

Research



Cite this article: Beckman EJ, Vargas Campos W, Benham PM, Schmitt CJ, Cheviron ZA, Witt CC. 2022 Selection on embryonic haemoglobin in an elevational generalist songbird. *Biol. Lett.* **18**: 20220105.
<https://doi.org/10.1098/rsbl.2022.0105>

Received: 27 February 2022

Accepted: 20 September 2022

Subject Areas:

evolution

Keywords:

adaptation, high elevation, hypoxia, haemoglobin, development, birds

Authors for correspondence:

Elizabeth J. Beckman

e-mail: libby.beckman@berkeley.edu

Christopher C. Witt

e-mail: cwitt@unm.edu

Electronic supplementary material is available online at <https://doi.org/10.6084/m9.figshare.c.6238138>.

Evolutionary biology

Selection on embryonic haemoglobin in an elevational generalist songbird

Elizabeth J. Beckman^{1,2}, Walter Vargas Campos³, Phred M. Benham^{1,2}, C. Jonathan Schmitt^{1,4}, Zachary A. Cheviron⁵ and Christopher C. Witt¹

¹Museum of Southwestern Biology and Department of Biology, University of New Mexico, Albuquerque, NM 87131, USA

²Museum of Vertebrate Zoology and Department of Integrative Biology, University of California, Berkeley, CA 94720, USA

³Centro de Ornitología y Biodiversidad, Calle Sta. Rita 105, Oficina 202, Santiago de Surco, Lima, Perú

⁴Department of Organismic and Evolutionary Biology and Museum of Comparative Zoology, Harvard University, Cambridge, MA 02138, USA

⁵Division of Biological Sciences, University of Montana, Missoula, MT, USA

id EJB, 0000-0002-8303-2475; CJS, 0000-0002-3100-7331; CCW, 0000-0003-2781-1543

Animals developing at high elevation experience a suite of environmental challenges, most notably the low partial pressure of oxygen (PO_2) in ambient air. In low PO_2 , bird species with high-elevation ancestry consistently demonstrate higher hatching success than lowland counterparts, suggesting highland birds are adapted to restricted O_2 (hypoxia) in early development. Haemoglobin (Hb), the critical oxygen-transport protein, is a likely target of PO_2 -related selection across ontogeny since Hb isoforms expressed at distinct developmental stages demonstrate different O_2 affinities. To test if Hb function is under PO_2 -related selection at different ontogenetic stages, we sampled a songbird, the hooded siskin (*Spinus magellanicus*), across two approximately 4000 m elevational transects. We sequenced all of the loci that encode avian Hb isoforms, and tested for signatures of spatially varying selection by comparing divergence patterns in Hb loci to other loci sampled across the genome. We found strong signatures of diversifying selection at non-synonymous sites in loci that contribute to embryonic (α^E , β^E) and definitive (β^A) Hb isoforms. This is the first evidence for selection on embryonic haemoglobin in high-elevation Neoaves. We conclude that selection on Hb function at brief, but critical stages of ontogeny may be a vital component to high elevation adaptation in birds.

1. Introduction

Selection pressures over the course of a lifetime determine whole-organism fitness. Animals developing at high elevation must surmount a number of environmental challenges. In particular, the decrease in the partial pressure of oxygen (PO_2) with increasing elevation is a chronic stressor for metabolically active animals [1]. Consequently, many high-elevation lineages have evolved genetic adaptations to cope with this environmental challenge [2]. The stress associated with low PO_2 (hypoxia) may be extreme at specific ontogenetic stages given the different energetic costs of growth and reproduction [3]. Further, the path of oxygen from the air to respiring tissues changes fundamentally from prenatal to postnatal life in many vertebrates including birds and mammals [4,5].

At the earliest life stages, birds breeding at high elevation are vulnerable to unique environmental stresses. Low barometric pressure impacts gas diffusion

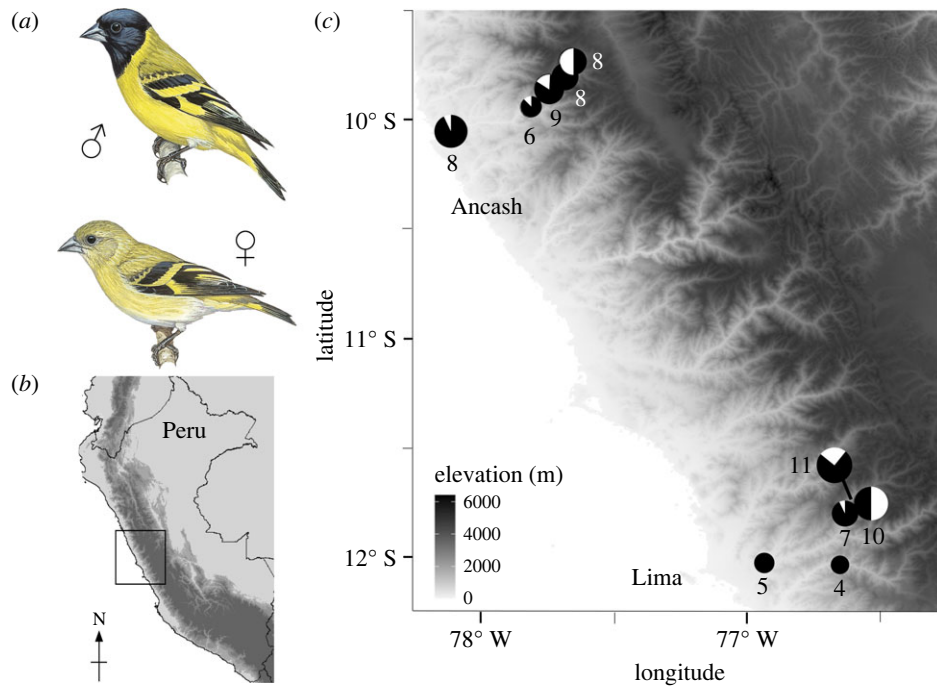


Figure 1. Peruvian hooded siskin sampling localities. (a) The hooded siskin; copyright © Lynx Edicions. (b) Peru; the study area in (c) is indicated with a black box. (c) Sample localities for Ancash and Lima transects. The number of individuals per locality is shown below each circle; coloration shows the proportion of $\beta^H135Val$ (black) and $\beta^H135Iso$ (white) alleles at each locality.

across the eggshell; consequently, water loss increases and the oxygen gradient between the environment and the egg is reduced [6,7]. Hatching success at low PO_2 is significantly reduced in birds with lowland ancestry [4,8,9]. However, high-elevation birds demonstrate embryonic growth rates, incubation times and hatching success *in situ* similar to lowland counterparts [6,9–11]. These differences may reflect evolved strategies to tolerate low barometric pressure during development. Compared to lowland relatives, embryos in high-elevation populations exhibit increased eggshell resistance to minimize water loss [6,12], enhanced oxygen delivery via the critical oxygen-transport protein haemoglobin (Hb) [13] and distinct transcript abundance profiles [14].

Little is known about the genetic basis of adaptations that improve embryonic survival at high elevation. However, haemoglobin, a tetramer comprised subunits from the α -globin and β -globin gene families, is a likely target of PO_2 -related selection across ontogeny. First, Hb isoforms with different O_2 affinities are expressed at different life stages [4]. Second, in the first week of incubation, embryonic Hb facilitates oxygen transport when diffusion is most restricted across the eggshell [7]. Third, birds at high elevation frequently demonstrate a stronger intrinsic Hb- O_2 binding affinity than lowland relatives due to spatially varying selection on the globins α^D , α^A and β^A which contribute to definitive Hb isoforms after the first week of incubation [14–17]. Last, in *Anas* ducks, the embryonic Hb locus β^E , along with β^A , is strongly differentiated at several non-synonymous sites between high and low populations, suggesting that embryonic Hb is under selection at high elevation [18]. However, there are fundamental differences in embryonic growth rates [19] and Hb transcript abundance profiles [20,21] in early development between Galloanserae (ducks, chickens and geese) and other birds. Selection on Hb function at different stages of ontogeny may be a potent mechanism for improving hypoxia tolerance in birds. However, to

understand its significance across the avian tree of life, this hypothesis must be evaluated in Neoaves, a large clade including over 95% of bird species [22].

To further our understanding of selection at high elevations over different ontogenetic stages, we tested for signatures of spatially varying selection in all Hb loci in a songbird, the hooded siskin (*Spinus magellanicus*). The hooded siskin occupies a broad elevational range in South America [23] and demonstrates little genetic structure across the west slope of the Peruvian Andes [24]. Natarajan *et al.* [17] identified a Hb β^A allele, present only in high-elevation individuals, that conferred an increase in Hb- O_2 binding affinity compared to other alleles. This suggests that adult-expressed Hb isoforms may be under selection across elevation in hooded siskins. To test the role of PO_2 -related selection on Hb loci, including those expressed during embryonic development, we sequenced all seven Hb loci using a custom capture array and tested for signatures of spatially varying selection by comparing patterns of divergence in Hb loci to other loci sampled across the genome.

2. Methods

We sampled hooded siskins, *Spinus magellanicus*, across two 75 km long transects spanning approximately 4000 m of elevation in the Peruvian Andes (figure 1; electronic supplementary material, appendix). We sampled 37 and 39 post-fledge individuals in the departments of Lima and Ancash, respectively. On each transect, we acquired an average of eight individuals from the following elevations: 0–400 m; 700–1000 m; 1400–2300 m; 2500–3000 m; and above 3700 m. To provide context for the intraspecific results, we sampled another seven South American *Spinus* species, the distinct genetic lineage *S. magellanicus alleni*, and *S. notatus*, sister to all South American *Spinus* species [24,25] (electronic supplementary material, appendix).

Table 1. Non-synonymous haemoglobin candidate SNP variation in *Spinus magellanicus*. Values greater than 97% or 99% of genome-wide SNPs are indicated by * and **, respectively, and are in bold. O₂ affinity summarized from [17]. Polarity change indicates a difference in polarity state (e.g. hydrophobic, polar, charged) between alternative amino acids.

	major allele < 1000 m	alternative allele	polarity change	change in O ₂ affinity	transect	allele frequency change	allele frequency change, 3000 to 3700 m	<i>F</i> _{ST}	PBS
$\alpha^{\pi 60}$	serine	phenylalanine	yes	—	Ancash	0.313	0.313	0.317*	0.286*
					Lima	0.05	0.05	—	—
$\beta^A 21$	alanine	threonine	yes	yes	Ancash	0.456*	0.5*	0.432*	0.625**
					Lima	0.21	0.16	0.132	0.099
$\beta^H 135$	valine	isoleucine	no	—	Ancash	0.409*	0.5*	0.316*	0.487**
					Lima	0.5*	0.25	0.447**	0.469**

We used a target-capture approach to sequence the seven complete Hb loci (α^{π} , α^D , α^A ; β^E , β^H , β^A , β^P) based on Hb locus alignments constructed from nine-primaried oscines. We also targeted one or two exons from 363 randomly selected genes distributed across the autosomal genome (electronic supplementary material, file S1) based on the genome of a near-relative, the common canary *Serinus canaria* (GCA_000534875.1) [26]. We extracted DNA from muscle with the Qiagen DNeasy Blood and Tissue kit and prepared individual genomic libraries with the NEBNext Ultra DNA library prep kit and NEBNext Multiplex Oligos for Illumina. We aimed for an insert size of 400–500 bp, enriched the libraries using an MYbaits custom target enrichment kit, and pooled samples in equimolar proportions. We sequenced all individuals in one Illumina HiSeq 2500 lane (160 bp, paired-end) at the Keck Center at the University of Illinois, Champaign-Urbana. Raw reads were demultiplexed; we retained reads with a PHRED score >30.

We built locus-specific *pseudo*-references with aTRAM v. 1.04 [27] using Velvet v. 1.2.10 [28]. For paralogous genes like Hb loci, multiple sequences may be recovered in an aTRAM analysis. We initiated aTRAM runs with *Spinus* Hb cDNA sequences, then alternated between by-eye evaluation in Geneious v. 6 [29] and aTRAM to identify seven Hb loci. For each autosomal locus, we initiated aTRAM with the original probe and selected the longest contig as the reference. We aligned samples to each *pseudo*-reference with a sensitive search in Bowtie2 v. 2.2.6 [30], indexed reads in SAMtools v. 1.3.1 [31], and used Angsd v. 0.934 [32] to call variants. We filtered SNPs with VCFtools v. 0.1.16 [33] to exclude sites with less than 70% of individuals at a locality, transect-specific minor allele frequency under 0.05 and, for genome-wide loci, high linkage disequilibrium ($LD \geq 0.5$). For interspecific data, we called SNPs using SAMtools and bcftools v. 1.3.1 [31].

We assessed population differentiation and selection among Peruvian hooded siskins. First, we conducted a principal component analysis using the genome-wide SNPs in R v. 4.1.0 [34]. Next, we calculated per-site *F*_{ST} [35] on each transect between individuals collected below 1000 m and those above 3700 m in VCFtools. To identify high-elevation population-specific allele frequency change on each transect, we calculated the per-site population branch statistic (PBS) [36]. We defined one focal high-elevation population, calculated pairwise *F*_{ST} among the focal population and the two low elevation populations (Lima < 1000 m; Ancash < 1000 m), then used a custom script (electronic supplementary material, file S2) in python v. 3.9.5 to compute the PBS. We estimated the total allele frequency change and the change observed from approximately 3000 m to over 3700 m on each transect. To assess significance, we asked if any non-synonymous sites in Hb loci demonstrated a value

that was greater than 95% of the SNPs distributed across the autosome for *F*_{ST}, PBS and total allele frequency change. Finally, we characterized the variation of non-synonymous Hb sites across the South American *Spinus* clade.

3. Results

We sequenced and aligned the complete coding sequences of all seven haemoglobin loci (electronic supplementary material, table S1). We recovered an average sequencing depth of 4.06× for Hb loci and 2.96× for genome-wide loci. We found high agreement between the *S. magellanicus* Hb genotypes inferred in this study and from published Sanger-sequenced cDNA (electronic supplementary material, file S3). After filtering, we recovered 249 variable loci from coding regions across the autosomal genome. Principal component analysis using the genome-wide loci revealed no structure within Peru in hooded siskins (electronic supplementary material, figure S1), concordant with previous work [24].

We tested for signatures of spatially varying selection by comparing the interrelated statistics of *F*_{ST}, PBS and total allele frequency change at non-synonymous SNPs in Hb loci to SNPs from across the autosome. We identified three non-synonymous Hb sites in the top 5% of all SNPs genome-wide in at least one of these transect-specific statistics: embryonic Hb sites $\alpha^{\pi 60}$ and $\beta^H 135$, and definitive Hb site $\beta^A 21$ (table 1; figure 2). All three Hb SNPs had a significant *F*_{ST} and PBS value in Ancash. In Lima, $\beta^H 135$ was the only significant outlier. For $\beta^A 21$, the high-elevation allele ($\beta^A 21$ Thr) in Ancash was also at a higher frequency in the Lima high-elevation population, but the frequency difference was not statistically significant in Lima. We plotted allele frequency by elevation for $\alpha^{\pi 60}$, $\beta^A 21$ and $\beta^H 135$, and discovered that a large proportion of the allele frequency change that occurred in Ancash was between the two highest sites (table 1; figure 2). By contrast, allele frequency in $\beta^H 135$ shifted as a linear function of elevation in Lima (adjusted $R^2 = 0.86$, *p*-value 0.014).

We examined interspecific variation at these three non-synonymous Hb sites in South American *Spinus* (electronic supplementary material, figures S2, S3). For two sites, the highland allele we found in Ancash and Lima ($\beta^A 21$ Thr; $\beta^H 135$ Iso) only occurred in *Spinus* species with an upper range limit of at least 3000 m (table 2). Species that only occupy below 3000 m

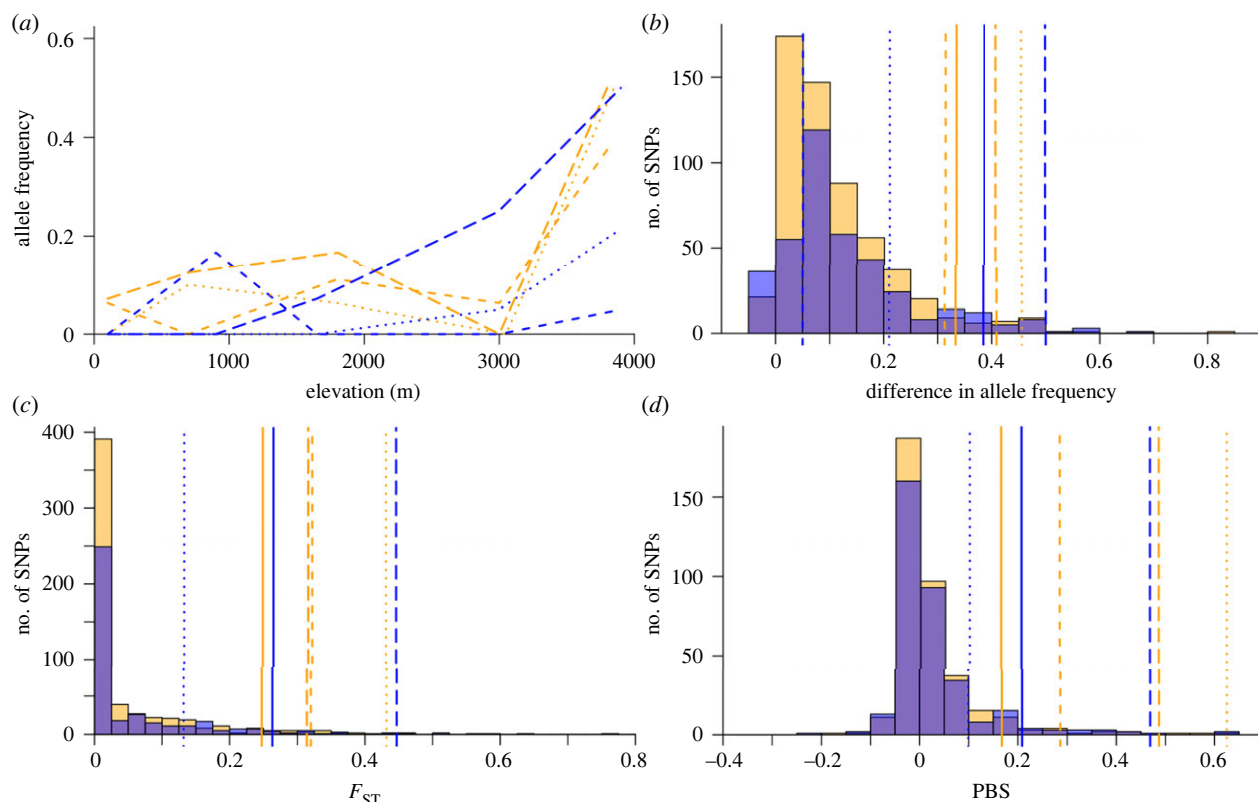


Figure 2. Haemoglobin non-synonymous candidate SNPs across elevation in hooded siskins. Results are coded by line type ($\alpha^{\pi 60}$, short-dashed; β^A21 , dotted; β^H135 , long-dashed) and colour (blue, Lima; orange, Ancash); overlap of the transect-specific histograms appears purple. (a) Allele frequency across elevation. Statistics in (b–d) calculated between populations below 1000 m and above 3700 m; 95% threshold shown as a solid line. (b) Allele frequency differences. (c) F_{ST} (d) PBS. Close values in (a) and (c) slightly offset for visibility.

Table 2. Variation of non-synonymous haemoglobin candidate SNPs across the *Spinus* clade. N indicates number of individuals. Alternative amino acids abbreviated as F: phenylalanine, T: threonine, I: isoleucine.

species	N	highest elevation (m)	$\alpha^{\pi 60}$	β^A21	β^H135
<i>Spinus notatus</i>	1	≤ 3000	serine	alanine	valine
<i>S. cucullatus</i>	1	≤ 3000	S	A	V
<i>S. barbatus</i>	1	≤ 3000	S	A	V
<i>S. olivaceus</i>	1	≤ 3000	S	A	V
<i>S. siemiradzki</i>	1	≤ 3000	S	A	V
<i>S. magellanicus alleni</i> , lowland	2	< 430	S	A	V
<i>S. magellanicus alleni</i> , Andes	1	~ 2500	S	A/T	I
<i>S. magellanicus</i> , Peru	76	≥ 3000	S/F	A/T	V/I
<i>S. uropygialis</i>	1	≥ 3000	S	A/T	I
<i>S. crassirostris</i>	2	≥ 3000	S	T	I
<i>S. atratus</i>	1	≥ 3000	S	T	I

were fixed for the common allele at low elevation in hooded siskins ($\beta^A21\text{Ala}$; $\beta^H135\text{Val}$). In *S. magellanicus alleni*, a genetic lineage observed from lowland Argentina into mid-elevation Bolivia, highland alleles were only present in individuals sampled near 2500 m. We observed the variation at $\alpha^{\pi 60}$ in hooded siskins from Peru alone; all other *Spinus* were fixed for $\alpha^{\pi 60}\text{Ser}$.

4. Discussion

We report the first evidence for selection on embryonic haemoglobin in high-elevation Neoaves. β^H , which encodes

the β -globin subunit of a major embryonic Hb isoform in passerines [21], contained the SNP β^H135 , that showed statistically significant population-specific differentiation on both transects. α^{π} encodes the α subunit of the embryonic Hb π ; the variant $\alpha^{\pi 60}$, which impacts amino acid side-chain polarity, demonstrated significant differentiation in Ancash, but not Lima. Last, a previously identified variant, the β^A21 allele, which also changes amino acid polarity, is known to alter the O_2 affinity of the definitive Hb isoforms HBD and HBA [17]. Here, we uncovered a striking signature of selection at β^A21 in Ancash, and a suggestive trend in Lima. Together, these

patterns suggest that the known functional effect at β^{A21} impacts whole-organism fitness. We conclude that hypoxic conditions likely exert selective pressure across the entire lifespan of this passerine, beginning in early development.

Avian embryonic haemoglobin isoforms contribute to O_2 transport over a brief window, from days 3–6 of incubation [37]. Our results highlight that early development is a sensitive life stage with high mortality risk [38]. Further, performance (e.g. the ability to cope with hypoxia) over short, critical windows can contribute to lifetime success. An analogous scenario is the extreme hypoxia experienced by the bar-headed goose (*Anser indica*) during its single-day Himalayan migration [39]. These conclusions fit the intraspecific and interspecific patterns in *Spinus*. Embryonic β^{H135} and definitive β^{A21} exhibit local adaptation in Peruvian hooded siskins and segregate by elevation across South American *Spinus*. *Spinus* is a recent, rapidly diverged clade with widespread introgression [24]. A plausible explanation for the observed pattern is a single origin for each Hb allele and some combination of ancestral polymorphism, inter-specific introgression and PO_2 -related selection at the species level.

Selection on embryonic Hb function may be a critical mechanism to improve oxygen transport in hypoxia in early avian development. Although deeply diverged from Neoaves, detailed studies in domestic fowl provide perspective on why this may be the case. In the chicken egg, oxygen in the first 6 days of incubation is severely limited due to the slow rate of gas diffusion across the aqueous inner membrane [4]. In the first week, the embryo requires little O_2 , and embryos with impaired Hb function may survive in normoxia [37]. However, in hypoxic conditions, lowland-ancestry embryo mortality is very high [40]. Many of the physiological mechanisms that allow embryos to tolerate hypoxia, like the synthesis of definitive Hb isoforms and their regulation through allosteric cofactors and changes to blood pH, develop at later ontogenetic stages [4]. Definitive Hb isoforms and their regulatory mechanisms develop earlier under hypoxic conditions [4,7,12,41]. However, oxygen transport from day 3–6 of incubation is unaltered by hypoxia exposure [37]; the growing embryo depends exclusively on embryonic Hb isoforms to transport diffused O_2 to respiring tissues [7]. Thus, modifications that facilitate O_2 binding and delivery for embryonic Hb likely contribute to improved embryo survivorship at low PO_2 . Studies that further examine the development of altricial passerines in hypoxic conditions will advance our mechanistic understanding of adaptation to hypoxia across the avian tree of life.

5. Conclusion

We report the first evidence of spatially varying selection associated with high elevation on Hb function at different stages of ontogeny in Neoaves. Our results dovetail with previous research that shows PO_2 -related selection on the primary sequence and relative isoform proportions of definitive Hb isoforms across the avian tree of life [15–17,42]. Embryonic Hb may be similarly predictable. In addition to our study, Graham *et al.* [18] showed significant differentiation between high and low lineages in embryonic Hb β^e in ducks (Galloanserae). We conclude that selection to improve hypoxia tolerance in avian embryos may be a critical, but understudied component to genetic adaptation to high elevation in birds.

Ethics. All fieldwork was conducted under the IACUC protocol 14-101228-MCC at the University of New Mexico and with the permission of Peruvian authorities (permits 0199-2012-AG-DGFFS-DGEFFS, 006-2013-MINAGRI-DGFFS/DGEFFS, and 280-2014-MINAGRI-DGFFS-DGEFFS). Specimens were deposited at the Centro de Ornitología y Biodiversidad (CORBIDI) in Lima, Peru and the Museum of Southwestern Biology (MSB) at University of New Mexico (UNM) in New Mexico, USA, with full data available in the Arctos database (arctosdb.org).

Data accessibility. Sequence data are available at NCBI Sequence Read Archive as BioProject ID PRJNA857269. Specimen data are available in the electronic supplementary material, appendix. Electronic supplementary material information includes a description of the data as well as all the code used (see electronic supplementary material, file S2). A version of this manuscript in Spanish (Español) is available as electronic supplementary material, file S4.

The data are provided in electronic supplementary material [43].

Authors' contributions. E.J.B.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, resources, software, visualization, writing—original draft, writing—review and editing; W.V.C.: data curation, resources, writing—review and editing; P.M.B.: data curation, methodology, resources, writing—review and editing; C.J.S.: data curation, resources, writing—review and editing; Z.A.C.: methodology, project administration, resources, writing—review and editing; C.C.W.: conceptualization, data curation, funding acquisition, methodology, project administration, resources, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This work was supported by the National Science Foundation (grant numbers DEB-1146491, DBI-1907358), the American Museum of Natural History and the University of New Mexico Department of Biology.

Acknowledgements. We gratefully thank SERFOR, Thomas Valqui, Emil Bautista O., Andrew B. Johnson, Alvaro Hernandez, and the associates of CORBIDI and UNM Ornithology for their many and varied contributions. We thank the Museum of Southwestern Biology, the Kansas University Biodiversity Institute, the National Museum of Natural History, the Burke Museum, the Field Museum of Natural History and the Louisiana State University Museum of Natural Science for tissue loans.

References

- Storz JF, Scott GR, Cheviron ZA. 2010 Phenotypic plasticity and genetic adaptation to high-altitude hypoxia in vertebrates. *J. Exp. Biol.* **213**, 4125–4136. (doi:10.1242/jeb.048181)
- Storz JF, Cheviron ZA. 2021 Physiological genomics of adaptation to high-altitude hypoxia. *Annu. Rev. Anim. Biosci.* **9**, 149–171. (doi:10.1146/annurev-animal-072820-102736)
- West G, Brown J, Enquist B. 2001 A general model for ontogenetic growth. *Nature* **413**, 628–631. (doi:10.1038/35098076)
- Baumann R, Meuer HJ. 1992 Blood oxygen transport in the early avian embryo. *Physiol. Rev.* **72**, 941–965. (doi:10.1152/PHYSREV.1992.72.4.941)
- Jauniaux E, Gulbis B, Burton GJ. 2003 The human first trimester gestational sac limits rather than facilitates oxygen transfer to the foetus—a review. *Placenta* **24**, S86–S93. (doi:10.1053/plac.2002.0932)
- Carey C, Garber SD, Thompson EL, James FC. 1983 Avian reproduction over an altitudinal gradient II. Physical characteristics and water loss of eggs. *Physiol. Zool.* **56**, 340–352. (doi:10.1086/physzool.56.3.30152599)
- León-Velarde F, Monge-C C. 2004 Avian embryos in hypoxic environments. *Respir. Physiol.*

- Neurobiol.* **141**, 331–343. (doi:10.1016/J.RESP.2004.02.010)
8. Giussani DA, Salinas CE, Villena M, Blanco CE. 2007 The role of oxygen in prenatal growth: studies in the chick embryo. *J. Physiol.* **585**, 911–917. (doi:10.1113/JPHYSIOL.2007.141572)
 9. Ho WC, Li D, Zhu Q, Zhang J, Zhang J. 2020 Phenotypic plasticity as a long-term memory easing readaptations to ancestral environments. *Sci. Adv.* **6**, eaba3388. (doi:10.1126/sciadv.aba3388)
 10. Carey C, Thompson EL, Vleck CM, James FC. 1982 Avian reproduction over an altitudinal gradient: incubation period, hatchling mass, and embryonic oxygen consumption. *Auk* **99**, 710–718. (doi:10.1093/AUK/99.4.710)
 11. Verbeek NA. 1967 Breeding biology and ecology of the horned lark in alpine tundra. *Wilson Bull.* **79**, 208–218.
 12. Black CP, Snyder GK. 1980 Oxygen transport in the avian egg at high altitude. *Integr. Comp. Biol.* **20**, 461–468. (doi:10.1093/ICB/20.2.461)
 13. Liu C, Zhang LF, Song ML, Bao HG, Zhao CJ, Li N. 2009 Highly efficient dissociation of oxygen from hemoglobin in Tibetan chicken embryos compared with lowland chicken embryos incubated in hypoxia. *Poult. Sci.* **88**, 2689–2694. (doi:10.3382/PS.2009-00311)
 14. Gou X, Li N, Lian L, Yan D, Zhang H, Wei Z, Wu C. 2007 Hypoxic adaptations of hemoglobin in Tibetan chick embryo: high oxygen-affinity mutation and selective expression. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* **147**, 147–155. (doi:10.1016/J.CBPB.2006.11.031)
 15. Projecto-Garcia J *et al.* 2013 Repeated elevational transitions in hemoglobin function during the evolution of Andean hummingbirds. *Proc. Natl Acad. Sci. USA* **110**, 20 669–20 674. (doi:10.1073/pnas.1315456110)
 16. Natarajan C *et al.* 2015 Convergent evolution of hemoglobin function in high-altitude Andean waterfowl involves limited parallelism at the molecular sequence level. *PLoS Genet.* **11**, e1005681. (doi:10.1371/journal.pgen.1005681)
 17. Natarajan C, Hoffmann FG, Weber RE, Fago A, Witt CC, Storz JF. 2016 Predictable convergence in hemoglobin function has unpredictable molecular underpinnings. *Science* **354**, 336–339. (doi:10.1126/science.aaf9070)
 18. Graham AM, Peters JL, Wilson RE, Muñoz-Fuentes V, Green AJ, Dorfsman DA, Valqui TH, Winker K, McCracken KG. 2021 Adaptive introgression of the beta-globin cluster in two Andean waterfowl. *Heredity* **127**, 107–123. (doi:10.1038/s41437-021-00437-6)
 19. Vleck CM, Hoyt DF, Vleck D. 1979 Metabolism of avian embryos: patterns in altricial and precocial birds. *Physiol. Zool.* **52**, 363–377. (doi:10.1086/physzool.52.3.30155757)
 20. Alev C, McIntyre BAS, Nagai H, Shin M, Shinmyozu K, Jakt LM, Sheng G. 2008 BetaA, the major beta globin in definitive red blood cells, is present from the onset of primitive erythropoiesis in chicken. *Dev. Dyn.* **237**, 1193–1197. (doi:10.1002/DVDY.21510)
 21. Alev C, Shinmyozu K, McIntyre BAS, Sheng G. 2009 Genomic organization of zebra finch alpha and beta globin genes and their expression in primitive and definitive blood in comparison with globins in chicken. *Dev. Genes Evol.* **219**, 353–360. (doi:10.1007/S00427-009-0294-8)
 22. Billerman SM, Keeney BK, Rodewald PG, Schulenberg TS (Editors) 2020 *Birds of the world*. Ithaca, NY: Cornell Laboratory of Ornithology. See <https://birdsoftheworld.org/bow/home>.
 23. Clement P. 2020 Hooded siskin (*Spinus magellanicus*), version 1.0. In *Birds of the world* (eds J del Hoyo, A Elliott, J Sargatal, DA Christie, E de Juana). Ithaca, NY: Cornell Lab of Ornithology.
 24. Beckman EJ, Benham PM, Cheviron ZA, Witt CC. 2018. Detecting introgression despite phylogenetic uncertainty: the case of the South American siskins. *Mol. Ecol.* **27**, 4350–4367. (doi:10.1111/mec.14795)
 25. Beckman EJ, Witt CC. 2015 Phylogeny and biogeography of the New World siskins and goldfinches: rapid, recent diversification in the Central Andes. *Mol. Phylogenet. Evol.* **87**, 28–45. (doi:10.1016/j.ympev.2015.03.005)
 26. Frankl-Vilches C *et al.* 2015 Using the canary genome to decipher the evolution of hormone-sensitive gene regulation in seasonal singing birds. *Genome Biol.* **16**, 19. (doi:10.1186/s13059-014-0578-9)
 27. Allen JM, Huang DI, Cronk QC, Johnson KP. 2015 aTRAM—automated target restricted assembly method: a fast method for assembling loci across divergent taxa from next-generation sequencing data. *BMC Bioinf.* **16**, 98. (doi:10.1186/s12859-015-0515-2)
 28. Zerbino DR, Birney E. 2008 Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res.* **18**, 821–829. (doi:10.1101/gr.074492.107)
 29. Kears M *et al.* 2012 Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647–1649. (doi:10.1093/bioinformatics/bts199)
 30. Langmead B, Salzberg SL. 2012 Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357–359. (doi:10.1038/nmeth.1923)
 31. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome project data processing subgroup. 2009 The sequence alignment/map (SAM) format and SAMtools. *Bioinformatics* **25**, 2078–2079. (doi:10.1093/bioinformatics/btp352)
 32. Kornelissen TS, Albrechtsen A, Nielsen R. 2014. ANGSD: analysis of next generation sequencing data. *BMC Bioinf.* **15**, 356. (doi:10.1186/s12859-014-0356-4)
 33. Danecek P *et al.* 2011 The variant call format and VCFtools. *Bioinformatics* **27**, 2156–2158. (doi:10.1093/bioinformatics/btr330)
 34. R Core Team. 2021 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. See <https://www.R-project.org/>.
 35. Weir BS, Cockerham CC. 1984 Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358–1370. (doi:10.1111/j.1558-5646.1984.tb05657.x)
 36. Yi X *et al.* 2010 Sequencing of 50 human exomes reveals adaptation to high altitude. *Science* **329**, 75–78. (doi:10.1126/science.1190371)
 37. Grotto C, Arangi I. 1989 How do avian embryos breathe? Oxygen transport in the blood of early chick embryos. *Comp. Biochem. Physiol. A: Physiol.* **94**, 607–613. (doi:10.1016/0300-9629(89)90602-6)
 38. Uchida Y, Uesaka M, Yamamoto T, Takeda H, Irie N. 2018 Embryonic lethality is not sufficient to explain hourglass-like conservation of vertebrate embryos. *EvoDevo* **9**, 7. (doi:10.1186/s13227-018-0095-0)
 39. Hawkes LA *et al.* 2011 The trans-Himalayan flights of bar-headed geese (*Anser indicus*). *Proc. Natl Acad. Sci. USA* **108**, 9516–9519. (doi:10.1073/pnas.1017295108)
 40. Bagley LG, Christensen VL, Bagley RA. 1990 Effect of altering eggshell permeability on the hatchability of turkey eggs incubated at high altitude. *Poult. Sci.* **69**, 451–456. (doi:10.3382/PS.0690451)
 41. Baumann R, Padeken S, Haller EA, Brilmayer T. 1983 Effects of hypoxia on oxygen affinity, hemoglobin pattern, and blood volume of early chicken embryos. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **244**, 733–741. (doi:10.1152/AJPREGU.1983.244.5.R733)
 42. Opazo JC, Hoffmann FG, Natarajan C, Witt CC, Berenbrink M, Storz JF. 2015 Gene turnover in the avian globin gene families and evolutionary changes in hemoglobin isoform expression. *Mol. Biol. Evol.* **32**, 871–887. (doi:10.1093/molbev/msu341)
 43. Beckman EJ, Vargas Campos W, Benham PM, Schmitt CJ, Cheviron ZA, Witt CC. 2022 Data from: selection on embryonic hemoglobin in an elevational generalist songbird. Figshare. (doi:10.6084/m9.figshare.c.6238138)