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Interactive effects of experimental heating rates, ontogeny and body mass on the upper thermal limits of anuran larvae

Gustavo A. Agudelo-Cantero[a,*], Carlos A. Navas[b]

[a] Graduate School Program in General Physiology, Institute of Biosciences, University of São Paulo, Rua do Matão 101, Travessa 14, CEP 05508-090, São Paulo, Brazil
[b] Department of Physiology, Institute of Biosciences, University of São Paulo, Rua do Matão 101, Travessa 14, CEP 05508-090, São Paulo, Brazil

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ABSTRACT

Biological and methodological factors influence the upper thermal limits (*UTL*) of ectothermic animals, but most factors have been studied independently. Few studies have integrated variables, so our understanding about sources of *UTL* variation remains fragmentary. Thereby, we investigated synergic effects of experimental protocols (heating rates, ΔTs) and biological factors (ontogeny and body mass) on the *UTL* on the larvae of two anuran species (*Physalaemus nattereri* and *Boana pardalis*), specifically their Critical Thermal Maximum (CT_{max}). The species displayed slightly different responses to ΔTs: In *B. pardalis* tadpoles both average and variance of CT_{max} increased at a fastest ΔT, the same response happened in *P. nattereri* tadpoles at slow and moderate ΔTs. Also, the CT_{max} of *P. nattereri* declined at the end of metamorphosis independently of ΔT, but tadpoles at all developmental stages still displayed higher heat tolerance at the slow ΔT. Finally, we detected small, synergic effects of body mass and ΔTs on the CT_{max} of both species. In small *B. pardalis* tadpoles and premetamorphic *P. nattereri* tadpoles, body mass had a positive effect on CT_{max}, but only at slow and moderate ΔTs, probably indicating physiological responses. A similar trend was observed in large *B. pardalis* tadpoles at the fast ΔT, but this result is likely to be influenced by thermal inertia. Our findings contribute to integrate the understanding of factors influencing *UTL* in small ectothermic animals. This understanding is critical to discuss the physiological component of vulnerability to climate change that is related to acute temperatures.

1. Introduction

Thermal limits have been proposed as proxies for analyzing a type of vulnerability to rising temperatures, particularly in some lineages of ectothermic animals (Deutsch et al., 2008; Huey et al., 2009). In this context, one view of warming tolerance (*WT*, Deutsch et al., 2008) focuses on the difference between habitat temperature (T_{hab}) and Critical Thermal Maximum (CT_{max}), an index of upper thermal limits for activity (*UTL* hereafter). This interpretation of *WT* assesses vulnerability of individuals to acute warming to presumably draw inference at the population/species levels. This index requires a conceptual expansion from the individual to the population level, and gains value when accurate *WT* values derive from T_{hab} and CT_{max} data that are appropriate to the scale that is adequate for a given study organism. For example, macroclimatic measurements of T_{hab} (e.g., 1 km^2) mask the spatiotemporal heterogeneity experienced by individuals in their microclimates (Camacho, 2012; Potter et al., 2013; Woods et al., 2015), thus biasing *WT* (Katzenberger et al., 2018; Scheffers et al., 2014, 2013). However, how intraspecific variation in CT_{max} affect *WT* remains elusive (Allen et al., 2016; Pincebourde and Casas, 2015). This is a critical consideration because there is not really a "species CT_{max}". This variable is plastic from several points of view (e.g., Brattstrom, 1968; Hutchison, 1976; Simon et al., 2015; see also next paragraph), and so it is *WT*; so this index cannot be seen as a fixed value for a given population (e.g., derived from averaging individual data).

UTL (e.g., CT_{max}) are influenced by intrinsic biological factors such as ontogeny (Delson and Whitford, 1973; Klok and Chown, 2001), so the notion of *WT* challenges a simplistic notion of "individual". In addition, this variable is influenced by sex (Lailvaux and Irschick, 2007; Winne and Keck, 2005), body mass (Becker and Genoway, 1979; Ribeiro et al., 2012) and physiological condition (Rezende et al., 2014, 2011). *UTL* may also depend on the interplay between internal

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Abbreviations: ΔTs, experimental heating rates (°C min^{-1}); *AIC*, Akaike information criterion; *AICc*, second-order Akaike information criterion; CT_{max}, Critical Thermal Maximum; *ER*, evidence ratio or relative likelihood of model pairs; *RI*, relative importance of predictive variables within a confidence set of models; T_b, body temperature; T_{hab}, habitat temperature; *UCI*, unconditional confidence intervals; *UTL*, upper thermal limits; *WT*, Warming Tolerance

[*] Corresponding author

E-mail addresses: gustavo.agudelo@ib.usp.br (G.A. Agudelo-Cantero), navas@usp.br (C.A. Navas).

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(physiology) and external (environmental) factors, for example via thermal history of individuals (Kellermann et al., 2017; Nyamukondiwa and Terblanche, 2010), photoperiod (Hutchison, 1961; Hutchison and Kosh, 1965) and daily/seasonal temperature cycles (Sanabria et al., 2013; Willhite and Cupp, 1982). Methods matter also, including thermal pre-treatments (Maness and Hutchison, 1980; Ribeiro et al., 2012), experimental endpoints (Lutterschmidt and Hutchison, 1997a, 1997b) and overall protocols (e.g., ramping or static) (Overgaard et al., 2012; Rezende et al., 2011), including the rate of temperature change (Ribeiro et al., 2012; Terblanche et al., 2007). Most of these variables have been studied independently and the synergic effects of biological and experimental factors on *UTL* remain poorly understood. This paper aims to advance in the integration of factors, and focuses on the interactions among experimental heating rates, ontogeny and body mass on CT_{max}. So, as a first step we shall discuss why we chose those variables among many options.

Researchers have used diverse experimental heating rates (ΔTs hereafter) to estimate *UTL*. Fast ΔTs (e.g., 1 °C min^{-1}) have been preferred presumably for filtering out physiological responses, and they inform about a baseline level of tolerance (Ribeiro et al., 2012). Yet, very fast ΔTs may fail to homogenize body temperature (T_b) and induce heat-shock deleterious effects (Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997b). In contrast, slow ΔTs may mimic ecological thermal shifts and favor physiological adjustment (Ribeiro et al., 2012; Terblanche et al., 2011), including thermal hardening (Hutchison, 1961; Ju et al., 2011; Overgaard et al., 2014). Still, they may also evoke confounding responses due to longer experimental time, including energy expenditure, desiccation, starvation (Rezende et al., 2011), or aerobic constraints (Pörtner, 2001, 2010). Under this framework, interspecific differences in ΔT-related effects on *UTL* are rather expected across ectothermic lineages (Allen et al., 2012; Chown et al., 2009; Peck et al., 2009; Terblanche et al., 2007; Vinagre et al., 2015). Since we are far from predicting effects of ΔTs on *UTL* across taxa, this topic needs to be further investigated in other species.

Upper thermal limits shift with ontogeny, as proved in insects (Klok and Chown, 2001; see review in Bowler and Terblanche, 2008), salamanders (Delson and Whitford, 1973; Hutchison, 1961), anurans (Cupp, 1980; Floyd, 1983; see review in Ultsch et al., 1999) and squamates (Liwanag et al., 2018; Winne and Keck, 2005). The mechanisms underlying ontogenetic effects are not easy to isolate, and may range from intrinsic ontogenetic shifts in physiology to parallel changes in natural history, including exposure to microclimate. For instance, in some anuran lineages heat tolerance decreases close to the climax of metamorphosis (Cupp, 1980; Floyd, 1983), presumably reflecting a peak of physiological stress (Sherman, 1980; Sherman and Levitis, 2003). Given that some lineages experience notable ecological shifts with ontogeny, more tolerant stages may be those experiencing the most extreme T_{hab} (Pincebourde and Casas, 2015; Terblanche et al., 2017). Equally important, individuals in nature might be exposed to diverse patterns of ΔTs along development. Then, if critical temperatures are an issue, adaptation of *UTL* may be influenced by both absolute T_{hab} and natural rates of thermal change. It is difficult to pose any generalization on this problem because most studies have used rapid and unrealistic ΔTs ranging from 0.5 to 1 °C min^{-1}, regardless of natural changes of ΔTs and *UTL* along ontogeny (but see for example Terblanche et al., 2017). Thus, the interaction between ontogeny and ΔTs deserves further attention.

Although body mass relates to most physiological variables (Calder, 1984; Peters, 1986; Schmidt-Nielsen, 1984) and must influence heat tolerance as well (Klockmann et al., 2017), it has been largely ignored (Chown et al., 2002; Peck et al., 2009; Ribeiro et al., 2012). Due to lack to studies, the current scenario is inconclusive regarding size-related effects on *UTL*: some studies with ectothermic animals report body mass effects on *UTL* (Messmer et al., 2017; Ribeiro et al., 2012; Verble-Pearson et al., 2015), others do not (Bury, 2008; Hutchison, 1961; Ospina and Mora, 2004). At this point generalizations are not only impossible, it is not even clear whether they should be looked for. An additional confounding factor is that size-related differences in *UTL* can reflect either physiological effects or methodological artifacts stemming from thermal inertia (Becker and Genoway, 1979; Ribeiro et al., 2012). These artifacts occur when larger individuals display higher than expected CT_{max} because their T_b does not match external temperature. The ΔT perceived by critical thermal receptors (e.g., peripheral and/or central) may lag behind the experimental ΔT, particularly fast ones (Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997b). Statistical interactions between ΔT and body mass may identify methodological artifacts (Ribeiro et al., 2012). Conversely, size-related effects in absence of a $\Delta T \times$ body mass interaction, likely signal true physiological effects (Ribeiro et al., 2012). Integrating these factors is a task in the making.

The main goal of this study is to test the hypothesis that variation in experimental protocols (ΔTs) and biological factors (ontogeny, body mass) influence synergistically the *UTL* (CT_{max}) of anuran larvae. We used aquatic larval stages as models because of theoretical and practical notions. First, the aquatic environment possesses a higher specific heat and conductivity than air, granting a greater thermal homogeneity required in *UTL* tests (Navas et al., 2010; Tejedo et al., 2012). Second, larval stages could be more prone to face overheating (i.e., stronger selection on *UTL*) than adults, because behavioural thermoregulation in natural conditions is likely more limited in aquatic larvae than in terrestrial forms (Gutiérrez-Pesquera et al., 2016; Oyamaguchi et al., 2018; Simon et al., 2015). We emphasize intraspecific variation in a study with two distantly related species that differ in life history, microhabitat, and typical exposure to thermal regimes. We expect ΔTs to elicit species-specific responses in CT_{max}. Also, we anticipate a decrease in CT_{max} at stages close to the climax of metamorphosis, a drop possibly enhanced at slow ΔTs. Since confounding factors (discussed above) are likely dependent on physiology (Rezende et al., 2011), we expect larger interindividual variability in CT_{max} when measured at slow vs. fast ΔTs, regardless of the species or developmental stage. Finally, we predict that large tadpoles will display higher CT_{max} than small tadpoles across all ΔTs, but if this effect is evident only at the fastest ΔT, methodological artifacts may explain patterns.

2. Materials and methods

2.1. Study sites, study species and acclimation treatment

The study comprised two localities at São Paulo State, southeastern Brazil. One is a pasture in the municipality of São José do Rio Preto (SJRP) under the domain of the Cerrado biome (20°42–43′ S, 49°18′ O, 495–518 m.a.s.l). The second site is an Atlantic Forest fragment in the municipality of São Luiz do Paraitinga (SLP; 23°12′ S, 45°16′ O, 769–829 m.a.s.l). Field work occurred during summer 2015 (January–March), at peak breeding season for most anuran species in these environments (Bertoluci, 1998; Bertoluci and Rodrigues, 2002; Vasconcelos and Rossa-Feres, 2005). We chose two common species as follows. In SJRP we collected four clutches of *Physalaemus nattereri* Steindachner (1983) in two different ephemeral ponds. This species is typical from savannah and grassland habitats in the Cerrado biome in central and southeastern Brazil, eastern lowlands of Bolivia and eastern areas of Paraguay, and breeds near of standing and temporary waterbodies that can reach 42 °C (Aquino et al., 2004; Freitas et al., 2016). In SLP we collected tadpoles of *Boana pardalis* Spix (1824) in three different waterbodies (stream and artificial dam in interior forest, and an open flooded area). This species is known from the Atlantic rainforest of southeastern Brazil and occurs on vegetation or on the ground near temporary or permanent waterbodies, where it breeds (Nascimento et al., 2004).

We transferred field-collected egg masses and tadpoles to aerated 5 L plastic containers with chlorine-free water. Once in the laboratory, we set photoperiod at 13L:11D (average of summer photoperiod of the last four years; Astronomical Applications Dept., USNO), and water temperature at 23 ± 1 °C. This is the minimum natural temperature experienced by tadpoles of *P. nattereri* (Freitas et al., 2016), and is slightly higher than mean temperatures registered in some waterbodies in the Atlantic Forest (Simon et al., 2015). We chose this temperature to reduce differentiation rates of both species during experiments. We mixed the clutches of *P. nattereri* to homogenize genetic variation among treatments, and then transferred to a climatic room three samples of about 200 eggs in 5 L plastic containers. The same procedure was followed for tadpoles of *B. pardalis*. *Physalaemus nattereri* embryos reached the Gosner stage 25 in the laboratory around 20 days after collection. For experiments, we chose tadpoles of both species at Gosner stage 25 and maintained them in density of 20 individuals/L. Tadpoles were fed *ad libitum* with fish food, and water was changed every other day.

2.2. Experimental design

Experiment 1 assessed how three different experimental ΔTs (1 °C min^{-1}, 0.1 °C min^{-1} and 0.05 °C min^{-1}) and body mass influenced the CT_{max} of both anuran species. We used tadpoles at Gosner stage 25 that were kept for two weeks under experimental conditions (see above). Experiment 2 tested how the CT_{max} of *P. nattereri* responded to ΔTs (as in Experiment 1) along ontogeny, and compared the dataset obtained in Experiment 1 for this species with comparable datasets obtained from tadpoles at intermediate (35–41) and advanced (42–44) Gosner stages. In this case, intermediate and advanced tadpoles reached those stages in captivity after 109–140 days. We also evaluated $\Delta T \times$ body mass interactions on the CT_{max} of intermediate and advanced *P. nattereri* tadpoles. Finally, in Experiment 3 we further explored the influence of body mass on CT_{max}, especially regarding potential methodological artifacts (see Introduction). Here we compared the dataset for *B. pardalis* obtained from Experiment 1 with that obtained from a second sample of tadpoles of the same species, same collection event, Gosner stage 25, but comparatively larger (Table 1). This size difference, with little differentiation in term of Gosner stages, was attained just by maintaining in captivity for longer times a second sample of *B. pardalis* tadpoles (110–160 days). Therefore, the size differences between the two groups of *B. pardalis* tadpoles did not result from any pre-experimental difference in growth or development.

2.3. Upper thermal limits

We based this study on the Hutchison's dynamic method (Hutchison, 1961) to estimate the CT_{max} of individual tadpoles, with some modifications of the experimental endpoint. All tests started between 0830 and 1000 h (UTC-2) and the initial temperature was always 23 ± 0.5 °C, following the previous thermal conditions experienced by the tadpoles (see above). Briefly, we used two large hand-controlled thermal baths for the slow (0.05 °C min^{-1}) and moderate (0.1 °C min^{-1}) ΔTs, which basically involved aquarium heaters coupled to a thermostat and a power regulator (Fig. S1). For the fast ΔT (1 °C min^{-1}), a smaller bath was heated by a hot plate with magnetic stirrer. In all cases, water inside the baths was constantly mixed to homogenize its temperature. We placed tadpoles individually inside plastic containers (300 mL containers and 100 mL water, large baths; 100 mL containers and 60 mL water, small bath) and then we randomly distributed these containers into the baths. In each *UTL* test, we also placed randomly one temperature datalogger (Onset HOBO®) into a container (without tadpole, same water volume) to register the ΔT potentially experienced by tadpoles (Table S1; Fig. S2). We continuously monitored water temperature as a proxy of tadpoles' T_b along experiments (Claussen, 1973; Navas et al., 2010). Individuals of both species mostly remained at the bottom of containers once tests began, but they became more active in terms of swimming and mouth movements as water temperature increased. In pilot tests, we failed to identify unambiguously muscular spasms in tadpoles of the studied species as the definitive end-point for their CT_{max} (Lutterschmidt and Hutchison, 1997a). So, we ended experiments when tadpoles became motionless and failed to respond after five consecutive taps applied each 2-s with the probe of a fast-reading thermometer, and then we recorded the water temperature beside the tadpole as its CT_{max} (Gutiérrez-Pesquera et al., 2016; Katzenberger et al., 2018, 2014). Afterwards, each tadpole was transferred individually into a plastic container with cool water (~23 °C) to test for recovery. We consider only the CT_{max} data of individuals that survived and appeared to display normal behavior 24 h after the tests (Table 1). After that time, we measured the body mass of each individual in an analytical balance to the nearest 0.001 g.

2.4. Data analyses

All analyses were performed in the R programming environment (version 3.5.1) (R Core Team, 2018). Despite slightly skewed distributions and different variances, the overall raw CT_{max} data approached normal distribution and homoscedasticity of errors. So we fitted general linear models to our data in order to explain CT_{max} variation according to our experimental design (Table S2). The categorical predictors were

Table 1

Summary statistics for the CT_{max} and body mass of tadpoles of *Physalaemus nattereri* and *Boana pardalis*, for each ontogenetic (Gosner) stage and heating rate (ΔT). N is the sample size (not counting deaths). † indicates the number of dead tadpoles after 24 h of tests. Min and Max are the minimum and maximum values for each variable, respectively. CV is the coefficient of variation.

Species	Gosner stage	ΔT (°C min^{-1})	N	†	CT_{max} (°C)				Body mass (mg)			
					Mean ± SD	Min	Max	CV (%)	Mean ± SD	Min	Max	CV (%)
P. nattereri	25	0.05	52	2	42.1 ± 0.8	40.8	43.6	1.9	21.9 ± 6.8	6.7	37.4	31.1
P. nattereri	25	0.1	53	1	41.8 ± 0.7	40.3	43.4	1.6	21.7 ± 6.0	10.5	38.8	27.4
P. nattereri	25	1	51	2	41.8 ± 0.5	40.7	42.9	1.2	21.4 ± 6.5	9.2	41.7	30.4
P. nattereri	35–41	0.05	15	1	41.9 ± 0.8	40.3	43.3	1.9	262.6 ± 79.9	147.4	436.4	30.4
P. nattereri	35–41	0.1	15	1	41.7 ± 1.0	39.8	43.3	2.4	254.8 ± 59.7	172.0	347.0	23.4
P. nattereri	35–41	1	15	0	41.5 ± 0.5	40.7	42.4	1.2	250.5 ± 98.9	134.2	471.7	39.5
P. nattereri	42–44	0.05	11	4	41.0 ± 1.0	39.1	42.7	2.4	278.7 ± 48.5	219.8	388.1	17.4
P. nattereri	42–44	0.1	10	3	39.8 ± 2.0	36.3	42.5	5.1	262.4 ± 67.5	168.0	420.0	25.7
P. nattereri	42–44	1	10	7	40.1 ± 0.9	38.4	41.5	2.2	259.8 ± 84.3	176.4	462.6	32.5
B. pardalis	25	0.05	51	13	41.1 ± 0.2	40.4	41.5	0.6	49.0 ± 19.2	19.0	101.5	39.2
B. pardalis	25	0.1	53	20	41.3 ± 0.2	40.6	41.8	0.5	54.9 ± 19.3	21.5	97.6	35.1
B. pardalis	25	1	54	0	41.9 ± 0.4	40.8	42.4	0.9	45.2 ± 14.9	22.5	83.1	32.9
B. pardalis[a]	25	1	36	0	41.6 ± 0.4	40.8	42.4	0.8	688.1 ± 506.4	212.4	2350.1	73.6

[a] This category represents the large-size group of *B. pardalis* tadpoles that was compared only with the small-size group of tadpoles of the same species, also tested at ΔT = 1 °C min^{-1}. See the text for more details.

'ΔT' (0.05 °C min^{-1}, 0.1 °C min^{-1}, 1 °C min^{-1}) and 'Ontogeny' (Gosner stages 25, 35–41, 42–44), whereas the continuous predictor was 'body mass' (mg). Three interactions were considered particularly informative given current theory: 1) The interaction 'ΔT × body mass' tested whether the effect of body mass on CT_{max} depended on the ΔT applied during the *UTL* experiments; 2) the interaction 'ΔT × Ontogeny' analyzed whether shifts in CT_{max} along the ontogeny of *P. nattereri* varied with ΔTs [Experiment 2]; and 3) the interaction 'Size class × body mass' assessed whether the effect of body mass on the CT_{max} of *B. pardalis* differed between small and large individuals when submitted to 1 °C min^{-1} [Experiment 3]. Additionally, we analyzed the 24-h survival of both species in response to our experimental ΔTs, comparing the proportions of alive (N) and dead individuals (†; Table 1) 24 h after the *UTL* tests. Thus, we fitted generalized linear models (binomial error distributions, logit link function) to test whether the 24-h survival after *UTL* tests 1) varied among ΔTs in *B. pardalis* tadpoles, and 2) whether this variable differed along the ontogeny and according to ΔTs in *P. nattereri* tadpoles (Table S2). To assess the extent to which CT_{max} variances differed among ΔTs in *B. pardalis* (Experiment 1), within each ontogenetic category of *P. nattereri* (Experiment 2), and between size classes of *B. pardalis* tadpoles (Experiment 3), we ran Levene's tests ($\alpha = 0.05$).

We adopted an information-theoretical approach (i.e., methods based on the Akaike information criterion, *AIC*; Akaike, 1974) for model selection and statistical inference. Specifically, we ranked competitive models fitted to explain CT_{max} or 24-h survival according to their second-order *AIC* value (*AICc*; Hurvich and Tsai, 1989; Sugiura, 1978) by using the 'aictab' function in the 'AICcmodavg' package (Mazerolle, 2017). The *AICc* penalizes models according to their number of estimated parameters (*k*) while correcting for small sample sizes (when $n/k < 40$, as in all our cases); as sample size increase, *AICc* values converges to *AIC* estimates (Burnham et al., 2011; Burnham and Anderson, 2002). For every case of model comparison (Table S2), we conducted model selection based on *AICc* differences ($\Delta AICc < 4.0$) and evidence ratios (*ER*, relative likelihood of model pairs, calculated with the 'evidence' function) between the top-ranked model and alternative candidate models, and on *AICc* weights of each candidate model. *AICc* weights indicate the evidence in favor of a candidate to best model describing the data (Burnham and Anderson, 2002). When our model selection procedure indicated that two or more models were equally fit to explain CT_{max} or 24-h survival, we computed the confidence set on the best model given the data by building profile likelihood intervals for the ratios of model likelihoods (cutoff value = 0.135, $\Delta AICc = 4.0$; 'confset' function, method = 'ratio'; Burnham and Anderson, 2002). Then we calculated the relative importance of variables (*RI*, the likelihood that a variable is part of the best approximating model) within the confidence set of models by summing the *AICc* weights over all models including the explanatory variable ('importance' function, 'MuMIn' package; Barton, 2018). Finally, in a model selection framework, we conducted multiple comparisons among levels of 'ΔT' and 'Ontogeny' ('multComp' function, 'AICcmodavg' package; Mazerolle, 2017). This method pooled levels of 'ΔT' or 'Ontogeny' in a model, updated the model for each grouping pattern possible and then ranked them according to their *AICc* weights. This approach also conducted model-averaged predictions and 95% unconditional confidence intervals (95% *UCI*) for each level of 'ΔT' and 'Ontogeny'.

3. Results

3.1. Effects of ΔTs and body mass on CT_{max} within species (Gosner stage 25)

The heat tolerance of *B. pardalis* was explained by diverse effects, as informed by three complementary models selected within the confidence set (Table 2). Overall, ΔT was the most important variable to explain CT_{max} variation in *B. pardalis* (*RI* = 100%), since there was a progressive decrease in heat tolerance with slower ΔTs (Multiple comparison among ΔTs, *AICc* weight = 99%; Table 1; Fig. 1A). Also, larger tadpoles were more tolerant than small counterparts only when exposed to the slow ΔT (0.05 °C min^{-1}), but this size effect was barely 0.06 °C by each 10 mg (Fig. 1B). Conversely, the CT_{max} of individuals exposed to a moderate (0.1 °C min^{-1}) or fast (1 °C min^{-1}) ΔT was unaffected by their body mass (Fig. 1B). Furthermore, *B. pardalis* tadpoles displayed more variable CT_{max} when exposed to the fast ΔT (Levene's test, $F_{(2,155)} = 9.55$, $P < 0.001$; Table 1; Fig. 1A).

For *P. nattereri* (Fig. 1A, C), we found strong support in favor of synergic effects between ΔT and body mass explaining most CT_{max} variation (*ER* of the top-ranked model = 19.3 against the second-ranked model; Table 2). Body mass had a positive effect on the heat tolerance of *P. nattereri* tadpoles exposed to slow and moderate ΔTs, as individuals increased their CT_{max} by 0.5 °C for every 10 mg of body mass (Fig. 1C). No size effect was evident in tadpoles submitted to fast ΔT. Average CT_{max} was 0.3 °C higher in *P. nattereri* tadpoles submitted to 0.05 °C min^{-1} than in individuals exposed to other ΔTs, and inter-individual variability of heat tolerance increased at slow and moderate ΔTs (Levene's test, $F_{(2,153)} = 9.52$, $P < 0.001$; Table 1; Fig. 1A).

3.2. Effects of ΔTs and body mass on the CT_{max} of *P. nattereri* along ontogeny

Regarding the heat tolerance of *P. nattereri* through larval development (Table 1; Fig. 2A), both ontogeny and ΔT were predictors of CT_{max} (*RI* = 100% for both variables), though their influence seemed independent (Table 2). According to model-averaged estimates, the mean CT_{max} of *P. nattereri* decreased about 1.5 °C close to the climax of metamorphosis (42–44; mean CT_{max} = 40.6 °C; 95% *UCI* = 40.3–41.0 °C) with respect to early (25; mean CT_{max} = 42.2 °C; 95% *UCI* = 42.0–42.4 °C) or intermediate (35–41; mean CT_{max} = 42.1 °C; 95% *UCI* = 41.8–42.3 °C) Gosner stages. Likewise, tadpoles exposed to the slow ΔT (mean CT_{max} = 42.2 °C; 95% *UCI* = 42.0–42.4 °C, *AICc* weight = 73%) increased their mean CT_{max} about 0.4 °C in comparison with individuals submitted to a moderate or fast thermal change (mean CT_{max} = 41.8 °C; 95% *UCI* = 41.6–41.9 °C), regardless of their developmental stage (Fig. 2A).

Like in tadpoles at Gosner stage 25, the CT_{max} of *P. nattereri* tadpoles at intermediate developmental stages increased according to their body mass only when submitted to slow (0.04 °C/10 mg) and moderate (0.1 °C/10 mg) ΔTs (Table 2; Fig. 2B). Individuals exposed to the fast ΔT did not display any size effect (Fig. 2B). Tadpoles of *P. nattereri* approaching metamorphic climax displayed high interindividual variation in CT_{max} (Table 1), so no model stood out as best (Table 2). Still, the most important variable for explaining CT_{max} in this case was ΔT (*RI* = 39%). Tadpoles of *P. nattereri*, even those close to metamorphic climax, increased their CT_{max} by almost 1 °C when exposed to slow ΔT, compared to other ΔTs (Multiple comparison among ΔTs, *AICc* weight = 49%; Table 1; Fig. 2A). Finally, *P. nattereri* tadpoles at intermediate (Levene's test, $F_{(2,42)} = 3.49$, $P = 0.04$) and advanced (Levene's test, $F_{(2,28)} = 6.80$, $P = 0.004$) Gosner stages also displayed higher CT_{max} variability at both slow and moderate ΔTs (Table 1; Fig. 2A).

3.3. Effects of body mass on the CT_{max} of two size classes of *B. pardalis* tadpoles exposed to ΔT = 1 °C min^{-1}

CT_{max} responses varied between the two size classes of *B. pardalis* tadpoles exposed to the fast ΔT (Table 2; Fig. 3A and B). Average CT_{max} was 0.3 °C higher in the small-size group of *B. pardalis* tadpoles compared to individuals of the large-size group, though CT_{max} variability was similar for tadpoles of both size groups (Levene's test, $F_{(1,88)} = 0.38$, $P = 0.539$; Table 1, Fig. 3A). Body mass exerted a small influence on the heat tolerance of *B. pardalis* tadpoles (Table 2) as

Table 2
Confidence set of selected models (cutoff value of 0.135, $\Delta AICc = 4.0$) for explaining variation in CT_{max} and 24-h survival after the *UTL* tests according to our experimental design.

Experiment	Confidence set of models	*k*	*AICc*	*ΔAICc*	*AICc* weight	*ER*
1) Effects of ΔT and body mass on CT_{max} within species (Gosner stage 25)						
Boana pardalis	$CT_{max} \sim \Delta T \times$ body mass	7	66.91	0	0.59	
	$CT_{max} \sim \Delta T +$ body mass	5	68.94	2.03	0.21	2.76
	$CT_{max} \sim \Delta T$	4	69.13	2.23	0.19	3.04
Physalaemus nattereri	$CT_{max} \sim \Delta T \times$ body mass	7	294.15	0	0.94	
2a) Effects of ΔT on the CT_{max} of *P. nattereri* along ontogeny	$CT_{max} \sim \Delta T +$ Ontogeny	6	571.18	0	0.72	
	$CT_{max} \sim \Delta T \times$ Ontogeny	10	573.21	2.03	0.26	2.76
2b) Effects of ΔT and body mass on the CT_{max} of *P. nattereri*						
Gosner stages 35–41	$CT_{max} \sim \Delta T \times$ body mass	7	102.51	0	0.82	
	$CT_{max} \sim$ body mass	3	106.47	3.96	0.11	7.25
Gosner stages 42–44	$CT_{max} \sim$ null	2	114.11	0	0.38	
	$CT_{max} \sim \Delta T$	4	114.75	0.65	0.27	1.38
	$CT_{max} \sim$ body mass	3	115.14	1.03	0.22	1.68
	$CT_{max} \sim \Delta T +$ body mass	5	116.58	2.48	0.11	3.45
3) Effects of body mass on the CT_{max} of two size classes of *B. pardalis* tadpoles exposed to $\Delta T = 1$°C min^{-1}	$CT_{max} \sim$ Size class + body mass	4	78.34	0	0.70	
	$CT_{max} \sim$ Size class $\times$ body mass	5	80.34	2.00	0.26	2.71
24-h survival after the *UTL* tests in response to ΔTs						
B. pardalis	Survival $\sim \Delta T$	4	158.51	0	1	
P. nattereri (along ontogeny)	Survival ~ Ontogeny	3	123.00	0	0.84	
	Survival $\sim \Delta T +$ Ontogeny	5	126.40	3.40	0.15	5.47

k = the number of estimated parameters for each model; *AICc* = second-order Akaike information criterion; *ΔAICc* = *AICc* differences between the top-ranked model and alternative candidate models; and *ER* = evidence ratio of the model weights between the top-ranked model and alternative candidate models.

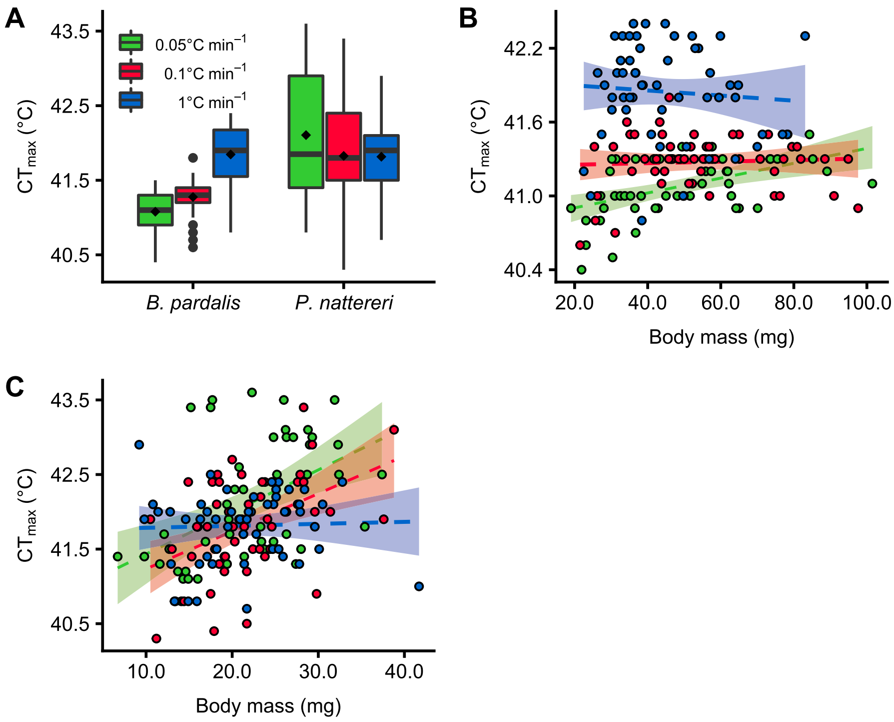

Fig. 1. Effects of ΔTs and body mass on CT_{max} within species (Gosner stage 25). (A) CT_{max} variation of both species in response to three ΔTs. Horizontal bars inside boxes are the median values, black diamonds indicate the mean values and whiskers are values within 1.5 × the inter-quartile range. Relationships between the CT_{max} and the body mass of tadpoles of *B. pardalis* (B) and *P. nattereri* (C), at three ΔTs. Color codes for ΔTs are the same in all cases.

follows: the CT_{max} of small *B. pardalis* tadpoles did not respond to body mass (Figs. 1B and 3B), but the CT_{max} of large tadpoles increased 0.003 °C for every 10 mg (or 0.3 °C g^{-1}; Fig. 3B).

3.4. 24-h survival after the UTL tests

The two species differed in survival according to heating rates. For *B. pardalis*, the 24-h survival decreased about 20% after slow (72.6% at 0.05 °C min^{-1}) and moderate (79.69% at 0.1 °C min^{-1}) ΔTs, compared to 100% survival at fast thermal rates (Tables 1 and 2). For *P. nattereri*, 24-h survival differed more markedly regarding ontogenetic stages, whereas ΔTs was not so influential (Table 2). Thus, average survival decreased from 97% to 96% at Gosner stages 25 and 35–41 (respectively), to 69% at Gosner stages 42–44 (Tables 1 and 2).

4. Discussion

As expected, the *UTL B. pardalis* and *P. nattereri* responded differently to experimental heating rates (ΔTs). Interspecific differences in central tendency (Allen et al., 2012; Peck et al., 2009; Tejedo et al., 2012; Terblanche et al., 2007) and variability (Chown et al., 2009; Vinagre et al., 2015) of *UTL* in response to ΔTs are expected and related to phylogenetic and ecological distances among the studied lineages. Changes in average CT_{max} for both species reported in this paper were

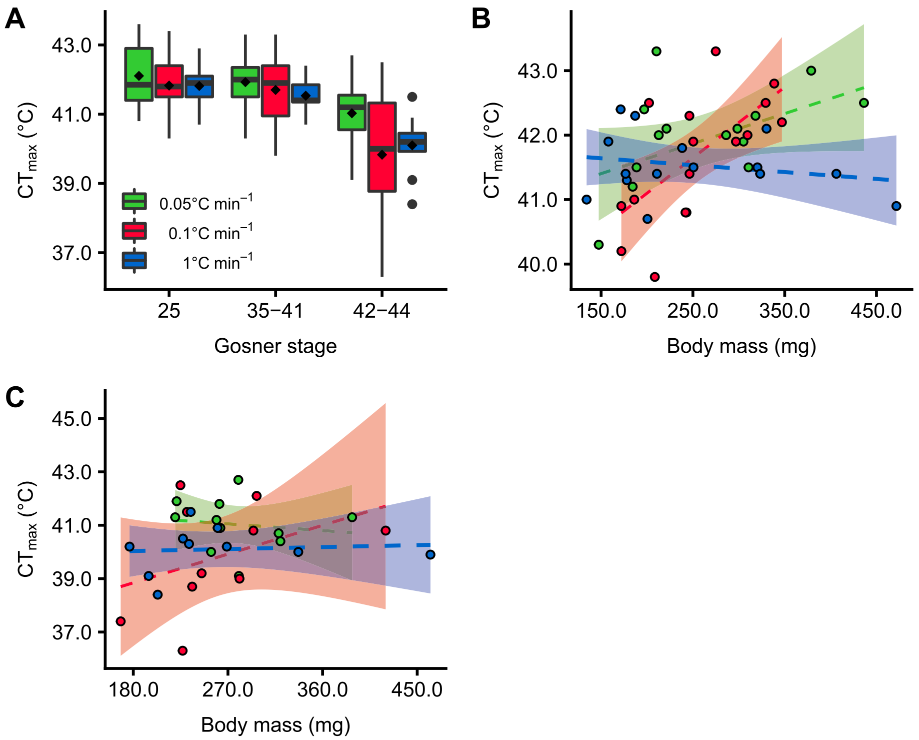

Fig. 2. Effects of *ΔTs* and body mass on the CT_{max} of *P. nattereri* along ontogeny. (A) CT_{max} variation along the ontogeny of *P. nattereri* in response to three *ΔTs*. Relationships between the CT_{max} and the body mass of *P. nattereri* tadpoles at stages 35–41 (B) and stages 42–44 (C), at three *ΔTs*. Figure details are the same than in Fig. 1.

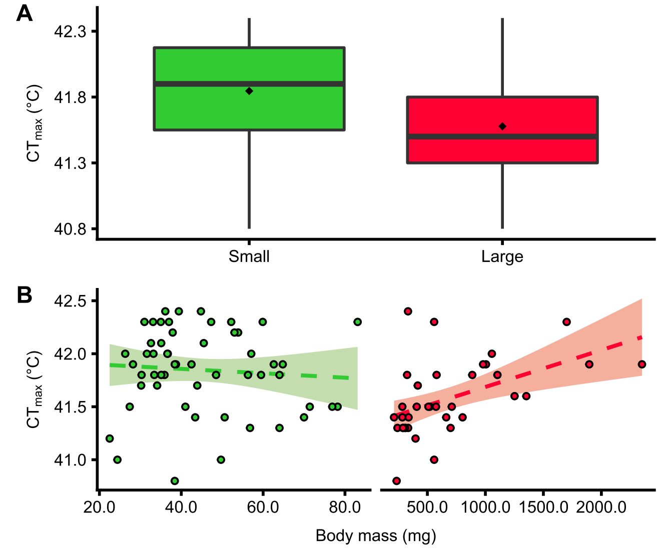

Fig. 3. Effects of body mass on the CT_{max} of two size classes of *B. pardalis* tadpoles exposed to $\Delta T = 1\,^{\circ}\mathrm{C}\ \mathrm{min}^{-1}$. (A) CT_{max} variation between two size classes of *B. pardalis* tadpoles. Boxplot elements are the same than in previous figures. (B) Relationships between the CT_{max} and the body mass of *B. pardalis* tadpoles classified into two size classes. Color codes are the same than in Fig. 3A.

lower than 2 °C. The magnitude of this value is small compared to patterns in distant taxa such as insects (Allen et al., 2012; Terblanche et al., 2007) and fishes (Mora and Maya, 2006), yet comparable with the observed in other arthropods (Vinagre et al., 2015) and anurans (Tejedo et al., 2012). Thus, the overall literature signals relevance of *ΔTs* as a factor influencing the thermal limits of ectothermic animals, but the state of art is premature for generalizations. The causes and consequences of this variation remain obscure, but some well-supported ad-hoc hypotheses can be brought about to illustrate the complexity of a system. A single factor, *ΔT*, may be associated to multifactorial impacts on reported thermal tolerances under experimental conditions, and impacts on variability must not be undervalued.

The higher variability of CT_{max} observed for *B. pardalis* only at the fast *ΔT* disagrees with our original prediction, although some arthropods show comparable responses (Chown et al., 2009; Vinagre et al., 2015). Although we are unable to pose underlying causes for this pattern, the pattern itself contains theory in the sense it shows relevant inter-individual variation in physiology under a given experimental condition. In contrast, slow and moderate thermal rates (longer experiments) apparently deteriorated the physiological condition of most *B. pardalis* tadpoles, a more uniform response. Health-influencing factors may include, among other, oxygen limitation, reserves depletion and metabolic imbalance (Pörtner, 2010; Rezende et al., 2011; Ribeiro et al., 2012). Regarding *P. nattereri*, tadpoles are naturally exposed to T_{hab} close to the CT_{max} we reported (Freitas et al., 2016), so selection on their *UTL* should be strong. Such conditions may induce differential heat hardening among individuals, with futher adjustment at slow experimental *ΔT* (e.g., last quarter of the CT_{max} distribution in Fig. 1A). The higher ecological heat tolerance (as in Ribeiro et al., 2012) of *P. nattereri* may involve antioxidant defenses (Freitas et al., 2016), heat shock proteins (Bozinovic et al., 2013; Williams et al., 2012), or metabolic acclimation (Feder, 1982, 1978). Independently of mechanisms, the bottom line is that, in terms of interindividual variability, CT_{max} was heavily and complexly influenced by *ΔT*. Research on ecological climate change must not ignore dispersion in critical temperatures caused by inter-individual variation. This dispersion is critical for a conceptual expansion from individual to population effects. Naturally, the expected impact of warming would not be equal for populations with same average CT_{max} but contrasting patterns of inter-individual variation.

P. nattereri tadpoles were more sensitive to warming at the end of metamorphosis independently of *ΔT*, partially supporting our prediction. A drop in *UTL* close to the metamorphic climax has been observed in other anuran species (Cupp, 1980; Enriquez-Urzelai et al., 2019; Floyd, 1983; Sherman, 1980; Sherman and Levitis, 2003), but the size of this effect differs among species (~1–5 °C). Some authors associate this pattern to the stress of approaching metamorphic climax (Sherman, 1980; Sherman and Levitis, 2003) and its concomitant and drastic remodeling on anatomy and physiology (Shi, 2000). In fact, tadpoles at their metamorphic climax have impaired physiological and ecological performance (Arnold and Wassersug, 1978; Denver, 1997; Floyd, 1984; Wilbur, 1980). But if tadpoles at these stages are unable to mobilize mechanisms to cope with high temperatures (Sherman and Levitis, 2003), slow thermal exposures would be expected to further reduce their heat tolerance, relative to fast exposures, and our results for *P. nattereri* suggest otherwise. So, we hypothesize that *P. nattereri* can display heat hardening throughout its larval development in response to ecologically relevant ΔTs (e.g., 0.05 °C min^{-1}).

We found small synergic effects of body mass and ΔTs on the *UTL* of both anuran species along our experimental design. Still, our outcomes diverge from previous studies that did not detect any size difference in the *UTL* of other aquatic ectothermic animals (Anttila et al., 2013; Bury, 2008; Ospina and Mora, 2004). Besides, ΔT × body mass interactions on *UTL* have been tested only in ants (Bentley et al., 2016; Ribeiro et al., 2012), and interpretations remain challenging. When ΔT × body mass interactions explain CT_{max} variation, size effects should be considered separately in order to discriminate their nature. For example, the size differences we observed in the CT_{max} of both species at slow and moderate ΔTs are probably physiological, because thermal inertia would not be an issue for individuals of such small size at such ΔTs (Becker and Genoway, 1979). Conversely, the size effect detected in large *B. pardalis* tadpoles at the fast ΔT may be a methodological artifact driven by their thermal inertia, because such effect was not observed for small counterparts at the same ΔT. Zhang and Kieffer (2014) found comparable results in the shortnose sturgeon, for large individuals were less heat tolerant than small ones, though they did find a consistent (therefore, physiological) positive influence of body mass on the *UTL* of these fish. Although the size effect in large *B. pardalis* tadpoles was small, it poses a caveat on the traditionally untested assumption that thermal inertia is negligible for estimating the *UTL* of small aquatic ectothermic animals of comparable sizes.

A final comment regards alternative factors that might account for our results. Captivity time elicit short and long term stress responses, some of which may reduce organismal performance (Assis et al., 2015; Moore et al., 1991; Navas and Gomes, 2001). Yet, thermal tolerance can even increase with captivity time, possibly because of acclimatory responses to laboratory conditions (Mora and Maya, 2006; Pintor et al., 2016). Interindividual differences in captivity time in Experiments 2 and 3 are consequences of our experimental design; however, it is unlikely that this is the main driver of our results. First, heat tolerance of *P. nattereri* tadpoles decreased only at advanced Gosner stages and not progressively, as would be expected by captivity time. Second, the two size classes of *B. pardalis* differed only in heat tolerance but not in 24-h survival, and we did not observe any mortality in individuals of the large-size group during their time in captivity. Despite the possible characterization of the two size classes of *B. pardalis* tadpoles as at the same Gosner stage, the discrete classification of the anuran development is arbitrary because this is a continuous process (Floyd, 1983; Sherman and Levitis, 2003). Accordingly, mean differences in heat tolerance between the two size classes of *B. pardalis* may reflect ontogenetic constraints on physiology that would be unseen through external morphology.

5. Conclusions

We demonstrated complex interactions among ΔTs, ontogeny and body mass that influenced the *UTL* of our studied species. Our findings also suggest that the thermal sensitivity of larval anurans to warming rate is species-specific, which may extend to other small ectothermic animals according to the current literature. Moreover, our data supports the view that heat tolerance of larval anurans decreases at the end of metamorphosis. But we also proved that, even at this developmental stage, *UTL* might be enhanced at the time course of slow, ecologically relevant ΔTs. So, we encourage next studies on ontogenetic shifts in the *UTL* of ectothermic animals to consider ΔTs. Finally, and unlike the current theory, we demonstrated that body mass is an important factor influencing the *UTL* of tadpoles. Thus, researchers should acknowledge this variable when assessing *UTL* in anuran larvae and, possibly, aquatic ectothermic species of comparable sizes, especially if multiple ΔTs are applied. Our findings contribute to integrate the understanding on the *UTL* of small ectothermic animals, which is critical to predict accurately their vulnerability to acute warming. In this context, we highlight that vulnerability assessments based on *WT* need to integrate factors influencing both the T_{hab} component (e.g., microclimate) and the CT_{max} component (e.g., ΔTs, ontogeny and body mass) of the equation, considering both the physiology and natural history of species. Also, we bring attention to the importance of inter-individual variation in critical temperatures, despite mean values, and to the importance that such source of variance may have in ecological climate change research.

Ethics statement

All samplings and captures were made under a SISBIO license (14836-1) from the Chico Mendes Institute for Biodiversity Conservation, Ministry of the Environment. All rearing and experimental procedures were reviewed and approved by the Animal Ethics Committee (license 269/2016) of the Institute of Biosciences, University of São Paulo. Access to the genetic heritage was registered in the Brazilian Genetic Heritage Management Council (CGEN) under a SISGEN register (A66D1F4).

Conflicts of interest

The authors declare no competing or financial interests.

Author contributions

G.A.A.C and C.A.N conceived and designed the study. G.A.A.C performed the experiments, collected the data, and led data analysis and manuscript writing. Both authors revised and edited the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.03.010.

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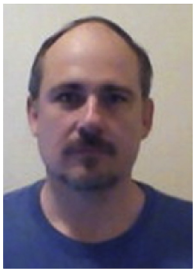
Gustavo A. Agudelo-Cantero: M.Sc. degree in 2016, University of São Paulo, Brazil. Currently Ph.D. student at the Graduate School Program in General Physiology, Institute of Biosciences, University of São Paulo, Brazil.



Carlos A. Navas: Ph.D. degree in 1995, University of Connecticut, USA. Currently Full Professor at the Department of Physiology, University of São Paulo, Brazil.