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

Jessie L. Williamson[1,4*], Ethan B. Linck[1], Emil Bautista[2], Ashley Smiley[1,3], Jimmy A. McGuire[3], Robert Dudley[3], and Christopher C. Witt[1*]

[1]Museum of Southwestern Biology and Department of Biology, University of New Mexico, 302 Yale Blvd NE, Albuquerque, NM 87131, USA

[2]Centro de Ornitología y Biodiversidad (CORBIDI), Lima, Perú

[3]Museum of Vertebrate Zoology and Department of Integrative Biology, University of California, Berkeley, Berkeley, CA 94720, USA

[4]Lead contact

*Corresponding authors: Jessie L. Williamson, williamson@unm.edu, @jl_williamson; Christopher C. Witt, cwitt@unm.edu, @MSBbirds). Address: Department of Biology, University of New Mexico, Castetter Hall 1480, MSC03-2020, 219 Yale Blvd. NE, Albuquerque, NM 87131, USA

SUMMARY

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, the rules by which this variation occurs have remained elusive. For example, elevational variation among conspecific individuals may not be comparable to variation among species that have evolved differences in traits, genes, and environmental niches. We sought to elucidate these eco-physiological rules in the hummingbird radiation by analyzing 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 and 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.

Key words: Andes, blood, blood-oxygen carrying capacity, cell, elevation, hummingbird, physiology, elevational gradient

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-

64 induced production of erythropoietin is expected to increase [Hb] and Hct. Birds that are
65 chronically exposed to high elevations have been found to display either normal or elevated
66 [Hb], Hct, and MCHC[25,26]. Evidence from high-altitude-adapted human populations and
67 theoretical work suggests that optimal [Hb] and Hct levels may be similar to those of sea-level
68 populations[8,13,27]. MCV typically increases in acclimatized, lowland-adapted animals undergoing
69 accelerated erythropoiesis[28]; however, bird and other vertebrate lineages that have evolved for
70 extended periods at altitude have often been found to have smaller cells, likely as part of a suite
71 of adaptive traits for efficient tissue oxygenation[23,29,30]. Plasticity in blood characteristics to low
72 partial pressure of O_2 over short-term timescales of acclimatization is well-documented[8,23]. Over
73 longer timescales, natural selection on genes that affect hypoxia performance leads predictably to
74 genetic adaptation[17–19,31], but the effects of this evolutionary process on the physical composition
75 of blood are unclear.

76 Systematic trait variation across environmental gradients reflects the outcome of
77 evolutionary and developmental processes, and explaining such 'trait gradients' will be essential
78 to predicting the functional consequence of climate warming for species and communities[32].
79 Despite its critical importance for tissue oxygenation, many questions remain unanswered
80 regarding elevational variation in blood traits that underpin blood-O_2 carrying capacity. Blood
81 traits vary among avian clades (e.g.,[20,33]) and exhibit elevational variation both within species
82 (e.g.,[4,25,34]) and among species (e.g.,[4,25,33,35]). Trait shifts with elevation are expected to differ
83 systematically for conspecific versus heterospecific comparisons; whereas within-species
84 variation should be driven primarily by developmental plasticity, among-species variation should
85 be influenced by genetic changes that have accrued during differences in elevational exposure
86 lasting thousands to millions of generations[36]. Linck et al.[34] estimated elevational effects on [Hb]

87 and Hct within each of 136 species of Andean birds and showed that, within the median species,
88 [Hb] increased by 0.7 g/dL per 1,000 m in elevation, while Hct increased by 2.0% per 1,000 m.
89 At least two additional recent studies have conducted broad-scale interspecific comparative
90 analyses of avian [Hb] and Hct data, but both datasets were compiled from heterogeneous
91 published data and lacked elevational sampling sufficient to estimate elevational effects[33,37].
92 Furthermore, no previous comparative analysis of hematological variation across elevation has
93 included the six key parameters that affect blood-O_2 carrying capacity per unit volume (Table
94 S1). We continue to lack a general understanding of within- and among-species effects of
95 elevation on blood traits, and specifically, an understanding of whether different rules govern
96 elevational variation at these different levels of biological organization.

97 Another outstanding question is the degree to which known genetic adaptations to
98 hematological function may predict blood-O_2 carrying capacity. Adaptive genetic changes to
99 hemoglobin proteins have been demonstrated in many high-altitude-native birds, including
100 hummingbirds, House Wrens, Andean waterfowl, Bar-headed Geese, and a few dozen other bird
101 taxa[17,19,38–41]. In hummingbirds, there is a highly predictable association between species-typical
102 Hb β^{A}_{13}–β^{A}_{83} genotype and elevation, with derived increases in Hb-O_2 affinity in highland
103 lineages and reductions in Hb-O_2 affinity in lowland lineages[17]. Hummingbirds occurring at high
104 altitudes exhibit two amino-acid substitutions on the β^{A}-globin subunit of Hb (Hb β^{A}-$13_{GLY\rightarrow SER}$
105 and Hb β^{A}-$83_{GLY\rightarrow SER}$) that are known to confer increased O_2-binding affinity[17]. In particular,
106 β^{A}-13_{SER} is known from only four hummingbird genera (*Patagona*, *Chalcostigma*, *Pterophanes*,
107 and *Oreotrochilus*), all of which occur at extreme high altitudes[3,19,42]. Both O_2-binding affinity
108 and blood-O_2 carrying capacity have critical effects on tissue oxygenation, but are part of a
109 complex O_2 uptake, transport, and delivery system that contains multiple convective and

110 diffusive steps[43]. It is plausible that species-typical Hb β^A genotypes associated with different
111 O_2-binding affinities predict hematological phenotypes that relate to blood-O_2 carrying functions;
112 such an association could reflect a direct functional tradeoff or an indirect effect of accrued
113 genetic adaptation to elevation at multiple loci.

114 The constraints on blood trait values that are imposed by physical tradeoffs and the
115 relative contributions of each blood trait to overall blood-O_2 carrying capacity remain poorly
116 understood. [Hb] defines O_2-carrying capacity per unit volume of blood, and it can be adjusted
117 via adjustments to cell number (TRBC), cell size (MCV), within-cell [Hb] (MCHC), or plasma
118 volume. MCHC tends to be relatively invariant across the vertebrate tree of life, as demonstrated
119 nearly a century ago by Wintrobe[44], with Hb typically comprising ~1/3 of erythrocytes. Plasma
120 volume adjustments can decouple Hb mass from [Hb] and, although plasma volume appears to
121 vary among human populations[9], we currently have insufficient data on elevational variation in
122 avian plasma volume to assess its importance. Cell number and cell size are both measurable
123 components that are variable among individuals and species, subject to plastic and evolutionary
124 adjustments, and have strong direct effects on [Hb]. Cell number and cell size are reciprocally
125 constrained by physical space available in the circulatory system, such that individual birds can
126 have a few large erythrocytes, many small erythrocytes, or an intermediate state for both
127 traits[44,45]. Quantifying the relative contributions of TRBC and MCV in driving [Hb] variation
128 within and among species may reveal that species utilize different mechanisms of adjusting
129 blood-O_2 carrying capacity; if such mechanisms are predictably associated with elevational
130 environments or evolutionary histories of altitude exposure, they could lead to new
131 biogeographic insights.

132 In this study, we sought to elucidate the rules governing variation in blood traits that
133 affect O_2-carrying functions, including the effects of environment, individual characteristics, and
134 evolved species differences. Specifically, we asked: (1) To what extent do blood traits shift
135 predictably with elevational changes in O_2 availability, while controlling for other sources of
136 variation?; (2) Do blood traits vary with O_2 availability differently over developmental (within
137 species) and evolutionary (among species) time scales?; (3) How are blood traits affected by
138 phylogenetic history and evolved species differences, such as body mass, metabolic intensity,
139 and known genetic variants that affect Hb-O_2 binding affinity?; (4) To what extent are individual
140 and species variation in O_2-carrying capacity per unit volume of blood attributable to changes in
141 cell number versus cell size? And (5) does manifestation of the cell number-size tradeoff vary
142 predictably among species?
143 We collected specimen-vouchered hematological data from hummingbirds between
144 2006–2020 across a ~4,600-meter elevational gradient in the Andes. Data for six blood traits
145 from 1,217 individuals of 77 species were used to model within- and among-species variation,
146 respectively (Figure 1). Hummingbirds are a useful system in which to study hematological
147 variation due to their documented evolutionary genetic responses to shifts in PO_2, high species
148 richness in mountains, phylogenetically conserved elevational ranges, and relative uniformity of
149 body size, clutch size, diet, mating system, and flight mode[19,42,46–48]. Notably, matching O_2
150 supply and demand is an acute challenge for all hummingbirds because they exhibit the highest
151 mass-specific metabolic rates among endotherms, and because they employ sustained hovering,
152 one of the most energetically demanding forms of animal locomotion[47,49].
153
154 **RESULTS AND DISCUSSION**

155 We quantified three primary ([Hb], Hct, and TRBC) and three secondary hematological indices
156 (MCV, MCH, and MCHC; Table S1), and separately analyzed two datasets: an individual-level
157 dataset, wherein each row contained data from one individual hummingbird (*n*=1,217), and a
158 species-level dataset, wherein each row contained mean blood values calculated for each species,
159 with a minimum of 2 individuals sampled (*n*=58–65 species, depending on blood trait modeled).
160 In the individual-level data, mean [Hb] was 18.84 g/dl (±1.65), mean Hct was 58.54% (±5.15),
161 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
162 fl (±17.1), mean MCH was 30.4 pg (±5.64), and mean MCHC was 32.44 g/dl (± 2.12). Complete
163 reference values—the first such values ever published for >90% of our study species—are
164 reported in detail in Table S2.
165
166 *Within-species blood comparison*
167 We used the individual-level dataset to model within species variation and accounted for
168 between-species differences by incorporating a species grouping variable. Using multilevel
169 Bayesian models for each of the six blood parameters, we estimated the effects of elevation,
170 body mass, precipitation, air temperature, species-typical Hb $\beta^A{}_{13}$–$\beta^A{}_{83}$ genotype, as well as
171 within-species variation in elevational position (an individual's sampled elevation relative to its
172 species elevational range), body mass, air temperature, and precipitation. Separately, we
173 excluded sex and wing-loading as potentially important predictors (see Methods). In total, we
174 compared six models per set using LOOIC and WAIC (Tables 1 and S5; Methods). In addition to
175 fixed effects, models included species as a random effect, and a phylogenetically correlated
176 random intercept (i.e., covariance matrix of phylogenetic distances among taxa). Thus, our full

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

179 Based on the top-performing model in each set, we found that for every 1,000-m increase
180 in elevation, [Hb] is expected to increase by 0.39 g/dl, Hct is expected to increase by 1.42%,
181 TRBC is expected to increase by 310,000 cells per µl, and MCH is expected to decrease by 0.48
182 pg. Elevation strongly predicted [Hb], Hct, TRBC, and MCH (Figures 2 A–F and 4A–F).
183 Elevational position was also a strong predictor of Hct, indicating that individuals located higher
184 in their species' elevational range tended to have higher Hct (Figure 4B). [Hb], TRBC, and MCH
185 also increased with increasing elevational position, although these relationships were subtle
186 (Figure 4A, C, E).

187 The finding that [Hb], Hct, and TRBC increase with elevation was consistent with
188 theoretical expectations for O_2-carrying capacity of blood and with prior empirical findings in
189 birds[3,4,25,33,37], humans[50,51], mammals[23,52], and other taxa[53,54]. In prior work, substantial variation
190 in slopes of these parameters across elevation may have been attributable to variation in
191 temporal, taxonomic, and spatial scales of comparison, as well as constraints on the time-course
192 of evolutionary adaptation[17,31,51]. We expect increases in Hct at higher elevations because an
193 increase in the total mass of Hb enhances oxygenation under lower ambient PO_2[13]; unless there
194 is a proportional increase in plasma volume or MCHC, an increase in Hb mass is expected to be
195 accompanied by an increase in the proportion of blood comprised of red blood cells. TRBC also
196 increased with elevation and was, along with MCV, one of two key drivers of [Hb] adjustment
197 (Figure 2, Figure 3, Figure 5C–D)[45]. MCV did not vary predictably in our linear models (Figure
198 2D). Although increasing within-cell [Hb], or MCHC, would seem to be an alternative
199 mechanism of increasing O_2-carrying capacity, MCHC was less variable than other blood

200 parameters (Table S2), likely due to physical constraints[55,56]; accordingly, MCH and MCV were
201 strongly correlated (Table S3; Figure 3C). Contrary to recent empirical findings[4,25], adjustments
202 to MCHC appeared to be neither substantial nor predictable with respect to elevation within or
203 among species (Figure 2F).

204 We found that MCV and MCH decreased in individuals occurring in warmer
205 environments (Figures 4D, E), and that MCHC decreased in individuals occurring at warmer
206 localities within a species' range (Figure 4F). The directions of these relationships were
207 consistent with previous experimental work: Comparisons of juvenile broiler chickens reared
208 from ~1-7 weeks of age in cool, ambient, and hot environments showed decreases in [Hb], Hct,
209 MCV, and TRBC as temperature increased[57,58]. Our findings add to a limited but intriguing pool
210 of evidence that warmer ambient temperatures may lead to developmental reductions in O_2-
211 carrying capacity of blood.

212 Models that included the species grouping variable fit substantially better than those
213 without; this effect explained 13–21% of the variance in the top model, depending on the model
214 set (Tables 1 and S5; Methods). Moderate effects of the species grouping variable suggest that
215 variation in blood traits is partly explained by aspects of species biology that evolve too fast to
216 have resulted in phylogenetic autocorrelation. Species-typical Hb β^{A}_{13}–β^{A}_{83} genotype did not
217 predict [Hb] or any other blood characteristics (Figure 4A–F), providing no evidence that
218 evolutionary adjustments to O_2-binding affinity affect the physical composition of blood or its
219 variation with elevation.

220

221 *Among-species blood comparison*

222 Using the species-level dataset, we evaluated the effects of elevation, body mass, species-typical
223 Hb β^{A}_{13}–β^{A}_{83} genotype, and ambient temperature and precipitation on each of our 6 blood
224 response variables. We compared sets of models with LOOIC and WAIC (Table 1),
225 incorporating a quadratic elevation effect for TRBC, MCV, MCH, and MCHC models based on
226 curvature evident in the data (Figure 2G–L; Methods).

227 Within- and among-species findings for elevational effects were similar (Figure 4). Based
228 on the top model in each set, for every 1,000-m increase in elevation, [Hb] is expected to
229 increase by 0.29 g/dl, and Hct is expected to increase by 1.55%. Elevation was a consistently
230 strong predictor of [Hb], Hct, and TRBC, all of which increased with increasing elevation
231 (Figures 2G–I and 4A–C). The importance of the quadratic effect of elevation for MCV and
232 MCH, and overall shape of the species-level data, suggest that O_2 carrying capacity adjustment
233 differs for middle-elevation species versus those found at low and high elevations (Figure 2J–K;
234 Figure 4D–E). Neither species-typical Hb $\beta^{A}13$–$\beta^{A}83$ genotype, body mass, environmental
235 temperature, nor precipitation explained variation in any species-mean blood characteristic
236 (Figure 4).

237

238 *Influence of cell number versus size on blood-O_2 carrying capacity*

239 The axis of variation in cell number versus size space was tightly constrained (Figure 3A–B); the
240 'banana' shape of Figure 3 mirrors patterns from Wintrobe[44] and Hawkey et al.[45], which present
241 similar plots based on species representing multiple vertebrate classes. The shape of this
242 relationship indicates that the highest levels of blood-O_2 carrying capacity per unit blood are
243 achieved at high values of TRBC or MCV, respectively. Individual hummingbirds can possess
244 few large erythrocytes or many small erythrocytes, but cell number and size must be balanced[45].

245 Because of the proportionately greater surface area for gas exchange, having many small cells is
246 predicted to be advantageous for fast metabolism or when the partial pressure of O_2 in inspired
247 air is low[45]. Similarly, having large cells may be advantageous for modulating the time-course of
248 gas exchange[59]. One way to shed light on this tradeoff is to test whether individuals and species,
249 respectively, adjust O_2 carrying capacity per unit blood primarily through increases in cell
250 number or size.

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

257 Using data for 32 well-sampled species, we estimated species-specific standardized
258 TRBC and MCV coefficients (β) in models with [Hb] as the response variable (Figure S2), and
259 we used these to calculate a 0–1 index that we refer to as the Cell Number-Size Index (hereafter
260 *CeNS*). *CeNS* represents the proportional contribution of TRBC versus MCV to increasing [Hb],
261 calculated as:

262 $$\frac{(\beta_{TRBC})}{(\beta_{TRBC} + \beta_{MCV})}$$

263

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

267 Extending our analysis, we designed a set of models to test potential predictors of *CeNS*:
268 elevation, a quadratic elevation effect (elevation^2; to account for notable curvature in the data),
269 body mass, wing loading, species-typical Hb β^{A}_{13}–β^{A}_{83} genotype, and phylogeny (see Methods).
270 Elevation, elevation^2, and body mass strongly predicted *CeNS* (Figure 5C-D; Tables S9–S10).
271 We found a trend of decreasing *CeNS* with higher wing loading (Figure 5F), but the 95%
272 credible intervals overlapped zero (Figure 5C). Species-typical Hb β^{A}_{13}–β^{A}_{83} genotype did not
273 predict *CeNS* (Figure S5), nor did we find phylogenetic signal in *CeNS* (Figure S4).
274 Examining the tradeoff between cell number and volume provides a potential explanation
275 for the striking pattern that middle elevation species tend to adjust [Hb] by different mechanisms
276 than low and high elevation species (Figure 5A–B). *CeNS* was uncorrelated with TRBC
277 coefficients ($R^2 = 0.07$, $r = -0.27$) but strongly correlated with MCV coefficients ($R^2 = 0.51$, $r = -$
278 0.71), indicating that variation in *CeNS* was primarily driven by the degree to which species
279 modulate cell size (Figure 5, Figure S3). We suggest that because low and high elevation species
280 tend to possess relatively small cells, they may be more able to rely on shifts in cell size, rather
281 than cell number, to increase [Hb] (Figure 2J; Figure 5). However, as cell size increased, species
282 were more likely to approach maximum size for red blood cells, beyond which they could incur a
283 functional detriment. As a result, species with larger red blood cells were more reliant on cell
284 number to adjust [Hb]. For middle elevation hummingbird species that have evolved under
285 moderate (~15–30%) reductions in O_2 availability, TRBC appears to be the primary axis for
286 adjustment of blood-O_2 carrying capacity per unit volume of blood. Species that have evolved at
287 the highest elevations are expected to be the best adapted to hypobaric hypoxia, and it is
288 therefore remarkable that their mode of [Hb] adjustment (primarily by MCV) resembles that of
289 hummingbird species in the lowlands, the putative ancestral elevation for hummingbirds[46].

290

291 *Hummingbirds at extreme elevations*

292 Do hummingbirds at extreme high elevations follow the same rules as hummingbirds
293 distributed at low and moderate elevations with respect to elevational blood variation? Our
294 models encompassed the entire elevational gradient and therefore could have missed important
295 phenomena such as threshold effects. To address this, we compared individual hummingbirds
296 sampled above and below 4,200 m elevation, a threshold that marks the upper ~10% of the
297 gradient. Hummingbirds sampled from these extreme high elevations had significantly smaller
298 cells and higher erythrocyte counts than others (Figure 6). This finding suggests that high-
299 altitude specialist hummingbirds tend to have smaller cells, consistent with findings in other high
300 altitude vertebrates[23,29,30,53,60]; however, this pattern was only detectable under the extreme
301 conditions at the uppermost limits of the Andean elevational gradient. We proceeded to formally
302 test for elevational breakpoints using segmented regression (see Methods). Evidence for
303 objective blood trait thresholds along the elevational gradient was mixed, with breakpoint
304 estimates for individual traits ranging from ~1,400 to ~4,000 m (Figure S6). Lack of consilience
305 in these estimates suggests that trait-specific thresholds are challenging to identify and may not
306 be generalizable across species and clades.

307

308 *Phylogenetic signal in blood characteristics*

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

312 sampled (Table S4; Figure S1). We additionally found no phylogenetic signal in *CeNS* (Figure
313 S4).

314 Non-existent to low phylogenetic signal in blood traits within and among hummingbird
315 species was surprising given demonstrated, phylogenetically conserved Hb genetic adaptations to
316 elevation[17,19] and in light of strong phylogenetic signal reported from recent comparative
317 analyses of blood traits that had broader taxonomic sampling[33,37]. However, it is likely that
318 diverse avian clades possess different blood trait optima; in previous comparative analyses that
319 included multiple families or orders, phylogenetic signal may have been driven primarily by
320 differences among clades. For example, Minias et al.[33] reported that small passerines had
321 consistently high [Hb] and Hct estimates, and that including the shorebird order,
322 Charadriiformes, which was characterized by high [Hb] but medium Hct values, strongly
323 influenced patterns of phylogenetic conservatism[33]. We tested for and found no interaction of
324 clade and elevation on the six studied blood traits, with the exception of the interaction of
325 elevation and the Hermit clade in the [Hb] model set (Figure S7). Interestingly, hermits are a
326 large, early diverging hummingbird clade that contains diminishing species diversity with
327 altitude (and no species that exceeds 3,000 m), and its apparent increased sensitivity of [Hb] to
328 elevation may hint at a functional explanation for this biogeographic tendency. The existence of
329 clade-specific trait optima[33] and strong species effects, demonstrated in this study, may at first
330 seem difficult to reconcile with our findings that there was no within-family phylogenetic signal
331 in hummingbirds; however, we view these findings as being consistent with steady evolution of
332 trait optima by a mean-reverting, Ornstein-Uhlenbeck process, as described by Minias et al.[33],
333 and marked by occasional major jumps that coincide with changes in body plan or respiratory
334 demands.

335 A strength of our study was that hummingbirds vary only subtly in aspects of their
336 biology that could plausibly confound our analyses, such as metabolic rate, energy budget, torpor
337 use, mode of locomotion, foraging strategy, or clutch size. Phylogenetic conservatism in these
338 traits facilitates comparisons of blood characteristics across the entire clade to an extent that
339 would be difficult in other taxa. A strong species effect in spite of these intrinsic controls hints
340 that species optimize O_2-carrying functions of blood for subtle changes in lifestyle and
341 environment, on rapid timescales. This finding is consistent with the rapid diversification of
342 human hematological responses to elevation, as illustrated by the copious variation among
343 populations in patterns of elevational variation[27,50,51,61–63].
344
345 *Conclusions*
346 Across ~22 Mya of the hummingbird evolution, ambient O_2-availability and species
347 idiosyncrasies—not phylogenetic affinities—were the primary drivers of variation in blood traits
348 affecting O_2-carrying functions. In response to elevational changes in O_2 partial pressure,
349 hummingbirds make predictable adjustments to the physical composition of blood. Remarkably,
350 after controlling for many potentially confounding variables, decreasing PO_2 drove increases in
351 [Hb] and Hct that were nearly identical in magnitude within and among hummingbird species,
352 respectively. This implies that developmental and evolutionary optimization of blood traits to
353 ambient PO_2 occurs in fundamentally similar ways, responding to the same set of physical rules
354 that govern gas exchange at the blood-gas barrier. In addition to O_2 availability, species identity
355 itself explained 13–21% of blood trait variation, suggesting that fast-evolving, unmodeled
356 aspects of species biology underpin substantial hematological variation. Our data revealed a
357 striking constraint space that defines the cell number-size tradeoff for hummingbird erythrocytes;

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

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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Table 1. Comparison of Bayesian models to evaluate within- and among-species variation in the 6 studied blood traits: hemoglobin concentration ([Hb]), hematocrit (Hct), total red blood cell count (TRBC), mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC). Only within species models included the species grouping variable and only among species TRBC, MCV, MCH, and MCHC models included a quadratic component ($elev^2$). Reduced models included the set of predictors from full models whose 95% credible intervals did not overlap zero. Dashes indicate that a reduced model was not present (i.e., all credible intervals from full models overlapped zero). Model fit was assessed using the widely applicable information criterion (WAIC) and leave-one-out cross-validation (LOO), which were consistent. The difference between the best fit model and each other model in the set is shown as ΔELPD with difference in standard error (ΔSE) in parentheses (LOOIC values are calculated as –2*ELPD, which converts ELPD to a deviance scale). ΔELPD < 4 indicates that differences between two models are small.

	Model description						**Response**					
	Predictors					**Grouping Variable**	**[Hb] ΔELPD (ΔSE)**	**Hct ΔELPD (ΔSE)**	**TRBC ΔELPD (ΔSE)**	**MCV ΔELPD (ΔSE)**	**MCH ΔELPD (ΔSE)**	**MCHC ΔELPD (ΔSE)**
	None (null)	**All (full)**	**Reduced**	**All + $elev^2$**	**Reduced + $elev^2$**	**Species**						
Within species	X			NA	NA	X	-29 (8.4)	-22.6 (8.2)	-3.9 (4.7)	-0.5 (1.0)	**0.0 (1.5)**	-11.5 (5.0)
		X		NA	NA	X	**0.0**	**0.0**	**0.0**	-0.9 (3.6)	-1.2 (3.1)	-0.1 (2.6)
			X	NA	NA	X	-3.3 (4.2)	-0.3 (2.8)	-0.1 (3.8)	**0.0**	**0.0**	**0.0**
	X			NA	NA		-85.5 (13.1)	-70.5 (13.6)	-34.6 (9.3)	-24.9 (6.4)	-19.6 (6.5)	-39.4 (8.7)
		X		NA	NA		-29.9 (7.6)	-29.9 (7.7)	-29.8 (7.7)	-26.5 (7.3)	-22.8 (6.7)	-29.3 (7.1)
			X	NA	NA		-29.7 (8.8)	-26.9 (7.9)	-25.0 (8.2)	-23.3 (7.1)	-20.1 (6.5)	-29.9 7.1
Among species	X					NA	-4.5 (3.9)	-2.3 (4.1)	**0.0**	**0.0**	-1.3 (2.2)	**0.0**
		X				NA	-0.1 (3.9)	**0.0**	-2.7 (2.6)	-3.6 (2.5)	-4.5 (3.5)	-5.4 (1.8)
			X			NA	**0.0**	0.1 (2.0)	-0.5 (0.8)	–	–	–
				X		NA	NA	NA	-2.0 (2.5)	-2.4 (2.7)	-2.8 (2.1)	-4.5 (2.1)
					X	NA	NA	NA	–	–	**0.0**	–

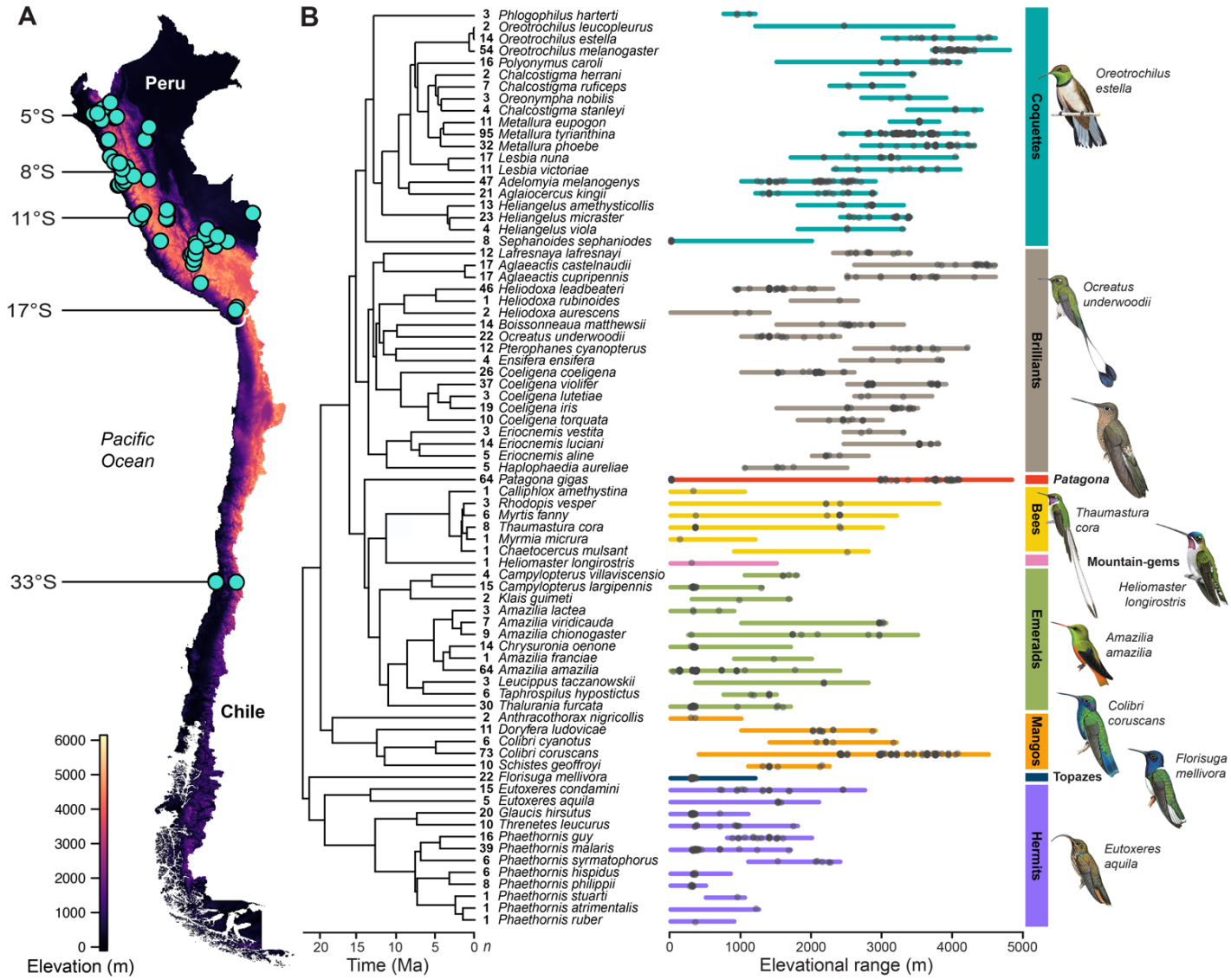
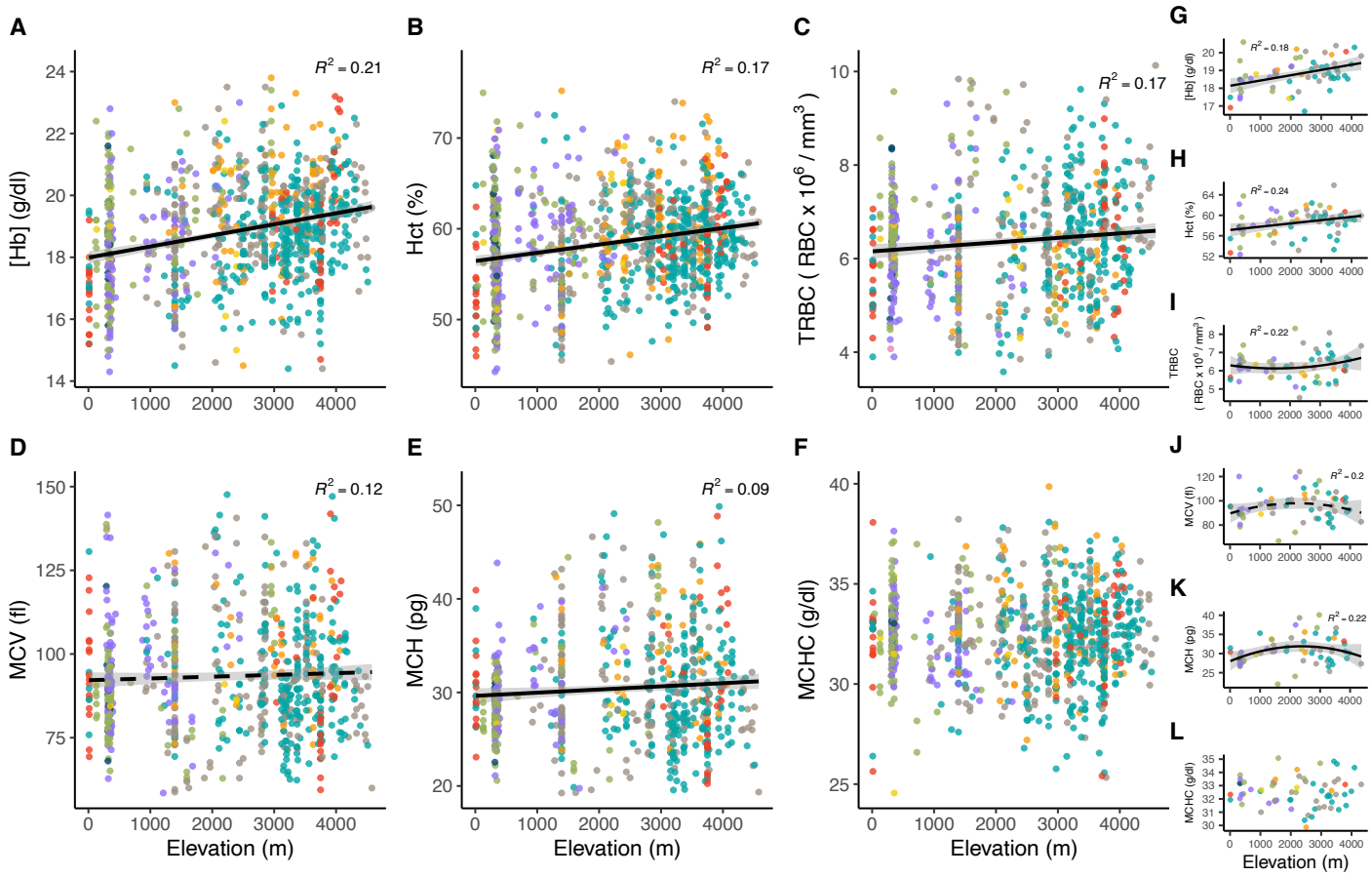
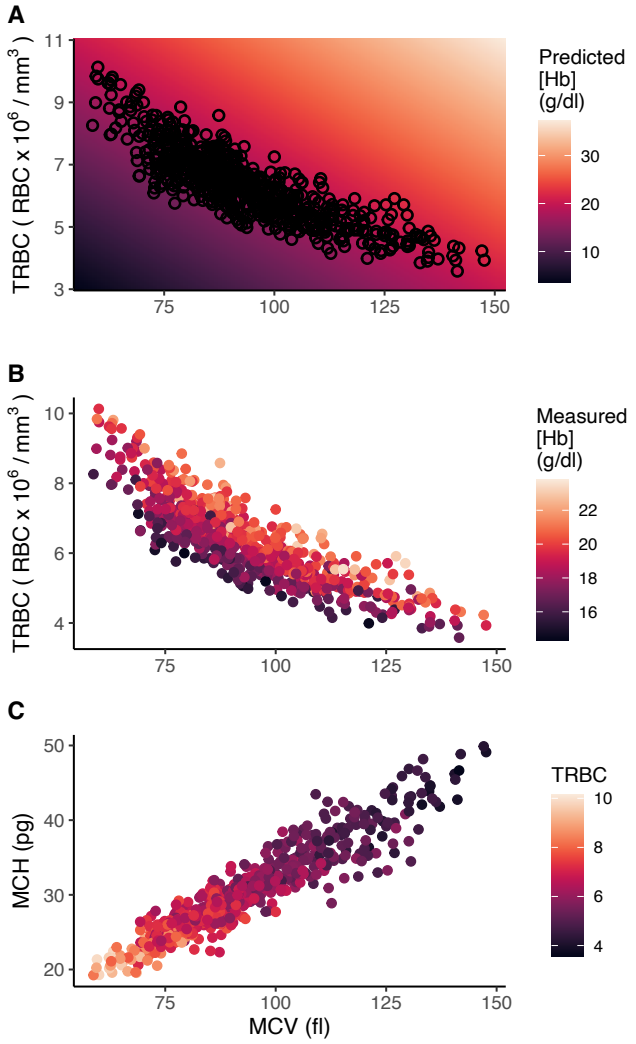


Figure 1. Elevational, latitudinal, and phylogenetic sampling from 2006–2020. **(A)** Sampling localities spanned 28° latitude and 4,578 meters in elevation in Peru and Chile. **(B)** We studied 77 species ('n' column indicates sampling depth) from all nine major hummingbird clades. Sampling (gray opaque points) is shown across each species' elevational range (colored horizontal bars). Time-calibrated phylogeny is modified from McGuire et al.[46]. Illustrations depicting a representative from each clade are from *Birds of the World* and are reproduced courtesy of the Cornell Lab of Ornithology (©).



650 **Figure 2.** Relationship between elevation and the six studied blood parameters (hemoglobin
651 concentration ([Hb]), hematocrit (Hct), total red blood cell count (TRBC), mean cell volume
652 (MCV), mean cell hemoglobin content (MCH), and mean cell hemoglobin concentration
653 (MCHC). **(A–F)** Within species comparisons, wherein each point represents a value from a
654 single individual. **(G–L)** Among species comparisons, wherein each point represents a mean
655 value from a single species. In all panels, solid trend lines correspond to relationships where 95%
656 posterior credible intervals in models did not overlap zero, while dashed trend lines correspond
657 to relationships where 89% credible intervals in models did not overlap zero (see Figure 4).
658 Credible intervals for both elevation (at the 95% level) and elevation^2 (at the 89% level) did not
659 overlap zero in the TRBC model (Panel I). Colors correspond to clades in Figure 1. R^2 values are
660 reported from Bayesian models reported in Figure 4.



676 **Figure 3.** Theoretical and empirical relationships among hummingbird blood parameters
677 underlying hemoglobin concentration ([Hb]) adjustment. **(A–B)** Hummingbirds have evolved
678 optimized blood-O_2 carrying capacity, achieved by balancing total red blood cell count (TRBC)
679 with mean cell volume (MCV). **(A)** Predictions for O_2 carrying-capacity. Background is shaded
680 by O_2 carrying-capacity, calculated as *MCV x TRBC x 1/3 scaling factor* to represent constant
681 MCHC. **(B)** Empirical findings. Points are colored by [Hb], with lighter colors representing
682 higher concentrations. **(C)** Mean cell hemoglobin (MCH) and MCV are tightly linked,
683 suggesting little variance in mean cell hemoglobin concentration (MCHC). Points are colored by
684 TRBC (units presented on the y-axis), with lighter colors representing higher erythrocyte counts.
685 In all panels, each point represents a value from a single individual.

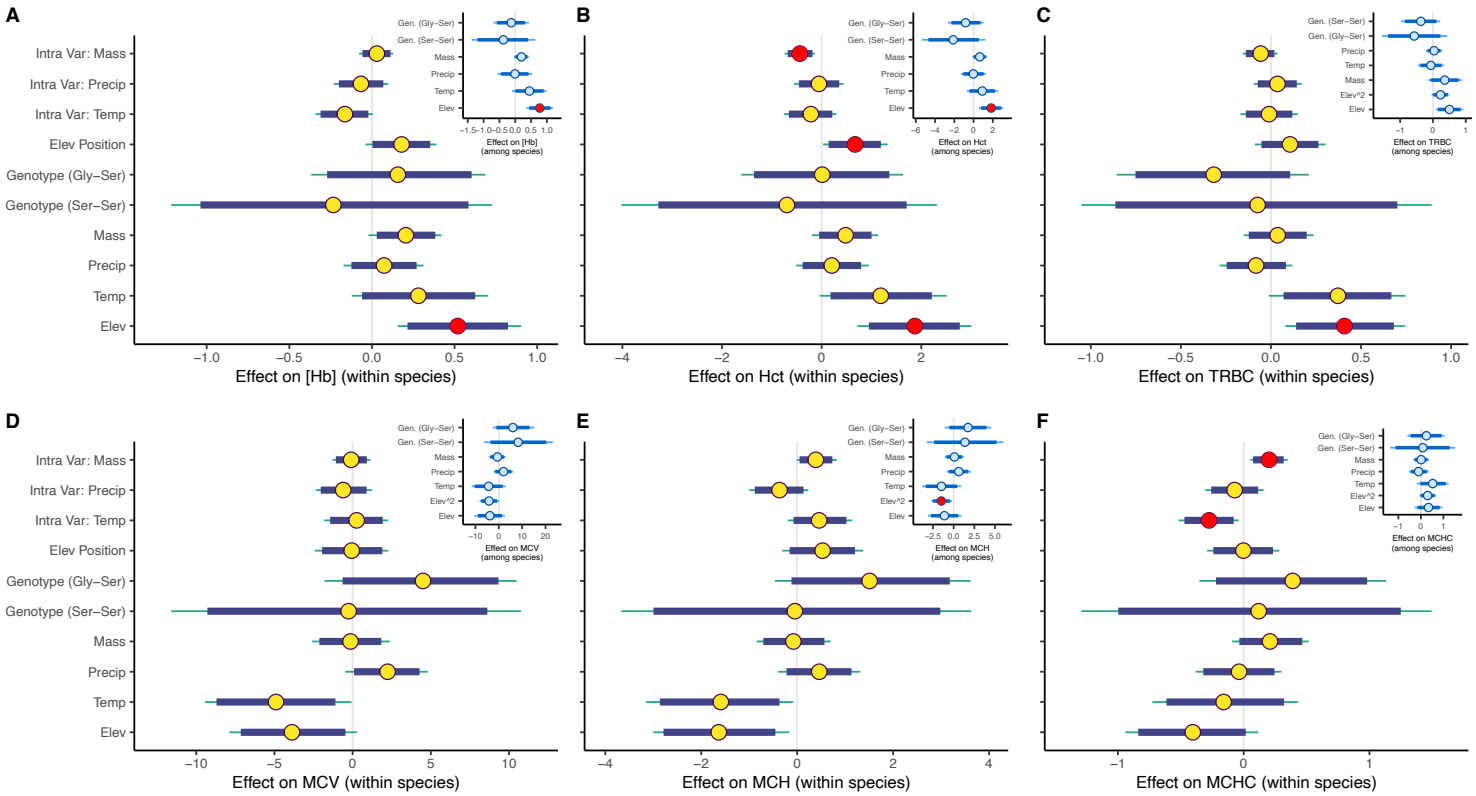


Figure 4. Posterior probabilities and credible intervals from full within-species (main plots) and among-species (insets) models for each of six blood traits: **(A)** hemoglobin concentration ([Hb]), **(B)** hematocrit (Hct), **(C)** total red blood cell (TRBC), **(D)** mean cell volume (MCV), **(E)** 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 1 and S5 for model comparison and parameter estimates from top models in other model sets.

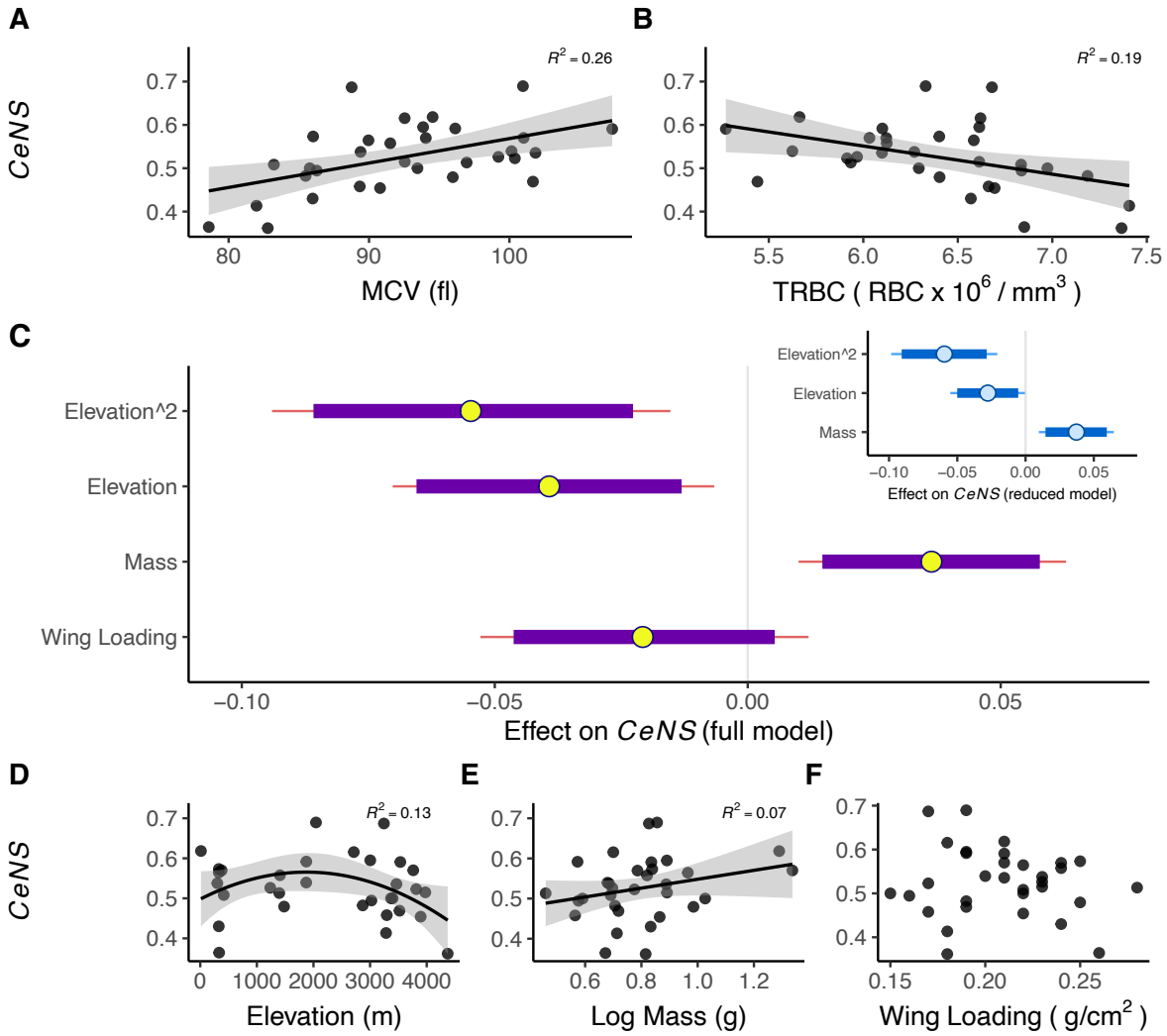


Figure 5. (A–B) Effects of mean cell volume (MCV) and total red blood cell count (TRBC) on *CeNS*, elucidating how, once cells reach a certain size, TRBC becomes the primary mechanism through which birds adjust [Hb]. **(C–F)** Results from *CeNS* models. **(C)** Posterior probabilities and credible intervals from the best fitting full model (main plot) and reduced model (inset) for *CeNS*. **(D)** *CeNS* shows a strong quadratic relationship with elevation, revealing that middle elevation species adjust blood-O_2 carrying capacity differently than low- and high-elevation species. **(E)** *CeNS* increases with increasing body mass. **(F)** *CeNS* shows a marginal decrease with increased wing loading. R^2 values are reported from generalized linear models of each *x* regressed on *y*, and thus do not account for other modeled variables.

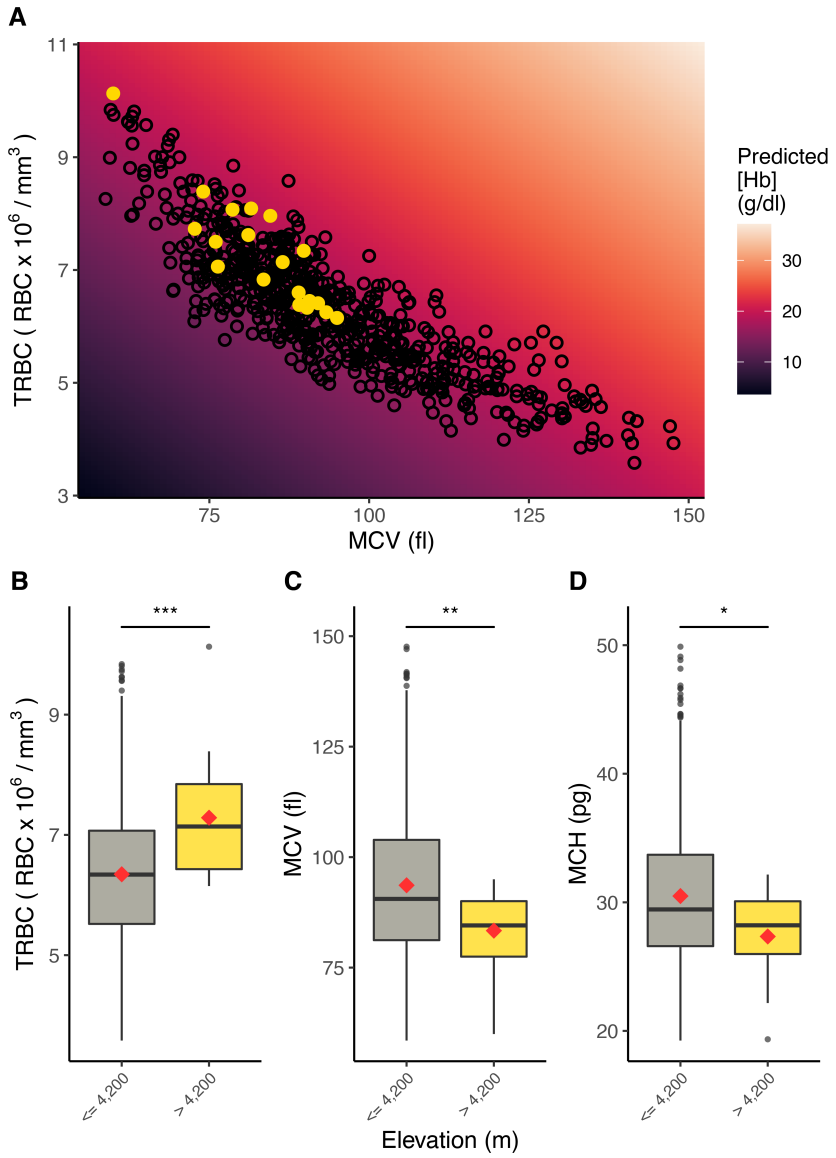


Figure 6. Blood values differ significantly at 'extreme' high elevations. **(A)** Total red blood cell count (TRBC) is significantly higher ≥4,200 m ($p < 0.001$). TRBC increases by 530,000 cells per µl with each 100-meter increase in elevation ≥4,200 m. **(B)** Mean cell volume (MCV) is significantly lower ≥4,200 m (p=0.010). MCV decreases by 5.52 fl with each 100-meter increase in elevation ≥4,200 m. **(C)** Mean cell hemoglobin content (MCH) is significantly lower ≥4,200 m (p=0.020). MCH decreases by 2.37 pg with each 100-meter increase in elevation ≥4,200 m. Data were analysed by subsetting the individual-level dataset by sampling elevation ("<4,200 m" or "≥4,200 m"), consistent with notable trends in the data (Figure 2). Significance and direction of relationships were assessed with one-way ANOVAs. Rates of increase and decrease were calculated using regression coefficients generalized linear models.

METHODS

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and code should be directed to and will be fulfilled by the Lead Contacts, Jessie Williamson (williamson@unm.edu) and Christopher Witt (cwitt@unm.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This paper analyzes original data collected from hummingbirds in Peru and Chile. All data are linked to vouchered specimens at the Museum of Southwestern Biology (MSB), Centro de Ornitología y Biodiversidad (CORBIDI), and the Universidad Pontificia Católica de Chile, accessible in the Arctos database (www.arctosdb.org). Detailed data on specimens, collection localities, and blood values are reported in a forthcoming data paper, Witt et al. (*In Prep*). Additional information about key resources is provided in the Key Resources Table. All code and extensive annotations for data processing, analyses, and figures are available on GitHub: https://github.com/jlwilliamson/ComparativeHummingbirdBlood. Any additional information required to reanalyze data reported in this paper is available from the lead contacts upon request.

METHOD DETAILS

Field Sampling

We collected data from 1,217 individuals of 77 hummingbird species from Peru and Chile during 2006–2020 (Figure 1). After filtering (see below), our final within-species dataset included 1,151 individuals of 77 species collected from 75 localities (Figure 1). 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). The majority of birds sampled were adults (936 adults, 179 juveniles, and 36 unknown). Detailed data on specimens, collection localities, and blood values are described in Witt et al. (*In prep*) and are available 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 (Hct) and Hemocue HB201+ cuvettes ([Hb]). Hct (%) was measured with digital calipers after centrifuging the sealed microcapillary tube for 5 min at 13,000 r.p.m. to separate the red blood cells and plasma. When >1 Hct sample was taken, values were averaged,

776 and weighted Hct values were used in analyses. [Hb] (g/dL blood) was measured on ~5 µL of
777 blood using a HemoCue HB201+ hemoglobin photometer. The HemoCue proprietary
778 photometric method produces values for avian blood that are ~1.0 g/dL greater than those
779 generated using cyanomethemoglobin spectrophotometry[64]. To account for this, we corrected
780 [Hb] values by subtracting 1.0 g/dL. When possible, at time of blood collection, 10 µl whole
781 blood was preserved in a 1:200 NaCl dilution to obtain TRBC estimates. Within 12-hours of
782 blood collection, we pipetted a 10 µl sub-sample of blood-NaCl dilution into a hemocytometer
783 and allowed cells to settle for 1 min. After focusing the microscope, one or more photos were
784 obtained of cells in the hemocytometer grid. Later, cells were manually counted in the ImageJ
785 software[65] and TRBC was estimated following standard protocols[20,21].
786 After drawing blood, birds were humanely killed by rapid cardiac compression. We
787 recorded collection elevation, latitude, longitude, sex, body mass, and date. Body mass was
788 typically taken prior to blood sampling; however, when individuals were massed after blood
789 sampling, body mass was corrected for estimated volume of blood drawn. Each collected bird
790 was prepared as a study skin. Specimens and tissues are housed at the Museum of Southwestern
791 Biology (MSB) at the University of New Mexico, USA, the Centro de Ornitología y
792 Biodiversidad (CORBIDI) in Peru, and the Universidad Pontificia Católica de Chile in Chile.
793 Secondary blood indices (mean cell volume (MCV), mean cell hemoglobin (MCH), and
794 mean cell hemoglobin concentration (MCHC)) were calculated from primary indices (Table S1)
795 following standard protocols[20]. We examined blood data distributions using histograms and
796 qqPlots and eliminated outliers based on distribution, in combination with known 'typical'
797 values[20,25,66]. To reduce the possibility of incorporating anomalous blood values resulting from
798 juvenile birds (i.e., age-related outliers), we examined values on the tail ends of distributions
799 associated with presence of a bursa measuring ~2 x 2 mm. All values fell within the expected
800 distribution of blood values. We did not age individuals by plumage, as juvenile plumage lasts
801 much longer than juvenile blood values, which stabilize one to several months after fledging.
802
803 **Environmental and life history variables**
804 We compiled elevational range limits for each species in our dataset using a combination of
805 published data, field guides, and specimen records from the Museum of Southwestern Biology
806 (MSB)[3,67–69]. We calculated each individual's elevational range position, hereafter 'elevational
807 position', as *1 – ((maximum species elevation – individual sampling elevation) / species*
808 *elevational range).* This 0.0–1.0 index allowed us to quantify each individual's position relative
809 to its species elevational range breadth. Generally, we expect that individuals sampled near the
810 higher end of their species' elevational range (i.e., that have higher elevational position values)
811 will have higher blood trait values for most parameters.
812 We incorporated species-typical Hb β^{A}_{13}–β^{A}_{83} genotypes using published sequence
813 values[42,46]. For *n*=7 species, we identified species-typical Hb β^{A}_{13}–β^{A}_{83} genotype from Hb β^{A}
814 sequences aligned to a GenBank reference from Projecto-Garcia et al.[42]. For *n*=12 species
815 (*Amazilia franciae, Campylopterus villaviscensio, Eutoxeres aquila, Klais guimeti, Leucippus*
816 *taczanowskii, Myrmia micrura, Phaethornis atrimentalis, Sephanoides sephaniodes,*
817 *Chaetocercus mulsant, Eriocnemis vestita, Myrtis fanny,* and *Oreotrochilus leucopleurus*)
818 species-typical Hb β^{A}_{13}–β^{A}_{83} genotypes were inferred from sister taxa or nearest relatives.
819 We characterized climatic variation among latitudinal and elevational gradients using 19
820 bioclimatic variables from the WorldClim database[70]. We downloaded rasters of each BioClim
821 variable using the 'raster' package in R (version 4.0.0), cropped them to our study areas of Peru

822 and Chile, and extracted temperature and precipitation values for each sampling locality. We
823 then used principal components analysis (PCA) for temperature (Bio1–11) and precipitation
824 variables (Bio12–19) to create a composite measure of temperature and precipitation across the
825 gradient. PC1 of the temperature variables (hereafter "temperature") explained 73.4% of the
826 variation in composite temperature, and loadings suggested that this axis corresponded to
827 increasing temperature across sites (see GitHub). PC1 of the precipitation variables (hereafter
828 "precipitation") explained 79.8% of the variation in composite precipitation, and loadings
829 suggested that this axis corresponded to increasing precipitation across sites (see GitHub).
830 To account for multiple measurement effects (i.e., traits measured from multiple
831 individuals within species), we calculated between- and within-species differences following
832 Villemereuil et al.[71]. This approach uses within-group centering to separate each predictor into
833 two components: We first calculated species mean values (between-species variability) and then
834 subtracted the mean value of individual observations (within-species differences). The latter
835 measures of within-species variation for temperature, precipitation, and body mass were included
836 in models. Because hummingbird body masses may differ by sex, within-species differences
837 were calculated using mean body mass of males for males, mean body mass of females for
838 females, and species-level means for birds of unknown sex (n=6).
839 We used wing area data from the 59 species from the Museum of Southwestern Biology
840 studied in Skandalis et al.[72] to calculate wing loading in g/cm^2, calculated as species mean body
841 mass (g) divided by total wing area (cm^2). We analyzed wing loading due its relevance to
842 aerodynamic theory and hummingbird flight at high altitudes[73–76].
843
844 **Hummingbird phylogeny**
845 We used the substitution rate-calibrated molecular phylogeny of hummingbirds from McGuire et
846 al.[46] as a backbone to generate a phylogeny pruned to the 77 species in our dataset using the R
847 package 'phytools'[77]. Two species, *Oreotrochilus leucopleurus* and *Phaethornis stuarti*, are
848 missing from the McGuire et al.[46] tree and were added to our phylogeny using the bind.tip()
849 function. Branch lengths and position of *O. leucopleurus* were calculated from genetic distances
850 and recent described sister relationships[78]. Approximate branch length and position of *P. stuarti*
851 was inferred from BirdTree.org[79,80] with divergence rate estimates from a multi-locus, time-
852 calibrated phylogeny of Hawaiian honeyceepers[81]. We generated other model-specific tree
853 subsets by including only those tips that corresponded to the species in each modeling subset
854 (see annotated GitHub analysis scripts).
855
856 **Phylogenetic signal**
857 Phylogenetic signal indicates the proportion of the total variance attributed to phylogenetic
858 variance. We originally ran sets of phylogenetic models (results not shown) and calculated
859 phylogenetic signal, or lambda (λ) from the models as the phylogenetic heritability described by
860 Lynch (1991). We estimated phylogenetic signal using the full and reduced models for each
861 blood parameter following the vignette and recommendations of P. Bürkner (https://cran.r-
862 project.org/web/packages/brms/ vignettes/brms_phylogenetics.html), using the 'hypothesis'
863 method. However, all estimates of phylogenetic signal for all model sets (full and reduced for
864 within- and among species analyses) were low ($\lambda < 0.004$); these estimates are supported by
865 visualizations of continuous trait maps of mean blood parameters (Figure S1). Because estimates
866 from preliminary models and continuous trait maps of mean blood values—across all datasets—
867 indicated low phylogenetic signal (Supplement; Figure S1), we excluded phylogeny as an effect

in models and evaluated phylogenetic signal prior to modeling using Blomberg's K and Pagel's λ. Results among the two tests were similar and consistent across model subsets (e.g., Table S4).

Within species analysis

We built generalized linear multilevel models using the R package brms[82] to analyze within species variation in blood characteristics. Brms uses Markov chain Monte Carlo (MCMC) sampling to obtain draws from posterior distributions and it easily interfaces with the R package loo[83] to compute different information criteria. 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 β^{A}_{13}–β^{A}_{83} 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.

For each response, we compared 6 models: (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, suggesting high importance. 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.

The full predictor models with species grouping variable had the lowest (best) WAIC and ELPD values for [Hb] (R^2 = 0.21), Hct (R^2 = 0.17), and TRBC (R^2 = 0.17) models. For MCV, the reduced model with temperature and the species grouping variable had the lowest scores (R^2 = 0.094) and for MCHC, the reduced model that included within species variation in both body mass and temperature plus the species grouping variable ranked the highest (R^2 = 0.12). Both top MCV and MCHC models ranked similarly to the full model with species grouping variable, as well as the null model. For MCH, the reduced model with elevational position, elevation, and the species grouping variable ranked the highest and was comparable to the null model (R^2 = 0.092; Table 1).

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 evaluated models and conducted model comparison as described for within and among species comparisons. 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 S7). 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 analysis

Our final among-species dataset included 58–65 species, depending on the subset for each blood characteristic ([Hb] = 60 species; Hct = 65 species; TRBC = 58 species, MCV = 60 species;

914 MCH = 59 species; MCHC = 64 species). Species mean values were calculated for all species
915 with *n*=2 or more individuals sampled. We split *Patagona gigas* into "north" and "south"
916 haplotypes in light of evidence that the complex may be comprised of two functionally and
917 genetically distinct populations separated by strong migratory divides[84], and we analyzed data
918 only from individuals sampled during the breeding months to ensure correct population
919 assignment. We designed model sets for each of our six continuous blood response variables. For
920 each response we analyzed distributions (qqPlots, Cook's Distance, leverage) and removed
921 outliers that strongly influenced fit. For each model, predictor variables included species-typical
922 Hb β^{A}_{13}–β^{A}_{83} genotype, mean elevation, mean body mass (log-transformed; one mean for all
923 individuals due to balanced sampling between sexes), mean air temperature, and mean
924 precipitation. All variables were standardized to a mean of zero and standard deviation of one.
925 For each response, we compared: (1) an intercept-only null model, (2) a model with all
926 predictors, and (3) a reduced-predictor model with only those predictors whose 95% CIs did not
927 overlap zero in full models (reduced model 2). Given the notable quadratic shape of species
928 mean data for TRBC, MCV, MCH, and MCHC (Figure 2G–L), for those model sets we
929 additionally compared (4) a model with all predictors plus a quadratic component of elevation
930 and (5) a reduced-predictor model with quadratic component including only those predictors
931 whose 95% CIs did not overlap zero in the full quadratic model (reduced model 4). Distribution
932 family differed by model set: Gaussian for [Hb; skew-normal for Hct; and student's *t*-distribution
933 for TRBC, MCV, MCH, and MCHC models. We ran four Markov chains for 20,000 iterations
934 with a burn-in period of 10,000, thinned every 10 steps.
935 We compared sets of models with LOOIC and WAIC (Table 1). The [Hb] model with the
936 best (lowest) WAIC and ELPD values was a reduced model with only elevation (R^2 = 0.18),
937 though this model was only marginally better than the full model with all predictors (R^2 = 0.33;
938 Table 1). The top Hct model was the full model with all predictors (R^2 = 0.24; Table 1). For
939 MCH, the top model was a reduced model containing elevation and a quadratic parameter for
940 elevation, both of which were important predictors (R^2 = 0.12; Tables 1 and S5; Figures 2K and
941 4E). For TRBC, MCV, and MCHC model sets, the intercept-only model received the strongest
942 support, though other models were closely ranked (Table 1).
943
944 **Cell number versus size analyses**
945 First, to assess within species variation in cell number versus size, we compared a null model
946 (intercept only) to a single full model *[Hb] ~ MCV + TRBC + (1|species)* for all individuals of
947 59 species that had [Hb], MCV, and TRBC measurements, distinguishing between *Patagona*
948 *gigas* "north" and "south". The species grouping variable allowed us to account for repeated
949 sampling of individuals within a species and to hold species identity constant. Second, to assess
950 among species cell number versus size variation, we generated a species mean dataset that
951 included data from all species with *n*=3 or more individuals sampled (48 species total). We again
952 compared a null (intercept only) model to a single full model *[Hb] ~ MCV + TRBC.* Third, to
953 assess how individual species differ in their relative contributions of MCV and TRBC to
954 increasing [Hb], we repeated our approach for individual species: For each species with *n*=8 or
955 more individuals sampled (32 species total), we generated a species-specific data subset,
956 carefully and conservatively evaluated influential outlier points, and ran a single model, *[Hb] ~*
957 *MCV + TRBC* (Figure S2). For all three model sets (within species, among species, and single
958 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 *TRBC coefficient / (MCV coefficient + TRBC coefficient)*; see 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.

For both within- and among-species *CeNS* models, full models fit substantially better than the intercept-only null model (full within species model $R^2 = 0.56$; full among species model $R^2 = 0.62$; Table S6). Based on model estimates, for every increase in TRBC of one million cells per µl, we expect that [Hb] will increase by 1.81 g/dl within species and 2.15 g/dl among species, and for every increase in MCV of one fl, we expect that [Hb] will increase by 0.12 g/dl within species and 0.14 g/dl among species.

We then designed a set of models to analyze what contributes to variation in *CeNS*. We pruned a phylogeny to the subset of 32 species with *CeNS* data for estimation of phylogenetic signal. Our model response variable was *CeNS* (0–1 index). Predictors, all species mean values, included elevation, a quadratic elevation term "elevation^2" to account for notable curvature in the data, body mass (log-transformed), wing loading, and species-typical Hb β^A_{13}–β^A_{83} 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 elevation^2, (4) a model with core predictors plus elevation^2 and species-typical Hb β^A_{13}–β^A_{83} 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 R^2 values from standardized species-specific *CeNS* models, unstandardized TRBC and MCV beta estimates, and raw TRBC and MCV values (Figure S3). 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.

In our *CeNS* analysis, we compared six models using LOOIC and WAIC (Table S7). The model with elevation, elevation^2, body mass, and wing loading received the strongest support ($R^2 = 0.38$) but was closely ranked with a model including only elevation, elevation^2, and body mass ($R^2 = 0.33$; Figure 5C; Table S7). Models with elevation^2 fit substantially better than those without. All but *Coeligena coeligena* showed a positive relationship between *CeNS* and predictors (Figure S2).

Model comparison and diagnostics

All models were run using the brm() function with default priors (weakly informative priors for the intercept and sigma; improper flat priors for predictors). For all models, to assess convergence we visually examined trace plots, checked diagnostics, and confirmed that Rhat values were less than 1.01[82]. 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 using the widely applicable information criterion (WAIC)[85] values as well as approximate leave-one-out cross-validation information criterion based on the posterior likelihoods (LOOIC) and Bayesian expected log pointwise predictive densities (ELPD)[83] to verify full model fits (Table 1). We estimated fixed effects (means and

95% CI) from the posterior distributions for each predictor. For relevant model sets, 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 with the exception of 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. Results are presented in Figure S6.