substitutions and/or parallel substitutions at sites that are not considered "key residues" (e.g., N/G83S in the β-chains of high-altitude hummingbirds and flowerpiercers; Fig. 1). Clearly, evolutionary increases in Hb-O_2 affinity can be produced by amino acid substitutions at numerous sites.

These findings expose a clear demarcation between the realms of chance and necessity at different hierarchical levels. At the level of biochemical phenotype, and even at the level of functional mechanism, evolutionary changes are highly predictable. At the amino acid level, in contrast, predictability breaks down.

In addition to the many-to-one mapping of genotype to phenotype, the phylogenetic distribution of affinity-enhancing parallel substitutions suggests another possible explanation for the limited contribution of such substitutions to convergent functional changes in the Hbs of distantly related species. The most striking functional parallelism at the amino acid level was concentrated in the hummingbird clade. Replicated G83S substitutions contributed to convergent increases in Hb-O_2 affinity in multiple high-altitude hummingbird species (table S5 and fig. S4) (*16*), and a convergent substitution at the same site (N83S) occurred in one other (nonhummingbird) high-altitude species: the black-throated flowerpiercer, *Diglossa brunneiventris*. One possible explanation for this phylogenetically concentrated pattern of parallelism is that the mutation's phenotypic effect is conditional on genetic background, so the same mutation produces different effects in different species.

To test this hypothesis, we used ancestral sequence reconstruction in combination with site-directed mutagenesis to test the effect of β83 substitutions in a set of distinct genetic backgrounds. We first resurrected HbA of the common ancestor of hummingbirds ("Anc hummingbird") (figs. S5 to S7), and we confirmed that G83S has a significant affinity-enhancing effect on this ancestral genetic background (Fig. 3A). This result is consistent with the affinity-enhancing effect of G83S in numerous descendant lineages of high-altitude hummingbirds (table S5 and fig. S4). In similar fashion, we resurrected HbA of the common ancestor of the high- and low-altitude flowerpiercers ("Anc flowerpiercer") to test the effect of N83S (fig. S7). Hbs of the two flowerpiercers differed at two sites because of substitutions in the *D. brunneiventris* lineage (V67A in α^A-globin, in addition to N83S in β^A-globin; Fig. 1). We therefore synthesized a total of four recombinant Hb mutants, representing each possible genotypic combination of the two substituted sites, to measure the relative contributions of V67A and N83S to the evolved increase in Hb-O_2 affinity in *D. brunneiventris* (table S2 and fig. S4). The tests showed that both mutations increased Hb-O_2 affinity in an additive fashion (Fig. 3B). We then engineered the same N83S mutation into resurrected ancestral Hbs representing two far more ancient nodes in the avian phylogeny: the reconstructed common ancestor of Neoaves ("Anc Neoaves") and the common ancestor of all extant birds ("Anc Neornithes") (Fig. 3C and figs. S5, S7, S8, and S9). In contrast to the highly significant effects of N/G83S in hummingbird and flowerpiercer Hbs, N83S produced no detectable effect in Anc Neoaves or Anc Neornithes (Fig. 3D and table S6). The ancestral hummingbird and flowerpiercer Hbs contained 18 and 32 amino acid states, respectively, that were not present in Anc Neornithes (fig. S7), representing net sequence differences that accumulated over a ~100-million-year time period. The context-dependent effects of N/G83S indicate that lineage-specific substitutions in the ancestry of hummingbirds and flowerpiercers produced a genetic background in which mutations at β83 could contribute to an adaptive increase in Hb-O_2 affinity. This adaptive solution was apparently not an option in the deeper ancestry of birds and may also represent a precluded possibility in contemporary, high-altitude members of other avian lineages.

These findings reveal a potentially important role of contingency in adaptive protein evolution. In different species that are adapting to the same selection pressure, the set of possible amino acids at a given site that have unconditionally beneficial effects may be contingent on the set of antecedent substitutions that have independently accumulated in the history of each lineage. Consequently, possible options for adaptive change in one species may be foreclosed options in other species.

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ACKNOWLEDGMENTS

This work was funded by grants from the U.S. NIH (HL087216), the U.S. NSF (IOS-0949931, MCB-1517636, and MCB-1516660), and the Danish Council for Independent Research (10-084-565 and 4181-00094). We thank E. Petersen, H. Moriyama, and A. Kumar for assistance in the laboratory and C. Meiklejohn and K. Montooth for helpful suggestions. All experimental data are tabulated in the supplementary materials, and sequence data are archived in GenBank under accession numbers KX240692 to KX241466.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6310/336/suppl/DC1
Materials and Methods
Figs. S1 to S9
Tables S1 to S6
References (*17*–*33*)

19 April 2016; accepted 20 July 2016
10.1126/science.aaf9070

Supplementary Materials for

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Chandrasekhar Natarajan, Federico G. Hoffmann, Roy E. Weber, Angela Fago, Christopher C. Witt, Jay F. Storz*

*Corresponding author. E-mail: jstorz2@unl.edu

Published 21 October 2016, *Science* **354**, 336 (2016)
DOI: 10.1126/science.aaf9070

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Materials and Methods

Specimen Collection

We preserved blood and tissue samples from vouchered bird specimens collected from localities spanning a broad range of elevations in the Andes and Southern Rocky Mountains (table S1). Collection and sample preservation protocols for all waterfowl taxa were described by Natarajan et al. (*12*). All specimens were live-trapped in mistnets and were bled and humanely killed in accordance with guidelines of the Ornithological Council (*17*), 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 research was conducted in accordance with permits issued by management authorities (Peru: 76-2006-INRENA-IFFS-DCB, 087-2007-INRENA-IFFS-DCB, 135-2009-AG-DGFFS-DGEFFS, 0377-2010-AG-DGFFS-DGEFFS, 0199-2012-AG-DGFFS-DGEFFS, and 006-2013-MINAGRI-DGFFS/DGEFFS; New Mexico, USA: NMDGF-3217 and USFWSMB094297-0).

For each individual bird specimen, we collected whole blood from the brachial or ulnar vein using heparinized microcapillary tubes. Red blood cells were separated from the plasma fraction via centrifugation, and the packed red cells were then flash-frozen in liquid nitrogen and were stored at -80°C prior to the isolation and purification of Hb components for the functional experiments. We collected liver and pectoral muscle from each specimen as sources of genomic DNA and globin mRNA, respectively. Tissue samples were either flash-frozen or preserved in RNAlater 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.

Cloning and Sequencing of Globin Genes

In 3-14 individual specimens from each of the nonwaterfowl species (median N = 7 individuals), including all specimens used as subjects in the experimental analyses of Hb function, we extracted RNA from whole blood using the RNeasy kit, and we amplified full-length cDNAs of the α^A-, α^D-, and β^A-globin genes using a OneStep RT-PCR kit (Qiagen, Valencia, CA, USA). Sample sizes for the waterfowl species are reported in Natarajan et al. (*12*). We designed paralog-specific primers using 5' and 3' UTR sequences, as described previously (*9-13*, *16, 18)*. We cloned reverse transcription (RT)-PCR products into pCR4-TOPO vector using the TOPO® TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA), and we sequenced at least five clones per gene in each individual in order to recover both alleles. This enabled us to determine full diploid genotypes for each of the three adult-expressed globin genes in each individual specimen. All new sequences were deposited in GenBank under the accessions numbers KX240692 to KX241466.

Characterization of Hb isoform Composition

We used a combination of tandem mass spectrometry (MS/MS) and isoelectric focusing (IEF) to characterize the Hb isoform composition of red blood cells from the same specimens used in the survey of DNA sequence variation. Native Hb components were separated by means of IEF using precast Phast gels (pH 3–9)(GE Healthcare Bio-

Sciences, Pittsburgh, PA, USA; 17-0543-01). IEF gel bands were then excised and digested with trypsin, and MS/MS was used to identify the resultant peptides, as described previously (*13*, *14*, *18*, *19*). Database searches of the resultant MS/MS spectra were performed using Mascot (Matrix Science, v1.9.0, London, UK); peptide mass fingerprints were queried against a custom database of avian globin sequences, including the full complement of embryonic and adult α- and β-type globin genes that we previously annotated in avian genome assemblies (*13*, *20*-*22*). We identified all significant protein hits that matched more than one peptide with $P<0.05$. After separating HbA and HbD isoforms by native gel IEF, the relative abundance of the two isoforms in each individual hemolysate was quantified densitometrically using Image J (*23*).

Protein Purification and *In Vitro* Analysis of Hb Function

Hemolysates of individual specimens were dialyzed overnight against 20 mM Tris buffer (pH 8.4), and the two tetrameric HbA and HbD isoforms were then separated using a HiTrap Q-HP column (GE Healthcare; 1 ml 17-1153-01) and equilibrated with 20 mM Tris buffer (pH 8.4). HbD was eluted against a linear gradient of 0-200 mM NaCl. The samples were desalted by means of dialysis against 10 mM HEPES buffer (pH 7.4) at 4°C, and were then concentrated by using a 30 kDa centrifuge filter (Amicon, EMD Millipore). In the case of hummingbirds and several of the small passerine species, HbA and HbD were isolated and purified from pooled hemolysates of 3-7 individuals that had identical globin genotypes (*10*, *11*, *16*).

We measured O_2-equilibria of 3 μL thin-film samples in a modified diffusion chamber where absorption at 436 nm was monitored during stepwise changes in equilibration gas mixtures generated by precision Wösthoff gas-mixing pumps (*9*, *18*, *24*). In order to characterize intrinsic Hb-O_2 affinities and mechanisms of allosteric regulatory control, we measured O_2-equilibria in the presence of Cl^- ions (0.1M KCl), in the presence of IHP (IHP/Hb tetramer ratio = 2.0), in the simultaneous presence of both effectors, and in the absence of both effectors (stripped). Free Cl^- concentrations were measured with a model 926S Mark II chloride analyzer (Sherwood Scientific Ltd, Cambridge, UK).

The two ground dove species (*Metriopelia melanoptera* and *Columbina cruziana*) expressed no trace of HbD, and several hummingbird species expressed HbD at exceedingly low levels (table S3). In such cases, sufficient quantities of HbD could not be purified for measures of O_2-equilibria, which is why sample sizes for measures of O_2-binding properties are larger for HbA than for HbD (table S2).

We previously reported O_2-binding data for several taxa that were included in the present study, including HbA data for seven of the 18 hummingbird species (*16*), and HbA and HbD data for rufous-collared sparrows (*Zonotrichia capensis*)(*10*), house wrens (*Troglodytes aedon*)(*11*), and all waterfowl taxa (*12*).

Ancestral Sequence Reconstruction

We reconstructed the α^A- and β^A-globin sequences of four ancestral Hbs (Anc Neornithes, Anc Neoaves, Anc flowerpiercer, Anc hummingbird, and Anc 1 (the common ancestor of the Andean hillstar hummingbird, *Oreotrochilus estella*, and the speckled hummingbird, *Adelomyia melanogenys*)(Fig. 3C). Anc flowerpiercer is the common ancestor of the black-throated flowerpiercer, *Diglossa* brunneiventris, and the deep blue flowerpiercer, *D. glauca*, and it also represents the common ancestor of flowerpiercers as a group. We estimated each of the ancestral amino acid sequences using the maximum likelihood (ML) approach implemented in PAML version 4.8 (*25*). To reconstruct α^A- and β^A-globin sequences of Anc Neornithes and Anc Neoaves, we selected a set of orthologous globins from a phylogenetically balanced set of avian taxa, and we included a diverse set of paralogous sequences from other birds and/or other sauropsid outgroup taxa. We included an especially diverse set of paralogous outgroup sequences in the reconstruction of ancestral β^A-globins sequences because avian β-type globin genes represent the products of repeated rounds of lineage-specific duplication events (*20*). In all cases we used annotated globin genes from high-coverage genome assemblies in addition to sequences that we generated for a number of key taxa.

For each of the ancestral reconstructions, globin sequences were arranged in accordance with well-supported species trees. For the various sets of orthologous bird sequences, we constructed supertrees by starting with a backbone provided by a total-evidence phylogeny from Jarvis *et al*. (*26*). We were able to unambiguously assign sequences from each species to its appropriate branch in this backbone tree. Subtrees for each branch were obtained from McGuire *et al*. (*27*) and the supertree of Jetz et al. (*28*), which was constructed using the Hackett *et al*. (*29*) backbone. Relationships among the major groups of sauropsids were based on the phylogeny in Green et al. (*30*). Tree topologies used for the sequence reconstructions of Anc hummingbird are shown in figs. S5 and S6. Those used for each of the sequence reconstructions of Anc Neornithes and Anc Neoaves are shown in figs. S8 and S9. The ancestral sequences were estimated with high levels of statistical confidence. Posterior probabilities for estimated character states at all sites in the globin sequences of Anc Neornithes, Anc Neoaves, Anc flowerpiercer, Anc hummingbird, and Anc 1 are reported in fig. S7. Since the reconstructed α- and β-chain sequences of Anc Neoaves (the clade containing all modern birds except Paleognathae [ratites and tinamous] and Galloanserae [landfowl and waterfowl]) were identical to the reconstructed sequences for the common ancestor of Neognathae (the clade containing all modern birds except Paleognathae), our experimental measurements of the Anc Neoaves rHb also apply to the more ancient 'Anc Neognathae'. Reconstructions of Anc 1 and Anc flowerpiercer were unambiguous. HbA isoforms of the two hummingbird species differed at three sites, and those of the two flowerpiercers differed at two (Fig. 1). In both pairs of species, each of the inferred substitutions occurred in the high-altitude lineage, so the ancestral genotypes were identical to the wildtype genotypes of the low-altitude members of each pair (*A. melanogenys* in the case of the hummingbirds, and *D. glauca* in the case of the flowerpiercers).

To infer the polarity of character-state changes for each amino acid replacement between each pair of high- and low-altitude sister taxa (Fig. 1), we estimated the relevant ancestral character states using tailored sets of sequence data for specific clades. For example, for the nine pairs of high- and low altitude hummingbirds, we aligned globin sequences from each of the 18 focal taxa with orthologous sequences from a phylogenetically balanced and diverse set of hummingbirds and non-hummingbird outgroup species (*13*, *16*), including the full set of sequence data used to estimate Anc hummingbird (figs. S5-S6). Likewise, for the nine pairs of passerine taxa, we aligned globin sequences from each of the 18 focal taxa with orthologous sequences from a phylogenetically balanced and diverse set of other passerines and non-passerine outgroup species (*11*, *13*), including all relevant sequences used in the ancestral state estimates for Anc Neornithes and Anc Neoaves (figs. S8-S9). We followed this same basic approach for the pair of ground dove species (Columbiformes), the pair of nightjar species (Caprimulgiformes), and the eight pairs of waterfowl taxa (Anseriformes). In this latter case, we aligned globin sequence data from the 16 focal taxa with the extensive set of waterfowl sequence data reported in Natarajan et al. (*12*). Inferences of character polarity were typically unambiguous, which is not surprising since the sister taxa comprising each pairwise comparison were very closely related. As would be expected, character polarity was particularly unambiguous in the 10 pairwise comparisons involving conspecific populations or nominal subspecies.

Vector Construction and Site-Directed Mutagenesis

The reconstructed α^A- and β^A-globin sequences of Anc Neornithes, Anc Neoaves, Anc flowerpiercer, Anc hummingbird, and Anc 1 were optimized according to *E. coli* codon preferences, and each α^A-β^A globin gene cassette was synthesized by Eurofins MWG Operon (Huntsville, AL, USA). The same procedure was followed for the α^A- and β^A-globin sequences of Anc 1 (the common ancestor of the hummingbirds *Oreotrochilus estella* and *Adelomyia melanogenys*). The α^A- and β^A-globin cassettes were cloned into a custom pGM vector system, as described previously (*31-33*). Codon changes were engineered using the QuikChange II XL Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA, USA); all such changes were verified by DNA sequencing.

Expression and Purification of Recombinant Hbs

Recombinant Hb expression was carried out in the JM109 (DE3) *E. coli* strain. To ensure that N-terminal methionines were post-translationally cleaved from the nascent globin chains, we co-transformed a plasmid (pCO-MAP) containing an additional copy of the *methionine aminopeptidase* (*MAP*) gene along with a kanamycin resistance gene (*16*, *31-33*). Both pGM and pCO-MAP plasmids were cotransformed and subject to dual selection in an LB agar plate containing ampicillin and kanamycin. The expression of each rHb mutant was carried out in 1.5 L of TB medium. Bacterial cells were grown in 37ºC in an orbital shaker at 200 rpm until absorbance values reached 0.60.8 at 600 nm. The bacterial cultures were induced by 0.2 mM IPTG and were then supplemented with hemin (50 µg/ml) and glucose (20 g/L). The bacterial culture conditions and the protocol for preparing cell lysates were described previously (*10-12*, *16*, *31-33*).

Bacterial cells were resuspended in lysis buffer (50 mM Tris, 1 mM EDTA, 0.5 mM DTT, pH 7.6) with lysozyme (1 mg/g wet cells) and were incubated in an ice bath for 30 min. Following sonication of the cells, 0.5-1.0% polyethyleneimine solution was added, and the crude lysate was then centrifuged at 13,000 rpm for 45 min at 4°C. The rHbs were purified by two-step ion-exchange chromatography. Using high-performance liquid chromatography, the samples were passed through a cation exchange-column (SP-Sepharose) followed by passage through an anion-exchange column (Q-Sepharose). The clarified supernatant was subjected to overnight dialysis in HEPES buffer (20 mM HEPES with 0.5mM EDTA, 1 mM DTT pH 7.0) at 4°C. We used prepackaged SP-Sepharose columns (HiTrap SPHP, 5 mL, 17-516101; GE Healthcare) equilibrated with HEPES buffer (20 mM HEPES with 0.5mM EDTA, 1 mM DTT pH 7.0). The *Diglossa* rHb mutants were purified using HEPES buffer with pH 7.4 and – due to differences in Hb net charge – the rHb mutants of Anc Neornithes, Anc Neoaves and Anc hummingbird were purified using HEPES buffer with pH 7.0. The samples were passed through the column and the rHb solutions were eluted against a linear gradient of 0-1.0 M NaCl. The eluted samples were desalted and dialyzed overnight against the Tris buffer (20 mM Tris, 0.5mM EDTA, 1 mM DTT pH 8.4) at 4°C for the second column. Dialyzed samples were then passed through a pre-equilibrated Q-Sepharose column (HiTrap QHP, 1 mL, 17-5158-01; GE Healthcare) with Tris buffer (20 mM Tris, 0.5mM EDTA, 1 mM DTT pH 8.4). The rHb samples were eluted with a linear gradient of 0-1.0 M NaCl. Samples were concentrated and desalted by overnight dialysis against 10 mM HEPES buffer (pH 7.4) and were stored at -80° C prior to the measurement of O_2-equilibrium curves.

The purified rHb samples were analyzed by means of sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis and IEF. After preparing rHb samples as oxyHb, deoxyHb, and carbonmonoxy derivatives, we measured absorbance at 450-600 nm to confirm that the absorbance maxima matched those of the native HbA samples. *In vitro* measurements of O_2-binding properties were conducted in the same manner for rHbs and native Hb samples.

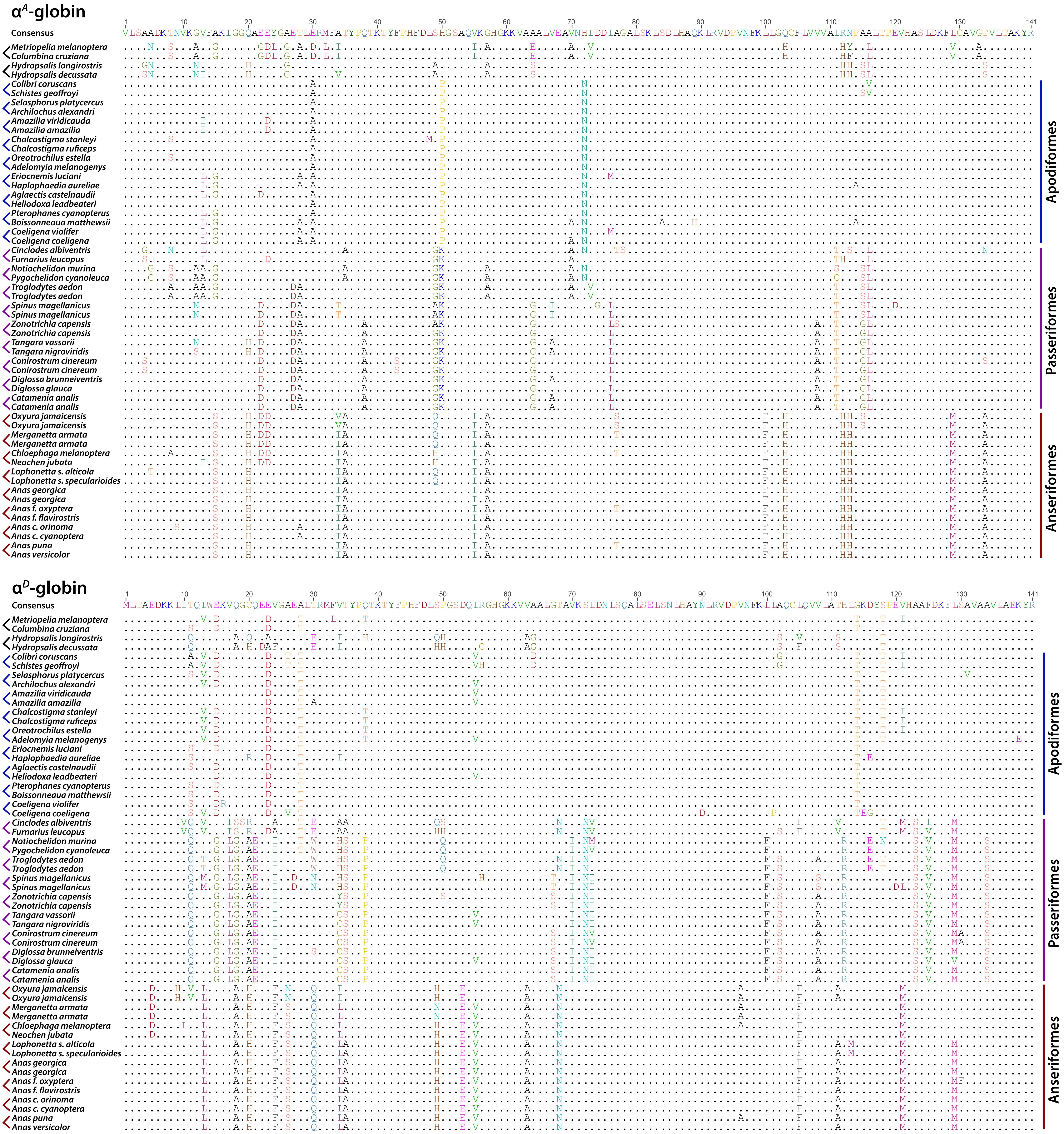

Fig. S1. Alignment of α^A- and α^D-globin amino acid sequences from Andean birds representing 28 matched pairs of high- and low-altitude taxa. The sequence for the high-altitude member of each taxon pair is shown in the top row and the sequence for the corresponding low-altitude taxon is shown in the bottom row. See Fig.1 for a depiction of phylogenetic relationships among these taxa.

Fig. S2. Alignment of β^{A}-globin amino acid sequences from Andean birds representing 28 matched pairs of high- and low-altitude taxa. The sequence for the high-altitude member of each taxon pair is shown in the top row and the sequence for the corresponding low-altitude taxon is shown in the bottom row. See Fig.1 for a depiction of phylogenetic relationships among these taxa.

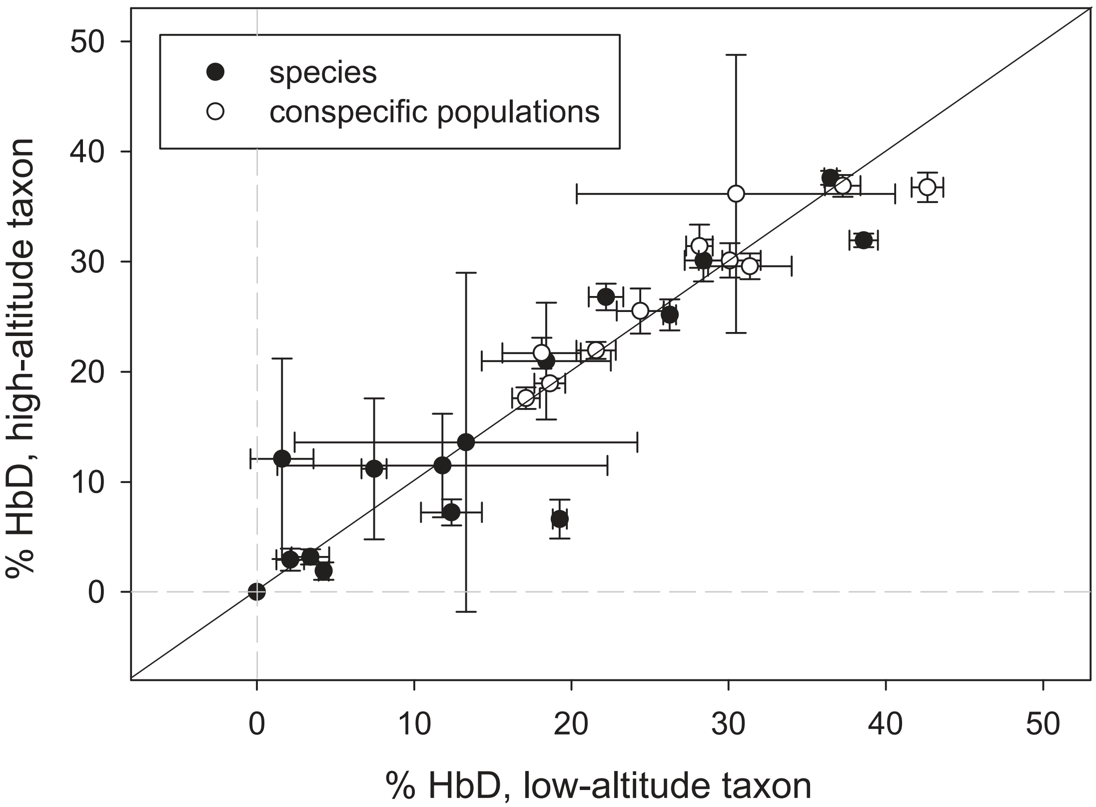

Fig. S3. No evidence for altitude-related differences in the relative abundance of HbA and HbD isoforms. Phylogenetically independent comparisons involving 26 matched pairs of high- and low-altitude taxa revealed no systematic difference in the relative expression level of the minor HbD isoform (Wilcoxon signed-ranks test, $Z = -0.775$, $P = 0.441$). The diagonal represents the line of equality (x=y).

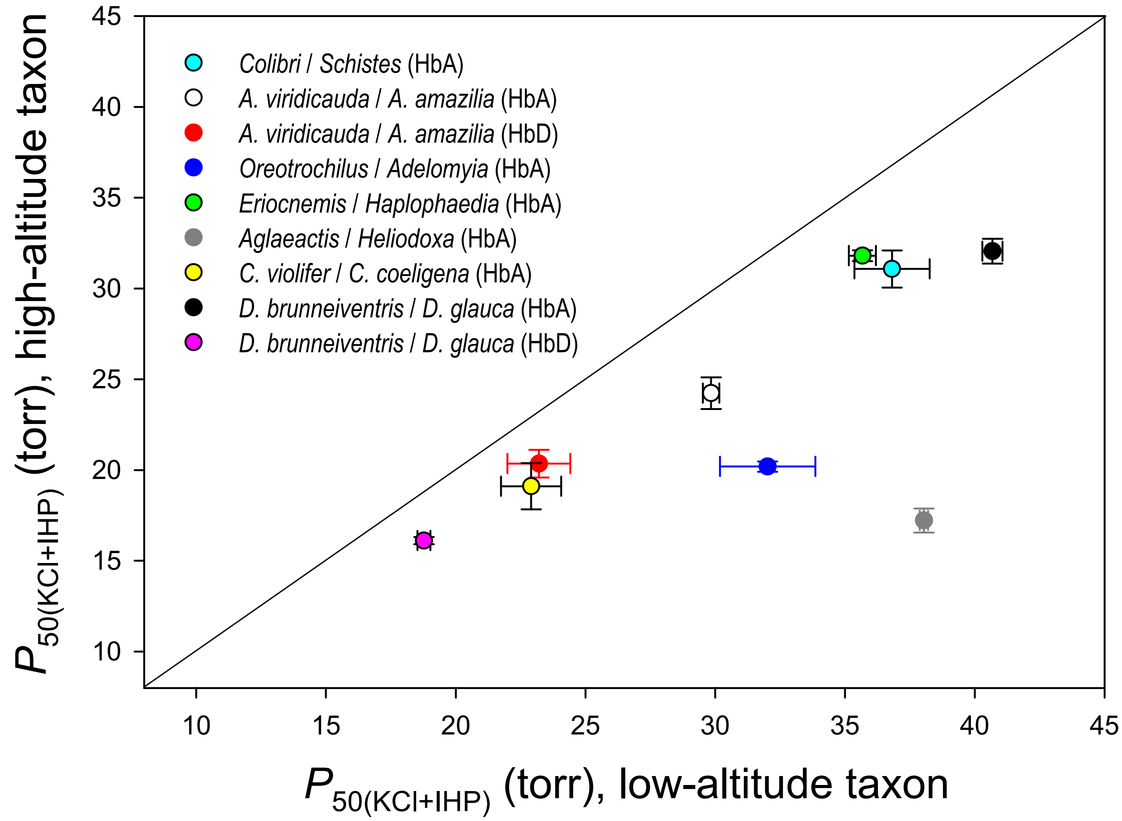

Fig. S4. Pairwise comparisons between matched pairs high- and low-altitude taxa reveal that replicated substitutions at β83 are consistently associated with derived increases in Hb-O_2 affinity in high-altitude hummingbirds and flowerpiercers (genus *Diglossa*). Shown is a plot of $P_{50(KCl+IHP)}$ (± 1 SE) for Hbs from six pairs of high- and low-altitude hummingbird species and one pair of high- and low-altitude flowerpiercers (*Diglossa brunneiventris* and *D. glauca*). Data points that fall below the diagonal denote cases in which the high-altitude member of a given taxon pair possesses a higher Hb-O_2 affinity (lower P_{50}). Hbs from each pair of high- and low-altitude hummingbird species are distinguished by a G83S substitution that occurred independently in each high-altitude lineage. Likewise, Hbs of the two *Diglossa* species are distinguished by an N83S substitution that occurred in the high-altitude *D. brunneiventris* lineage. Data for the major HbA isoform are shown for each comparison, and data for the minor HbD isoform are shown for the pair of *Amazilia* species (the high-altitude *A.viridicauda* and the low-altitude *A. amazilia*) and the two *Diglossa* species. Since the β-chain subunits are shared by both HbA and HbD, effects of the N/G83S substitutions are manifest in both isoforms. HbD data are reported for only one of the six pairs of hummingbird species that differ at β83 because in all hummingbird taxon pairs other than *Amazilia viridicauda*/*A. amazilia*, measures of HbD O_2-affinity were not available for one or both members of the pair. This was because some species expressed HbD at an exceedingly low level, so sufficient quantities of HbD could not be purified for measures of O_2-equilibria. In addition to the β-chain N/G83S substitutions, the HbA and HbD isoforms of each pair of taxa also differ at one or more additional sites (see Fig. 1).

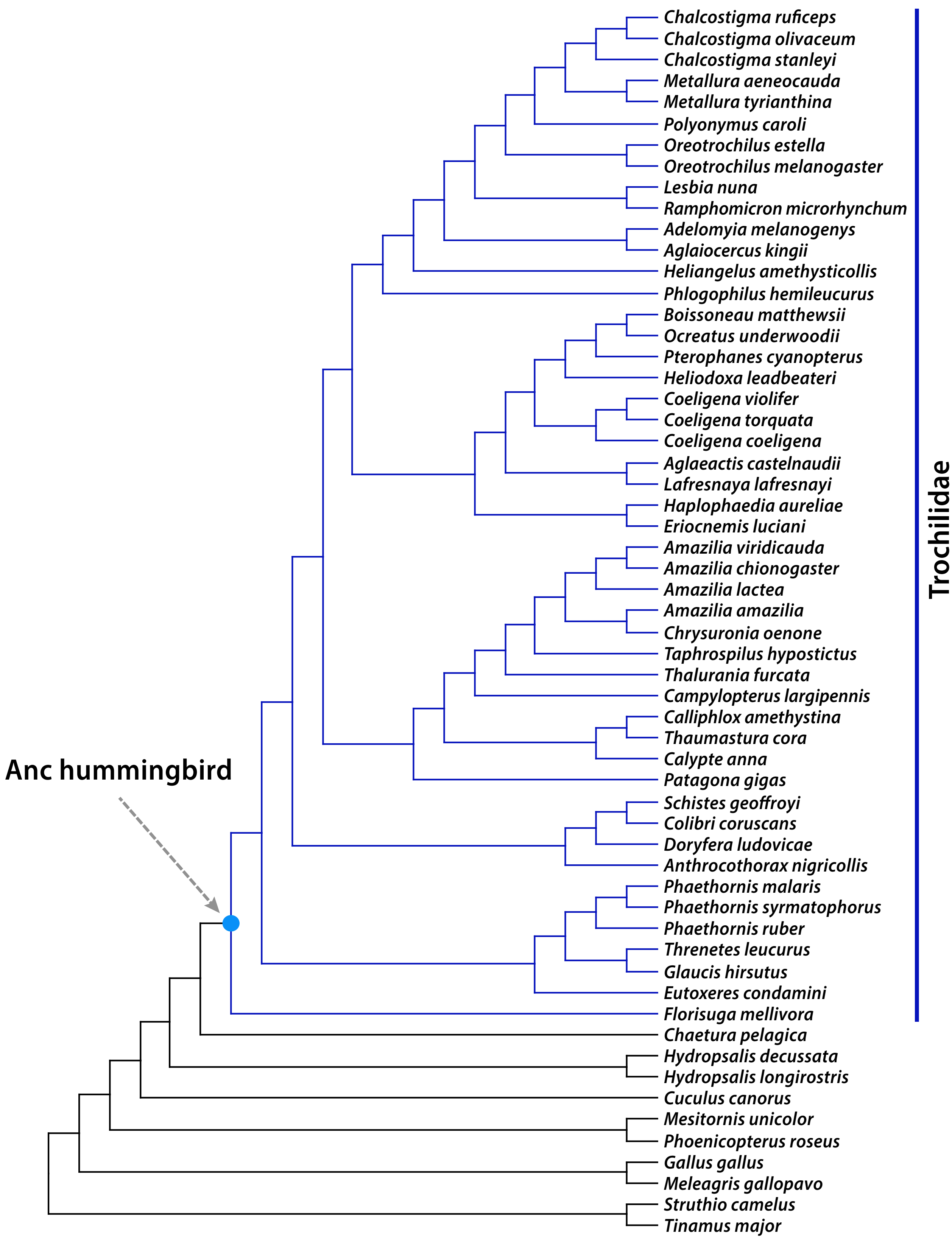

Fig. S5. Phylogenetic tree of avian α^A-globin sequences that were used to reconstruct the sequence of the most recent common ancestor of modern hummingbirds. See *SI Methods* for a description of methods used to construct the supertree.

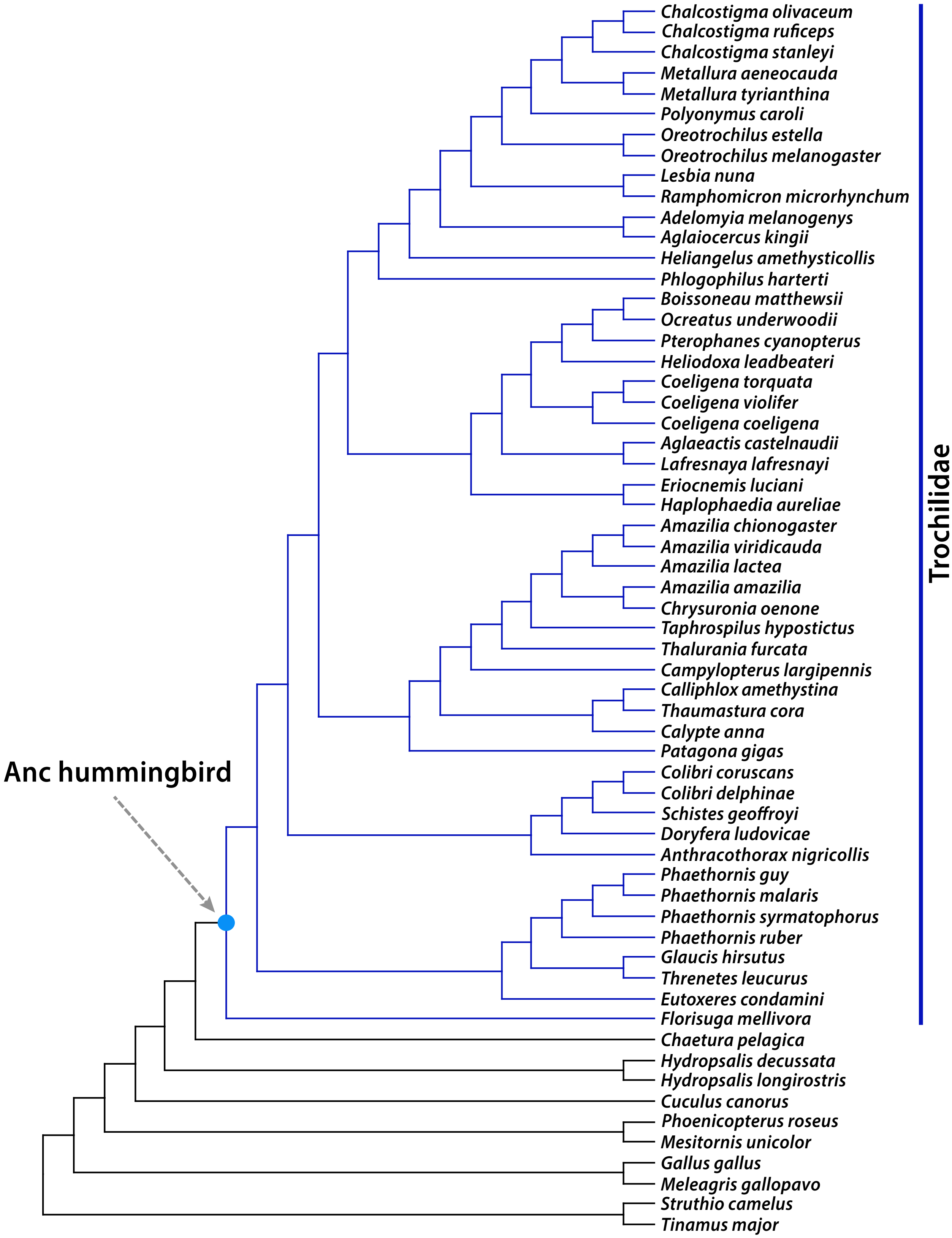

Fig. S6. Phylogenetic tree of avian β^A-globin sequences that were used to reconstruct the sequence of the most recent common ancestor of modern hummingbirds. See *SI Methods* for a description of methods used to construct the supertree.

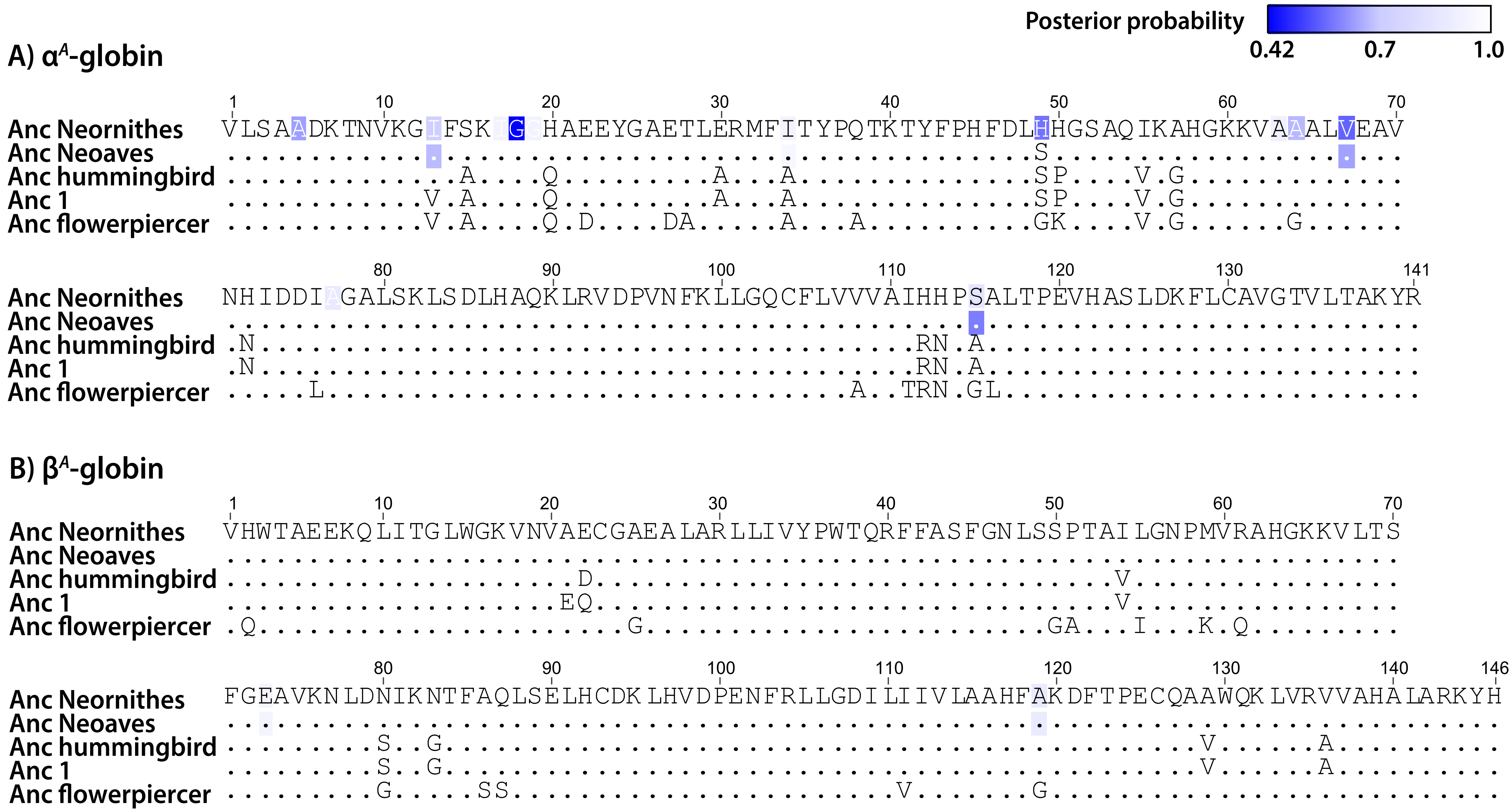

Fig. S7. Maximum likelihood (ML) ancestral state estimates for avian globin sequences. (**A**) ML sequences of α^A-globin representing five internal nodes of the avian phylogeny (Fig. 3C). Posterior probabilities of estimated character states were 1.00 for the vast majority of sites in the ML sequences. In the ML sequence for Anc Neornithes, character states at 135 of 141 sites had posterior probabilities >0.90. The following character states had posterior probabilities <0.90: 5A (0.73), 13I (0.87), 18G (0.42), 49H (0.64), 64A (0.80), and 67V (0.61). In the ML sequence for Anc Neoaves, character states at 138 of 141 sites had posterior probabilities >0.90. The following character states had posterior probabilities <0.90: 13I (0.81), 67V (0.74), and 115S (0.66). In the other three ancestral sequences, estimated character states for all sites had posterior probabilities ≥0.99. (**B**) Reconstructed sequences of β^A-globin representing the same nodes of the avian phylogeny mentioned above (Fig. 3C). Similar to the case for α^A-globin, posterior probabilities of estimated character states were 1.00 for the vast majority of sites in the ML β^A-globin sequences. In both Anc Neornithes and Anc Neoaves, ancestral state estimates for sites β73 and β119 had posterior probabilities of 0.94 and 0.89, respectively. All other sites in these two ML sequences had posterior probabilities ≥0.99. In the other three ancestral sequences, character states for all sites had posterior probabilities of 1.00. See *SI methods* for a description of the ML approach used to estimate ancestral states.

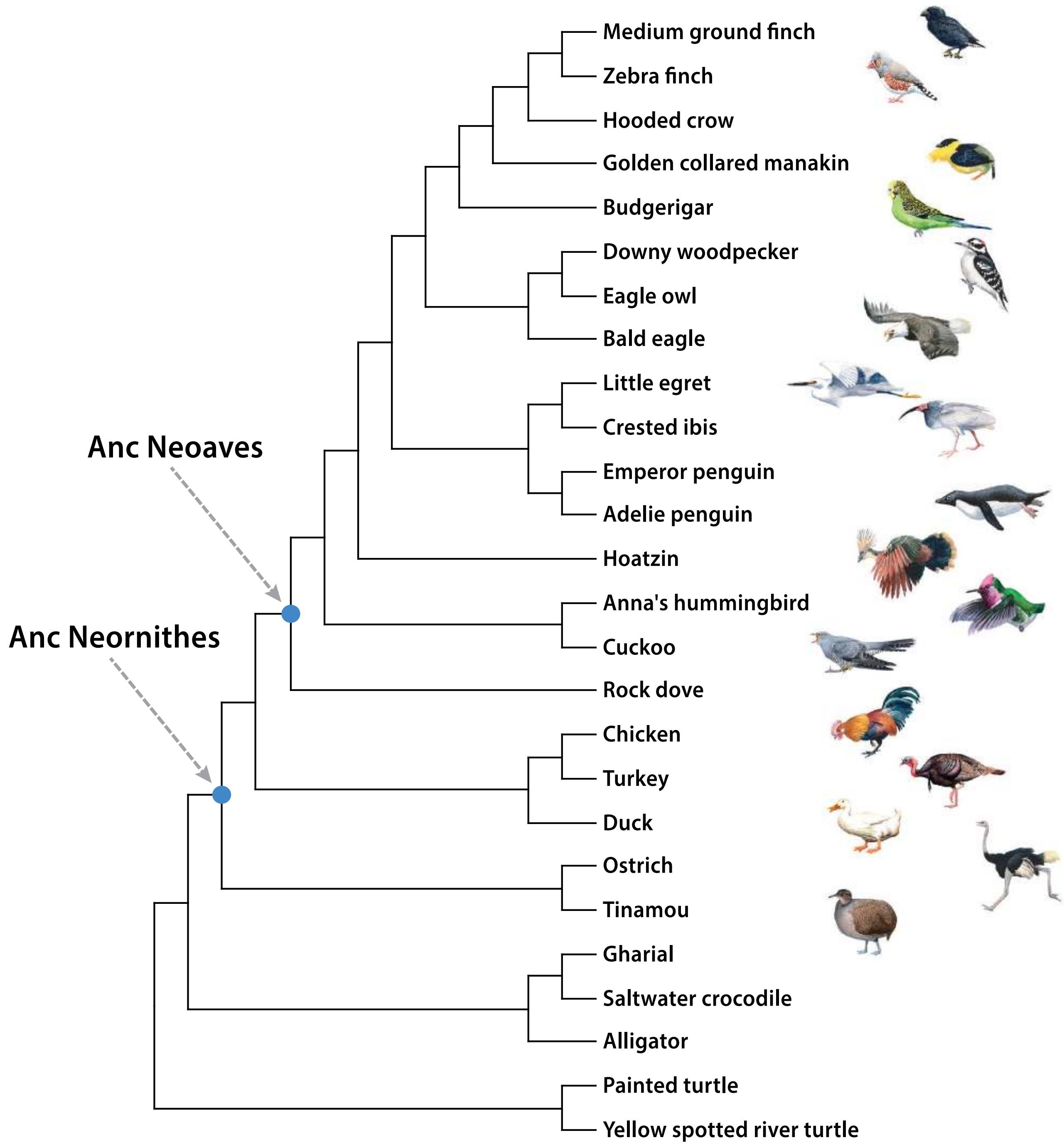

Fig. S8. Phylogenetic tree of orthologous α^A-globin sequences from birds, crocodilians, and turtles that were used to reconstruct ancestral avian sequences. Using a maximum likelihood approach (*SI Methods*), this set of sequences was used to reconstruct α^A-globin sequences of the ancestor of Neoaves ('Anc Neoaves'), the clade that contains all modern birds with the exception of Paleognathae (ratites and tinamou) and Galloanserae (landfowl and waterfowl), and the ancestor of Neornithes ('Anc Neornithes'), the clade that contains all modern birds. See *SI Methods* for a description of methods used to construct the supertree of orthologous α^A-globin sequences.

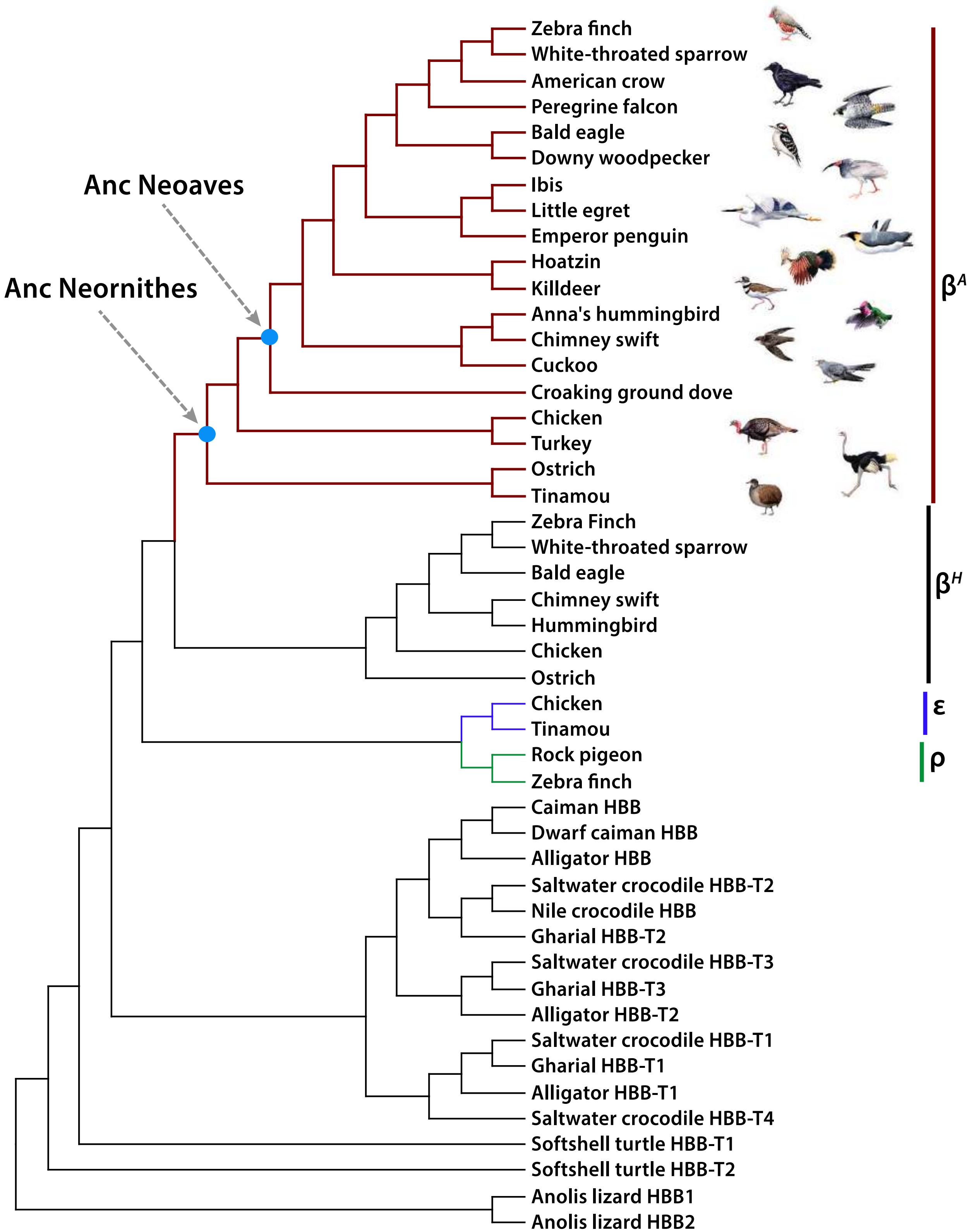

Fig. S9. Phylogenetic tree of β-type globin sequences of birds and other sauropsid taxa that were used to reconstruct ancestral avian β^A-globin sequences. The paralogous β^H-, ε-, and ρ-globin genes encode β-chain subunits of Hb isoforms that are not expressed at appreciable levels in definitive red blood cells; the ε- and ρ-globin genes are exclusively expressed during early embryogenesis. Since the avian β-type globins are products of multiple, bird-specific duplication events, we used a diversity of paralogous outgroup sequences from crocodilians, turtles, and lizards to reconstruct the ancestral β^A-globin sequences of Neoaves and Neornithes. See *SI Methods* for a description of methods used to construct the supertree of sauropsid β-type globin sequences.

Table S1. Museum-vouchered tissue specimens used in the survey of Hb variation in high- and low-altitude Neoaves taxa. The URL associated with each individual specimen provides a link to complete data on the open-access Arctos database. Frozen tissue and voucher specimens are stored at the Museum of Southwestern Biology (New Mexico, USA) and CORBIDI (Lima, Peru).

Family	Species	Elevation (m)	NK Tissue number	URL with MSB Catalog Number
Columbidae	*Metriopelia melanoptera*	4178	168686	http://arctos.database.museum/guid/MSB:Bird:33467
Columbidae	*Metriopelia melanoptera*	4150	168640	http://arctos.database.museum/guid/MSB:Bird:33421
Columbidae	*Metriopelia melanoptera*	4131	168678	http://arctos.database.museum/guid/MSB:Bird:33459
Columbidae	*Metriopelia melanoptera*	4131	168721	http://arctos.database.museum/guid/MSB:Bird:33502
Columbidae	*Metriopelia melanoptera*	4093	168528	http://arctos.database.museum/guid/MSB:Bird:33309
Columbidae	*Metriopelia melanoptera*	4093	168644	http://arctos.database.museum/guid/MSB:Bird:33425
Columbidae	*Metriopelia melanoptera*	3967	163044	http://arctos.database.museum/guid/MSB:Bird:31480
Columbidae	*Metriopelia melanoptera*	3563	172687	http://arctos.database.museum/guid/MSB:Bird:35872
Columbidae	*Columbina cruziana*	372	162986	http://arctos.database.museum/guid/MSB:Bird:31422
Columbidae	*Columbina cruziana*	372	163004	http://arctos.database.museum/guid/MSB:Bird:31440
Columbidae	*Columbina cruziana*	357	171634	http://arctos.database.museum/guid/MSB:Bird:34908
Columbidae	*Columbina cruziana*	352	168134	http://arctos.database.museum/guid/MSB:Bird:32962
Columbidae	*Columbina cruziana*	309	171404	http://arctos.database.museum/guid/MSB:Bird:34678
Columbidae	*Columbina cruziana*	309	171493	http://arctos.database.museum/guid/MSB:Bird:34767
Columbidae	*Columbina cruziana*	132	168977	http://arctos.database.museum/guid/MSB:Bird:33751
Columbidae	*Columbina cruziana*	309	171423	http://arctos.database.museum/guid/MSB:Bird:34697
Caprimulgidae	*Hydropsalis longirostris*	4401	169353	http://arctos.database.museum/guid/MSB:Bird:34127
Caprimulgidae	*Hydropsalis longirostris*	4401	169354	http://arctos.database.museum/guid/MSB:Bird:34128
Caprimulgidae	*Hydropsalis longirostris*	4384	169307	http://arctos.database.museum/guid/MSB:Bird:34081
Caprimulgidae	*Hydropsalis longirostris*	3940	168583	http://arctos.database.museum/guid/MSB:Bird:33364
Caprimulgidae	*Hydropsalis longirostris*	3931	173825	http://arctos.database.museum/guid/MSB:Bird:35994
Caprimulgidae	*Hydropsalis longirostris*	3927	168535	http://arctos.database.museum/guid/MSB:Bird:33316
Caprimulgidae	*Hydropsalis longirostris*	3300	162746	http://arctos.database.museum/guid/MSB:Bird:28187
Caprimulgidae	*Hydropsalis longirostris*	3120	159748	http://arctos.database.museum/guid/MSB:Bird:27091
Caprimulgidae	*Hydropsalis decussata*	309	171446	http://arctos.database.museum/guid/MSB:Bird:34720
Caprimulgidae	*Hydropsalis decussata*	309	171469	http://arctos.database.museum/guid/MSB:Bird:34743

Caprimulgidae	*Hydropsalis decussata*	309	171509	http://arctos.database.museum/guid/MSB:Bird:34783
Caprimulgidae	*Hydropsalis decussata*	309	171510	http://arctos.database.museum/guid/MSB:Bird:34784
Caprimulgidae	*Hydropsalis decussata*	309	171511	http://arctos.database.museum/guid/MSB:Bird:34785
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Trochilidae	*Colibri coruscans*	3750	163372	http://arctos.database.museum/guid/MSB:Bird:31700
Trochilidae	*Colibri coruscans*	3750	163377	http://arctos.database.museum/guid/MSB:Bird:31705
Trochilidae	*Colibri coruscans*	3840	163408	http://arctos.database.museum/guid/MSB:Bird:31736
Trochilidae	*Colibri coruscans*	3750	163422	http://arctos.database.museum/guid/MSB:Bird:31750
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Trochilidae	*Colibri coruscans*	3931	173807	http://arctos.database.museum/guid/MSB:Bird:35976
Trochilidae	*Colibri coruscans*	3120	159753	http://arctos.database.museum/guid/MSB:Bird:27095
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Trochilidae	*Schistes geoffroyi*	1304	161085	http://arctos.database.museum/guid/MSB:Bird:27315
Trochilidae	*Schistes geoffroyi*	1395	161125	http://arctos.database.museum/guid/MSB:Bird:27353
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Trochilidae	*Amazilia amazilia*	366	162026	http://arctos.database.museum/guid/MSB:Bird:31222
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Trochilidae	*Chalcostigma stanleyi*	4030	168268	http://arctos.database.museum/guid/MSB:Bird:33049
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Trochilidae	*Adelomyia melanogenys*	2144	163756	http://arctos.database.museum/guid/MSB:Bird:32084
Trochilidae	*Adelomyia melanogenys*	2147	163838	http://arctos.database.museum/guid/MSB:Bird:32166
Trochilidae	*Eriocnemis luciani*	3520	168230	http://arctos.database.museum/guid/MSB:Bird:27023
Trochilidae	*Eriocnemis luciani*	3520	168241	http://arctos.database.museum/guid/MSB:Bird:27034
Trochilidae	*Eriocnemis luciani*	3520	168242	http://arctos.database.museum/guid/MSB:Bird:27035
Trochilidae	*Eriocnemis luciani*	3520	168243	http://arctos.database.museum/guid/MSB:Bird:27036
Trochilidae	*Eriocnemis luciani*	3520	168244	http://arctos.database.museum/guid/MSB:Bird:27037
Trochilidae	*Eriocnemis luciani*	3680	169181	http://arctos.database.museum/guid/MSB:Bird:33955
Trochilidae	*Eriocnemis luciani*	3520	220353	http://arctos.database.museum/guid/MSB:Bird:31131
Trochilidae	*Eriocnemis luciani*	3520	220354	http://arctos.database.museum/guid/MSB:Bird:31132
Trochilidae	*Eriocnemis luciani*	3520	220367	http://arctos.database.museum/guid/MSB:Bird:31145
Trochilidae	*Eriocnemis luciani*	3520	220368	http://arctos.database.museum/guid/MSB:Bird:31146
Trochilidae	*Eriocnemis luciani*	3520	220371	http://arctos.database.museum/guid/MSB:Bird:31149
Trochilidae	*Haplophaedia aureliae*	1051	175908	http://arctos.database.museum/guid/MSB:Bird:36728
Trochilidae	*Haplophaedia aureliae*	1740	176743	http://arctos.database.museum/guid/MSB:Bird:41711
Trochilidae	*Haplophaedia aureliae*	1582	176864	http://arctos.database.museum/guid/MSB:Bird:41832
Trochilidae	*Haplophaedia aureliae*	1517	176868	http://arctos.database.museum/guid/MSB:Bird:41836
Trochilidae	*Aglaeactis castelnaudii*	4470	159782	http://arctos.database.museum/guid/MSB:Bird:27124
Trochilidae	*Aglaeactis castelnaudii*	4330	159783	http://arctos.database.museum/guid/MSB:Bird:27125
Trochilidae	*Aglaeactis castelnaudii*	4400	159798	http://arctos.database.museum/guid/MSB:Bird:27140
Trochilidae	*Aglaeactis castelnaudii*	4330	159801	http://arctos.database.museum/guid/MSB:Bird:27143
Trochilidae	*Aglaeactis castelnaudii*	4470	159808	http://arctos.database.museum/guid/MSB:Bird:27149
Trochilidae	*Aglaeactis castelnaudii*	4300	159809	http://arctos.database.museum/guid/MSB:Bird:27150
Trochilidae	*Aglaeactis castelnaudii*	4578	169373	http://arctos.database.museum/guid/MSB:Bird:34147
Trochilidae	*Heliodoxa leadbeateri*	1395	161046	http://arctos.database.museum/guid/MSB:Bird:27279
Trochilidae	*Heliodoxa leadbeateri*	1395	161048	http://arctos.database.museum/guid/MSB:Bird:31204
Trochilidae	*Heliodoxa leadbeateri*	1395	161049	http://arctos.database.museum/guid/MSB:Bird:27281
Trochilidae	*Heliodoxa leadbeateri*	1395	161052	http://arctos.database.museum/guid/MSB:Bird:27284
Trochilidae	*Heliodoxa leadbeateri*	1395	161077	http://arctos.database.museum/guid/MSB:Bird:31206
Trochilidae	*Heliodoxa leadbeateri*	1395	161107	http://arctos.database.museum/guid/MSB:Bird:27337

Trochilidae	*Heliodoxa leadbeateri*	1395	161153	http://arctos.database.museum/guid/MSB:Bird:27380
Trochilidae	*Heliodoxa leadbeateri*	1890	167994	http://arctos.database.museum/guid/MSB:Bird:32822
Trochilidae	*Heliodoxa leadbeateri*	938	175905	http://arctos.database.museum/guid/MSB:Bird:36725
Trochilidae	*Pterophanes cyanopterus*	3332	162553	http://arctos.database.museum/guid/MSB:Bird:28046
Trochilidae	*Pterophanes cyanopterus*	3369	162688	http://arctos.database.museum/guid/MSB:Bird:28150
Trochilidae	*Pterophanes cyanopterus*	3250	162794	http://arctos.database.museum/guid/MSB:Bird:28222
Trochilidae	*Pterophanes cyanopterus*	3710	163499	http://arctos.database.museum/guid/MSB:Bird:31827
Trochilidae	*Pterophanes cyanopterus*	3710	163503	http://arctos.database.museum/guid/MSB:Bird:31831
Trochilidae	*Pterophanes cyanopterus*	4200	168304	http://arctos.database.museum/guid/MSB:Bird:33085
Trochilidae	*Pterophanes cyanopterus*	3779	169153	http://arctos.database.museum/guid/MSB:Bird:33927
Trochilidae	*Boissonneaua matthewsii*	2076	167880	http://arctos.database.museum/guid/MSB:Bird:32708
Trochilidae	*Boissonneaua matthewsii*	2850	171131	http://arctos.database.museum/guid/MSB:Bird:34405
Trochilidae	*Boissonneaua matthewsii*	2850	171151	http://arctos.database.museum/guid/MSB:Bird:34425
Trochilidae	*Boissonneaua matthewsii*	2600	171213	http://arctos.database.museum/guid/MSB:Bird:34487
Trochilidae	*Boissonneaua matthewsii*	2850	171355	http://arctos.database.museum/guid/MSB:Bird:34629
Trochilidae	*Boissonneaua matthewsii*	2514	218813	http://arctos.database.museum/guid/MSB:Bird:42185
Trochilidae	*Boissonneaua matthewsii*	2529	219476	http://arctos.database.museum/guid/MSB:Bird:42538
Trochilidae	*Coeligena violifer*	2798	163129	http://arctos.database.museum/guid/MSB:Bird:31564
Trochilidae	*Coeligena violifer*	2778	163210	http://arctos.database.museum/guid/MSB:Bird:31645
Trochilidae	*Coeligena violifer*	2810	163213	http://arctos.database.museum/guid/MSB:Bird:31648
Trochilidae	*Coeligena violifer*	3710	163485	http://arctos.database.museum/guid/MSB:Bird:31813
Trochilidae	*Coeligena violifer*	2858	168451	http://arctos.database.museum/guid/MSB:Bird:33232
Trochilidae	*Coeligena violifer*	3688	169121	http://arctos.database.museum/guid/MSB:Bird:33895
Trochilidae	*Coeligena violifer*	3688	169124	http://arctos.database.museum/guid/MSB:Bird:33898
Trochilidae	*Coeligena violifer*	3779	169232	http://arctos.database.museum/guid/MSB:Bird:34006
Trochilidae	*Coeligena coeligena*	2052	163658	http://arctos.database.museum/guid/MSB:Bird:31986
Trochilidae	*Coeligena coeligena*	2052	163741	http://arctos.database.museum/guid/MSB:Bird:32069
Trochilidae	*Coeligena coeligena*	2131	163914	http://arctos.database.museum/guid/MSB:Bird:32242
Trochilidae	*Coeligena coeligena*	2100	163915	http://arctos.database.museum/guid/MSB:Bird:32243
Trochilidae	*Coeligena coeligena*	2052	167517	http://arctos.database.museum/guid/MSB:Bird:32345
Trochilidae	*Coeligena coeligena*	2051	167534	http://arctos.database.museum/guid/MSB:Bird:32362
Trochilidae	*Coeligena coeligena*	2052	167823	http://arctos.database.museum/guid/MSB:Bird:32651

Furnariidae	*Cinclodes albiventris*	4401	169350	http://arctos.database.museum/guid/MSB:Bird:34124
Furnariidae	*Cinclodes albiventris*	4391	169358	http://arctos.database.museum/guid/MSB:Bird:34132
Furnariidae	*Cinclodes albiventris*	4385	169379	http://arctos.database.museum/guid/MSB:Bird:34153
Furnariidae	*Cinclodes albiventris*	4385	169386	http://arctos.database.museum/guid/MSB:Bird:34160
Furnariidae	*Cinclodes albiventris*	4363	169261	http://arctos.database.museum/guid/MSB:Bird:34035
Furnariidae	*Cinclodes albiventris*	4200	168293	http://arctos.database.museum/guid/MSB:Bird:33074
Furnariidae	*Cinclodes albiventris*	4030	168286	http://arctos.database.museum/guid/MSB:Bird:33067
Furnariidae	*Furnarius leucopus*	350	162114	http://arctos.database.museum/guid/MSB:Bird:27674
Furnariidae	*Furnarius leucopus*	348	162034	http://arctos.database.museum/guid/MSB:Bird:27615
Furnariidae	*Furnarius leucopus*	143	169095	http://arctos.database.museum/guid/MSB:Bird:33869
Furnariidae	*Furnarius leucopus*	140	169113	http://arctos.database.museum/guid/MSB:Bird:33887
Furnariidae	*Furnarius leucopus*	135	169090	http://arctos.database.museum/guid/MSB:Bird:33864
Furnariidae	*Furnarius leucopus*	135	169091	http://arctos.database.museum/guid/MSB:Bird:33865
Furnariidae	*Furnarius leucopus*	133	169051	http://arctos.database.museum/guid/MSB:Bird:33825
Hirundinidae	*Orochelidon murina*	4300	159788	http://arctos.database.museum/guid/MSB:Bird:27130
Hirundinidae	*Orochelidon murina*	4470	159792	http://arctos.database.museum/guid/MSB:Bird:27134
Hirundinidae	*Orochelidon murina*	3360	159863	http://arctos.database.museum/guid/MSB:Bird:27199
Hirundinidae	*Orochelidon murina*	3959	163096	http://arctos.database.museum/guid/MSB:Bird:31532
Hirundinidae	*Orochelidon murina*	3750	163370	http://arctos.database.museum/guid/MSB:Bird:31698
Hirundinidae	*Orochelidon murina*	3910	168573	http://arctos.database.museum/guid/MSB:Bird:33354
Hirundinidae	*Orochelidon murina*	3940	168577	http://arctos.database.museum/guid/MSB:Bird:33358
Hirundinidae	*Orochelidon murina*	4391	169391	http://arctos.database.museum/guid/MSB:Bird:34165
Hirundinidae	*Pygochelidon cyanoleuca*	2500	172006	http://arctos.database.museum/guid/MSB:Bird:35280
Hirundinidae	*Pygochelidon cyanoleuca*	2550	172041	http://arctos.database.museum/guid/MSB:Bird:35315
Hirundinidae	*Pygochelidon cyanoleuca*	2550	172060	http://arctos.database.museum/guid/MSB:Bird:35334
Troglodytidae	*Troglodytes aedon*	3967	163033	http://arctos.database.museum/guid/MSB:Bird:31469
Troglodytidae	*Troglodytes aedon*	3959	163046	http://arctos.database.museum/guid/MSB:Bird:31482
Troglodytidae	*Troglodytes aedon*	4300	159789	http://arctos.database.museum/guid/MSB:Bird:27131
Troglodytidae	*Troglodytes aedon*	4300	159790	http://arctos.database.museum/guid/MSB:Bird:27132
Troglodytidae	*Troglodytes aedon*	4030	168338	http://arctos.database.museum/guid/MSB:Bird:33119
Troglodytidae	*Troglodytes aedon*	4056	168635	http://arctos.database.museum/guid/MSB:Bird:33416
Troglodytidae	*Troglodytes aedon*	4375	169335	http://arctos.database.museum/guid/MSB:Bird:34109

Troglodytidae	*Troglodytes aedon*	935	168074	http://arctos.database.museum/guid/MSB:Bird:32902
Troglodytidae	*Troglodytes aedon*	372	162982	http://arctos.database.museum/guid/MSB:Bird:31418
Troglodytidae	*Troglodytes aedon*	322	162535	http://arctos.database.museum/guid/MSB:Bird:28029
Troglodytidae	*Troglodytes aedon*	352	163018	http://arctos.database.museum/guid/MSB:Bird:31454
Troglodytidae	*Troglodytes aedon*	352	163020	http://arctos.database.museum/guid/MSB:Bird:31456
Troglodytidae	*Troglodytes aedon*	352	168139	http://arctos.database.museum/guid/MSB:Bird:32967
Troglodytidae	*Troglodytes aedon*	143	169115	http://arctos.database.museum/guid/MSB:Bird:33889
Fringillidae	*Spinus magellanicus*	3573	168879	http://arctos.database.museum/guid/MSB:Bird:33653
Fringillidae	*Spinus magellanicus*	3808	163380	http://arctos.database.museum/guid/MSB:Bird:31708
Fringillidae	*Spinus magellanicus*	3500	159836	http://arctos.database.museum/guid/MSB:Bird:27176
Fringillidae	*Spinus magellanicus*	3945	163061	http://arctos.database.museum/guid/MSB:Bird:31497
Fringillidae	*Spinus magellanicus*	3840	163413	http://arctos.database.museum/guid/MSB:Bird:31741
Fringillidae	*Spinus magellanicus*	3750	163376	http://arctos.database.museum/guid/MSB:Bird:31704
Fringillidae	*Spinus magellanicus*	3905	168627	http://arctos.database.museum/guid/MSB:Bird:33408
Fringillidae	*Spinus magellanicus*	39	169477	http://arctos.database.museum/guid/MSB:Bird:34251
Fringillidae	*Spinus magellanicus*	39	169475	http://arctos.database.museum/guid/MSB:Bird:34249
Fringillidae	*Spinus magellanicus*	372	162993	http://arctos.database.museum/guid/MSB:Bird:31429
Fringillidae	*Spinus magellanicus*	372	163003	http://arctos.database.museum/guid/MSB:Bird:31439
Fringillidae	*Spinus magellanicus*	935	168079	http://arctos.database.museum/guid/MSB:Bird:32907
Fringillidae	*Spinus magellanicus*	935	168109	http://arctos.database.museum/guid/MSB:Bird:32937
Fringillidae	*Spinus magellanicus*	935	168110	http://arctos.database.museum/guid/MSB:Bird:32938
Emberizidae	*Zonotrichia capensis*	4079	168727	http://arctos.database.museum/guid/MSB:Bird:33508
Emberizidae	*Zonotrichia capensis*	3548	168882	http://arctos.database.museum/guid/MSB:Bird:33656
Emberizidae	*Zonotrichia capensis*	3573	168888	http://arctos.database.museum/guid/MSB:Bird:33662
Emberizidae	*Zonotrichia capensis*	3573	168890	http://arctos.database.museum/guid/MSB:Bird:33664
Emberizidae	*Zonotrichia capensis*	2168	169279	http://arctos.database.museum/guid/MSB:Bird:34053
Emberizidae	*Zonotrichia capensis*	3931	173811	http://arctos.database.museum/guid/MSB:Bird:35980
Emberizidae	*Zonotrichia capensis*	39	169485	http://arctos.database.museum/guid/MSB:Bird:34259
Emberizidae	*Zonotrichia capensis*	352	168113	http://arctos.database.museum/guid/MSB:Bird:32941
Emberizidae	*Zonotrichia capensis*	352	168121	http://arctos.database.museum/guid/MSB:Bird:32949
Thraupidae	*Tangara vassorii*	3358	162565	http://arctos.database.museum/guid/MSB:Bird:28056
Thraupidae	*Tangara vassorii*	3421	162631	http://arctos.database.museum/guid/MSB:Bird:28104

Thraupidae	*Tangara vassorii*	3250	162640	http://arctos.database.museum/guid/MSB:Bird:28112
Thraupidae	*Tangara vassorii*	3279	162665	http://arctos.database.museum/guid/MSB:Bird:28132
Thraupidae	*Tangara vassorii*	3212	162723	http://arctos.database.museum/guid/MSB:Bird:28168
Thraupidae	*Tangara vassorii*	3220	162808	http://arctos.database.museum/guid/MSB:Bird:28229
Thraupidae	*Tangara vassorii*	3220	162809	http://arctos.database.museum/guid/MSB:Bird:28230
Thraupidae	*Tangara nigroviridis*	1395	161158	http://arctos.database.museum/guid/MSB:Bird:27385
Thraupidae	*Tangara nigroviridis*	1395	161342	http://arctos.database.museum/guid/MSB:Bird:27563
Thraupidae	*Tangara nigroviridis*	2111	163588	http://arctos.database.museum/guid/MSB:Bird:31916
Thraupidae	*Tangara nigroviridis*	2131	163703	http://arctos.database.museum/guid/MSB:Bird:32031
Thraupidae	*Tangara nigroviridis*	2100	163795	http://arctos.database.museum/guid/MSB:Bird:32123
Thraupidae	*Tangara nigroviridis*	2085	168003	http://arctos.database.museum/guid/MSB:Bird:32831
Thraupidae	*Tangara nigroviridis*	2698	218718	http://arctos.database.museum/guid/MSB:Bird:42091
Thraupidae	*Conirostrum cinereum*	3835	168319	http://arctos.database.museum/guid/MSB:Bird:33100
Thraupidae	*Conirostrum cinereum*	4030	168308	http://arctos.database.museum/guid/MSB:Bird:33089
Thraupidae	*Conirostrum cinereum*	3602	168891	http://arctos.database.museum/guid/MSB:Bird:33665
Thraupidae	*Conirostrum cinereum*	3907	168563	http://arctos.database.museum/guid/MSB:Bird:33344
Thraupidae	*Conirostrum cinereum*	3602	168878	http://arctos.database.museum/guid/MSB:Bird:33652
Thraupidae	*Conirostrum cinereum*	3905	168569	http://arctos.database.museum/guid/MSB:Bird:33350
Thraupidae	*Conirostrum cinereum*	4056	168636	http://arctos.database.museum/guid/MSB:Bird:33417
Thraupidae	*Conirostrum cinereum*	352	163024	http://arctos.database.museum/guid/MSB:Bird:31460
Thraupidae	*Conirostrum cinereum*	352	168137	http://arctos.database.museum/guid/MSB:Bird:32965
Thraupidae	*Conirostrum cinereum*	352	168129	http://arctos.database.museum/guid/MSB:Bird:32957
Thraupidae	*Conirostrum cinereum*	352	168148	http://arctos.database.museum/guid/MSB:Bird:32976
Thraupidae	*Conirostrum cinereum*	352	168161	http://arctos.database.museum/guid/MSB:Bird:32989
Thraupidae	*Conirostrum cinereum*	352	168131	http://arctos.database.museum/guid/MSB:Bird:32959
Thraupidae	*Conirostrum cinereum*	352	168132	http://arctos.database.museum/guid/MSB:Bird:32960
Thraupidae	*Diglossa brunneiventris*	4300	159802	http://arctos.database.museum/guid/MSB:Bird:27144
Thraupidae	*Diglossa brunneiventris*	3973	163042	http://arctos.database.museum/guid/MSB:Bird:31478
Thraupidae	*Diglossa brunneiventris*	3967	163051	http://arctos.database.museum/guid/MSB:Bird:31487
Thraupidae	*Diglossa brunneiventris*	3945	163093	http://arctos.database.museum/guid/MSB:Bird:31529
Thraupidae	*Diglossa brunneiventris*	3710	163496	http://arctos.database.museum/guid/MSB:Bird:31824
Thraupidae	*Diglossa brunneiventris*	4088	168650	http://arctos.database.museum/guid/MSB:Bird:33431

Thraupidae	*Diglossa brunneiventris*	3548	168872	http://arctos.database.museum/guid/MSB:Bird:33646
Thraupidae	*Diglossa brunneiventris*	4385	169387	http://arctos.database.museum/guid/MSB:Bird:34161
Thraupidae	*Diglossa glauca*	1395	161139	http://arctos.database.museum/guid/MSB:Bird:27366
Thraupidae	*Diglossa glauca*	1304	161326	http://arctos.database.museum/guid/MSB:Bird:27548
Thraupidae	*Diglossa glauca*	1395	161341	http://arctos.database.museum/guid/MSB:Bird:27562
Thraupidae	*Catamenia analis*	3120	159727	http://arctos.database.museum/guid/MSB:Bird:27071
Thraupidae	*Catamenia analis*	3120	159713	http://arctos.database.museum/guid/MSB:Bird:27058
Thraupidae	*Catamenia analis*	3548	168873	http://arctos.database.museum/guid/MSB:Bird:33647
Thraupidae	*Catamenia analis*	3548	168874	http://arctos.database.museum/guid/MSB:Bird:33648
Thraupidae	*Catamenia analis*	3548	168875	http://arctos.database.museum/guid/MSB:Bird:33649
Thraupidae	*Catamenia analis*	3573	168871	http://arctos.database.museum/guid/MSB:Bird:33645
Thraupidae	*Catamenia analis*	39	169488	http://arctos.database.museum/guid/MSB:Bird:34262
Thraupidae	*Catamenia analis*	39	169490	http://arctos.database.museum/guid/MSB:Bird:34264
Thraupidae	*Catamenia analis*	350	162985	http://arctos.database.museum/guid/MSB:Bird:31421
Thraupidae	*Catamenia analis*	372	162994	http://arctos.database.museum/guid/MSB:Bird:31430
Thraupidae	*Catamenia analis*	372	162990	http://arctos.database.museum/guid/MSB:Bird:31426
Thraupidae	*Catamenia analis*	372	162991	http://arctos.database.museum/guid/MSB:Bird:31427
Thraupidae	*Catamenia analis*	372	163000	http://arctos.database.museum/guid/MSB:Bird:31436

Table S2. O_2 affinities (P_{50}, torr) and cooperativity coefficients (n_{50}) of purified HbA and HbD isoforms from highland and lowland Andean birds. High- and low-altitude populations of the same species are denoted by a parenthetical 'H' or 'L', respectively. O_2 equilibria were measured in 0.1 mM HEPES buffer at pH 7.4 (± 0.01) and 37ºC in the absence (stripped) and presence of Cl^- ions (0.1 M KCl]) and IHP (at two-fold molar excess over tetrameric Hb). P_{50} and n_{50} values were derived from single O_2 equilibrium curves, where each value was interpolated from linear Hill plots (correlation coefficient r> 0.995) based on 4 or more equilibrium steps between 25 and 75% saturation. Due to allelic polymorphism, two main genotypes were present in the high-altitude sample of speckled teal (H1 and H2) and in the low-altitude sample of ruddy ducks (L1 and L2).

Taxon	IsoHb	Stripped		+ KCl		+ IHP		+ KCl + IHP	
		P_{50}	n_{50}	P_{50}	n_{50}	P_{50}	n_{50}	P_{50}	n_{50}
Metriopelia melanoptera	HbA	3.70 ± 0.03	2.00 ± 0.03	5.05 ± 0.09	2.26 ± 0.10	34.49 ± 0.43	2.77 ± 0.09	26.86 ± 0.61	2.67 ± 0.13
	HbD	-	-	-	-	-	-	-	-
Columbina cruziana	HbA	4.76 ± 0.09	1.53 ± 0.05	5.83 ± 0.04	1.67 ± 0.02	43.03 ± 2.54	1.86 ± 0.16	28.38 ± 0.18	2.23 ± 0.03
	HbD	-	-	-	-	-	-	-	-
Hydropsalis longirostris	HbA	2.48 ± 0.03	1.77 ± 0.04	3.73 ± 0.04	1.97 ± 0.05	43.55 ± 1.06	2.35 ± 0.11	30.21 ± 0.82	2.29 ± 0.14
	HbD	2.30 ± 0.05	1.67 ± 0.06	3.58 ± 0.10	1.81 ± 0.07	23.72 ± 0.92	2.32 ± 0.17	19.30 ± 0.55	2.34 ± 0.13
Hydropsalis decussata	HbA	2.77 ± 0.02	1.87 ± 0.02	4.24 ± 0.04	2.12 ± 0.04	50.38 ± 1.64	2.01 ± 0.10	36.12 ± 1.30	2.13 ± 0.14
	HbD	2.07 ± 0.02	1.51 ± 0.03	3.13 ± 0.04	1.86 ± 0.04	32.88 ± 1.24	2.33 ± 0.17	21.54 ± 0.88	2.40 ± 0.24
Colibri coruscans	HbA	3.31 ± 0.10	1.83 ± 0.08	4.39 ± 0.00	1.99 ± 0.00	41.24 ± 0.44	2.51 ± 0.07	31.08 ± 1.02	2.40 ± 0.18
	HbD	-	-	-	-	-	-	-	-
Schistes geoffroyi	HbA	3.85 ± 0.03	1.92 ± 0.04	5.30 ± 0.02	2.11 ± 0.02	44.95 ± 0.07	2.59 ± 0.11	36.81 ± 1.45	2.50 ± 0.22
	HbD	-	-	-	-	-	-	-	-
Selasphorus platycercus	HbA	3.69 ± 0.03	1.91 ± 0.03	5.99 ± 0.16	2.09 ± 0.13	46.02 ± 1.19	2.37 ± 0.12	38.25 ± 0.27	2.61 ± 0.05
	HbD	2.88 ± 0.06	1.91 ± 0.08	3.48 ± 0.05	1.91 ± 0.05	29.93 ± 0.79	2.34 ± 0.12	21.45 ± 0.53	2.11 ± 0.12
Archilochus alexandri	HbA	3.97 ± 0.05	2.03 ± 0.05	5.58 ± 0.10	2.23 ± 0.09	47.93 ± 0.82	2.63 ± 0.11	39.12 ± 0.23	2.81 ± 0.05
	HbD	3.10 ± 0.10	2.00 ± 0.13	4.07 ± 0.08	2.01 ± 0.08	31.55 ± 0.29	2.47 ± 0.07	26.19 ± 0.76	2.32 ± 0.15
Amazilia viridicauda	HbA	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
	HbD	2.78 ± 0.10	1.34 ± 0.07	3.90 ± 0.10	1.64 ± 0.13	21.83 ± 0.23	2.22 ± 0.02	20.36 ± 0.76	2.29 ± 0.05
Amazilia amazilia	HbA	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
	HbD	3.36 ± 0.07	1.70 ± 0.05	4.79 ± 0.06	2.08 ± 0.05	28.61 ± 0.80	2.63 ± 0.29	23.20 ± 1.21	2.40 ± 0.19
Chalcostigma stanleyi	HbA	3.52 ± 0.13	1.88 ± 0.21	4.57 ± 0.04	2.17 ± 0.04	38.79 ± 0.40	2.83 ± 0.08	32.34 ± 0.16	2.80 ± 0.04
	HbD	-	-	-	-	-	-	-	-
Chalcostigma ruficeps	HbA	3.70 ± 0.10	1.61 ± 0.09	5.34 ± 0.23	1.75 ± 0.14	43.20 ± 1.23	2.43 ± 0.17	33.02 ± 0.99	2.11 ± 0.14
	HbD	-	-	-	-	-	-	-	-
Oreotrochilus estella	HbA	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
	HbD	-	-	-	-	-	-	-	-
Adelomyia melanogenys	HbA	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 ± 1.84	2.31 ± 0.07
	HbD	-	-	-	-	-	-	-	-
Eriocnemis luciani	HbA	3.16 ± 0.03	1.91 ± 0.05	4.55 ± 0.04	2.19 ± 0.04	45.46 ± 0.68	2.62 ± 0.10	31.81 ± 0.30	2.34 ± 0.06
	HbD	3.08 ± 0.05	1.77 ± 0.06	3.52 ± 0.07	1.56 ± 0.05	28.99 ± 0.17	2.41 ± 0.04	23.19 ± 0.30	2.36 ± 0.07

Haplophaedia aurelieae	HbA	3.52 ± 0.02	1.91 ± 0.03	5.11 ± 0.08	2.24 ± 0.08	43.04 ± 0.40	2.63 ± 0.07	35.67 ± 0.52	2.62 ± 0.10
	HbD	-	-	-	-	-	-	-	-
Aglaeactis castelnaudii	HbA	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
	HbD	-	-	-	-	-	-	-	-
Heliodoxa leadbeateri	HbA	3.93 ± 0.09	2.46 ± 0.15	5.20 ± 0.05	2.15 ± 0.05	47.66 ± 0.18	2.95 ± 0.03	38.04 ± 0.16	2.96 ± 0.03
	HbD	3.74 ± 0.06	2.35 ± 0.09	4.86 ± 0.04	2.48 ± 0.05	36.61 ± 0.47	2.40 ± 0.07	30.51 ± 0.48	2.60 ± 0.11
Pterophanes cyanopterus	HbA	3.48 ± 0.04	2.01 ± 0.05	4.50 ± 0.03	2.20 ± 0.04	38.69 ± 0.25	2.69 ± 0.06	30.43 ± 0.48	2.73 ± 0.13
	HbD	-	-	-	-	-	-	-	-
Boissonneaua matthewsii	HbA	4.22 ± 0.11	2.13 ± 0.11	5.68 ± 0.04	2.43 ± 0.04	42.59 ± 0.40	2.70 ± 0.07	36.51 ± 0.06	2.75 ± 0.12
	HbD	-	-	-	-	-	-	-	-
Coeligena violifer	HbA	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
	HbD	2.48 ± 0.07	1.40 ± 0.01	3.65 ± 0.06	1.80 ± 0.07	17.71 ± 0.38	2.30 ± 0.04	17.01 ± 0.09	2.46 ± 0.04
Coeligena coeligena	HbA	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 ± 1.16	2.19 ± 0.11
	HbD	-	-	-	-	-	-	-	-
Cinclodes albiventris	HbA	2.47 ± 0.03	1.56 ± 0.04	3.24 ± 0.03	1.76 ± 0.03	30.77 ± 0.78	1.99 ± 0.14	25.12 ± 1.45	1.95 ± 0.23
	HbD	2.15 ± 0.03	1.63 ± 0.04	2.82 ± 0.03	1.82 ± 0.04	25.33 ± 0.52	2.28 ± 0.11	21.59 ± 0.70	2.13 ± 0.15
Furnarius leucopus	HbA	3.85 ± 0.12	1.90 ± 0.10	4.99 ± 0.11	1.74 ± 0.09	63.87 ± 2.63	2.00 ± 0.21	44.69 ± 1.26	1.99 ± 0.15
	HbD	2.97 ± 0.07	2.02 ± 0.10	3.36 ± 0.03	1.88 ± 0.03	39.78 ± 0.53	2.37 ± 0.08	29.14 ± 0.27	2.47 ± 0.07
Notiochelidon murina	HbA	3.07 ± 0.03	1.94 ± 0.04	4.21 ± 0.02	2.03 ± 0.02	46.86 ± 1.53	2.36 ± 0.16	30.89 ± 0.44	1.96 ± 0.07
	HbD	2.17 ± 0.01	1.91 ± 0.02	2.69 ± 0.02	2.12 ± 0.04	23.74 ± 0.94	2.29 ± 0.20	16.05 ± 0.31	2.56 ± 0.13
Pygochelidon cyanoleuca	HbA	3.77 ± 0.13	1.92 ± 0.15	5.16 ± 0.16	1.99 ± 0.12	55.47 ± 1.51	2.37 ± 0.15	39.59 ± 0.41	2.34 ± 0.06
	HbD	2.38 ± 0.11	2.02 ± 0.02	3.18 ± 0.05	2.31 ± 0.08	25.10 ± 0.74	3.06 ± 0.25	17.79 ± 0.06	2.52 ± 0.03
Troglodytes aedon (H)	HbA	2.47 ± 0.07	1.53 ± 0.04	2.96 ± 0.20	1.36 ± 0.09	21.39 ± 0.32	1.37 ± 0.14	17.07 ± 0.79	1.36 ± 0.01
	HbD	1.59 ± 0.03	1.36 ± 0.10	2.47 ± 0.04	1.81 ± 0.02	17.54 ± 0.31	2.22 ± 0.14	13.45 ± 0.29	2.28 ± 0.10
Troglodytes aedon (L)	HbA	2.80 ± 0.25	1.48 ± 0.15	4.57 ± 0.01	1.91 ± 0.07	33.90 ± 1.61	1.98 ± 0.25	25.88 ± 1.22	2.11 ± 0.13
	HbD	1.58 ± 0.03	1.47 ± 0.07	2.67 ± 0.09	1.92 ± 0.11	22.60 ± 0.74	2.39 ± 0.06	16.29 ± 0.19	2.36 ± 0.12
Spinus magellanica (H)	HbA	2.54 ± 0.02	1.69 ± 0.03	4.26 ± 0.10	2.04 ± 0.09	38.82 ± 1.26	2.47 ± 0.18	27.82 ± 0.72	2.58 ± 0.18
	HbD	1.51 ± 0.05	1.31 ± 0.07	2.51 ± 0.03	1.76 ± 0.03	24.34 ± 0.89	2.55 ± 0.20	13.57 ± 0.87	1.98 ± 0.24
Spinus magellanica (L)	HbA	3.16 ± 0.03	1.61 ± 0.03	5.30 ± 0.13	1.85 ± 0.08	42.56 ± 2.60	2.16 ± 0.24	31.53 ± 2.11	2.07 ± 0.27
	HbD	2.07 ± 0.02	1.45 ± 0.02	3.20 ± 0.03	1.88 ± 0.03	26.86 ± 1.18	2.18 ± 0.18	20.63 ± 0.23	2.50 ± 0.06
Zonotrichia capensis (H)	HbA	3.09 ± 0.11	1.87 ± 0.12	4.78 ± 0.14	1.99 ± 0.11	49.57 ± 2.03	2.25 ± 0.17	39.98 ± 1.82	2.31 ± 0.23
	HbD	1.88 ± 0.03	1.71 ± 0.04	2.90 ± 0.07	2.02 ± 0.09	26.69 ± 1.43	2.44 ± 0.36	18.56 ± 0.86	2.22 ± 0.22
Zonotrichia capensis (L)	HbA	3.34 ± 0.06	1.72 ± 0.05	5.69 ± 0.15	1.97 ± 0.09	53.18 ± 7.22	1.78 ± 0.29	36.28 ± 1.85	2.26 ± 0.22
	HbD	1.80 ± 0.06	1.71 ± 0.09	2.87 ± 0.04	1.96 ± 0.05	31.58 ± 0.96	2.39 ± 0.15	22.42 ± 1.02	2.58 ± 0.28
Tangara vassorii	HbA	4.13 ± 0.01	2.18 ± 0.02	5.53 ± 0.11	1.81 ± 0.08	60.59 ± 0.81	2.68 ± 0.10	43.09 ± 0.63	2.74 ± 0.10
	HbD	2.66 ± 0.06	2.04 ± 0.09	3.39 ± 0.01	2.04 ± 0.01	33.28 ± 0.21	2.87 ± 0.07	24.61 ± 0.90	2.64 ± 0.25
Tangara nigroviridis	HbA	3.91 ± 0.10	2.32 ± 0.14	4.41 ± 0.01	2.11 ± 0.02	52.75 ± 0.19	2.83 ± 0.03	43.22 ± 0.16	3.00 ± 0.04
	HbD	2.11 ± 0.04	1.81 ± 0.07	2.68 ± 0.05	1.86 ± 0.07	28.89 ± 0.54	2.49 ± 0.11	23.52 ± 0.55	2.88 ± 0.18
Conirostrum cinereum (H)	HbA	3.63 ± 0.05	1.94 ± 0.06	4.90 ± 0.02	2.07 ± 0.02	48.84 ± 0.89	2.41 ± 0.09	39.47 ± 0.33	2.72 ± 0.07
	HbD	2.07 ± 0.01	2.00 ± 0.02	3.06 ± 0.04	2.37 ± 0.08	28.35 ± 0.74	2.73 ± 0.18	20.80 ± 0.71	2.57 ± 0.22

Conirostrum cinereum (L)	HbA	3.98 ± 0.06	1.98 ± 0.06	5.67 ± 0.17	2.06 ± 0.13	47.80 ± 0.52	2.42 ± 0.08	40.50 ± 1.17	2.93 ± 0.25
	HbD	2.17 ± 0.02	1.94 ± 0.05	2.80 ± 0.02	2.19 ± 0.05	25.46 ± 0.43	2.86 ± 0.13	19.06 ± 0.06	2.94 ± 0.03
Diglossa brunneiventris	HbA	2.74 ± 0.03	1.71 ± 0.04	3.98 ± 0.03	1.85 ± 0.03	39.51 ± 0.42	2.54 ± 0.07	32.06 ± 0.68	2.53 ± 0.14
	HbD	1.84 ± 0.02	1.79 ± 0.04	2.73 ± 0.02	2.10 ± 0.04	22.87 ± 0.29	2.89 ± 0.10	16.11 ± 0.20	2.62 ± 0.08
Diglossa glauca	HbA	3.61 ± 0.02	2.00 ± 0.03	5.04 ± 0.10	1.97 ± 0.08	52.75 ± 0.66	2.43 ± 0.08	40.68 ± 0.39	2.56 ± 0.06
	HbD	2.51 ± 0.03	1.85 ± 0.05	3.12 ± 0.04	2.13 ± 0.06	24.35 ± 0.48	2.61 ± 0.13	18.77 ± 0.25	2.77 ± 0.11
Catamenia analis (H)	HbA	3.20 ± 0.07	1.99 ± 0.07	4.73 ± 0.06	2.24 ± 0.06	46.65 ± 0.36	2.45 ± 0.04	37.46 ± 0.15	2.62 ± 0.03
	HbD	2.04 ± 0.04	1.88 ± 0.08	3.02 ± 0.05	1.98 ± 0.07	26.24 ± 0.42	2.42 ± 0.10	21.52 ± 0.38	2.61 ± 0.12
Catamenia analis (L)	HbA	3.12 ± 0.05	1.87 ± 0.07	4.39± 0.04	2.11 ± 0.05	43.50 ± 0.54	2.54 ± 0.08	37.95 ± 0.68	2.54 ± 0.10
	HbD	2.44 ± 0.05	2.09 ± 0.10	3.10 ± 0.10	1.92 ± 0.13	34.01 ± 0.12	2.68 ± 0.03	24.36 ± 0.65	2.18 ± 0.15
Oxyura jamaicensis (H)	HbA	2.84 ± 0.05	2.10 ± 0.07	3.98 ± 0.03	2.44 ± 0.04	42.11 ± 0.85	3.21 ± 0.20	30.05 ± 0.72	2.75 ± 0.17
	HbD	2.14 ± 0.05	1.75 ± 0.06	2.94 ± 0.08	1.98 ± 0.11	29.22 ± 0.17	2.93 ± 0.06	20.28 ± 0.34	2.55 ± 0.13
Oxyura jamaicensis (L1)	HbA	2.42 ± 0.06	1.78 ± 0.08	3.39 ± 0.07	2.00 ± 0.08	38.19 ± 1.14	2.56 ± 0.18	28.52 ± 0.18	2.93 ± 0.06
	HbD	2.38 ± 0.05	1.44 ± 0.05	2.84 ± 0.01	1.42 ± 0.01	27.76 ± 0.96	1.98 ± 0.15	19.34 ± 0.61	1.99 ± 0.12
Oxyura jamaicensis (L2)	HbA	3.62 ± 0.07	1.55 ± 0.05	4.75 ± 0.17	1.66 ± 0.09	42.58 ± 2.47	1.38 ± 0.12	28.61 ± 1.56	1.83 ± 0.20
	HbD	-	-	-	-	-	-	-	-
Merganetta armata (H)	HbA	2.01 ± 0.01	1.87 ± 0.02	3.03 ± 0.02	2.18 ± 0.03	33.45 ± 0.28	2.56 ± 0.07	26.60 ± 0.30	3.05 ± 0.10
	HbD	1.80 ± 0.03	1.27 ± 0.04	2.68 ± 0.02	1.79 ± 0.02	21.57 ± 0.06	2.64 ± 0.02	17.88 ± 0.16	2.92 ± 0.09
Merganetta armata (L)	HbA	2.42 ± 0.02	1.98 ± 0.03	3.48 ± 0.03	2.30 ± 0.04	34.95 ± 0.16	2.72 ± 0.04	27.97 ± 0.06	3.05 ± 0.03
	HbD	1.90 ± 0.02	1.50 ± 0.03	2.86 ± 0.04	1.77 ± 0.05	22.44 ± 0.24	2.48 ± 0.08	18.50 ± 0.01	3.08 ± 0.01
Chloephaga melanoptera	HbA	2.88 ± 0.07	1.79 ± 0.06	4.34 ± 0.13	2.07 ± 0.11	34.53 ± 1.10	2.30 ± 0.14	27.64 ± 0.92	2.41 ± 0.17
	HbD	1.97 ± 0.03	1.55 ± 0.03	2.99 ± 0.05	1.84 ± 0.05	22.78 ± 0.52	2.41 ± 0.12	17.12 ± 0.63	2.45 ± 0.20
Neochen jubata	HbA	3.14 ± 0.11	1.75 ± 0.09	5.31 ± 0.18	1.88 ± 0.09	41.17 ± 3.35	1.78 ± 0.17	35.31 ± 2.52	1.70 ± 0.16
	HbD	2.14 ± 0.03	1.62 ± 0.03	3.32 ± 0.06	1.92 ± 0.06	22.52 ± 0.87	2.21 ± 0.16	20.63 ± 0.84	2.46 ± 0.21
Lophonetta s. alticola	HbA	2.66 ± 0.02	1.54 ± 0.03	4.33 ± 0.03	2.18 ± 0.03	34.63 ± 0.05	3.07 ± 0.02	25.14 ± 0.25	3.01 ± 0.10
	HbD	1.95 ± 0.02	1.54 ± 0.04	2.93 ± 0.05	1.83 ± 0.07	13.24 ± 0.62	1.73 ± 0.19	10.51 ± 0.08	2.32 ± 0.06
Lophonetta s. specularioides	HbA	3.45 ± 0.02	2.01 ± 0.03	4.87 ± 0.00	2.22 ± 0.00	52.40 ± 0.30	2.52 ± 0.04	37.98 ± 0.46	2.83 ± 0.12
	HbD	1.93 ± 0.01	1.81 ± 0.03	3.16 ± 0.05	1.90 ± 0.07	25.90 ± 0.32	2.63 ± 0.10	20.35 ± 0.24	2.84 ± 0.12
Anas georgica (H)	HbA	2.61 ± 0.02	1.73 ± 0.02	4.11 ± 0.05	2.25 ± 0.06	40.00 ± 0.17	3.03 ± 0.06	35.62 ± 0.40	2.78 ± 0.09
	HbD	2.26 ± 0.05	1.37 ± 0.09	3.04 ± 0.05	1.99 ± 0.08	22.25 ± 0.91	2.47 ± 0.30	17.41 ± 0.20	2.74 ± 0.11
Anas georgica (L)	HbA	3.26 ± 0.03	2.13 ± 0.06	5.01 ± 0.07	2.05 ± 0.06	56.51 ± 0.91	2.41 ± 0.10	42.20 ± 0.23	2.77 ± 0.05
	HbD	2.64 ± 0.03	2.05 ± 0.08	4.35 ± 0.04	2.55 ± 0.07	28.26 ± 0.34	3.43 ± 0.14	17.02 ± 0.88	1.46 ± 0.18
Anas f. oxyptera (H)	HbA	2.14 ± 0.07	1.73 ± 0.11	3.48 ± 0.00	2.10 ± 0.00	33.91 ± 0.17	3.16 ± 0.06	30.08 ± 0.18	2.99 ± 0.07
	HbD	1.74 ± 0.02	1.36 ± 0.04	2.38 ± 0.05	1.66 ± 0.07	18.65 ± 0.09	3.18 ± 0.06	13.52 ± 0.13	3.18 ± 0.11
Anas f. flavirostris (L1)	HbA	2.93 ± 0.01	2.14 ± 0.03	4.00 ± 0.02	2.23 ± 0.03	33.29 ± 0.40	3.26 ± 0.15	30.96 ± 0.19	3.47 ± 0.09
	HbD	1.57 ± 0.08	1.49 ± 0.18	2.73 ± 0.03	1.82 ± 0.05	22.00 ± 0.12	3.35 ± 0.07	17.64 ± 0.11	3.20 ± 0.09
Anas f. flavirostris (L2)	HbA	2.85 ± 0.10	1.50 ± 0.14	5.64 ± 0.01	2.53 ± 0.02	58.30 ± 0.81	2.94 ± 0.19	41.71 ± 0.19	3.27 ± 0.08
	HbD	2.87 ± 0.02	1.50 ± 0.02	4.10 ± 0.03	2.37 ± 0.04	32.28 ± 0.19	3.45 ± 0.08	23.81 ± 0.20	3.04 ± 0.09
Anas c. orinoma	HbA	2.37 ± 0.02	1.86 ± 0.04	3.59 ± 0.02	2.26 ± 0.04	34.79 ± 0.19	2.99 ± 0.05	29.36 ± 0.23	3.10 ± 0.10
	HbD	2.13 ± 0.01	1.84 ± 0.01	3.16 ± 0.06	2.41 ± 0.10	36.55 ± 0.36	3.49 ± 0.12	26.23 ± 0.10	3.45 ± 0.06

Anas c. cyanoptera	HbA	3.00 ± 0.03	2.06 ± 0.06	4.24 ± 0.05	2.15 ± 0.08	47.71 ± 0.35	2.79 ± 0.07	37.43 ± 0.13	3.04 ± 0.05
	HbD	2.24 ± 0.04	1.60 ± 0.05	3.23 ± 0.07	2.02 ± 0.09	30.66 ± 0.92	2.32 ± 0.17	22.28 ± 0.34	2.71 ± 0.12
Anas puna	HbA	3.38 ± 0.01	2.31 ± 0.75	4.35 ± 0.01	2.18 ± 0.01	33.29 ± 0.34	2.89 ± 0.11	27.32 ± 0.33	2.91 ± 0.11
	HbD	2.06 ± 0.01	1.63 ± 0.04	3.09 ± 0.01	1.87 ± 0.01	24.61 ± 0.44	2.81 ± 0.16	17.98 ± 0.13	3.49 ± 0.10
Anas versicolor	HbA	3.93 ± 0.02	2.34 ± 0.03	5.80 ± 0.04	2.34 ± 0.05	55.55 ± 0.48	2.97 ± 0.07	39.66 ± 1.01	2.62 ± 0.20
	HbD	1.97 ± 0.01	1.81 ± 0.03	3.67 ± 0.06	2.35 ± 0.13	32.60 ± 0.03	2.84 ± 0.01	28.54 ± 0.15	3.12 ± 0.05

Table S3. Relative percentage concentrations of the minor HbD isoform (mean ± SD) in the red blood cells of highland and lowland Andean birds.

Taxon	% HbD	*N*
Metriopelia melanoptera	0	7
Columbina cruziana	0	7
Hydropsalis longirostris	21.0 ± 5.3	6
Hydropsalis decussata	18.4 ± 4.1	5
Colibri coruscans	3.2 ± 0.7	7
Schistes geoffroyi	3.4 ± 1.2	7
Selasphorus platycercus	7.2 ± 1.2	4
Archilochus alexandri	12.4 ± 1.9	8
Amazilia viridicauda	11.5 ± 4.7	7
Amazilia amazilia	11.8 ± 10.5	7
Chalcostigma stanleyi	1.9 ± 0.8	5
Chalcostigma ruficeps	4.2 ± 0.3	5
Oreotrochilus estella	13.6 ± 15.4	7
Adelomyia melanogenys	13.3 ± 10.9	7
Eriocnemis luciani	6.6 ± 1.8	11
Haplophaedia aurelieae	19.3 ± 0.5	2
Aglaeactis castelnaudii	11.2 ± 6.4	6
Heliodoxa leadbeateri	7.5 ± 0.8	4
Pterophanes cyanopterus	2.9 ± 1.0	3
Boissonneaua matthewsii	2.12 ± 0.9	3
Coeligena violifer	12.1 ± 9.1	7
Coeligena coeligena	1.6 ± 2.0	7
Cinclodes albiventris	31.9 ± 0.6	6
Furnarius leucopus	38.6 ± 0.9	7
Notiochelidon murina	30.1 ±1.9	8
Pygochelidon cyanoleuca	28.4 ±1.2	4
Troglodytes aedon (H)	36.8 ± 1.3	3
Troglodytes aedon (L)	42.6 ± 1.0	4
Spinus magellanica (H)	21.7 ± 1.4	4
Spinus magellanica (L)	18.1 ± 2.5	5
Zonotrichia capensis (H)	36.2 ± 12.6	13
Zonotrichia capensis (L)	30.5 ± 10.1	17
Tangara vassorii	37.6 ± 0.6	3
Tangara nigroviridis	36.48 ± 0.4	2
Conirostrum cinereum (H)	36.9 ± 1.0	7
Conirostrum cinereum (L)	37.3 ± 1.1	7
Diglossa brunneiventris	26.8 ± 1.2	8
Diglossa glauca	22.2 ± 1.1	4
Catamenia analis (H)	31.4 ± 2.0	5
Catamenia analis (L)	28.1 ± 0.8	7
Oxyura jamaicensis (H)	19.0 ±0.4	9
Oxyura jamaicensis (L)	18.6 ± 1.0	5
Merganetta armata (H)	17.6 ± 1.0	7
Merganetta armata (L)	17.1 ± 0.9	7
Chloephaga melanoptera	18.1 ± 2.0	7
Neochen jubata	-	-
Lophonetta s. alticola	25.5 ± 2.1	8
Lophonetta s. specularioides	24.4 ± 1.5	8
Anas georgica (H)	30.1 ± 1.6	8

Anas georgica (L)	30.1 ± 2.0	8
Anas f. oxyptera	29.6 ± 1.2	7
Anas f. flavirostris	31.4 ± 2.7	8
Anas c. orinoma	22.0 ± 0.7	7
Anas c. cyanoptera	21.6 ± 1.3	6
Anas puna	25.2 ± 1.4	8
Anas versicolor	26.3 ± 0.4	3

Table S4. Allosteric regulation of Hb-O_2 affinity in highland and lowland Andean birds. O_2 affinities (P_{50}, torr) were measured in 0.1 mM HEPES buffer at pH 7.4 (± 0.01) and 37ºC in the absence (stripped) and presence of Cl^- ions (0.1 M KCl]) and IHP (at two-fold molar excess over tetrameric Hb). Sensitivity to allosteric effectors is indexed by the difference in log-transformed P_{50} values measured for stripped Hb samples in the presence and absence of Cl^- ions and IHP. $\Delta\log P_{50(KCl\text{-}str)}$ measures Cl^- sensitivity, $\Delta\log P_{50(IHP\text{-}str)}$, and $\Delta\log P_{50([KCl+IHP]\text{-}str)}$ measures sensitivity to both effectors together.

Taxon	IsoHb	$\Delta\log P_{50(KCl\text{-}str)}$	$\Delta\log P_{50(IHP\text{-}str)}$	$\Delta\log P_{50([KCl+IHP]\text{-}str)}$
Metriopelia melanoptera	HbA	0.135	0.970	0.861
	HbD	-	-	-
Columbina cruziana	HbA	0.088	0.956	0.775
	HbD	-	-	-
Hydropsalis longirostris	HbA	0.177	1.245	1.086
	HbD	0.192	1.013	0.924
Hydropsalis decussata	HbA	0.185	1.260	1.115
	HbD	0.180	1.201	1.017
Colibri coruscans	HbA	0.123	1.096	0.973
	HbD	-	-	-
Schistes geoffroyi	HbA	0.139	1.067	0.981
	HbD	-	-	-
Selasphorus platycercus	HbA	0.210	1.096	1.016
	HbD	0.082	1.017	0.872
Archilochus alexandri	HbA	0.148	1.082	0.994
	HbD	0.118	1.008	0.927
Amazilia viridicauda	HbA	0.232	1.036	0.966
	HbD	0.147	0.895	0.865
Amazilia amazilia	HbA	0.226	1.069	0.978
	HbD	0.154	0.930	0.839
Chalcostigma stanleyi	HbA	0.113	1.042	0.963
	HbD	-	-	-
Chalcostigma ruficeps	HbA	0.159	1.067	0.950
	HbD	-	-	-
Oreotrochilus estella	HbA	0.194	1.002	0.969
	HbD	-	-	-
Adelomyia melanogenys	HbA	0.208	1.005	1.051
	HbD	-	-	-
Eriocnemis luciani	HbA	0.158	1.158	1.003
	HbD	0.058	0.974	0.877
Haplophaedia aurelieae	HbA	0.162	1.087	1.006
	HbD	-	-	-
Aglaeactis castelnaudii	HbA	0.173	1.015	0.900
	HbD	-	-	-
Heliodoxa leadbeateri	HbA	0.122	1.084	0.986
	HbD	0.114	0.991	0.912
Pterophanes cyanopterus	HbA	0.112	1.046	0.942
	HbD	-	-	-
Boissonneaua matthewsii	HbA	0.129	1.004	0.937
	HbD	-	-	-
Coeligena violifer	HbA	0.247	1.046	0.955
	HbD	0.168	0.854	0.836
Coeligena coeligena	HbA	0.229	1.048	0.964
	HbD	-	-	-
Cinclodes albiventris	HbA	0.118	1.095	1.007

	HbD	0.118	1.071	1.002
Furnarius leucopus	HbA	0.113	1.220	1.065
	HbD	0.054	1.127	0.992
Notiochelidon murina	HbA	0.137	1.184	1.003
	HbD	0.093	1.039	0.869
Pygochelidon cyanoleuca	HbA	0.136	1.168	1.021
	HbD	0.126	1.023	0.874
Troglodytes aedon (H)	HbA	0.079	0.938	0.840
	HbD	0.191	1.043	0.927
Troglodytes aedon (L)	HbA	0.213	1.083	0.966
	HbD	0.228	1.156	1.013
Spinus magellanica (H)	HbA	0.225	1.184	1.040
	HbD	0.221	1.207	0.954
Spinus magellanica (L)	HbA	0.225	1.129	0.999
	HbD	0.189	1.113	0.999
Zonotrichia capensis (H)	HbA	0.189	1.205	1.112
	HbD	0.188	1.152	0.994
Zonotrichia capensis (L)	HbA	0.231	1.202	1.036
	HbD	0.203	1.244	1.095
Tangara vassorii	HbA	0.127	1.167	1.018
	HbD	0.105	1.097	0.966
Tangara nigroviridis	HbA	0.052	1.130	1.044
	HbD	0.104	1.137	1.047
Conirostrum cinereum (H)	HbA	0.130	1.129	1.036
	HbD	0.170	1.137	1.002
Conirostrum cinereum (L)	HbA	0.154	1.080	1.008
	HbD	0.111	1.069	0.944
Diglossa brunneiventris	HbA	0.162	1.159	1.068
	HbD	0.171	1.094	0.942
Diglossa glauca	HbA	0.145	1.165	1.052
	HbD	0.095	0.987	0.874
Catamenia analis (H)	HbA	0.170	1.164	1.068
	HbD	0.170	1.109	1.023
Catamenia analis (L)	HbA	0.148	1.144	1.085
	HbD	0.104	1.144	0.999
Oxyura jamaicensis (H)	HbA	0.147	1.171	1.025
	HbD	0.138	1.135	0.977
Oxyura jamaicensis (L)	HbA	0.146	1.198	1.071
	HbD	0.077	1.067	0.910
Merganetta armata (H)	HbA	0.178	1.221	1.122
	HbD	0.173	1.079	0.997
Merganetta armata (L)	HbA	0.158	1.160	1.063
	HbD	0.178	1.072	0.988
Chloephaga melanoptera	HbA	0.178	1.079	0.982
	HbD	0.181	1.063	0.939
Neochen jubata	HbA	0.228	1.118	1.051
	HbD	0.191	1.022	0.984
Lophonetta s. alticola	HbA	0.212	1.115	0.975
	HbD	0.177	0.832	0.732
Lophonetta s. specularioides	HbA	0.150	1.182	1.042
	HbD	0.214	1.128	1.023
Anas georgica (H)	HbA	0.197	1.185	1.135
	HbD	0.129	0.993	0.887
Anas georgica (L)	HbA	0.187	1.239	1.112
	HbD	0.217	1.030	0.809

Anas f. oxyptera	HbA	0.211	1.200	1.148
	HbD	0.136	1.030	0.890
Anas f. flavirostris	HbA	0.296	1.311	1.165
	HbD	0.155	1.051	0.919
Anas c. orinoma	HbA	0.180	1.167	1.093
	HbD	0.171	1.235	1.090
Anas c. cyanoptera	HbA	0.150	1.201	1.096
	HbD	0.159	1.136	0.998
Anas puna	HbA	0.110	0.993	0.908
	HbD	0.176	1.077	0.941
Anas versicolor	HbA	0.169	1.150	1.004
	HbD	0.270	1.219	1.161

Table S5. Phenotypic effects of phylogenetically replicated β-chain substitutions in the Hbs of highland and lowland Andean birds. For each of the listed substitutions in each pair of taxa, the Hbs of the high-altitude taxon always possesses the derived amino acid. In addition to the replicated substitutions listed below, the HbA and HbD isoforms of each pair of taxa often differ at one or more additional sites (see Fig. 1). Detailed experimental data for each of the native HbA and HbD variants are provided in table S2. Causative effects of each of the N/G83S, A86S, D94E, and A116S substitutions have been confirmed by experimental tests involving purified, native Hb variants as well as recombinant Hb mutants that were engineered via site-directed mutagenesis (Fig. 3A,B)[*12*, *16*].

β-chain substitution	Taxon pair (highland/lowland)	Family	Hb isoform	$\Delta P_{50(\mathrm{KCl+IHP})}$ (%)
G83S	*Colibri coruscans* / *Schistes geoffroyi*	Trochilidae	HbA	-15.6
G83S	*Amazilia viridicauda* / *A. amazilia*	Trochilidae	HbA	-18.8
G83S			HbD	-12.2
G83S	*Oreotrochilus estella* / *Adelomyia melanogenys*	Trochilidae	HbA	-36.9
G83S	*Eriocnemis luciani* / *Haplophaedia aurelieae*	Trochilidae	HbA	-10.8
G83S	*Aglaeactis castelnaudii* / *Heliodoxa leadbeateri*	Trochilidae	HbA	-54.7
G83S	*Coeligena violifer* / *C. coeligena*	Trochilidae	HbA	-16.5
N83S	*Diglossa brunneiventris* / *D. glauca*	Thraupidae	HbA	-21.2
N83S			HbD	-14.2
A86S	*Cinclodes albiventris* / *Furnarius leucopus*	Furnariidae	HbA	-43.8
A86S			HbD	-25.9
A86S	*Chloephaga melanoptera* / *Neochen jubata*	Anatidae	HbA	-21.7
A86S			HbD	-17.0
D94E	*Metriopelia melanoptera* / *Columbina cruziana*	Columbidae	HbA	-5.4
D94E	*Lophonetta specularioides alticola* / *L. s. specularioides*	Anatidae	HbA	-33.8
D94E			HbD	-48.4
D94E	*Anas puna* / *Anas versicolor*	Anatidae	HbA	-31.1
D94E			HbD	-37.0
A116S	*Anas georgica* (high) / *A. georgica* (low)	Anatidae	HbA	-15.6
A166S			HbD	+2.3
A116S	*Anas flavirostris oxyptera* / *A.f. flavirostris*	Anatidae	HbA	-27.9
A166S			HbD	-43.2

Table S6. O_2 affinities (P_{50}, torr) of purified recombinant avian Hbs representing a diverse range of ancestral genotypes. On each background, we tested the effect of β-chain G/N83S substitutions and, in the case of Anc flowerpiercer, the effect of α-chain V67A substitutions. For each genotype, the derived amino acid is underlined in bold. O_2 equilibria were measured in 0.1 mM HEPES buffer at pH 7.4 (± 0.01) and 37ºC in the absence (stripped) and presence of IHP (at two-fold molar excess over tetrameric Hb) and in the simultaneous presence of IHP and Cl^- ions (0.1 M KCl]). P_{50} values were derived from O_2 equilibrium curves, where each value was interpolated from linear Hill plots based on 4 or more equilibrium steps between 25 and 75% saturation.

		Stripped	+IHP	+KCl + IHP
Ancestral background	Genotype	P_{50}	P_{50}	P_{50}
Anc 1	β83G	2.74 ± 0.03	24.22 ± 0.90	19.38 ± 0.41
Anc 1	β83**S**	3.08 ± 0.06	16.73 ± 0.65	11.76 ± 1.09
Anc Hummingbird	β83G	3.83 ± 0.10	25.97 ± 0.37	16.95 ± 0.58
Anc Hummingbird	β83**S**	5.51 ± 0.25	18.26 ± 0.26	12.42 ± 0.22
Anc Flowerpiercer	α67V, β83N	3.09 ± 0.03	29.58 ± 1.47	26.27 ± 0.49
Anc Flowerpiercer	α67**A**, β83N	3.22 ± 0.06	21.78 ± 0.72	21.66 ± 1.46
Anc Flowerpiercer	α67V, β83**S**	3.67 ± 0.09	26.12 ± 0.49	19.15 ± 1.16
Anc Flowerpiercer	α67**A**, β83**S**	2.67 ± 0.04	17.27 ± 0.05	12.57 ± 0.70
Anc Neoaves	β83N	4.03 ± 0.14	26.48 ± 0.34	21.12 ± 0.40
Anc Neoaves	β83**S**	3.64 ± 0.08	26.95 ± 0.44	21.32 ± 0.44
Anc Neornithes	β83N	3.60 ± 0.28	28.21 ± 0.65	19.26 ± 1.57
Anc Neornithes	β83**S**	4.48 ± 0.14	25.73 ± 0.48	18.96 ± 0.94

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