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Acclimation scope of the critical thermal limits in *Agalychnis spurrelli* (Hylidae) and *Gastrotheca pseustes* (Hemiphractidae) and their implications under climate change scenarios

Disertación previa a la obtención del título de Licenciada en Ciencias Biológicas

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“La ciencia es bella y por esa belleza es que debemos trabajar en ella,
y quizás, y algún día, un descubrimiento científico como el
radio, puede llegar a beneficiar a la humanidad”

-Madame Curie-

Este trabajo de tesis está dedicado a mi madre; gracias a su apoyo y dedicación, me ha enseñado que todo es posible de cumplir.

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SÍMBOLOS Y ABREVIACIONES

Abreviación/Símbolo	Significado
Acclim	Aclimatación
ARR_{max}	Radio de respuesta de aclimatación del CTmax
ARR_{min}	Radio de respuesta de aclimatación del CTmin
ASP	*Agalychnis spurrelli*
BIO 1	Temperatura media anual
BIO 5	Temperatura máxima del mes más cálido en el año
BIO 6	Temperatura mínima del mes más frío en el año
CTM	Limites críticos térmicos (máximo o mínimo)
CTmax	Tolerancia térmica crítica máxima
CTmin	Tolerancia térmica crítica mínima
CVH	Hipótesis de la variabilidad climática
GPS	*Gastrotheca pseustes*
HadGEM2	Modelo global ambiental del centro Hadley
L:D	Luz y oscuridad
LRR	Pérdida de la respuesta de enderezamiento
MS22	Metanosulfonato tricaína (TMS)
RCP	Vía de concentración representativa
SD	Desviación estándar
Te	Temperatura de exposición
To	Temperatura óptima
CVH	Hipótesis de variación de clima
WT	Tolerancia de calentamiento
Δ	Variación
$\bar{x}$	Promedio

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1. RESUMEN

Se analizó el alcance de aclimatación de los límites críticos térmicos (CTmax y CTmin) en renacuajos de dos especies de ranas ecuatorianas, que se diferencian principalmente por las condiciones térmicas del hábitat y sus rangos de distribución altitudinal: (1) *Agalychnis spurrelli* (Hylidae) de Durango, Provincia de Esmeraldas, 200 msnm y (2) *Gastrotheca pseustes* (Hemiphractidae) de la carretera Ambato-Guaranda, provincia de Bolívar, 3467 msnm. Los renacuajos pasaron por un periodo de aclimatación de tres días y fueron sometidas a pruebas de tolerancias térmicas a través de un baño térmico que cambiaba a una tasa de 0.25 °C/min. Ambas especies aumentaron su CTmax y CTmin acorde al incremento de la temperatura de aclimatación. *Agalychnis spurrelli* obtuvo una mejor tolerancia al calor, mientras que *Gastrotheca pseustes* obtuvo una mayor tolerancia al frío. *Gastrotheca pseustes* posee rangos de tolerancias térmicas más amplios que *Agalychnis spurrelli*. El ARR_{max} fue mayor en *Agalychnis spurrelli* (0.14) que en *Gastrotheca pseustes* (0.05). La temperatura máxima de exposición (Te) en los microhábitats en la actualidad es de 26.19 °C en *Agalychnis spurrelli* y 20.52 °C en *Gastrotheca pseustes*. En los escenarios RCP 2.6 y 8.5, la temperatura de exposición en ambas especies, mostró una tendencia de aumento hacia el futuro. A pesar de que *Agalychnis spurrelli* es más tolerante al calor y posee un alcance mayor de aclimatación que *Gastrotheca pseustes* en escenarios presentes y futuros, fue la especie más vulnerable debido a que vive en un ambiente con temperaturas más cercanas a sus tolerancias. Este resultado difiere de lo esperado ya que se ha asumido que las especies de zonas altas son más vulnerables al cambio climático. Los resultados sugieren revaluar esos supuestos en base a información eco-fisiológica.

2. ABSTRACT

We analyzed the acclimation scope in the critical thermal limits (CTmax and CTmin) in tadpoles of two Ecuadorian frog species which strongly differ in thermal habitat conditions and altitudinal distribution ranges: (1) *Agalychnis spurrelli* (Hylidae), Durango, Esmeraldas province, 200 m.a.s.l. and (2) *Gastrotheca pseustes* (Hemiphractidae), Ambato-Guaranda road, Bolivar province, 3467 m.a.s.l. The tadpoles were acclimated for a three days period and then their thermal tolerances were tested through a thermal bath which rise 0.25 °C/min. In both species, the CTmax and CTmin increased along with the acclimation temperature. *Agalychnis spurrelli* was tolerant to higher temperatures; however, *Gastrotheca pseustes* was a better cold tolerant. *Gastrotheca pseustes* had a wider tolerance range than *Agalychnis spurrelli*. The ARR_{max} was higher in *Agalychnis spurrelli* (0.14) than *Gastrotheca pseustes* (0.05). The maximum exposure temperature (Te) in microhabitat at present conditions was 26.19 °C for *Agalychnis spurrelli* and 20.52 °C for *Gastrotheca pseustes*. In RCP 2.6 and 8.5 scenarios, the Te in both species, tended to increase in the future. In spite of the fact that *Agalychnis spurrelli* was a heat tolerant and had a greatest acclimation scope than *Gastrotheca pseustes* in present and future scenarios, it was the most vulnerable. This result was different to the one expected because we assume that high altitude species are more vulnerable to climate change. The results suggest that the vulnerability of the frogs of different altitude have to be reevaluated in base to eco-physiological information.

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First Title: Acclimation scope of the critical thermal limits in *Agalychnis spurrelli* (Hylidae) and *Gastrotheca pseustes* (Hemiphractidae) and their implications under climate change scenarios.

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Running Title: Acclimation scope of CTM in two tropical frogs

Abstract

We analyzed the acclimation scope in the critical thermal limits (CTmax and CTmin) in tadpoles of two Ecuadorian frog species which strongly differ in thermal habitat conditions and altitudinal distribution ranges: (1) *Agalychnis spurrelli* (Hylidae), Durango, Esmeraldas province, 200 m.a.s.l. and (2) *Gastrotheca pseustes* (Hemiphractidae), Ambato-Guaranda road, Bolivar province, 3467 m.a.s.l. The tadpoles were acclimated for a three days period and then their thermal tolerances were tested through a thermal bath which rise 0.25 °C/min. In both species, the CTmax and CTmin increased along with the acclimation temperature. *Agalychnis spurrelli* was tolerant to higher temperatures; however, *Gastrotheca pseustes* was a better cold tolerant. *Gastrotheca pseustes* had a wider tolerance range than *Agalychnis spurrelli*. The $\mathrm{ARR}_{\mathrm{max}}$ was higher in *Agalychnis spurrelli* (0.14) than *Gastrotheca pseustes* (0.05). The maximum exposure temperature (Te) in microhabitat at present conditions was 26.19 °C for *Agalychnis spurrelli* and 20.52 °C for *Gastrotheca pseustes*. In RCP 2.6 and 8.5 scenarios, the Te in both species, tended to increase in the future. In spite of the fact that *Agalychnis spurrelli* was a heat tolerant and had a greatest acclimation scope than *Gastrotheca pseustes* in present and future scenarios, it was the most vulnerable. This result was different to the one expected because we assume that high altitude species are more vulnerable to climate change. The results suggest that the vulnerability of the frogs of different altitude have to be reevaluated in base to eco-physiological information.

***Keywords*:** Acclimation scope; critical thermal limits; tropical anurans; climate change.

3.1 INTRODUCTION

Temperature is the abiotic factor which most strongly influences fitness in ectothermic organisms. Ectotherms depend on the environment to regulate their own body temperature (Glanville and Seebacher, 2006; Huey and Stevenson, 1979). Any changes in the external temperature can affect the performance of biological functions such as locomotion, reproduction, growth, behavior, or even ecological interactions (Deutsch et al., 2008; Huey and Berrigan, 2001). Climate change is increasing mean environmental temperatures (0.6°C since the mid-1990s to present) and the frequency of extreme thermal events (Pachauri et al., 2014; Parmesan and Matthews, 2006). As a consequence, organisms, specially ectotherms, could experiment temperatures beyond their physiological thermal limits (Duarte et al., 2012; Gutiérrez-Pesquera et al., 2016) affecting the phenology and geographic distribution of many species (Bernal and Lynch, 2013). Many populations may be forced to displace to higher altitudes or latitudes to compensate the increase in temperature (Parmesan, 2006; Urban et al., 2013; Wilson et al., 2005).

It is believed that tropical species will be more vulnerable than those at higher latitudes because they are currently exposed to temperatures close to their physiological thermal limits (Deutsch et al., 2008; Duarte et al., 2012; Gerick et al., 2014; Tewksbury et al., 2008). In addition, tropical species are more vulnerable than temperate ones because they have evolved in unseasonal environments and experienced relatively constant temperatures throughout the year. Consequently, they have narrow thermal regimens (Janzen, 1967). Climate change could affect dramatically species at different altitudes (Bernal and Lynch, 2013; Buckley et al., 2013; Navas et al., 2008). The predicted temperature increase can limit the distributional range for some species. For example, species living in mountain tops may become endangered because they cannot escape to higher places (Wilson et al., 2005). These effects may be even greater in small ectotherms with limited dispersal ability; such as amphibians (Sinsch, 1991).

Amphibians are the most threatened vertebrate group. Approximately 41% are threatened with extinction (IUCN, 2015; Stuart, 2004). Population declines in amphibians have not only been attributed to climate change but also to other agents such as habitat loss and fragmentation, increased pollution, over-exploitation, and emergent diseases (e.g. chytriodiomycosis) (Beebee and Griffiths, 2005; Parmesan and Matthews, 2006; Walther et al., 2002). Some of these factors may be acting synergically. Researchers have demonstrated, for example, that changes in environmental temperatures can depress the immune system of amphibians (Lips et al., 2008). Thus, emergent diseases produced by pathogens can take advantage of a weakened immunological system and spread among populations (Raffel et al., 2012).

The thermal performance curves show how temperature can affect physiological functions. This curve represents the variation of a specific function (locomotion, metabolic rate, fecundity, critical thermal limits (CTM), survival, etc.) expressed in percentage through a temperature range (Huey and Stevenson, 1979). The critical thermal maximum (CTmax) and critical thermal minimum (CTmin) were described as the temperature that a specific physiological function (e.g. locomotion) became disorganized (e.g. the loss of righting response -LRR-, immobility, muscle spasms) and once reached this endpoint, the individual will be unable to escape from any biotic or abiotic threat (Cowles and Bogert, 1944; Lutterschmidt and Hutchison, 1997).

Plasticity or acclimation ability allows organisms to modify their phenotypic characteristics (e.g. thermal tolerance) in response to different environmental conditions (Huey et al., 1999; Thomson et al., 2001; Wilson and Franklin, 2002). The mechanism of thermal acclimation could adjust physiological functions of ectotherms exposed to variable thermal conditions; for example: locomotion performance, metabolism, thermal tolerance, etc. (Navas et al., 2008). Acclimation can shift thermal optima and performance breadth, which may increase fitness and improve the viability of populations during warming (Burger and Lynch, 1995; Fry and Hart, 1948; Plaut, 2001; Scott and Johnston, 2012). For example, in tadpoles of *Xenopus laevis* acclimated at cold-temperatures had a greater swimming performance than the ones acclimated to warm-temperatures in the cold laboratory conditions (Wilson and Johnston, 2000). According to Brattstrom (1968, 1970), tropical anurans have lower acclimation capacity than temperate ones. Species of high altitude show acclimation abilities similar to temperate species because the mountains expose them to variable thermal conditions that some species at low altitude do not experiment in their environment (Brattstrom, 1968). Negative impacts due to global warming are expected to affect individuals that are thermally specialized with limited acclimation capacity and restricted geographical ranges (Buckley et al., 2013; Tewksbury et al., 2008; Urban et al., 2013). Claussen (1977) proposed as a measure of an acclimation ability as the differences of the higher and lower values of critical thermal tolerances and acclimation temperature or the change in the critical thermal limit per degree in acclimation temperature (Angue and Bennett, 2003; Cuculescu et al., 1998). If ARR value was 1, it was considered as positive, so the organism was able to a complete compensation for acclimation temperatures (1 °C of CTmax for 1 °C of acclimation temperature). In the other hand, if the ARR value was 0, the organism was not able to acclimate.

The aim of this study is to determine though physiological tests in the laboratory the acclimation scope of the critical thermal limits of two tropical frogs: *Agalychnis spurrelli,* a lowland species and *Gastrotheca pseustes,* a highland species. Assessment of the influence of acclimation range temperatures on critical thermal limits will provide information about the plasticity in thermal tolerances of species with distinct altitudinal ranges. By comparing those limits with the environmental temperatures to which each species is exposed, we will estimated their vulnerability to climate change (warming tolerance) in order to delineate and improve conservation strategies for these species.

3.2 MATERIALS AND METHODS

a. Study organisms

Gastrotheca pseustes larvae were collected in a roadside pond at 3467 m.a.s.l. on the Ambato-Guaranda road, Bolivar province (1.3367° S; 78.7594° W). It is a highland species that lives between 2200 and 4080 m.a.s.l. It occurs in the Andes from northern to southern of Ecuador, between the provinces of Pichincha and Loja. Females of *Gastrotheca pseustes* carry their embryos in a pouch in their backs. After they hatch, she lays them in temporary ponds (Ron et al., 2014).

Agalychnis spurrelli larvae were born at "Balsa de los Sapos" Conservation Initiative facilities at Pontificia Universidad Católica del Ecuador in Quito. The temperature conditions at which the eggs and the newborns of *Agalychnis spurrelli* were kept in "Balsa de los Sapos" laboratory was: Mean temperature = 27.9 °C, maximum temperature = 29.7 °C and minimum temperature = 23.7 °C. The parental pair was collected in a pond at 200 m.a.s.l. in Durango, Esmeraldas province (1.0303° N; 78.5918° W). This species inhabits altitudes below 885 m in tropical moist lowland forests of the Pacific from southern Costa Rica to central-western Ecuador. The eggs of *Agalychnis spurrelli* are laid in vegetation over temporary ponds (Ortega-Andrade et al., 2011). When the tadpoles hatch they drop into the pond. We used 80 larvae for each species at Gosner stages between 27 and 36 (Gosner, 1960).

b. Acclimation treatments

Larvaes were kept at “Balsa de los Sapos” Conservation Initiative in Quito at Pontificia Universidad Católica del Ecuador (2800 m.a.s.l). Twenty individuals per species were acclimatized at four temperatures, 15 °C, 20 °C, 27 °C and 32 °C with a photoperiod of 12:12 L:D in a period of acclimation of three days (Hutchison, 1961). Tadpoles were randomly assigned to each acclimation temperature. Each larva was individualized in a plastic beaker of 400 mL and set into the corresponding thermal bath. They were fed *ad libitum* during the period of acclimation. Within each of the four acclimation temperatures, 10 individuals were tested for upper thermal limit (CTmax) and 10 for lower thermal limit (CTmin). Each individual was examined only in a single physiological test.

c. Thermal tolerances

After the acclimation treatments both CTmax and CTmin were obtained for both species. Thermal tolerance tests were developed using a thermal water bath of 15 L (HUBER K15-cc-NR) with an initial temperature of 20 °C. Thermal tolerance limits were carried out using a dynamic method (Lutterschmidt and Hutchison, 1997) by promoting a ramp that increase or decrease the temperature at a constant rate of 0.25 °C min^{-1}. Each individual was tested in a plastic beaker with 100 mL of dechlorinated water and exposed to this thermal ramp.

The endpoint of the experiment was set at the explicit temperature at which the animal was unable to move and respond to 10 gentle and consecutive strokes using a wooden stick. At this point, the critical temperatures were obtained from the surrounding water with a Miller and Webber quick-response mercury thermometer (Lutterschmidt and Hutchison, 1997).

Then, each individual was transferred immediately to a plastic beaker with water at room temperature. The experiment was considered valid if the individual survived at least 24 hours after the experiment (Brattstrom, 1968). The mass of the tadpoles was obtained after experimental assays in a BOECO (BBI-41) electronic balance to the nearest 0.001 g. All of the tadpoles were euthanized at the conclusion of the thermal tolerance assays. We used a lethal dose of MS22 anesthesia to euthanize the tadpoles according to the protocol of the laboratory (CCAC, 2003; Simmons, 2002). CTmax and CTmin are presented as the mean of valid experiments for each acclimation temperature and species.

A Shapiro-Wilk's test was used to evaluate the normality and Levene's test was used to prove the homogeneity of variance of the CTmax and CTmin data between the treatments. If these statistical assumptions were violated in the case of a methodological mistake, outliers were removed. The tests of normality and homogeneity of variance were performed again and if the results also revealed that assumptions were not fitted, a log 10 transformation of the data was performed to adjust and improve normality and homoscedasticity. We used Analysis of Covariance (ANCOVA) to test for differences between the mean values of the thermal tolerances at the different temperatures of acclimation (as fixed factor). Tadpole mass was included as a covariate to control possible effects on the response variable. In the case that assumptions were not fitted, a non-parametric Kruskal-Wallis test was used. Finally, based on the results obtained, the *post hoc* Tukey HSD test or a Dunn test (depending of the case) was used to test for differences in temperatures of acclimation.

d. Thermal breadth and acclimation scope

The difference between the CTmax and CTmin for each species gave an estimate of the thermal breadth (Navas et al., 2008). This value showed if the studied species have similar thermal ranges of tolerances.

The acclimation scope was calculated as Acclimation ratio response (ARR) values. Acclimation ratio response values represent the range of CTmax or CTmin showed by the species divided by the range of acclimation temperatures (Claussen, 1977; Gunderson and Stillman, 2015).

e. Current climate conditions

The current microenvironmental data was obtained though temperature data loggers (HOBO pendant ®, Onset Computer Corporation (UA-001-64)) set at collection ponds of both species. *Gastrotheca pseustes* pond was in the roadside exposed to open conditions and *Agalychnis spurrelli* pond was located in a forested border covered by foliage. The sensors collected data every 15 minutes from August to December 2014. The data gave information of the thermal exposure extremes experimented by both species in their own natural microhabitats. The fluctuation of the temperatures in both microhabitats was compared with an F test. The results showed which microhabitat was the most variable.

The macroenvironmental data was obtained from present bioclimatic layers from WorldClim website (Hijmans et al., 2005) for each collection locality. We used 30" resolution bioclimatic layers. Bioclimatic variables were BIO1 (Annual mean temperature), BIO5 (Maximum temperature of the warmest month) and BIO6 (Minimum temperature of the coolest month) (Varela et al., 2015).

f. Future climate conditions

The projections of temperature for 2070 were used to estimate how the temperature will change in the future at tadpoles' collection localities and to test if this change could affect the relative vulnerability to changes in the thermal environment of both species. The future climatic conditions were obtained from 30" bioclimatic layers available at WorldClim (Hijmans et al., 2005). The bioclimatic layers used for these future conditions were the same as the present conditions (BIO1, BIO5 and BIO6). We used global climate model HadGEM2 (Hadley Centre Global Environment Model) because it considers the atmosphere interactions, the vegetation, the ocean and the chemistry of the atmosphere (IPCC, 2013). Two scenarios were considered: Representative Concentration Pathway (RCP) 2.6 and 8.5 (Low emission and high emission respectively) (Bjørnæs, 2009). For both study localities, the future temperatures of each bioclimatic layers were extracted by Spatial Analyst tool in ArcGIS 10.3 (Childs, 2011).

The maximum and minimum potential temperature changes in macroenvironment temperature to 2070 were calculated as the difference between the maximum and minimum temperatures in the future and the present. This difference was added to the microenvironment temperatures measured in the field.

g. Relative vulnerability to environmental temperature changes

For each species' maximum tolerance, we determined the warming tolerance (WT) as the difference between the critical thermal maxima (CTmax) and the maximum exposure temperature (Te) at microenvironmental scale (Deutsch et al., 2008; Wilson et al., 2005, Duarte et al., 2012, Gutiérrez-Pesquera et al., 2016).

The value of WT in the future was recalculated with each maximum temperature of exposure (Te) of the future microenvironmental conditions in scenarios 2.6 and 8.5 (CTmax-maxTe$_{(2.6/8.5)}$) to determinate if the relative vulnerability of any species will change in the future.

3.3 RESULTS

a. Thermal tolerances

Agalychnis spurrelli

Mean CTmax increased from the lowest to the highest acclimation temperatures (15 °C, 20 °C, 27 °C, and 32 °C; Fig. A1, Table 1). Mean CTmin increased from 20 °C to 32 °C acclimation treatments. The 15 °C acclimation treatment had a different behavior; because the CTmin value for this treatment was higher than 20 °C treatment (Fig. A2, Table 1).

ANCOVA tests (*F*=32.727, $p<0.001$ and *F*=70.6733, $p<0.001$ for CTmax and CTmin, respectively) showed that the critical thermal limits differed between acclimation treatments. Body mass was not statistically significant ($p> 0.062$).

Post hoc Tuckey Test for CTmax and CTmin showed that the treatment pair-comparisons were significant, with the exception of T20 vs. T15 pair of *Post hoc* Tuckey Test for CTmin which did not differ (Appendix F).

The breadth of thermal tolerances for the four acclimation temperatures is shown in Table 3. The smallest range of tolerance was 34.2 °C at 32 °C and the greatest range was 37.7 °C at 20 °C.

Gastrotheca pseustes

Both the ANCOVA results for CTmax ($F=30.1352$, $p < 0.001$) and Kruskal-Wallis results for CTmin (chi-squared = 36.8968, DF = 3, p-value < 0.001) showed that the critical thermal tolerances differed between acclimation treatments (Appendix G). Body mass was not significant ($p > 0.638$) The *post-hoc* Tuckey HSD in CTmax showed that all treatment pair-comparisons were significantly different, with the exception of T20 vs. T15 which did not differ. The Dunn test for CTmin showed that treatment pair comparisons T32 vs. T20, T27 vs. T20, and T15 vs. T20 were different (Appendix H). However, the CTmin values in the 20 °C and 15 °C treatments for *Gastrotheca pseustes* were underestimated because during the experiments the water reached its crystallization point before the animal locomotor functions ceased.

The breadth of the thermal tolerances for the four treatments is shown in Table 3. The smallest range of tolerance was 39.6 °C at 32 °C and the greatest range was 41.5 °C at 20 °C.

b. Acclimation scope

Acclimation response ratio (ARR) of both species showed in Table 2, proved that *Agalychnis spurrelli* had greater acclimation capacity than *Gastrotheca pseustes* ($\mathrm{ARR}_{\mathrm{max}}$=0.14; $\mathrm{ARR}_{\mathrm{max}}$=0.05, respectively). The $\mathrm{ARR}_{\mathrm{min}}$ value for *Agalychnis spurrelli* was 0.29. The $\mathrm{ARR}_{\mathrm{min}}$ for *Gastrotheca pseustes* was not considered because the CTmin values in treatments 20 °C and 15 °C were underestimated.

c. Present environmental conditions

Environmental data

The thermal conditions of macro environment data based on WorldClim bioclimatic layers showed that the maximum and minimum Te were 29.76 °C and 20.29 °C in Durango-Esmeraldas and 14.19 °C and 2.7 °C in Ambato-Guaranda road-Bolivar (Table 4). Local Annual Mean Temperature ($\bar{x}$) for *A. spurrelli* was 24.88 °C and 8.23 °C for *G. pseustes*.

For both species, the raw data collected from August to December was summarized in Table 5. The exposure temperature (Te) in *Agalychnis spurrelli* microhabitat was 26.19 °C and the minimum was 23.97 °C while the maximum exposure temperature in *Gastrotheca pseustes* microhabitat was 20.52° C and its minimum was 7.98° C (Table 5). Thermal range for *Gastrotheca pseustes* microhabitat showed to be more variable than the microhabitat for *A. spurrelli* (Table 6).

d. Future environmental conditions

Environmental data in 2070's

The maximum and minimum temperatures in macroenvironmental and microenvironmental future conditions are summarized in Tables 4 and 5. The magnitude of the change of temperature in both collection localities was similar in both cases. The maximum increase in Te in RCP 2.6 scenario is a proximally 1 °C and in RCP 8.5 scenario is proximally 2 °C. Future maximum microenvironmental temperatures for *Agalychnis spurrelli* for RCP 2.6 and RCP 8.5 scenarios were 27.2 °C and 28.3 °C, while for *Gastrotheca pseustes* the temperature were 21.73 °C and 22.73 °C respectively.

e. Relative vulnerability to environmental temperature change

Warming tolerances at present conditions

The warming tolerance (difference between CTmax and maximum exposure temperature) showed that *Agalychnis spurrelli* values were lower (15.21 at 20 °C acclimation treatment) than the *Gastrotheca pseustes* ones (17.38 at 20 °C acclimation treatment); consequently, the most vulnerable species was *Agalychnis spurrelli* (Figure B; Table 7). Both species show not to be at imminent risk to suffer heat shocks, because none of both species is close to its thermal physiological limits.

Warming tolerances in 2070's scenarios

The increase of the maximum exposure temperature in both microenvironments showed to be similar (Table 5). This increase did not promote changes in the relative vulnerability to climate change (Table 7). Even with the projected increase in temperature, both species will not be at risk to suffer heat shocks at their collection localities under the climate change scenarios used here (Figure B).

3.4 DISCUSSION

The CTmax and CTmin values of both species were higher at the highest acclimation temperature; however, *Agalychnis spurrelli* got CTmax and CTmin values higher than *Gastrotheca pseustes*. High CTmax and CTmin values in *A. spurrelli* made this species more tolerant to heat and less tolerant to cold conditions; on the other hand, the lower CTmax and CTmin values in *Gastrotheca pseustes* made it more tolerant to cold and less tolerant to heat conditions. As mentioned before, The CTmin values for 20 °C and 15 °C treatments in *Gastrotheca pseustes* were underestimated because during the experiments the water reached its crystallization point before the animal locomotor functions ceased. The final temperature taken for these two treatments was before the water froze however the tadpoles were still swimming at this point. We could not find a methodological strategy to find its real CTmin value without the water freezing. For this reason, the CTmin values for 20 °C and 15 °C treatments could be lower than CTmin values showed in this document.

In both species, the CTmin values described an interesting pattern in the acclimation treatments of 15 °C. Contrary to expectations, the organisms acclimated at 20 °C showed lower CTmin values than the ones acclimated at 15 °C (reversal acclimation). The acclimation response to a chronic exposure of stressful temperatures, probably determine deleterious effects that could low the potential for acclimation as a consequence to stressful temperatures (such as 15 °C) (Bevelhimer and Bennett, 2000; Podrabsky and Somero, 2004; Wilson and Franklin, 2002; Niehaus et al., 2012). The higher CTmin value at 15 °C was even more unexpected in the case of *Gastrotheca pseustes*, because this species is able to occur in cool environments (Annual mean temperature = 8.23 °C), however the growing optimal temperature (To) for this species is 23 °C (P. Pintanel, M. Tejedo, and A. Merino-Viteri, unpublished data). Truthfully, this species bears low temperatures, but it requires warmer temperatures to have the best grow rate (consequently better functional metabolism).

Brattstrom (1968) proposed that some high-elevation species of the tropics could behavior thermophysiologically as a temperate one. Heatwole et al. (1965) reported that *Eleutherodactylus portoricens* a high-elevation tropical species from Puerto Rico, showed a trend in temperature tolerance similar to temperate species; where they observed that individuals of *E. portoricensis* restricted at high-elevation had lower CTmax values than individuals of the same genus from low elevation. In our case, *Gastrotheca pseustes* is high-elevation species, which in fact, showed critical thermal tolerances lower than the low-elevation species, *Agalychnis spurrelli*.

Between the two species, *Gastrotheca pseustes* had a wider thermal tolerance range than *Agalychnis spurrelli*. The variability of the temperature in the microenvironment of *Gastrotheca pseustes* could make that the breadth between the CTmax and CTmin values were greater (12.72 °C) than for *Agalychnis spurrelli* (2.22 °C). Our results support the Climate Variability Hypothesis (CVH) which suggest a positive relationship between thermal tolerance range and the level of climatic variability (Stevens, 1989). The variation of the microenvironment temperatures in *Gastrotheca pseustes* provides it the ability to support warm and cold temperatures in a short period of time. On the other hand, the microenvironment temperatures in *Agalychnis spurrelli* were more stable, so this species only supports a small breadth of temperatures.

The relationship between the increase of the thermal tolerance and the acclimation temperature in anurans was previously examined by Brattstrom and Lawrence (1962), Brattstrom (1968, 1970), Hutchison and Maness (1979) and, Marshall and Grigg (1980) who observed that the beneficial increase in the CTmax or CTmin at different temperatures allows the organism to maximize the use of energy and increase the possibilities to survive at extreme thermal conditions. Our results agreed with this proposal, *Agalychnis spurrelli* and *Gastrotheca pseustes,* experimented higher CTmax values at higher acclimation temperatures in a short period of time.

Some ectotherms, such as the crabs *Cancer pagurus*, showed a positive response between their thermal tolerances (CTmax) and their acclimation temperature (ARR_{max}=0.46 in summer and ARR_{max}=0.61 in winter). According to proposed by Claussen (1977) this species is able to increase 1 °C of CTmax for 1 °C acclimation temperature (Cuculescu et al., 1998). However, in amphibians, the ARR_{max} proved in ambystomatid salamanders were typically 0.07 to 0.44, where the range of acclimation temperatures varied between 5 to 25 °C (Claussen, 1977). We have to consider that de ARR values vary depending on the variation of the thermal limit along the range of acclimation temperatures. The ARR values found in this study are within the range proved for salamanders, possibly due to the experimental acclimation ranges were partially overlapped. Although the ARR values were not 1, a compensatory acclimation response of CTmax is real in amphibians, but the changes in this ability were small in magnitude compared with other groups (Feder, 1992). Our results showed a relation between the critical thermal tolerances and acclimation temperatures; however, *Agalychnis spurrelli* compared with *Gastrotheca pseustes* had a greater acclimation scope (ARR_{max}=0.14 and 0.05, respectively). The magnitude of the acclimation scope of both species was small compared with some temperate anuran species (e.g. ARR_{max}=0.40 for *Cyclorana brevipes*, *Rana clamitans* and *Hyla californiae*). Our results follow predictions for tropical species that live in more stable thermal conditions than temperate ones (Brattstrom and Lawrence 1962; Brattstrom 1968, 1970). According to predicted global change, the increment of the temperature could affect the performance of many organisms, especially those in the tropics that have low acclimation response (Stillman, 2003, 2004; Gunderson and Stillman, 2015).

High-elevation species could get higher acclimation ability than low-elevations species because of the exposure to a greater temperature fluctuations in their habitat and provide them the capacity to acclimate to a wider temperature ranges (Temperature variation hypothesis- TVH) (Janzen, 1967; Lacey et al., 2010). However, our results showed that *Gastrotheca pseustes* got a lower acclimation scope compared to *Agalychnis spurrelli*. The low acclimation scope in *Gastrotheca pseustes* could be explained by several reasons: (1) Variable environments: Organisms, which were exposed to wide daily temperature fluctuations, had to decrease the thermal sensibility of many metabolic functions to buffer the effect of the temperature variability. This reduction of the thermal sensibility allowed the organisms to widen the performance breadth. Probably, the organisms exposed to a daily temperature fluctuation do not have the need to develop plasticity strategies as acclimation to survive in this thermal fluctuating environments (e.g. Killifish *Fundulus heteroclitus*) (Niehaus et al., 2011; Temple and Johnson, 1998) (2) The thermal natural history of the species: The acclimation responses depend of the previous and current thermal conditions to modify performance functions (Precht, 1958). So an evolutionary response could influence the low acclimation in *Gastrotheca pseustes* (3) Specie-specific acclimation response: Organisms like diving-beetles (*Agabus brunneus* group) are not able to acclimate like other groups of insects of the same latitude because they have limited phenotypic plasticity of thermal tolerances, so their physiological response is not immediate and could only express in adaptive evolution (Calosi et al., 2008).

The differences between the current and future temperatures in both collection localities were similar, so the relative projected vulnerability of both species would not change in the future. *Gastrotheca pseustes* vulnerability was the opposite of expected because it has been suggested that mountain top ectotherms could not disperse to upper and colder locations (Buckley et al., 2013; Wilson et al., 2005) and consequently to be more vulnerable to climate change. However, the thermal microenvironmental conditions and its wide thermal breadth give *Gastrotheca pseustes* the advantage to support the increase of temperature without getting close to its thermal tolerance. Tropical species have been proposed to be more vulnerable to climate change, because their Te is closer to their thermal physiological limits than in temperate species (Deutsch et al., 2008; Duarte et al., 2012; Gerick et al., 2014; Tewksbury et al., 2008). Our two study species, in current and future conditions, did not exhibit a significant closeness between their CTmax with their Te, so both species will not be directly affected by climate change.

For future thermal conditions in the collection ponds, we used the projected change in air temperature (Hijmans et al., 2005); however the increase of the temperature in future scenarios will be different in water than air. In thermodynamics, the specific heat is higher in water (4190 J/Kg °C) than in air (1050 J/Kg °C) (Hewitt, 1999). The specific heat capacity of a substance is defined as the amount of heat required to raise the temperature of a unit mass of a substance by 1 degree (Nahle, 2006). The transfer of heat is defined as the formula $Q=mc\Delta T$; where m=mass of the substance, c=specific heat and ΔT=variation of temperature (Tippens, 1992). If the specific heat is higher in some substances than other, the raise of the heat dissipation of these substances is slow and the variation of the temperature decreases. So the temperature of water rises more slowly than air. Our predictions for the rise of temperature in the future in the ponds will be slower than the predictions for the air made in this study by 2070. But, an increase of temperature may affect other factors in the water which may be thermal induced stressors to tadpoles, such as a decrease in water dissolved oxygen affecting their aerobic performance or an increase in osmotic stress may interact in synergy with thermal tolerances (Gómez-Mestre and Tejedo, 2003, 2004; Katzenberger, 2009; Pörtner and Knust, 2007; Re et al., 2006).

Of the 577 species of amphibians in Ecuador, the east and west lowlands of Andes which represent 56.2% of Ecuador continental area, houses 260 species, the most of them (97%) are present in lowlands tropical wet forest (Ron et al., 2014). If the projections for these lowland species are similar to the situation found for *Agalychnis spurrelli*, we could assume that many of them could be at risk. For this reason, it is of substantial importance to create conservation programs for all vulnerable species that will be exposed to an imminent heat damage induced by the increase of temperature in their environments. Conservation strategies should be focus on this situation and promote the gathering of additional scientific information in order to complete this assessment and save amphibians from potential local extinctions.

SYMBOLS AND ABBREVIATIONS

Abbreviation/Symbols	Significance
Acclim	Acclimation
ARR_{max}	Acclimation ratio response to CTmax
ARR_{min}	Acclimation ratio response to CTmin
ASP	*Agalychnis spurrelli*
BIO 1	Mean annual temperature
BIO 5	Maximum temperature of the warmest month of the year
BIO 6	Minimum temperature of the coolest month of the year
CTM	Critical thermal limits (maximum or minimum)
CTmax	Maximum critical thermal tolerances
CTmin	Minimum critical thermal tolerances
CVH	Climate Variability Hypothesis
GPS	*Gastrotheca pseustes*
HadGEM2	Hadley Centre Global Environment Model
L:D	Light and Dark
LRR	Loss of righting response
MS22	Tricaine methanesulfonate (TMS)
RCP	Representative Concentration Pathway
SD	Standard deviation
Te	Exposure temperature
To	Optimal temperature
CVH	Climate variation hypothesis
WT	Warming tolerance
Δ	Variation
$\bar{x}$	Mean

4. FIGURES

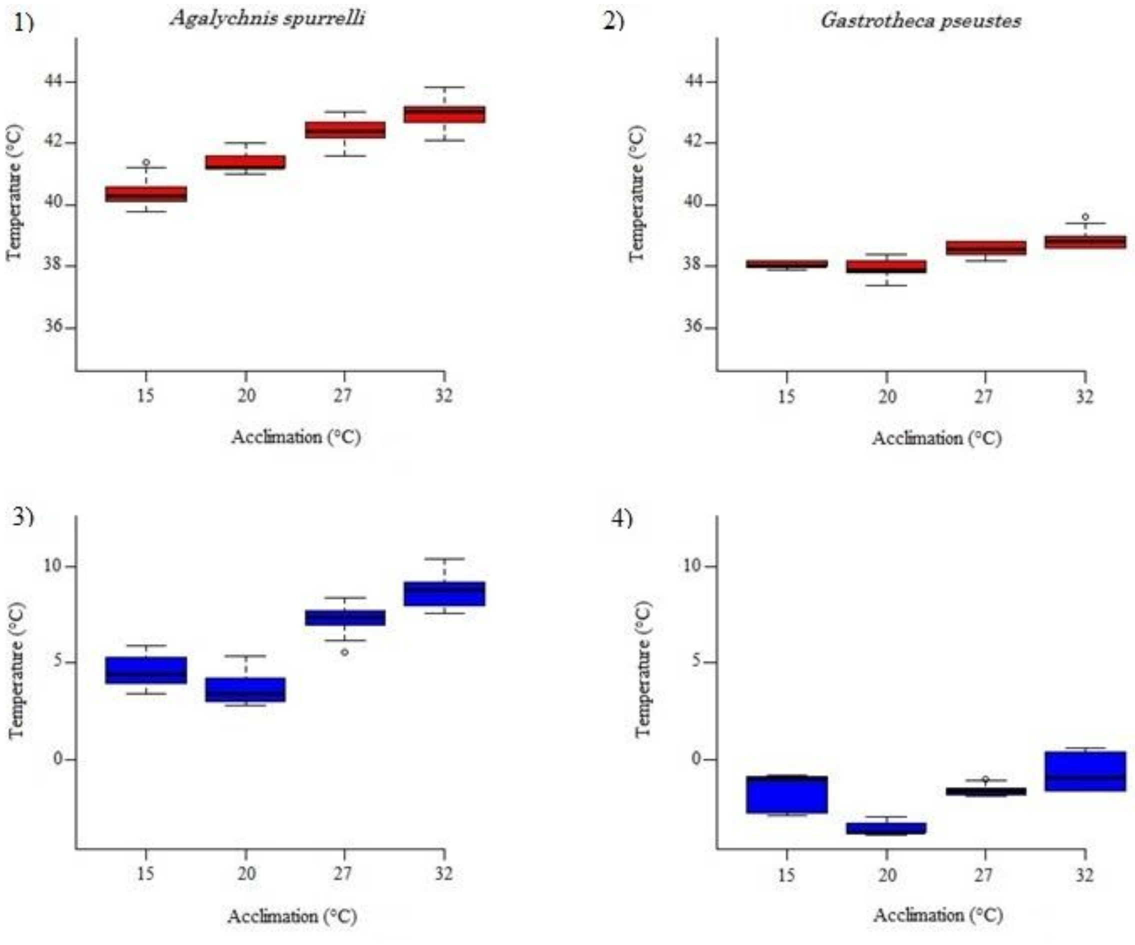


Figure A. Variation of critical thermal maximum (1-2) and critical thermal minimum (3-4) of two tropical species of different altitude in Ecuador at four temperatures of acclimation (15 °C, 20 °C, 27 °C and 32 °C) for the lowland species *Agalychnis spurrelli* (left panels) and the highland species *Gastrotheca pseustes* (right panels).

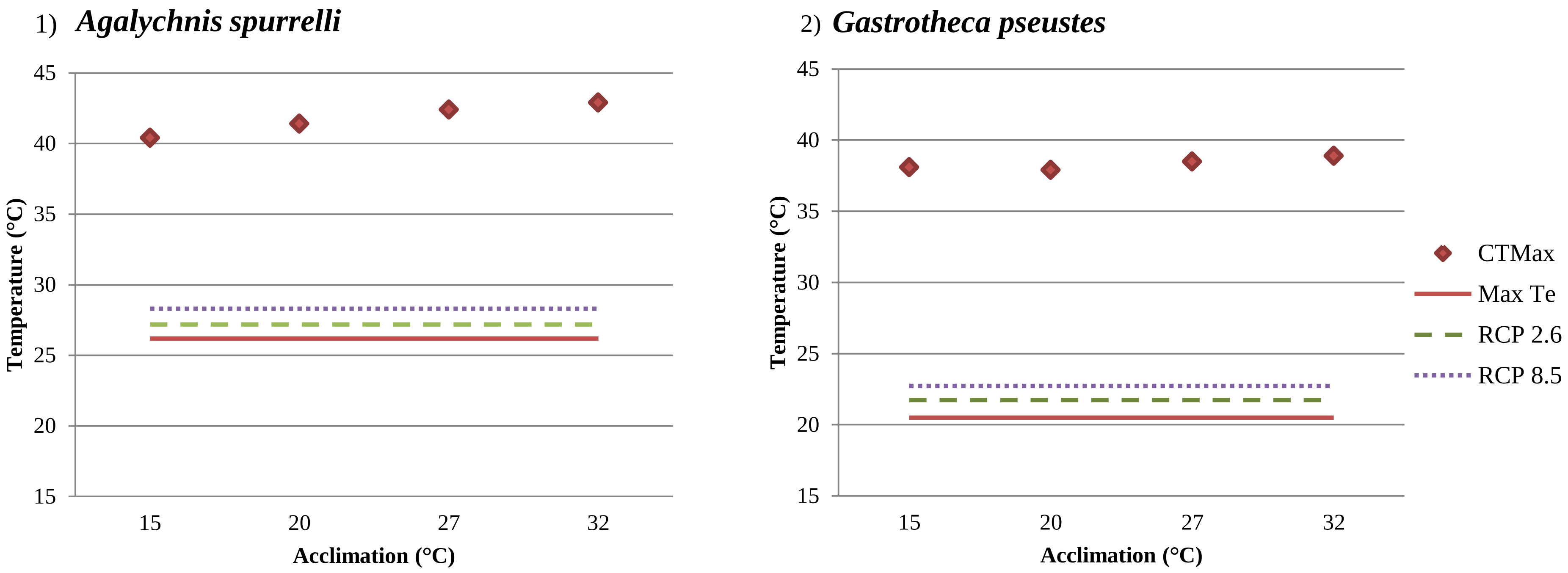


Figure B. Relative vulnerability in the present and future of both study species (CTmax-MaxTe) at four different acclimation temperatures in *Agalychnis spurrelli* and *Gastrotheca pseustes*.

5. TABLES

Table 1. Summary of the CTmax and CTmin (mean ± SD) of each acclimation temperature treatment of both species *Agalychnis spurrelli* and *Gastrotheca pseustes*.

Species	Acclimation temperature	CTmax (°C)	N	CTmin(°C)	N
Agalychnis spurrelli	32°C	42.9±0.09°C	18	8.7±0.287	10
	27°C	42.4±0.089	17	7.3±0.245	11
	20°C	41.4±0.098	13	3.7±0.268	11
	15°C	40.4±0.109	16	4.6±0.265	11
Gastrotheca pseustes	32°C	38.9±0.121	10	-0.7±0.284	10
	27°C	38.5±0.073	10	-1.6±0.097	10
	20°C	37.9±0.072	19	-3.6±0.084	19
	15°C	38.1±0.037	10	-1.5±0.293	10

Table 2. Acclimation response ratio (ARR=range of CTmax or CTmin/ range of acclimation temperatures) in *Agalychnis spurrelli* and *Gastrotheca pseustes*.

Species	Range acclimation temperature	Range CTmax	ARR_{max}	Range CTmin	ARR_{min}
Agalychnis spurrelli	17	2.5	0.14	5	0.29
Gastrotheca pseustes	17	1	0.05	2.9	

Table 3. Thermal breadth (difference between CTmax and CTmin) of each acclimation temperatures in *Agalychnis spurrelli* and *Gastrotheca pseustes*.

	Acclimation Temperatures			
Species	**15°C**	**20°C**	**27°C**	**32°C**
Agalychnis spurrelli	35.8	37.7	35.1	34.2
Gastrotheca pseustes	39.6	41.5	40.1	39.6

Table 4. Macroenvironmental data at present and future scenarios and future variation from present maximum exposure temperature.

Location	Maximum Exposure Temperature (°C)						
	Present			**RCP 2.6**	**RCP 8.5**	**ΔT 2.6**	**ΔT 8.5**
	$\bar{x}$T	**T_{max}**	**T_{min}**	**T_{max}**	**T_{max}**		
Durango	24.88	29.79	20.29	30.8	31.9	1.01	2.11
Ambato-Guaranda road	8.23	14.19	2.7	15.4	16.4	1.21	2.21

Table 5. Microenvironmental data gathered between August to December 2014 at the two collection localities.

Location/Altitude (m.a.s.l)	$\bar{x}$ T (°C)	T Max (°C)	T Min (°C)	Maximum Temperature Change (°C) in 2070	
				2.6	**8.5**
Durango, (200)	24.68	26.19	23.97	27.2	28.3
Ambato-Guaranda, (3467)	13.02	20.52	7.8	21.73	22.73

Table 6. Mean of maximum and minimum of the temperatures and thermal breadth in microenvironmental of the two collection localities.

Location	$\bar{x}$ T Max (°C)	$\bar{x}$ T Min (°C)	Breadth (°C)	$\bar{x}$ Breadth (°C)
Durango	25.54	24.11	2.22	1.13
Ambato-Guaranda	16.08	9.19	12.72	7.54

Table 7. Relative vulnerability (WT=CTmax-MaxTe) of *Agalychnis spurrelli* and *Gastrotheca pseustes* in current and future conditions

	Relative Vulnerability (Current climate conditions)				Relative Vulnerability (RCP 2.6)				Relative Vulnerability (RCP 8.5)			
Species	**15°C**	**20°C**	**27°C**	**32°C**	**15°C**	**20°C**	**27°C**	**32°C**	**15°C**	**20°C**	**27°C**	**32°C**
Agalychnis spurrelli	14.21	15.21	16.21	16.71	13.2	14.2	15.2	15.7	12.1	13.1	14.1	14.6
Gastrotheca pseustes	17.58	17.38	17.98	18.38	16.37	16.17	16.77	17.17	15.37	15.17	15.77	16.17

6. APPENDIXES

Appendix A: Analysis of the Covariance (ANCOVA), Normal distribution and Homogeneity of the variance of CTmax~Acclimation in *Agalyschnis spurrelli*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
ACLIM	3	10,9347	3,6449	32,0727	3,197e-07	***
WEIGHT	1	0,4538	0,4538	3,9929	0,06195	.
ACLIM:WEIGHT	2	0,2192	0,1096	0,9642	0,40119	
RESIDUALS	17	1,9320	0,1136			

Significance 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

Shapiro-Wilk	**Levene Test**	
p-value=0.905	F=0.1973	p>0.8978

Appendix B: Analysis of the Covariance (ANCOVA), Normal distribution and Homogeneity of the variance of CTmin~Acclimation in *Agalyschnis spurrelli*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
ACLIM	3	167,465	55,822	70,6733	1,085e-14	***
WEIGHT	1	0,684	0,684	0,8666	0,3585	
ACLIM:WEIGHT	3	1,597	0,532	0,6738	0,5741	
RESIDUALS	34	26,855	0,790			

Significance 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

Shapiro-Wilk	**Levene Test**	
p-value=0.2284	F=0.1528	p>0.9273

Appendix C: Analysis of the Covariance (ANCOVA), Normal distribution and Homogeneity of the variance of CTmax~Acclimation in *Gastrotheca pseustes*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
ACLIM	3	7,0339	2,34462	30,1352	2,351e-10	***
WEIGHT	1	0,0175	0,01754	0,2255	0,6375	
ACLIM:WEIGHT	3	0,2863	0,09542	1,2264	0,3127	
RESIDUALS	40	3,1121	0,07780			

Significance 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

Shapiro-Wilk	**Levene Test**	
p-value=0.2416	F=2.0323	p>0.1232

Appendix D: Kruskal-Wallis (Non-parametric test) of CTmin~Acclimation in *Gastrotheca pseustes*

Chi-squared	36.8968
df	3
p-value	4.839e-08

Appendix E: *Post hoc* Tuckey Test of CTmax~Acclimation in *Agalychnis spurrelli*

Tukey multiple comparisons of means 95% family-wise confidence level				
Treatments	difference	lower	upper	p adj
T20-T15	0.9923077	0.6094206	1.3751948	0.0000000
T27-T15	2.0411765	1.6840057	2.3983472	0.0000000
T32-T15	2.5555556	2.2032280	2.9078831	0.0000000
T27-T20	1.0488688	0.6710637	1.4266739	0.0000000
T32-T20	1.5632479	1.1900181	1.9364776	0.0000000
T32-T27	0.5143791	0.1675810	0.8611772	0.0012866

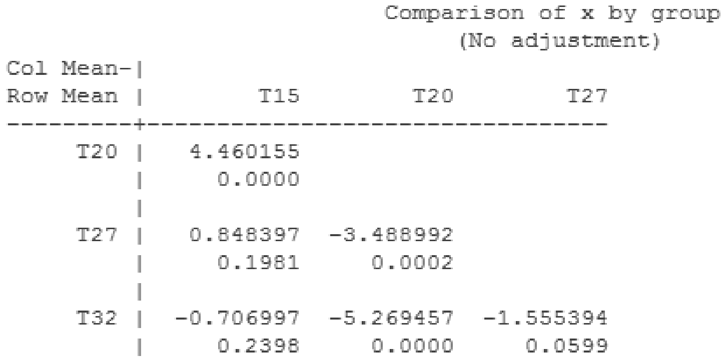
Appendix F: *Post hoc* Tuckey Test of CTmin~Acclimation in *Agalychnis spurrelli*

Tukey multiple comparisons of means 95% family-wise confidence level				
Treatments	difference	lower	upper	p adj
T20-T15	-0.9181818	-1.9148983	0.07853462	0.0803971
T27-T15	2.6454545	1.6487381	3.64217098	0.0000001
T32-T15	4.1009091	3.0795787	5.12223952	0.0000000
T27-T20	3.5636364	2.5669199	4.56035280	0.0000000
T32-T20	5.0190909	3.9977605	6.04042134	0.0000000
T32-T27	1.4554545	0.4341241	2.47678497	0.0025047

Appendix G: *Post hoc* Tuckey Test of CTmax~Acclimation in *Gastrotheca pseustes*

Tukey multiple comparisons of means 95% family-wise confidence level				
Treatments	difference	lower	upper	p adj
T20-T15	-0.1184211	-0.40906632	0.1722242	0.6986952
T27-T15	0.4800000	0.14729751	0.8127025	0.0020615
T32-T15	0.8611111	0.51929179	1.2029304	0.0000002
T27-T20	0.5984211	0.30777579	0.8890663	0.0000107
T32-T20	0.9795322	0.67849368	1.2805706	0.0000000
T32-T27	0.3811111	0.03929179	0.7229304	0.0235299

Appendix H: Dunn Test of Multiple Comparisons Using Rank Sums of CTmin~Acclimation in *Gastrotheca pseustes*



```
                           Comparison of x by group
                               (No adjustment)
Col Mean-|
Row Mean |        T15        T20        T27
---------+---------------------------------
     T20 |   4.460155
         |     0.0000
         |
     T27 |   0.848397  -3.488992
         |     0.1981     0.0002
         |
     T32 |  -0.706997  -5.269457  -1.555394
         |     0.2398     0.0000     0.0599
```

Appendix I: Relation between the CTmax and the acclimation temperatures in *Agalychnis spurrelli*

Regression Statistics	
Multiple correlation coefficient	0,99143863
R^2	0,98295056
R^2 adjust	0,97442583
Typical error	0,17729893
Observations	4

	Coefficients	*Typical Error*	*Statistics t*	*Probability*	*Inferior 95%*	*Superior 95%*	*Inferior 95,0%*	*Superior 95,0%*
Interception	0,63343195	0,33253602	1,90485214	0,19709084	0,79735507	2,06421897	0,79735507	2,06421897

Appendix J: Relation between the CTmax and the acclimation temperatures in *Gastrotheca pseustes*

Regression Statistics	
Multiple correlation coefficien	0,89129331
R^2	0,79440377
R^2 adjust	0,69160566
Typical error	0,24627401
Observations	4

	Coefficients	*Typical error*	*Statistics t*	*Probability*	*Inferior 95%*	*Superior 95%*	*Inferior 95,0%*	*Superior 95,0%*
Intercepción	16,612426	0,4619034	35,9651522	0,00077221	14,6250161	18,599836	14,6250161	18,599836

Appendix K: F test to compare two variances

Data	**ASP$Range, GPS$Range**
F	15.393
num df	17
denom df	14
p-value	5.87e-06

Alternative hypothesis: true ratio of variances is not equal to 1

95 percent confidence interval: 5.307403; 42.370958

Sample estimates: ratio of variances=15.39284

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Competing interests

No competing interests declared

Author contributions

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