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Oxygen availability drives blood traits and the cell number-size tradeoff across Andean hummingbirds

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ABSTRACT

For endotherms with sustained high metabolism, like hummingbirds, blood-oxygen (O₂) carrying capacity should be finely tuned to supply O₂ to respiring tissues. Hematological adjustments are expected in response to changes in O₂ availability that occur along elevational gradients; however, eco-physiological rules by which this variation occurs have remained elusive. If evolutionary divergence in elevational niches is accompanied by predictable shifts in blood trait reaction norms, we would expect different patterns of elevational variation for within-species versus among-species comparisons; but this has not been systematically tested. Here, we sought to elucidate these rules using hummingbirds as a model clade. We measured blood traits that affect O₂-carrying functions: total hemoglobin concentration ([Hb]), hematocrit (Hct), erythrocyte count (TRBC), mean cell volume (MCV), mean cellular hemoglobin content (MCH), and mean cellular hemoglobin concentration (MCHC). For 1,217 Andean hummingbirds representing 77 species, spanning sea level to 4,578 meters in elevation, we modeled blood traits as responses to environmental conditions and individual and species traits. [Hb] and Hct increased with elevation similarly within species and across the phylogeny—for every 1,000-m ascent, [Hb] increased by one third of a gram per deciliter and Hct increased by one and a half percentage points, corresponding to about three-hundred thousand additional cells per microliter. Hummingbirds adjusted blood cell number and cell size by similar proportions to modulate blood-O₂ carrying capacity; however, species at the lowest and highest elevations relied relatively more on adjustments to cell volume, revealing qualitatively different evolutionary responses elicited by moderate versus extreme reductions in O₂ availability.

22 **SIGNIFICANCE STATEMENT**

23 Trait variation along environmental gradients reveals how individuals adjust or populations
 24 evolve to improve performance, foreshadowing climate change effects. For blood traits such as
 25 hemoglobin concentration ([Hb]), rules of elevational variation have remained elusive; it is
 26 unknown whether species adaptation affects reaction of blood phenotypes to local oxygen
 27 availability. We tested this in a model clade, hummingbirds, that have undergone many
 28 elevational shifts. Elevational variation was predictable, but similar within and among species,
 29 indicating no predictable evolution of reaction norms. However, for the cell number-size tradeoff
 30 that underpins [Hb], species varied by elevational range. Cell number explained [Hb] at
 31 intermediate elevations, while cell size was more important at extremes, indicating qualitative
 32 differences in respiratory adaptation to moderate versus high elevations.

INTRODUCTION

Organisms that sustain high levels of aerobic activity under hypoxia, such as birds flying at high altitude, must supply oxygen (O_2) to respiring tissues despite reduced O_2 availability in ‘thin’ air. To achieve this, the O_2 transport functions of blood must be finely tuned to match O_2 supply and demand under a variety of ambient conditions (1–6). Most O_2 in the blood is carried by hemoglobin (Hb; (7), the concentration of which ([Hb]) is a key determinant of blood- O_2 carrying capacity. Under hypobaric hypoxia, animals experience reduced arterial O_2 saturation; to compensate, they undergo erythropoiesis, which can increase [Hb] and therefore blood- O_2 carrying capacity, enhancing O_2 delivery to the tissues and compensating for reduced arterial O_2 saturation (8). In the absence of compensatory increases in plasma volume (9), increased Hb mass leads to elevated hematocrit (Hct) and, beyond a certain threshold, corresponding increases in blood viscosity will begin to compromise O_2 transport (10). A suite of interrelated physical characteristics of blood—total red blood cell count (TRBC), mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC; Table S1)—can be modulated to optimize blood- O_2 transport, or to compensate for other cardiac, vascular, or hematological responses to elevation.

Birds are well-known for their abilities to maintain tissue oxygenation in hypoxic environments, which can be attributed to both plastic and constitutive aspects of respiratory physiology that enhance O_2 uptake, transport, and delivery (3, 11–19). Along with many factors that have been shown to affect the physical characteristics of avian blood, including age, sex, body condition, hormones, environmental factors, and disease (20, 21), high-altitude hypoxia has predictable short-term effects on blood composition that are mediated by the hypoxia-inducible factor (HIF) pathway (8, 22–24). Accordingly, when lowland-adapted birds ascend to altitude,

HIF-induced production of erythropoietin is expected to increase [Hb] and Hct. Birds that are chronically exposed to high elevations have been found to display either normal or elevated [Hb], Hct, and MCHC (25, 26). Evidence from high-altitude-adapted human populations and theoretical work suggests that optimal [Hb] and Hct levels may be similar to those of sea-level populations (8, 13, 27). MCV typically increases in acclimatized, lowland-adapted animals undergoing accelerated erythropoiesis (28); however, bird and other vertebrate lineages that have evolved for extended periods at altitude have often been found to possess smaller cells, likely as part of a suite of adaptive traits for efficient tissue oxygenation (23, 29, 30). Sensitivity of blood traits to low partial pressure of O₂ on the short timescale of acclimatization is well-documented (8, 23). Over longer timescales, natural selection on genes that affect hypoxia performance leads predictably to genetic adaptation (18, 19, 31, 32), but it is not known how this evolutionary process may affect the reaction norms that govern elevational variation in blood traits.

Systematic trait variation across environmental gradients reflects the outcome of evolutionary and developmental processes, and explaining such ‘trait gradients’ will be essential to predicting the functional consequence of climate warming for species and communities (33). Despite its critical importance for tissue oxygenation, many questions remain unanswered regarding elevational variation in blood traits that underpin blood-O₂ carrying capacity. Blood traits vary among avian clades (e.g., 20, 33) and exhibit elevational variation both within species (e.g., 4, 25, 34) and among species (e.g., 4, 25, 33, 35). Trait shifts with elevation are expected to differ systematically for conspecific versus heterospecific comparisons; whereas within-species variation should be driven primarily by developmental plasticity, among-species variation should be influenced by genetic changes that have accrued during differences in elevational exposure lasting thousands to millions of generations (37). Linck et al. (35) estimated elevational effects

on [Hb] and Hct within each of 136 species of Andean birds and showed that, within the median species, [Hb] increased by 0.7 g/dL per 1,000 m in elevation, while Hct increased by 2.0 percentage points per 1,000 m. At least two additional recent studies have conducted broad-scale interspecific comparative analyses of avian [Hb] and Hct data, but both datasets were compiled from heterogeneous published data and lacked elevational sampling sufficient to estimate elevational effects (34, 38). Furthermore, no previous comparative analysis of hematological variation across elevation has included the six key parameters that affect blood-O₂ carrying capacity per unit volume (Table S1). We continue to lack a general understanding of within- and among-species effects of elevation on blood traits, and specifically, an understanding of whether different rules govern elevational variation at these different levels of biological organization.

Another outstanding question is the degree to which known genetic adaptations to hematological function may predict blood-O₂ carrying capacity. Adaptive genetic changes to hemoglobin proteins have been demonstrated in many high-altitude-native birds, including hummingbirds, House Wrens, Andean waterfowl, Bar-headed Geese, and a few dozen other bird taxa (18, 19, 39–41). In hummingbirds, there is a highly predictable association between species-typical Hb β^A_{13} – β^A_{83} genotype and elevation, with derived increases in Hb-O₂ affinity in highland lineages and reductions in Hb-O₂ affinity in lowland lineages (19). Hummingbirds occurring at high altitudes exhibit two amino-acid substitutions on the β^A -globin subunit of Hb (Hb β^A -13_{GLY→SER} and Hb β^A -83_{GLY→SER}) that are known to affect O₂ binding (19). In particular, β^A -13_{SER} is known from only four hummingbird genera (*Patagona*, *Chalcostigma*, *Pterophanes*, and *Oreotrochilus*), all of which occur at extreme high altitudes (3, 18, 19). Both O₂-binding affinity and blood-O₂ carrying capacity have critical effects on tissue oxygenation, but are part of a complex O₂ uptake, transport, and delivery system that contains multiple convective and

diffusive steps (42). It is plausible that species-typical Hb β^A genotypes associated with different O₂-binding properties induce predictable differences in blood traits and their reaction norms to elevation; such an association could reflect a direct functional tradeoff or an indirect effect of accrued genetic adaptation to elevation at multiple loci.

The constraints on blood trait values that are imposed by physical tradeoffs and the relative contributions of each blood trait to overall blood-O₂ carrying capacity remain poorly understood. [Hb] defines O₂-carrying capacity per unit volume of blood, and it can be adjusted via adjustments to cell number (TRBC), cell size (MCV), within-cell [Hb] (MCHC), or plasma volume. MCHC tends to be relatively invariant across the vertebrate tree of life, as demonstrated nearly a century ago by Wintrobe (43), with Hb typically comprising ~1/3 of erythrocytes. Plasma volume adjustments can decouple Hb mass from [Hb] and, although plasma volume appears to vary among human populations (9), we currently have insufficient data on elevational variation in avian plasma volume to assess its importance. Cell number and cell size are both measurable components that are variable among individuals and species, subject to plastic and evolutionary adjustments, and have strong direct effects on [Hb]. Cell number and cell size are reciprocally constrained by physical space available in the circulatory system, such that individual birds can have a few large erythrocytes, many small erythrocytes, or an intermediate state for both traits (43, 44). Quantifying the relative contributions of TRBC and MCV in driving [Hb] variation within and among species may reveal that species utilize different mechanisms of adjusting blood-O₂ carrying capacity; if such mechanisms are predictably associated with elevational environments or evolutionary histories of altitude exposure, they could lead to new biogeographic insights.

In this study, we sought to elucidate the rules governing variation in blood traits that affect O₂-carrying functions, including the effects of environment, individual characteristics, and evolved species differences. Specifically, we asked: 1) To what extent do blood traits shift predictably with elevational changes in O₂ availability, while controlling for other sources of variation?; 2) Do blood traits vary with O₂ availability differently across developmental (within species) and evolutionary (among species) time scales?; 3) How are blood traits affected by phylogenetic history and evolved species differences, such as body mass, metabolic intensity, and known genetic variants that affect Hb-O₂ binding affinity?; 4) To what extent are individual and species variation in O₂-carrying capacity per unit volume of blood attributable to changes in cell number versus cell size? And 5) does manifestation of the cell number-size tradeoff vary predictably among species?

We collected specimen-vouchered hematological data from hummingbirds between 2006–2020 across a ~4,600-meter elevational gradient in the Andes. Data for six blood traits from 1,217 individuals of 77 species were used to model within- and among-species variation, respectively (Figure 1). Hummingbirds could be considered a model clade in which to study hematological variation due to their documented evolutionary genetic responses to shifts in *PO*₂, high species richness in mountains, phylogenetically conserved elevational ranges, and relative uniformity of body size, clutch size, diet, mating system, and flight mode (18, 19, 45–47). Notably, matching O₂ supply and demand is an acute challenge for all hummingbirds because they exhibit the highest mass-specific metabolic rates among endotherms, and because they employ sustained hovering, one of the most energetically demanding forms of animal locomotion (46, 48).

RESULTS AND DISCUSSION

We quantified three primary ([Hb], Hct, and TRBC) and three secondary hematological indices (MCV, MCH, and MCHC; Table S1), and separately analyzed two datasets: an individual-level dataset, wherein each row contained data from one individual hummingbird ($n=1,217$), and a species-level dataset, wherein each row contained mean blood values calculated for each species, with a minimum of 2 individuals sampled ($n=58$ – 65 species, depending on blood trait modeled). In the individual-level data, mean [Hb] was 18.84 g/dl (± 1.65), mean Hct was 58.54% (± 5.15), mean TRBC was $6.37 \text{ RBC} \times 10^6/\text{mm}^3$ or 6,370,000 cells per μl (± 1.16), mean MCV was 93.36 fl (± 17.1), mean MCH was 30.4 pg (± 5.64), and mean MCHC was 32.44 g/dl (± 2.12). Complete reference values—the first such values ever published for >90% of our study species—are reported in detail in Table S2.

Within-species blood comparison

We used the individual-level dataset to model within species variation and accounted for between-species differences by incorporating a species grouping variable. Using multilevel Bayesian models for each of the six blood parameters, we estimated the effects of elevation, body mass, precipitation, air temperature, species-typical Hb β^A13 – β^A83 genotype, as well as within-species variation in elevational position (an individual's sampled elevation relative to its species elevational range), body mass, air temperature, and precipitation. Separately, we excluded sex and wing-loading as potentially important predictors (see Methods). In total, we compared six models per set using LOOIC and WAIC (Tables S3-S4; Methods). In addition to fixed effects, models included species as a random effect, and a phylogenetically correlated random intercept (i.e., covariance matrix of phylogenetic distances among taxa). Thus, our full

model in each set reflects the hypothesis that all predictors, phylogeny, and species identity influence blood characteristics.

Based on the top-performing model in each set, we found that for every 1,000-m increase in elevation, [Hb] is expected to increase by 0.39 g/dl, Hct is expected to increase by 1.42 percentage points, TRBC is expected to increase by 310,000 cells per μl , and MCH is expected to decrease by 0.48 pg. Elevation strongly predicted [Hb], Hct, TRBC, and MCH (Figures 2 A–F and 3A–F). Elevational position was also a strong predictor of Hct, indicating that individuals located higher in their species' elevational range tended to have higher Hct (Figure 3B). [Hb], TRBC, and MCH also increased with increasing elevational position, although these relationships were subtle (Figure 3A, C, E).

The finding that [Hb], Hct, and TRBC increase with elevation was consistent with theoretical expectations for O_2 -carrying capacity of blood and with prior empirical findings in birds (3, 4, 25, 34, 38), humans (49, 50), mammals (23, 51), and other taxa (52, 53). In prior work, substantial variation in slopes of these parameters across elevation may have been attributable to variation in temporal, taxonomic, and spatial scales of comparison, as well as constraints on the time-course of evolutionary adaptation (19, 32, 50). We expect increases in Hct at higher elevations because an increase in the total mass of Hb enhances oxygenation under lower ambient PO_2 (13); unless there is a proportional increase in plasma volume or MCHC, an increase in Hb mass is expected to be accompanied by an increase in the proportion of blood comprised of red blood cells. TRBC also increased with elevation and was, along with MCV, one of two key drivers of [Hb] adjustment (Figure 2, Figure 4; 45). MCV did not vary predictably in our linear models (Figure 2D). Although increasing within-cell [Hb], or MCHC, would seem to be an alternative mechanism of increasing O_2 -carrying capacity, MCHC was less

variable than other blood parameters (Table S2), likely due to physical constraints (54, 55); accordingly, MCH and MCV were strongly correlated (Table S5; Figure 4C). Contrary to recent empirical findings (4, 25), adjustments to MCHC appeared to be neither substantial nor predictable with respect to elevation within or among species (Figure 2F).

We found that MCV and MCH decreased in individuals occurring in warmer environments (Figures 3D, E), and that MCHC decreased in individuals occurring at warmer localities within a species' range (Figure 3F). The directions of these relationships were consistent with previous experimental work: Comparisons of juvenile broiler chickens reared from ~1-7 weeks of age in cool, ambient, and hot environments showed decreases in [Hb], Hct, MCV, and TRBC as temperature increased (56, 57). Our findings add to a limited but intriguing pool of evidence that warmer ambient temperatures may lead to developmental reductions in O₂-carrying capacity of blood.

Models that included the species grouping variable fit substantially better than those without; this effect explained 13–21% of the variance in the top model, depending on the model set (Tables S3-S4; Methods). Moderate effects of the species grouping variable suggest that variation in blood traits is partly explained by aspects of species biology that evolve too fast to have resulted in phylogenetic autocorrelation. Species-typical Hb β^A13 – β^A83 genotype did not predict [Hb] or any other blood characteristics (Figure 3A–F), providing no evidence that evolutionary adjustments to O₂-binding affinity affect the physical composition of blood or its variation with elevation.

Among-species blood comparison

Using the species-level dataset, we evaluated the effects of elevation, body mass, species-typical Hb β^A13 – β^A83 genotype, and ambient temperature and precipitation on each of our 6 blood response variables. We compared sets of models with LOOIC and WAIC (Table S3), incorporating a quadratic elevation effect for TRBC, MCV, MCH, and MCHC models based on curvature evident in the data (Figure 2G–L; Methods).

Within- and among-species findings for elevational effects were similar (Figure 3). Based on the top model in each set, for every 1,000-m increase in elevation, [Hb] is expected to increase by 0.29 g/dl, and Hct is expected to increase by 1.55 percentage points. Elevation was a consistently strong predictor of [Hb], Hct, and TRBC, all of which increased with increasing elevation (Figures 2G–I and 3A–C). The importance of the quadratic effect of elevation for MCV and MCH, and overall shape of the species-level data, suggest that O₂ carrying capacity adjustment differs for middle-elevation species versus those found at low and high elevations (Figure 2J–K; Figure 3D–E). Neither species-typical Hb β^A13 – β^A83 genotype, body mass, environmental temperature, nor precipitation explained variation in any species-mean blood characteristic (Figure 3).

Influence of cell number versus size on blood-O₂ carrying capacity

The axis of variation in cell number versus size space was tightly constrained (Figure 4A–B); the ‘banana’ shape of Figure 4A–B mirrors patterns from Wintrobe (43) and Hawkey et al. (44), which present similar plots based on species representing multiple vertebrate classes. The shape of this relationship indicates that the highest levels of blood-O₂ carrying capacity per unit blood are achieved at high values of TRBC or MCV, respectively. Individual hummingbirds can possess few large erythrocytes or many small erythrocytes, but cell number and size must be

balanced (44). Because of the proportionately greater surface area for gas exchange, having many small cells is predicted to be advantageous for fast metabolism or when the partial pressure of O₂ in inspired air is low (44). Similarly, having large cells may be advantageous for modulating the time-course of gas exchange (58). One way to shed light on this tradeoff is to test whether individuals and species, respectively, adjust O₂ carrying capacity per unit blood primarily through increases in cell number or size.

We designed models to understand the relative contributions of cell number versus size to blood O₂-carrying capacity per unit volume ([Hb]), within and among species over ~22 Mya of hummingbird evolution. These models demonstrated that hummingbirds co-equally modulated cell number and cell size to adjust [Hb] (Table S6). The fact that this was true both within and among species suggests that developmental and evolutionary fine-tuning of blood-O₂ carrying capacity occur similarly.

Using data for 32 well-sampled species, we estimated species-specific standardized TRBC and MCV coefficients (β) in models with [Hb] as the response variable (Figure S1), and we used these to calculate a 0–1 index that we refer to as the Cell Number-Size Index (hereafter *CeNS*). *CeNS* represents the proportional contribution of TRBC versus MCV to increasing [Hb], calculated as:

$$\frac{(\beta_{TRBC})}{(\beta_{TRBC} + \beta_{MCV})}$$

Species specific *CeNS* values ranged from 0.36-0.69 ($\bar{x} = 0.52$), with higher values indicating a proportionally greater contribution of TRBC than MCV in adjusting [Hb]; conversely, lower values indicate a proportionally greater contribution of MCV than TRBC.

Extending our analysis, we designed a set of models to test potential predictors of *CeNS*: elevation, a quadratic elevation effect (elevation²; to account for notable curvature in the data), body mass, wing loading, species-typical Hb β^A_{13} – β^A_{83} genotype, and phylogeny (see Methods). Elevation, elevation², and body mass strongly predicted *CeNS* (Figure 4D-F; Table S7). We found a trend of decreasing *CeNS* with higher wing loading, but the 95% credible intervals overlapped zero (Figure S2A–B). Species-typical Hb β^A_{13} – β^A_{83} genotype did not predict *CeNS* (Figure S3), nor did we find phylogenetic signal in *CeNS* (Figure S4).

Examining the tradeoff between cell number and volume provides a potential explanation for the striking pattern that middle elevation species tend to adjust [Hb] by different mechanisms than low and high elevation species (Figure 4E). *CeNS* was uncorrelated with TRBC coefficients ($R^2 = 0.07$, $r = -0.27$) but strongly correlated with MCV coefficients ($R^2 = 0.51$, $r = -0.71$), indicating that variation in *CeNS* was primarily driven by the degree to which species modulate cell size (Figure S2, Figure S5). We suggest that because low and high elevation species tend to possess relatively small cells, they may be more able to rely on shifts in cell size, rather than cell number, to increase [Hb] (Figure 2J; Figure 4). However, as cell size increased, species were more likely to approach maximum size for red blood cells, beyond which they could incur a functional detriment. As a result, species with larger red blood cells were more reliant on cell number to adjust [Hb]. For middle elevation hummingbird species that have evolved under moderate (~15–30%) reductions in O₂ availability, TRBC appears to be the primary axis for adjustment of blood-O₂ carrying capacity per unit volume of blood. Species that have evolved at the highest elevations are expected to be the best adapted to hypobaric hypoxia, and it is therefore remarkable that their mode of [Hb] adjustment (primarily by MCV) resembles that of hummingbird species in the lowlands, the putative ancestral elevation for hummingbirds (45).

Hummingbirds at extreme elevations

Do hummingbirds at extreme high elevations follow the same rules as hummingbirds distributed at low and moderate elevations with respect to elevational blood variation? Our models encompassed the entire elevational gradient and therefore could have missed important phenomena such as threshold effects. To address this, we compared individual hummingbirds sampled above and below 4,200 m elevation, a threshold that marks the upper ~10% of the gradient. Hummingbirds sampled from these extreme high elevations had significantly smaller cells and higher erythrocyte counts than others (Figure S6). This finding suggests that high-altitude specialist hummingbirds tend to have smaller cells, consistent with findings in other high altitude vertebrates (23, 29, 30, 52, 59); however, this pattern was only detectable under the extreme conditions at the uppermost limits of the Andean elevational gradient. We proceeded to formally test for elevational breakpoints using segmented regression (see Methods). Evidence for objective blood trait thresholds along the elevational gradient was mixed, with breakpoint estimates for individual traits ranging from ~1,400 to ~4,000 m (Figure S7). Lack of consistency in these estimates suggests that trait-specific thresholds are challenging to identify and may not be generalizable across species and clades.

Phylogenetic signal in blood characteristics

Phylogenetic signal was low ($\lambda = 0.0$; K range = 0.14–0.21) across all blood traits for both within- and among-species models. Blomberg's K and Pagel's λ results were consistent with visual assessment of continuous trait maps, even after removing species with <3 individuals

sampled (Table S8; Figure S8). We additionally found no phylogenetic signal in *CeNS* (Figure S4).

Non-existent to low phylogenetic signal in blood traits within and among hummingbird species was surprising given demonstrated, phylogenetically conserved Hb genetic adaptations to elevation (18, 19) and in light of strong phylogenetic signal reported from recent comparative analyses of blood traits that had broader taxonomic sampling (34, 38). However, it is likely that diverse avian clades possess different blood trait optima; in previous comparative analyses that included multiple families or orders, phylogenetic signal may have been driven primarily by differences among clades. For example, Minias et al. (34) reported that small passerines had consistently high [Hb] and Hct estimates, and that including the shorebird order, Charadriiformes, which was characterized by high [Hb] but medium Hct values, strongly influenced patterns of phylogenetic conservatism (34). We tested for and found no interaction of clade and elevation on the six studied blood traits, with the exception of the interaction of elevation and the Hermit clade in the [Hb] model set (Figure S9). Interestingly, hermits are a large, early diverging hummingbird clade that contains diminishing species diversity with altitude (and no species that exceeds 3,000 m), and its apparent increased sensitivity of [Hb] to elevation hints at a functional explanation for this biogeographic tendency. The existence of clade-specific trait optima (34) and strong species effects, demonstrated in this study, may at first seem difficult to reconcile with our findings that there was no within-family phylogenetic signal in hummingbirds; however, we view these findings as being consistent with steady evolution of trait optima by a mean-reverting, Ornstein-Uhlenbeck process, as described by Minias et al. (34), and marked by occasional major jumps that coincide with changes in body plan or respiratory demands.

A strength of our study was that hummingbirds vary only subtly in aspects of their biology that could plausibly confound our analyses, such as metabolic rate, energy budget, torpor use, mode of locomotion, foraging strategy, or clutch size. Phylogenetic conservatism in these traits facilitates explanation of blood trait variation across the entire clade. A strong effect of species identity in spite of these intrinsic controls hints that species optimize O₂-carrying functions of blood for subtle changes in lifestyle and environment, and on rapid timescales. This finding is consistent with the rapid diversification of human hematological responses to elevation, as illustrated by the copious variation among populations in patterns of elevational variation (27, 49, 50, 60–62).

Conclusions

Across ~22 Mya of the hummingbird evolution, ambient O₂-availability and species idiosyncrasies—not phylogenetic affinities—were the primary drivers of variation in blood traits affecting O₂-carrying functions. In response to elevational changes in O₂ partial pressure, hummingbirds make predictable adjustments to the physical composition of blood. Remarkably, after controlling for many potentially confounding variables, decreasing PO₂ drove increases in [Hb] and Hct that were nearly identical in magnitude within and among hummingbird species, respectively. This implies that developmental and evolutionary optimization of blood traits to ambient PO₂ occurs in fundamentally similar ways, responding to the same set of physical rules that govern gas exchange at the blood-gas barrier. In addition to O₂ availability, species identity itself explained 13–21% of blood trait variation, suggesting that fast-evolving, unmodeled aspects of species biology underpin substantial hematological variation. Our data revealed a striking constraint space that defines the cell number-size tradeoff for hummingbird erythrocytes;

within this space, individuals and species co-equally modulated cell number and size to adjust [Hb]. However, species varied in their proportional reliance on adjustments to cell number versus size (*CeNS*) to increase [Hb], and both elevation and body mass were strongly predictive of species' tendencies. Middle-elevation species that have been evolving under moderate hypoxia adjusted [Hb] primarily by adjusting cell number; in contrast, low and high elevation species tended to adjust [Hb] by adjusting cell size, a pattern partly underpinned by hummingbirds at extreme elevations exhibiting smaller cells. Together, these results suggest that evolutionary solutions to reduced O₂ availability can differ qualitatively in conjunction with species-specific histories of exposure to hypoxia challenges of varying severity.

MATERIALS AND METHODS

Field Sampling

We collected data from 1,217 individuals of 77 hummingbird species from Peru and Chile from 2006–2020 (Figure 1). After filtering, our final within-species dataset included 1,151 individuals of 77 species from 75 localities. The best-sampled clades were the Coquettes (387 individuals, 20 species) and Brilliants (269 individuals, 19 species). The species with greatest sampling depth were *Metallura tyrianthina* (95 individuals), *Colibri coruscans* (73 individuals), and *Amazilia amazilia* and *Patagona gigas* (64 individuals each; Figure 1). Sampling across sexes was balanced (553 females, 592 males, and 6 unknown). Most birds sampled were adults (936 adults, 179 juveniles, and 36 unknown). Detailed data on specimens, collection localities, and blood traits are available in Witt et al. (*In prep*) and in the ARCTOS database (arctosdb.org) for reproducibility and extension of our work.

Hematological measurements

To calculate primary indices (hemoglobin concentration ([Hb]), hematocrit (Hct), and total red blood cell count (TRBC)), we obtained whole blood samples within ~5 mins to ~3 hours of capture by venipuncture on the underside of the wing and collection with heparinized microcapillary tubes and Hemocue HB201+ cuvettes. Hct (%) was measured with digital calipers after centrifuging the sealed microcapillary tube for 5 min at 13,000 r.p.m. When >1 Hct sample was taken, weighted average values were used in analyses. [Hb] (g/dL blood) was measured on ~5 µL of blood using a HemoCue HB201+ hemoglobin photometer. The HemoCue proprietary photometric method produces values for avian blood that are ~1.0 g/dL greater than those generated using cyanomethemoglobin spectrophotometry (63). Accordingly, we corrected [Hb] values by subtracting 1.0 g/dL. When possible, at the time of blood collection, 10 µl whole blood was preserved in a 1:200 NaCl dilution to obtain TRBC estimates. Within 12-hours of blood collection, we pipetted a 10 µl sub-sample of blood-NaCl dilution into a hemocytometer and allowed cells to settle for 1 min. After focusing the microscope, one or more photos were obtained of cells in the hemocytometer grid. Later, cells were manually counted in the ImageJ software (64) and TRBC was estimated following standard protocols (20, 21).

After drawing blood, birds were humanely killed by rapid cardiac compression. We recorded collection elevation, latitude, longitude, sex, body mass, and date. Body mass was typically recorded prior to blood sampling; however, body mass was corrected for estimated volume of blood drawn for any individuals massed after blood sampling. Each collected bird was prepared as a study skin. Specimens and tissues are housed at the Museum of Southwestern Biology (MSB) at the University of New Mexico, USA, the Centro de Ornitología y Biodiversidad (CORBIDI) in Peru, and the Universidad Pontificia Católica de Chile in Chile.

Secondary blood indices (mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC)) were calculated from primary indices (Table S1) and following standard protocols (20). We examined distributions of the data using histograms and qqPlots, and we eliminated outliers that fell outside of the expected distribution and that differed from known ‘typical’ values (20, 25, 65). To reduce age-related outliers, we examined values at the tails of each blood trait distribution that were associated with presence of a bursa measuring ~2 x 2 mm. All juvenile values fell within the expected distribution of blood values. We did not age individuals by plumage, as juvenile plumage lasts much longer than juvenile blood values, which stabilize one to several months after fledging.

Environmental and life history variables

We compiled elevational range limits for each species using a combination of published data, field guides, and specimen records from the MSB (3, 66–68). We calculated each individual’s elevational range position relative to its species elevational range breadth (hereafter ‘elevational position’) as $1 - ((\text{maximum species elevation} - \text{individual sampling elevation}) / \text{species elevational range})$.

We incorporated species-typical Hb β^{A13} – β^{A83} genotypes from published data (19, 45). For 7 species, we identified these genotypes from Hb β^A sequences aligned to a GenBank reference from Projecto-Garcia et al. (19). For 12 species (*Amazilia franciae*, *Campylopterus villaviscensio*, *Eutoxeres aquila*, *Klais guimeti*, *Leucippus taczanowskii*, *Myrmia micrura*, *Phaethornis atrimentalis*, *Sephanoides sephaniodes*, *Chaetocercus mulsant*, *Eriocnemis vestita*, *Myrtis fanny*, and *Oreotrochilus leucopleurus*) species-typical Hb β^{A13} – β^{A83} genotypes were inferred from sister taxa or nearest relatives.

We characterized climatic variation among latitudinal and elevational gradients using 19 bioclimatic variables from the WorldClim database (69). We downloaded rasters of each BioClim variable using the ‘raster’ package in R (version 4.0.0), cropped them to our study areas of Peru and Chile, and extracted temperature and precipitation values for each sampling locality. We used principal components analysis (PCA) for temperature (Bio1–11) and precipitation (Bio12–19) variables to create a composite measure of temperature and precipitation across the gradient. PC1 of the temperature variables (hereafter “temperature”) explained 73.4% of the variation in composite temperature, and loadings suggested that this axis corresponded to increasing temperature across sites (see GitHub). PC1 of the precipitation variables (hereafter “precipitation”) explained 79.8% of the variation in composite precipitation, and loadings suggested that this axis corresponded to increasing precipitation across sites (see GitHub).

To account for multiple measurement effects (i.e., traits measured from multiple individuals within species), we calculated between- and within-species differences following Villemereuil et al. (70). This approach uses within-group centering to separate each predictor into two components: We first calculated species mean values (i.e., between-species variability) and then subtracted the mean value of individual observations (i.e., within-species differences). These measures of within-species variation for temperature, precipitation, and body mass were included in models. Because hummingbird body masses may differ by sex, within-species differences were calculated using mean body mass of males for males, mean body mass of females for females, and species-level means for individuals of unknown sex ($n=6$).

We used wing area data from the 59 MSB species studied in Skandalis et al. (71) to calculate wing loading in g/cm^2 , calculated as species mean body mass (g) divided by total wing

area (cm²). We analyzed wing loading due its relevance to aerodynamic theory and hummingbird flight at high altitudes (72–75).

Hummingbird phylogeny

We used the rate-calibrated molecular hummingbird phylogeny from McGuire et al. (45) as a backbone to generate a phylogeny pruned to the 77 species in our dataset using the R package ‘phytools’ (76). Two species, *Oreotrochilus leucopleurus* and *Phaethornis stuarti*, were missing from the McGuire et al. (45) tree and were added to our phylogeny using the `bind.tip()` function. Branch lengths and position of *O. leucopleurus* were calculated from genetic distances and recently described sister relationships (77). Approximate branch length and position of *P. stuarti* were inferred from BirdTree.org (78, 79) with divergence rate estimates from a multi-locus, time-calibrated phylogeny of Hawaiian honeycreepers (80). We generated model-specific tree subsets by including only those tips that corresponded to the species in each modeling subset.

Phylogenetic signal

We initially constructed phylogenetic models (results not shown), and calculated phylogenetic signal using the full and reduced models for each blood parameter, following the vignette and recommendations of P. Bürkner (https://cran.r-project.org/web/packages/brms/vignettes/brms_phylogenetics.html), using the ‘hypothesis’ method. Estimates of phylogenetic signal for all model sets (full and reduced in both within- and among species analyses) were low ($\lambda < 0.004$); these estimates are supported by visualizations of continuous trait maps of mean blood parameters (SI Appendix; Figure S8). Accordingly, we excluded phylogeny as an effect in

models and evaluated phylogenetic signal using Blomberg's K and Pagel's λ . Results from the two tests were concordant and consistent across model subsets (Table S8).

Within species models

We analyzed within species variation in blood characteristics using generalized linear multilevel models in the R package brms (81). Brms uses Markov chain Monte Carlo (MCMC) sampling to obtain draws from posterior distributions. We designed hierarchical models for each of six continuous responses variables: [Hb], Hct, TRBC, MCV, MCH, and MCHC. For each model, predictor variables included one species-level categorical variable (species-typical Hb $\beta^{A13-\beta^{A83}}$ genotype) and eight individual-level continuous variables (elevation, elevational position, body mass (log-transformed), precipitation, temperature, and within species variation in body mass, temperature, and precipitation). All variables were standardized to a mean of zero and standard deviation of one.

We compared 6 models in each model set: 1) an intercept-only null model, 2) an intercept-only model with a species grouping variable (1|species), 3) a model with all predictors and no grouping variable, 4) a model with all predictors and the species grouping variable, 5) a reduced-predictor model with no grouping variable (reduced model 3), 6) a reduced-predictor model with the species grouping variable (reduced model 4). Reduced models included only predictors whose 95% credible intervals (CIs) did not overlap zero in full models. Distribution family differed by model set: Gaussian for [Hb], Hct, and TRBC models; skew-normal for MCV and MCH models; and student's t -distribution for MCHC models. We ran four Markov chains for 10,000 iterations with a burn-in period of 5,000, thinned every 10 steps.

We tested for interactions between clade and elevation by comparing three models: 1) the top within-species model for each blood parameter; 2) the top model + (1|Clade); and 3) the top model with an elev:Clade interaction. We found no meaningful interaction of elevation and clade except for elev:CladeHermit in the [Hb] model, whose 95% credible intervals did not overlap zero (Figure S9). In accordance with lack of phylogenetic signal in our data, the effect of clade explained minimal variation in model sets for each parameter (Hb=13%; Hct=8%; TRBC=4%; MCV=2%; MCH=2%; MCHC=4%).

Among species models

Final among-species datasets varied in the number of species they contained: ([Hb]=60 species; Hct=65 species; TRBC=58 species, MCV=60 species; MCH=59 species; MCHC=64 species). Species mean values were calculated for all species with $n=2$ or more individuals sampled. We split *Patagona gigas* into “north” and “south” haplotypes in light of evidence that the complex may be comprised of two functionally and genetically distinct populations separated by strong migratory divides (82), and we analyzed data only from individuals sampled during the breeding months to ensure correct population assignment. We designed model sets for each of our six blood traits. For each response, we analyzed distributions (qqPlots, Cook’s Distance, leverage) and removed outliers that strongly influenced fit. For each model, predictor variables included species-typical Hb β^{A13} – β^{A83} genotype, mean elevation, mean body mass (log-transformed; one mean for all individuals due to balanced sampling between sexes), mean temperature, and mean precipitation. All variables were standardized to a mean of zero and standard deviation of one.

For each response, we compared: 1) an intercept-only null model, 2) a model with all predictors, and 3) a reduced-predictor model with only those predictors whose 95% CIs did not

overlap zero in full models (reduced model 2). Given the notable quadratic shape of species mean data for TRBC, MCV, MCH, and MCHC (Figure 2G–L), for those model sets we additionally compared 4) a model with all predictors plus a quadratic component of elevation and 5) a reduced-predictor model with quadratic component including only those predictors whose 95% CIs did not overlap zero in the full quadratic model (reduced model 4). Distribution family differed by model set: Gaussian for [Hb]; skew-normal for Hct; and student's *t*-distribution for TRBC, MCV, MCH, and MCHC models. We ran four Markov chains for 20,000 iterations with a burn-in period of 10,000, thinned every 10 steps.

Cell number versus size analyses

First, to assess within species variation in cell number versus size, we compared a null model (intercept only) to a single full model $[Hb] \sim MCV + TRBC + (1|species)$ for all individuals of 59 species that had [Hb], MCV, and TRBC measurements, distinguishing between *Patagona gigas* “north” and “south”. The species grouping variable allowed us to account for repeated sampling of individuals within a species and to hold species identity constant. Second, to evaluate among-species variation in cell number versus size, we generated a mean dataset for each species with ≥ 3 individuals sampled (48 species total). We again compared a null (intercept only) model to a single full model $[Hb] \sim MCV + TRBC$. Third, to assess how individual species differ in their relative contributions of MCV and TRBC to increasing [Hb], we repeated our approach for individual species: For each species with ≥ 8 sample individuals (32 species total), we generated a species-specific data subset, carefully and conservatively evaluated influential outlier points, and ran a single model: $[Hb] \sim MCV + TRBC$ (Figure S1). For all three model sets

(within species, among species, and single species), both predictors were standardized to a mean of zero and standard deviation of one.

Using standardized, model-estimated coefficients from each single-species model, for each species we calculated the Cell Number-Size Index (hereafter *CeNS*) as described in the main text. *CeNS* ranged from 0.0–1.0 and represented the proportional contribution of TRBC relative to MCV in driving [Hb], with higher values corresponding to greater relative contributions of cell number versus cell size.

We then analyzed the factors that contribute to variation in *CeNS*. Our model response variable was *CeNS* (0–1 index). Predictors, all species mean values, included elevation, a quadratic elevation term “elevation²” to account for notable curvature in the data, body mass (log-transformed), wing loading, and species-typical Hb β^{A13} – β^{A83} genotype. We compared six models: 1) an intercept-only null model, 2) a model with core predictors (elevation, body mass, and wing loading), 3) a model with core predictors plus elevation², 4) a model with core predictors plus elevation² and species-typical Hb β^{A13} – β^{A83} genotype, 5) a reduced version of model 3, and 6) a reduced version of model 4. Reduced models included only those predictors whose 95% CIs did not overlap zero in full models. In addition to model fit diagnostics, we assessed correlations between variables by analyzing correlation matrices and by regressing *CeNS* on each R² values from standardized species-specific *CeNS* models, unstandardized TRBC and MCV beta estimates, and raw TRBC and MCV values (Figure S5). Distribution family differed by model set: Gaussian for individual-level, species mean, and *CeNS* prediction models, and student’s *t*-distribution for species-specific models. For all *CeNS* models we ran four Markov chains for 20,000 iterations with a burn-in period of 10,000, thinned every 10 steps. We assessed phylogenetic signal with Blomberg’s *K*, Pagel’s λ , and visually with contmaps (Figure S4).

554

555 *Model comparison and diagnostics*

556 All models and sets were run using the `brm()` function with default priors (weakly informative
557 priors for the intercept and sigma; improper flat priors for predictors). To assess convergence we
558 visually examined trace plots, checked diagnostics, and confirmed that R_{hat} was <1.01 (81). We
559 used a posterior predictive check to assess whether model assumptions yielded good
560 approximations of the data generating process. There were no problems in any of our models
561 resulting from Bayesian fraction of missing information or divergent transitions. We compared
562 models and verified fits using the widely applicable information criterion (WAIC; (83), Bayesian
563 expected log pointwise predictive densities (ELPD; (84), and approximate leave-one-out cross-
564 validation information criterion based on the posterior likelihoods (LOOIC; Tables S3, S6). We
565 estimated fixed effects (means and 95% CIs) from the posterior distributions for each predictor.
566 For relevant models, we estimated the proportion of the total variance attributed to the species
567 grouping variable, which accounts for unique aspects of blood parameters that are not captured
568 by the individual, life history, or environmental predictors.

569

570 *Breakpoint analysis*

571 We tested for objective thresholds in each blood trait using piecewise linear regression of
572 elevation (standardized) regressed on each blood trait. In this procedure, we first fit a simple
573 linear regression model, iteratively searched for objective “breakpoints” along our sampled
574 elevational gradient, selected the breakpoint with the lowest residual error, and refit the linear
575 regression model with separate estimates for slopes and intercepts below and above the
576 breakpoint, respectively. We constrained the model to estimate breakpoints that were each 400 m

from the lowest and highest points in our data (i.e., from 400 m to 4,178 m in elevation), to avoid biasing breakpoints towards small sample sizes and each low and high elevations, and anomalous species at both ends of the distribution. We evaluated goodness of fit using fit metrics and by visually assessing QQplots, Cook's D, leverage, and residual distributions. We compared models using AIC values. All breakpoint models fit significantly better than simple linear regression models except for the TRBC breakpoint model; this model did fit better in all respects than the simple linear regression model, but the fit was not significant at the $p=0.05$ level. We used a one-way ANOVA to compare whether individuals below and above model-estimated breakpoints differed in their blood characteristics (Figure S7).

Data and code availability

Specimen information is available from the Arctos database (<https://www.arctosdb.org>). Detailed data on specimens, collection localities, and blood values are reported in a forthcoming data paper, Witt et al. (*In Prep*). All data and code for analyses are available in an open GitHub repository: <https://github.com/jlwilliamson/ComparativeHummingbirdBlood>.

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REFERENCES

1. J. S. Krause, *et al.*, Annual Hematocrit Profiles in Two Subspecies of White-Crowned Sparrow: A Migrant and a Resident Comparison. *Physiol. Biochem. Zool.* **89**, 51–60 (2015).
2. J. F. Storz, G. R. Scott, Life Ascending: Mechanism and Process in Physiological Adaptation to High-Altitude Hypoxia. *Annu. Rev. Ecol. Evol. Syst.* **50**, 1–24 (2019).
3. J. L. Williamson, C. C. Witt, Elevational niche-shift migration: Why the degree of elevational change matters for the ecology, evolution, and physiology of migratory birds. *Ornithology* **138**, 1–26 (2021).
4. S. Barve, A. A. Dhondt, V. B. Mathur, Z. A. Cheviron, Life-history characteristics influence physiological strategies to cope with hypoxia in Himalayan birds. *Proc. R. Soc. B* **283**, 1–8 (2016).
5. S. L. Laguë, High-altitude champions: birds that live and migrate at altitude. *J. Appl. Physiol.* **123**, 942–950 (2017).
6. J. F. Storz, Hemoglobin–oxygen affinity in high-altitude vertebrates: is there evidence for an adaptive trend? *J. Exp. Biol.* **219**, 3190–3203 (2016).
7. R. N. Pittman, “Chapter 4: Oxygen Transport” in *Regulation of Tissue Oxygenation*, (Morgan & Claypool Life Sciences, 2011) (June 23, 2021).
8. J. F. Storz, G. R. Scott, Z. A. Cheviron, Phenotypic plasticity and genetic adaptation to high-altitude hypoxia in vertebrates. *J. Exp. Biol.* **213**, 4125–36 (2010).
9. M. Stembridge, *et al.*, The overlooked significance of plasma volume for successful adaptation to high altitude in Sherpa and Andean natives. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 16177–16179 (2019).

- 646 10. J. F. Storz, Genes for High Altitudes. *Science* (80-.). **329**, 40–41 (2010).
- 647 11. S. L. Laguë, *et al.*, Divergent respiratory and cardiovascular responses to hypoxia in bar-
648 headed geese and Andean birds. *J. Exp. Biol.* **220**, 4186–4194 (2017).
- 649 12. F. M. Faraci, Adaptations to hypoxia in birds: how to fly high. *Annu. Rev. Physiol.* **53**, 59–
650 70 (1991).
- 651 13. G. R. Scott, W. K. Milsom, Flying high: A theoretical analysis of the factors limiting
652 exercise performance in birds at altitude. *Respir. Physiol. Neurobiol.* **154**, 284–301
653 (2006).
- 654 14. C. M. Bishop, Heart mass and the maximum cardiac output of birds and mammals:
655 Implications for estimating the maximum aerobic power input of flying animals. *Philos.*
656 *Trans. R. Soc. B Biol. Sci.* **352**, 447–456 (1997).
- 657 15. B. R. Grubb, Allometric relations of cardiovascular function in birds. *Am. J. Physiol.* **245**,
658 H567–H572 (1983).
- 659 16. G. R. Scott, *et al.*, How Bar-Headed Geese Fly Over the Himalayas. *Physiology* **30**, 107–
660 115 (2015).
- 661 17. M. C. W. Lim, C. C. Witt, C. H. Graham, L. M. Dávalos, Parallel Molecular Evolution in
662 Pathways, Genes, and Sites in High-Elevation Hummingbirds Revealed by Comparative
663 Transcriptomics. *Genome Biol. Evol.* **11**, 1552–1572 (2019).
- 664 18. C. Natarajan, *et al.*, Predictable convergence in hemoglobin function has unpredictable
665 molecular underpinnings. *Science* (80-.). **354**, 336–339 (2016).
- 666 19. J. Projecto-Garcia, *et al.*, Repeated elevational transitions in hemoglobin function during
667 the evolution of Andean hummingbirds. *Proc. Natl. Acad. Sci.* **110**, 20669–20674 (2013).
- 668 20. T. W. Campbell, C. K. Ellis, *Avian and Exotic Animal Hematology and Cytology*

(Blackwell Publishing Inc., 2007).

21. J. Samour, “Diagnostic Value of Hematology” in *Clinical Avian Medicine, Volume II*, G. Harrison, T. Lightfoot, Eds. (2005), pp. 587–610.
22. A. Borrás, J. Cabrera, J. C. Senar, Hematocrit Variation in Response to Altitude Changes in Wild Birds: A Repeated-Measures Design. *Condor* **112**, 622–626 (2010).
23. D. M. Tufts, *et al.*, Phenotypic plasticity in blood-oxygen transport in highland and lowland deer mice. *J. Exp. Biol.* **216**, 1167–1173 (2013).
24. D. Levett, *et al.*, Acclimatization of skeletal muscle mitochondria to high-altitude hypoxia during an ascent of Everest. *FASEB J.* **26**, 1431–1441 (2012).
25. S. G. Dubay, C. C. Witt, Differential high-altitude adaptation and restricted gene flow across a mid-elevation hybrid zone in Andean tit-tyrant flycatchers. *Mol. Ecol.* **23**, 3551–3565 (2014).
26. D. T. Clemens, Interspecific variation and effects of altitude on blood properties of rosy finches (*Leucosticte arctoa*) and House Finches (*Carpodacus mexicanus*). *Physiol. Zool.* **62**, 288–307 (1990).
27. C. M. Beall, Two routes to functional adaptation: Tibetan and Andean high-altitude natives. *Proc. Natl. Acad. Sci.* **104**, 8655–8660 (2007).
28. K. Heinicke, *et al.*, Long-term exposure to intermittent hypoxia results in increased hemoglobin mass, reduced plasma volume, and elevated erythropoietin plasma levels in man. *Eur. J. Appl. Physiol.* **88**, 535–543 (2003).
29. G. Ruiz, M. Rosenmann, A. Veloso, Altitudinal distribution and blood values in the toad, *Bufo spinulosus* Wiegmann. *Comp. Biochem. Physiol. -- Part A Physiol.* **94**, 643–646 (1989).

30. H. Zhang, C. X. Wu, Y. Chamba, Y. Ling, Blood characteristics for high altitude adaptation in Tibetan chickens. *Poult. Sci.* **86**, 1384–1389 (2007).
31. Y. Hao, *et al.*, Comparative transcriptomics of 3 high-altitude passerine birds and their low-altitude relatives. *Proc. Natl. Acad. Sci.* **116**, 11851–11856 (2019).
32. N. J. Dawson, L. Alza, G. Nandal, G. R. Scott, K. G. McCracken, Convergent changes in muscle metabolism depend on duration of high-altitude ancestry across andean waterfowl. *Elife* **9**, 1–35 (2020).
33. B. J. Enquist, *et al.*, “Scaling from Traits to Ecosystems: Developing a General Trait Driver Theory via Integrating Trait-Based and Metabolic Scaling Theories” in *Advances in Ecological Research*, A. I. D. Samraat Pawar, Guy Woodward, Ed. (Elsevier Ltd., 2015), pp. 249–318.
34. P. Minias, Ecology and evolution of blood oxygen-carrying capacity in birds. *Am. Nat.* **195**, 788–801 (2020).
35. E. B. Linck, *et al.*, Blood variation implicates respiratory limits on elevational ranges of Andean birds. *bioRxiv*, 1–28 (2021).
36. C. Carey, M. L. Morton, Aspects of circulatory physiology of montane and lowland birds. *Comp. Biochem. Physiol. Part A* **54**, 61–74 (1976).
37. C. J. Axelrod, F. Laberge, B. W. Robinson, Intraspecific brain size variation between coexisting sunfish ecotypes. *Proc. R. Soc. B Biol. Sci.* **285** (2018).
38. K. N. Yap, O. H.-I. Tsai, T. D. Williams, Haematological traits co-vary with migratory status, altitude and energy expenditure: a phylogenetic, comparative analysis. *Sci. Rep.* **9**, 6351 (2019).
39. C. Natarajan, *et al.*, Convergent Evolution of Hemoglobin Function in High-Altitude

- Andean Waterfowl Involves Limited Parallelism at the Molecular Sequence Level. *PLoS Genet.* **11**, 1–25 (2015).
40. J. F. Storz, H. Moriyama, Mechanisms of hemoglobin adaptation to high altitude hypoxia. *High Alt. Med. Biol.* **9**, 148–157 (2008).
41. X. Zhu, *et al.*, Elevational divergence in the great tit complex revealed by major hemoglobin genes. *Curr. Zool.* **64**, 455–464 (2017).
42. J. F. Storz, High-Altitude Adaptation: Mechanistic Insights from Integrated Genomics and Physiology. *Mol. Biol. Evol.* (2021) <https://doi.org/10.1093/molbev/msab064>.
43. M. M. Wintrobe, Variation in the size and hemoglobin content of erythrocytes in the blood of various vertebrates. *Folia Haematol.* **51**, 32–59 (1933).
44. C. M. Hawkey, P. M. Bennett, S. C. Gascoyne, M. G. Hart, J. K. Kirkwood, Erythrocyte size, number and haemoglobin content in vertebrates. *Br. J. Haematol.* **77**, 392–397 (1991).
45. J. A. McGuire, *et al.*, Molecular phylogenetics and the diversification of hummingbirds. *Curr. Biol.* **24**, 1–7 (2014).
46. R. K. Suarez, Hummingbird flight: Sustaining the highest mass-specific metabolic rates among vertebrates. *Experientia* **48**, 565–570 (1992).
47. C. H. Graham, J. L. Parra, C. Rahbek, J. A. McGuire, Phylogenetic structure in tropical hummingbird communities. *Proc. Natl. Acad. Sci.* **106**, 19673–19678 (2009).
48. W. Buermann, *et al.*, Projected changes in elevational distribution and flight performance of montane Neotropical hummingbirds in response to climate change. *Glob. Chang. Biol.* **17**, 1671–1680 (2011).
49. C. M. Beall, *et al.*, Hemoglobin concentration of high-altitude Tibetans and Bolivian

- Aymara. *Am. J. Phys. Anthropol.* **106**, 385–400 (1998).
50. C. M. Beall, Andean, Tibetan, and Ethiopian patterns of adaptation to high-altitude hypoxia. *Integr. Comp. Biol.* **46**, 18–24 (2006).
51. K. A. Hammond, J. O. E. Szewczak, E. Król, Effects of altitude and temperature on organ phenotypic plasticity along an altitudinal gradient. *J. Exp. Biol.* **204**, 1991–2000 (2001).
52. S. Lu, *et al.*, Differences in hematological traits between high- and low-altitude lizards (Genus *Phrynocephalus*). *PLoS One* **10**, 1–16 (2015).
53. N. F. Hadley, T. A. Burns, Intraspecific Comparison of the Blood Properties of the Side-Blotched Lizard, *Uta stansburiana*. *Copeia* **1968**, 737–740 (1968).
54. S. L. Fischer, S. P. Fischer, Mean Corpuscular Volume. *JAMA Intern. Med.* **143**, 282–283 (1983).
55. M. S. Rose, Epitaph for the M.C.H.C. *Br. Med. J.* **4**, 169 (1971).
56. A. Donkoh, Ambient temperature: a factor affecting performance and physiological response of broiler chickens. *Int. J. Biometeorol.* **33**, 259–265 (1989).
57. R. J. Moye, K. W. Washburn, T. M. Huston, Effects of environmental temperature on erythrocyte numbers and size. *Poult. Sci.* **48**, 1683–1686 (1969).
58. C. A. Glomski, A. Pica, *The Avian Erythrocyte: Its Phylogenetic Odyssey*, 1st Ed. (Science Publishers, 2011).
59. K. Yamaguchi, K. D. Jürgens, H. Bartels, J. Piiper, Oxygen transfer properties and dimensions of red blood cells in high-altitude camelids, dromedary camel and goat. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **157**, 1–9 (1987).
60. T. D. Brutsaert, M. Araoz, R. Soria, H. Spielvogel, J. D. Haas, Higher arterial oxygen saturation during submaximal exercise in Bolivian Aymara compared to European

- sojourners and Europeans born and raised at high altitude. *Am. J. Phys. Anthropol.* **113**,
169–181 (2000).
61. J. Grimmering, *et al.*, Thin Air Resulting in High Pressure: Mountain Sickness and
Hypoxia-Induced Pulmonary Hypertension. *Can. Respir. J.*, 1–17 (2017).
62. Y.-B. Guo, *et al.*, GCH1 plays a role in the high-altitude adaptation of Tibetans. *Zool. Res.*
38, 155–162 (2017).
63. P. Simmons, A. Lill, Development of parameters influencing blood oxygen carrying
capacity in the welcome swallow and fairy martin. *Comp. Biochem. Physiol. - A Mol.*
Integr. Physiol. **143**, 459–468 (2006).
64. W. S. Rasband, ImageJ.
65. N. Elarabany, A comparative study of some haematological and biochemical parameters
between two species from the Anatidae family within migration season. *J. Basic Appl.*
Zool. **79** (2018).
66. T. A. I. Parker, D. F. Stotz, J. W. Fitzpatrick, “Ecological and Distributional Databases” in
Neotropical Birds: Ecology and Conservation, D. K. M. D.F. Stotz, J.W. Fitzpatrick, T.A.
Parker, III, Ed. (University of Chicago Press, 1996).
67. A. Jaramillo, *Birds of Chile* (Princeton University Press, 2003).
68. T. S. Schulenberg, D. F. Stotz, D. F. Lane, J. P. O’Neill, T. A. P. III, *Birds of Peru*
(Princeton University Press, 2010).
69. R. J. Hijmans, S. E. Cameron, J. L. Parra, P. G. Jones, A. Jarvis, Very high resolution
interpolated climate surfaces for global land areas. *Int. J. Climatol.* **25**, 1965–1978 (2005).
70. P. de Villemereuil, S. Nakagawa, Modern phylogenetic comparative methods and their
application in evolutionary biology. *Mod. Phylogenetic Comp. Methods their Appl. Evol.*

784 *Biol.*, 1–552 (2014).

785 71. D. A. Skandalis, *et al.*, The biomechanical origin of extreme wing allometry in
786 hummingbirds. *Nat. Commun.* **8**, 1–9 (2017).

787 72. R. Dudley, Mechanisms and Implications of Animal Flight Maneuverability. *Integr.*
788 *Comp. Biol.* **42**, 135–140 (2002).

789 73. D. L. A. Roslyn Dakin, Paolo S. Segre, Andrew D. Straw, Morphology, muscle capacity,
790 skill, and maneuvering ability in hummingbirds. *Science (80-.)*. **657**, 653–657 (2018).

791 74. S. Y. Lee, G. R. Scott, W. K. Milsom, Have wing morphology or flight kinematics
792 evolved for extreme high altitude migration in the bar-headed goose? *Comp. Biochem.*
793 *Physiol. Part C* **148**, 324–331 (2008).

794 75. D. L. Altshuler, R. Dudley, J. A. McGuire, Resolution of a paradox: Hummingbird flight
795 at high elevation does not come without a cost. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 17731–
796 17736 (2004).

797 76. L. J. Revell, phytools: an R package for phylogenetic comparative biology (and other
798 things). *Methods Ecol. Evol.* **3**, 217–223 (2012).

799 77. F. Sornoza-Molina, J. F. Freile, J. Nilsson, N. Krabbe, E. Bonaccorso, A striking,
800 critically endangered, new species of hillstar (Trochilidae: Oreotrochilus) from the
801 southwestern Andes of Ecuador. *Auk* **135**, 1146–1171 (2018).

802 78. D. Rubolini, A. Liker, L. Z. Garamszegi, A. P. Moller, N. Saino, Using the BirdTree. org
803 website to obtain robust phylogenies for avian comparative studies: A primer. *Curr. Zool.*
804 **61**, 959–965 (2015).

805 79. W. Jetz, G. H. Thomas, J. B. Joy, K. Hartmann, A. O. Mooers, The global diversity of
806 birds in space and time. *Nature* **491**, 444–498 (2012).

80. H. R. L. Lerner, M. Meyer, H. F. James, M. Hofreiter, R. C. Fleischer, Multilocus resolution of phylogeny and timescale in the extant adaptive radiation of Hawaiian honeycreepers. *Curr. Biol.* **21**, 1838–1844 (2011).
81. P. C. Bürkner, brms: An R package for Bayesian multilevel models using Stan. *J. Stat. Softw.* **80** (2017).
82. J. L. Williamson, C. C. Witt, A lightweight backpack harness for tracking hummingbirds. *J. Avian Biol.*, e02802 (2021).
83. S. Watanabe, Asymptotic equivalence of Bayes cross validation and widely applicable information criterion in singular learning theory. *J. Mach. Learn. Res.* **11**, 3571–3594 (2010).
84. A. Vehtari, A. Gelman, J. Gabry, Practical Bayesian model evaluation using leave-one-out cross-validation and WAIC. *Stat. Comput.*, 1413–1432 (2017).

Figure 1. Elevational, latitudinal, and phylogenetic sampling from 2006–2020. **(A)** Sampling localities spanned 28° latitude and 4,578 meters in elevation in Peru and Chile. **(B)** We studied 77 species ('n' column indicates sampling depth) from all nine major hummingbird clades. Sampling (gray opaque points) is shown across each species' elevational range (colored horizontal bars). Time-calibrated phylogeny is modified from McGuire et al. (45). Illustrations depicting a representative from each clade are from *Birds of the World* and are reproduced courtesy of the Cornell Lab of Ornithology (©).

Figure 2. Relationship between elevation and the six studied blood parameters (hemoglobin concentration ([Hb]), hematocrit (Hct), total red blood cell count (TRBC), mean cell volume (MCV), mean cell hemoglobin content (MCH), and mean cell hemoglobin concentration (MCHC). (A–F) Within species comparisons, wherein each point represents a value from a single individual. (G–L) Among species comparisons, wherein each point represents a mean value from a single species. In all panels, solid trend lines correspond to relationships where 95% posterior credible intervals in models did not overlap zero, while dashed trend lines correspond to relationships where 89% credible intervals in models did not overlap zero (see Figure 3). Credible intervals for both elevation (at the 95% level) and elevation² (at the 89% level) did not overlap zero in the TRBC model (Panel I). Colors correspond to clades in Figure 1. R^2 values are reported from Bayesian models reported in Figure 3.

Figure 3. Posterior probabilities and credible intervals from full within-species (main plots) and among-species (insets) models for each of six blood traits: **(A)** hemoglobin concentration ([Hb]), **(B)** hematocrit (Hct), **(C)** total red blood cell (TRBC), **(D)** mean cell volume (MCV), **(E)** mean cell hemoglobin (MCH), **(F)** mean cell hemoglobin concentration (MCHC). Circles indicate beta estimates, thick and thin bars illustrate 89% and 95% credible intervals, respectively. Red circles denote parameters that did not overlap zero in top-ranked models. For Hb, Hct, and TRBC within-species analyses, the full model was the top model. See Tables S3-S4 for model comparison and parameter estimates from top models in other model sets.

Figure 4. Theoretical and empirical relationships among hummingbird blood parameters underlying hemoglobin concentration ([Hb]) adjustment and predictors of the Cell Number-Size Index (*CeNS*). **(A–B)** Hummingbirds have evolved optimized blood- O_2 carrying capacity, achieved by balancing total red blood cell count (TRBC) with mean cell volume (MCV). **(A)** Predictions for O_2 carrying-capacity. Background is shaded by O_2 carrying-capacity, calculated as $\text{MCV} \times \text{TRBC} \times 1/3 \text{ scaling factor}$ to represent constant MCHC. **(B)** Empirical findings. Points are colored by [Hb], with lighter colors representing higher concentrations. **(C)** Mean cell hemoglobin (MCH) and MCV are tightly linked, suggesting little variance in mean cell

854 hemoglobin concentration (MCHC). Points are colored by TRBC (units presented on the y-axis),
855 with lighter colors representing higher erythrocyte counts. In panels A–C, each point represents a
856 value from a single individual. **(C)** Posterior probabilities and credible intervals from the best
857 fitting reduced *CeNS* model. **(D)** *CeNS* shows a strong quadratic relationship with elevation,
858 revealing that middle elevation species adjust blood-O₂ carrying capacity differently than low-
859 and high-elevation species. **(E)** *CeNS* increases with increasing body mass. In panels D–E, R²
860 values are reported from generalized linear models of each *x* regressed on *y*, and thus do not
861 account for other modeled variables.