Hummingbird blood traits track oxygen availability across space and time

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Abstract

Predictable trait variation across environmental gradients suggests that adaptive evolution repeatedly finds the same solution to a challenge. Trait-environment associations can reflect long-term, genetic evolution across phylogenies, or short-term, plastic responses of individuals. When phylogenetic and population-level patterns match, it implies consistency between the long timescale of adaptation and the short timescale of acclimatization. Alternatively, genetic adaptation can find solutions that 'break the rules' of trait-environment covariation. For example, blood-hemoglobin concentration ([Hb]) increases at high elevations in animals, but genetic adaptations in some populations have been shown to augment tissue oxygenation while curtailing hemoglobin production, altering this predictable [Hb]-elevation association. Here, we tested whether species adaptation to elevation generally alters trait-environment relationships for blood. To do this, we measured blood traits of 1,217 individuals representing 77 species of Andean hummingbirds, across a 4,600 m elevational gradient. We used hierarchical Bayesian modeling to estimate blood trait responses to elevation, environmental temperature, precipitation, individual and species characteristics, and phylogeny. Strikingly, the effects of elevation on [Hb] and hematocrit (Hct) were nearly identical for individuals and species, implying that rules of elevational blood variation are set by physics of gas exchange in the hummingbird respiratory system and are unchanged by species adaptation. However, when we looked at mechanisms of [Hb] adjustment—by changes in red blood cell size or number—we did find a signal of species adaptation: To adjust [Hb], species at low and high elevations, respectively, tended to adjust cell size, whereas species at mid-elevations tended to adjust cell number. Despite scale-independent elevational variation in [Hb] and Hct, the species-specific balance of red blood cell size versus

number appears to have been affected by adaptations that distinguish hummingbird species living at moderate versus high elevations.

Keywords: Andes, blood-oxygen, cell size, ecophysiology, elevation, hummingbird

Introduction

Predictable variation of traits across environmental gradients can reveal both the power and limitations of adaptive evolution. Trait-environment relationships among species tend to reflect long-term, genetically fixed trait differences across phylogenies; in contrast, trait-environment relationships within species tend to reflect short-term, plastic responses of individuals with shared genetic backgrounds. When within-species and among-species patterns for a specific trait match, it implies a single, shared solution to an environmental challenge. For example, cooler waters at high latitudes are consistently associated with higher numbers of vertebrae in fish, whether comparing species or conspecific individuals (Jordan's Rule) [1,2]. When within-species and among-species patterns conflict, it implies that genetic adaptation has found a qualitatively different solution to the same environmental challenge, altering the trait-environment relationship. The tendency for larger body sizes at colder ambient temperatures among species (Bergmann's Rule) and within species (James's Rule; [3]) is frequently subject to conflicting patterns at these two scales of comparison; it is detectable in only a subset of animal clades, and is reversed in others [4]. These conflicting trait patterns have been attributed to multifarious selection pressures impacting species differences in body size and thermoregulatory physiology [3], and they reflect the expected erosion of trait-environment (or trait-trait) associations over macroevolutionary time [5]. Trait-trait relationships are similarly scale-dependent [6]; for example, individual common milkweed (*Asclepia syriaca*) plants that produce higher concentrations of cardenolide toxins have reduced growth rates [7], while other species of milkweed that produce up to seven times the concentrations of cardenolide toxins as *A. syriaca* show no reduction in growth rate [8]. In this way, genetic adaptations can 'break the rules' that govern trait relationships. Explaining such 'trait gradients' across biological scales will be

65 essential not only for understanding the functional basis of adaptation, but for predicting the
66 functional consequence of global change for species and communities [9].

67 In animals, blood traits associated with oxygen transport change predictably with
68 declining oxygen (O_2) availability at elevation [10–13]. Most O_2 in the blood is carried by
69 hemoglobin (Hb) [14], the concentration of which ([Hb]) is a key determinant of blood-O_2
70 carrying capacity. When lowland-adapted birds ascend to altitude, they experience reduced
71 arterial O_2 saturation; to compensate, they undergo erythropoiesis, which can increase [Hb] and
72 therefore blood-O_2 carrying capacity, enhancing O_2 delivery to the tissues and compensating for
73 reduced arterial O_2 saturation [13]. In the absence of compensatory increases in plasma volume
74 [15], increased Hb mass leads to elevated hematocrit (Hct) [16]. A suite of interrelated physical
75 characteristics of blood—total red blood cell count (TRBC), mean cell volume (MCV), mean
76 cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC; Table S1)—can be
77 modulated to optimize blood-O_2 transport, or to compensate for other cardiac, vascular, or
78 hematological responses to elevation. For example, MCV typically increases in acclimatized,
79 lowland-adapted animals undergoing accelerated erythropoiesis [17].

80 However, genetic adaptation to high elevation can break the 'rules' of trait-environment
81 covariation in blood traits. For example, evidence from high-altitude-adapted human populations
82 and theoretical work suggests that optimal [Hb] and Hct levels may be similar to those of sea-
83 level populations [13,18–20]. Adaptive genetic changes to hemoglobin proteins have been
84 demonstrated in many high-altitude-native birds, including hummingbirds, House Wrens,
85 Andean waterfowl, Bar-headed Geese, and a few dozen other bird taxa [21–25]. In
86 hummingbirds, there is a highly predictable association between species-typical Hb β^A13–β^A83
87 genotype and elevation, with derived increases in Hb-O_2 affinity in highland lineages and

88 reductions in Hb-O_2 affinity in lowland lineages [22]. Hummingbirds occurring at high altitudes
89 exhibit two amino-acid substitutions on the β^A-globin subunit of Hb (Hb β^A-$13_{GLY \rightarrow SER}$ and Hb
90 β^A-$83_{GLY \rightarrow SER}$) that are known to affect O_2 binding [22]. In particular, β^A-13_{SER} is known from
91 only four hummingbird genera (*Patagona*, *Chalcostigma*, *Pterophanes*, and *Oreotrochilus*), all
92 of which occur at extreme high altitudes [21,22,26]. O_2-binding affinity of hemoglobin has
93 critical effects on tissue oxygenation, and, in birds, binding affinity increases predictably with
94 elevations [21,27]. It is not yet clear whether the genetic adaptations that change Hb-O_2 binding
95 affinity predictably affect other blood traits or their covariation with elevation. It is plausible that
96 species-typical Hb β^A genotypes associated with different O_2-binding affinities induce
97 predictable differences in blood traits and their reaction norms to elevation; such an association
98 could reflect a direct functional tradeoff or an indirect effect of accrued, multi-locus adaptation
99 to elevation at multiple loci.

100 The macro-physiological 'rules' governing blood trait variation over phylogenetic and
101 population timescales have remained elusive because adequate comparative data are scarce.
102 Blood traits are known to vary among avian clades (e.g., [28,29]) and exhibit elevational
103 variation both within species (e.g., [30–32]) and among species (e.g., [30,31]). At least two
104 additional recent studies have conducted broad-scale interspecific comparative analyses of avian
105 [Hb] and Hct data, but both datasets were compiled from heterogeneous published data and
106 lacked elevational sampling sufficient to estimate elevational effects [33,34]. Furthermore, no
107 previous comparative analysis of hematological variation across elevation has included the six
108 key parameters that affect blood-O_2 carrying capacity per unit volume (Table S1). Thus, we
109 continue to lack a general understanding of within- and among-species blood trait-environment
110 relationships.

111 Examining the underlying mechanisms of adjustment for blood-oxygen carrying capacity
112 can offer insight on trait-environment relationships. [Hb] is equivalent to O_2-carrying capacity
113 per unit volume of blood, and it can be adjusted via adjustments to cell number (TRBC), cell size
114 (MCV), within-cell [Hb] (MCHC), or plasma volume. Cell number and cell size are both
115 measurable components that vary among individuals and species, are 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 [35,36]. MCHC tends to be relatively invariant across the vertebrate tree of
120 life [35], with Hb typically comprising ~1/3 of erythrocytes. Plasma volume adjustments can
121 decouple Hb mass from [Hb] and, although plasma volume appears variable among human
122 populations [15], we currently have insufficient data on elevational variation in avian plasma
123 volume to assess its importance. Quantifying the relative contributions of TRBC and MCV in
124 driving [Hb] variation within and among species may show that species use different
125 mechanisms of adjusting blood-O_2 carrying capacity, revealing signatures of adaptation that
126 distinguish hummingbirds living at moderate versus high elevations.
127 In this study, we sought to test whether phylogenetic and population patterns match for
128 blood-trait variation with environment. Specifically, we asked: Does species adaptation change
129 trait-environment relationships? To address this question, we spent 2006–2020 collecting
130 specimen-vouchered hematological data from 1,217 wild hummingbirds of 77 species,
131 representing all 9 clades, and spanning ~4,600 meters in elevation. Using these data, we then
132 constructed hierarchical Bayesian models to estimate the responses of six blood traits to
133 elevation, climate, individual and species characteristics, and phylogeny. The hummingbirds are

134 a potential model clade in which to study rules of hematological variation due to their
135 documented evolutionary genetic responses to shifts in PO_2, high species richness in mountains,
136 phylogenetically conserved elevational ranges, and relative uniformity of body size, clutch size,
137 diet, mating system, and flight mode [21,22,37–39]. Notably, matching O_2 supply and demand is
138 an acute challenge for all hummingbirds because they exhibit the highest mass-specific
139 metabolic rates among endotherms, and because they employ sustained hovering, one of the
140 most energetically demanding forms of animal locomotion [38,40]. This study posits new rules
141 of elevational blood trait variation, at least for hummingbirds, providing new insights into the
142 origins and maintenance of trait-environment relationships.
143

144 Results and Discussion

145 We quantified three primary ([Hb], Hct, and TRBC) and three secondary hematological indices
146 (MCV, MCH, and MCHC; Table S1), and separately analyzed two datasets: an individual-level
147 dataset, wherein each row contained data from one individual hummingbird (*n*=1,217), and a
148 species-level dataset, wherein each row contained mean blood values calculated for each species,
149 with a minimum of 2 individuals sampled (*n*=58–65 species, depending on blood trait modeled).
150 In the individual-level data, mean [Hb] was 18.84 g/dl (±1.65), mean Hct was 58.54% (±5.15),
151 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
152 fl (±17.1), mean MCH was 30.4 pg (±5.64), and mean MCHC was 32.44 g/dl (± 2.12). Complete
153 reference values—the first such values ever published for >90% of our study species—are
154 reported in detail in Table S2.
155

156 **Within-species blood trait-environment variation**

157 We used the individual-level dataset to model within species (i.e., interspecific) variation
158 using multilevel Bayesian models. For each of six blood parameters, we estimated the effects of
159 elevation, body mass, precipitation, air temperature, species-typical Hb β^A13–β^A83 genotype, as
160 well as within-species variation in elevational position (an individual's sampled elevation
161 relative to its species elevational range), body mass, air temperature, and precipitation. We
162 accounted for between-species differences by incorporating a species grouping variable.
163 Separately, we excluded sex and wing-loading as potentially important predictors (see Methods).
164 In total, we compared six models per set using LOOIC and WAIC (Tables S3-S4; Methods). In
165 addition to fixed effects, models included species as a random effect, and a phylogenetically
166 correlated random intercept (i.e., covariance matrix of phylogenetic distances among taxa). Thus,
167 our full model in each set reflects the hypothesis that all predictors, phylogeny, and species
168 identity influence blood characteristics.
169 Based on the top-performing model in each set, we found that for every 1,000-m increase
170 in elevation, [Hb] is expected to increase by 0.39 g/dl, Hct is expected to increase by 1.42
171 percentage points, TRBC is expected to increase by 310,000 cells per µl, and MCH is expected
172 to decrease by 0.48 pg. Elevation strongly predicted [Hb], Hct, TRBC, and MCH (Figs 2 A–F
173 and 3A–F). Elevational position was also a strong predictor of Hct, indicating that individuals
174 located higher in their species' elevational range tended to have higher Hct (Fig 3B). [Hb],
175 TRBC, and MCH also increased with increasing elevational position, although these
176 relationships were subtle (Fig 3A, C, E).
177 The finding that [Hb], Hct, and TRBC increase with elevation was consistent with
178 theoretical expectations for O_2-carrying capacity of blood and with prior empirical findings in
179 birds [26,30,31,33,34], humans [41,42], mammals [11,43], and other taxa [44,45]. In prior work,

180 substantial variation in slopes of these parameters across elevation may have been attributable to
181 variation in temporal, taxonomic, and spatial scales of comparison, as well as constraints on the
182 time-course of evolutionary adaptation [22,42,46]. We expect increases in Hct at higher
183 elevations because an increase in the total mass of Hb enhances oxygenation under lower
184 ambient PO_2 [18]; unless there is a proportional increase in plasma volume or MCHC, an
185 increase in Hb mass is expected to be accompanied by an increase in the proportion of blood
186 comprised of red blood cells. TRBC also increased with elevation and was, along with MCV,
187 one of two key drivers of [Hb] adjustment (Fig 2, Fig 4; [38]. MCV did not vary predictably in
188 our linear models (Fig 2D). Although increasing within-cell [Hb], or MCHC, would seem to be
189 an alternative mechanism of increasing O_2-carrying capacity, MCHC was less variable than other
190 blood parameters (Table S2), likely due to physical constraints [47,48]; accordingly, MCH and
191 MCV were strongly correlated (Table S5; Fig 4C). Contrary to recent empirical findings [30,31],
192 adjustments to MCHC appeared to be neither substantial nor predictable with respect to elevation
193 within or among species (Fig 2F).

194 We found that MCV and MCH decreased in individuals occurring in warmer
195 environments (Figs 3D, E), and that MCHC decreased in individuals occurring at warmer
196 localities within a species' range (Fig 3F). The directions of these relationships were consistent
197 with previous experimental work: Comparisons of juvenile broiler chickens reared from ~1-7
198 weeks of age in cool, ambient, and hot environments showed decreases in [Hb], Hct, MCV, and
199 TRBC as temperature increased [49,50]. These findings add to a limited but intriguing pool of
200 evidence that warmer ambient temperatures may lead to developmental reductions in O_2-carrying
201 capacity of blood.

202 Models that included the species grouping variable fit substantially better than those
203 without; this effect explained 13–21% of the variance in the top model, depending on the model
204 set (Tables S3-S4; Methods). Moderate effects of the species grouping variable suggest that
205 variation in blood traits is partly explained by aspects of species biology that evolve too fast to
206 have resulted in phylogenetic autocorrelation. Species-typical Hb β^A13–β^A83 genotype did not
207 predict [Hb] or any other blood characteristics (Fig 3A–F), providing no evidence that
208 evolutionary adjustments to O_2-binding affinity affect the physical composition of blood or its
209 variation with elevation.
210
211 **Among-species blood trait-environment variation**
212 Using the species-level dataset, we evaluated the effects of elevation, body mass, species-typical
213 Hb β^A13–β^A83 genotype, and ambient temperature and precipitation on each of our 6 blood
214 response variables. We compared sets of models with LOOIC and WAIC (Table S3),
215 incorporating a quadratic elevation effect for TRBC, MCV, MCH, and MCHC models based on
216 curvature evident in the data (Fig 2G–L; Methods).
217 Within- and among-species findings for elevational effects were similar (Fig 3). Based on
218 the top model in each set, for every 1,000-m increase in elevation, [Hb] is expected to increase
219 by 0.29 g/dl, and Hct is expected to increase by 1.55 percentage points. Elevation was a
220 consistently strong predictor of [Hb], Hct, and TRBC, all of which increased with increasing
221 elevation (Figs 2G–I and 3A–C). The importance of the quadratic effect of elevation for MCV
222 and MCH, and overall shape of the species-level data, suggest that O_2 carrying capacity
223 adjustment differs for middle-elevation species versus those found at low and high elevations
224 (Fig 2J–K; Fig 3D–E). Neither species-typical Hb β^A13–β^A83 genotype, body mass,

225 environmental temperature, nor precipitation explained variation in any species-mean blood
226 characteristic (Fig 3).
227
228 **Mechanisms of blood-O_2 carrying capacity adjustment**
229 The 'banana' shape of the relationship between cell number versus size indicates that the highest
230 levels of blood-O_2 carrying capacity per unit blood are achieved at high values of TRBC or
231 MCV, respectively. Our data support that the axis of variation in cell number versus size space is
232 tightly constrained (Fig 4A–B), mirroring patterns evident from comparisons across multiple
233 vertebrate classes [35,36]. Individual hummingbirds can possess few large erythrocytes or many
234 small erythrocytes, but cell number and size must be balanced [36]. Because of the
235 proportionately greater surface area for gas exchange, having many small cells is predicted to be
236 advantageous for fast metabolism or when the partial pressure of O_2 in inspired air is low [36].
237 Similarly, having large cells may be advantageous for modulating the time-course of gas
238 exchange [51]. One way to shed light on this tradeoff is to test whether individuals and species,
239 respectively, adjust O_2 carrying capacity per unit blood primarily through increases in cell
240 number or size.
241 We designed models to understand the relative contributions of cell number versus size to
242 blood O_2-carrying capacity per unit volume ([Hb]), within and among species over ~22 Mya of
243 hummingbird evolution. These models demonstrated that hummingbirds co-equally modulated
244 cell number and cell size to adjust [Hb] (Table S6). The fact that this was true both within and
245 among species suggests that developmental and evolutionary fine-tuning of blood-O_2 carrying
246 capacity occur similarly.

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

252 $$\frac{(\beta_{TRBC})}{(\beta_{TRBC} + \beta_{MCV})}$$

253

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

257 Extending our analysis, we designed a set of models to test potential predictors of *CeNS*:
258 elevation, a quadratic elevation effect (*elevation*^{2}; to account for notable curvature in the data),
259 body mass, wing loading, species-typical Hb β^A13–β^A83 genotype, and phylogeny (see
260 Methods). Elevation, *elevation*^{2}, and body mass strongly predicted *CeNS* (Fig 4D-F; Table S7).
261 We found a trend of decreasing *CeNS* with higher wing loading, but the 95% credible intervals
262 overlapped zero (Fig S2A–B). Species-typical Hb β^A13–β^A83 genotype did not predict *CeNS*
263 (Fig S3), nor did we find phylogenetic signal in *CeNS* (Fig S4).

264 Examining the tradeoff between cell number and volume provides a potential explanation
265 for the striking pattern that middle elevation species tend to adjust [Hb] by different mechanisms
266 than low and high elevation species (Fig 4E). *CeNS* was uncorrelated with TRBC coefficients
267 (R^2 = 0.07, r = -0.27) but strongly correlated with MCV coefficients (R^2 = 0.51, r = -0.71),
268 indicating that variation in *CeNS* was primarily driven by the degree to which species modulate
269 cell size (Fig S2, Fig S5. We suggest that because low and high elevation species tend to possess

270 relatively small cells, they may be more able to rely on shifts in cell size, rather than cell number,
271 to increase [Hb] (Fig 2J; Fig 4). However, as cell size increased, species were more likely to
272 approach maximum size for red blood cells, beyond which they could incur a functional
273 detriment. As a result, species with larger red blood cells were more reliant on cell number to
274 adjust [Hb]. For middle elevation hummingbird species that have evolved under moderate (~15–
275 30%) reductions in O_2 availability, TRBC appears to be the primary axis for adjustment of
276 blood-O_2 carrying capacity per unit volume of blood. Species that have evolved at the highest
277 elevations are expected to be the best adapted to hypobaric hypoxia, and it is therefore
278 remarkable that their mode of [Hb] adjustment (primarily by MCV) resembles that of
279 hummingbird species in the lowlands, the putative ancestral elevation for hummingbirds [37].
280

281 **Hummingbirds at extreme elevations**

282 Do hummingbirds at extreme high elevations follow the same rules as hummingbirds
283 distributed at low and moderate elevations with respect to elevational blood variation? Our
284 models encompassed the entire elevational gradient and therefore could have missed important
285 phenomena such as threshold effects. To address this, we compared individual hummingbirds
286 sampled above and below 4,200 m elevation, a threshold that marks the upper ~10% of the
287 gradient. Hummingbirds sampled from these extreme high elevations had significantly smaller
288 cells and higher erythrocyte counts than others (Fig S6). This finding suggests that high-altitude
289 specialist hummingbirds tend to have smaller cells, consistent with findings in other high altitude
290 vertebrates [11,29,44,52,53]; however, this pattern was only detectable under the extreme
291 conditions at the uppermost limits of the Andean elevational gradient. We proceeded to formally
292 test for elevational breakpoints using segmented regression (see Methods). Evidence for

293 objective blood trait thresholds along the elevational gradient was mixed, with breakpoint
294 estimates for individual traits ranging from ~1,400 to ~4,000 m (Fig S7). Lack of consilience in
295 these estimates suggests that trait-specific thresholds are challenging to identify and may not be
296 generalizable across species and clades.
297
298 **Phylogenetic signal in blood traits**
299 Phylogenetic signal was low (λ = 0.0; K range = 0.14–0.21) across all blood traits for both
300 within- and among-species models. Blomberg's K and Pagel's λ results were consistent with
301 visual assessment of continuous trait maps, even after removing species with <3 individuals
302 sampled (Table S8; Fig S8). We additionally found no phylogenetic signal in *CeNS* (Fig S4).
303 Non-existent to low phylogenetic signal in blood traits within and among hummingbird
304 species was surprising given demonstrated, phylogenetically conserved Hb genetic adaptations to
305 elevation [21,22] and in light of strong phylogenetic signal reported from recent comparative
306 analyses of blood traits that had broader taxonomic sampling [33,34]. However, it is likely that
307 diverse avian clades possess different blood trait optima; in previous comparative analyses that
308 included multiple families or orders, phylogenetic signal may have been driven primarily by
309 differences among clades. For example, Minias et al. [33] reported that small passerines had
310 consistently high [Hb] and Hct estimates, and that including the shorebird order,
311 Charadriiformes, which was characterized by high [Hb] but medium Hct values, strongly
312 influenced patterns of phylogenetic conservatism [33]. We tested for and found no interaction of
313 clade and elevation on the six studied blood traits, with the exception of the interaction of
314 elevation and the Hermit clade in the [Hb] model set (Fig S9). Interestingly, hermits are a large,
315 early diverging hummingbird clade that contains diminishing species diversity with altitude (and

316 no species that exceeds 3,000 m), and its apparent increased sensitivity of [Hb] to elevation hints
317 at a functional explanation for this biogeographic tendency. The existence of clade-specific trait
318 optima [33] and strong species effects, demonstrated in this study, may at first seem difficult to
319 reconcile with our findings that there was no within-family phylogenetic signal in
320 hummingbirds; however, we view these findings as being consistent with steady evolution of
321 trait optima by a mean-reverting, Ornstein-Uhlenbeck process, as described by Minias et al. [33],
322 and marked by occasional major jumps that coincide with changes in body plan or respiratory
323 demands.

324 A strength of our study was that hummingbirds vary only subtly in aspects of their
325 biology that could plausibly confound our analyses, such as metabolic rate, energy budget, torpor
326 use, mode of locomotion, foraging strategy, or clutch size. Phylogenetic conservatism in these
327 traits facilitates explanation of blood trait variation across the entire clade. A strong effect of
328 species identity in spite of these intrinsic controls hints that species optimize O_2-carrying
329 functions of blood for subtle changes in lifestyle and environment, and on rapid timescales. This
330 finding is consistent with the rapid diversification of human hematological responses to
331 elevation, as illustrated by the copious variation among populations in patterns of elevational
332 variation [19,41,42,54–56].

333

334 **Conclusions**

335 Across ~22 Mya of the hummingbird evolution, ambient O_2-availability and species
336 idiosyncrasies—not phylogenetic affinities—were the primary drivers of variation in blood traits
337 affecting O_2-carrying functions. In response to elevational changes in O_2 partial pressure,
338 hummingbirds make predictable adjustments to the physical composition of blood. Remarkably,

339 after controlling for many potentially confounding variables, decreasing PO_2 drove increases in
340 [Hb] and Hct that were nearly identical in slope within and among hummingbird species,
341 respectively. This implies that developmental and evolutionary optimization of blood traits to
342 ambient PO_2 occurs in fundamentally similar ways, responding to the same set of physical rules
343 that govern gas exchange at the blood-gas barrier. In addition to O_2 availability, species identity
344 itself explained 13–21% of blood trait variation, suggesting that fast-evolving, unmodeled
345 aspects of species biology underpin substantial hematological variation. Our data revealed a
346 striking constraint space that defines the cell number-size tradeoff for hummingbird erythrocytes;
347 within this space, individuals and species co-equally modulated cell number and size to adjust
348 [Hb]. However, species varied in their proportional reliance on adjustments to cell number
349 versus size (*CeNS*) to increase [Hb], and both elevation and body mass were strongly predictive
350 of species' tendencies. Middle-elevation species that have been evolving under moderate
351 hypoxia adjusted [Hb] primarily by adjusting cell number; in contrast, low and high elevation
352 species tended to adjust [Hb] by adjusting cell size, a pattern partly underpinned by
353 hummingbirds at extreme elevations exhibiting smaller cells. Together, these results suggest that
354 evolutionary solutions to reduced O_2 availability can differ qualitatively in conjunction with
355 species-specific histories of exposure to hypoxia challenges of varying severity.
356

357 # Materials and Methods

358 **Field Sampling**

359 We collected data from 1,217 individuals of 77 hummingbird species from Peru and Chile from
360 2006–2020 (Fig 1). After filtering, our final within-species dataset included 1,151 individuals of
361 77 species from 75 localities. The best-sampled clades were the Coquettes (387 individuals, 20

species) and Brilliants (269 individuals, 19 species). The species with greatest sampling depth were *Metallura tyrianthina* (95 individuals), *Colibri coruscans* (73 individuals), and *Amazilia amazilia* and *Patagona gigas* (64 individuals each; Fig 1). Sampling across sexes was balanced (553 females, 592 males, and 6 unknown). Most birds sampled were adults (936 adults, 179 juveniles, and 36 unknown). Detailed data on specimens, collection localities, and blood traits are available in Witt et al. (*In prep*) and in the ARCTOS database (arctosdb.org) for reproducibility and extension of our work.

Blood trait measurements

To calculate primary indices (hemoglobin concentration ([Hb]), hematocrit (Hct), and total red blood cell count (TRBC)), we obtained whole blood samples within ~5 mins to ~3 hours of capture by venipuncture on the underside of the wing and collection with heparinized microcapillary tubes and Hemocue HB201+ cuvettes. Hct (%) was measured with digital calipers after centrifuging the sealed microcapillary tube for 5 min at 13,000 r.p.m. When >1 Hct sample was taken, weighted average values were used in analyses. [Hb] (g/dL blood) was measured on ~5 µL of blood using a HemoCue HB201+ hemoglobin photometer. The HemoCue proprietary photometric method produces values for avian blood that are ~1.0 g/dL greater than those generated using cyanomethemoglobin spectrophotometry [57]. Accordingly, we corrected [Hb] values by subtracting 1.0 g/dL. When possible, at the time of blood collection, 10 µl whole blood was preserved in a 1:200 NaCl dilution to obtain TRBC estimates. Within 12-hours of blood collection, we pipetted a 10 µl sub-sample of blood-NaCl dilution into a hemocytometer and allowed cells to settle for 1 min. After focusing the microscope, one or more photos were

384 obtained of cells in the hemocytometer grid. Later, cells were manually counted in the ImageJ
385 software [58] and TRBC was estimated following standard protocols [28,59].
386 After drawing blood, birds were humanely killed by rapid cardiac compression. We
387 recorded collection elevation, latitude, longitude, sex, body mass, and date. Body mass was
388 typically recorded prior to blood sampling; however, body mass was corrected for estimated
389 volume of blood drawn for any individuals massed after blood sampling. Each collected bird was
390 prepared as a study skin. Specimens and tissues are housed at the Museum of Southwestern
391 Biology (MSB) at the University of New Mexico, USA, the Centro de Ornitología y
392 Biodiversidad (CORBIDI) in Peru, and the Universidad Pontificia Católica de Chile in Chile.
393 Secondary blood indices (mean cell volume (MCV), mean cell hemoglobin (MCH), and
394 mean cell hemoglobin concentration (MCHC)) were calculated from primary indices (Table S1)
395 and following standard protocols [28]. We examined distributions of the data using histograms
396 and qqPlots, and we eliminated outliers that fell outside of the expected distribution and that
397 differed from known 'typical' values [28,31,60]. To reduce age-related outliers, we examined
398 values at the tails of each blood trait distribution that were associated with presence of a bursa
399 measuring ~2 x 2 mm. All juvenile values fell within the expected distribution of blood values.
400 We did not age individuals by plumage, as juvenile plumage lasts much longer than juvenile
401 blood values, which stabilize one to several months after fledging.
402
403 **Environmental and life history traits**
404 We compiled elevational range limits for each species using a combination of published data,
405 field guides, and specimen records from the MSB [26,61–63]. We calculated each individual's
406 elevational range position relative to its species elevational range breadth (hereafter 'elevational

407 position') as *1 – ((maximum species elevation – individual sampling elevation)/species*
408 *elevational range).*
409 We incorporated species-typical Hb β^A13–β^A83 genotypes from published data [22,37].
410 For 7 species, we identified these genotypes from Hb β^A sequences aligned to a GenBank
411 reference from Projecto-Garcia et al. [22]. For 12 species (*Amazilia franciae, Campylopterus*
412 *villaviscensio, Eutoxeres aquila, Klais guimeti, Leucippus taczanowskii, Myrmia micrura,*
413 *Phaethornis atrimentalis, Sephanoides sephaniodes, Chaetocercus mulsant, Eriocnemis vestita,*
414 *Myrtis fanny,* and *Oreotrochilus leucopleurus*) species-typical Hb β^A13–β^A83 genotypes were
415 inferred from sister taxa or nearest relatives.
416 We characterized climatic variation among latitudinal and elevational gradients using 19
417 bioclimatic variables from the WorldClim database [64]. We downloaded rasters of each
418 BioClim variable using the 'raster' package in R (version 4.0.0), cropped them to our study areas
419 of Peru and Chile, and extracted temperature and precipitation values for each sampling locality.
420 We used principal components analysis (PCA) for temperature (Bio1–11) and precipitation
421 (Bio12–19) variables to create a composite measure of temperature and precipitation across the
422 gradient. PC1 of the temperature variables (hereafter "temperature") explained 73.4% of the
423 variation in composite temperature, and loadings suggested that this axis corresponded to
424 increasing temperature across sites (see GitHub). PC1 of the precipitation variables (hereafter
425 "precipitation") explained 79.8% of the variation in composite precipitation, and loadings
426 suggested that this axis corresponded to increasing precipitation across sites (see GitHub).
427 To account for multiple measurement effects (i.e., traits measured from multiple
428 individuals within species), we calculated between- and within-species differences following
429 Villemereuil et al. [65]. This approach uses within-group centering to separate each predictor

430 into two components: We first calculated species mean values (i.e., between-species variability)
431 and then subtracted the mean value of individual observations (i.e., within-species differences).
432 These measures of within-species variation for temperature, precipitation, and body mass were
433 included in models. Because hummingbird body masses may differ by sex, within-species
434 differences were calculated using mean body mass of males for males, mean body mass of
435 females for females, and species-level means for individuals of unknown sex (n=6).
436 We used wing area data from Skandalis et al. [66] to calculate wing loading in g/cm^2,
437 calculated as species mean body mass (g) divided by total wing area (cm^2). We analyzed wing
438 loading due its relevance to aerodynamic theory and hummingbird flight at high altitudes [67–
439 70].
440
441 **Hummingbird evolutionary history**
442 We used the rate-calibrated molecular hummingbird phylogeny from McGuire et al. [37] as a
443 backbone to generate a phylogeny pruned to the 77 species in our dataset using the R package
444 'phytools' [71]. Two species, *Oreotrochilus leucopleurus* and *Phaethornis stuarti*, were missing
445 from the McGuire et al. [37] tree and were added to our phylogeny using the bind.tip() function.
446 Branch lengths and position of *O. leucopleurus* were calculated from genetic distances and
447 recently described sister relationships [72]. Approximate branch length and position of *P. stuarti*
448 were inferred from BirdTree.org [73,74] with divergence rate estimates from a multi-locus, time-
449 calibrated phylogeny of Hawaiian honeycreepers [75]. We generated model-specific tree subsets
450 by including only those tips that corresponded to the species in each modeling subset.
451
452 **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; Fig S8). Accordingly, we excluded phylogeny as an effect in models and evaluated phylogenetic signal using Blomberg's K and Pagel's λ. Results from the two tests were concordant and consistent across model subsets (Table S8).

Within species trait-environment models

We analyzed within species variation in blood characteristics using generalized linear multilevel models in the R package brms [80]. Brms uses Markov chain Monte Carlo (MCMC) sampling to obtain draws from posterior distributions. We designed hierarchical models for each of six continuous responses variables: [Hb], Hct, TRBC, MCV, MCH, and MCHC. For each model, predictor variables included one species-level categorical variable (species-typical Hb β^A13–β^A83 genotype) and eight individual-level continuous variables (elevation, elevational position, body mass (log-transformed), precipitation, temperature, and within species variation in body mass, temperature, and precipitation). All variables were standardized to a mean of zero and standard deviation of one.

We compared 6 models in each model set: 1) an intercept-only null model, 2) an intercept-only model with a species grouping variable (1|species), 3) a model with all predictors and no grouping variable, 4) a model with all predictors and the species grouping variable, 5) a

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

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

489

490 **Among species trait-environment models**

491 Final among-species datasets varied in the number of species they contained: ([Hb]=60 species;
492 Hct=65 species; TRBC=58 species, MCV=60 species; MCH=59 species; MCHC=64 species).
493 Species mean values were calculated for all species with *n*=2 or more individuals sampled. We
494 split *Patagona gigas* into "north" and "south" haplotypes in light of evidence that the complex
495 may be comprised of two functionally and genetically distinct populations separated by strong
496 migratory divides [77], and we analyzed data only from individuals sampled during the breeding
497 months to ensure correct population assignment. We designed model sets for each of our six
498 blood traits. For each response, we analyzed distributions (qqPlots, Cook's Distance, leverage)

499 and removed outliers that strongly influenced fit. For each model, predictor variables included
500 species-typical Hb β^A13–β^A83 genotype, mean elevation, mean body mass (log-transformed; one
501 mean for all individuals due to balanced sampling between sexes), mean temperature, and mean
502 precipitation. All variables were standardized to a mean of zero and standard deviation of one.
503 For each response, we compared: 1) an intercept-only null model, 2) a model with all
504 predictors, and 3) a reduced-predictor model with only those predictors whose 95% CIs did not
505 overlap zero in full models (reduced model 2). Given the notable quadratic shape of species
506 mean data for TRBC, MCV, MCH, and MCHC (Fig 2G–L), for those model sets we additionally
507 compared 4) a model with all predictors plus a quadratic component of elevation and 5) a
508 reduced-predictor model with quadratic component including only those predictors whose 95%
509 CIs did not overlap zero in the full quadratic model (reduced model 4). Distribution family
510 differed by model set: Gaussian for [Hb; skew-normal for Hct; and student's *t*-distribution for
511 TRBC, MCV, MCH, and MCHC models. We ran four Markov chains for 20,000 iterations with
512 a burn-in period of 10,000, thinned every 10 steps.
513
514 **Cell number versus size analyses**
515 First, to assess within species variation in cell number versus size, we compared a null model
516 (intercept only) to a single full model *[Hb] ~ MCV + TRBC + (1|species)* for all individuals of
517 59 species that had [Hb], MCV, and TRBC measurements, distinguishing between *Patagona*
518 *gigas* "north" and "south". The species grouping variable allowed us to account for repeated
519 sampling of individuals within a species and to hold species identity constant. Second, to
520 evaluate among-species variation in cell number versus size, we generated a mean dataset for
521 each species with ≥3 individuals sampled (48 species total). We again compared a null (intercept

522 only) model to a single full model *[Hb] ~ MCV + TRBC.* Third, to assess how individual species
523 differ in their relative contributions of MCV and TRBC to increasing [Hb], we repeated our
524 approach for individual species: For each species with ≥8 sample individuals (32 species total),
525 we generated a species-specific data subset, carefully and conservatively evaluated influential
526 outlier points, and ran a single model: *[Hb] ~ MCV + TRBC* (Fig S1). For all three model sets
527 (within species, among species, and single species), both predictors were standardized to a mean
528 of zero and standard deviation of one.

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

534 We then analyzed the factors that contribute to variation in *CeNS*. Our model response
535 variable was *CeNS* (0–1 index). Predictors, all species mean values, included elevation, a
536 quadratic elevation term "elevation^2" to account for notable curvature in the data, body mass
537 (log-transformed), wing loading, and species-typical Hb β^A13–β^A83 genotype. We compared six
538 models: 1) an intercept-only null model, 2) a model with core predictors (elevation, body mass,
539 and wing loading), 3) a model with core predictors plus elevation^2, 4) a model with core
540 predictors plus elevation^2 and species-typical Hb β^A13–β^A83 genotype, 5) a reduced version of
541 model 3, and 6) a reduced version of model 4. Reduced models included only those predictors
542 whose 95% CIs did not overlap zero in full models. In addition to model fit diagnostics, we
543 assessed correlations between variables by analyzing correlation matrices and by regressing
544 *CeNS* on each R^2 values from standardized species-specific *CeNS* models, unstandardized TRBC

545 and MCV beta estimates, and raw TRBC and MCV values (Fig S5). Distribution family differed
546 by model set: Gaussian for individual-level, species mean, and *CeNS* prediction models, and
547 student's *t*-distribution for species-specific models. For all *CeNS* models we ran four Markov
548 chains for 20,000 iterations with a burn-in period of 10,000, thinned every 10 steps. We assessed
549 phylogenetic signal with Blomberg's K, Pagel's λ, and visually with contmaps (Fig S4).
550
551 **Model comparison and diagnostics**
552 All models and sets were run using the brm() function with default priors (weakly informative
553 priors for the intercept and sigma; improper flat priors for predictors). To assess convergence we
554 visually examined trace plots, checked diagnostics, and confirmed that Rhat was <1.01 [76]. We
555 used a posterior predictive check to assess whether model assumptions yielded good
556 approximations of the data generating process. There were no problems in any of our models
557 resulting from Bayesian fraction of missing information or divergent transitions. We compared
558 models and verified fits using the widely applicable information criterion (WAIC) [78], Bayesian
559 expected log pointwise predictive densities (ELPD) [79], and approximate leave-one-out cross-
560 validation information criterion based on the posterior likelihoods (LOOIC; Tables S3, S6). We
561 estimated fixed effects (means and 95% CIs) from the posterior distributions for each predictor.
562 For relevant models, we estimated the proportion of the total variance attributed to the species
563 grouping variable, which accounts for unique aspects of blood parameters that are not captured
564 by the individual, life history, or environmental predictors.
565
566 **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 (Fig S7).

Data and code availability

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

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Author Contributions

JLW, EBL, EB, JAM, RD, and CCW designed the research and methods; JLW, EB, AS, JAM, RD, and CCW collected the data; JLW analyzed the data; JLW, JAM, RD, and CCW acquired funds and resources; and JLW and CCW wrote the paper with input from all authors.

Competing interests

The authors declare no competing interests.

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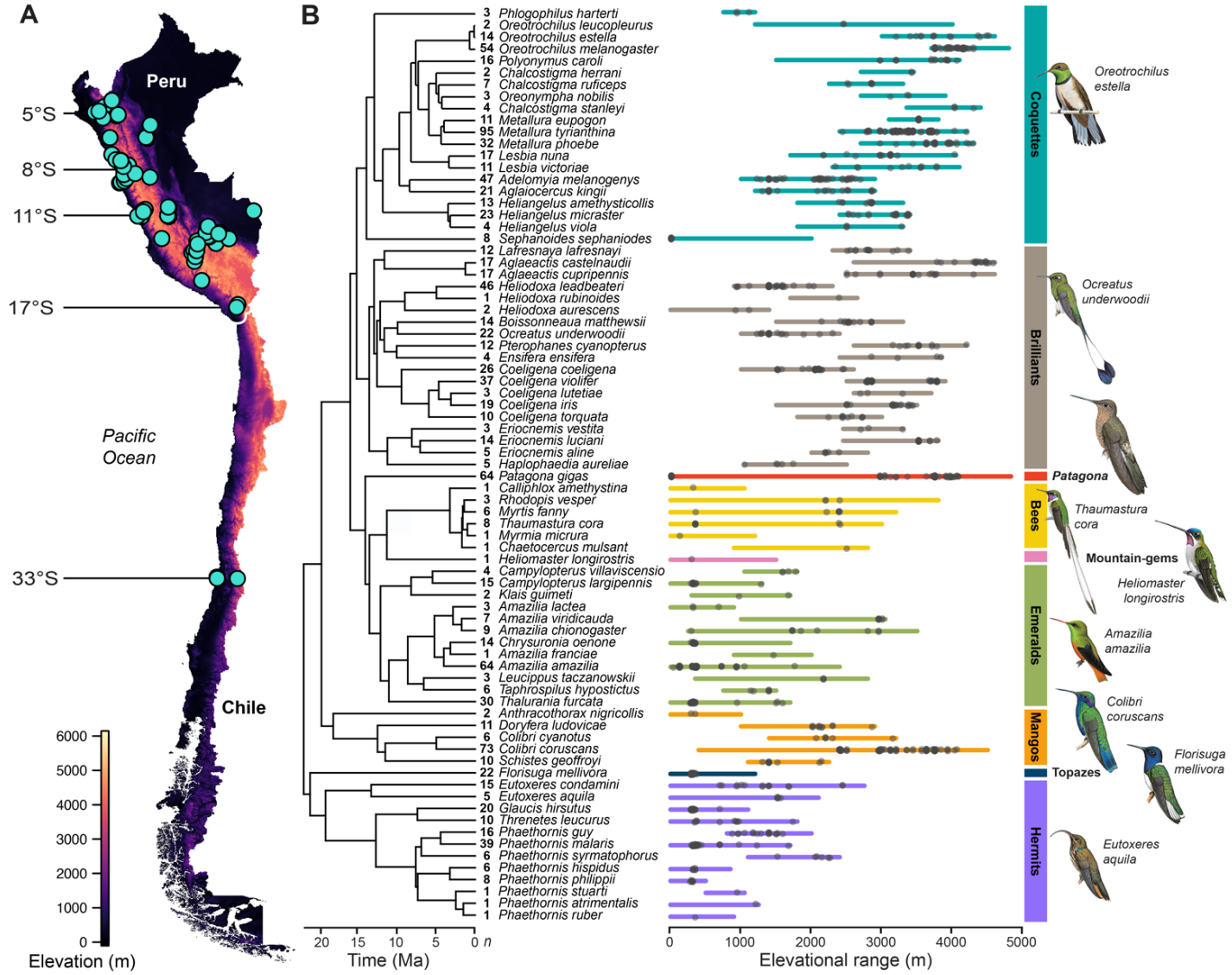

Fig 1. Elevational, latitudinal, and phylogenetic sampling from 2006–2020. **(A)** Sampling localities spanned 28° latitude and 4,578 meters in elevation in Peru and Chile. **(B)** We studied 77 species ('n' column indicates sampling depth) from all nine major hummingbird clades. Sampling (gray opaque points) is shown across each species' elevational range (colored horizontal bars). Time-calibrated phylogeny is modified from McGuire et al. [37]. Illustrations depicting a representative from each clade are from *Birds of the World* and are reproduced courtesy of the Cornell Lab of Ornithology (©).

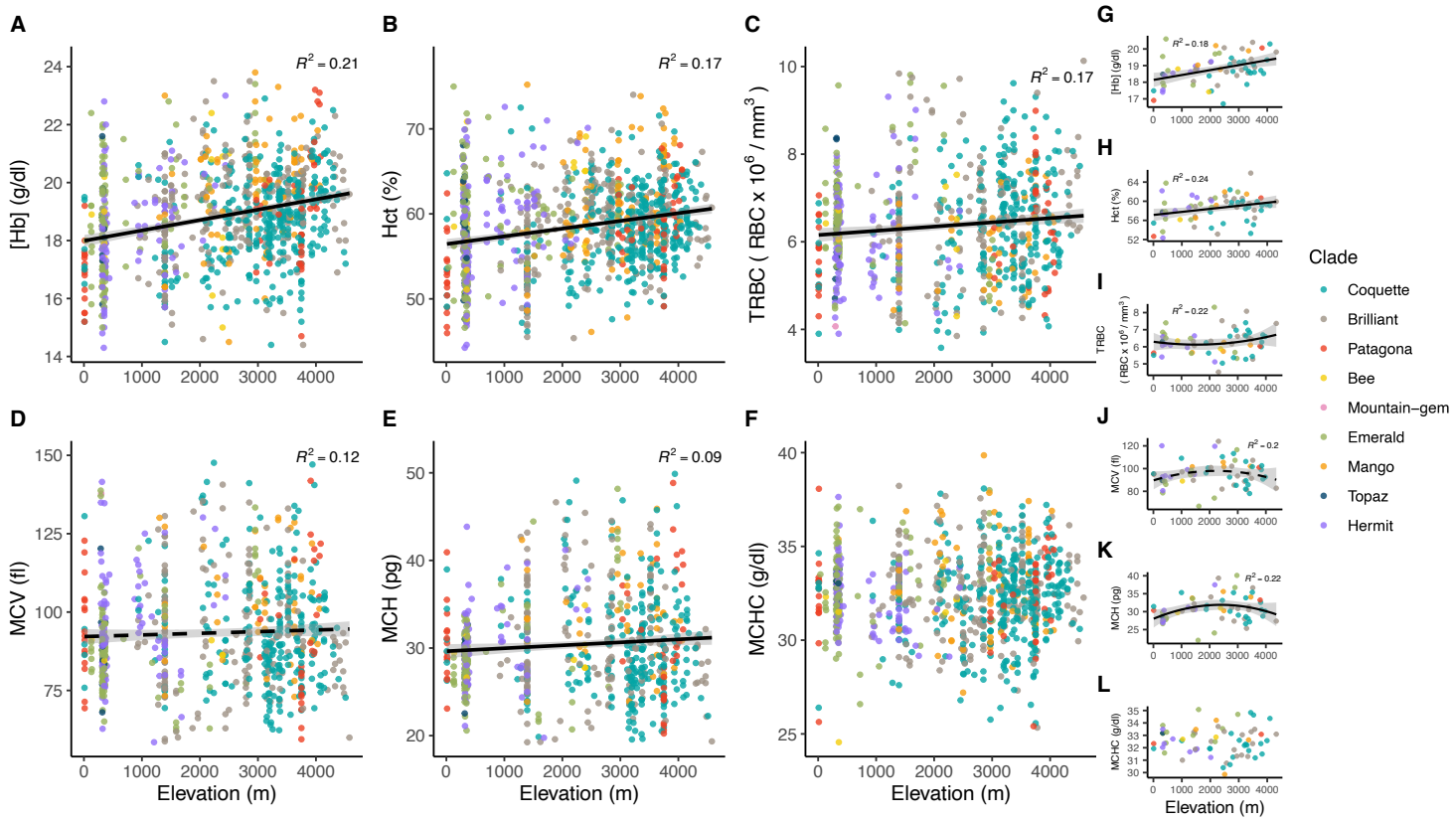

Fig 2. Relationship between elevation and the six studied blood parameters (hemoglobin concentration ([Hb]), hematocrit (Hct), total red blood cell count (TRBC), mean cell volume (MCV), mean cell hemoglobin content (MCH), and mean cell hemoglobin concentration (MCHC). **(A–F)** Within species comparisons, 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 Fig 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 Fig 1. R^2 values are reported from Bayesian models reported in Fig 3.

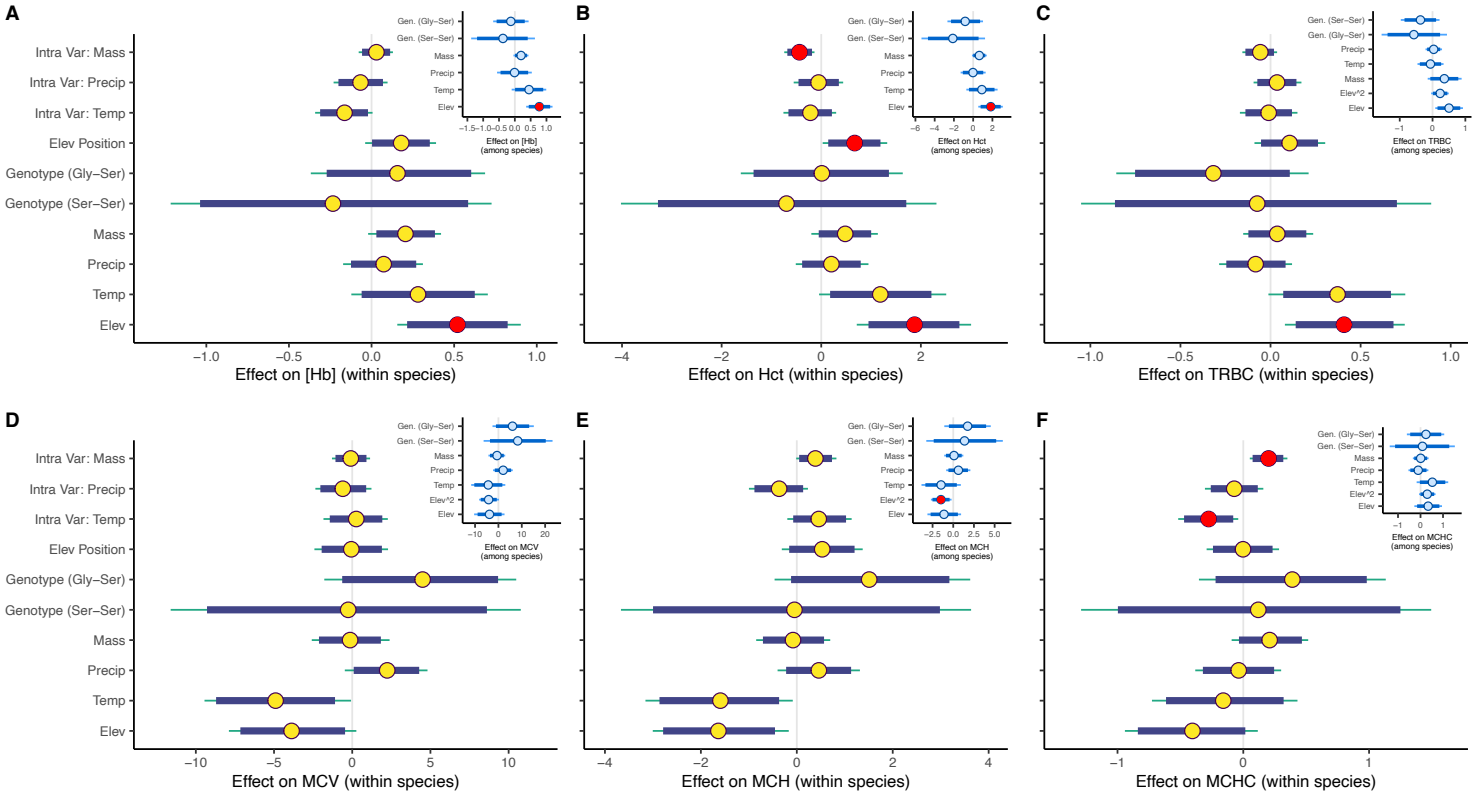

Fig 3. Posterior probabilities and credible intervals from full within-species (main plots) and among-species (insets) models for each of six blood traits: **(A)** hemoglobin concentration ([Hb]), **(B)** hematocrit (Hct), **(C)** total red blood cell (TRBC), **(D)** mean cell volume (MCV), **(E)** mean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

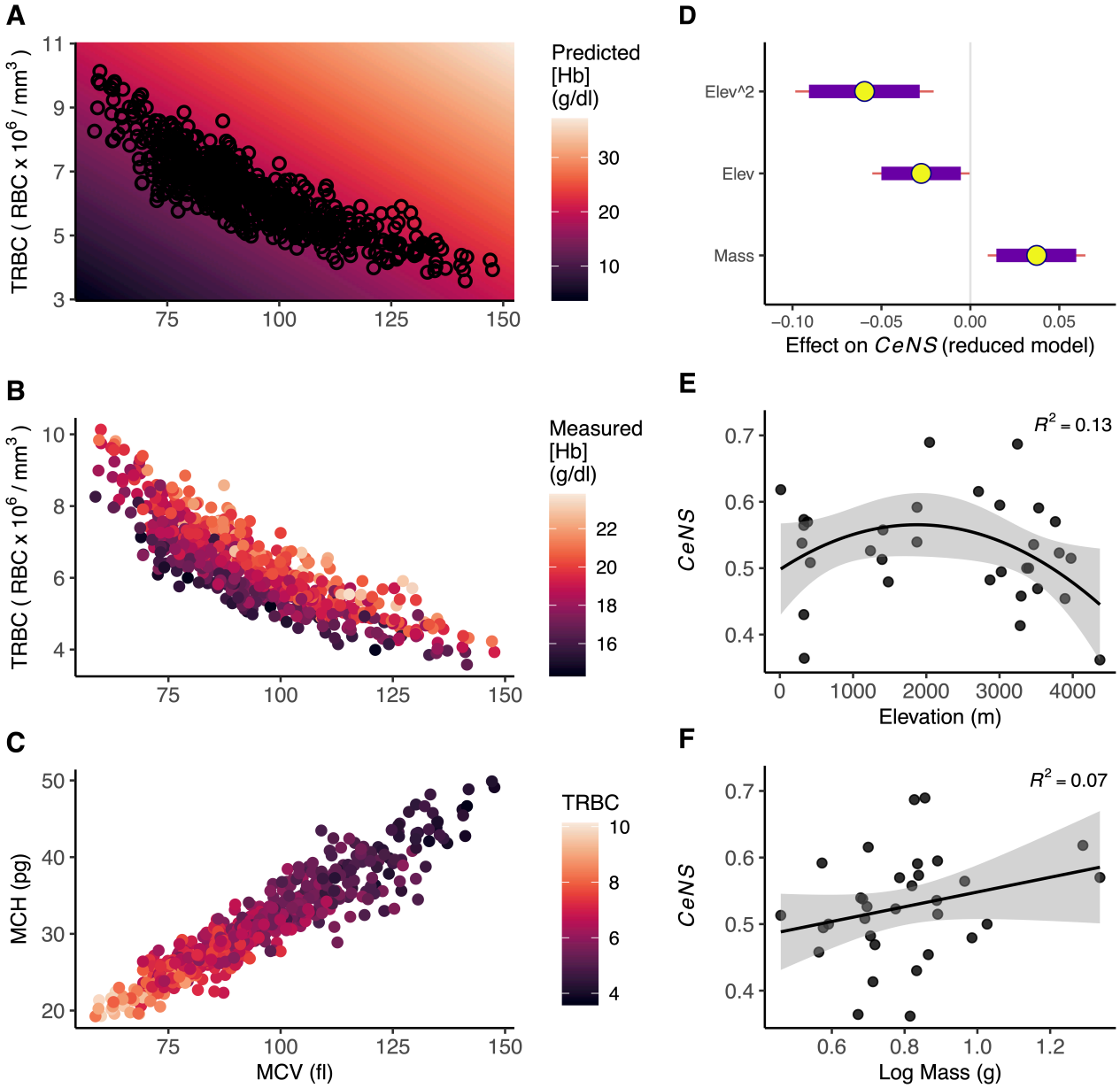

Fig 4. Theoretical and empirical relationships among hummingbird blood parameters underlying hemoglobin concentration ([Hb]) adjustment and predictors of the Cell Number-Size Index (*CeNS*). **(A–B)** Hummingbirds have evolved optimized blood-O_2 carrying capacity, achieved by balancing total red blood 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 hemoglobin concentration

876 (MCHC). Points are colored by TRBC (units presented on the y-axis), with lighter colors
877 representing higher erythrocyte counts. In panels A–C, each point represents a value from a
878 single individual. **(C)** Posterior probabilities and credible intervals from the best fitting reduced
879 *CeNS* model. **(D)** *CeNS* shows a strong quadratic relationship with elevation, revealing that
880 middle elevation species adjust blood-O_2 carrying capacity differently than low- and high-
881 elevation species. **(E)** *CeNS* increases with increasing body mass. In panels E–F, R^2 values are
882 reported from generalized linear models of each *x* regressed on *y*, and thus do not account for
883 other modeled variables.