Thermal tolerance across latitudinal and altitudinal gradients in tadpoles

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Memoria presentada por Luis Miguel Gutiérrez Pesquera para optar al grado de Doctor en Biología Molecular y Biomedicina: línea de Fisiología Animal, por la Universidad de Sevilla.

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“Ignorance more frequently begets confidence than does knowledge”

—Charles Darwin, 1809 - 1882—

"Temperature is not just a property of life; it is a property of matter.

Nothing escapes its control"

—Michael J. Angilletta Jr.—

"Es necesario explorar sistemáticamente el azar"

—Graffiti de mayo del 68—

A mis padres

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INDICE

INTRODUCCIÓN GENERAL

LOS ANFIBIOS ANTE EL CAMBIO CLIMÁTICO

La temperatura es una condición abiótica fundamental que afecta a todos los niveles de organización biológica determinando, por ejemplo, desde la velocidad a la que ocurren las reacciones químicas en el interior de la célula (Hochachka & Somero, 2002) hasta las interacciones ecológicas en el seno de las comunidades (Dunson & Travis, 1991). El cambio global es probablemente una de las mayores amenazas para el mantenimiento de la biodiversidad (Sala *et al.*, 2000), particularmente para los anfibios, los cuales presentan una serie de características biológicas que los hacen especialmente vulnerables a los cambios ambientales, como su condición de ectotermos, una piel permeable y un ciclo vital complejo que les lleva a ocupar secuencialmente ambientes acuáticos (durante su fase larvaria) y terrestres (durante su vida adulta) (Wells, 2007). Se calcula que aproximadamente el 41% de las especies conocidas de anfibios de las que se tiene información, presentan algún tipo de riesgo para su conservación (Hoffmann *et al.*, 2010). Entre las causas principales de su declive podríamos citar la destrucción y fragmentación de sus hábitats, el aumento de la radiación ultravioleta, la contaminación de las aguas, la introducción de especies alóctonas, su explotación directa y el aumento de las temperaturas (Beebee & Griffiths, 2005). Especial mención merece el caso de la mortalidad causada por el hongo patógeno *Batrachochytrium dendrobatidis* y otras enfermedades emergentes similares (Daszak *et al.*, 1999; Bosch *et al.*, 2007; Seimon *et al.*, 2007). Todos estos factores, lejos de actuar aisladamente,

presentan sinergias que exacerban sus efectos negativos sobre las poblaciones de anfibios (Brook *et al.*, 2008).

El calentamiento global ha provocado ya un incremento confirmado de 0.85 °C de la temperatura media del planeta durante el periodo 1880 a 2012, previéndose mayores aumentos de las temperaturas medias ambientales (de entre 2 a 4 °C para finales de siglo en función del escenario de emisiones) y la frecuencia de eventos térmicos extremos en el futuro como las olas de calor (Pachauri *et al.*, 2014). El calentamiento climático de origen antropogénico está ocurriendo a una velocidad sin parangón comparado con los cambios climáticos ocurridos en el pasado, y ha tenido ya consecuencias ecológicas detectables para la biodiversidad en general y para los anfibios en particular (Southward *et al.*, 1995; Walther *et al.*, 2002; Root *et al.*, 2003) causando, por ejemplo, el desplazamiento del área de distribución de las especies, cambios fenológicos o desajustes en las interacciones biológicas (Parmesan, 2006). Frente a este desafío, las especies cuentan básicamente con tres estrategias para contrarrestar los efectos del calentamiento (Bradshaw & Holzapfel, 2006; Chown *et al.*, 2010; Somero, 2010): 1) modificar su rango de distribución siguiendo sus necesidades térmicas en las nuevas condiciones generadas por el cambio climático; 2) cambios plásticos, reversibles o no, de su fisiología térmica (plasticidad fenotípica) (Calosi *et al.*, 2008a); y/ó 3) la evolución adaptativa de sus características fisiológicas mediante cambio en las frecuencias génicas por selección natural (Huey & Kingsolver, 1993). Este último supuesto puede resultar más improbable debido a la rápida naturaleza del cambio y a factores intrínsecos de las especies como por ejemplo la cantidad de varianza genética aditiva en el seno de las poblaciones (Hoffmann *et al.*, 2013).

Tratar de predecir y, cuando sea posible, mitigar los efectos negativos del cambio climático, es un reto importante y urgente que actualmente afrontan los

biólogos. Ello requiere, al menos, conocer cómo varía la fisiología de los organismos a través del *espacio* y del *tiempo* y, en segundo lugar, determinar el rango de temperaturas a la que las especies están expuestas en su ambiente. En otras palabras, la susceptibilidad de una población, especie o comunidad de recibir un impacto negativo debido al cambio climático dependerá de la combinación de dos factores: la *sensibilidad* de los organismos, controlada por factores intrínsecos como pueden ser los límites de tolerancia térmica fisiológicos (CT_{max} y CT_{min} , Fig. 1); y la cantidad y variación en la *exposición* a factores ambientales extrínsecos potencialmente estresantes, como pueden ser las temperaturas extremas (Deutsch *et al.*, 2008; Williams *et al.*, 2008; Duarte *et al.*, 2012; Foden *et al.*, 2013).

EL ESTUDIO DE LA FISIOLOGÍA TÉRMICA EN ANFIBIOS

La fisiología térmica hunde sus raíces en los trabajos pioneros que empezaron a publicarse a mediados del siglo pasado (Cowles & Bogert, 1944; Brett, 1956; Janzen, 1967; Brattstrom, 1968) y que sentaron sus bases conceptuales y experimentales. Dado que los ectotermos dependen fuertemente de las temperaturas ambientales para llevar a cabo sus principales funciones vitales (Angilletta *et al.*, 2002), uno de los objetivos de la fisiología térmica es describir cómo la temperatura corporal afecta al desempeño de dichas funciones. Típicamente, las curvas de desempeño (*termal performance curves*, TPC) son funciones no lineales y asimétricas, caracterizadas por un aumento progresivo en la eficacia de la función fisiológica a medida que aumenta la temperatura y un rápido declive en el desempeño cuando la temperatura corporal supera un determinado valor óptimo (Fig. 1). Algunos ejemplos de variables biológicas comúnmente analizadas incluyen: la locomoción, el crecimiento, la fecundidad, el desarrollo o la supervivencia de los organismos.

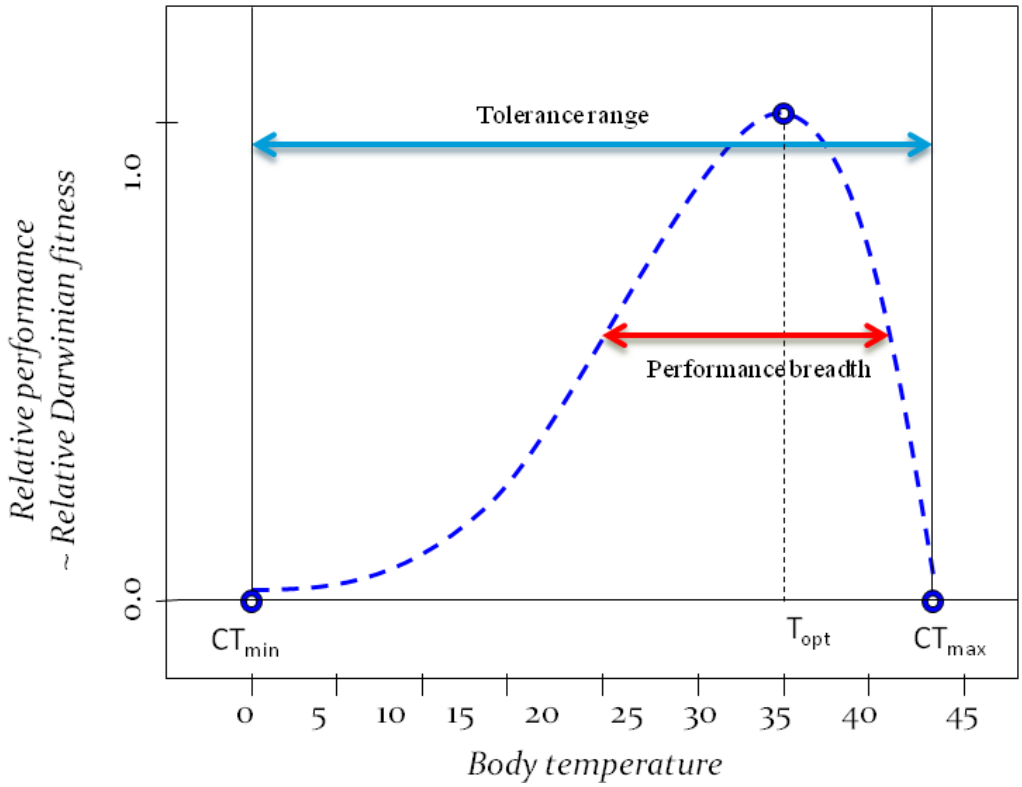

Figura 1. Ejemplo de una curva de desempeño en ectotermos indicando sus principales parámetros: temperatura óptima de desempeño (T_{op}), temperatura crítica mínima (CT_{min}), temperatura crítica máxima (CT_{max}), rango de tolerancia (*tolerance range*) y amplitud de desempeño (*performance breadth*).

Los límites de tolerancia son parámetros claves de las curvas de desempeño que delimitan el rango de temperaturas corporales dentro del cual pueden tener lugar una función biológica determinada (Huey & Stevenson, 1979). Básicamente, existen dos aproximaciones diferentes para el estudio de las tolerancias térmicas. La primera, denominada *método estático* se basa en la determinación de la temperatura letal (LT_{50}), por su equivalencia al LD_{50} empleado en farmacología, mediante la exposición de un determinado número de individuos a una serie *constante* de temperaturas experimentales. De esta forma, el UTL (*upper thermal limit*) y el LTL (*lower thermal limit*) se calculan a partir del tiempo de exposición a las que un 50% de la población sobrevive a las distintas temperaturas experimentales. El *método dinámico* se basa en la

estimación de los límites críticos térmicos (CT_{max} y CT_{min}) mediante la exposición de los individuos a una determinada tasa de calentamiento/enfriamiento hasta que el animal muestra una secuencia de respuestas que incluye la pérdida de equilibrio, el inicio de espasmos musculares, la inmovilidad y finalmente la muerte. La temperatura crítica máxima (CT_{max}) y, de forma análoga, la temperatura crítica mínima (CT_{min}) se definieron inicialmente en lagartos basándose en la capacidad de movimiento, como la temperatura en la que la locomoción se vuelve descoordinada y los animales son incapaces de escapar a situaciones que, en condiciones naturales, los conduciría a la muerte (Cowles & Bogert, 1944; Lutterschmidt & Hutchison, 1997a), por ejemplo, al no poder conseguir su alimento o escapar de sus depredadores. El método dinámico presenta una serie de ventajas respecto al LT_{50}. En primer lugar, al obtenerse un valor de tolerancia para cada animal analizado, generalmente requiere de unos tamaños muestrales muy inferiores. Esto puede resultar especialmente relevante cuando es necesario trabajar con individuos adultos o poblaciones de especies amenazadas o difíciles de obtener. En segundo lugar, los procedimientos estáticos implican generalmente la muerte de los especímenes. Finalmente, el método dinámico es más parecido a los fenómenos de calentamiento/enfriamiento naturales pudiendo usarse también para simular condiciones más cercanas a la realidad.

Los límites críticos de tolerancia térmica de las especies pueden variar en función de diversos factores metodológicos, como por ejemplo la tasa de calentamiento/enfriamiento escogida (Mitchell & Hoffmann, 2010), la duración del periodo previo de aclimatación (Brattstrom, 1968), la temperatura de aclimatación (Loeschcke & Sorensen, 2005), el fotoperiodo (Floyd, 1985), la selección del punto final (Lutterschmidt & Hutchison, 1997b; Chown *et al.*, 2009b) y el estado de desarrollo

(Floyd, 1983). Las tasas de calentamiento tradicionalmente empleadas oscilan entre los 0.5 a 1.5 °C min^{-1}. En general, se recomienda el uso de tasas rápidas para evitar procesos de aclimatación o *hardening* durante la duración del ensayo que puedan interferir en la interpretación del resultado (Lutterschmidt & Hutchison, 1997a). También se ha visto, por ejemplo, que las estimas de heredabilidad dan valores cercanos a cero cuando se emplean tasas lentas de calentamiento (Rezende *et al.*, 2011). No obstante, bajo determinadas circunstancias el uso de tasas más lentas, ecológicamente relevantes, puede ser recomendable cuando se trata de simular condiciones naturales. Aunque originalmente el criterio utilizado para designar el punto final de las pruebas de tolerancia térmica fue la inmovilidad (Cowles & Bogert, 1944), trabajos posteriores han demostrado la idoneidad de utilizar el comienzo de los espasmos musculares como la respuesta a observar para marcar el final del ensayo (Lutterschmidt & Hutchison, 1997b). La preferencia por el empleo de una respuesta u otra también ha variado en función del grupo analizado. Así en peces, por ejemplo, abundan los estudios que utilizan la pérdida de equilibrio (*loss of righting response*) (Becker & Genoway, 1979; Díaz & Bückle, 1999; Beitinger *et al.*, 2000). El empleo de la inmovilidad implica, no obstante, una serie de ventajas respecto a los otros criterios. En primer lugar, resulta fácilmente observable y medible en diferentes tipos de organismos. En segundo lugar, encontramos la misma respuesta cuando los organismos se exponen tanto a bajas como altas temperaturas, haciendo ambos límites térmicos comparables bajo el mismo criterio.

Finalmente, el historial de temperaturas a las que un organismo ha estado expuesto influye de manera determinante en los resultados obtenidos en sus tolerancias térmicas (Brattstrom, 1970). Es por ello que cualquier estudio de tolerancia implica someter a los organismos a un periodo previo de *aclimatación* a una determinada

temperatura de control que hagan comparables entre sí los resultados obtenidos para diferentes individuos, poblaciones o especies (Angilletta, 2009).

El análisis de las tolerancias térmicas en anfibios fue desarrollado inicialmente por Hutchison (1961) en salamandras y por Brattstrom (1968) en anuros. Tradicionalmente, la base funcional de los límites de tolerancia térmica ha sido examinada desde la perspectiva de la fisiología comparada, mediante estudios intra- o inter-específicos basados en una o pocas especies (Feder & Hofmann, 1999; Hochachka & Somero, 2002; Podrabsky & Somero, 2004). Probablemente impulsados por la necesidad de comprender los efectos provocados por la amenaza del cambio climático, se está produciendo un renovado interés por la fisiología térmica y los límites de tolerancia (Huey *et al.*, 2009; Kearney & Porter, 2009; Somero, 2010). En particular, el examen de hipótesis macroecológicas y reglas ecogeográficas incluye, implícita o explícitamente, dentro de sus predicciones el análisis de los rangos térmicos y su plasticidad (Janzen, 1967; Brattstrom, 1968; Rapoport, 1975; Gaston *et al.*, 2009).

Dos aspectos íntimamente relacionados con la capacidad de las especies para resistir al cambio climático son la variación en el espacio de los límites de tolerancia y su plasticidad. Diversas hipótesis macrofiosiológicas (Gaston *et al.*, 2009) han sido planteadas analizando estas cuestiones. Basándose en la menor variabilidad térmica estacional en las zonas tropicales, Janzen (1967) propuso que los «pasos de montaña eran más altos en los trópicos». Janzen observó que, en las regiones tropicales, el rango de temperaturas experimentado en una localidad situada a una determinada altitud es más o menos constante a lo largo del año, de manera que existe escaso solapamiento térmico entre localidades situadas a diferentes alturas. Así, las especies que habitan en diferentes pisos altitudinales habrían evolucionado como especialistas con márgenes

estrechos de tolerancia y con capacidad reducida para desplazarse a localidades situadas a diferentes alturas. Por el contrario, en las latitudes templadas, con una marcada variación estacional, existe un mayor grado de solapamiento térmico entre localidades situadas a diferente altitud. Es decir, la misma temperatura que encontramos en las zonas bajas para una época del año puede ser encontrada en las zonas altas en una época distinta, lo que permitiría a los organismos adaptados a estas condiciones desplazarse a lo largo del gradiente. La hipótesis de Janzen fue más tarde generalizada por Stevens (1989) en su *hipótesis de variabilidad climática* como un mecanismo que explique el patrón descrito por la *regla de Rapoport*, el incremento del rango de distribución de las especies con la latitud (Rapoport, 1975). Esta hipótesis básicamente sostiene que existe una relación positiva entre el rango de tolerancia térmica y el grado de variabilidad climática experimentado por los taxones al aumentar la latitud (Bozinovic *et al.*, 2011b).

Relacionado con el riesgo de sufrir extinciones debido al estrés térmico, ha sido propuesto también que las especies con una capacidad de tolerancia mayor podrían tener a su vez una menor capacidad de aclimatación (Stillman, 2004). En conjunto, una exposición a temperaturas ambientales más altas, unos reducidos márgenes de seguridad y una supuesta menor capacidad de aclimatación hacen a las especies tropicales especialmente vulnerables al calentamiento climático (Stillman, 2003; Deutsch *et al.*, 2008; Duarte *et al.*, 2012).

En un extenso trabajo, Brattstrom (1968) analizó el potencial de aclimatación de anfibios adultos y propuso que la capacidad para ajustar su fisiología térmica sería sólo ventajosa en las latitudes sometidas a una mayor variabilidad térmica (hipótesis de la aclimatación beneficiosa) (Kingsolver & Huey, 1998; Wilson & Franklin, 2002). Por lo tanto, basándonos en la mayor estabilidad térmica de los trópicos, deberíamos esperar

una mayor capacidad de aclimatación en las especies templadas que en las tropicales. La hipótesis de Brattstrom predice un aumento en la plasticidad de la tolerancia térmica con la latitud relacionada con en el concomitante incremento de la estacionalidad en las temperaturas. Estas dos hipótesis constituyen el eje central en torno al cual gira el presente trabajo.

ESTRUCTURA DE LA TESIS DOCTORAL

Del mismo modo que la temperatura y los efectos del cambio climático varían con el espacio y el tiempo, esta tesis doctoral se centra en conocer la variación en los límites fisiológicos a diferentes escalas espacio-temporales así como caracterizar el ambiente térmico al que actualmente se encuentran expuestos las poblaciones y especies de anfibios analizadas. Ello nos permitirá, en primer lugar, aportar información básica para tratar de predecir las consecuencias del calentamiento global, y en segundo lugar, poner a prueba algunas de las principales hipótesis macrofisiológicas expuestas anteriormente. Así, los capítulos 1, 2 y 3 se centran en la variabilidad espacial de los límites de tolerancia térmicos en anfibios (a lo largo de gradientes altitudinales y latitudinales). Dado que existe cierta controversia acerca del grado de variación encontrada entre poblaciones de la misma especie y entre especies distintas, los capítulos 1 y 3 adoptan un enfoque interespecífico mientras que el capítulo 2 se ocupa de analizar la variabilidad intraspecífica encontrada para una serie de poblaciones de *Rana temporaria* a lo largo de un gradiente climático altitudinal. Los capítulos 4, 5 y 6 se centran en analizar el cambio en los límites de tolerancia en el tiempo ya sea a través de procesos de aclimatación (capítulos 4 y 5) o el cambio en las tolerancias térmicas en diferentes estadios de desarrollo (capítulo 6).

La primera parte de la tesis abarca el análisis de la variación espacial en los límites de tolerancia térmicos. Así:

En el capítulo 1: **"Testing the climate variability hypothesis in thermal tolerance limits of tropical and temperate tadpoles"**, analizamos la variación latitudinal en los CT_{max}/CT_{min} de 47 sp. de anfibios, durante su estapa larvaria, comparando dos comunidades procedentes de un ambiente tropical, Brasil (Bahía), y un ambiente templado, la Península Ibérica y Norte de África. En este trabajo ponemos a prueba la *hipótesis de variabilidad climática* en renacuajos, analizando además la relevancia de la información térmica a escalas micro y macroclimáticas como predictores de estos parámetros que delimitan las curvas de *performance* de los ectotermos.

En el capítulo 2: **"Can breeding phenology and plasticity prevent local adaptation in thermal tolerance?"**, estudiamos la variación altitudinal de los límites de tolerancia térmica en once poblaciones de *Rana temporaria*, al tiempo que caracterizamos su ambiente térmico teniendo en cuenta la fenología de la especie, y discutimos los aspectos que pueden estar afectando al grado de diferenciación en las tolerancias térmicas mostrado entre las poblaciones, así como si esta diferenciación puede responder a procesos de adaptación local.

En el capítulo 3: **"Altitudinal variation of the critical thermal limits in 20 species of tadpoles in the tropical Andes"**, realizamos una comparación altitudinal interespecífica en los límites térmicos de 20 especies de anuros andinos, durante su etapa larvaria, y una valoración preliminar del riesgo de extinción de estas especies, basándonos en las estimas de su tolerancia al calentamiento. Del mismo modo que en el capítulo 1, comprobaremos además como la hipótesis de variabilidad climática puede explicar los resultados obtenidos.

La segunda parte de la tesis trata de los efectos sobre las tolerancias térmicas de la variación temporal, mediante cambios en las temperaturas de aclimatación y el estadio de desarrollo ontogénico:

En el capítulo 4: **"Acclimation of critical thermal limits in temperate and tropical tadpoles"**, analizamos las supuestas diferencias en el potencial de aclimatación de anfibios tropicales y templados, sometidos a temperaturas constantes en el laboratorio (hipótesis de Brattstrom, Brattstrom (1968)).

En el capítulo 5: **"The effect of constant vs fluctuating acclimation on critical thermal limits in three temperate tadpoles"**, comparamos los efectos que la aclimatación puede tener sobre las tolerancia térmica de tres especies de anfibios de la Península Ibérica cuando se usan a) unas temperaturas de aclimatación constantes, o b) simulando unas condiciones similares a las que pueden ocurrir en la naturaleza, mediante el empleo de aclimataciones con temperaturas diarias fluctuantes, con valores medios equivalentes a los tratamientos de aclimatación a temperatura constante.

En el capítulo 6: **"Ontogenetic shifts in thermal tolerances in temperate anurans. Does metamorphosis impose a thermal constraint that may affect vulnerability to global warming?"**, analizamos los cambios que tienen lugar a lo largo del desarrollo ontogenético (larvas, metamórficos y juveniles) en la tolerancia térmica de los individuos de seis especies de anuros procedentes de Marruecos y la Península Ibérica.

Finalmente la sección de conclusiones generales recoge y resume los principales resultados obtenidos en esta tesis doctoral.

CHAPTER 1

“Testing the climate variability hypothesis in thermal tolerance limits of tropical and temperate tadpoles”

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Testing the climate variability hypothesis in thermal tolerance limits of tropical and temperate tadpoles

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ABSTRACT

The climate variability hypothesis (CVH) states that a positive relationship may exist between the breadth of thermal tolerance range and the level of climatic variability experienced by taxa with increasing latitude, especially in terrestrial ectotherms. Under CVH, we expected to find a correspondence between both thermal tolerance limits (CT_{max} and CT_{min}), ambient extreme temperature and the range sizes of species. We examined the validity of these predictions in a lowland tropical (Bahia, Brazil) and a temperate tadpole assemblage (Iberian Peninsula and North Africa). We employed phylogenetic eigenvector regression (PVR) and Pagel's lambda to analyze phylogenetic signals in CT_{max} and CT_{min}. We used phylogenetic regression analyses (PGLS) to test the relationships between thermal limits, range size and temperature predictors (measured at the macroscale and microhabitat levels) and phy-ANOVA to compare both the physiological traits and thermal regimen in both tropical and temperate assemblages. We documented moderate-to-strong phylogenetic signal in both heat and cold tolerance. Temperate-zone tadpoles had broader thermal tolerances than tropical ones. Thermal tolerance range was correlated with range sizes and was explained by seasonal thermal

range predictors at the global scale. Both macro- and microclimate temperature variables provided the best predictive multivariate models of thermal limits at the global scale. Microclimatic predictors, however, were the main determinants of CT_{max} and CT_{min} variation at the local level of tropical and temperate communities, respectively. Thermal tolerance range increases with latitude in tadpoles due to the higher increase in cold tolerance in temperate tadpoles. At the global scale, both macro and microenvironment thermal information were reliable predictors of critical thermal limits and thermal tolerance range, as CVH predicts. However, thermal limits were best predicted by temperatures of the micro-habitat at the regional level, thus suggesting that physiological thermal boundaries may be governed by thermal selection.

Keywords CT_{max}, CT_{min}, latitudinal variation, macrophysiology, Rapoport's rule, amphibians

INTRODUCTION

Temperature has pervasive effects on life, affecting organisms at different scales ranging from the molecular level, i.e. rates of chemical reaction (Hochachka & Somero, 2002), to the ecosystem level, i.e. ecological interactions (Dunson & Travis, 1991). The study of physiological tolerance ranges, especially thermal ones, is essential to understand many aspects of the biology of organisms. This includes the conditions that define their fundamental niches, geographic distribution and evolutionary dynamics, which in turn allows us to determine their vulnerability to climate change (Hutchinson, 1981; Kearney & Porter, 2009; Soberón & Nakamura, 2009; Peterson, 2011; Duarte *et al*., 2012; Seebacher & Franklin, 2012).

The interest in the study of the evolution and functionality of thermal limits has led to the development of numerous biogeographical hypotheses (Gaston *et al*., 2009). In a seminal paper, Janzen (1967) suggested that mountains would act as physiological barriers to the dispersal of tropical species. This prediction originally derived from the observation that annual climatic variation is relatively lower in the tropics than at higher latitudes, so that tropical organisms should have narrower physiological thermal breadths compared to organisms in temperate zones. This idea was later on generalized with the climate variability hypothesis (CVH) proposed by Stevens as a potential explanation for Rapoport's rule (Stevens, 1989). Climate variability hypothesis predicts that temperate species would be able to inhabit larger geographic ranges than tropical ones (Stevens, 1989) suggesting a positive relationship between thermal tolerance range and the level of climatic variability experienced by taxa with increasing latitude (Bozinovic *et al*., 2011b).

The CVH hypothesis has been tested for insects (Addo-Bediako *et al*., 2000; Calosi *et al*., 2010; Sheldon & Tewksbury, 2014), bivalves (Compton *et al*., 2007), lizards (Van Berkum, 1986; Cruz et al., 2005; Clusella-Trullas et al., 2011) and amphibians during their adult terrestrial stage (Snyder & Weathers, 1975; Ghalambor *et al.*, 2006, both reanalyzing the original data set by Brattstrom, 1968; John-Alder *et al.*, 1988). Two recent global-scope publications have synthesized our knowledge on this topic: Sunday *et al*. (2014) for aquatic and terrestrial ectothermic animals and Araújo *et al*. (2013) for terrestrial ectotherms, including plants. Most of these studies suggest that increasing thermal tolerance in extratropical ectotherms basically depends on the extreme reduction in CT_{min} with latitude, attributed to a rapid decline in minimum ambient temperatures. Contrarily, the variation in maximum ambient temperature seems to be relatively independent of latitude, making CT_{max} geographically invariant (Gaston *et al*., 2009). This asymmetric pattern appears to be a general trend (Addo-Bediako *et al*., 2000; Huey *et al*., 2009; Clusella-Trullas *et al*., 2011; Araújo *et al*., 2013) and has even been considered a rule in terrestrial ectotherms (Brett's rule, Gaston *et al*., 2009). However, quite interestingly, this trend is not supported by aquatic marine organisms which exhibit similar reductions in both thermal limits with latitude (Sunday *et al*., 2011). This asymmetry may have important biogeographical implications. For instance, the ability of species to evolve physiological tolerance to cold may condition the migration of lowland tropical lineages to temperate zones or highland tropical climates (e.g Wiens *et al*., 2006).

In this study we test the CVH prediction of an asymmetrical latitudinal variation for thermal tolerance limits and explore their relationship with climatic determinants in two larval amphibian assemblages from different climatic regimes: a temperate and a lowland tropical assemblage. Aquatic larval amphibians are an ideal model to analyze

thermal adaptations. Despite being capable of regulating their body temperatures (Hutchison & Dupré, 1992), tadpoles may be limited in their search for favorable microhabitats, for instances when trapped in shallow heated ponds where tadpoles are obligated to act as thermoconformers (Balogová & Gvoždík, 2015). Thus, their physiological resistances may have been adjusted to the local thermal extremes experienced in their ponds through thermal selection.

It is expected that local thermal conditions drive the evolution of thermal tolerance limits which ultimately result in thermal adaptations (Angilletta, 2009; Bozinovic et al., 2011b). Assuming that natural selection governs the evolution of thermal resistances, an asymmetric geographical variation in thermal tolerance limits will emerge if two predictions are met. First, there exists a correspondence between either thermal tolerance limits with ambient extreme temperature and, second, that such correspondence is steeper for cold tolerance and weaker for warming tolerance (Kellermann *et al*., 2012b; Araújo *et al*., 2013; Sunday *et al*., 2014). One way to determine whether thermal selection is prone to drive thermal tolerance limits is by assessing the risk of populations and species to suffer heat or cold impacts. An operative metric to estimate the eventual occurrence of heat shock is warming tolerance —i.e. the difference between CT_{max} and Tmax— (Deutsch *et al*., 2008; Duarte *et al*., 2012). Similarly, we can define cooling tolerance as the risk to suffer cold shocks and measure it as the difference between the minimum recorded temperature, Tmin, and the CT_{min}.

Previous approaches dealing with CVH predictions may be weakened by some concerns. These rely on meta-analyses employing heterogeneous and non-standardized procedures that may yield biased estimates of thermal tolerances (Rezende *et al*., 2011), and also depend on limited phylogenetic controls. Additionally, most approaches dealing with the relationship between thermal resistance traits and environmental

variables rely on macroclimatic information from large-scale databases or repositories —e.g. WorldClim— (Clusella-Trullas *et al*., 2011; Kellermann *et al*., 2012a, 2012b; Araújo *et al*., 2013). These may not fairly reflect the operative temperatures to which ectotherms are exposed, especially when dealing with extreme temperatures where thermoregulation may play a significant role (Sunday *et al*., 2014). Therefore, macroclimate estimators should provide lower explanatory power than microclimate data, especially when analyzing thermotolerance on an individually-based local area (Angilletta *et al*., 2002; Graae *et al*., 2012; Navas *et al*., 2013; Potter *et al*., 2013). In order to better characterize environmental thermal conditions, we gathered temperature data from both macroclimate (WorldClim) and microclimate —water temperature measurements— during the time tadpoles are present in the ponds.

A key factor to determine species vulnerability to climate change is their capacity to adapt their thermal tolerance limits through plastic and/or evolutionary responses (Williams *et al*., 2008; Hoffmann *et al*., 2013). As a result of climate niche conservatism, however, closely related species may display similar physiological resemblance and limited adaptive potential (Losos, 2008). A way to test the evolutionary constraints of physiological traits is the study of phylogenetic signal through comparative methods. For example, CT_{min} has been considered a more labile evolutionary trait than CT_{max} in ectotherms (Araújo *et al*., 2013; Grigg & Buckley, 2013). Here we use a statistically-based phylogenetic eigenvector regression method (Diniz-Filho *et al*., 1998) to infer the relative weight of heredity versus adaptation on thermal limits.

We aim to answer the following related questions and predictions:

i) Did temperate tadpoles evolve wider thermal and geographical ranges through increasing cold tolerance as expected from CVH (Janzen, 1967) and Rapoport's rule

(Stevens, 1989)? ii) What set of thermal predictors —macro or micro— have a higher explanatory power to describe the variation in thermal limits and thermal range of species at both large latitudinal and regional ranges? iii) Given the latitudinal variation of seasonality, should we expect stronger correlations between lower thermal limits and minimum environmental temperature than for upper thermal resistance and maximum temperature?

MATERIAL AND METHODS

Amphibian tadpole surveys and estimates of thermal tolerance

Thermal tolerance was determined for tadpoles of 47 species. We examined 27 tropical species in the state of Bahía (Brazil) between latitudes 13^{o}-15^{o} S from November 2011 to January 2012. The studied species occupy different aquatic environments with contrasting temperatures and thermal variability (see Table S2, Appendix S2 in Annexe 1) such as forest ponds and streams in the Atlantic Forest (Mata Atlântica) and open forest ponds both in the semi-arid Caatinga (Contendas do Sincorá FLONA reserve), and in deforested areas of Mata Atlântica. We also included two species inhabiting phytotelmata associated to open forest Atlantic coast restingas (Table S2 in Appendix S2, Annexe 1). We studied 20 temperate species in the Iberian Peninsula and northern Morocco ranging from latitude 29^{o}-43^{o} N, from 2011-2013 (Table S2, Appendix S2, Annexe 1). These temperate species show different breeding phenologies and also inhabit a variety of thermal environments from mountain ponds, streams, Mediterranean lowland ponds to semi-arid sub-Saharan species (Table S2, Appendix S2, Annexe 1). All examined tadpoles were collected in their natural ponds and transported to one of the two reference laboratories at particular study sites (Brazil,

UESC, Ilhéus, November 2011 to January 2012; Spain, EBD-CSIC, Sevilla, 2011-2013). During all the experiments, larvae were maintained at a similar and constant room temperature of 20 °C, with a natural photoperiod ca. 12:12 light: dark. Tadpoles were placed in plastic containers with similar densities, for a minimum of three-four days. This acclimation period was chosen as previous research in adult amphibians revealed that between 2-3 days was the time required to stabilize CT_{max} and CT_{min} after a large change in acclimation temperature, such as field and laboratory environments (Brattstrom, 1968). Larvae were tested individually between 25 and 38 Gosner stages (Gosner, 1960). Tadpoles over 38 Gosner stage were excluded because near the metamorphic climax they tend to have reduced thermal tolerances (Floyd, 1983).

Whenever possible, we used a minimum sample of 16 individual tadpole replicates per species and thermal tolerance limit —although for some species, mostly tropical, the available number of individuals examined was lower, Table S1, Appendix S2, Annexe 1—. Each tadpole was weighed immediately before the beginning of the test. Both thermal tolerance limits were determined using the Hutchison's dynamic method (Lutterschmidt & Hutchison, 1997a) in which each animal was exposed to a constant heating/cooling rate (ΔT=0.25 °C min^{-1}) until an end point is attained. The end-point was signalled for both thermal limits as the point at which the tadpoles become motionless and fail to respond to external stimuli by prodding 10 consecutive hits applied each two seconds with a wooden stick. Each tested tadpole was placed individually in 100 mL containers with dechlorinated tap water in a refrigerated heating bath of 15 L (HUBER K15-cc-NR) at a start temperature of 20 °C (temperature of acclimation). Because of the small size of tadpoles we assumed that body temperature was equivalent to water temperature (Lutterschmidt & Hutchison, 1997b) and then

CT_{max} and CT_{min} were recorded as the water temperature beside the tadpole measured with a Miller & Weber quick-recording thermometer (to the 0.1 °C). After a tolerance limit was determined, we immediately transferred tadpoles to water at the acclimation temperature (20 °C) to allow for recovery, after which their Gosner stage was registered. Tadpole survival was verified a few minutes and 24 hours after the end of the heating/cooling assays. Each individual was tested only once. To ensure that lethal temperature was not exceeded only those individuals who recovered 24 h after the test were included in subsequent analyses. Although we only examined a single population for each species, we assumed that response variation among species is larger than variation within species (cf. Klok & Chown, 2003). In a large multi-species comparison, any intraspecific difference in CT_{max} and CT_{min} would be overwhelmed by the high number of species and the great diversity of thermal environments examined. To analytically address this potential issue, we explored inter-populational variation for those species in our dataset for which we had available data. Analyses on different populations of both temperate and tropical species (*Rana temporaria*, *Bufo bufo*, *Epidalea calamita*, *Pelodytes ibericus*, *Rana arvalis* and *Dendropsophus novaisi*) revealed that intraspecific variability in thermal limits is rather limited (around 1 °C) compared to the variation observed among species from the same region (5.9 °C and 5.3 °C for CT_{max} in temperate and tropical communities, respectively, and 4.7 °C and 5.7 °C for CT_{min} in temperate and tropical communities respectively) or differences between temperate-tropical regions (8.3 ºC for CT_{max} and 11.8 ºC for CT_{min}). Finally, we calculate the thermal tolerance range as the difference between CT_{max} and CT_{min} for each species.

Climatic determinants of thermal tolerance limits

To obtain macroclimatic determinants of thermal tolerance limits we used digital distribution maps from IUCN (IUCN and Nature Serve, 2006) and processed them in QGIS to obtain the following geographical variables for each species: geographic range size, longitude and latitude of the centroid, maximum and minimum values of latitude, and latitudinal range. Latitudinal and longitudinal coordinates of population samples were taken to extract and summarize the climatic information from WorldClim layers —30 ” spatial resolutions; records from 1950 to 2000 (Hijmans *et al*., 2005)—. Although WorldClim climatic variables correspond to air measurements, previous studies have shown that air temperatures correlate well with water temperature in streams and lakes (Livingstone & Lotter, 1998; Webb *et al*., 2003), being reliable predictors used in biogeographical and conservation analyses with aquatic organisms such as continental fishes (Chessman, 2013) and tadpoles (Gerick *et al*., 2014). We selected the following four macroclimatic variables: TMAX (Maximum of the Average monthly maximum temperature); TMIN (Minimum of the Average monthly minimum temperature); TMEAN (Monthly Mean Temperature) and Temperature SEASONAL RANGE (TMAX-TMIN). For each species only the months in which tadpoles were present in the ponds were taken into account to perform the analyses —for further details see Appendix S1 and S2 in Annexe 1—.

To assess the explanatory power of microclimatic habitat conditions on thermal tolerance limits, we included temperature data obtained for each sample points directly from dataloggers (HOBO pendant) that were deployed at the bottom of diverse aquatic microenvironments such as ponds, streams and phytotelmata. Temperature was recorded every 15 min. We analyzed mean (tmean), maximum (tmax) and minimum (tmin) daily temperatures, average daily range and seasonal range (tmax-tmin) from

each water body for the period in which tadpoles were present in the aquatic habitat. The number of sampling days ranged from 30-497 days (see Table S2 Appendix S2). To evaluate the risk of each species suffering cold and heat acute shocks, we estimated both warming and cooling tolerances (sensu Deutsch *et al*., 2008; Duarte *et al*., 2012) as (CT_{max} - tmax) and (tmin - CT_{min}), respectively.

Statistical analyses

Phylogeny

We constructed a phylogenetic tree containing the 47 studied species from Pyron & Wiens (2011). For 16 species not included in this phylogeny, we used the position of a known sister-taxon that does appear in the phylogeny. In some cases the position of certain species within the genus could not be resolved and therefore appear as polytomies (Fig. S1, Appendix S3, Annexe 1).

Phylogenetic signal on thermal limits

We tested for phylogenetic signal in each trait calculating Pagel's λ —with the fitContinuous function in the R package GEIGER (Harmon *et al*., 2008)—, which assumes a Brownian motion model of character evolution. Apart from this model-based metric, we also incorporated a statistical-based phylogenetic method that makes no assumptions on the underlying evolutionary model to evaluate the weight of heredity versus adaptation in the critical thermal limits in our data. Phylogenetic eigenvector Regression (PVR) (Diniz-Filho *et al*., 1998), allowed us to partition the components of variance attributable to ecological (S) and phylogenetic effects (P). We used the function PVRdecomp from the PVR package (Santos *et al*., 2013) in R (see Appendix S1, Annexe 1).

Phylogenetic comparative analyses

To evaluate the correlations between thermal physiology variables (CT_{max}, CT_{min} and thermal tolerance range), and geographical variables (area and latitudinal range of species distribution, latitude of the centroid distribution and maximum latitude), we used phylogenetic generalized least squares (PGLS) analyses under a Brownian motion model of evolution using the R package CAIC (Orme *et al*., 2009). We also used PGLS to test for correlation between CT_{max} and CT_{min}. A multiple regression approach by PGLS, was used to examine relationships between the physiology variables and the environmental temperature variables (both for WorldClim and dataloggers). We selected the best model using the lowest Akaike information criterion (AIC) (Burnham & Anderson, 2002). See Table S7-S9, Appendix S2 in Anexxe 1 for a summary of the models used.

To analyze the influence of climatic region (tropical versus temperate) on the variation of both physiological traits —CT_{max}, CT_{min}, Thermal tolerance range, and Warming and Cooling Tolerances— and environmental temperature variables, we used phylogenetic ANOVA models (phy-ANOVA) in CAIC. Previous analyses revealed that tadpole mass did not explain significant CT_{max} nor CT_{min} variation and, hence, was not included in the models. We employed ANCOVA to compare differences in the regression slopes of CT_{max}/CT_{min} with latitude using the basic R package (see Appendix S1, Annexe 1).

RESULTS

Climate Variability and Rapoport's Hypothesis in tadpoles: comparison of thermal tolerance between temperate and tropical geographical areas

The two methods we used to estimate phylogenetic signal (Pagel's lambda and PVR) identified strong phylogenetic effects on CT_{min} ($\lambda = 0.97$, P component = 0.80), whereas CT_{max} showed only moderate levels of phylogenetic signal ($\lambda = 0.67$, P = 0.50) (Table S5, Appendix S2, Annexe 1). Therefore, PGLS was used in all subsequent analyses to control for the phylogenetic structure in the data.

Temperate species showed on average greater thermal tolerance ranges than tropical species (phy-ANOVA, $P < 0.01$) (Fig. 1 and Table 1). Broader thermal breadths in temperate species were mainly attributed to their higher resistance to cold (phy-ANOVA, $P < 0.001$, Table 1), which offsets their lower values for CT_{max} (phy-ANOVA, $P < 0.01$, Table 1). Both CT_{max} and CT_{min} decreased with latitude (Fig. 2a, Table S4 Appendix S2, Annexe 1), but the slope of the variation in CT_{min} (-0.20 ± 0.02) was significantly steeper than that of CT_{max} (-0.08 ± 0.02) when both are compared against species' latitudinal centroids (ANCOVA: CT [min/max] x Latitude, $F_{1,86} = 7.38$, $P < 0.01$). However, neither CT_{max} nor CT_{min} were correlated with latitude when each geographical area was analyzed separately.

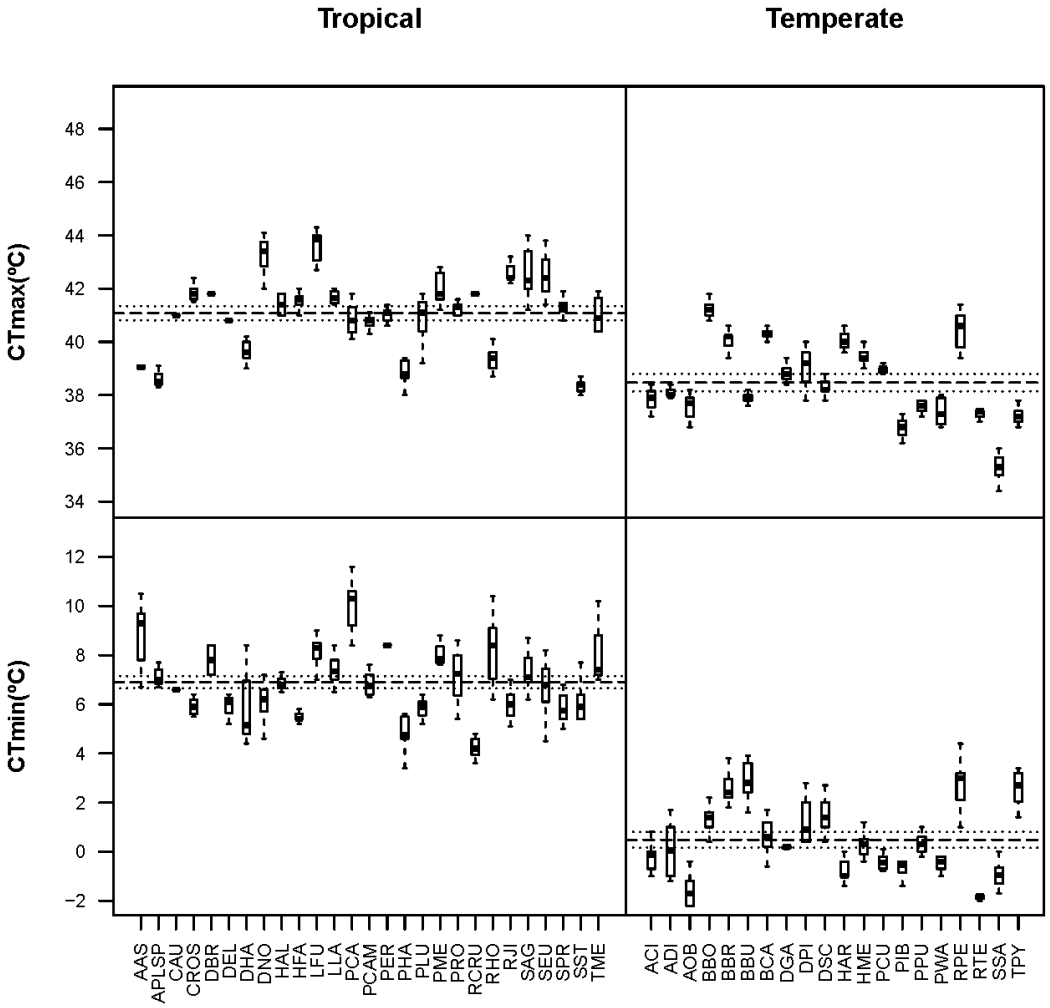

Figure 1. Upper and lower critical thermal limits (CTmax an CTmin) for tropical and temperate tadpole assemblages. Dashed lines indicate the average CTmax/CTmin and 95% CI, respectively, for the overall geographical area. Species codes, ordered alphabetically within region, see Table S1.

Analyses performed with combined data (i.e. tropical plus temperate) showed that thermal tolerance range was correlated with species maximum latitude, latitudinal range and range size (log transformed) (PGLS, $F_{1,36}$ = 43.85, P <0.01; $F_{1,36}$ = 123.4, P <0.01, $F_{1,36}$ = 148.8, P <0.01, respectively) (Table S4, Appendix S2 in Anexxe 1, Fig. 2b and Fig. 3). However, when analyses were restricted to a particular area, only the

temperate assemblage marginally exhibited the trend with latitudinal range (PGLS, $F_{1,15}$ = 4.19, P = 0.06).

Table 1. Phylogenetic ANOVAs comparing physiological traits (CTmax, CTmin, tolerance range, warming and cooling tolerance) and climatic predictors (macroclimate WorldClim estimators in caps and microenvironment predictors from dataloggers, in lowercase), considering only the reproductive period where tadpoles are present, between tropical and temperate tadpole assemblages. TMAX (maximum of the average monthly maximum temperature); TMIN (minimum of the average monthly minimum temperature); TMEAN (mean of the average monthly temperature); SEASONAL RANGE (TMAX-TMIN); tmax (maximum temperature); tmin (minimum temperature); tmean (mean temperature); daily seasonal range (mean seasonal daily tmax-tmin); seasonal range (seasonal absolute tmax - absolute tmin).

	Temperate			Tropical				
Physiological traits	N	$\bar{X}$	SD	N	$\bar{X}$	SD	F-value	Phy-p
1. CT_{max}	20	38.5	1.46	27	41.10	1.37	39.371	0.01
2. CT_{min}	20	0.50	1.43	27	6.90	1.29	260.51	0.001
3. Tolerance Range	20	38.0	1.57	27	34.2	1.89	54.028	0.004
4. Warming Tolerance (WT)	17	9.24	1.41	27	10.06	1.22	0.185	0.89
5. Cooling Tolerance (CT)	17	3.10	0.31	27	13.15	0.35	389.72	0.001
Macroclimatic temperatures (WorldClim)								
1.TMAX	20	25.12	5.345	27	29.51	0.45	18.277	0.11
2.TMIN	20	3.99	3.39	27	18.71	1.19	435.75	0.001
3.TMEAN	20	12.83	2.29	27	24.3	0.5	645.10	0.001
4. SEASONAL RANGE	20	21.1	6.03	27	10.8	1.5	73.64	0.001
Microclimatic temperatures (Dataloggers)								
1.tmax	17	30.04	6.27	27	31.03	7.18	0.22	0.891
2.tmin	17	3.35	2.17	27	20.03	1.29	1024.7	0.001
3.tmean	17	12.52	2.57	27	24.07	1.91	291.1	0.001
4.daily seasonal range	17	4.74	1.94	27	2.48	2.70	8.9092	0.315
5.seasonal range	17	26.69	6.12	27	11.00	7.45	52.801	0.01

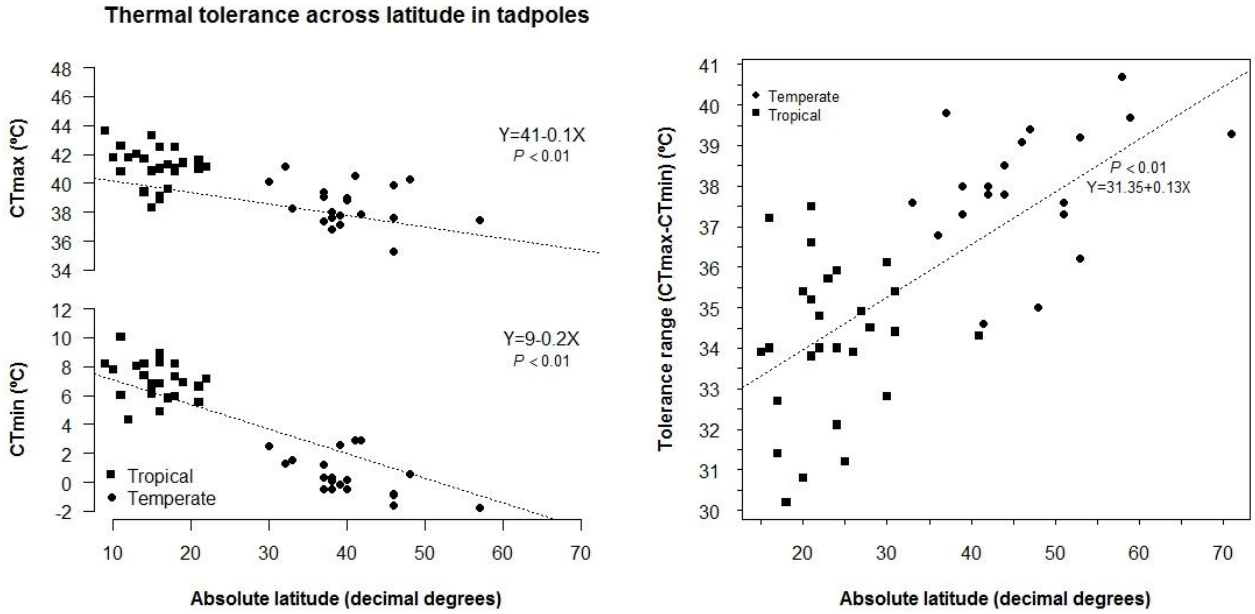

Figure 2. (Left) CTmax and CTmin variation in tadpoles with the absolute latitude (ºN or ºS) of the centroid´s distribution. The change is steeper for CTmin than CTmax (ANCOVA p<0.01). (Right) Phylogenetic generalized least squares for tolerance range and absolute latitude of the poleward limit of distribution.

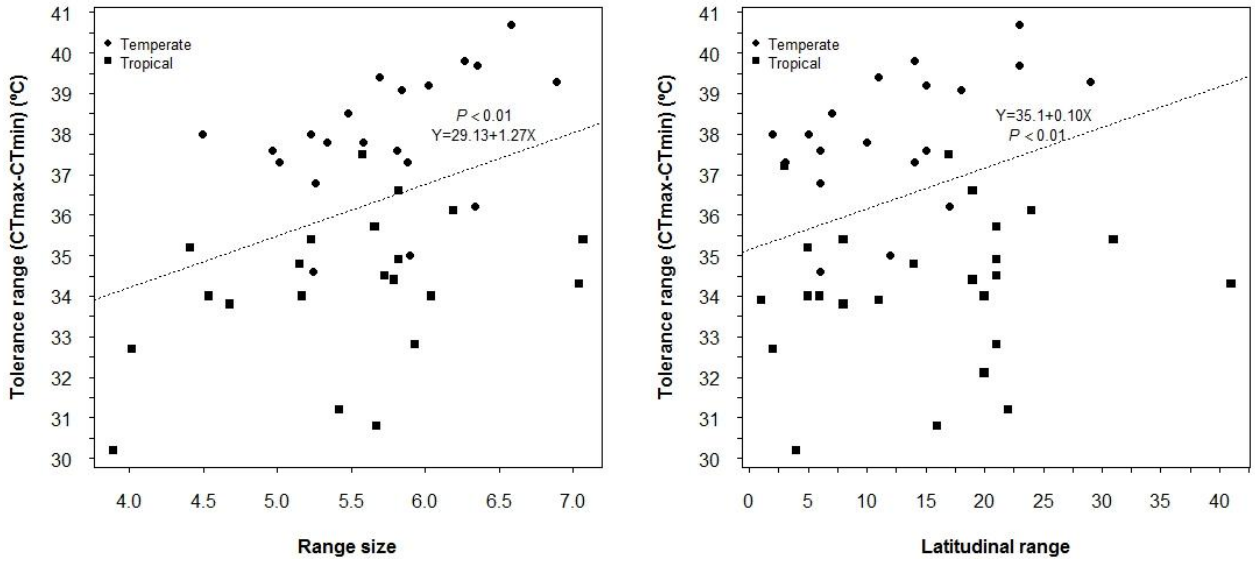

Figure 3. Phylogenetic generalized least squares for thermal range and the log-transformed of the area distribution (range size) (a) and latitudinal range (b) of the analyzed tadpoles.

Table 2. Multiple regression models (PGLS) used to predict physiological variables (CTmax, CTmin and tolerance range) with environmental thermal information at different spatial scales in tadpoles. For each variable in the model we include their standardized coefficients to evaluate the relative importance of each one. Climatic predictors (macroclimate WorldClim estimators in caps and microenvironment predictors from dataloggers, in lowercase), considering only the period where tadpoles were present. TMAX (maximum of the average monthly maximum temperature); TMIN (minimum of the average monthly minimum temperature); TMEAN (mean of the average monthly temperature); SEASONAL RANGE (TMAX-TMIN); tmax (maximum temperature); tmin (minimum temperature); tmean (mean temperature); daily range (mean seasonal daily tmax-tmin); seasonal range (seasonal absolute tmax - absolute tmin). λ = Pagel's lambda, AIC = Akaike Information Criterion, w_i = Akaike weight. Akaike weights were calculated from AIC values of all models for each trait. Models are ranked by AIC and only models with ΔAIC < 2 are presented. See Appendix S2 in Annexe 1 for a complete list of analyzed models.

		Predictors in the model													F		
Physiological trait	Region	TMAX	tmax	TMIN	tmin	TMEAN	tmean	SEASONAL RANGE	seasonal range	daily range	λ	AIC	ΔAIC	w_i	d.f	Value	P
CTmax	Global		0.487			1.111					0.73	113.1	0	0.16	2,35	18.56	<0.01
			0.659			0.945			-0.248		0.7	114.9	2	0.06	3,34	12.29	<0.01
	Temperate							0.950		0.724	0.85	53.1	0	0.07	2,14	4.50	0.03
								1.053	0.753		0.97	53.6	0.5	0.05	2,14	7.28	<0.01
								1.056	-0.910	1.397	0.79	53.7	0.6	0.05	3,13	3.14	0.06
	Tropical		0.653								0	54.2	0	0.07	1,19	13.07	<0.01
									0.905		0	54.7	0.5	0.06	1,19	12.34	<0.01
								2.159	1.395	-0.383	0	55.0	0.8	0.05	3,17	5.47	<0.01
CTmin	Global			1.628	1.867						0	128.2	0	0.1	2,35	153.80	<0.01
				1.304	1.322		0.881				0	128.3	0.1	0.1	3,34	105.10	<0.01
				1.453			1.746		-0.463		0	128.8	0.6	0.08	3,34	103.70	<0.01
	Temperate				3.204						0	55.2	0	0.07	1,15	7.69	0.01
					2.120		1.008				0	55.6	0.4	0.06	2,14	4.63	0.03
					3.228	0.931					0	55.7	0.5	0.06	2,14	4.54	0.03
	Tropical						3.334	-5.638	-0.738		0.77	69.0	0	0.06	3,17	4.02	0.02
				4.501			1.381				0.76	69.2	0.2	0.06	2,18	4.78	0.02
						7.894					0.7	69.8	0.8	0.04	1,19	6.43	0.02
Tolerance Range	Global							1.155	1.205		0	158.1	0	0.48	2,35	17.85	<0.01
								1.024	1.566	-0.389	0	159.4	1.4	0.24	3,34	11.93	<0.01
	Temperate								0.622		0.22	65	0	0.27	1,15	0.92	0.35
	Tropical							5.166	0.749		0.43	86.5	0	0.28	2,18	2.58	0.1

Macro and microclimatic predictors for the thermal tolerance traits and estimates of warming and cooling tolerances

Maximum ambient temperatures did not differ between geographical areas (in both macroclimatic and microclimatic approaches) (Table 1), (see also Fig. S3a, Appendix S3, Annexe 1). Mean and minimum temperature (for both macro and microenvironmental scales) were significantly lower for the temperate assemblage (phy-ANOVA, P <0.001, Table 1, Fig. S3b, Appendix S3, Annexe 1). Although average daily range did not differ between regions (Table 1), both SEASONAL RANGE (from macroclimate) and seasonal range (microclimate) was significantly higher for temperate latitudes (phy-ANOVA, P <0.01) (Table 1). Multivariate models that combined macro- and microclimatic temperature variables, showed the best predictive power for physiological traits at the extent of whole latitudinal range (Table 2). CT_{max} was best predicted with TMEAN and tmax (CT_{max} ~ TMEAN + tmax, AIC = 113.1, $F_{2.35}$ = 18.56, P <0.01, Table 2). The best model for CT_{min} included minimum temperature from macro- and microclimate (CT_{min} ~ TMIN + tmin, AIC = 128.2, $F_{2.35}$ = 153.80, P <0.01, Table 2). Thermal tolerance range was best predicted by seasonal range (macro and microclimate) (Tolerance Range ~ SEASONAL RANGE + seasonal range, AIC = 158.05, $F_{2.35}$ = 17.85, P <0.01, Table 2). By examining the slopes of climatic predictors of tolerance limits for pooled communities, we found steeper trends for CT_{min} predictors than for CT_{max} (Table 2).

Within-region analyses revealed that thermal limits correlated better with micro- than macroclimatic temperature predictors for the most extreme temperature in each zone: tmax with CT_{max} (tropical assemblage) and tmin with CT_{min} (temperate assemblage). Thermal tolerance range was not correlated with either micro- or

macroclimatic ambient temperature at local scales (Table 2). Similar to the pooled analyses, the slopes of climatic predictors within particular communities were higher for CT_{min} than for CT_{max} (Tables 2, Fig. S3, Appendix S3, Annexe 1). The analyses of warming tolerance between communities revealed that tropical and temperate species did not differ (phy-ANOVA, $F_{1,42}$ = 0.185, P = 0.89, Table 1). However, by distinguishing open and forest pond microenvironments within the tropical assemblage, we found that open forest pond species had lower warming tolerances than both temperate species and tropical forest pond species (phy-ANOVA, $F_{2,41}$ = 28.253, P = 0.01, Table S6, Appendix S2 in Anexxe 1, Fig. S4, Appendix S3 in Anexxe 1). Cooling tolerance was much lower for temperate assemblage species than tropical ones at both microenvironments (phy-ANOVA $F_{2,41}$= 192.53, P < 0.0001; Table S6, Appendix S2, Fig S4, Appendix S3 in Anexxe 1).

DISCUSSION

Thermal tolerance range and the distribution of species: CVH as an explanation for Rapoport's rule

Our overall analyses revealed that temperate species have greater thermal tolerance ranges than tropical ones, thus supporting the climatic variability hypothesis (Janzen, 1967; Stevens, 1989). The observed increase in thermal tolerance range for temperate species compared to tropical ones stems from their greater cold resistance, rather than an equivalent increase in upper tolerances. This asymmetric latitudinal trend in lower and upper thermal resistance limits is concordant with previous analyses for amphibians during their adult terrestrial stage (Snyder & Weathers, 1975; Ghalambor *et al*., 2006), who reanalysed Brattstrom's (1968) dataset, thus suggesting a similar trend for both aquatic and terrestrial amphibian life stages. Similarly, other terrestrial

ectotherms exhibit clear latitudinal clines in CT_{min}, whereas weaker or even no latitudinal variation for CT_{max} (Addo-Bediako *et al.*, 2000; Huey *et al*., 2009; Clusella-Trullas *et al.*, 2011; Sunday *et al.*, 2011; Araújo *et al*., 2013). This pattern is considered a macrophysiological rule in terrestrial ectotherms although it was originally formulated in fishes (Brett, 1956; Gaston *et al*., 2009) and it is argued that the increased thermal tolerance range in temperate species mainly results from higher temperature seasonalities as they move away from the equator. Such seasonality emerges due to the rapid drop of minimum temperatures whereas maxima are relatively independent of latitude (Chown *et al*., 2004; Olalla-Tárraga *et al*., 2011; Whitton *et al*., 2012).

Thermal tolerance range was predicted by thermal variability from both macro- and microclimate estimators, which is consistent with CVH predictions. Although tropical and temperate regions showed a similar daily thermal range at the micro scale, seasonal range (both at macro- and microclimatic levels) was higher for the temperate area. Interestingly, the level of daily thermal variation at the micro scale for the tropical assemblage was twice than that found in the temperate one (CV 108.9 % and 40. 9%, respectively). This reflects the high diversity of breeding habitats of tropical amphibians (Scheffers *et al*., 2013). Within the studied lowland tropical area (where minimum temperatures did not drop below 17ºC), we found habitats of high thermal stability (e.g. ponds and streams located under the forest canopy of Mata Atlântica) with mean daily ranges extending from 0.1 ºC – 1.6 ºC, well below the average range of the temperate zone (4.7 ºC). However, other biomes and microenvironments studied in the tropical region, such as open ponds in Mata Atlântica, Caatinga, and the specialized phytotelmata showed similar or even greater temperature daily ranges to that found in the temperate assemblage (they can reach up to 41.3 ºC, with a daily oscillation of 7.3-10.3 ºC) (Table S2, Appendix S2 in Anexxe 1). In contrast, in the temperate zone,

seasonal range was greater due to the drop of minimum temperatures during autumn-winter in contrast to the higher temperatures reached in spring and early summer. Thus, increased thermal variability in the temperate zone was mainly due to the seasonal component associated to minimum temperature decreases in winter (to 0.4 ºC).

Our overall results controlling for the phylogenetic structure in the data show that thermal tolerance range may explain a significant variability of both range size and latitudinal extent, especially for temperate species. This supports CVH as an explanation for Rapoport's rule. Similar results have been found in adult aquatic insects (Calosi *et al*., 2008b, 2010) and terrestrial ectotherms such as *Liolaemus* lizards (Cruz *et al*., 2005).

Average CT_{max} for temperate species was lower than expected by maximum ambient temperatures, which showed no significant differences between tropical and temperate areas. Interestingly, tropical and temperate species exhibit a parallel trend of CT_{max} with tmax but tropical species have relative higher tolerances (conventional ANOVA, $F_{1,41}$ = 70.28, P <0.01; LS means (mean ± SE, N), Tropical: 40.99ºC ± 0.22ºC, N=27; Temperate: 38.37ºC ± 0.28ºC, N=17, Fig S3, Appendix S3). Two complementary arguments may account for the pattern of lower CT_{max} found for temperate species. The first argument is ecological: while maximum temperature values did not differ between both environments, the timing of ocurrence of heat peaks over the larval season shows differences. High temperatures in the ponds can be reached at any time in the tropics, but the occurrence of maximum temperatures is nonetheless predictable and mainly restricted to late spring and early summer at temperate latitudes. In addition, warming tolerances of the tropical open forest species lay near the lethal threshold in which physiological thermal mechanisms could hardly overcome high temperatures (Fig. S3, S4, Appendix S3 in Annexe 1). Thus, selection on upper thermal

limits is probably more intense for tropical tadpole species, whereas temperate amphibians can temporarily tune their larval period and shift their breeding time to avoid risky heating peaks, which may explain their greater safety margins. Thereby, we found better fitting between CT_{max} and tmax for the tropical species (Table 2 and Fig. S3a, Appendix S3 in Annexe 1). The second explanation may rely on the existence of a physiological trade-off between thermal limits, so that achieving greater cold tolerance has an associated cost in tolerance to high temperatures, as suggested by the positive correlation found between CT_{max} and CT_{min} in the pooled sample. This trade-off has been explained by molecular mechanisms such as changes in the structure of enzymes and other proteins, when adjusted to low or high temperatures; the modification of fluidity and viscosity of the cell membrane through its lipid composition; or oxygen limitation (Angilletta, 2009). Terrestrial and aquatic ectotherms seem to show opposite conclusions regarding this trade-off (Hoffmann *et al*., 2013). While marine species tend to have coupled physiological limits (Portner *et al*., 2006; Sunday *et al*., 2011), such correlation has not been detected in terrestrial species —mainly insects— (Alford *et al*., 2012), but see Anderson *et al.* (2003) for the molecular basis of a trade-off in thermal tolerance in *Drosophila* and an example of evolutionary thermal specialization in *Escherichia coli* (Bennett & Lenski, 1993). Our results are in consonance with those obtained for other aquatic organisms.

Macroclimatic and microclimatic predictors of thermal tolerance

Models combining climatic predictors at both macro and microclimate scales provided the best explanatory power of physiological thermal limits for global comparisons. Interestingly, analyses restricted to a single region concluded that microclimate variables were more predictive for thermal limits (CT_{max} in the tropical

assemblage and CT_{min} in the temperate assemblage), which are presumably under high thermal selection, as the lower values of warming and cooling tolerance may suggest. This implies that the evolution of heat tolerance for tropical and especially cold resistance for temperate tadpoles may be governed by thermal extremes experienced in the pond microenvironments.

Given the closer relationship of latitude with lower tolerance, we predicted a stronger correlation between environmental predictors and CT_{min} than with CT_{max} (Araújo *et al*., 2013). Accordingly, we found weaker correlation coefficients for CT_{max} and temperature variables predictors. As we expected, the best predictor for CT_{min} was minimum temperature both from micro- and macroclimatic data. Our results agree with those previously found for adult amphibians, reptiles, and *Drosophila*, where CT_{min} was related to minimum or mean temperatures (Clusella-Trullas *et al*., 2011; Kellermann *et al*., 2012a; Araújo *et al*., 2013). CT_{max} was correlated, in our study, with both maximum temperatures and mean temperature at the micro- and macroclimatic scale. By contrast, in reptiles, CT_{max} was more related to predictors of temperature variation such as seasonality or diurnal temperature range (Clusella-Trullas *et al*., 2011). In adult amphibians, Araújo *et al*. (2013) found a positive correlation between CT_{max} and Tmax whereas the slope was nearly zero for Tmean. In *Drosophila*, Kellermann *et al*. (2012b) found an interaction between maximum temperatures and precipitation as the best predictor. Therefore, this diversity in climatic predictors of upper thermal resistance limits in different ectotherms may probably be conditioned by the specific physical environment (terrestrial or aerial) to which they are exposed, which also limits their ability to use behavioural compensation (the Bogert effect, Huey *et al*. 2012; Sunday *et al*, 2014), so that they cannot be generalized for all ectotherms.

Phylogenetic considerations

Recent studies have found strong phylogenetic signal in the realized climatic niches of amphibians worldwide (Hof *et al*., 2010; Olalla-Tárraga *et al*., 2011; Gouveia *et al*., 2014). Using taxonomic and phylogenetic analyses, Olalla-Tarraga *et al*. (2011) found that the climatic niche dimension that tends to be most phylogenetically conserved is cold tolerance. Only a few exceptions within each taxon (termed as 'escapee' lineages) have apparently been able to shift their cold tolerance and occupy northern latitudes. Strong phylogenetic conservatism in cold tolerance has previously been reported for hylid frogs as well (Smith *et al*., 2005; Wiens *et al*., 2006). Our results on the fundamental thermal niche for tropical and temperate amphibian tadpoles are in agreement with these findings and showed moderate phylogenetic signal for CT_{max} and strong signal for CT_{min}. Contrastingly, previous work on lizards has shown lower values of phylogenetical signal for CT_{min} (Grigg & Buckley, 2013), although Kellermann *et al*. (2012a) also found a moderate signal for *Drosophila*. Overall, it is often assumed that CT_{max} is an evolutionary conservative trait (Hoffmann *et al*., 2013), whereas CT_{min} tends to be more plastic.

CONCLUDING REMARKS

Previous works have analyzed how macroclimatic conditions determine thermal physiology traits. However, most organisms, especially small ectotherms with limited dispersal ability, do not experience climate on geographical scales much larger than organismal size. Rather, they live under microclimatic conditions, which can be highly heterogeneous and differ from surrounding macroclimates (Potter *et al*., 2013). In this study both macro- and microclimate temperature variables were reliable predictors of thermal limits at the global scale and, as CVH's hypothesis predicts, thermal range scale

with species geographical range. However, physiological limits variation at specific regional community is much better explained by microclimate variables related to the prevalent local most stressful thermal conditions —heat in the tropical and cold in the temperate region, respectively— which suggest both the mismatch between organismal scale and climatic data and that physiological thermal boundaries may be governed by thermal selection operating at the local environment. Further studies are required to establish how the tolerance–habitat condition–macroclimate relationships are interrelated and scales up from physiological parameters to the geographical distribution of the species.

Under current scenarios of climatic change it is expected greater increases in mean environmental temperatures and the frequency of extreme thermal events in the future (Pachauri *et al*., 2014). Given the conservatism of thermal limits, especially upper tolerance (Hoffmann *et al*., 2013), and the low overall plasticity potential of ectotherms (Gunderson & Stillman, 2015), it seems unlikely that amphibians may mitigate climatic changes relying solely on thermal adaptation of critical thermal limits. Upward and poleward migration, tracking suitable temperatures may be a chance for certain ectotherms (Parmesan, 2006), but probably not for organisms with poor dispersal ability such as amphibians (Sinsch, 1991). This limitation is evidenced, for example, by the fact that current distributions of amphibians display high levels of non-equilibrium with current climate (Araújo & Pearson, 2005). New thermal conditions created by climatic change surely will limit southern boundaries of taxa's latitudinal range or even threat population with local extinctions, especially lowland tropical species, as for example those inhabiting the tropical open forest Caatinga. However it could eventually allow the poleward expansion of other species currently limited by low temperatures with the existence of suitable pathways for dispersal (Araújo *et al*., 2006).

CHAPTER 2

“Can breeding phenology and plasticity prevent local adaptation in thermal tolerance?”

Can breeding phenology and plasticity prevent local adaptation in thermal tolerance?

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ABSTRACT

We analyzed vulnerability to climatic change in 11 local populations of tadpoles of *Rana temporaria* across an altitudinal gradient from 40 to 1800 (m.a.s.l.) in the southern limit of its geographical range. The existence of local adaptation in critical thermal maxima and minima (CT_{max} and CT_{min}) was examined through a P_{ST}-F_{ST} comparison using six microsatellite loci as neutral markers in a common garden environment. In addition, we studied plasticity in thermal limits for five populations from low, intermediate and high altitude. Finally, we assessed warming tolerance of populations considering breeding phenology and developmental periods for larvae, and thermal data for air and water measures. Temperature data were gathered from both, macroclimatic repositories (WorldClim) and microclimatic water temperatures using dataloggers deployed in ponds. Although our study revealed significantly differences in critical thermal limits for some populations, this divergence could not be unambiguously attributed to selection. Neither CT_{max} nor CT_{min} were related to altitude or temperature variation. However, our results suggest that phenological shifts through the altitudinal gradient and plasticity may explain the lack of local adaptation for physiological thermal traits.

Keywords: CT_{max}, CT_{min}, altitudinal variation, P_{ST}-F_{ST} comparison, local adaptation, *Rana temporaria*, phenological shifts, plasticity

INTRODUCTION

Climate change is affecting ecosystems worldwide and its impacts have promoted shifts in species' ranges towards the poles and higher altitudes in response to increasing environmental temperatures (Wilson *et al.*, 2005; Parmesan, 2006; Hill *et al.*, 2011; Thomas *et al.*, 2012; Parmesan *et al.*, 2013). The vulnerability of particular organisms will depend on its sensitivity, exposure and potential to adapt to this change (Williams *et al.*, 2008; Foden *et al.*, 2013). In a scenario of global warming, species basically relies on three strategies to cope with this crisis: evolutionary changes in their tolerance limits, thermal acclimation (phenotypic plasticity) and shifts in seasonal timing and geographical ranges (Walther *et al*., 2002; Angilletta, 2009; Hoffmann & Sgrò, 2011). Hence, the study of population variation and plasticity in physiological traits and the characterization of the thermal environment and species phenology can be decisive to understand the ecological and evolutionary impacts of global warming (Garland *et al*., 1991; Somero, 2010; Huey *et al*., 2012), especially for populations inhabiting at the edges of their geographical ranges (Hill *et al.*, 2011).

Critical thermal limits (CT_{max} and CT_{min}) are key parameters of the performance curves in ectothermic animals. These are useful tools to understand the occurrence and evolution of species, and also to determine the vulnerability to directional climate change (Deutsch *et al*., 2008; Duarte *et al.*, 2012; Gutiérrez-Pesquera *et al.*, 2016, Chapter 1). Geographical variation in the thermal physiology has been thoroughly described on an intraspecific level in terrestrial and aquatic ectotherms (Klok & Chown, 2003; Fangue *et al.*, 2006; Gaitán-Espitia *et al.*, 2013), particularly in insects (Chown, 2001; Sorensen *et al.*, 2005; Overgaard *et al.*, 2011a; Higgins *et al.*, 2014). This variation of thermal limits in relation with latitude and altitude is associated with environmental thermal heterogeneity (Bozinovic *et al.*, 2011b). Compared to latitudinal gradients, altitudinal clines integrate substantial climatic variation over much shorter geographical distances, and thus altitudinal gradients represent an ideal choice for detecting fine-scale variation in physiological traits and its

evolutionary response to thermal variation (Sorensen *et al.*, 2005; Körner, 2007). Despite clinal variation in thermal tolerance traits is often assumed to be the rule in altitudinal analyses —due basically to the adiabatic change of temperature with elevation— other factors beyond thermal conditions may prevent adaptive differentiation in thermal limits as thermoregulatory behaviour (Hertz & Huey, 1981; Hertz *et al.*, 1983; Huey *et al.*, 2003, 2012; Buckley *et al.*, 2015), phenological changes (Phillimore *et al.*, 2010; Álvarez *et al.*, 2012; Álvarez, 2013), or physiological plasticity (Menke & Claussen, 1982; Ultsch *et al.*, 1999; Price *et al.*, 2003; Chevin *et al.*, 2010; Kolbe *et al.*, 2010; Nyamukondiwa *et al.*, 2010). For example, in the southwestern end of its range, *Rana temporaria* occurs from the coast to over 2200 meters above sea level. Reproductive timing of these populations is strongly conditioned by altitude (Álvarez *et al.*, 2012): in the lowlands (below 600 m), reproduction typically begins in late summer and autumn lasting until early February. By contrast, in mountain populations (above 1200 m), breeding season is constrained by the melt of snow and the availability of water in the ponds, showing an explosive breeding pattern (1-2 wks) from spring to early summer (see Fig. 1). Thus, although thermal environment varies dramatically with altitude, the actual thermal exposure among populations could be buffered by shifts in breeding time.

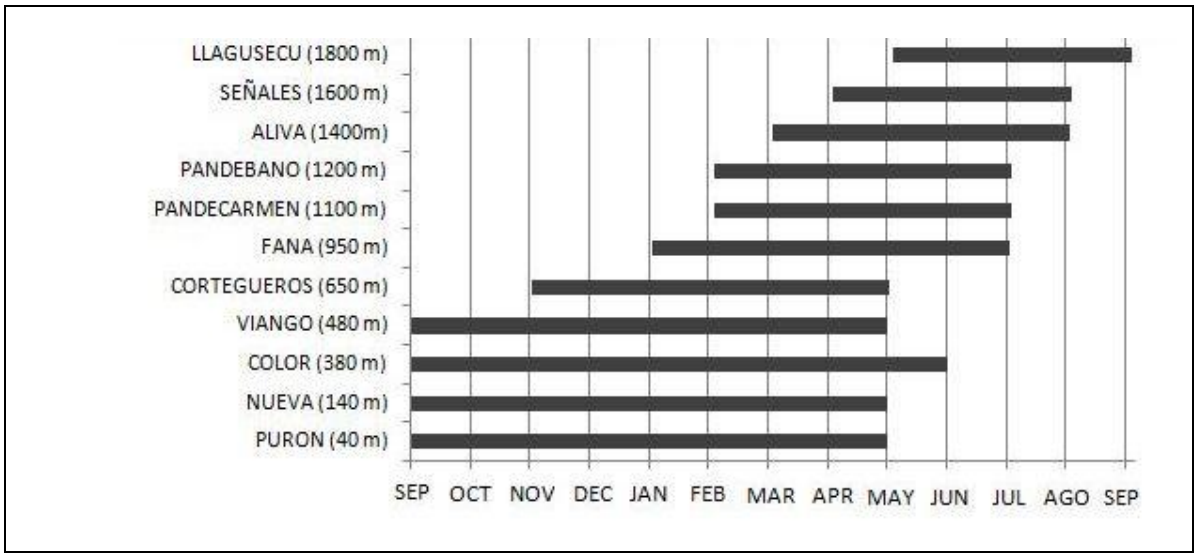


Figure 1. Observed temporal variation in the breeding period of *Rana temporaria* along the altitudinal gradient for the analyzed populations.

Geographical variation in thermal tolerance limits and its acclimation response have been documented in a number of anuran (Brattstrom, 1968, 1970; Miller & Packard, 1974, 1977; Hoppe, 1978) and salamander species (Howard *et al.*, 1983), but geographical differentiation was not evident in other cases (Delson & Whitford, 1973; Zheng & Liu, 2010; Richter-Boix *et al.*, 2015). In *Rana temporaria*, several works have studied the geographical variation in life history traits and countergradient variation (Laugen *et al.*, 2002, 2003; Palo *et al.*, 2003; Lindgren & Laurila, 2009; Phillimore *et al.*, 2010), but information on the geographical variation of thermal physiology is more disperse and scarce (Beattie, 1987; Stahlberg *et al.*, 2008; Sorensen *et al.*, 2009b), especially for altitude gradients (Muir *et al.*, 2014a).

One commonly applied method to detect selective pressures and potential adaptation in any trait is studying the interpopulation quantitative variation observed (Q_{ST} or its phenotypic analogue P_{ST}) compared with a neutral expectation of stochastic variation —as the population differentiation in a putative neutral molecular marker, e.g. F_{ST}—. This approach has previously been used for a number of traits of *Rana temporaria* (Cano *et al*., 2004; Alho *et al*., 2010; Muir *et al*., 2014b). Several caveats about the use of P_{ST} as an approach to Q_{ST} are well known: non-additive genetic variance (epistasis or dominance effects), maternal effects or environmental factors and genotype-environment interaction, can lead to a distorted picture of additive genetic variation when studying only phenotypic variation in natural conditions (Pujol *et al.*, 2008; Brommer, 2011; Leinonen *et al.*, 2013). Nonetheless, here we used a *common garden* approach, where individuals from different populations were raised and analyzed under the same environmental conditions, which allow us to control some of these unwanted effects. Local adaptation in thermal limits (CT_{max} and CT_{min}) was assessed using P_{ST}-F_{ST} comparisons across eleven populations originated along an altitudinal range from 40 m to 1800 m and raised in a common garden environment in the laboratory. We used six microsatellite loci to assess neutral variation. In addition, because the finding of greater variation than expected by chance alone is not enough to conclude local adaptation, we also gathered data for

both, air (WorldClim) and water (dataloggers) temperatures; taking into account the species phenology (breeding and larval periods). These data can help us to identify the selective thermal pressures that might have lead to divergence in studied traits.

Locally adapted populations may not only differ in their basal tolerance but also in their acclimation capacity (Lind *et al.*, 2011; Seebacher *et al.*, 2012). Thus, we analysed potential plasticity in thermal limits for five populations corresponding to the lower (140 m and 650 m), middle (1100 m) and upper (1400 m and 1800 m) levels along the altitudinal gradient. We hypothesized that mountain populations, exposed to wider thermal variation, could show a greater acclimation capacity to overcome a greater thermal stress at seasonal and daily scales (Hoffmann & Watson, 1993; Kingsolver & Huey, 1998).

Populations living at the species' range margins are especially vulnerable to suffer thermal stress and, eventually, extinction by the increase in temperatures and the frequency in extreme thermal events caused by climate change (Colwell *et al.*, 2008; Hill *et al.*, 2011; Pachauri *et al.*, 2014). Thus, we assessed thermal stress across the altitudinal gradient based on estimates of warming tolerance (WT), which is the difference between species' upper critical thermal limit (CT_{max}) and its current maximum environmental temperature (T_{max}) (Deutsch *et al.*, 2008; Duarte *et al.*, 2012; Gutiérrez-Pesquera *et al.*, 2016).

MATERIALS AND METHODS

Study area and sampling

The European common frog (*Rana temporaria*) is one the most widely studied amphibian species. Its distributional range covers almost the entire Euro-Siberian region (from the north of the Iberian Peninsula to the Urals) occupying all sorts of habitats: lowlands, mountains, deciduous and coniferous forests, farmlands and even cities, resulting in a widespread and locally abundant species. The Cantabrian Mountains, in the northwest of the Iberian Peninsula, represent the south-western

limit of distribution of the common frog. In this area, characterized by a marked altitudinal gradient, the species occurs in wetlands from the sea level to altitudes above 2100 m. This sheer slope generates great environmental variability representing an excellent scenario to detect population differentiation at a fine spatial scale as a result of local adaptation to native climatic conditions.

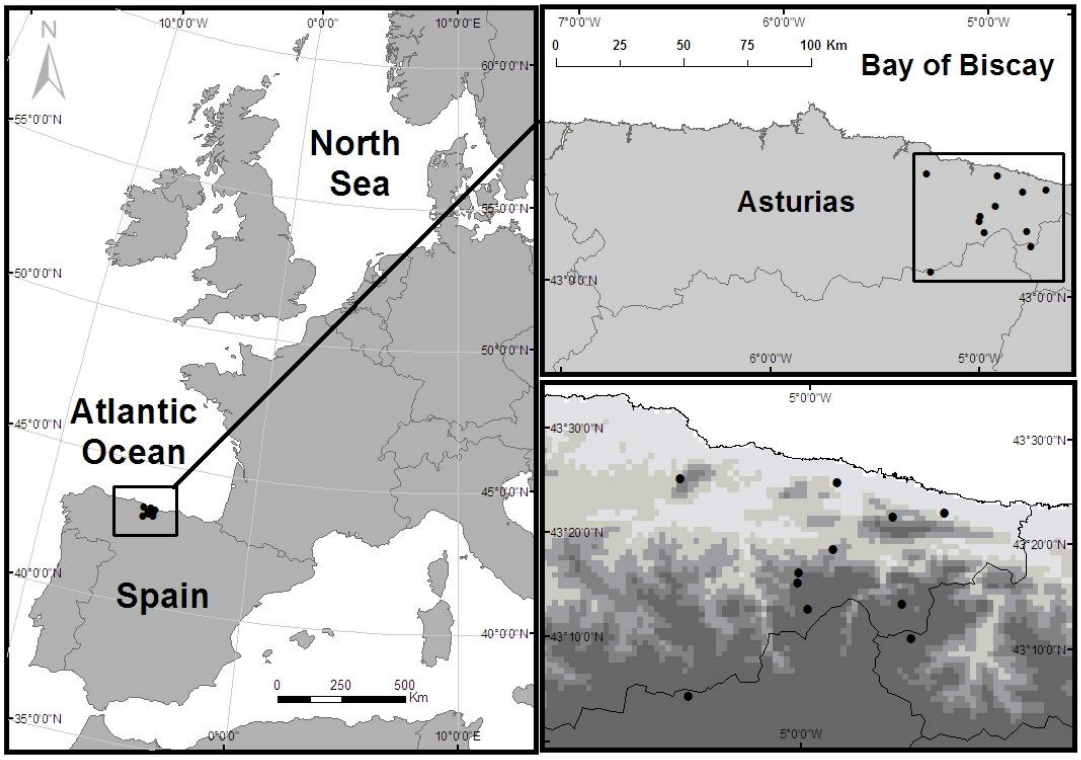


Figure 2. Sample points and study area.

Sampling was carried out between April 2012 and March 2014. We selected a total of 11 sampling sites along an altitudinal transect between 40-1800 m.a.s.l. in Picos de Europa and surrounding areas, covering a relatively reduced geographical space (see Table 1, Annexe 2, and Fig. 2). For each site, we haphazardly collected 5-7 clutches to ensure that obtained individuals could be representative of the population. In order to disentangle environmental and genetics effects in the tolerance thermal limits, all populations were raised under the same environmental conditions in the laboratory (*common garden*). Clutches were transported to one of the two reference laboratories:

Departmento de Organismos y Sistemas, University of Oviedo, or the Doñana Biological Station (EBD-CSIC), Sevilla, and kept in trays or plastic buckets with a similar larval density inside climatic chambers under constant conditions of photoperiod (12:12 L:D) and temperature (15 °C) until tadpoles reached Gosner stage 26.

Temperature data

We used HOBO Pendant Temperature data loggers to obtain a continuous record of water temperature at each sampling site. Temperatures were recorded during the breeding and larval periods every 10-30 min (see Table 2, Annexe 2, for details). For one of the studied populations (Aliva, 1420 m), the datalogger was lost and therefore we used temperature information from a nearby population (Pandébano 1320 m). For each locality, maximum and minimum daily temperatures were calculated for the period of larval presence (see Table 3, Annexe 2).

To characterized thermal environment through the altitudinal gradient, we used the 'extract' function, in the R package RASTER (Hijmans, 2014; R Core Team, 2014), to obtain the climatic information from WorldClim layers (30 " or 1 km^2 spatial resolutions; records from 1950 to 2000 (Hijmans *et al.*, 2005) for the geographical coordinates of each populations: mean (TMEAN), maximum (TMAX) and minimum (TMIN) monthly temperatures). We calculated monthly thermal range as the difference between maximum and minimum temperatures (see Annexe 2, Tables 4-7). To characterize the thermal environment for the study locations, we took into account the variation in breeding period of the species in function of altitude (Fig. 1). Although WorldClim climatic variables correspond to air measurements, previous studies have shown that air temperatures correlate well with water temperature in streams and lakes (Livingstone & Lotter, 1998; Webb *et al.*, 2003), being reliable predictors used in biogeographical and conservation analyses with aquatic organisms such as continental fishes (Chessman, 2013) and tadpoles (Gerick *et al.*, 2014; Gutiérrez-Pesquera *et al.*, 2016).

Experimental procedures

Determination of critical thermal limits

Tadpoles were acclimated to a constant temperature of 20 °C for at least 4 days inside environmental chambers (FitoClima, Aralab). Larvae were individualized in 400 mL plastic glasses and fed *ad libitum*. During this time, the excess food was removed each day to control for bacterial oxygen consumption. Oxygen saturation in the vessels was daily monitored with a laboratory multi-parameter sonde (Multi 340i). Recorded values were always over 60%. For each population, we analyzed 32 larvae within Gosner stages 26-30 (Gosner, 1960). Around half of the larvae, (16) were used to determine CT_{max} and the same number used for CT_{min} estimation. Each tadpole was weighed immediately before the beginning of the test, individualized in 100 mL containers with dechlorinated tap water, and placed inside a refrigerated heating bath of 15 L (HUBER K15-cc-NR) previously stabilized to 20 °C (temperature of acclimation) for five minutes. Both thermal tolerance limits (CT_{max} and CT_{min}) were determined using the Hutchison's dynamic method (Lutterschmidt & Hutchison, 1997a) in which each animal was exposed to a constant heating / cooling rate (ΔT = 0,25 °C min^{-1}) until an end point is attained. The end-point was signalled for both thermal limits as the point at which the tadpoles become motionless and failed to respond to external stimuli by prodding 10 consecutive hits applied each two seconds with a wooden stick. Because of the small size of tadpoles, we assumed that body temperature was equivalent to water temperature (Lutterschmidt & Hutchison, 1997b) and then CT_{max} and CT_{min} were recorded as the water temperature beside the tadpole measured with a Miller & Weber quick-recording thermometer (to the 0.1 °C). After a tolerance limit was determined, we immediately transferred tadpoles to water at 20 °C to allow recovery and their Gosner stage was recorded. Tadpole survival was verified a few minutes and 24 hours after the end of the heating/cooling assays. Each individual was tested only once, and only those individuals who recovered 24 h after the test were included in subsequent analyses.

Phenotypic plasticity in CT_{max}/CT_{min}

We also studied plastic changes in thermal limits to different temperatures in five populations: Nueva (140 m), Cortegueros (650 m), Pandecarmen (1100 m), Aliva (1400 m), and Llagusecu (1800 m) (corresponding to low, intermediate and high altitude of the study area) acclimated to three different constant temperature treatments (6 °C, 13 °C and 27 °C), and photoperiod (12:12 L:D) for a minimum period of four days. A total of 32 tadpoles were randomly assigned to each acclimation temperature and individually maintained in 400 mL plastic vessels. Individuals acclimated to 6 °C were placed inside a different climatic chamber (BINDER) but with similar range of variation (see Table 8, Annexe 2). Tadpoles assigned to the 13 °C temperature treatment, were acclimated in an environmental chamber (FitoClima, Aralab). The 27 °C acclimation treatment was achieved by employing Portable Fluid Heaters with Regulation Adjustment, (patent licensing U201431698) inside a FitoClima chamber set to 20 °C (see Table 8, Annexe 2). CT_{max} and CT_{min} were determined following the above protocol.

Molecular markers

Sampling extended from early September in coastal and lowland populations to late June in the highest locations. In general, samples were collected from breeding adults, unlike tadpoles, to reduce the likelihood of including related individuals. However, for a reduced number of localities, we sampled tadpoles or embryos. In these cases, to reduce the risk of collecting highly related individuals, we obtained eggs or tadpoles from several ponds within each location. We obtained the material for genetic analyses either as buccal swabs (Pidancier *et al*., 2003) or by cutting the tip of a toe on the hind foot. All samples were stored at low temperature in 100% EtOH.

Whole genomic DNA was isolated from samples with either standard Chelex extraction (500 µL of a 10% Chelex solution (Chelex-100, Bio-Rad) incubate with 7 µg Proteinase K at 55 °C

for 60 min and 100 °C for 20 min) or an E.Z.N.A kit of DNA extraction. We selected 11 polymorphic microsatellite loci whose primers were originally developed for *Rana temporaria*. These markers included different degrees of polymorphism (Annexe 2, Table 9, for details see (Choda, 2015).

Four microsatellites (Rtempμ1, Rtempμ2, Rtempμ4, RtμB) were amplified by using 6-20 ng of template DNA, 0.3-0.7 μM of primer, 250 μM of dNTPs (PROMEGA, USA) and Go Taq ® Flexi DNA Polymerase of Promega, USA: 0.5U of Go Taq Polymerase, 2.0-2.5 mM of Mg2+, 2 μl of 5x colorless Go Taq Flexi Buffer and 2 μl of 5x Green Go Taq Flexi Buffer. PCR cycles were starting with 5 min at 94 °C and 40 cycles that consisted of: denaturation, 30 s at 94 °C; annealing, 30 s at 46 or 58 °C (depending on primer); and extending, 30 s at 72 °C. After 40 cycles, twenty minutes at 72 °C were left for elongation. The remainder seven primers were processed with multiplex PCR; we used 6-20 ng of template DNA, 0.3-0.7 μM of primer (RtμH, RtU4, and RtU7 for A-plex, and BFG072, BFG093, BFG183, and BFG241 for B-plex PCR), 5 μl of Qiagen Multiplex PCR Master Mix (Quiagen GmbH, Hilden, Germany). PCR cycles were starting with 15 min at 95 °C and 38 cycles that consisted of: denaturation, 30 s at 94 °C; annealing, 30 s at 55 °C; and extending, 30 s at 72 °C; and finally elongation during 30 min at 60 °C. PCR reactions were performed on Applied Biosystems 2720 Thermal (Applied Biosystems, Inc.), GeneAmp®PCR Systems 9700 (Applied Biosystems, Inc.) and Bio-Rad My Cycler™ (Bio-Rad Laboratories, Inc). PCR products were segregated and detected by capillary electrophoresis on an ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems).

Estimates of neutral genetic and phenotypic divergences (F_{ST} and P_{ST})

We used the MICRO-CHECKER software (Oosterhout *et al*., 2004) to check for genotyping errors and null alleles. We found no evidence for the presence of scoring alleles and large alleles dropout, but MICRO-CHECKER indicated a possibility of null alleles in RtU7, and then this marker

was excluded. In addition, four markers (Rtempμ1, RtU4, RtμH, and RtμB) were discarded due to failed amplifications. The remainder six markers (Rtempμ2, Rtempμ4, BFG072, BFG093, BFG183, and BFG241) were quantifiable for the 11 experimental populations and then used to estimate F_{ST} values.

Exact tests for departure from Hardy-Weinberg equilibrium (HWE) and tests for linkage disequilibrium were performed for each population across all loci, and at each locus individually, using GENEPOP v2.1 (Raymond & Rousset, 1995). Significance was evaluated using the Markov chain methods (Guo & Thompson, 1992) with 5000 dememorizations steps and 1000 batches of 10000 interactions per batch for HWE, and 5000 interactions for linkage disequilibrium tests. To obtain estimates of the degree of genetic differentiation among populations, we calculated F_{ST} values according to Weir and Cockerham (1984) by using FSTAT 2.9.3.2 (Goudet, 2002). Comparison of these values with Q_{ST} (a measure of the amount of genetic variance between populations relative to the total of the genetic variance for a quantitative trait) allow us to discern whether trait differences observed between populations obeys to single stochastic variation due to drift or, alternatively, it may be the result of natural selection (Leinonen *et al*., 2008). Because of information about the genetic variation of quantitative traits is not always available, P_{ST} (a measure of phenotypic divergence of a trait) is often used as a surrogate of Q_{ST} (Brommer, 2011).

When $P_{ST} \approx F_{ST}$, the differences observed between populations may be due solely to genetic drift. If $P_{ST} \geq F_{ST}$, the difference in the feature exceeds that expected by simple neutral variation, and therefore we may infer the action of directional selection. If $P_{ST} \leq F_{ST}$, divergence between populations is lower than expected only by genetic drift, and then this pattern may suggest the action of stabilizing selection between populations.

The P_{ST} estimate is based on measuring a phenotypic characteristic in a set of individuals from a number of populations and it is calculated by the equation (Leinonen *et al.*, 2006):

$$P_{ST} = \frac{c\sigma_B^2}{c\sigma_B^2 + 2h^2\sigma_w^2}$$

where σ_B^2 denotes phenotypic variation between populations, σ_w^2 denotes phenotypic variation within- population, and h^2 is the character heritability —the proportion of phenotypic variance that is because of additive genetic effects—. The constant *c* represents the proportion of the total variance due to additive genetic effects across populations.

To obtain the P_{ST} values for each couple of populations, we used a mixed model, with population defined as a random factor, using the 'lmer' function in R (LME4 packeage): CT_{max}/CT_{min}~1+(1|Population) (Bates *et al*., 2013). We used the error variance as a proxy for σ_w^2 (within-population variance) and the intercept variation for σ_B^2 (variance between populations). Thus, P_{ST} estimates depend on the ratio $\frac{c}{h^2}$. Since these parameters are extremely changing to obtain in the wild and usually unknown (Pujol *et al.*, 2008), we considered a set of values to calculate P_{ST} (Brommer, 2011). We constructed several matrices for the P_{ST} values obtained for different values of *c* and h^2. For each possible combination, the global mean values and their 95% confidence intervals were calculated using a nonparametric bootstrap and compared with the upper limit of the confidence interval for F_{ST} (see Tables 13 and 14 in Annexe 2). The value of the c/h^2 ratio at which the lower confidence interval for P_{ST} and the upper F_{ST} estimates overlap, can be interpreted as a measure of the robustness of the difference between F_{ST} and P_{ST} estimates (see, Bromer 2011). The correlation between P_{ST} and F_{ST} pairwise population matrices were examined with Mantel tests in R with 999 permutations (VEGAN package).

Warming tolerance of the populations

Warming tolerance (WT) is defined as the difference between the maximum ambient temperature (T_{max}) and CT_{max} (Deutsch *et al.*, 2008; Duarte *et al.*, 2012; Gutiérrez-Pesquera *et al.*, 2016):

$$WT = CT_{max} - T_{max}$$

To determine warming tolerance, we used both WorldClim air temperature (WT) and microenvironmental pond water maximum temperatures (wt) recorded during the larval period at the study populations.

Statistical analyses

We used one-way ANOVA to analyse population variation in CT_{max} and CT_{min}. To evaluate whether acclimation effects differ among populations, we used a two-way analysis of variance of CT_{max} and CT_{min} with temperature and population as fixed factors. To compare thermal values between the coastal and montane populations, we used the Mann-Whitney U-test. We used ordinary linear regression model to correlate temperatures, physiological thermal limits, warming tolerances and altitude. All the analyses were performed in R (R Core Team, 2014).

RESULTS

Thermal characterization of the altitudinal gradient

In a first approach, we used WorldClim database to extract temperature data for each local population averaged over the entire year. We found that maximum, mean and minimum monthly temperatures decreased along the altitudinal gradient (TMAX_A: $R^2 = 0.97$, $P < 0.001$; TMEAN_A: $R^2 = 0.98$, $P < 0.001$; TMIN_A: $R^2 = 0.98$, $P < 0.001$) (Fig. 3, left, see Table 15, Annexe 2). However, this trend was not supported when the analyses were restricted to the period of larval activity —i.e., the temperatures at which tadpoles are effectively exposed—. For populations below 900-1000 m, reproduction typically begins in autumn (Sep-Nov), lasting until spring. On the other hand, high elevation populations delay breeding until the time of snow melting, and larval phase can extend until August, when they can be exposed to relatively high temperatures (Fig. 1). As a consequence, TMAX and TMEAN experienced by tadpoles did not differ between populations

below and above 1000 m (Wilcoxon test: TMAX, $W = 509$, $P = 0.70$; TMEAN, W = 420.5, $P = 0.14$, n low=47, n high= 23, Fig. 3 right, Fig. 4). Montane populations experienced, nevertheless, lower minimum temperatures and broader thermal ranges than coastal populations (Wilcoxon test: TMIN, $W = 299$, $P < 0.01$; Range, $W = 983$, $P < 0.01$), (Fig. 4).

Local adaptation in thermal tolerance limits

Critical thermal limits (Table 1) significantly differed between populations (CT_{max} ANOVA $F_{10,160} = 8.97$, $P < 0.01$, CT_{min} ANOVA $F_{10,160} = 14.52$, $P < 0.01$) (Table 2, Fig. 5). However, these variations were not related to altitude nor maximum or minimum reported temperatures (see Table 15, Annexe 2). Maximum differences found across the gradient were slightly larger for CT_{min} (1.4 °C) than for CT_{max} (1.1 °C). The population at the lower end of the gradient, Purón, showed the lowest CT_{max} (36.4 °C) and highest CT_{min} values (-0.9 °C). One of the mountain populations (Aliva, 1400 m) showed the highest values of CT_{max} (37.5 °C), whereas Color (380 m) had the lower CT_{min} (-2.3 °C). In fact, CT_{min} for Color was not significantly different from those found for high-elevation populations (Tukey test; P>0.05).

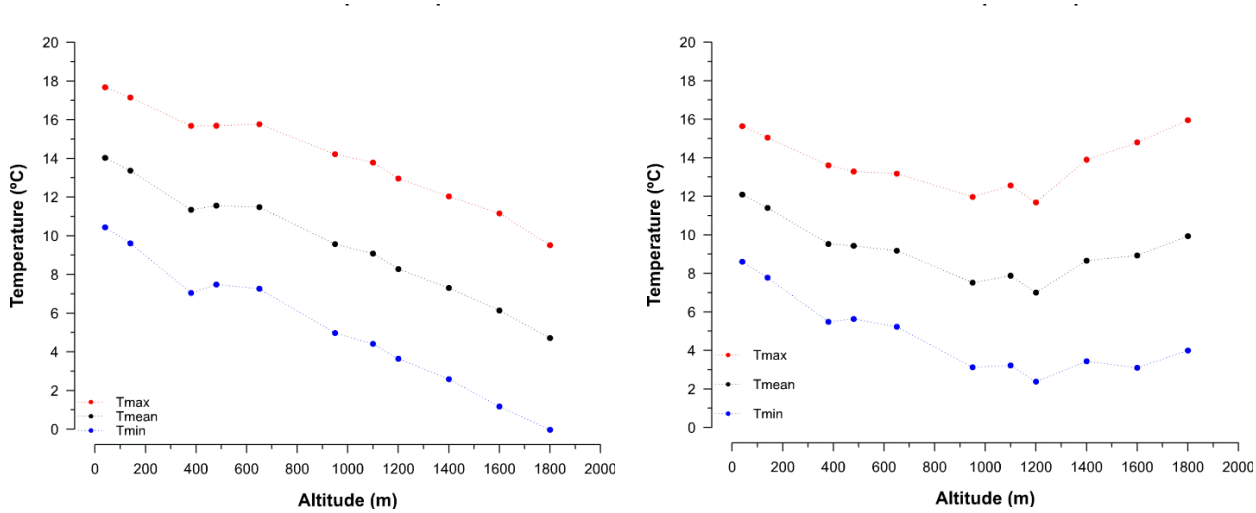


Figure 3. Thermal profile across the altitudinal gradient from WorldClim database. (Left) Mean yearly variation in maximum, mean and minimum temperatures with altitude. (Right) Mean sesasonal (presence of larvae in ponds) variation in maximum, mean and minimum temperatures with altitude.

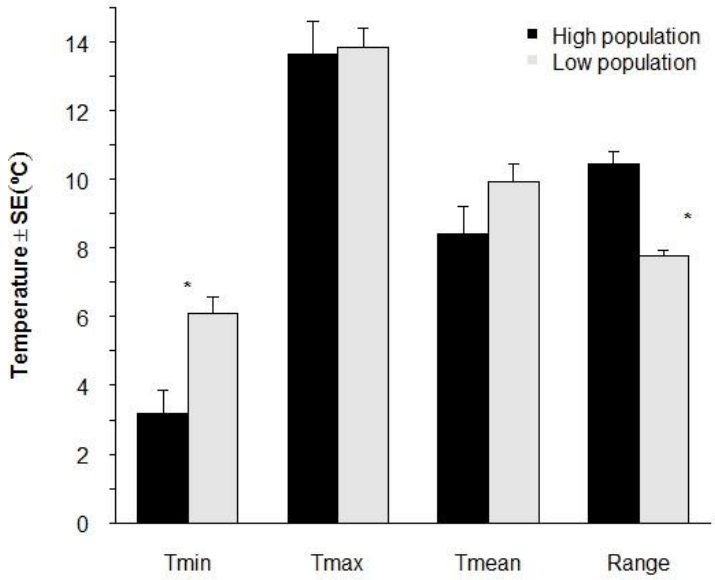


Figure 4. Comparison of minimum (Tmin), maximum (Tmax), mean temperatures (Tmean) and thermal range (Range) (monthly average for reproductive period from WorldClim) for populations below and above 1 000 m (high-low populations). * Differences were significant for minimum temperatures and thermal range but not for the maximum and average temperatures (see text).

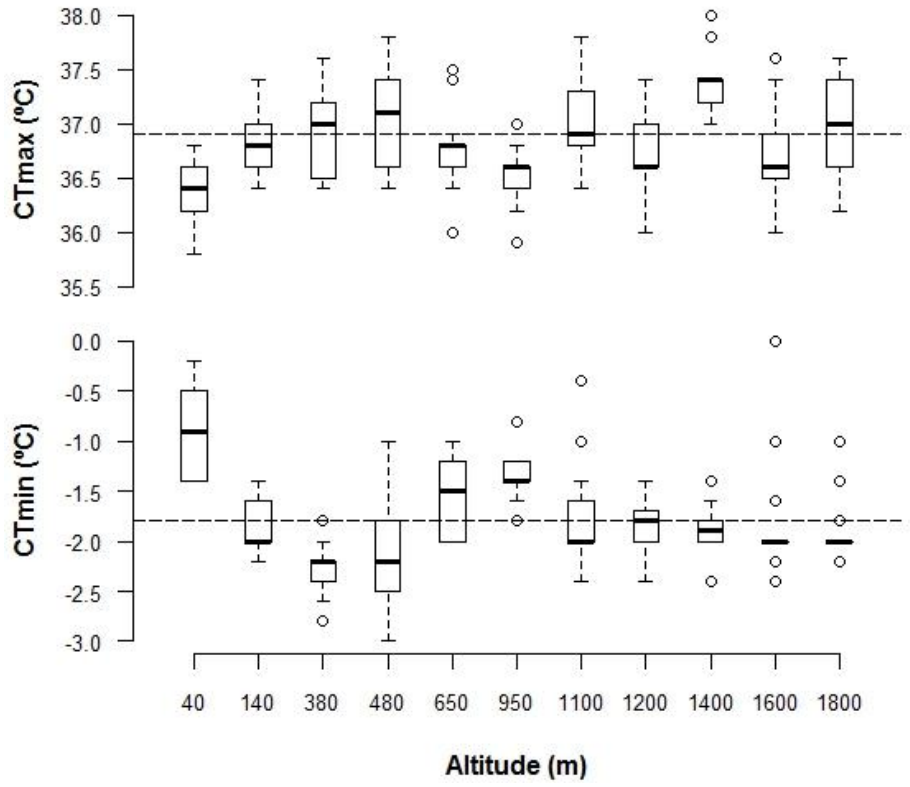


Figure 5. Boxplot showing the variation of thermal tolerance limits across the altitudinal gradient. First and third quartile ("hinges") and 95% confidence interval of median ("notches") are showed. Dashed line denotes global mean of populations.

Table 1. Critical thermal limits (CTmax and CTmin) for the eleven analyzed populations acclimated to 20 °C. (N) number of tadpoles examined.

Population	Altitude (m)	CTmax (°C)			CTmin (°C)		
		N	$\bar{X}$	SE	N	$\bar{X}$	SE
Purón	40	16	36.4	0.1	16	-0.9	0.1
Nueva	140	16	36.8	0.1	13	-1.9	0.1
Color	380	16	36.9	0.1	16	-2.3	0.1
Viango	480	16	37.1	0.1	15	-2.1	0.2
Cortegueros	650	16	36.8	0.1	12	-1.6	0.1
Fana	950	16	36.5	0.1	16	-1.3	0.1
Pandecarmen	1100	16	37.0	0.1	16	-1.8	0.1
Pandébano	1200	16	36.7	0.1	16	-1.9	0.1
Aliva	1400	14	37.5	0.1	16	-1.9	0.1
Señales	1600	15	36.7	0.1	16	-1.8	0.1
Llagusecu	1800	14	37.0	0.1	14	-1.9	0.1

The overall value of neutral differentiation between populations (F_{ST}), estimated from six microsatellite loci, was 0.066 (95% CI: 0.058 - 0.075) (Table 10, Annexe 2). Under the null hypothesis $c = h^2$, both CT_{max} and CT_{min} showed higher P_{ST} values than the upper confidence interval for F_{ST} (P_{ST} CT_{max}, 95% CI: 0.12 - 0.21; P_{ST} CT_{min}, 95% CI: 0.17-0.30) (Fig. 6 and Tables 11-14 in Annexe 2). However, the significance of this difference was not very robust, as the lower confidence estimate of P_{ST} overlaps with the upper limit of F_{ST} when c/h^2= 0.51 for CT_{max}, and when c/h^2= 0.29 for CT_{min}. The pairwise P_{ST} and F_{ST} matrices were not correlated etiher CT_{max} or CT_{min} under the null hypothesis ($c = h^2$) (Mantel´s Test CT_{max} r = 0.066, P = 0.40; CT_{min} r = -0.1695, P = 0.75).

Table 2. ANOVA for CTmax and CTmin between populations (as fixed factor).

	CTmax				
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Population	10	12.707	1.27074	8.9728	<0.001
Residuals	160	22.659	0.14162		
	CTmin				
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Population	10	22.603	2.2603	14.521	<0.001
Residuals	155	24.126	0.15565		

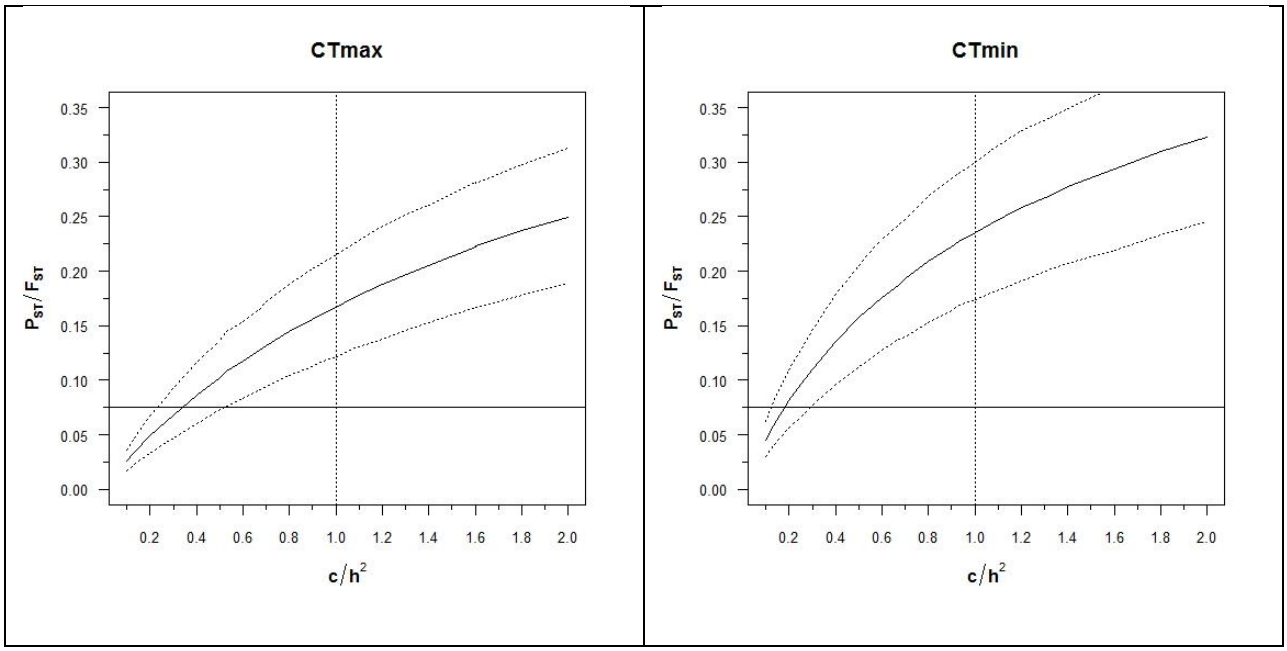


Figure 6. Comparison between neutral differentiation and the estimate of P_{ST} depends on the c / h^2 ratio. CTmax (left), CTmin (right). In each plot the dashed vertical line denotes the 'null assumption' $c = h^2$ for estimating P_{ST}. The horizontal solid line marks the upper confidence estimate of the neutral divergence estimated as F_{ST} (= 0.075). Estimates of P_{ST} and its lower and upper 95% confidence intervals are plotted. Both CTmax and CTmin clearly differ from F_{ST} under the null hypothesis ($P_{ST} > F_{ST}$).

Phenotypic plasticity in critical thermal limits

Because all tadpoles acclimated to low temperatures (6 °C and 13 °C) reached a freezing exotherm (around -2.0 ºC), the nearly instantaneous rise in temperature associated with the onset of water crystallization, before the attaining of tadpole cold resistance end-point, we could not assess potential populational divergence in CTmin for these acclimation temperatures. Thus, we analyzed populational variation at the acclimation temperatures of 20 °C and 27 °C (Table 4, Fig. 8). We found interpopulational variation in plasticity in both critical thermal limits as a result of the acclimation treatment, as revealed by the significant interaction between population and acclimation (CT_{max}, $F_{12, 274} = 2.54$, $P = 0.003$; CT_{min} $F_{4,130} = 11.906$, $P < 0.001$) (Tables 3-5, Fig. 7-8). Moreover, acclimation to these moderate and high temperatures resulted in higher CT_{max}, but also CT_{min} was increased (Fig. 8). High-altitude populations (Pandecarmen, Aliva, Llagusecu) showed the highest plasticity for CT_{min} (Table 4). However, for CT_{max}, mid-elevation Pandecarmen (1300 m) showed the highest plasticity (Fig. 7).

Table 3. Variation in upper thermal limits for five populations acclimated to four constant temperatures (6 °C, 13 °C, 20 °C, 27 °C). Plasticity is estimated as the differences between mean CTmax values at 27 °C and 6 °C acclimation treatments.

	CTmax (°C)														
	NUEVA			CORTEGUEROS			PANDECARMEN			ALIVA			LLAGUSECU		
Acclimation	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE
6 °C	11	36.1	0.1	16	35.9	0.1	17	35.6	0.1	15	36.2	0.1	14	36.3	0.1
13 °C	12	36.4	0.1	16	36.2	0.1	17	36.0	0.1	14	36.7	0.1	14	36.5	0.1
20 °C	16	36.8	0.1	16	36.8	0.1	16	37.0	0.1	14	37.5	0.1	14	37.0	0.1
27 °C	16	38.2	0.1	16	38.1	0.1	17	38.3	0.1	12	38.3	0.1	14	38.4	0.1
PLASTICITY	2.1			2.2			2.7			2.1			2.1		

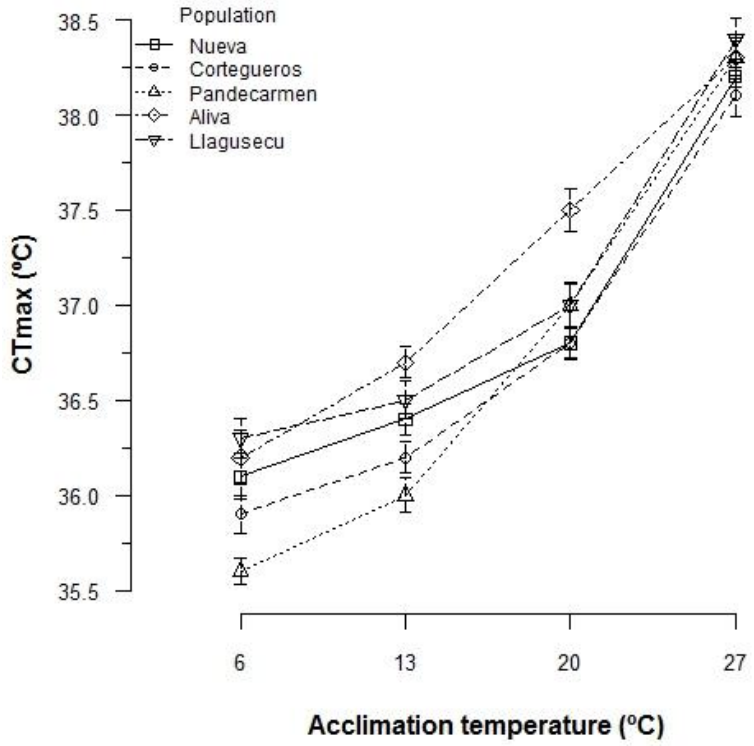


Figure 7. Phenotypic plasticity of upper critical thermal limits (CTmax) in five *Rana temporaria* populations: Nueva (40 m), Cortegueros (650 m), Pandecarmen (1100 m), Aliva (1400 m) and Llagusecu (1800 m), previously acclimated to constant temperatures (6 °C, 13 °C, 20 °C, 27 °C) during four days .

Table 4. Variation in lower thermal limits for five populations acclimated to four constant temperatures (20 °C, and 27 °C). Plasticity is estimated as the differences between mean CTmin values at 27 °C and 20 °C acclimation treatments.

	CTmin (°C)														
	NUEVA			CORTEGUEROS			PANDECARMEN			ALIVA			LLAGUSECU		
Acclimation	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE
20 °C	13	-1.9	0.1	12	-1.6	0.1	16	-1.8	0.1	16	-1.9	0.1	14	-1.9	0.1
27 °C	12	-0.8	0.2	12	-0.4	0.2	17	0.5	0.1	14	0.0	0.1	14	0.6	0.2
PLASTICITY	1.1			1.2			2.3			1.9			2.5		

Table 5. Two way ANOVA for changes in critical thermal limits (CTmax and CTmin) in five populations acclimated to several tempatures (6 °C,13 °C,20 °C and 27 °C for CTmax; 20 °C and 27 °C for CTmin).

			CTmax		
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Population	4	6.298	1.574	10.957	<0.001
Temperature	3	222.322	74.107	515.7394	<0.001
Population X Temperature	12	4.382	0.365	2.5413	0.003
Residuals	274	39.371	0.144		
			CTmin		
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Population	4	10.342	2.585	11.087	<0.001
Temperature	1	116.51	116.51	499.652	<0.001
Population X Temperature	4	11.105	2.776	11.906	<0.001
Residuals	130	30.314	0.2333		

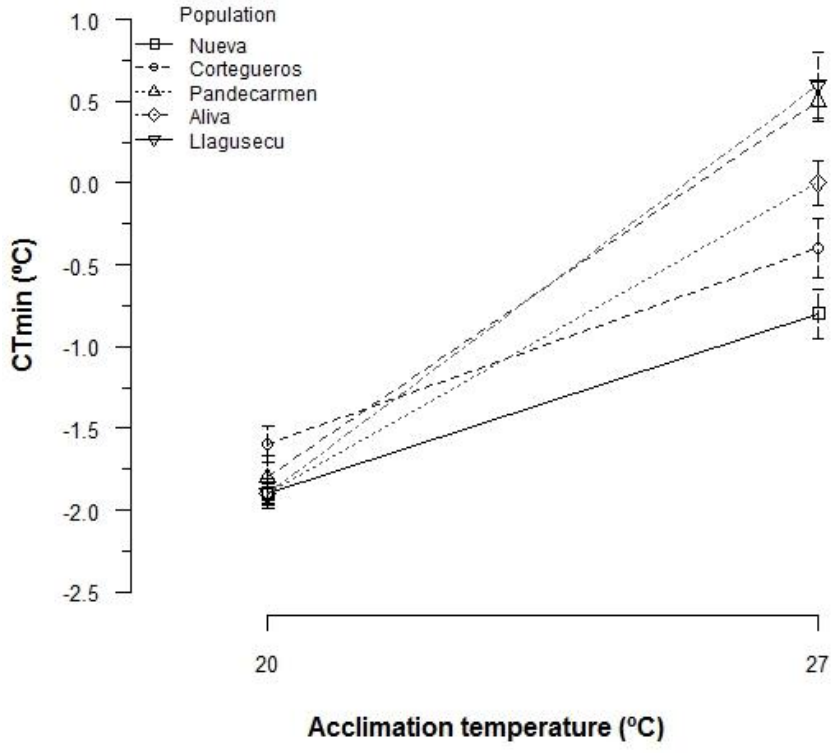
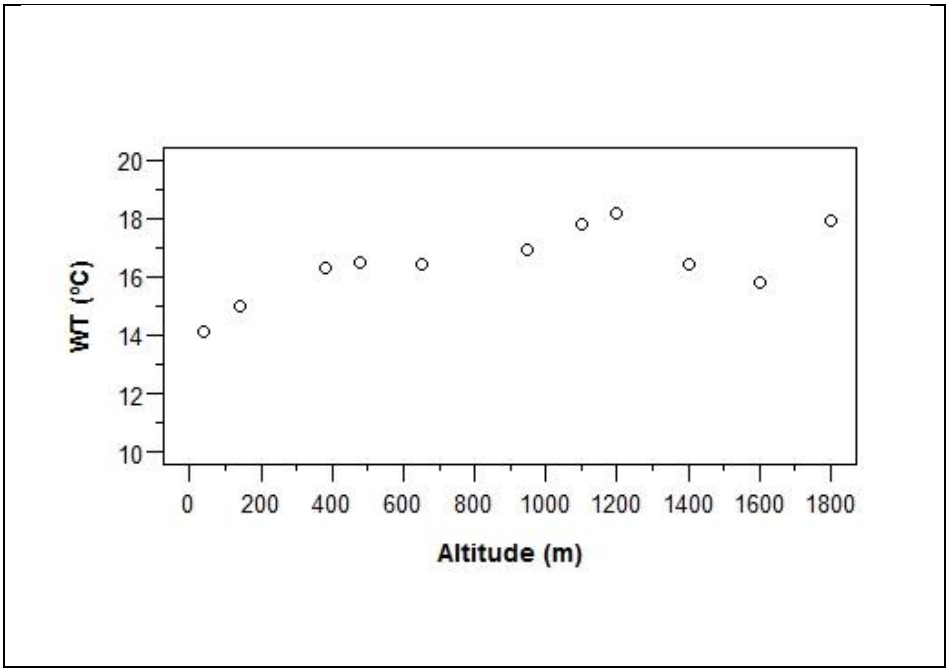


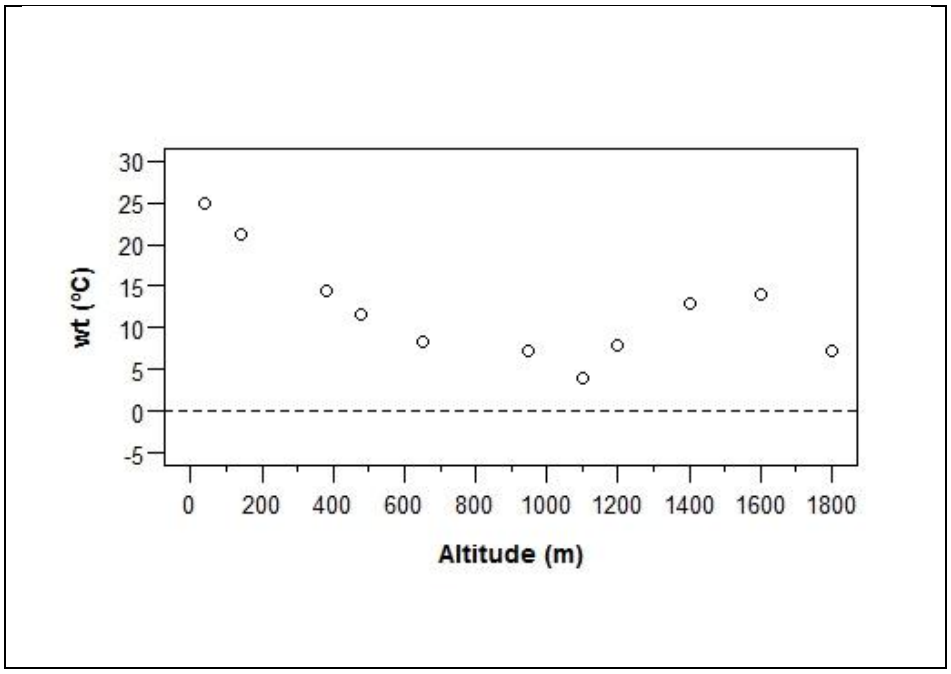
Figure 8. Phenotypic plasticity of lower critical thermal limits (CTmin) in five *R.temporaria* populations: Nueva (40 m), Cortegueros (650 m), Pandecarmen (1100 m), Aliva (1400 m) and Llagusecu (1800 m), previously acclimated to constant temperatures (20 °C and 27 °C) during four days .

Warming tolerance

The analysis of pond thermal profiles during the larval period did not suggest thermal stress for any of the tested populations. However, we found some differences between the warming tolerance estimates based on WorldClim thermal data (WT) and microenvironmental temperatures monitored directly in ponds (wt). First, WT was higher —with values ever exceeding/above 14 ºC— than wt — with values as small as 4 °C, Fig. 9—. Second, WT significantly increased with altitude (OLS, R^2= 0.44, P = 0.03), whereas wt decreased linearly for populations located below 1000 m (Fig. 9 and Table 14, in Annexe 2). Based on these wt estimates, frogs from the intermediate part of the gradient (mid-elevation populations) had the lowest warming tolerance and then will be exposed to potential acute heat stress at present (Table 15, Annexe 2).

Figure 9. Estimates of Warming tolerance for the analyzed populations, only considering larval period: (Up) Warming tolerance estimates based on air temperature measurements from WorldClim. (Down) Warming tolerance estimates based on water temperatures registered in ponds. Dashed line denotes equality between maximum temperatures and CTmax.





DISCUSSION

Environmental temperatures and Warming Tolerances

The contrasting estimates of warming tolerances derived from both WorldClim, which provides regional air temperatures, and dataloggers, that measure microenvironmental pond temperatures, highlight the importance of monitoring microclimatic variables in studies of vulnerability to global warming. In fact, these differences stem on the different nature of the data employed for calculation of maximum temperatures. First, maximum temperatures from WorldClim repositories represent air temperatures while dataloggers register water temperatures. Although, in general, air and water temperatures should be consistent because most of the aquatic habitats used by *R. temporaria* are shallow water bodies, some differences can arise due to fine-grain, microclimatic variation —e.g. underwater sources and springs, topographic shadow, plant cover— that can be precisely captured by dataloggers. Second, while WorldClim values correspond to monthly averages from fifty years of the

past century (1950-2000), maximum temperatures of dataloggers have a daily basis and are the result of a few recent years (2002-2014), so their values will be necessarily more extreme, considering that recent years may be warmer than previous years of the twentieth century based on climatic change predictions (Pachauri *et al*., 2014), but also the lower effect of averaging over much shorter periods. WorldClim values have the advantage to integrate a wider period of potential environmental influence on the past evolution of the populations, but in a context of rapid change in temperature that also could interfere with the analysis of thermal stress on actual populations.

Thus, the estimates obtained from WorldClim can be overestimating warming tolerances, whereas the analyses based on dataloggers alone could be temporally insufficient in order to understand adaptive variation. Based on microclimate conditions, our study revealed that populations from the intermediate part of the altitudinal gradient were exposed to higher temperatures than populations from the gradient extremes. This is not surprising because these mid altitude wetlands are located in open sunlit ponds without forest canopy, so that water is fully exposed to solar irradiance. High altitude wetlands are also exposed to direct irradiance, but average temperatures are lower because of adiabatic cooling. Finally, in the Cantabrian region, lowland and coastal wetlands are often located in areas with some degree of plant cover that may limit maximum temperatures.

Since we used different time periods for different locations —i.e., to adjust the exposition period to the breeding and larval phenology—, we consider that the pattern showed in Fig. 9 is not conclusive (see Table 2, Annexe 2). The general pattern based on WorldClim database (WT) agrees with previous results obtained from an interspecific comparison of warming tolerance in an altitudinal gradient in Ecuador (see Chapter 3), in which species from low altitudes showed lower warming tolerance than

species from upper areas. Considering that, for the less tolerant population, WT was 4 °C and, under laboratory conditions, the capacity to increase WT by short-term plasticity was 2 °C, it seems unlikely that populations will suffer heat stress during the larval stages in the short-term.

Lack of local adaptation in the critical thermal limits

The populations examined in this study differed in upper and lower thermal limits. The degree of phenotypic differentiation (P_{ST}) was greater than null expectation for both CT_{max} and CT_{min}. However, the comparison of P_{ST} with neutral differentiation did not provide a conclusive result (CT_{max}, critical c/h^2 = 0.51; CT_{min}, critical c/h^2 = 0.29). Finally, we were unable to demonstrate a sound relationship between thermal tolerance and altitude, and neither thermal tolerance limits were related to maximum or minimum temperatures during the breeding season. Therefore, we cannot conclude that the above mentioned differences in physiological traits can obey to a process of local adaptation. In fact, the observed microgeographic variation and the differences among populations could be the result of genetic drift alone. However, because the differentiation matrices F_{ST}-P_{ST} were not correlated, there are alternative explanations for the lack of clinal variation in thermal limits.

In the Cantabrian region, *Rana temporaria* populations exhibit a marked phenological shift in the breeding season with altitude (see Álvarez *et al.*, 2012; Choda, 2015) with fall and winter breeding for lowland populations and late spring and summer reproduction for upper mountain ones. As a result, maximum temperature experienced by larvae do not differ significantly between high and low altitude populations. The expected thermal gradient, unequivocal when estimates are based on temperatures all around the year, disappears when one takes into account only the periods for larval

activity. Therefore, the absence of an altitudinal pattern for larval CT_{max} does not imply necessarily the lack of local adaptation to thermal environments; rather, differences in breeding phenology across the elevation gradient can act as a buffer for strong thermal variation, thus precluding the occurrence of population adaptive differentiation in thermal tolerance.

Interestingly, the altitudinal variation in timing for reproduction seems to be conditioned by the hydroperiod in montane areas rather than temperature itself. Mountain populations must delay breeding until the snow melt (Álvarez *et al*., 2012; Corn, 2003). In addition, the time of spawning may be genetically determined in mountain populations of *Rana temporaria* (Álvarez *et al.*, 2012; see also Phillimore *et al.*, 2010); during warm winters with early melting, frogs appears to delay reproduction until a fixed date (A.G. Nicieza, unpublished data). This depicts a scenario where pond availability constraints reproductive phenology which, in turn, will ultimately determine the effective temperatures experienced by tadpoles.

Behavioural thermoregulation is considered a mechanism that allows organisms to mitigate the negative effects of global warming when different microclimatic conditions are available (Kearney *et al*., 2009; Huey *et al*., 2012). However, a potential side effect of behavioural adjustments is to lessen the selective pressures on physiological traits (e.g. critical thermal limits), by reducing the exposure to extreme temperatures. This phenomenon has been described as the 'Bogert effect' (Bogert, 1949; Huey *et al*., 2003). As a consequence, Bogert effect might jeopardize population viability in the long-term if thermal changes proceed rapidly, because it would limit the evolution of adaptive physiological responses through natural selection (Buckley *et al*., 2015; Gunderson & Stillman, 2015).

As other boreoatlantic species, *Rana temporaria* showed strong resistance to cold conditions, with CT_{min} values in the freezing zone. This can explain both, its widespread distribution in the northern hemisphere, and the colonization of habitats at high latitude and altitude. Moreover, larval resistance to freezing conditions conforms well with early breeding in mountains or high latitude areas, which can have important ecological implications. For instance, in the Cantabrian Mountains, *R. temporaria* breeds earlier than any other amphibian species, which renders a space free of competitors for the early larval phases and competitive advantage through priority effects. In turn, in the lowlands, breeding starts by late summer/early autumn, several months before larvae of any other amphibian (with the exception of *Alytes obstetricans*, a prolonged breeder with extended larval phase) can occupy the aquatic habitat. Thus, also for lowland populations, this breeding phenology confers a space free of competitors for young larvae, and competitive advantage for older larvae. Therefore, in *R. temporaria*, cold resistance might have a twofold evolutionary significance: first, it can be involved in the expansion to new habitats as a response to climatic change; second, it could affect the species persistence in a part of its actual range due to differentiation of the temporal niche and increased competitive ability. In a context of climate warming, this can have two major consequences for the expected changes in *R. temporaria* spatial distribution. Firstly, rising temperatures could prompt local extinctions due to intensification of competitive interactions before upper thermal limits can be reached. Secondly, as temperature increases, cold resistance can allow the species expansion towards higher latitudes and altitudes where most of the amphibian species will be thermally constrained.

In cold adapted species, the crystallization point of water set an absolute limit for the directional selection of its cold tolerance. The absence of liquid water prevents

breeding, and tadpole survival. In this study, some of the populations of *R. temporaria* reached this extreme value during the CT_{min} trials; the water vessel froze around -2.0 °C after which we transferred the tadpoles to a recovering chamber at 20 °C. In most cases, these 'frozen' tadpoles recovered activity after a period of 24-h indicating resistance to freezing and extreme cold tolerance, especially if the animals were exposed to a period of acclimation to low temperatures (most of the tadpoles from all tested populations survived a freezing event after acclimation to 6 °C and 13 °C). This suggests that *R. temporaria* might have evolved to a point near the maximum cold tolerance. If so, adaptive differentiation of CT_{min} is unlikely because there would be no scope for a further lowering of CT_{min} in mountain populations. This hypothesis was supported by our finding that lowland populations showed extreme cold tolerances (e.g., Color, 380 m) despite minimum temperatures decrease along the altitudinal gradient.

Populations from intermediate and high altitudes showed a greater plasticity for both CT_{max} and CT_{min} than low elevation populations. Our results support the hypothesis that plasticity in critical thermal limits can be a response to an increase of the environment thermal variability (expressed as thermal range) with altitude. It should be pointed out that the level of phenotypic plasticity in a population can affect adaptive genetic evolution. High levels of plasticity may increase the probability of population persistence in the short and mid-term, but can reduce the likelihood of local adaptation by buffering environmental variation and thus lowering the intensity of selection pressures (Schlichting *et al*., 1998). Thus, in a context of thermal tolerance, plasticity can prevent or slow down local adaptation along thermal gradients and genetic response to climate change (see Price *et al.*, 2003; Gunderson and Stillman, 2015). Then, we can conclude that the concurrence of 1) a higher plasticity in populations living in more variable environments, and 2) different seasonal timings across the

altitudinal gradient (“Bogert effect”) may hinder the adaptive differentiation of thermal limits even when gene flow among populations is restricted, and this could impose a limit to long-term adaptation to climate change.

CHAPTER 3

“Altitudinal variation of the critical thermal limits in 20 species of tadpoles in the tropical Andes”

Altitudinal variation of the critical thermal limits in 20 species of tadpoles in the tropical Andes

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ABSTRACT

Here we analyzed the interspecific variation in the critical thermal limits (CT_{max} and CT_{min}) of 20 species of tadpoles in the tropical Andes of Ecuador (along an altitudinal gradient ranging from 20 to 3500 m.a.s.l.). We also assessed vulnerability of species to receive heat impacts due to global warming through the estimate of its warming tolerance based on maximum temperatures obtained from both microenvironmental measures of water temperature in breeding habitats (dataloggers) and air temperature (WorldClim data layers). We found a clinal variation of both critical thermal limits with altitude, in parallel to the decline in environmental maximum and minimum temperatures. In addition, we found an increase in thermal tolerance range along the elevational gradient that is explained by an asymmetric clinal variation in thermal limits, implying faster decline of cold thermal limit with altitude. The level of divergence showed in thermal tolerance between lowland and mountain top species, especially for CT_{min}, is analogous to that found when comparing tadpole amphibian communities from different latitudes. As in that case, we found a relationship between thermal breadth and species distribution range, thus supporting climate variability hypothesis for altitudinal gradients. Due to its greater exposure to higher temperatures, species from the lowlands showed narrower warming tolerance than those from upper localities. Our results suggested that, given the predicted increase of mean temperatures

and extreme heat episodes with climate change, some species from the low altitude will suffer thermal stress at the short-term. Upland species appears to be relatively safe to currently suffer acute heat impacts. However, information is lacking regarding the possible incidence of non-lethal chronic stress in high altitude frog communities, as well as the consequences of potential specific interactions with the eventual upward migration of other lowland species that may threat its survival in the long/medium term.

Keywords: CT_{max}, CT_{min}, altitudinal variation, tadpoles, tropical mountains, warming tolerance, Janzen hypothesis

INTRODUCTION

Temperature is the most important single factor limiting amphibian and reptiles fauna in tropical Andes (Navas, 2002). Janzen (1967) seminal paper proposed, almost fifty years ago, that tropical species are more physiologically constrained by climatic barriers in their environment than temperate species ('mountain passes are higher in the tropics'). This physiological limitation is one of the mechanisms that may explain the increase in species turnover along elevational gradients in the tropics (Ghalambor *et al*., 2006; Jankowski *et al*., 2013; Zuloaga & Kerr, 2016). Thus, tropical species, characterized by extreme high degree of endemism and limited distribution, are exposed to a relatively uniform and narrow temperature range, making them among the most sensitive organisms to climate change impacts (Deutsch *et al*., 2008; Laurance *et al*., 2011). Species are expected to be displaced towards the poles and higher altitudes to compensate the forecasted increase in environmental temperatures (Wilson *et al*., 2005; Parmesan, 2006; Hill *et al*., 2011). This pursuit of suitable thermal conditions would require species to shift hundreds of meters upward, given typical adiabatic lapse rates of ~5– 6 °C/1000 m (Jankowski *et al*., 2013). Because no species from lower latitudes or below sea level are available to replace species that move upslope, lowland tropical rainforests are expected to suffer a net loss of species (Colwell *et al*., 2008). Species in tropical montane systems are likely to be even more vulnerable than lowland species, because 1) changes in climate appear to be more pronounced at higher elevations (Larsen *et al*., 2011) and 2) mountaintop species are physically constrained to track suitable thermal conditions. Recent elevational range shifts have been reported for amphibians in tropical latitudes (Pounds *et al*., 2005; Seimon *et al*., 2007; Raxworthy *et al*., 2008). Comparisons of ecological and physiological traits among taxa can help to

disentangle the causes of biodiversity patterns over altitudinal gradients (Navas, 2002). In conclusion, to understand, prevent and, when necessary, mitigate the impacts of climate change it is of paramount importance to characterize the thermal environment, thermal physiology, thermoregulatory behaviour and activity patterns of species (Huey *et al*., 2012).

Compared with latitudinal gradients (Addo-Bediako *et al*., 2000; Hoffmann *et al*., 2002; Deutsch *et al*., 2008; Huey *et al*., 2009; Calosi *et al*., 2010; Sunday *et al*., 2011; Diamond *et al*., 2012; Araújo *et al*., 2013; Chown *et al*., 2015), less attention has been paid to the altitudinal variation in ectotherms critical thermal limits, especially in the interspecific analyses of cold themal resistance (Gaston & Chown, 1999; Sorensen *et al*., 2005; Bridle *et al*., 2009; Verdú, 2011; Muñoz *et al*., 2014). Regarding amphibians, pioneering works focused mainly in latitudinal variation and were only conducted in adult terrestrial stage (Brattstrom, 1968; Snyder & Weathers, 1975; Christian *et al*., 1988; John-Alder *et al*., 1988). To our knowledge, this is the first attempt to characterize the altitudinal variation in the critical thermal limits in a community of tropical amphibians during their aquatic tadpole stage.

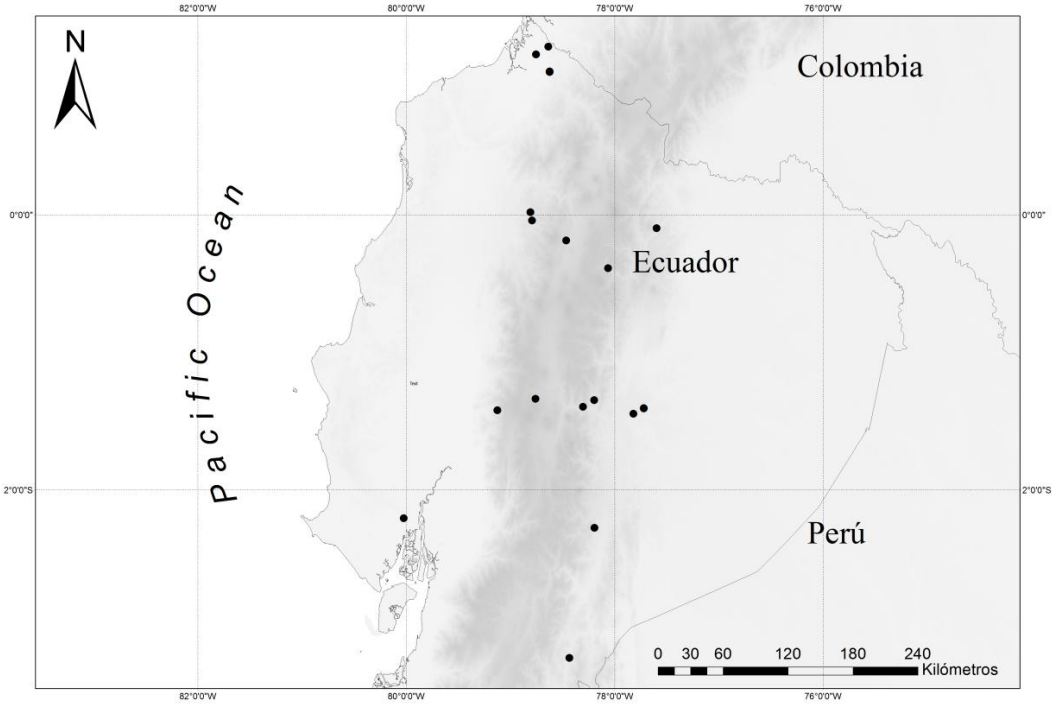
Following Janzen (1967) and the climate variability hypothesis (CVH) applied to altitudinal gradients (Stevens 1992), several predictions can be posed in the analysis of altitudinal variation in thermal boundaries. First, minimum temperatures limit species upward distribution and, thus, a correspondence between the critical thermal limits and environmental temperatures with altitude it is expected, especially for CT_{min}. Second, because daily temperature variation in tropical regions increase with altitude (Navas, 1997; Soobramoney *et al*., 2003; Sheldon & Tewksbury, 2014), species from higher elevations should have broader thermal tolerances than those from lower elevations. As an extension of Rapoport´s rule (Rapoport, 1975) applied to altitude, it is predicted that

tropical mountain top species exposed to variable climatic conditions will have larger altitudinal ranges than lowland species (Stevens, 1992). Finally, we assessed vulnerability of species to climate changes along the altitudinal gradient through the estimates of its warming tolerance based on maximum temperatures from both microclimatic water and air measuremtents —WorldClim datasets, (Hijmans *et al*., 2005)—.

MATERIAL AND METHODS

Study area and sampling

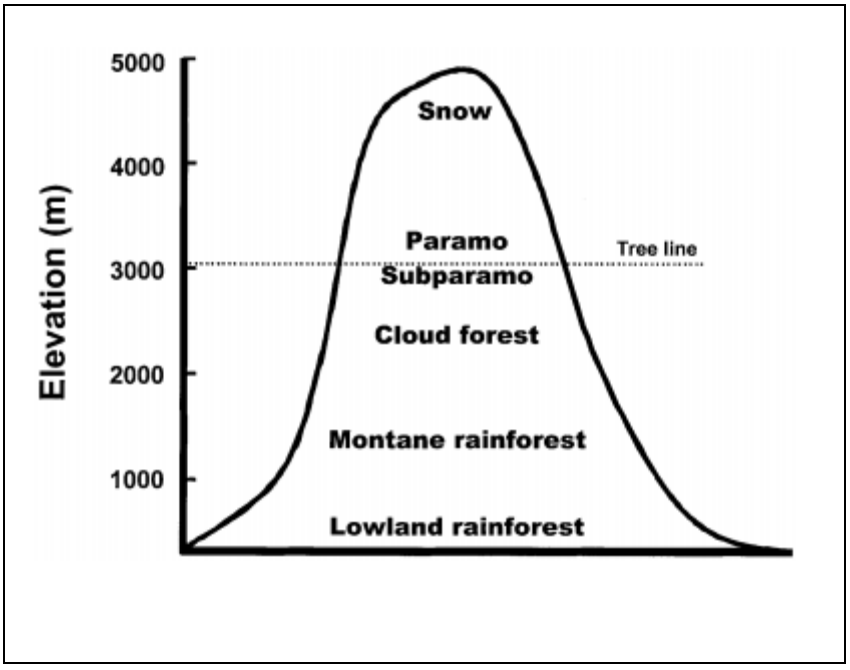
Figure 1. Sample points and study area. Greyscale indicates elevation. The shaded region represents the Andes mountain ranges.



Ecuador is an ideal region to analyze the effects of altitude on the thermal tolerance of tropical amphibian communities. It is among the 17 countries considered mega-diverse by the International Union for Conservation of Nature (IUCN) (Mittermeier *et al*., 1997; Myers *et al*., 2000). Ecuador hosts the third largest amphibian fauna of the world with a total number of 539 species, only surpassed by Brazil and Colombia (Amphibiaweb, 2015, accesed in February 2016). Taking into account its surface, Ecuador has the highest species richness per unit area (~ 139 documented spp/6.5 km^2, for example, in the Tiputini Biodiverity Station) which makes it the world region with the most varied concentration of anurans (Duellman, 1999; Bass *et al*., 2010). Unfortunately, Ecuadorian amphibians are suffering decline processes with

extinctions of populations, especially in areas of montane forest and andean páramo (Bustamante *et al.*, 2005). Further, intermontane valleys have been greatly altered by land use, with much of Ecuadorian biodiversity concentrated in the forests located on the outer wet flanks of both sides of the Andes (Young, 2011). Overall, it is estimated that 32% of the species that inhabit Ecuador are in serious danger of extinction (Stuart *et al*., 2004; Lips *et al*., 2005). Ecuador is crossed from north to south by the Andes providing an elevational gradient from sea level to 4500 m in which analyze the upslope shifts of biotic communites due to global warming (e.g. Morueta-Holme *et al.*, 2015), and the evolutionary changes in thermal tolerance limits and vulnerability to climate warming of biotic communities in different altitudinal tiers (Fig. 1). The ecological communities in the Andes range from extensive rainforests at low elevations to permanent snow (Fig. 2) (Navas, 1997).

Figure 2. Habitat types with elevation along tropical altitudinal gradients in the Andes, (Navas, 2002).



Montane forests cover most of the region. Lowland forests are characterized by constant warm temperatures throughout the year and present a vertically stratified

vegetation (Smith & Smith, 2001). Transition from cloud forests to the páramo occurs around 3000 m of altitude. This uncover habitat is characterized by an extreme thermal regime (Navas, 1997) and the landscape is typically dominated by open shrublands in a matrix of grasslands and other vegetation adapted to cold and very wet conditions.

Tadpole samplings were carried out between June 2014 and April 2015. We analyzed thermal tolerance limits of 20 species of tadpoles along an altitudinal transect between 20-3500 m.a.s.l. (see Fig. 1 and Table S1, Annexe 3). The studied species have free aquatic laravae and occupy different aquatic environments with contrasting temperatures and thermal variability, such as lowland open ponds, streams and mountain pools (see Table 1). Most of examined tadpoles were collected in their natural habitat and transported to the reference laboratory: Balsa de los Sapos- Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ). Some of the species, (*Atelopus limon, Engystomops guayaco, Epipedobates tricolor*) were, however, obtained from captivity through reproductive couples at the Balsa de los Sapos facilities. In these cases, we assumed that the results did not differ from those obtained in natural populations given the limited number of generations spent in captivity in the laboratory (F1 or F2).

Thermal data

According to the records of tadpoles' samplings in QCAZ database (Ron *et al.*, 2014), the analyzed species showed a continuous or prolonged breeding season —see also Navas, (1996)—. Thus, to characterize thermal environment through the altitudinal gradient, we used the 'extract' function, in the R package RASTER (Hijmans, 2014; R Core Team, 2014), to obtain the climatic information from WorldClim layers —30 " or 1 km^2 spatial resolutions; records from 1950 to 2000 (Hijmans *et al.*, 2005)—for the

geographical coordinates of each populations: BIO1 (annual mean temperature), BIO5 (maximum temperature of the warmest month) and BIO6 (minimum temperature of the coldest month) (see Table S1, Annexe 3). Although WorldClim climatic variables correspond to air measurements, previous studies have shown that air temperatures correlate well with water temperature in streams and lakes (Livingstone & Lotter, 1998; Webb *et al*., 2003), being reliable predictors used in biogeographical and conservation analyses with aquatic organisms such as continental fishes (Chessman, 2013) and tadpoles (Gerick *et al*., 2014; Gutiérrez-Pesquera *et al*., 2016). To assess microclimatic habitat conditions, we used HOBO Pendant temperature data loggers to obtain a continuous record of water temperature at each sampling site. Temperature was recorded every 15 min. We analyzed mean (tmean), maximum (tmax) and minimum (tmin) daily temperatures from each water body. The number of sampling days ranged from 31 to 456 days (Table 1).

Estimates of critical thermal limits and warming tolerances

Larvae were maintained at a similar and constant room temperature of 20 °C, with a natural photoperiod ca. 12:12 light: dark. Tadpoles were placed in plastic containers with similar densities, for a minimum of three-four days. This acclimation period was chosen as previous research in adult amphibians revealed that between 2-3 days was the time required to stabilize CT_{max} and CT_{min} after a large change in acclimation temperature, such as field and laboratory environments (Brattstrom, 1968). Larvae were tested individually between 25 and 38 Gosner stages (Gosner, 1960). Tadpoles over 38 Gosner stage were excluded because near the metamorphic climax they tend to have reduced thermal tolerances (Floyd, 1983, see Chapter 6, this thesis).

Whenever possible, we used a minimum sample of 16 individual tadpole replicates per species and thermal tolerance limit (for some species the available number of individuals examined was lower, Table S1, Annexe 3). Each tadpole was weighed immediately before the beginning of the test. Both thermal tolerance limits were determined using the Hutchison's dynamic method (Lutterschmidt & Hutchison, 1997a) in which each animal was exposed to a constant heating / cooling rate (ΔT=0.25 °C min^{-1}) until an end point was attained. The end-point was signalled for both thermal limits as the point at which the tadpoles become motionless and fail to respond to external stimuli by prodding 10 consecutive hits applied each two seconds with a wooden stick. Each tested tadpole was placed individually in 100 mL containers with dechlorinated tap water in a refrigerated heating bath of 15 L (HUBER K15-cc-NR) at a start temperature of 20 °C (temperature of acclimation). Because of the small size of tadpoles, we assumed that body temperature was equivalent to water temperature (Lutterschmidt & Hutchison, 1997b), and then CT_{max} and CT_{min} were recorded as the water temperature beside the tadpole measured with a Miller & Weber quick-recording thermometer (to the 0.1 °C). After a tolerance limit was determined, we immediately transferred tadpoles to water at the acclimation temperature (20 °C) to allow for recovery, after which their Gosner stage was registered. Tadpole survival was verified a few minutes and 24 hours after the end of the heating/cooling assays. Each individual was tested only once. To ensure that lethal temperature was not exceeded, only those individuals who recovered 24 h after the test were included in subsequent analyses. Although we only examined a single population for each species, we assumed that response variation among species is larger than variation within species (cf. Klok & Chown, 2003). Species' tolerance range was calculated as the difference of CT_{max} – CT_{min}. To evaluate the risk of each species suffering thermal stress, we estimated

warming tolerances (*sensu* Deutsch *et al*., 2008; Duarte *et al*., 2012) based on both macroclimatic (BIO5, WorldClim) and microhabitat temperature data (tmax) as the difference of CT_{max} – Tmax.

Statistical analyses

We constructed a phylogenetic tree containing the 20 studied species from Pyron & Wiens (2011). For some species not included in this phylogeny, we used the position of a known sister-taxon that does appear in the original tree. In some cases the position of certain species within the genus could not be resolved and therefore appear as polytomies (Fig. S1, Annexe 3). The altitudinal range of species was obtained from both IUCN and Amphibian Web databases (IUCN and Nature Serve, 2006; Amphibiaweb, 2015). To evaluate the correlations between thermal physiology variables (CT_{max}, CT_{min}, tolerance range and warming tolerance), with altitude and thermal data, we used both ordinary least squares regression (OLS) and phylogenetic generalized least squares (PGLS) analyses under a Brownian motion model of evolution using 'pgls' function in the R package GEIGER (Harmon *et al*., 2008). We employed ANCOVA to compare differences in the regression slopes of CT_{max}/CT_{min} and CT_{max}/BIO5 with altitude using the basic R package.

RESULTS

(a) Thermal habitat

We visited 16 localities in Ecuador distributed in an altitudinal range comprising between sea level and 3500 m.a.s.l., and a variety of habitats from lowland rainforest to streams and open ponds in mountain páramos (Fig. 1). Tropical regions are generally characterized by a great thermal stability with little annual variability in temperature.

Specially, we found little thermal variation in the water temperatures registered in streams and shaded ponds under canopy in the lowland tropical forest, on both a daily and seasonal basis (tmax-tmin) (Table 1). As expected by the adiabatic change of temperature with elevation, temperature of the sampling locations showed a clinal variation with altitude (Fig. S2, Annexe 3) independently of the sort of thermal data employed: WorldClim thermal variables or microenvironmental water temperature obtained by dataloggers (OLS: tmax, $R^2 = 0.45$, $P < 0.01$; BIO5, $R^2 = 0.92$, $P < 0.001$). Maximum temperature (36.8 °C) was recorded in a lowland temporary pond (DUR_2) and minimum temperature (7.9 °C) in a highland temporary pond in the páramo.

Table 1. Summary of water temperatures in each sampling site using dataloggers. N: number of days. tmin (minimum temperature); tmean (mean temperature); tmax (maximum temperature) and daily range are in °C. tmax-tmin represents seasonal absolute thermal range. Latitude and longitude in decimal degrees (WGS 84).

Location	Description	Latitude	Longitude	Altitude (m)	N	tmin	tmean	tmax	daily range	tmax-tmin
DUR_1 (Niñas)	Permanent pond	1.2218	-78.636	227	117	23.96	24.5	26.2	0.74	2.24
DUR_2 (Yanez)	Temporary pond	1.1653	-78.7527	250	180	24.64	28.5	36.84	6.34	12.2
Caimán	Temporary pond	-1.4117	-77.705	850	353	19.85	22.7	28.34	1.61	8.49
MIN_2	Stream	0.0182	-78.8076	1066	80	19.19	19.93	20.9	0.42	1.71
MIN_3	Pond	0.0182	-78.8076	1066	80	17.85	22.01	32.6	3.68	14.75
PUY_3(CUNETA)	Ditch road	-1.4052	-77.7185	1068	456	18.71	20.62	23.1	1.02	4.39
PL_3	Temporary pond	-1.3674	-78.0559	1250	36	16.81	19.84	22.14	1.06	5.33
PL_4	Temporary pond	-1.3674	-78.0559	1250	36	16.9	20.29	26.97	1.83	10.07
PL_1	Permanent pond	-1.3587	-78.0528	1300	31	16.81	20.89	22.43	0.68	5.62
PL_2	Permanent pond	-1.3587	-78.0528	1300	41	16.08	20.64	22.91	0.93	6.83
PL_5	Artificial pool	-1.362	-78.0522	1300	45	16.62	20.53	23.00	0.93	6.38
TOPO_1	Stream	-1.3903	-78.1921	1540	223	17.48	17.75	18.14	0.06	0.66
REV_1	Temporary pond	-0.097	-77.5962	1820	213	15.19	17.3	21.09	0.99	5.90
PAPALLAC_1	Temporary pond	-0.3877	-78.0619	2800	129	11.33	12.77	15.37	1.19	4.04
GAS_2	Temporary pond	-0.1873	-78.4639	2969	154	8.48	15.04	25.31	4.91	16.83
Guaranda	Temporary pond	-1.3367	-78.7594	3500	128	7.98	12.23	19.66	4.04	12.20

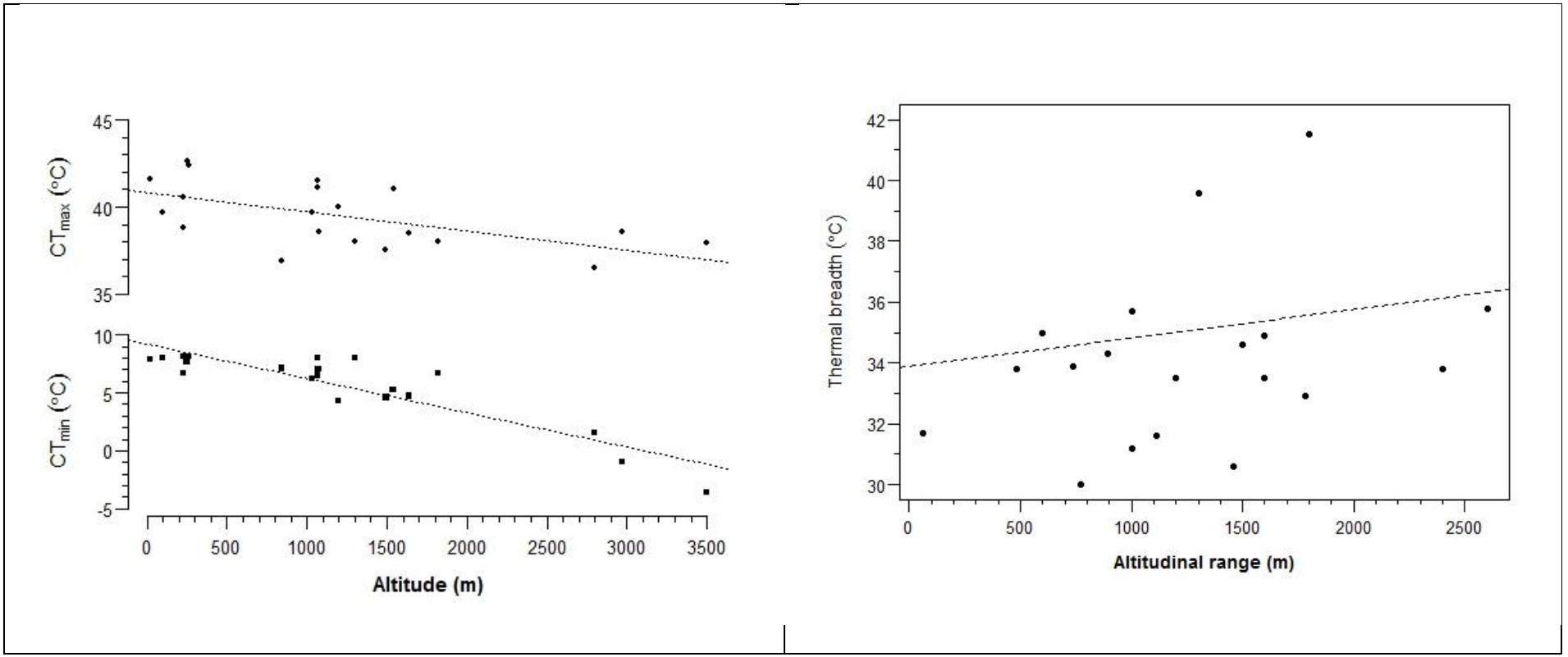
(b) Analysis of thermal physiology

Table S1, Annexe 3, gathers the physiological and environmental information obtained for the 20 analyzed amphibian species. CT_{max} values ranged from 36.9 °C, for the lesser thermotolerant species —*Atelopus limon*, which inhabits cool streams— to 42.6 °C of sunlit pond breeder, *Smilisca phaeota*. Species with lower CT_{max}, generally breed in cold streams and rivers, characterized by low and constant temperatures throughout the year (i.e., *Hyloscirtus* spp., *Hyloxalus* spp., and *Atelopus* spp.) (MIN_2, TOPO_1, see Table 1). By contrast, high thermotolerant species were found in lowlands, temporary open ponds, frequently located in disturbed areas such as deforested lands for agriculture and animal husbandry or roadsides. The most cold tolerant species was *Gastrotheca pseustes* (CT_{min}: -3.6 °C) whose distribution ranges from 2.200 to 4.000 m.a.s.l. in the Andes. We found a negative relationship between CT_{max} and altitude (OLS: R^2 = 0.35, P < 0.01) even after correcting by phylogeny (PGLS: P < 0.01). This correlation was even steeper for CT_{min} (OLS: R^2 = 0.81, P < 0.001; PGLS: P < 0.001; ANCOVA: P < 0.001). Similarly, tolerance range was positively correlated with the altitude of the sampling point (OLS: R^2 = 0.39, P < 0.01; PGLS: P < 0.001) and the altitudinal range of species (PGLS: P < 0.001) (Table 2 and Fig. 3). Both thermal limits were also correlated respectively with maximum and minimum temperatures found along the altitudinal gradient (CT_{max} ~tmax, R^2 = 0.50, P < 0.01; CT_{min}~tmin, R^2 = 0.80 P < 0.01) (Table 2).

Table 2. Results from linear regressions assessing the relationship between physiological traits (critical thermal maximum, CTmax; critical thermal minimum, CTmin, tolerance range (CTmax-CTmin) and warming tolerance, (WT, WorldClim; or wt, microenvironmental temperature), thermal environment (maximum temperatures of water, tmax; minimum temperatures of water, tmin; maximum temperature of warmest month BIO5 and minimum temperature of coldest month, BIO6) and altitude using a) OLS: ordinary least squares regression and b) PGLS: phylogenetic generalized least squares.

	a) OLS				b) PGLS			
	Intercept ± SE	Slope ± SE	R^2	*P*	Intercept ± SE	Slope ± SE	R^2	*P*
CTmax								
~ altitude	40.81 ± 0.55	-0.001 ± 0.001	0.35	<0.01	41.29 ± 0.58	-0.001 ± 0.001	0.52	<0.01
~ tmax	34.78 ± 1.30	0.19 ± 0.05	0.50	<0.01	34.65 ± 1.32	0.19 ± 0.05	0.50	<0.01
~ BIO5 (Max. Temperature)	34.39 ± 2.27	0.20 ± 0.09	0.20	<0.05	33.91 ± 1.86	0.23 ± 0.07	0.40	<0.01
CTmin								
~ altitude	9.18 ± 0.52	-0.003 ± 0.001	0.81	<0.001	8.70 ± 0.74	-0.003 ± 0.001	0.82	<0.001
~ tmin	-5.06 ± 1.42	0.56 ± 0.07	0.80	<0.001	-5.80 ± 1.33	0.60 ± 0.07	0.83	<0.001
~ BIO6 (Min. Temperature)	-2.37 ± 1.15	0.54 ± 0.07	0.75	<0.001	0.24 ± 0.84	0.31 ± 0.01	0.99	<0.001
Tolerance range								
~altitude	31.62 ± 0.84	0.001 ± 0.001	0.39	<0.01	32.9 ± 0.01	0.001 ± 0.001	0.99	<0.001
~altitudinal range	32.32 ± 1.43	0.001 ± 0.001	0.11	0.184	33.87 ± 0.01	0.001 ± 0.001	0.99	<0.001
WT								
~ altitude	9.63 ± 0.85	0.003 ± 0.001	0.70	<0.001	10.29 ± 0.84	0.003 ± 0.001	0.80	<0.001
wt								
~ altitude	9.80 ± 1.89	0.003 ± 0.001	0.30	0.02	4.91 ± 2.17	0.006 ± 0.001	1.00	<0.001

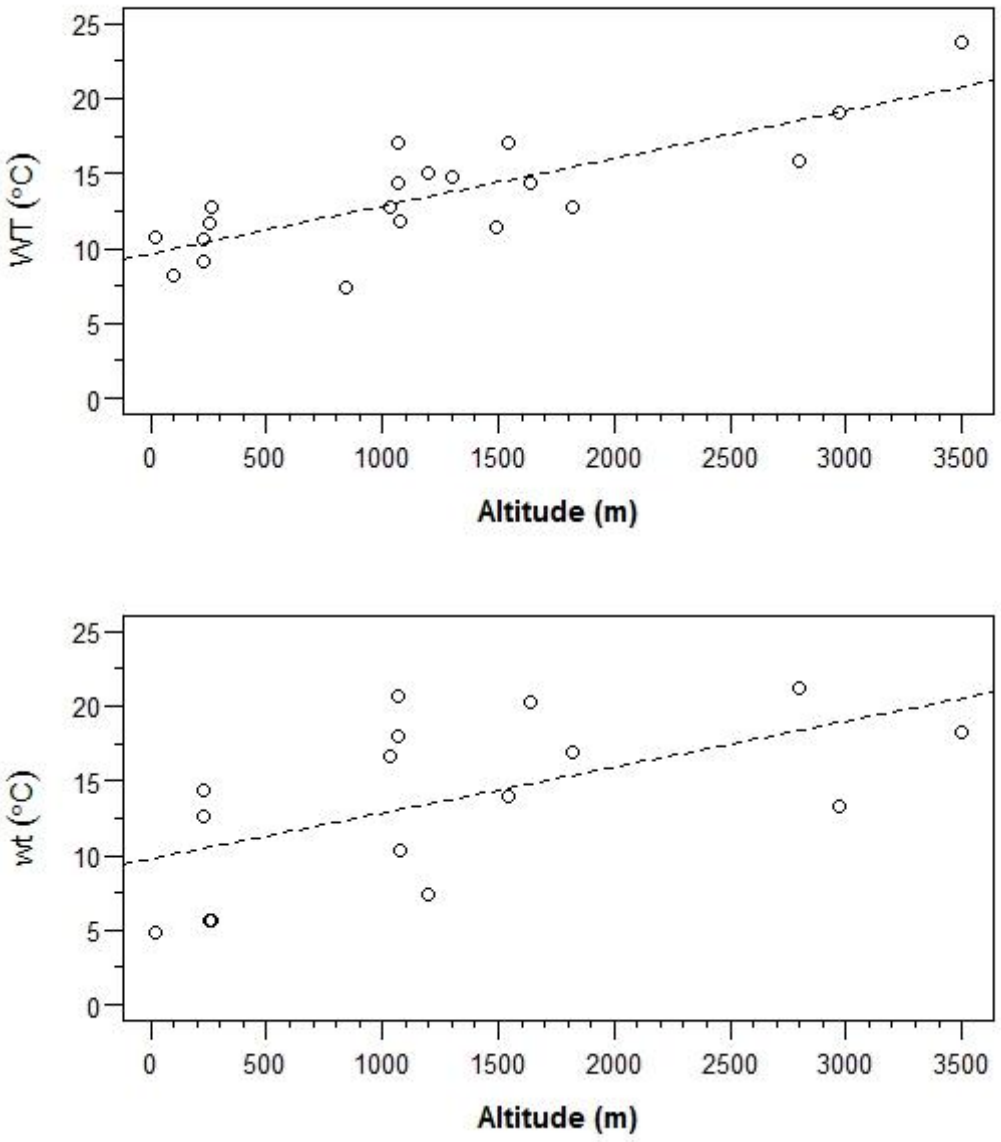
Figure 3. (Left) CTmax and CTmin variation in 20 tadpole species with its altitude sample points in the Ecuadorian Andes. Each point represents mean value of the species. Dashed lines represent OLS results from Table 2. (Rigth) Relationship between the thermal breadth (CTmax-CTmin) and altitudinal range of species is plotted. Dashed line represents significant PGLS coefficients from Table 2.



(c) Warming tolerance

Taking into account either, maximum air temperatures (WorldClim) (WT) and maximum water temperature (wt), none of the analyzed species appear to be under risk of suffering from acute heat stress under current conditions. However, regardless of the indicator used, we found a positive correlation between warming tolerance and altitude (WT: R^2 = 0.70, P <0.001; wt: R^2 = 0.30, P <0.05, Fig. 4). Thus, lowland species resulted more vulnerable to suffer heat impacts, obtaining lower values of warming tolerances. Generally, microhabitat temperatures were more variable and extreme than those corresponding to air measurements data. Thus, warming tolerances based on the water temperature (wt) yield narrower margins than those based on the air temperature (WT) (Table S1, Annexe 3).

Figure 4. Species warming tolerances means are given for (up) macroclimatic data estimations (WorldClim) and (down) microclimatic estimates based on maximum temperatures registered in ponds. Dashed lines denote significant linear regression results (see Table 2).



DISCUSSION

Altitudinal variation of the critical thermal limits

Because altitudinal clines affect species in different ways (Körner, 2007), different factors may limit amphibians distribution through the Andes mountain ranges. For example, the loss of biodiversity of anurans along southern altitudinal clines seems highly influenced by water limitations that reduce both number and diversity of potential sites for reproduction (Navas, 2002). However, given the absence of hydric constraints near the equator, reduced daily temperatures and increased thermal variance are the most important factors limiting the distribution of amphibians along equatorial elevational gradients (Janzen, 1967; Navas, 2002). Unlike lizards, amphibians are provided with a wet permeable skin which enables them carry out cutaneous respiration but hinders behavioural thermoregulation due to rapid loss of water by evaporation. Hereby, amphibian have limited opportunities for behavioural thermoregulation but incentives to increase the importance of physiological adjustments to tolerate a wide thermal range (Navas, 1996). Coincident with the decline in species richness with increasing elevation, we found an increase in the altitudinal range of species. This pattern is analogous to the relationship between the latitudinal range of species and latitude (Rapoport's latitudinal rule, Chapter 1) (Stevens, 1992).

The Andean genus *Gastrotheca* (Fam. Hemiphractidae), have colonized high-elevations through dramatic evolutionary shifts in thermal physiology (Navas, 1996). Similar temperature-related shifts in anuran thermal physiology have been reported also for Holartic treefrogs of the genus *Hyla*, supposedly derived from central American tropical ancestors (Smith *et al.*, 2005), especially for critical thermal limits (Gutiérrez-Pesquera *et al.*, 2016, see Chapter 1). In both cases, these new environments are

characterized by greater daily/seasonal thermal variability and lower minimum temperatures. Compared to latitudinal gradients, altitudinal clines integrate substantial climatic variation over much shorter geographical distances (Sorensen *et al*., 2005; Körner, 2007). As Janzen proposed, we found a correspondence between the thermal physiology (thermal tolerance range), the level of thermal variability experienced by taxa and the altitudinal range of species. Thus climate variability hypothesis may also explain the species turn-over and biodiversity patterns showed in the tropics (Stevens, 1992). Physiological thermal variation found for amphibians species between 13°S – 43°N and spaced more than 5000 km (Chapter 1) matched that found for an altitudinal transect between 0-3500 m in a single country. This awesome evolutionary divergence has occurred for some lineage even when critical thermal limits are considered highly conservatives (Wiens *et al*., 2006; Olalla-Tárraga *et al*., 2011; Hoffmann *et al*., 2013).

Warming tolerance and the impact of climate change

No organism is an ecological island (Huey *et al*., 2012). Climate often determines the geographic range of species and thus, where species overlap geographically and interact (Janzen, 1967; Bozinovic *et al*., 2011b; Olalla-Tárraga *et al*., 2011; Whitton *et al*., 2012; Gutiérrez-Pesquera *et al*., 2016). Altitudinal species turnover in the tropical Andes has been argued to result from interactions in which some species are excluded from particular elevations by competitively superior species, while the elevational range of the latter species is limited by climatic factors (Herzog *et al*., 2011). Traditionally, mountaintop species have been considered at higher risk of extinction than lowlands ones because they are physically constrained (they have nowhere to go) (Raxworthy *et al*., 2008; Laurance *et al*., 2011). Although none analyzed species seem to be exposed to maximum temperatures higher than its thermal

tolerance limit (CT_{max}), the predicted increase in temperatures by the end of the 21st century is until 3.1 °C under moderate scenarios (Pachauri *et al*., 2014). Thus, our results suggested that some lowland species will suffer thermal stress at the short-term. Also, we must keep in mind that many of studied species occupy disturbed areas (most sample points were within 25 m of a road) (Haddad *et al*., 2015). Because forest species, where temperatures are lower and constants, are low thermotolerant; our estimates of warming tolerance for lowland species are indeed overestimated, especially if fragmentation and habitat destruction continue in the future (Tuff *et al*., 2016), exposing forest species to high temperatures. Taxa from upper parts of the gradient showed wider warming tolerance because of the steeper decrease of maximum temperature with altitude than the CT_{max} values (ANCOVA, $P < 0.001$, Fig. S3). Nonetheless, species' responses to warming can indirectly lead to shifts in community dynamics through direct changes in biotic interactions (Gilman *et al*., 2010; Sheldon *et al*., 2011). Higher temperatures may allow or force species from lower elevations to migrate upwards leading to negative interspecific interactions through competition for resources (food, shelter, breeding sites, etc.) (Brooker *et al*., 2007; Gilman *et al*., 2010; Urban *et al*., 2012) or pathogen transmission (Freed *et al*., 2005; Seimon *et al*., 2007). In amphibians, for example, Beebee (1995) described a case of increased competition due to changes in phenology directly causes by climate change. Warmer winters in Britain resulted in earlier breeding period in newts (*Triturus* spp.) but no similar response occurred in frogs (*Rana temporaria*). As a consequence, early developmental stages of the latter species are exposed to higher predation pressure by newts. Similar responses could be found in the tropics. Thereby, although upland species appears to be safe to ongoing heat impacts, we do not know whether nonlethal effects induced by increased temperatures could determine chronic stress affecting performance —thermal

safety margins, sensu (Deutsch *et al.*, 2008)— as well as the consequences of potential specific interactions with the eventual upward migration of other lowland species that may threat its survival in the long/medium term. Further efforts must been increased in order to fully characterize thermal environments and thermal sensitivity along altitudinal gradients in amphibians and other ectothermic taxa occupying different thermal habitats, especially those from well conserved patches. In addition, we should explore the interaction between species in the context of thermal physiology and climate change as, for example, the effects of temperature on predation and competition. We should also explore the synergies between global warming and habitat fragmentation in the Ecuadorian region that may limit the potential dispersion of species and the effects of deforestation over canopy species. Finally, little is known about the activity patterns and behavioural thermoregulation that may grant more accurate predictions of the effects of climate change on the conservation of Andean amphibians.

CONCLUDING REMARKS

Both thermal limits (CT_{max} and CT_{min}) showed a clinal variation with altitude associated to a parallel decline in both minimum and maximum temperatures through the altitudinal gradient. This variation was steeper for the cold tolerance. As Janzen (1967) proposed, we found a correspondence between the environmental thermal variability, the tolerance range of species and their altitudinal range. This result supports the climate variability hypothesis as an explanation to Rapoport's rule through altitudinal gradients. The thermo-tolerance divergence found between upper and lower species in the altitudinal gradient was similar to that obtained, through a latitudinal comparison, between lowland tropical and temperate species, especially for CT_{min}. None of the analyzed tadpole species are currently exposed to environemental

temperatures higher than its CT_{max}. A positive trend between warming tolerance and altitude was found, thus suggesting that lowland species could be at higher risk in the short-term given the forecasted increase in temperatures. Mountaintop species could be, nevertheless, threatened at the middle/long-term due to the non lethal effects of warmer temperatures or through negative interactions with species migrations from the lowlands.

CHAPTER 4

“Acclimation of critical thermal limits in temperate and tropical tadpoles”

Acclimation of critical thermal limits in temperate and tropical tadpoles

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ABSTRACT

Thermal acclimation is one of the mechanisms by which species may face the predicted increase in temperatures due to global warming by rapid changes in thermal tolerance limits. Two complementary hypotheses have been proposed to explain broad patterns of variation in thermal plasticity among ectotherms. First, the *latitudinal hypothesis* predicts that organisms living in more variable environments could have greater capacity to adjust its thermal sensitivity through plasticity than those inhabiting more stable thermal environments. Because their greater seasonal thermal exposure, temperate ectotherms are predicted to have greater acclimation capacity than tropical exposed to narrower thermal boundaries. Second, it has been proposed that species with inherent higher basal thermal tolerance may reduce potential acclimation capacity (*trade-off hypothesis*). Here we compared critical thermal limits (both CT_{max} and CT_{min}) and Acclimation Ratio Response (ARR) of 12 tadpole species from temperate (Spain and Morocco, N = 5 spp.) and tropical latitudes (Ecuador, N = 7 spp.). Although temperate species are exposed to higher thermal variability than tropical ones, ARR_{min} did not differ between temperate and tropical species whereas ARR_{max} was slightly higher in tropical species. In any case, acclimation response found for CT_{max} was rather small in magnitude and probably insufficient to compensate alone, increased temperatures caused by climate change. We found a positive relationship between basal

CT_{max} and ARR_{max}, indicating that species with high thermal tolerance can also show higher plasticity. We did not find a trade-off between plasticity in lower and upper thermal limits.

Keywords: thermal acclimation, CT_{max}, CT_{min}, latitude, tadpoles

INTRODUCTION

Amphibians are the most vulnerable group of vertebrates, one-third or more of the 6,300 species are threatened with extinction (Wake & Vredenburg, 2008). Some causes of amphibian decline are, between others: habitat destruction, UV-B irradiation, emerging diseases (chytridiomycosis), the introduction of alien species, direct exploitation, and climate change (Beebee & Griffiths, 2005). Global warming has already increased 0.85 °C the mean Earth's temperature over the period 1880 to 2012 and it is predicted greater increases in mean environmental temperatures and the frequency of extreme thermal events in the future (Pachauri *et al*., 2014). Mitigating strategies in ectotherms to challenging increasing heating, imply a source of intrinsic biological mechanisms such as thermal acclimation plasticity, dispersal ability and fast microevolutionary shifts, which will presumably follow a temporal sequence of action and effectiveness in the process of environmental warming (Huey *et al*., 2012).

As ectotherms, amphibians strongly depend on environmental temperatures to perform its major biological functions such as: locomotion, growth, development, reproduction, survival, etc (Angilletta *et al*., 2002). Critical temperatures (CT_{max} and CT_{min}) delimit the range of body temperatures within which these processes can occur (Huey & Stevenson, 1979). The ability of organisms to deal physiologically with global warming basically relies on two factors: (i) how close organisms are to their thermal limits in nature and, (ii) the degree to which organisms are able to adjust, or acclimatize, their thermal sensitivity (Stillman, 2003; Somero, 2005). We define thermal acclimation, a form of phenotypic plasticity, as the capacity of organisms to modify their physiological characteristics in response to environmental temperatures to which they are exposed (Angilletta, 2009). As any other physiological traits, critical thermal

limits are susceptible to acclimate in response to different thermal environments (Somero, 2005). It has been shown that tropical organisms are currently living at habitat temperatures near upper thermal tolerance limits (Deutsch *et al*., 2008; Duarte *et al*., 2012). On the other side, temperate ectotherms are closer to their lower thermal limit than tropical species (at least those from lowland tropics) (Gutiérrez-Pesquera *et al*., 2016, Chapter 1). Regarding the relative proximity of physiological resistance with climatic extreme exposures, it has been proposed that species with the highest thermal tolerance, will have the lowest tolerance plasticity (*trade-off hypothesis*) (Stillman, 2004; Gunderson & Stillman, 2015). However the opposite trend has been observed in other taxa (Calosi *et al*., 2008a; Strachan *et al*., 2011), thus the question remains unresolved.

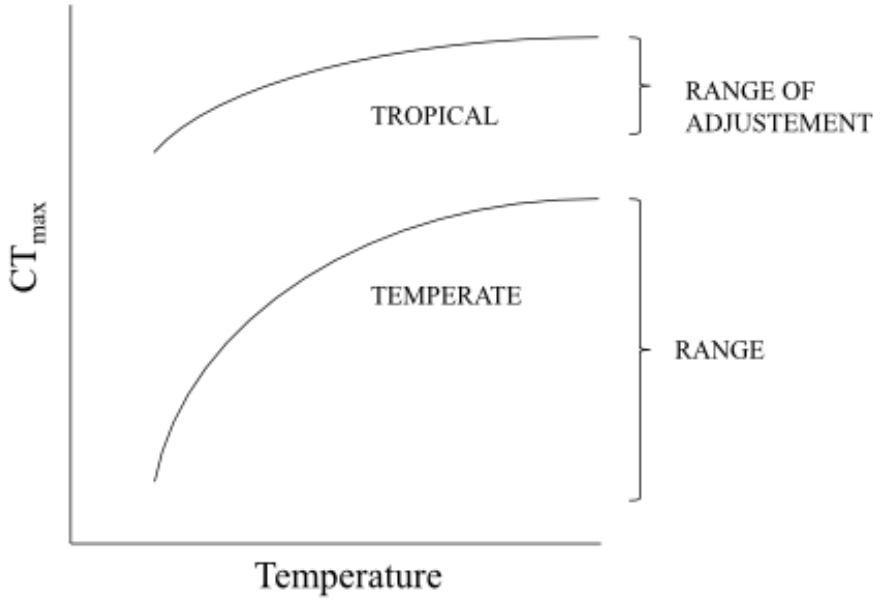


Figure 1. Expected acclimation scope response in tropical vs. temperate species. Adapted from Brattstrom (1968).

In two extensive studies, Brattstrom (1968;1970) analyzed potential acclimation response in adult amphibians and proposed that physiological adjustments would only have a selective advantage in the more variable temperate latitudes. Thereby, based on

the thermal stability of tropics, we would expect to find a greater capacity for acclimation in temperate species than in tropical ones (Fig. 1) (*latitudinal hypothesis*) (Gunderson & Stillman, 2015). *Latitudinal hypothesi*s predicts a pattern of increasing thermal tolerance plasticity as one moves from the equator to the poles based on the concomitant increase in thermal seasonality. This idea can be nested within the Climate Variability Hypothesis conceptual framework, developed by Stevens (1989), that basically states that a positive relationship exists between thermal tolerance range and the level of climatic variability experienced by taxa with increasing latitude (Bozinovic *et al*., 2011b). Interestingly, Brattstrom's analyses of two amphibian communities along two independent latitudinal gradients, North and Central America (1968) and Australia (1970), respectively, found no difference in the ability of acclimation between temperate and tropical species. However, he detected in both communities a positive relationship between thermal acclimation ability and geographic range size.

Considering that thermal seasonality at higher latitudes is mainly mediated by a disproportional decline in minimum temperatures than maximum temperatures (Addo-Bediako *et al*., 2000; Ghalambor *et al*., 2006), we should expect greater plasticity in cold tolerance rather than to heat resistance, especially in the temperate species (Addo-Bediako *et al*., 2000; Hoffmann *et al*., 2013). However, in a recent review analyzing global variation of acclimation response in insects, crustaceans, fish, amphibians and reptiles, no relationship was found between plasticity for both upper and lower thermal limits and latitude, although a trend was found for CTmin plasticity with thermal seasonality in some habitat types (Gunderson & Stillman, 2015). In addition, these authors found that the potential acclimation capacity was generally low and possibly will result insufficient to cope alone with the predicted increase in temperatures.

Our analysis focus on the larval stage of amphibians. Tadpoles can be considered as ideal organisms to examine the evolution of thermal tolerances because they are easy to obtain and maintain in large numbers in the laboratory. Tadpoles are aquatic and their body temperatures are equal to the temperature of surrounding environment, since the high heat capacity and conductivity of water conductivity, 23 times higher than air (Hillman *et al*. 2009). In addition, they do not suffer the associated dehydration response of terrestrial adult stages when heating. Despite being capable of regulating their body temperatures (Hutchison & Dupré, 1992), tadpoles may be limited in their search for favorable microhabitats. Thus, selection on both critical thermal limits and its plasticity may be stronger in aquatic larvae than in terrestrial adult amphibians (Feder & Hofmann, 1999; Huey *et al*., 2012).

Here we compared the critical thermal limits (CT_{max} and CT_{min}) and their acclimation scopes in 12 tadpole species from two different latitudinal communities: tropical (Ecuador, N = 7 spp.) and temperate (Iberian Peninsula and Morocco, N = 5 spp.). We analyzed the plasticity of CT_{max} and CT_{min} for individuals exposed to a broad gradient of experimental temperatures ranging between 18- 19 °C in the laboratory. To obtain the greatest level in acclimation scope, we select a range of acclimation temperature that presumably represents the broadest thermal conditions that each species were able to survive during four days of constant temperature acclimation. Therefore, we are able to obtain the maximum acclimation scope for both thermal limits in each species in order to examine the following hypotheses: First, do tadpole species from thermally variable environments acclimate more than those from more stable environments? (*latitudinal hypothesis*); second, is there a relationship between basal thermal tolerance and acclimation capacity? (*trade-off hypothesis*). Third, does

acclimation capacity to high temperatures impairs its counterpart tolerance plasticity at low temperatures, and *vice versa*?

MATERIAL AND METHODS

Sampling and study species

We studied 12 anuran species belonging to temperate and tropical latitudes, five temperate from the Iberian Peninsula and Morocco (between 27 °N and 43 °N, 0-2750 m.a.s.l) – *Bufo bufo*, *Barbarophryne brongersmai*, *Hyla meridionalis, Pelodytes ibericus* and *Rana temporaria* – and seven from Ecuador (between 1 °N and 1 °S, 0-3500 m.a.s.l.) – *Agalychnis spurrelli*, *Smilisca phaeota*, *Engystomops* sp., *Hypsiboas geographicus*, *Rhinella marina*, *Gastrotheca riobambae* and *Gastrotheca pseustes*. All tested species follow similar reproductive strategies with free aquatic larvae occupying temporary or permanent ponds. Tropical species were obtained along an altitudinal transect from 20 to 3500 m.a.s.l (see Table 1). Tadpoles were directly sampled in the field or raised from clutches in the laboratory within Balsa de los Sapos, Pontificia Universidad Católica, PUCE, Quito, captive breeding program. Whenever possible, we collected more than one clutch per species. Although we only examined a single population for each species, we assumed that response variation among species is larger than variation within species (cf. Klok & Chown, 2003). All examined tadpoles were collected in their natural ponds and transported to one of the two reference laboratories at particular study sites (Ecuador, Balsa de los Sapos, Pontificia Universidad Católica, PUCE, Quito, June 2014-August 2015 and Spain, EBD-CSIC, Sevilla, 2013-2014). Before the experiments, larvae were maintained at a similar and constant room temperature of 20 °C, with a natural 12 L:12 D photoperiod. Tadpoles were placed in plastic containers with similar densities and fed *ad libitum*. In the case of clutches, tadpoles were raised until Gosner stage 26 was reached.

Table 1. Sampling localities of the twelve analyzed species.

Species	Locality	Longitude	Latitude	Altitude (m)
Bufo bufo	Cabra, Córdoba (Spain)	-4.3686	37.4974	960
Hyla meridionlis	Toba, Córdoba (Spain)	-4.9014	37.9938	580
Barbarophryne brongersmai	Megouss (Morocco)	-9.3253	29.6747	500
Pelodytes ibericus	Toba, Córdoba (Spain)	-4.9014	37.9938	580
Rana temporaria	Cortegueros, Asturias (Spain)	-4.9396	43.3174	650
Agalychnis spurrelli	Durango (Ecuador)	-78.6232	1.0412	200
Smilisca phaeota	Durango (Ecuador)	-78.6196	1.0363	20
Engystomops sp.	Puyo (Ecuador)	-77.7203	-1.4063	1000
Hypsiboas geographicus	Puyo (Ecuador)	-77.8206	-1.4443	1000
Rhinella marina	Mindo (Ecuador)	-78.7700	-0.06143	1300
Gastrotheca riobambae	Quito (Ecuador)	-78.4639	-0.1873	2900
Gastrotheca pseustes	Guaranda (Ecuador)	-78.75938	-1.33673	3500

Acclimation treatments

Previously to conduct tolerance assays, tadpoles were acclimated to constant temperatures during four days. This acclimation period was chosen as the minimum time required to stabilize CT_{max} and CT_{min} after a large change in acclimation temperature (Hutchison, 1961). Additionally, this relatively short time of acclimation may avoid mortality and other undesirable effects due to long exposure to constant temperatures, especially at extreme temperatures. All species were acclimated to a similar range of temperatures (18 °C or 19 °C), ranging from 9 °C to 27 °C for temperate species and 15 °C to 34 °C for tropical ones with the exception of stenothermal species: *Engystomops* sp., which showed higher mortality after two days of acclimation at the lowest temperature (15 °C) and no tadpole survival at the highest

temperature (34 °C). Plasticity response in critical thermal limits was studied through four constant acclimation treatments: 9 °C - 15 °C - 20 °C and 27 °C for temperate and 15 °C - 20 °C - 27 °C and 34 °C for tropical species.

Table 2. Mean ± SD (°C) temperature for the constant acclimation treatments during four days.

Acclimation Treatment	$\bar{X}$ ± SD (°C)
9	8.6 ± 0.5
15	14.9 ± 0.4
20	20.1 ± 0.1
27	27.0 ± 0.1
34	33.8 ± 0.3

We haphazardly collected up to 128 tadpoles (N=16 tadpoles per treatment) of each species previously maintained at 20 °C. Then, we randomly distributed them into the four acclimation temperatures. Larvae were individualized in 400 mL plastic glasses filled with 300 mL of dechlorinated tap water that was continuously aerated with an air pump system and fed *ad libitum*. Experimental acclimation temperatures were achieved with a portable fluid heater with regulation adjustment, utility model Nr: U201431698, inside a chamber setting to 20 °C (see Table 2, for mean values and standard deviation of the temperatures for each treatment) and a constant photoperiod of 12 L: 12 D.

Determination of critical thermal limits and plasticity

We examined between 10-18 tadpoles for each thermal acclimation treatment and estimate (CT_{max} and CT_{min}). Some of the species showed low survivorship during acclimation period when exposed to the extreme temperatures (15 °C and 34 °C); resulting in a reduced number of observations for some treatments (see Table S1 and S2 in Annexe 4 for details). Each tadpole was weighed immediately before the beginning of the test. Both thermal tolerance limits were determined using the Hutchison's

dynamic method (Lutterschmidt & Hutchison, 1997a) in which each animal was exposed to a constant heating/cooling rate (ΔT=0.25 °C min^{-1}) until an end point is attained. The end-point was signalled for both thermal limits as the point at which the tadpoles become motionless and fail to respond to external stimuli by prodding 10 consecutive hits applied each two seconds with a wooden stick. Each tested tadpole was placed individually in 100 mL containers with dechlorinated tap water in a refrigerated heating bath of 15 L (HUBER K15-cc-NR) at a start temperature of 20 °C. Because of the small size of tadpoles we assumed that body temperature was equivalent to water temperature (Lutterschmidt & Hutchison, 1997b) and then CT_{max} and CT_{min} were recorded as the water temperature beside the tadpole measured with a Miller & Weber quick-recording thermometer (to the 0.1 °C). After a tolerance limit was determined, we immediately transferred tadpoles to water at the room temperature (20 °C) to allow for recovery, after which their Gosner stage was registered. Tadpole survival was verified a few minutes and 24 hours after the end of the heating/cooling assays. Each individual was tested only once.

Plasticity for both thermal limits ($CT_{max/min}$) was calculated for each species as the acclimation response ratio (ARR) as the slope of the line describing the change in thermal tolerance with a given change in acclimation temperature, calculated as the difference between the mean resistance value for the highest and lowest experimental acclimation temperature divided by the thermal acclimation range: $\Delta CT_{max-min}/\Delta T$, or, in other words, as the change in the CT_{max}/CT_{min} per degree change in acclimation temperature (Claussen, 1977; Gunderson & Stillman, 2015). Therefore, ARR is a dimensionless variable. An ARR of 1, indicates a positive, 1 °C shift in thermal limit for each 1 °C shift in acclimation temperature, suggesting complete compensation for

acclimation temperature. In contrast, an ARR of 0 indicates that the trait is unaffected by the acclimation treatments (Kingsolver & Huey, 1998; Beitinger *et al*., 2000).

Statistical analyses

To check for possible effects of non-independence due to evolutionary relationship between studied species, all statistical analyses were controlled by phylogeny. We extracted a phylogenetic tree containing the 12 analyzed species from Pyron & Wiens (2011) (see Fig. S1, Annexe 4). Given that phylogentic comparative methods can present problems when estimating the phylogenetic signal (λ) when there are less than 30 species (Martins, 1996; Blomberg *et al*., 2003; Münkemüller *et al*., 2012), we reported the results for both conventional and comparative approaches. To contrast the trade-off hypothesis, we analyze correlations between ARRs and basal critical thermal limits by employing both OLS, and phylogenetic generalized least squares (PGLS) analyses under a Brownian motion model of evolution using the R package CAPER (Orme *et al*., 2013). We used conventional and phylogenetic ANOVA, R package GEIGER (Harmon *et al*., 2008), to compare ARR values between tropical and temperate species. To compare the amount of plasticity of each critical thermal limit (CTs) in tropical and temperate communities, we apply the following mixed model ANOVA: CTs = Acclimation + Community + Community (Spp) + Acclimation * Community + Acclimation * Community (Spp) + Error. Community (Spp) with Spp as random factor nested inside Community and Community and Acclimation as fixed effect. Acclimation temperatures were codified as follows: 1 (9 °C temperate and 15 °C tropical), 2 (15 °C temperate and 20 °C tropical), 3 (20 °C temperate and 27 °C tropical), and 4 (27 °C temperate and 34 °C tropical). The interaction Community x Acclimation explicitly tests the difference in CTs plasticity between tropical and

temperate species, i.e. whether the slopes of the reaction norms across acclimation temperatures differ between regions (tropical or temperate).

RESULTS

General description

Both thermal limits increased with acclimation temperature, although CT_{min} varied near linearly in temperate species, three tropical species showed ,contrarily to expected, a reversal acclimation response to the lowest temperature (15 °C) (see *Agalichnys spurrelli*, *Hypsiboas geographicus* and *Rhinella marina,* in Fig. 3 right). Mean CT_{max} ranged from 36.1 ± 0.1 °C in *Rana temporaria* to 44.4 ± 0.1 °C in *Rhinella marina*, while mean CT_{min} fell between -3.6 ± 0.1 °C in *Gastrotheca pseustes* and 9.8 ± 0.2 °C in *Hypsiboas geographicus* (Fig. 2 and 3). Interestingly, two species reached crystallization point of water during CT_{min} trials at low acclimation temperatures: one temperate species, *Rana temporaria*, and, the tropical species inhabiting high páramos of Ecuadorian Andes, *Gastrotheca pseustes.* Crystallization point of water coincides with an exothermic reaction at the onset of water freezing determining a quick raise in water temperature and, thus, invalidating the cold tolerant assay when it happens. Acclimation response ratios were generally low in both geographical regions, ranging between 0.04 to 0.20 for CT_{max}, and between 0.02 and 0.22 for ARR_{min} (Table 3). Temperate species showed the lesser plastic response for both CT_{max}, *Bufo bufo*, and CT_{min}, *Hyla meridionalis*. The greatest acclimation capacities for both limits were found in two tropical species (*Rhinella marina* and *Agalychnis spurrelli,* respectively). A tropical species (*Engypstomops* sp.) was, however, especially stenotherm. Individuals of this species did not survive to acclimation temperatures above 27 ° C and had a low survival rate for the treatment at 15 ° C.

Figure 2. Acclimation scope for CT_{max} in temperate (left) and tropical (right) tadpoles. Means ± standard errors.

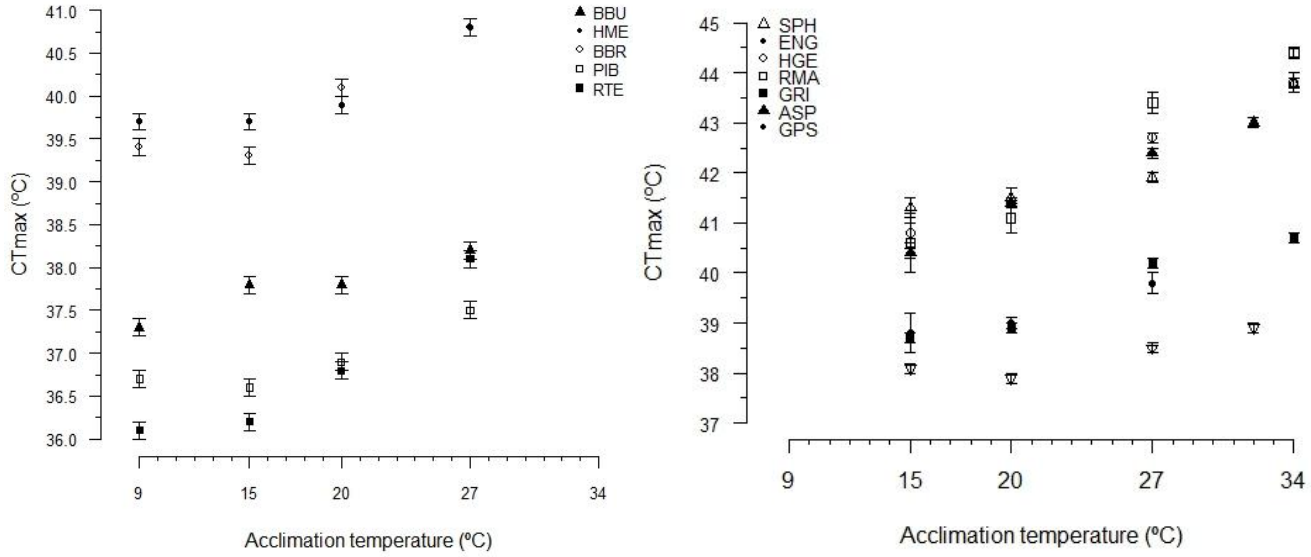


Figure 3. Acclimation scope for CT_{min} in temperate (left) and tropical (right) tadpoles. Means ± standard errors. Dashed lines denotes absolute minimum (crystallization temperature).

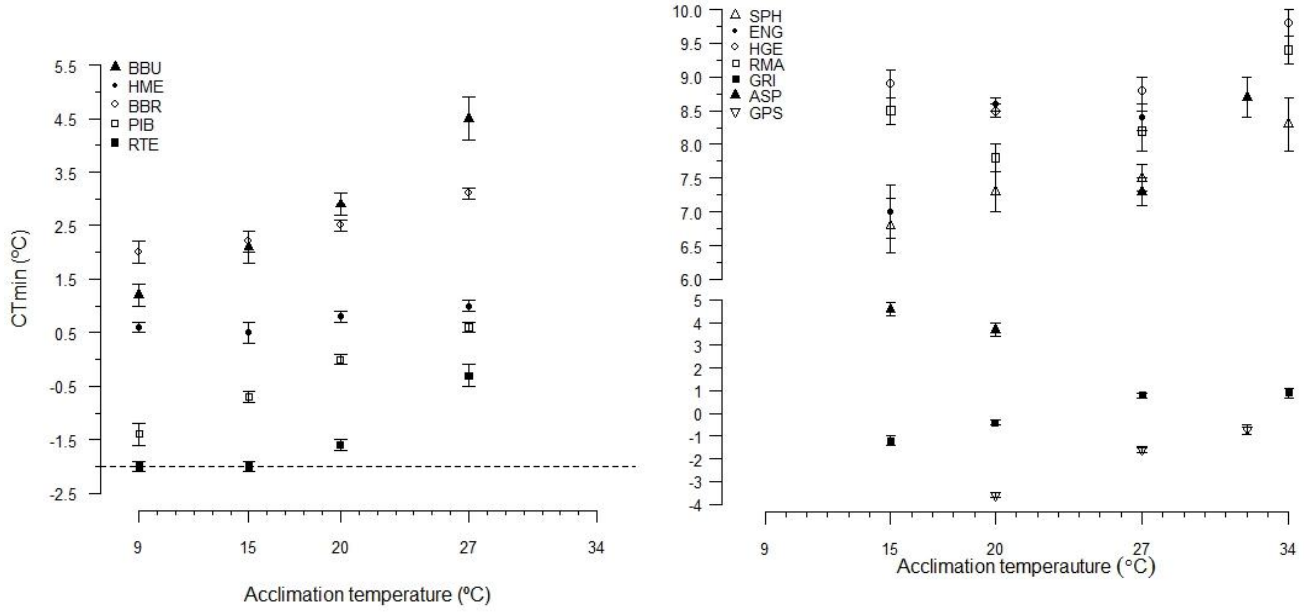


Table 3. Plasticity for both thermal limits CT_{max} (ARR_{max}) and CT_{min} (ARR_{min}) for each analyzed species and the mean value for each geographical region. * denotes marginally significant differences between communities.

Species	Community	ARR_{max}	Mean ARR_{max}	ARR_{min}	Mean ARR_{min}
Bufo bufo	Temperate	0.05	0.07*	0.18	0.10
Hyla meridionalis		0.06		0.02	
Barbarophryne brongersmai		0.08		0.06	
Pelodytes ibericus		0.04		0.11	
Rana temporaria		0.11		0.14	
Smilisca phaeota	Tropical	0.13	0.12*	0.08	0.12
Engystomops sp.		0.09		0.11	
Hypsiboas geographicus		0.16		0.05	
Rhinella marina		0.20		0.05	
Gastrotheca riobambae		0.11		0.11	
Gastrotheca pseustes		0.04		0.21	
Agalychnis spurrelli		0.14		0.22	

Table 4. Comparison between communities of the variability in the environmental temperatures for some of the analyzed species. Temperature data obtained from microenvironment (dataloggers). All values are in °C. * Significant t-test ($P < 0.001$), $^{+}$ marginally significant result for t-test ($P = 0.09$).

Species	Community	tmax	tmin	seasonal range	daily range	Mean seasonal range	Mean daily range
Bufo bufo	Temperate	25.1	7.5	17.7	3.4	27.8*	5.3^{+}
Hyla meridionlis		36.1	5.9	30.2	7.2		
Pelodytes ibericus		33.3	1.4	31.8	5.0		
Rana temporaria		32.1	0.5	31.6	5.6		
Agalychnis spurrelli	Tropical	26.2	24.0	2.2	0.7	8.2*	2.9^{+}
Gastrotheca pseustes		19.7	8.0	11.7	4.0		
Gastrotheca riobambae		25.3	8.5	16.8	4.9		
Hypsiboas geographicus		23.1	18.7	4.4	1.0		
Rhinella marina		20.9	19.2	1.7	0.4		
Smilisca phaeota		36.8	24.6	12.2	6.3		

Latitudinal hypothesis

Tropical and Temperate species differed in their critical thermal limits and plasticity (Table 4). However the magnitude of the ARR_{max} was small and the differences were only marginally significant (ANOVA, $F_{1,10} = 4.90$, $P = 0.05$, *phy-P* = 0.07) and no significant differences was found for ARR_{min} between regions (ANOVA, $F_{1,10} = 0.18$, $P = 0.69$, *phy-P* = 0.74) (Table 3).

Table 4. Influence of community (COM), species identity (SP) and acclimation temperature (ACCL) on a) upper thermal tolerance and b) lower thermal tolerance of the analyzed species.

	Source	Df	SS	MS	F-value	P
(a)	Intercept	1	864784.9	864784.9	7318.05	<0.001
	COM	1	893.8	893.8	7.48	0.021
	COM (SP)	10	1246.2	124.6	53.34	<0.001
	ACCL	3	309.1	103.0	44.30	<0.001
	COM x ACCL	3	43.8	14.6	6.23	0.002
	COM(SP) x ACCL	29	69.5	2.4	6.75	<0.001
	Error	571	202.9	0.4		
(b)	Intercept	1	5265.4	5265.4	9.51	0.01
	COM	1	2806.9	2806.9	5.00	0.05
	COM (SP)	10	5624.3	562.4	96.16	<0.001
	ACCL	3	249.5	83.2	14.18	<0.001
	COM x ACCL	3	10.05	3.3	0.57	0.64
	COM(SP) x ACCL	29	173.2	5.9	11.43	<0.001
	Error	535	279.58	0.5		

Figure 4. Acclimation of upper thermal limits (CT_{max}) in temperate (left) and tropical tadpoles (right). Slopes represent the differences between CT_{max} values form the lower acclimation temperature (T °C) and the higher acclimation treatment (T + 18/19 °C), for temperate and tropical species, respectively (see Table 5). BBU: *Bufo bufo*, HME: *Hyla meridionalis*, BBR: *Barbarophryne brongersmai*, PIB: *Pelodytes ibericus*, RTE: *Rana temporaria*, SPH: *Smilisca phaeota*, ENG: *Engystomops* sp., HGE: *Hypsiboas gepgraphicus*, RMA: *Rhinella marina*, GRI: *Gastrotheca riobambae*, GPS: *Gastrotheca pseustes*, ASP*: Agalychnis spurrelli*.

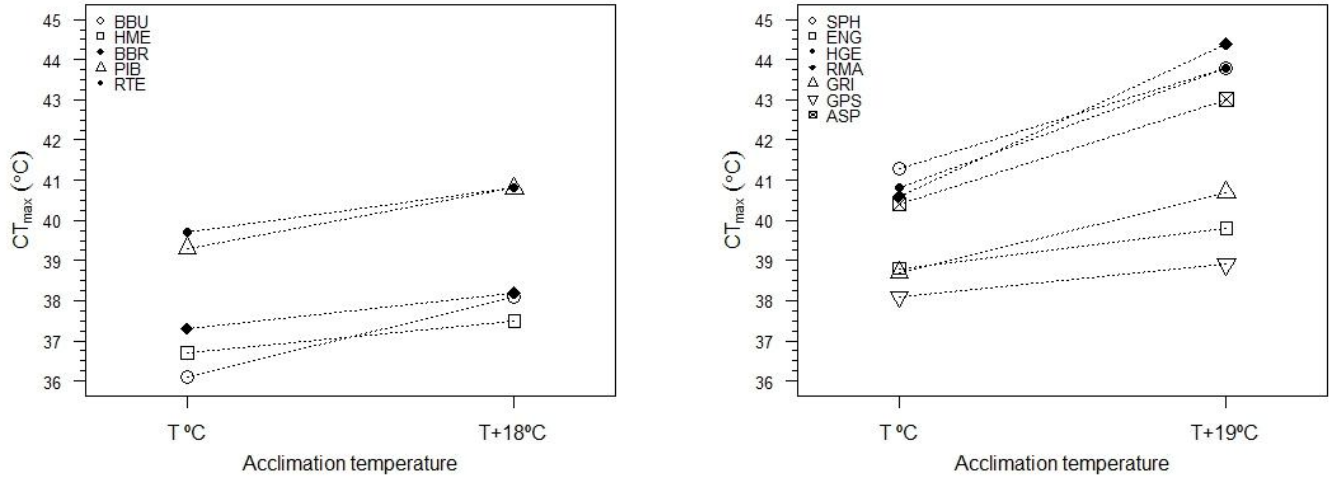
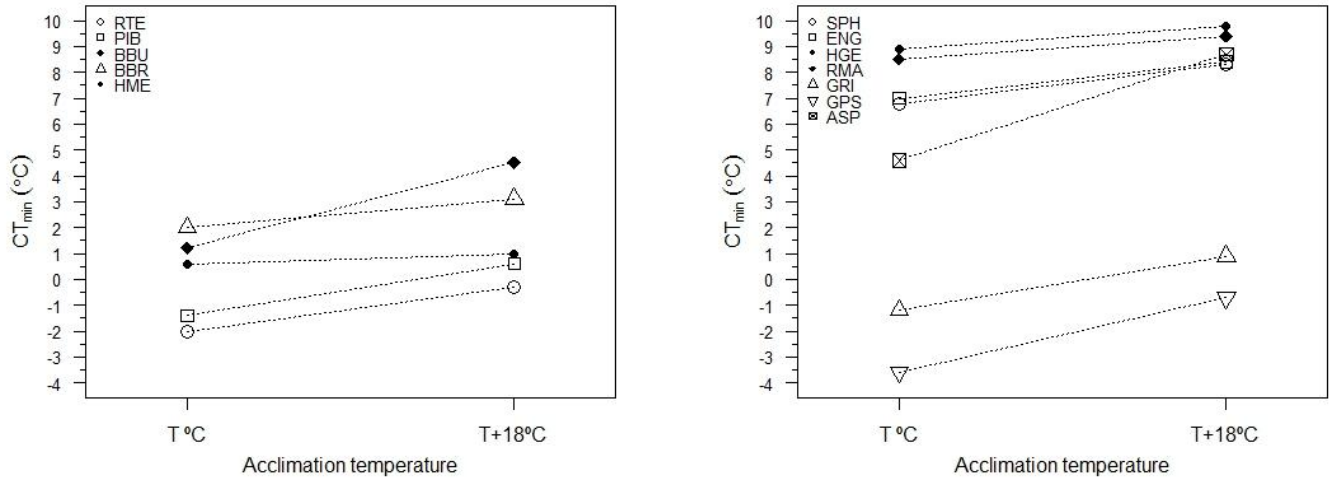


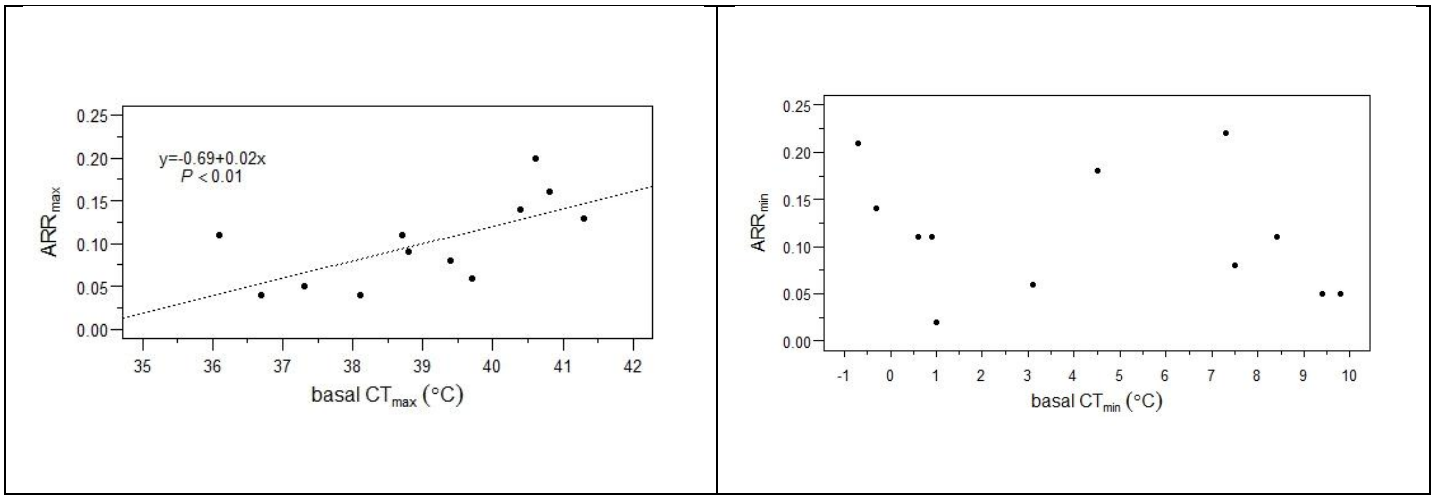
Figure 5. Acclimation of lower thermal limits (CT_{min}) in temperate (left) and tropical tadoples (right). Slopes represents the differences between CT_{min} values form the lower acclimation temperature (T °C) and the higher acclimation treatment (T + 18/19 °C), for temperate and tropical species, respectively (see Table 5). BBU: *Bufo bufo*, HME: *Hyla meridionalis*, BBR: *Barbarophryne brongersmai*, PIB: *Pelodytes ibericus*, RTE: *Rana temporaria*, SPH: *Smilisca phaeota*, ENG: *Engystomops* sp., HE: *Hypsiboas geographicus*, RMA: *Rhinella marina*, GRI: *Gastrotheca riobambae*, GPS: *Gastrotheca pseustes*, ASP*: Agalychnis spurrelli*.

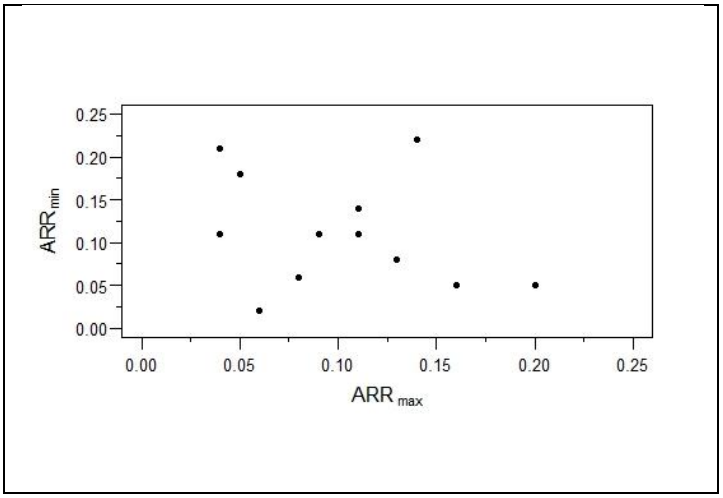


Trade-off hypotheses

Species with the highest basal CT_{max} (acclimated to the lowest temperatures) also possess the greatest acclimation capacity (ARR_{max}) (Fig. 6a), a significant positive relationship was found between basal CT_{max} and ARR_{max} (PGLS, $F_{1,10} = 8.13$, $P < 0.05$; OLS, $F_{1,10} = 7.122$, $P < 0.05$, $R^2 = 0.42$). By contrast, no relationship was detected between basal CT_{min} and ARR_{min} (Fig. 6b). Acclimation capacity to upper and lower thermal limits (ARR_{max} and ARR_{min}) was also decoupled (Fig. 6c).

Figure 6. Relationships between (a) critical thermal maxima (CT_{max} °C) and its acclimation ability (ARR_{max}), (b) critical thermal minima (CT_{min} °C) and its acclimation ability (ARR_{min}) and (c) acclimation ability to upper and lower thermal limits. Dashed lines in (a) represents significant relationships (PGLS).





DISCUSSION

Acclimation of thermal physiology involves a plethora of proximal biochemical processes such as: the expression of allozymes (Baldwin & Hochachka, 1970), modifications in fluidity and viscosity of the cell membrane through its lipid composition (Cossins & Prosser, 1978), oxygen limitation (Portner & Knust, 2007) or alterations to the intracellular environment mediated by the synthesis of heat shock proteins (Horowitz, 2001). Some of these changes may be irreversible involving enhanced performance to high temperatures at the expense of reduced performance to low temperatures and *vice versa* (Angilletta, 2009). Thereby, acclimation to higher temperatures usually determines, in most analyzed species, higher absolute values of both CT_{max} and CT_{min}, and *vice versa*. We did not found, however, a trade-off between acclimation scopes (ARRs) of cold and heat tolerances, which suggests that the underlying processes determining acclimation capacity may be qualitatively different in both resistance limits (Sinclair & Roberts, 2005).

Our data show that tropical species display higher ARR_{max} than temperate species. Yet, this result needs to be interpreted with caution because: (1) The absolute difference in slopes of the tropical and temperate species reaction norms (ARR) was only 0.05°C (Table 3) and, (2) we found high interspecific variability in slopes within region. Thus, acclimation response varied greatly between species (Fig. 2-5) and it was not related to expected environmental variability (Table 4). ARR_{min} did not differ, in any way, between communities. The weak latitudinal variation in ARR_{max}, agrees with former studies, including adult amphibians (Brattstrom, 1968) and other ectotherms (Stillman & Somero, 2000; Overgaard *et al*., 2011b; Cooper *et al*., 2012) but contrast to that found in tidal organisms, where tropical taxa exhibited lower acclimatory potential

than temperate counterparts (Stillman, 2003; Vinagre *et al*., 2015). Additionally, we could not find latitudinal divergence in ARR_{min}, although a positive relationship with seasonality was found in reptiles (Gunderson & Stillman, 2015). An absence of adaptive plasticity in upper thermal tolerances was also found for some tadpole species, between two contrasting thermal environments, the Atlantic rain Forest, Mata Atlantica and the dry open forest Caatinga (Simon *et al*., 2015). Our results and these previous evidences revealed that acclimation responses are small and possibly insufficient to offset possible increases in mean and extreme temperature due to global warming, especially in the case of tropical species living at temperatures close to theirphysiological resistances, but also, this low mitigating response is found for cold resistance in some temperate species that may be exposed to peak low temperatures.

The adaptive thermal hypothesis would predict that plasticity must have advantages over individuals or species that do not present such mechanisms (*beneficial acclimation hypothesis*, Wilson & Franklin, 2002) . Thus, we could expect that those species subjected to greater environmental variation must have greater capacity for acclimation (Janzen, 1967; Brattstrom, 1968, 1970; Stevens, 1989). However, thermal acclimation scope may have limits and incur in disadvantages, such as energetic costs and time lags which may constrain its evolution (DeWitt *et al*., 1998). The net benefit of acclimation depends, for example, on the energy required for change and the predictability of the environment. An additional potential constraint is the amount of genetic variation in critical thermal limits and its plasticity to allow evolution (Hoffmann *et al*., 2013). At a macroevolutive perspective, it has been widely recognized that CT_{max} is a conservative trait with little or no variation across latitudinal gradient (Addo-Bediako *et al*., 2000; Kellermann *et al*., 2012b; Gutiérrez-Pesquera *et al*., 2016).

An important concern regarding the ecological relevance of thermal acclimation in organisms, is that ectotherms usually are exposed to thermal variability in their environment (Angilletta *et al*., 2006), whereas the traditional approach in the study of acclimation is by generally exposing the organisms to a constant temperature that possible yields inaccurate information about its real adaptation capacity in its natural environment (Niehaus *et al*., 2006). Acclimation response based in constant temperatures may differ from natural fluctuating acclimatization, for example, by inducing deleterious effects to chronic exposure, especially at stressful temperatures, that could overwhelm any beneficial effect of thermal plasticity (Bevelhimer & Bennett, 2000; Wilson & Franklin, 2002; Podrabsky & Somero, 2004; Niehaus *et al*., 2012). Further efforts must been made in order to analyze the changes in thermal tolerance of organisms acclimated to simulated natural conditions (see Chapter 5) and additionally by employing ecologically relevant rates of heating/cooling for the estimates of critical thermal limits (Chown *et al*., 2009b; Rezende *et al*., 2011).

The trade-off hypothesis between basal tolerance and acclimation capacity of the thermal limits remains controversial. Unlike the results obtained for intertidal porcelain-crabs (Stillman, 2003), or in upper limits in *Drosophila* (Hoffmann *et al*., 2005), we did not find a trade-off between basal maximum tolerance and potential of acclimation. On the contrary, species with higher CT_{max} also showed higher acclimation capacity. Our results are similar to those obtained for other aquatic organisms such as diving beetles (Calosi *et al*., 2008a) and lower thermal limits in *Drosophila* (Strachan *et al*., 2011). Rather than respond to general trends, it seems that the relationship between basal thermal tolerance and acclimation capacities rely on species-specific particularities. Summarizing, not all species with higher intrinsic values of tolerance should have a reduced capacity for acclimation.

CHAPTER 5

“The effect of constant vs fluctuating acclimation on critical thermal limits in three temperate tadpoles”

The effect of constant vs fluctuating acclimation on critical thermal limits in three temperate tadpoles

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ABSTRACT

Most global warming scenarios suggest that ectotherms are prone to suffer thermal extreme conditions, especially through the increment in the frequency and duration of hot waves. Thermal acclimation is one of the mechanisms by which species could face with the predicted increase in thermal stress through rapid changes in thermal tolerance limits. However, current estimates rely on unrealistic constant temperature treatments that may inaccurately predict the acclimatory scope of organisms. Here, we examined acclimation effects on the critical thermal limits (CT_{max} and CT_{min}) in three species of temperate tadpoles differing in basal thermotolerance and thermal exposure: *Rana temporaria (Rte)*, *Pelodytes ibericus* (*Pib*) and *Hyla meridionalis (Hme)*. Individuals were acclimated during four days to several constant treatments ranging from 9ºC to 27ºC, (C9-C15-C20-C24-C25-C27) and variable acclimation regimes, simulating natural daily thermal fluctuations in ponds (DTF), under three different scenarios: *cool* (16-24 ºC, F24), *warm* (17-30ºC, F30) and, additionally for *Hme*, *hot* (17-35ºC, F35) days. Results indicate species-specific responses; with insensitive responses in both CT_{min} in thermophylic *Hme* and CT_{max} for the low heat tolerant *Pib*. Acclimation under *hot* and *warm* DTF regimens increases heat tolerance in *Hme*, but decreased it in *Pib*, respectively, with no effects of warm DTF in *Rte*. Otherwise, *cool* DTF, increase cold resistance in both *Pib* and *Hme*, whereas no effect was found in *Rte* in either *warm* and *cool* DTFs. Finally, multiple daily heating episodes increase CTmax in *Hme* and *Pib*

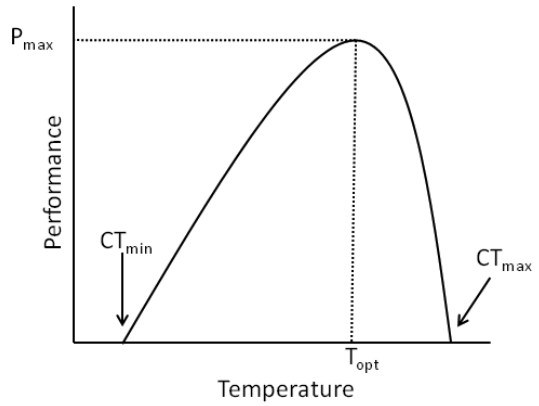
and decrease CT_{min} in *Hme*. These results illustrate how DTF affect differentially thermal resistance limits and suggest that warming vulnerability based on thermal limits under constant acclimation conditions could be imprecise when estimating the risk of ectotherms to suffer from acute thermal stress.

Keywords: CT_{max}, CT_{min}, acclimation, thermal variability, amphibians

INTRODUCTION

Temperature has pervasive effect on life, affecting organisms at different scales ranging from the molecular level, i.e. rates of chemical reaction (Hochachka & Somero, 2002), to the ecosystem level, i.e. ecological interactions (Dunson & Travis, 1991). In ectotherms, thermal performance curve describe how physiological functions are affected by body temperature (Angilletta *et al*., 2002). Typical performance curves in ectotherms are non-linear and asymmetric with a rapid decline in fitness once temperatures exceed the thermal optimum (Fig. 1). Some common measures of performance include locomotion, assimilation, growth, development, fecundity and survivorship (Angilletta, 2009).

Figure 1. A typical thermal performance curve in ectotherms. CT_{max}, critical thermal maxima; CT_{min}, critical thermal minima; T_{op}, optimum temperature; P_{max}, maximum performance.



Critical thermal maxima (CT_{max}) and, in an analogous way, critical thermal minimum (CT_{min}), was initially defined in lizards as the temperature at which locomotor activity becomes disorganized and the animal loses the ability to escape situations that in natural conditions lead to death (Cowles & Bogert, 1944; Lutterschmidt & Hutchison, 1997a). Thus, critical thermal limits are key parameters of thermal

performance curve of ectotherms determining the thermal range of temperatures within a species may grow, reproduce or survive (John-Alder *et al*., 1988; Deutsch *et al*., 2008).

As other traits, critical thermal limits may be subjected to phenological plasticity, named as thermal acclimation, or the capacity of organisms to changes their physiological characteristics in response to environmental temperatures to which they are exposed (Lagerspetz, 2006; Angilletta, 2009). Acclimation of thermal physiology involves a plethora of proximal processes: expression of allozymes (Baldwin & Hochachka, 1970), modifications in fluidity and viscosity of the cell membrane through its lipid composition (Cossins & Prosser, 1978), oxygen limitation (Portner & Knust, 2007) or alterations to the intracellular environment as the synthesis of heat shock proteins (Horowitz, 2001), between others. Some of these changes may be irreversible and involve enhanced performance to high temperatures at the expense of reduced performance to low temperatures and *vice versa* (Angilletta, 2009).

Although beneficial acclimation plasticity has been considered the most direct and effective mechanisms that ectotherms may employ to face heat impacts through predicted increase in the frequency and duration of heat waves (Somero, 2005; Chown *et al*., 2010; Huey *et al*., 2012; Pachauri *et al*., 2014), recent comparative analysis has shown the limited capacity of ectotherms to acclimate, which may compromise the net benefit of this compensatory mechanism (Chapter 4, Gunderson & Stillman, 2015). However, most current performance curves and critical thermal limits have traditionally been estimated undergoing organisms to a certain range of constant temperatures in the laboratory (Niehaus *et al*., 2012). This methodological procedure can be subjected to some theoretical and practical criticism (Chown *et al*., 2009a). Because ectotherms usually are exposed to thermal variability in their natural environment (Angilletta *et al*.,

2006; Niehaus *et al*., 2006, 2012), the traditional constant temperature acclimation approach may yield unaccurate responses about the real adaptation capacity of ectotherms in nature. Environmental variance can have important and predictable biological consequences that cannot be inferred from average environmental conditions (Ruel & Ayres, 1999). In addition, it is expected that climate change not only affects mean environmental temperatures but also thermal variability with predicted increase in the frequency and duration of extreme events (Folguera *et al*., 2009; Diffenbaugh & Field, 2013; Pachauri *et al*., 2014; Vasseur *et al*., 2014). Heat waves lasting several days or even weeks may impose recurrent heating stress and organisms may respond by inducing both medium-term developmental acclimation or short-term hardening (Hercus *et al*., 2003; Hoffmann *et al*., 2003; Loeschcke & Sorensen, 2005) occurring at consecutive days. We predict that both developmental acclimation and short-term daily hardening can incur in costs and benefits whose differential balance will determine the final expression of upper and lower thermal limits (DeWitt *et al*., 1998; Krebs & Feder, 1998; Hoffmann & Loeschcke, 2002; Wilson & Franklin, 2002). Therefore, to understand how species would respond to the effects of global warming, it is necessary to analyze the effects of thermal variation on the plasticity of the thermal limits (Bozinovic *et al*., 2011a; Overgaard *et al*., 2011b; Paaijmans *et al*., 2013).

Despite the paucity of previous work (Hutchison & Ferrance, 1970; Feldmeth *et al*., 1974; Feder, 1985a, 1985b; Houston & Gingras-Bedard, 1994; Roberts *et al*., 1997), some recent studies have recovered interest to examine the effects of thermal variability in physiological traits plasticity in ectotherms in the last years (Folguera *et al*., 2009; Paaijmans *et al*., 2010, 2013; Bozinovic *et al*., 2011a; Overgaard *et al*., 2011b; Kellermann *et al*., 2012b; Niehaus *et al*., 2012) including tadpoles (Turriago *et al*., 2015). Additionally, recurrent peaks of heating may determine differential CT_{max}

response and have implication in lower thermal resistance. Although some research on repeated freezing and cold stress has been conducted in insects (e.g. Marshall & Sinclair, 2009), to our knowledge this is the first time than the effect of constant versus variable acclimation and the consequences of repeated heating stress on thermal resistance limits have been assessed in vertebrates and, specifically, in amphibian tadpoles.

We studied three temperate amphibian species during their aquatic larval stage: *Pelodytes ibericus*, *Rana temporaria* and *Hyla meridionalis* which differ in their thermal exposure due to contrasting breeding phenologies and geographical distributions. Additionally, the examined species exhibit different thermal physiology both in basal upper and lower temperature tolerances (Gutiérrez-Pesquera *et al*., 2016), and thermal sensitivity in several life history traits and survival (Katzenberger, 2014; Tejedo, Gutiérrez-Pesquera *et al*., unpublished data). Each species were undergone to constant and variable acclimation regimes. Based on previous thermal profiles obtained from natural breeding ponds, variable acclimation treatments simulated natural daily thermal fluctuations (DTF), representing *cool* or early season thermal oscillations (*cool days*), and hot fluctuations typical at the end of the larval season when ponds are drying (see Annexe 5, Fig. A1-A3). In order to compare constant vs fluctuatuing acclimation effects, we incorporate in the experimental design constant acclimation treatments representing the mean temperature of DTF regimes (see Annexe 5, Fig. B1-B3). In addition, we examined the effect of recurrent multiple heat stress by subjecting tadpoles to either a single at the last day or four consecutive heating peaks during the four days of pre-test acclimation.

These set of thermal treatments allow us to examine the following specific issues:

a) Analyze the interspecific variation in thermal sensitivity of both thermal limits through constant temperatures over a broad natural range (9ºC-27ºC, C9-C27).

b) Whether the acclimation scope differed interspecifically by increasing either constant or pond daily fluctuating temperatures (DTF), simulating *cool* (16-24 ºC, F24), *warm* (17-30ºC, F30) and, *hot* (17-35ºC, F35) days, whose average values are equivalent to either constant C20, C23.5 and C25, respectively), and

c) whether medium-term fluctuating acclimation at high temperatures differed of short-term hardening occurring at a single or multiple consecutive days, by examining hot DTF reaching sublethal but stressful temperatures.

MATERIAL AND METHODS

Sampling of study species and environmental thermal data

Samplings of tadpoles and determination of their thermal limits were carried out between February and May 2013. Although only one population of each species was analyzed, we assumed that thermal tolerance variation among species is larger than within species (c.f Klok and Chown, 2003). *Rana temporaria* (*Rte*) was obtained from the locality of Purón (North of Spain, 43°22'45."N; 4°41'55."O). *Pelodytes ibericus* (*Pib*) and *Hyla meridionalis* (*Hme*) were sampled from Toba locality (Córdoba, South of Spain, 37°59'4"N, 4°54'5."O). Water temperature was monitored by dataloggers (HOBO pendant) deployed in the bottom of the breeding ponds recording temperature every 15 min. We determined temperature profile and the absolute maximum pond temperature (T_{max}) from a set of ponds monitored during several years (2009-2015) for *Pib* and *Hme* and for different ponds and years (2002-2014) for *Rte*. All species breed in temporary ponds, but reproductive timing and thermal environments do differ between them. *Rte* is a widespread Eurosiberian anuran that breeds in northern Spain from

autumn in lowlands lasting until summer at high altitudes. Tadpoles are exposed to low temperatures, especially at the start of breeding season, at high altitudes, when the pond can get frozen (Gutiérrez-Pesquera L.M., personal observation). Based on previous analyses, maximum temperatures recorded in the ponds were 32 ºC (Fig.A.1, Annexe 5) and thermal optimum for both growth and locomotion performance were around 24 ºC (Katzenberger, 2014; Tejedo *et al.*, unpublished data). *Pib* and *Hme* are southwestern Europe distributed species that occupy syntopic ponds but differ in breeding phenology. *Pib* is an early breeder from October to early May when the last larvae generally reach metamorphosis before pond desiccate, with optimal growth and swimming performance for 24ºC and maximum environmental temperature of 34 ºC (Fig.A.2, Annexe 5). Finally, *Hme* is a late breeder with a larval period lasting until pond desiccate in May and early June, and therefore, their larvae are exposed to higher temperatures (until 36 ºC) (Fig.A.3, Annexe 5). *Hme* can be considered a warm-adapted species, showing high thermal tolerance to heat (Duarte et al., 2012; Gutiérrez-Pesquera et al., 2016), optimum temperature performance (26 ºC and 35 °C for growth and swimming, respectively) and a wider thermal breadth (Tejedo *et al*, unpublished data). Tadpoles were collected in temporary ponds using sampling nets and transported to the reference laboratory at Seville (Spain, EBD-CSIC), being maintained in trays or plastic buckets with a similar larval density placed inside climatic chambers under constant conditions photoperiod (12:12 L: D) and temperature (20 °C), and feed *ad libitum* until the beginning of the experiment.

Acclimation treatments

In order to examine acclimation scope in thermal limits, we acclimated tadpoles during four days previous thermal tolerance assays. We used several constant

temperatures over a broad natural range (9ºC, 15ºC, 20ºC, 23.5ºC, 25ºC and 27ºC; C9-C27 treatments) To examine the acclimation response under thermal fluctuating regimens, we employ three daily thermal fluctuations (DTF) treatments, simulating natural daily cycle based on the temperature profiles registered in the ponds (Fig. A.1-3, Annexe 5), corresponding to *cool* (16.5-24 ºC, F24), *warm* (17.5-30ºC, F30) and, additionally a third DTF treatment for *Hme*, *hot* (17.5-35ºC, F35) days, whose average values are equivalent to either constant C20, C23.5 and C25, respectively (Fig. B.1-3, Annexe 5).

Higher DTF oscillations, F30 for *Pib* and *Rte* and F35 for *Hme*, respectively, simulated the thermal profile experienced for each species at an extreme peak day, usually occurring at the end of the breeding season for each species. We selected a lower DTF peak day for *Pib* and *Rte* (warm DTF) than for *Hme* (hot DTF) because maximum water temperature in the natural ponds (T_{max}) differed between species (Table 1 and Fig. 3), due to differences in breeding phenologies between *Pib* and *Hme*, and higher latitudes and altitudes for *Rte*. Fig. 4 also show the interspecific differences between Pib and Hme in the frequency distributions and relationship between natural daily Tmax and daily thermal fluctuations (DTF).

Tadpoles of all species were acclimated at thermal chambers (FitoClima, Aralab) at the reference laboratory of the EBD-CSIC at Seville, Spain. Constant temperature treatments (C9-C27) were distributed in two chambers set at 9ºC (9ºC ± 0.5ºC) and 20ºC (20 ± 0.2 ºC). The remaining C15, C23.5 and C25 treatments were obtained by heating baths located within the thermal chambers with controlled 1500 W heating resistors (U201431698) that maintain temperature to the nearest ± 0.2 ºC). DTF treatments were achieved by heating a water pool with controlled 1500 W heating resistors (U201431698). Resistors were controlled with a clock that switched on at

07:30 am reaching selected peak maximum temperatures for each DTF around 2:30 pm, with a differential ramping rate set at 1.07 ºC h^{-1}, 1.79 ºC h^{-1} and 2.5ºC h^{-1} for F24, F30 and F35 DTFs, respectively. Once peak temperature was reached, this value was kept constant for 1 h and then the watch switched off the resistors, dropping the temperature until the new cycle started. The duration of acclimation period previous to thermal tolerance assays was four days. In order to examine whether multiple fluctuating daily heating episodes for the most stressful temperatures (F30 and F35), increases CTmax and alter CTmin, we used two different acclimation periods: four days (M) —multiple heating peaks— and a single day of fluctuating acclimation, simulating a single heating peak (S). In the last acclimation treatment, we kept tadpoles at constant treatments C23.5 for *Pib* and *Rte* and C25 for *Hme*, during day 1-3 of pre-test acclimation and shifting to either F30 or F35 DTF during the fourth day, completing the acclimation period previous thermal tolerance assays. All acclimation treatments involved 12D:12 L photoperiod to limit any confounding factors of light cycle in the experiments.

Determination of critical thermal limits

For each species and treatment, a number between 12-16 tadpoles were tested, except in *Rana temporaria* for C20 and C24 treatment, in which we had only eight valid measures due to technical problems (see Table A1, Annexe 5, for details). All examined tadpoles were within 26 and 30 Gosner stage (Gosner, 1960). Each tadpole were weighed immediately before the beginning of the test, individualized in 100 mL containers with dechlorinated tap water and placed inside a refrigerated heating bath of 15 L (HUBER K15-cc-NR), previously stabilized to 20 ºC for five minutes. Both thermal tolerance limits (CT_{max} and CT_{min}) were determined using the Hutchison's dynamic method (Lutterschmidt & Hutchison, 1997a) in which each larvae was exposed

to a constant heating/cooling rate (ΔT=0,25 °C min^{-1}) until an end point was attained. The end-point was signalled for both thermal limits as the point at which the tadpoles become motionless and failed to respond to external stimuli by prodding 10 consecutive hits applied each two seconds with a wooden stick. Because of the small size of tadpoles, we assumed that body temperature was equivalent to water temperature (Lutterschmidt & Hutchison, 1997b) and then, CT_{max} and CT_{min} were recorded as the water temperature beside the tadpole and measured with a Miller & Weber quick-recording thermometer (to the 0.1 ^{o}C). After a tolerance limit was determined, we immediately transferred tadpoles to water at the experiment starting temperature (20 °C) to allow recovery, after which their Gosner stage was registered. Tadpole survival was verified a few minutes and 24 hours after the end of the heating/cooling assays. Each individual was tested only once, and only those individuals who recovered 24 h after the test were included in subsequent analyses.

Statistical analyses

We searched for normality and homogeneity of variance of the data using Shapiro-Wilk and Bartlett tests. Once normality and homocedasticity was assumed, we used one-way ANOVA, fixed factor acclimation temperature, followed by Tukey post hocs to analyze differences in CT_{max} and CT_{min} values between treatments for each species and post hoc Scheffé test, after Bonferroni correction, for thermal data. All analyzes were performed in R (R Core Team, 2014).

RESULTS

Fig. 2-4 and Table 1, show the frequency distribution and the basic statistics of T_{max} and T_{min} experienced for each species in a set of natural ponds monitored for several years. *Hme* tadpoles received the highest mean peak temperatures followed by *Pib* whose larvae were exposed to lower T_{max}, as they metamorphosed earlier in the season, whereas *Rte* ponds reached the lowest T_{max} (ANOVA, $F_{2,68}$ = 25.641, P = 0.000001; Post hoc Scheffé test, after Bonferroni correction, $P < 0.001$). T_{min} was colder both in *Pib* and *Rte* whereas late breeder *Hme* was exposed to the warmest T_{min} (Table 1).

Figure 2. Frequency distribution of minimum pond temperatures for *Hyla meridionalis* (green bars), *Rana temporaria* (brown) and *Pelodytes ibericus* (grey).

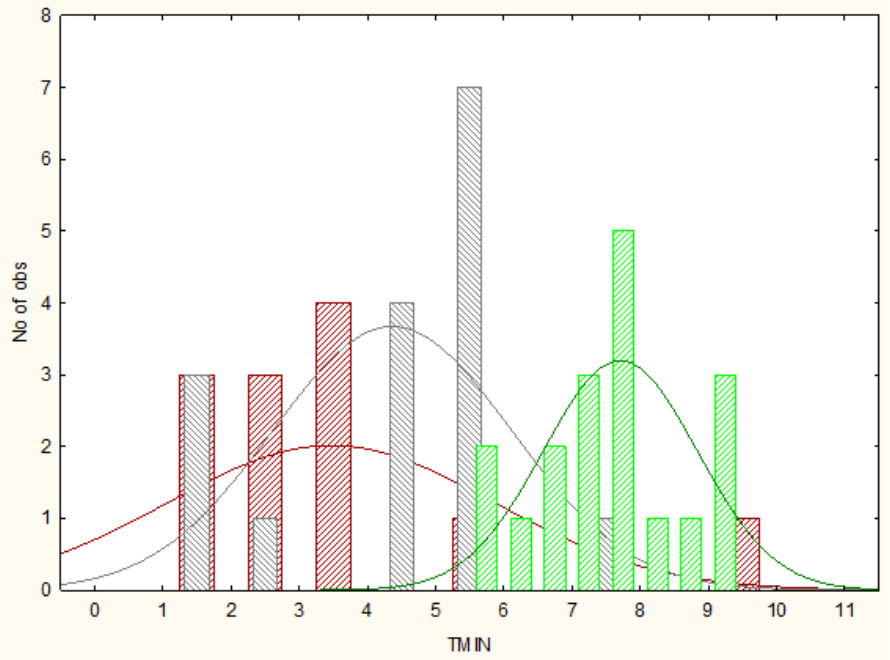


Figure 3. Frequency distribution of maximum pond temperatures for *Hyla meridionalis* (green bars), *Rana temporaria* (brown) and *Pelodytes ibericus* (grey).

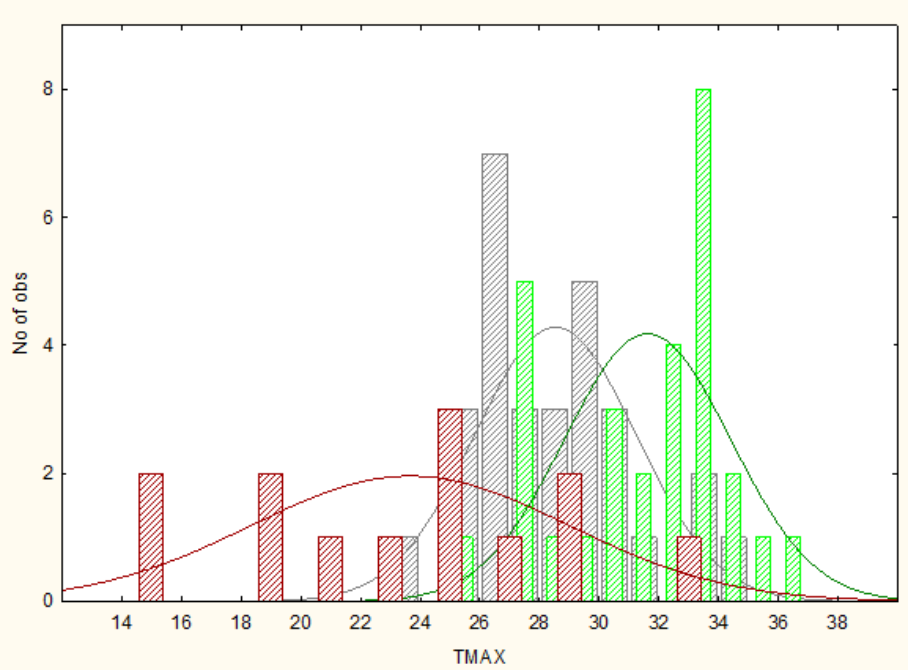


Table 1. Basic statistics (mean, SD, range and CV) for maximum temperature (T_{max}) and minimum temperature (T_{min}) in natural breeding ponds for the three analyzed species. These peak temperatures were obtained only during tadpole presence in the water.

Species		MEAN	SD	Tmax / Tmin Range	N	CV
Pelodytes ibericus	Tmax	28.45	2.7	23.58- 34.90	29	9.49
	Tmin	4.34	1.7	1.00 - 7.48	16	40.11
Hyla meridionalis	Tmax	31.52	2.8	25.80 - 36.08	29	8.77
	Tmin	7.66	1.1	5.55-9.47	18	14.67
Rana temporaria	Tmax	23.69	5.5	15.66 - 32.29	12	23.28
	Tmin	3.45	2.4	1.00 - 9.87	12	69.03

Acclimation under constant temperature regimens determined a contrasting pattern in the plastic response of each resistance boundaries between species (ANOVA, Acclimation x Species x Thermal limit: $F_{8,406}$ = 5.69, P = 0.000001, Fig. 5). Higher acclimation temperature increases CT_{max} in all species, whereas lower thermal acclimation strongly augments cold resistance in *Rte* and *Pib* but not in *Hme* (Fig. 5).

Figure 4. Frequency distributions and scatterplot between daily maximum pond temperature (Tmax) and daily thermal fluctuations (DTF), pooled for six different breeding ponds for *Hme* (R^2 = 0.807, t = 43.35, P < 0.00001, N = 451 days, DTF = -5.89 + 0.632 Tmax), and *Pib* (R^2 = 0.635, t = 30.58, P < 0.00001, N = 538 days, DTF = -3.113 + 0.510 Tmax).

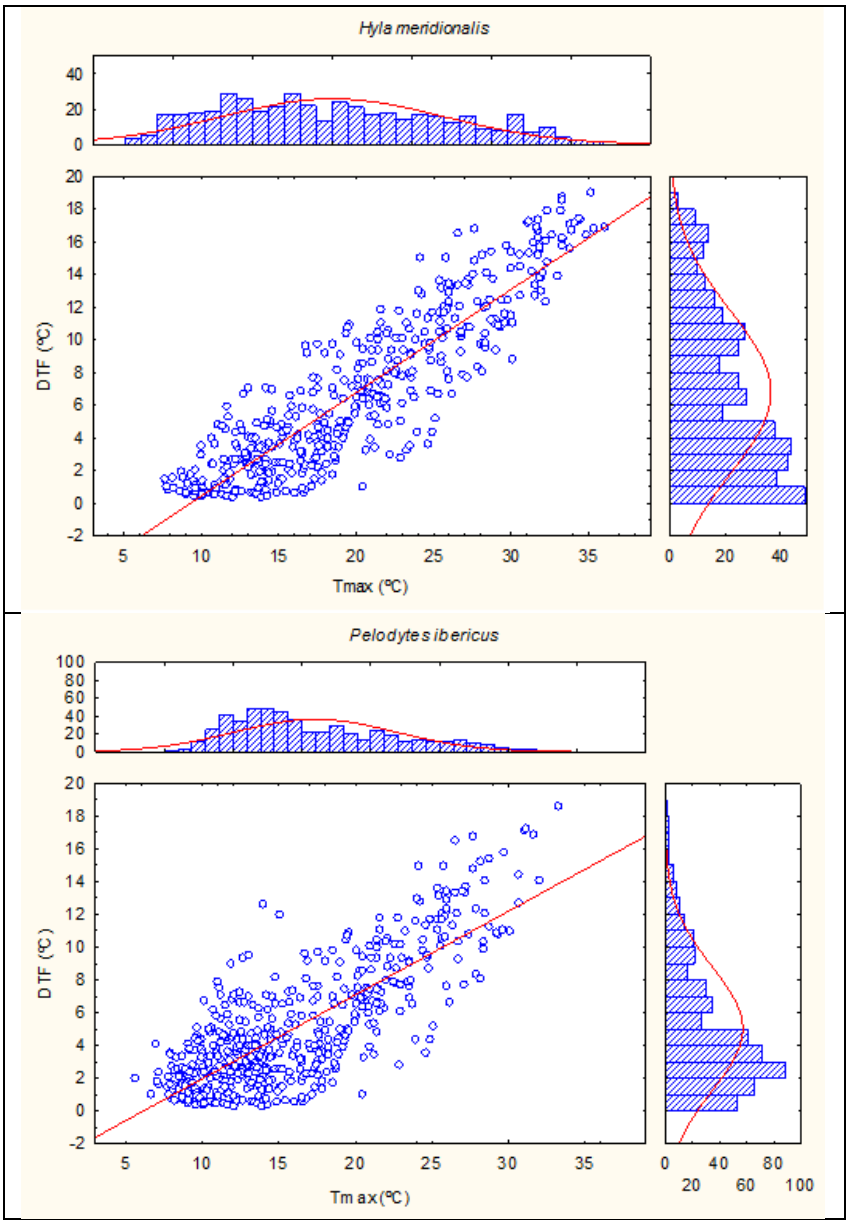
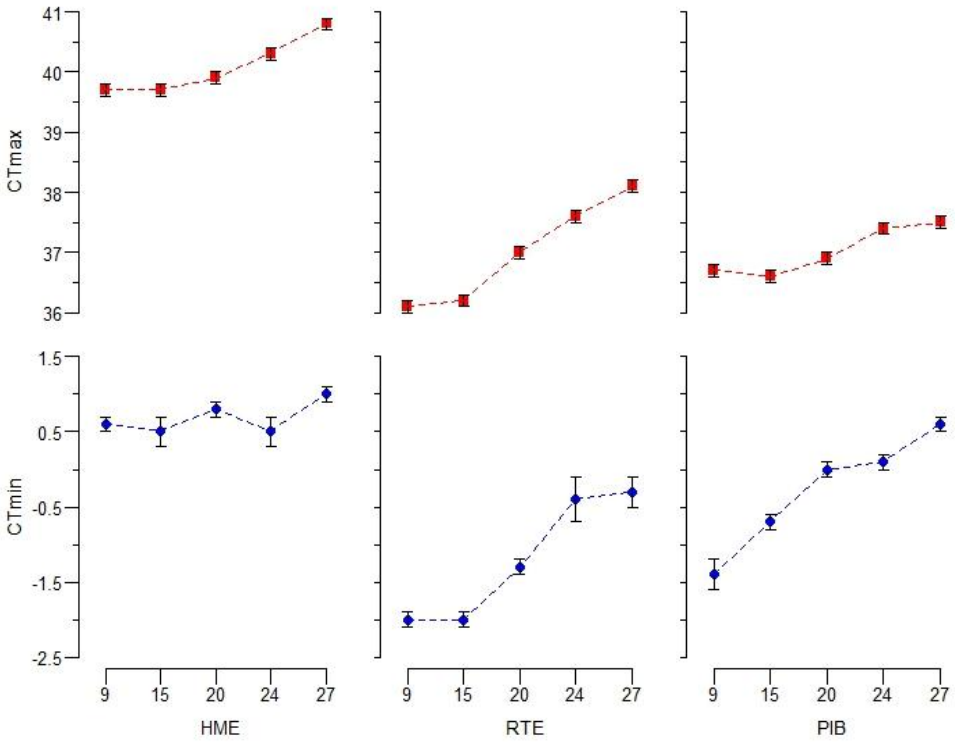


Figure 5. Acclimation scope for both upper (CT_{max}) and lower (CT_{min}) thermal limits (mean ± SE) in *Hyla meridionalis* (HME), *Rana temporaria* (RTE) and *Pelodytes ibericus* (PIB) under increasing acclimation regimens at constant temperature.

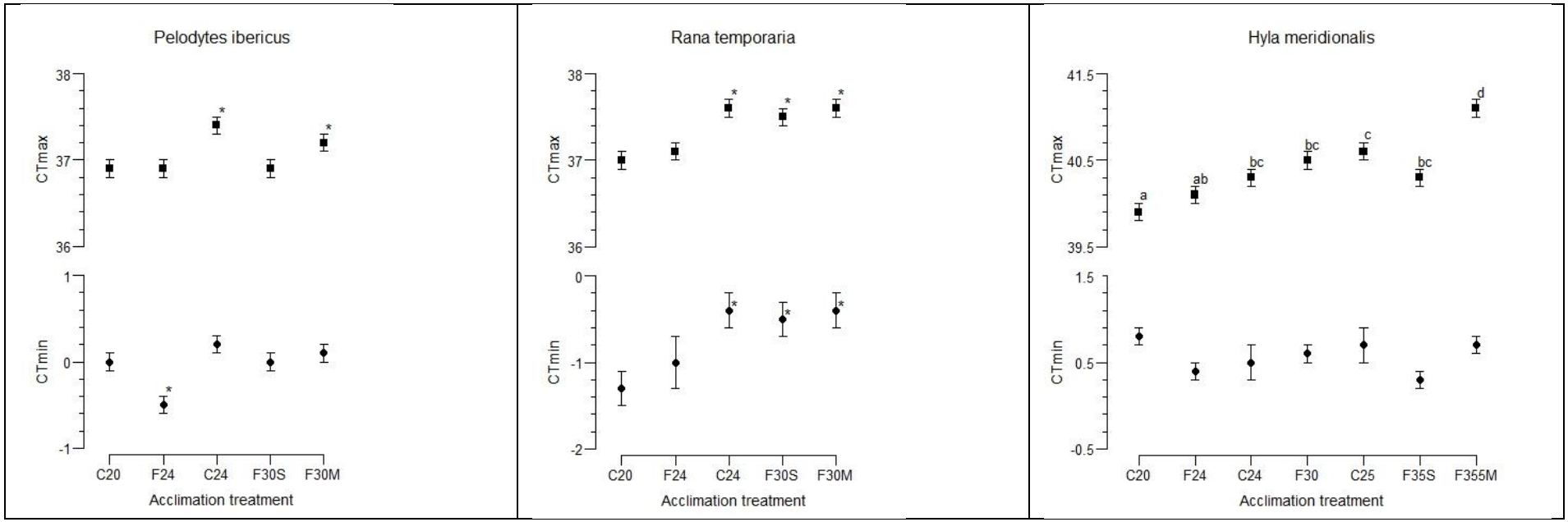


Acclimation under constant and daily fluctuating regimens varied between species (Fig. 6). At the highest DTF regimens, heat tolerance is increased with respect to similar constant regimens, in *Hme*, but contrarily, DTF decreases CT*max* in Pib (ANOVA, $P < 0.05$). Cooler DTFs, otherwise, increase cold resistance in both *Pib* and *Hme* with respect to constant regimens, whereas no effect was found in *Rte*, in either warm and cool DTFs.

Medium-term acclimation at multiple consecutive DTFs, differed of short-term hardening occurring at a single DTF. Sublethal DTF peak temperatures occurring in a single episode previous to CT_{max} assays, reduce upper thermal tolerance in *Hme* and *Pib*, but increase CT_{min} in *Hme* (ANOVA, $P < 0.05$, Fig. 6). Finally, multiple daily

heating episodes increase CT_{max} with respect equal mean constant temperature in *Hme* and determined higher upper tolerance than single heating peak in both *Hme* and *Pib*. No changes in thermal limits were observed in *Rte* between these thermal regimens.

Figure 6. The plots show (1) the acclimation scope of both CT_{max} and CT_{min} (mean values ± standard error) under increasing daily thermal fluctuations (DTF) and mean constant regimens in *Hme*, *Rte* and *Pib*, and (2) the effect on the thermal boundaries of either multiple consecutive (four days) (M) or single day heat peaks by using the highest DTFs (S)(F30S-F30M, for *Pib* and *Rte*, and F35S-F35M, for *Hme*). Letters denote results from Tukey post-hoc analysis.



DISCUSSION

As expected, higher acclimation temperature under constant and fluctuating conditions determined increased response in upper resistance. However, we found species-specific resistance sensitivities to acclimation under fluctuating thermal regimens. First, low tolerant species (*Pib* and *Rte*) had a similar resistance performance under constant and variable thermal acclimation regimens, but a differential increase in tolerance for CT_{max} was found for thermophilic *Hme* under variable thermal conditions. Second, in high tolerant *Hme*, we found that thermal resistance of those tadpoles raised under maximum level fluctuactions (18ºC - 35ºC) occurring repeatedly during four days contrast to that found when this fluctuation affected punctually during a single day. This suggests that tadpoles are able to harden their upper thermal limit by +0.8ºC. Although the molecular bases of our results require further research beyond the scope of this chapter, this enhanced tolerance probably involve the up-regulation of heat-shock proteins (Feder & Hofmann, 1999; Hoffmann *et al*., 2003; Sorensen *et al*., 2003, 2009a; Loeschcke & Sorensen, 2005; Angilletta, 2009).

Interestingly, our results disagree with some previous studies that analyze the effects of variability in other performance traits such as survival or growth rate (Houston & Gingras-Bedard, 1994; Paaijmans *et al*., 2010; Folguera *et al*., 2011) including thermal limits, where thermal oscillations beyond optimum temperature had detrimental effects on performance traits and thermal resistance (Paaijmans *et al*., 2013). But agree with others (Díaz & Bückle, 1999; Terblanche *et al*., 2010; Bozinovic *et al*., 2011a) which found no differences, or even greater tolerance, in fluctuating acclimation to high temperatures. In addition, our results show that thermal plasticity in CT_{max} differs between species related to the level of exposure to high temperatures.

Recurrent multiple heating peaks determine beneficial "hardening" in CT_{max} in the thermotolerant and thermophilic *Hme*, that metamorphose later and it is exposed to higher T_{max} and DTFs (see Fig. 3-4 and Table 1), and contrarily, a loss of upper resistance in less tolerant *Pib*. These differences have been inferred by employing ecologically realistic, non-constant, daily fluctuating acclimations. Thus warming vulnerability to ongoing heat impacts based on thermal limits under constant acclimation conditions, could be imprecise when estimating the risk of ectotherms to suffer acute thermal stress.

Current evidences suggest the existence of an evolutionary trade-off between heat and cold resistance in ectotherms (Portner *et al*., 2006; Sunday *et al*., 2011; Gutiérrez-Pesquera *et al*., 2016). This trade-off would be based on the molecular proximal mechanisms of thermal adaptation. Thus plasticity to high temperatures, for example, may cause missadaptation to low temperatures and *vice versa* (Angilletta, 2009). The pattern observed when acclimation is conducted under constant temperatures through a 9 °C-27 °C thermal range, revealed, however, an asymmetric pattern with greater cold acclimation scope in *Pib* and *Rte*, whereas a reversal pattern is expressed in *Hme* with no cold acclimation under this thermal range. This contrasting pattern suggests thermal adaptation in the cold tolerant species which are exposed to lower peak temperatures than thermophilic and late breeder *Hme*. Contrarily, in this species, especially by acclimating under hot DTF days, thermal tolerance limits appears asymmetric, with a disproportionally increase in heat resistance with neutral variation in cold tolerance. This differential pattern in CT_{max} acclimation appears as an adaptive response in tadpoles of *Hme* to cope with heating peaks (> 30 °C) that occurs frequently in their breeding ponds when desiccate at early June. Our DTFs treatments were markedly designed to simulate heat stress conditions and, therefore, we could not

properly test whether cold stressful DTFs may promote equivalent asymmetry in thermal limits. The differential increase in resistance scope in thermotolerant *Hme* tadpoles acclimated to hot DTF contradicts the trade-off hypothesis (see Chapter 4). Thus plasticity observed in CT_{max} differed from that observed in cold tolerance, probably because of the dissimilar nature of the underlying processes involved in heat and cold resistance acclimations (Sinclair & Roberts, 2005; Lagerspetz, 2006).

CHAPTER 6

“Ontogenetic shifts in thermal tolerances in temperate anurans. Does metamorphosis impose a thermal constraint that may affect vulnerability to global warming?”

Ontogenetic shifts in thermal tolerances in temperate anurans. Does metamorphosis impose a thermal constraint that may affect vulnerability to global warming?

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ABSTRACT

Sequential niche transitions typical from complex life organisms are considered adaptive strategies to occupy environments undergoing contrasting selective pressures, including ontogenetic divergence in exposure to thermal stress. Thus, thermal selection may drive physiological resistance to face thermal extremes experienced for each stage at its particular environment. In addition, we can predict that during the transition climax, organisms may be physiologically unpaired due to increased maintenance and physiological costs at this stage that will ultimately reduce their thermal tolerances.

We examined these predictions by determining thermal tolerance limits (CT_{max} and CT_{min}) in six temperate frog species at three discrete life stages: aquatic larvae, semi-aquatic metamorphs at climax and terrestrial juveniles. Additionally, we monitored extreme maximum pond temperatures (T_{max}) to estimate warming (WT) (CT_{max} - T_{max}) as heat stress predictors, for the aquatic stages, larvae and metamorphs.

Overall, thermal breadth was wider for the larval stage. Upper thermal resistance resulted higher for the aquatic tadpoles whereas cold tolerance was maximal at the juvenile, terrestrial stage. Thermal resistance was lower during metamorphic climax, a general trend suggested by meta-analysis, although metamorphs were equally resistant

to heat and cold than both terrestrial juveniles and aquatic tadpoles, respectively. This pattern of ontogenetic variation was, however, not general and there were species-specific responses such as high heat tolerant metamorphs and juveniles in *Pelobates cultripes* and high cold tolerant *Rana temporaria* tadpoles, probably reflecting stage-specific thermal selection to face extreme temperature stress occurring at these stages.

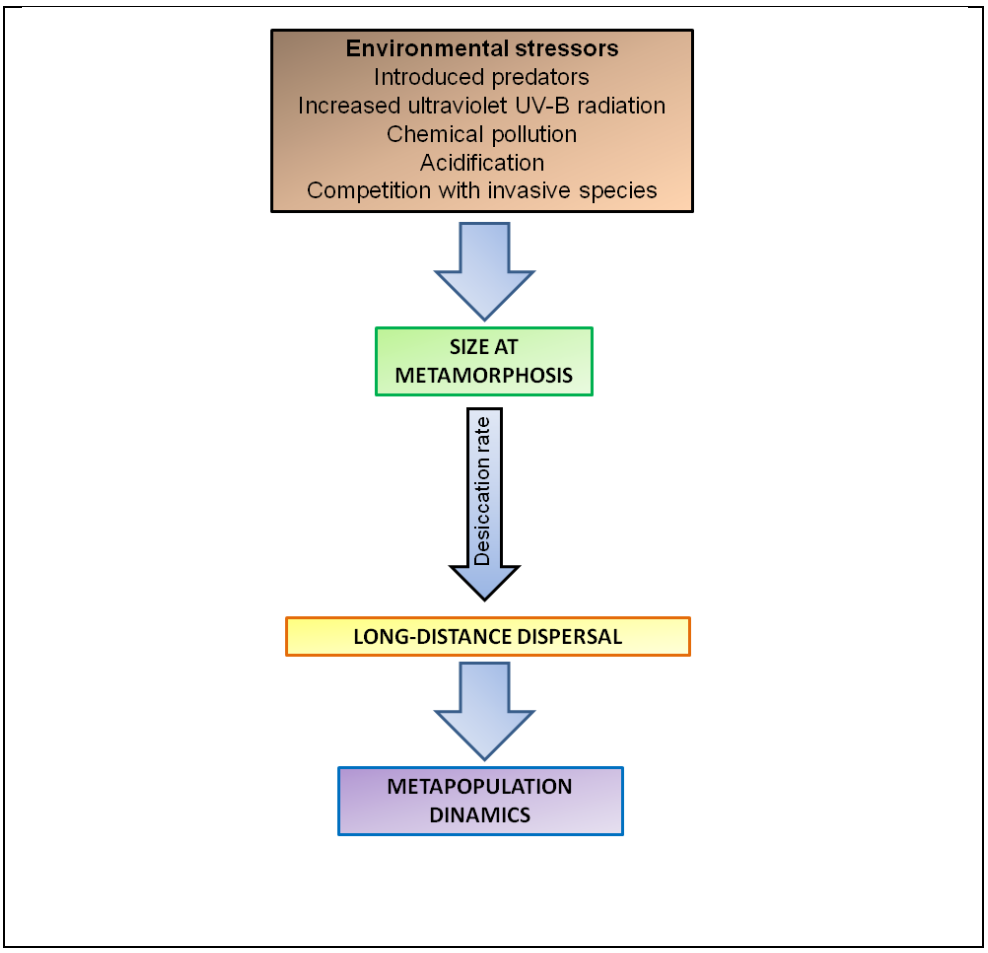
Thermal risk to suffer heat impacts was maximal at metamorphosis for all analysed species, except for the high tolerant *P. cultripes* metamorphs that exhibited similar expected risk to suffer acute heat impacts than larvae. Since metamorphs are temporally concentrated at the end of the season, coinciding with maximum pond temperatures, their higher heat exposure together lower tolerances determine the lowest warming tolerances and thus maximum susceptibility to receive heat acute stress.

Keywords: metamorphosis, amphibians, thermal tolerance, warming tolerance, complex life organisms

INTRODUCTION

Many organisms –insects, marine invertebrates, fishes, amphibians and parasites– have complex life cycles with distinct life stages that may differ in morphology, physiology and behaviour occupying different microhabitats which may be exposed to contrasting climatic and microclimatic conditions (Huey *et al.*, 2012). Sequential niche transitions, typical from complex life organisms, are considered adaptive strategies that exploit transient opportunities for growth or dispersal and may, therefore, involve contrasting selective pressures including ontogenetic divergence in thermal stress exposure (Wilbur, 1980; Crozier *et al.*, 2008; Kingsolver *et al.*, 2011).

Figure 1. A schematic example about how environmental stressors and carryover effects after metamorphosis may affect amphibian fitness from individual to population dynamics.



During metamorphosis, organisms usually experience morphological remodeling and physiological and biochemical adjustments that may impose internal stressful conditions (Wilbur, 1980; Brown & Cai, 2007; Campero *et al.*, 2008; Costantini, 2014). Thus, metamorphosis may involve associated energetic demands that ultimately reduce the vitality and resistance of individuals to external challenges. Environmental stressors may act synergistically with internal conditions determining widely detrimental biotic consequences for postmetamorphic stages, including individual functional aspects with demographic consequences (carryover effects), such as recruitment oscillations that may ultimately determine local extinctions (Fig. 1) (Bridges, 2000; Chelgren *et al.*, 2006; Pechenik, 2006; Tejedo *et al.*, 2010).

Most of the studies analyzing species response to temperature were clearly restricted to a single life stage (Brattstrom, 1968; Currie *et al.*, 1998; Hoffmann *et al.*, 2002; Kolbe *et al.*, 2010; Diamond *et al.*, 2012; Duarte *et al.*, 2012). Amphibians have a general biphasic aquatic-terrestrial life cycle, and previous research suggested different thermal tolerance to cold and heat stress for aquatic tadpoles and terrestrial juveniles in several frog species (e.g. Delson & Whitford, 1973a; Cupp Jr, 1980; Sherman & Levitis, 2003, see Table S2 in Annexe 6). Our ability to predict how climate change will affect populations in the future, therefore, depends on our understanding of the key effects of temperature at the level of each stage of the life cycle, as they will all combine to determine local population demography, and hence population dynamics and viability (Kingsolver *et al.*, 2011; Radchuk *et al.*, 2013).

The understanding of how biodiversity may be altered by climate change (Thompson *et al.*, 2013; Lawson *et al.*, 2015) have relied primarily on mean temperatures (García *et al.*, 2014). However, measures of climate variability of acute thermal stress through the occurrence of extreme temperature events may be equally or

more relevant (Smith, 2011; Buckley & Huey, 2016). From an ecological and evolutionary perspective, such extreme events can trigger stress or physiological damage, which may cause death, becoming major selective factors that influence the evolution of physiological capacities and resistances (Gutschick & BassiriRad, 2003; Denny *et al.*, 2009; Somero, 2010; Hoffmann *et al.*, 2013).

Temporary ponds used by amphibians to reproduce are considered an extreme environment, especially when desiccating, with deep alterations in water chemistry, temperature and oxygen, that result highly stressful and detrimental to resident organisms (Lillywhite & Navas, 2006). Most temperate amphibians are seasonal winter-spring breeders and developing larvae may be exposed to double thermal stress, to cold extreme values, at the start of development, and to heat stress at the warmer end of the season, that will be harder especially to those late and slow developing tadpoles that may be exposed to heat stress and direct mortality by premature desiccation of ponds (Griffiths, 1997; McMenamin *et al.*, 2008; Rittenhouse *et al.*, 2008). Individuals at metamorphosis may avoid mortality in some degree when ponds start to dry because they are air-breathers at the end point of the aquatic larval development. However, most metamorphosing individuals concentrate late in the season, and thus, they are prone to be exposed to high and stressful temperatures. Post-metamorphic terrestrial juveniles may be exposed to higher temperatures in land but they are able of adopting two physiological-behavioral mechanisms to reduce heat stress. First, spatial behavioral selection of cooler microhabitats is enhanced in land because its higher thermal spatial heterogeneity than water since the high heat capacity and conductivity of the latter (Feder & Hofmann, 1999; Angilletta, 2009; Huey *et al.*, 2012). Second, enough hydrated juvenile frogs are able to evaporative cooling in aerial environments that may ameliorate extreme environmental temperatures (Navas *et al.*, 2008; Köhler *et al.*, 2011;

Tracy *et al.*, 2013). Also, juveniles may be under the risk of suffering cold stress once temperature drops in autumn and winter (Voituron *et al.*, 2002; Muir *et al.*, 2014; Ludwig *et al.*, 2015; Williams *et al.*, 2015). By contrast, wintering or early-breeder larvae can be sheltered from the rigours of winter, especially extreme freezing temperatures, due to the higher specific heat of water and the formation of an ice layer on water surface (Gutiérrez-Pesquera, L.M., González Nicieza, A., Tejedo, M., personal observations).

Considering these ontogenetic scenarios of environmental exposure to extreme temperatures and the mechanisms above mentioned for post-metamorphic stages to thermoregulate, we can predict that thermal selection have determined the evolution of wider thermal breadths, with higher heat tolerances, for larvae and more cold resistance for juveniles (able of effective behavior to buffer heat stress). Additionally, functional constraints during the metamorphic climax may result detrimental to the physiological resistance at this stage (Krakauer, 1970; Delson & Whitford, 1973b; Cupp Jr, 1980; Sherman, 1980; Noland & Ultsch, 1981; Menke & Claussen, 1982; Floyd, 1983; Sherman & Levitis, 2003) and, ultimately, making this life-stage the most vulnerable to the forecasted increase in ongoing temperatures.

We will examine these predictions by determining thermal tolerance limits (CT_s, CT_{max} and CT_{min}) in six Paleartic frog species from Iberian Peninsula and north Africa, which inhabit diverse temperate climates, subdesert Marocco, Mediterranean and Atlantic biomes, at three discrete life stages: aquatic larvae, semi-aquatic metamorphs at climax and terrestrial juveniles, by employing the dynamic method which estimate CT_s with ramping heating procedures (Lutterschmidt & Hutchison, 1997, see Methods). Although the less tolerant developmental amphibian stages are eggs and embryos, we discard these stages from the analyses because in these sessile vital stages, thermal

resistances estimates are limited to static temperature methods yielding lethal rather than critical thermal limits (Moore, 1939; Gosner & Black, 1955; Zweifel, 1968; Kuramoto, 1978; Turriago *et al.*, 2015). Additionally, we will obtain warming (WT) (CT_{max} - T_{max}) (Deutsch *et al.*, 2008; Duarte *et al.*, 2012) for larval and metamorphic stages by monitoring micro-environmental pond thermal profiles.

MATERIAL AND METHODS

Species, husbandry and field monitoring

We analysed thermal resistances in six anuran species through their ontogeny, during their aquatic tadpole, metamorphic and the early terrestrial juvenile stages. Four Spanish and southern Moroccan endemics (*Pelobates cultripes*, *Pelodytes ibericus*, *Hyla meridionalis*, and *Barbarophryne brongersmai*, respectively) and, two widely distributed Paleartic species (*Bufo bufo* and *Rana temporaria*) were examined. These species exhibit a contrasting thermal physiology both in upper and lower thermal tolerances (Gutiérrez-Pesquera *et al.*, 2016) and sensitivities in growth rate and survival (Tejedo, Gutiérrez-Pesquera *et al.* unpublished data) during their aquatic larval stage, that may reflect divergent exposure to thermal stress. *P. ibericus*, *P. cultripes*, and *H. meridionalis* ranges through Southwestern Europe at low altitudes, occupying syntopic temporary ponds but differing in their breeding phenology. *P. ibericus* is an early breeder, from October to April or early May, when tadpoles leave the ponds, usually before they desiccate and then avoiding risky peak temperatures. *P. cultripes* and *H. meridionalis* are early and late breeder species, respectively but both species prolong their larval period reaching metamorphosis mostly when pond desiccates in late May and early June, being, therefore, exposed to the highest peak temperatures. *R. temporaria* and *B. bufo* are widespread Eurosiberian frogs that may reach high altitudes

in mountains. Reproduction typically begins in autumn in lowlands, lasting until summer at high altitudes. However, both species differ in the breeding habitat that may confer differential thermal extremes exposures. *R. temporaria* breeds in temporary ponds that may be exposed to very low temperatures, especially at the start of breeding season, at high latitudes or altitudes, when the pond can get frozen, but also they may be exposed to relatively high temperatures in summer at high mountains (Nicieza, A.G., Gutiérrez-Pesquera, L.M., personal observation, see Chapter 2). However, *B. bufo*, breeds in permanent ponds, lakes and streams that are thermally buffered and showing less extreme temperatures. Finally, *B. brongersmai* inhabits lowland semidesert areas of southern and eastern Morocco breeding in temporary ponds and streams, which flood temporarily during winter and early spring rains (Donaire, D. personal observations). This species appears to have a fast developmental rate (Delfino *et al.*, 2009) and metamorphs may occur in early April lasting until May (Schleich *et al.*, 1996; García-Muñoz *et al.*, 2009). This species is a warm but not cold resistant (Gutiérrez-Pesquera *et al.*, 2016), suggesting that their breeding ponds are presumably exposed to high temperatures.

Tadpoles and embryos were field collected using wading nets at the following localities. *R. temporaria* larvae were obtained from the locality of Purón (Asturias, North of Spain, 43°22'45."N; 4°41'55"W, 40 m.a.s.l.) on January 2013. *P. ibericus* and *H. meridionalis* larvae were sampled from Toba locality (Córdoba, 37°59'4" N, 4°54'5"W, 560 m.a.s.l.) on 17th April 2013. *P. cultripes* tadpoles were sampled from Navas del Berrocal population (Seville, 37°46' 58"N, 6°5'5" W, 510 m.a.s.l.) on 28 th May 2013. *B. bufo* larvae was sampled from la Nava de Cabra (Córdoba, 37°29'51"N , 4°22' 6"W, 967 m.a.s.l.) on 8th April 2013. *B. brongersmai* recently laid eggs, were

collected in a temporary pond in Megouss, Anzi, southern Morocco (29°40'32"N-9°19'3" W, 479 m.a.s.l.), on 20th February 2013.

All sampled individuals were transported to the reference laboratory at Seville (Spain, EBD-CSIC) where they were maintained in trays or plastic buckets with a similar larval density inside climatic chambers under constant conditions:photoperiod (12:12 L: D) and temperature (20 °C), and fed *ad libitum* to the beginning of the experiment.

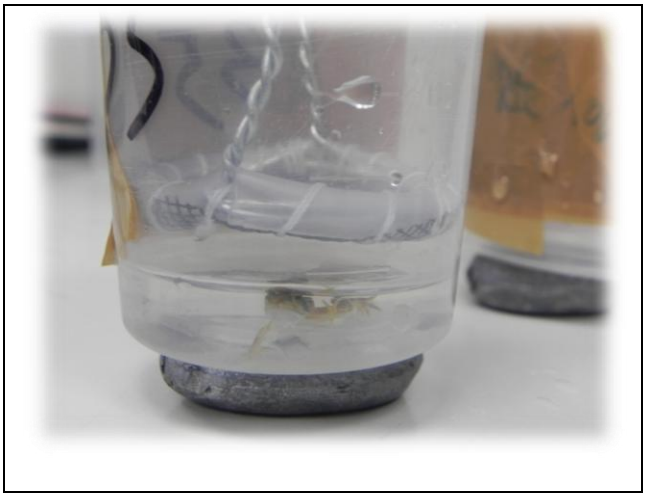
Estimates of critical thermal limits

We estimated both CT_{max} and CT_{min} in tadpoles within a developmental Gosner's stage not exceeding 38 Gosner (Gosner, 1960). Metamorphs were tested at Gosner stage 43-44, coinciding with maximum level of tail resorption rate (see van Buskirk & Saxer, 2001) that sharply reduces swimming speed efficiency (Huey, 1980). In addition, mortality usually peaks at these stages, thus indicating possible sensitivity to internal and external stressors (Tejedo, M., Gutiérrez-Pesquera, L.M. et al, unpublished data). Juveniles were examined one-two days after full tail resorption (46 Gosner stage). Because experimental groups occurs sequentially, and in order to avoid any potential bias induced by different time of pre-test lab acclimation, we selected experimental animals belonging to the different ontogenetic stages fitting the following schedule. Once any tadpole reached pre-metamorphic climax stage (Gosner 40-41), we alternatively allotted it to be tested either as metamorph or juvenile. Simultaneously, we haphazardly selected a less developed individual to be tested as larvae. Tested animals were acclimated at 20 °C, for a minimum of three-four days previous to the assays. This acclimation period was chosen as previous research in adult amphibians revealed that between 2-3 days was the time required to stabilize both CT_{max} and CT_{min} after a large

change in acclimation temperature, such as field and laboratory environments (Brattstrom, 1968).

Both thermal tolerance limits were determined using the Hutchison's dynamic method (Lutterschmidt & Hutchison, 1997) (ranging between N = 10-23 individuals for stage and species) in which each animal was exposed to a constant heating/cooling rate ($\Delta T = 0.25$ °C min^{-1}) until an end point is attained. The end-point was signalled for both thermal limits as the point at which individuals become motionless and fail to respond to external stimuli by prodding five consecutive hits applied each two seconds with a wooden stick. Each tested tadpole or metamorph was placed individually in 100 mL vessel with 80 mL of dechlorinated tap water in a refrigerated heating bath of 15 L (HUBER K15-cc-NR) at a start temperature of 20 °C (temperature of acclimation). Terrestrial juveniles were tested in the same 100 mL container but with only 2 mm of water to maintain tested animals partially submerged in water but avoiding/precluding drowning. To keep juveniles partially submerged at the bottom of the container, avoiding their climbing through the walls, which would expose the tested individuals to varied thermal ramping conditions and, ultimately, preventing the escape of animals, we built a plastic cap covered with a 1 mm mesh net that allowed to check the state of the animal, and, simultaneously to monitor water temperature (Fig. 2).

Figure 2. Detail of experimental vessel containing a metamorph of *R. temporaria* with the plastic cap built with a mesh attached to a strand of wire. Note the fishing sinker to keep the vessel well submerged in the water bath.



Because of the small size of tadpoles, metamorphs and juveniles, we assumed that body temperature was equivalent to water temperature (Lutterschmidt & Hutchison, 1997b) and then CT_{max} and CT_{min} were recorded as the water temperature beside the tadpole measured with a Miller & Weber quick-recording thermometer (to the 0.1 °C). After a tolerance limit was determined, we immediately transferred tadpoles to water at the acclimation temperature (20 °C) to allow for recovery, after which their Gosner stage was registered. Tadpole survival was verified a few minutes and 24 hours after the end of the heating/cooling assays. Each individual was tested only once. To ensure that lethal temperature was not exceeded only those individuals who recovered a few minutes after the test were included in subsequent analyses. Although we only examined a single population for each species, we assumed that response variation among species is larger than variation within species (cf. Klok & Chown, 2003) Species' tolerance range was calculated as the difference of $CT_{max} - CT_{min}$.

Estimates of warming and cooling tolerances

To evaluate the risk of each species suffering thermal stress, we estimated warming tolerances (WT) (sensu Deutsch *et al.*, 2008; Duarte *et al.*, 2012) which is the measurement of an organism's thermal buffer between the habitat maximum current exposure temperatures and its maximal thermal limits, and can be defined as the average amount of environmental temperature change an organism can tolerate before performance drops to fatal levels (Deutsch *et al.*, 2008, Gutiérrez Pesquera *et al.* 2016). We calculated WT metrics for the aquatic tadpoles and metamorphic ontogenetic stages based on microhabitat maximum water temperature data (T_{max}), as the difference of $CT_{max} - T_{max}$, where CT_{max} is mean species physiological thermal limit, determined for each ontogenetic stage in experiments described above, and T_{max} is the metric (median, 95th centile, 99th centile and maximum) of breeding pond water temperatures, estimated from a set of ponds monitored during several years: 2009-2015, for *P. ibericus, P. cultripes and H. meridionalis;* 2002-2014, for *R. temporaria;* and, 2004-2013 for *B. bufo*, obtained by dataloggers (HOBO pendant). These dataloggers were deployed in the bottom of the breeding ponds recording temperature every 15 min. We do not analyse WT's for terrestrial juveniles because the difficulty to monitor terrestrial microenvironments, including aestivating refuges and hibernacula.

To determine thermal exposure at specific larval and metamorph life stages, we monitored the presence of each stage in each sampling pond and, therefore, we could assign seasonally relevant thermal data for each ontogenetic stage. Each breeding pond, with the exception of *B. brongersmai* and *B. bufo*, was visited at day time (10-12 am) two-three times during the reproductive period. The presence of particular species life-stages was based on visual or photographic recordings of dip-netted samplings taken directly in the ponds to check for larvae or metamorphosing individuals. Once any

metamorph was spotted in a particular sampling, we assume their presence from this moment onwards until the next sampling revealed the absence of tadpoles for the target species.

Statistical analyses

We conducted conventional (non-corrected by phylogeny) two-way ANOVAs to analyze CT_{max} and CT_{min} tolerance limits variation in function of species and developmental stages. Pair-wise comparisons were performed with Scheffé test. All statistical tests were obtained using Statistica statistical software (StatSoft 2007). To evaluate whether amphibians during metamorphosis stage lose upper thermal resistance than aquatic tadpoles and terrestrial juveniles, we conducted a meta-analysis comprising our experimental data and those studies, collected from the literature, that employ the same Hutchison's dynamic method of CT_{max} estimates and a similar acclimation temperature (20 °C to 22 °C) (see Table S2, Annexe 6). Effect sizes were considered significant if the 95% confidence intervals did not cross zero. The magnitude of the overall effect size is generally interpreted as "small" if $d+ = 0.2$, "medium" if $d+ = 0.5$ and "large" if $d+ \geq 0.8$ (Cohen, 1988). Effect sizes within analyses were considered different from one another if their 95% confidence intervals did not overlap. All statistical analyses were performed using MetaWin 2.1 statistical program (Rosenberg *et al.*, 2000).

RESULTS

Critical Thermal limits

Upper thermal resistance limit overally ranged 6.67 ºC, from 33.8 ºC, for *R. temporaria* juvenile, to 40.4 ºC, for *P. cultripes* juveniles (Table 1, Fig. 3). CT_{max} varies

between species and stages (Table 2, Fig. 3), being tadpoles more heat tolerant that both metamorph and juveniles (Fig. 4). Interestingly, we found species-specific response in ontogenetic thermal resistance, with a significant Species x Stage interaction (Table 2) due basically to the contrasting pattern of metamorphs and juveniles of *P. cultripes*, which did not reduce their heat resistance with respect to larvae (Fig. 3).

Table 1. Mean critical thermal limits ($\overline{X}$) obtained for the different species throughout the three analyzed life stages.

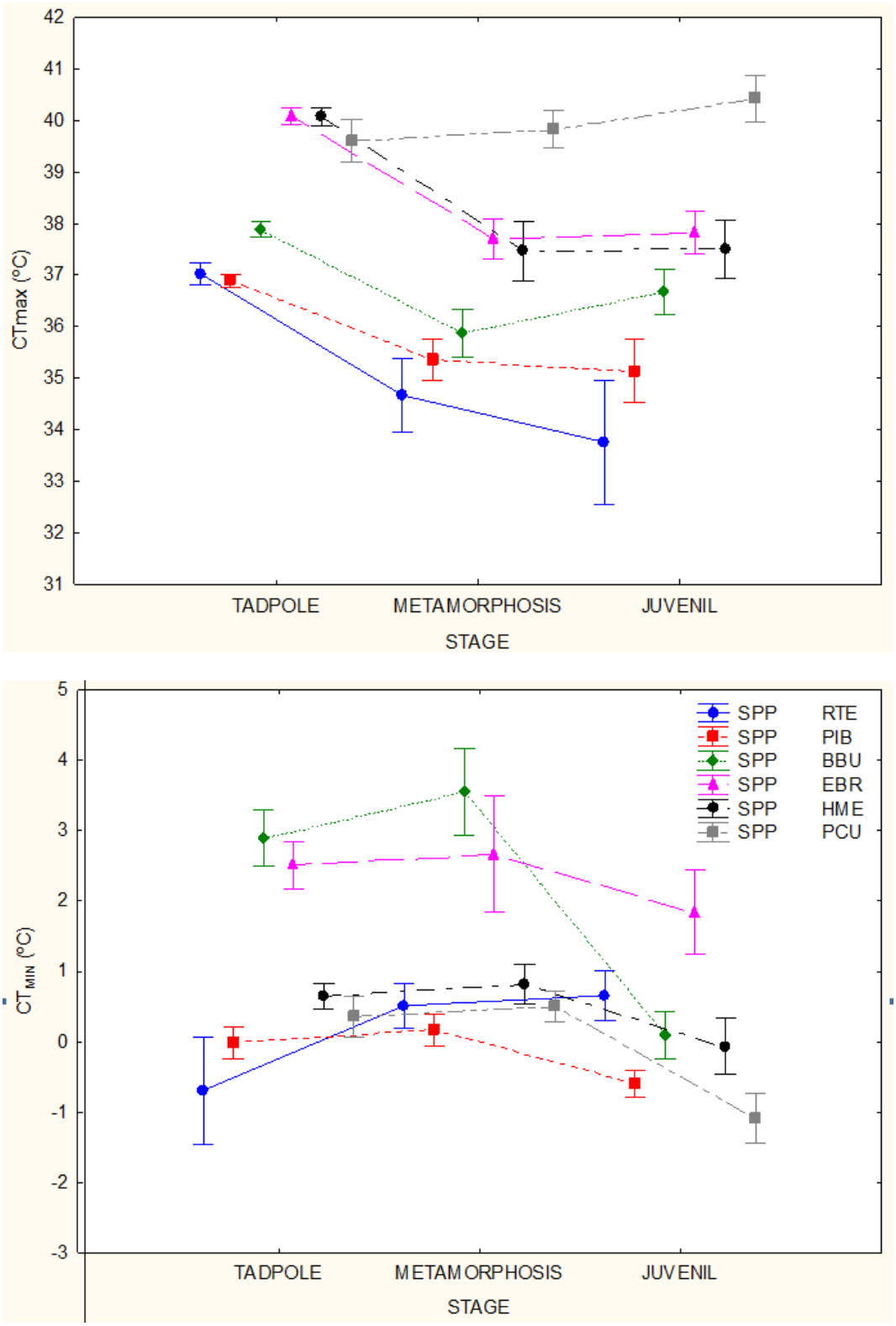
SE, standard error. N, sample size.

Species	Stage	CTmax $\overline{X}$	CTmax SE	N	CTmin $\overline{X}$	CTmin SE	N
R. temporaria	Larvae	37.0	0.1	14	-0.7	0.3	11
R. temporaria	Metamorph	34.7	0.3	16	0.5	0.2	16
R. temporaria	Juvenile	33.8	0.5	12	0.7	0.2	14
P. ibericus	Larvae	36.9	0.1	16	0.0	0.1	16
P. ibericus	Metamorph	35.4	0.2	15	0.2	0.1	15
P. ibericus	Juvenile	35.1	0.3	14	-0.6	0.1	15
B. bufo	Larvae	37.9	0.1	16	2.9	0.2	16
B. bufo	Metamorph	35.9	0.2	23	3.5	0.3	21
B. bufo	Juvenile	36.7	0.2	15	0.1	0.2	15
B. brongersmai	Larvae	40.1	0.1	16	2.5	0.2	16
B. brongersmai	Metamorph	37.7	0.2	15	2.7	0.4	15
B. brongersmai	Juvenile	37.8	0.2	15	1.8	0.3	15
H. meridionalis	Larvae	40.1	0.1	16	0.6	0.1	16
H. meridionalis	Metamorph	37.5	0.3	18	0.8	0.1	15
H. meridionalis	Juvenile	37.5	0.3	11	-0.1	0.2	15
P. cultripes	Larvae	39.6	0.2	10	0.4	0.1	9
P. cultripes	Metamorph	39.8	0.2	12	0.5	0.1	14
P. cultripes	Juvenile	40.4	0.2	16	-1.1	0.2	15

Table 2. Two way ANOVAs for CTmax and CTmin variation.

	CTmax					CTmin				
	SS	df	MS	F	P	SS	df	MS	F	P
SPP	679.5	5	135.9	178.4	<0.001	293.8516	5	58.7703	94.7435	<0.001
STAGE	176.3	2	88.1	115.7	<0.001	70.6640	2	35.3320	56.9586	<0.001
SPP*STAGE	72.4	10	7.2	9.5	<0.001	84.8472	10	8.4847	13.6782	<0.001
Error	192.0	252	0.8			155.6978	251	0.6203		

Figure 3. Critical thermal limits (CTmax, upper panel, CTmin, lower panel) across the different ontogenic stages in tadpoles of six frog species: RTE, *Rana temporaria*; PIB, *Pelodytes ibericus*; BBU, *Bufo bufo*; EBR, *Barbarophryne brongersmai*, HME, *Hyla meridionalis*, and PCU, *Pelobates cultripes*.



Cold thermal resistance exhibits a lower range than CTmax (4.64 ºC) from -1.1 ºC for *P. cultripes* juveniles, to +3.6, for *B. bufo* metamorphs (Fig. 3). We also found a significant effect of species and stages (Table 2) being juveniles more cold tolerant than both larvae and metamorph (Fig. 4). There were also different species pattern in cold resistance through ontogeny, with highest cold resistance of *R. temporaria* tadpoles than juveniles and the extreme cold tolerance increase (Δ 3.4 ºC) of *B. bufo* juveniles with respect to the lowest response of tadpole and metamorph stages. There are not correlations between upper and lower thermal tolerances within particular stage (r_s > 0.20), thus suggesting that there is not a compromise between both resistance limits. Thermal breadth, considering all life stages, differed between species with *Pelobates cultripes* showing the broadest thermal boundaries and *Rana temporaria* the narrowest (Fig. 5).

Figure 4. Variation in thermal tolerance limits across developmental stages . Means are given for the pooled data (regardless of species). Different letters denotes significant differences (Scheffé test, P < 0.05).

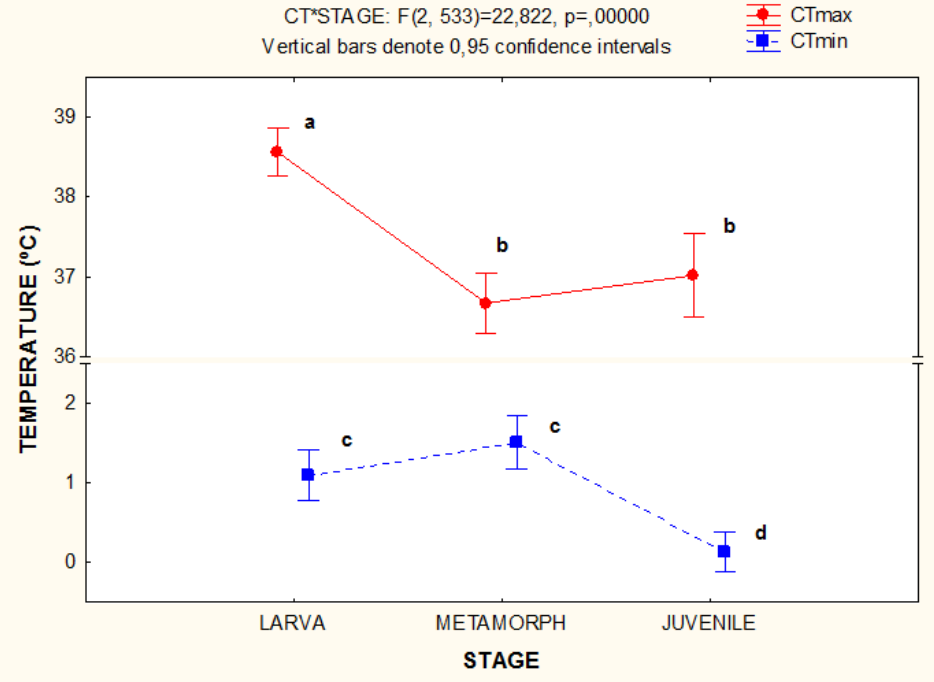
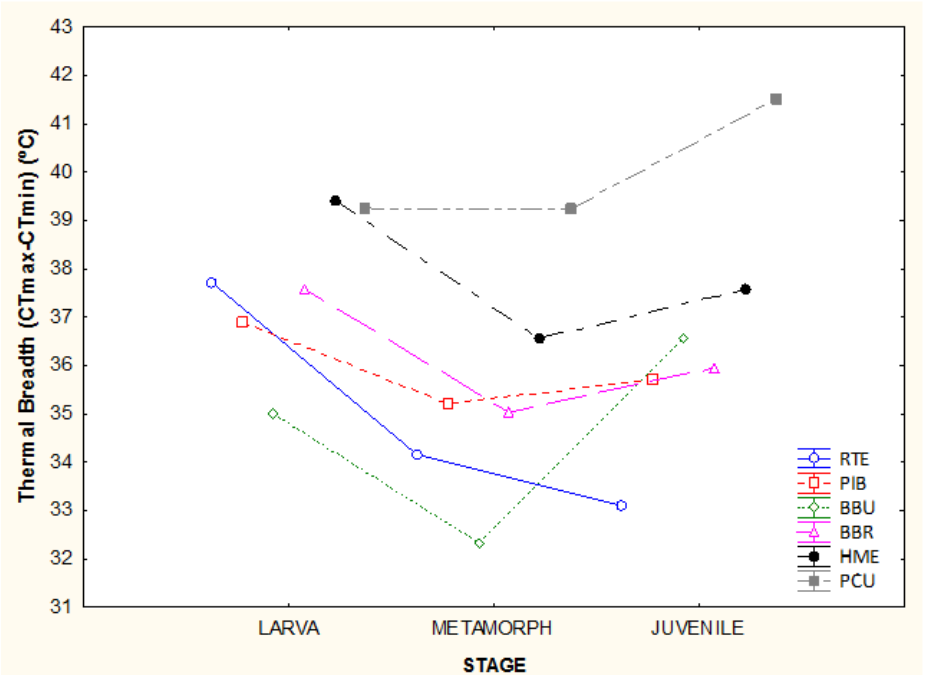
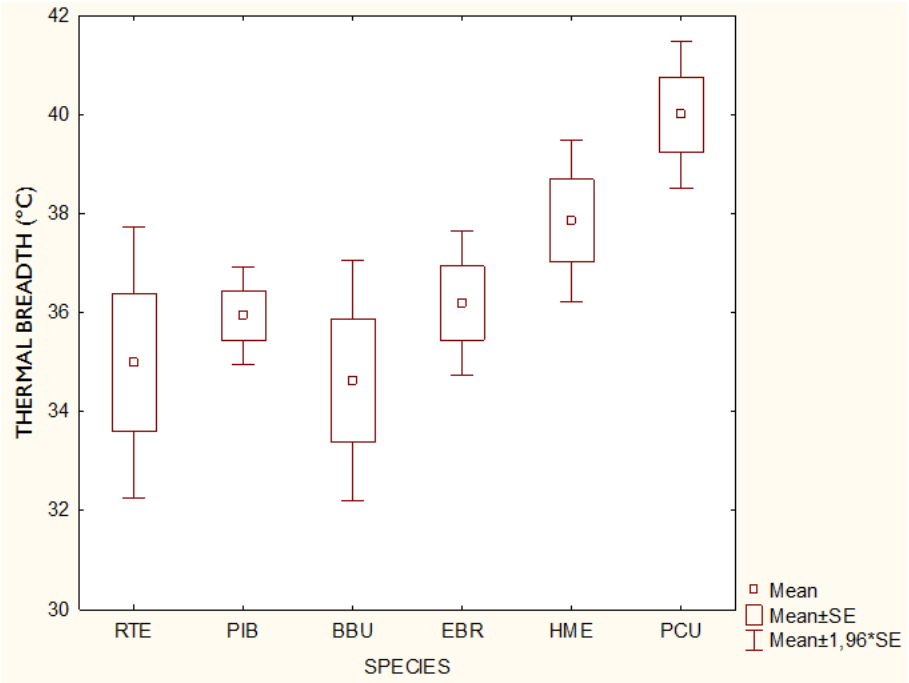


Figure 5. Variation in the Thermal Breadth (CTmax- CTmin) for each tadpole species and particular developmental stage (upper panel) and, overall species Thermal Breadth for the complete life cycle by pooling mean stage values . RTE, *Rana temporaria*; PIB, *Pelodytes ibericus*; BBU, *Bufo bufo*; BBR, *Barbarophryne brongersmai*, HME, *Hyla meridionalis*, and, PCU, *Pelobates cultripes*.





Warming tolerances

We estimate warming tolerances for larval and metamorphic stages for each species (Tables 3-4, Fig. S1, Annexe 6) by using current micro-environmental breeding pond information (T_{max}) (Table S1, Annexe 6). Warming tolerance estimates for terrestrial juveniles were not provided because we have no reliable thermal data available for terrestrial microenvironments. Considering the median pond T_{max}, all the species are relatively safe through the different ponds and years examined, because their WT are ever >5.0 °C. However, when we consider extreme heat conditions, based in yearly pond T_{max}, WT are below 3 °C for all the species in some moment through their ontogeny, with the exception of *B. bufo* (Table 3). Given that larvae and metamorphs are exposed to the same maximum temperatures, when considering the full breading season, metamorphs are, more prone to suffer acute heat impacts because of their lesser tolerance to heat, with values of WT as small as 1.4 °C, 2.2 °C and 2.5 °C for *P. ibericus*, *H. meridionalis* and *R. temporaria*, respectively, at the 95^{th} percentile (Table 3).

Table 3. Warming tolerance (WT, ºC) estimates (defined as CTmax − pond Tmax, median, 95th centile, 99th centile and absolute maximum temperature for all the sampled pond) for tadpole and metamorph stages of five of the examined species, and throughout different ponds and years. In parentheses appears, for each species, the number of pond/year samples. See Table 1 for CTmax values. Bold, WT values < 4ºC. Bold and red values are those with a WT < 3ºC, expected mean temperature increase by IPCC models (IPCC 2013) by the end of 21th century.

Species	Ontogenetic stage	Tmax			
		Median	95th centile	99th centile	Absolute max
Pelodytes ibericus (N = 30)	Tadpole	8.14	**2.94**	**1.99**	**1.99**
	Metamorph	6.61	**1.41**	**0.46**	**0.46**
Pelobates cultripes (N = 37)	Tadpole	6.49	**3.21**	**2.99**	**2.99**
	Metamorph	6.71	**3.42**	**3.20**	**3.20**
Hyla meridionalis (N = 29)	Tadpole	7.67	4.85	**3.99**	**3.99**
	Metamorph	5.07	**2.24**	**1.39**	**1.39**
Rana temporaria (N = 13)	Tadpole	12.55	4.74	4.74	4.74
	Metamorph	10.19	**2.38**	**2.38**	**2.38**
Bufo bufo (N = 6)	Tadpole	15.27	12.76	12.76	12.76
	Metamorph	13.25	10.74	10.74	10.74

Table 4. Warming tolerance (WT, ºC) estimates (defined as CTmax − daily Tmax, median, 95th centile, 99th centile and absolute maximum temperature for all the sampled ponds) for tadpole and metamorph stages of four of the examined species, sampled daily during the breeding season of 2009, 2010, 2011 and 2013 at the Toba breeding site (Córdoba) for *Pelodytes ibericus* and *Hyla meridionalis*; 2009, 2010 and 2013 for *Pelobates cultripes*; and finally, *Rana temporaria*, examined during 2012 at the Señales locality (Asturias). See Table 1 for CTmax values. Bold, WT values < 4 °C. Bold and red values are those with a WT < 3 °C, expected mean temperature increase by IPCC models (IPCC 2013) by the end of 21th century.

Species	Ontogenetic stage	Tmax			
		Median	95th centile	99th centile	Absolute max
Pelodytes ibericus	Tadpole	24.98	10.89	7.44	**3.57**
	Metamorph	15.46	6.61	**3.68**	**2.03**
Pelobates cultripes	Tadpole	25.62	8.75	5.76	**3.53**
	Metamorph	10.78	5.66	**3.75**	**3.75**
Hyla meridionalis	Tadpole	21.07	8.19	5.90	**3.99**
	Metamorph	9.11	**3.62**	**1.39**	**1.39**
Rana temporaria	Tadpole	22.18	12.77	10.54	10.54
	Metamorph	15.39	8.67	8.18	8.18

P. cultripes metamorphs exhibit, however, similar or even higher WT than larvae both at the median and extreme T_{max} values (Table 3). This divergence between larval and metamorph results more acute when we conduct a daily Tmax analysis which incorporates the seasonal component of larval and metamorph differential exposure through considering their particular temporal presence in the ponds (Table 4). In this case, all species at metamorphosis, including *P. cultripes* has lower WT than tadpoles for both median and extreme T_{max}. This lower WT can be attributed to the fact that metamorphs occur later in the season that determine contrasting maximum temperatures distribution, with much higher daily Tmax for metamorph than larvae (Fig. S2-S3, Annexe 6).

DISCUSSION

Life stages in complex life organisms often occupies contrasting thermal environment that may drive stage-specific thermal sensitivities and physiological and biochemical mechanisms for adaptation to local climatic conditions (Kingsolver *et al.*, 2011). As we have shown, amphibians at metamorphic climax are more sensitive to heat impacts than tadpoles, but similar to terrestrial juveniles (Table S2 and S3, Annexe 6), with some exceptions which will be discussed later. This conclusion is supported by two facts:

1) Metamorphosis climax is a stressful period that involve between others, a reduction, especially, in the upper thermal tolerance. This result emerged as a possible general rule in amphibians as shown by the meta-analysis (see Table S2 and S3, Annexe 6).

2) As a novel contribution of our approach, the thermal characterization of the water environment disclosed the contrasting pattern of distribution for maximum temperatures between the whole larvae and metamorphic stages (concentrated in late summer) (Fig. S2-S3, Annexe 6).

The combination of both: lower thermal tolerance (higher *sensitivity*) and higher maximum temperatures (higher *exposition*) yields, as a consequence, an overall reduction in warming tolerances (Deutsch *et al.*, 2008; Williams *et al.*, 2008).

Recent analyses predict that the increasing frequency of high temperatures during ongoing climate warming is likely to decrease mean fitness of tropical ectotherms but, conversely, it may promote benefits for temperate ones (Deutsch *et al.* 2008). To gather rarely available thermal physiology data of risky populations, species and communities, and environmental temperature of microhabitats, are fundamental tool

to establish a biologically sounded vulnerability assessment, and specifically to assess the endangered degree of amphibians forces through their contrasting life stages. Our results suggested differences in exposure and sensitivity to high temperatures for particular amphibian life phase that could alter our predictions about the fitness consequences of climate change commonly based only on adult individuals (Crozier *et al.*, 2008; Kingsolver *et al.*, 2011; Radchuk *et al.*, 2013).

Our WT estimates have focused basically on the aquatic tadpoles and metamorphs obviating the crucial transition and occupation of land environment by postmetamorphic juveniles. The higher thermal heterogeneity of air compared to water environments and the specific thermoregulatory and activity pattern of juveniles, make highly complex a fair approach to assess their environmental exposure. We could suggest a crucial role of behavioural thermoregulation at this vital phase, since the narrower thermal breadth found in juveniles compared to tadpoles. More effort is necessary to provide evaluations of heat impacts on amphibian during the terrestrial stage

Because the expected higher risk to suffer heat impacts during the metamorphic transitional phase, we could expect behavioural compensatory options such as water pond premature evasion to seek for cooler aerial/terrestrial microenvironments. These environments, unlike aquatic ones, allow the possibility of adopting two physiological-behavioural mechanisms that may reduce the possibility to suffer heat stress. First, spatial behavioural selection of cooler microhabitats that is enhanced in land because its higher thermal spatial heterogeneity compared to water since the high heat capacity and conductivity of the latter (Angilletta, 2009; Hillman *et al.*, 2009; Huey *et al.*, 2012). Second, amphibians are able to evaporative cooling in aerial environments and the interface water-land may be a very suitable microenvironment that eases this

physiological accommodation. Behavioural thermoregulation in aerial juveniles and metamorphs stages involves, however, to avoid desiccation (Tracy *et al.*, 1993). In Brattstrom's own words: "Pity the poor frog, his behavioural and physiological problems are so complicated and interrelated, it is amazing that we can understand them and that he is alive at all!! (Brattstrom, 1979). Air breathing is possible during metamorphosis climax and this may allow metamorphs and early transformed juveniles to adopt evaporative cooling in the shoreline and, once dehydrated, they could return to the pond to influx water. This physiological adjustment may be fundamental to face this crucial niche transition in amphibian life cycle that may complemented by behavioural thermoregulation (Navas, 2002; Navas *et al.*, 2007, 2008). Desiccation risk played a more important role than lethal temperatures, for example, in the spatial distribution of metamorphs in the lands around pounds in *Bufo marinus* (Child *et al.*, 2008). Nevertheless, diurnal activity is described in bufonids, even in hot biomes, such as tropical Brazilian Caatinga (Navas *et al.*, 2007).

Given that in species with complex life histories, selection due to climate change can act simultaneously on multiple traits in ways that differ through the life cycle (in Crozier *et al.*, 2008; see also Marshall & Morgan, 2011), another important question to answer is whether physiological responses are phenotypically or genetically correlated across life stages (Kingsolver *et al* 2011, Marshall *et al* 2011), for example, by the emergence of trade-offs in the selective pressures that may constraint the expression of thermal tolerance at different life stages.

The paradoxical high heat tolerance in *Pelobates cultripes* metamorphs and juveniles could be the exception to the rule raised at the beginning of this discussion. Unlike other species, low tolerance to heat at metamorphosis and juvenile phase seemed to be canalized. What evolutionary pressures have promoted this heat resistance? It is

hard to say based on our current knowledge including the analysis of sister species such as *P. varaldii*, *P. fuscus* and *P. syriacus*, in order to examine the eventual common resistance for all the species belonging to this clade. Thus, further efforts must been made in order to first, fully characterize the thermal environment of metamorphs and juveniles, especially air temperatures. Second, to describe and understand thermoregulatory behaviour and habitat occupation of these stages and finally, a thorough analysis about the phenotypic and genetic links across life stages within individual species.

GENERAL CONCLUSIONS

1) According to climate variability hypothesis, species exposed to greater environmental temperature variability showed greater thermal tolerance breadth (Chapters 1 and 3).

2) This increase in thermal tolerance breadth was mainly achieved by an improvement in cold tolerance both across altitudinal and latitudinal gradients. Thus, as Janzen proposed, only few species were able to colonize upper parts of the Andes through evolutionary changes in their CT_{min}. Species from mountain-top in the tropics exhibited tolerance ranges similar to temperate species (Chapters 1 and 3).

3) Both thermal limits (CT_{max} and CT_{min}) showed moderate to high levels of conservatism (Chapter 1).

4) Microclimatic thermal data were better reliable predictors of the physiological traits, especially when we analyzed restricted geographical areas (Chapter 1).

5) Generally, warming tolerance estimates based on water temperature (from dataloggers) yield narrower margins than those based on WorldClim data layers. Thus, forecasts made using only macro-climatic information may underestimate the risk of species to suffer from thermal stress (Chapters 1, 2 and 3).

6) Based on warming tolerances, most sensitive species to climate change were those once from lowland and open forest tropics (Chapter 1 and 3).

7) Interspecific variation in critical thermal limits was much greater that intraspecific variation, especially for CT_{min} (Chapters 1, 2 and 3)

8) At least in *Rana temporaria* under an extreme temperate altitudinal gradient, populations showed little amount of differentiation in their thermal tolerance. This

conservatism in the critical thermal limits could be partially explained by behavioural phenological shifts and plasticity (Chapter 2).

9) Analyzed species showed limited capacity to modify their critical thermal limits through plasticity when exposed to constant acclimation treatment (classical approach). In addition, no conclusive differences were found between tropical and temperate communities in their potential to acclimate both thermal limits (Chapter 4).

10) However, some species (*Hyla meridionalus*) were able to increase their tolerance to heat when exposed to repeated simulated hot days. Thus, we should be cautious when trying to predict aclimation capacity of species in nature without considering realistic protocols implying daily thermal fluctuations (Chapter 5).

11) The thermal risk to suffer from heat impacts was maximal at metamorphosis for all analysed species, except for *P. cultripes* metamorphs that exhibit similar expected risk to suffer from acute heat impacts than larvae (Chapter 6).

CONCLUDING REMARKS

Based on previous research and the results presented in this thesis, the climate variability hypothesis rises as a generalized pattern in ectotherms, and in particular amphibians during their aquatic tadpole stage. We should encourage the use and inclusion of microclimatic data in any research about the impact of global warming, the establishment of ecologically sounding experimental protocols that including thermal variability to study the responses of crucial physiological traits to heat impacts, and highlight the importance –in complex life cycle organisms– of characterizing the thermal sensitivity throughout their entire life cycle to correctly predict the effects of climate change. However, some of the hypothesis analyzed in this thesis yield non-conclusive results, thus, further research is needed to a better understanding of the evolution and the effects of plasticity of thermal physiology in the adaptation to a challenging environment.

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Esta aventura comenzó a principios del año 2010. Ahora seis años después y más de 64.000 kilómetros recorridos, llega a su fin. Además de un desafío académico y profesional, cualquier proyecto de esta envergadura se convierte necesariamente en una vivencia personal única. Una experiencia con altibajos en el que da tiempo a conocer nuevos países y gentes, hacer nuevos amigos —y a veces también enemigos—, enamorarse, desenemarorase y volverse a enamorar, despedir con tristeza para siempre a varios seres queridos y recibir a los nuevos; sudar, mancharse, llorar, reír, ilusionarse, desesperarse y volverse a motivar… pero nunca aburrirse. En resumen, ya sabéis, la vida es eso que pasa mientras haces la tesis.

Tratar de recordar a todas aquellas personas con las que, de una manera u otra, he quedado en deuda después de tanto tiempo se me antoja ahora una tarea abrumadora. Algunas personas tienen la buena costumbre de elaborar desde el principio una lista donde van apuntando sus agradecimientos —qué útil es Facebook—. Yo, que suelo ser sistemático —algunos dirán cuadriculado— no soy detallista y no me queda otra que recurrir a mi memoria. Procederé, por tanto, del mismo modo en que he organizado este trabajo: siguiendo criterios espaciales y temporales, pidiendo perdón de antemano por cualquier omisión.

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A todos aquellos que de una manera u otra han contribuido a que esta tesis haya llegado a buen puerto, GRACIAS.

if it doesn’t come bursting out of you

in spite of everything,

don’t do it.

unless it comes unasked out of your

heart and your mind and your mouth

and your gut,

don’t do it.

if you’re doing it for money or

fame,

don’t do it.

if you’re doing it because you want

women in your bed,

don’t do it.

if it’s hard work just thinking about doing it,

don’t do it.

unless it comes out of

your soul like a rocket,

unless being still would

drive you to madness or

suicide or murder,

don’t do it.

—Charles Bukowski, 1920 - 1994—

BIBLIOGRAPHIC REFERENCES

Addo-Bediako A., Chown S.L., & Gaston K.J. (2000) Thermal tolerance, climate variability and latitude. *Proceedings of The Royal Society B Biological Sciences*, **267**, 739–745.

Alford L., Blackburn T.M., & Bale J.S. (2012) Effect of latitude and acclimation on the lethal temperatures of the peach-potato aphid *Myzus persicae*. *Agricultural and Forest Entomology*, **14**, 69–79.

Alho J.S., Herczeg G., Söderman F., Laurila A., Jönsson K.I., & Merilä J. (2010) Increasing melanism along a latitudinal gradient in a widespread amphibian: local adaptation, ontogenic or environmental plasticity? *BMC Evolutionary Biology*, **10**, 317.

Álvarez D. (2013) Rana bermeja - Rana temporaria. *Enciclopedia Virtual de Vertebrados Españoles* (ed. by A. Salvador and I. Martínez-Solano), Museo Natural de Ciencias Naturales, Madrid.

Álvarez D., Choda M., Viesca L., Cano J.M., Bañuelos M.J., Matsuba C., & Nicieza A.G. (2012) Variación genética adaptativa en gradientes altitudinales: efectos sobre la viabilidad de poblaciones subdivididas en escenarios de cambio climático. *Proyectos de Investigación en parques nacionales: 2008-2011. Naturaleza y Parques Nacionales. Serie investigación en la red.* (ed. by Ramírez L & Asensio B (Eds.)), pp. 125–150.

Amphibiaweb (2015) Available at: http://amphibiaweb.org/.

Anderson A., Collinge J., Hoffmann A., Kellett M., & McKechnie S. (2003) Thermal tolerance trade-offs associated with the right arm of chromosome 3 and marked by the hsr-omega gene in *Drosophila melanogaster*. *Heredity*, **90**, 195–202.

Angilletta M.J., Bennett A.F., Guderley H., Navas C.A., Seebacher F., & Wilson R.S. (2006) Coadaptation: a unifying principle in evolutionary thermal biology. *Physiological and Biochemical Zoology : PBZ*, **79**, 282–94.

Angilletta M.J.J. (2009) *Thermal adaptation: a theoretical and empirical synthesis*. Oxford University Press, Oxford.

Angilletta M.J.J., Niewiarowski P.H., & Navas C.A. (2002) The evolution of thermal biology in ectotherms. *Journal of Thermal Biology*, **27**, 249–268.

Araújo M.B., Ferri-Yáñez F., Bozinovic F., Marquet P.A., Valladares F., & Chown S.L. (2013) Heat freezes niche evolution. *Ecology Letters*, **16**, 1206–1219.

Araújo M.B. & Pearson R.G. (2005) Equilibrium of species' distributions with climate. *Ecography*, **28**, 693–695.

Araújo M.B., Thuiller W., & Pearson R.G. (2006) Climate warming and the decline of amphibians and reptiles in Europe. *Journal of Biogeography*, **33**, 1712–1728.

Baldwin J. & Hochachka P.W. (1970) Functional significance of isoenzymes in thermal acclimatization. Acetylcholinesterase from trout brain. *Biochemical Journal*, **116**, 883–887.

Balogová M. & Gvoždík L. (2015) Can newts cope with the heat? disparate thermoregulatory strategies of two sympatric species in water. *PLoS ONE*, **10**, e0128155.

Bass M.S., Finer M., Jenkins C.N., Kreft H., Cisneros-Heredia D.F., McCracken S.F., Pitman N.C.A., English P.H., Swing K., Villa G., Di Fiore A., Voigt C.C., & Kunz T.H. (2010) Global conservation significance of Ecuador's Yasuní National Park. *PLoS ONE*, **5**, e8767.

Bates D., Maechler M., Bolker B., & Walker S. (2013) lme4: Linear mixed-effects models using Eigen and S4. R package version 1.0-4. *Accessed online: December*,

.

Beattie R.C. (1987) The reproductive biology of Common frog (*Rana temporaria*) populations from different altitudes in northern England. *Journal of Zoology*, **211**, 387–398.

Becker C.D. & Genoway R.G. (1979) Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. *Environmental Biology of Fishes*, **4**, 245–256.

Beebee T.J.C. (1995) Amphibian breeding and climate. *Nature*, **374**, 219–220.

Beebee T.J.C. & Griffiths R.A. (2005) The amphibian decline crisis: A watershed for conservation biology? *Biological Conservation*, **125**, 271–285.

Beitinger T.L., Bennett W.A., & McCauley R.W. (2000) Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Environmental Biology of Fishes*, **58**, 237–275.

Bennett A. & Lenski R. (1993) Evolutionary Adaptation to Temperature II. Thermal Niches of Experimental Lines of *Escherichia coli*. *Evolution*, **47**, 1–12.

van Berkum F.H. (1986) Evolutionary patterns of the thermal sensitivity of sprint speed in Anolis lizards. *Evolution*, **40**, 594–604.

Bevelhimer M. & Bennett W. (2000) Assessing cumulative thermal stress in fish during chronic intermittent exposure to high temperatures. *Environmental Science & Policy*, **3**, 211–216.

Blomberg S.P., Garland T., & Ives A.R. (2003) Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution*, **57**, 717–745.

Bogert C.M. (1949) Thermoregulation in reptiles, a factor in evolution. *Evolution*, **3**, 195–211.

Bosch J., Carrascal L.M., Duran L., Walker S., & Fisher M.C. (2007) Climate change

and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society of London B: Biological Sciences*, **274**, 253–260.

Bozinovic F., Bastias D.A., Boher F., Clavijo-Baquet S., Estay S.A., & Angilletta Jr M.J. (2011a) The mean and variance of environmental temperature interact to determine physiological tolerance and fitness. *Physiological and Biochemical Zoology*, **84**, 543–552.

Bozinovic F., Calosi P., & Spicer J.I. (2011b) Physiological correlates of geographic range in animals. *Annual Review of Ecology, Evolution, and Systematics, Vol 42* (ed. by D.J. Futuyma, H.B. Shaffer, and D. Simberloff), pp. 155–179.

Bradshaw W.E. & Holzapfel C.M. (2006) Climate change - Evolutionary response to rapid climate change. *Science*, **312**, 1477–1478.

Brattstrom B.H. (1968) Thermal acclimation in anuran amphibians as a function of latitude and altitude. *Comparative Biochemistry and Physiology*, **24**, 93–111.

Brattstrom B.H. (1970) Thermal acclimation in Australian amphibians. *Comparative Biochemistry and Physiology*, **35**, 69–103.

Brett J.R. (1956) Some principles in the thermal requirements of fishes. *Quarterly Review of Biology*, **31**, 75–87.

Bridle J.R., Gavaz S., & Kennington W.J. (2009) Testing limits to adaptation along altitudinal gradients in rainforest *Drosophila*. *Proceedings. Biological sciences / The Royal Society*, **276**, 1507–15.

Brommer J.E. (2011) Whither Pst? The approximation of Qst by Pst in evolutionary and conservation biology. *Journal of Evolutionary Biology*, **24**, 1160–8.

Brook B.W., Sodhi N.S., & Bradshaw C.J.A. (2008) Synergies among extinction drivers under global change. *Trends in ecology & evolution*, **23**, 453–460.

Brooker R.W., Travis J.M.J., Clark E.J., & Dytham C. (2007) Modelling species' range shifts in a changing climate: the impacts of biotic interactions, dispersal distance and the rate of climate change. *Journal of Theoretical Biology*, **245**, 59–65.

Buckley L.B., Ehrenberger J.C., & Angilletta M.J. (2015) Thermoregulatory behavior limits local adaptation of thermal niches and confers sensitivity to climate change. *Functional Ecology*, **29**, 1038-1047.

Burnham K.P. & Anderson D.R. (2002) *Model selection and multimodel inference: a practical information-theoretic approach.* Springer Science & Business Media, New York.

Bustamante M.R., Ron S.R., & Coloma L.A. (2005) Cambios en la Diversidad en Siete Comunidades de Anuros en los Andes de Ecuador1. *Biotropica*, **37**, 180–189.

Calosi P., Bilton D.T., & Spicer J.I. (2008a) Thermal tolerance, acclimatory capacity and vulnerability to global climate change. *Biology Letters*, **4**, 99–102.

Calosi P., Bilton D.T., Spicer J.I., & Atfield A. (2008b) Thermal tolerance and geographical range size in the *Agabus brunneus* group of European diving beetles (Coleoptera: Dytiscidae). *Journal of Biogeography*, **35**, 295–305.

Calosi P., Bilton D.T., Spicer J.I., Votier S.C., & Atfield A. (2010) What determines a species' geographical range? Thermal biology and latitudinal range size relationships in European diving beetles (Coleoptera: Dytiscidae). *Journal of Animal Ecology*, **79**, 194–204.

Cano J.M., Laurila A., Palo J., & Merilä J. (2004) Population differentiation in G matrix structure due to natural selection in *Rana temporaria*. *Evolution*, **58**, 2013–2020.

Chessman B.C. (2013) Do protected areas benefit freshwater species? A broad-scale assessment for fish in Australia's Murray–Darling Basin. *Journal of Applied Ecology*, **50**, 969–976.

Chevin L.-M., Lande R., & Mace G.M. (2010) Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory. *PLoS Biol*, **8**, e1000357.

Choda M. (2015) *Genetic variation and local adaptations of* Rana temporaria *in the Cantabrian Mountains.* Tesis Doctoral. Universidad de Oviedo.

Chown S.L. (2001) Physiological variation in insects: hierarchical levels and implications. *Journal of Insect Physiology*, **47**, 649–660.

Chown S.L., Duffy G.A., & Sørensen J.G. (2015) Upper thermal tolerance in aquatic insects. *Current Opinion in Insect Science*, **11**, 78–83.

Chown S.L., Hoffmann A.A., Kristensen T.N., Anguiletta M.J.J., Stenseth N.C., & Pertoldi C. (2010) Adapting to climate change: a perspective from a evolutionary physiology. *Climate Research*, **43**, 3–15.

Chown S.L., Jumbam K.R., Sørensen J.G., & Terblanche J.S. (2009a) Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. *Functional Ecology*, **23**, 133–140.

Chown S.L., Keafon R.J., Sorensen J.G., & Terblanche J.S. (2009b) Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. *Functional Ecology*, **23**, 133–140.

Chown S.L., Sinclair B.J., Leinaas H.P., & Gaston K.J. (2004) Hemispheric asymmetries in biodiversity—a serious matter for ecology. *PLoS Biol*, **2**, e406.

Christian K.A., Nunez F., Clos L., & Diaz L. (1988) Thermal relations of some tropical frogs along an altitudinal gradient. *Biotropica*, 236–239.

Claussen (1977) Thermal acclimation in ambystomatid salamanders. *Comparative Biochemistry and Physiology--Part A: Physiology*, **58**, 333–340.

Clusella-Trullas S., Blackburn T.M., & Chown S.L. (2011) Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses

to climate change. *The American Naturalist*, **177**, 738–751.

Colwell R.K., Brehm G., Cardelús C.L., Gilman A.C., & Longino J.T. (2008) Global warming, elevational range shifts, and lowland biotic attrition in the wet tropics. *Science*, **322**, 258–261.

Compton T.J., Rijkenberg M.J.A., Drent J., & Piersma T. (2007) Thermal tolerance ranges and climate variability: A comparison between bivalves from differing climates. *Journal of Experimental Marine Biology and Ecology*, **352**, 200–211.

Cooper B.S., Tharp J.M., Jernberg I.I., & Angilletta M.J. (2012) Developmental plasticity of thermal tolerances in temperate and subtropical populations of *Drosophila melanogaster*. *Journal of Thermal Biology*, **37**, 211–216.

Corn P.S. (2003) Amphibian breeding and climate change: importance of snow in the mountains. *Conservation Biology*, **17**, 622–625.

Cossins A.R. & Prosser C.L. (1978) Evolutionary adaptation of membranes to temperature. *Proceedings of the National Academy of Sciences*, **75**, 2040–2043.

Cowles R.B. & Bogert C.M. (1944) A preliminary study of the thermal requirements of desert reptiles. *Bulletin of the American Museum of Natural History*, **83**, 263–296.

Cruz F.B., Fitzgerald L.A., Espinoza R.E., & Schulte J.A. (2005) The importance of phylogenetic scale in tests of Bergmann's and Rapoport's rules: lessons from a clade of South American lizards. *Journal of evolutionary biology*, **18**, 1559–74.

Daszak P., Berger L., Cunningham A.A., Hyatt A.D., Green D.E., & Speare R. (1999) Emerging infectious diseases and amphibian population declines. *Emerging infectious diseases*, **5**, 735.

Delson J. & Whitford W.G. (1973) Critical thermal maxima in several life history stages in desert and montane populations of *Ambystoma tigrinum*. *Herpetologica*, **29**, 352–355.

Deutsch C.A., Tewksbury J.J., Huey R.B., Sheldon K.S., Ghalambor C.K., Haak D.C., & Martin P.R. (2008) Impacts of climate warming on terrestrial ectotherms across latitude. *PNAS*, **105**, 6668–6672.

DeWitt T.J., Sih A., & Wilson D.S. (1998) Costs and limits of phenotypic plasticity. *Trends in Ecology & Evolution*, **13**, 77–81.

Diamond S.E., Sorger D.M., Hulcr J., Pelini S.L., Del Toro I., Hirsch C., Oberg E., & Dunn R.R. (2012) Who likes it hot? A global analysis of the climatic, ecological, and evolutionary determinants of warming tolerance in ants. *Global Change Biology*, **18**, 448–456.

Díaz F. & Bückle L.F. (1999) Effect of the critical thermal maximum on the preferred temperatures of *Ictalurus punctatus* exposed to constant and fluctuating temperatures. *Journal of Thermal Biology*, **24**, 155–160.

Diffenbaugh N.S. & Field C.B. (2013) Changes in ecologically critical terrestrial climate conditions. *Science*, **341**, 486–492.

Diniz-Filho J.A.F., Sant´Ana R. De, & Bini L.M. (1998) An eigenvector method for estimating phylogenetic inertia. *Evolution*, **52**, 1247–1262.

Duarte H., Tejedo M., Katzenberguer M., Marangoni F., Baldo D., Beltrán J.F., Martí D.A., Richter-Boix A., & Gonzalez-Voyer A. (2012) Can amphibians take the heat? Vulnerability to climate warming in subtropical and temperate larval amphibian communities. *Global Change Biology*, **18**, 412–421.

Duellman W.E. (1999) *Patterns of distribution of amphibians: a global perspective*. JHU Press,

Dunson W.A. & Travis J. (1991) The role of abiotic factors in community organization. *American Naturalist*, **138**, 1067–1091.

Fangue N.A., Hofmeister M., & Schulte P.M. (2006) Intraspecific variation in thermal

tolerance and heat shock protein gene expression in common killifish, Fundulus heteroclitus. *The Journal of experimental biology*, **209**, 2859–72.

Feder M.E. (1985a) Acclimation to constant and variable temperatures in plethodontid salamanders—I. Rates of oxygen consumption. *Comparative Biochemistry and Physiology Part A: Physiology*, **81**, 673–682.

Feder M.E. (1985b) Acclimation to constant and variable temperatures in plethodontid salamanders. II. Time course of acclimation to cool and warm temperatures. *Herpetologica*, 241–245.

Feder M.E. & Hofmann G.E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual Review of Physiology*, **61**, 243–282.

Feldmeth C.R., Stone E.A., & Brown J.H. (1974) An increased scope for thermal tolerance upon acclimating pupfish (*Cyprinodon*) to cycling temperatures. *Journal of Comparative Physiology*, **89**, 39–44.

Floyd R.B. (1983) Ontogenetic change in the temperature tolerance of larval *Bufo marinus* (Anura, Bufonidae). *Comparative Biochemistry and Physiology Part A: Physiology*, **75**, 267–271.

Floyd R.B. (1985) Effects of photoperiod and starvation on the temperature tolerance of larvae of the giant toad, *Bufo marinus*. *Copeia*, 625–631.

Foden W.B., Butchart S.H.M., Stuart S.N., Vié J.-C., Akçakaya H.R., Angulo A., DeVantier L.M., Gutsche A., Turak E., Cao L., & others (2013) Identifying the world's most climate change vulnerable species: a systematic trait-based assessment of all birds, amphibians and corals. *PLoS ONE*, **8**, e65427.

Folguera G., Bastías D.A., & Bozinovic F. (2009) Impact of experimental thermal amplitude on ectotherm performance: Adaptation to climate change variability?

Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology, **154**, 389–93.

Folguera G., Bastías D.A., Caers J., Rojas J.M., Piulachs M.-D., Bellés X., & Bozinovic F. (2011) An experimental test of the role of environmental temperature variability on ectotherm molecular, physiological and life-history traits: implications for global warming. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, **159**, 242–246.

Freed L.A., Cann R.L., Goff M.L., Kuntz W.A., & Bodner G.R. (2005) Increase in avian malaria at upper elevation in Hawaii. *The Condor*, **107**, 753–764.

Gaitán-Espitia J.D., Arias M.B., Lardies M.A., & Nespolo R.F. (2013) Variation in thermal sensitivity and thermal tolerances in an invasive species across a climatic gradient: lessons from the land snail *Cornu aspersum*. *PloS ONE*, **8**, e70662.

Garland T., Adolph S.C., & Adolph C. (1991) Physiological differentiation of vertebrate populations. *Annual Review of Ecology and Systematics*, **22**, 193–228.

Gaston K.J. & Chown S.L. (1999) Elevation and climatic tolerance: a test using dung beetles. *Oikos*, **86**, 584–590.

Gaston K.J., Chown S.L., Calosi P., Bernardo J., Bilton D.T., Clarke A., Clusella-Trullas S., Ghalambor C.K., Konarzewski M., Peck L.S., Porter W.P., Portner H.O., Rezende E.L., Schulte P.M., Spicer J.I., Stillman J.H., Terblanche J.S., & van Kleunen M. (2009) Macrophysiology: A Conceptual Reunification. *The American Naturalist*, **174**, 595–612.

Gerick A.A., Munshaw R.G., Palen W.J., Combes S.A., & O'Regan S.M. (2014) Thermal physiology and species distribution models reveal climate vulnerability of temperate amphibians. *Journal of Biogeography*, **41**, 713–723.

Ghalambor C.K., Huey R.B., Martin P.R., Tewksbury J.J., & Wang G. (2006) Are

mountain passes higher in the tropics? Janzen's hypothesis revisited. *Integrative and Comparative Biology*, **46**, 5–17.

Gilman S.E., Urban M.C., Tewksbury J., Gilchrist G.W., & Holt R.D. (2010) A framework for community interactions under climate change. *Trends in Ecology & Evolution*, **25**, 325–331.

Gosner (1960) A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, **16**, 183–190.

Goudet J. (2002) FSTAT, a program to estimate and test gene diversities and fixation indices. .

Gouveia S.F., Hortal J., Tejedo M., Duarte H., Cassemiro F.A.S., Navas C.A., & Diniz-Filho J.A.F. (2014) Climatic niche at physiological and macroecological scales: the thermal tolerance--geographical range interface and niche dimensionality. *Global ecology and biogeography*, **23**, 446–456.

Graae B.J., De Frenne P., Kolb A., Brunet J., Chabrerie O., Verheyen K., Pepin N., Heinken T., Zobel M., Shevtsova A., Nijs I., & Milbau A. (2012) On the use of weather data in ecological studies along altitudinal and latitudinal gradients. *Oikos*, **121**, 3–19.

Grigg J.W. & Buckley L.B. (2013) Conservatism of lizard thermal tolerances and body temperatures across evolutionary history and geography. *Biology letters*, **9**, 20121056.

Gunderson A.R. & Stillman J.H. (2015) Plasticity in thermal tolerance has limited potential to buffer ectotherms from global warming. *Proceedings of the Royal Society of London B: Biological Sciences*, **282**, 20150401.

Guo S.W. & Thompson E.A. (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*, 361–372.

Gutiérrez-Pesquera L.M., Tejedo M., Olalla-Tárraga M.Á., Duarte H., Nicieza A., & Solé M. (2016) Testing the climate variability hypothesis in thermal tolerance limits of tropical and temperate tadpoles. *Journal of Biogeography*, .

Haddad N.M., Brudvig L.A., Clobert J., Davies K.F., Gonzalez A., Holt R.D., Lovejoy T.E., Sexton J.O., Austin M.P., Collins C.D., & others (2015) Habitat fragmentation and its lasting impact on Earth's ecosystems. *Science Advances*, **1**, e1500052.

Harmon L.J., Weir J.T., Brock C.D., Glor R.E., & Challenger W. (2008) GEIGER: investigating evolutionary radiations. *Bioinformatics*, **24**, 129–131.

Hercus M.J., Loeschcke V., & Rattan S.I.S. (2003) Lifespan extension of *Drosophila melanogaster* through hormesis by repeated mild heat stress. *Biogerontology*, **4**, 149–156.

Hertz P.E. & Huey R.B. (1981) Compensation for altitudinal changes in the thermal environment by some *Anolis* lizards on Hispaniola. *Ecological Society of America*, **62**, 515–521.

Hertz P.E., Huey R.B., & Nevo E. (1983) Homage to Santa Anita: Thermal sensitivity of sprint speed in agamid lizards. *Evolution*, **37**, 1075–1084.

Herzog S.K., Martínez R., Jørgensen P.M., & Tiessen H. (2011) *Climate change and biodiversity in the tropical Andes.* Inter-American Institute for Global Change Research Sao José dos Campos,

Higgins J.K., MacLean H.J., Buckley L.B., & Kingsolver J.G. (2014) Geographic differences and microevolutionary changes in thermal sensitivity of butterfly larvae in response to climate. *Functional Ecology*, **28**, 982–989.

Hijmans R.J. (2014) Raster: Geographic data analysis and modeling. .

Hijmans R.J., Cameron S.E., Parra J.L., Jones P.G., & Jarvis A. (2005) Very high

resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, **25**, 1965–1978.

Hill J.K., Griffiths H.M., & Thomas C.D. (2011) Climate change and evolutionary adaptations at species' range margins. *Annual Review of Entomology*, **56**, 143–159.

Hochachka P.W. & Somero G.N. (2002) *Biochemical Adaptation.* Oxford University Press, Oxford.

Hof C., Rahbek C., & Araújo M.B. (2010) Phylogenetic signals in the climatic niches of the world's amphibians. *Ecography*, **33**, 242–250.

Hoffmann A.A., Anderson A., & Hallas R. (2002) Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. *Ecology Letters*, **5**, 614–618.

Hoffmann A.A., Chown S.L., & Clusella-Trullas S. (2013) Upper thermal limits in terrestrial ectotherms: how constrained are they? *Functional Ecology*, **27**, 934–949.

Hoffmann A.A. & Loeschcke V. (2002) The detrimental acclimation hypothesis. *Trends in ecology and evolution*, **17**, 407–408.

Hoffmann A.A. & Sgrò C.M. (2011) Climate change and evolutionary adaptation. *Nature*, **470**, 479–85.

Hoffmann A.A., Sorensen J.G., & Loeschcke V. (2003) Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology*, **28**, 175–216.

Hoffmann A.A. & Watson M. (1993) Geographical variation in the acclimation responses of *Drosophila* to temperature extremes. *The American naturalist*, S93–S113.

Hoffmann A.A.., Shirriffs J., & Scott M. (2005) Relative importance of plastic vs genetic factors in adaptive differentiation: geographical variation for stress resistance in *Drosophila melanogaster* from eastern Australia. *Functional Ecology*,

19, 222–227.

Hoffmann M., Hilton-Taylor C., Angulo A., Böhm M., Brooks T.M., Butchart S.H.M., Carpenter K.E., Chanson J., Collen B., Cox N.A., & others (2010) The impact of conservation on the status of the world's vertebrates. *Science*, **330**, 1503–1509.

Hoppe D.M. (1978) Thermal tolerance in tadpoles of the chorus frog Pseudacris triseriata. *Herpetologica* , **34**, 318–321.

Horowitz M. (2001) Heat acclimation: phenotypic plasticity and cues to the underlying molecular mechanisms. *Journal of Thermal Biology*, **26**, 357–363.

Houston A.H. & Gingras-Bedard J.H. (1994) Variable versus constant temperature acclimation regimes: effects on hemoglobin isomorph profile in goldfish, *Carassius auratus*. *Fish Physiology and Biochemistry*, **13**, 445–450.

Howard J.H., Wallace R.L., & Stauffer R. (1983) Critical thermal maxima in populations of *Ambystoma macrodactylum* from different elevations. *Journal of Herpetology*, **17**, 400–402.

Huey R.B., Deutsch C.A., Tewksbury J.J., Vitt L.J., Hertz P.E., Perez H.J.A., & Garland T. (2009) Why tropical forest lizards are vulnerable to climate warming. *Proceedings of the Royal Society B-Biological Sciences*, **276**, 1939–1948.

Huey R.B., Hertz P.E., & Sinervo B. (2003) Behavioral drive versus behavioral inertia in evolution: a null model approach. *The American Naturalist*, **161**, 357–366.

Huey R.B., Kearney M.R., Krockenberger A., Holtum J.A.M., Jess M., & Williams S.E. (2012) Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **367**, 1665–1679.

Huey R.B. & Kingsolver J.G. (1993) Evolution of resistance to high temperature in ectotherms. *The American Naturalist*, S21–S46.

Huey R.B. & Stevenson R.D. (1979) Integrating thermal physiology and ecology of ectotherms: A discussion of approaches. *Integrative and Comparative Biology*, **19**, 357–366.

Hutchinson G.E. (1981) *Introducción a la ecología de poblaciones.* Editorial Blume, Barcelona.

Hutchison V.H. (1961) Critical thermal maxima in salamanders. *Physiological Zoology*, **34**, 92–125.

Hutchison V.H. & Dupré R.K. (1992) Thermoregulation. *Environmental Physiology of the Amphibians* (ed. by M.E. Ferder and W.M. Burggren), The University of Chicago Press, Chicago.

Hutchison V.H. & Ferrance M.R. (1970) Thermal tolerances of *Rana pipiens* acclimated to daily temperature cycles. *Herpetologica*, **26**, 1–8.

IUCN and Nature Serve C.I. (2006) Global Amphibian Assessment. .

Jankowski J.E., Londoño G.A., Robinson S.K., & Chappell M.A. (2013) Exploring the role of physiology and biotic interactions in determining elevational ranges of tropical animals. *Ecography*, **36**, 1–12.

Janzen D.H. (1967) Why mountain passes are higher in the tropics. *The American Naturalist*, **101**, 233–249.

John-Alder H.B., Morin P.J., & Lawler S. (1988) Thermal physiology, phenology, and distribution of tree frogs. *The American Naturalist*, **132**, 506–520.

Katzenberger M. (2014) *Impact of global warming in Holarctic and Neotropical communities of amphibians.*Tesis Doctoral. Universidad de Sevilla.

Kearney M. & Porter W. (2009) Mechanistic niche modelling: combining physiological and spatial data to predict species' ranges. *Ecology letters*, **12**, 334–50.

Kearney M., Shine R., & Porter W.P. (2009) The potential for behavioral

thermoregulation to buffer "cold-blooded" animals against climate warming. *Proceedings of the National Academy of Sciences*, **106**, 3835–3840.

Kellermann V., Loeschcke V., Hoffmann A.A., Kristensen T.N., Fløjgaard C., David J.R., Svenning J.-C., & Overgaard J. (2012a) Phylogenetic constraints in key functional traits behind species' climate niches: patterns of desiccation and cold resistance across 95 *Drosophila* species. *Evolution*, **66**, 3377–3389.

Kellermann V., Overgaard J., Hoffmann A.A., Flojgaard C., Svenning J.C., & Loeschcke V. (2012b) Upper thermal limits of *Drosophila* are linked to species distributions and strongly constrained phylogenetically. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 16228–16233.

Kingsolver J.G. & Huey R.B. (1998) Evolutionary analyses of morphological and physiological plasticity in thermally variable environments. *American Zoologist*, **38**, 545–560.

Klok C.J. & Chown S.L. (2003) Resistance to temperature extremes in sub-Antarctic weevils: interspecific variation,population differentiation and acclimation. *Biological Journal of the Linnean Society*, **78**, 401–414.

Kolbe J.J., Kearney M., & Shine R. (2010) Modeling the consequences of thermal trait variation for the cane toad invasion of Australia. *Ecological Applications*, **20**, 2273–2285.

Körner C. (2007) The use of "altitude"in ecological research. *Trends in ecology & evolution*, **22**, 569–574.

Krebs R.A. & Feder M.E. (1998) Experimental manipulation of the cost of thermal acclimation in *Drosophila melanogaster*. *Biological Journal of the Linnean Society*, **63**, 593–601.

Lagerspetz K.Y.H. (2006) What is thermal acclimation? *Journal of Thermal Biology*,

31, 332–336.

Larsen T.H., Brehm G., Navarrete H., Franco P., Gomez H., Mena J.L., Morales V., Argollo J., Blacutt L., & Canhos V. (2011) Range shifts and extinctions driven by climate change in the tropical Andes: synthesis and directions. In: *Climate Change and Biodiversity in the Tropical Andes: Inter-American Institute for Global Change Research (IAI) and Scientific Committee on Problems of the Environment (SCOPE)*, Sebastian K. Herzog, Rodney Martínez, Peter M. Jørgensen, Holm Tiessen (eds.), pp. 47-67..

Laugen A.T., Laurila A., & Merila J. (2002) Maternal and genetic contributions to geographical variation in Rana temporaria larval life-history traits. *Biological Journal of the Linnean Society*, **76**, 61–70.

Laugen A.T., Laurila A., Rasanen K., & Merila J. (2003) Latitudinal countergradient variation in the common frog (*Rana temporaria*) development rates - evidence for local adaptation. *Journal of Evolutionary Biology*, **16**, 996–1005.

Laurance W.F., Useche D.C., Shoo L.P., Herzog S.K., Kessler M., Escobar F., Brehm G., Axmacher J.C., Chen I.C., Gamez L.A., Hietz P., Fiedler K., Pyrcz T., Wolf J., Merkord C.L., Cardelus C., Marshall A.R., Ah-Peng C., Aplet G.H., Arizmendi M.D., Baker W.J., Barone J., Bruhl C.A., Bussmann R.W., Cicuzza D., Eilu G., Favila M.E., Hemp A., Hemp C., Homeier J., Hurtado J., Jankowski J., Kattan G., Kluge J., Kromer T., Lees D.C., Lehnert M., Longino J.T., Lovett J., Martin P.H., Patterson B.D., Pearson R.G., Peh K.S.H., Richardson B., Richardson M., Samways M.J., Senbeta F., Smith T.B., Utteridge T.M.A., Watkins J.E., Wilson R., Williams S.E., & Thomas C.D. (2011) Global warming, elevational ranges and the vulnerability of tropical biota. *Biological Conservation*, **144**, 548–557.

Leinonen T., Cano J.M., Mäkinen H., & Merilä J. (2006) Contrasting patterns of body

shape and neutral genetic divergence in marine and lake populations of threespine sticklebacks. *Journal of evolutionary biology*, **19**, 1803–1812.

Leinonen T., McCairns R.J.S., O'Hara R.B., & Merilä J. (2013) Q(ST)-F(ST) comparisons: evolutionary and ecological insights from genomic heterogeneity. *Nature reviews. Genetics*, **14**, 179–90.

Leinonen T., O'Hara R.B., Cano J.M., & Merilä J. (2008) Comparative studies of quantitative trait and neutral marker divergence: a meta-analysis. *Journal of evolutionary biology*, **21**, 1–17.

Lind M.I., Ingvarsson P.K., Johansson H., Hall D., & Johansson F. (2011) Gene flow and selection on phenotypic plasticity in an island system of *Rana temporaria*. *Evolution; international journal of organic evolution*, **65**, 684–97.

Lindgren B. & Laurila A. (2009) Physiological variation along a geographical gradient: is growth rate correlated with routine metabolic rate in *Rana temporaria* tadpoles? *Biological Journal of the Linnean Society*, **98**, 217–224.

Lips K.R., Burrowes P.A., Mendelson J.R., & Parra-Olea G. (2005) Amphibian declines in Latin America: widespread population declines, extinctions, and impacts. *Biotropica*, **37**, 163–165.

Livingstone D.M. & Lotter A.F. (1998) The relationship between air and water temperatures in lakes of the Swiss Plateau: a case study with paleolimnological implications. *Journal of Paleolimnology*, **19**, 181–198.

Loeschcke, V. and Hoffmann, A.A. (2002) The detrimental acclimation hypothesis. *Trends in Ecology and Evolution*, **17**, 407-408

Loeschcke V. & Sorensen J.G. (2005) Acclimation, heat shock and hardening - a response from evolutionary biology. *Journal of thermal biology*, **30**, 255–257.

Losos J.B. (2008) Phylogenetic niche conservatism, phylogenetic signal and the

relationship between phylogenetic relatedness and ecological similarity among species. *Ecology letters*, **11**, 995–1003.

Lutterschmidt W.I. & Hutchison V.H. (1997a) The critical thermal maximum: history and critique. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, **75**, 1561–1574.

Lutterschmidt W.I. & Hutchison V.H. (1997b) The critical thermal maximum: data to support the onset of spasms as the definitive end point. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, **75**, 1553–1560.

Marshall K.E. & Sinclair B.J. (2009) Repeated stress exposure results in a survival--reproduction trade-off in *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, rspb20091807.

Martins E.P. (1996) Phylogenies, spatial autoregression, and the comparative method: a computer simulation test. *Evolution*, 1750–1765.

Menke M.E. & Claussen D.L. (1982) Thermal acclimation and hardening in tadpoles of the bullfrog, *Rana catesbeiana*. *Journal of thermal biology*, **7**, 215–219.

Miller K. & Packard G.C. (1974) Critical thermal maximum: ecotypic variation between montane and piedmont chorus frogs (*Pseudacris triseriata*, Hylidae). *Experientia*, **30**, 355–356.

Miller K. & Packard G.C. (1977) Altitudinal cline in critical thermal maxima of chorus frogs (Pseudacris triseriata). *The American Naturalist*, **111**, 267–277.

Mitchell K.A. & Hoffmann A.A. (2010) Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in *Drosophila*. *Functional Ecology*, **24**, 694–700.

Mittermeier R.A., Robles-Gil P., & Mittermeier C.G. (1997) *Megadiversity: Earth's biologically wealthiest nations.* CEMEX/ Agrupación Sierra Madre, Mexico City.

Morueta-Holme N., Engemann K., Sandoval-Acuña P., Jonas J.D., Segnitz R.M., & Svenning J.-C. (2015) Strong upslope shifts in Chimborazo's vegetation over two centuries since Humboldt. *Proceedings of the National Academy of Sciences*, **112**, 12741–12745.

Muir A.P., Biek R., & Mable B.K. (2014a) Behavioural and physiological adaptations to low-temperature environments in the common frog, *Rana temporaria*. *BMC evolutionary biology*, **14**, 1.

Muir A.P., Biek R., Thomas R., & Mable B.K. (2014b) Local adaptation with high gene flow: temperature parameters drive adaptation to altitude in the common frog (*Rana temporaria*). *Molecular ecology*, **23**, 561–574.

Münkemüller T., Lavergne S., Bzeznik B., Dray S., Jombart T., Schiffers K., & Thuiller W. (2012) How to measure and test phylogenetic signal. *Methods in Ecology and Evolution*, **3**, 743–756.

Muñoz M.M., Stimola M.A., Algar A.C., Conover A., Rodriguez A.J., Landestoy M.A., Bakken G.S., & Losos J.B. (2014) Evolutionary stasis and lability in thermal physiology in a group of tropical lizards. *Proceedings of the Royal Society of London B: Biological Sciences*, **281**, 20132433.

Myers N., Mittermeier R.A., Mittermeier C.G., Da Fonseca G.A.B., & Kent J. (2000) Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853–858.

Navas C.A. (1996) Implications of microhabitat selection and patterns of activity on the thermal ecology of high elevation neotropical anurans. *Oecologia*, **108**, 617–626.

Navas C.A. (1997) Thermal extremes at high elevations in the Andes: physiological ecology of frogs. *Journal of thermal Biology*, **22**, 467–477.

Navas C.A. (2002) Herpetological diversity along Andean elevational gradients: links with physiological ecology and evolutionary physiology. *Comparative*

Biochemistry and Physiology Part A: Molecular & Integrative Physiology, **133**, 469–485.

Navas C.A., Carvajalino-Fernández J.M., Saboyá-Acosta L.P., Rueda-Solano L.A., & Carvajalino-Fernández M.A. (2013) The body temperature of active amphibians along a tropical elevation gradient: patterns of mean and variance and inference from environmental data. *Functional Ecology*, **27**, 1145–1154.

Niehaus A.C., Angilletta M.J., Sears M.W., Franklin C.E., & Wilson R.S. (2012) Predicting the physiological performance of ectotherms in fluctuating thermal environments. *The Journal of Experimental Biology*, **215**, 694–701.

Niehaus A.C., Wilson R.S., & Franklin C.E. (2006) Short- and long-term consequences of thermal variation in the larval environment of anurans. *Journal of Animal Ecology*, **75**, 686–692.

Noland R. & Ultsch G.R. (1981) The roles of temperature and dissolved oxygen in microhabitat selection by the tadpoles of a frog (*Rana pipiens*) and a toad (*Bufo terrestris*). *Copeia*, **1981**, 645–652.

Nyamukondiwa C., Kleynhans E., & Terblanche J.S. (2010) Phenotypic plasticity of thermal tolerance contributes to the invasion potential of Mediterranean fruit flies (*Ceratitis capitata*). *Ecological Entomology*, **35**, 565–575.

Olalla-Tárraga M.Á., McInnes L., Bini L.M., Diniz-Filho J. a. F., Fritz S. a., Hawkins B. a., Hortal J., Orme C.D.L., Rahbek C., Rodríguez M.Á., & Purvis A. (2011) Climatic niche conservatism and the evolutionary dynamics in species range boundaries: global congruence across mammals and amphibians. *Journal of Biogeography*, **38**, 2237–2247.

Oosterhout C., Hutchinson W.F., Wills D.P.M., & Shipley P. (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in

microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Orme D., Freckleton R., Thomas G., & S. P.T.& F. (2009) CAIC: Comparative analyses using independent contrasts.

Orme D., Freckleton R., Thomas G., Petzoldt T., Fritz S., Isaac N., & Pearse W. (2013) caper: Comparative analyses of phylogenetics and evolution in R. .

Overgaard J., Hoffmann A.A., & Kristensen T.N. (2011a) Assessing population and environmental effects on thermal resistance in *Drosophila melanogaster* using ecologically relevant assays. *Journal of Thermal Biology*, **36**, 409–416.

Overgaard J., Kristensen T.N., Mitchell K.A., & Hoffmann A.A. (2011b) Thermal tolerance in widespread and tropical *Drosophila* species: does phenotypic plasticity increase with latitude? The *American Naturalist*, **178**, S80–S96.

Paaijmans K.P., Blanford S., Bell A.S., Blanford J.I., Read A.F., & Thomas M.B. (2010) Influence of climate on malaria transmission depends on daily temperature variation. *Proceedings of the National Academy of Sciences*, **107**, 15135–15139.

Paaijmans K.P., Heinig R.L., Seliga R.A., Blanford J.I., Blanford S., Murdock C.C., & Thomas M.B. (2013) Temperature variation makes ectotherms more sensitive to climate change. *Global change biology*, **19**, 2373–2380.

Pachauri R.K., Allen M.R., Barros V.R., Broome J., Cramer W., Christ R., Church J.A., Clarke L., Dahe Q., Dasgupta P., & others (2014) Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. .

Palo J.U., O'Hara R.B., Laugen A.T., Laurila A., Primmer C.R., & Merila J. (2003) Latitudinal divergence of common frog (*Rana temporaria*) life history traits by natural selection: evidence from a comparison of molecular and quantitative genetic data. *Molecular Ecology*, **12**, 1963–1978.

Parmesan C. (2006) Ecological and evolutionary responses to recent climate change. *Annual Review of Ecology Evolution and Systematics*, **37**, 637–669.

Parmesan C., Burrows M.T., Duarte C.M., Poloczanska E.S., Richardson A.J., Schoeman D.S., & Singer M.C. (2013) Beyond climate change attribution in conservation and ecological research. *Ecology letters*, **16**, 58–71.

Peterson A.T. (2011) Ecological niche conservatism: a time-structured review of evidence. *Journal of Biogeography*, **38**, 817–827.

Phillimore A.B., Hadfield J.D., Jones O.R., & Smithers R.J. (2010) Differences in spawning date between populations of common frog reveal local adaptation. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 8292–7.

Pidancier N., Miquel C., & Miaud C. (2003) Buccal swabs as a non-destructive tissue sampling method for DNA analysis in amphibians. *Herpetological Journal*, **13**, 175–178.

Podrabsky J.E. & Somero G.N. (2004) Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish Austrofundulus limnaeus. *Journal of Experimental Biology*, **207**, 2237–2254.

Portner H.O., Bennett A.F., Bozinovic F., Clarke A., Lardies M.A., Lucassen M., Pelster B., Schiemer F., & Stillman J.H. (2006) Trade-offs in thermal adaptation: The need for a molecular to ecological integration. *Physiological and Biochemical Zoology*, **79**, 295–313.

Portner H.O. & Knust R. (2007) Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science*, **315**, 95–97.

Potter K.A., Arthur Woods H., & Pincebourde S. (2013) Microclimatic challenges in

global change biology. *Global change biology*, **19**, 2932–9.

Pounds J.A., Fogden M.P.L., & Masters K.L. (2005) Responses ofnatural communities to climate change in a highland tropical forest. *Climate change and biodiversity* (ed. by T.E. Lovejoy and L. Hannah), pp. 70–74. Yale University Press, New Haven.

Price T.D., Qvarnström A., & Irwin D.E. (2003) The role of phenotypic plasticity in driving genetic evolution. *Proceedings of the Royal Society of London B: Biological Sciences*, **270**, 1433–1440.

Pujol B., Wilson A.J., Ross R.I.C., & Pannell J.R. (2008) Are Q(ST)-F(ST) comparisons for natural populations meaningful? *Molecular ecology*, **17**, 4782–5.

Pyron R.A. & Wiens J. (2011) A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. *Molecular Phylogenetics and Evolution*, **61**, 543–583.

R Core Team (2014) R: A Language and Environment for Statistical Computing. .

Rapoport E.H. (1975) *Aerografía: Estrategias Geográficas de las Especies.* Fondo de Cultura Económica,

Raxworthy C.J., Pearson R.G., Rabibisoa N., Rakotondrazafy A.M., Ramanamanjato J. B., Raselimanana A.P., Wu S., Nussbaum R.A., & Stone D.A. (2008) Extinction vulnerability of tropical montane endemism from warming and upslope displacement: a preliminary appraisal for the highest massif in Madagascar. *Global Change Biology*, **14**, 1703–1720.

Raymond M. & Rousset F. (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rezende E.L., Tejedo M., & Santos M. (2011) Estimating the adaptive potential of critical thermal limits: methodological problems and evolutionary implications.

Functional Ecology, **25**, 111–121.

Richter-Boix A., Katzenberger M., Duarte H., Quintela M., Tejedo M., & Laurila A. (2015) Local divergence of thermal reaction norms among amphibian populations is affected by pond temperature variation. *Evolution*, **69**, 2210–2226.

Roberts D.A., Hofmann G.E., & Somero G.N. (1997) Heat-shock protein expression in *Mytilus californianus*: acclimatization (seasonal and tidal-height comparisons) and acclimation effects. *The Biological Bulletin*, **192**, 309–320.

Ron S.R., Guayasamin J.M., Yánez-Muñoz M.H., Merino-Viteri A., Ortiz D.A., & Nicolalde D.A. (2014) AmphibiaWebEcuador. Version 2013.0. Museo de Zoología, Pontificia Universidad Católica del Ecuador. .

Root T.L., Price J.T., Hall K.R., Schneider S.H., Rosenzweig C., & Pounds J.A. (2003) Fingerprints of global warming on wild animals and plants. *Nature*, **421**, 57–60.

Ruel J.J. & Ayres M.P. (1999) Jensen's inequality predicts effects of environmental variation. *Trends in Ecology & Evolution*, **14**, 361–366.

Sala O.E., Chapin F.S., Armesto J.J., Berlow E., Bloomfield J., Dirzo R., Huber-Sanwald E., Huenneke L.F., Jackson R.B., Kinzig A., & others (2000) Global biodiversity scenarios for the year 2100. *science*, **287**, 1770–1774.

Santos T., Diniz-Filho J.A., & e Luis Mauricio Bini T.R. (2013) PVR: Computes phylogenetic eigenvectors regression (PVR) and phylogenetic signal-representation curve (PSR) (with null and Brownian expectations). .

Scheffers B.R., Brunner R.M., Ramirez S.D., Shoo L.P., Diesmos A., & Williams S.E. (2013) Thermal buffering of microhabitats is a critical factor mediating warming vulnerability of frogs in the philippine biodiversity hotspot. *Biotropica*, **45**, 628–635.

Schlichting C.D., Pigliucci M., & others (1998) *Phenotypic evolution: a reaction norm*

perspective. Sinauer Associates Incorporated,

Seebacher F. & Franklin C.E. (2012) Determining environmental causes of biological effects: the need for a mechanistic physiological dimension in conservation biology. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, **367**, 1607–14.

Seebacher F., Holmes S., Roosen N.J., Nouvian M., Wilson R.S., & Ward A.J.W. (2012) Capacity for thermal acclimation differs between populations and phylogenetic lineages within a species. *Functional Ecology*, **26**, 1418-1428.

Seimon T.A., Seimon A., Daszak P., Halloy S.R.P., Schloegel L.M., Aguilar C.A., Sowell P., Hyatt A.D., Konecky B., & Simmons J.E. (2007) Upward range extension of Andean anurans and chytridiomycosis to extreme elevations in response to tropical deglaciation. *Global Change Biology*, **13**, 288–299.

Sheldon K.S. & Tewksbury J.J. (2014) The impact of seasonality in temperature on thermal tolerance and elevational range size. *Ecology*, **95**, 2134–2143.

Sheldon K.S., Yang S., & Tewksbury J.J. (2011) Climate change and community disassembly: impacts of warming on tropical and temperate montane community structure. *Ecology Letters*, **14**, 1191–1200.

Simon M.N., Ribeiro P.L., & Navas C.A. (2015) Upper thermal tolerance plasticity in tropical amphibian species from contrasting habitats: Implications for warming impact prediction. *Journal of thermal biology*, **48**, 36–44.

Sinclair B.J. & Roberts S.P. (2005) Acclimation, shock and hardening in the cold. *Journal of Thermal Biology*, **30**, 557–562.

Sinsch U. (1991) Mini-review: the orientation behaviour of amphibians. *Herpetological Journal*, **1**, 1–544.

Smith R.L. & Smith T.M. (2001) *Ecología.* Pearson Educación, Madrid.

Smith S.A., Stephens P.R., & Wiens J.J. (2005) Replicate patterns of species richness, historical biogeography, and phylogeny in Holarctic treefrogs. *Evolution*, **59**, 2433–2450.

Snyder G.K. & Weathers W.W. (1975) Temperature adaptations in amphibians. *The American Naturalist*, **109**, 93–101.

Soberón J. & Nakamura M. (2009) Niches and distributional areas: concepts, methods, and assumptions. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 19644–19650.

Somero G.N. (2005) Linking biogeography to physiology: evolutionary and acclimatory adjustments of thermal limits. *Front Zool*, **2**, 1.

Somero G.N. (2010) The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine "winners" and "losers." *The Journal of experimental biology*, **213**, 912–920.

Soobramoney S., Downs C.T., & Adams N.J. (2003) Physiological variability in the fiscal shrike *Lanius collaris* along an altitudinal gradient in South Africa. *Journal of Thermal Biology*, **28**, 581–594.

Sorensen J.G., Kristensen T.N., & Loeschcke V. (2003) The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, **6**, 1025–1037.

Sorensen J.G., Norry F.M., Scannapieco A.C., & Loeschcke V. (2005) Altitudinal variation for stress resistance traits and thermal adaptation in adult *Drosophila buzzatii* from the New World. *Journal of Evolutionary Biology*, **18**, 829–37.

Sorensen J.G., Pekkonell M., Beatrice L.C., Loeschcke V., Laurila A., & Merila J. (2009a) Complex patterns of geographic variation in heat tolerance and Hsp70 expression levels in the common frog Rana temporaria. *Journal of Thermal Biology*, **34**, 49–54.

Sorensen J.G., Pekkonen M., Lindgren B., Loeschcke V., Laurila A., & Merilä J. (2009b) Complex patterns of geographic variation in heat tolerance and Hsp70 expression levels in the common frog Rana temporaria. *Journal of Thermal Biology*, **34**, 49–54.

Southward A.J., Hawkins S.J., & Burrows M.T. (1995) 70 Years observations of changes in distribution and abundance of zooplankton and intertidal organisms in the Western English-channel in relation to rising sea temperature. *Journal of Thermal Biology*, **20**, 127–155.

Stahlberg F., Olsson M., & Uller T. (2008) Population divergence of developmental thermal optima in Swedish common frogs, *Rana temporaria*. *Journal of Evolutionary Biology*, **14**, 755–762.

Stevens G.C. (1989) The latitudinal gradient in geographical range – how so many species coexist in the tropics. *The American Naturalist*, **133**, 240–256.

Stevens G.C. (1992) The elevational gradient in altitudinal range – an extension of Rapoport latitudinal rule to altitude. *The American Naturalist*, **140**, 893–911.

Stillman J.H. (2003) Acclimation capacity underlies susceptibility to climate change. *Science (New York, N.Y.)*, **301**, 65.

Stillman J.H. (2004) A comparative analysis of plasticity of thermal limits in porcelain crabs across latitudinal and intertidal zone clines. *Animals and Environments* (ed. by S. Morris and A. Vosloo), pp. 267–274.

Stillman J.H. & Somero G.N. (2000) A comparative analysis of the upper thermal tolerance limits of eastern Pacific porcelain crabs, genus Petrolisthes: Influences of latitude, vertical zonation, acclimation and phylogeny. *Physiological and Biochemical Zoology*, **73**, 200–208.

Strachan L.A., Tarnowski-Garner H.E., Marshall K.E., & Sinclair B.J. (2011) The

evolution of cold tolerance in *Drosophila* larvae. *Physiological and Biochemical Zoology*, **84**, 43–53.

Stuart S.N., Chanson J.S., Cox N.A., Young B.E., Rodrigues A.S.L., Fischman D.L., & Waller R.W. (2004) Status and trends of amphibian declines and extinctions worldwide. *Science*, **306**, 1783–1786.

Sunday J.M., Bates A.E., & Dulvy N.K. (2011) Global analysis of thermal tolerance and latitude in ectotherms. *Proceedings of the Royal Society B-Biological Sciences*, **278**, 1823–1830.

Sunday J.M., Bates A.E., Kearney M.R., Colwell R.K., Dulvy N.K., Longino J.T., & Huey R.B. (2014) Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. *Proceedings of the National Academy of Sciences of the United States of America*, **111**, 5610–5615.

Terblanche J.S., Nyamukondiwa C., & Kleynhans E. (2010) Thermal variability alters climatic stress resistance and plastic responses in a globally invasive pest, the Mediterranean fruit fly (*Ceratitis capitata*). *Entomologia Experimentalis et Applicata*, **137**, 304–315.

Thomas M.K., Kremer C.T., Klausmeier C.A., & Litchman E. (2012) A global pattern of thermal adaptation in marine phytoplankton. *Science*, **338**, 1085–1088.

Tuff K.T., Tuff T., & Davies K.F. (2016) A framework for integrating thermal biology into fragmentation research. *Ecology Letters*, **19**, 361-374. .

Turriago J.L., Parra C.A., & Bernal M.H. (2015) Upper thermal tolerance in anuran embryos and tadpoles at constant and variable peak temperatures. *Canadian Journal of Zoology*, **93**, 267-272..

Ultsch G.R., Bradford D.F., & Freda J. (1999) Physiology: coping with the environment. *Tadpoles: the biology of anuran larvae*, 189–214.

Urban M.C., Tewksbury J.J., & Sheldon K.S. (2012) On a collision course: competition and dispersal differences create no-analogue communities and cause extinctions during climate change. *Proceedings of the Royal Society of London B: Biological Sciences*, **279**, 2072–2080.

Vasseur D.A., DeLong J.P., Gilbert B., Greig H.S., Harley C.D.G., McCann K.S., Savage V., Tunney T.D., & O'Connor M.I. (2014) Increased temperature variation poses a greater risk to species than climate warming. *Proceedings of the Royal Society of London B: Biological Sciences*, **281**, 20132612.

Verdú J.R. (2011) Chill tolerance variability within and among populations in the dung beetle *Canthon humectus hidalgoensis* along an altitudinal gradient in the mexican semiarid high plateau. *Journal of Arid Environments*, **75**, 119–124.

Vinagre C., Leal I., Mendonça V., Madeira D., Narciso L., Diniz M.S., & Flores A.A. V (2015) Vulnerability to climate warming and acclimation capacity of tropical and temperate coastal organisms. *Ecological Indicators*, **62**, 317-327.

Wake D.B. & Vredenburg V.T. (2008) Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 11466–11473.

Walther G.-R., Post E., Convey P., Menzel A., Parmesan C., Beebee T.J.C., Fromentin J.-M., Hoegh-Guldberg O., & Bairlein F. (2002) Ecological responses to recent climate change. *Nature*, **416**, 389–95.

Webb B.W., Clack P.D., & Walling D.E. (2003) Water–air temperature relationships in a Devon river system and the role of flow. *Hydrological Processes*, **17**, 3069–3084.

Weir B.S. & Cockerham C.C. (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Wells K.D. (2007) *The ecology and behavior of amphibians*. The University of Chicago Press, United States of America.

Whitton F.J.S., Purvis A., Orme C.D.L., & Olalla-Tárraga M.Á. (2012) Understanding global patterns in amphibian geographic range size: does Rapoport rule? *Global Ecology and Biogeography*, **21**, 179–190.

Wiens J.J., Graham C.H., Moen D.S., Smith S.A., & Reeder T.W. (2006) Evolutionary and ecological causes of the latitudinal diversity gradient in hylid frogs: treefrog trees unearth the roots of high tropical diversity. *The American Naturalist*, **168**, 579–596.

Williams S.E., Shoo L.P., Isaac J.L., Hoffmann A.A., & Langham G. (2008) Towards an integrated framework for assessing the vulnerability of species to climate change. *PLoS biology*, **6**, 2621–6.

Wilson R.J., Gutiérrez D., Gutiérrez J., Martínez D., Agudo R., & Monserrat V.J. (2005) Changes to the elevational limits and extent of species ranges associated with climate change. *Ecology Letters*, **8**, 1138–1146.

Wilson R.S. & Franklin C.E. (2002) Testing the beneficial acclimation hypothesis. *Trends in Ecology & Evolution*, **17**, 66–70.

Young K.R. (2011) Introduction to Andean Geographies. *Climate Change and Biodiversity in the Tropical Andes* (ed. by S.K. Herzog, R. Martinez, P.M. Jorgensen, and H. Tiessen), pp. 128–140. Inter-American Institute for Global Change Research (IAI) and Scientific Committee on Problems of the Environment (SCOPE),

Zheng R.-Q. & Liu C.-T. (2010) Giant spiny-frog (*Paa spinosa*) from different populations differ in thermal preference but not in thermal tolerance. *Aquatic Ecology*, **44**, 723–729.

Zuloaga J. & Kerr J.T. (2016) Over the top: do thermal barriers along elevation gradients limit biotic similarity? *Ecography*.

ANNEXE 1

Supporting Information for Chapter 1

APPENDIX S1 SUPPLEMENTARY METHODS

Digital distribution maps of the analyzed species were obtained from IUCN (IUCN and Nature Serve, 2006). We used these layers mapped on a cylindrical equal area projection to obtain the following geographical variables in QGIS for each taxon: total area of the geographic range, longitude and latitude of the centroid, maximum and minimum values of latitude, and latitudinal range. We used both latitudinal and longitudinal coordinates of the population samples as well as their total polygon distribution to extract and summarize the climatic information from WorldClim layers (30 ” spatial resolutions; records from 1950 to 2000 (Hijmans *et al*., 2005). We obtained six bioclimatic variables: annual mean temperature (BIO1), mean diurnal range (BIO2), seasonality (bio4) maximum temperature of the warmest month (BIO5), minimum temperature of the coldest month (BIO6) and temperature annual range (BIO7). Additionally, we examined monthly variables: mean, maximum and minimum temperatures considering only the reproductive period of the species.

Although WorldClim climatic variables correspond to air measurements, previous studies have shown that air temperatures correlate well with water temperature in streams and lakes (Livingstone & Lotter 1998; Pilgrim *et al*., 1998; Webb *et al*., 2003), being reliable predictors used in biogeographical and conservation analyses with aquatic organisms such as continental fishes (Li *et al*., 2009; Schaefer & Arroyave 2010; Chessman, 2013) and amphibian tadpoles (Gerick *et al*., 2014).

Our previous analyses found that many of the WorldClim variables were highly correlated (R^2>0.80) and that using climatic information from WorldClim estimated at the sampling point or for the whole distribution was redundant (Table S3, Appendix S2). Also, because WorldClim BIO variables do not consider reproductive period of

species, we reduced the initial variable set to the following four macroclimatic variables: TMAX (Maximum of the Average monthly maximum temperature); TMIN (Minimum of the Average monthly minimum temperature); TMEAN (Monthly Mean Temperature) and Temperature Annual Range (TMAX-TMIN) for the sample point.

To assess the explanatory power of microclimatic habitat conditions on thermal tolerance limits, we included the microenvironment thermal information obtained for each sampling point directly from the dataloggers (HOBO pendant). Water temperature was recorded every 15 min. We limited our analyses to the larval period and discarded thermal records when the pond had dried. This condition was easily recognizable by the appearance of erratic peaks typical of air measurement against a smooth profile characteristic of a water thermal record. We analyzed mean (tmean), maximum (tmax) and minimum (tmin) daily temperature, average daily range and seasonal range (tmax-tmin) from each pond. The number of sampling days ranged from 30-497 days (see Table S2, Appendix S2 Supporting information). We used function VIF in R-package (fmsb) to evaluate multicolinearity in our final models (Table 2). If VIF is more than 10, multicolinearity is strongly suggested. We found no strong colinearity in any of the models presented in our results. For example, CTmax~TMEAN+tmax VIF=2.83, CTmin~TMIN + tmin VIF=9.04, TR~SR+sr VIF=2.25.

Phylogenetic comparative analyses

Data collected across multiple species violate the basic assumption of statistical independence of observations (Felsenstein 1985; Harvey & Pagel 1991; Garland *et al*., 1992); therefore, all statistical analyses were undertaken incorporating phylogenetic information. To evaluate the correlations between thermal physiology variables (CTmax, CTmin and Thermal Tolerance Range), geographical variables (area and

latitude of species distribution), and the temperature variables (both at the macro and the microenvironmental scale), we used phylogenetic generalized least squares (PGLS) analyses under a Brownian motion model of evolution using the package CAIC (Orme *et al*., 2009) in R. We also use PGLS to detect correlation between CTmax and CTmin. The PGLS model incorporates a parameter lambda (λ), which adjusts the variance-covariance matrix so that the model fits the assumptions of the Brownian model of phenotypic evolution. A high value of lambda (i.e. $\lambda = 1$) indicates that the covariance between the traits follows that predicted under a Brownian model of trait evolution, where variance in traits accumulates with time since divergence from a common ancestor, whereas values of $\lambda < 1$ indicate that the actual covariance between the traits is lower than would be expected under a Brownian model (Freckleton *et al*., 2002). We select the best model in the set employing the lowest Akaike Information Criterion (AIC) and Akaike weights (w_i) (Burnham & Anderson, 2002). Akaike weights provide the probability that a model is the best fit among those tested, with values close to 1 being the best models and models with similar weights having similar levels of support in the data (Clusella-Trullas *et al*., 2011).

Phylogenetic signal on thermal limits

Phylogenetic signal can be defined as the tendency for related species to resemble each other more than they resemble species drawn at random from the phylogenetic tree (Blomberg & Garland 2002; Losos, 2008). It may arise due to two causes: phylogenetic inertia, because of conservation of the trait throughout the phylogeny or convergent evolution owing to adaptation to similar environments (adaptation vs. constraint) (Freckleton *et al*. 2002; Losos, 2008). Thus, related species may exhibit similar physiological tolerance limits (phylogenetic signal) due to either

evolutionary phylogenetic constraints (phylogenetic inertia) or spatial proximity, which may determine common selection regimes (Kellermann *et al*., 2012b)

To untangle this, we assessed the phylogenetic signal in physiological resistance traits by using two different methods. We used the fitContinuous function in GEIGER (R package) assuming a Brownian motion model of character evolution, to estimate the Pagel's λ for each trait. As in the case of PGLS, this parameter indicates the degree of phylogenetic correlation in the data (ranging from 0, no phylogenetic effects, to 1 strong phylogenetic inertia). In addition, Phylogenetic eigenvectors Regression method (PVR) (Diniz-Filho *et al*., 1998), allowed us to partition the components of variance attributable to ecological (S) and phylogenetic effects (P).

We used the function PVRdecomp from the PVR package in R to obtain a set of orthogonal eigenvectors based on our phylogenetic distance matrix. Eigenvector selection was implemented using a non-sequential method that minimizes residual phylogenetic autocorrelation, an approach that has shown to be robust to accurately quantify phylogenetic signal with PVR under different evolutionary scenarios (Diniz Filho *et al*., 2012). We used Moran's I smaller than 0.05 as a stopping rule for our iterative search (Diniz Filho *et al*., 2012). The selected eigenvectors were then used as explanatory variables in a standard OLS multiple regression. Coefficients of determination of these models provide an estimate of the amount of phylogenetic signal in the trait.

References in Supporting Information

Blomberg S.P. & Garland T. (2002) Tempo and mode in evolution: phylogenetic inertia, adaptation and comparative methods. *Journal of Evolutionary Biology*, **15**, 899–910.

Burnham K.P. & Anderson D.R. (2002) *Model selection and multi-model inference: a practical information-theoretic approach*. Springer, New York.

Clusella-Trullas S., Blackburn T.M., & Chown S.L. (2011) Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change. *The American Naturalist*, **177**, 738–751.

Chessman B.C. (2013) Do protected areas benefit freshwater species? A broad-scale assessment for fish in Australia's Murray–Darling Basin. *Journal of Applied Ecology*, **50**, 969–976.

Diniz Filho J.A.F., Rangel T.F., Santos T., & Bini L.M. (2012) Exploring patterns of interspecific variation in quantitative traits using sequential phylogenetic eigenvector regressions. *Evolution; International Journal of Organic Evolution*, **66**, 1079–90.

Diniz-Filho J.A.F., Sant´Ana R. De, & Bini L.M. (1998) An eigenvector method for estimating phylogenetic inertia. *Evolution*, **52**, 1247–1262.

Felsenstein J. (1985) Phylogenies and the comparative method. *The American Naturalist*, **125**, 1–15.

Freckleton R., Harvey P.H., & Pagel M. (2002) Phylogenetic analysis and comparative data: a test and review of evidence. *The American Naturalist*, **160**, 712–726.

Garland T., Harvey P., & Ives A. (1992) Procedures for the analysis of comparative data using phylogenetically independent contrasts. *Sistematic Biology*, **41**, 18–32.

Gerick A.A., Munshaw R.G., Palen W.J., Combes S.A., & O'Regan S.M. (2014) Thermal physiology and species distribution models reveal climate vulnerability of temperate amphibians. *Journal of Biogeography*, **41**, 713–723.

Harvey P. & Pagel M. (1991) *The comparative method in evolutionary biology*. Oxford University Press, Oxford.

Hijmans R.J., Cameron S.E., Parra J.L., Jones P.G., & Jarvis A. (2005) Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, **25**, 1965–1978.

IUCN and Nature Serve C.I. (2006) Global Amphibian Assessment. Available at http://www.iucnredlist.org/technical-documents/spatial-data (accessed 16 Nov 2015).

Kellermann V., Loeschcke V., Hoffmann A.A., Kristensen T.N., Fløjgaard C., David J.R., Svenning J.-C., & Overgaard J. (2012) Phylogenetic constraints in key functional traits behind species' climate niches: patterns of desiccation and cold resistance across 95 *Drosophila* species. *Evolution*, **66**, 3377–3389.

Li J., He Q., Hua X., Zhou J., Xu H., Chen J., & Fu C. (2009) Climate and history explain the species richness peak at mid-elevation for *Schizothorax* fishes (Cypriniformes: Cyprinidae) distributed in the Tibetan Plateau and its adjacent regions. *Global Ecology and Biogeography*, **18**, 264–272.

Livingstone D.M. & Lotter A.F. (1998) The relationship between air and water temperatures in lakes of the Swiss Plateau: a case study with paleolimnological implications. *Journal of Paleolimnology*, **19**, 181–198.

Losos J.B. (2008) Phylogenetic niche conservatism, phylogenetic signal and the relationship between phylogenetic relatedness and ecological similarity among species. *Ecology letters*, **11**, 995–1003.

Orme D., Freckleton R., Thomas G., & S. P.T.& F. (2009) CAIC: Comparative analyses using independent contrasts. R package version 1.0.4-94/r94. Available at: http://R-Forge.R-project.org/projects/caic/ (accessed 16 Nov 2015).

Pilgrim J.M., Fang X., & Stefan H.G. (1998) Stream temperature correlations with air temperatures in Minnesota: implications for climate warming. *Journal of the American Water Resources Association*, **34**, 1109–1121.

Schaefer S.A. & Arroyave J. (2010) Rivers as islands: determinants of the distribution of Andean astroblepid catfishes. *Journal of Fish Biology*, **77**, 2373–2390.

Webb B.W., Clack P.D., & Walling D.E. (2003) Water–air temperature relationships in a Devon river system and the role of flow. *Hydrological Processes*, **17**, 3069–3084.

APPPENDIX S2. SUPPLEMENTARY TABLES

Table S1. Summary of the physiological traits and geographical sampling location of the 47 studied species. The coordinates of the sample point are in decimal degrees. WT: warming tolerance expressed as CTmax-tmax, see Table S2 (Deutsch *et al*. 2008, Duarte *et al*. 2012); CT: cooling tolerance, expressed as tmin-CTmin, see Table S2). NA: not available data.

Specie	SPP. Code	Region	Sample point		CTmax			CTmin			Tolerance	WT	CT
			Longitude	Latitude	N	$\bar{X}$	SD	N	$\bar{X}$	SD	Range		
Agalychnis aspera	AAS	Tropical	-39.2210	-14.6475	2	39.1	0.07	10	8.9	1.20	30.2	15.04	10.29
Alytes cisternasii	ACI	Temperate	-6.0799	37.7929	16	37.8	0.37	16	-0.2	0.54	38.0	7.85	4.51
Alytes dickhilleni	ADI	Temperate	-3.5594	37.0462	16	38.0	0.32	16	0.1	1.09	38.0	17.67	1.56
Alytes obstetricans	AOB	Temperate	-4.9304	43.3202	16	37.6	0.41	16	-1.6	0.62	39.2	19.02	2.52
Aplastodiscus sp	APLSP	Tropical	-39.2210	-14.6475	7	38.6	0.31	11	7.2	0.50	31.4	14.54	11.99
Bufo boulengeri	BBO	Temperate	-2.4216	34.6696	14	41.2	0.29	14	1.3	0.47	39.8	NA	NA
Bufo brongersmai	BBR	Temperate	-9.3254	29.6747	16	40.1	0.30	16	2.5	0.64	37.6	NA	NA
Bufo bufo	BBU	Temperate	-4.3686	37.4974	16	37.9	0.27	16	2.9	0.74	35.0	12.77	4.58
Bufo calamita	BCA	Temperate	-6.0780	37.7912	15	40.3	0.28	16	0.6	0.64	39.7	4.86	1.7
Ceratophrys aurita	CAU	Tropical	-41.1176	-13.9223	2	41.0	0.00	1	6.6	NA	34.4	6.1	16.21
Crossodactylus sp	CROS	Tropical	-39.5412	-15.4219	8	41.8	0.29	10	5.9	0.31	35.9	17.16	13.76
Dendropsophus branneri	DBR	Tropical	-39.0626	-14.5932	1	41.8	NA	2	7.8	0.85	34.0	0.46	11.67
Dendropsophus elegans	DEL	Tropical	-39.1727	-14.7960	2	40.8	0.00	3	5.9	0.62	34.9	16.45	14.62
Dendropsophus haddadi	DHA	Tropical	-39.1727	-14.7960	9	39.6	0.42	8	5.8	1.43	33.8	15.25	14.72
Dendropsophus novaisi	DNO	Tropical	-41.1160	-13.9219	24	43.3	0.56	24	6.1	0.72	37.2	1.96	15.56
Discoglossus galganoi	DGA	Temperate	-5.5735	43.4274	16	38.8	0.36	16	0.2	0.27	38.5	18.45	3.23
Discoglossus pictus	DPI	Temperate	2.7191	41.8311	16	39.1	0.73	16	1.2	0.85	37.8	11.1	1.8
Discoglossus scovazzi	DSC	Temperate	-5.3818	35.8640	16	38.3	0.39	16	1.5	0.66	36.8	NA	NA
Hyla arborea	HAR	Temperate	-5.9260	42.9847	16	39.9	0.52	14	-0.8	0.46	40.7	15.6	5.06
Hyla meridionalis	HME	Temperate	-4.9014	37.9939	16	39.4	0.37	16	0.3	0.50	39.1	3.32	5.56
Hypsiboas albomarginatus	HAL	Tropical	-39.2299	-13.8923	2	41.4	0.57	3	6.9	0.40	34.5	5.86	12.29

Specie	SPP. Code	Region	Sample point		CTmax			CTmin			Tolerance	WT	CT
			Longitude	Latitude	N	$\bar{X}$	SD	N	$\bar{X}$	SD	Range		
Hypsiboas faber	HFA	Tropical	-39.5412	-15.4219	11	41.6	0.27	12	5.5	0.18	36.1	16.96	14.16
Leptodactylus fuscus	LFU	Tropical	-39.0718	-14.6662	12	43.6	0.56	12	8.2	0.51	35.4	2.26	11.27
Leptodactylus latrans	LLA	Tropical	-39.0626	-14.5932	4	41.7	0.28	8	7.4	0.60	34.3	0.36	12.07
Pelobates cultripes	PCU	Temperate	-6.0846	37.7830	16	39.0	0.16	16	-0.5	0.29	39.4	3.35	1.94
Pelodytes ibericus	PIB	Temperate	-8.2629	37.6692	11	36.8	0.36	12	-0.5	0.53	37.3	3.54	1.94
Pelodytes punctatus	PPU	Temperate	-2.7019	38.4917	16	37.6	0.20	16	0.3	0.36	37.3	10.72	3.49
Phasmahyla spectabilis	PHA	Tropical	-39.5457	-15.4178	11	38.9	0.45	10	4.9	0.95	33.9	14.84	14.29
Phyllodytes luteolus	PLU	Tropical	-38.9989	-15.0879	9	40.9	0.82	11	6.1	0.99	34.8	0.99	11.98
Phyllodytes melanomystax	PME	Tropical	-39.0688	-14.6834	5	42.0	0.68	4	8.0	0.53	34.0	5.04	9.13
Phyllomedusa rohdei	PRO	Tropical	-39.1727	-14.7960	5	41.1	0.55	12	7.1	1.04	34.0	16.75	13.42
Physalaemus camacan	PCAM	Tropical	-39.0629	-14.5910	12	40.8	0.29	12	6.8	0.46	33.9	11.95	12.96
Physalaemus erikae	PER	Tropical	-39.1726	-14.7962	6	41.0	0.28	5	8.3	0.33	32.7	12.15	11.46
Pipa carvalhoi	PCA	Tropical	-39.1736	-14.7947	12	40.8	0.58	14	10.0	0.92	30.8	12.44	10.71
Pleurodeles waltl	PWA	Temperate	-6.0846	37.7830	8	37.4	0.50	11	-0.5	0.30	37.8	2.92	2.8
Rana perezi	RPE	Temperate	-6.0846	37.7830	9	40.5	0.69	16	2.9	0.98	37.6	6.02	4.08
Rana temporaria	RTE	Temperate	-4.8095	43.3710	14	37.5	0.43	16	-1.8	0.22	39.3	5.41	2.25
Rhinella crucifer	RCRU	Tropical	-39.5412	-15.4219	2	41.8	0.00	12	4.3	0.40	37.5	17.16	15.36
Rhinella hoogmoedi	RHO	Tropical	-39.0636	-14.5896	21	39.4	0.38	23	8.2	1.32	31.2	12.23	13.75
Rhinella jimi	RJI	Tropical	-41.1116	-13.9758	12	42.6	0.34	12	6.0	0.57	36.6	8.01	16.62
Salamandra salamandra	SSA	Temperate	-6.5690	37.9175	16	35.3	0.49	16	-0.9	0.47	36.2	11.14	2.34
Scinax agilis	SAG	Tropical	-39.0718	-14.6662	10	42.5	0.97	13	7.3	0.84	35.2	1.16	12.17
Scinax eurydice	SEU	Tropical	-39.0612	-14.6094	25	42.4	0.67	27	6.8	1.12	35.7	1.06	12.67
Scinax strigilatus	SST	Tropical	-39.0636	-14.5896	12	38.3	0.32	12	6.2	1.08	32.1	14.24	12.99
Sphaenorhynchus prasinus	SPR	Tropical	-39.1727	-14.7960	15	41.3	0.42	16	5.8	0.51	35.4	16.95	14.72
Trachycephalus mesophaeus	TME	Tropical	-39.1727	-14.7960	4	41.0	0.75	3	8.2	1.74	32.8	13.83	13.75
Triturus pygmaeus	TPY	Temperate	-4.8482	37.9735	14	37.1	0.47	16	2.6	0.68	34.6	3.25	3.26

Table S2. Summary of the microenvironments, biomes, reproductive season of each species studied and thermal information from the dataloggers placed in the ponds of origin of each species (N number of days of temperature records). daily range: mean daily tmax - tmin. Microenvironments-Biomes: Tropical FP, forest pond Mata Atlântica; RIA, stream ("riacho"), Mata Atlântica; OFMA, open forest, Mata Atlântica; OFCAA, open forest Caatinga, PHY, phytotelmata in restingas, Mata Atlântica. Temperate: MED, lowland pond mediterranean, MOU, mountain ponds, Mediterranean or Atlantic; SDES, subdesert Marocco, STR; stream, Mediterranean. Season: breeding period months with tadpole presence. If not indicated for Mata Atlântica species, it indicates that breeding and tadpole presence may occur throughout the year. NA: not available data.

Specie	Microenv-Biomes	Season	Location	Longitude	Latitude	Region	N	tmean	tmax	tmin	daily range
Agalychnis aspera	FP		Uruçuca	-39.2209	-14.6474	Tropical	359	21.83	24.06	19.19	0.82
Alytes cisternasii	STR	Oct-Apr	Navas del Berrocal	-6.0799	37.7929	Temperate	165	13.22	29,95	4.31	5.70
Alytes dickhilleni	MOU	Jan-Dic	Sierra Nevada	-3.5594	37.0462	Temperate	497	9.64	20.33	1.66	1.27
Alytes obstetricans	MOU	Jan-Dic	Julagua	-4.9304	43.3202	Temperate	412	7.98	18.58	0.92	1.13
Aplastodiscus sp	FP		Uruçuca	-39.2209	-14.6474	Tropical	359	21.83	24.06	19.19	0.82
Bufo boulengeri	SDES		Marruecos	-2.4215	34.6696	Temperate	NA	NA	NA	NA	NA
Bufo brongersmai	SDES	Jan-Apr	Marruecos	-9.3253	29.6747	Temperate	NA	NA	NA	NA	NA
Bufo bufo	STR	Dic-May	Cabra	-4.3686	37.4974	Temperate	164	12.31	25.13	7.48	3.43
Bufo calamita	MED	Jan-May	Navas del Berrocal	-6.0780	37.7912	Temperate	138	12.60	35.44	2.30	7.31
Ceratophrys aurita	OFCAA	Nov-MAy	CA2	-41.1176	-13.9223	Tropical	49	26.39	34.90	22.81	3.05
Crossodactylus sp	RIA		SB_Poza	-39.5412	-15.4219	Tropical	359	22.16	24.64	19.66	0.34
Dendropsophus branneri	OFMA		CH_Jacaré	-39.0626	-14.5932	Tropical	342	26.39	41.34	19.47	3.95
Dendropsophus elegans	FP		UESC_Cabruca	-39.1727	-14.796	Tropical	309	22.76	24.35	20.52	0.11
Dendropsophus haddadi	FP		UESC_Cabruca	-39.1727	-14.796	Tropical	309	22.76	24.35	20.52	0.11
Dendropsophus novaisi	OFCAA	Nov-May	CA1;CA2	-41.1160	-13.9219	Tropical	47	27.14	41.34	21.66	7.31

Annexe 1

Specie	Microenv-Biomes	Season	Location	Longitude	Latitude	Region	N	tmean	tmax	tmin	daily range
Discoglossus galganoi	MED	Jan-Apr	Asturias	-5.5735	43.4274	Temperate	122	11.50	20.35	3.43	3.58
Discoglossus pictus	MED	Feb-Apr	Riudarenes	2.7191	41.8311	Temperate	67	16.12	28.00	3.00	5.45
Discoglossus scovazzi	MED	Dic-Apr	Ceuta	-5.3818	35.864	Temperate	NA	NA	NA	NA	NA
Hyla arborea	MOU	Apr-Jul	Cubilla	-5.9260	42.9847	Temperate	81	13.46	24,30	4.26	6.51
Hyla meridionalis	MED	Feb-Jun	Toba cordoba	-4.9014	37.9938	Temperate	97	16.75	36.08	5.86	7.18
Hypsiboas albomarginatus	OFMA		MCH2	-39.2299	-13.8923	Tropical	137	22.35	35.54	19.19	0.88
Hypsiboas faber	FP		SB_P	-39.5412	-15.4219	Tropical	359	22.16	24.64	19.66	0.34
Leptodactylus fuscus	OFMA		CH_Jacaré	-39.0718	-14.6662	Tropical	342	26.38	41.34	19.47	3.95
Leptodactylus latrans	OFMA		CH_Jacaré	-39.0626	-14.5932	Tropical	342	26.38	41.34	19.47	3.95
Pelobates cultripes	MED	Nov-Jun	Navas del Berrocal	-6.0845	37.7829	Temperate	154	11.76	35.65	1.44	5.67
Pelodytes ibericus	MED	Nov-Apr	Toba/Cabra	-8.2629	37.6692	Temperate	145	11.22	33.26	1.44	4.97
Pelodytes punctatus	MED	Feb-Jun	Jaén	-2.7019	38.4917	Temperate	30	10.24	26.88	3.79	5.06
Phasmahyla spectabilis	RIA		SB_R	-39.5457	-15.4178	Tropical	359	21.83	24.06	19.19	0.82
Phyllodytes luteolus	PHY		Mirco_H	-38.9989	-15.0879	Tropical	374	25.39	39.91	18.08	10.34
Phyllodytes melanomystax	PHY		P.melanomystax	-39.0688	-14.6834	Tropical	370	25.18	36.96	17.13	8.04
Phyllomedusa rohdei	FP		UESC_Cabruca	-39.1727	-14.796	Tropical	309	22.76	24.35	20.52	0.11
Physalaemus camacan	FP		Cabruca	-39.0629	-14.591	Tropical	351	23.17	28.85	19.76	1.98
Physalaemus erikae	FP		UESC_Phy	-39.1726	-14.7962	Tropical	351	23.17	28.85	19.76	1.98
Pipa carvalhoi	FP		UESC_Pipa	-39.1736	-14.7947	Tropical	352	24.58	28.36	20.71	0.55
Pleurodeles waltl	MED	Nov-Jun	Navas del Berrocal	-6.0846	37.7830	Temperate	223	13.02	34.48	2.30	6.07
Rana perezi	MED	Apr-Aug	Navas del Berrocal	-6.0846	37.7830	Temperate	160	15.51	34.48	6.98	2.20
Rana temporaria	MOU	Nov-Apr	Purón	-4.8095	43.3710	Temperate	357	8.53	32.09	0.45	5.60
Rhinella crucifer	FP		SB_P	-39.5412	-15.4219	Tropical	359	22.16	24.64	19.66	0.34

Specie	Microenv-Biomes	Season	Location	Longitude	Latitude	Region	N	tmean	tmax	tmin	daily range
Rhinella hoogmoedi	FP		Riacho cabruca	-39.0636	-14.5896	Tropical	226	24.57	27.17	21.95	1.61
Rhinella jimi	OFCAA	Nov-May	CA4	-41.1116	-13.9758	Tropical	45	26.73	34.59	22.62	5.18
Salamandra salamandra	STR	Nov-May	Aracena, La Sorda	-6.5690	37.9175	Temperate	320	13.33	24.16	1.44	3.13
Scinax agilis	OFMA		Charca Jacaré	-39.0718	-14.6662	Tropical	342	26.38	41.34	19.47	3.95
Scinax eurydice	OFMA		Charca Jacaré	-39.0612	-14.6094	Tropical	342	26.38	41.34	19.47	3.95
Scinax strigilatus	RIA		Uruçuca	-39.2209	-14.6474	Tropical	359	21.83	24.06	19.19	0.82
Sphaenorhynchus prasinus	FP		UESC_Cabruca	-39.1727	-14.796	Tropical	309	22.76	24.35	20.52	0.11
Trachycephalus mesophaeus	FP		Riacho Cabruca	-39.1727	-14.796	Tropical	226	24.57	27.17	21.95	1.61
Triturus pygmaeus	MED	Dic-May	Charca eucalipto	-4.8482	37.9735	Temperate	85	15.69	33.85	5.86	6.28

Table S3. Summary of the coefficient of determination (R^2) between the different variables of WorldClim (in caps) and WorldClim and the microenvironmental variables (in lowercase) provided by datalogger data. BIO1 = Annual Mean Temperature; BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp)); BIO4 = Temperature Seasonality (standard deviation *100); BIO5 = Max Temperature of Warmest Month; BIO6 = Min Temperature of Coldest Month; BIO7 = Temperature Annual Range (BIO5-BIO6); *monthly variables consider only the reproductive period (tadpole presence in ponds). Sample point variables only summarise climatic information from the coordinates of the pond, distribution takes into account the total area distribution.

	BIO2_sample_point	BIO2_distribution	Monthly range (TMax-Tmin RP)	BIO4_distribution	BIO4_sample_point	BIO1_distribution	BIO5_sample_point	BIO5_distribution	Tmax_RP	BIO1_sample_point	BIO6_point	BIO6_distribution	Tmin_RP	BIO7_point	BIO7_distribution
BIO2_sample_point															
BIO2_distribution	0.41														
Monthly range (TMax-Tmin RP)	0.84	0.19													
BIO4_distribution	0.74	0.38	0.75												
BIO4_sample_point	0.87	0.36	0.89	0.92											
BIO1_distribution	-0.59	-0.15	-0.72	-0.86	-0.77										
BIO5_sample_point	0.44	0.20	0.33	-0.01	0.28	0.21									
BIO5_distribution	-0.08	0.50	-0.30	-0.26	-0.15	0.57	0.34								
Tmax_RP*	-0.16	-0.28	0.00	-0.59	-0.36	0.49	0.51	0.05							
BIO1_sample_point	-0.71	-0.27	-0.79	-0.94	-0.90	0.89	0.16	0.33	0.58						
BIO6_point	-0.83	-0.34	-0.85	-0.96	-0.96	0.85	-0.03	0.25	0.50	0.98					
BIO6_distribution	-0.71	-0.35	-0.74	-0.97	-0.87	0.91	0.08	0.42	0.55	0.93	0.93				
Tmin_RP*	-0.80	-0.31	-0.85	-0.95	-0.95	0.86	-0.01	0.28	0.53	0.97	0.98	0.92			
BIO7_point	0.92	0.38	0.90	0.89	0.99	-0.73	0.35	-0.13	-0.30	-0.86	-0.95	-0.84	-0.93		
BIO7_distribution	0.75	0.57	0.71	0.98	0.90	-0.80	0.03	-0.10	-0.59	-0.90	-0.92	-0.95	-0.91	0.88	
Dataloggers															
tmax	0.07	0.10	-0.04	-0.06	-0.03	0.18	0.20	0.17	0.16	0.12	0.08	0.08	0.11	-0.01	-0.04
tmin	-0.71	-0.23	-0.84	-0.96	-0.93	0.86	-0.01	0.35	0.49	0.94	0.95	0.91	0.95	-0.89	-0.93
tmean	-0.68	-0.19	-0.79	-0.93	-0.88	0.88	0.05	0.41	0.54	0.92	0.90	0.93	-0.85	-0.85	-0.90
average daily range	0.42	0.04	0.38	0.40	0.42	-0.24	0.16	-0.02	-0.14	-0.35	-0.40	-0.33	-0.39	0.43	0.38

Table S4. PGLS Results for some of the Stevens' CVH assumptions and trade-off between both thermal limits.

PGLS	λ	AIC	Slope ± SE	Intercept ± SE	$F_{1,36}$	p
CTmax ~ Centroid latitude	0.68	101.5	-0.08 ± 0.02	41.02 ± 0.81	28.92	<0.01
CTmin ~ Centroid latitude	0.92	125.7	-0.20 ± 0.02	8.97 ± 1.48	135.9	<0.01
Tolerance range ~ Max Latitude (poleward)	0.00	136.2	0.13 ± 0.02	31.35 ± 0.83	43.85	<0.01
Tolerance range ~ Latitudinal range	1.00	145.0	0.10 ± 0.01	35.16 ± 2.30	123.4	<0.01
Tolerance range ~ Range size	1.00	141.0	1.27 ± 0.12	29.13 ± 2.27	148.8	<0.01
CTmax ~ CTmin	0.61	107.5	0.30 ± 0.06	37.60 ± 0.60	20.7	<0.01

Table S5. Values of phylogenetic signal for the physiological traits: λ Pagel's lambda, AICc for lambda values and P components from PVR analyses.

	λ	AICc	P
CTmax	0.67	177.71	0.50
CTmin	0.97	221.82	0.80
TR	0.89	220.87	0.52

Table S6. Warming and cooling tolerances for tropical and temperate species. We distinguish the tropical community into two thermal microenvironments: Tropical Open Forest pond species and Tropical Forest pond and stream species. Different superscripts indicate significant differences between groups (Tukey Test, $P<0.05$).

Community-microenvironment	Cooling Tolerance (CT)(ºC)	SE CT (ºC)	Warming Tolerance(WT) (ºC)	SE WT (ºC)	N species
Tropical-Open Forest pond	12.88^{A}	0.69	3.02^{A}	0.82	11
Tropical-Forest pond-stream	13.31^{A}	0.37	14.87^{C}	0.48	16
Temperate	3.09^{B}	0.31	9.23^{B}	1.41	17

Table S7. Single and multivariate models (PGLS) used to predict physiological traits (CT_{max}, CT_{min} and Tolerance Range) at the global scale. Models are ranked in each trait by AIC.

Models	λ	AIC	w_i
Critical thermal maximum (CT_{max}):			
CTmax ~ TMEAN + tmax	0.72	113.06	0.16
CTmax ~ TMEAN + tmax + sr	0.70	114.90	0.06
CTmax ~ TMEAN + sr	0.78	114.97	0.06
CTmax ~ TMEAN + tmax + SR	0.76	114.97	0.06
CTmax ~ TMAX + TMEAN + tmax	0.70	115.01	0.06
CTmax ~ TMEAN + tmax + tmean	0.71	115.04	0.06
CTmax ~ TMEAN + tmax + dr	0.73	115.06	0.06
CTmax ~ TMEAN + tmean + sr	0.72	115.93	0.04
CTmax ~ tmax + sr	0.53	116.57	0.03
CTmax ~ tmax + tmean	0.57	116.65	0.03
CTmax ~ TMEAN + dr	0.69	116.66	0.03
CTmax ~ TMAX + TMEAN + sr	0.73	116.72	0.03
CTmax ~ tmean	0.55	116.73	0.03
CTmax ~ TMEAN + sr + dr	0.77	116.92	0.02
CTmax ~ TMEAN + SR + sr	0.78	116.97	0.02
CTmax ~ tmean + sr	0.58	117.23	0.02
CTmax ~ TMEAN + tmean + dr	0.63	117.50	0.02
CTmax ~ TMAX + tmax + sr	0.66	117.65	0.02
CTmax ~ tmean + dr	0.56	117.74	0.02
CTmax ~ tmax + tmean + sr	0.54	117.97	0.01
CTmax ~ TMEAN + tmean	0.58	118.21	0.01
CTmax ~ TMAX + tmax + tmean	0.64	118.27	0.01
CTmax ~ TMAX + tmean	0.61	118.47	0.01
CTmax ~ tmax + SR + sr	0.56	118.49	0.01
CTmax ~ tmax + sr + dr	0.52	118.50	0.01
CTmax ~ tmean + SR	0.59	118.55	0.01
CTmax ~ tmax + tmean + dr	0.57	118.58	0.01
CTmax ~ TMAX + TMEAN + dr	0.66	118.61	0.01
CTmax ~ TMEAN + SR + dr	0.71	118.61	0.01
CTmax ~ tmax + tmean + SR	0.57	118.65	0.01
CTmax ~ TMAX + tmean + sr	0.63	118.92	0.01
CTmax ~ TMAX + tmax + SR	0.70	119.18	0.01
CTmax ~ tmean + sr + dr	0.58	119.23	0.01
CTmax ~ tmean + SR + sr	0.58	119.23	0.01
CTmax ~ TMAX + tmean + dr	0.62	119.35	0.01
CTmax ~ TMEAN	0.69	119.61	0.01
CTmax ~ tmean + SR + dr	0.57	119.71	0.01
CTmax ~ TMEAN + tmean + SR	0.65	119.81	0.01

Models	λ	AIC	w_i
CTmax ~ TMAX + TMEAN + tmean	0.61	120.16	0.00
CTmax ~ TMAX + tmean + SR	0.61	120.46	0.00
CTmax ~ TMEAN + SR	0.76	121.40	0.00
CTmax ~ TMAX + TMEAN	0.71	121.60	0.00
CTmax ~ TMAX + SR + sr	0.70	122.31	0.00
CTmax ~ TMAX + TMEAN + SR	0.77	122.54	0.00
CTmax ~ TMAX + SR + dr	0.66	122.74	0.00
CTmax ~ TMAX + SR	0.70	124.25	0.00
CTmax ~ TMAX + tmax + dr	0.89	126.24	0.00
CTmax ~ TMAX + tmax	0.90	127.33	0.00
CTmax ~ tmax + SR + dr	0.59	128.46	0.00
CTmax ~ tmax + dr	0.84	129.00	0.00
CTmax ~ tmax + SR	0.51	129.09	0.00
CTmax ~ TMAX	0.92	130.15	0.00
CTmax ~ TMAX + sr	0.92	132.13	0.00
CTmax ~ tmax	0.86	132.40	0.00
CTmax ~ TMAX + dr	0.87	132.58	0.00
CTmax ~ TMAX + sr + dr	0.89	133.26	0.00
CTmax ~ SR	0.54	134.00	0.00
CTmax ~ SR + dr	0.53	135.28	0.00
CTmax ~ SR + sr	0.54	135.83	0.00
CTmax ~ SR + sr + dr	0.52	137.04	0.00
CTmax ~ sr	0.84	137.74	0.00
CTmax ~ dr	0.87	137.76	0.00
CTmax ~ sr + dr	0.79	138.54	0.00
Critical thermal minimum (CT_{min}):			
CTmin ~ TMIN + tmin	0.00	128.17	0.10
CTmin ~ TMIN + tmin + tmean	0.00	128.34	0.10
CTmin ~ TMIN + tmean + sr	0.00	128.80	0.08
CTmin ~ TMIN + tmean	0.00	129.43	0.06
CTmin ~ TMIN + tmin + dr	0.00	129.59	0.05
CTmin ~ TMIN + tmin + SR	0.00	129.65	0.05
CTmin ~ TMIN + tmin + sr	0.00	129.84	0.05
CTmin ~ tmin + tmean	0.00	129.99	0.04
CTmin ~ TMIN + tmean + dr	0.00	130.08	0.04
CTmin ~ TMIN + TMEAN + tmin	0.00	130.13	0.04
CTmin ~ TMEAN + tmin	0.00	130.22	0.04
CTmin ~ TMEAN + tmin + tmean	0.00	130.23	0.04
CTmin ~ TMIN + tmean + SR	0.00	131.32	0.02
CTmin ~ TMIN + TMEAN + tmean	0.00	131.42	0.02
CTmin ~ TMEAN + tmin + dr	0.00	131.43	0.02
CTmin ~ TMEAN + tmean + sr	0.00	131.47	0.02
CTmin ~ tmin + tmean + sr	0.00	131.62	0.02
CTmin ~ tmean + sr	0.00	131.65	0.02

Models	λ	AIC	w_i
CTmin ~ TMEAN + tmin + sr	0.00	131.73	0.02
CTmin ~ TMEAN + tmin + SR	0.00	131.74	0.02
CTmin ~ tmin + tmean + SR	0.00	131.74	0.02
CTmin ~ tmin + tmean + dr	0.00	131.85	0.02
CTmin ~ tmin	0.00	132.18	0.01
CTmin ~ tmean + SR + sr	0.00	133.05	0.01
CTmin ~ TMEAN + tmean	0.00	133.07	0.01
CTmin ~ tmin + dr	0.00	133.10	0.01
CTmin ~ TMEAN + tmean + SR	0.00	133.14	0.01
CTmin ~ tmin + sr	0.00	133.18	0.01
CTmin ~ TMEAN + tmean + dr	0.00	133.19	0.01
CTmin ~ tmean + sr + dr	0.00	133.60	0.01
CTmin ~ tmin + SR	0.00	133.93	0.01
CTmin ~ TMIN	0.00	133.95	0.01
CTmin ~ tmean + dr	0.00	133.98	0.01
CTmin ~ tmean + SR + dr	0.00	134.80	0.00
CTmin ~ tmin + SR + dr	0.00	134.89	0.00
CTmin ~ tmin + SR + sr	0.00	134.93	0.00
CTmin ~ tmin + sr + dr	0.00	135.00	0.00
CTmin ~ TMIN + TMEAN	0.00	135.25	0.00
CTmin ~ TMIN + sr	0.00	135.35	0.00
CTmin ~ TMIN + SR	0.00	135.46	0.00
CTmin ~ TMIN + dr	0.00	135.95	0.00
CTmin ~ TMIN + sr + dr	0.00	136.48	0.00
CTmin ~ TMIN + TMEAN + sr	0.00	136.74	0.00
CTmin ~ TMIN + SR + sr	0.00	136.74	0.00
CTmin ~ TMEAN + SR	0.00	137.15	0.00
CTmin ~ tmean + SR	0.00	137.23	0.00
CTmin ~ TMIN + TMEAN + SR	0.00	137.24	0.00
CTmin ~ TMIN + TMEAN + dr	0.00	137.24	0.00
CTmin ~ TMIN + SR + dr	0.00	137.46	0.00
CTmin ~ tmean	0.00	137.68	0.00
CTmin ~ TMEAN + SR + sr	0.00	138.89	0.00
CTmin ~ TMEAN + SR + dr	0.00	139.12	0.00
CTmin ~ TMEAN	0.00	139.32	0.00
CTmin ~ TMEAN + sr	0.00	140.52	0.00
CTmin ~ TMEAN + sr + dr	0.00	141.19	0.00
CTmin ~ TMEAN + dr	0.00	141.32	0.00
CTmin ~ SR + sr	0.32	172.24	0.00
CTmin ~ SR + sr + dr	0.00	172.45	0.00
CTmin ~ SR	0.42	175.06	0.00
CTmin ~ SR + dr	0.45	175.81	0.00
CTmin ~ sr	0.94	178.74	0.00
CTmin ~ sr + dr	0.90	179.24	0.00

Models	λ	AIC	w_i
CTmin ~ dr	0.97	183.25	0.00
Tolerance Range (CTmax-CTmin):			
TR ~ SR + sr	0.00	158.05	0.48
TR ~ SR + dr + sr	0.00	159.44	0.24
TR ~ sr	0.00	161.10	0.10
TR ~ dr + sr	0.00	161.13	0.10
TR ~ SR + dr	0.38	162.66	0.05
TR ~ SR	0.36	164.24	0.02
TR ~ dr	1.00	169.43	0.00

Note: Macroclimatic predictors: TMAX (maximum of the average monthly maximum temperature); TMIN (minimum of the average monthly minimum temperature); TMEAN (mean of the average monthly temperature); (SR) SEASONAL RANGE (TMAX-TMIN). Microclimatic predictors: tmax (maximum temperature); tmin (minimum temperature); tmean (mean temperature); (dr) daily range (mean seasonal daily tmax-tmin); (sr) seasonal range (seasonal absolute tmax - absolute tmin). λ = Pagel's lambda, AIC=Akaike Information Criterion, w_i=Akaike weight. Akaike weights were calculated from AIC values of all models for each trait.

Table S8. Single and multivariate models (PGLS) used to predict physiological traits (CT_{max}, CT_{min} and Tolerance Range) at the temperate region. Models are ranked in each trait by AIC.

Models	λ	AIC	w_i
Critical thermal maximum (CT_{max}):			
CTmax ~ SR + dr	0.85	53.08	0.07
CTmax ~ SR + sr	0.97	53.57	0.05
CTmax ~ SR + sr + dr	0.79	53.68	0.05
CTmax ~ dr	0.49	54.06	0.04
CTmax ~ tmax + SR + dr	0.78	54.51	0.03
CTmax ~ TMEAN + dr	0.71	54.68	0.03
CTmax ~ TMEAN + SR + dr	0.89	54.69	0.03
CTmax ~ TMAX + SR + dr	0.83	54.70	0.03
CTmax ~ TMAX + dr	0.75	54.72	0.03
CTmax ~ TMAX + TMEAN + SR	1.00	54.94	0.03
CTmax ~ tmean + SR + dr	0.86	55.02	0.03
CTmax ~ tmax + SR	1.00	55.05	0.03
CTmax ~ TMEAN	0.94	55.13	0.02

Models	λ	AIC	w_i
CTmax ~ TMEAN + SR	1.00	55.26	0.02
CTmax ~ TMEAN + tmax + SR	1.00	55.28	0.02
CTmax ~ TMEAN + tmax	0.95	55.58	0.02
CTmax ~ TMAX + sr + dr	0.71	55.68	0.02
CTmax ~ sr + dr	0.48	55.69	0.02
CTmax ~ TMEAN + sr + dr	0.66	55.78	0.02
CTmax ~ TMEAN + SR + sr	0.99	55.85	0.02
CTmax ~ tmax + dr	0.47	55.90	0.02
CTmax ~ SR	1.00	56.01	0.02
CTmax ~ tmean + dr	0.50	56.04	0.02
CTmax ~ TMAX	0.95	56.06	0.02
CTmax ~ TMEAN + sr	0.93	56.08	0.02
CTmax ~ tmax	0.60	56.18	0.01
CTmax ~ TMAX + tmax	0.95	56.24	0.01
CTmax ~ TMAX + tmax + dr	0.70	56.29	0.01
CTmax ~ TMEAN + tmax + dr	0.64	56.37	0.01
CTmax ~ TMAX + TMEAN + dr	0.77	56.44	0.01
CTmax ~ TMAX + sr	0.95	56.44	0.01
CTmax ~ sr	0.59	56.50	0.01
CTmax ~ TMEAN + tmean + dr	0.70	56.61	0.01
CTmax ~ TMAX + tmean + dr	0.76	56.67	0.01
CTmax ~ tmax + tmean + SR	1.00	56.70	0.01
CTmax ~ TMAX + TMEAN	0.96	56.87	0.01
CTmax ~ tmax + SR + sr	1.00	56.98	0.01
CTmax ~ TMEAN + tmean	0.95	57.03	0.01
CTmax ~ TMAX + tmax + SR	1.00	57.05	0.01
CTmax ~ TMEAN + tmean + SR	1.00	57.21	0.01
CTmax ~ tmean + SR + sr	0.98	57.24	0.01
CTmax ~ TMAX + SR + sr	0.98	57.24	0.01
CTmax ~ tmean	0.58	57.27	0.01
CTmax ~ TMAX + TMEAN + tmax	0.97	57.32	0.01
CTmax ~ TMEAN + tmax + tmean	0.94	57.36	0.01
CTmax ~ TMEAN + tmax + sr	1.00	57.62	0.01
CTmax ~ TMAX + SR	1.00	57.64	0.01
CTmax ~ tmax + sr + dr	0.49	57.67	0.01
CTmax ~ tmean + sr + dr	0.49	57.67	0.01
CTmax ~ TMAX + tmax + tmean	0.96	57.72	0.01
CTmax ~ TMAX + TMEAN + sr	0.95	57.74	0.01
CTmax ~ tmean + SR	1.00	57.77	0.01
CTmax ~ tmax + tmean + dr	0.49	57.80	0.01
CTmax ~ TMAX + tmean	0.93	58.00	0.01
CTmax ~ TMEAN + tmean + sr	0.93	58.08	0.01
CTmax ~ tmax + tmean	0.60	58.14	0.01
CTmax ~ tmax + sr	0.60	58.18	0.01

Models	λ	AIC	w_i
CTmax ~ tmean + sr	0.60	58.19	0.01
CTmax ~ TMAX + tmax + sr	0.95	58.22	0.01
CTmax ~ TMAX + tmean + sr	0.95	58.42	0.00
CTmax ~ TMAX + TMEAN + tmean	0.96	58.84	0.00
CTmax ~ TMAX + tmean + SR	1.00	59.54	0.00
CTmax ~ tmax + tmean + sr	0.60	60.13	0.00
Critical thermal minimum (CT_{min}):			
CTmin ~ tmin	0.00	55.18	0.07
CTmin ~ tmin + tmean	0.00	55.58	0.06
CTmin ~ TMEAN + tmin	0.00	55.71	0.06
CTmin ~ tmean	0.00	55.94	0.05
CTmin ~ TMIN + tmin	0.00	56.05	0.05
CTmin ~ tmin + dr	0.00	56.33	0.04
CTmin ~ tmin + SR	0.00	56.67	0.04
CTmin ~ TMIN + tmin + SR	0.00	57.07	0.03
CTmin ~ TMEAN + tmin + dr	0.00	57.09	0.03
CTmin ~ TMEAN + tmin + tmean	0.00	57.12	0.03
CTmin ~ tmin + tmean + SR	0.00	57.13	0.03
CTmin ~ tmean + sr	0.00	57.14	0.03
CTmin ~ tmin + sr	0.00	57.16	0.03
CTmin ~ TMIN + tmin + tmean	0.00	57.32	0.03
CTmin ~ tmin + tmean + sr	0.00	57.34	0.03
CTmin ~ tmin + tmean + dr	0.00	57.42	0.02
CTmin ~ tmin + sr + dr	0.00	57.43	0.02
CTmin ~ TMIN + TMEAN + tmin	0.00	57.49	0.02
CTmin ~ tmean + SR	0.00	57.59	0.02
CTmin ~ tmin + SR + dr	0.00	57.61	0.02
CTmin ~ TMEAN + tmin + sr	0.00	57.66	0.02
CTmin ~ tmean + sr + dr	0.00	57.69	0.02
CTmin ~ TMEAN + tmin + SR	0.00	57.70	0.02
CTmin ~ TMIN + tmin + dr	0.00	57.72	0.02
CTmin ~ tmean + dr	0.00	57.92	0.02
CTmin ~ TMIN + tmean	0.00	57.94	0.02
CTmin ~ TMEAN + tmean	0.00	57.94	0.02
CTmin ~ TMIN + tmin + sr	0.00	57.94	0.02
CTmin ~ tmin + SR + sr	0.00	58.65	0.01
CTmin ~ tmean + SR + sr	0.00	58.75	0.01
CTmin ~ TMIN + tmean + sr	0.00	59.08	0.01
CTmin ~ TMEAN + tmean + sr	0.00	59.09	0.01
CTmin ~ TMEAN + tmean + SR	0.00	59.46	0.01
CTmin ~ tmean + SR + dr	0.00	59.53	0.01
CTmin ~ TMIN + tmean + SR	0.00	59.58	0.01
CTmin ~ TMIN + tmean + dr	0.00	59.91	0.01
CTmin ~ TMEAN + tmean + dr	0.00	59.92	0.01

Models	λ	AIC	w_i
CTmin ~ TMIN + TMEAN + tmean	0.00	59.93	0.01
CTmin ~ dr	0.00	60.72	0.00
CTmin ~ sr + dr	0.00	60.93	0.00
CTmin ~ TMEAN + sr + dr	0.00	61.03	0.00
CTmin ~ TMEAN	0.00	61.35	0.00
CTmin ~ TMIN	0.00	61.40	0.00
CTmin ~ SR + sr + dr	0.00	61.65	0.00
CTmin ~ SR	0.00	61.80	0.00
CTmin ~ SR + dr	0.00	62.05	0.00
CTmin ~ TMEAN + dr	0.00	62.07	0.00
CTmin ~ sr	0.00	62.20	0.00
CTmin ~ TMIN + sr + dr	0.00	62.24	0.00
CTmin ~ TMIN + dr	0.00	62.47	0.00
CTmin ~ TMIN + SR	0.00	62.64	0.00
CTmin ~ TMIN + TMEAN	0.00	63.14	0.00
CTmin ~ TMEAN + SR	0.00	63.31	0.00
CTmin ~ TMEAN + sr	0.00	63.32	0.00
CTmin ~ TMIN + sr	0.00	63.32	0.00
CTmin ~ TMIN + SR + dr	0.00	63.62	0.00
CTmin ~ SR + sr	0.00	63.78	0.00
CTmin ~ TMEAN + SR + dr	0.00	63.87	0.00
CTmin ~ TMIN + TMEAN + dr	0.00	64.07	0.00
CTmin ~ TMIN + SR + sr	0.00	64.49	0.00
CTmin ~ TMIN + TMEAN + SR	0.00	64.54	0.00
CTmin ~ TMIN + TMEAN + sr	0.00	65.03	0.00
CTmin ~ TMEAN + SR + sr	0.00	65.29	0.00
Tolerance Range (CTmax-CTmin):			
TR ~ sr	0.22	65.03	0.27
TR ~ dr	0.23	65.69	0.20
TR ~ SR	0.16	65.82	0.18
TR ~ SR + sr	0.14	66.75	0.12
TR ~ dr + sr	0.21	66.92	0.11
TR ~ SR + dr	0.18	67.56	0.08
TR ~ SR + dr + sr	0.08	68.45	0.05

Note: Macroclimatic predictors: TMAX (maximum of the average monthly maximum temperature); TMIN (minimum of the average monthly minimum temperature); TMEAN (mean of the average monthly temperature); (SR) SEASONAL RANGE (TMAX-TMIN). Microclimatic predictors: tmax (maximum temperature); tmin (minimum temperature); tmean (mean temperature); (dr) daily range (mean seasonal

daily tmax-tmin); (sr) seasonal range (seasonal absolute tmax - absolute tmin). λ = Pagel's lambda, AIC=Akaike Information Criterion, w_i=Akaike weight. Akaike weights were calculated from AIC values of all models for each trait.

Table S9. Single and multivariate models used to predict physiological traits (CT_{max}, CT_{min} and Tolerance Range) at the tropical region. Models are ranked in each trait by AIC.

Models	λ	AIC	w_i
Critical thermal maximum (CT_{max}):			
CTmax ~ tmax	0.00	54.21	0.07
CTmax ~ sr	0.00	54.69	0.06
CTmax ~ SR + sr + dr	0.00	55.02	0.05
CTmax ~ SR + sr	0.00	55.04	0.05
CTmax ~ TMAX + sr + dr	0.00	55.12	0.05
CTmax ~ TMAX + sr	0.00	55.36	0.04
CTmax ~ tmax + SR	0.00	55.43	0.04
CTmax ~ tmax + dr	0.00	55.52	0.04
CTmax ~ TMAX + tmax	0.00	55.71	0.03
CTmax ~ sr + dr	0.00	55.90	0.03
CTmax ~ TMEAN + tmax	0.00	55.98	0.03
CTmax ~ tmax + tmean	0.00	56.19	0.03
CTmax ~ tmax + sr	0.00	56.21	0.03
CTmax ~ tmean + sr	0.00	56.26	0.03
CTmax ~ tmax + SR + dr	0.00	56.39	0.02
CTmax ~ TMEAN + sr	0.00	56.46	0.02
CTmax ~ TMAX + tmax + dr	0.00	56.63	0.02
CTmax ~ TMAX + TMEAN + sr	0.00	56.67	0.02
CTmax ~ TMAX + tmax + tmean	0.00	56.82	0.02
CTmax ~ TMAX + SR + sr	0.00	56.92	0.02
CTmax ~ TMAX + tmean + sr	0.00	56.95	0.02
CTmax ~ TMEAN + SR + sr	0.00	57.01	0.02
CTmax ~ tmean + SR + sr	0.00	57.04	0.02
CTmax ~ tmax + SR + sr	0.00	57.04	0.02
CTmax ~ tmean + sr + dr	0.00	57.13	0.02
CTmax ~ TMAX + TMEAN + tmax	0.00	57.26	0.02
CTmax ~ tmax + tmean + SR	0.00	57.34	0.01
CTmax ~ TMAX + tmax + sr	0.00	57.35	0.01
CTmax ~ TMEAN + tmax + dr	0.00	57.37	0.01
CTmax ~ TMAX + tmax + SR	0.00	57.42	0.01
CTmax ~ TMEAN + tmax + SR	0.00	57.43	0.01
CTmax ~ tmax + tmean + dr	0.00	57.47	0.01
CTmax ~ tmax + sr + dr	0.00	57.48	0.01
CTmax ~ TMEAN + tmean + sr	0.00	57.60	0.01
CTmax ~ TMEAN + sr + dr	0.00	57.75	0.01
CTmax ~ TMEAN + tmax + tmean	0.00	57.84	0.01
CTmax ~ TMEAN + tmax + sr	0.00	57.98	0.01
CTmax ~ tmean	0.00	57.98	0.01
CTmax ~ tmax + tmean + sr	0.00	58.19	0.01
CTmax ~ TMEAN + tmean	0.00	59.08	0.01

Models	λ	AIC	w_i
CTmax ~ TMAX + tmean	0.00	59.40	0.01
CTmax ~ tmean + dr	0.00	59.93	0.00
CTmax ~ tmean + SR	0.00	59.98	0.00
CTmax ~ TMEAN + tmean + SR	0.00	60.32	0.00
CTmax ~ TMAX + TMEAN + tmean	0.00	60.45	0.00
CTmax ~ TMAX + tmean + SR	0.00	60.63	0.00
CTmax ~ TMEAN + tmean + dr	0.00	61.04	0.00
CTmax ~ TMAX + tmean + dr	0.00	61.40	0.00
CTmax ~ dr	0.00	61.80	0.00
CTmax ~ tmean + SR + dr	0.00	61.93	0.00
CTmax ~ TMAX + dr	0.00	63.50	0.00
CTmax ~ SR + dr	0.00	63.66	0.00
CTmax ~ TMEAN + dr	0.00	63.79	0.00
CTmax ~ TMAX	0.00	64.30	0.00
CTmax ~ TMEAN	0.00	65.00	0.00
CTmax ~ SR	0.00	65.01	0.00
CTmax ~ TMAX + TMEAN + dr	0.00	65.46	0.00
CTmax ~ TMAX + SR + dr	0.00	65.50	0.00
CTmax ~ TMEAN + SR + dr	0.00	65.63	0.00
CTmax ~ TMAX + SR	0.00	66.21	0.00
CTmax ~ TMAX + TMEAN	0.00	66.25	0.00
CTmax ~ TMEAN + SR	0.00	66.38	0.00
CTmax ~ TMAX + TMEAN + SR	0.00	67.49	0.00
Critical thermal minimum (CT_{min}):			
CTmin ~ tmean + SR + sr	0.77	68.99	0.06
CTmin ~ TMIN + tmean	0.76	69.20	0.06
CTmin ~ TMEAN	0.70	69.75	0.04
CTmin ~ tmean + SR	0.74	69.84	0.04
CTmin ~ TMIN + tmin + tmean	0.78	69.92	0.04
CTmin ~ TMIN + tmean + sr	0.78	69.93	0.04
CTmin ~ tmin + tmean + SR	0.75	69.96	0.04
CTmin ~ TMIN	0.63	70.05	0.04
CTmin ~ TMIN + tmin	0.66	70.35	0.03
CTmin ~ TMIN + tmin + dr	0.73	70.56	0.03
CTmin ~ TMIN + tmean + dr	0.79	70.58	0.03
CTmin ~ TMIN + tmin + sr	0.75	70.69	0.03
CTmin ~ TMIN + SR	0.69	70.75	0.03
CTmin ~ TMIN + TMEAN	0.68	70.79	0.03
CTmin ~ TMEAN + SR	0.68	70.81	0.03
CTmin ~ tmean + SR + dr	0.77	70.94	0.02
CTmin ~ tmin + SR + dr	0.69	71.20	0.02
CTmin ~ TMIN + tmean + SR	0.76	71.30	0.02
CTmin ~ TMIN + TMEAN + tmean	0.76	71.31	0.02
CTmin ~ TMEAN + tmean + SR	0.75	71.33	0.02

Models	λ	AIC	w_i
CTmin ~ TMEAN + tmean	0.72	71.56	0.02
CTmin ~ TMEAN + sr	0.72	71.59	0.02
CTmin ~ TMIN + dr	0.66	71.71	0.02
CTmin ~ TMEAN + tmin	0.70	71.73	0.02
CTmin ~ TMEAN + dr	0.69	71.74	0.02
CTmin ~ TMIN + sr	0.65	71.80	0.02
CTmin ~ TMEAN + tmin + SR	0.69	71.85	0.02
CTmin ~ TMIN + TMEAN + tmin	0.69	71.89	0.01
CTmin ~ tmin + SR + sr	0.70	71.94	0.01
CTmin ~ SR	0.59	71.96	0.01
CTmin ~ TMIN + tmin + SR	0.70	71.97	0.01
CTmin ~ TMIN + TMEAN + SR	1.00	72.08	0.01
CTmin ~ tmin + SR	0.62	72.17	0.01
CTmin ~ TMIN + SR + sr	0.72	72.58	0.01
CTmin ~ TMIN + TMEAN + sr	0.70	72.64	0.01
CTmin ~ TMEAN + SR + sr	0.70	72.66	0.01
CTmin ~ TMIN + SR + dr	0.71	72.67	0.01
CTmin ~ TMIN + TMEAN + dr	0.69	72.76	0.01
CTmin ~ TMEAN + SR + dr	0.69	72.79	0.01
CTmin ~ TMEAN + sr + dr	0.73	73.12	0.01
CTmin ~ TMEAN + tmean + dr	0.72	73.30	0.01
CTmin ~ TMEAN + tmin + sr	0.73	73.44	0.01
CTmin ~ SR + dr	0.63	73.47	0.01
CTmin ~ SR + sr	0.62	73.57	0.01
CTmin ~ TMEAN + tmin + tmean	0.72	73.57	0.01
CTmin ~ TMEAN + tmean + sr	0.72	73.58	0.01
CTmin ~ TMEAN + tmin + dr	0.70	73.73	0.01
CTmin ~ TMIN + sr + dr	0.66	73.74	0.01
CTmin ~ tmean	0.65	74.15	0.00
CTmin ~ sr	0.63	75.09	0.00
CTmin ~ SR + sr + dr	0.63	75.48	0.00
CTmin ~ dr	0.62	75.58	0.00
CTmin ~ tmin	0.61	75.75	0.00
CTmin ~ tmean + dr	0.66	75.76	0.00
CTmin ~ tmin + tmean	0.65	76.03	0.00
CTmin ~ tmean + sr	0.65	76.16	0.00
CTmin ~ tmin + tmean + dr	0.67	76.75	0.00
CTmin ~ tmin + sr	0.64	77.01	0.00
CTmin ~ sr + dr	0.63	77.01	0.00
CTmin ~ tmin + tmean + sr	0.62	77.25	0.00
CTmin ~ tmin + dr	0.62	77.58	0.00
CTmin ~ tmean + sr + dr	0.67	77.76	0.00
CTmin ~ tmin + sr + dr	0.64	78.92	0.00

Tolerance Range (CTmax-CTmin):

Models	λ	AIC	w_i
TR ~ SR + sr	0.43	86.52	0.28
TR ~ SR	0.60	86.77	0.24
TR ~ SR + dr + sr	0.51	87.71	0.15
TR ~ SR + dr	0.52	88.37	0.11
TR ~ sr	0.46	88.49	0.10
TR ~ dr	0.48	89.17	0.07
TR ~ dr + sr	0.47	90.48	0.04

Note: Macroclimatic predictors: TMAX (maximum of the average monthly maximum temperature); TMIN (minimum of the average monthly minimum temperature); TMEAN (mean of the average monthly temperature); (SR) SEASONAL RANGE (TMAX-TMIN). Microclimatic predictors: tmax (maximum temperature); tmin (minimum temperature); tmean (mean temperature); (dr) daily range (mean seasonal daily tmax-tmin); (sr) seasonal range (seasonal absolute tmax - absolute tmin). λ = Pagel's lambda, AIC=Akaike Information Criterion, w_i=Akaike weight. Akaike weights were calculated from AIC values of all models for each trait.

APPENDIX S3. SUPPLEMENTARY FIGURES

Figure S1. Phylogenetic tree of the species included in this study from Pyron & Wiens 2011.

Figure S2. Phylogenetic generalized least squares for CTmax and CTmin.

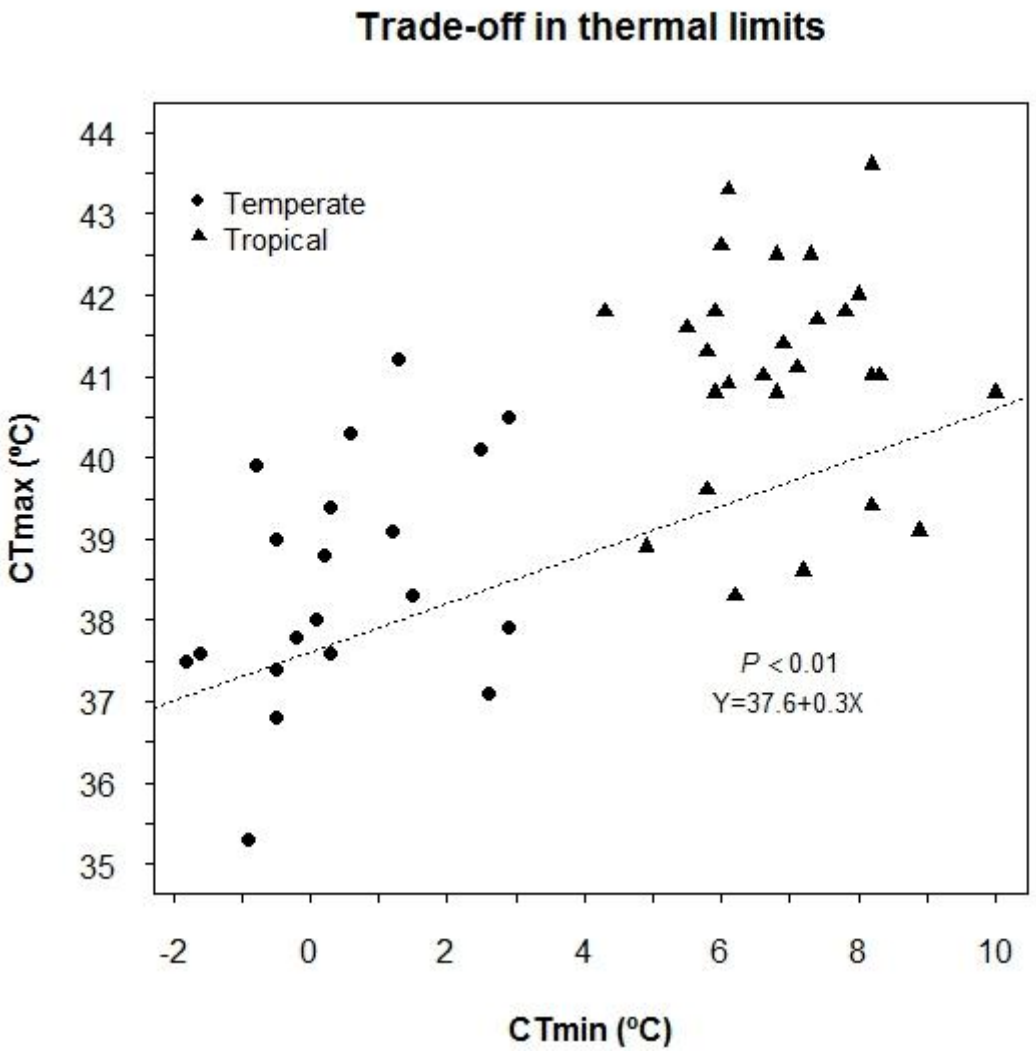


Figure S3. Phylogenetic correlation between (a) CTmax and maximum temperature and (b) CTmin and minimum temperature recorded in the aquatic microenvironments with dataloggers. Dotted line implies equality between microenvironmental temperature and physiological limit and thus representing a lethal threshold where either warming tolerance (CTmax - tmax) or cooling tolerance (tmin - CTmin), are equal to zero (see text).

A B

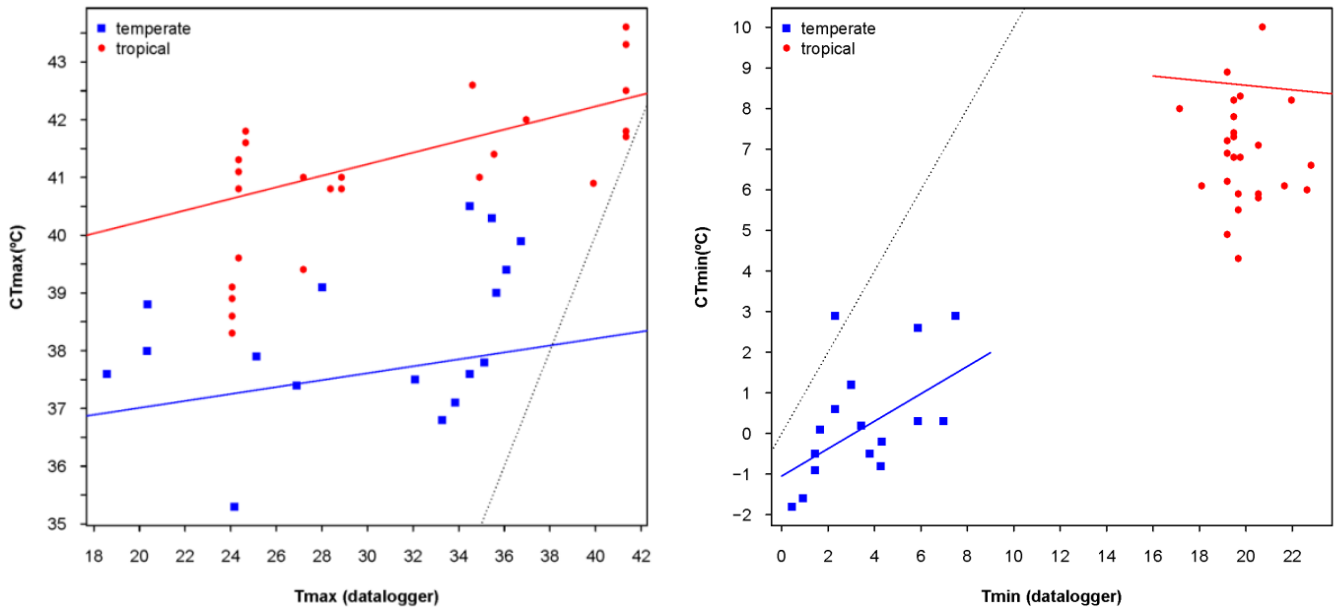
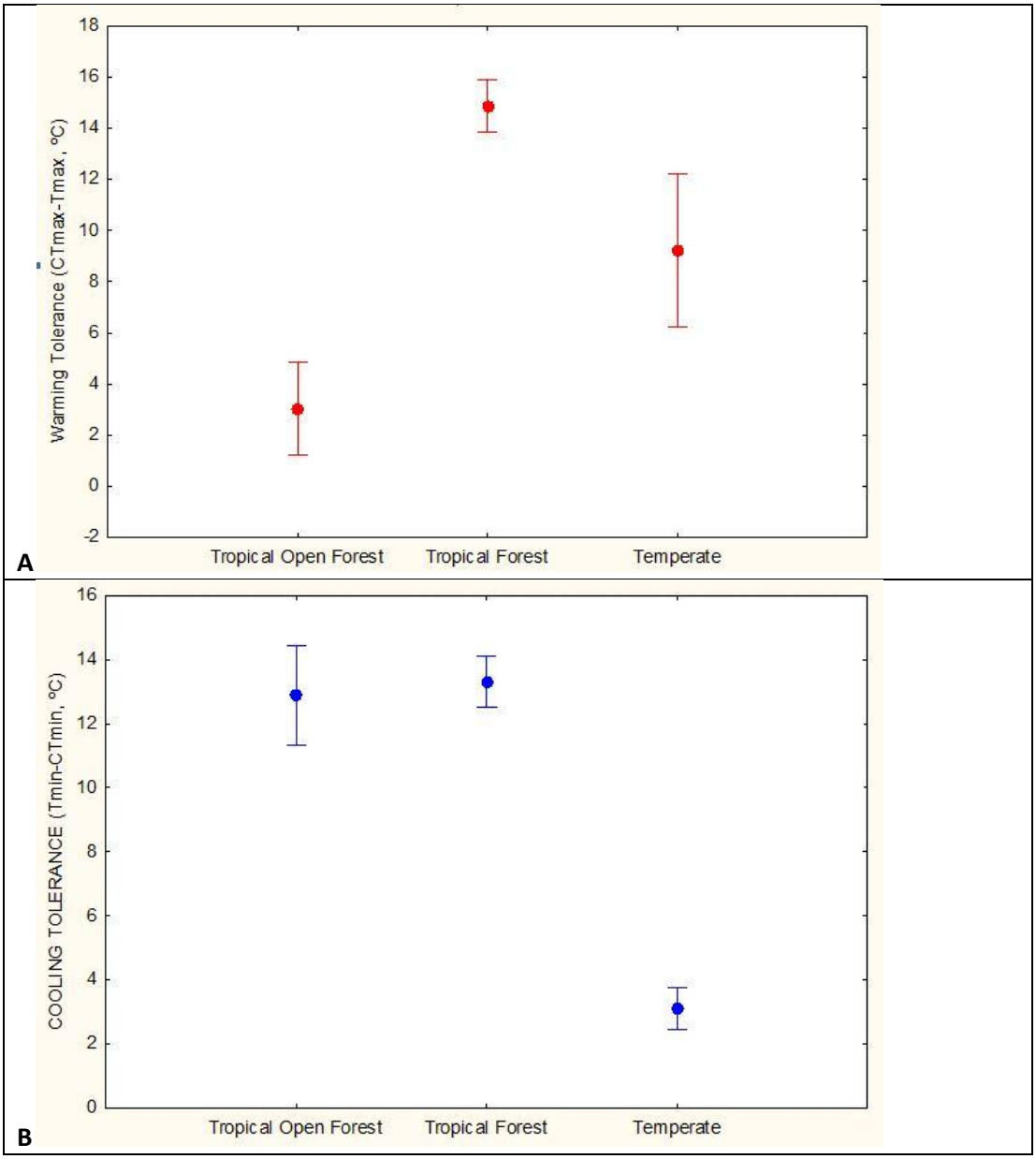


Figure S4. Mean Warming (A) and cooling tolerances (B) for tropical and temperate species. Vertical bars denote 0.95 confidence intervals. We distinguish the tropical community into two thermal microenvironments: Tropical Open Forest pond species and Tropical Forest pond and stream species.



ANNEXE 2

Supporting Information for Chapter 2

Table 2. Longitude, latitude (in decimal degrees, WGS84) and altitude of the eleven analyzed populations.

Location	Longitude	Latitude	Altitude
PURON	-4.6987	43.3794	40
NUEVA	-4.9335	43.4251	140
COLOR	-5.2768	43.4251	380
VIANGO	-4.8095	43.3710	480
CORTEGUEROS	-4.9396	43.3174	650
FANA	-5.0139	43.2803	950
PANDECARMEN	-5.0143	43.2633	1100
PANDÉBANO	-4.7868	43.2326	1200
ALIVA	-4.7648	43.1788	1400
SEÑALES	-5.2465	43.0792	1600
LLAGUSECU	-4.9921	43.2226	1800

Table 2. Periods of temperature recording in temporary ponds. n: number of registered days. The table lists the first and last dates of the analyzed data, as well as the exact day when the maximum and minimum values were logged.

Location	Altitude	n	Start Date	Finish Date	minT Date	maxT Date
Purón	40	101	08/02/2013	23/05/2013	24/02/2013	28/04/2013
Nueva	140	80	06/02/2013	26/04/2013	23/02/2013	25/04/2013
Color	380	506	01/09/2008	31/05/2010	08/01/2010	30/05/2010
Viango	480	81	09/02/2013	30/04/2013	11/02/2013	25/04/2013
Cortegueros	650	140	09/02/2013	28/06/2013	27/02/2013	12/06/2013
Fana	950	42	26/02/2014	08/04/2014	23/03/2014	06/04/2014
Pandecarmen	1100	40	07/03/2014	15/05/2014	15/03/2014	18/04/2014
Pandébano	1200	113	10/03/2014	30/06/2014	30/03/2014	13/06/2014
Aliva	1400	82	29/04/2002	18/07/2002	09/05/2002	17/06/2002
Señales	1600	83	02/05/2012	23/04/2013	03/05/2012	18/07/2012
Llagusecu	1800	114	11/08/2009	01/08/2010	06/05/2010	19/08/2009

Table 3. Summaries of daily water temperatures in each sampling site.

Location	Altitude	Tmin		Tmax	
		min	$\bar{X}$ ± SE	max	$\bar{X}$ ± SE
Purón	40	9.9	10.3 ± 0.1	11.4	10.7 ± 0.1
Nueva	140	6.4	9.3 ± 0.1	15.7	11.0 ± 0.2
Color	380	3.8	9.9 ± 0.1	22.5	11.4 ± 0.1
Viango	480	2.4	7.4 ± 0.3	25.6	12.3 ± 0.5
Cortegueros	650	1.0	7.6 ± 0.5	28.4	13.9 ± 0.5
Fana	950	1.5	4.4 ± 0.3	29.3	13.3 ± 1.0
Pandecarmen	1100	-1.6	2.7 ± 0.5	33.0	15.8 ± 1.5
Pandébano	1200	0.6	7.8 ± 0.3	28.9	15.6 ± 0.6
Aliva	1400	4.3	10.5 ± 0.4	24.5	17.0 ± 0.5
Señales	1600	4.0	9.0 ± 0.4	22.6	14.0 ± 0.4
Llagusecu	1800	0.5	6.8 ± 0.5	29.7	12.4 ± 0.9

Table 4. Values of the average minimum monthly temperatures obtained for the different analyzed populations in WorldClim throughout the year. The shaded values correspond to the period of presence of larvae in ponds. Mean and minimum values have been calculate only with shaded data.

Location	Altitude	longitude	latitude	JAN tmin_01	FEB tmin_02	MAR tmin_03	APR tmin_04	MAY tmin_05	JUN tmin_06	JUL tmin_07	AGO tmin_08	SEP tmin_09	OCT tmin_10	NOV tmin_11	DIC tmin_12	$\bar{X}$	MIN
PURON	40	-4.698653	43.3794037	5.4	5.8	7.5	8.6	10.9	13.8	15.7	16	14.6	11.5	8.6	6.9	8.6	5.4
NUEVA	140	-4.933516	43.4251387	4.6	5.0	6.7	7.7	10.1	13	14.8	15.2	13.8	10.7	7.7	6	7.8	4.6
COLOR	380	-5.276755	43.4251387	2.0	2.6	4.2	5.1	7.6	10.3	12.2	12.6	11.4	8.1	5	3.4	5.5	2.0
VIANGO	480	-4.809492	43.3710238	2.5	2.7	4.5	5.5	8	10.8	12.8	13.2	11.7	8.5	5.6	4	5.6	2.5
CORTEGUEROS	650	-4.939555	43.3174303	2.1	2.5	4.3	5.3	7.8	10.7	12.6	12.9	11.5	8.3	5.4	3.7	5.2	2.1
FANA	950	-5.0131	43.279605	-0.2	0.2	2	2.8	5.4	8.6	10.5	10.8	9.3	6	2.9	1.3	3.1	-0.2
PANDECARMEN	1100	-5.014256	43.263259	-0.9	-0.4	1.4	2.2	4.9	8	9.9	10.4	8.8	5.4	2.4	0.8	3.2	-0.4
PANDEBANO	1200	-4.786842	43.232574	-1.6	-1.2	0.4	1.3	4.1	7.3	9.3	9.7	8	4.7	1.7	0	2.4	-1.2
ALIVA	1400	-4.764802	43.1788459	-2.7	-2.3	-0.6	0.1	3	6.4	8.3	8.8	6.9	3.6	0.5	-0.9	3.4	-0.6
SEÑALES	1600	-5.246499	43.0791979	-4.2	-3.8	-1.9	-1.2	1.6	5	7	7.4	5.6	2.2	-1.1	-2.6	3.1	-1.2
LLAGUSECU	1800	-4.992107	43.22258	-5.1	-4.9	-3.3	-2.6	0.3	3.7	5.8	6.2	4.4	1	-2.2	-3.7	4.0	0.3

Table 5. Values of the average monthly temperatures obtained for the different analyzed populations in WorldClim throughout the year. The shaded values correspond to the period of presence of larvae in ponds.

Total Mean values have been calculate only with shaded data.

Location	Altitude	longitude	latitude	JAN	FEB	MAR	APR	MAY	JUN	JUL	AGO	SEP	OCT	NOV	DIC	$\bar{X}$
				tmean_01	tmean_02	tmean_03	tmean_04	tmean_05	tmean_06	tmean_07	tmean_08	tmean_09	tmean_10	tmean_11	tmean_12	
PURON	40	-4.698653	43.3794037	8.5	9.1	11.1	12.4	14.5	17.6	19.7	19.9	18.4	15.2	12	10	12.1
NUEVA	140	-4.933516	43.4251387	7.8	8.4	10.4	11.6	13.9	17	19	19.3	17.8	14.6	11.3	9.3	11.4
COLOR	380	-5.276755	43.4251387	5.5	6.3	8.1	9.5	12	15.2	17.4	17.7	16	12.4	9	7	9.5
VIANGO	480	-4.809492	43.3710238	5.8	6.2	8.4	9.7	12.2	15.4	17.7	18	16.1	12.6	9.3	7.3	9.4
CORTEGUEROS	650	-4.939555	43.3174303	5.5	6.1	8.2	9.7	12.1	15.5	17.8	17.9	16.1	12.6	9.2	7.1	9.2
FANA	950	-5.0131	43.279605	3.2	4	6.1	7.5	10.2	14.1	16.5	16.5	14.5	10.6	6.9	4.7	7.5
PANDECARMEN	1100	-5.014256	43.263259	2.6	3.4	5.6	7	9.8	13.6	16	16.3	14.1	10	6.4	4.2	7.9
PANDEBANO	1200	-4.786842	43.232574	1.8	2.5	4.6	6	9	12.9	15.5	15.6	13.3	9.2	5.6	3.3	7.0
ALIVA	1400	-4.764802	43.1788459	0.7	1.5	3.6	4.9	8	12.1	14.7	14.9	12.3	8.2	4.4	2.3	8.7
SEÑALES	1600	-5.246499	43.0791979	-0.6	0.2	2.4	3.8	6.9	11.1	13.9	14	11.4	7	2.9	0.7	8.9
LLAGUSECU	1800	-4.992107	43.22258	-1.7	-1.1	0.9	2.2	5.3	9.5	12.4	12.5	9.9	5.5	1.6	-0.5	9.9

Table 6. Values of the average maximum monthly temperatures obtained for the different analyzed populations in WorldClim throughout the year. The shaded values correspond to the period of presence of larvae in ponds. Mean and maximum values have been calculate only with shaded data.

Location	Altitude	longitude	latitude	JAN	FEB	MAR	APR	MAY	JUN	JUL	AGO	SEP	OCT	NOV	DIC	$\bar{X}$	MAX
				tmax_01	tmax_02	tmax_03	tmax_04	tmax_05	tmax_06	tmax_07	tmax_08	tmax_09	tmax_10	tmax_11	tmax_12		
PURON	40	-4.698653	43.3794037	11.7	12.4	14.8	16.3	18.2	21.4	23.7	23.8	22.3	19	15.5	13.1	15.6	22.3
NUEVA	140	-4.933516	43.4251387	11.1	11.8	14.1	15.6	17.7	21	23.3	23.4	21.8	18.5	14.9	12.6	15.1	21.8
COLOR	380	-5.276755	43.4251387	9.1	10.0	12.1	13.9	16.4	20.2	22.7	22.8	20.6	16.8	13	10.6	13.6	20.6
VIANGO	480	-4.809492	43.3710238	9.1	9.8	12.3	14	16.4	20.1	22.7	22.8	20.6	16.8	13.1	10.6	13.3	20.6
CORTEGUEROS	650	-4.939555	43.3174303	8.9	9.8	12.2	14.1	16.5	20.4	23.1	23	20.8	16.9	13	10.5	13.2	20.4
FANA	950	-5.0131	43.279605	6.7	7.8	10.3	12.3	15.1	19.6	22.5	22.3	19.7	15.2	10.9	8.2	12.0	19.6
PANDECARMEN	1100	-5.014256	43.263259	6.1	7.3	9.8	11.8	14.7	19.2	22.2	22.2	19.4	14.7	10.4	7.6	12.7	19.2
PANDEBANO	1200	-4.786842	43.232574	5.3	6.3	8.9	10.8	13.9	18.5	21.7	21.5	18.6	13.8	9.5	6.7	11.7	18.5
ALIVA	1400	-4.764802	43.1788459	4.2	5.3	7.8	9.8	13	17.8	21.1	21	17.8	12.8	8.3	5.6	13.9	21.1
SEÑALES	1600	-5.246499	43.0791979	3	4.2	6.8	8.8	12.2	17.3	20.9	20.7	17.2	11.8	6.9	4.1	14.8	20.9
LLAGUSECU	1800	-4.992107	43.22258	1.8	2.7	5.1	7	10.4	15.4	19.1	18.9	15.5	10.1	5.4	2.7	16.0	19.1

Table 7. Monthly range of temperatures, calculated as the difference between the average maximum and minimum. The shaded values correspond to the period of presence of larvae in ponds.

Mean range values have been calculate only with shaded data.

Location	Altitude	longitude	latitude	JAN range_01	FEB range_02	MAR range_03	APR range_04	MAY range_05	JUN range_06	JUL range_07	AGO range_08	SEP range_09	OCT range_10	NOV range_11	DIC range_12	$\bar{X}$
PURON	40	-4.698653	43.3794037	6.3	6.6	7.3	7.7	7.3	7.6	8	7.8	7.7	7.5	6.9	6.2	7.0
NUEVA	140	-4.933516	43.4251387	6.5	6.8	7.4	7.9	7.6	8	8.5	8.2	8	7.8	7.2	6.6	7.3
COLOR	380	-5.276755	43.4251387	7.1	7.4	7.9	8.8	8.8	9.9	10.5	10.2	9.2	8.7	8	7.2	8.0
VIANGO	480	-4.809492	43.3710238	6.6	7.1	7.8	8.5	8.4	9.3	9.9	9.6	8.9	8.3	7.5	6.6	7.7
CORTEGUEROS	650	-4.939555	43.3174303	6.8	7.3	7.9	8.8	8.7	9.7	10.5	10.1	9.3	8.6	7.6	6.8	7.9
FANA	950	-5.0131	43.279605	6.9	7.6	8.3	9.5	9.7	11	12	11.5	10.4	9.2	8	6.9	8.4
PANDECARMEN	1100	-5.014256	43.263259	7	7.7	8.4	9.6	9.8	11.2	12.3	11.8	10.6	9.3	8	6.8	8.4
PANDEBANO	1200	-4.786842	43.232574	6.9	7.5	8.5	9.5	9.8	11.2	12.4	11.8	10.6	9.1	7.8	6.7	8.3
ALIVA	1400	-4.764802	43.1788459	6.9	7.6	8.4	9.7	10	11.4	12.8	12.2	10.9	9.2	7.8	6.5	8.4
SEÑALES	1600	-5.246499	43.0791979	7.2	8	8.7	10	10.6	12.3	13.9	13.3	11.6	9.6	8	6.7	8.7
LLAGUSECU	1800	-4.992107	43.22258	6.9	7.6	8.4	9.6	10.1	11.7	13.3	12.7	11.1	9.1	7.6	6.4	8.3

Table 8. Mean values temperature ± SD (°C) for the constant acclimation treatments.

Acclimation Treatment	$\overline{X}$ ± SD (°C)
6	5.92 ± 0.32
13	12.75 ± 0.18
20	19.96 ± 0.26
27	26.83 ± 0.19

Table 9. Markers used in this study and the PCR conditions.

					PCR conditions			
Locus	**Repeat motif**	**Allele size range (bp)**	**Number of alleles**	**Dye**	**Polymerase**	**T_a (°C)**	**cycles**	**Gene Bank no.**
Rtempµ1	$(CA)_4GG(CA)_{24}$	92-130	21	PET	GoTaq	46	40	AF297972[1]
Rtempµ2	$(AT)_8AT(AC)_{22}$	83-140	7	PET	GoTaq	46	40	AF297973[1]
Rtempµ4	$(AC)_{16}$	106-142	15	VIC	GoTaq	58	40	AF297975[1]
RtµB	$(CA)_{14}$	246	27	VIC	GoTaq	58	40	AF489577[2]
A-plex					Qiagen	55	40	
RtµH	$(CA)_7$	206	4	NED				AF489579[2]
RtU4	$(GT)_{23}(T)_{13}$	75-108	14	VIC				AF257481[3]
RtU7	$(GATA)_{37}$	152-295	57	FAM				AF257482[3]
B-plex					Qiagen	55	38	
BFG072	$(TGTA)_{13}$	104-132	2	PET				EU334947[4]
BFG093	$(TG)_{21}$	116-142	20	FAM				EU334958[4]
BFG183	$(TG)_9$	112-158	41	NED				EU335004[4]
BFG241	$(CATA)_8$	107-152	21	VIC				EU335033[4]

Table 10. Pairwise F_{ST} values for the eleven populations included in the study.

	PUR	NUE	COL	VIA	COR	FAN	PAC	PAN	ALI	SEN	LLA
PUR	0.0000	0.0437	0.0670	0.0201	0.0542	0.0465	0.0363	0.0851	0.0578	0.0817	0.0701
NUE	0.0437	0.0000	0.0365	0.0393	0.0253	0.0178	0.0125	0.1024	0.1081	0.0622	0.0708
COL	0.0670	0.0365	0.0000	0.0603	0.0536	0.0378	0.0501	0.1002	0.0936	0.0642	0.1175
VIA	0.0201	0.0393	0.0603	0.0000	0.0627	0.0499	0.0247	0.0714	0.0601	0.0907	0.0560
COR	0.0542	0.0253	0.0536	0.0627	0.0000	0.0143	0.0334	0.1152	0.1165	0.0350	0.0934
FAN	0.0465	0.0178	0.0378	0.0499	0.0143	0.0000	0.0164	0.0802	0.0958	0.0389	0.0777
PAC	0.0363	0.0125	0.0501	0.0247	0.0334	0.0164	0.0000	0.0781	0.1006	0.0627	0.0330
PAN	0.0851	0.1024	0.1002	0.0714	0.1152	0.0802	0.0781	0.0000	0.0700	0.1115	0.0928
ALI	0.0578	0.1081	0.0936	0.0601	0.1165	0.0958	0.1006	0.0700	0.0000	0.0896	0.1322
SEN	0.0817	0.0622	0.0642	0.0907	0.0350	0.0389	0.0627	0.1115	0.0896	0.0000	0.1293
LLA	0.0701	0.0708	0.1175	0.0560	0.0934	0.0777	0.0330	0.0928	0.1322	0.1293	0.0000

Table 11. Pairwise PST values for CTmax under null assumption $c=h^2=1$.

	PUR	NUE	COL	VIA	COR	FAN	PAC	PAN	ALI	SEN	LLA
PUR	0.0000	0.3468	0.3451	0.4301	0.2286	0.0226	0.4293	0.1774	0.6887	0.1596	0.4204
NUE	0.3468	0.0000	0.0000	0.0495	0.0000	0.2192	0.0378	0.0035	0.4018	0.0000	0.0294
COL	0.3451	0.0000	0.0000	0.0091	0.0000	0.2314	0.0000	0.0279	0.3130	0.0162	0.0000
VIA	0.4301	0.0495	0.0091	0.0000	0.1001	0.3386	0.0000	0.1319	0.1427	0.1112	0.0000
COR	0.2286	0.0000	0.0000	0.1001	0.0000	0.1060	0.0882	0.0000	0.4403	0.0000	0.0782
FAN	0.0226	0.2192	0.2314	0.3386	0.1060	0.0000	0.3344	0.0617	0.6445	0.0539	0.3250
PAC	0.4293	0.0378	0.0000	0.0000	0.0882	0.3344	0.0000	0.1203	0.1707	0.0999	0.0000
PAN	0.1774	0.0035	0.0279	0.1319	0.0000	0.0617	0.1203	0.0000	0.4667	0.0000	0.1096
ALI	0.6887	0.4018	0.3130	0.1427	0.4403	0.6445	0.1707	0.4667	0.0000	0.4295	0.1720
SEN	0.1596	0.0000	0.0162	0.1112	0.0000	0.0539	0.0999	0.0000	0.4295	0.0000	0.0892
LLA	0.4204	0.0294	0.0000	0.0000	0.0782	0.3250	0.0000	0.1096	0.1720	0.0892	0.0000

Table 12. Pairwise PST values for CTmin under null assumption $c=h^2=1$.

	PUR	NUE	COL	VIA	COR	FAN	PAC	PAN	ALI	SEN	LLA
PUR	0.0000	0.6171	0.7715	0.5746	0.3566	0.2590	0.4345	0.6232	0.6419	0.4391	0.6057
NUE	0.6171	0.0000	0.3939	0.0485	0.1759	0.5517	0.0000	0.0000	0.0000	0.0000	0.0000
COL	0.7715	0.3939	0.0000	0.0000	0.5650	0.7791	0.2776	0.3513	0.3894	0.1789	0.2828
VIA	0.5746	0.0485	0.0000	0.0000	0.2282	0.4360	0.0759	0.0466	0.0507	0.0312	0.0242
COR	0.3566	0.1759	0.5650	0.2282	0.0000	0.0778	0.0177	0.1827	0.2060	0.0441	0.1786
FAN	0.2590	0.5517	0.7791	0.4360	0.0778	0.0000	0.2023	0.5241	0.5717	0.2233	0.4995
PAC	0.4345	0.0000	0.2776	0.0759	0.0177	0.2023	0.0000	0.0000	0.0000	0.0000	0.0000
PAN	0.6232	0.0000	0.3513	0.0466	0.1827	0.5241	0.0000	0.0000	0.0000	0.0000	0.0000
ALI	0.6419	0.0000	0.3894	0.0507	0.2060	0.5717	0.0000	0.0000	0.0000	0.0000	0.0000
SEN	0.4391	0.0000	0.1789	0.0312	0.0441	0.2233	0.0000	0.0000	0.0000	0.0000	0.0000
LLA	0.6057	0.0000	0.2828	0.0242	0.1786	0.4995	0.0000	0.0000	0.0000	0.0000	0.0000

Table 13. Confidence interval in global P_{ST} for CTmax estimates with different values of c y h^2 using non parametric bootstrap.

c	h=1			h=0.75			h=0.5			h=0.25		
	Low	Mean	Up	Low	Mean	Up	Low	Mean	Up	Low	Mean	Up
1	0.12	0.17	0.21	0.15	0.20	0.25	0.19	0.25	0.31	0.27	0.35	0.42
0.9	0.11	0.16	0.20	0.14	0.19	0.24	0.18	0.24	0.30	0.26	0.33	0.41
0.8	0.10	0.14	0.19	0.13	0.17	0.22	0.17	0.22	0.28	0.24	0.32	0.39
0.7	0.09	0.13	0.17	0.12	0.16	0.21	0.15	0.21	0.26	0.23	0.30	0.37
0.6	0.08	0.12	0.15	0.10	0.15	0.19	0.14	0.19	0.24	0.21	0.27	0.34
0.5	0.07	0.10	0.14	0.09	0.13	0.17	0.12	0.17	0.22	0.19	0.25	0.32
0.4	0.06	0.09	0.12	0.08	0.11	0.14	0.10	0.14	0.19	0.17	0.22	0.28
0.3	0.05	0.07	0.09	0.06	0.09	0.12	0.08	0.12	0.16	0.14	0.19	0.24
0.2	0.03	0.05	0.07	0.04	0.06	0.08	0.06	0.09	0.12	0.10	0.15	0.19
0.1	0.02	0.03	0.04	0.02	0.03	0.05	0.03	0.05	0.07	0.06	0.09	0.12

Table 14. Confidence interval in global P_{ST} for CTmin estimates with different values of c y h^2 using non parametric bootstrap.

c	h=1			h=0.75			h=0.5			h=0.25		
	Low	Mean	Up	Low	Mean	Up	Low	Mean	Up	Low	Mean	Up
1	0.17	0.24	0.30	0.20	0.27	0.34	0.25	0.32	0.40	0.32	0.41	0.50
0.9	0.16	0.22	0.28	0.19	0.26	0.33	0.23	0.31	0.39	0.31	0.40	0.49
0.8	0.15	0.21	0.27	0.18	0.24	0.31	0.22	0.29	0.37	0.29	0.38	0.47
0.7	0.14	0.19	0.25	0.17	0.23	0.29	0.21	0.28	0.35	0.28	0.37	0.45
0.6	0.13	0.18	0.23	0.15	0.21	0.27	0.19	0.26	0.33	0.27	0.35	0.43
0.5	0.11	0.16	0.21	0.13	0.19	0.24	0.17	0.23	0.30	0.24	0.32	0.40
0.4	0.10	0.14	0.18	0.12	0.16	0.21	0.15	0.21	0.27	0.22	0.29	0.37
0.3	0.08	0.11	0.15	0.10	0.14	0.18	0.13	0.18	0.23	0.19	0.26	0.33
0.2	0.06	0.08	0.11	0.07	0.10	0.13	0.10	0.14	0.18	0.15	0.21	0.27
0.1	0.03	0.05	0.06	0.04	0.06	0.08	0.06	0.08	0.11	0.10	0.14	0.18

Table 15. Results of the linear regression for temperature data and thermal limits with altitude and physiological traits with environmental temperature for each populations: TMAX (maximum monthly temperature); TMIN (minimum monthly temperature) and TMEAN (average monthly temperature) estimated for _A (full year) and _BS (for the breeding season only). WT (Warming Tolerance based on WorldClim temperature data), wt (warming tolerance based on temperature from water).

OLS		Intercept	SE	Slope	SE	F	df	R^2	P
TMAX_A	Altitude	17.91517	0.25	-0.0043	0.0002	310.20	1,9	0.97	**<0.001**
TMIN_A	Altitude	10.30937	0.26	-0.0056	0.0003	505.10	1,9	0.98	**<0.001**
TMEAN_A	Altitude	14.09026	0.24	-0.0049	0.0002	467.80	1,9	0.98	**<0.001**
TMAX_BS	Altitude	13.87478	0.85	-0.0001	0.0008	0.02	1,9	0.00	0.902
TMIN_BS	Altitude	7.27427	0.65	-0.0029	0.0006	21.36	1,9	0.70	**<0.01**
TMEAN_BS	Altitude	10.55129	0.74	-0.0015	0.0007	4.42	1,9	0.33	*0.06*
CTmax	Altitude	36.69	0.16	0.0002	0.0002	1.61	1,9	0.15	0.24
CTmin	Altitude	-1.62050	0.22	-0.0002	0.0002	0.55	1,9	0.06	0.48
CTmax	TMAX_A	37.52295	0.50	-0.0471	0.0347	1.85	1,9	0.17	0.21
CTmax	TMAX_BS	36.81	0.94	0.00	0.0677	0.00	1,9	0.00	0.96
CTmin	TMIN_A	-1.95	0.22	0.04	0.0356	1.05	1,9	0.10	0.33
CTmin	TMIN_BS	-2.03	0.30	0.06	0.06	0.96	1,9	0.96	0.35
WT	Altitude	15.26	0.55	0.001	0.001	7.01	1,9	0.44	**0.03**
wt	Altitude	18.11	2.920	-0.007	0.003	5.84	1,9	0.39	**0.04**

Table 16. Maximum temperatures during breeding season and estimated warming tolerance for eleven studied populations. TMAX (maximum monthly temperature, air-measurement from WordlClim), tmax (maximum daily temperature recorded in ponds). WT=CTmax-TMAX, wt=CTmax-tmax.

Population	Altitude	CTmax	TMAX	tmax	WT	wt
PURON	40	36.4	22.3	11.4	14.1	25.0
NUEVA	140	36.8	21.8	15.7	15.0	21.1
COLOR	380	36.9	20.6	22.5	16.3	14.4
VIANGO	480	37.1	20.6	25.6	16.5	11.5
CORTEGUEROS	650	36.8	20.4	28.4	16.4	8.4
FANA	950	36.5	19.6	29.3	16.9	7.2
PANDECARMEN	1100	37.0	19.2	33.0	17.8	4.0
PANDEBANO	1200	36.7	18.5	28.9	18.2	7.8
ALIVA	1400	37.5	21.1	24.5	16.4	13.0
SENALES	1600	36.7	20.9	22.6	15.8	14.1
LLAGUSECU	1800	37.0	19.1	29.7	17.9	7.3

ANNEXE 3

Supporting Information for Chapter 3

TABLES

Table S1. Species sampled, locality coordinates (in decimal degrees), altitude and thermal and physiological data. Mean critical thermal maximum (CTmax), critical thermal minimum (CTmin) and tolerance range (TR: CTmax-CTmin) are given for each species. Units for physiological metrics and thermal data are in degrees Celsius (°C). N: sample size. BIO6: minimum temperature of coldest month, BIO1: annual mean temperature, BIO5: maximum temperature of warmest month, tmin: minimum temperature from ponds, tmean: mean temperature from ponds, tmax: maximum temperature registered by dataloggers. WT: warming tolerance estimate based on WorldClim temperature data (BIO5), wt: warming tolerance estimate based on maximum water temperatures (tmax).

Species	Family	Label	Altitude (m)	Latitude	Longitude	CTmax(ºC)			CTmin (ºC)			TR	BIO6	BIO1	BIO5	tmin	tmean	tmax	WT	wt
						N	$\bar{X}$	SE	N	$\bar{X}$	SE									
Scinax_quinquefasciatus	Hylidae (subfam.Hylinae)	SQU	23	1.1653	-78.7527	8	41.6	0.2	10	7.9	0.1	33.8	21.4	25.9	30.9	24.6	28.5	36.8	10.7	4.8
Engystomops_guayaco	Leptodactylidae	EGU	100	-2.2056	-80.0225	14	39.7	0.1	8	8	0.3	31.7	19.4	25.5	31.5	NA	NA	NA	8.2	NA
Agalychnis_spurrelli	Hylidae (subfam.Phyllomedusinae)	ASP	227	1.2218	-78.636	37	40.6	0.1	31	6.7	0.1	33.9	20.8	25.2	30	24	24.5	26.2	10.6	14.4
Epipedobates_boulengeri	Dendrobatidae	EBO	227	1.0413	-78.6232	2	38.8	0.1	4	8.1	0.3	30.6	20.1	24.7	29.7	24	24.5	26.2	9.1	12.6
Smilisca_phaeota	Hylidae (subfam.Hylinae)	SPH	250	1.1653	-78.7527	35	42.6	0.2	57	7.7	0.1	34.9	21.4	25.9	30.9	24.6	28.5	36.8	11.7	5.7
Hypsiboas_rosenbergi	Hylidae (subfam.Hylinae)	HRO	263	1.0374	-78.6221	16	42.4	0.1	16	8.1	0.1	34.3	20.1	24.7	29.7	24.6	28.5	36.8	12.7	5.6
Atelopus_limon	Bufonidae	ALI	840	-3.221	-78.4347	15	36.9	0.1	15	7.1	0.1	29.8	17.3	23	29.5	NA	NA	NA	7.4	NA
Hypsiboas_geographicus	Hylidae (subfam.Hylinae)	HGE	1034	-1.4444	-77.8206	13	39.7	0.1	12	6.2	0.1	33.5	15.4	21	26.9	18.7	20.6	23.1	12.8	16.6
Rhinella_marina	Bufonidae	RMA	1066	0.0182	-78.8076	16	41.5	0.4	24	8	0.2	33.5	13.4	19.1	24.4	19.2	19.9	20.9	17.1	20.6
Hypsiboas_lanciformis	Hylidae (subfam.Hylinae)	HLA	1068	-1.4053	-77.7186	13	41.1	0.1	11	6.5	0.1	34.6	15.3	20.9	26.8	18.7	20.6	23.1	14.3	18
Engystomops_petersi	Leptodactylidae	EPE	1072	-1.4064	-77.7204	15	38.6	0.1	16	7	0.2	31.6	15.3	20.9	26.8	19.9	22.7	28.3	11.8	10.3
Dendrosophus_carnifex	Hylidae (subfam.Hylinae)	DCA	1200	-0.0411	-78.7917	18	40	0	18	4.3	0.2	35.7	14.1	19.7	24.9	17.9	22	32.6	15.1	7.4
Epipedobates_tricolor	Dendrobatidae	ETR	1300	-1.4197	-79.125	10	38	0.2	10	8	0.2	30.0	13.1	18.2	23.2	NA	NA	NA	14.8	NA
Hyloscirtus_gr.phyllognathus	Hylidae (subfam.Hylinae)	HPH	1495	-2.274	-78.1922	9	37.5	0.1	18	4.6	0.1	32.9	13.5	19.4	26.1	NA	NA	NA	11.4	NA
Scinax_ruber	Hylidae (subfam.Hylinae)	SRU	1540	-1.3954	-78.3027	4	41	0.1	5	5.2	0.1	35.8	12.6	18.1	23.9	16.1	20.4	27	17.1	14
Rhinella_margaritifera	Bufonidae	RMG	1638	-1.3468	-78.1966	7	38.5	0.1	8	4.7	0.1	33.8	12.4	18.1	24.2	17.5	17.8	18.1	14.3	20.3
Hyloxalus_bocagei	Dendrobatidae	HBO	1820	-0.097	-77.5962	16	38	0.1	16	6.7	0.1	31.2	12.7	19	25.2	15.2	17.3	21.1	12.8	16.9
Hyloscirtus_lindae/ psarolaimus	Hylidae (subfam.Hylinae)	HLI	2800	-0.3877	-78.0619	17	36.5	0.1	15	1.5	0.1	35.0	7.3	14	20.7	11.3	12.8	15.4	15.8	21.2
Gastrotheca_riobambe	Hemiphractidae	GRI	2969	-0.1873	-78.4639	16	38.6	0.1	16	-1	0.1	39.6	6.4	13	19.6	8.5	15	25.3	19	13.3

Species	Family	Label	Altitude (m)	Latitude	Longitude	CTmax(ºC)			CTmin (ºC)			TR	BIO6	BIO1	BIO5	tmin	tmean	tmax	WT	wt
						N	$\bar{X}$	SE	N	$\bar{X}$	SE									
Gastrotheca_pseustes	Hemiphractidae	GPS	3500	-1.3367	-78.7594	19	37.9	0.1	19	-3.6	0.1	41.5	2.7	8.2	14.2	8	12.2	19.7	23.7	18.3

FIGURES

Figure S1. Phylogenetic tree of the 20 analyzed species based on Pyron & Wiens 2011.

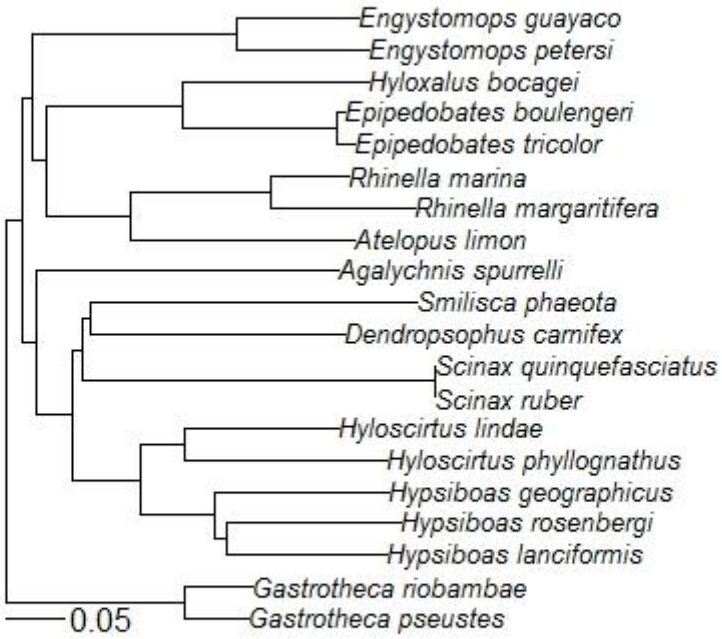


Figure S2. Thermal profile across the altitudinal gradient for the sample points of the 20 analyzed species. (Up) Variation in average, maximum and minimum temperatures with altitude based on temperature of water (dataloggers). (Down) Variation in average (BIO1), maximum (BIO5) and minimum (BIO6) temperatures with altitude based on WorldClim temperature database. Dashed lines denote significant correlations (OLS, $P < 0.01$)

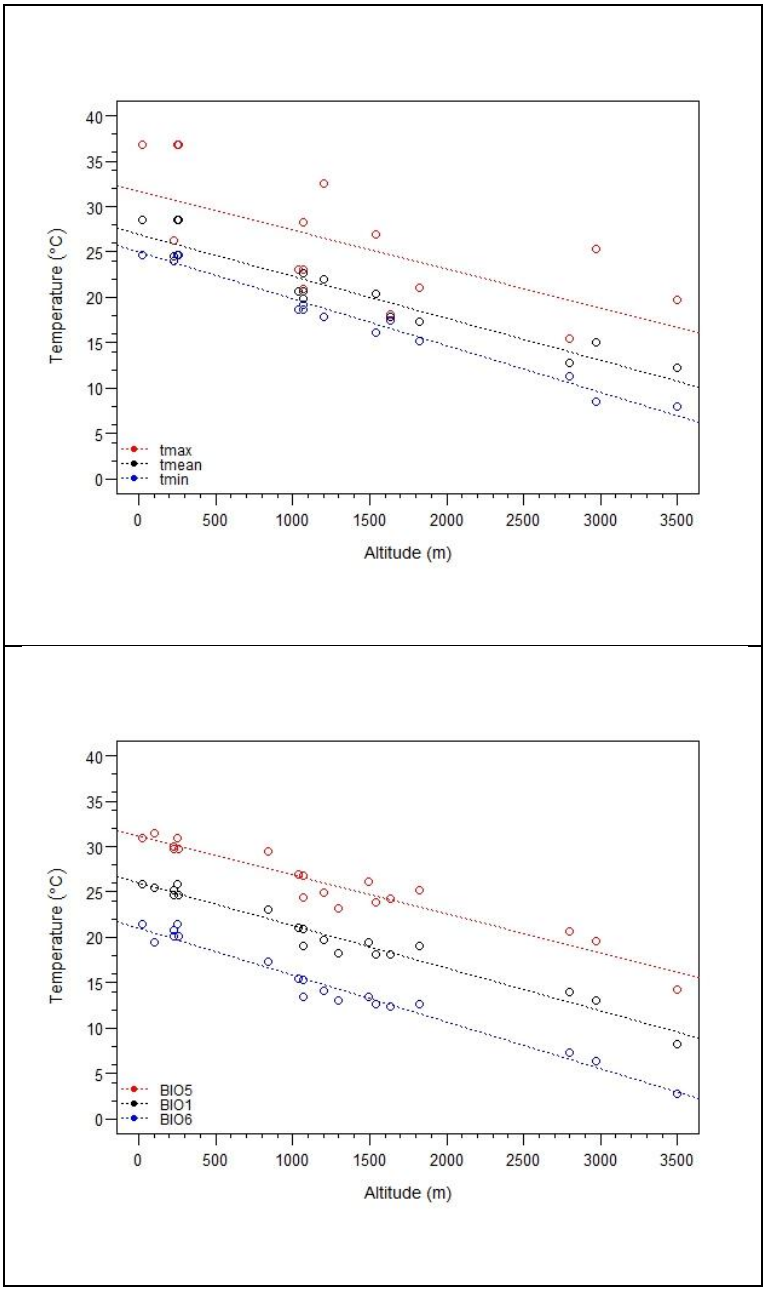
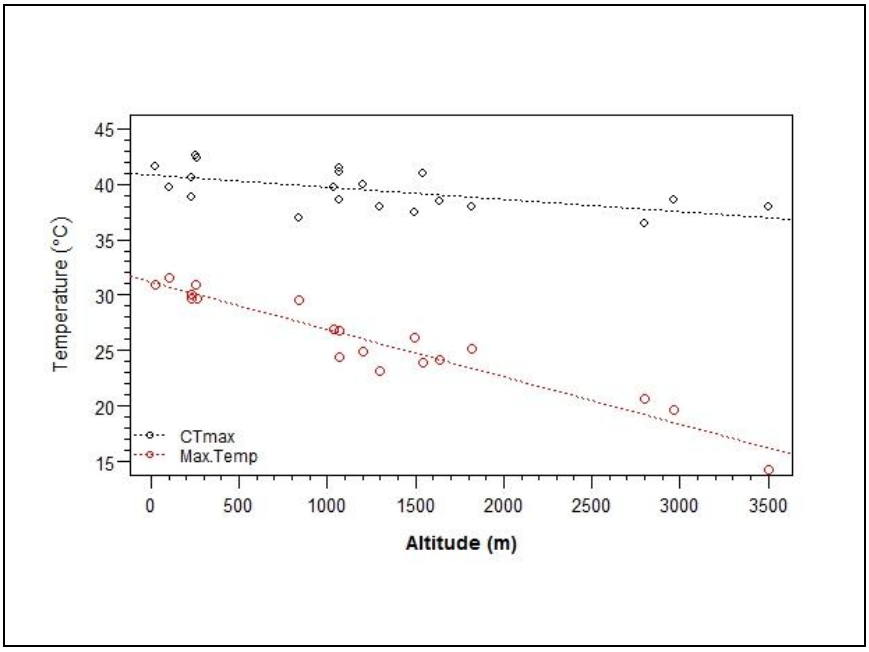


Figure S3. Maximum temperatures of the macroclime (BIO5) decreases steeper than species tolerance to high temperatures (CTmax) through the altitudinal gradient (ANCOVA, $P < 0.05$). As consequence, warming tolerance decreased significantly with altitude.



ANNEXE 4

Supporting Information for Chapter 4

TABLES

Table S1. Summaries of the CT_{max} values for the twelve analyzed species in relation to the acclimation temperature. N: number of valid observation for CT_{max}.

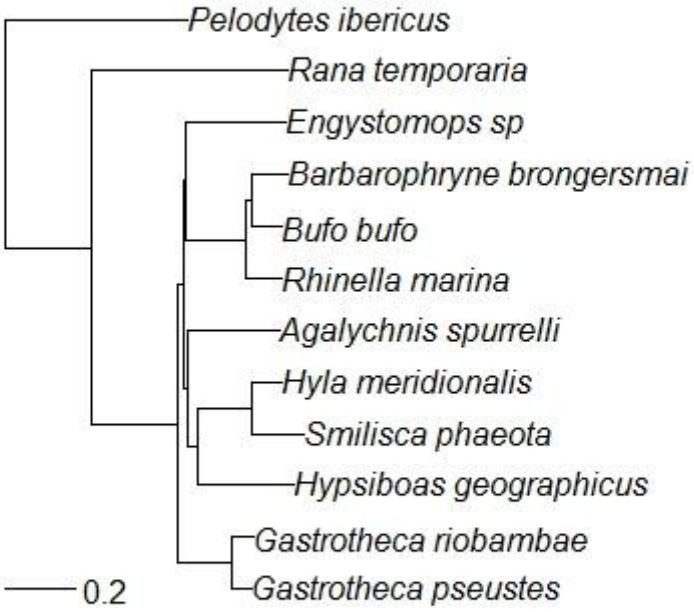
	9			15			20			27			34		
Species	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE
Bufo bufo	16	37.3	0.1	14	37.8	0.1	14	37.8	0.1	12	38.2	0.1			
Hyla meridionlis	11	39.7	0.1	14	39.7	0.1	14	39.9	0.1	14	40.8	0.1			
Barbarophryne brongersmai	15	39.4	0.1	16	39.3	0.1	16	40.1	0.1	16	40.8	0.1			
Pelodytes ibericus	16	36.7	0.1	16	36.6	0.1	16	36.9	0.1	16	37.5	0.1			
Rana temporaria	14	36.1	0.1	14	36.2	0.1	16	36.8	0.1	16	38.1	0.1			
Agalychnis spurrelli				16	40.4	0.1	13	41.4	0.1	17	42.4	0.1	18	43.0	0.1
Smilisca phaeota				6	41.3	0.2	8	41.5	0.2	7	41.9	0.1	7	43.8	0.1
Engystomops sp.				2	38.8	0.4	10	39.0	0.1	9	39.8	0.2			
Hypsiboas geographicus				6	40.8	0.2	10	41.4	0.1	9	42.7	0.1	5	43.8	0.2
Rhinella marina				4	40.6	0.6	9	41.1	0.3	9	43.4	0.2	10	44.4	0.1
Gastrotheca riobambae				16	38.7	0.1	10	38.9	0.1	14	40.2	0.1	15	40.7	0.1
Gastrotheca pseustes				10	38.1	0.1	19	37.9	0.1	10	38.5	0.1	10	38.9	0.1

Table S2. Summaries of the CT_{min} values for the twelve analyzed species in relation to the acclimation temperature. N: number of valid observation for CT_{min}.

	9			15			20			27			34		
Species	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE
Bufo bufo	16	1.2	0.2	16	2.1	0.3	16	2.9	0.2	14	4.5	0.4			
Hyla meridionlis	14	0.6	0.1	14	0.5	0.2	14	0.8	0.1	14	1.0	0.1			
Barbarophryne brongersmai	16	2.0	0.2	16	2.2	0.2	16	2.5	0.2	16	3.1	0.1			
Pelodytes ibericus	16	-1.4	0.2	16	-0.7	0.1	16	0.0	0.1	16	0.6	0.1			
Rana temporaria	16	-2.0	0.1	14	-2.0	0.1	14	-1.6	0.1	16	-0.3	0.2			
Agalychnis spurrelli				11	4.6	0.3	11	3.7	0.3	11	7.3	0.2	10	8.7	0.3
Smilisca phaeota				6	6.8	0.4	7	7.3	0.3	8	7.5	0.2	6	8.3	0.4
Engystomops sp.				5	7.0	0.4	9	8.6	0.1	10	8.4	0.2			
Hypsiboas geographicus				8	8.9	0.2	10	8.5	0.1	10	8.8	0.2	7	9.8	0.2
Rhinella marina				9	8.5	0.2	10	7.8	0.2	9	8.2	0.3	7	9.4	0.2
Gastrotheca riobambae				16	-1.2	0.2	16	-0.4	0.1	16	0.8	0.1	8	0.9	0.2
Gastrotheca pseustes							19	-3.6	0.1	10	-1.6	0.1	10	-0.7	0.2

FIGURES

Figure S1. Phylogenetic tree of the species included in this study from Pyron & Wiens 2011.



ANNEXE 5

Supporting Information for Chapter 5

FIGURES

A) Temperature profiles in the ponds

Fig.A.1. Temperature profile in a breeding pond of *Rana temporaria*. Dashed line indicates maximum temperature employed in fluctuating acclimation regime (T_{max}). Dotted line denotes optimum growth temperature (T_{op}).

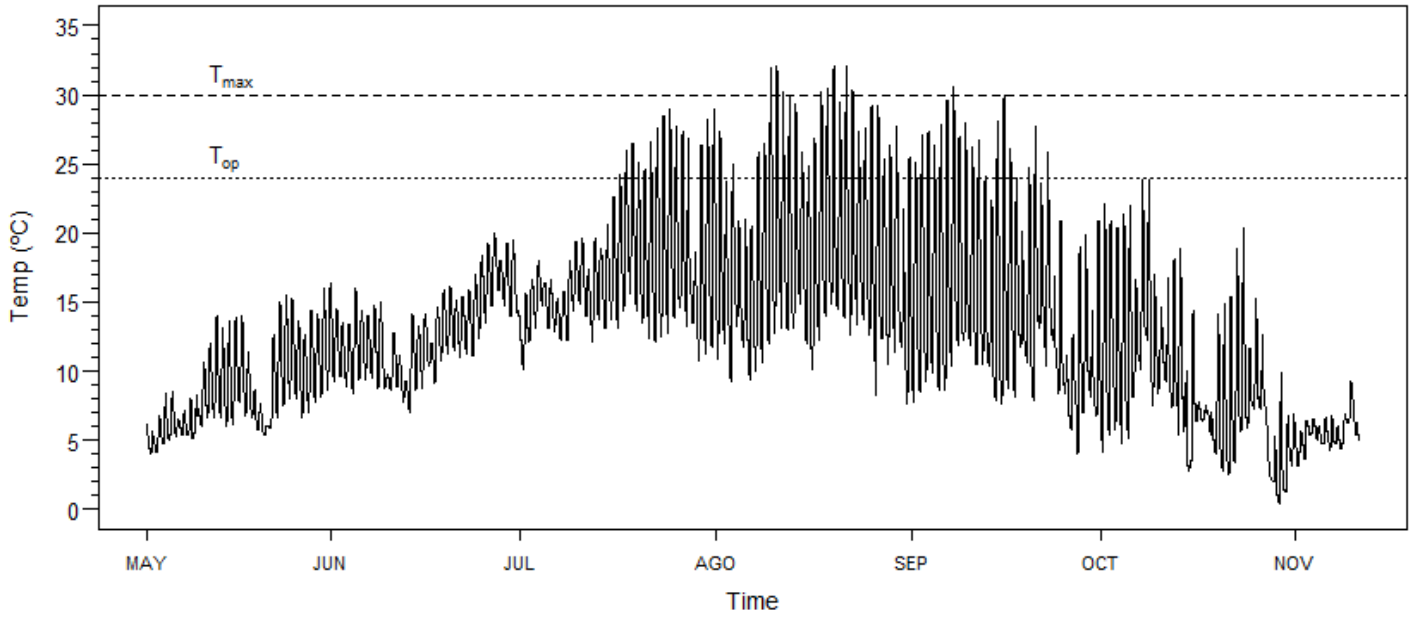


Fig.A.2. Temperature profile in a breeding pond of *Pelodytes ibericus*. Dashed line indicates maximum temperature employed in fluctuating acclimation regime (T_{max}). Dotted line denotes optimum growth temperature (T_{op}).

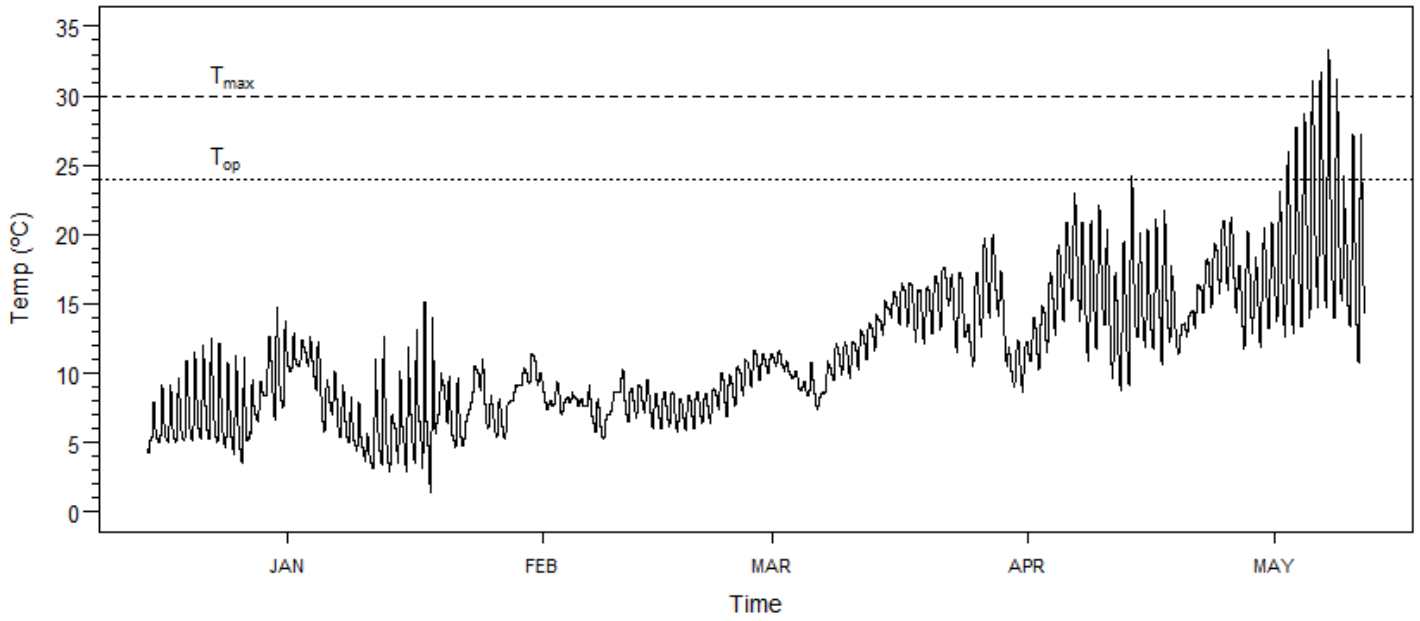
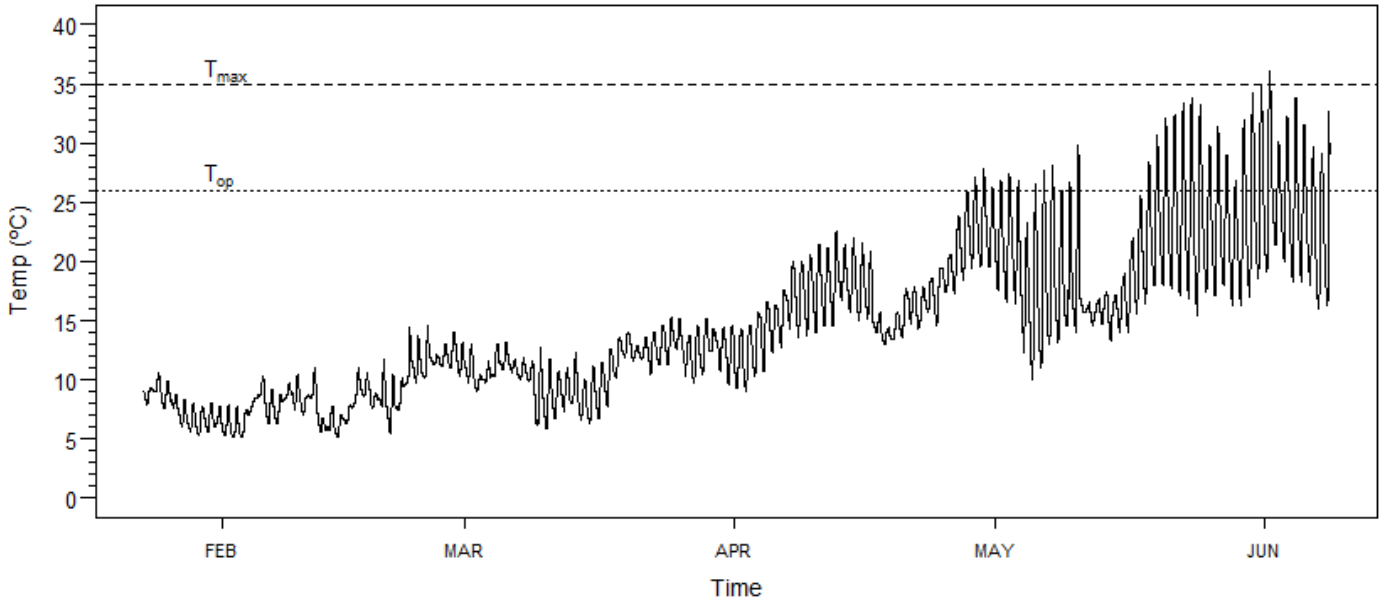


Fig.A.3. Temperature profile in a breeding pond of *Hyla meridionalis*. Dashed line indicates maximum temperature employed in fluctuating acclimation regime (T_{max}). Dotted line denotes optimum growth temperature (T_{op}).



B. Temperature profiles of the fluctuating treatments vs. its equivalent constant temperature acclimation.

Fig.B.1) Temperature profile for treatments F24 and C20. Point line represents real temperature values from data loggers during the acclimation period. Solid and dashed lines denotes mean ± SD of the constants treatments.

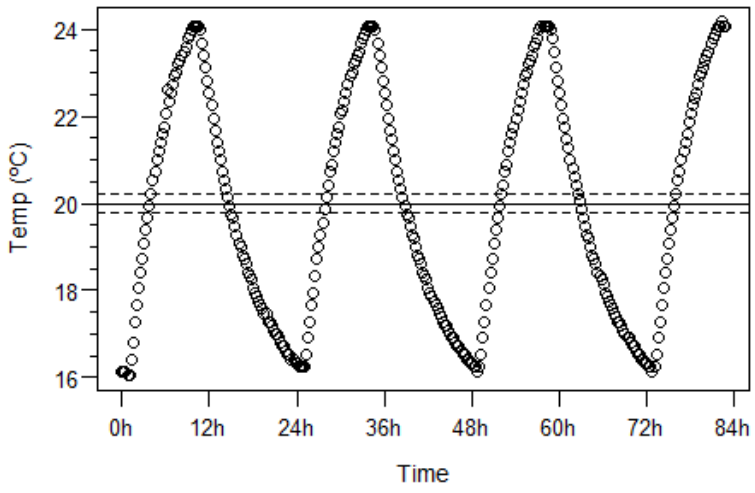


Fig.B.2) Temperature profile for treatments F30 and C24. Point line represents real temperature values from data loggers during the acclimation period. Solid and dashed lines denotes mean ± SD of the constants treatments.

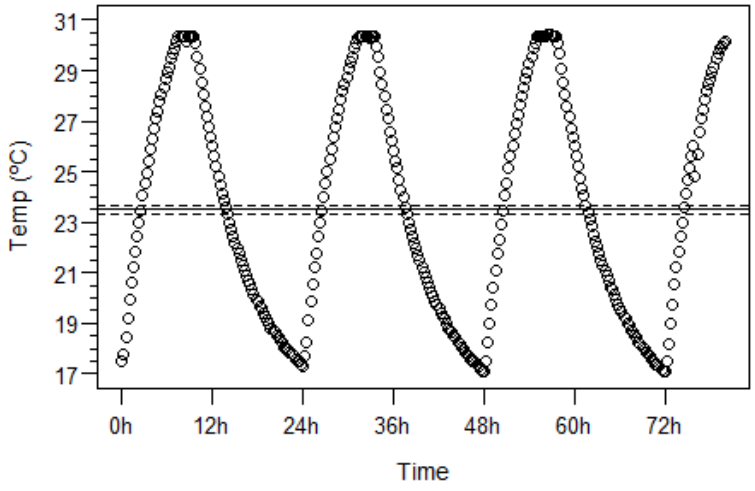
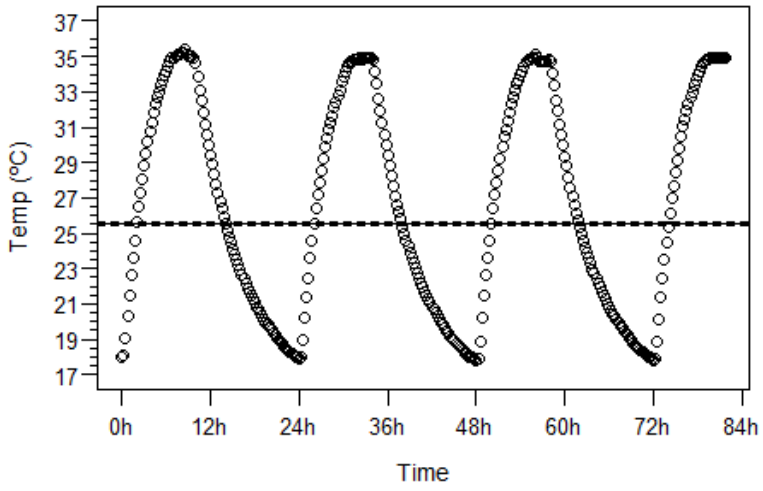


Fig.B.3) Temperature profile for treatments F35 and C25. Point line represents real temperature values from data loggers during the acclimation period. Solid and dashed lines denotes mean ± SD of the constants treatments.



TABLES

Table A1. Mean values for the effects for the constant and its equivalent DTF acclimation treatments on thermal limits and results of ANOVA tests. n sample size, $\bar{X}$ mean value, SE standard error. C denotes constant acclimation temperatures. Fuctuating acclimation (DTF): F24 (16-24 ºC), F30 (17-30ºC), F35 (17-35 ºC). The time of acclimation was 4 days except for (S) treatments that simulates only a single peak of hot. (M) indicates multiple peaks hot (4 days) in fluctuating treatments.

	Temperature of Acclimation																							
	C20			**F24**			**C23.5**			**F30S**			**F30M**			**C25**			**F35S**			**F35M**			**ANOVA**	
CT_{max}	n	$\bar{X}$	SE	n	$\bar{X}$	SE	n	$\bar{X}$	SE	n	$\bar{X}$	SE	n		SE	n	$\bar{X}$	SE	n	$\bar{X}$	SE	n	$\bar{X}$	SE		
Hyla meridionalis	14	39.9	0.1	12	40.1	0.1	14	40.3	0.1				12	40.5	0.1	14	40.6	0.1	15	40.3	0.1	14	41.1	0.1	$F_{6,81}$= 22.25	*P<0.01*
Pelodytes ibericus	16	36.9	0.1	16	36.9	0.1	16	37.4	0.1	16	36.9	0.1	16	37.2	0.1										$F_{4,74}$= 10.11	*P<0.01*
Rana temporaria	14	37.0	0.1	13	37.1	0.1	14	37.6	0.1	15	37.5	0.1	16	37.6	0.1										$F_{4,67}$= 5.8405	*P<0.01*
CT_{min}	n	$\bar{X}$	SE	n	$\bar{X}$	SE	n	$\bar{X}$	SE	n	$\bar{X}$	SE	n		SE	n	$\bar{X}$	SE	n	$\bar{X}$	SE	n	$\bar{X}$	SE		
Hyla meridionalis	14	0.8	0.1	14	0.4	0.1	13	0.5	0.2				14	0.6	0.1	13	0.7	0.2	15	0.3	0.1	14	0.7	0.2	$F_{6,90}$= 2.14	P= 0.06
Pelodytes ibericus	16	0.0	0.1	16	-0.5	0.1	16	0.2	0.1	16	0.1	0.1	16	0.1	0.1										$F_{4,75}$= 5.65	*P<0.01*
Rana temporaria	8	-1.3	0.2	12	-1.0	0.2	8	-0.4	0.3	14	-0.5	0.1	15	-0.4	0.1										$F_{4,52}$= 4.48	*P<0.01*

B. Results of Tukey post-hoc comparisons.

Table.B.1) Results of pairwise Tukey's post-hoc comparisons for CT_{max} in *P.ibericus.* In bold is highlighted the comparison between constant and variable acclimation treatment.

	Estimate	Std. Error	t value	Pr(>|t|)
C24 -C 20 == 0	0.49375	0.10361	4.765	< 1e-04***
F24 - C20 == 0	**-0.03125**	**0.10361**	**-0.302**	**0.99816**
F30M - C20 == 0	0.33958	0.10532	3.224	0.01566*
F30S - C20 == 0	0.04375	0.10361	0.422	0.99322
F24 - C24 == 0	-0.525	0.10361	-5.067	< 1e-04***
F30M - C24 == 0	**-0.15417**	**0.10532**	**-1.464**	**0.58891**
F30S - C24== 0	**-0.45**	**0.10361**	**-4.343**	**0.00041*** **
F30M – F24 == 0	0.37083	0.10532	3.521	0.00644**
F30S – F24 == 0	0.075	0.10361	0.724	0.95032
F30S – F30M == 0	-0.29583	0.10532	-2.809	0.04841*

Table.B. 2) Results of pairwise Tukey's post-hoc comparisons for CT_{max} in *R. temporaria*. In bold is highlighted the comparison between constant and variable acclimation treatment.

	Estimate	Std.error	t value	Pr(>|t|)
C24 - C20 == 0	0.55714	0.16193	3.441	0.00859**
F24 -C 20 == 0	**0.10989**	**0.16502**	**0.666**	**0.96295**
F30M - C20 == 0	0.60893	0.15679	3.884	0.00216**
F30S - C20 == 0	0.45143	0.15921	2.835	0.04617*
F24 - C24 == 0	-0.44725	0.16502	-2.71	0.06296
F30M - C24== 0	**0.05179**	**0.15679**	**0.33**	**0.99737**
F30S - C24 == 0	**-0.10571**	**0.15921**	**-0.664**	**0.96334**
F30M – F24 == 0	0.49904	0.15997	3.12	0.02174*
F30S – F24 == 0	0.34154	0.16235	2.104	0.23044
F30S – F30M == 0	-0.1575	0.15398	-1.023	0.84384

Table.B.3) Results of pairwise Tukey's post-hoc comparisons for CT_{max} in *H. meridionalis.* In bold is highlighted the comparison between constant and variable acclimation treatment.

	Estimate	Std.error	t value	Pr(>|t|)
C24 - C20 == 0	0.37857	0.11296	3.352	0.01998*
C25 - C20 == 0	0.72857	0.11757	6.197	< 0.001***
F24 - C20 == 0	**0.20635**	**0.12768**	**1.616**	**0.67144**
F30 - C20 == 0	0.5869	0.11757	4.992	< 0.001***
F35M - C20 == 0	1.24524	0.11757	10.592	< 0.001***
F35S - C20 == 0	0.43524	0.11106	3.919	0.0033**
C25 – C24 == 0	0.35	0.11757	2.977	0.05617
F24 – C24 == 0	-0.17222	0.12768	-1.349	0.82584
F30 – C24 == 0	**0.20833**	**0.11757**	**1.772**	**0.56934**
F35M - C24 == 0	0.86667	0.11757	7.372	< 0.001***
F35S – C24 == 0	0.05667	0.11106	0.51	0.99863
F24 - C25 == 0	-0.52222	0.13178	-3.963	0.00289**
F30 - C25 == 0	-0.14167	0.12201	-1.161	0.90598
F35M - C25 == 0	**0.51667**	**0.12201**	**4.235**	**0.00119****
F35S - C25 == 0	**-0.29333**	**0.11574**	**-2.534**	**0.16043**
F30- F24 == 0	0.38056	0.13178	2.888	0.07047
F35M – F24 == 0	1.03889	0.13178	7.883	< 0.001***
F35S – F24 == 0	0.22889	0.12601	1.816	0.53994
F35M – F30 == 0	0.65833	0.12201	5.396	< 0.001***
F35S – F30 == 0	-0.15167	0.11574	-1.31	0.84455
F35S – F35M == 0	-0.81	0.11574	-6.998	< 0.001***

Table.B.4) Results of pairwise Tukey's post-hoc comparisons for CT_{min} in *P.ibericus*. In bold is highlighted the comparison between constant and variable acclimation treatment.

	Estimate	Std. Error	t value	Pr(>|t|)
C24 - C20 == 0	0.16875	0.14762	1.143	0.783119
F24 - C20 == 0	**-0.4625**	**0.14762**	**-3.133**	**0.020247***
F30M - C20 == 0	0.09375	0.14762	0.635	0.968858
F30S - C20 == 0	0.03125	0.14762	0.212	0.999543
F24 – C24 == 0	-0.63125	0.14762	-4.276	0.000532***
F30M – C24 == 0	**-0.075**	**0.14762**	**-0.508**	**0.98633**
F30S - C24 == 0	**-0.1375**	**0.14762**	**-0.931**	**0.883794**
F30M – F24 == 0	0.55625	0.14762	3.768	0.002982**
F30S – F24 == 0	0.49375	0.14762	3.345	0.010976*
F30S – F30M == 0	-0.0625	0.14762	-0.423	0.993152

Table.B.5) Results of pairwise Tukey's post-hoc comparisons for CT_{min} in *R. temporaria*. In bold is highlighted the comparison between constant and variable acclimation treatment.

	Estimate	Std. Error	t value	Pr(>|t|)
C24 -C 20 == 0	0.925	0.30191	3.064	0.027*
F24 -C 20 == 0	**0.25833**	**0.27561**	**0.937**	**0.8798**
F30M -C 20 == 0	0.83214	0.26762	3.109	0.024*
F30S -C 20 == 0	0.80833	0.26435	3.058	0.0274*
F24 - C24 == 0	-0.66667	0.27561	-2.419	0.1251
F30M - C24 == 0	**-0.09286**	**0.26762**	**-0.347**	**0.9967**
F30S - C24 == 0	**-0.11667**	**0.26435**	**-0.441**	**0.9918**
F30M – F24 == 0	0.57381	0.23754	2.416	0.126
F30S – F24 == 0	0.55	0.23386	2.352	0.1438
F30S – F30M == 0	-0.02381	0.22439	-0.106	1

ANNEXE 6

Supporting Information for Chapter 6

ANNEXE 6SUPPLEMENTARY FIGURES

Figure S1. Maximum pond temperature distributions for four of the analyzed species. Dashed vertical lines denotes most limiting CTmax values (either metamorphs for *Pelodytes ibericus*,, *Hyla meridionalis* and *Rana temporaria*, or tadpoles for *Pelobates cultripes*) (see Table 1).

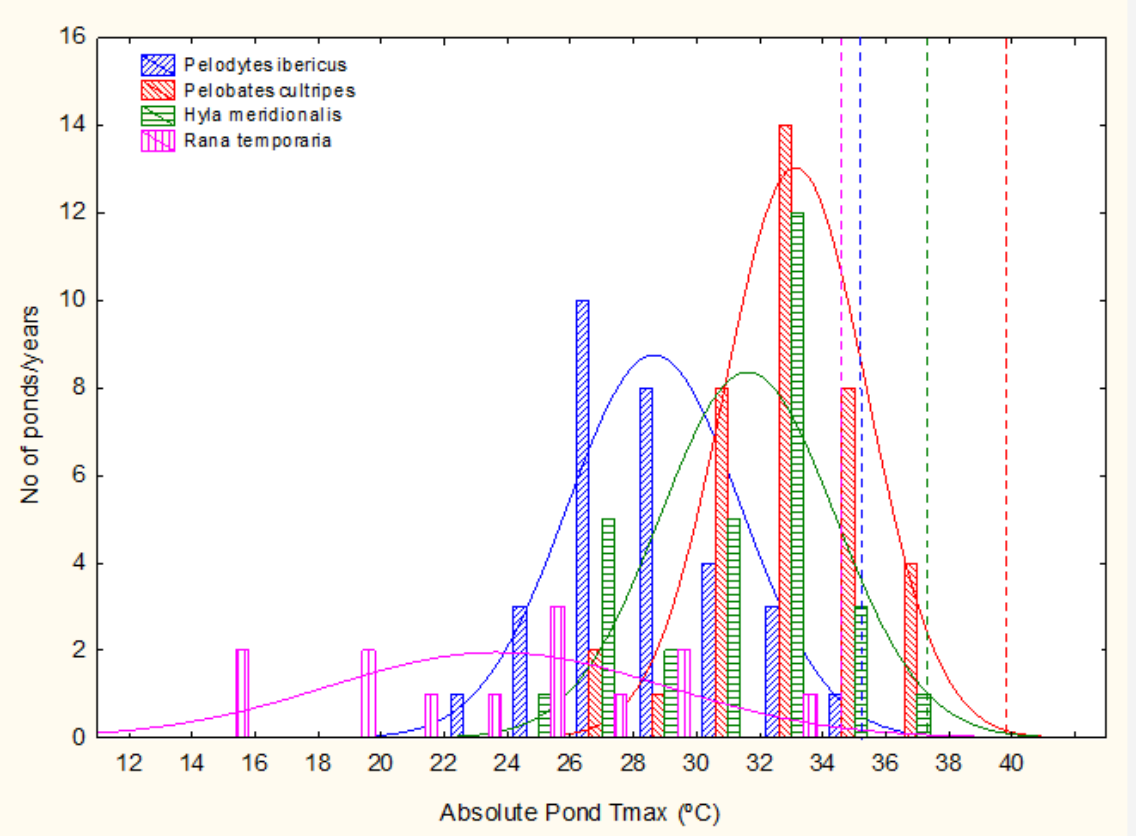
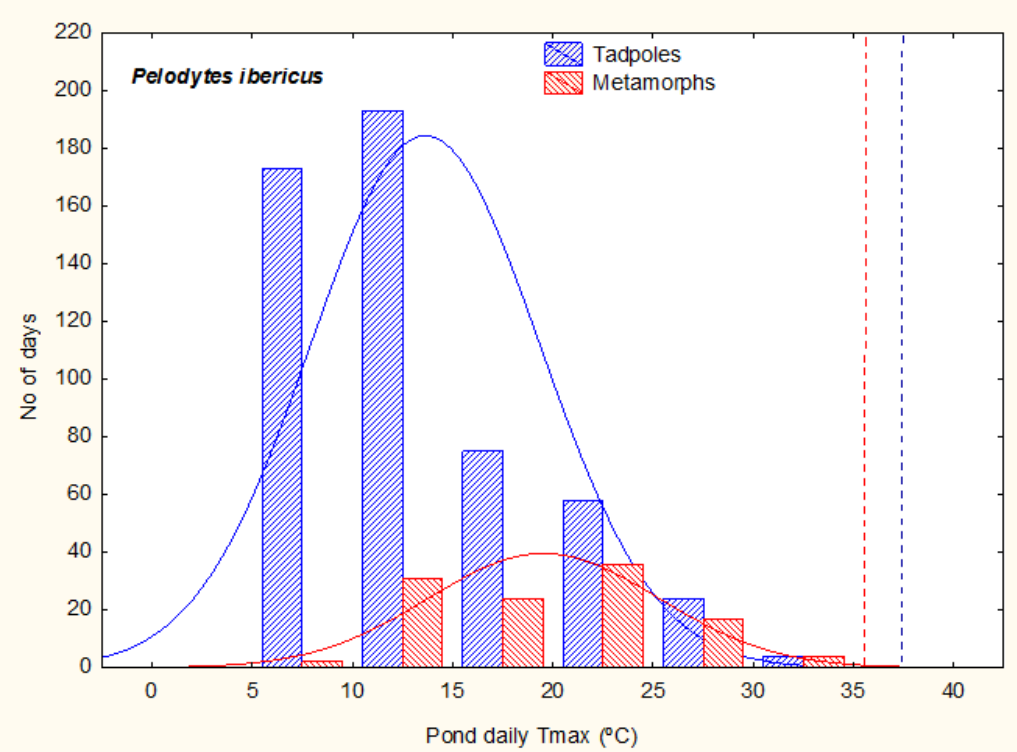


Figure S2. Contrasting patterns of daily maximum temperatures distribution (obtained by pooling all the sampled ponds) between tadpoles and metamorphs of *Pelodytes ibericus* (Up) and *Pelobates cultripes* (Down). Vertical dashed lines denote CTmax values for each developmental stage (see Table 1).



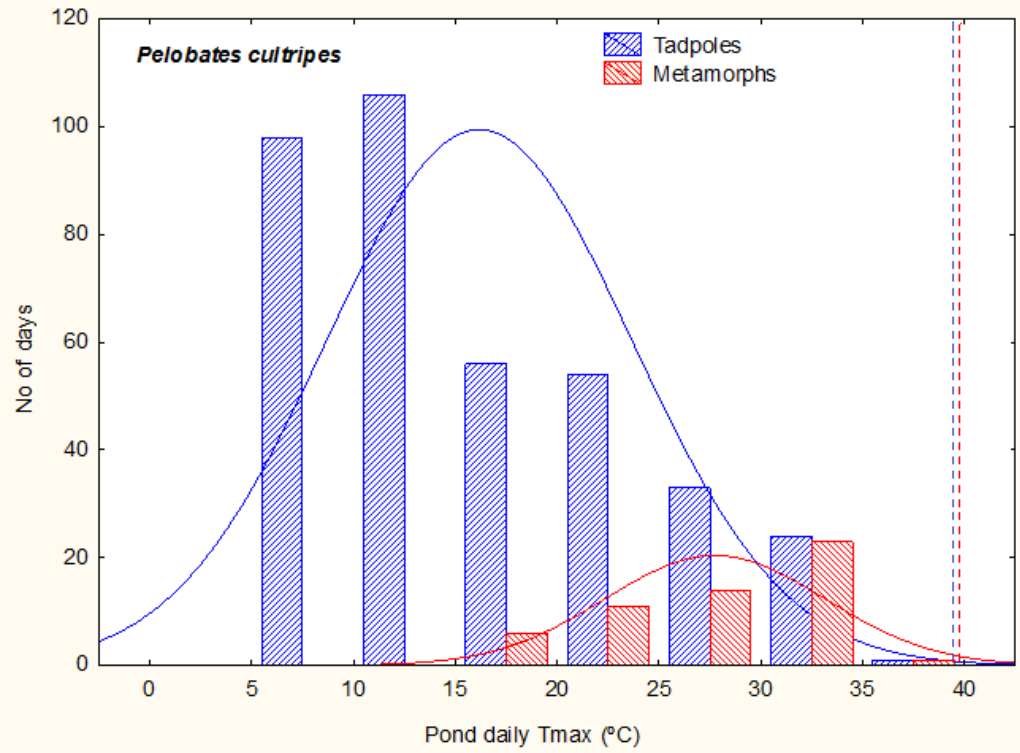
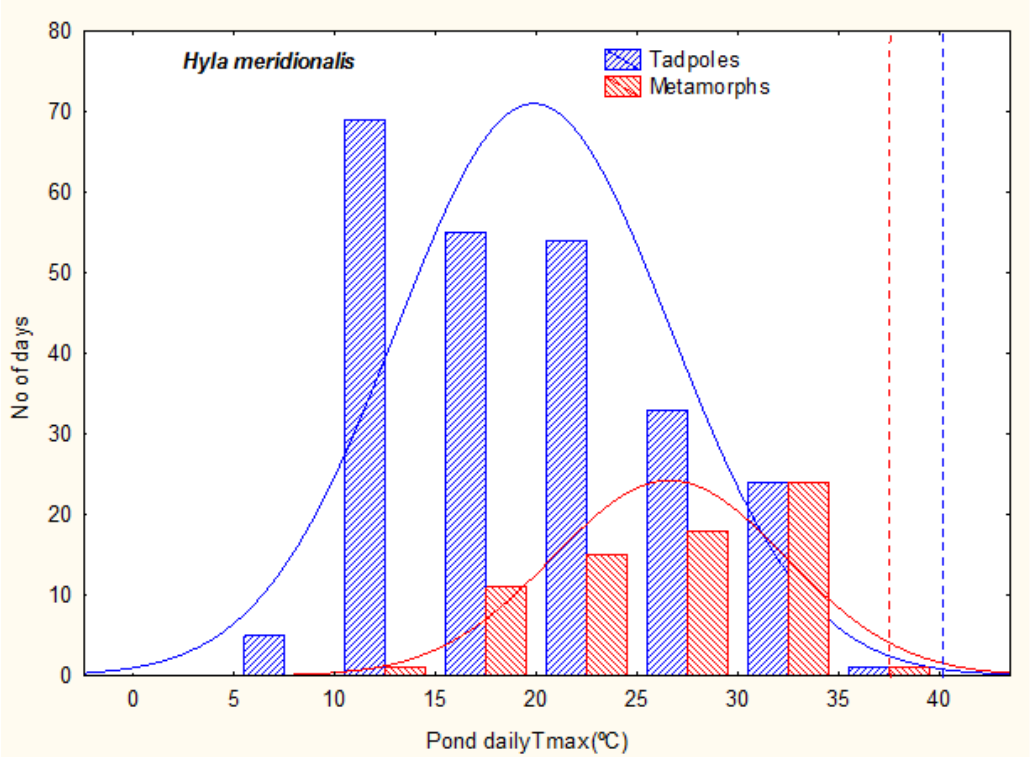
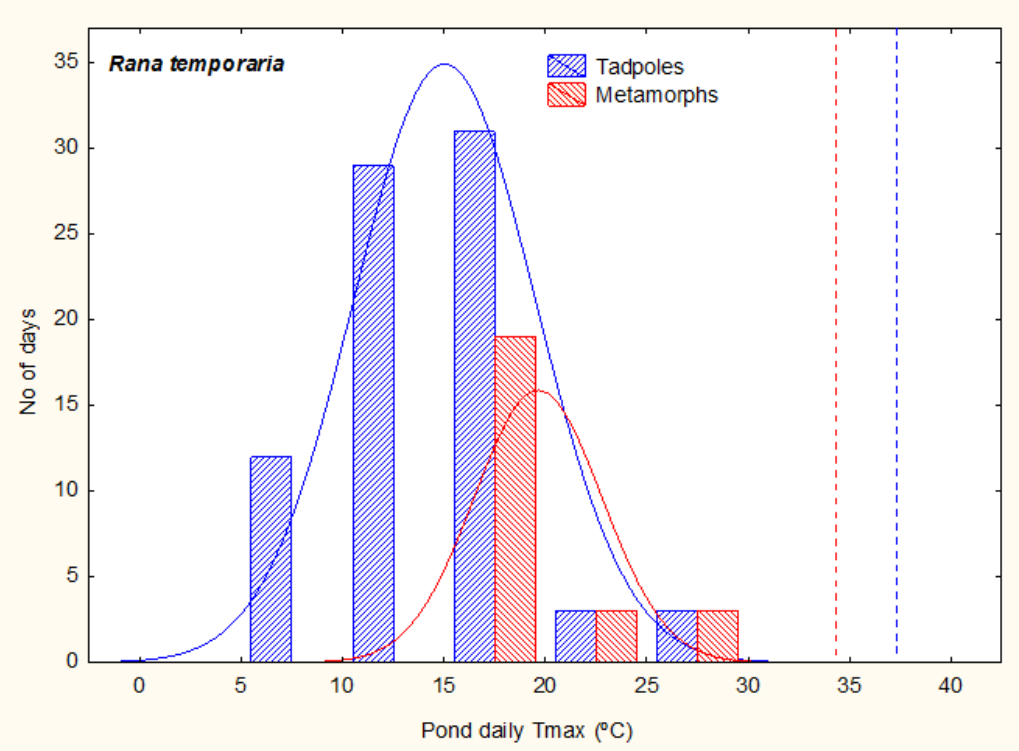


Figure S3. Contrasting patterns of maximum temperatures distribution, (obtained by pooling all the sampled ponds), between tadpoles and metamorphs of *Hyla meridionalis* (Up) and *Rana temporaria* (Down). Vertical dashed lines denote CTmax values for each particular stage (see Table 1).





SUPPLEMENTARY TABLES

Table S1. Summary of maximum pond temperature (Tmax) of monitored ponds.

Species	Tmax	Pond name	Year
Pelodytes ibericus	33.326	SURA	2009
Pelodytes ibericus	33.953	MAIN	2009
Pelodytes ibericus	27.862	SURA	2010
Pelodytes ibericus	26.692	C3	2010
Pelodytes ibericus	25.125	SURA	2011
Pelodytes ibericus	31.37	C3	2011
Pelodytes ibericus	26.39	MAIN	2011
Pelodytes ibericus	26.488	RASO	2011
Pelodytes ibericus	26.097	C3	2013
Pelodytes ibericus	26.585	RASO	2013
Pelodytes ibericus	26.683	TUMBA	2013
Pelodytes ibericus	28.555	SURA	2013
Pelodytes ibericus	30.154	SURA	2014
Pelodytes ibericus	28.953	NORTE	2014
Pelodytes ibericus	34.903	Z4	2009
Pelodytes ibericus	30.356	Z4	2010
Pelodytes ibericus	28.953	Z3	2011
Pelodytes ibericus	29.053	Z4	2011
Pelodytes ibericus	30.154	Z4B	2011
Pelodytes ibericus	29.452	MEANDRO	2010
Pelodytes ibericus	29.953	MEANDRO	2011
Pelodytes ibericus	25.319	CERCADO	2011
Pelodytes ibericus	29.053	MEANDRO	2013
Pelodytes ibericus	29.252	GRAZ	2010
Pelodytes ibericus	32.021	GRAZ	2011
Pelodytes ibericus	23.581	GRAZ	2013
Pelodytes ibericus	25.137	JEREZ	2010
Pelodytes ibericus	26.231	JEREZ	2011
Pelodytes ibericus	27.85	TREB	2010
Pelodytes ibericus	27.456	TREB	2011
Hyla meridionalis	33.326	SURA	2009
Hyla meridionalis	33.953	MAIN	2009
Hyla meridionalis	36.079	SURA	2010
Hyla meridionalis	27.961	C3	2010
Hyla meridionalis	32.394	MAIN	2010
Hyla meridionalis	25.805	JARAS	2010
Hyla meridionalis	35.222	SURA	2011
Hyla meridionalis	33.118	C3	2011
Hyla meridionalis	33.118	RASO	2011
Hyla meridionalis	27.862	JARAS	2011

Species	Tmax	Pond name	Year
Hyla meridionalis	31.983	SURA	2013
Hyla meridionalis	33.014	NORTE	2013
Hyla meridionalis	30.558	C3	2013
Hyla meridionalis	31.888	RASO	2013
Hyla meridionalis	27.37	EUCALIPTOS	2013
Hyla meridionalis	32.911	SURA	2014
Hyla meridionalis	33.535	NORTE	2014
Hyla meridionalis	33.43	JARAS	2015
Hyla meridionalis	34.903	Z4	2009
Hyla meridionalis	30.356	Z4	2010
Hyla meridionalis	32.497	Z3	2011
Hyla meridionalis	29.053	Z4	2011
Hyla meridionalis	30.154	Z4B	2011
Hyla meridionalis	27.862	ARROY ACI	2012
Hyla meridionalis	34.691	ARROY ACI	2015
Hyla meridionalis	32.086	Z3	2015
Hyla meridionalis	33.222	GRAZ	2010
Hyla meridionalis	28.357	BOD	2010
Hyla meridionalis	27.37	BOD2	2014
Rana temporaria	15.664	PURÓN	2013
Rana temporaria	18.55	COLOR	2009
Rana temporaria	22.49	COLOR	2010
Rana temporaria	25.708	VIANGO	2013
Rana temporaria	32.291	CORTEGUEROS	2013
Rana temporaria	18.806	FANA	2014
Rana temporaria	15.996	PANDECARMEN	2014
Rana temporaria	21.885	PANDÉBANO	2014
Rana temporaria	24.48	ALIVA	2002
Rana temporaria	28.953	SEÑALES	2013
Rana temporaria	24.91	LLAGUSECU	2008
Rana temporaria	29.652	LLAGUSECU	2009
Rana temporaria	27.468	LLAGUSECU	2010
Pelobates cultripes	33.326	SURA	2009
Pelobates cultripes	33.953	MAIN	2009
Pelobates cultripes	36.079	SURA	2010
Pelobates cultripes	27.961	C3	2010
Pelobates cultripes	32.394	MAIN	2010
Pelobates cultripes	35.222	SURA	2011
Pelobates cultripes	33.118	C3-MAIN	2011
Pelobates cultripes	33.118	RASO	2011
Pelobates cultripes	30.558	C3	2013
Pelobates cultripes	31.888	RASO	2013
Pelobates cultripes	31.983	SURA	2013
Pelobates cultripes	33.014	NORTE	2013

Species	Tmax	Pond name	Year
Pelobates cultripes	32.911	SURA	2014
Pelobates cultripes	33.535	NORTE	2014
Pelobates cultripes	34.903	Z4	2009
Pelobates cultripes	33.953	LLANO1	2009
Pelobates cultripes	33.743	Z1-centro	2009
Pelobates cultripes	34.903	Z4	2009
Pelobates cultripes	33.639	LLANO1	2010
Pelobates cultripes	34.479	Z1	2010
Pelobates cultripes	30.356	Z4	2010
Pelobates cultripes	35.435	LLANO1	2011
Pelobates cultripes	36.62	LLANO2	2011
Pelobates cultripes	30.457	Z1	2011
Pelobates cultripes	32.188	Z2	2011
Pelobates cultripes	32.497	Z3	2011
Pelobates cultripes	29.053	Z4	2011
Pelobates cultripes	30.154	Z4B	2011
Pelobates cultripes	36.403	LLANO2	2012
Pelobates cultripes	27.862	ARROY ACI	2012
Pelobates cultripes	35.328	LLANO2	2013
Pelobates cultripes	31.88	LLANO2	2014
Pelobates cultripes	34.691	ARROY ACI	2015
Pelobates cultripes	32.086	Z3	2015
Pelobates cultripes	31.166	LLANO2	2015
Pelobates cultripes	34.903	Z1	2015
Pelobates cultripes	36.295	Z2	2015
Bufo bufo	22.537	Lag Grande	2006
Bufo bufo	21.951	Lag. Los Pájaros	2013
Bufo bufo	22.705	Lag. Los Pájaros	2006
Bufo bufo	25.125	Charca Pilar Cabra	2010
Bufo bufo	22.046	Charca Pilar Cabra	2013
Bufo bufo	23.84	Color Cuneta	2004

Table S2. Summary dataset of mean, standard error and sample size of upper thermal tolerances (CTmax, ºC) estimates across ontogeny in amphibians. d+, var, means and variances Hedges' *d*+ estimate of effect sizes, obtained with MetaWin 2.1 (Rosenberg et al. 2000) that allow to contrast ontogenetic divergences between tadpole, metamorphs and juveniles of reported species

	TADPOLE			METAMORPH			JUVENILE			TAD vs MET		TAD vs JUV		JUV vs MET	
SPECIES	**MEAN**	**SD**	**N**	**MEAN**	**SD**	**N**	**MEAN**	**SD**	**N**	**d+**	**var**	**d+**	**var**	**d+**	**var**
Ambystoma tigrinum Delson & Whitford 1973	38.081	0.1976	10	36.598	0.7335	8	37.254	0.4593	10	-2.7841	0.4403	-2.2403	0.3255	1.05	0.2556
Barbarophryne brongersmai This study	40.087	0.3030	16	37.700	0.7091	15	37.820	0.7389	15	-4.3159	0.4296	-3.9594	0.382	0.1612	0.1338
Bufo americanus Cupp 1980	40.730	0.3335	10	38.770	0.8010	7	39.320	0.5067	4	-3.2714	0.5576	-3.4358	0.7716	0.7019	0.4152
Bufo americanus Sherman & Levitis 2003	40.731	0.2448	18	39.307	0.9608	10	39.807	0.2660	7	-2.3083	0.2507	-3.567	0.4529	0.622	0.2542
Bufo bufo This study	37.887	0.2655	16	35.869	1.0678	23	36.667	0.7970	15	-2.3515	0.1769	-2.0297	0.1956	0.8032	0.1186
Bufo marinus Krakauer 1970	42.470	0.0848	72				39.950	1.0730	20						
Bufo marinus Floyd 1983	42.300	0.1186	10	37.900	0.1785	10				-27.8086	19.5329				
Bufo terrestris Noland & Ultsch 1981	39.896	0.4650	13	39.140	0.5260	18				-1.4677	0.1672				
Bufo woodhousei Cupp 1980	41.140	0.2586	10	40.130	0.5691	10	40.150	0.3232	10	-2.1885	0.3197	-3.2395	0.4624	0.0414	0.2

	TADPOLE			METAMORPH			JUVENILE			TAD vs MET		TAD vs JUV		JUV vs MET	
SPECIES	**MEAN**	**SD**	**N**	**MEAN**	**SD**	**N**	**MEAN**	**SD**	**N**	**d+**	**var**	**d+**	**var**	**d+**	**var**
Bufo woodhousei fowleri Sherman 1980	42,880	0,4210	20	37,235	2,6306	5	37,788	1,1410	12	-4,6984	0,6915	-6,4642	0,7862	0,3137	0,2862
Gastrophryne carolinensis Cupp 1980	42,32	0,2727	8	38,803	0,5151	10	38,710	0,4848	3	-7,8561	1,9394	-9,9485	4,9571	-0,1697	0,4344
Hyla meridionalis This study	40,069	0,3219	16	37,467	1,1717	18	37,500	0,8414	11	-2,8805	0,2401	-4,2385	0,4861	0,0305	0,1465
Pelobates cultripes This study	39,610	0,5782	10	39,825	0,5658	12	40,431	0,8467	16	0,3619	0,1863	1,0502	0,1837	0,7942	0,1571
Pelodytes ibericus This study	36,894	0,2265	16	35,360	0,7346	15	35,136	1,0595	14	-2,7879	0,2545	-2,3094	0,2228	-0,2407	0,1391
Pseudacris triseriata Cupp 1980	38,730	0,2971	10	37,820	0,3823	10	38,060	0,3820	10	-2,5457	0,362	-1,8752	0,2879	0,6015	0,209
Rana catesbeiana Menke & Claussen 1982	39,450	0,3356	8	37,930	0,4446	2	39,260	0,6124	8	-3,9105	1,3896	-0,3638	0,2541	2,0223	0,8295
Rana pipiens Noland & Ultsch 1981	38,014	0,4957	13	37,507	0,5850	17				-0,8993	0,1492				
Rana sylvatica Cupp 1980	38,160	0,3364	10	37,490	0,5889	10	36,750	0,5887	10	-1,3381	0,2448	-2,8166	0,3983	-1,2037	0,2362
Rana temporaria This study	37,028	0,3730	14	34,668	1,3494	16	33,758	1,8961	12	-2,2513	0,2184	-2,4126	0,2667	-0,5512	0,1513
Xenopus laevis Sherman & Levitis 2003	37,627	0,3530	13	35,509	0,4350	9	37,235	0,3390	12	-5,253	0,8152	-1,0944	0,1842	4,3334	0,6415

Table S3. Hedges' *d* effect sizes summary analysis (E+) for different contrasts. Bias CI: bias-corrected 95% bootstrap confidence intervals. Negative values of effect sizes indicate that the CTmax decreases at the metamorphosis. Effect sizes are considered significant if 95% confidence intervals did not overlap with zero (both Tadpole vs Metamorph .and Tadpole vs Juveniles, but not Juvenile vs Metamorph)

Contrast	No. of studies	E+	df	Bias CI
TADPOLE vs METAMORPH	19	-2,1704	18	-2,8271 to -1,5446
TADPOLE vs JUVENILE	17	-2,3245	16	-3,2776 to -1,3649
JUVENILE vs METAMORPH	16	-0,3169	15	-0,7004 to 0,0176

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