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Ontogenetic variation of thermal tolerance in two anuran species of Ecuador: *Gastrotheca pseustes* (Hemiphractidae) and *Smilisca phaeota* (Hylidae) and their relative vulnerability to environmental temperature change

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A mi familia

Ontogenetic variation of thermal tolerance in two anuran species of Ecuador: *Gastrotheca pseustes* (Hemiphractidae) and *Smilisca phaeota* (Hylidae) and their relative vulnerability to environmental temperature change

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Abstract

Thermal tolerance and morphological plasticity have allowed amphibians to survive in many unusual habitats. Amphibians show a particular life cycle, presenting different ranges of thermal regimes in their different ontogenetic stages. In this study we show the critical thermal maximum (CTmax) and critical thermal minimum (CTmin) of three different developmental stages: larvae, metamorphic and post-metamorphic for two species of frog *Gastrotheca pseustes*, a high altitude species and *Smilisca phaeota*, a low altitude species of Ecuador. The CTmax values were similar for both species and higher in larvae: 37.9^{o}C for *Gastrotheca psesutes* and 44.0^{o}C for *Smilisca phaeota.* A decrease was observed at metamorphosis climax (stages 43-44): 37.6^{o}C for *Gastrotheca pseustes* and 36.4^{o}C for *Smilisca phaeota*. The data showed that after metamorphosis thermal limit decrease gradually. The high altitude species showed very low CTmin (lowest value for metamorphic: -3.4^{o}C) and consequently a wider thermal tolerance range for the three ontogenetic stages (wider range for larvae: 41.5^{o}C). The comparison between CTmax and maximum exposure temperature (Te) showed that metamorphic and post-metamorphic stages of *Smilisca phaeota* are the most vulnerable specie to change in environmental temperature living apparently over their upper thermal limit. According to this study the conservation strategies should focus on amphibian species of low altitude, in order to mitigate the effects of climate change in this group of animals.

Keywords: Ontogenetic stages, thermal tolerance, exposure temperature, vulnerability to climate change.

1. INTRODUCTION

Body temperature of amphibians is strongly affected by environmental temperature because they are ectothermic organisms (Duellman and Trueb, 1994). In amphibians, temperature acts as a controlling factor for many physiological processes, including rates of oxygen uptake, heart rate, locomotion, water balance, digestion, developmental rate, sex determination, and immune function (Blaustein et al. 2010; Corn, 2005). Some amphibians show a particular life cycle, with early stages of life taking place in water and the adult stage taking place partially or completely on land (Duellman and Trueb, 1994). The different ontogenetic stages are exposes to different ambient temperature conditions, depending on the microhabitat and geographical area where they occur. As a result, the thermal environment to which these frogs are exposed changes during their life cycle (Sherman, 1979). Thermal tolerance and morphological plasticity have allowed amphibians to survive in many unusual habitats such as arid environments and high elevations (Wu and Kam, 2005). Critical thermal limits define the range of thermal tolerance of an organism. If the temperature increases or declines below these limits, effects on physiological functions may even cause the death of an organism (Katzenberger et al. 2012). The study of the thermal tolerance range is essential for understanding many aspects of the organisms' biology, because this represents the conditions that limit their fundamental niche and therefore, their presence and evolution in a specific habitat and geographic area (Hutchinson, 1961). If the thermal range is wide the organism will have more chances to survive climatic variations (Williams et al. 2008).

Floyd (1983), Herreid and Kinney (1967), Whitford and Delson (1973) and Sherman (1979) found that during the stage of metamorphosis, thermal tolerance is greatly reduced. Cupp (1980) suggested that this reduction in thermal tolerance during metamorphosis is indicative of changes within the organism. Floyd (1983) and Sherman (1979) suggested that because the metamorphosis is a period of great biochemical and morphological rearrangement, the animals are under stress and they are unable to tolerate external environmental changes such as temperature, making them more vulnerable than during other ontogenetic stages.

Organisms' thermal tolerance generally varies depending on the geographical area occupied by the species. It has been proposed that ectothermic animals living in the tropics are more vulnerable to environmental changes (Deutsch et al. 2008; Huey et al. 2009). Reasons include the tropics having minimum annual seasonality, their species are exposed to higher temperatures than temperate regions, and the organisms have narrow ranges of thermal tolerance (Deutsch et al. 2008; Sunday et al. 2011). In addition, they are exposed to environmental conditions closer their thermal limits (Tewksbury et al. 2008). Several studies indicate that the thermal limits show a correlation with environmental conditions, habitat and geographical distribution of the species (Wu and Kam, 2005). Little research has been focused on thermal tolerances of species in tropical highlands (Navas 2006; Navas et al. 2010) where the environments present greater daily fluctuations in temperature (Dangles et al. 2008).

Over Earth's history, climate has considerably changed due to natural processes, but in present time a general warming is happening much faster than any change the planet has experimented in recorded history (IPCC, 2007). An increase of 0.6 to 0.7°C has been observed over the last century, affecting biochemical and physiological processes and life cycles of organisms (Beitinger et al. 2000; Castañeda et al. 2004; Corn, 2005; Mora and Maya, 2006; Nguyen et al. 2011). When a species confront adverse changes in climate, it has some potential options: adjusting their behavior (rapid response), physiological adjustments as acclimation (slow response), evolutionary adjustments through natural selection, change its distribution range and the species goes extinct (Blaustein et al. 2010; Deutsch et al. 2008). The organisms that may face extinction are those with low or null adaptability (Hughes, 2000; Williams et al. 2008). Determining how changes in environmental temperature affect organisms requires an understanding of species thermal limits (including at different ontogenetic stages) and their ability to respond to such changes (Blaustein et al. 2010; Nguyen et al. 2011).

This study aims to determine the thermal tolerance of larval, metamorphic and post-metamorphic individuals of two species of Ecuadorian frogs: *Gastrotheca pseustes* and *Smilisca phaeota,* the first a high altitude species and the second a low altitude species. Both species have similar life cycles, with activity during night and are found in disturbed areas (Duellman, 1970). Both are arboreal, morphologically similar, and deposit the eggs (*S* phaeota) or tadpoles (*G. pseustes*) in small temporary pools of water (Duellman, 1970; Ron et al. 2014; Savage, 2002). The thermal limits will provide evidence of the thermal specialization between species of tropical environments occurring in different altitudes. Linking these thermal parameters to actual thermal exposure conditions from microenvironments used by each stage and species, will allow us to determine their relative vulnerability to changes in the thermal regimes of their environments, resulting from anthropogenic climate change. This exercise can guide environmental authorities to promote focused conservation strategies on specific developmental stages, species and geographical regions.

2. MATERIALS AND METHODS

2.1 Study organisms

Ninety individuals, eggs or tadpoles, of each species, *Smilisca phaeota* and *Gastrotheca pseustes*, were collected in roadside ponds at 120 m.a.s.l. in Durango, Esmeraldas province (1.0374°N; 78.6221°W) and at 3467 m.a.s.l. on the Ambato-Guaranda road, Bolivar province (1.3367°S; 78.7594°W), respectively. The experimental individuals for each species were collected in the same pond. Thirty individuals were designated for experiments at each developmental stage. Within each of the three developmental stages, 15 individuals were tested for upper thermal limit and 15 for lower thermal limit. Larvae individuals were tested while in 30-36 Gosner stages, metamorphic individuals between 43-44 Gosner stages and post-metamorphic individuals in 46 Gosner stage (Gosner, 1960).

The eggs, tadpoles, and metamorphosing individuals were kept at the "Balsa de los Sapos" Conservation Initiative facilities at Pontificia Universidad Católica del Ecuador in Quito (2800 m.a.s.l.), at room temperature (aprox. 20^{o}C) until experimentation. Time for experimentation varied depending on species and stage, from 3 to 92 days after collection. Each individual was examined in a single physiological test.

2.2 Thermal tolerance

Critical thermal maximum (CTmax) and critical thermal minimum (CTmin) were obtained for each developmental stage for both species. All the individuals were acclimatized during at least three days at 20^{o}C before experimentation, following published protocols (Brattstrom, 1968; Hutchinson, 1961; Lutterschmidt and Hutchison, 1997a, 1997b; Navas, 2010).

Thermal tolerance tests were carried out using a thermal water bath (HUBER D77656 Offenburg), which had a fixed software mechanism to raise or to decrease water temperature at a rate of 0.25^{o}C/min. Animals were placed in plastic beakers with different volumes of water, depending on their developmental stage (100 ml for larvae and 10 ml for metamorphic and post-metamorphic individuals), to avoid desiccation. The endpoint of the experiment was the lack of movement of the specimen, which was evaluated by touching it with a wooden stick every 10 minutes at the start of the experiment and every 30 seconds when the specimen started lack of mobility. The critical temperatures were obtained from a rapid response mercury thermometer at the moment when the individuals did not respond to the stimulus. Later, the individuals were placed in containers with water at room temperature until they recovered normal movement. The value of each parameter for each stage represented the average of all valid experimental values. An experiment was considered valid if the individual survived 24 hours after the test.

A Shappiro Wilks test was used to evaluate data normality. A Bartlett test was used to check for variance-homogeneity. If the results were not significant, outliers were removed (they existed), because they could represent experiments with problems. Both tests were ran again and if the results did not change, a log10 transformation of the data was performed to adjust and improve normality and variance-homogeneity. Depending on statistical signification the Analysis of Variance (ANOVA) or a Kruskal-Wallis non-parametric test was used to check for differences between the mean values of the thermal tolerance of different ontogenetic stages. Finally, depending on previous results, the *post-hoc* Tukey HSD test or a Dunn test was applied (depending on the case) to determine which ontogenetic stage is different.

An estimation of the thermal range for both species was defined by the difference between CTmax and CTmin. This parameter showed if specific stages or species at different altitudes presented narrower physiological tolerances.

2.3 Microenvironmental data

Two temperature loggers (HOBO Pendant temp) were placed at the *Smilisca phaeota* collection site. One sensor was placed in the pond where tadpoles were found and other near ground level (aprox. 60 cm) under surrounding vegetation close to the pond. For *Gastrotheca pseustes*, one logger was set at the tadpoles' collection pond. The ground level logger at this location disappeared, so an estimation of air temperature near the ground at this place was obtained from a logger set in a different site where this species has also been collected: Cashca Totoras Protected Forest, 50 km (straight-line) southwest from the tadpoles collection site (1.7177°S; -78.9778°W; 3100 m.a.s.l.). The sensors collected data every 15 minutes, between August and December 2014, in order to estimate the thermal exposure extremes experienced by these species in their natural habitats. Maximum and minimum weekly temperature averages were obtained based on daily data. Microhabitat thermal variation (pond and air near ground) was obtained as the mean of the difference of the maximum and minimum weekly temperatures.

2.4 Exposure conditions (Te)

For both species, tadpoles' range of exposure conditions was the maximum and minimum weekly temperatures in the ponds during the monitoring period. During the beginning of metamorphosis, both species remain in the water. They leave the pond when the process is close to its end. We assumed that the metamorphic and post-metamorphic individuals presented a circadian behavior similar to adults when they are outside the water.

Smilisca phaeota is active during the nighttime. During the day, it rests on the upper side of big leaves above ground, at least throughout the wet season (Savage, 2002). Therefore, we assumed that the maximum exposure temperature, for both metamorphic and post-metamorphic individuals, was the maximum weekly air temperature recorded by the logger near the ground.

Gastrotheca pseustes is a nocturnal species, during daytime it looks for refuges, such as bromeliads or under logs (Carvajal-Endara, 2010; Merino-Viteri, A., unpublished observations). For this study, the maximum exposure temperature for the metamorphic and post-metamorphic individuals was the maximum weekly air temperature for the nocturnal data only (activity period: 6pm-6am) recorded by the logger near the ground.

2.5 Relative vulnerability to environmental temperature changes

To determine the relative vulnerability to environmental temperature changes, the difference between the thermal critical maximum and maximum exposure temperature for each species at every stage was calculated: (CTmax – maximum Te). Only the warmest limit of the tolerance was used because the predictions of climate change for the region show a future increase in temperature (Wilson et al. 2005).

3. RESULTS

3.1 Thermal tolerances and ontogenetic stages

3.1.1 *Gastrotheca pseustes*:

The mean CTmax for the different ontogenetic stages is 37.8±0.2°C (n=14) for larvae, 37.6±0.2°C (n=15) for metamorphic individuals, and 37.1±0.2°C (n=12) for post-metamorphic individuals (Fig. A.1; Table A). The data did not show a normal distribution and homogeneity of variance after eliminating outliers and performing log10 transformation. The results are summarized in Appendix A. The Kruskal-Wallis test (Chi-squared = 22.1578, DF = 2, p = 0.009) and Dunn´s test proved that the post-metamorphic is different to the others.

The mean CTmin for the three developmental stages are -3.7±0.5°C (n=15) for larvae, -3.4±0.5°C (n=15) for metamorphic individuals and -3.6±0.5°C (n=15) for post-metamorphic individuals (Fig. A.2; Table A). However, it is necessary to consider that these values are underestimated because the experiments were stopped before the animals reached the endpoint. This situation was caused by the freezing of the water inside of the plastic beakers, containing the animals.

Despite of this situation, the thermal tolerance ranges for the three stages were calculated and are summarized in Table B. The post-metamorphic stage showed the lowest range of thermal tolerance (40.5°C), and the larvae stage showed the greatest range of thermal tolerance (41.5°C).

3.1.2 *Smilisca phaeota:*

The mean CTmax for the different ontogenetic stages is 44.0±0.4°C (n=8) for larvae, 36.4±0.4°C (n=12) for metamorphic individuals, and 36.0±0.4°C (n=14) for post-metamorphic individuals (Fig. A.3; Table A). The data showed a normal distribution and homogeneity of variance, so an ANOVA was performed. The results are summarized in Appendix B. The ANOVA results ($F = 1143.5$ $p < 0.0001$) proved that at least one of the stages is different. The *post-hoc* Tukey HSD test showed the larvae stage was different to the other stages.

The mean CTmin for the three developmental stages is 8.44±0.3°C (n=10) for larvae, 8.42±0.3°C (n=14) for metamorphic individuals, and 6.9±0.3°C (n=14) for post-metamorphic individuals (Fig. A.4; Table A). The data did not show a normal distribution and homogeneity of variance. The results are summarized in Appendix C. The Kruskal-Wallis test (Chi-squared = 28.038, DF = 2, $p = 0.0008$) and Dunn's test proved that the post-metamorphic stage was different to the other stages.

The thermal tolerance ranges for the three stages were calculated and are summarized in Table B. The results for this species showed that the metamorphic stage had the lowest range of thermal tolerance (27.9°C) and the larvae stages had also the greatest range of thermal tolerance (35.6°C).

3.2 Microenvironmental data and exposure conditions

3.2.1 Microenvironmental data

For both species the raw data was summarized weekly, resulting in 18 weeks data for *Gastrotheca pseustes* and 16 weeks data for *Smilisca phaeota* (Appendices D and E). The weekly microenvironmental data showed that the ponds were thermally more stable environments for both species. Also, as expected, the lowland locality showed warmer maximum conditions than the highland site (Appendices D and E).

3.2.2 Maximum exposure conditions

Table C summarizes the maximum exposure temperature for each ontogenetic stage for *Gastrotheca pseustes* and *Smilisca phaeota.* The maximum exposure conditions for *Gastrotheca pseustes* in the three ontogenetic stages are lower than for *Smilisca phaeota* (Fig. A.1 and A.3; Table C).

3.3 Vulnerability to climate change

For each ontogenetic stage of both species, the temperature difference between the CTmax and the maximum Te is summarized in Table D. *Smilisca phaeota* shows lower values than *Gastrotheca pseustes* for all the stages. *Smilisca phaeota*'s metamorphic and post-metamorphic stages are the most vulnerable ones. The results suggest that even now they are exposed to conditions above their thermal tolerance (negative values). The larval stage of *Gastrotheca pseustes* is the most vulnerable stage for this species, however its warming tolerance is extremely high (21.0°C).

4. DISCUSSION

When comparing the CTmax results between *Gastrotheca pseustes* and *Smilisca phaeota*, they show higher values in larval stage compared with metamorphic and post-metamorphic stages. This result may be explained by the restriction of larvae to temporary ponds (Ron et al. 2014), which can be subjected to extreme temperatures when they are small or are drying out. The other two developmental stages may have the option of looking for refuges and buffer from warm conditions. It has been observed that amphibian larvae select specific water temperatures in the ponds, preferring warmer and shallow water (Mullally, 1953; Brattstrom, 1962; Brown, 1969). This exposure may allow an early metamorphic, to develop faster, to leave the ponds before it dries out, and also allowing them to escape from aquatic predators sooner (Brattstrom, 1962; Heatwole et al, 1968; Sherman, 1979). In addition, our results for the low altitude species (CTmax = 44.0^{o}C) support the assumption that tropical species larvae should exhibit CTmax above 40.0^{o}C (Abe and Neto, 1991). However, the highest CTmax ever reported for a tadpole is 43.3^{o}C in *Gastrophryne carolinensis* for a temperate species (Cupp, 1974), and 44.7^{o}C in *Lepidobatrachus llanensis* for a subtropical species (Duarte et al. 2012). This data may suggest that the CTmax is not linked to latitude. Our results may also suggest that there are not high differences considering the altitude since our high altitude species has a CTmax of 37.9^{o}C.

Floyd (1983), Herreid and Kinney (1967), Whitford and Delson (1973) and Sherman (1979) have demonstrated a decrease in thermal tolerance in the amphibian metamorphic stage. It is a time of great stress, morphological changes and biochemical reorganization (Cupp, 1974; Sherman, 1979). Here we demonstrate a decrease of CTmax in metamorphic individuals compared to the larval stage. In *Gastrotheca pseustes* the difference is 0.3^{o}C, and for *Smilisca phaeota* the difference is 7.6^{o}C. It also has been proposed that after metamorphosis the upper thermal limit decrease gradually until adult stage, when the tolerance increases again (Cupp, 1974; Heatwole et al. 1968; Sherman, 1979). This study shows that the lowest CTmax is the post-metamorphic stage in *Gastrotheca pseustes* and in *Smilisca phaeota*. A possible cause is the change in microhabitat presented by anurans at this stage and because the physiological system decays in the stage of metamorphosis. Our data are consistent with other ontogenetic studies, including reports on *Bufo fowleri* *woodhousii*, which has a thermal tolerance of 42.5°C for larvae, 37.9°C for metamorphic and 37.7°C for post-metamorphic stage (Sherman, 1979).

In the other hand, CTmin values show differences between the studied species (Fig. A.3 and A.4). This results support the influence of environmental conditions on the lower thermal limit, as suggested by Deutsch et al. (2008) and Katzenberger et al. (2012). CTmin in *Gastrotheca pseustes* needs further attention. The values are extremely low compared even with high latitude species. The lowest CTmin reported for a temperate species is 2°C in *Hyla chrysoscelis* (Layne and Romano, 1985). Additionally, a different methodological approach is necessary to assess a more accurate CTmin values. CTmin in *Smilisca phaeota* shows a similar pattern than CTmax having the larvae stage the highest tolerance and the post-metamorphic stage with the lowest tolerance.

The high altitude species shows the wider tolerance for all the developmental stages (Table B). This pattern may be explained by the effect of the great daily fluctuation of temperature in highland environments. This result also suggests that tropical species not necessarily present narrow thermal tolerances. Here, we present differences in thermal tolerances depending on the altitude.

Tolerance to high temperatures reflects adaptations to local thermal environments throughout their evolutionary history (Christian et al. 1988), which would mean that the species adapt and evolve to withstand environmental changes. This may be the case for *Gastrotheca pseustes*, which occurs at high altitude in distribution and may be adapted to live in a wider range of environments because its range of thermal tolerance is wide compared to the lowland species.

Deutsch et al. (2008) and Huey et al. (2009) suggest the tolerance to environmental warming comes from the difference between the species physiological limit (CTmax in this paper) and the environmental conditions experienced by the organism (maximum Te in this paper). *Smilisca phaeota* is the most vulnerable species to changes in environmental temperature, because it experiences daily ambient temperatures closer to its thermal tolerance than the high altitude species. The results show that *Smilisca phaeota* in metamorphic and post-metamorphic stages, experience environmental temperatures that exceed their thermal limits. It is possible because the individuals in these stages behave differently to adults (our *a priori* assumption) looking for refuges during day time or adjusting its physiology to these new conditions differently to adults.

The climate change impacts depend not only on the thermal tolerance of the organism, but also the size of the organism, the microenvironment, macrophysiological and thermoregulatory behavior (Corn, 2005). In addition, the vulnerability to this change will depend on the species susceptibility and the exposure degree to that change (Williams et al. 2008). As shown with our results, we should pay more attention to the gather this kind of information for more accurate vulnerability analysis to climate change and to focus conservation strategies in lowland species.

5. CONCLUSIONS

The results showed that there is a difference between thermal tolerances in different ontogenetic stages, being the most tolerant the larval stage and having a reduced tolerance in CTmax in the metamorphic stages in both species. *Gastrotheca pseustes* showed higher tolerance to cold environments conditions suggesting that it may be an adaptation to extreme cold environments. *Smilisca phaeota* also showed smaller thermal tolerance ranges maybe because it lives in more stable thermal environments than highland species that experience daily extreme condition. *Smilisca phaeota* showed to be the metamorphic and post-metamorphic were the most vulnerable stages. These stages experiencing thermal conditions close or over their thermal limits, possibly they are require buffering these warm conditions through behavior. This study shows the importance of accurate climate change vulnerability analysis based on thermal physiology and specific exposure conditions. It is possible that conservation strategies should focus on lowland amphibian’s species, in order to mitigate climate change impacts in this group of animals. It is possible to think that highland species would be the most threatened by climate change because they have not colder places to migrate if warming occurs. However, our data shows that they are less threatened that the lowland species because of its exposure conditions.

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7. FIGURES

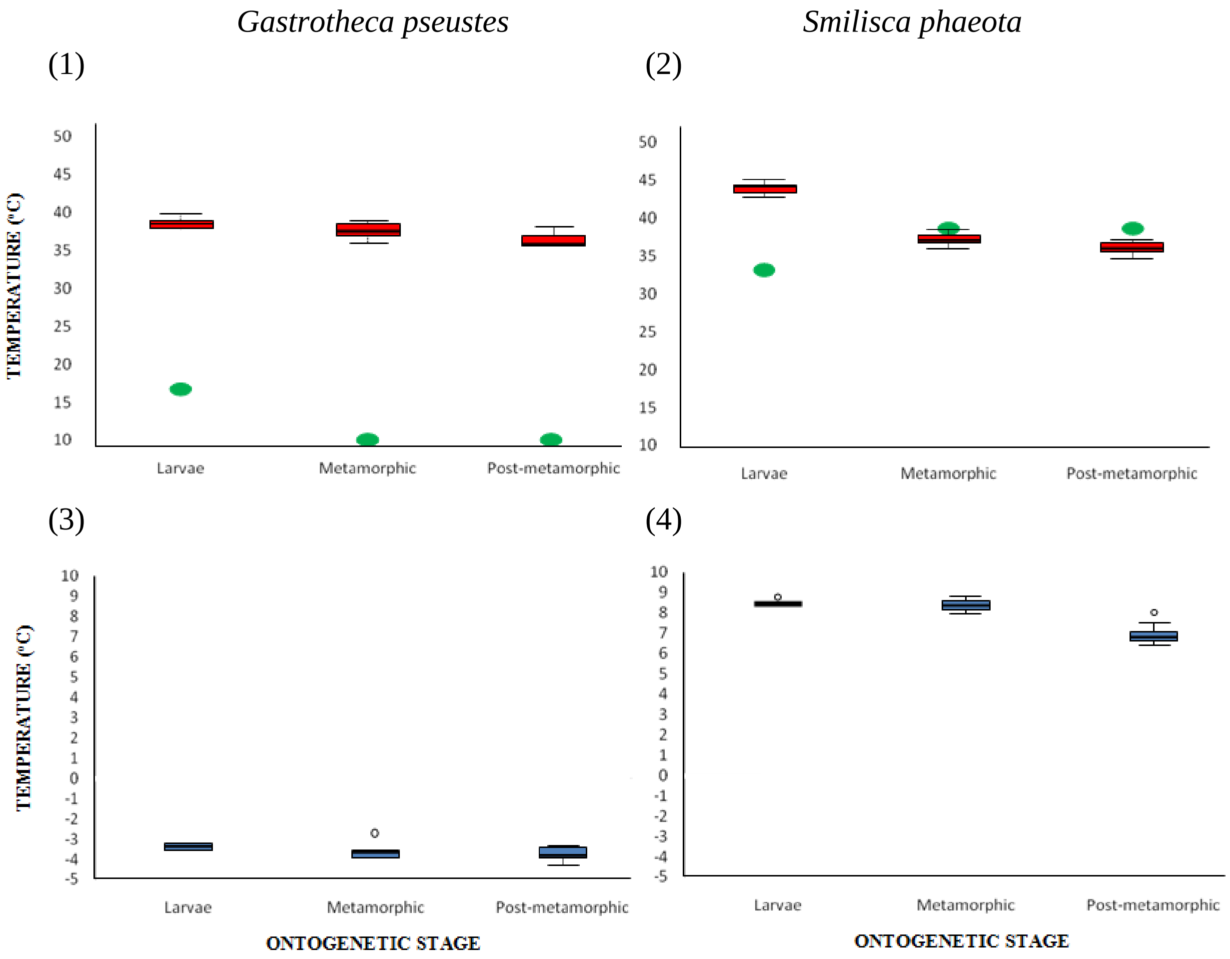

Fig. A. Variation of critical thermal maxima (CTmax) (1-2) and critical thermal minimum (CTmin) (3-4) in three different ontogenetic stages (larvae, metamorphic and post-metamorphic) for both studied species *Gastrotheca pseustes* (left panels) and *Smilisca phaeota* (right panels). The distance between CTmax and CTmin represents the thermal range for each developmental stage and species. The green circles show the maximum exposure temperature (Te) in the microenvironment for each developmental stage and species. The distance between CTmax and the correspondent green circle represents the tolerance to changes in environmental temperature.

8. TABLES

Table A. Summary of mean of critical thermal maxima (CTmax) and critical thermal minimum (CTmin) for the three ontogenetic stages for *Gastrotheca pseustes* and *Smilisca phaeota.*

SPECIES	ONTOGENETIC STAGE	CTmax	N	CTmin	N
Gastrotheca pseustes	Larvae	37.8±0.2	14	-3.7±0.5	15
	Metamorphic	37.6±0.2	15	-3.4±0.5	15
	Post-metamorphic	37.1±0.2	12	-3.6±0.5	15
Smilisca phaeota	Larvae	44.0±0.4	8	8.44±0.3	10
	Metamorphic	36.4±0.4	12	8.42±0.3	14
	Post-metamorphic	36.0±0.4	14	6.9±0.3	14

Table B. Thermal tolerance ranges (CTmax – CTmin) for the three ontogenetic stages for *Gastrotheca pseustes* and *Smilisca phaeota* (the smaller thermal ranges for each species are in bold).

SPECIES	**ONTOGENETIC STAGE**		
	Larvae	Metamorphic	Post-metamorphic
Gastrotheca pseustes	41.5	40.9	**40.5**
Smilisca phaeota	35.6	**27.9**	29.1

Table C. Maximum exposure temperature for each developmental stage and studied species, based on specific microhabitat occupied and behavior.

SPECIES	ONTOGENETIC STAGE		
	Larvae	Metamorphic	Post-metamorphic
Gastrotheca pseustes	16.9	10.8	10.8
Smilisca phaeota	34.2	38.0	38.0

Table D. Index of vulnerability to change in environmental temperature based on the difference between CTmax and the maximum Te, for the three ontogenetic stages and two studied species (the most vulnerable stages are in bold).

SPECIES	ONTOGENETIC STAGE		
	Larvae	Metamorphic	Post-metamorphic
Gastrotheca pseustes	21.0	26.8	26.1
Smilisca phaeota	9.8	**-1.6**	**-2.0**

9. APPENDIX

Appendix A. Result of statistical test, for normality (Shapiro Wilks test) and homogeneity of variance (Bartlett test) with outliers, without outliers and log10 transformation for critical thermal maximum (CTmax) of *Gastrotheca pseustes*.

	Shappiro Wilks test		**Bartlett test**
	Ontogenetic Stages	*p*-values	*p*-values
With outliers	Larvae	p = 0.04	p = 0.006
	Metamorphic	p = 0.03	
	Post-metamorphic	p = 0.01	
Without outliers	Larvae	p = 0.009	p = 0.01
	Metamorphic	p = 0.03	
	Post-metamorphic	p = 0.001	
Trasformation log10	Larvae	p = 0.008	p = 0.004
	Metamorphic	p = 0.03	
	Post-metamorphic	p = 0.001	

Appendix B. Result of statistical test, for normality (Shapiro Wilks test) and homogeneity of variance (Bartlett test) for critical thermal tolerance maximum (CTmax) of *Smilisca phaeota*.

Shappiro Wilks test		**Bartlett test**
Ontogenetic Stages	*p*-values	*p*-values
Larvae	p = 0.18	p = 0.4
Metamorphic	p = 0.04	
Post-metamorphic	p = 0.48	

Appendix C. Result of statistical test, for normality (Shapiro Wilks test) and homogeneity of variance (Bartlett test) with complete data and log10 transformation for critical thermal minimum (CTmin) of *Smilisca pseustes.*

	Shappiro Wilks test		**Bartlett test**
	Ontogenetic Stages	*p*-values	*p*-values
Complete data*	Larvae	p = 0.005	p = 0.02
	Metamorphic	p = 0.14	
	Post-metamorphic	p = 0.03	
Trasformation log10	Larvae	p = 0.004	p = 0.0000003
	Metamorphic	p = 0.16	
	Post-metamorphic	p = 0.10	

*No outliers were present.

Appendix D. Weekly microenvironmental data for *Gastrotheca pseustes* collection site (Altitude: 3467 m.a.s.l.). Columns represent maximum, minimum and range of temperature in pond and air near ground. For logger near the ground also maximum and minimum were calculated but considering only the nocturnal period between 6pm and 6am. Last row shows an estimation of the thermal variation of each microhabitat (the maximum weekly temperature averages are in bold).

Week	Date of week's first day	Pond			Air Near ground				
		Max T (^{o}C)	Min T (^{o}C)	Range (^{o}C)	Max T (^{o}C) on day	Min T (^{o}C) on day	Range (^{o}C)	Max T (^{o}C) at night	Min T (^{o}C) at night
1	04/08/2014	11.8	9.1	2.7	16.0	6.7	9.3	10.1	6.5
2	11/08/2014	11.7	8.9	2.8	20.0	7.1	12.9	10.2	5.8
3	18/08/2014	13.0	9.7	3.3	19.3	6.2	13.1	10.6	6.1
4	25/08/2014	14.2	10.4	2.8	17.9	6.3	11.6	9.7	6.2
5	01/09/2014	14.9	10.1	4.8	20.0	6.0	14.0	10.6	5.9
6	08/09/2014	13.0	9.7	3.3	18.7	6.7	12.0	10.1	6.3
7	15/09/2014	15.4	10.7	4.7	16.7	7.0	9.7	10.0	6.9
8	22/09/2014	15.9	11.2	4.7	17.7	6.1	11.6	9.7	6.2
9	29/09/2014	14.3	9.4	4.9	21.3	6.9	14.4	**10.8**	6.1
10	06/10/2014	13.2	10.2	3.0	15.6	7.5	8.1	9.6	7.4
11	13/10/2014	12.0	10.1	1.9	19.4	7.7	11.7	10.2	7.3
12	20/10/2014	14.6	11.8	2.8	15.7	7.4	8.3	9.6	7.3
13	27/10/2014	15.9	11.7	4.2	20.3	7.4	12.9	10.1	6.8
14	03/11/2014	**16.9**	12.2	4.7	21.9	7.5	14.4	10.6	7.1
15	10/11/2014	16.4	11.3	5.1	18.2	6.8	11.2	9.4	6.4
16	17/11/2014	14.3	9.8	4.5	**22.8**	7.4	15.4	10.4	7.2
17	24/11/2014	15.8	10.8	5.0	21.6	7.8	13.8	10.1	7.3
18	01/12/2014	15.5	10.1	5.4	19.5	7.9	11.6	10.1	8.0
Mean Range:				3.9			12.0		

Appendix E. Weekly microenvironmental data for *Smilisca phaeota* collection site (Altitude: 120 m.a.s.l.). Columns represent maximum, minimum and range of temperature in pond and air near ground. For logger near the ground also maximum and minimum were calculated but considering only the nocturnal period between 6pm and 6am. Last row shows an estimation of the thermal variation of each microhabitat (the maximum weekly temperature averages are in bold).

Week	Date of week's first day	Pond			Air Near ground				
		Max T (^{o}C)	Min T (^{o}C)	Range (^{o}C)	Max T (^{o}C) on day	Min T (^{o}C) on day	Range (^{o}C)	Max T (^{o}C) at night	Min T (^{o}C) at night
1	25/08/2014	31.7	24.2	7.5	35.3	23.3	12.0	**28.1**	24.5
2	01/09/2014	32.0	25.4	6.6	34.5	23.3	11.2	26.9	23.3
3	08/09/2014	33.7	25.5	8.2	37.8	23.1	14.7	27,3	23.0
4	15/09/2014	33.0	26.4	6.6	37.3	23.5	13.8	27,9	23.5
5	22/09/2014	32.8	25.9	6.9	**38.0**	23.8	14.2	27.8	23.3
6	29/09/2014	**34.2**	26.4	7.8	36.9	23.4	13.5	26.7	23.4
7	06/10/2014	31.5	26.3	5.2	35.8	23.5	12.3	26,6	23.6
8	13/10/2014	31.7	26.7	5.0	35.6	23.7	11.9	26.4	23.5
9	20/10/2014	32.0	26.7	5.3	34.7	23.9	10.8	26,7	23,6
10	27/10/2014	34.1	26.3	7.8	36.6	23.7	12.9	27.7	23.9
11	03/11/2014	32.7	26.3	6.4	34.2	23.7	10.5	26.4	23.7
12	10/11/2014	33.3	26.4	6.9	33.7	23.7	10.0	27,6	23,8
13	17/11/2014	32.6	26.3	6.3	33.6	23.6	10.0	26,6	23,4
14	24/11/2014	32.3	25.9	6.4	32.7	23.7	9.0	26.1	23.4
15	01/12/2014	32.1	26.3	5.8	33.5	23.8	9.7	26.0	23.9
16	08/12/2014	31.0	26.1	4.9	35.3	23.9	11.4	27.0	23.5
Mean Range:				6.5			11.7		