Received: 9 January 2023 | Revised: 20 March 2023 | Accepted: 13 April 2023

DOI: 10.1111/ele.14235

ECOLOGY LETTERS cnrs WILEY

LETTER

# Hummingbird blood traits track oxygen availability across space and time

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**Funding information**
American Museum of Natural History, Grant/Award Number: Chapman Grants (2017 & 2019); American Philosophical Society, Grant/Award Number: Lewis & Clark Grant; Explorers Club; National Science Foundation, Grant/Award Number: DEB-0543556, DEB-1146491, DBI-1907353 and DBI-2208924; Nuttall Ornithological Club, Grant/Award Number: Blake-Nuttall Fund (2016 & 2018); University of New Mexico Biology Graduate Student Association; University of New Mexico Department of Biology; University of New Mexico Graduate and Professional Student Association; University of New Mexico Latin American and Iberian Institute; Cornell Lab of Ornithology

**Editor:** Julien Cote

## Abstract

Predictable trait variation across environments suggests shared adaptive responses via repeated genetic evolution, phenotypic plasticity or both. Matching of trait–environment associations at phylogenetic and individual scales implies consistency between these processes. Alternatively, mismatch implies that evolutionary divergence has changed the rules of trait–environment covariation. Here we tested whether species adaptation alters elevational variation in blood traits. We measured blood for 1217 Andean hummingbirds of 77 species across a 4600-m elevational gradient. Unexpectedly, elevational variation in haemoglobin concentration ([Hb]) was scale independent, suggesting that physics of gas exchange, rather than species differences, determines responses to changing oxygen pressure. However, mechanisms of [Hb] adjustment did show signals of species adaptation: Species at either low or high elevations adjusted cell size, whereas species at mid-elevations adjusted cell number. This elevational variation in red blood cell number versus size suggests that genetic adaptation to high altitude has changed how these traits respond to shifts in oxygen availability.

**KEYWORDS**
Andes, blood, blood–oxygen, ecophysiology, elevation, haematology, haemoglobin, hummingbird, phylogeny, trait–environment association

  

## INTRODUCTION

Predictable variation of traits across environmental gradients can reveal both the power and limitations of adaptive mechanisms, but the evolutionary implications of such patterns depend on their scale. Trait–environment relationships *among* species tend to reflect long-term, genetically fixed trait differences across phylogenies. By contrast, trait–environment relationships *within* species tend to reflect short-term, plastic responses of individuals with shared genetic backgrounds. When within- and among-species patterns match (e.g. Jordan, 1891; McDowall, 2003), it implies a shared solution to an environmental challenge. When trait–environment patterns conflict among scales or clades (e.g. Blackburn et al., 1999; De Queiroz & Ashton, 2004), it implies that genetic adaptation has taken a qualitatively different path when faced with the same environmental challenge, breaking the 'rules' that govern the trait–environment relationship. As varied adaptations accrue over macroevolutionary time, predictable trait–environment (or trait–trait) associations are expected to erode (Agrawal, 2020; McGlothlin et al., 2018). Testing the consistency of trait associations across biological scales can provide insights into the functional basis of adaptation, as well as the functional consequences of global environmental change (Enquist et al., 2015).

In animals, blood traits associated with oxygen ($O_2$) transport change predictably with declining $O_2$ availability at elevation (Borras et al., 2010; Levett et al., 2012; Storz et al., 2010; Tufts et al., 2013). Most $O_2$ in the blood is carried by haemoglobin (Hb) (Pittman, 2011), the concentration of which ([Hb]) is a key determinant of blood–$O_2$-carrying capacity. When lowland-adapted birds ascend to altitude, they 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 (Storz et al., 2010). In the absence of compensatory increases in plasma volume (Stembridge et al., 2019), increased Hb mass leads to elevated haematocrit (Hct; Storz, 2010). A suite of interrelated physical characteristics of blood—total red blood cell count (TRBC), mean cell volume (MCV), mean cell haemoglobin (MCH) and MCH concentration (MCHC; Table S1)—can be adjusted to optimize blood–$O_2$ transport, or to compensate for other cardiac, vascular or haematological responses to elevation.

However, genetic adaptation to high elevation can 'break the rules' of trait–environment covariation in blood traits. For example, evidence from high-altitude-adapted human populations and theoretical work suggests that optimal [Hb] and Hct levels may be similar to those of sea-level populations (Beall, 2007; Scott & Milsom, 2006; Simonson et al., 2010; Storz et al., 2010). Adaptive genetic changes to haemoglobin proteins have been demonstrated in several high-altitude-native bird clades, including hummingbirds (e.g. Natarajan et al., 2016). In hummingbirds, there is a highly predictable association between species-typical Hb $\beta^A13$–$\beta^A83$ genotype and elevation, with derived increases in Hb-$O_2$ affinity in highland lineages and reductions in Hb-$O_2$ affinity in lowland lineages; importantly, hummingbirds occurring at high altitudes exhibit two amino-acid substitutions on the $\beta^A$-globin subunit of Hb (Hb $\beta^A$-$13_{GLY\rightarrow SER}$ and Hb $\beta^A$-$83_{GLY\rightarrow SER}$) that are known to affect $O_2$ binding (Projecto-Garcia et al., 2013). $O_2$-binding affinity of haemoglobin has critical effects on tissue oxygenation and, in birds, binding affinity increases predictably with elevation (Natarajan et al., 2016; Storz, 2016). It is not yet clear whether the genetic adaptations that change Hb-$O_2$ binding affinity predictably affect other blood traits or their covariation with elevation. It is plausible that species-typical Hb $\beta^A$ genotypes associated with different $O_2$-binding affinities induce predictable differences in blood traits and their reaction norms to elevation; such an association could reflect a direct functional trade-off or an indirect effect of accrued, multi-locus adaptation to elevation.

The macro-physiological 'rules' governing blood trait variation over phylogenetic and population timescales have remained elusive because adequate comparative data are scarce. Blood traits are known to vary among avian clades (e.g. Campbell & Ellis, 2007; Zhang et al., 2007) and exhibit elevational variation both within (e.g. Barve et al., 2016; Dubay & Witt, 2014; Linck et al., 2023) and among species (e.g. Barve et al., 2016; Dubay & Witt, 2014). At least two additional recent studies have conducted broad-scale interspecific comparative analyses of avian [Hb] and Hct data, but both datasets were compiled from heterogeneous published data and lacked elevational sampling sufficient to estimate elevational effects (Minias, 2020; Yap et al., 2019). Furthermore, no previous comparative analysis of haematological variation across elevation has included the six key parameters that affect blood–$O_2$-carrying capacity per unit volume (Table S1). Thus, we continue to lack definitive understanding of blood trait–environment relationships, their possible scale dependence and the effects of known genetic adaptations.

Examining the mechanisms underlying blood–$O_2$-carrying capacity adjustment can offer further insight into blood trait–environment relationships. [Hb] is equivalent to $O_2$-carrying capacity per unit volume of blood, and it can be altered via adjustments to cell number (TRBC), cell size (MCV), within-cell [Hb] (MCHC) or plasma volume. Cell number and size are both measurable components that vary among individuals and species, are subject to plastic and evolutionary adjustments, and have strong direct effects on [Hb]. Both are reciprocally constrained by physical space available in the circulatory system, such that individual birds can have a few large erythrocytes, many small erythrocytes or intermediate values for both

traits (Hawkey et al., 1991; Wintrobe, 1933). MCHC tends to be relatively invariant across vertebrates (Wintrobe, 1933), with Hb typically comprising ~1/3 of erythrocytes (Linck et al., 2023). Quantifying the relative contributions of TRBC and MCV in driving [Hb] variation within and among species may show that species use different mechanisms of adjusting blood–$O_2$-carrying capacity, revealing signatures of adaptation that correspond with species-specific histories of altitude exposure.

In this study, we sought to test whether phylogenetic and population patterns for blood-trait–environment variation match. Specifically, we asked: Does species adaptation change trait–environment relationships? To address this question, we first needed to describe these relationships. We spent 2006–2020 collecting specimen-vouchered haematological data from 1217 wild hummingbirds of 77 species, representing all 9 clades and spanning ~4600 meters in elevation. Using these data, we then constructed hierarchical Bayesian models to estimate the responses of six blood traits to elevation while accounting for other sources of variation. Hummingbirds are a potential model clade in which to study haematological variation due to their documented evolutionary genetic responses to shifts in $PO_2$, high species richness in mountains, phylogenetically conserved elevational ranges and relative life-history uniformity (Graham et al., 2009; McGuire et al., 2014; Natarajan et al., 2016; Projecto-Garcia et al., 2013; Suarez, 1992). Matching $O_2$ supply and demand is an acute challenge for hummingbirds because they employ sustained hovering, which is highly energetically demanding (Buermann et al., 2011; Suarez, 1992). This study posits new patterns of elevational blood trait variation. The scale independence of some rules and scale dependence of others have implications for the power and limitations of adaptive evolution.

# MATERIALS AND METHODS

## Field sampling

We collected data from 1217 individuals of 77 South American hummingbird species from 2006 to 2020 (Figure 1). Sampling across sexes was balanced (553 females, 592 males and 6 unknown). Most birds sampled were adults (936 adults, 179 juveniles and 36 unknown). Detailed data on specimens, collection localities and blood traits are available in the ARCTOS database (arctosdb.org) and will be archived on Dryad.

## Blood trait measurements

To calculate primary indices ([Hb], Hct and TRBC), we obtained whole blood samples within ~5 min to ~3 h of capture by venipuncture on the wing underside and collection with heparinized microcapillary tubes and Hemocue HB201+ cuvettes. Hct (%) was measured with digital callipers after centrifuging the sealed microcapillary tube for 5 min at 13,000 r.p.m. When >1 Hct sample was taken, weighted averages were used in analyses. [Hb] (g/dL) was measured on ~5 μL of blood using a HemoCue HB201+ haemoglobin photometer, with a correction for avian blood (Simmons & Lill, 2006). We sampled 10 μL whole blood diluted in 1:200 in NaCl solution; we then pipetted a sub-sample of the dilution into a haemocytometer, allowing cells to settle for 1 min, before photographing at 100× magnification. We estimated TRBC (Campbell & Ellis, 2007; Samour, 2005) by counting cells in ImageJ (Rasband, n.d.).

After blood sampling, birds were humanely killed and prepared as study skins. Specimens and tissues are housed at the Museum of Southwestern Biology (MSB) at the University of New Mexico, Centro de Ornitología y Biodiversidad (CORBIDI) and Pontificia Universidad Católica de Chile.

Secondary blood indices (MCV, MCH and MCHC) were calculated from primary indices following standard protocols (Campbell & Ellis, 2007). The data were carefully analysed for outliers (Supporting Information).

## Environmental and life-history traits

We compiled species elevational ranges using published data, field guides and MSB specimen records (Jaramillo, 2003; Parker et al., 1996; Schulenberg et al., 2010; Williamson & Witt, 2021b). We calculated each individual's elevational range position relative to its species elevational range breadth (hereafter 'elevational position') as *1 – ((maximum species elevation – individual sampling elevation)/species elevational range)*. We obtained species-typical Hb $\beta^A13$–$\beta^A83$ genotypes from published data (McGuire et al., 2014; Projecto-Garcia et al., 2013), reference sequences and expert inference (Supporting Information).

We characterized latitudinal and elevational climatic variation using WorldClim data (Hijmans et al., 2005). We downloaded rasters of each bioclimatic variable using the R package 'raster' (v4.0.0), cropped them to study areas of Peru and Chile, and extracted temperature and precipitation values for each sampling locality. We used principal component analysis (PCA) to create a composite measure of temperature (BIO1-11) and precipitation (BIO12-19) across the gradient. PC1 of temperature variables (hereafter 'temperature') explained 73.4% of the variation in composite temperature; loadings suggested that this axis corresponded to increasing temperature across sites (see GitHub). PC1 of precipitation variables (hereafter 'precipitation') explained 79.8% of the variation in composite precipitation; loadings suggested that this axis corresponded to increasing precipitation across sites (see GitHub).

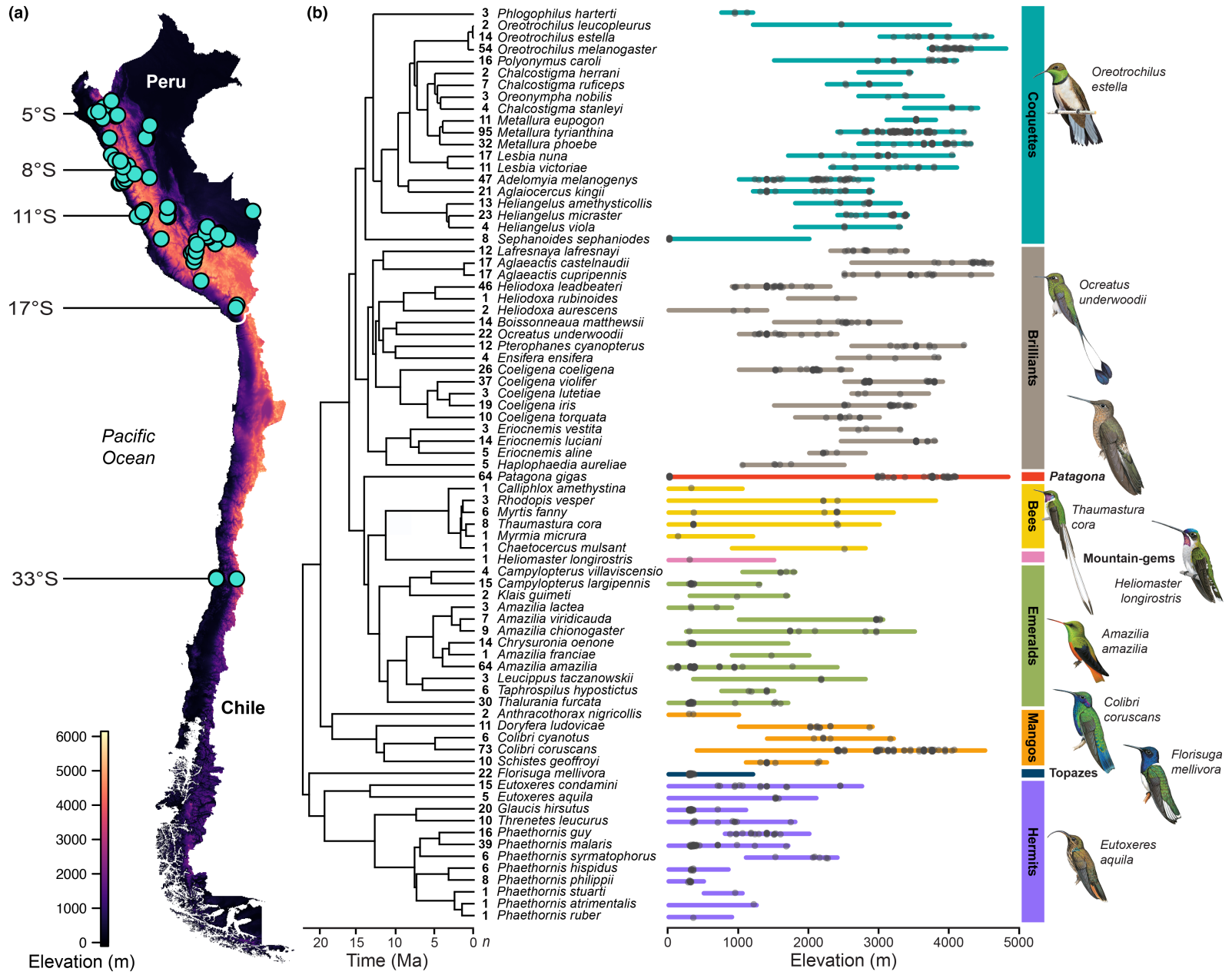


**FIGURE 1** Elevational, latitudinal and phylogenetic sampling from 2006 to 2020. (a) Sampling localities spanned 28° latitude and 4578 meters in elevation in Peru and Chile. (b) We studied 77 species ('*n*' column indicates sampling depth) from all nine major hummingbird clades. Sampling (grey opaque points) is shown across each species' elevational range (coloured horizontal bars). Time-calibrated phylogeny is modified from McGuire et al. (2014). Illustrations depicting a representative from each clade are from *Birds of the World* and are reproduced courtesy of the Cornell Lab of Ornithology (Birds of the World, 2022).

To account for multiple measurement effects (i.e. traits measured from multiple individuals within species), we calculated between- and within-species differences following De Villemereuil and Nakagawa (2014). Because hummingbird body mass may differ by sex, within-species differences were calculated using mean mass of males for males, mean mass of females for females and species-level means for individuals of unknown sex ($n=6$). We calculated and analysed wing loading (g/cm$^2$) from published data (Skandalis et al., 2017), as previous work suggests that wing shape and flight cost vary with elevation (e.g. Altshuler et al., 2004; Supporting Information).

## Hummingbird phylogeny

We used a time-calibrated hummingbird phylogeny (McGuire et al., 2014) to prune our 77-tip tree using the R package 'phytools' (Revell, 2012), adding two missing taxa (Supporting Information). We generated model-specific tree subsets by including only those tips that corresponded to the species in each subset.

## Trait–environment models

To evaluate the effects of species and environmental characteristics on blood traits, we built Bayesian multilevel models in the R package brms (Bürkner, 2017). All predictors were standardized to a mean of zero and standard deviation of one. Distribution family differed by model set (Supporting Information). For within and among species models, we ran four Markov chains for 10,000 iterations with a burn-in of 5000, thinned every 10 steps; for cell size versus number models, we ran four Markov chains for 20,000 iterations with a burn-in of 10,000, thinned every 10 steps. Reduced models included only predictors whose 95% credible intervals (CIs) did not overlap zero in full models.

## Within species models

Our final within species (individual level) dataset included 1151 individuals of 77 species after filtering for blood trait outliers. We designed models for six continuous response variables: [Hb], Hct, TRBC, MCV, MCH and MCHC; each dataset contained $n=703$–$1086$ individuals of $n=69$–76 species, depending on the trait. For each model set, predictors included species-typical Hb $\beta^A13$–$\beta^A83$ genotype and eight individual-level continuous variables (elevation, elevational position, body mass (log-transformed), precipitation, temperature, and within species variation in body mass, temperature and precipitation). We compared six models in each set: (1) intercept-only, (2) intercept-only + (1|species), (3) all predictors, (4) all predictors + (1|species), (5) reduced-predictor model and (6) reduced-predictor model + (1|species). We tested whether hummingbird clades respond differently to elevation by analysing clade*elevation interactions. We additionally searched for objective thresholds, or 'breakpoints', in each blood trait to analyse whether hummingbirds at extreme high elevations follow the same rules with respect to elevational blood variation as hummingbirds distributed across low to moderate elevations (Supporting Information).

## Among-species models

Our final among-species (species-level) datasets included $n=58$–$65$ species, depending on the blood trait modelled. In each, each row contained mean blood values for species with ≥2 individuals sampled, with *Patagona* evaluated as two species (Williamson & Witt, 2021a, 2021b) (Supporting Information). We designed models for six blood traits following within-species model protocols. Predictor variables included species-typical Hb $\beta^A13$–$\beta^A83$ genotype, and means of elevation, body mass (log-transformed; one mean for all individuals), temperature and precipitation. For each response, we compared (1) intercept-only null model, (2) full predictor model and (3) reduced-predictor model. Given the notable quadratic shape of species mean data for TRBC, MCV, MCH and MCHC (Figure 2g–l), for these model sets we additionally compared: (4) full predictor model plus a quadratic component of elevation (*elevation*$^2$) and (5) reduced-predictor model with *elevation*$^2$.

## Phylogenetic signal

We initially calculated phylogenetic signal from full and reduced phylogenetic models for each blood parameter. However, phylogenetic signal for all model sets (full and reduced in both within- and among-species analyses) was low ($\lambda<0.004$; Supporting Information). Accordingly, we excluded phylogeny from models and evaluated phylogenetic signal using Blomberg's K and Pagel's λ with concordant results across model subsets (Table S2, Figure S1).

## Cell number versus size analyses

We designed models to understand the relative contributions of cell number versus size (*CeNS*) to blood–$O_2$-carrying capacity per unit volume ([Hb]) at two different timescales (Supporting Information). The first scale assessed was variation within species, and we compared an intercept-only null model to a full model, $[Hb] \sim MCV + TRBC$ + (1|species), for 640 individuals of 59 species. The second scale assessed was variation among species, and we compared null and full models using species-mean values for 48 species with ≥3 individuals sampled (Supporting Information).

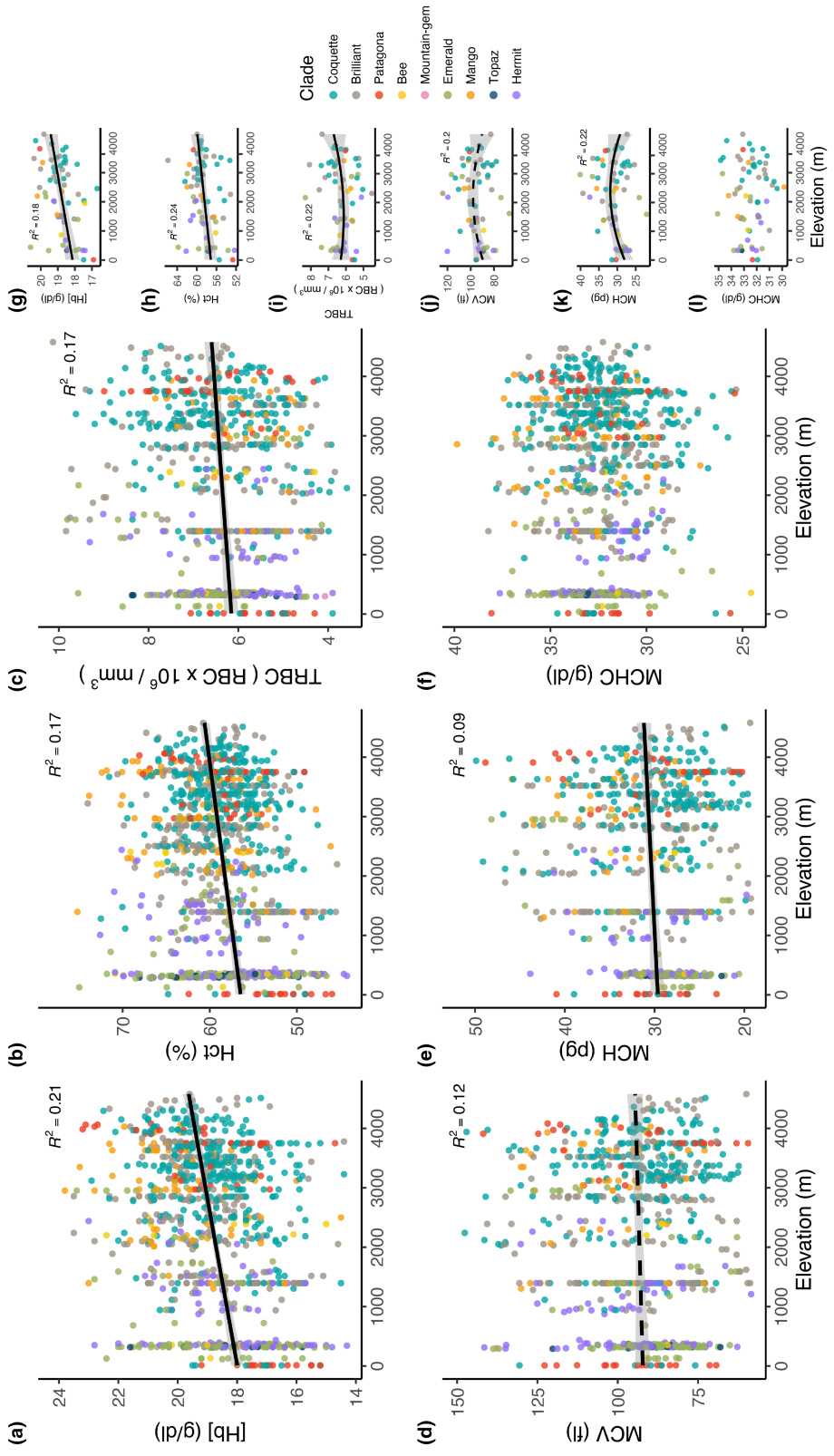


**FIGURE 2** Relationship between elevation and each of the six studied blood parameters (haemoglobin concentration ([Hb]), haematocrit (Hct), total red blood cell count (TRBC), mean cell volume (MCV), mean cell haemoglobin content (MCH) and mean cell haemoglobin concentration (MCHC)). (a–f) Within species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

After establishing *CeNS* patterns across all hummingbird individuals and species, respectively, we asked whether species vary with respect to *CeNS*. Using data for 32 well-sampled species, we estimated species-specific standardized TRBC and MCV coefficients ($\beta$) from the model $[Hb] \sim MCV + TRBC$ (Figure S2). We used these coefficients to calculate an index that describes, for each species, the relative use of these two alternative mechanisms in adjusting $O_2$-carrying capacity; we call it the 'Cell Number-Size Index' (hereafter *CeNS* Index). The *CeNS* Index represents the proportional (0–1) contribution of TRBC versus MCV to variation in [Hb], with higher values (closer to one) corresponding to greater relative contributions of cell number versus cell size. The index is calculated as:

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

Next, we asked whether we could predict species tendencies for the *CeNS* Index by designing a set of models to test potential predictors: Elevation, $elevation^2$, body mass, wing loading, species-typical Hb $\beta^A13$–$\beta^A83$ genotype and phylogeny. We compared six models: (1) intercept-only, (2) core predictors (elevation, body mass and wing loading), (3) core predictors plus $elevation^2$, (4) core predictors plus $elevation^2$ and species-typical Hb $\beta^A13$–$\beta^A83$ genotype, (5) reduced model 3 and (6) reduced model 4 (Supporting Information).

## Model comparison and diagnostics

All models incorporated default priors. To assess convergence, we examined trace plots, checked diagnostics and confirmed that Rhat, a convergence diagnostic, was <1.01 (Bürkner, 2017). We assessed whether model assumptions yielded good approximations of the data generating process with posterior predictive checks. We identified no problems in any model resulting from Bayesian fraction of missing information or divergent transitions. Models were compared using widely applicable information criterion (Watanabe, 2010), the Bayesian extension of AIC, Bayesian expected log pointwise predictive densities (Vehtari et al., 2017) and approximate leave-one-out cross-validation information criterion (Tables S3 and S4; Supporting Information).

# RESULTS

## Data summary

In the individual-level dataset, mean [Hb] was 18.84 g/dL (±1.65), mean Hct was 58.54% (±5.15), mean TRBC was 6.37 RBC × $10^6$/$mm^3$ or 6,370,000 cells per μl (±1.16), mean MCV was 93.36 fl (±17.1), mean MCH was 30.4 pg (±5.64) and mean MCHC was 32.44 g/dL (±2.12). Complete reference values—the first such values published for >90% of our study species—are reported in detail in Table S5.

## Within-species blood trait–environment variation

Based on the top-performing model in each set, we found that for every 1000-m increase in elevation, [Hb] is expected to increase by 0.39 g/dL (95% CI 0.12–0.67), Hct is expected to increase by 1.42 percentage points (95% CI 0.55–2.29), TRBC is expected to increase by 310,000 cells per μl (95% CI 60,000–550,000) and MCH is expected to decrease by 0.48 pg (95% CI −1.2 to 0.27 pg; Table S6). Elevation strongly predicted [Hb], Hct, TRBC and MCH (Figures 2a–f, 3a–f). Elevational position was a strong predictor of Hct, indicating that individuals located higher in their species' elevational range tended to have higher Hct (Figure 3b). [Hb], TRBC and MCH also increased subtly with increasing elevational position (Figure 3a,c,e). TRBC increased with elevation; along with MCV, it was a key driver of [Hb] adjustment (Figures 2, 4). MCV did not vary predictably in linear models (Figure 2d). MCV and MCH decreased in individuals occurring in warmer environments (Figure 3d,e), and MCHC decreased in individuals occurring at warmer localities across a species' range (Figure 3f).

Models that included the species grouping variable fit substantially better than those without; this effect explained 13–21% of the variation in the top model, depending on the set (Tables S3, S6; Supporting Information). Species-typical Hb $\beta^A13$–$\beta^A83$ genotype did not predict [Hb] or any other blood characteristics (Figure 3a–f).

## Among-species blood trait–environment variation

Among-species findings for elevational effects were similar to within-species findings (Figure 3). Based on the top model in each set, for every 1000-m increase in elevation, [Hb] is expected to increase by 0.29 g/dL (95% CI 0.13–0.46), and Hct is expected to increase by 1.55 percentage points (95% CI 0.54–2.58). Elevation was a consistently strong predictor of [Hb], Hct and TRBC, all of which increased with increasing elevation (Figures 2g–i, 3a–c). We found that the quadratic effect of elevation was strongly important for MCV and MCH (Figure 2j,k; Figure 3d,e). Neither species-typical Hb $\beta^A13$–$\beta^A83$ genotype, body mass, environmental temperature, nor precipitation explained variation in any species-mean blood characteristic (Figure 3).

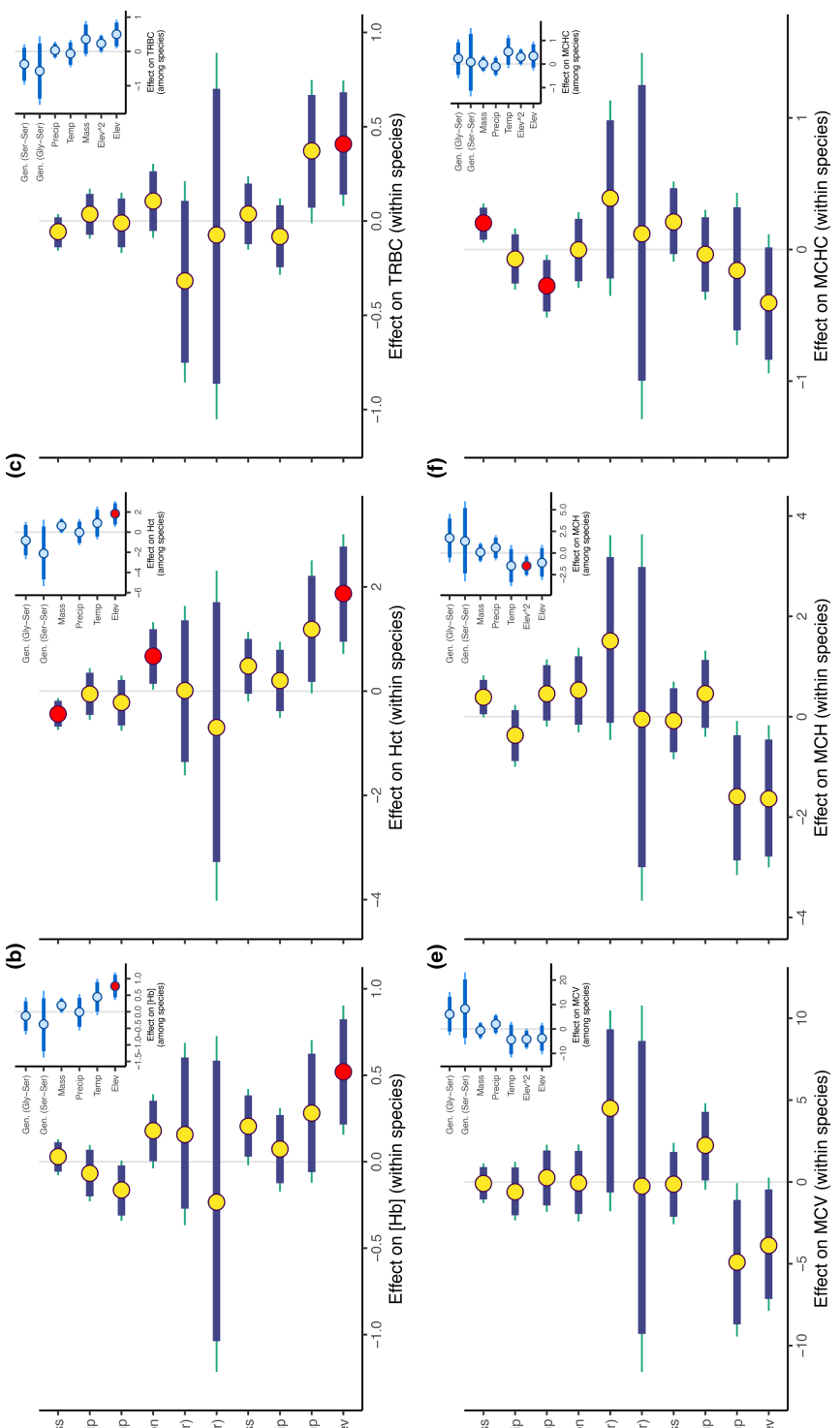


**FIGURE 3** Posterior probabilities and credible intervals from full within-species (main plots) and among-species (insets) models for each of six blood traits: (a) haemoglobin concentration ([Hb]), (b) haematocrit (Hct), (c) total red blood cell (TRBC), (d) mean cell volume (MCV), (e) mean cell haemoglobin (MCH) and (f) mean cell haemoglobin 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 and S6 for model comparison and parameter estimates from top models in other model sets.

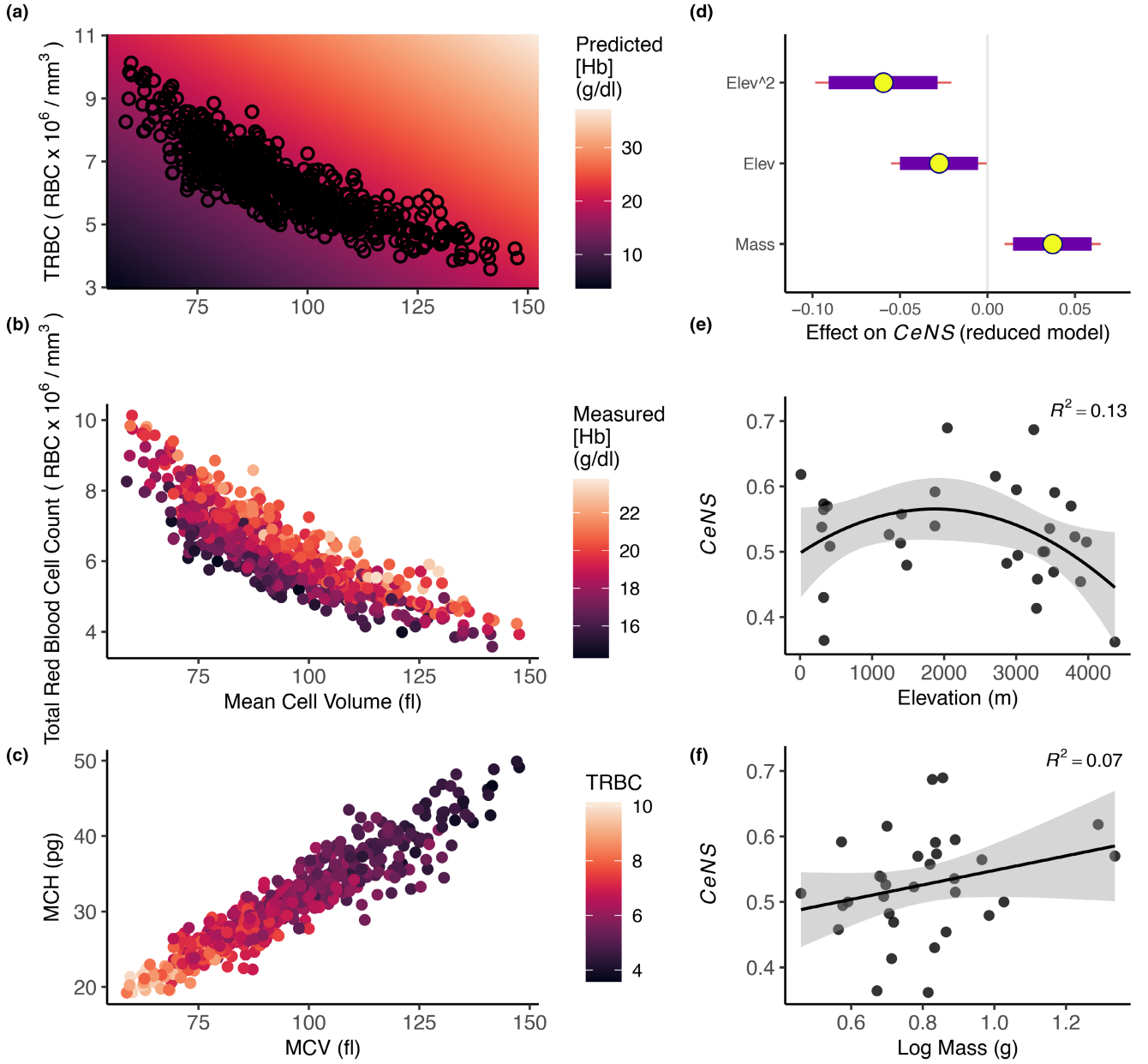


**FIGURE 4** Theoretical and empirical relationships among hummingbird blood parameters underlying haemoglobin concentration ([Hb]) adjustment and predictors of the Cell Number-Size (*CeNS*) Index. (a, b) Hummingbirds have evolved optimized blood–$O_2$-carrying capacity, achieved by balancing total red 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 coloured by [Hb], with lighter colours representing higher concentrations. (c) Mean cell haemoglobin (MCH) and MCV are tightly linked, suggesting little variance in mean cell haemoglobin concentration (MCHC). Points are coloured by TRBC (units presented on the y-axis), with lighter colours representing higher erythrocyte counts. In panels (a–c), each point represents a value from a single individual. (d) Posterior probabilities and credible intervals from the best fitting reduced *CeNS* Index model. (e) The *CeNS* Index 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. (f) The *CeNS* Index increases with increasing body mass. In panels (e–f), $R^2$ values are reported from generalized linear models of each $x$ regressed on $y$, and thus do not account for other modelled variables.

## Mechanisms of blood–$O_2$-carrying capacity adjustment

The axis of variation in cell number versus size space is tightly constrained (Figure 4a,b). Models demonstrated that hummingbirds, overall, co-equally modulated cell number and size to adjust [Hb] (Table S4). Species-specific *CeNS* Index values ranged from 0.36 to 0.69 ($\bar{x}$=0.52), with higher values indicating a proportionally greater contribution of TRBC than MCV in adjusting [Hb]; conversely, lower values indicated a proportionally greater contribution of MCV than TRBC in adjusting [Hb]. Species-specific *CeNS* Index values were strongly predicted by elevation (negative effect),

*elevation*$^2$ (negative effect) and body mass (positive effect; Figure 4d–f; Figure S3; Table S7).

### Hummingbirds at extreme elevations

Hummingbirds sampled from extreme high elevations had significantly smaller cells and higher erythrocyte counts than others (Figure S4). Our breakpoint analysis identified no consistent thresholds across which blood traits shifted; breakpoint estimates for various blood traits ranged from ~1400 to ~4000 m (Figure S5; Supporting Information).

### Phylogenetic signal in blood traits

Phylogenetic signal was low ($\lambda = 0.0$; $K$ range = 0.14–0.21) across all blood traits for both within- and among-species models. Blomberg's K and Pagel's λ results were consistent with visual assessment of continuous trait maps, even after removing species with <3 individuals sampled (Table S2; Figure S1). We found modest phylogenetic signal in the *CeNS* Index (Figure S6), but phylogeny was not an important predictor of variation. We found no interaction of clade and elevation on the six studied blood traits, with the exception of the interaction of elevation and the Hermit clade in the [Hb] model set (Figure S7; Supporting Information).

## DISCUSSION

### Within-species blood trait–environment variation

Our findings that [Hb], Hct and TRBC generally increase with elevation is consistent with theoretical expectations for $O_2$-carrying capacity of blood and with prior empirical findings in birds (Barve et al., 2016; Dubay & Witt, 2014; Minias, 2020; Williamson & Witt, 2021b; Yap et al., 2019), humans (Beall, 2006; Beall et al., 1998), mammals (Hammond et al., 2001; Tufts et al., 2013) and other taxa (González-Morales et al., 2017; Hadley & Burns, 1968; Lu et al., 2015). In prior work, substantial variation in the slopes of these parameters across elevation may have been attributable to variation in temporal, taxonomic and spatial scales of comparison, as well as constraints on the time course of evolutionary adaptation (Beall, 2006; Dawson et al., 2020; Projecto-Garcia et al., 2013). Increased total mass of Hb enhances oxygenation under lower ambient $PO_2$ (Scott & Milsom, 2006). Unless there is a proportional increase in plasma volume or MCHC, such an increase in total Hb mass is expected to be accompanied by an increase in the proportion of blood comprised of red blood cells; as a result, increased Hct is expected as elevation increases. Although increasing within-cell [Hb], or MCHC, would seem to be an alternative mechanism of increasing $O_2$-carrying capacity, MCHC was less variable than other blood parameters (Table S5), likely due to physical constraints (Fischer & Fischer, 1983; Rose, 1971); accordingly, the mean amount of haemoglobin per cell (MCH) was strongly correlated with MCV (Table S8; Figure 4c). Contrary to recent empirical findings (Barve et al., 2016; Dubay & Witt, 2014), adjustments to MCHC appeared to be neither substantial nor predictable with respect to elevation within or among species (Figure 2f,l).

Our finding that increasing temperatures resulted in decreased blood values for several parameters was consistent with previous experimental findings: Comparisons of juvenile broiler chickens reared from ~1 to 7 weeks of age in cool, ambient and hot environments showed decreases in [Hb], Hct, MCV and TRBC as temperature increased (Donkoh, 1989; Moye et al., 1969). These findings add to an intriguing pool of evidence that warmer ambient temperatures may lead to developmental reductions in $O_2$-carrying capacity of blood.

Moderate effects of the species grouping variable suggest that variation in blood traits is partly explained by aspects of species biology that evolve too fast to have resulted in phylogenetic signal. The fact that species-typical Hb $\beta^A13$–$\beta^A83$ genotype did not predict [Hb], nor any other blood characteristics (Figure 3a–f), suggests that evolutionary adjustments to $O_2$-binding affinity do not affect the physical composition of blood nor its variation with elevation.

### Among-species blood trait–environment variation

Some blood-elevation relationships were consistent across within- and among-species scales. Rates of [Hb] and Hct change with elevation were nearly identical within and among species, consistent with scale independence, and with a shared solution to the same environmental challenge, regardless of the degree of evolutionary divergence. Other blood-elevation relationships were unique to the among-species scale of comparison: The quadratic effect of elevation for MCV and MCH, which leant a humped shape to the species-level data, suggests that middle-elevation species and high-elevation species differ qualitatively in their haematological responses to hypoxia (Figures 2j,k, 3d,e).

### Mechanisms of blood–$O_2$-carrying capacity adjustment

The 'banana' shape of the cell number versus size relationship demonstrates that the highest levels of

blood–$O_2$-carrying capacity per unit blood are achieved at high values of TRBC or MCV, respectively. Our data support this tightly constrained trade-off (Figure 4a,b), mirroring patterns demonstrated across vertebrate classes (Hawkey et al., 1991; Wintrobe, 1933). Individual hummingbirds can possess few large erythrocytes or many small erythrocytes, but cell number and size must be balanced (Hawkey et al., 1991). Because of the proportionately greater surface area for gas exchange, having many small cells may be advantageous for fast metabolism, or, when the $PO_2$ in inspired air is low (Hawkey et al., 1991). Similarly, having large cells may be advantageous for modulating the time course of gas exchange (Glomski & Pica, 2011). To shed light on this trade-off, we tested whether individuals and species, respectively, adjust $O_2$-carrying capacity per unit blood primarily through increases in TRBC or MCV. We found that hummingbirds use both mechanisms coequally to adjust [Hb].

To examine how individual species vary with respect to [Hb] adjustment, we developed the *CeNS* Index, a 0–1 measure of the relative contribution of cell number versus size to variation in [Hb]. The results uncovered a fascinating biogeographical pattern. We discovered that differences in the mechanisms through which species adjusted [Hb] depended on where they occur along the Andean elevational gradient. Low- and high-elevation species, respectively, relied more on shifts in cell *size*, whereas middle-elevation species relied more on shifts in cell *number* to modulate [Hb]. Our results highlight a likely proximate explanation for these striking patterns, and one that may hint at underlying, ultimate mechanisms (Figure 4e). The *CeNS* index was uncorrelated with TRBC coefficients ($R^2 = 0.07$, $r = -0.27$) but strongly correlated with MCV coefficients ($R^2 = 0.51$, $r = -0.71$), indicating that variation in the *CeNS* Index was primarily driven by differential reliance on cell size adjustment (Figure S3, Figure S8). Because low- and high-elevation species tend to possess relatively small cells, we posit that they may be more able to rely on shifts in cell size, rather than number, to increase [Hb] (Figures 2j, 4). However, as cell size increases, species may approach a maximum size for erythrocytes, beyond which they may incur a functional detriment. As a result, species with larger red blood cells were more reliant on cell number for [Hb] adjustments. For middle-elevation hummingbird species that have evolved under moderate (~15–30%) reductions in $O_2$ availability, TRBC appears to be the primary axis for adjustment of blood–$O_2$-carrying capacity per unit volume of blood. Species that have evolved at the highest elevations are expected to be the best adapted to hypobaric hypoxia, and it is therefore remarkable that their mode of [Hb] adjustment (primarily by MCV) resembles that of species in the lowlands, the ancestral elevation for hummingbirds (McGuire et al., 2014).

## Hummingbirds at extreme elevations

Our models encompassed the entire elevational gradient, and it is possible they could have missed important phenomena, such as threshold effects. To address this, we compared individual hummingbirds sampled above and below 4200 m, a threshold that marks the upper ~10% of the Andean elevational gradient (Figures S4, S5). Our finding that high-altitude specialist hummingbirds tended to have smaller cells is consistent with findings from other high-altitude vertebrates (Lu et al., 2015; Ruiz et al., 1989; Tufts et al., 2013; Yamaguchi et al., 1987; Zhang et al., 2007). However, this pattern was only detectable at extreme high elevations. Lack of consilience in 'breakpoint' threshold estimates suggests that trait-specific thresholds are challenging to identify and may not be generalizable across species and clades.

## Phylogenetic signal in blood traits

Low phylogenetic signal in blood traits within and among hummingbird species was surprising given demonstrated, phylogenetically conserved Hb genetic adaptations to elevation (Natarajan et al., 2016; Projecto-Garcia et al., 2013) and in light of strong phylogenetic signal reported from recent comparative blood trait analyses with broader taxonomic sampling (Minias, 2020; Yap et al., 2019). However, diverse avian clades likely possess different blood trait optima, and this may have driven phylogenetic signal in previous comparative analyses including multiple families or orders. For example, Minias (2020) reported that small passerines had consistently high [Hb] and Hct, and that including the shorebird order, Charadriiformes, characterized by high [Hb] but medium Hct, strongly influenced patterns of phylogenetic conservatism. Our findings are consistent with steady evolution of trait optima by a mean-reverting, Ornstein–Uhlenbeck process, as described by Minias (2020). Such a process would be marked by occasional major jumps that coincide with changes in body plan or respiratory demands, but there were simply no such changes in bauplan or lifestyle during hummingbird evolution.

That hummingbirds vary only subtly in aspects of their biology, such as metabolic rate, energy budget, torpor use, mode of locomotion, foraging strategy or clutch size, is a strength of our study because those variables could otherwise confound analyses. The moderate effect of species identity that we uncovered hints that species optimize $O_2$-carrying functions of blood for subtle changes in lifestyle and environment, and on rapid timescales, a result consistent with the rapid diversification of human haematological responses to elevation (Beall, 2006, 2007; Beall et al., 1998; Brutsaert et al., 2000; Grimminger et al., 2017; Guo et al., 2017).

## Conclusions

Across ~22 Mya of hummingbird evolution, ambient $O_2$ availability and species idiosyncrasies—not phylogenetic affinities—were the primary drivers of variation in blood traits affecting $O_2$-carrying functions. In response to elevational changes in $O_2$ partial pressure, hummingbirds make predictable adjustments to the physical composition of blood. Remarkably, decreasing $PO_2$ drove increases in [Hb] and Hct that were nearly identical in slope within and among hummingbird species, respectively. This scale-independent trait–environment association implies that developmental and evolutionary optimization of blood traits to ambient $PO_2$ occurs in fundamentally similar ways, responding to the same set of physical rules that govern gas exchange at the blood–gas barrier. In addition to $O_2$ availability, species identity itself explained 13–21% of blood trait variation, suggesting that fast-evolving, unmodeled aspects of species biology underpin substantial haematological variation. Our data revealed a striking constraint space that defines the cell number versus size (*CeNS*) trade-off for hummingbird erythrocytes; within this space, individuals and species co-equally modulated cell number and size to adjust [Hb]. However, species varied in their proportional reliance on adjustments to *CeNS* to increase [Hb], and both elevation and body mass were strongly predictive of species' tendencies. Middle-elevation species that have been evolving under moderate hypoxia adjusted [Hb] primarily by adjusting cell number; in contrast, species at lower or higher elevations tended to adjust [Hb] via cell size, and the latter species also exhibited smaller cells. These results suggest that evolutionary solutions to reduced $O_2$ availability can differ qualitatively in conjunction with evolutionary histories of exposure to hypoxia challenges of varying severity. In this way, our results demonstrate that species adaptation alters predictable trait–environment relationships in some cases but not in others.

### 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 analysed the data and made tables and figures; JLW, JAM, RD and CCW acquired funds and resources; and JLW and CCW wrote the paper with input from all authors.

### ACKNOWLEDGEMENTS

We thank Luis Alza, Christopher Barger, Selina Bauernfeind, Matthew Baumann, Elizabeth Beckman, Phred Benham, José Miguel Bogdanovich, Francisco Bozinovic, Homan Castillo Benitez, Jessica Castillo, Marlon Chagua, Jennifer Clark, Mariela Combe, Lida Crooks, Robert Driver, Shane DuBay, Margarita Espinoza, L. Monica Flores, Chauncey Gadek, Ariel Gaffney, Spencer Galen, Avia González-Méndez, Paige Handley, Zachary Hanna, Mike Hartshorne, José Ernesto Huaroto Tornero, Andrew Johnson, Matthew Jones, Daniel Lane, LyAndra Lujan, Mattías Marzfeld, Sabrina McNew, Kyana Montoya, Jano Nuñez-Zapata, Iris Olivas, Paloma Ordoñez, Alessandra Quiñonez, Javier Reinoso, Natalia Ricote, C. Gregory Schmitt, Donna Schmitt, C. Jonathan Schmitt, Frank Solano Bravo, Dora Susanibar, William Talbot, Jorge Tiravanti C., Abraham Urbay T., Thomas Valqui, Walter Vargas Campos, Karen Verde-Guerra, Natalie Wright and Alfredo Zelada. We are grateful to the following communities in Peru for site access: San Pedro de Casta, Comunidad Campesina Santiago de Carampoma, Ocros, Macate, Oncoy, Yanahara, Huacarpay, Sianbal, Plataforma, Agua Azul, Incahuasi and Anchicha. This research was funded by the National Science Foundation (DEB-0543556, DEB-1146491, DBI-1907353, and DBI-2208924), Cornell Lab of Ornithology (an Edward W. Rose Postdoctoral Fellowship), and research grants to JLW from the Nuttall Ornithological Club (Blake-Nuttall Fund Grants), American Philosophical Society (Lewis & Clark Fund Grant), Explorers Club (Exploration Fund Grant), American Museum of Natural History (Frank M. Chapman Memorial Grants), UNM Biology Graduate Student Association, UNM Graduate and Professional Student Association, UNM Latin American & Iberian Institute, and UNM Department of Biology (Grove, Melinda Bealmear Memorial, and Dr. Jones & Dr. Wong Scholarships). Permits in Chile were granted by Servicio Agrícola y Ganadero (SAG; RE No°s: 7593/2016, 6817/2017, 7903/2018, 6691/2018, 6692/2018, 6693/2018, 6694/2018). Permits in Peru were granted by Servicio Nacional Forestal y de Fauna Silvestre (SERFOR; RDG No°s: 004-2007-INRENA-IFFS-DCB, 135-2009-AG-DGFFS-DGEFFS, 0377-2010-AG-DGFFS-DGEFFS, 0199-2012-AG-DGFFS-DGEFFS, and 006-2013-MINAGRI-DGFFS/DGEFFS, 244-2020-MINAGRI-SERFOR/DGGSPFFS-DGSPFS). UNM IACUC approval was granted under protocols 16-200596-MC and 19-200804-MC.

### CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

### PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ele.14235.

### DATA AVAILABILITY STATEMENT

Data are archived on Dryad (https://doi.org/10.5061/dryad.mkkwh714z). Analysis code is available on GitHub: 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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## REFERENCES


Agrawal, A.A. (2020) A scale-dependent framework for trade-offs, syndromes, and specialization in organismal biology. *Ecology*, 101, 1–24.

Altshuler, D.L., Dudley, R. & McGuire, J.A. (2004) Resolution of a paradox: hummingbird flight at high elevation does not come without a cost. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 17731–17736.

Barve, S., Dhondt, A.A., Mathur, V.B. & Cheviron, Z.A. (2016) Life-history characteristics influence physiological strategies to cope with hypoxia in Himalayan birds. *Proceedings of the Royal Society B*, 283, 1–8.

Beall, C.M. (2006) Andean, Tibetan, and Ethiopian patterns of adaptation to high-altitude hypoxia. *Integrative and Comparative Biology*, 46, 18–24.

Beall, C.M. (2007) Two routes to functional adaptation: Tibetan and Andean high-altitude natives. *Proceedings of the National Academy of Sciences*, 104, 8655–8660.

Beall, C.M., Brittenham, G.M., Strohl, K.P., Blangero, J., Williams-Blangero, S., Goldstein, M.C. et al. (1998) Hemoglobin concentration of high-altitude Tibetans and Bolivian Aymara. *American Journal of Physical Anthropology*, 106, 385–400.

Birds of the World. (2022) In: Billerman, S.M., Keeney, B.K., Rodewald, P.G. & Schulenberg, T.S. (Eds.). Ithaca: Cornell Laboratory of Ornithology. Available from: https://birdsoftheworld.org/bow/home [Accessed 15th March 2023].

Blackburn, T.M., Gaston, K.J. & Loder, N. (1999) Geographic gradients in body size: a clarification of Bergmann's rule. *Diversity and Distributions*, 5, 165–174.

Borras, A., Cabrera, J. & Senar, J.C. (2010) Hematocrit variation in response to altitude changes in wild birds: a repeated-measures design. *The Condor*, 112, 622–626.

Brutsaert, T.D., Araoz, M., Soria, R., Spielvogel, H. & Haas, J.D. (2000) Higher arterial oxygen saturation during submaximal exercise in Bolivian Aymara compared to European sojourners and Europeans born and raised at high altitude. *American Journal of Physical Anthropology*, 113, 169–181.

Buermann, W., Chaves, J.A., Dudley, R., Mcguire, J.A., Smith, T.B. & Altshuler, D.L. (2011) Projected changes in elevational distribution and flight performance of montane Neotropical hummingbirds in response to climate change. *Global Change Biology*, 17, 1671–1680.

Bürkner, P.C. (2017) Brms: an R package for Bayesian multilevel models using Stan. *Journal of Statistical Software*, 80, 1–28.

Campbell, T.W. & Ellis, C.K. (2007) *Avian and exotic animal hematology and cytology*. Ames: Blackwell Publishing Professional.

Dawson, N.J., Alza, L., Nandal, G., Scott, G.R. & McCracken, K.G. (2020) Convergent changes in muscle metabolism depend on duration of high-altitude ancestry across Andean waterfowl. *eLife*, 9, 1–35.

De Queiroz, A. & Ashton, K.G. (2004) The phylogeny of a species-level tendency: species heritability and possible deep origins of Bergmann's rule in tetrapods. *Evolution (N. Y)*, 58, 1674–1684.

de Villemereuil, P. & Nakagawa, S. (2014) Chapter 11: General quantitative genetic methods for comparative biology. In: Garamszegi, L.Z. (Ed.) *Modern phylogenetic comparative methods and their application in evolutionary biology*. Berlin; Heidelberg: Springer-Verlag, pp. 287–303. https://doi.org/10.1007/978-3-662-43550-2_11

Donkoh, A. (1989) Ambient temperature: a factor affecting performance and physiological response of broiler chickens. *International Journal of Biometeorology*, 33, 259–265.

Dubay, S.G. & Witt, C.C. (2014) Differential high-altitude adaptation and restricted gene flow across a mid-elevation hybrid zone in Andean tit-tyrant flycatchers. *Molecular Ecology*, 23, 3551–3565.

Enquist, B.J., Norberg, J., Bonser, S.P., Violle, C., Webb, C.T., Henderson, A. et al. (2015) Scaling from traits to ecosystems: developing a general trait driver theory via integrating trait-based and metabolic scaling theories. In: Pawar, S. & Guy Woodward, A.I.D. (Eds.) *Advances in ecological research*. Waltham: Academic Press (imprint of Elsevier), pp. 249–318.

Fischer, S.L. & Fischer, S.P. (1983) Mean Corpuscular Volume. *JAMA Internal Medicine*, 143, 282–283.

Glomski, C.A. & Pica, A. (2011) *The avian erythrocyte: its phylogenetic odyssey*, 1st edition. Enfield, New Hampshire, USA: Science Publishers.

González-Morales, J.C., Beamonte-Barrientos, R., Bastiaans, E., Guevara-Fiore, P., Quintana, E. & Fajardo, V. (2017) A mountain or a plateau? Hematological traits vary nonlinearly with altitude in a Highland lizard. *Physiological and Biochemical Zoology*, 90, 638–645.

Graham, C.H., Parra, J.L., Rahbek, C. & McGuire, J.A. (2009) Phylogenetic structure in tropical hummingbird communities. *Proceedings of the National Academy of Sciences*, 106, 19673–19678.

Grimminger, J., Richter, M., Tello, K., Sommer, N., Gall, H. & Ghofrani, H.A. (2017) Thin air resulting in high pressure: mountain sickness and hypoxia-induced pulmonary hypertension. *Canadian Respiratory Journal*, 2017, 1–17.

Guo, Y.-B., He, Y.-X., Cui, C.-Y., Zhuluobu, O., Makangzhuo, B., Jizhuoma, D. et al. (2017) GCH1 plays a role in the high-altitude adaptation of Tibetans. *Zoological Research*, 38, 155–162.

Hadley, N.F. & Burns, T.A. (1968) Intraspecific comparison of the blood properties of the side-blotched lizard, Uta stansburiana. *Copeia*, 1968, 737–740.

Hammond, K.A., Szewczak, J.O.E. & Król, E. (2001) Effects of altitude and temperature on organ phenotypic plasticity along an altitudinal gradient. *The Journal of Experimental Biology*, 204, 1991–2000.

Hawkey, C.M., Bennett, P.M., Gascoyne, S.C., Hart, M.G. & Kirkwood, J.K. (1991) Erythrocyte size, number and haemoglobin content in vertebrates. *British Journal of Haematology*, 77, 392–397.

Hijmans, R.J., Cameron, S.E., Parra, J.L., Jones, P.G. & Jarvis, A. (2005) Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, 25, 1965–1978.

Jaramillo, A. (2003) *Birds of Chile*. Princeton, New Jersey, USA: Princeton University Press.

Jordan, D.S. (1891) Relations of temperature to vertebrae among fishes. *Proceedings of the United States National Museum*, 14, 107–120.

Levett, D., Radford, E., Menassa, D., Graber, E., Morash, A., Hoppeler, H. et al. (2012) Acclimatization of skeletal muscle mitochondria to high-altitude hypoxia during an ascent of Everest. *The FASEB Journal*, 26, 1431–1441.

Linck, E.B., Williamson, J.L., Bautista, E., Beckman, E.J., Benham, P.M., DuBay, S.G. et al. (2023) Blood variation implicates respiratory limits on elevational ranges of Andean birds. *The American Naturalist*, 201(5), 741–754.

Lu, S., Xin, Y., Tang, X., Yue, F., Wang, H., Bai, Y. et al. (2015) Differences in hematological traits between high- and low-altitude lizards (genus Phrynocephalus). *PLoS One*, 10, 1–16.

McDowall, R.M. (2003) Variation in vertebral number in galaxiid fishes (Teleostei: Galaxiidae): a legacy of life history, latitude and length. *Environmental Biology of Fishes*, 66, 361–381.

McGlothlin, J.W., Kobiela, M.E., Wright, H.V., Mahler, D.L., Kolbe, J.J., Losos, J.B. et al. (2018) Adaptive radiation along a deeply conserved genetic line of least resistance in Anolis lizards. *Evolution Letters*, 2, 310–322.

McGuire, J.A., Witt, C.C., Remsen, J.V., Corl, A., Rabosky, D.L., Altshuler, D.L. et al. (2014) Molecular phylogenetics and the diversification of hummingbirds. *Current Biology*, 24, 1–7.

Minias, P. (2020) Ecology and evolution of blood oxygen-carrying capacity in birds. *The American Naturalist*, 195, 788–801.

Moye, R.J., Washburn, K.W. & Huston, T.M. (1969) Effects of environmental temperature on erythrocyte numbers and size. *Poultry Science*, 48, 1683–1686.

Natarajan, C., Hoffmann, F.G., Weber, R.E., Fago, A., Witt, C.C. & Storz, J.F. (2016) Predictable convergence in hemoglobin function has unpredictable molecular underpinnings. *Science*, 354, 336–339.

Parker, T.A.I., Stotz, D.F. & Fitzpatrick, J.W. (1996) Ecological and distributional databases. In: Stotz, D.F., Fitzpatrick, J.W., Parker, T.A., III & Moskovits, D.K. (Eds.) *Neotropical birds: ecology and conservation*. Chicago, IL, USA: University of Chicago Press.

Pittman, R.N. (2011) Chapter 4: Oxygen Transport. In: *Regulation of tissue Oxygentation*. San Rafael: Morgan & Claypool Life Sciences.

Projecto-Garcia, J., Natarajan, C., Moriyama, H., Weber, R.E., Fago, A., Cheviron, Z.A. et al. (2013) Repeated elevational transitions in hemoglobin function during the evolution of Andean hummingbirds. *Proceedings of the National Academy of Sciences*, 110, 20669–20674.

Rasband, W.S. (n.d.) ImageJ.

Revell, L.J. (2012) Phytools: an R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution*, 3, 217–223.

Rose, M.S. (1971) Epitaph for the M.C.H.C. *British Medical Journal*, 4, 169.

Ruiz, G., Rosenmann, M. & Veloso, A. (1989) Altitudinal distribution and blood values in the toad, Bufo spinulosus Wiegmann. *Comparative Biochemistry and Physiology. A, Comparative Physiology*, 94, 643–646.

Samour, J. (2005) Diagnostic value of hematology. In: Harrison, G. & Lightfoot, T. (Eds.) *Clinical avian medicine, Volume II*. Palm Beach: Spix Publishing, Inc., pp. 587–610.

Schulenberg, T., Thomas, T. & Stotz, D. (2010) *Birds of Peru: revised and updated edition*. Princeton: Princeton University Press.

Scott, G.R. & Milsom, W.K. (2006) Flying high: a theoretical analysis of the factors limiting exercise performance in birds at altitude. *Respiratory Physiology & Neurobiology*, 154, 284–301.

Simmons, P. & Lill, A. (2006) Development of parameters influencing blood oxygen carrying capacity in the welcome swallow and fairy martin. *Comparative Biochemistry and Physiology. A, Comparative Physiology*, 143, 459–468.

Simonson, T.S., Yang, Y., Huff, C.D., Yun, H., Qin, G., Witherspoon, D.J. et al. (2010) Genetic evidence for high-altitude adaptation in Tibet. *Science*, 329, 72–75.

Skandalis, D.A., Segre, P.S., Bahlman, J.W., Groom, D.J.E., Welch, K.C., Witt, C.C. et al. (2017) The biomechanical origin of extreme wing allometry in hummingbirds. *Nature Communications*, 8, 1–9.

Stembridge, M., Williams, A.M., Gasho, C., Dawkins, T.G., Drane, A., Villafuerte, F.C. et al. (2019) The overlooked significance of plasma volume for successful adaptation to high altitude in Sherpa and Andean natives. *Proceedings of the National Academy of Sciences of the United States of America*, 116, 16177–16179.

Storz, J.F. (2010) Genes for high altitudes. *Science*, 329, 40–41.

Storz, J.F. (2016) Hemoglobin–oxygen affinity in high-altitude vertebrates: is there evidence for an adaptive trend? *The Journal of Experimental Biology*, 219, 3190–3203.

Storz, J.F., Scott, G.R. & Cheviron, Z.A. (2010) Phenotypic plasticity and genetic adaptation to high-altitude hypoxia in vertebrates. *The Journal of Experimental Biology*, 213, 4125–4136.

Suarez, R.K. (1992) Hummingbird flight: sustaining the highest mass-specific metabolic rates among vertebrates. *Experientia*, 48, 565–570.

Tufts, D.M., Revsbech, I.G., Cheviron, Z.A., Weber, R.E., Fago, A. & Storz, J.F. (2013) Phenotypic plasticity in blood-oxygen transport in highland and lowland deer mice. *The Journal of Experimental Biology*, 216, 1167–1173.

Vehtari, A., Gelman, A. & Gabry, J. (2017) Practical Bayesian model evaluation using leave-one-out cross-validation and WAIC. *Statistics and Computing*, 27, 1413–1432.

Watanabe, S. (2010) Asymptotic equivalence of Bayes cross validation and widely applicable information criterion in singular learning theory. *Journal of Machine Learning Research*, 11, 3571–3594.

Williamson, J.L. & Witt, C.C. (2021a) A lightweight backpack harness for tracking hummingbirds. *Journal of Avian Biology*, 52, 1–9.

Williamson, J.L. & Witt, C.C. (2021b) Elevational niche-shift migration: why the degree of elevational change matters for the ecology, evolution, and physiology of migratory birds. *Ornithology*, 138, 1–26.

Wintrobe, M.M. (1933) Variation in the size and hemoglobin content of erythrocytes in the blood of various vertebrates. *Folia Haematologica*, 51, 32–59.

Yamaguchi, K., Jürgens, K.D., Bartels, H. & Piiper, J. (1987) Oxygen transfer properties and dimensions of red blood cells in high-altitude camelids, dromedary camel and goat. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, 157, 1–9.

Yap, K.N., Tsai, O.H.-I. & Williams, T.D. (2019) Haematological traits co-vary with migratory status, altitude and energy expenditure: a phylogenetic, comparative analysis. *Scientific Reports*, 9, 6351.

Zhang, H., Wu, C.X., Chamba, Y. & Ling, Y. (2007) Blood characteristics for high altitude adaptation in Tibetan chickens. *Poultry Science*, 86, 1384–1389.

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Williamson, J.L., Linck, E.B., Bautista, E., Smiley, A., McGuire, J.A., Dudley, R. et al. (2023) Hummingbird blood traits track oxygen availability across space and time. *Ecology Letters*, 26, 1223–1236. Available from: https://doi.org/10.1111/ele.14235