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Divergence of thermal physiological traits in terrestrial breeding frogs along a tropical elevational gradient

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33   Data Archival location: GenBank (molecular sequence data), Dryad (phylogenetic trees).

34   **Abstract**

35

36   Critical thermal limits are thought to be correlated with the elevational distribution of species

37   living in tropical montane regions, but with upper limits being relatively invariant compared to

38   lower limits. To test this hypothesis, we examined the variation of thermal physiological traits in

39   a group of terrestrial breeding frogs (Craugastoridae) distributed along a tropical elevational

40   gradient. We measured the critical thermal maximum (CT_{max}; n = 22 species) and critical

41   thermal minimum (CT_{min}; n = 14 species) of frogs captured between the Amazon floodplain (250

42   m asl) and the high Andes (3800 m asl). After inferring a multi-locus species tree, we conducted

43   a phylogenetically informed test of whether body size, body mass, and elevation contributed to

44   the observed variation in CT_{max} and CT_{min} along the gradient. We also tested whether CT_{max} and

45   CT_{min} exhibit different rates of change given that critical thermal limits (and their plasticity) may

46   have evolved differently in response to different temperature constraints along the gradient.

47   Variation of critical thermal traits was significantly correlated with species' elevational midpoint,

48   their maximum and minimum elevations, as well as the maximum air temperature and the

49   maximum operative temperature as measured across this gradient. Both thermal limits showed

50   substantial variation, but CT_{min} exhibited relatively faster rates of change than CT_{max}, as

51   observed in other taxa. Nonetheless, our findings call for caution in assuming inflexibility of

52   upper thermal limits, and underscore the value of collecting additional empirical data on species'

53   thermal physiology across elevational gradients.

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Introduction

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In a rapidly changing world, many species are faced with shrinking habitat and novel climatic
conditions. As a result, there has been widespread interest in understanding species responses to
past and present climatic variation in order to predict how best to conserve species in future
climatic conditions (e.g., Sinervo et al. 2010, Moritz & Agudo 2013). While much attention has
been given to modeling and predicting elevational range shifts in montane organisms, especially
in the context of climate change, most predictions about future geographic ranges are based on
correlative models that ignore species' evolutionary history and eco-physiology (Colwell et al.
2008, Laurance et al. 2011, VanDerWal et al. 2012). Tropical montane regions are of special
concern because they are centers of biodiversity and endemism (Graham et al. 2014). Mountain
uplift, climatic fluctuations, and the emergence of new ecological conditions have been
hypothesized to promote the diversification of organisms at high elevations (Moritz et al. 2000,
Hoorn et al. 2010). As a result, species living at high elevation often exhibit narrowly
overlapping (i.e., parapatric) distributions, many of which are assumed to have greater tolerance
to cold (Janzen 1967, Navas 2005, Ghalambor et al. 2006). However, empirical data on critical
thermal limits of most tropical montane taxa remain unknown. Furthermore, tropical lowland
taxa, especially ectotherms, are thought to live near their thermal optimum, so increased
temperatures due to changing climates would lead to decreased fitness (Colwell et al. 2008, Huey
et al. 2009, Sunday et al. 2014). As with species living at higher elevations, empirical data on
species' critical thermal limits are not available for most tropical lowland taxa.

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Several hypotheses have been proposed to explain the potential causes of diversity patterns along
elevational gradients (Terborgh 1970, MacArthur 1972, Hofer et al. 1999, Lomolino 2001,
McCain & Grytnes 2010, McCain & Colwell 2011, Graham et al. 2014, Peters et al. 2016). One
of these hypotheses proposes that climatic conditions along the gradient restrict species'
distributions (von Humboldt 1849, Janzen 1967). Air temperature is the main environmental
factor that predictably decreases with increasing elevation as a result of adiabatic cooling (on
average 5.2−6.5 °C decrease per 1000 m elevation; Colwell et al. 2008). Critical thermal
maximum (CT_{max}) and critical thermal minimum (CT_{min}) are two measures that have been used

88 to infer species' critical thermal limits. Numerous studies have shown that ectotherms exhibit a

89 general trend of decreasing critical thermal limits with elevation (Heatwole et al. 1965, Christian

90 et al. 1988, Gaston & Chown 1999, Navas 2003, Catenazzi et al. 2014). Moreover, it is likely

91 that critical thermal limits change at different rates in response to different temperature

92 constraints along elevational gradients (McCain & Grytnes 2010). Specifically, CT_{max} is thought

93 to be relatively inflexible across elevation (e.g., Hoffman et al. 2013, Muñoz et al. 2014, 2016),

94 with a narrow upper limit and low plasticity (Sunday et al. 2011; Gunderson & Stillman 2015).

95

96 Although many researchers have examined the relationship between critical thermal limits and

97 the elevational distribution of species living in montane gradients, only a few have combined

98 empirical (CT_{max} and CT_{min}) data and accounted for the effect of phylogenetic relatedness

99 among species (Muñoz et al. 2014, 2016, Sheldon et al. 2015). Phylogenetic comparative

100 methods are particularly useful for this purpose because they allow researchers to examine

101 evolutionary transitions in physiological traits and account for statistical non-independence of

102 interspecific data when studying life history evolution among closely related species (Harvey &

103 Pagel 1991, Garland et al. 1992, Revell 2008).

104

105 We investigated the role of physiological divergence among closely related species distributed

106 along an elevational gradient of > 3500 m in southern Peru. Although 80% of Peruvian Andean

107 frogs (ca. 250 species) occur within relatively narrow elevational ranges (Aguilar et al. 2010),

108 little is known about the relationship between their critical thermal limits and their elevational

109 distributions. We focused on 22 species of terrestrial-breeding frogs, Craugastoridae, the most

110 diverse amphibian family in the Tropical Andes (Hedges et al. 2008, Duellman & Lehr 2009,

111 Padial et al. 2014). These direct-developing frogs (Figure 1) are ideal model organisms in which

112 to test hypotheses about divergence across environmental gradients because they have low

113 vagility (resulting in local genetic structure), small body size (a trait that makes them amenable

114 for physiological experiments), and limited geographic and elevational ranges (suggesting strong

115 potential for local adaptation).

116

117 Our goal was to examine how CT_{max} and CT_{min} vary in relation to the elevational distribution of

118 species and to test whether life history traits such as body size and body mass, and elevational

119   range midpoint explain differences in CT_{max} and CT_{min} among species. Altogether, we used four

120   metrics relating to elevation (elevational minimum, maximum, midpoint, and range) and two

121   metrics relating to temperature (maximum air temperature and maximum operative temperature)

122   as proxy for thermal environments. We reconstructed a phylogeny to determine the evolutionary

123   relatedness among species and to evaluate the relationship between critical thermal limits and

124   elevation using phylogenetic comparative methods. We tested for phylogenetic signal in all life

125   history traits to infer the role of niche conservatism, which is when related species resemble each

126   other more than expected under a Brownian motion model of trait evolution (Losos 2008). We

127   also tested whether CT_{max} and CT_{min} are correlated with one another, and determined which life

128   history traits can explain the observed variation in CT_{max} and CT_{min}. Furthermore, given that

129   recent studies focusing on thermal niche evolution of terrestrial ectotherms showed that tolerance

130   to cold changes more than tolerance to heat (Sunday et al. 2011, Araújo et al. 2013, Hoffman et

131   al. 2013, Muñoz et al. 2014), we evaluated whether CT_{max} and CT_{min} exhibited different rates of

132   thermal physiological change.

133

134   **Material and Methods**

135

136   **Study area.** We worked in Manu National Park and its surrounding habitat in southern Peru.

137   Key study sites along the elevational transect included in this study are Acjanaco (13°11'56" S,

138   71°37'03" W, 3700 m elev.), Wayqecha Biological Station (13°10'29" S, 71°35'14" W, 2900 m

139   elev.), San Pedro Cock of the Rock Biological Station (13°03'16" S, 71°32'45" W, 1400 m elev.),

140   Villa Carmen Biological Station (12°53'44" S, 71°24'14" W, 530 m elev.), and Los Amigos

141   Biological Station (12°34'07" S, 70°05'57" W, 250 m elev.). A general overview of the study

142   sites and local climate was provided by Catenazzi et al. (2011) and von May et al. (2009), and

143   Catenazzi et al. (2013) provided an inventory of the herpetofauna in this region.

144

145   **Field surveys and critical thermal limits.** All species surveyed in this study are distributed

146   within the watershed of the Madre de Dios river and along a single montane gradient. Data

147   collected for this study were obtained from multiple surveys conducted along the elevational

148   gradient from Los Amigos Biological Station at 250 m (von May et al. 2009, 2010; von May &

149   Donnelly 2009) to Tres Cruces at 3800 m (Catenazzi & Rodriguez 2001; Catenazzi et al. 2011,

150 2013, 2014). We measured CT_{max} and CT_{min} in 22 and 14 species, respectively, expanding the

151 taxonomic diversity, number of individuals sampled per species, and elevational coverage of a

152 previous study (Catenazzi et al. 2014). Animals were captured in the field and transported to a

153 field laboratory, where they were kept in individual containers with water *ad libitum*. All

154 individuals were housed at 16–21 °C for 2-3 days prior to measurements. Thus, our measures

155 relate to thermal limits under field conditions, and are likely influenced by both plasticity and

156 adaptation. We used non-lethal experiments to evaluate critical thermal maxima (CT_{max}) and

157 minima (CT_{min}). CT_{max} and CT_{min} were measured as the point where frogs lost their righting

158 response, defined as the moment when a frog cannot right itself from being placed venter-up for

159 a period longer than 5 sec (Navas 1997, Navas 2003, Catenazzi et al. 2014). We placed each

160 individual in a plastic cup with a thin layer of water (3-5 mm) and immersed the cups in a water

161 bath. For CT_{max}, the bath temperature was progressively increased from 18°C to up to ~35°C at a

162 rate of ~1°C/minute by adding warm water. For CT_{min}, the temperature was progressively

163 decreased from 18°C to ~0°C by adding ice to the water bath (Christian et al. 1988). We forced

164 animals to a venter-up position; whenever animals were unable to right themselves for 5 sec, we

165 used a quick-reading thermometer to measure temperature against the body of the frog immersed

166 in the thin layer of water. Given the small size of these frogs, we assumed that this temperature is

167 equivalent to the core temperature of frogs (Navas et al. 2007). The righting response is relevant

168 for considering selection on thermal physiology, because a frog that is unable to display their

169 automatic righting reflex will likely be unable to escape predators. We measured CT_{max} in 768

170 individuals (22 species) and CT_{min} in 196 individuals (14 of the 22 species). Even though there

171 are fewer data points for CT_{min}, our sampling covered the entire gradient for both critical thermal

172 traits.

173

174 **Laboratory methods.** We collected DNA sequence data for two mitochondrial and two nuclear

175 genes in order to determine the phylogenetic relationships among focal species. The

176 mitochondrial genes were a fragment of the 16S rRNA gene and the protein-coding gene

177 cytochrome c oxidase subunit I (COI). The nuclear protein-coding genes were the

178 recombination-activating protein 1 (RAG1) and Tyrosinase precursor (Tyr). Extraction,

179 amplification, and sequencing of DNA followed protocols previously used for terrestrial

180 breeding frogs (Lehr et al. 2005, Hedges et al. 2008). Primers used are listed in Table S1 and we

181 employed the following thermocycling conditions to amplify DNA from each gene using the

182 polymerase chain reaction (PCR). For 16S, we used: 1 cycle of 96°C/3 min; 35 cycles of

183 95°C/30 s, 55°C/45 s, 72°C/1.5 min; 1 cycle 72°C/7 min. For RAG, we used: 1 cycle of 96°C/2

184 min; 40 cycles of 94°C/30 s, 52°C/30 s, 72°C/1.5 min; 1 cycle 72°C/7 min. For Tyr, we used: 1

185 cycle of 94°C/5 min; 40 cycles of 94°C/30 s, 54°C/30 s, 72°C/1 min; 1 cycle 72°C/7 min. We

186 performed the cycle sequencing reactions using BigDye Terminator 3.1 (Applied Biosystems)

187 and ran the purified reaction products on an ABI 3730 Sequence Analyzer (Applied Biosystems).

188 Newly obtained sequences generated in this study were deposited in GenBank (Table S2).

189

190 **Phylogenetic analysis.** We used Geneious R6, version 6.1.8 (Biomatters 2013;

191 http://www.geneious.com/) to align the sequences using the built-in multiple alignment program.

192 For 16S, we visualized the alignment, trimmed the ends, and removed the highly variable non-

193 coding loop regions (to avoid alignment artifacts). Prior to conducting phylogenetic analysis, we

194 used PartitionFinder, version 1.1.1 (Lanfear 2012) to select the appropriate models of nucleotide

195 evolution. We used the Bayesian information criterion (BIC) to determine the best partitioning

196 scheme and substitution model for each gene. The best fitting substitution model for 16S was

197 GTR+I+G. The best partitioning scheme for COI and both nuclear genes included specific sets

198 according to codon positions. For COI, the best partitioning scheme included three sets of sites

199 (substitution models in parentheses): the first set with 1st codon position (K80+G), the second set

200 with 2nd codon position (HKY), and the third set with the 3rd codon position (TrN+G). For RAG,

201 the best partitioning scheme included two sets of sites: the first set with 1st and 2nd codon

202 positions together (HKY+I) and the second set with only the 3rd codon position (K80+G).

203 Likewise, for Tyr, the best partitioning scheme included two sets of sites: the first set with 1st and

204 2nd codon positions together (K80+I) and the second set with only the 3rd codon position

205 (K80+G). We inferred nuclear haplotypes from genotype data using PHASE version 2.1

206 (Stephens et al. 2001; Stephens & Scheet 2005) and processed the input and output files with

207 SEQPHASE (Flot 2010).

208

209 We used a multispecies coalescent approach implemented in *BEAST v1.6.2 (Drummond &

210 Rambaut 2007) to infer a Bayesian multilocus timetree of the 22 focal taxa. The primary goal of

211 the analysis was to obtain an ultrametric tree to be used for phylogenetic comparative analyses

212   (see below). Our analyses only depend on the relative branch lengths of the tree, but we preferred

213   to illustrate our tree in rough units of time. Therefore, we used an uncorrelated relaxed molecular

214   clock with the rate of nucleotide substitution for 16S was set at 1% per million years. However,

215   we note that the dates associated with the tree should only be viewed as very approximate and

216   that there can be multiple sources of error when calibrating phylogenies (Arbogast et al. 2002).

217   The analysis in *BEAST included two independent runs, each with 100 million generations and

218   sampled every 10000 generations. Following the completion of the analysis, we used Tracer v1.5

219   (Rambaut & Drummond 2007) to examine effective sample sizes, verify convergence of the

220   runs, and to ensure the runs had reached stationarity. Observed effective sample sizes were

221   sufficient for most parameters (ESS >200) except for substitution rates for a few partitions. We

222   discarded the first 10% of samples from each run as burn-in. Subsequently, we used

223   LogCombiner v1.6.2 to merge all remaining trees from both runs and used TreeAnnotator v1.6.2

224   (Drummond & Rambaut 2007) to summarize trees and obtain a Maximum Clade Credibility tree

225   (available at the Dryad Digital Repository: doi:10.5061/dryad.84bp7). We visualized the MCC

226   tree and the associated node support values in FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

227

228   **Phylogenetic signal.** For a given quantitative trait, phylogenetic signal is present when related

229   species tend to resemble one another (Harvey & Pagel 1991, Blomberg et al. 2003). We tested

230   for phylogenetic signal by calculating the K (Blomberg et al. 2003) and λ statistics (Pagel 1999)

231   in the R package 'phytools' (Revell 2012). Both methods are commonly used to account for non-

232   independence of interspecific data resulting from shared ancestry (Ashton 2004, Revell 2008,

233   Corl et al. 2010). For K, values smaller than 1 indicate that related species are less similar than

234   expected under a Brownian motion model of trait evolution whilst values greater than 1 indicate

235   that related species resemble each other more than expected under a Brownian motion model of

236   trait evolution (Blomberg et al. 2003). The value of λ typically ranges between 0, indicating no

237   phylogenetic signal, and 1, indicating strong phylogenetic signal (i.e., when related species

238   resemble each other more than expected under a Brownian motion model of evolution) (Pagel et

239   al. 1999). For CT_{max} and CT_{min}, phylogenetic signal tests were done both considering and not

240   considering intraspecific measurement error in either CT_{max} or CT_{min} values. Given that

241   considering measurement error did not affect the results, only results from tests with no

242   measurement error are included in the Results section.

243

244 **Rates of thermal physiological change.** Prior to comparing the rates of physiological change

245 for CT_{max} and CT_{min}, we searched for a model of evolution that best explains the variation in the

246 observed data. We used the fitContinuous function in GEIGER (Harmon et al. 2008) to fit three

247 models of evolution: Brownian Motion (BM), Ornstein-Uhlenbeck (OU), and Early Burst (EB).

248 The Brownian motion model assumes a constant rate of change, such that the differences

249 between species will (on average) be proportional to the time since their divergence. The

250 Ornstein-Uhlenbeck model assumes a stationary distribution, such that the differences between

251 species will not necessarily relate to their time since divergence. Finally, the Early Burst model

252 assumes an exponential decline in rates through time. This means that species with recent

253 divergence times will be very similar, while species with deeper divergences will be relatively

254 independent of one another. After determining the best fitting model of evolution for each trait,

255 we used the R package 'APE' (Paradis et al. 2004) and code developed by Adams (2013) to

256 estimate the rates of change.

257

258 **Correlates of CT_{max} and CT_{min}.** We explored the relationship between critical thermal traits

259 and other life history characteristics (body size and body mass) as well as four metrics relating to

260 elevation —minimum, maximum, midpoint, and range— collected from 22 species of

261 craugastorid frogs. We also considered maximum air temperatures (T_a) and maximum operative

262 temperatures (T_e), both of which were previously estimated for the same montane gradient

263 (Catenazzi et al. 2014). The T_a data were inferred by regressing daily average temperatures vs.

264 elevation from four weather stations operated by Peru's national weather service (SENAMHI =

265 Servicio Nacional de Meteorología e Hidrología del Perú) from 520 to 3485 m. The T_e data were

266 inferred from field measurements taken with 21 iButtons (Maxim Integrated Products,

267 Sunnyvale, California, U.S.A.) placed in forest microhabitats used by frogs at five sites between

268 1525 and 3500 m. For two species that are primarily distributed in the Andean grassland

269 (*Bryophryne cophites* and *Psychrophrynella usurpator*), T_e data were inferred from

270 measurements taken with 12 iButtons placed in this microhabitat from 2800 to 3450 m.

271 Furthermore, as in Catenazzi et al. (2014), we calculated operative warming tolerances (OWTs)

272 by subtracting the average maximum T_e from CT_{max}. We also considered the thermal range,

273 defined as the difference between CT_{max} and CT_{min}. We examined a correlogram displaying the

274 relationships between pairs of variables (Figure S1) to determine which predictor variables were

275 highly correlated with each other. We used the R package 'phylolm' (Ho & Anné 2014ab) to fit

276 phylogenetic generalized linear regression models. This package implements a phylogenetic

277 regression under various models for the residual error, including Brownian Motion (BM) and

278 Ornstein-Uhlenbeck (OU; these models were implemented with a constant selection strength α

279 and variance rate σ^2). We used the AIC value to identify the model that best explains the

280 variation of observed data (Ho & Anné 2014b).

281

282 **Results**

283

284 **Phylogenetic relatedness and elevational distribution.** We recovered a well-supported

285 phylogenetic tree (Figure 2 and Figure S2; node support values shown in Figure S2) that was

286 generally congruent with previous trees (Padial et al. 2014). Seventeen out of 21 nodes had

287 Bayesian posterior probabilities greater than 0.95 (Figure S2). We mapped elevational data on to

288 the species tree obtained with *BEAST to visually assess the patterns of elevational distribution

289 and phylogenetic relatedness (Figure 2).

290

291 We observed that closely related, congeneric species exhibit generally parapatric distributions

292 with respect to elevation; an exception to this pattern was seen in some species of *Pristimantis*

293 (e.g., *P. platydactylus* and *P. salaputium*) that exhibit broader elevational overlap (Figure 2). A

294 congruent and similarly well-supported phylogeny was obtained with a concatenated partitioned

295 dataset analyzed with MrBayes (Ronquist & Huelsenbeck 2003; see Supplementary Information

296 and Figure S3).

297

298 **Critical thermal traits.** We observed substantial differences in CT_{max} values (from 24.8 °C to

299 34.8 °C) among both closely- and distantly-related species (Figure 3; Table S3). In five cases,

300 close relatives had non-overlapping CT_{max} values and non-overlapping elevational distributions.

301 The highest CT_{max} was found in *Oreobates cruralis*, an exclusively lowland species, and the

302 lowest CT_{max} was found in *Bryophryne hanssaueri*, a species distributed in highland forests just

303 below the treeline. CT_{min} also varied substantially across the gradient (from 1.6 °C to 15.2 °C;

304 Table S3). In three cases, close relatives exhibited non-overlapping CT_{min} values (Figure S4).

305

Phylogenetic signal. No phylogenetic signal was detected for CT_{max}, in tests both considering
and not considering intraspecific measurement error in CT_{max} values (Table 1; only results from
tests with no measurement error are shown). This result infers that, for CT_{max}, closely related
species are less similar than expected from a Brownian motion model of evolution along the tree.
Likewise, no phylogenetic signal was detected for CT_{min}, based on a test using the reduced
dataset (14 species). In contrast, a strong phylogenetic signal was detected for both SVL and
body mass, and a moderate phylogenetic signal for minimum elevation, maximum elevation,
elevational midpoint, and elevational range (Table 1). The only discrepancy observed between
the two phylogenetic signal statistics was observed for maximum elevation (λ non-significant)
and elevational range (λ marginally non-significant).

Rates of thermal physiological change. Results of fitting tests for the three models of trait
evolution showed that BM was the best model for both CT_{max} and CT_{min} (Table S4). The method
used for estimating the rates of evolution (Adams 2013) assumes a constant rate of change (BM),
and we performed this test assuming BM for both traits and using the reduced dataset (14
species). We found that CT_{max} exhibits a slower rate of change than CT_{min} ($\sigma^2 = 0.686$ and $\sigma^2 = 1.353$, respectively; likelihood ratio test, LRT = 4.443, AICc = 128.319, P = 0.035).

Correlates of CT_{max} and CT_{min}. Phylogenetic linear regression models indicated that CT_{max}
and CT_{min} were significantly correlated with all proxies of thermal environment—minimum
elevation, maximum elevation, elevational midpoint, maximum air temperature, and maximum
operative temperature (Table 2, Table 3). In all cases, increasing elevation led to decreasing
CT_{max} and CTmin (Figure 4, Table 2, Table 3). Body size, body mass, and elevational range did
not explain the variation in CT_{max} and CT_{min} (Table 2, Table 3). Models with two or more
variables did not provide a better fit compared to univariate models (i.e., AIC values of models
with two or more variables were greater than AIC values of univariate models; Table S5).
Further, CT_{max} and CT_{min} were significantly correlated with one another (AIC = 53.46, Log
likelihood = -23.73, P = 0.0003; reduced dataset of 14 species).

334

335 Our data also showed that operative warming tolerance (OWT) increased with elevation (AIC =

336 86.90, Log likelihood = -40.45, $P < 0.001$; Figure 5). Therefore, the distance between CT_{max} and

337 maximum operative temperature (T_e) of high-elevation species is greater that that of species

338 distributed at lower elevations. We also observed a consequent increase in thermal range (=

339 $CT_{max} - CTmin$) at higher elevations, although this relationship was marginally non-significant

340 (AIC = 59.87, Log likelihood = -26.94, $P < 0.0831$; Figure 5).

341

342 **Discussion**

343

344 Our findings suggest that thermal physiology is relevant in determining where species live, and

345 provide further evidence that local adjustment to the thermal environment, whether by plasticity

346 or adaptation, is an important process in tropical mountains (Cadena et al. 2012). Overall, critical

347 thermal limits decreased with elevation as well as with decreasing air (T_a) and operative (T_e)

348 temperatures, a pattern exhibited by other terrestrial ectotherms living along montane gradients

349 (Christian et al. 1988, Gaston & Chown 1999, Navas 2003, Muñoz et al. 2014).

350

351 Importantly, the high variability observed in both CT_{max} and CT_{min} among closely related species

352 (Figures 3 and S4) supports the idea that thermal traits in ectotherms can adjust through

353 evolutionary time. In contrast to studies focusing on thermal physiology across distantly related

354 taxa (i.e., different families) and/or larger geographic scales (e.g., Kellermann et al. 2012a,b,

355 Araújo et al. 2013, Sunday et al. 2014), we investigated species within a single family distributed

356 along a steep elevational gradient. We believe this approach can be used to refine predictions and

357 to test further hypotheses regarding physiological divergence among montane taxa, especially if

358 such studies incorporate knowledge of phylogenetic relatedness among species. Synthesizing this

359 information is essential for understanding historical patterns and processes determining species'

360 elevational distributions and for predicting species' responses to climate change (Moritz &

361 Agudo 2013).

362

363 Our tests of phylogenetic signal focusing on CT_{max} indicated that closely related species are less

364 similar than expected under a Brownian motion model of evolution, supporting the idea that even

365 upper thermal limits can change rapidly in this diverse amphibian clade. This finding, along with

366 those from Neotropical plethodontid salamanders (Kozak & Wiens 2007), suggests that niche

367 divergence in tolerance to heat may be common among montane amphibians (e.g., Navas 1997,

368 Navas 2003). Our tests of phylogenetic signal focusing on CT_{min} based on a reduced dataset (14

369 species) also suggested that closely related species tend to differ in their tolerance to cold. The

370 reduced dataset for CT_{min} spans the full elevational range, but had few species distributed at high

371 elevation (e.g., only one species of *Bryophryne* and only one *Psychrophrynella*), so an expanded

372 dataset is required to examine this pattern more thoroughly. Given that CT_{max} and CT_{min} are

373 significantly correlated with one another, and that each of these traits is significantly correlated

374 with elevational midpoint, maximum elevation, and minimum elevation, we predict that an

375 expanded dataset for CT_{min} will support the hypothesis that tolerance to cold has changed rapidly

376 in this clade. Given that the Andes have experienced multiple uplift events since the Miocene

377 (Hoorn et al. 2010), the emergence of colder environments along the montane gradient might

378 have promoted rapid divergence in species' thermal physiological traits. These observations for

379 amphibians contrast with experimental studies of *Drosophila*, were there appears to be strong

380 phylogenetic constraint on both cold and heat tolerance (Kellermann et al. 2012a,b).

381

382 Nevertheless, observing strong correlations does not necessarily imply that either the lower or

383 upper bound of the elevational range of montane frog species is constrained by their critical

384 thermal limits (Navas 1997, Catenazzi 2011). In addition to species' thermal physiology, factors

385 such as availability of breeding sites, competition, predation, and other biotic interactions may

386 play an important role in restricting species' elevational distribution (Hutchinson 1957, Terborgh

387 & Weske 1975, Wake & Lynch 1976). Likewise, other climatic factors such as rainfall, relative

388 humidity, and availability of microrefugia in the dry season may also play a role in determining

389 the upper and lower elevational range limits in some species (Wake & Lynch 1976, McCain &

390 Grytnes 2010, Graham et al. 2014).

391

392 Our finding that CT_{min} has faster rates of change than CT_{max} is consistent with results from

393 phylogenetic comparisons of sets of related lizards distributed across elevational gradients in the

394 tropics (e.g., Muñoz et al. 2014, 2016). Nevertheless, differences in species distributions and in

395 species' thermoregulation strategies between frogs and lizards might reflect contrasting patterns

396 of physiological evolution. Whilst lizards tend to occur in warm places where they can actively

397   thermoregulate, frogs occur in greater numbers in cold environments and most species are

398   considered to be thermoconformers (Navas 2003)—with the notable exceptions of some high-

399   elevation frog species that thermoregulate opportunistically (Navas 1997). For example, the

400   mountaintop at our study site (~ 3500 m elevation) is inhabited by eight frog species of three

401   families, but only one lizard species. Therefore, the selective pressures on thermal limits are

402   likely to differ largely between frogs and lizards.

403

404   Several studies focusing on terrestrial ectotherms have suggested that plasticity may not play an

405   important role in shaping inter-specific variation in critical thermal limits. For example, a recent

406   meta-analysis by Gunderson & Stillman (2015) found that terrestrial ectotherms exhibit low

407   acclimation potential (i.e., low plasticity) for heat resistance. However, this hypothesis requires

408   further testing and the group of tropical frogs studied here represents a suitable study system to

409   examine the contribution of plasticity vs. genetic effects. Future studies should examine variation

410   in the acclimation potential of montane and high-elevation tropical frogs, complementing

411   previous studies that found no such capacity, or very low acclimation potential, in frogs

412   (Brattstrom 1968, Christian et al. 1988, Gunderson & Stillman 2015).

413

414   Our findings do not support a broad assumption of niche conservatism in research aimed at

415   examining species' responses to environmental change. Many researchers have used species

416   distribution modeling approaches to predict whether species will experience range shifts or

417   extinction in the face of climate warming (Chen et al. 2011, Laurance et al. 2011, VanDerWal et

418   al. 2012). The assumption underlying many of these studies is that climatic niches have not

419   changed along the history of species, both within and among closely related species (Wiens et al.

420   2010). However, our results call for caution in assuming inflexibility of thermal limits, especially

421   CT_{max}, in montane anurans, and underscore the value of collecting additional empirical data on

422   species' thermal physiology (Perez et al. 2016). It is worth noting that while our results suggest

423   that thermal limits may change rapidly on the time scale of the formation of new species, it is

424   still an open question about whether thermal physiology will be able to keep pace with future

425   global climate change that may be more rapid than in the recent past (Gunderson & Stillman

426   2015). Our data on operative warming tolerance (Figure 5) support the idea that tropical lowland

427 species might be more sensitive to increased temperatures than high-elevation species, because

428 they live at ambient conditions that are closer to their critical thermal limits (Colwell et al. 2008,

429 Huey et al. 2009, Sunday et al. 2014). In turn, tropical amphibians living at high elevation might

430 be more buffered from increased temperatures, as their CT_{max} values are farther away from the

431 maximum temperatures that they regularly experience in the wild (Catenazzi et al. 2014). More

432 studies on populations/species that have recently diverged along montane gradients are needed to

433 help estimate maximal rates of change of thermal limits.

434

435 **Acknowledgments**

436

458

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806 **Figure legends**

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808 **Figure 1.** (A) Female *Bryophryne cophites* attending a clutch of direct-developing embryos at

809 high elevation (above 3200 m a.s.l.). These frogs tolerate near-freezing temperatures (which they

810 experience during the dry season) as well as moderately high temperatures (which they may

811 experience during sunny days). (B) *Bryophryne hanssaueri* individuals have bright orange

812 coloration ventrally, including the throat. These frogs live under mosses and leaf litter in the

813 high-elevation cloud forest between 3195 and 3430 m, just below the treeline. Like other

814 *Bryophryne* species, females attend clutches of direct-developing embryos until they hatch into

815 tiny froglets. Photographs by A. Catenazzi.

816

817 **Figure 2.** Elevational divergence in terrestrial breeding frogs along a tropical montane gradient.

818 Species tree (obtained with *BEAST) depicting the relationship among the 22 species included

819 in this study (top) and their elevational distribution along the study transect (bottom). The

820 elevational midpoint is denoted by a black bar. Species are color-coded according to genus.

821

822 **Figure 3.** Divergence in CT_{max} in terrestrial breeding frogs along a tropical montane gradient.

823 Species tree (obtained with *BEAST) depicting the relationship among the 22 species included

824 in this study (top) and box plots depicting their CT_{max} values (bottom). The box plots show the

825 median (black bar), interquartile range (box), and 1.5 times the inter-quartile range (bars); circles

826 represent outliers. Species are color-coded according to genus.

827

828   **Figure 4.** Correlation between CT_{max} and elevational midpoint (left) and between CT_{min} and

829   elevational midpoint (right). Species are color-coded according to genus (see Figures 2 and 3).

830   The slopes of the regression lines reflect the phylogenetic corrections in each model.

831

832   **Figure 5.** Correlation between operative warming tolerance and elevational midpoint (left) and

833   correlation between thermal range (= $CT_{max} - CT_{min}$) and elevational midpoint (right). Species

834   are color-coded according to genus and the regression lines reflect the phylogenetic correction.

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852   **Tables**

853

854   **Table 1.** Results from the tests for phylogenetic signal based on two statistics, K and λ. Log

855   likelihood values included correspond to the λ estimates. Phylogenetic signal tests were done

856   with the full dataset (22 species) for all traits except for CT_{min}. Phylogenetic signal tests were

857   conducted for CT_{min} and repeated for CT_{max} with the reduced dataset (14 species).

Trait	K	P-value $_{(K)}$	λ	P-value $_{(λ)}$	lnL

Analyses with full dataset (22 species)					
CT$_{max}$	0.3955	0.1572	0.0626	0.8202	-49.22
SVL	0.9548	**0.0010**	1.0352	**0.0003**	-64.71
Mass	0.7589	**0.0030**	1.0560	**0.0031**	-24.61
Minimum elevation	0.7011	**0.0020**	0.7291	**0.0055**	-179.52
Maximum elevation	0.5233	**0.0140**	0.3854	0.1559	-181.17
Elevational midpoint	0.6115	**0.0060**	0.5903	**0.0307**	-180.03
Elevational range	0.4944	**0.0280**	0.4999	0.0635	-160.54
Analyses with reduced dataset (14 species)					
CT$_{min}$	0.7019	0.0631	1.1339	0.0681	-35.68
CT$_{max}$	0.5279	0.2302	0.0001	1.000	-30.25

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Table 2. Results from phylogenetic generalized linear regression models for CT$_{max}$, fitted assuming the Brownian Motion (BM) model of evolution. Model fitting was done with the full dataset (22 species). Similar results were obtained with the Ornstein-Uhlenbeck (OU) model (results not shown). T$_a$ = maximum air temperature; T$_e$ = maximum operative temperature.

Model	Evol. model	Coefficient	P-value	AIC	lnL
CT$_{max}$ ~ min_elev	BM	-0.0023	**<0.001**	90.02	-42.01
CT$_{max}$ ~ max_elev	BM	-0.0020	**<0.001**	93.24	-43.62
CT$_{max}$ ~ elev_midpoint	BM	-0.0022	**<0.001**	90.21	-42.10
CT$_{max}$ ~ elev_range	BM	-0.0002	0.881	110.27	-52.13
CT$_{max}$ ~ T$_a$	BM	0.3542	**<0.001**	89.09	-41.55
CT$_{max}$ ~ T$_e$	BM	0.4782	**<0.001**	69.28	-31.64
CT$_{max}$ ~ svl	BM	-0.1844	0.136	107.78	-50.89
CT$_{max}$ ~ mass	BM	-1.191	0.115	107.49	-50.75

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Table 3. Results from phylogenetic generalized linear regression models for CT_{min}, fitted assuming the Brownian Motion (BM) model of evolution. Model fitting was done with the reduced dataset (14 species). Similar results were obtained with the Ornstein-Uhlenbeck (OU) model (results not shown). T_a = maximum air temperature; T_e = maximum operative temperature.

Model	Evol. model	Coefficient	P-value	AIC	lnL
CT_{min} ~ min_elev	BM	-0.0031	**<0.001**	58.99	-26.50
CT_{min} ~ max_elev	BM	-0.0026	**<0.001**	56.39	-25.20
CT_{min} ~ elev_midpoint	BM	-0.0029	**<0.001**	56.14	-25.07
CT_{min} ~ elev_range	BM	0.0041	0.081	75.17	-34.59
CT_{min} ~ T_a	BM	0.4728	**<0.001**	51.74	-22.87
CT_{min} ~ T_e	BM	0.5998	**<0.001**	58.26	-26.13
CT_{min} ~ svl	BM	-0.2730	0.138	76.19	-35.10
CT_{min} ~ mass	BM	-1.2024	0.358	77.83	-35.92

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