Gustavo Adolfo Agudelo Cantero

Methodological assessment of the Critical Thermal Maximum (CT_{max}) of anuran larvae: interaction among the experimental heating rates, ontogeny and body mass.

Estudo metodológico da Temperatura Crítica Máxima (CT_{max}) de larvas de anfíbios anuros: interação entre as taxas de aquecimento experimental, ontogenia e massa corpórea.

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Adviser: Carlos Arturo Navas Iannini

São Paulo

2016

Dissertation Committee:

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Prof. Dr. Carlos Arturo Navas Iannini

Adviser

Dedication

This dissertation, as everything in my entire life, is primarily dedicated to my faithful God and Father. All what I am, what I have, and what I attain is to Him and because of Him.

To my beloved wife. She is the love of my life, my confident, my loyal friend, my adventure partner. Achieve my goals in life is completely pleasant with her by my side.

To my wonderful parents and my adored sister. They are my motivation to strive and go on every day. Without their love, patience and support I would not be where I am. All my achievements are also theirs.

Epigraph

About faith

«Now *faith* is confidence in what we hope for and assurance about what we do not see (…) By *faith* we understand that the universe was formed at God's command, so that what is seen was not made out of what was visible. »

[Hebrews 11:1, 3 (NIV)]

«I'm an evolutionary biologist and a Christian. »

Joan Roughgarden (2006). *Evolution and Christian faith: Reflections of an evolutionary biologist*, pp. 3. Island Press, USA.

«I am an ordinary scientist (…) [also] I am an ordinary person of faith (…) My faith is not baseless or irrational, but neither is it scientific. ***I believe in God as both creator and friend. That is, I believe that God is personal and interacts with us*** (…)

As an experimental physicist, I require hard evidence, reproducible experiments, and rigorous logic to support any scientific hypothesis. How can such a person base belief on faith? In fact, there are two questions: "**How** *can* **I believe in God?**" and "**Why** *do* **I believe in God?**"

On the first question: ***a scientist can believe in God because such belief is not a scientific matter***. Scientific statements must be "falsifiable." (…) There is no requirement that every statement be a scientific statement. Nor are non-scientific statements worthless or irrational simply because they are not scientific. Science is not the only useful way of looking at life (…)

[On the second question] As a physicist, (…) I see an orderly, beautiful universe in which nearly all physical phenomena can be understood from a few simple mathematical equations. I see a universe that, had it been constructed slightly differently, would never

have given birth to stars and planets, let alone bacteria and people (…) Many good scientists have concluded from these observations that an intelligent God must have chosen to create the universe with such beautiful, simple, and life-giving properties. Many other equally good scientists are nevertheless atheists. ***Both conclusions are positions of faith*** (…)

I believe in God because I can feel God's presence in my life, because I can see the evidence of God's goodness in the world, because I believe in Love and because I believe that God is Love. Does this belief make me a better person or a better physicist than others? Hardly. I know plenty of atheists who are both better people and better scientists than I. ***I do think that this belief makes me better than I would be if I did not believe***. Am I free of doubts about God? Hardly.

I do believe, more because of science than in spite of it, but ultimately just because I believe. »

William D. Phillips, Nobel Prize in Physics (1997)

Does science make belief in God obsolete? Big Questions Essay Series, John Templeton Foundation.

(2006) *Ordinary faith, ordinary science*. In: J. Staune (ed.) Science and the search for meaning: Perspectives from international scientists. Cap. 12. Templeton Foundation Press, USA.

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First at all, I want sincerely to thank my Heavenly Father and Friend for giving me the strength and courage every day, and for bringing me to this point of my career. He is my source of inspiration, who leads my life beyond I can desire, plan and imagine, so all the honor that this dissertation may deserve is primary to Him.

Likewise, I am especially grateful with my wife, my parents and my sister for their company, for believing in me and for encouraging me to surpass myself every day. Also my wife's family, who is my second family. I am very privileged for having all them in my life, they are the strongest motivation I have to strive, go on and succeed. That all my achievements be not just source of proud for them, but also contribute to improve their lives.

Many thanks to my adviser, Prof. Dr. Carlos Navas, not just for his invaluable guidance through my career, which makes this dissertation possible, but also for his wise advices in many aspects of my life, for trusting me, for guiding me in a new country, for opening the doors of his house and family to his students, for all the funny times with pizza, beer, stories, colombian food, etc. He is not only my adviser or my professor, but my master and friend, and I still have and want to learn so many things of him. Thanks 'profe'.

I want to briefly thank the people that in one way or another have contributed to my Master dissertation and to the path I have walked in Brazil to get here. My aunt Luz Encir and the family Villegas Cantero for all their trust and support to me and my wife. The professors Fernando Ribeiro Gomes and Renata Guimarães Moreira Whitton, who have taught me and inspired me a lot, I am very grateful for their support, kindness and advices. My dear friend Carla Madelaire, a woman who I admire a lot, many thanks for her true friendship, for all the laughs and also our discussions about science and life. Thanks to all my partners of the 'LEFE' and the staff of the Department of Physiology for all their support and contributions through this time. Many thanks to all people of the ICT São Paulo for their affection and friendship, especially Miguel Hernández and his family for their guide and company.

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Abstract

Thermal limits for ectothermic animals displays a picture of the range of body temperatures that is tolerable by individuals before their locomotory capacity is impaired. However, thermal limits are not fixed and specific traits, but labile ones subjected to plastic adjustments and evolutionary change, and also are influenced by intrinsic and extrinsic factors of organisms, as well as by methodological factors inherent to experimental protocols. Even more, the influences of these factors on thermal limits have been commonly addressed independently in different taxa, and the extent by which multiple factors interact and affect thermal limits within taxa is poorly understood. Thus, the main aim of this work was to conduct a methodological assessment of the Critical Thermal Maximum (CT_{max}) by studying the influences of different experimental heating rates ($\Delta T's$), ontogeny, body mass, and the interaction among these factors on this trait. This matter was addressed on larvae of *Physalaemus nattereri* and *Hypsiboas pardalis*, two anuran species from the São Paulo State, southeastern Brazil, that differ in their phylogenetic background, ecological and life-history characteristics and inhabit environments with different thermal regimes. First, $\Delta T's$ did affect averages and variances of CT_{max} in a species-specific manner. In addition, it was found a ΔT-dependent decreasing in CT_{max} at the end of metamorphosis in tadpoles of *P. nattereri*, because only the metamorphosing tadpoles exposed to the acute ΔT were more sensitive to high temperature than premetamorphic tadpoles. Finally, body mass and $\Delta T's$ interacted on the CT_{max} of both species along our experimental design. In *P. nattereri*, body mass affected CT_{max} through physiology at the slow $\Delta T's$, whereas in *H. pardalis* body mass affected CT_{max} at the acute ΔT through a methodological artifact driven by higher thermal inertia in the group of large tadpoles. This study revealed that $\Delta T's$, ontogeny and body mass interact on the CT_{max} of our studied species, and these interactive effects could not have been elucidated by the independent study of each factor. It also highlights the importance of integrating the factors that influence thermal limits of ectothermic animals, especially in the context of climate change.

Resumo

Os limites térmicos para animais ectotérmicos mostram uma imagem do intervalo de temperaturas corporais que é tolerável pelos indivíduos antes de sua capacidade locomotora ser prejudicada. Porém, os limites térmicos não são características fixas e específicas, mas traços lábeis sujeitos tanto a ajustes plásticos quanto a mudanças evolutivas, e são influenciados por fatores intrínsecos e extrínsecos dos organismos, e também por fatores metodológicos associados aos protocolos experimentais. Ainda mais, as influências desses fatores sobre os limites térmicos têm sido comumente abordadas de forma independente em diferentes espécies, e o grau pelo qual múltiplos fatores interagem e afetam os limites térmicos dentro das espécies é pouco compreendido. Assim, o principal objetivo deste trabalho foi conduzir uma avaliação metodológica da Temperatura Crítica Máxima (CT_{max}) estudando as influências de diferentes taxas de aquecimento experimental ($\Delta T's$), ontogenia, massa corpórea e a interação entre esses fatores sobre esta característica fisiológica. Este assunto foi abordado em larvas de *Physalaemus nattereri* e *Hypsiboas pardalis*, dois espécies de anfíbios anuros encontrados no Estado de São Paulo, sudeste do Brasil, que diferem em sua origem filogenética, características ecológicas e de história de vida, e também habitam ambientes com diferentes regimes térmicos. Primeiro, foi encontrado que as $\Delta T's$ afetaram tanto os valores médios quanto as variâncias da CT_{max} em ambas as espécies de maneira específica. Além disso, achou-se uma diminuição em CT_{max} no final da metamorfose que foi dependente da ΔT em larvas de *P. nattereri*, dado que nessa espécie só os girinos em metamorfose que foram expostos à ΔT aguda foram mais sensíveis às altas temperaturas do que os girinos premetamórficos. Finalmente, a massa corpórea e as $\Delta T's$ interagiram sobre a CT_{max} em ambas as espécies ao longo do desenho experimental. Em *P. nattereri*, o efeito da massa corpórea sobre a CT_{max} foi fisiológico nas $\Delta T's$ lentas, enquanto que em *H. pardalis* o efeito da massa corpórea na ΔT aguda foi devido a um artefato metodológico causado por maior inércia térmica no grupo de girinos maiores. Este estudo revelou que as $\Delta T's$, a ontogenia e a massa corpórea interagem sobre a CT_{max} das espécies estudadas, e estes efeitos interativos não poderiam ter sido elucidados pelo estudo independente de cada fator. Também é salientada a importância de integrar os fatores que influenciam os limites térmicos dos animais ectotérmicos, especialmente no contexto das mudanças climáticas.

General Introduction

Among the many abiotic factors that compound the Earth's climate, temperature is maybe the one with the most pervasive influences on organisms, and thermal biology is a discipline with a long-standing history within the broad areas of comparative and environmental physiology (Cossins and Bowler, 1987; Somero, 2011). The influences of environmental temperature on life permeate all levels of biological organization, from molecular interactions to biogeographic patterns (Hochachka and Somero, 2002; Somero, 2011). Organismal physiology emerges as the interface that integrates the mechanisms evident in lower levels of organisms and the ecological patterns seen in higher levels (Bartholomew, 1966), constituting so the keystone of the evolutionary theory of thermal adaptation (Angilletta, 2009; Cossins and Bowler, 1987). In this context, shifts in the thermal environment may have strong ecological impacts particularly in ectothermic animals, who constitute the vast majority of animal diversity. This is so because body temperature, which is typically influenced by environmental temperature, affects virtually all the physiological functions and, ultimately, the behavior of ectotherms (Angilletta et al., 2002).

How temperature affects the activities and survivorship of animals is a long-standing question that have drawn the interest of researchers since the era of naturalism (maybe before), and it still does today. Regarding extreme temperatures (a term exclusively taxon-dependent), by the second half of the 18th century the priest and naturalist Lazzaro Spallanzani exposed insect eggs and larvae, as well as salamanders, anuran embryos and adults to high temperatures and registered their lethal temperatures (Spallanzani, 1777). On account of the discussion about spontaneous generation, at this time it was already known that temperatures close to the boiling point of water were deleterious to most organisms (Bastian, 1874). By the end of the 19th century, Davenport & Castle published a great work on thermal acclimation of organisms to high temperature, in which they pointed out: "(…) For every individual there is, *as is well known*, an optimum temperature for its activities. As we elevate the temperature above this, we eventually reach a point where motion, after having somewhat diminished, suddenly ceases, producing what is known as "***heat rigor***", a condition from which the organism will return to activity upon lowering the temperature. A few degrees above the heat rigor point is that of death (…)" (Davenport and Castle, 1895). Thus, Davenport and Castle discriminated two different

states on organisms caused by high temperatures: the *heat-rigor point*, which sets the maximum tolerance, is reversible and characterized by the stopping of locomotion; and *death* caused by heat exposure when the heat-rigor point is exceeded, obviously irreversible. Davenport and Castle's contribution is very important because it represents the cornerstone that precedes a lot of literature on thermal tolerance of ectothermic animals that would be published afterward in the 20th century.

Much of what we currently know about the thermal tolerance of ectothermic animals was initially addressed directly in fish (Carter, 1887; Hathaway, 1927; Heath, 1884; Loeb and Wasteneys, 1912), and indirectly through studies on anuran development (Hathaway, 1927; Moore, 1939; Moore, 1942; Schechtman and Olson, 1941). In this sense, Brett's work and Fry and colleagues' work on fish established the framework of the *static method* to study thermal tolerance by estimating *individual lethal temperatures*, exposing individuals suddenly to fixed and unchangeable thermal conditions until they die (Brett, 1944; Fry, 1947; Fry et al., 1946). But it was not until Dr. Raymond B. Cowles and Dr. Charles M. Bogert published their classic work on desert reptiles that concrete metrics of thermoregulation were introduced in literature, including some additional for thermal tolerance (Cowles and Bogert, 1944). They proposed *critical thermal points* (both minimum and maximum) of individual tolerance, that differed fundamentally of the lethal temperatures in that the former did not involve the death of the organism, but the thermal points that prevent locomotion (like the heat rigor of Davenport & Castle). Regarding the **Critical Thermal Maximum** (CT_{max}), Cowles and Bogert defined it as "the thermal point at which *locomotory activity becomes disorganized and the animal loses its ability to escape from conditions that will promptly lead to its death*. Recovery from this incapacitation is the criterion for determining whether the animal has been exposed to unnecessarily high temperatures. *From the ecological viewpoint it is the lethal temperature* (…)" (Cowles and Bogert, 1944). They also set the basis of the *dynamic method* to estimate *individual critical temperatures* on ectothermic animals, which involves the gradual change of the experimental temperature until an endpoint is reached by individuals.

Cowles and Bogert's classic propelled definitely later studies on thermal tolerance of other ectothermic tetrapods, led mainly by Dr. Victor H. Hutchison and Dr. Bayard H. Brattstrom. Their research were remarkable in elucidating the taxonomic and biogeographic patterns on thermal tolerance and its acclimatory capacity (Brattstrom, 1963; Brattstrom, 1965; Brattstrom, 1968; Brattstrom, 1970; Brattstrom and Lawrence,

1962), as well as the standardization of the dynamic method (Hutchison, 1961), daily and seasonal variation in thermal tolerance (Hutchison and Kosh, 1965; Kosh and Hutchison, 1968; Mahoney and Hutchison, 1969), heat hardening (Maness and Hutchison, 1980), the physiological basis of the onset of muscular spasms as determining endpoint of CT_{max} (Paulson and Hutchison, 1987), the role of behavior (Hutchison and Maness, 1979), etc. All this happened when experimental ecologists and comparative physiologists were meeting together into fruitful and more integrative collaborations, which allowed to move from pattern descriptions toward a stronger evolutionary framework on thermal tolerance (and other topics, of course) in the following decades (see Feder et al. 1987 for a revision on the restructuring on Ecological Physiology).

The thermal performance curve (TPC) was initially proposed in the context of the benefits and costs of lizard thermoregulation as a model to explain the energetic gain per unit time of a lizard which is active along a given range of body temperatures (Huey and Slatkin, 1976). Later, the TPC was expanded conceptually to ectotherms in general to describe the thermal sensitivity of a specific type of function when it is measured during acute exposures along a broad range of body temperatures (Huey and Stevenson, 1979). Thus, the critical temperatures (as proposed by Cowles & Bogert) were contextualized into the TPC as the ***thermal limits*** of the ectotherm performance (e.g., energetic gain), that is the body temperatures at which physiological performance is null (Angilletta, 2009; Angilletta et al., 2002). The ultimate causes that set the thermal limits have been much debated in literature regarding the effects of temperature on proteins (particularly on enzymes), lipids and membrane functions, and temperature-pH interactions (Angilletta 2009; see Hochachka & Somero 2002 and Somero 2011 for comprehensive reviews on these topics). Pörtner and colleagues proposed recently an integrative model based on the whole-animal aerobic scope as the first process limited at thermal extremes, linked to the progressively insufficient capacity of circulatory, ventilatory and mitochondrial functions, and they called this model *the oxygen and capacity limitation of thermal tolerance* (Pörtner, 2001; Pörtner et al., 2000). They also have presented several theoretical and empirical evidences supporting their hypothesis (Pörtner, 2002; Pörtner, 2010; Pörtner et al., 2006), though contrasting evidences against also exist (Motyka et al. in press; Verberk et al. 2016).

In the recent years, thermal limits of ectothermic animals have gained additional attention as physiological traits linked to the distribution and resilience of some species to the ongoing climate change (Deutsch et al., 2008; Duarte et al., 2012; Helmuth et al.,

2002; Huey et al., 2009; Kingsolver et al., 2013). At the same time, some researchers have warned about experimental issues that affect estimates of thermal limits, which if not accounted in tests may yield misleading outcomes, modeling and conclusions (Rezende et al., 2011; Ribeiro et al., 2012; Terblanche et al., 2007). Even more, thermal limits are also affected by many intrinsic and extrinsic factors of organisms (this is further discussed in the paper) (Hutchison, 1976), which researchers should also recognize and control when delineating their experimental designs. However, the influences of intrinsic, extrinsic and methodological factors on thermal limits have been commonly addressed independently in different taxa, hence we lack of a cohesive framework that integrates different 'sources of variation' on thermal limits within taxa. Additionally, the extent by which sources of variation interact and affect thermal limits is poorly understood. This knowledge is necessary for a better understanding of the evolution of thermal limits (e.g., trade-offs), as well as for making more realistic predictions of species distributions and responses to changing thermal conditions under climate change scenarios, which is paramount for conservation purposes. All this was the motivation to conduct this work.

Considering both the historical and theoretical context about thermal limits of ectothermic animals, especially the knowledge gaps presented above, the main aim of this work was to conduct a methodological assessment of the Critical Thermal Maximum (CT_{max}) by studying the influences of different experimental heating rates, ontogeny and body mass on this trait. To do so, this matter was addressed on larvae of two anuran species from São Paulo State, southeastern Brazil, which were chosen based on their abundance in the field and availability during sampling. These anuran species also differ fundamentally in their phylogenetic background, ecological and life-history characteristics and inhabit environments with different thermal regimes.

The following manuscript presents more detail on the theoretical framework supporting the research question, the hypothesis, and the experimental and analytical methods employed. This manuscript was prepared to be submitted to the scientific journal *Journal of Experimental Biology* (http://jeb.biologists.org/), basically because this is a high-quality scientific journal in comparative physiology that has published other papers in thermal tolerance of ectothermic animals from different perspectives.

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

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Key-words: Anuran metamorphosis, climate change, CT_{max}, ectothermic animals, thermal inertia, thermal tolerance.

Running title: Interactive effects on thermal limits

Summary statement

Thermal limits of ectothermic animals are influenced by many sources of variation not only in isolation, but also by the interaction among them in a species-specific manner.

Abstract

Thermal limits of ectothermic animals are influenced by intrinsic and extrinsic factors of organisms, as well as by the interplay between physiology and methodological factors. Several studies have addressed independently some of these factors across different species, but little is known about how different sources of variation interact on thermal limits within taxa. We investigated the effects of experimental heating rates ($\Delta T's$), ontogeny, body mass, and their interactions on upper thermal limits (i.e., CT_{max}) in tadpoles of *Physalaemus nattereri* and *Hypsiboas pardalis*. We found that $\Delta T's$ affected averages and variances of CT_{max} in a species-specific manner. Also, we observed a ΔT-dependent decreasing in CT_{max} at the end of metamorphosis in *P. nattereri*, because only the metamorphosing tadpoles exposed to the acute ΔT were more sensitive to high temperature than premetamorphic tadpoles. Finally, we detected synergic effects of body mass and $\Delta T's$ on the CT_{max} of both species along our experimental design. In *P. nattereri* body mass affected CT_{max} through physiology at the slow $\Delta T's$, whereas in *H. pardalis* body mass affected CT_{max} at the acute ΔT through a methodological artifact driven by higher thermal inertia in the group of large tadpoles. Our study revealed interactive effects of $\Delta T's$, ontogeny and body mass on the upper thermal limits of our studied species that could not have been elucidated by the independent study of each factor. We highlight the importance of integrating the factors that influence thermal limits of ectothermic animals, especially in the context of climate change.

Introduction

In the recent years, thermal limits of ectothermic animals have gained increased attention as physiological traits linked to the distribution and resilience of some species to the ongoing climate change (Deutsch et al., 2008; Duarte et al., 2012; Helmuth et al., 2002; Huey et al., 2009; Kingsolver et al., 2013). As a corollary, the metric of 'Warming Tolerance' (WT, the difference between the Critical Thermal Maximum [CT_{max}] and maximum habitat temperature) was proposed to estimate species vulnerability to climate warming (Deutsch et al., 2008). But the above only applies in the precise cases of populations/species that are exposed in nature to extreme environmental temperatures (T_e, specifically at the individual scale) near their thermal limits (Deutsch et al., 2008; Duarte et al., 2012; Helmuth et al., 2002; Hoffmann et al., 2013; Nguyen et al., 2011), particularly those populations/species with stable or declining geographic range both in the tropics and high latitudes (Lancaster, 2016). However, the estimate of WT requires to consider thermal limits as traits with fixed values across species. This is more a mathematical convenience than a physiological truth for thermal limits are prone to both individual adjustments and evolutionary change (Brattstrom, 1968; Geerts et al., 2015; Healy and Schulte, 2012; Simon et al., 2015; Snyder and Weathers, 1975), and many sources of variation have been identified (Hutchison, 1976).

Thermal limits may be influenced by intrinsic factors of organisms, such as ontogenetic stage (Cupp, 1980; Floyd, 1983; Sherman, 1980), body mass (Becker and Genoway, 1979; Ospina and Mora, 2004; Ribeiro et al., 2012), physiological condition (Rezende et al., 2011), thermal history (Nyamukondiwa and Terblanche, 2010), among others. Thermal limits also vary because of extrinsic factors to organisms, such as photoperiod (Brattstrom, 1968; Hoar, 1956; Hutchison, 1961), seasonal and daily temperature cycles (Hopkin et al., 2006; Kosh and Hutchison, 1968; Sharma et al., 2015; Willhite and Cupp, 1982), geographic range (Baldanzi et al., 2015; Coyne et al., 1983; Sorte et al., 2011), among others. Finally, thermal limits are influenced by the interplay between physiology and experimental factors, such as thermal pre-treatments (Rajamohan and Sinclair, 2009; Ribeiro et al., 2012; Zheng et al., 2011), experimental endpoints of tests (Lutterschmidt and Hutchison, 1997a; Lutterschmidt and Hutchison, 1997b), experimental protocols (e.g., ramping or static) (Overgaard et al., 2012; Rezende et al., 2011), rates of temperature change (Allen et al., 2012; Mora and Maya, 2006; Ribeiro et al., 2012; Terblanche et al., 2007), among others. These sources of variation

have been largely studied independently in different taxa, therefore more complex and integrative studies are much needed within taxa to understand how different variables interact on estimates of thermal limits (Ribeiro et al., 2012).

Rate of temperature change (i.e., temperature time^{-1}, ΔT henceforth) matters on thermal-limits tests because heating and cooling occur at concrete rates in the field, and experiments may be performed at similar or alternative rates. On the one hand, in natural settings the ΔT measured at individual scales varies in time and space, even at very short scales. For instance, in a region of the Atlantic Forest (southeastern Brazil), water temperature in a bromeliad increased from 19.9°C early in the morning to 50.3°C at midday ($\Delta T \approx 0.11$°C min^{-1}), whereas in a permanent pond not further than 1 km away water temperature increased only 0.48°C for nearly 11 h ($\Delta T \approx 0.001$°C min^{-1}) in the same day (G.A. Agudelo and C.A. Navas, unpublished data). On the other hand, researchers have used diverse ΔT's in thermal-limits tests depending on their research question or on methodological issues (Lutterschmidt and Hutchison, 1997b; Rezende et al., 2011; Terblanche et al., 2011). The magnitude of ΔT influences negatively the duration of thermal-limits tests (i.e., the faster the ΔT, the shorter the experimental test, and vice versa), so researchers should consider the biological and methodological implications of a given ΔT. Too fast ΔT's may produce a lag in the homogenization of body temperature (T_b) (in function of individual body size) and induce heat-shock effects (Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997b). Yet, fast ΔT's may inform about baseline thermal limits (Ribeiro et al., 2012), but they may be ecologically unrealistic of ΔT's in field scenarios (i.e., ecological ΔT's) (Rezende et al., 2011; Terblanche et al., 2011). Alternatively, slow ΔT's can closely mimic ecological ΔT's, and therefore they do inform about ecological thermal limits (Ribeiro et al., 2012). Moreover, slow ΔT's can either enhance thermal limits via acute acclimation processes (Hutchison, 1961; Lutterschmidt and Hutchison, 1997b), or reduce them by exacerbating energy expenditure, desiccation, starvation (Rezende et al., 2011) and oxygen- and aerobic capacity-limitation (Pörtner, 2001; Pörtner, 2010). The final occurrence (or lack) of impacts of ΔT's on thermal limits differ greatly among lineages in terms of average and variance values (Allen et al., 2012; Chown et al., 2009; Peck et al., 2009; Terblanche et al., 2007; Vinagre et al., 2015), so it is not possible to anticipate the impact of ΔT's on thermal limits of new organisms to be tested (Ribeiro et al., 2012).

Ontogenetic effects on thermal tolerance have been observed in insects (Coyne et al., 1983; Klok and Chown, 2001; Krebs et al., 1998; Pincebourde and Casas, 2015; see

revision in Bowler and Terblanche, 2008), salamanders (Berkhouse and Fries, 1995; Delson and Whitford, 1973; Hutchison, 1961), anurans (Cupp, 1980; Floyd, 1983; Sherman, 1980; see revision in Ultsch et al., 1999) and some reptiles (Winne and Keck, 2005; Xu and Ji, 2006). The patterns showed by each contribution are associated with differences in natural history and microhabitat use among life stages within the studied species, since the ontogenetic stage with the highest thermal tolerance does experience the highest T_e (with an exception showed by Berkhouse and Fries, 1995). However, if life stages within a species do differ in morphology, microhabitat use and natural history traits, then an individual animal might experience different ecological $\Delta T's$ along its lifespan. Hence, differences in thermal limits along ontogeny within a species might indicate that selection would act differentially on life stages not only via absolute values of extreme $T_e's$, but also by the velocity as these temperatures are perceived by organisms in time. Most studies that examined ontogenetic effects on upper thermal limits used a single and constant ΔT ranging between 0.5 - 1°C min^{-1} (Delson and Whitford, 1973; Hutchison, 1961; Klok and Chown, 2001; Sherman, 1980; Winne and Keck, 2005), but the interaction between ontogeny and multiple $\Delta T's$ on thermal tolerance is poorly understood.

Body mass has been an underestimated variable in thermal-limits tests (Chown et al., 2002; Peck et al., 2009; Ribeiro et al., 2012), and even body mass values are rarely reported. Few studies have examined the effects of body mass on upper thermal limits, and no clear trend emerges from these contributions among different taxa (Berkhouse and Fries, 1995; Hutchison, 1961; Ribeiro et al., 2012; Verble-Pearson et al., 2015; Zhang and Kieffer, 2014). The concern is that body mass may influence thermal limits via true physiological effects or through methodological artifacts (Becker and Genoway, 1979; Ribeiro et al., 2012). Body mass affects the actual ΔT experienced by individuals, since T_b of small organisms equilibrates with T_e more rapidly than T_b of large organisms, which possess higher thermal inertia because of a lower surface-to-volume ratios (Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997b; Seebacher and Shine, 2006; Seebacher et al., 1999). Potential interferences of body mass on thermal limits are more amplified at fast $\Delta T's$ than at slower ones, and researchers might identify whether 'size differences' for a particular ΔT are methodological artifacts by tracking lags between internal T_b and temperature of the external media in function of body mass (Becker and Genoway, 1979). Yet, the lag only describes the dynamics of the experimental system for a given size, and it tells whether animals of different sizes are heated (or cooled) at

different ΔT's, but it informs little about the intrinsic effect of body mass on thermal limits. Size differences in thermal limits are better discriminated through ΔT-body mass interactions in a covariance design (Ribeiro et al., 2012). If ΔT-body mass interactions explain variance in thermal limits, it is likely that size differences are methodological artifacts because actual ΔT's vary among individuals of different sizes (Ribeiro et al., 2012). But if size differences persist in absence of significant ΔT-body mass interactions (i.e., all individuals are heated [or cooled] at the same ΔT), body mass would have a physiological effect on thermal limits (Ribeiro et al., 2012). However, such an experiment requires a large sample size, which could be non-viable for some organisms of interest. We propose a related approach to test and discriminate potential artifacts of body mass for a particular ΔT by exposing individuals of different size classes at the same ΔT (controlling for other potential sources of variation, see above).

The main goal of this work was to investigate the effects of ΔT's, ontogeny, body mass, and the interactions among these factors on the upper thermal limits (i.e., CT_{max}) of anuran larvae. We chose anuran larvae as models because of two theoretical and practical conceptions. First, water has a much higher specific heat and conductivity than air, and that means a higher homogeneity in thermal variation and easier experimental control of water temperature (Navas et al., 2010; Tejedo et al., 2012). Second, given that small body sizes make anuran larvae practically isothermic with the aquatic environment, and behavioral thermoregulation is more limited in larvae when compared with juveniles or adult individuals, selection on upper thermal limits should be higher in aquatic larvae than in terrestrial frogs (Gutiérrez-Pesquera et al., 2016; Simon et al., 2015; Tejedo et al., 2012). We studied two species of anuran larvae which differ in life history traits, microhabitat use and that inhabit environments with different thermal regimes. Our approach involved first to compare the CT_{max} of tadpoles of both species at the same ontogenetic stage after they were exposed to three different experimental ΔT's. Second, we measured the effects of these three ΔT's on the CT_{max} for tadpoles of the same species but at different developmental stages. Finally, we classified tadpoles of the same species from the same ontogenetic stage into two different body size classes and then we exposed those individuals to the fastest ΔT we used in our experiments. Next we scaled CT_{max} and body mass for every size class, and lastly we compared the CT_{max} between the body size classes. We tested for ΔT-body mass interactions along all our experimental design in order to identify and discriminate possible size differences in CT_{max}. Thus, we hypothesized that: 1) the effects of ΔT's on CT_{max} (average and variances) will be species-

specific; 2) $\Delta T's$ will affect CT_{max} along ontogeny; and 3) body mass will have a physiological effect on CT_{max} if this effect is consistent between size classes or involves only the small individuals. Yet, body mass will affect CT_{max} through a methodological artifact (i.e. thermal inertia) if this effect is not consistent between size classes and involves only the large individuals.

Materials and methods

Study sites, study species and acclimation treatment.

The study sites comprise two localities of the São Paulo State, southeastern Brazil. The first site was 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), and the second site was an Atlantic Forest fragment in the municipality of São Luiz do Paraitinga (SLP; 23°12' S, 45°16' O, 769-829 m.a.n.m). Field sampling was done in both sites during the summer of 2015, between January – March, because that period matches with the peak of breeding season of most anuran species from the Cerrado and the Atlantic Forest in southeastern Brazil (Bertoluci, 1998; Bertoluci and Rodrigues, 2002; Vasconcelos and Rossa-Feres, 2005). The studied species were chosen for their abundance in the field. Thus, in SJRP we collected nine clutches of *Physalaemus nattereri* (Steindachner, 1983) in a temporary pond and also in a permanent pond. 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 it breeds near of standing and temporary waterbodies (Aquino et al., 2004). On the other hand, in SLP we collected tadpoles of *Hypsiboas pardalis* (Spix, 1824) in three different sites (stream and artificial dam in interior forest, and an open flooded area), and selected tadpoles in stage 25 after in the laboratory. This species is known from the Atlantic rainforest of southeastern Brazil, and it 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. In the laboratory, we mixed the clutches of *P. nattereri* in order to minimize the genetic variances between experimental treatments, and then we transferred three samples of about 200 eggs into three plastic containers inside a climatic room. Water temperature was set at $23 \pm 1°C$, 13L:11D photoperiod (average of summer photoperiod of the last four years; Astronomical Applications Dept., USNO).

The same procedure was followed for *H. pardalis* tadpoles. *Physalaemus nattereri* embryos reached the Gosner stage 25 in the laboratory around 20 days after collected. Tadpoles at Gosner stage 25 of both species were fed *ad libitum* with fish food flakes, water was changed every other day, and the individuals were maintained in the acclimation treatment for two weeks until we began upper thermal limits tests.

Experimental design.

Experiment 1 assessed the effects of ΔT's on upper thermal limits applying $\Delta T = 1°C$ min^{-1}, $\Delta T = 0.05°C$ min^{-1} and $\Delta T = 0.1°C$ min^{-1} to tadpoles at Gosner 25 stage, both species. Time of captivity was the same (two weeks) for individuals of both species in experiment 1. Experiment 2 assessed the interaction between ΔT's and ontogeny at the same ΔT's than Experiment 1, but we used only tadpoles of *P. nattereri* at intermediate (35-41) and advanced (42-44) Gosner stages, to be compared with data on stage 25 (Experiment 1). Time of captivity was not the same for all groups in experiment 2, because intermediate and advanced tadpoles of *P. nattereri* reached those stages in captivity 109 - 140 days after we performed the experiment 1. Experiment 3 analyzed the influence of body mass on upper thermal limits measured only at 1°C min^{-1}. This experiment applied to a second sample of tadpoles of *H. pardalis* (Gosner stage 25) whose body mass ranged from 212.4 - 2350.1 mg to be compared with those tested at same ΔT in Experiment 1 (Body mass range 23.5 - 83.1 mg, Welch's ANOVA, $F_{(1,47)} = 489.71$, $P < 0.01$; Table S2). Time of captivity for the large group of tadpoles of *H. pardalis* ranged from 110 - 160 days.

Upper thermal limits.

We used 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 – 1000 h (UTC-2), and the initial temperature always was 23 ± 0.5 °C. We placed the tadpoles individually inside plastic containers and then we randomly distributed these containers into three temperature-controlled baths (two large baths for $\Delta T = 0.05°C$ min^{-1} and $\Delta T = 0.1°C$ min^{-1} and one smaller for $\Delta T = 1°C$ min^{-1}, Fig. S1), where water temperature was constantly increased and registered (Table S1; Fig. S2). Individuals of both species mostly remained at the bottom of containers once tests began, but as water temperature increased, they became more active in terms of swimming and mouth movements. We continuously monitored water temperature as a proxy of tadpoles

T_b along the tests (Navas et al., 2010). In pilot tests, we failed to identify unambiguously muscular spasms (Lutterschmidt and Hutchison, 1997a), then we ended the tests when we observed immobility after five consecutive taps. Once an individual reached its CT_{max}, we transferred it into plastic containers with cool water (~23°C) to allow recovery. We consider only the CT_{max} data of individuals that remained alive 24 h after the tests (Table 1). We measured the body mass of each individual in an analytical balance to the nearest 0.001 g.

Statistical analyses.

CT_{max} determinations involve both temperature and time in which an individual animal reach the endpoint, and we explored the relationships between both variables through Spearman Rank Correlations. We provided descriptive statistics for both variables in Table 1. As we expected, CT_{max} and time of resistance were positively correlated in most experimental groups (Spearman Rank Correlations, $P < 0.05$) (oddly not at $\Delta T = 1°C$ min^{-1}), thus we reported our results mainly in terms of temperature as the most ecologically meaningful variable. CT_{max} data were either normally distributed or negatively-skewed and CT_{max} variances were heterogeneous in most group comparisons. Thus, we used Welch's Analysis of Variances (Welch's ANOVA) to compare CT_{max} heteroscedastic data (McDonald, 2014) despite non-normality given robustness associated with large samples (Feir-Walsh and Toothaker, 1974; McDonald, 2014; Schmider et al., 2010). Then, we applied Games-Howell tests for multiple comparisons. When CT_{max} variances were homogeneous, we compared CT_{max} data through One-Way Analysis of Covariance (ANCOVA), with body mass as covariate. We tested ΔT-body mass interactions through two complementary statistic approaches. First, we employed Tests for Homogeneity of Slopes, which run a customized ANCOVA design for testing the interaction between ΔT (fixed factor) and body mass (covariate) (Hill and Lewicki, 2007). Second, we conducted Linear Regressions or Spearman Rank Correlations to test the relationship between CT_{max} and body mass, depending on fulfillment of assumptions. We used a significance level of 0.05 and performed the analyses and figures in Statistica 8 (Hill and Lewicki, 2007) and Excel packages.

Results

Heating rates ($\Delta T's$)

At Gosner stage 25, species responded differently to $\Delta T's$ in both average and variance of CT_{max}. For example, in comparison to *H. pardalis*, the tadpoles of *P. nattereri* displayed a broader range of CT_{max} at any ΔT (Table 1, Fig. S3). In addition, and regarding variability of CT_{max}, each species responded differently to $\Delta T's$, for tadpoles of *P. nattereri* displayed more variable CT_{max} under slow $\Delta T's$ (Levene's test, $F_{(2,118)} = 4.35$, $P = 0.01$; Fig. S3A), whereas tadpoles of *H. pardalis* showed more variable CT_{max} under the acute ΔT (Levene's test, $F_{(2,117)} = 10.69$, $P < 0.01$; Fig. S3B). Regarding average tolerance to heating (mean CT_{max}, see Fig. 1A), tadpoles of *H. pardalis* were comparatively more sensitive than tadpoles of *P. nattereri* (Welch's ANOVA, $F_{(5,100)} = 43.17$, $P < 0.01$) given their lower CT_{max} at slow $\Delta T's$ (Games-Howell test, $P < 0.05$). Differently, tadpoles of *P. nattereri* exhibited similar average CT_{max} at any ΔT (Games-Howell test, $P > 0.05$).

Ontogeny, ΔT and body mass

At intermediate (35-41) and advanced (42-44) Gosner stages, tadpoles of *P. nattereri* were also affected by $\Delta T's$ in both average and variances of CT_{max}. Regarding CT_{max} variability, intermediate and metamorphosing *P. nattereri* tadpoles displayed more variable CT_{max} under slow $\Delta T's$ (Gosner 35-41, Levene's test, $F_{(2,42)} = 3.49$, $P = 0.04$; Gosner 42-44, Levene's test, $F_{(2,28)} = 6.79$, $P < 0.01$; Table 1). Regarding average tolerance to heating (Fig. 2A), tadpoles of *P. nattereri* exhibited a ΔT-dependent decreasing in CT_{max} along ontogeny (Welch's ANOVA, $F_{(8,49)} = 6.72$, $P < 0.01$), for metamorphosing tadpoles were more sensitive to high temperatures than premetamorphic tadpoles only when the former were exposed to the acute ΔT (Games-Howell test, $P < 0.05$). Also, only intermediate tadpoles of *P. nattereri* were influenced by synergic effects of body mass and $\Delta T's$ (Test for Homogeneity of Slopes, $F_{(2,39)} = 6.29$, $P < 0.01$), for their CT_{max} was positively correlated with their body mass only at $\Delta T = 0.1°C$ min^{-1} (Spearman Rank Correlation, $r_s = 0.64$, $P = 0.01$; Fig. 2B). Upper thermal limits of metamorphosing tadpoles of *P. nattereri* were not influenced by body mass at any ΔT (Test for Homogeneity of Slopes, $F_{(2,25)} = 1.12$, $P = 0.34$; Spearman Rank Correlations at any ΔT, $P > 0.05$; Fig. 2C).

Δ*T*-body mass interactions

Also at stage 25 Gosner, upper thermal limits were affected differently between species by synergic effects of body mass and Δ*T's* (or Δ*T*-body mass interactions). First, tadpoles of *P. nattereri* tadpoles were affected by Δ*T*-body mass interactions (Test for Homogeneity of Slopes, $F_{(2,115)} = 5.54$, $P < 0.01$) for their CT_{max} was positively correlated with body mass only at slow Δ*T's* (Δ*T* = 0.05°C min^{-1}, Spearman Rank Correlation, $r_s = 0.61$, $P < 0.01$; Δ*T* = 0.1°C min^{-1}, Spearman Rank Correlation, $r_s = 0.53$, $P < 0.01$; Fig. 1B). The relationship between CT_{max} and time of resistance is illustrative for the previous trend at Δ*T* = 0.05°C min^{-1}, because it split tadpoles of *P. nattereri* into two groups with different mean tolerance to heating (CT_{max}, ANOVA, $F_{(1,32)} = 154.5$, $P < 0.01$; Time of resistance, Welch's ANOVA, $F_{(1,18)} = 95.74$, $P < 0.01$; Fig. S4). Interestingly, the more tolerant tadpoles were significantly larger than the more sensitive ones (ANOVA, $F_{(1,32)} = 9.67$, $P < 0.01$), therefore we reassessed the relationship between CT_{max} and body mass for each group separately (Fig. 3A). Body mass was positively associated with the CT_{max} of the more sensitive tadpoles (i.e., the small ones; Linear Regression, $R^2 = 0.21$, $F = 5.07$, $P = 0.04$), but was not associated with the CT_{max} of the more tolerant tadpoles (Linear Regression, $R^2 = 0.01$, $F = 0.14$, $P = 0.72$).

Second, upper thermal limits of tadpoles of *H. pardalis* were unaffected by Δ*T*-body mass interactions when we considered all the Δ*T's* (Test for Homogeneity of Slopes, $F_{(2,114)} = 0.3$, $P = 0.73$; Spearman Rank Correlations at any Δ*T*, $P > 0.05$; Fig. 1C). However, when the two size classes of *H. pardalis* were compared, a synergic effect of body mass and the acute Δ*T* emerged. Despite one analysis suggested no Δ*T*-body mass interaction at this Δ*T* (Test for Homogeneity of Slopes, $F_{(1,86)} = 0.5$, $P = 0.47$), the other one showed an effect only in the group of large tadpoles, because *within* that group the larger individuals were more tolerant to high temperatures than the small ones (Linear Regression, $R^2 = 0.25$, $F = 11.37$, $P < 0.01$; Fig. 3B). The group of large tadpoles also resisted more time in experiments than the group of small tadpoles (Welch's ANOVA, $F_{(1,86)} = 83.03$, $P < 0.01$). Yet, after we accounted for body mass in CT_{max} comparisons *between* size classes, the group of small tadpoles of *H. pardalis* was actually more tolerant to high temperatures than the group of large tadpoles (ANCOVA, $F_{(1,87)} = 19.9$, $P < 0.01$; Table 1).

Discussion

Our data demonstrated species-specific effects of the experimental heating rates ($\Delta T's$) we used regarding average and variability of upper thermal limits in tadpoles of *P. nattereri* and *H. pardalis*. Regarding mean CT_{max} of tadpoles of *H. pardalis*, perhaps the thermal stress accumulated during tests at slow $\Delta T's$ surpassed baseline thermal protection (Rezende et al., 2011), and acclimatory processes (e.g., heat hardening) could be insufficient at the time course of these $\Delta T's$ to overcome thermal stress. As ours, other studies have also reported contrasting effects of $\Delta T's$ on mean upper thermal limits among species (Allen et al., 2012; Peck et al., 2009; Tejedo et al., 2012; Terblanche et al., 2007; Vinagre et al., 2015), and such physiological diversity is expected given the distant phylogenetic position of the studied species. However, most of these studies have focused on mean thermal limits, neglecting variances (Chown et al., 2009). Chown et al. (2009) also found a similar outcome of contrasting variances between two phylogenetically and ecologically distant insect species.

Likely, patterns of averages and variances in upper thermal limits might reflect both the action of natural selection on species' thermal physiology via absolute $T_e's$ and ecological $\Delta T's$ (two different aspects of environmental thermal variability), as well as past thermal history (Giomi et al., 2016). Tadpoles of *P. nattereri* typically develop in ephemeral waterbodies which can be in open areas exposed to high and rapid daily thermal variation, whereas tadpoles of *H. pardalis* develop in more thermally stable waterbodies inside the forest. In adult insects, fluctuating thermal regimes that remain within tolerant physiological ranges generally enhance performance and thermal tolerance (Colinet et al., 2015), likely because of increased upregulation of heat shock proteins (HSPs) and induction of metabolic depression in thermally variable conditions (Bozinovic et al., 2013; Williams et al., 2012). Whether similar mechanisms are responsible for enhancing thermal tolerance in anuran species inhabiting thermally variable environments is a hypothesis to be tested, since the capacity for thermal acclimation of metabolic rate seems to be positively correlated with the degree of environmental thermal variability in amphibians (adults and larvae) (Feder, 1978; Feder, 1982).

Experimental heating rates ($\Delta T's$) affected the upper thermal limits of *P. nattereri* along ontogeny. Previous contributions also observed a diminishing on the upper thermal tolerance at the end of metamorphosis in other anuran species (Cupp, 1980; Floyd, 1983;

Sherman, 1980; Sherman and Levitis, 2003). However, we showed that in *P. nattereri* this trend was dependent of the ΔT. This means on the one hand that metamorphosing tadpoles of this species are able to cope with slow thermal exposures with similar physiological scope than individuals at early or intermediate ontogenetic stages do. This is a striking outcome, because one would expect that slow thermal exposures would be more deleterious for thermal tolerance of tadpoles close the climax of metamorphosis than acute exposures. One possible explanation is that tadpoles of *P. nattereri* might exhibit heat hardening even at the end of metamorphosis, like seen in *Xenopus laevis* (Sherman and Levitis, 2003). On the other hand, a trade-off arises in advanced ontogenetic stages when individuals are exposed to acute thermal changes (as proposed by Rezende et al., 2014). Although this trend is particular for *P. nattereri*, ΔT-ontogeny interactions are predicted to occur in other anuran species and even other ectothermic taxa, therefore this matter should be further investigated.

Some final considerations about ontogeny and thermal tolerance that require attention are the nature itself of anuran metamorphosis and the associated HSPs expression. The drop in upper thermal limits at the end of anuran metamorphosis has been seen as a sign of the stressful nature of this process (Sherman, 1980), resulting from the drastic changes and remodeling of essentially every organ/tissue of the tadpole (Shi, 2000). Neurohormonal, behavioral and ecological observations also agree with the notion of anuran metamorphosis as a stressful process (Arnold and Wassersug, 1978; Denver, 1997; Floyd, 1984; Wilbur, 1980). Thus, metamorphosing tadpoles are hypothesized to be less able to mobilize mechanisms to cope with high temperatures when compared with premetamorphic tadpoles (Sherman and Levitis, 2003). However, our results in metamorphosing tadpoles of *P. nattereri* challenge the previous claim, for the degree of the thermal exposure may be a determinant for the scope of responses in these 'naturally-stressed' individuals. In terms of mechanisms, metamorphosing tadpoles might increase HSPs levels without eliciting a heat shock response, as evidenced in *Rana temporaria* (Nikinmaa et al., 2008). HSPs are hypothesized to play a role in the restructuring of tissues during anuran metamorphosis, given that the inherent anatomical and physiological changes imply both the degradation of proteins characteristic of the larval phenotype and the *de novo* synthesis of proteins of the adult phenotype (Helbing et al., 1996). Yet, the role of HSPs during thermal stress along anuran metamorphosis is poorly understood and deserves more investigation. This matter could benefit from studies on HSPs regulation and overexpression such as those performed in *Drosophila* larvae (Krebs

and Feder, 1997; Krebs et al., 1998), which may shed light into the function of these molecular chaperones along anuran metamorphosis, as well as the trade-offs of their expression between development and physiological stress.

We found synergic effects of body mass and ΔT's on the upper thermal limits of *P. nattereri* and *H. pardalis* along our experimental design, and this contrasts with previous contributions that did not detect any size difference in upper thermal tolerance in other amphibian larvae (Bury, 2008) and small fish (Anttila et al., 2013; Ospina and Mora, 2004). Ribeiro et al. (2012) indicated that if ΔT-body mass interactions explain variance in CT_{max}, likely experimental correlates of body mass are complicating factors. However, discriminating size differences in thermal limits may require also considering the nature of the interaction itself. For instance, we argue that the size differences in the CT_{max} of tadpoles of *P. nattereri* (premetamorphic stages) at the slow ΔT's are true physiological effects. It is unlikely that thermal inertia was an issue at our slow ΔT's for the tadpoles of *P. nattereri* we used, considering both their small size (Table S2) and that body mass effects involved the small individuals (Becker and Genoway, 1979). Furthermore, the body mass effect on CT_{max} only in the smaller tadpoles of *P. nattereri* (stage 25 Gosner) at $\Delta T = 0.05°C\ min^{-1}$ reflects that upper thermal tolerance is possibly constrained early in the tadpole development during the embryo-larval transition and matures as tadpoles grow and develop.

On the other hand, we argue that the effect of body mass on CT_{max} in the group of large tadpoles of *H. pardalis* at the acute ΔT is a methodological artifact. The discrepancy between time of resistance and mean upper thermal tolerance between size classes is illustrative for higher thermal inertia in the large tadpoles of this species. These findings suggest a caveat on the traditional assumption that thermal inertia is a negligible aspect when testing thermal limits of ectothermic animals at size scales comparable with tadpoles, even when dealing with aquatic organisms. Despite the ΔT used in experimental tests, researchers should take into account possible ΔT-body mass interactions in their experimental designs and may opt by use an experimental approach similar than ours to discriminate size differences in thermal limits. Other authors adopted similar approaches before with the same purpose. For instance, Miller and Packard (1977) classified adult frogs of *Pseudacris triseriata* into two size classes and did not find any relationship between CT_{max} and body mass in either size class at $\Delta T = 0.5 \pm 0.2°C\ min^{-1}$. Also, Zhang and Kieffer (2014) compared the CT_{max} of the shortnose sturgeon *Acipenser brevirostrum* across two size classes and did find a positive relationship between CT_{max} and body mass

that was consistent between size classes at $\Delta T = 0.1°C$ min^{-1}. As we saw for tadpoles of *H. pardalis*, Zhang and Kieffer (2014) found that the group of small fish was more tolerant to high temperatures than the group of large fish, and they attributed these size differences to ontogenetic effects in this species. Although our body size categories represent tadpoles at the same ontogenetic stage, the discrete classification of the development of anuran larvae is arbitrary because this is a continuous process (Floyd, 1983; Sherman and Levitis, 2003). Therefore, the mean differences in upper thermal tolerance we observed between size classes in tadpoles of *H. pardalis* may reflect true physiological differences hidden by external morphology.

A final important consideration is regarding time of captivity. Plasma concentrations of corticosterone increase in some vertebrate species under short-term and long-term captivity stress (Assis et al., 2015; Lattin and Romero, 2014; Moore et al., 1991; Pankhurst and Sharples, 1992), and this variable may induce species-specific changes in amphibian physiology and behavior (Navas and Gomes, 2001). Few studies have examined the effect of time of captivity on thermal tolerance, and the resulting impacts are not obvious. For example, Mora & Maya (2006) found in a blenny fish species that upper thermal tolerance actually increased with time of captivity. This 'beneficial effect' might be partially explained by acclimation responses during captivity, which may occur in laboratory conditions with a time course species-specific (Pintor et al., 2016). It is unlikely that time of captivity affect the outcomes of our interspecific comparison, because in these cases (experiment 1) time of captivity was the same for both species. Although time of captivity was not the same in intraspecific comparisons (experiments 2 and 3), it is also unlikely this variable interfered in the ontogenetic trends we observed, because the less tolerant tadpoles of *P. nattereri* were actually those exposed to the less stressful (ΔT). Also, the high thermal inertia in large tadpoles of *H. pardalis* is strictly consequence of their body mass and the physical dynamics of energy exchange with the external medium. We do not know to which extent mean differences in upper thermal tolerance between size classes of *H. pardalis* could be affected by time of captivity. This is an uncontrolled variable in our design, but no other alternative was viable and an ideal experiment to control it would be impractical for requiring many more individuals.

In conclusion, data from this study show that $\Delta T's$, ontogeny and body mass interact in our studied species to influence their upper thermal limits. The relevance of our findings is that the main trends resulting from these interactive effects could not have been elucidated by the independent study of each factor. We highlight that researchers

interested in estimating thermal limits of ectothermic animals through the dynamic method should consider the $\Delta T's$ very carefully, particularly when the primary focus is on ecological thermal limits (Ribeiro et al., 2012). Also, the general trend of decreasing upper thermal tolerance in developing anurans should be tested rather than assumed, considering both the baseline thermal limits and the ecological. Since many species of ectothermic animals exhibit substantial variation in thermal sensitivity among life stages, and thermal conditions experienced during ontogeny may strongly affect the thermal tolerance of individual adults (Cavieres et al., 2016), it is paramount to include ontogeny into the predictions of the impacts of climate warming on ectothermic species (Klockmann et al., in press). Body mass effects, either physiological or via thermal inertia, coexist in nature, but the extent to which this variable favor or constrain adaptive responses in heat tolerance is just beginning to be understood (Klockmann et al., in press). Our experimental approach was successful in discriminating size effects on upper thermal limits of our studied species, so researchers interested in this matter may adopt similar approaches if apply on their experimental subjects. Yet, if researchers desire to break free from size effects on thermal limits as much as possible, they may opt by select individuals with small body size range (Anttila et al., 2013; Simon et al., 2015). Finally, our study presents some caveats that worth consideration when assessing thermal limits of ectothermic animals. It is crucial to develop an integrative understanding of the factors influencing thermal tolerance, specially to forecast the impacts/responses of ectothermic species to the ongoing climate change.

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Competing interests

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, collected and analysed the data. G.A.A.C wrote the initial draft, and both authors revised the manuscript. G.A.A.C and C.A.N gave final approval for publication.

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Supplementary information

Supplementary information available online at…

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Table 1. Values of CT_{max} and Time of resistance for tadpoles of *Physalaemus nattereri* and *Hypsiboas pardalis*, for each ontogenetic stage and heating rate (ΔT). *N* is the sample size (not counting deaths). † indicates the number of dead tadpoles after 24 h of tests, which were discarded for data analysis. Min and Max are the minimum and maximum values for each variable, respectively. CV is the coefficient of variation.

Species	Ontogenetic stage	ΔT (°C min^{-1})	N	†	CT_{max} (°C)				Time to CT_{max} (min)			
					Mean ± s.d.	Min	Max	CV (%)	Mean ± s.d.	Min	Max	CV (%)
P. nattereri	Stage 25	0.05	34	2	41.9 ± 0.7	40.8	43.4	1.7	391.9 ± 20.8	364	441	5.3
P. nattereri	Stage 25	0.1	36	1	41.9 ± 0.7	40.3	43.4	1.7	210.2 ± 10.5	190	230	5.0
P. nattereri	Stage 25	1	51	2	41.8 ± 0.5	40.7	42.9	1.2	18.2 ± 1.2	16	20	6.8
P. nattereri	Stages 35-41	0.05	15	1	41.9 ± 0.8	40.3	43.3	1.9	365.2 ± 22.6	329	401	6.1
P. nattereri	Stages 35-41	0.1	15	1	41.7 ± 1.0	39.8	43.3	2.4	205.6 ± 18.5	178	235	9.0
P. nattereri	Stages 35-41	1	15	0	41.5 ± 0.5	40.7	42.4	1.2	20.5 ± 1.2	19	23	6.1
P. nattereri	Stages 42-44	0.05	11	4	41.0 ± 1.0	39.1	42.7	2.4	349.4 ± 27.6	304	392	7.9
P. nattereri	Stages 42-44	0.1	10	3	39.8 ± 2.0	36.3	42.5	5.1	193.0 ± 33.4	145	235	17.3
P. nattereri	Stages 42-44	1	10	7	40.1 ± 0.9	38.4	41.5	2.2	19.1 ± 1.6	17	21	8.4
H. pardalis	Stage 25	0.05	20	13	41.1 ± 0.1	40.9	41.3	0.4	333.7 ± 4.1	324	341	1.2
H. pardalis	Stage 25	0.1	46	20	41.3 ± 0.2	40.6	41.6	0.5	191.7 ± 4.1	182	201	2.1
H. pardalis	Stage 25	1	54	0	41.8 ± 0.4	40.8	42.4	0.9	18.2 ± 1.0	17	20	5.4
*H. pardalis**	Stage 25	1	36	0	41.6 ± 0.4	40.8	42.4	0.8	19.9 ± 0.7	18	21	3.8

*This category represents the group of large *H. pardalis* tadpoles at stage 25 Gosner (212.4 - 2350.1 mg), which was compared only with the other group of *H. pardalis* tadpoles tested at $\Delta T = 1$°C min^{-1} (i.e. the group of small individuals, 23.5 - 83.1 mg). See the text for more details.

Figure legends

Figure 1. Effects of ΔT on the CT_{max} of tadpoles (stage 25 Gosner) of *P. nattereri* and *H. pardalis*. (A) CT_{max} comparisons between tadpoles of *P. nattereri* and *H. pardalis* at three ΔT. Symbols indicate the averages; error bars indicate the standard deviations (s.d.). Sample size for each group is given above error bars. Relationships between the CT_{max} and the body mass of individual tadpoles of *P. nattereri* (B) and *H. pardalis* (C), at three ΔT. Trend lines: solid line for $\Delta T = 0.05$°C min^{-1}, dashed line for $\Delta T = 0.1$°C min^{-1}, dotted line for $\Delta T = 1$°C min^{-1}.

Figure 2. Interaction between ΔT's and ontogeny in tadpoles of *P. nattereri*. (A) CT_{max} comparisons between tadpoles at stage 25, stages 35-41 and stages 42-44, at three ΔT. Relationships between the CT_{max} and the body mass of individual tadpoles of *P. nattereri* at stages 35-41 (B) and stages 42-44 (C), at three ΔT. Figure details are the same that in Figure 1.

Figure 3. Body mass affects CT_{max} at determined ΔT through either physiology or methodological artifacts. (A) Relationship between the CT_{max} and the body mass in tadpoles of *P. nattereri* (stage 25 Gosner) at $\Delta T = 0.05$°C min^{-1} (same data set than that depicted in Figure 1B), showing a physiological effect of body mass on CT_{max} only in the small tadpoles. Closed squares and the dotted thin line represent tadpoles with CT_{max} ranging from 40.8 - 41.8, whereas open squares and the solid line represent tadpoles with CT_{max} ranging from 42.3 - 43.4. The dotted thick line is the trend for the whole data. (B) Relationships between the CT_{max} and the body mass of *H. pardalis* tadpoles (stage 25 Gosner) classified into two different body size classes. CT_{max} was tested at $\Delta T = 1$°C min^{-1}. The effect of body mass on the CT_{max} of the large size class is a methodological artifact. Closed triangles, the dotted line and the bottom x-axis represent tadpoles of 23.5 - 83.1 mg; open triangles, the dashed line and the top x-axis represent tadpoles of 212.4 - 2350.1 mg. See the text for more explanation.
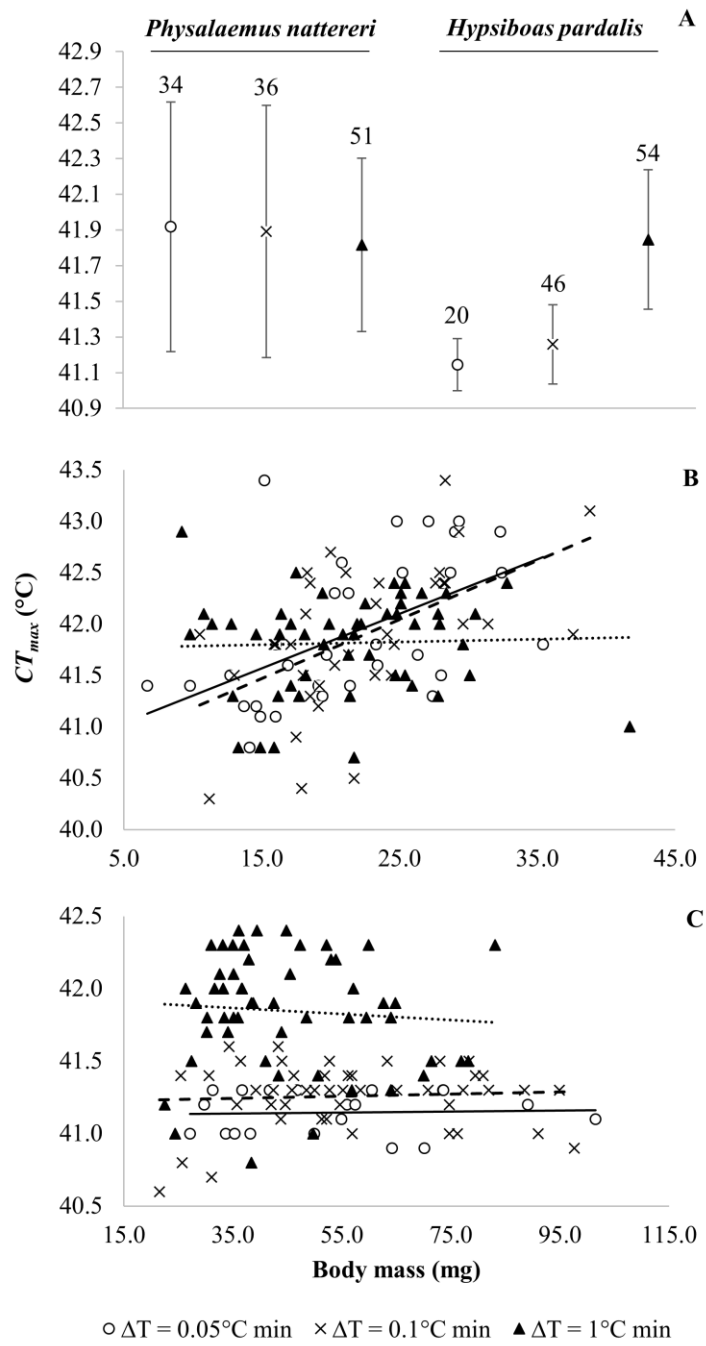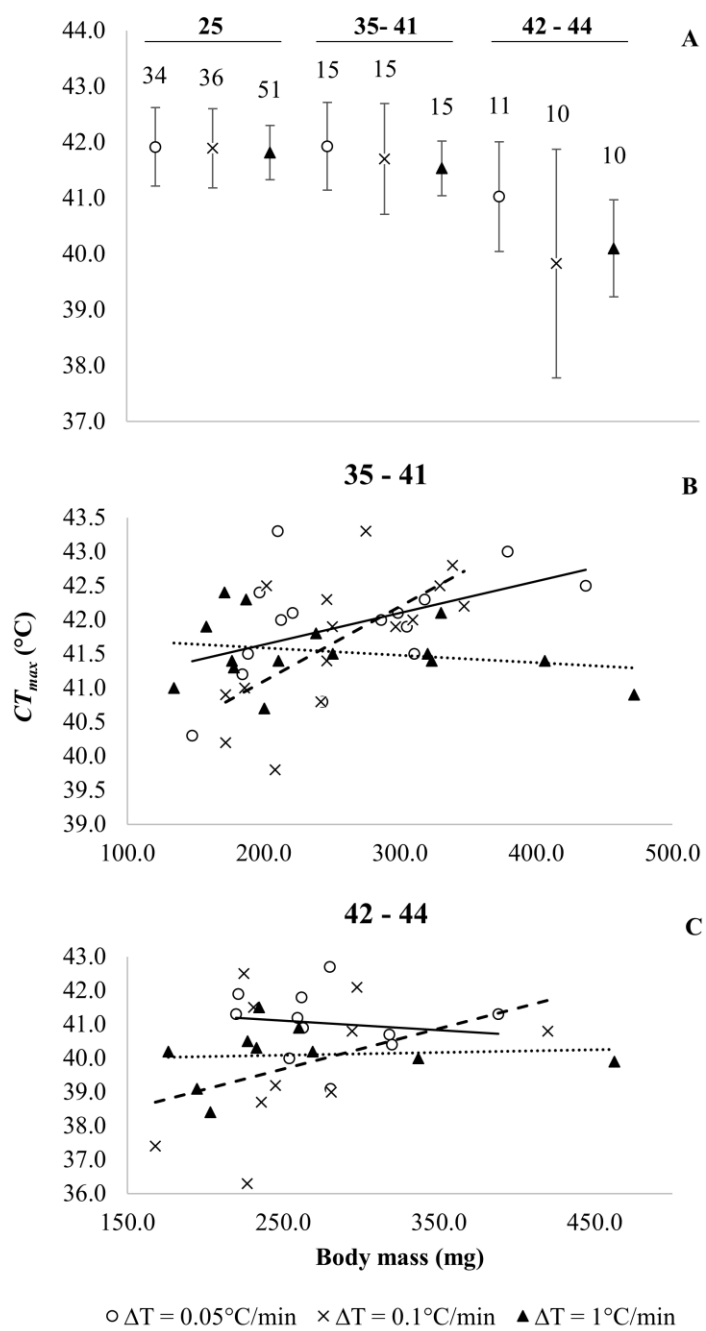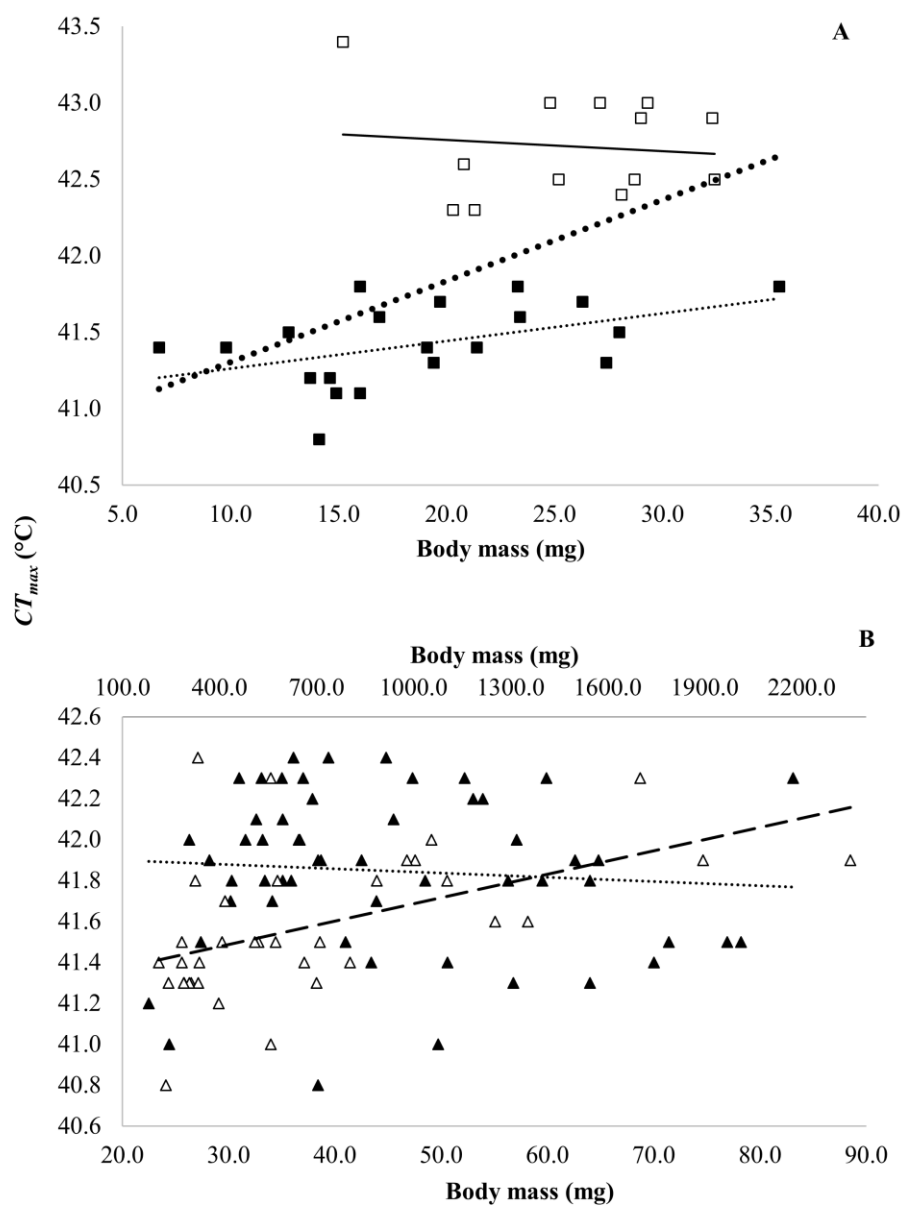
Figures

Figure 1

Figure 2

Figure 2

Figure 3

Supplementary information

Supplementary information available online at…

Table S1. Summary of the experimental tests for upper thermal limits in *Physalaemus nattereri* tadpoles and *Hypsiboas pardalis* tadpoles. We provide the mean and standard deviations for the heating rates (ΔT) registered in the experimental tests for each experimental group. N is the number of experimental tests.

Experimental group	ΔT (°C min^{-1})	N	Mean	SD
P. nattereri - Stage 25 Gosner	0.05	2	0.05	0.001
	0.1	2	0.09	0.001
	1	17	0.98	0.032
H. pardalis - Stage 25 Gosner	0.05	2	0.05	0.002
	0.1	5	0.10	0.003
	1	17	1.04	0.025
	1*	13	1.01	0.020
P. nattereri - 35-41 and 42-44 stages Gosner	0.05	3	0.05	0.001
	0.1	3	0.10	0.006
	1	11	1.01	0.040

*This category represents the group of large *H. pardalis* tadpoles at stage 25 Gosner (212.4 - 2350.1 mg), which was compared only with the other group of *H. pardalis* tadpoles tested at $\Delta T = 1$°C min^{-1} (i.e. the group of small individuals, 23.5 - 83.1 mg). See the text for more details.

Table S2. Summary of the body mass data (mg) within each experimental group. N is the sample size. Min and Max are the minimum and maximum values of body mass, respectively. CV is the coefficient of variation.

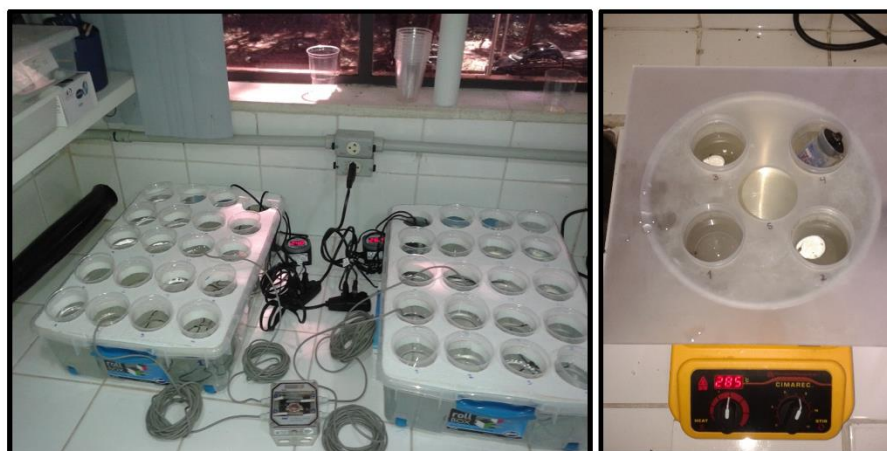| Experimental group	ΔT (°C min^{-1})	N	Mean ± s.d.	Min	Max	CV (%)
P. nattereri - Stage 25 Gosner	0.05	34	21.6 ± 6.9	6.7	35.4	32.2
	0.1	36	22.3 ± 6.5	10.5	38.8	29.2
	1	51	21.4 ± 6.5	9.2	41.7	30.4
H. pardalis - Stage 25 Gosner	0.05	20	53.6 ± 20.6	27.1	101.5	38.4
	0.1	46	56.5 ± 19.6	21.5	97.6	34.7
	1	54	45.2 ± 14.9	22.5	83.1	32.9
	1*	36	688.1 ± 506.4	212.4	2350.1	73.6
P. nattereri - 35-41 stages Gosner	0.05	15	262.6 ± 79.9	147.4	436.4	30.4
	0.1	15	254.8 ± 59.7	172.0	347.0	23.4
	1	15	250.5 ± 98.9	134.2	471.7	39.5
P. nattereri - 42-44 stages Gosner	0.05	11	278.7 ± 48.5	219.8	388.1	17.4
	0.1	10	262.4 ± 67.5	168.0	420.0	25.7
	1	10	259.8 ± 84.3	176.4	462.6	32.5

*As in Table S1.

Figure S1. Experimental settings for the upper thermal limits tests. Three temperature-controlled baths were used in order to measure the CT_{max} of the tadpoles, targeting three different experimental heating rates ($\Delta T = 0.05°C$ min^{-1} and $\Delta T = 0.1°C$ min^{-1} in the larger baths, *left*, and $\Delta T = 1°C$ min^{-1} in the smaller bath, *right*. See the text for more explanation). The thermal conditions experienced by the individuals were measured with temperature dataloggers in all experimental tests.

Figure S2. Example for the estimation of the heating rates (ΔT) in the experimental tests for upper thermal limits. Temperature data were registered with dataloggers set at one-minute interval. A) Experimental test set at $\Delta T = 0.05°C$ min^{-1}. B) Experimental test set at $\Delta T = 0.1°C$ min^{-1}. C) Experimental test set at $\Delta T = 1°C$ min^{-1}. The regression slopes indicate the corresponding experimental ΔT.
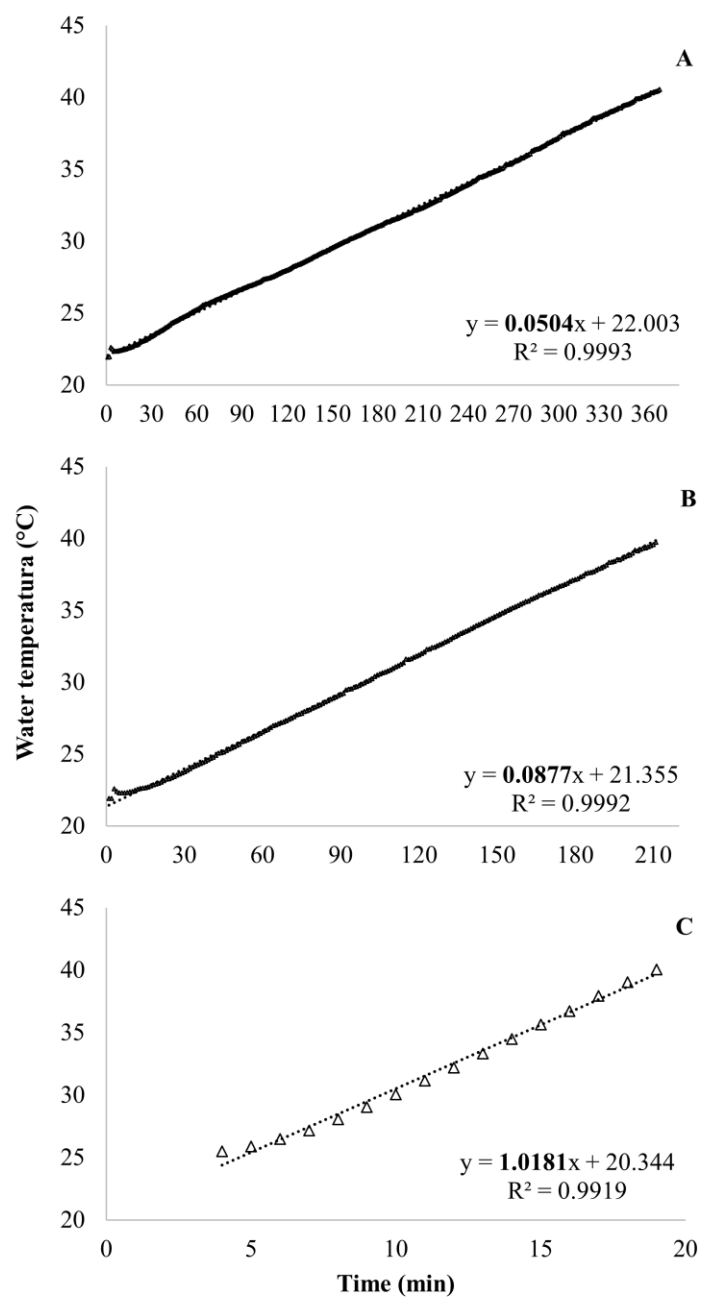
Figure S3. Frequency distributions of CT_{max} at three different ΔT for tadpoles of *Physalaemus nattereri* (A) and *Hypsiboas pardalis* (B), following acclimation to 23°C.

Figure S4. Relationship between the CT_{max} and the Time of resistance for *Physalaemus nattereri* tadpoles (Stage 25, Gosner) tested at $\Delta T = 0.05°C$ min^{-1} (Spearman Rank Correlation, $r_s = 0.96$, $P < 0.01$). Data points clustered into two groups: a first group of tadpoles with CT_{max} ranging from 40.8 - 41.8°C, and a second group with CT_{max} ranging from 42.3 - 43.4°C. See the text for more explanation.

Supplementary Figure S1.

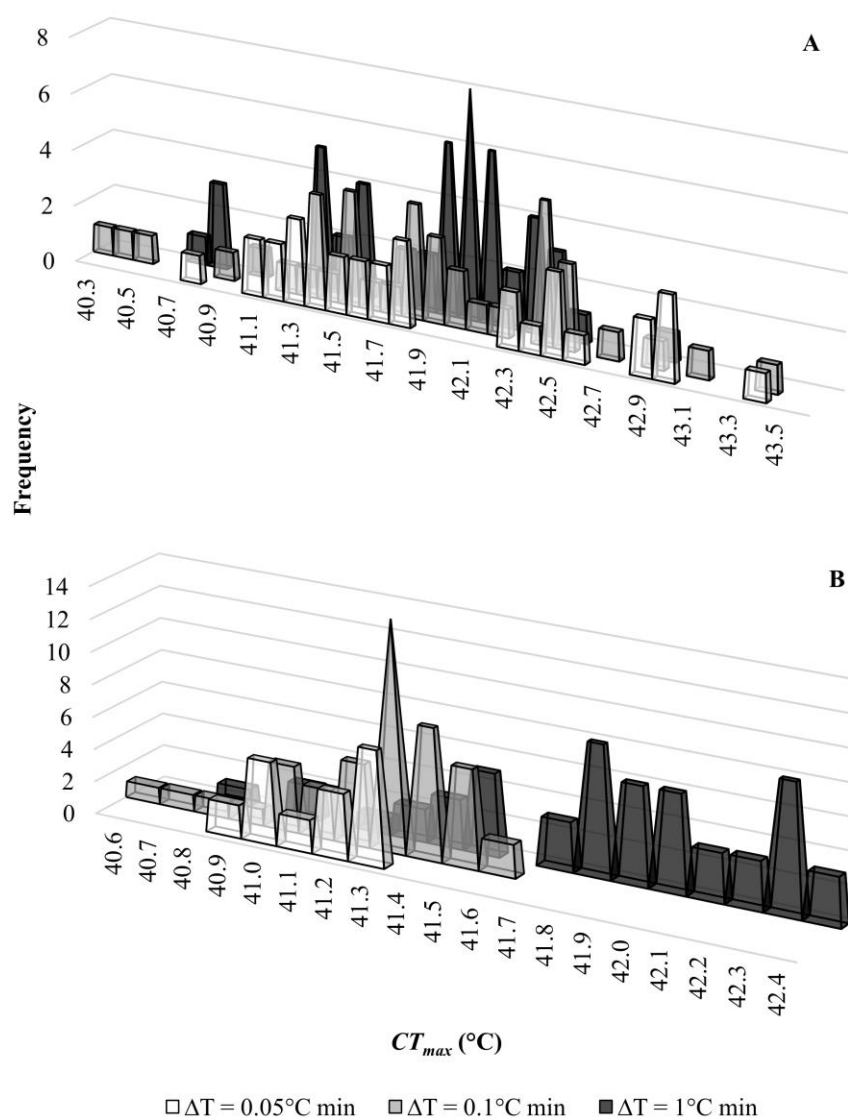Supplementary Figure S2.

$y = \mathbf{0.0504}x + 22.003$
$R^2 = 0.9993$

A

$y = \mathbf{0.0877}x + 21.355$
$R^2 = 0.9992$

B

$y = \mathbf{1.0181}x + 20.344$
$R^2 = 0.9919$

C

Water temperatura (°C)

Time (min)

Supplementary Figure S3.

CT_{max} (°C)

□ ΔT = 0.05°C min   ▨ ΔT = 0.1°C min   ■ ΔT = 1°C min

Supplementary Figure S4.
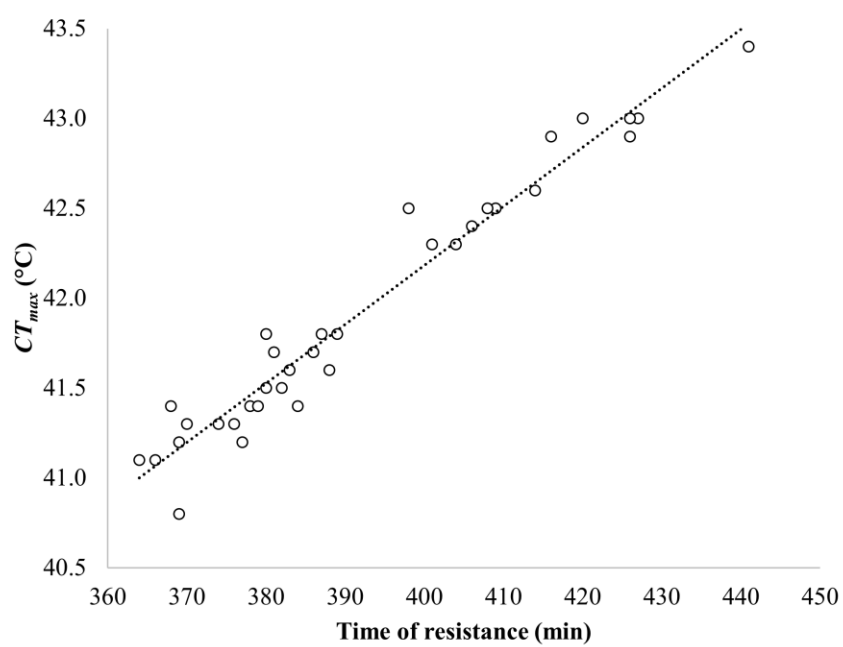
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