



Elevational and microclimatic drivers of thermal tolerance in Andean *Pristimantis* frogs

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Abstract

Aim: We analysed elevational and microclimatic drivers of thermal tolerance diversity in a tropical mountain frog clade to test three macrophysiological predictions: less spatial variation in upper than lower thermal limits (Bretts' heat-invariant hypothesis); narrower thermal tolerance ranges in habitats with less variation in temperature (Janzen's climatic variability hypothesis); and higher level of heat impacts at lower elevations.

Location: Forest and open habitats through a 4,230-m elevational gradient across the tropical Andes of Ecuador.

Method: We examined variability in critical thermal limits (CTmax and CTmin) and thermal breadth (TB; CTmax–CTmin) in 21 species of *Pristimantis* frogs. Additionally, we monitored maximum and minimum temperatures at the local scale (tmax, tmin) and estimated vulnerability to acute thermal stress from heat (CTmax–tmax) and cold (tmin–CTmin), by partitioning thermal diversity into elevational and microclimatic variation.

Results: Our results were consistent with Brett's hypothesis: elevation promotes more variation in CTmin and tmin than in CTmax and tmax. Frogs inhabiting thermally variable open habitats have higher CTmax and tmax and greater TBs than species restricted to forest habitats, which show less climatic overlap across the elevational gradient (Janzen's hypothesis). Vulnerability to heat stress was higher in open than forest habitats and did not vary with elevation.

Main conclusions: We suggest a mechanistic explanation of thermal tolerance diversity in elevational gradients by including microclimatic thermal variation. We propose that the unfeasibility to buffer minimum temperatures locally may explain the rapid increase in cold tolerance (lower CTmin) with elevation. In contrast, the relative invariability in heat tolerance (CTmax) with elevation may revolve around the organisms' habitat selection of open- and canopy-buffered habitats. Secondly, on the basis of microclimatic estimates, lowland and upland species may be equally vulnerable to temperature increase, which is contrary to the pattern inferred from regional interpolated climate estimators.



KEYWORDS

amphibians, climatic gradients, critical thermal limits, elevation, Janzen's hypothesis, microclimate variation, *Pristimantis*, tropical mountains

1 | INTRODUCTION

Alexander von Humboldt noted that species were distributed across distinct elevational zones and proposed climate as the primary driver of this pattern (Figure 1; von Humboldt, 1849; von Humboldt & Bonpland, 2009). After Humboldt's elevational zonation hypothesis, several patterns have been described relating climate with species geographical distribution (Chown & Gaston, 2016; Gaston et al., 2009). Some of those patterns pertain variation in critical thermal limits (CT_{max} and CT_{min}) which delimit the temperature range within which ectotherms can perform, disperse and survive. Thermal limits, thus, determine their fundamental niche and, therefore, influence species potential distribution (Angilletta, 2009).

Tropical mountains are amenable to study patterns of variation in thermal limits because reduced seasonality leads to lower overlap in temperatures across elevation bands compared to temperate mountains (Figure 1). This climate stratification is predicted to cause physiological barriers through specialization to narrow climatic conditions (Janzen, 1967; Sheldon, Huey, Kaspary, & Sanders, 2018; Stevens, 1989). As a consequence, thermal limits are expected to evolve by thermal adaptation in order to fit the temperature extremes to which organisms are exposed (Ghalambor, Huey, Martin, Tewksbury, & Wang, 2006; Kingsolver & Buckley, 2017). The main aim of this research is to examine three hypotheses of variation in thermal limits at large geographical scales (Chown & Gaston, 2016; Sheldon et al., 2018). First, that upper thermal limits are less spatially variable than lower thermal limits at a range of scales: Brett's heat-invariant hypothesis (Bozinovic, Orellana, Martel, & Bogdanovich, 2014; Brett, 1956). This hypothesis predicts a stronger response of cold tolerance limits than heat tolerance limits with increasing elevation and latitude (Bishop, Robertson, Van Rensburg, & Parr, 2017; Gaston & Chown, 1999; von May et al., 2017; Muñoz et al., 2014; Sunday et al., 2014). The lower spatial variation in heat tolerance is argued to be due to intrinsic and extrinsic mechanisms. An intrinsic mechanism is the constrained evolutionary potential of CT_{max} (Beacham & Withler, 1991; Blackburn, van Heerwaarden, Kellermann, & Sgrò, 2014; Grigg & Buckley, 2013). An extrinsic mechanism is the apparent greater potential for behavioural thermoregulation to face heat rather than cold stress (Muñoz & Bodensteiner, 2019; Muñoz et al., 2014). However, most times, this pattern may simply be the consequence of the use of macroclimate data lacking resolution to quantify the thermal heterogeneity faced by organisms (Gutiérrez-Pesquera et al., 2016; Potter, Woods, & Pincebourde, 2013; Sunday et al., 2014). Recent studies have shown high variability in upper thermal tolerance in lowland tropical environments where heat impacts are expected, especially in open microhabitats where forest

canopy buffers are absent (Duarte et al., 2012; Gutiérrez-Pesquera et al., 2016; Huey et al., 2009; Kaspary, Clay, Lucas, Yanoviak, & Kay, 2015). Forests, especially in the tropics, host a variety of microhabitats with lower temperatures relative to adjacent open areas (Bader, Rietkerk, & Bregt, 2007; Frishkoff, Hadly, & Daily, 2015; Jose, Gillespie, George, & Kumar, 1996). These microhabitats provide protection against heat impacts (Scheffers, Edwards, Diesmos, Williams, & Evans, 2014). Thus, thermal habitat heterogeneity can potentially become an important selective source in the evolution of thermal physiology, and therefore, any spatial analysis of variation in thermal limits must consider how much of its overall diversity is attained by local-scale drivers.

Given their thermal buffering capacity, there is less thermal variability in forests than in open environments, and as a result, forest ectotherms should exhibit narrower thermal breadths (TBs) than open-habitat species throughout the elevational gradient (Janzen, 1967). As a consequence, we can predict that physiological barriers to dispersal will be stronger for thermally specialized forest species, and as a corollary, forest species will have narrower elevational ranges than open-habitat species (Ghalambor et al., 2006; Sheldon et al., 2018). Janzen's hypothesis pertains latitudinal comparisons between mountains (Gill et al., 2016; Polato et al., 2018). Our hypothesis is derived from the same underlying physiological principle; species are adapted to temperatures normally encountered in their temporal and geographical habitat or microhabitat (Janzen, 1967). Similar hypothesis has been proposed for a vertical climatic gradient in rain forest (Scheffers & Williams, 2018), and circadian climatic gradients with contrasting daytime and night-time thermal variability (Muñoz & Bodensteiner, 2019).

Finally, greater heat impacts are expected to occur in low elevational and latitudinal areas as species inhabiting these environments experience temperatures closer to their upper tolerance limits (Colwell, Brehm, Cardelús, Gilman, & Longino, 2008; Diamond et al., 2012). Consequently, these species are predicted to be forced to disperse upwards or, in the absence of mountains, disperse latitudinally in order to match their historical climates (Bonebrake & Deutsch, 2012; Colwell et al., 2008; Loarie et al., 2008). Alternatively, tropical organisms could persist under current global warming in situ by selecting adjacent thermally buffered habitats (Suggitt et al., 2018). Additionally, the intense habitat loss and fragmentation of tropical forests are changing microclimates within forest remnants that may exacerbate the risk posed by global warming for those species adapted to local thermal and hydric forest conditions (Tuff, Tuff, & Davies, 2016).

We examine these predictions by partitioning drivers of thermal tolerance diversity into local habitat versus elevational gradients in peak temperatures in the species-rich *Pristimantis* frogs

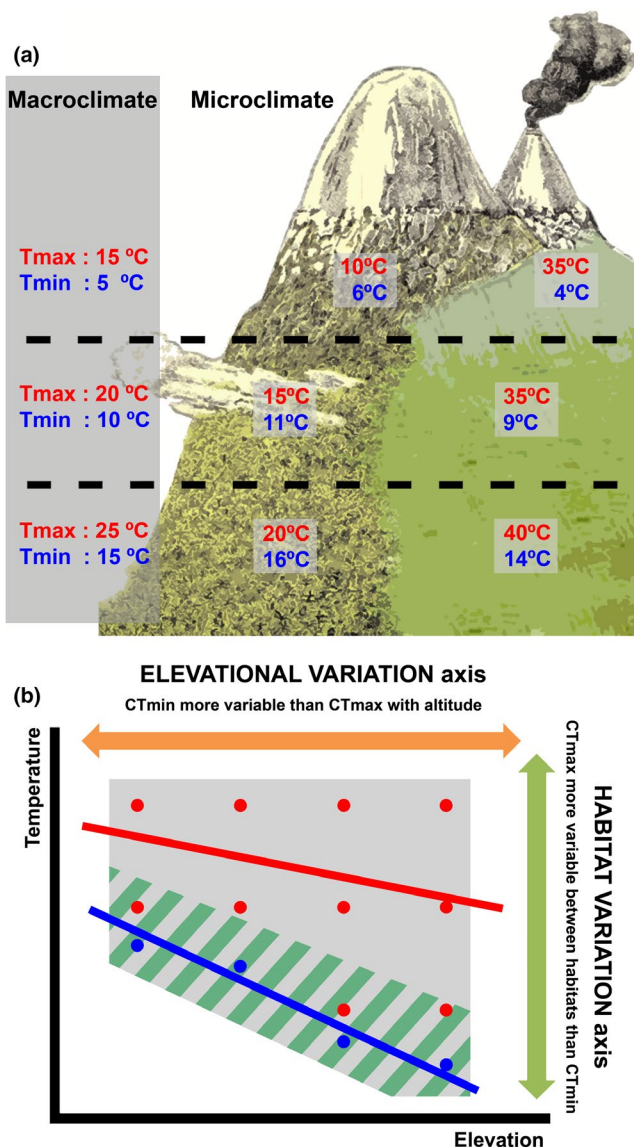


FIGURE 1 Hypothetical variation of extreme temperatures; tmax (red) and tmin (blue), along a tropical elevational gradient, modified from von Humboldt and Bonpland's (2009). (a) The traditional view of thermal variation in tropical mountains is seen as non-overlapping ranges of temperatures through elevation. This is the pattern found when examining macroenvironmental estimates of temperature, obtained from WorldClim, that basically follow a lapse rate variation. However, this pattern of climatic bands need not coincide with the patterns of microclimatic variation (forest vs open habitats) that possibly better reflects the operative temperatures to which organisms are actually exposed. (b) Temperature variation in tropical mountains through elevational and habitat axes. Macrophysiology predicts (1) an elevational axis with an asymmetric pattern of variation between critical thermal limits with a more variable CTmin (and their climatic predictor tmin) (Brett's heat-invariant hypothesis). (2) The axis of habitat variation determines a reversed second asymmetry, with CTmax and tmax being locally more variable between habitats than CTmin and tmin. Habitat thermal heterogeneity with higher tmax in open environments will fuel greater CTmax, determining the presence of thermal generalist organisms with broader thermal breadth (grey frame) than in less thermally variable forest, inhabited by thermal specialists with narrow TBs (green-striped frame) (Janzen's hypothesis, thought 3 in Janzen 1967 and assumption 2, in Sheldon et al. 2018) [Colour figure can be viewed at wileyonlinelibrary.com]

included forest *Pristimantis* species; thus, we build on this previous work by considering microhabitats as potential drivers of thermal tolerance diversity.

2 | MATERIAL AND METHODS

2.1 | Study sites, thermal variability and habitats

We sampled adult *Pristimantis* frogs covering their entire elevational range within the Ecuadorian Andes (between latitudes 1°N and 4°S and elevations 23 and 4,130 masl) and their main habitats (forest and open land) from November 2014 to March 2017 to examine the overall range of thermal limits of the clade. In order to match species' thermal limits and extreme cold and heat peak temperatures to what each *Pristimantis* frog is actually exposed to, we characterized microclimatic shelter temperature where frogs were sampled ($N = 9$ open and $N = 7$ forest locations, respectively) (see Appendix S3 and Appendix S1: Table S1). We used HOBO Pendant temperature dataloggers to obtain continuous recordings of shelter temperature (every 15 min). In some instances, we could not recover all the loggers due to landslides or other unexpected losses (ca. 25%). In these cases, we used data from loggers located in the same habitat and at similar elevations to the missing loggers (see correspondence in Tables S1 and S2). Although *Pristimantis* frogs occupy diverse microhabitats (Carvajalino-Fernández, Bonilla, & Navas, 2011; Navas, 1996; Navas, Carvajalino-Fernández, Saboyá-Acosta, Rueda-Solano, & Carvajalino-Fernández, 2013), we set one logger per site within shelters used by frogs during the daytime (inside bromeliads and underneath rocks or fallen leaves or logs). We assumed that these shelters are probably being selected by frogs in order to avoid

(Strabomantidae), with roughly 520 species (AmphibiaWeb, 2018), mostly distributed through the tropical Andes, ranging from lowland rain forest to the cold páramos up to 4,500 masl (Hedges, Duellman, & Heinicke, 2008; Meza-Joya & Torres, 2016). These terrestrial breeding frogs are nocturnal thermoconformers with low thermoregulatory ability (Navas, 1996, 1997) and are usually distributed in small elevational ranges (Bernal & Lynch, 2008), thus are potentially exposed to a narrow range of operative temperatures. In addition, they occupy a wide array of forested habitats, such as moist lowland and montane forest, but are also widespread in open habitats – pasture, grassland and páramos (Lynch & Duellman, 1997). Former studies in Strabomantidae frogs revealed elevational variation in CTmax (Catenazzi, Lehr, & Vredenburg, 2014; Christian, Nunez, Clos, & Diaz, 1988; Heatwole, Mercado, & Ortiz, 1965), and more recently, von May et al. (2017) showed elevational variation in thermal tolerance limits, with faster rates of change for cold than for heat tolerances, with lowland species being more prone to suffer heat impacts. The latter study only



extreme heat and cold peak temperatures, and hence, these shelter temperatures will better represent the body temperature of frogs at extreme thermal conditions than air temperatures (Carvajalino-Fernández et al., 2011; Navas et al., 2013). We assigned species elevational range and habitat type into two categories, either forest (for forest-restricted species) or open habitats (for open exploiters or generalist species) (e.g. grasslands, potreros, páramos) obtained from our field surveys and complemented with publications and well-supported observations from museums (see Supplementary information in Appendix S3 and Appendix S1: Table S2). For each frog shelter datalogger, we obtained three microclimatic thermal variables: mean temperature (tmean), minimum temperature (tmin) and maximum temperature (tmax). The difference between tmax and tmin gives the absolute range of temperature, tr. Additionally, we also gathered macroclimatic air temperature measurements for the same coordinates where the loggers were located by extracting the following thermal variables: bio1-TMEAN, bio5-TMAX and bio6-TMIN (see Table S1) from the WorldClim layers. These air temperatures are usually taken in shade at 1 to 2 m height and interpolated with a 1-km² spatial resolution (Fick & Hijmans, 2017). Current evidence reveals that these large-scale and readily available air temperatures are loose and uninformative predictors of either the body temperature of active frogs through a tropical elevational gradient (Navas et al., 2013) or amphibian thermal tolerance limits (Gutiérrez-Pesquera et al., 2016).

By assuming that habitats (forest and open) show contrasting level of thermal variability (e.g. relatively narrower temperature range in forest habitats), we predicted less climatic overlap in forest locations than equivalently separated open area localities. *Ceteris paribus*, forest frogs dispersing up or down will more likely encounter habitats with different climates than an open area disperser and therefore would face a stronger physiological barrier to dispersal. In order to test this, we calculated an index of thermal overlap between sampling localities along the elevational gradient, following a modified version of Janzen's (1967) and Buckley, Miller, and Kingsolver's (2013) overlap indexes:

$$\text{overlap} = d / (R_1 R_2)^{1/2}$$

where d is the amount of thermal overlap between sites, as the upland maximum temperature – lowland minimum temperature. R_1 and R_2 are the difference (°C) between tmax and tmin for the upland and lowland sites, respectively. Thus, the metric actually represents a relative climatic overlap. A negative value of d expresses that both localities do not share temperatures at any point.

2.2 | Estimates of critical thermal limits and vulnerability to thermal stress

To determine how thermal limits vary among *Pristimantis* species with elevation and habitat, we measured CTmax and CTmin for 148 individuals (75 CTmax and 73 CTmin) across 21 species from 25 populations. For four species, two distinct populations were examined and, given their large genetic and/or elevational differences,

were treated as independent entities for statistical analysis (see Appendix S2: Figure S1 and Appendix S1: Table S2). We also included a species of the *Craugastor* genus because of its phylogenetic closeness and ecological similarities to *Pristimantis*. The frogs were placed at a constant temperature of 20°C with a photoperiod of 12L:12D for at least 3 days before conducting the tolerance assays, which is enough time to stabilize CTmax and CTmin after a large change in acclimation temperatures between field and laboratory environments (Brattstrom, 1968). Each tested individual was placed in a plastic cup with a thin layer of water (<1 mm) in a 15 l HUBER K15-cc-NR bath at a starting temperature of 20°C. We increased or decreased the temperature at a constant rate of 0.25°C/min using the dynamic method of Lutterschmidt and Hutchison (1997) until the frogs did not respond to any physically stimuli with total immobility. This was defined as the end point because it is reproducible for both thermal limits. At this end point, CTmax and CTmin were measured by touching the individual with a Miller & Weber quick-recording thermometer (to the nearest 0.1°C). Given the small size of these frogs, we assumed that skin temperature is equivalent to body temperature (Navas et al., 2013). After a tolerance limit was determined, we immediately transferred the frogs to a plastic cup with a thin layer of water at the acclimation temperature, allowing for recovery within two hours. After the test, each frog was wet weighed to the nearest 0.001 g. Finally, we calculated the TB as the difference between CTmax and CTmin for each species.

One way to determine whether thermal selection is prone to drive thermal tolerance limits through elevation and between habitats is by assessing the risk of species to suffer heat or cold impacts. An operative metric to estimate the eventual occurrence of acute heat stress is the warming tolerance (i.e. the difference between CTmax and the maximum exposure temperature) taken at both the micro (substrate temperature, tmax)- and the macro (air temperature, TMAX)-climatic scales (Duarte et al., 2012; Gutiérrez-Pesquera et al., 2016; Hoffmann, Chown, & Clusella-Trullas, 2013). Similarly, we can define cooling tolerance as the risk to suffer cold shocks measured as the difference between the minimum exposure temperature, also taken at both micro (substrate, tmin)- and macro (air, TMIN)-climatic scales, and CTmin (Gutiérrez-Pesquera et al., 2016; Sunday et al., 2014).

2.3 | Phylogenetic reconstruction

We obtained a phylogeny for all analysed species based on newly generated DNA sequences for mitochondrial genes 12S rRNA (12S), tRNA-Valine and 16S rRNA (16S). DNA was extracted from muscle or liver tissue preserved in 95% ethanol or tissue storage buffer, using standard phenol–chloroform extraction protocols (Sambrook, Fritsch, & Maniatis, 1989). We used a polymerase chain reaction (PCR) to amplify DNA fragments. PCR was performed under standard protocols, and amplicons were sequenced by the MacroGen Sequencing Team (MacroGen Inc.). The combined DNA matrix had up to 2,608 bp.

The newly generated DNA sequences will be available on GenBank under accession numbers (Table S7). We also included available sequences from GenBank. *Craugastor longirostris*, *Eleutherodactylus atkinsi*, *Leptodactylus melanonotus*, *Mannophryne trinitatis* and *Odontophrynus occidentalis* were included as outgroups. The forward and reverse chromatograms were assembled in GENEIOUS 9.1.8 (Kearse et al., 2012) and edited manually as required. Alignment was done with MAFFT 7.2 software with the L-INS-i algorithm (Katoh & Standley, 2013).

Phylogenetic trees were obtained using maximum likelihood with software GARLI 2.0 (Zwickl, 2006). We made 40 independent searches, 20 starting from random trees and 20 from stepwise addition trees. The number of generations without topology improvement required for termination (genthreshfortopterm) was set to 15,000. Other settings were set to default values. Node support was assessed with 200 pseudoreplicate nonparametric bootstraps (npb), configured with the same settings of the full search, but with two replicates per run.

2.4 | Statistical analyses

To estimate the phylogenetic dependence of the data, we used the Pagel's lambda (Pagel, 1999) and Blomberg's *K* (Blomberg, Garland, & Ives, 2003) approaches in the R package "PHYTOOLS" (Revell, 2012). Pagel's lambda varies from 0 (phylogenetic independence) to 1 (strong phylogenetic signal). On the other hand, *K* varies from 0 to infinite with values <1 indicating that closely related species resemble each other less than expected under the Brownian motion model of trait evolution, while values >1 indicate that closely related species are more similar than predicted by the model.

We also used the function "ratebytree" from the R package "PHYTOOLS" (Revell, 2012) to evaluate whether there are differences in the rate of CTmax and CTmin evolution. This function allows the comparison of phenotypic evolution of continuous traits between trees under different models of evolution: "random walk" (Brownian motion, BM) and the adaptive models Ornstein-Uhlenbeck (OU) and early burst (EB) (Revell et al., 2018). We first fitted the three different models of evolution to each physiological trait (CTmax and CTmin) using the "fitContinuous" function from the R package geiger (Harmon, Weir, Brock, Glor, & Challenger, 2008). We then used the Akaike information criterion corrected (AICc: Sugiura, 1978) for small sample size to identify the best model (Burnham & Anderson, 2002). If OU or EB models were not significantly better ($\Delta AIC < 2$; Burnham & Anderson, 2002) than the simpler model (i.e. BM), we used the simpler model. Once we determined the best evolutionary model for each variable (see Appendix S1: Table S3), we performed the test for the whole dataset. We then repeated the analysis using only the 20 species that had both CTmax and CTmin estimates for consistency purposes.

We used an ANCOVA to test the effect of elevation and habitat on micro- and macroenvironmental temperatures: maximum temperature (tmax, TMAX), minimum temperature (tmin, TMIN) and absolute thermal range for the sampling period (tr = tmax - tmin, TR = TMAX - TMIN). In order to examine whether forest and open

environments have a similar pattern of thermal overlap through elevation, we conducted a homogeneity of slopes test, comparing the slopes of overlap with elevation for both habitats. We ran these analyses for micro- and macroclimatic temperature estimators. To test whether forest and open areas species exhibit contrasting elevational distributional ranges, we conducted a phylogenetic least squares (PGLS) approach to compare species elevational ranges with habitat, using "pgls" function in the R package caper (Freckleton, Harvey, & Pagel, 2002). We also used PGLS analyses to test the effects of elevation and habitat on thermal physiology variables (CTmax, CTmin and TB) and vulnerability risk (warming tolerance and cooling tolerance). We found an effect of body size on thermal tolerance limits. However, the inclusion of weight did not change the conclusions, and hence, it was not included in the models shown here (but see Appendix S1: Tables S4 and S5). To examine the congruence of macro- and microclimatic information as predictors of the examined macrophysiological trend, we evaluated the correlations between thermal physiology variables (CTmax, CTmin and TB) and vulnerability indices (warming and cooling tolerance) with environmental extreme temperatures at both microclimatic substrate (tmax, tmin, tr) and macroclimatic air (TMAX, TMIN, TR) temperatures. Finally, to examine the consequences of intense deforestation on forest species vulnerability to acute heat impacts, we obtained a new index of deforested warming tolerance ($wt_{\text{deforested}} = CT_{\text{max}} - t_{\text{max,deforested}}$), by considering $t_{\text{max,deforested}}$ as the maximum temperatures recorded from the open-habitat loggers closest in elevation. All values in the main text are expressed as mean and standard error. All analyses were done in R (R Core Team, 2014).

3 | RESULTS

We found a strong phylogenetic signal for CTmax ($\lambda = 0.807$; $K = 0.911$) and CTmin ($\lambda = 1$; $K = 1.032$). The Brownian motion (BM) model of evolution was the most supported model for both thermal limits (see Appendix S1: Table S3). We found no differences in the rate of evolutionary change for both thermal tolerance limits under the BM model of evolution, using either the reduced or the whole dataset (only whole dataset results are shown; $\sigma^2_{CT_{\text{max}}} = 14.798$, $\sigma^2_{CT_{\text{min}}} = 20.44$, LRT = 0.571, $p = 0.45$).

Micro- as opposed to macroenvironmental temperatures are better predictors for CTmax and TB; however, for CTmin, both temperature estimates were equally good predictors (see Appendix S1: Table S6). Contrasting patterns of variation of both thermal limits and peak temperatures occurred at elevational and habitat scales (Figure 2). Both CTmax and CTmin decreased with elevation, with a faster decline in CTmin (PGLS, slope \pm SE: CTmin: -0.003 ± 0.0003 ; CTmax: -0.0007 ± 0.0004 ; heterogeneity of slopes test, elevation \times CT[min/max], $F_{1,40} = 10.23$, $p = 0.0027$; Figure 2a, b). Thermal predictors exhibit a similar pattern, with faster decline in tmin (-0.006 ± 0.0003 , Figure 2e) than in tmax (-0.003 ± 0.001 , Figure 2d) (heterogeneity of slopes test for thermal limits: elevation \times temperature

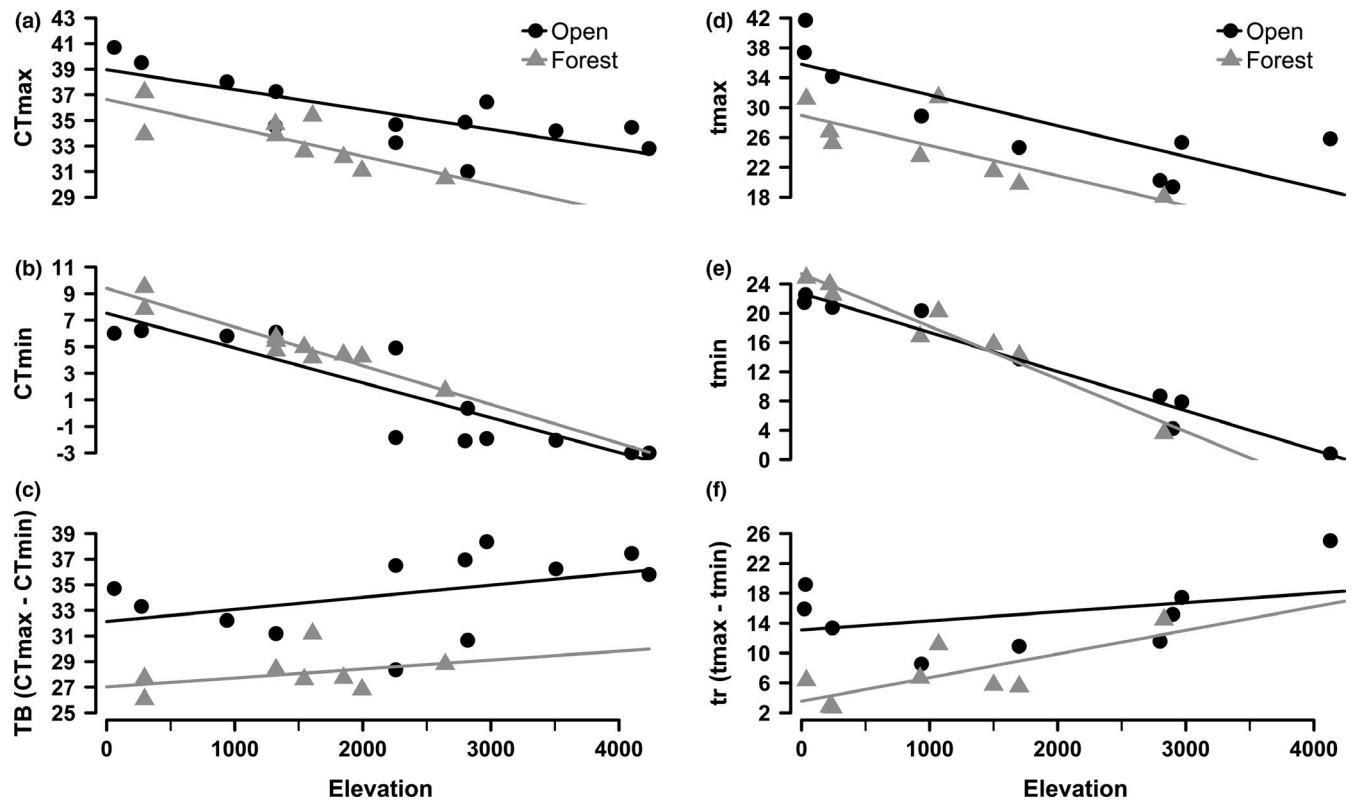


FIGURE 2 Relationship between physiological variables in Ecuadorian *Pristimantis* (CTmax, CTmin and thermal breadth (TB); (a–c) and microenvironmental temperatures (tmax, tmin and absolute thermal range (tr); (d–f) with elevation. Regression lines reflect the significant phylogenetic corrections in each model. Open: Species that can exploit open environments. Forest: Species restricted to forest habitats (see Appendix S1 and S3)

[min/max], $F_{1,40} = 16.83$, $p = 0.0002$). These asymmetries resulted in an increase in both TB and tr with elevation (Figure 2c, f).

A contrary pattern appeared when comparing open and forest habitats at the local scale. Habitat explains a significant source of variation in CTmax and tmax but not in CTmin and tmin (Figure 2; Tables 1 and 2). Frogs inhabiting open environments have higher CTmax (Table 2, Figure 2a; PGLS means. Open: $35.77^{\circ}\text{C} \pm 0.11$, $N = 13$; and forest: $32.73^{\circ}\text{C} \pm 0.68$, $N = 9$) and were exposed to warmer tmax than frogs limited to forest habitats (LS means. Open: $28.36 \pm 0.86^{\circ}\text{C}$, $N = 13$; forest: $20.26 \pm 1.04^{\circ}\text{C}$, $N = 9$; Figure 2d, Table 1). This environmental signal of habitat on CTmax variation appears to be mediated by maximum tmax. When tmax is introduced in the model, the difference in CTmax between forest and open environments disappears, thus suggesting that CTmax varies as a function of the actual tmax to which species are exposed to and have to face (PGLS: $F_{1,18} = 0.676$, $p = 0.422$). Yet, a discrepancy is revealed when employing macroclimate temperatures. As expected, no habitat variation was found in TMAX, TMIN and TR (Table 1).

The absolute temperature range (tr) variation was smaller for forest-restricted frogs, which also exhibit a narrower TB (Figure 2c, f; Tables 1 and 2), and this divergence was kept constant throughout the elevational gradient ($t_{16} = 0.707$, $p = 0.49$; $t_{16} = 0.191$, $p = 0.851$, respectively). Forest and open habitats also display a different degree of climatic overlap between equally separated low- and

high-elevation sites along the elevational gradient. ANCOVA analyses conclude that the level of overlap decreases with elevation and does so at a faster rate in forest localities (test of homogeneity of slopes, $F_{1,59} = 6.22$, $p = 0.015$) with no overlap in climatic distinctions ($d < -0.5$) for those localities separated over 1,500 m of elevational slope, the elevational distance at which $d = 0$. However, most open-habitat localities do overlap in temperatures, with the elevational distance at which $d = 0$ at a much greater, 3,500 m, elevational slope (Figure 3). A contrasting pattern was found by employing macroclimatic temperatures. In this case, overlap declines similarly for both habitats with climatic distinction occurring in localities spaced more than 2,500 m (Figure S2). Elevational ranges did not differ between forest and open habitats ($F_{1,18} = 0.82$, $p = 0.377$, forest 1159.8 ± 165.3 m, $N = 9$; open habitat, 1361.64 ± 149.5 m, $N = 11$).

Thermal tolerance limits did not exhibit a physiological trade-off (PGLS: $F_{1,18} = 0.077$, $p = 0.785$). Nonetheless, when we included habitat in the model, thermal tolerance limits covaried positively (PGLS: $F_{1,17} = 13.837$, $p = 0.002$; see Appendix S2: Figure S3), suggesting a trade-off in thermal resistance. Increased cold tolerance implies a reduction in heat resistance that was parallel in both habitats, although higher heat resistance was found in open habitats for similar cold resistance.

Increasing elevation increases risk of cold stress (Figure 4b) because tmin decreases with elevation at a faster rate than CTmin (Figure 2b, e; heterogeneity of slopes test:

TABLE 1 Microenvironmental (lower case) and macroenvironmental (caps) maximum, minimum and temperature range (dependent variables) in relation to elevation and habitat

Climatic data	df	Sum Sq	F value	p (>F)
tmax ($R^2 = 0.634$)				
Elevation (E)	1	414.39	25.681	<0.001
Habitat (H)	1	178.37	11.054	0.005
Residuals	14	225.91		
tmin ($R^2 = 0.955$)				
Elevation (E)	1	319.77	116.589	<0.001
Habitat (H)	1	12.23	4.458	0.055
E × H	1	15.77	5.749	0.032
Residuals	13	35.66		
tr (tmax–tmin) ($R^2 = 0.547$)				
Elevation (E)	1	72.123	4.232	0.058
Habitat	1	199.732	11.719	0.004
Residuals	14	238.605		
TMAX ($R^2 = 0.94$)				
Elevation (E)	1	549.72	221.337	0.001
Habitat	1	4.88	1.965	0.183
Residuals	14	34.77		
TMIN ($R^2 = 0.98$)				
Elevation (E)	1	626.04	707.149	<0.001
Habitat E × H	1	0.91	1.03	0.327
Residuals	14	12.39		
TR (TMAX–TMIN) ($R^2 = 0.321$)				
Elevation (E)	1	2.48	1.901	0.19
Habitat E × H	1	1.572	1.206	0.291
Residuals	14	18.26		

Note: Interaction between factors was only included if models were significantly better.

$F_{1,40} = 51.21$, $p < 0.001$). Contrarily, tmax does decrease at the same rate as CTmax (Figure 2a, d; heterogeneity of slopes test, $F_{1,40} = 0.67$, $p = 0.419$) and thus, no elevational trend in heat stress was found (Figure 4a). However, a contrasting elevational decline in heating risk was obtained when employing macroclimatic TMAX (Figure 4a), as it decreases at a faster rate than CTmax (heterogeneity of slopes: $F_{1,40} = 37.28$, $p < 0.001$). Finally, macro- and microclimatic estimates reveal divergent patterns with elevation when assessing frog vulnerability to suffer heat stress (Figure 4a; Appendix S1: Table S4). Habitat buffers the vulnerability to heat stress. Forest species have higher warming tolerance ($12.11 \pm 0.61^\circ\text{C}$, $N = 9$) than open-habitat frogs ($7.91 \pm 0.99^\circ\text{C}$, $N = 13$), ($F_{1,20} = 8.72$, $p = 0.0079$), whereas cooling tolerance did not differ across habitats ($F_{1,20} = 0.052$, $p = 0.822$). This pattern of lower vulnerability of forest species is reverted when considering habitat conversion with deforestation; that is, forest frogs will exhibit much lower warming tolerances than open species (forest $wt_{\text{deforested}} = 3.53 \pm 0.77^\circ\text{C}$, $N = 9$, $F_{1,20} = 10.44$, $p = 0.004$).

4 | DISCUSSION

Macrophysiological rules seek to link organismal physiological features with temporal, spatial and climatic predictors that are assumed to correlate with organism's body temperatures (Chown & Gaston, 2016; Gaston et al., 2009). Yet, several concerns cast doubt on the use of geographical variables, such as elevation or latitude, to explain broadscale patterns of physiological variation because they only represent a partial description of the environmental gradient (Hawkins & Diniz-Filho, 2004; Körner, 2007). Hence, unravelling the actual physical drivers of physiological variation is of major importance (Chown & Gaston, 2016). Our results appear to uncover two weaknesses of current

TABLE 2 Physiological variables in relation to elevation of the population sample point and habitat (open versus forest) in direct-developing frogs using a PGLS approach

Physiological traits	Sum Sq	df	F value	p (>F)
CTmax ($R^2 = 0.61$, $\lambda = 0$)				
Elevation	74.729	1	26.893	<0.001
Habitat	53.413	1	19.222	<0.001
Residuals	52.796	19		
CTmin ($R^2 = 0.836$, $\lambda = 0.438$)				
Elevation	261.563	1	105.567	<0.001
Habitat	9.47	1	3.822	0.065
Residuals	47.076	19		
TB ($R^2 = 0.631$, $\lambda = 0$)				
Elevation	81.817	1	13.451	0.0019
Habitat	128.235	1	21.082	<0.001
Residuals	103.406	17		

Note: Interaction was not included as its addition in the models was not significantly better.

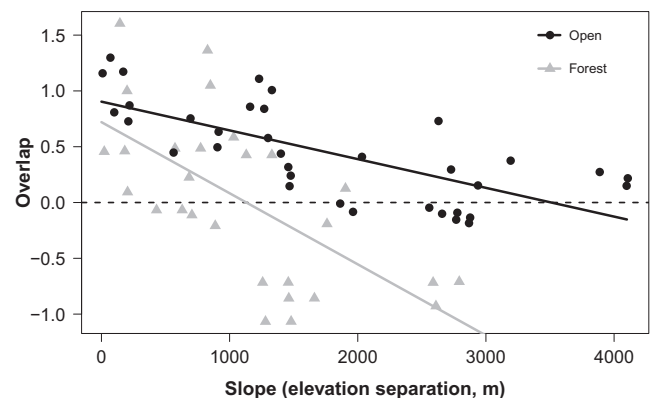
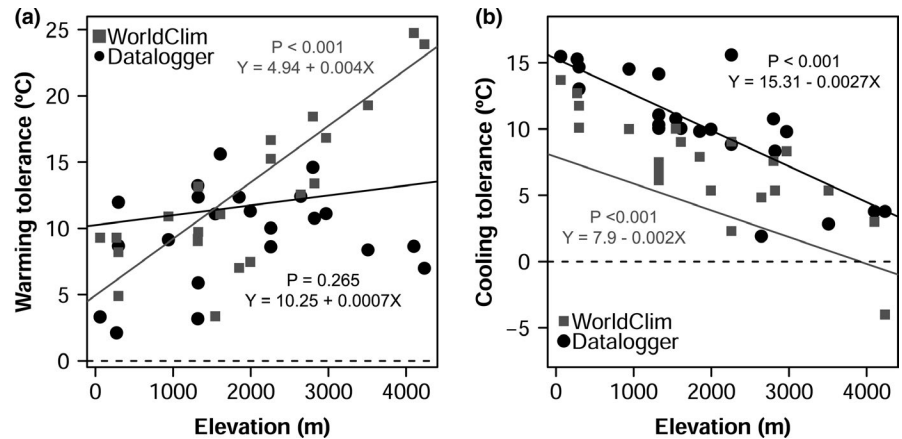
**FIGURE 3** Regression lines of temperature overlap (microclimate shelters) with elevational separation between pairs of localities in Ecuador, for forest (grey triangles, overlap = 0.7203–0.0006 slope; $R = -0.646$; $p = 0.0002$; $R^2 = 0.417$, $N = 28$) and open (black dots, overlap = 0.9929–0.0003 slope; $R = -0.695$; $p = 0.00000$; $R^2 = 0.483$; $N = 35$) habitat locations. Dashed lines, overlap = 0



FIGURE 4 Phylogenetic generalized least squares for (a) warming tolerance and (b) cooling tolerance for Ecuadorian *Pristimantis* using macroclimatic (grey squares) and microclimatic (black dots) predictors. Dashed lines represent a lethal threshold (WT or CT = 0)



macrophysiological trend analyses. First, the analyses of physiological variation through geographical gradients do not usually account for the thermal heterogeneity associated with microenvironments where organisms actually live (Potter et al., 2013; Sears, Raskin, & Angilletta, 2011) which, otherwise, may represent a main driver of thermal tolerance variance (Gutiérrez-Pesquera et al., 2016; Kaspari et al., 2015). We found that habitat thermal diversity (forest versus open areas) appears to drive CTmax variation, and its link to tmax suggests local thermal adaptation to the significantly different peak temperatures experienced in both habitats (around 8°C higher in open as opposed to forest environments) (also see Muñoz et al., 2016). Additionally, the fact that both thermal limits exhibit a similar rate of evolutionary change and that CTmax exhibits a slightly lower phylogenetic signal, at least in Pagel's λ , contradicts the general view of conservatism of upper thermal tolerance assuming depauperate evolutionary potential to face upcoming increasing heat (Araújo et al., 2013; Grigg & Buckley, 2013; Hoffmann et al., 2013; Muñoz et al., 2014). Second, the assumed relevance of interpolated coarse-resolution climate to organismal body temperatures is in most cases unsustainable, exhibiting high spatial bias in topographically complex and environmentally heterogeneous areas, such as mountain regions (Dobrowski, 2011; Faye, Herrera, Bellomo, Silvain, & Dangles, 2014; Fridley, 2009). This inaccurate mapping of actual exposure temperatures at the local scale may compromise our ability to project vulnerability assessments to ongoing global climatic predictions (Sunday et al., 2014). For instance, coarse-resolution climatic layers may overestimate risk to suffer heat impacts as estimated thermal regimens exceed species upper thermal tolerances (Buckley, Cannistra, & John, 2018; Storlie et al., 2014). Our results revealed that the probability to suffer acute heat impacts was similar across elevations and habitats. This pattern contrasts the often predicted higher vulnerability in lowland species found using low-resolution macroclimatic estimators (Colwell et al., 2008; Diamond et al., 2012; Sunday et al., 2014). Contrary to expectations, forest frogs, although exhibiting lower heat tolerances, are less prone to suffer heat impacts because forest canopy shields ground temperatures yielding cooler tmax. Similarly,

lower risk to heat impacts was found in forest amphibian tadpole communities with lower pond tmax than open ponds (Duarte et al., 2012; Gutiérrez-Pesquera et al., 2016) and in terrestrial ectotherms inhabiting areas with diverse vegetation coverage resulting in contrasting temperatures (Kaspari et al., 2015; Kearney, Shine, & Porter, 2009). Given *Pristimantis*' direct development, we believe that these same results would apply across the whole life cycle as eggs are usually laid within the same shelters as those used by adults.

The crucial role of forests as a structural biotic buffer of climatic variability, initially proposed by von Humboldt (1849), has a correlated signal on species' physiological thermal variation. Forest species of *Pristimantis* experience less climatic overlap with elevation than open-habitat species which is linked to both their environmental thermal stability and narrow range of physiological tolerances. These premises are in agreement with Janzen's climatic variability hypothesis, initially thought to propose limited dispersal in tropical compared to temperate organisms across elevational climatic bands due to "higher" (or more inhibiting) climatic barriers (Janzen, 1967). We propose a variant of Janzen's hypothesis applied to tropical mountains, implying a horizontal axis of climatic habitat variation (i.e. open versus forest habitats) that crosses with the traditional axis of climatic elevational gradient (Figure 1b). Habitat thermal variation determines climatic and physiological barriers in forests, but less in open habitats, which reveal weaker climatic distinctions. This asymmetry in dispersal capacity should result in smaller elevational ranges for forest thermal specialists than open-habitat generalists (Sheldon et al., 2018). However, we could not corroborate this trend, which has otherwise been seen when comparing height of barriers through latitudinal gradients (McCain, 2009). Local climatic fluctuations, such as habitat variation, may also explain the absence of the expected divergence in dispersal capacity (see Chan et al., 2016). It is possible that horizontal movements of organisms will represent a climate sorting process allowing organisms to increase resilience to climatic changes under current upcoming heating as is proposed in the analogous vertical climatic variation within tropical forest (Scheffers &

Williams, 2018; Scheffers et al., 2017). Habitat-driven thermal variation may exceed the expected rise of temperatures, and thermally buffered habitats can become climatic shelters for the organisms most exposed to the thermal shocks (see Lenoir & Svenning, 2013). For instance, this scenario has been suggested in high-risk open lowland tropical lizards that will move to cooler forest environments leading to negative interspecific interactions (Huey et al., 2009; but see Logan, Huynh, Precious, & Calsbeek, 2013). Furthermore, when incorporating current rates of deforestation to our climate change vulnerability predictions, the consequences for forest amphibian species may be severe (Hof, Araujo, Jetz, & Rahbek, 2011).

Finally, the existence of local climatic heterogeneity throughout the elevational gradient, in addition to the comparatively lower dispersal ability of forest species, should increase their potential for genetic isolation. This, in turn, may result in higher levels of speciation in tropical mountainous populations (see Claramunt, Derryberry, Remsen, & Brumfield, 2012), especially within this extremely species-rich group of terrestrial breeding frogs. However, this hypothesis remains to be tested. As a final conclusion, we suggest a habitat-driven mechanistic explanation for local variation in heat tolerance which promotes the large-scale thermal tolerance diversity in tropical elevational gradients.

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CONFLICT OF INTEREST

We declare no competing of interests.

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BIOSKETCH

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SUPPORTING INFORMATION

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

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