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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 (O_2) carrying capacity should be finely tuned to supply O_2 to respiring tissues. Hematological adjustments are expected in response to changes in O_2 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 O_2-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-O_2 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 O_2 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.

33 **INTRODUCTION**

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

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

56 HIF-induced production of erythropoietin is expected to increase [Hb] and Hct. Birds that are
57 chronically exposed to high elevations have been found to display either normal or elevated
58 [Hb], Hct, and MCHC (25, 26). Evidence from high-altitude-adapted human populations and
59 theoretical work suggests that optimal [Hb] and Hct levels may be similar to those of sea-level
60 populations (8, 13, 27). MCV typically increases in acclimatized, lowland-adapted animals
61 undergoing accelerated erythropoiesis (28); however, bird and other vertebrate lineages that have
62 evolved for extended periods at altitude have often been found to possess smaller cells, likely as
63 part of a suite of adaptive traits for efficient tissue oxygenation (23, 29, 30). Sensitivity of blood
64 traits to low partial pressure of O_2 on the short timescale of acclimatization is well-documented
65 (8, 23). Over longer timescales, natural selection on genes that affect hypoxia performance leads
66 predictably to genetic adaptation (18, 19, 31, 32), but it is not known how this evolutionary
67 process may affect the reaction norms that govern elevational variation in blood traits.
68 Systematic trait variation across environmental gradients reflects the outcome of
69 evolutionary and developmental processes, and explaining such 'trait gradients' will be essential
70 to predicting the functional consequence of climate warming for species and communities (33).
71 Despite its critical importance for tissue oxygenation, many questions remain unanswered
72 regarding elevational variation in blood traits that underpin blood-O_2 carrying capacity. Blood
73 traits vary among avian clades (e.g., 20, 33) and exhibit elevational variation both within species
74 (e.g., 4, 25, 34) and among species (e.g., 4, 25, 33, 35). Trait shifts with elevation are expected to
75 differ systematically for conspecific versus heterospecific comparisons; whereas within-species
76 variation should be driven primarily by developmental plasticity, among-species variation should
77 be influenced by genetic changes that have accrued during differences in elevational exposure
78 lasting thousands to millions of generations (37). Linck et al. (35) estimated elevational effects

79 on [Hb] and Hct within each of 136 species of Andean birds and showed that, within the median
80 species, [Hb] increased by 0.7 g/dL per 1,000 m in elevation, while Hct increased by 2.0
81 percentage points per 1,000 m. At least two additional recent studies have conducted broad-scale
82 interspecific comparative analyses of avian [Hb] and Hct data, but both datasets were compiled
83 from heterogeneous published data and lacked elevational sampling sufficient to estimate
84 elevational effects (34, 38). Furthermore, no previous comparative analysis of hematological
85 variation across elevation has included the six key parameters that affect blood-O_2 carrying
86 capacity per unit volume (Table S1). We continue to lack a general understanding of within- and
87 among-species effects of elevation on blood traits, and specifically, an understanding of whether
88 different rules govern elevational variation at these different levels of biological organization.
89 Another outstanding question is the degree to which known genetic adaptations to
90 hematological function may predict blood-O_2 carrying capacity. Adaptive genetic changes to
91 hemoglobin proteins have been demonstrated in many high-altitude-native birds, including
92 hummingbirds, House Wrens, Andean waterfowl, Bar-headed Geese, and a few dozen other bird
93 taxa (18, 19, 39–41). In hummingbirds, there is a highly predictable association between species-
94 typical Hb β^A13–β^A83 genotype and elevation, with derived increases in Hb-O_2 affinity in
95 highland lineages and reductions in Hb-O_2 affinity in lowland lineages (19). Hummingbirds
96 occurring at high altitudes exhibit two amino-acid substitutions on the β^A-globin subunit of Hb
97 (Hb β^A-$13_{GLY\rightarrow SER}$ and Hb β^A-$83_{GLY\rightarrow SER}$) that are known to affect O_2 binding (19). In particular,
98 β^A-13_{SER} is known from only four hummingbird genera (*Patagona*, *Chalcostigma*, *Pterophanes*,
99 and *Oreotrochilus*), all of which occur at extreme high altitudes (3, 18, 19). Both O_2-binding
100 affinity and blood-O_2 carrying capacity have critical effects on tissue oxygenation, but are part of
101 a complex O_2 uptake, transport, and delivery system that contains multiple convective and

102 diffusive steps (42). It is plausible that species-typical Hb β^A genotypes associated with different
103 O_2-binding properties induce predictable differences in blood traits and their reaction norms to
104 elevation; such an association could reflect a direct functional tradeoff or an indirect effect of
105 accrued genetic adaptation to elevation at multiple loci.

106 The constraints on blood trait values that are imposed by physical tradeoffs and the
107 relative contributions of each blood trait to overall blood-O_2 carrying capacity remain poorly
108 understood. [Hb] defines O_2-carrying capacity per unit volume of blood, and it can be adjusted
109 via adjustments to cell number (TRBC), cell size (MCV), within-cell [Hb] (MCHC), or plasma
110 volume. MCHC tends to be relatively invariant across the vertebrate tree of life, as demonstrated
111 nearly a century ago by Wintrobe (43), with Hb typically comprising ~1/3 of erythrocytes.
112 Plasma volume adjustments can decouple Hb mass from [Hb] and, although plasma volume
113 appears to vary among human populations (9), we currently have insufficient data on elevational
114 variation in avian plasma volume to assess its importance. Cell number and cell size are both
115 measurable components that are variable among individuals and species, subject to plastic and
116 evolutionary adjustments, and have strong direct effects on [Hb]. Cell number and cell size are
117 reciprocally constrained by physical space available in the circulatory system, such that
118 individual birds can have a few large erythrocytes, many small erythrocytes, or an intermediate
119 state for both traits (43, 44). Quantifying the relative contributions of TRBC and MCV in driving
120 [Hb] variation within and among species may reveal that species utilize different mechanisms of
121 adjusting blood-O_2 carrying capacity; if such mechanisms are predictably associated with
122 elevational environments or evolutionary histories of altitude exposure, they could lead to new
123 biogeographic insights.

124 In this study, we sought to elucidate the rules governing variation in blood traits that
125 affect O_2-carrying functions, including the effects of environment, individual characteristics, and
126 evolved species differences. Specifically, we asked: 1) To what extent do blood traits shift
127 predictably with elevational changes in O_2 availability, while controlling for other sources of
128 variation?; 2) Do blood traits vary with O_2 availability differently across developmental (within
129 species) and evolutionary (among species) time scales?; 3) How are blood traits affected by
130 phylogenetic history and evolved species differences, such as body mass, metabolic intensity,
131 and known genetic variants that affect Hb-O_2 binding affinity?; 4) To what extent are individual
132 and species variation in O_2-carrying capacity per unit volume of blood attributable to changes in
133 cell number versus cell size? And 5) does manifestation of the cell number-size tradeoff vary
134 predictably among species?
135 We collected specimen-vouchered hematological data from hummingbirds between
136 2006–2020 across a ~4,600-meter elevational gradient in the Andes. Data for six blood traits
137 from 1,217 individuals of 77 species were used to model within- and among-species variation,
138 respectively (Figure 1). Hummingbirds could be considered a model clade in which to study
139 hematological variation due to their documented evolutionary genetic responses to shifts in PO_2,
140 high species richness in mountains, phylogenetically conserved elevational ranges, and relative
141 uniformity of body size, clutch size, diet, mating system, and flight mode (18, 19, 45–47).
142 Notably, matching O_2 supply and demand is an acute challenge for all hummingbirds because
143 they exhibit the highest mass-specific metabolic rates among endotherms, and because they
144 employ sustained hovering, one of the most energetically demanding forms of animal
145 locomotion (46, 48).
146

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 RBC x 10^6/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

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

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

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

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

197 We found that MCV and MCH decreased in individuals occurring in warmer
198 environments (Figures 3D, E), and that MCHC decreased in individuals occurring at warmer
199 localities within a species' range (Figure 3F). The directions of these relationships were
200 consistent with previous experimental work: Comparisons of juvenile broiler chickens reared
201 from ~1-7 weeks of age in cool, ambient, and hot environments showed decreases in [Hb], Hct,
202 MCV, and TRBC as temperature increased (56, 57). Our findings add to a limited but intriguing
203 pool of evidence that warmer ambient temperatures may lead to developmental reductions in O_2-
204 carrying capacity of blood.

205 Models that included the species grouping variable fit substantially better than those
206 without; this effect explained 13–21% of the variance in the top model, depending on the model
207 set (Tables S3-S4; Methods). Moderate effects of the species grouping variable suggest that
208 variation in blood traits is partly explained by aspects of species biology that evolve too fast to
209 have resulted in phylogenetic autocorrelation. Species-typical Hb β^A13–β^A83 genotype did not
210 predict [Hb] or any other blood characteristics (Figure 3A–F), providing no evidence that
211 evolutionary adjustments to O_2-binding affinity affect the physical composition of blood or its
212 variation with elevation.

213

214 *Among-species blood comparison*

215 Using the species-level dataset, we evaluated the effects of elevation, body mass, species-typical
216 Hb β^A13–β^A83 genotype, and ambient temperature and precipitation on each of our 6 blood
217 response variables. We compared sets of models with LOOIC and WAIC (Table S3),
218 incorporating a quadratic elevation effect for TRBC, MCV, MCH, and MCHC models based on
219 curvature evident in the data (Figure 2G–L; Methods).
220 Within- and among-species findings for elevational effects were similar (Figure 3). Based
221 on the top model in each set, for every 1,000-m increase in elevation, [Hb] is expected to
222 increase by 0.29 g/dl, and Hct is expected to increase by 1.55 percentage points. Elevation was a
223 consistently strong predictor of [Hb], Hct, and TRBC, all of which increased with increasing
224 elevation (Figures 2G–I and 3A–C). The importance of the quadratic effect of elevation for
225 MCV and MCH, and overall shape of the species-level data, suggest that O_2 carrying capacity
226 adjustment differs for middle-elevation species versus those found at low and high elevations
227 (Figure 2J–K; Figure 3D–E). Neither species-typical Hb β^A13–β^A83 genotype, body mass,
228 environmental temperature, nor precipitation explained variation in any species-mean blood
229 characteristic (Figure 3).
230
231 *Influence of cell number versus size on blood-O_2 carrying capacity*
232 The axis of variation in cell number versus size space was tightly constrained (Figure 4A–B); the
233 'banana' shape of Figure 4A–B mirrors patterns from Wintrobe (43) and Hawkey et al. (44),
234 which present similar plots based on species representing multiple vertebrate classes. The shape
235 of this relationship indicates that the highest levels of blood-O_2 carrying capacity per unit blood
236 are achieved at high values of TRBC or MCV, respectively. Individual hummingbirds can
237 possess few large erythrocytes or many small erythrocytes, but cell number and size must be

238 balanced (44). Because of the proportionately greater surface area for gas exchange, having
239 many small cells is predicted to be advantageous for fast metabolism or when the partial pressure
240 of O_2 in inspired air is low (44). Similarly, having large cells may be advantageous for
241 modulating the time-course of gas exchange (58). One way to shed light on this tradeoff is to test
242 whether individuals and species, respectively, adjust O_2 carrying capacity per unit blood
243 primarily through increases in cell number or size.

244 We designed models to understand the relative contributions of cell number versus size to
245 blood O_2-carrying capacity per unit volume ([Hb]), within and among species over ~22 Mya of
246 hummingbird evolution. These models demonstrated that hummingbirds co-equally modulated
247 cell number and cell size to adjust [Hb] (Table S6). The fact that this was true both within and
248 among species suggests that developmental and evolutionary fine-tuning of blood-O_2 carrying
249 capacity occur similarly.

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

255 $$\frac{(\beta_{TRBC})}{(\beta_{TRBC} + \beta_{MCV})}$$

256

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

260 Extending our analysis, we designed a set of models to test potential predictors of *CeNS*:
261 elevation, a quadratic elevation effect (elevation^2; to account for notable curvature in the data),
262 body mass, wing loading, species-typical Hb β^A13–β^A83 genotype, and phylogeny (see
263 Methods). Elevation, elevation^2, and body mass strongly predicted *CeNS* (Figure 4D-F; Table
264 S7). We found a trend of decreasing *CeNS* with higher wing loading, but the 95% credible
265 intervals overlapped zero (Figure S2A–B). Species-typical Hb β^A13–β^A83 genotype did not
266 predict *CeNS* (Figure S3), nor did we find phylogenetic signal in *CeNS* (Figure S4).
267 Examining the tradeoff between cell number and volume provides a potential explanation
268 for the striking pattern that middle elevation species tend to adjust [Hb] by different mechanisms
269 than low and high elevation species (Figure 4E). *CeNS* was uncorrelated with TRBC coefficients
270 ($R^2 = 0.07$, $r = -0.27$) but strongly correlated with MCV coefficients ($R^2 = 0.51$, $r = -0.71$),
271 indicating that variation in *CeNS* was primarily driven by the degree to which species modulate
272 cell size (Figure S2, Figure S5. We suggest that because low and high elevation species tend to
273 possess relatively small cells, they may be more able to rely on shifts in cell size, rather than cell
274 number, to increase [Hb] (Figure 2J; Figure 4). However, as cell size increased, species were
275 more likely to approach maximum size for red blood cells, beyond which they could incur a
276 functional detriment. As a result, species with larger red blood cells were more reliant on cell
277 number to adjust [Hb]. For middle elevation hummingbird species that have evolved under
278 moderate (~15–30%) reductions in O_2 availability, TRBC appears to be the primary axis for
279 adjustment of blood-O_2 carrying capacity per unit volume of blood. Species that have evolved at
280 the highest elevations are expected to be the best adapted to hypobaric hypoxia, and it is
281 therefore remarkable that their mode of [Hb] adjustment (primarily by MCV) resembles that of
282 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 consilience 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 (λ = 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

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

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

328 A strength of our study was that hummingbirds vary only subtly in aspects of their
329 biology that could plausibly confound our analyses, such as metabolic rate, energy budget, torpor
330 use, mode of locomotion, foraging strategy, or clutch size. Phylogenetic conservatism in these
331 traits facilitates explanation of blood trait variation across the entire clade. A strong effect of
332 species identity in spite of these intrinsic controls hints that species optimize O_2-carrying
333 functions of blood for subtle changes in lifestyle and environment, and on rapid timescales. This
334 finding is consistent with the rapid diversification of human hematological responses to
335 elevation, as illustrated by the copious variation among populations in patterns of elevational
336 variation (27, 49, 50, 60–62).
337
338 *Conclusions*
339 Across ~22 Mya of the hummingbird evolution, ambient O_2-availability and species
340 idiosyncrasies—not phylogenetic affinities—were the primary drivers of variation in blood traits
341 affecting O_2-carrying functions. In response to elevational changes in O_2 partial pressure,
342 hummingbirds make predictable adjustments to the physical composition of blood. Remarkably,
343 after controlling for many potentially confounding variables, decreasing PO_2 drove increases in
344 [Hb] and Hct that were nearly identical in magnitude within and among hummingbird species,
345 respectively. This implies that developmental and evolutionary optimization of blood traits to
346 ambient PO_2 occurs in fundamentally similar ways, responding to the same set of physical rules
347 that govern gas exchange at the blood-gas barrier. In addition to O_2 availability, species identity
348 itself explained 13–21% of blood trait variation, suggesting that fast-evolving, unmodeled
349 aspects of species biology underpin substantial hematological variation. Our data revealed a
350 striking constraint space that defines the cell number-size tradeoff for hummingbird erythrocytes;

351 within this space, individuals and species co-equally modulated cell number and size to adjust
352 [Hb]. However, species varied in their proportional reliance on adjustments to cell number
353 versus size (*CeNS*) to increase [Hb], and both elevation and body mass were strongly predictive
354 of species' tendencies. Middle-elevation species that have been evolving under moderate
355 hypoxia adjusted [Hb] primarily by adjusting cell number; in contrast, low and high elevation
356 species tended to adjust [Hb] by adjusting cell size, a pattern partly underpinned by
357 hummingbirds at extreme elevations exhibiting smaller cells. Together, these results suggest that
358 evolutionary solutions to reduced O_2 availability can differ qualitatively in conjunction with
359 species-specific histories of exposure to hypoxia challenges of varying severity.
360

361 **MATERIALS AND METHODS**

362 *Field Sampling*

363 We collected data from 1,217 individuals of 77 hummingbird species from Peru and Chile from
364 2006–2020 (Figure 1). After filtering, our final within-species dataset included 1,151 individuals
365 of 77 species from 75 localities. The best-sampled clades were the Coquettes (387 individuals,
366 20 species) and Brilliants (269 individuals, 19 species). The species with greatest sampling depth
367 were *Metallura tyrianthina* (95 individuals), *Colibri coruscans* (73 individuals), and *Amazilia*
368 *amazilia* and *Patagona gigas* (64 individuals each; Figure 1). Sampling across sexes was
369 balanced (553 females, 592 males, and 6 unknown). Most birds sampled were adults (936 adults,
370 179 juveniles, and 36 unknown). Detailed data on specimens, collection localities, and blood
371 traits are available in Witt et al. (*In prep*) and in the ARCTOS database (arctosdb.org) for
372 reproducibility and extension of our work.
373

374 *Hematological measurements*

375 To calculate primary indices (hemoglobin concentration ([Hb]), hematocrit (Hct), and total red
376 blood cell count (TRBC)), we obtained whole blood samples within ~5 mins to ~3 hours of
377 capture by venipuncture on the underside of the wing and collection with heparinized
378 microcapillary tubes and Hemocue HB201+ cuvettes. Hct (%) was measured with digital calipers
379 after centrifuging the sealed microcapillary tube for 5 min at 13,000 r.p.m. When >1 Hct sample
380 was taken, weighted average values were used in analyses. [Hb] (g/dL blood) was measured on
381 ~5 µL of blood using a HemoCue HB201+ hemoglobin photometer. The HemoCue proprietary
382 photometric method produces values for avian blood that are ~1.0 g/dL greater than those
383 generated using cyanomethemoglobin spectrophotometry (63). Accordingly, we corrected [Hb]
384 values by subtracting 1.0 g/dL. When possible, at the time of blood collection, 10 µl whole blood
385 was preserved in a 1:200 NaCl dilution to obtain TRBC estimates. Within 12-hours of blood
386 collection, we pipetted a 10 µl sub-sample of blood-NaCl dilution into a hemocytometer and
387 allowed cells to settle for 1 min. After focusing the microscope, one or more photos were
388 obtained of cells in the hemocytometer grid. Later, cells were manually counted in the ImageJ
389 software (64) and TRBC was estimated following standard protocols (20, 21).

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

397 Secondary blood indices (mean cell volume (MCV), mean cell hemoglobin (MCH), and
398 mean cell hemoglobin concentration (MCHC)) were calculated from primary indices (Table S1)
399 and following standard protocols (20). We examined distributions of the data using histograms
400 and qqPlots, and we eliminated outliers that fell outside of the expected distribution and that
401 differed from known 'typical' values (20, 25, 65). To reduce age-related outliers, we examined
402 values at the tails of each blood trait distribution that were associated with presence of a bursa
403 measuring ~2 x 2 mm. All juvenile values fell within the expected distribution of blood values.
404 We did not age individuals by plumage, as juvenile plumage lasts much longer than juvenile
405 blood values, which stabilize one to several months after fledging.
406
407 *Environmental and life history variables*
408 We compiled elevational range limits for each species using a combination of published data,
409 field guides, and specimen records from the MSB (3, 66–68). We calculated each individual's
410 elevational range position relative to its species elevational range breadth (hereafter 'elevational
411 position') as *1 – ((maximum species elevation – individual sampling elevation)/species*
412 *elevational range).*
413 We incorporated species-typical Hb β^A13–β^A83 genotypes from published data (19, 45).
414 For 7 species, we identified these genotypes from Hb β^A sequences aligned to a GenBank
415 reference from Projecto-Garcia et al. (19). For 12 species (*Amazilia franciae, Campylopterus*
416 *villaviscensio, Eutoxeres aquila, Klais guimeti, Leucippus taczanowskii, Myrmia micrura,*
417 *Phaethornis atrimentalis, Sephanoides sephaniodes, Chaetocercus mulsant, Eriocnemis vestita,*
418 *Myrtis fanny,* and *Oreotrochilus leucopleurus*) species-typical Hb β^A13–β^A83 genotypes were
419 inferred from sister taxa or nearest relatives.

420 We characterized climatic variation among latitudinal and elevational gradients using 19
421 bioclimatic variables from the WorldClim database (69). We downloaded rasters of each
422 BioClim variable using the 'raster' package in R (version 4.0.0), cropped them to our study areas
423 of Peru and Chile, and extracted temperature and precipitation values for each sampling locality.
424 We used principal components analysis (PCA) for temperature (Bio1–11) and precipitation
425 (Bio12–19) variables to create a composite measure of temperature and precipitation across the
426 gradient. PC1 of the temperature variables (hereafter "temperature") explained 73.4% of the
427 variation in composite temperature, and loadings suggested that this axis corresponded to
428 increasing temperature across sites (see GitHub). PC1 of the precipitation variables (hereafter
429 "precipitation") explained 79.8% of the variation in composite precipitation, and loadings
430 suggested that this axis corresponded to increasing precipitation across sites (see GitHub).
431 To account for multiple measurement effects (i.e., traits measured from multiple
432 individuals within species), we calculated between- and within-species differences following
433 Villemereuil et al. (70). This approach uses within-group centering to separate each predictor
434 into two components: We first calculated species mean values (i.e., between-species variability)
435 and then subtracted the mean value of individual observations (i.e., within-species differences).
436 These measures of within-species variation for temperature, precipitation, and body mass were
437 included in models. Because hummingbird body masses may differ by sex, within-species
438 differences were calculated using mean body mass of males for males, mean body mass of
439 females for females, and species-level means for individuals of unknown sex (n=6).
440 We used wing area data from the 59 MSB species studied in Skandalis et al. (71) to
441 calculate wing loading in g/cm^2, calculated as species mean body mass (g) divided by total wing

area (cm^2). 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

464 models and evaluated phylogenetic signal using Blomberg's K and Pagel's λ. Results from the
465 two tests were concordant and consistent across model subsets (Table S8).
466
467 *Within species models*
468 We analyzed within species variation in blood characteristics using generalized linear multilevel
469 models in the R package brms (81). Brms uses Markov chain Monte Carlo (MCMC) sampling to
470 obtain draws from posterior distributions. We designed hierarchical models for each of six
471 continuous responses variables: [Hb], Hct, TRBC, MCV, MCH, and MCHC. For each model,
472 predictor variables included one species-level categorical variable (species-typical Hb β^A13–
473 β^A83 genotype) and eight individual-level continuous variables (elevation, elevational position,
474 body mass (log-transformed), precipitation, temperature, and within species variation in body
475 mass, temperature, and precipitation). All variables were standardized to a mean of zero and
476 standard deviation of one.
477 We compared 6 models in each model set: 1) an intercept-only null model, 2) an
478 intercept-only model with a species grouping variable (1|species), 3) a model with all predictors
479 and no grouping variable, 4) a model with all predictors and the species grouping variable, 5) a
480 reduced-predictor model with no grouping variable (reduced model 3), 6) a reduced-predictor
481 model with the species grouping variable (reduced model 4). Reduced models included only
482 predictors whose 95% credible intervals (CIs) did not overlap zero in full models. Distribution
483 family differed by model set: Gaussian for [Hb], Hct, and TRBC models; skew-normal for MCV
484 and MCH models; and student's *t*-distribution for MCHC models. We ran four Markov chains
485 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

509 overlap zero in full models (reduced model 2). Given the notable quadratic shape of species
510 mean data for TRBC, MCV, MCH, and MCHC (Figure 2G–L), for those model sets we
511 additionally compared 4) a model with all predictors plus a quadratic component of elevation and
512 5) a reduced-predictor model with quadratic component including only those predictors whose
513 95% CIs did not overlap zero in the full quadratic model (reduced model 4). Distribution family
514 differed by model set: Gaussian for [Hb; skew-normal for Hct; and student's *t*-distribution for
515 TRBC, MCV, MCH, and MCHC models. We ran four Markov chains for 20,000 iterations with
516 a burn-in period of 10,000, thinned every 10 steps.
517
518 *Cell number versus size analyses*
519 First, to assess within species variation in cell number versus size, we compared a null model
520 (intercept only) to a single full model *[Hb] ~ MCV + TRBC + (1|species)* for all individuals of
521 59 species that had [Hb], MCV, and TRBC measurements, distinguishing between *Patagona*
522 *gigas* "north" and "south". The species grouping variable allowed us to account for repeated
523 sampling of individuals within a species and to hold species identity constant. Second, to
524 evaluate among-species variation in cell number versus size, we generated a mean dataset for
525 each species with ≥3 individuals sampled (48 species total). We again compared a null (intercept
526 only) model to a single full model *[Hb] ~ MCV + TRBC.* Third, to assess how individual species
527 differ in their relative contributions of MCV and TRBC to increasing [Hb], we repeated our
528 approach for individual species: For each species with ≥8 sample individuals (32 species total),
529 we generated a species-specific data subset, carefully and conservatively evaluated influential
530 outlier points, and ran a single model: *[Hb] ~ MCV + TRBC* (Figure S1). For all three model sets

531 (within species, among species, and single species), both predictors were standardized to a mean
532 of zero and standard deviation of one.
533 Using standardized, model-estimated coefficients from each single-species model, for
534 each species we calculated the Cell Number-Size Index (hereafter *CeNS*) as described in the
535 main text. *CeNS* ranged from 0.0–1.0 and represented the proportional contribution of TRBC
536 relative to MCV in driving [Hb], with higher values corresponding to greater relative
537 contributions of cell number versus cell size.
538 We then analyzed the factors that contribute to variation in *CeNS*. Our model response
539 variable was *CeNS* (0–1 index). Predictors, all species mean values, included elevation, a
540 quadratic elevation term "elevation^2" to account for notable curvature in the data, body mass
541 (log-transformed), wing loading, and species-typical Hb β^A13–β^A83 genotype. We compared six
542 models: 1) an intercept-only null model, 2) a model with core predictors (elevation, body mass,
543 and wing loading), 3) a model with core predictors plus elevation^2, 4) a model with core
544 predictors plus elevation^2 and species-typical Hb β^A13–β^A83 genotype, 5) a reduced version of
545 model 3, and 6) a reduced version of model 4. Reduced models included only those predictors
546 whose 95% CIs did not overlap zero in full models. In addition to model fit diagnostics, we
547 assessed correlations between variables by analyzing correlation matrices and by regressing
548 *CeNS* on each R^2 values from standardized species-specific *CeNS* models, unstandardized TRBC
549 and MCV beta estimates, and raw TRBC and MCV values (Figure S5). Distribution family
550 differed by model set: Gaussian for individual-level, species mean, and *CeNS* prediction models,
551 and student's *t*-distribution for species-specific models. For all *CeNS* models we ran four Markov
552 chains for 20,000 iterations with a burn-in period of 10,000, thinned every 10 steps. We assessed
553 phylogenetic signal with Blomberg's K, Pagel's λ, and visually with contmaps (Figure S4).

Model comparison and diagnostics

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

Breakpoint analysis

We tested for objective thresholds in each blood trait using piecewise linear regression of elevation (standardized) regressed on each blood trait. In this procedure, we first fit a simple linear regression model, iteratively searched for objective "breakpoints" along our sampled elevational gradient, selected the breakpoint with the lowest residual error, and refit the linear regression model with separate estimates for slopes and intercepts below and above the 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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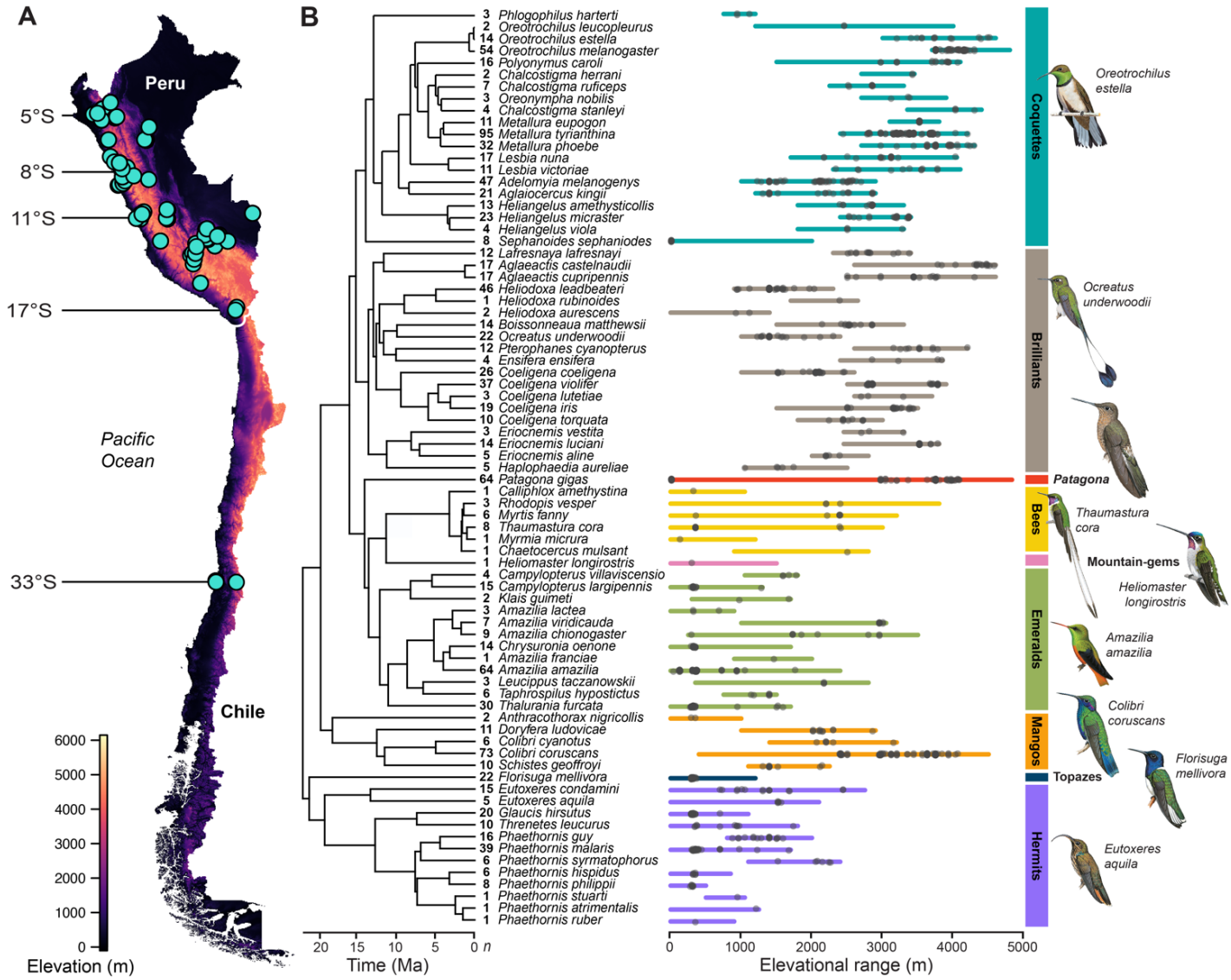

Figure 1. Elevational, latitudinal, and phylogenetic sampling from 2006–2020. **(A)** Sampling localities spanned 28° latitude and 4,578 meters in elevation in Peru and Chile. **(B)** We studied 77 species ('n' column indicates sampling depth) from all nine major hummingbird clades. Sampling (gray opaque 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 (©).

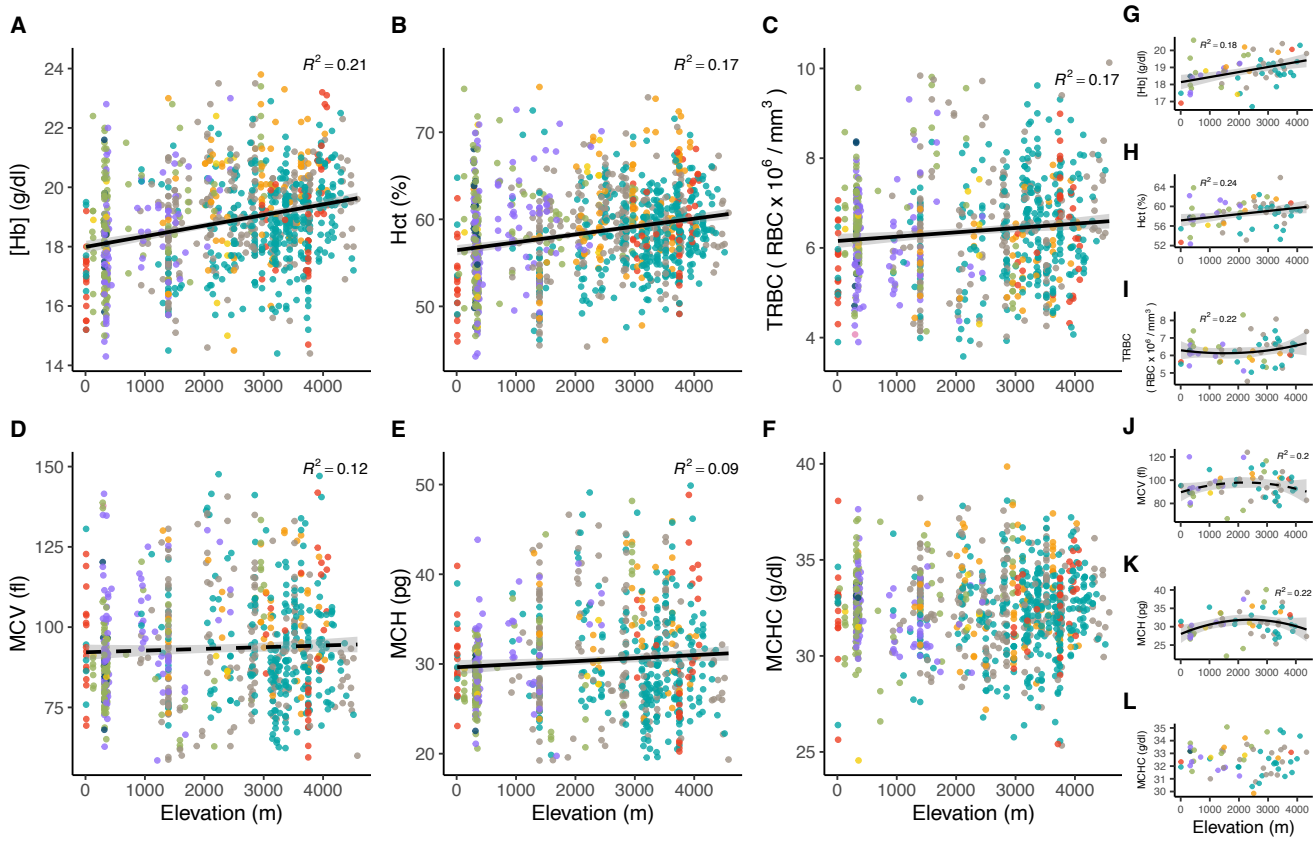

Figure 2. Relationship between elevation and the six studied blood parameters (hemoglobin concentration ([Hb]), hematocrit (Hct), total red blood cell count (TRBC), mean cell volume (MCV), mean cell hemoglobin content (MCH), and mean cell hemoglobin concentration (MCHC). **(A–F)** Within species comparisons, 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 elevation^2 (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.

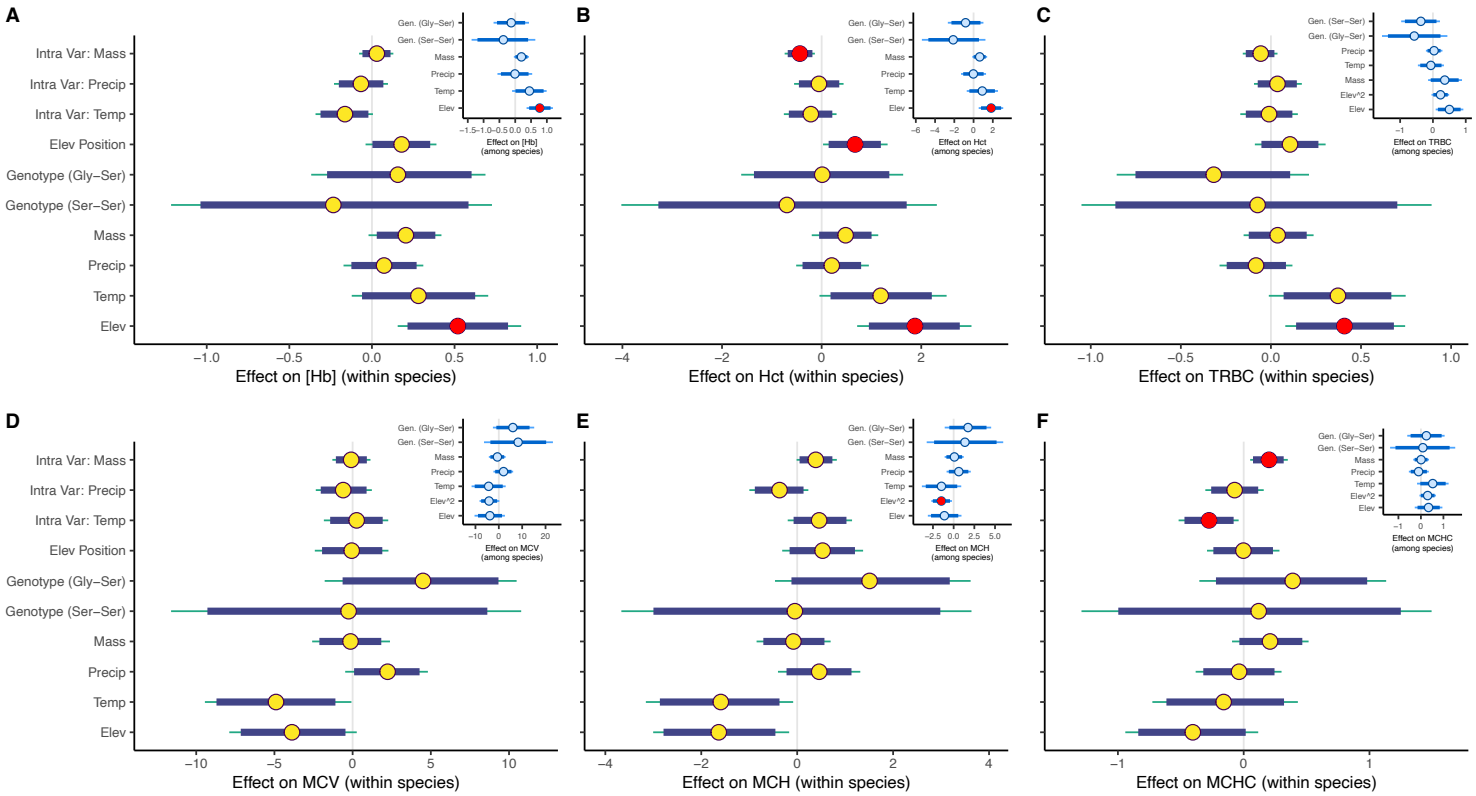

Figure 3. Posterior probabilities and credible intervals from full within-species (main plots) and among-species (insets) models for each of six blood traits: **(A)** hemoglobin concentration ([Hb]), **(B)** hematocrit (Hct), **(C)** total red blood cell (TRBC), **(D)** mean cell volume (MCV), **(E)** 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.

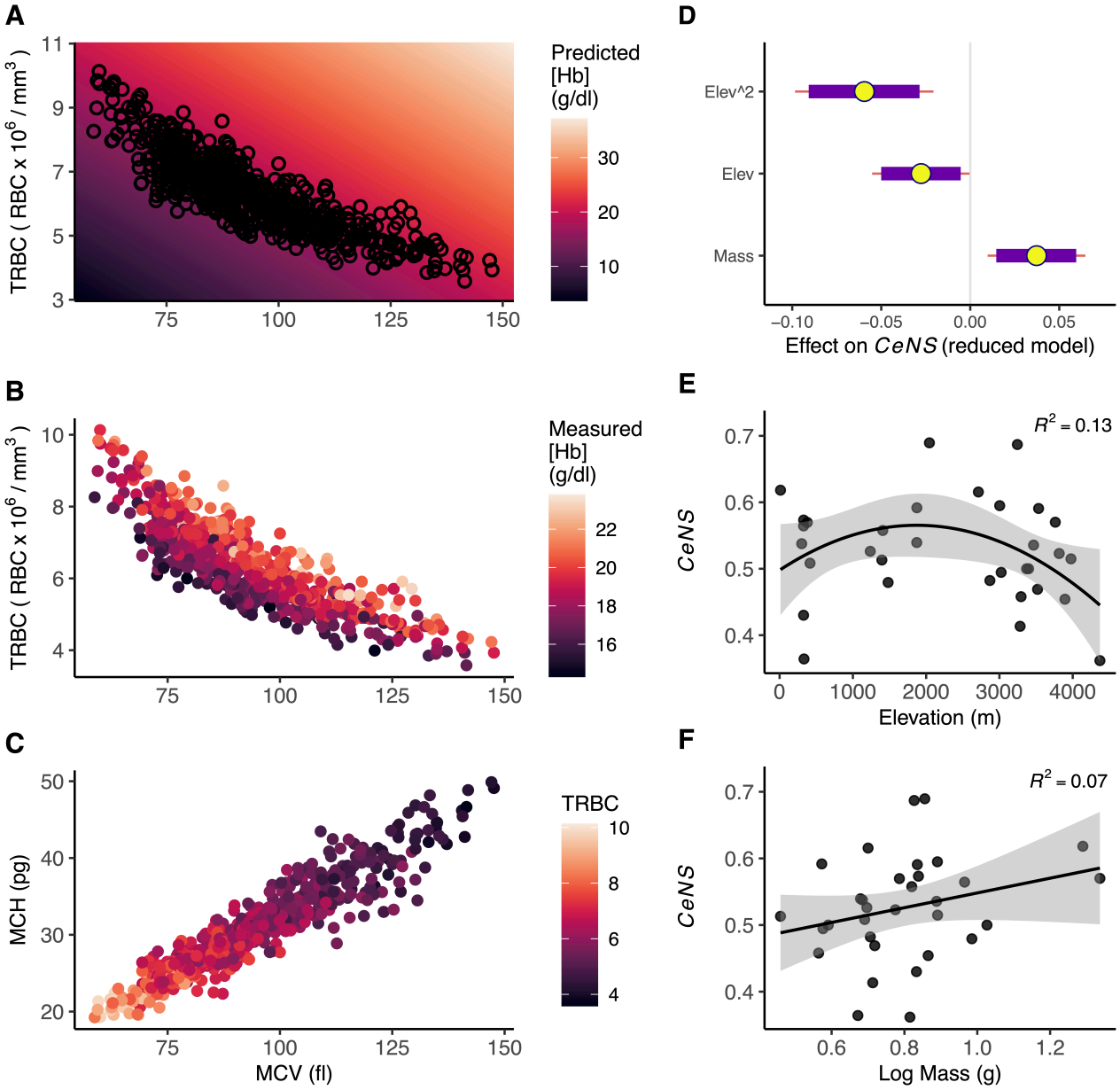

Figure 4. Theoretical and empirical relationships among hummingbird blood parameters underlying hemoglobin concentration ([Hb]) adjustment and predictors of the Cell Number-Size Index (*CeNS*). **(A–B)** Hummingbirds have evolved optimized blood-O_2 carrying capacity, achieved by balancing total red 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 *MCV x TRBC x 1/3 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-O_2 carrying capacity differently than low-
859 and high-elevation species. **(E)** *CeNS* increases with increasing body mass. In panels D–E, R^2
860 values are reported from generalized linear models of each *x* regressed on *y*, and thus do not
861 account for other modeled variables.