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Integrating ecophysiology, complex life histories, and niche models to forecast the impacts of climate change:

A mechanistic assessment of the vulnerability of European common frogs (*Rana temporaria*)

Integrando ecofisiología, historias de vida complejas y modelos de nicho para pronosticar los impactos del cambio climático:

Una evaluación mecanicista de la vulnerabilidad de la rana bermeja (*Rana temporaria*)

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Urtzi Enriquez-Urzelai

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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2.- Autor	
Nombre: Eder Urtzi Enríquez Urcelay	DNI/Pasaporte/NIE:
Programa de Doctorado: Biogeociencias	
Órgano responsable: Universidad de Oviedo	

RESUMEN (en español)

El clima de la Tierra está cambiando a tasas sin precedentes, amenazando la biodiversidad. En este contexto, es de vital importancia evaluar la vulnerabilidad de especies y poblaciones, lo que depende de su exposición al cambio, su sensibilidad intrínseca (estrechamente relacionado con la tolerancia fisiológica de las especies), y su capacidad de respuesta incluyendo la adaptación (p.ej. evolución del nicho) y respuestas plásticas (tanto fisiológicas como comportamentales). Si estos mecanismos se quedan cortos y los individuos no son capaces de dispersar a localidades con condiciones climáticas favorables, las poblaciones se extinguirían. Además, los ciclos de vida complejos suponen otro reto ya que distintos estadios del ciclo de vida podrían ser desproporcionadamente vulnerables a la extinción. Desgraciadamente, la mayoría de los intentos para predecir las consecuencias del cambio climático han ignorado cómo la capacidad de respuesta y vulnerabilidad cambia a lo largo de la ontogenia. En esta tesis, adopto una aproximación multidisciplinar para pronosticar el impacto del cambio climático en un anfibio: la rana bermeja (*Rana temporaria*). Específicamente, exploro la información que proveen las distribuciones actuales de los anfibios europeos, y, además, combino experimentos ecofisiológicos realizados sobre distintos estadios de *R. temporaria* (i.e. renacuajos, juveniles y adultos) que originan de poblaciones a lo largo de gradientes ambientales con modelado correlativo y mecanicista de distribución de especies. Aunque la adaptación a condiciones ambientales nuevas (i.e. evolución de nicho) parece relativamente común al nivel de especie, a nivel poblacional, la evolución del nicho térmico parece limitado, especialmente en juveniles y adultos. Estos resultados sugieren que el ritmo de evolución podría ser demasiado lento para contrarrestar el cambio climático. Por otro lado, la aclimatación a temperaturas cálidas modificó la tolerancia térmica de renacuajos y juveniles de *R. temporaria*. Sin embargo, la exposición temprana a altas temperaturas (i.e. durante la fase de renacuajo) no tuvo efectos arrastrados en fases posteriores. En lugar de eso, resulto en juveniles más pequeños, lo que podría reducir su supervivencia hasta la madurez. Probablemente, la aclimatación del desarrollo no va a beneficiar a esta especie de anfibio. Es más, el cambio fenológico conseguido mediante la aclimatación a corto plazo fue menor que los incrementos de temperatura esperados. Alternativamente, los modelos mecanicistas de nicho revelaron que los ajustes comportamentales (termorregulación en este caso) son clave en *R. temporaria* para persistir en áreas con distintos climas, a lo largo de su distribución. Por un lado, la termorregulación comportamental podría ser la responsable de la desdeñable divergencia en nichos térmicos observados en juveniles y adultos – en el caso de los renacuajos, limitaciones para termorregular en charcas someras podría favorecer la adaptación local en ese estadio. Por otro lado, la termorregulación será esencial para protegerse de los futuros incrementos de las temperaturas. Integramdo rasgos funcionales específicos de cada estadio del ciclo de vida, modelos mecanicistas de nicho y modelos correlativos de distribución de especies, demostramos que los renacuajos son el estadio climáticamente más sensible y que, en combinación con las temperaturas máximas de las charcas subyacen a la distribución geográfica de *R. temporaria*. Los pronósticos de la distribución de *R. temporaria* bajo climas en cambio arrojaron extinciones extensivas en el sur de Europa, donde alberga la mayor parte de la diversidad genética de esta especie. Por lo tanto, los planes de conservación podrían incorporar medidas dirigidas a asegurar que los puntos de aguas garantizan condiciones térmicas favorables para renacuajos de *R. temporaria*.

RESUMEN (en Inglés)

Earth's climate is changing at unprecedented rates, threatening biodiversity. In this context, it is of uppermost importance to assess the vulnerability of species and populations, which depends on the exposure to change, the intrinsic sensitivity (tightly linked to species physiological tolerances), and the response capacity including adaptation (e.g. niche evolution) and plastic responses (either physiological or behavioral). If these mechanisms fall short and individuals are not able to disperse to locations with suitable climatic conditions, populations would go extinct. Furthermore, complex life histories pose another challenge since different lifecycle stages may be disproportionately vulnerable to extinction. Unfortunately, most attempts to predict the impacts of climate change largely ignore response capacities and how vulnerability changes through ontogeny. In this thesis, I embrace a multidisciplinary approach to forecast the impacts of climate change on a temperate amphibian: the European common frog (*Rana temporaria*). Specifically, I explored the information that European amphibians' current distributions provide, and further, I combined targeted ecophysiological experiments on different lifecycle stages of *R. temporaria* (i.e. tadpoles, juveniles, and adults) originating from populations along environmental gradients, with mechanistic and correlative species distribution modelling. Although adaptation to novel environmental conditions (i.e. niche evolution) seemed relatively widespread at the species level, at the population level, thermal niche evolution seemed limited, especially for juveniles and adults. These results suggest that the pace of niche evolution may be too slow to counteract climate change. Besides, acclimation to warm temperatures modified the thermal tolerance of tadpole and juvenile *R. temporaria*. However, early exposure to warm temperatures (i.e. at the tadpole stage) did not carry-over to later stages, but rather, it resulted in smaller juveniles which may have a reduced survival to maturity. Thus, developmental acclimation is not likely to benefit this amphibian species. Additionally, the phenotypic change produced by 'short-term' acclimation is lower than expected temperature increases. Alternatively, mechanistic niche models revealed that behavioral adjustments (thermoregulation in this case) are key for *R. temporaria* to cope with contrasting climates across its distributions. On the one hand, behavioral thermoregulation may be responsible for the negligible divergence in thermal niches observed at the juvenile and adult stages – in the case of tadpoles, a more limited opportunity for behavioral thermoregulation in shallow ponds may promote local adaptation. On the other hand, I show that thermoregulation will be essential to buffer future air temperature rises. By integrating stage-specific functional traits, mechanistic niche models, and correlative species distribution models, we demonstrated that tadpole stages are the most climatically sensitive stage and that in combination with maximum pond temperatures may underpin the geographic distribution limits of *R. temporaria*. Forecasts of the distribution of *R. temporaria* under changing climates yielded extensive local extinctions in southern Europe, where most of the genetic diversity of this species is to be found. Hence, conservation plans could incorporate directed measurements to ensure thermally suitable water bodies for tadpole *R. temporaria*.

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La investigación presentada en esta Tesis Doctoral se ha desarrollado en el Departamento de Biología de Organismos y Sistemas de la Universidad de Oviedo y la Unidad Mixta de Investigación en Biodiversidad (UMIB, UO-CSIC-PA). Esta tesis fue financiada por el proyecto CGL2012-40246-C02-02, que incluía una beca predoctoral de Formación de Personal Investigador (ref: BES-2013-063203) del ministerio de Economía y Competitividad (MINECO). Además, fue financiada con una ayuda para estancias breves dentro del programa estatal de movilidad (ref: EEBB-I-17-12497), realizada en la University of Melbourne (School of BioSciences).

We just can't seem to stop burning up all those buried trees from way back in the carboniferous age, in the form of coal, and the remains of ancient plankton, in the form of oil and gas. If we could, we'd be home free climate wise. Instead, we're dumping carbon dioxide into the atmosphere at a rate the Earth hasn't seen since the great climate catastrophes of the past, the ones that led to mass extinctions. We just can't seem to break our addiction to the kinds of fuel that will bring back a climate last seen by the dinosaurs, a climate that will drown our coastal cities and wreak havoc on the environment and our ability to feed ourselves. All the while, the glorious sun pours immaculate free energy down upon us, more than we will ever need. Why can't we summon the ingenuity and courage of the generations that came before us? The dinosaurs never saw that asteroid coming. What's our excuse?

Neil deGrasse Tyson – Cosmos: A Spacetime Odyssey 2014

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ABSTRACT

Earth's climate is changing at unprecedented rates, threatening biodiversity. In this context, it is of uppermost importance to assess the vulnerability of species and populations, which depends on the exposure to change, the intrinsic sensitivity (tightly linked to species physiological tolerances), and the response capacity including adaptation (e.g. niche evolution) and plastic responses (either physiological or behavioral). If these mechanisms fall short and individuals are not able to disperse to locations with suitable climatic conditions, populations would go extinct. Furthermore, complex life histories pose another challenge since different lifecycle stages may be disproportionately vulnerable to extinction. Unfortunately, most attempts to predict the impacts of climate change largely ignore response capacities and how vulnerability changes through ontogeny. In this thesis, I embrace a multidisciplinary approach to forecast the impacts of climate change on a temperate amphibian: the European common frog (*Rana temporaria*). Specifically, I explored the information that European amphibians' current distributions provide, and further, I combined targeted ecophysiological experiments on different lifecycle stages of *R. temporaria* (i.e. tadpoles, juveniles, and adults) originating from populations along environmental gradients, with mechanistic and correlative species distribution modelling. Although adaptation to novel environmental conditions (i.e. niche evolution) seemed relatively widespread at the species level, at the population level, thermal niche evolution seemed limited, especially for juveniles and adults. These results suggest that the pace of niche evolution may be too slow to counteract climate change. Besides, acclimation to warm temperatures modified the thermal tolerance of tadpole and juvenile *R. temporaria*. However, early exposure to warm temperatures (i.e. at the tadpole stage) did not carry-over to later stages, but rather, it resulted in smaller juveniles which may have a reduced survival to maturity. Thus, developmental acclimation is not likely to benefit this amphibian species. Additionally, the phenotypic change produced by 'short-term' acclimation is lower than expected temperature increases. Alternatively, mechanistic niche models revealed that behavioral adjustments (thermoregulation in this case) are key for *R. temporaria* to cope with contrasting climates across its distributions. On the one hand, behavioral thermoregulation may be responsible for the negligible divergence in thermal niches observed at the juvenile and adult stages – in the case of tadpoles, a more limited opportunity for behavioral thermoregulation in shallow ponds may promote local adaptation. On the other hand, I show that thermoregulation will be essential to buffer future air temperature rises. By integrating stage-specific functional traits, mechanistic niche models, and correlative species distribution models, we demonstrated that tadpole stages are the most climatically sensitive stage and that in combination with maximum pond temperatures may underpin the geographic distribution limits of *R. temporaria*. Forecasts of the distribution of *R. temporaria* under changing climates yielded extensive local extinctions in southern Europe, where most of the genetic diversity of this species is to be found. Hence, conservation plans could incorporate directed measurements to ensure thermally suitable water bodies for tadpole *R. temporaria*.

GENERAL INTRODUCTION

Earth's climate is changing at unprecedented rates, threatening biodiversity, ecosystem functioning, and even human well-being (Williams et al., 2008; Pecl et al., 2017). Most of our activities, directly or indirectly, involve the emission of greenhouse gases into the atmosphere, which prevent heat from radiating towards space. As a consequence, the atmosphere and oceans have warmed, sea level has risen, and snow and ice have contracted (IPCC5; Walther et al., 2002; Karl & Trenberth, 2003). All life forms on Earth are already responding to these profound environmental changes, and numerous studies have reported changes in phenology (e.g. advancing the breeding or flowering) and species' interactions (Beebee, 1995; Visser & Both, 2005). Yet, changes in species' distributions stand out among the observed responses to recent climate change (Parmesan, 2006; Tingley et al., 2009; Moreno-Rueda et al., 2011). Most species' distributions are shifting towards cooler regions: poleward and to higher altitudes on land, and poleward and to greater depths on water. Such is the generality of this observation, that some authors consider that a universal biodiversity redistribution is underway (Sunday et al., 2012; Pecl et al., 2017). Thus, a deeper understanding of the underpinnings of species' distributions will be essential if we are to predict the likely impacts of climate change.

The niche is a recurrent concept central to modern evolutionary biology, ecology, and biogeography, which relates species to their distributions (Kearney et al., 2010a; Wiens, 2011). Over the last century, it remained a vague concept, most often referring to the 'place' a species occupies in its ecosystem; i.e. a property of the environment. In the late 50s, G. Evelyn Hutchinson reasoned that the niche is a property of populations or species, and his revolutionary view of the niche opened new opportunities to explore associations between niches and species' distributions. With that, he prepared the ground to develop ways with which to predict species' distributions under different environmental conditions. Due to current threats to biodiversity, especially climate change, the Hutchinsonian niche and its many applications remain more topical than ever (Hutchinson, 1957; Colwell, 1992; Pulliam, 2000; Kearney & Porter, 2009; Araújo et al., 2013).

Hutchinson formally defined the niche as a n-dimensional hypervolume in multidimensional environmental space, delimited by species' tolerances along each dimension. All the combinations of the environmental space that fall within that hypervolume would allow the persistence of stable populations without immigration – the so-called fundamental or physiological niche (Soberón & Nakamura, 2009; Kearney et al., 2010a). However, species rarely occupy all the places on Earth that could sustain its populations. Some areas will be inaccessible due to dispersal limitations, either internal (e.g. low locomotor capacity) or external (e.g. configuration of land masses). In other areas, species may be unable to persist due to the effect of biotic interaction with competitors, predators, or pathogens. Hence, species occupy areas with suitable abiotic and biotic conditions that, moreover, have been able to colonize through dispersal – this subset of the fundamental niche is often referred to as the realized niche (Hutchinson, 1957; Soberón, 2007). Presumably, while biotic interactions play a key role at the fine-scale in setting species' occupancies, abiotic (e.g. climatic) constraints on the fundamental niche – mainly mediated by species' physiology – may govern the broader limits of species' distributions (Soberón & Nakamura, 2009; Sunday et al., 2012; Araújo et al., 2013; but see Gotelli et al., 2010).

To predict the impacts of climate change on species' distributions, several authors have proposed an integrative framework (**Fig. I.1A**). According to this framework, the vulnerability (or probability of extinction) of any given species or population will depend on its exposure to climate change, intrinsic sensitivity, and response capacity (Williams et al., 2008; Nadeau et al., 2017). If local climatic conditions change (*exposure*) to the point that they fall outside the niche of a population (*sensitivity*), individuals may *respond* either dispersing to locations that hold suitable conditions or in situ through adaptation to novel environmental conditions (e.g. niche evolution), phenotypic plasticity, and behavioral responses (Chevin et al., 2010; Dawson et al., 2011; Moritz & Agudo, 2013). Thus, to assess the vulnerability of species or populations, we need robust measurements of the exposure to climate change at scales relevant to the individual, the climatic limitations of species, directly related to the niche, and their capacity to respond to changing climates, related but not limited to the niche (Kearney et al., 2012; Moritz & Agudo, 2013). All these aspects are challenging.

Most species have complex life histories and, thus, exposure, sensitivity, and response capacity may change during the life-span of individuals (**Fig. I.1B**; Kingsolver et al., 2011; Briscoe et al., 2012; MacLean et al., 2016). In species with complex lifecycles, which represent the vast majority of species, each lifecycle stage (or ontogenetic stage) inhabits different microhabitats, with specific microclimates and climate extremes (Wilbur, 1980). Furthermore, different stages may also vary in their ability to move. For instance, while egg

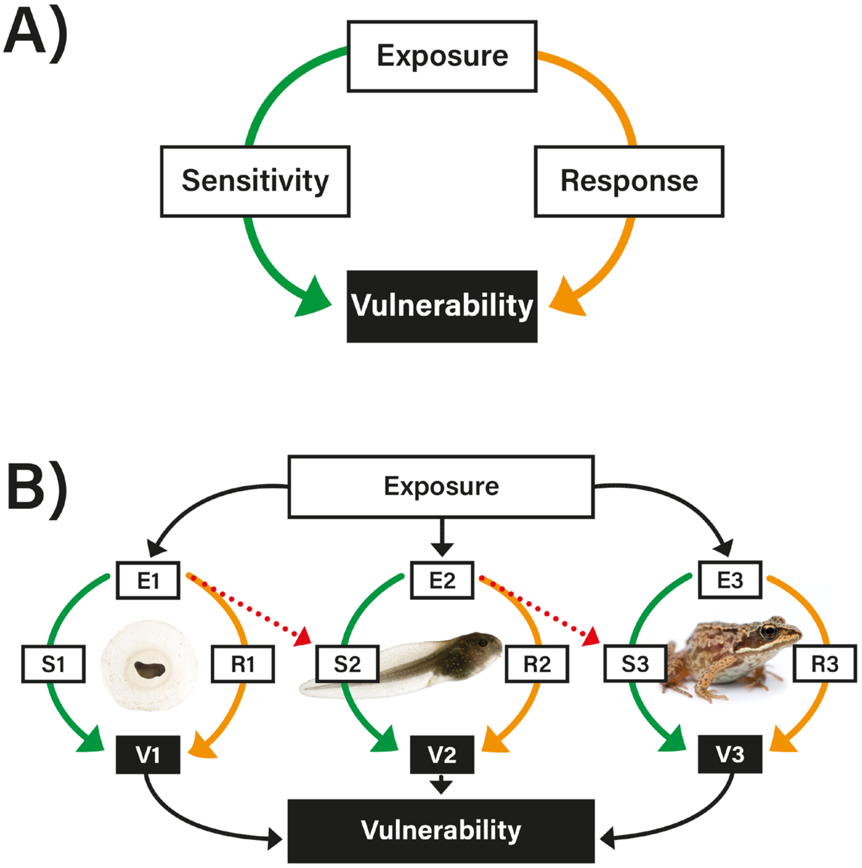

Figure I.1: **(A)** The vulnerability framework proposed by several authors, where exposure, sensitivity, and response capacity are taken into account (modified from Williams et al 2008; Moritz and Agudo 2013). And **(B)** the extended vulnerability framework embraced in this thesis. Exposure is expected to impact on the microclimates of all lifecycle stages, which may be differentially sensitive and responsive. Thus different stages may be disproportionatelly vulnerable and partially determine the whole species' vulnerability (see main text).

stages are sessile, most adult individuals are vagile. To a large extent, this determines the ability of each stage to behaviorally avoid unwanted conditions. Altogether, this suggests that different stages may encounter different (micro)climatic challenges and, as expected, different stages of many species show specific physiological limits (Ragland & Kingsolver, 2008; Potter et al., 2010; Pincebourde & Casas, 2015). The vulnerability of each lifecycle stage will vary to the degree that specific microclimates change due to climate change, stage-specific physiological limits, and their capacity to respond (including behavioral thermo- or hydroregulation). In turn, the vulnerability of any given population of a species may depend on the vulnerability of particular lifecycle stages (Radchuk et al., 2012; McDermott Long et al., 2016).

Furthermore, the (micro)climatic conditions encountered during early stages of ontogeny can condition later stages, via the co-called 'carry-over' effects, which can have profound effects on population dynamics, ultimately, affecting the long-term persistence of populations (**Fig. I.1B**; Pechenik, 2006; Braña & Ji, 2007; Gomez-Mestre et al., 2010; Tejedo et al., 2010). Life history transitions involve a plethora of morphological, physiological, and behavioral changes, oftentimes accompanied by shifts in (micro)habitats (Wilbur, 1980). However, even in the most extreme transitions (e.g. from larvae to juveniles), each lifecycle stage may not be completely 'autonomous' (Podolsky & Moran, 2006). For instance, temperatures during early stages impact on the timing of lifecycle transitions by altering growth and developmental rates, and may even shape the morphology, physiological limits (e.g. thermal tolerance), and survival of later stages (Podolsky & Moran, 2006; Levy et al., 2015; Kellermann et al., 2017). In the context of climate change, increased temperatures during early life could increase the thermal tolerance of individuals, that if carried over to later stages, could confer resistance to heat extremes (Beaman et al., 2016). However, smaller sizes at transitions, commonplace at high temperatures, would result in lower survival to maturity due to a reduced ability to forage and escape predators (Berven, 1981; Smith, 1987). Including these effects could utterly change our predictions on the impacts of climate change (Levy et al., 2015; Carlo et al., 2017). Accordingly, in addition to the actual impacts of climate change upon each lifecycle stage, we need to get insights into how exposure at early stages may influence later stages.

Most attempts to foresee the impacts of climate change, however, rely on 'correlational' descriptions of species' niches, rather than on an understanding of the physiology, morphology, and behavior during the ontogeny of species. By linking widely available fine-resolution global climate and terrain maps and georeferenced species' occurrences (sometimes absences), ecologists have been able to 'empirically' quantify realized niches (Guisan & Thuiller, 2005; Elith et al., 2006; Pacifici et al., 2015). Some studies simply extract the environmental conditions of the locations a species occupies (Olalla-Tárraga et al., 2011; Quintero & Wiens, 2013). Alternatively, most approaches embrace correlative species distribution models (correlative SDMs) to seek for statistical relationships between occurrences and environmental layers. Then, these SDMs can be projected onto current and future climates to predict changes in species' distributions (Elith & Leathwick, 2009; Thuiller et al., 2011; Broennimann et al., 2012). In addition, these niche estimates together with large-scale phylogenies have proven powerful tools to explore the impacts of historical (e.g. fingerprints of allopatric or sympatric speciation) and eco-evolutionary factors (e.g. above-species level niche evolution) in species distributions (Ahmadzadeh et al., 2013; Quintero & Wiens, 2013; Bonetti & Wiens, 2014; Gómez-Rodríguez et al., 2014). This information is of great value for conservation and species management, but unfortunately, it has several shortcomings. On the one hand, they rest upon climatic layers at coarse temporal (e.g. monthly or annual means) and spatial scales (e.g. several kilometers). These layers, overlook the microclimates that different lifecycle stages of species experience, both from the temporal and spatial point of view (Kearney et al., 2012; Potter et al., 2013; Buckley & Huey, 2016). Thus, they might represent poor descriptors of the actual exposure to climate change that species will face. On the other hand, most of these approaches ignore the intrinsic sensitivity of different lifecycle stages (e.g. physiology) and their response capacity through niche evolution, phenotypic plasticity, and behavioral responses (Araújo et al., 2006; Deutsch et al., 2008; Schloss et al., 2012). Mechanistic niche models are key to integrate these factors in climate change forecasts.

To explain species distributions, mechanistic niche models explicitly incorporate the interactions between species' functional traits (i.e. stage-specific morphology, physiology, and behavior) and the microclimates available to them (Kearney et al., 2008). Many mechanistic models, but not all of them (for thorough reviews see Kearney et al., 2010a; Pacifici et al., 2015), apply the principles of biophysical ecology to model heat and mass transfer between individuals and their physical environment (Kearney & Porter, 2009; Buckley et al., 2010; Riddell et al., 2017). To model the microclimatic conditions, biophysical models converts climate, terrain (i.e. slope, aspect, and hillshade), and vegetation data into radiation, air temperature, wind speed, humidity, substrate temperature and soil moisture at locations relevant for the focal species (**Fig. I.2**; Porter et al., 1973; Kearney & Porter, 2016). Moreover, it is possible to estimate key parameters for animals, by solving balance equations of energy (i.e. exchange of heat through conduction, convection, radiation, and evaporation) and mass (i.e. gas, matter, and water exchanges), while accounting for their morphology (size, shape, and solar reflectivity), physiology (e.g. thermal tolerance), and behavior (e.g. retreat to burrows if temperatures exceed their tolerance). For instance, we can estimate body temperatures and water balance for ectotherms (Tracy, 1976; Buckley & Kingsolver, 2012; Riddell et al., 2017) and metabolic rates to maintain homeostasis for

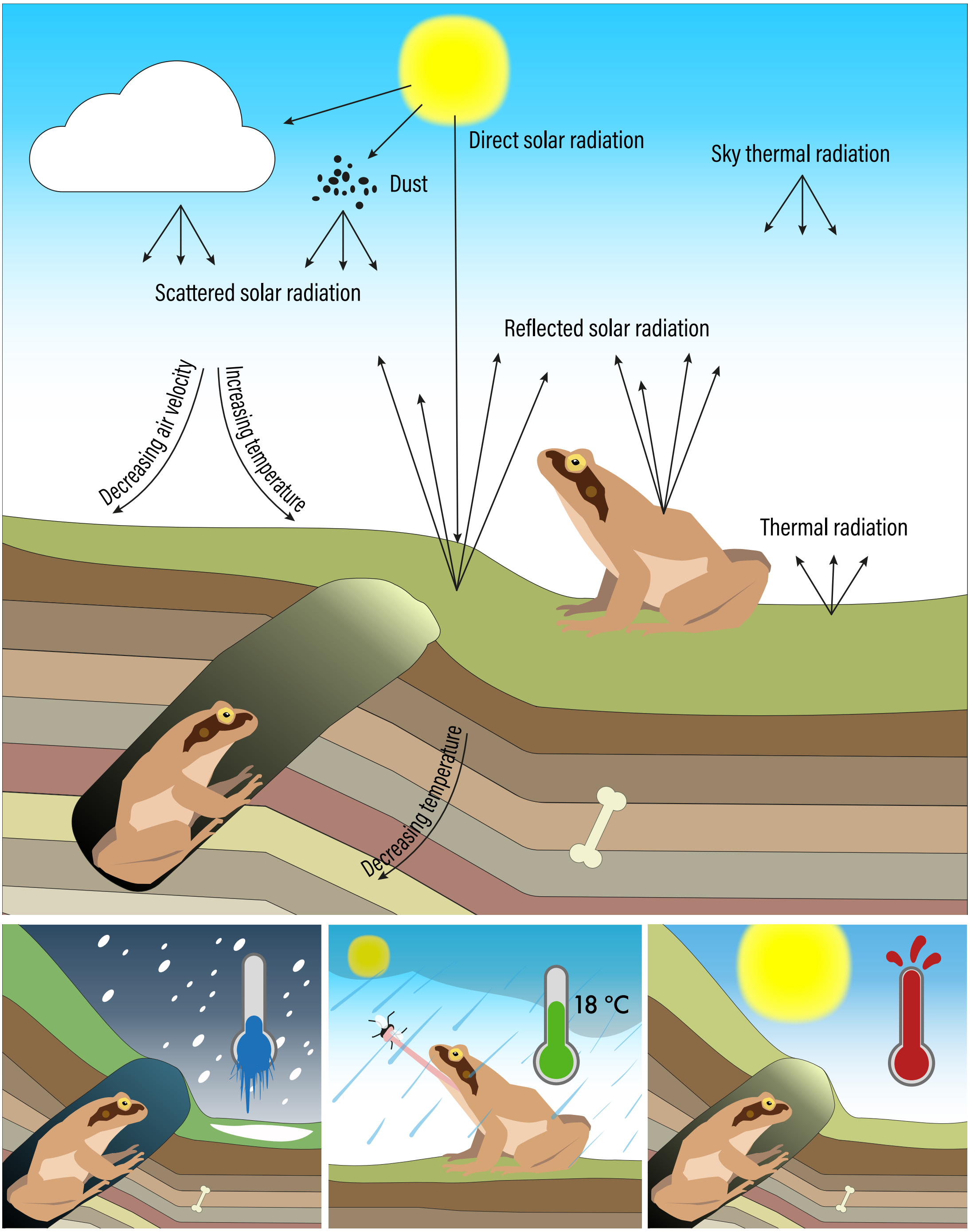

Figure I.2: The upper panel shows the microclimatic processes determining energy and mass balance of organisms (after Porter et al. 1973). This balances are integrated in NicheMapR and coupled with an animal model to account for specific morphologies, physiologies, and behaviours (see Chapters 5 and 6). This way we can get hourly estimates of body temperatures, water balance, and activity patterns. For instance, if body temperatures exceed the minimum (left-bottom panel) or maximum (right-bottom panel), animals would retreat to shelters. Alternatively, if body temperatures and water balance is adequate for activity (central-bottom panel), animals would be predicted to be active.

endotherms (Kearney et al., 2010b; Briscoe et al., 2016; Mathewson et al., 2017). All these mechanistic interactions can be then translated into fitness components (e.g. activity time at a given location) and mapped into the landscape to foresee the impacts of climate change. Due to the flexibility of mechanistic models, it is possible to integrate stage-specific functional traits and microclimates, as well as other mechanisms, such as niche evolution, phenotypic plasticity, carry-over effects, or within-species geographic phenotypic variation to better assess the vulnerability of species to climate change (Briscoe et al., 2012; Maino et al., 2016; Moran et al., 2016).

Although mechanistic niche models may provide causal and robust insights into the impacts of climate change, their use lags behind the use of correlative SDMs. This stems from the fact that mechanistic models require from a detailed species-specific information, which in most cases is lacking (Dormann et al., 2012; Pacifici et al., 2015; Cabral et al., 2017). This realization, instead of discouraging researches, should motivate collaborative efforts between ecologists, physiologists, evolutionary biologists, and modellers. Yet, approaches that combine targeted ecophysiological experiments, correlative SDMs, and mechanistic niche models are relatively scant (Holt, 2009; Jackson et al., 2009; Evans et al., 2015).

THESIS OUTLINE AND OBJECTIVES

In this thesis, I embrace a multidisciplinary approach to forecast the impacts of climate change on a temperate amphibian: the European common frog (*Rana temporaria* L., 1758; **Fig. I.3** and **I.4**). Previous attempts to anticipate amphibians' distributions in the face of climate change have drawn a pessimistic picture, predicting severe range contractions (Araújo et al., 2006; Girardello et al., 2009; Carvalho et al., 2010; Lawler et al., 2010). Nevertheless, to improve our predictive ability, we need thorough assessments of the vulnerability of species, including better estimates of species' exposure to climate change, intrinsic sensitivity, and response capacity (**Fig. I.1**). In part because of a high site-fidelity, amphibians are often considered poor dispersers (Araújo et al., 2006; Zeisset & Beebee, 2008). Consequently, the response capacity of most amphibians may largely depend on *in situ* adaptation to environmental change (e.g. niche evolution) and plastic responses (phenotypic plasticity and behavioral adjustments) (Chevin et al., 2010; Dawson et al., 2011; Moritz & Agudo, 2013). If these mechanisms fall short, populations would perish.

To explore the vulnerability of *R. temporaria* – and the likely responses to climate change – I take advantage of the information that current species' distributions provide, and further, I conduct a set of directed experiments with different lifecycle stages coupled with mechanistic and correlative species distribution modelling.

To quantify species' niches and their climatic limitations, researchers have developed two main alternatives. One of these alternatives consists on stablishing associations between where a species occurs currently and the climatic conditions at those locations, most often based on macroclimatic layers (e.g. WorldClim; Hijmans et al., 2005). The other alternative is to experimentally 'measure' the niche (or physiologic limitations) of species (Angilletta, 2009; Holt, 2009). Experimentation can be expensive and time consuming, and additionally, it may not be feasible to measure the tolerance along all the axes that define the niche of species. Oftentimes, experimental quantifications limit to the thermal (or hydric) niche due to the direct impact of temperature on species' physiological performance, especially in ectotherms (e.g. amphibians; Kearney et al., 2008; Tingley et al., 2012; Titon & Gomes, 2015; Carlo et al., 2017). Oppositely, due to the wide availability of diverse macroclimatic layers and species' occurrences, now it is possible to measure the conditions that any given species "experiences" along many axes of the niche simultaneously (e.g. temperature, precipitation, soil, radiation), in a few hours. As explained before, this approach has many shortcomings and may not be enough to assess the vulnerability of species. Nonetheless, together with phylogenies it has proven useful to shed light into past patterns of climatic niche evolution (or conservatism!), which inform us about the potential of niche evolution to protect species from climate change (Quintero & Wiens, 2013; Schwallier et al., 2015; **chapter 1**).

However, only through experiments can we unveil how the niche of species change with ontogeny or geography, to perform robust examinations of the likely impacts of climate change. *R. temporaria*, as most amphibians, possess a complex life history, with aquatic egg and tadpole stages, a semiaquatic metamorphic stage, and fully terrestrial juveniles and adults (**Fig. I.3**), which face different climatic challenges. Further, it spans across vast geographical clines and hence, it may encounter radically different climatic conditions across its distribution (**Fig. I.4**). All these aspects make *R. temporaria* a good model to experimentally explore how different lifecycle stages originating from populations with contrasting climates (e.g. at the extremes of altitudinal or latitudinal clines) cope with

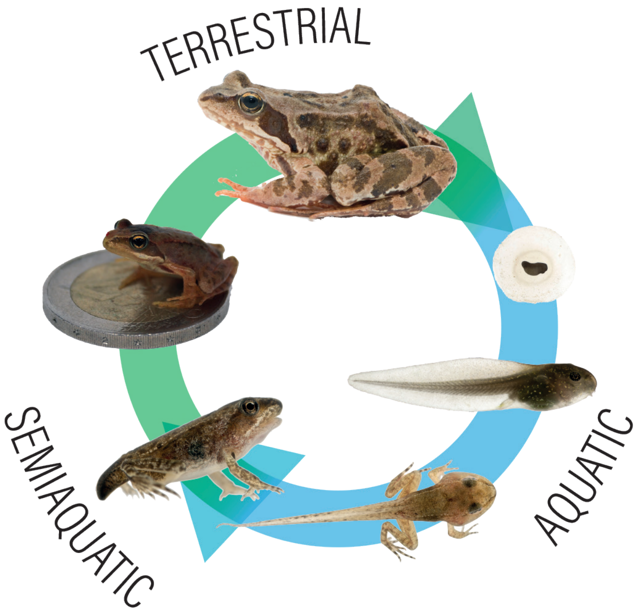

Figure I.3: Lifecycle of the European common frog (*Rana temporaria*), which shows aquatic (i.e. egg and tadpoles) and terrestrial or semiaquatic stages (newly metamorphosed individuals, juveniles, and adults).

their environments. Due to the pervasive effects of temperature on all biological functions (Ojanguren & Braña, 2000; Angilletta, 2009; Kingsolver, 2009), I focus on the thermal niche. Roughly, the thermal niche depicts how the fitness of a species (most often a fitness related function) changes with temperature.

By comparing proxies of the thermal niche (thermal sensitivity of specific functions, preferred temperatures, or thermal tolerance) of different lifecycle stages from populations along environmental gradients, I intend to unravel whether *R. temporaria* copes with contrasting climate through local (thermal) adaptation, phenotypic plasticity, or a combination of both (**chapters 2, 3, and 5**). This population-level design allows us to explore the response capacity of *R. temporaria* populations to climate change via adaptation or plasticity. Also, it might shed further light into the extent to which *R. temporaria* consists on locally adapted populations with different potential for plastic responses, which could alter our forecasts under climate change (Valladares et al., 2014; Richter-Boix et al., 2015). Further, to assess whether exposure to warm temperatures at early live can shape the thermal tolerance of later stages, I explore potential carry-over effects on thermal tolerance between the larval, metamorphic, and juvenile stages (**chapter 4**).

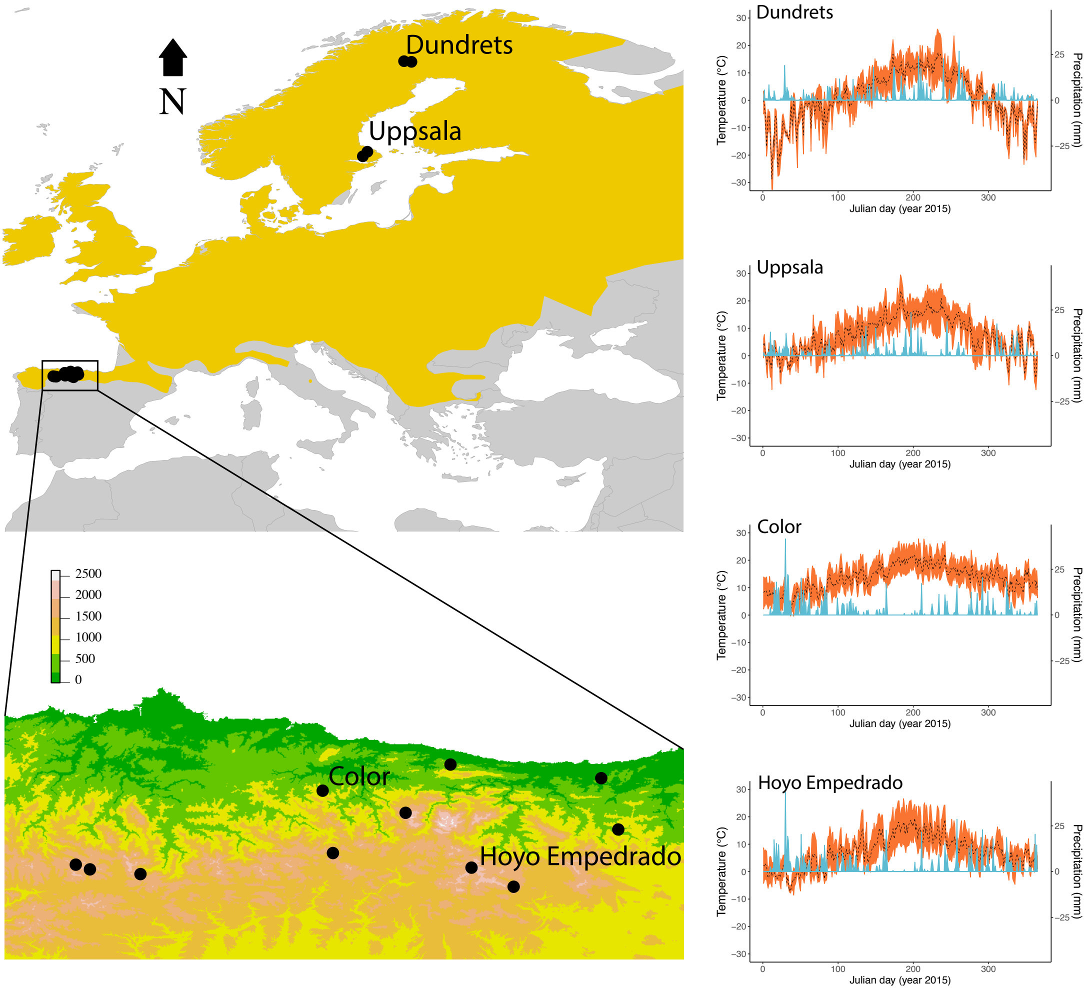

Figure I.4: Geographic distribution (yellow polygon over Europe's map; obtained from IUCN) of *R. temporaria* and the populations studied during this thesis. The panels in the right represent the climograms (temperature and precipitation mean and extremes) of selected populations (labelled in maps).

Mechanistic niche models are invaluable tools to perform better assessment of the vulnerability of species, because they allow a better rendering of the exposure, intrinsic sensitivity, and response capacity. To that end, mechanistic models explicitly reconstruct relevant microclimates (under current and future climates) and offer the possibility to explicitly integrate experimentally measured species-, population-, or stage-specific traits (e.g. physiology and morphology) and key processes that will modulate the impacts of environmental change (e.g. niche evolution, phenotypic plasticity, carry-over effects, geographic phenotypic variation, or behavioral choices). Unfortunately, mechanistic models have barely been employed with amphibians (Winter et al., 2016). To produce a robust appraisal of the vulnerability of *R. temporaria*, I integrate stage-specific thermal niche parameters with mechanistic (biophysical) niche models using NicheMapR (Kearney & Porter, 2016). Specifically, I explore the vulnerability of juveniles *R. temporaria* at different altitudes by integrating population-specific thermal tolerances and its potential to adjust through plasticity (i.e. acclimation), in addition to the potential to buffer air temperature rises through behavioral thermoregulation (**chapter 5**). Finally, to evaluate potential range shifts due to anthropogenic climate change, I combine stage-specific mechanistic models (i.e. for larvae, juvenile, and adult *R. temporaria*) with correlative species distribution models and associated methods (i.e. permutation-based variable importance tests). This approach is aimed at unravelling which lifecycle stage (or biophysical process) limits the geographic distribution of *R. temporaria* under current climates, to perform physiologically informed projections under climate change (**chapter 6**).

OBJECTIVES

1 To unveil past dynamics of species-level climatic niche evolution in European amphibians from current species' distributions (chapter 1).

2 To characterize aspects of the thermal niche of different lifecycle stages of *Rana temporaria* and disentangle how they might cope with their thermal environments.

2.1 To explore the degree of local thermal adaptation and the potential for phenotypic plasticity in growth and developmental rates of larvae *R. temporaria* that face contrasting eco-climatic challenges (i.e. originating from different altitudes and latitudes) (chapter 2).

2.2 To explore patterns of thermal niche evolution in juvenile and adult *R. temporaria* from populations with different thermal environments (i.e. at different altitudes) (chapter 3).

2.3 To examine the consequences of exposure to warm temperatures at early stages (larvae) in thermal tolerance, and potential 'carry-over' effects in later stages (metarmophic and juvenile individuals) (chapter 4).

3 To perform robust assessments of the vulnerability of *Rana temporaria* to climate change by bridging population- and stage-specific parameters with mechanistic niche models.

3.1 To determine the vulnerability to climate change of juveniles of *R. temporaria* at different altitudes using biophysical models that account for population-specific thermal tolerances, capacity for acclimation, behavioral thermoregulation, and their interconnections (chapter 5).

3.2 To foresee potential range shifts – and the underlying mechanisms – in *R. temporaria* through a combination of stage-specific mechanistic niche models and correlative species distribution models (chapter 6).

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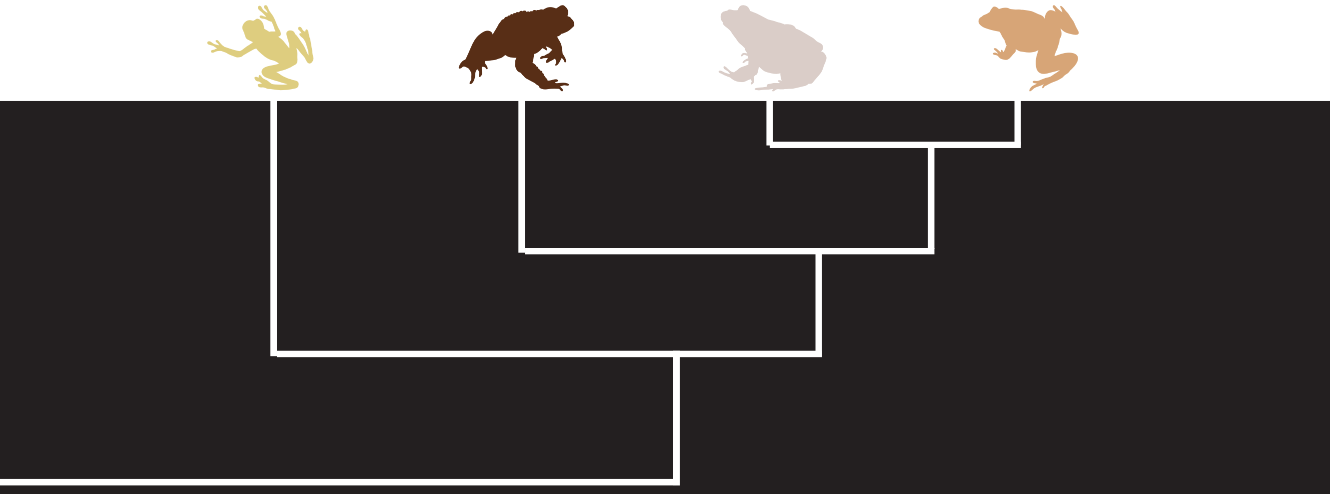
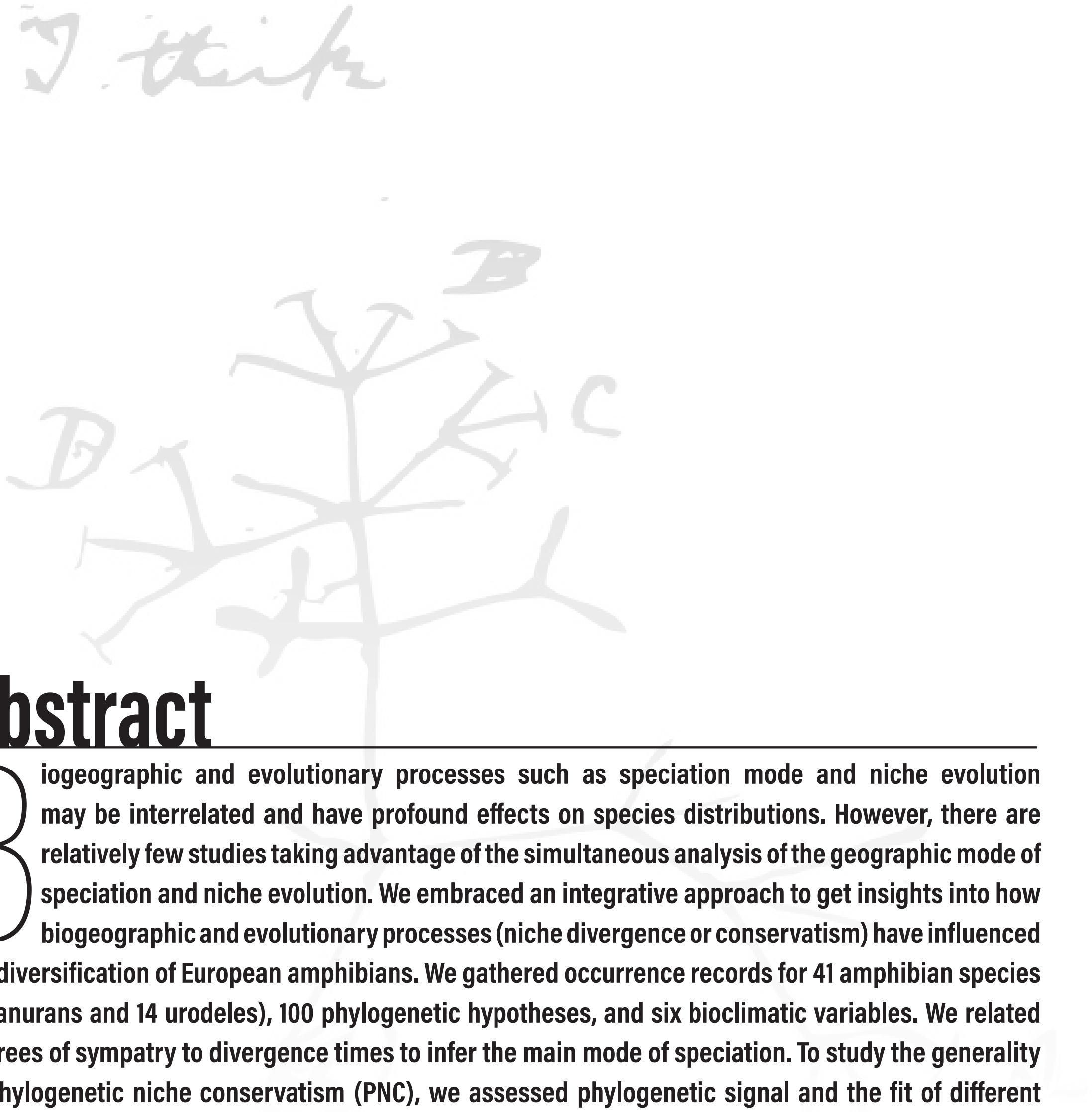
CHAPTER 1

Influence of biogeographic and evolutionary processes on European amphibians' diversification

URTZI ENRIQUEZ-URZELAI - NEFTALÍ SILLERO - ANTIGONI KALIONTZOPOULOU - ALFREDO G. NICIEZA





SOURCE: DARWIN'S NOTEBOOK 1837

Abstract

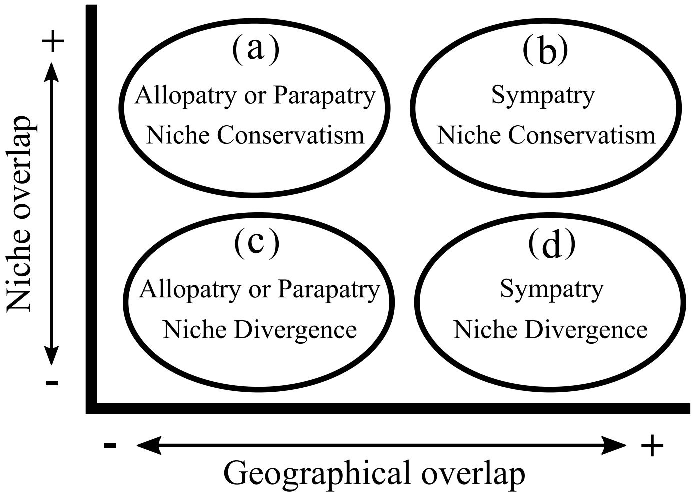
Biogeographic and evolutionary processes such as speciation mode and niche evolution may be interrelated and have profound effects on species distributions. However, there are relatively few studies taking advantage of the simultaneous analysis of the geographic mode of speciation and niche evolution. We embraced an integrative approach to get insights into how biogeographic and evolutionary processes (niche divergence or conservatism) have influenced the diversification of European amphibians. We gathered occurrence records for 41 amphibian species (27 anurans and 14 urodeles), 100 phylogenetic hypotheses, and six bioclimatic variables. We related degrees of sympatry to divergence times to infer the main mode of speciation. To study the generality of phylogenetic niche conservatism (PNC), we assessed phylogenetic signal and the fit of different evolutionary models on different macroclimatic niche traits (i.e. position and breadth) at the assemblage-tree level, and we compared the multivariate niche in a pairwise fashion. Further, we analysed disparity changes through time to examine diversification rates in macroclimatic niche traits. European amphibians show reduced degrees of sympatry and a lack of correlation between range overlap and divergence time. Despite a lack of support for PNC at the assemblage-tree level (phylogenetic signal and evolutionary models), pairwise niche comparisons yielded mixed results of niche conservatism and divergence. We observed high diversification associated to macroclimatic niche traits, especially for water requirements and tolerances in anurans, and temperature tolerance in urodeles. Allopatric speciation during climatic oscillations triggered climatic niche diversification, favouring the accumulation of amphibian species in southern Europe. However, many of these species showed signs of conserved macroclimatic niches. This, together with small geographic ranges and poor dispersal abilities, raises the possibility that global change can have substantial effects on future distributions.

1.1. Introduction

Species are not randomly distributed in space, but rather they occur unevenly, yielding striking geographical patterns of species richness (Buckley et al., 2010; Fritz & Rahbek, 2012; Chejanovski & Wiens, 2014). Population processes such as extinction and dispersal dependent on climate, and other climatic and historical factors ultimately determine the mode of speciation and can shape the observed patterns of diversity (Ricklefs, 2004; Wiens, 2007). These processes in turn are largely driven by climate through mechanisms such as niche divergence or conservatism (Hua & Wiens, 2013; Duran & Pie, 2015). Among biogeographical (i.e. historical) processes, the geographic mode of speciation (i.e. allopatric, parapatric, or sympatric) may be especially relevant. In the case of allopatric and parapatric speciation, initial overlap values are generally negligible, whereas in sympatric speciation the degree of geographical overlap would be essentially complete (**Fig. 1.1**). After speciation, independent range shifts may modify the degree of overlap over time (Barraclough et al., 1998; Barraclough & Vogler, 2000; Graham et al., 2004). Thus, biogeographical processes alone may generate a non-random distribution of species. Species-rich areas could be associated with areas of diversification during strong climatic oscillations, which may have provided abundant opportunities for allopatric speciation (Veith et al., 2003). Interestingly, rapid niche evolution between allopatric species might promote high diversification rates (Kozak & Wiens, 2010). Most likely, the interplay between the geography of speciation and spatial patterns of climatic niche evolution may explain both global and regional diversity patterns (Warren et al., 2014).

Due to the inherent difficulty of measuring fundamental climatic niches, most studies of climatic niche evolution focus on realised niches (Olalla-Tárraga et al., 2011; Chejanovski & Wiens, 2014). The realised climatic niche is the subset of the fundamental niche that a species occupies when biotic interactions (i.e. competition, predation, mutualism, and parasitism) and biogeographical factors (e.g. geography of speciation, plate tectonics, and dispersal) are taken into account (Soberón, 2007; Sillero, 2011). Given that environmental conditions change with geographical distance (i.e. spatial autocorrelation; see Diniz Filho et al., 2003), the geographic patterns of speciation and dispersal capabilities may determine which set of environmental conditions are available for incipient species. In the case of allopatric speciation, available environmental conditions may differ greatly from the ancestral niche. Thus, incipient species may either evolve to tolerate the new

Figure 1.1: Species distributions provide valuable information on the degree of geographical and niche overlap. Combining these sources of information, we may gain insights into the mechanisms underlying diversity patterns. Lower degree of geographical overlap with increasing phylogenetic relatedness **(a and c)** may suggest allopatry or parapatry, while the opposite pattern **(b and d)** may suggest sympatry as the main mode of speciation. High niche overlap values **(a and b)** would suggest a prevalence of phylogenetic niche conservatism (PNC) while low values **(c and d)** would suggest a predominance of niche divergence.



available environmental conditions (**Fig. 1.1c**), or show niche conservatism (**Fig. 1.1a**) and persist within the ancestral climatic zone. According to the phylogenetic niche conservatism hypothesis (PNC), species may show a tendency to retain ancestral niches (Wiens et al., 2010), which could in turn impede the invasion of novel environments (e.g. biomes). Ultimately this can lead to an increase of species richness within the ancestral climatic zone (Wiens & Donoghue, 2004; Wiens et al., 2006). Alternatively, rapid niche shifts (e.g. niche diversification) could also explain species accumulation, mainly due to increased speciation rates (Kozak & Wiens, 2010). In this context, the interaction between biogeographical and evolutionary processes may partly determine the sensitivity of species to changing climates. Under a scenario of allopatry as the main geographic mode of speciation, low dispersal ability, and the presence of PNC, we would expect species to be restricted to the subset of the available environmental space that more closely resembles their ancestral niche. By contrast, niche shifts could both facilitate the invasion of new environments and allow species to adapt to environmental change (Holt, 1990; Kozak & Wiens, 2010).

The current distributions of European amphibians show imprints of both paleogeography and paleoclimate (Rage & Roček, 2003; Martínez-Solano et al., 2004; Araújo et al., 2008). Thus, they provide a good model for studying how the interplay between important biogeographical and evolutionary processes has generated the pattern of impoverished northernmost and increased southernmost gamma diversity in European faunas. The pronounced climatic cycles during the Pliocene and Pleistocene, with subsequent glacial and interglacial periods, profoundly impacted species' distributions (Schmitt, 2007; Araújo et al., 2008; Zeisset & Beebee, 2008). Glacial periods forced species to retreat to southern glacial refugia and favoured speciation (Veith et al., 2003). Although some amphibians recolonised Europe during the interglacial periods (Schmitt, 2007; Zeisset & Beebee, 2008; Teacher et al., 2009), most narrow-ranging species are restricted to geographical areas associated with putative Pleistocene refugia in southern Europe (Sillero et al., 2014; Trakimas et al., 2016). Moreover, anurans and urodeles differ in their biogeographic origin: while the European anuran fauna is derived mostly from Asian immigrants diversified in Europe (Rage & Roček, 2003), European urodeles originated in Europe *in situ* (Zhang et al., 2008). Thus, due to potential differences in biogeographical origin, historical and evolutionary processes may have played a different role in the two groups. Since many European amphibians are presumably poor dispersers (Araújo et al., 2006; Trakimas et al., 2016) and endemic to the Mediterranean hotspot, understanding the degree of niche conservatism may inform us about the sensitivity of European amphibian biodiversity to environmental change.

In this paper we analyse the distributions of the European amphibian assemblage based on occurrence records (Sillero et al., 2014) to reveal the imprints of the interplay between the geography of speciation and climatic niche evolution. Although we acknowledge that the scenarios outlined in **Fig. 1.1** may correspond to the extremes of a continuum, they may help us hypothesize how the pattern of impoverished northernmost and increased southernmost gamma diversity might have been generated. Low overlap values in geographical and high overlap values in niche space may indicate a predominance of speciation in isolation during climatic oscillations (i.e. allopatric or parapatric) and the presence of PNC (**Fig. 1.1a**), which may have favoured an accumulation of species in southernmost latitudes. If during isolation climatic niches evolved to match the prevailing environmental conditions, we would retrieve low overlap values in niche space too (**Fig. 1.1c**). High overlap in geographical space would indicate a predominance of sympatric speciation (**Fig 1.1b, d**), which could also favour the accumulation of species in a certain area. A number of previous studies have suggested that the diversification of amphibian species and lineages and species has occurred mainly in allopatry (Steinfartz et al., 2000; Veith et al., 2003; Martínez-Solano et al., 2004; Gómez & Lunt, 2007). However, to our knowledge, the prevalence of allopatry as the main mode of speciation has not been assessed yet. We hypothesize that after allopatric speciation, niche diversification due to niche divergence, as opposed to niche conservatism, has shaped the amphibian biodiversity pattern in Europe (i.e. scenario 'c' in **Fig. 1.1**), with many species contributing to the Mediterranean hotspot. First, we test the prevalence of allopatry as the main mode of speciation and the degree of subsequent range shifts, by regressing the geographic overlap on divergence time using age-range correlation analyses. Second, we test the presence of climatic niche conservatism or divergence at the assemblage-tree level using phylogenetic comparative methods on niche descriptors (i.e., niche position and breadth). In addition, we examined niche conservatism/divergence in a pairwise fashion controlling for the available environmental space (Broennimann et al., 2012), to account for the effects of the geography of speciation on the potential for niche evolution or conservatism. Finally, we examine the diversification of niche descriptors to examine the rate of climatic niche evolution in European amphibians.

1.2. Materials and Methods

We obtained occurrence records for European amphibians from the most recent published atlas (Sillero et al., 2014) at 50-km² resolution. This new European atlas represents the most comprehensive effort made to date in order to merge all available sources of information and to accurately describe the geographical distributions of amphibian and reptile species in this region. We only examined those species that are native to Europe and for which at least five presence data points were available, resulting in a data set with 41 amphibian species (27 anurans and 14 urodeles; **Table S1.1**). The least and most represented species had 20 and 2254 unique occurrence points respectively.

We obtained 100 dated phylogenetic trees from Roquet et al., (2014), available at Dryad (http://dx.doi.org/10.5061/dryad.11609), which we trimmed to exclude species lacking occurrence data. We run all the analyses involving the use of a phylogenetic tree (all except niche equivalency and similarity, see further on) with the obtained 100 dated phylogenetic trees to control for phylogenetic uncertainty. Due to differences in dispersal requirements and biogeographical origin, all analyses were performed on the entire data set, and on anurans and urodeles separately.

To describe the realised climatic niche of each species, we first obtained climatic data from the WorldClim database (Hijmans et al., 2005) at a resolution of ~1 km², which were then resampled using the *resample()* function in R to match the spatial resolution of the occurrence data set (50 km^2). We focused on six climatic variables (see **Table S1.2** for their correlation): annual mean temperature (Bio1), maximum temperature of the warmest month (Bio5), minimum temperature of the coldest month (Bio6), annual mean precipitation (Bio12), precipitation of the wettest quarter (Bio16), and precipitation of the driest quarter (Bio17). We chose these variables because they reflect energy and water properties of climate, and may limit distribution areas due to shared physiological constraints (Carey & Alexander, 2003; Araújo et al., 2006; Quintero & Wiens, 2013). Then, we described species realised climatic niches (*sensu* Sillero, 2011) using the outlying mean index approach (OMI; Dolédec et al., 2000) implemented in the ade4 R-package (Dray & Dufour, 2007). OMI is a particularly suitable ordination approach to describe species' niches, due to two main properties: it makes no assumption about the shape of species' response curves and it gives equal weight to presence records independently of species richness (Thuiller et al., 2004; Hof et al., 2010). The analysis provides measures of niche position and breadth along main environmental gradients, represented by OMI axes. Niche position expresses the distance between the environmental conditions experienced by a species and the mean environmental conditions of the study area, and niche breadth captures the environmental variation along the respective OMI axes (Dolédec et al., 2000;

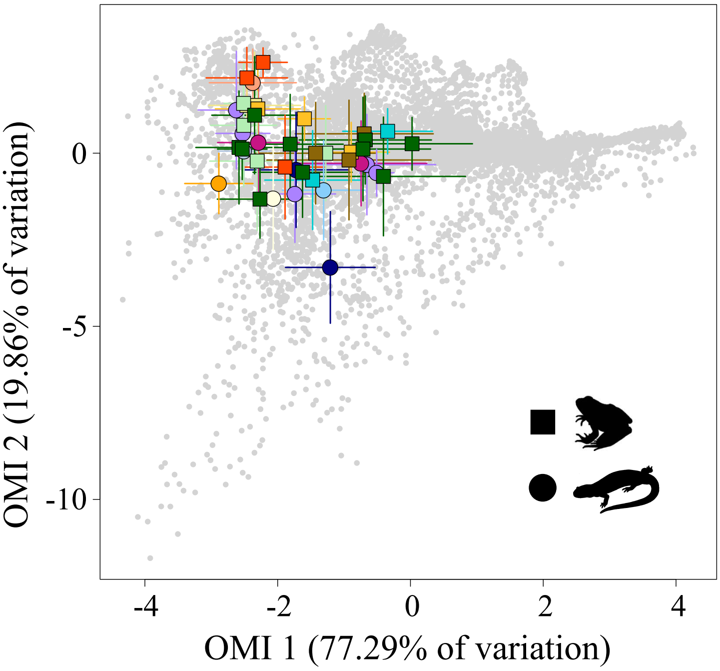


Figure 1.2: Niche position and breadth of each species along the first (OMI1) and second (OMI2) main environmental gradients in Europe. Squares represent anurans and circles represent urodele species. Different colours denote different genera. Grey dots represent the scores of the available environmental space in Europe.

Hof et al., 2010). We extracted niche position (mean) and breadth (standard deviation) along the first and second OMI axes: Pos1, Pos2, Bre1, and Bre2 (**Fig. 1.2**).

GEOGRAPHY OF SPECIATION AND RANGE SHIFTS

In order to detect imprints of the geography of speciation and subsequent range shifts on current species distributions, we correlated degrees of geographic overlap (i.e. degree of sympatry) to phylogenetic relatedness (Fitzpatrick & Turelli, 2006; Warren et al., 2014). First, we estimated the degree of geographic overlap between species pairs. We used the *lets.overlap()* function of the letsR R-package (Vilela & Villalobos, 2015) to estimate the proportion of the smaller range that overlapped with the larger range based on occurrence points (Chesser & Zink, 1994). With those geographic overlap values we performed age-range correlation analyses (ARC; Fitzpatrick & Turelli, 2006; Warren et al., 2014) as implemented in the phyloclim R-package (Heibl & Calenge, 2013). ARC is particularly suitable for our purpose because of its robustness to incomplete taxon sampling, common at the assemblage level (Warren et al., 2008; Pearman et al., 2014). Rather than attempting to reconstruct ancestral ranges, this method produces topologically weighted averages along phylogenetic trees to take shared ancestry into account, and then fits linear regressions between those range overlap values and relative node ages (Fitzpatrick & Turelli, 2006). If the regression intercept is significantly lower than 0.5 and the slope is positive, we would infer that allopatric speciation is most common (Fitzpatrick & Turelli, 2006). This approach is built on the idea that allopatric speciation results in low degrees of range overlap and subsequent niche shifts lead to secondary contacts, therefore increasing the degree of sympatry (Warren et al., 2014). It is noteworthy that allopatric and parapatric speciation are not easily distinguishable; abutting ranges are compatible with both modes of speciation (Cracraft, 1982). Besides, very limited or non-existent overlap values would yield similar statistical results. Although non-significant correlations are usually interpreted as inconclusive, predominance of small overlap values would also suggest allopatric speciation followed by limited range shifts.

CLIMATIC NICHE CONSERVATISM OR DIVERGENCE

We tested for the presence or absence of PNC at the assemblage-tree level using phylogenetic comparative methods. More precisely, we examined climatic niche parameters that describe each species' position and breadth along the most pronounced climatic gradients (Pos1, Pos2, Bre1, and Bre2). First, we tested for phylogenetic signal in each of these variables using Blomberg's *K* (Blomberg et al., 2003) as implemented in the phytools R-package (Revell, 2012) with a randomisation procedure of 1000 simulations. Further, we fit four different models of phenotypic evolution – White-noise (*WN*), Brownian motion (*BM*), Orstein-Uhlenbeck (*OU*), and *Kappa* – to Pos1, Pos2, Bre1, and Bre2. *WN* is a model where phylogenetic relatedness is not relevant for the distribution of the examined phenotypic trait; *BM* is a model of neutral drift; single-peak *OU* model is a model of directional or stabilising selection; and Pagel's *Kappa* is a punctuational model of phenotypic evolution. Support for *BM* or *OU* models could be consistent with niche conservatism. On the contrary, *WN* would indicate a lack of conservatism (Wiens et al., 2010). Support for a *Kappa* model with a *kappa* statistic of 1 would indicate that traits change proportional to divergence time, as expected under PNC (Wiens et al., 2010), while a *kappa* statistic of 0 would suggest rapid change at or immediately following speciation (Pearman et al., 2014). We evaluated the relative fit of each model using mean ΔAICc and Akaike weights across all replicate runs.

To compare the characteristics of the niche occupied by different species while accounting for the effects of biogeographic factors and given the spatial autocorrelation in environmental variables, we applied the PCA-env framework proposed by Broennimann et al. (2012). Specifically, we examined niche equivalency (i.e., whether niches of two species are identical) and similarity (i.e., whether the observed niche overlap is a product of the available environmental space or active habitat selection) in a pairwise fashion within families or genera, for anurans and urodeles respectively. We used the ecospat R-package (Broennimann et al., 2015) for that purpose. As a first step, we computed the density of occurrences in PCA space using a kernel density function ($R = 100$) and we calculated niche overlap in terms of Schoener's *D* (Schoener, 1968) in a pairwise fashion. We condensed overlap values into discrete classes (0–0.2 = no or very limited overlap, 0.2–0.4 = low overlap, 0.4–0.6 = moderate overlap, 0.6–0.8 = high overlap, 0.8–1.0 = very high overlap) as proposed by Rödder & Engler (2011). To assess the significance of niche equivalency and niche similarity tests, we used 100 permutations to obtain an empirical distribution of expected niche overlap values and compare them to the values observed in our dataset. To test for niche equivalency all occurrences were pooled and then randomly split into two datasets, while preserving the number of observations as in the original data sets. Then, Schoener's *D* is recalculated for each random dataset, thus providing an empirical distribution of values. In the case of niche similarity, niche overlaps are computed between the environmental conditions at the occurrences of a species and records randomly generated within the available environmental space of other species. Because these analyses are sensitive to the definition of the geographic background (Mateo et al., 2014), we repeated these analyses using either the entire study area (i.e. Europe) or Olson's biomes (Olson et al., 2001) as background. In the latter case, we only contemplated as available environmental space the biomes exploited for each species, with at least four occurrences.

DIVERSIFICATION OF CLIMATIC NICHES

To explore rates of climatic niche diversification we examined how climatic niche disparity has changed through time. For this, we performed disparity-through-time analyses (DTT; Harmon et al., 2003) on Pos1, Pos2, Bre1, and Bre2, using Graham Slater's code (downloaded from http://fourdimensionalbiology.com/code/; Slater et al., 2010). We compared the among- and within-clade disparity through time both at the empirical and 1000 simulated data sets along phylogenetic hypotheses under a *BM* model of phenotypic evolution (Harmon et al., 2003). Then, we plotted mean subclade disparity values for the empirical and simulated data sets against the corresponding node age and we calculated *MDI* statistics (minimum discrimination index). *MDI* measures the overall difference between the observed and simulated relative disparity. Among-clade disparity values above null expectations lead to positive *MDI* values, while those below expectations are given negative values. Therefore, positive deviations indicate a lower overlap in niche-space within subclades than expected under *BM* and suggest rapid niche shifts (Harmon et al., 2003).

1.3. Results

GEOGRAPHY OF SPECIATION AND RANGE SHIFTS

The degree of sympatry between contemporary species was low (median ± SE geographic overlap value for amphibians: 0.320 ± 0.013; anurans: 0.305 ± 0.020; urodeles: 0.357 ± 0.040). Furthermore, we did not found any significant correlation between divergence time and the degree of sympatry (ARC amphibians: $F = 0.352 \pm 0.001$, $P = 0.704$

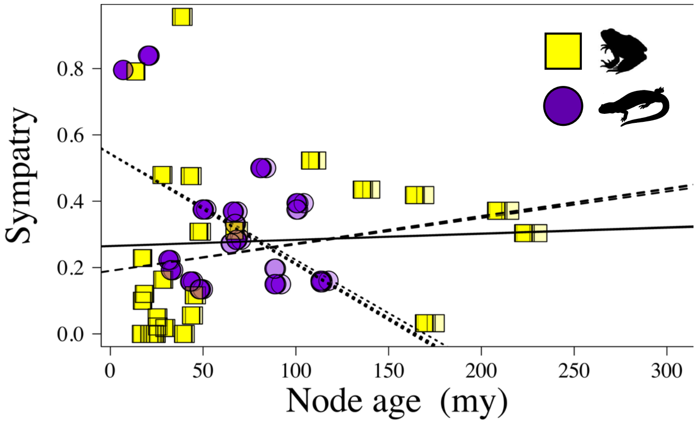


Figure 1.3: Linear regressions between the degree of sympatry (i.e. geographic overlap) and node age (i.e. time since divergence). Yellow squares correspond to the nodes in the anuran assemblage tree and purple dots nodes within urodeles' tree. The solid, dashed, and dotted lines represent the regression lines for all amphibians, anurans, and urodeles respectively. For graphical purposes, we superimposed all the regression lines and sympatry values for each node (using transparency) for all 100 phylogenetic hypotheses. Note that phylogenetic uncertainty mostly affected node age in deep branches.

Table 1.1: Results from phylogenetic signal tests for all niche traits (i.e., Pos1, Pos2, Bre1, and Bre2) and taxonomic levels (i.e., all European amphibians, anurans, and urodeles). We display Blomberg's K statistic and the associated p-value. Additionally, we provide the range of values (minimum – maximum) obtained across the replicate analyses run on the 100 phylogenetic hypotheses (Roquet et al. 2014) to account for phylogenetic uncertainty.

	Pos1		Pos2		Bre1		Bre2	
	K	*p*-value	*K*	*p*-value	*K*	*p*-value	*K*	*p*-value
Amphibians	0.183 (0.183 – 0.183)	0.265 (0.232 – 0.303)	0.221 (0.221 – 0.222)	0.086 (0.064 – 0.109)	0.121 (0.121 – 0.121)	0.928 (0.908 – 0.949)	0.128 (0.128 – 0.129)	0.894 (0.868 – 0.915)
Anurans	0.194 (0.194 – 0.194)	0.460 (0.416 – 0.493)	0.220 (0.220 – 0.220)	0.267 (0.228 – 0.304)	0.239 (0.239 – 0.240)	0.152 (0.124 – 0.178)	0.160 (0.160 – 0.161)	0.861 (0.837 – 0.888)
Urodeles	0.692 (0.691 – 0.695)	0.145 (0.114 – 0.176)	0.557 (0.556 – 0.558)	0.359 (0.327 – 0.409)	0.135 (0.134 – 0.135)	0.991 (0.984 – 0.998)	0.312 (0.311 – 0.312)	0.756 (0.713 – 0.794)

± 0.003; ARC anurans: $F = 0.083 \pm 0.001$, $P = 0.166 \pm 0.002$; ARC urodeles: $F = 0.873 \pm 0.001$, $P = 0.255 \pm 0.002$; **Fig. 1.3**).

CLIMATIC NICHE CONSERVATISM OR DIVERGENCE

The first and second OMI axes explained 77.29 and 19.87% of the climate variation throughout Europe respectively (**Table S1.3**; **Fig. S1.1**). The OMI1 axis correlated negatively to mean (Bio1: r = -0.761) and minimum temperatures (Bio6: r = -0.780). The OMI2 axis correlated weakly to minimum precipitations (Bio17: r = -0.442). We obtained species' climatic niche positions and breadths along those environmental gradients (i.e., Pos1, Pos2, Bre1, and Bre2; Fig. 1.2).

We did not find any evidence for PNC in the climatic niche traits at the amphibian,

Table 1.2: Summary of comparisons of evolutionary model fit for all niche traits and taxonomic levels. We fitted White-noise (WN), Brownian-motion (BM), Orstein-Uhlenbeck (OU), and Kappa models of phenotypic evolution. Since the best model was unambiguously inferred due to the low phylogenetic uncertainty (see Table S4 for standard error estimates), we provide mean ΔAICc and Akaike weights values obtained across the 100 phylogenetic hypotheses (Roquet et al. 2014) to evaluate the fit of the models.

		Pos1		Pos2		Bre1		Bre2	
		ΔAICc	Akaike weights	ΔAICc	Akaike weights	ΔAICc	Akaike weights	ΔAICc	Akaike weights
Amphibians	**WN**	0.000	0.627	0.000	0.683	0.000	0.761	0.000	0.762
	BM	23.340	5.359×10^{-6}	15.120	3.559×10^{-4}	40.493	1.226×10^{-9}	37.816	4.684×10^{-9}
	OU	1.055	0.370	1.549	0.315	2.333	0.237	2.333	0.238
	Kappa	10.884	2.717×10^{-3}	12.404	1.383×10^{-3}	12.200	1.708×10^{-3}	23.186	7.038×10^{-6}
Anurans	**WN**	0.000	0.736	0.000	0.754	0.000	0.681	0.000	0.781
	BM	16.606	1.825×10^{-4}	14.997	4.176×10^{-4}	11.778	1.885×10^{-3}	22.237	1.158×10^{-5}
	OU	2.439	0.217	2.281	0.241	2.352	0.210	2.543	0.219
	Kappa	5.543	0.046	10.134	4.751×10^{-3}	3.690	0.108	17.120	1.496×10^{-4}
Urodeles	**WN**	0.000	0.551	0.000	0.712	0.000	0.838	0.000	0.818
	BM	2.816	0.135	3.705	0.112	24.170	4.727×10^{-6}	12.767	1.382×10^{-3}
	OU	1.308	0.287	3.061	0.154	3.309	0.160	3.309	0.156
	Kappa	6.010	0.027	6.965	0.022	11.868	2.218×10^{-3}	7.066	0.024

anuran or urodele assemblage-tree levels. Climatic niche traits did not exhibit any evidence for phylogenetic signal (**Table 1.1**). Additionally, *WN* exhibited the best fit among all the fitted models of phenotypic evolution for all niche traits and taxonomic levels. Based on the criteria of ΔAICc > 4, some models were indistinguishable from *WN* models (all *OU* models, *Kappa* for anurans' Bre1, and *BM* for urodeles' Pos1 and Pos2). However, Akaike weights largely supported *WN* models (**Table 1.2**).

In general, niche overlap values among pairs of species were rather variable, from very limited to moderate (median value for amphibians = 0.314; anurans = 0.344; urodeles = 0.195; **Fig. 1.4**). However, four Iberian endemic species, namely two distantly related Alytidae (*Alytes cisternasii* and *Discoglossus galganoi*) and Ranidae (*Pelophylax perezi* and *Rana iberica*), showed high niche overlap values. Niche overlap values were very similar when using biomes as background (**Table S1.5**). Although niche equivalency was rejected in all pairwise comparisons (**Table S1.5**), niche similarity tests showed mixed results of niche conservatism and divergence (**Fig. 1.4**). Although the definition of the background (i.e. all Europe vs. Olson's biomes) did not dramatically affect the results, using biomes as the

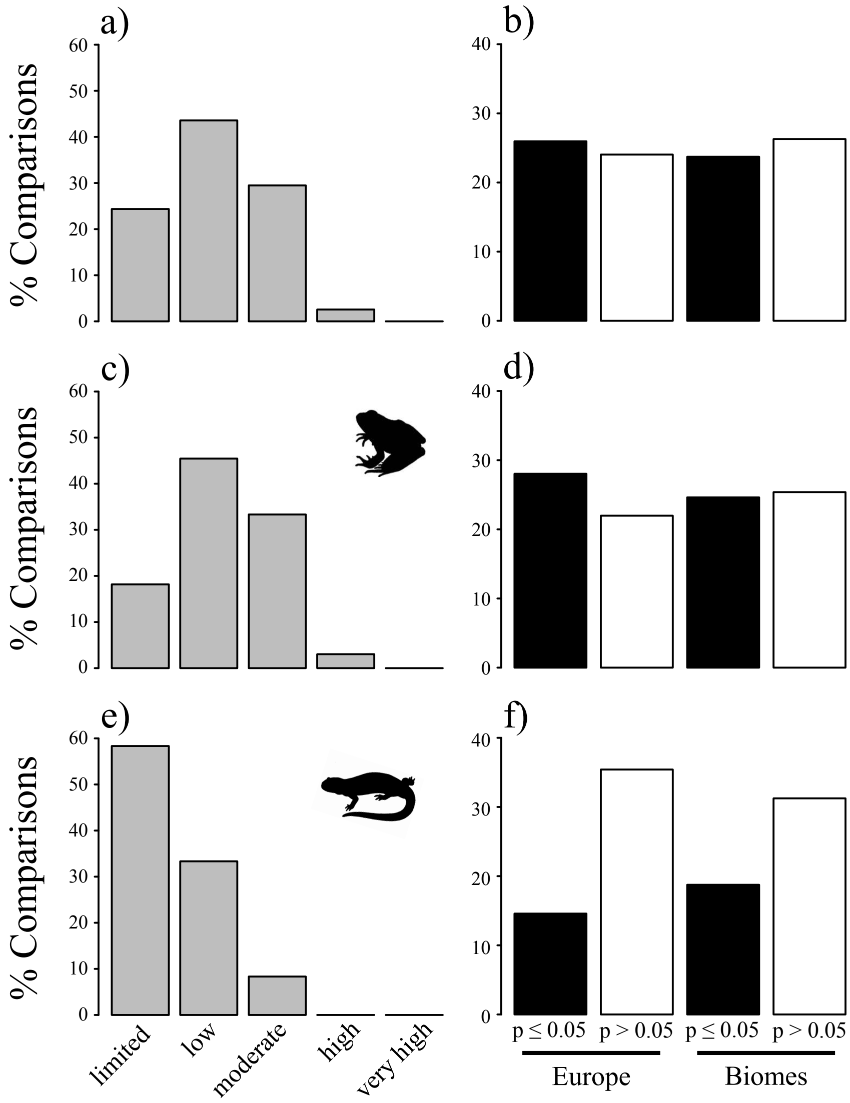


Figure 1.4: Percentage of pairwise comparisons yielding climatic niche overlap values (Schoener's *D*) that fall into the discrete categories proposed by Rödder and Engler (2011) for **(a)** all amphibians, **(c)** anurans, and **(e)** urodeles. Number of comparisons with similarity tests indicating niche conservatism (i.e. $p \leq 0.05$; black bars) or divergence (i.e. $p > 0.05$; white bars) for **(b)** all amphibians, **(d)** anurans, and **(f)** urodeles, using all Europe or Olson's biomes as background.

Table 1.3: Summary of disparity through time (DTT) analyses for each niche trait and taxonomic level. We provide mean values (± SE) of *MDI* statistic across all runs. *MDI* values were significantly greater than 0 in all replicate runs performed on the distribution of 100 phylogenies sampled to account for phylogenetic uncertainty.

	Pos1	Pos2	Bre1	Bre2
Amphibians	0.493 ($\pm 3.185 \times 10^{-4}$)	0.545 ($\pm 2.822 \times 10^{-4}$)	0.543 ($\pm 1.908 \times 10^{-4}$)	0.861 ($\pm 2.412 \times 10^{-4}$)
Anurans	0.429 ($\pm 2.068 \times 10^{-4}$)	0.706 ($\pm 1.950 \times 10^{-4}$)	0.306 ($\pm 1.883 \times 10^{-4}$)	0.907 ($\pm 1.627 \times 10^{-4}$)
Urodeles	0.343 ($\pm 3.272 \times 10^{-4}$)	0.486 ($\pm 8.824 \times 10^{-4}$)	1.359 ($\pm 3.388 \times 10^{-4}$)	0.540 ($\pm 2.246 \times 10^{-4}$)

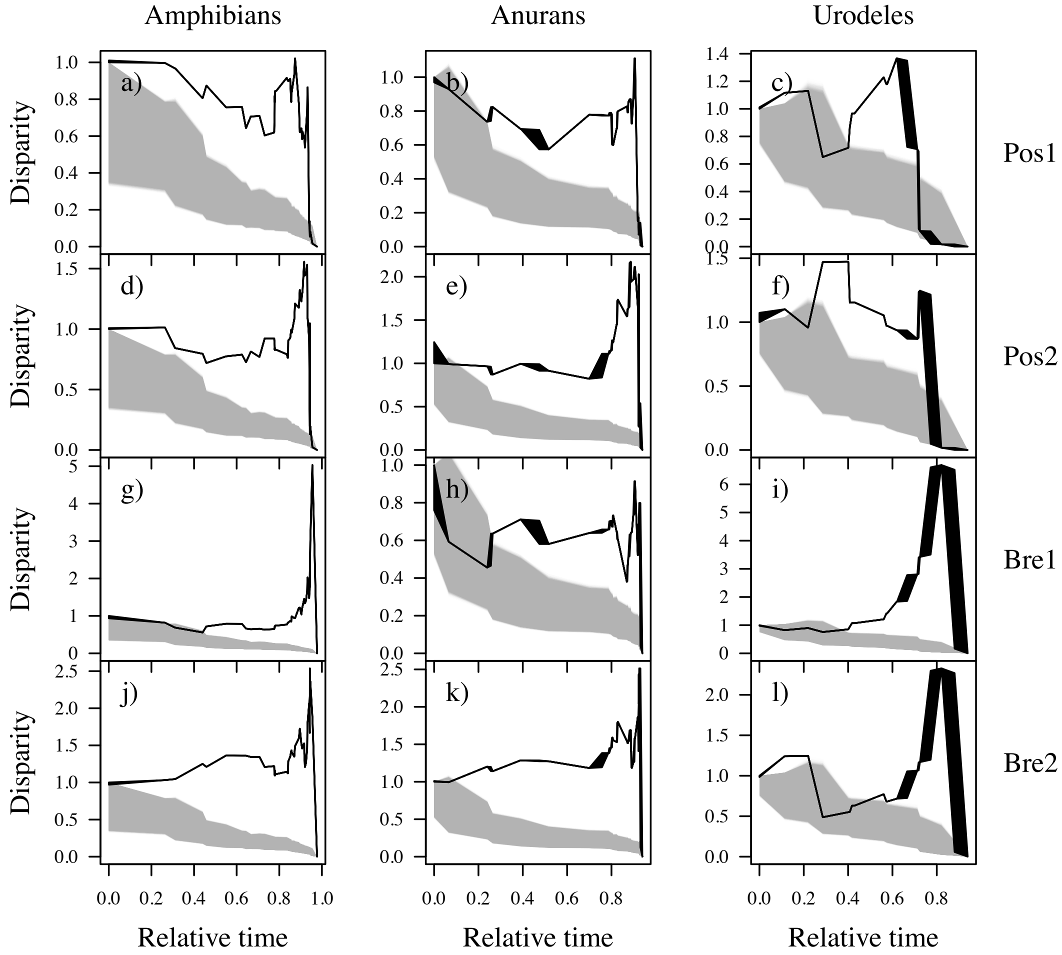


Figure 1.5: Plots of mean subclade disparity through time (DTT) for observed (*black polygons*) and simulated traits under a BM model of phenotypic evolution (*grey polygons*) for all climatic niche traits (i.e., Pos1 **[a-c]**, Pos2 **[d-f]**, Bre1 **[g-i]**, and Bre2 **[j-l]**) and taxonomic levels (i.e., all European amphibians **[a, d, g, j]**, anurans **[b, e, h, k]**, and urodeles **[c, f, i, l]**).

background slightly increased the number of similarity tests indicating niche divergence within anuran families (**Fig. 1.4**).

DIVERSIFICATION OF CLIMATIC NICHES

Within-clade niche disparity remained high through time (**Fig. 1.5**). Subclade disparity of climatic niche was always higher than expected under a *BM* model of phenotypic evolution, as indicated by positive *MDI* values (**Table 1.3**). Urodeles' Bre1 exhibited particularly high *MDI* values (**Table 1.3**) and a remarkable increase in disparity through time following major speciation events (**Fig. 1.5i**). Anurans' Pos2 and Bre2 also showed a marked increase in diversity through time (**Fig. 1.5e, k**) and high MDI values.

1.4. Discussion

Integrative approaches combining biogeographical and evolutionary evidence may shed light on the formation of distribution patterns, and inform about potential future responses to environmental change. Based on levels of sympatry and age-range correlation (ARC) analyses, our results suggest that most speciation events in the European amphibian assemblage occurred in isolation (allopatry or parapatry; scenarios ‘a’ and ‘c’ in **Fig. 1.1**). Additionally, current distributions exhibit little imprints of past shifts, in accordance with studies showing that a limited number of European amphibians possesses functional traits associated to rapid expansion, allowing them to disperse and recolonise Europe (Trakimas et al., 2016). Under allopatric speciation and overall limited dispersal, the environmental space may have varied greatly from the ancestral niche. Interestingly, niche equivalency and similarity tests showed that European amphibians exhibited both conservatism and divergence of macroclimatic niches, which may have generated the apparent lack of phylogenetic niche conservatism (PNC) obtained at the assemblage-tree levels using phylogenetic comparative methods. Seemingly, the positive deviation of disparity-through-time (DTT) analyses showed that the diversification of different niche traits depart from neutral evolution, supporting the apparent lack of phylogenetic niche conservatism (PNC). Therefore, we conclude that the most frequent speciation mode in European amphibians is allopatry/parapatry coupled with niche divergence mechanisms (scenario ‘c’ in **Fig. 1.1**).

Biogeographical processes can promote a non-random distribution of species and produce specific diversity patterns even in the absence of any directional evolutionary influence (Barraclough et al., 1998; Warren et al., 2014). Most evidence suggests that allopatric speciation largely predominates over sympatric speciation (Coyne & Orr, 1989; Fitzpatrick et al., 2009). Although the results obtained from ARC analyses are inconclusive (*sensu* Fitzpatrick & Turelli, 2006; **Fig. 1.3**) the observed low degrees of sympatry (i.e. geographic overlap) observed point to allopatry (or parapatry) as the main mode of speciation, followed by limited range shifts. Accordingly, phylogeographic studies conducted on European amphibians have revealed that climatic oscillations promoted isolation between and within climatic refugia, and favoured the diversification of many species and lineages (Steinfartz et al., 2000; Veith et al., 2003; Martínez-Solano et al., 2004; Gómez & Lunt, 2007). Indeed, many endemic amphibian species are distributed close to past refugia and contribute to the accumulation of *gamma* diversity in southern

Europe (Sillero et al., 2014; Trakimas et al., 2016). Recently, Trakimas et al. (2016) linked life-history traits with range sizes in European amphibians, and demonstrated that only a few species in the assemblage possessed the specific combination of traits required for rapid range expansion and colonization after glacial periods. Our results may support this notion, suggesting that after speciation in isolation limited range movements have probably shaped amphibian distribution patterns in Europe (scenario 'c' of **Fig. 1.1**). Thus, we can reject scenario 'd' in **Fig. 1.1** where sympatry should occur with high levels of divergence.

Under such a scenario of allopatric/parapatric speciation and low dispersal ability, the available environmental space for incipient species may differ greatly from their ancestral niche. In such circumstances, the degree of climatic niche conservatism may influence the proportion of the environmental space that a species can occupy. Further, geographically restricted species with conserved niches may result more sensitive to environmental change. While the tendency of species to retain ancestral niches (i.e. PNC) seems widespread at global scales (Hof et al., 2010; Wiens et al., 2010), several studies have shown that some niche traits do not follow the predictions of PNC (Cooper et al., 2011; Olalla-Tárraga et al., 2011). Similarly, the European amphibian assemblage does not follow the expectations of PNC, as our results indicate that they do not show phylogenetic signal (**Table 1.1**), and that assemblage-level phylogenetic trees seem uncoupled from the evolutionary history of climatic niches (**Table 1.2**). Moreover, niche overlap values range from very limited to moderate. Yet, similar to other studies (Ahmadzadeh et al., 2013; Rato et al., 2015; Ahmadzadeh et al., 2016; Posso-Terranova & Andrés, 2016) pairwise comparisons of the multivariate climatic niche yielded mixed results of niche conservatism and divergence (**Fig. 1.4**; **Table S1.5**). This could explain why significant phylogenetic signal could not be detected in niche traits, and why phylogeny seemed uncoupled from niche evolution. These results did not change even with our most restrictive background definition, where we only considered as available environmental space that encountered within the biomes exploited by each species. Put together, these results show that some species exhibit climatic niche divergence from their ancestors, but others persisted in well restricted areas that closely resemble the ancestral niche. It should be pointed that several geographically restricted species (e.g. *Alytes cisternasii*, *Discoglossus galganoi*, *Rana graeca*, and *R. iberica*), show signs of a reduced tendency to evolve towards new climatic niches (**Table S1.5**). This, along with a limited dispersal ability, may increase the extinction risk of these species, distributed along southern Europe and closely related to the Mediterranean hotspot.

Further, DTT analyses suggest that rapid climatic shifts have influenced the amphibian diversification within Europe (**Fig. 1.5**), a conclusion also reported for the European avifauna (Pearman et al., 2014) and American *Oophaga* poisson frogs (Posso-Terranova & Andrés, 2016). Interestingly, our results also suggest that the degree of diversification varies across climatic niche traits (i.e., Pos1, Pos2, Bre1, and Bre2) and between clades (i.e. anurans and urodeles; **Table 1.3**), providing more insights to the possible mechanisms driving diversification. In the case of anurans, we detected higher levels of diversification in niche position and breadth (i.e. tolerance) along OMI2, which was correlated to minimum precipitation (**Table S1.3**). This may indicate that specialization with respect to water requirements and tolerance are the main drivers of the anuran diversity gradient found from northern Europe to the Mediterranean hotspot, in accordance with the observation that amphibians tend to become more specialised as they adapt to warmer and drier environments (Bonetti & Wiens, 2014). In the case of urodeles, we find higher levels of diversification in niche breadth along OMI1, which corresponds to the temperature gradient (**Table S1.3**). Indeed, geographically restricted, more specialized, species of European urodeles occur in southern Europe, which translates into a tendency towards higher diversity in areas where species show narrow niche breadths (Chejanovski & Wiens, 2014). Such a pattern may be associated to the higher diversification rates observed in areas with low geographical overlap, possibly as the consequence of allopatric speciation (Kozak & Wiens, 2010).

In conclusion, the results obtained through this study provide multifaceted insights into the biogeographic and evolutionary processes underlying current European amphibian biodiversity. Although some diversity patterns are congruent across the globe (e.g. latitudinal gradients of diversity), vast differences in diversity between continents and ecoregions suggest that region-specific geographical and evolutionary idiosyncrasies may be decisive (Qian & Ricklefs, 2000; Kozak & Wiens, 2010). In the case of European amphibian assemblage, allopatric or parapatric speciation may have played a central role (namely, scenario ‘c’ in **Fig. 1.1**). Similarly, temperate plants are more diverse in Asia compared to North America, most probably due to the abundant opportunity for allopatric speciation that Asian paleogeography provided (Qian & Ricklefs, 2000). Further, we show that speciation was followed by high levels of niche diversification and specialization in European amphibians. The diversification of water requirements and tolerances may have allowed the emergence and coexistence of anuran species in southern Europe. Urodele diversification arises from divergence in the tolerance ranges of temperature requirements. However, we want to highlight that many species showed signs of conserved macroclimatic niches. This, together with restricted geographic distributions and poor dispersal abilities, raises the possibility that global environmental change can have substantial effects on future distributions. Studies addressing species potential for niche evolution and behavioural buffering may shed additional light on the actual impact of global climate change. The mixed results of niche conservatism and divergence suggest that the formation of biodiversity may be triggered by a mosaic of both mechanisms, in line with other studies (Rato et al., 2015; Ahmadzadeh et al., 2016).

1.5. References

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Supplementary Material for Chapter 1

Table S1.1: Species list, abbreviation in Sillero et al. (2014) and number of occurrences.

Species	Code in Sillero et al. 2014	Occurrence nº
Anurans		
Alytes cisternasii	aly_cis	93
Alytes dickhilleni	aly_dick	20
Alytes obstetricans	aly_obs	477
Bombina bombina	bom_bom	783
Bombina variegata	bom_var	526
Bufo bufo	buf_buf	2254
Bufo calamita	buf_cal	968
Bufo viridis	buf_vir	1332
Discoglossus galganoi	dis_gal	210
Discoglossus sardus	dis_sar	26
Hyla arborea	hyl_arb	1117
Hyla intermedia	hyl_int	134
Hyla meridionalis	hyl_mer	201
Hyla sarda	hyl_sar	28
Pelobates cultripes	pelb_cul	253
Pelobates fuscus	pelb_fusc	432
Pelobates syriacus	perlb_syr	35
Pelophylax perezi	pelp_per	306
Pelophylax ridibundus	pelp_ribe	1443
Rana arvalis	ran_arv	1254
Rana dalmatina	ran_dal	614
Rana graeca	ran_grae	65
Rana iberica	ran_ibe	78
Rana italic	ran_ita	67
Rana latastei	ran_lat	29
Rana macrocnemis	ran_macro	67
Rana temporaria	ran_temp	1979
Urodeles		
Calotriton asper	cal_asp	28
Chioglossa lusitanica	chio_lus	40
Ichthyosaura alpestris	icht_alp	524
Lissotriton boscai	liss_bos	119
Lissotriton helveticus	liss_hel	546
Lissotriton italicus	liss_ital	39
Lissotriton montandoni	liss_mon	44
Lissotriton vulgaris	liss_vul	1691
Pleurodeles waltl	pleu_wal	166
Salamandra atra	sal_atr	62
Salamandra salamandra	sal_sal	833
Salamandrina perspisillata	sal_pete	58
Triturus cristatus	tri¬_cris	1368
Triturus marmoratus	tri_mar	332

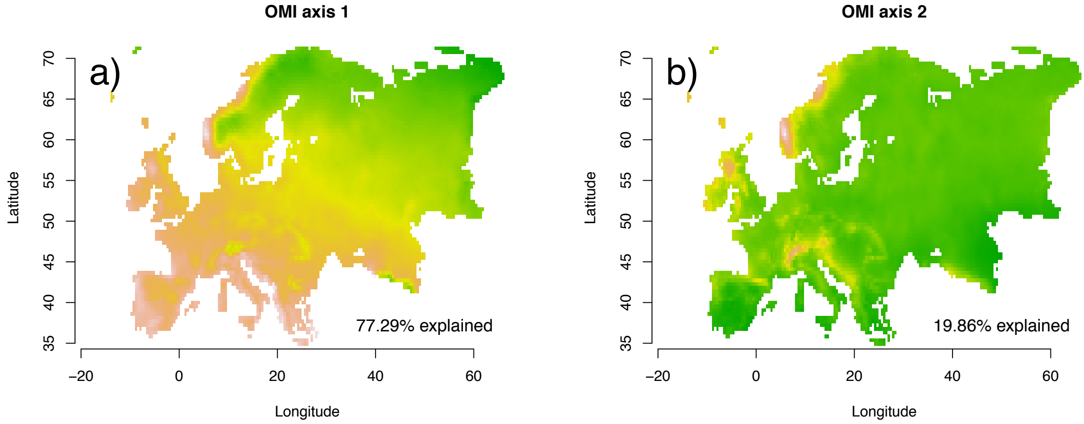


Figure S1.1: **a)** First and **b)** second OMI axes normed scores projected onto Europe.

Table S1.2: Pearson's correlation coefficient between included bioclimatic variables.

	Bio01	Bio05	Bio06	Bio12	Bio16	Bio17
Bio01	-					
Bio05	0.693	-				
Bio06	0.920	0.367	-			
Bio12	0.178	-0.324	0.395	-		
Bio16	0.185	-0.286	0.381	0.950	-	
Bio17	0.116	-0.352	0.327	0.882	0.713	-

Table S1.3: Each bioclimatic variable's normed scores on the first four OMI axes and the variance explained by those four OMI axes.

Variable	OMI 1	OMI 2	OMI 3	OMI 4
Bio01	-0.609	0.158	0.124	-0.048
Bio05	-0.366	0.461	0.126	0.709
Bio06	-0.625	-0.003	-0.054	-0.569
Bio12	-0.209	-0.435	-0.356	0.153
Bio16	-0.177	-0.295	-0.676	0.304
Bio17	-0.171	-0.698	0.619	0.236
% Variance	77.29	19.86	2.07	0.72

Table S1.4: The range of values (minimum – maximum) obtained across the replicate analyses run on the 100 phylogenetic hypotheses (Roquet et al. 2014) for the fit of evolutionary models.

	Pos1		Pos2		Bre1		Bre2	
	ΔAICc	Akaike weights	ΔAICc	Akaike weights	ΔAICc	Akaike weights	ΔAICc	Akaike weights
Amphibians								
WN	0.000 – 0.000	0.627 – 0.628	0.000 – 0.000	0.682 – 0.684	0.000 – 0.000	0.761 – 0.761	0.000 – 0.000	0.762 – 0.762
BM	23.323 – 23.377	5.266×10^{-6} – 5.406×10^{-6}	15.050 – 15.156	3.498×10^{-4} – 3.682×10^{-4}	40.466 – 40.503	1.220×10^{-9} – 1.243×10^{-9}	37.778 – 37.827	4.657×10^{-9} – 4.773×10^{-9}
OU	1.054 – 1.060	0.370 – 0.370	1.536 – 1.553	0.315 – 0.316	2.333 – 2.333	0.237 – 0.237	2.333 – 2.333	0.237 – 0.238
Kappa	10.854 – 10.891	2.707×10^{-3} – 2.759×10^{-3}	12.347 – 12.423	1.372×10^{-3} – 1.421×10^{-3}	12.189 – 12.202	1.706×10^{-3} – 1.717×10^{-3}	23.183 – 23.200	6.990×10^{-6} – 7.049×10^{-6}
Anurans								
WN	0.000 – 0.000	0.736 – 0.736	0.000 – 0.000	0.753 – 0.754	0.000 – 0.000	0.681 – 0.681	0.000 – 0.000	0.781 – 0.781
BM	16.598 – 16.634	1.799×10^{-4} – 1.832×10^{-4}	14.955 – 15.008	4.153×10^{-4} – 4.259×10^{-4}	11.772 – 11.800	1.865×10^{-3} – 1.891×10^{-3}	22.225 – 22.240	1.157×10^{-5} – 1.165×10^{-5}
OU	2.439 – 2.439	0.217 – 0.217	2.270 – 2.284	0.241 – 0.242	2.351 – 2.355	0.210 – 0.210	2.543 – 2.543	0.219 – 0.219
Kappa	5.543 – 5.543	0.046 – 0.046	10.134 – 10.134	4.744×10^{-3} – 4.753×10^{-3}	3.690 – 3.690	0.108 – 0.108	17.120 – 17.120	1.496×10^{-4} – 1.496×10^{-4}
Urodeles								
WN	0.000 – 0.000	0.551 – 0.552	0.000 – 0.000	0.711 – 0.713	0.000 – 0.000	0.838 – 0.838	0.000 – 0.000	0.818 – 0.818
BM	2.804 – 2.822	0.135 – 0.135	3.689 – 3.727	0.111 – 0.113	24.152 – 24.181	4.702×10^{-6} – 4.769×10^{-6}	12.740 – 12.779	1.374×10^{-3} – 1.401×10^{-3}
OU	1.306 – 1.309	0.287 – 0.287	3.060 – 3.063	0.154 – 0.154	3.309 – 3.309	0.160 – 0.160	3.309 – 3.309	0.156 – 0.156
Kappa	6.005 – 6.013	0.027 – 0.027	6.951 – 6.986	0.022 – 0.022	11.861 – 11.870	2.216×10^{-3} – 2.225×10^{-3}	7.061 – 7.087	0.024 – 0.024

Table S1.5: Niche similarity and equivalency tests following Broennimann et al (2012). Without '*': using all Europe as background. With '*': using Olson's biomes as background.

Comparison	Overlap	Overlap*	Equivalency	Similarity		Similarity*	
				Sp1 ► Sp2	Sp2 ► Sp1	Sp1 ► Sp2	Sp2 ► Sp1
Alytidae							
Alycis – Alydick	0.545	0.457	0.02	0.010	0.010	0.010	0.050
Alycis – Alyobs	0.318	0.442	0.02	0.089	0.089	0.010	0.010
Alycis – Disgal	0.625	0.654	0.02	0.010	0.010	0.010	0.010
Alycis – Dissar	0.342	0.296	0.02	0.050	0.050	0.416	0.445
Alydick – Alyobs	0.152	0.180	0.02	0.119	0.198	0.030	0.574
Alydick – Disgal	0.348	0.332	0.02	0.010	0.010	0.030	0.050
Alydick – Dissar	0.249	0.209	0.02	0.050	0.089	0.327	0.683
Alyobs – Disgal	0.554	0.557	0.02	0.010	0.010	0.040	0.010
Alyobs – Dissar	0.240	0.284	0.02	0.089	0.089	0.327	0.059
Disgal – Dissar	0.267	0.224	0.02	0.050	0.030	0.554	0.089
Bombinatoridae							
Bombom – bomvar	0.207	0.217	0.02	0.594	0.307	0.752	0.347
Bufonidae							
Bufbuf – bufcal	0.576	0.582	0.02	0.010	0.010	0.010	0.010
Hylidae							
Hylarb - hylint	0.473	0.522	0.02	0.020	0.010	0.010	0.010
Hylarb - hylmer	0.494	0.478	0.02	0.010	0.040	0.010	0.059
Hylarb - hylsar	0.180	0.221	0.02	0.297	0.188	0.247	0.050
Hylint - hylmer	0.587	0.568	0.02	0.010	0.010	0.010	0.010
Hylint - hylsar	0.207	0.202	0.02	0.079	0.040	0.277	0.119
Hylmer - hylsar	0.298	0.269	0.02	0.040	0.010	0.396	0.069
Pelobatidae							
Pelbcult – pelbfusc	0.192	0.200	0.02	0.307	0.238	0.020	0.376
Pelbcult – pelbsyr	0.369	0.170	0.02	0.050	0.050	0.010	0.129
Pelbfusc – pelbsyr	0.243	0.183	0.02	0.307	0.168	0.396	0.069
Ranidae							
Pelpper – pelpribe	0.358	0.372	0.02	0.010	0.050	0.010	0.099
Pelpper – ranarv	0.061	0.078	0.02	0.346	0.723	0.941	0.960
Pelpper – randal	0.398	0.351	0.02	0.020	0.238	0.010	0.089
Pelpper - rangrae	0.579	0.595	0.02	0.030	0.010	0.010	0.010

Table S1.5: Continued

Comparison	Overlap	Overlap*	Equivalency	Similarity		Similarity*	
				Sp1 ► Sp2	Sp2 ► Sp1	Sp1 ► Sp2	Sp2 ► Sp1
Pelpper - ranibe	0.644	0.626	0.02	0.010	0.010	0.010	0.010
Pelpper - ranita	0.298	0.281	0.02	0.059	0.089	0.129	0.040
Pelpper - ranlat	0.467	0.279	0.02	0.010	0.119	0.010	0.099
Pelpper - ranmacro	0.346	0.212	0.02	0.030	0.010	0.010	0.554
Pelpper - rantemp	0.139	0.145	0.02	0.099	0.911	0.168	0.554
Pelpribe - ranarv	0.420	0.414	0.02	0.218	0.238	0.060	0.257
Pelpribe - randal	0.548	0.549	0.02	0.040	0.010	0.010	0.010
Pelpribe - rangrae	0.525	0.536	0.02	0.050	0.010	0.010	0.010
Pelpribe - ranibe	0.338	0.371	0.02	0.267	0.010	0.010	0.010
Pelpribe - ranita	0.209	0.224	0.02	0.416	0.010	0.535	0.020
Pelpribe - ranlat	0.357	0.257	0.02	0.307	0.010	0.129	0.010
Pelpribe - ranmacro	0.508	0.278	0.02	0.010	0.010	0.010	0.020
Pelpribe - rantemp	0.414	0.418	0.02	0.099	0.050	0.406	0.030
Ranarv - randal	0.238	0.232	0.02	0.515	0.287	0.752	0.327
Ranarv - rangrae	0.162	0.179	0.02	0.802	0.446	0.495	0.792
Ranarv - ranibe	0.039	0.052	0.02	0.970	0.406	0.960	0.980
Ranarv - ranita	0.038	0.039	0.02	0.951	0.426	0.950	0.970
Ranarv - ranlat	0.107	0.090	0.02	0.851	0.376	0.921	0.782
Ranarv - ranmacro	0.215	0.146	0.02	0.376	0.653	0.792	0.406
Ranarv - rantemp	0.297	0.300	0.02	0.317	0.257	0.554	0.010
Randal - rangrae	0.577	0.497	0.02	0.020	0.010	0.475	0.010
Randal - ranibe	0.419	0.389	0.02	0.020	0.010	0.040	0.010
Randal - ranita	0.364	0.340	0.02	0.089	0.010	0.079	0.010
Randal - ranlat	0.594	0.436	0.02	0.010	0.010	0.010	0.010
Randal - ranmacro	0.387	0.240	0.02	0.168	0.139	0.050	0.673
Randal - rantemp	0.419	0.437	0.02	0.030	0.010	0.089	0.020
Rangrae - ranibe	0.538	0.535	0.02	0.010	0.010	0.010	0.010
Rangrae - ranita	0.415	0.365	0.02	0.010	0.020	0.030	0.188
Rangrae - ranlat	0.533	0.321	0.02	0.010	0.010	0.010	0.762
Rangrae - ranmacro	0.431	0.281	0.02	0.030	0.010	0.010	0.010
Rangrae - rantemp	0.287	0.271	0.02	0.030	0.040	0.099	0.703

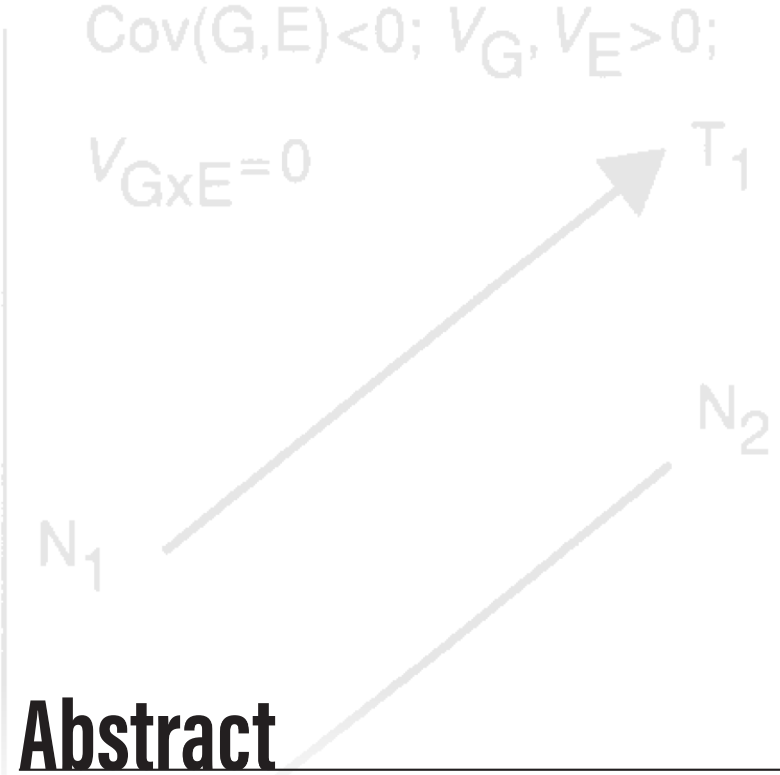
Table S1.5: Continued

Comparison	Overlap	Overlap*	Equivalency	Similarity		Similarity*	
				Sp1 ► Sp2	Sp2 ► Sp1	Sp1 ► Sp2	Sp2 ► Sp1
Ranibe - ranita	0.273	0.298	0.02	0.020	0.059	0.050	0.099
Ranibe - ranlat	0.580	0.384	0.02	0.010	0.010	0.010	0.030
Ranibe - ranmacro	0.283	0.158	0.02	0.030	0.109	0.020	0.287
Ranibe - rantemp	0.126	0.125	0.02	0.089	0.198	0.099	0.208
Ranita - ranlat	0.307	0.437	0.02	0.069	0.040	0.020	0.030
Ranita - ranmacro	0.156	0.154	0.02	0.050	0.406	0.099	0.228
Ranita - rantemp	0.085	0.084	0.02	0.109	0.673	0.148	0.851
Ranlat - ranmacro	0.313	0.215	0.02	0.109	0.020	0.020	0.356
Ranlat - rantemp	0.265	0.213	0.02	0.010	0.059	0.050	0.257
Ranmacro - rantemp	0.373	0.181	0.02	0.030	0.644	0.673	0.040
Lissotriton							
Lissbos - lisshel	0.301	0.284	0.02	0.030	0.426	0.059	0.247
Lissbos - lissital	0.269	0.246	0.02	0.059	0.168	0.762	0.119
Lissbos - lissmon	0.005	0.005	0.02	0.624	0.851	0.842	0.990
Lissbos - lissvul	0.200	0.201	0.02	0.020	0.752	0.069	0.525
Lisshel - lissital	0.177	0.303	0.02	0.188	0.099	0.050	0.109
Lisshel - lissmon	0.105	0.093	0.02	0.238	0.327	0.673	0.663
Lisshel - lissvul	0.555	0.567	0.02	0.010	0.010	0.010	0.010
Lissital - lissmon	0.010	0.060	0.02	0.515	0.683	0.683	0.030
Lissital - lissvul	0.163	0.247	0.02	0.069	0.396	0.010	0.020
Lissmon - lissvul	0.189	0.174	0.02	0.010	0.099	0.010	0.148
Salamandra							
Salatr - salsal	0.390	0.302	0.02	0.079	0.050	0.198	0.020
Triturus							
Tricris - trimar	0.315	0.304	0.02	0.129	0.010	0.366	0.010

CHAPTER 2

Local adaptation in response to eco-climatic factors along macro- and micro-geographical gradients: can spatial scale prevent convergence?

URTZI ENRIQUEZ-URZELAI - ALFREDO G. NICIEZA



SOURCE: BOX 2 IN CONOVER AND SCHULTZ 1995

Abstract

Within a species, phenotypes vary geographically, depending on the environment, genetic variation, phenotypic plasticity, and their interconnections. Since physiological functions decrease at lower temperatures, at higher latitudes and altitudes, populations of widespread species should show compensatory mechanisms. However, not only temperature changes with latitude or altitude: at the cold extreme of these clines species are also subjected to severe temporal constraints. Further, the spatial scale at which altitudinal and latitudinal clines occur differ. While latitudinal clines involve highly isolated populations, oftentimes belonging to different lineages, altitudinal clines occur over much smaller distances, where gene flow cannot be completely excluded. Here, we study the phenotypic variation associated to altitude and latitude in tadpoles of the European common frog (*Rana temporaria*) to assess the potential role of convergent evolution driven by eco-climatic factors (i.e. temperature conditions and temporal constraints). Our results show that larval growth rates, developmental rates, size at metamorphosis, and their plasticity vary along latitude and altitude in *R. temporaria*. Notably, we observed signs of countergradient variation in developmental rates – higher physiological rates at unfavourable environments – associated to shorter time windows at higher latitudes and altitudes. Alternatively, growth rates decreased with latitude and correlated with thermal variation. Furthermore, plasticity increased towards both extremes of the latitudinal gradient, but decreased with altitude. Although we observed signs of convergent evolution between latitudinal and altitudinal clines in *R. temporaria* (mainly driven by shorter growing seasons), we show that the phenotypic consequences of altitudinal clines do not perfectly mirror the effects of latitudinal clines. We argue that thermal conditions, temporal constraints, and other idiosyncratic factors (phylogeographic structure or dispersal abilities) influence microevolutionary processes, ultimately, yielding disparate patterns of phenotypic variation along different environmental clines.

2.1. Introduction

Patterns of geographic phenotypic variation result from complex relationships between genotypes and environments (Aitken & Whitlock, 2013). Phenotypes depend on the effects of the environment, genetic variation, phenotypic plasticity, and their interconnections. Ultimately, the partial contributions of each of these factors are responsible for the observable patterns of geographic variation along environmental gradients (Conover & Schultz, 1995). Latitudinal and altitudinal gradients are coupled with profound environmental changes and contrasting thermal environments are amongst the most striking environmental correlates of geographical clines (Sunday et al., 2011; 2014). From a macroclimatic perspective, higher latitudes and altitudes are characterized by relatively low temperatures and high seasonal and daily variation, but also short growing seasons (Angilletta, 2009; Sunday et al., 2011). This can impose strong physiological stress, severe temporal constraints, or both, and therefore may act as a major selective agent promoting intraspecific (e.g. population level) divergence in behaviour, physiology, and life-history (Schmidt et al., 2005; Moran et al., 2016).

Since physiological functions decrease at lower temperatures, in the absence of adaptive compensatory mechanisms, growth and developmental rates should decrease towards higher latitudes and altitudes (i.e. colder environments; Hertz et al., 1983; Huey & Kingsolver, 1989; Oufiero & Angilletta, 2006). Moreover, any potential adaptive responses is likely to be masked by the environmental forces operating along these gradients, so that the realized performance usually decreases with increasing latitude and altitude. Even so, it is expected that populations under strong physiological or temporal constraints have evolved to counteract environmental pressure. Two main hypotheses have been issued to explain geographic variation in thermal related traits. Local adaptation to prevailing temperatures (Levinton 1983, Lonsdale and Levinton 1985, 1989), although tempting, has received mixed support (Angilletta, 2009; Laiolo & Obeso, 2015; Drakulić et al., 2016). Alternatively, the countergradient variation (CgV) hypothesis (Levins, 1969) states that populations living in colder environments (i.e., high altitude or latitude) will outperform those from warmer environments over a wide range of conditions. CgV was postulated in relation to environmental harshness in general, but it has received support from a number of studies analysing geographic variation in developmental rates or growth potential along altitudinal or latitudinal clines (Berven et al. 1979; Nicieza et al. 1994; Conover & Schultz, 1995; Oufiero & Angilletta, 2006; Iraeta et al., 2006; Orizaola et al., 2013; but see Jensen et al., 2000). In fact, CgV can be more of a response to the occurrence of eco-temporal constraints (Orizaola et al. 2012, 2013) than a way of response to thermal variation in itself. This makes sense because seasonal cold, northern/highland environments have also shorter growing seasons than warmer, southern/lowland environments. Therefore, shorter temporal windows and lower mean temperatures for growth and development at higher latitudes and altitudes can result in strong selection for genotypes developing and growing faster (thus compensating for a low growth opportunity), which may favour higher intrinsic growth- and developmental-rates (Laugen et al., 2003b; Dahl et al., 2012).

Because temporal variability in thermal conditions changes drastically, the potential for phenotypic plasticity also varies along environmental gradients (Valladares et al., 2014; Seebacher et al., 2015). Most often, high thermal variability (e.g. towards higher latitudes and altitudes) promotes high plasticity (Hendry, 2015; but see Condon et al., 2014). This can be important because high levels of plasticity in some traits can hamper further microevolution of traits directly involved in fitness. However, this is more likely to affect the tolerance limits than the temperatures for maximum performance.

Due to the parallel changes in temperature and growing seasons, latitudinal and altitudinal clines could promote convergent evolution (Lencioni, 2004). However, despite they can incorporate common temporal constraints, these two geographic clines may not be as similar as previously thought. Indeed, many environmental factors change dissimilarly with latitude and altitude (e.g. temporal patterns of radiation or short term thermal variation; see Angilletta, 2009; Pitchers et al., 2013; Buckley et al., 2013). In addition, constraints for local adaptation are expected to differ between latitude and elevation gradients. Latitudinal changes involve long distances, so that populations facing contrasting climatic and thermal conditions are often isolated (i.e., due to barriers or IBD). In contrast, thermal and climatic conditions can shift rapidly with elevation. In the gradient extremes, local populations can be exposed to very different pressures, but high levels of gene flow might prevent any form of local adaptation (Allendorf & Luikart 2007). Finally, latitudinal differentiation of populations often occurs over a large spatial scale where hierarchical genetic structure is more likely than in the case of altitudinal differentiation, which usually proceed over a relatively small spatial scale where historical divergence (e.g., lineage separation) can be uncommon.

The European common frog (*Rana temporaria*) is one of the most widespread amphibian species in Europe, spanning from northern Spain to northern Scandinavia, and from sea level to up to 3000 m.a.s.l. (**Fig. 2.1**; García-París et al., 2004). Thus, it may face similar time constraints at the extremes of both altitudinal and latitudinal clines, although environmental conditions may differ during activity periods. Here we conducted two linked, common garden experiments to study the geographic variation associated to altitude and latitude, and to assess the potential convergence in life history traits derived from similar time constraints. More specifically, we hypothesise that 1) populations exposed to short growing seasons will exhibit faster growth and developmental rates as an adaptive response to strong temporal constraints, and therefore 2) response patterns in altitude and latitude clines will converge at the upper extremes, but 3) convergence can be hampered by a lack of adaptive response in the altitudinal gradient derived from reduced spatial distances between populations and high levels of gene flow.

2.2. Materials and Methods

We conducted two experiments to test hypotheses of convergence and local adaptation. In the first experiment (LATITUDE), we compared the thermal performance of six populations distributed along a latitudinal cline. We selected two populations from northern Sweden, two from central Sweden, and two from northern Spain (**Fig. 2.1A**). The Spanish populations originated from high altitudes, and thus face similar time constraints

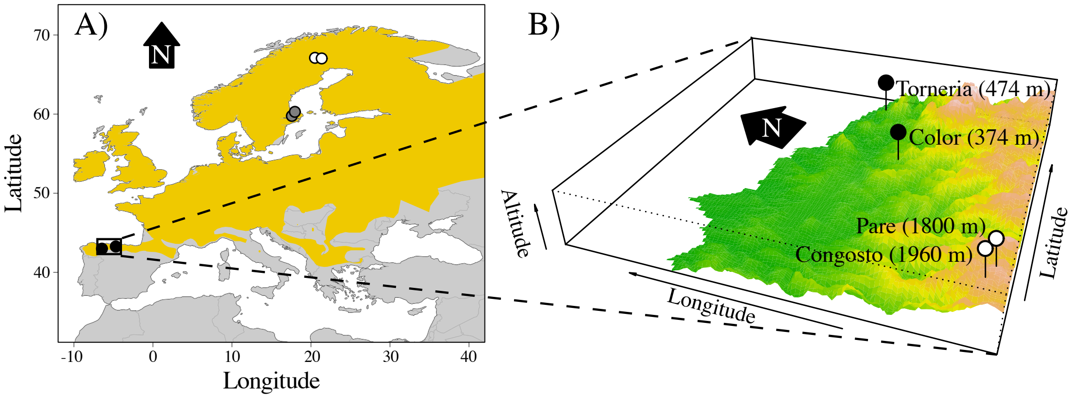


Figure 2.1: **(A)** Location of *Rana temporaria* populations included in the experiment regarding latitudinal clines (*white*: northernmost Sweden populations, *gray*: central Sweden populations, *black*: southernmost populations, *yellow polygon*: distribution of the species obtained from IUCN) and **(B)** populations included in the experiment regarding altitudinal clines (*white*: high altitude, *black*: low altitude populations).

Table 2.1: General information of the selected populations. Names, abbreviation codes, number of the experiment in which the population was involved (1 = LATITUDE; 2 = ALTITUDE), coordinates, altitude in metres, collection date, and growth season length (GSL) are given for each population. We measured growth season length as the number of months with mean temperature above 5 (according to the WoldClim layers; Hijmans et al., 2005). Further, we present descriptive thermal parameters (*Tmean*: mean, *Tmax*: maximum, *Tmin*: minimum, and *max⊿T*: maximum monthly variation) during the period when tadpoles are present at each locality (A. G. Nicieza and G. Orizaola, personal observation).

Population	Code	Exp.	Coordinates	Alt. (m)	Collection date	GSL	*Tmean*	*Tmax*	*Tmin*	*max⊿T*
Leipojärvi	LEI	1	67° 03’N, 21° 13’E	268	June 2014	4	10.17	18.29	-0.18	9.31
Dundrets	DUN	1	67° 06’N, 20° 39’E	432	June 2014	4	9.61	17.70	-0.61	9.39
Österbybruk	OST	1	60° 11’N, 17° 51’E	36	April 2014	6	6.70	14.68	-0.14	10.20
Uppsala	UPP	1	59° 50’N, 17° 30’E	30	April 2014	6	6.97	14.99	0.10	10.30
Llagusecu	LLA	1	43° 13’N, 4° 59’W	1910	June 2014	4	10.95	18.48	3.13	13.23
Paré del agua	PAR	1-2	43° 2’N, 6° 9’W	1800	May 2014-2015	6	10.08	20.08	1.16	13.46
Congosto	CON	2	43° 1’N, 6° 6’W	1960	June 2015	6	11.60	19.36	3.62	13.44
Tornería	TOR	2	43° 23’N, 4° 49’W	474	January 2015	12	10.05	21.05	3.13	8.68
Color	COL	2	43° 17’N, 5° 16’W	374	January 2015	12	9.42	21.16	2.08	9.66

but higher maximum temperatures and wider thermal variations than the Scandinavian populations (**Table 2.1**). In the second experiment (ALTITUDE), we used four populations from the extremes of an altitudinal cline in the Cantabrian Range (**Fig. 2.1B**). At the extremes of this elevation range, mean, maximum, and minimum temperatures are similar during the larval growing season, but in high altitudes thermal variation is larger and growing season is much shorter (**Table 2.1**).

During 2014 (LATITUDE experiment) and 2015 (ALTITUDE experiment), we collected seven to ten freshly laid egg-clutches in each population and transported them to the facilities of the Research Unit of Biodiversity (University of Oviedo-CSIC-PA, Mieres). We reared egg clutches in a constant temperature room set at 14 ± 1°C. When the embryos hatched, we removed the egg jelly from clutches and we randomly distributed tadpoles from different clutches. We placed each group at similar densities in 4 to 6 10-L plastic containers with dechlorinated tap water and we made partial water changes every fourth day. When tadpoles reached developmental stage 25 (feeding and free-swimming larvae; Gosner, 1960), we individually placed them in a rack system of 0.8-L PMMA (Poly(methyl methacrylate)) containers. We randomly selected 120 tadpoles from each population for LATITUDE, and 80 tadpoles for the ALTITUDE experiment. We reared each half of the tadpoles (60 or 40) at 13 ºC or 18ºC. A constant water-flow system renewed the water in the PMMA containers and we kept rearing temperatures constant using water coolers (± 0.5ºC; Teco TC20). The photoperiod was set to 12:12 h light/dark and we fed larvae ad libitum with rabbit chow (Nanta S.A.; 18,5% crude fiber, 16% protein, 4.8% lipids) throughout the experiments.

To explore patterns in growth rates, we weighed tadpoles at Gosner stage 27-28 (day 0) and then at days 16, 23, and 30 (LATITUDE) or 7, 14, and 21 (ALTITUDE) to the nearest 0.0001 g (Mettler Toledo PL83-S) after gently removing the excess of water. We calculated daily growth rates (Δw) as the increase in mass from day 0 to 14-16, divided by the number of days: $\Delta w = (w_i - w_0) / (t_i - t_0)$; we selected this time interval because developmental differentiation is minimal during this period and therefore we can exclude potential developmental noise affecting growth rates.

We used the length of the larval period as a proxy for developmental rates. When tadpoles approached the start of metamorphosis (Gosner stage 42) we checked all the containers daily and recorded the day of forelimb emergence (stage 42). We computed larval period as the number of days from developmental stage 27-28 to stage 42. Further, we weighed metamorphic individuals (Gosner stage 42) to the nearest 0.001 g to analyse the potential effects of geographic origin and rearing temperatures in body size at metamorphosis.

We examined the effects of macro and micro-geographical gradients (latitude and altitude, respectively) and rearing temperatures on growth-rates, larval periods, and size at metamorphosis by mixed effects model ANCOVAs. We included initial body size (weight at day 0) as a covariate and latitude/altitude, rearing temperature, and their interaction as fixed effects. We included population of origin (nested within latitude/altitude) as a random factor. If interactions between factors were nonsignificant, we reduced the model excluding interactions. Slopes homogeneity was tested with the full model including the covariate by effect interaction. Alternatively, when these interactions were significant, we explored the effects of latitude/altitude and initial body size within each temperature treatment separately (see **Supplementary Material**). We used the 'nlme' R-package (v. 3.1-127; Pinheiro et al., 2016) to fit mixed effects models using maximum likelihood estimations and type III sum of squares. We visually inspected normality and homogeneity of variances on model residuals. To reduce potential bias derived from variance heterogeneity, we used a heterogeneous variance model (*varIdent* in 'nlme'). When we observed indications of potential deviations from normality we log-transformed the dependent variables.

To explore potential adaptations to local conditions, we used Pearson's correlations between population-specific mean life history traits (growth rate, larval period, and weight at metamorphosis) and local thermal descriptors. We obtained different thermal parameters during the months of occurrence of tadpoles in the ponds (A. G. Nicieza and G. Orizaola, personal observation). We downloaded maximum (T_{max}), minimum (T_{min}), and mean air temperature (T_{mean}) layers at a resolution of ~1 km^2 from the WorldClim database (Hijmans et al., 2005) and extracted the values within a buffer (5 km radius) around each population's coordinates. We computed the average T_{mean}, maximum T_{max}, minimum T_{min}, and maximum monthly thermal variation ($max\Delta T$ = max [T_{max} (month) – T_{min} (month)]) during tadpole's activity periods. Although these layers represent air temperatures, correlate well with water temperatures in the ponds *R. temporaria* uses for breeding (Gutiérrez-Pesquera et al., 2016).

2.3. Results

LATITUDINAL CLINE

We found differences in the plasticity of growth-rates and larval periods along the latitudinal cline (significant latitude × temperature interaction; **Table 2. 2**). Apparently, populations at the extremes of the latitudinal cline (i.e. the northernmost and southernmost populations) showed greater plasticity than populations from central Sweden (**Fig. 2.1**-**2.2**). Due to significant interactions, we explored the effects of body size and latitude within each temperature treatment separately. We only found differences in growth rates across latitudes at 18 °C (**Table S2.1**; **Fig. 2.2A**). Although initial size also affected subsequent growth, at 18 °C southernmost (Spanish) populations grew unequivocally faster than the rest of populations (**Fig. S2.1**). Similarly, we only found differences in larval periods at 18 °C (**Table S2.2**; **Fig. 2.2B**). The two populations from central Sweden showed the longest larval periods, followed by northernmost populations, and reaching to the shortest larval periods in southernmost populations (Tuckey's HSD; $P < 0.006$ in all cases; **Fig. 2.2B**).

Latitude, rearing temperature, and initial body size affected size at metamorphosis (**Table 2.2**, **S2.3**). In fact, body mass at metamorphosis differed among all latitudes (Tukey's

HSD; $P < 0.001$ in all cases). The froglets from central Sweden were the largest (mean ± sd: 492.99 ± 155.67), followed by those from northern Sweden (mean ± sd: 355.32 ± 127.06). The froglets from the two southern populations had the smallest sizes (mean mass ± sd: 307.22 ± 67.92). Regardless of latitude, froglets were consistently smaller at warmer rearing temperatures (mean ± sd at 13°C: 445.19 ± 133.82; at 18°C: 321.72 ± 118.75).

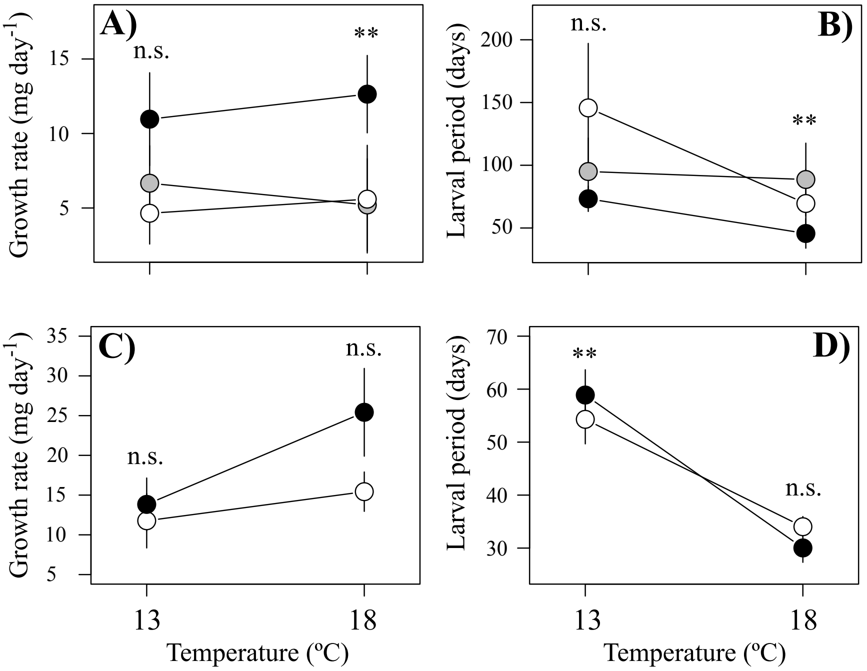


Figure 2.2: Mean ± SE of **(A)** growth rates and **(B)** developmental rates at 13 and 18°C of populations across latitudes (*white*: northernmost Sweden populations, *gray*: central Sweden populations, *black*: southernmost populations), and **(C)** growth rates and **(D)** developmental rates of populations distributed along an altitudinal gradient (*white*: high altitude, *black*: low altitude populations).

Table 2.2: Mixed effect model ANCOVA for growth rates, larval periods, and weight at metamorphosis including weight (covariate), latitude, temperature, and their interactions. *ndf*: numerator's degrees of freedom, *ddf*: denominator's degrees of freedom.

	Growth rate		Larval period		Weight at metamorphosis	
	F (ndf, ddf)	P	F (ndf, ddf)	P	F (ndf, ddf)	P
Weight (W)	6.725 (1, 282)	0.010	99.829 (1, 282)	< 0.001	33.94 (1, 282)	< 0.001
Latitude	8.115 (2, 3)	0.062	78.933 (2, 3)	0.003	65.20 (2, 3)	0.003
Temperature	2.648 (1, 282)	0.105	309.145 (1, 282)	< 0.001	126.98 (1, 282)	< 0.001
W × Latitude	3.720 (2, 282)	0.025	0.361 (2, 282)	0.697	0.35 (2, 282)	0.707
W × Temperature	13.557 (1, 282)	< 0.001	18.638 (1, 282)	< 0.001	1.41 (1, 282)	0.235
Latitude × Temperature	5.347 (2, 282)	0.005	25.067 (2, 282)	< 0.001	0.43 (2, 282)	0.652
W × Latitude × Temperature	4.770 (2, 282)	0.009	0.429 (2, 282)	0.652	0.35 (2, 282)	0.708

Table 2.3: Mixed effect model ANCOVA for growth rates, larval periods, and weight at metamorphosis including weight (covariate), altitude, temperature, and their interactions. *ndf*: numerator's degrees of freedom, *ddf*: denominator's degrees of freedom.

	Growth rate		Larval period		Weight at metamorphosis	
	F (ndf, ddf)	P	F (ndf, ddf)	P	F (ndf, ddf)	P
Weight (W)	97.858 (1, 123)	< 0.001	193.25 (1, 123)	< 0.001	55.006 (1, 123)	< 0.001
Altitude	6.319 (1, 2)	0.129	88.25 (1, 2)	0.011	0.097 (1, 2)	0.785
Temperature	99.428 (1,123)	< 0.001	2596.97 (1, 123)	< 0.001	22.238 (1, 123)	< 0.001
W × Altitude	6.712 (1, 123)	0.011	3.77 (1, 123)	0.055	0.646 (1, 123)	0.423
W × Temperature	2.925 (1, 123)	0.090	44.65 (1, 123)	< 0.001	48.732 (1, 123)	< 0.001
Altitude × Temperature	14.941 (1, 123)	< 0.001	11.19 (1, 123)	0.001	4.590 (1, 123)	0.034
W × Altitude × Temperature	1.673 (1, 123)	0.198	2.65 (1, 123)	0.106	4.288 (1, 123)	0.041

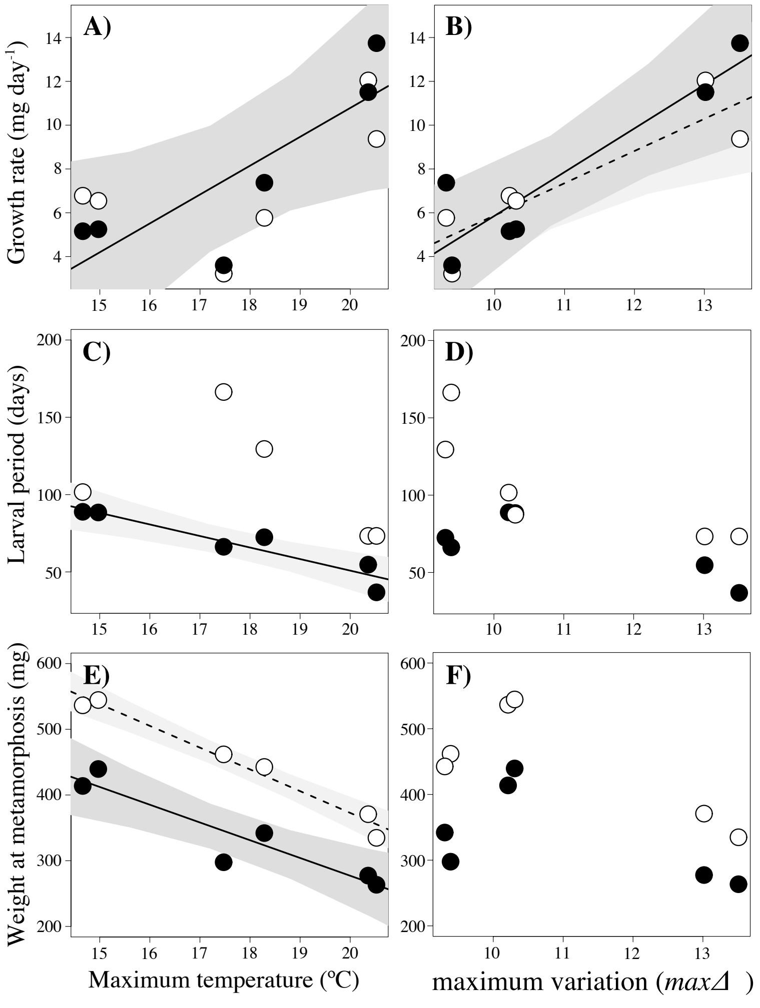


Figure 2.3: Relationships between **(A-B)** growth rates, **(C-D)** larval periods, and **(E-F)** weight at metamorphosis and two environmental correlates (*Tmax* and *max∆T*) (*White* dots represent mean values of individuals reared at 13 º C and *Black* dots mean values at 18ºC). Only significant correlations are plotted. (*Dashed lines* and *light gray polygons*: at 13ºC; *Solid lines* and *dark gray polygons*: at 18ºC)

Table 2.4: Pearson's correlations between life history traits (growth rates, larval periods, and weight at metamorphosis at 13 and 18ºC) and environmental correlates. Significant values (p<0,05) are indicated in bold.

	Tmean	*Tmax*	*Tmin*	*max∆T*
GR_{13}	0.60	0.57	0.93	0.88
GR_{18}	0.70	0.83	0.73	0.91
LP_{13}	-0.19	-0.25	-0.67	-0.80
LP_{18}	-0.78	-0.93	-0.53	-0.76
$W42_{13}$	-0.87	-0.98	-0.63	-0.75
$W42_{18}$	-0.86	-0.93	-0.50	-0.56

ALTITUDINAL CLINE

We also found differences in the plasticity of growth rates, larval periods, and size at metamorphosis across altitudes (significant altitude × temperature interaction; **Table 2.3**). Lowland populations showed greater plasticity than high altitude populations (**Fig. 2.2C-D**). Due to significant interactions, we explored the effects of body size and altitude within each temperature treatment separately. We found no differences in growth across altitudes regardless of temperature (**Table S2.4**; **Fig. 2.2C**). We only detected differences in larval periods at 13 ºC (**Table S2.5**). Although size interacted with altitude, we observed shorter larval periods in high altitude populations compared to lowland conspecifics (**Fig. 2.2D**, **S2.2**). Body size at metamorphosis did not differ between altitudes (**Table 2.3**, **S2.6**).

ENVIRONMENTAL CORRELATES

Growth rates at both rearing temperatures positively correlated with maximum monthly thermal variation (*maxΔT*; **Table 2.4**; **Fig. 2.3B**). Further, growth rates at 18 and 13ºC positively correlated with maximum (T_{max}; **Fig. 2.3A**) and minimum temperatures (T_{min}) respectively. We only found a negative correlation between larval periods at 18ºC and T_{max} (**Fig. 2.3C-D**). Weight at metamorphosis at both rearing temperatures negatively correlated to T_{mean} and T_{max}, but not with *maxΔT* (**Table 2.4**, **Fig. 2.3E-F**).

2.4. Discussion

Geographical gradients often promote intraspecific phenotypic divergence (Laugen et al., 2003a; Oufiero & Angilletta, 2006; Iraeta et al., 2006). Our results show that key life-history traits (i.e. larval growth and developmental rates, size at metamorphosis, and plasticity in all these traits) vary along latitude and altitude in *Rana temporaria* in a complex way. Apparently, populations of *R. temporaria* have evolved strategies to cope with divergent thermal landscapes and temporal constraints during larval development (Palo et al., 2003; Dahl et al., 2012; Orizaola et al., 2013). Remarkably, we observed signs of countergradient variation (CgV) in developmental rates, and increased plasticity in growth and developmental rates towards the extremes of geographic clines. We argue that this pattern of convergent evolution emerged mainly as a response to shorter growing seasons at higher latitudes and elevations. However, the phenotypic consequences of the altitudinal cline did not recapitulate the effects of the latitudinal cline studied here. It is likely that the spatial scale at which latitudinal and altitudinal gradients occur, shape the response of populations of *R. temporaria* to environmental gradients and prevent complete convergence. Also, we cannot discard the existence of genetic differences associated with the large historical differentiation of the Spanish populations from the rest of the *R. temporaria* populations across Europe (see Vences et al. 2013, 2017).

At higher latitudes and elevations, shared shorter growing seasons and lower mean temperatures may favour genotypes that optimize resources and opportunities for growth and development (Laugen et al., 2003b; Liess et al., 2015). In line with this expectation, we found that at the warm temperature treatment (i.e. 18ºC), tadpoles from populations with severe time restrictions (i.e. northern Sweden and southernmost populations from high altitudes) showed higher intrinsic developmental rates than central Sweden populations (Laugen et al., 2003b). In addition, in the altitudinal cline at the southern distribution limit of the common frog, high altitude populations developed faster at the cold treatment (i.e. 13ºC) than lowland counterparts, in agreement with previous studies (Choda, 2014). These results suggest that convergent evolution leads to CgV in developmental rates as a response to shorter growth seasons both in latitudinal and altitudinal clines.

Growth rates, however, showed a different geographic pattern. Strikingly, we did not find any difference in growth rates linked to altitudinal clines, although it has been previously reported in numerous species (Oufiero & Angilletta, 2006) including among populations of this species distributed along altitudinal clines (Muir et al., 2014a; 2014b; Oromí et al., 2015). Oppositely, in the latitudinal cline, southernmost populations grew faster than central and northern Sweden populations at high rearing temperatures. This pattern conforms to the cogradient variation – higher intrinsic rates in populations from warmer environments (Conover & Schultz, 1995; Conover et al., 2009)–, and matches the findings of Palo et al. (2003), who also observed CgV in developmental rates but not growth rates in tadpoles from a latitudinal gradient in Scandinavia. Thus, factors other than temporal constraints may be the proximate drivers of the observed patterns of growth rate variation (Palo et al., 2003). In fact, growth rates correlated well with maximum thermal variation. It is worth mentioning that thermal variability increases both with latitude and altitude. Yet, growth rates appeared associated to thermal variation at our latitudinal gradient but did not vary along the altitudinal gradient at southern Europe. We attribute this difference to the spatial scale at which environmental gradients occur in latitudinal and altitudinal clines: while at large scales (e.g. latitudinal cline), populations belong to well differentiated and isolated lineages (Vences et al., 2013; 2017), at finer scales (e.g. altitudinal cline) gene flow could prevent local adaptation (but see Richter-Boix et al., 2010; Muir et al., 2014b). Hence, we hypothesize that the spatial scale, involving phylogeographic and dispersal factors that dictate the genetic constitution and gene flow between populations, can rule adaptive responses in *R. temporaria*.

Similar to the pattern found for developmental rates, plasticity in growth and developmental rates resulted higher at the extremes of the latitudinal cline (i.e. in northern Sweden and sourthermonst, high altitude populations), as already demonstrated by Orizaola & Laurila (2016) in other European frog species (i.e. *Pelophylax lessonae*). As discussed for the pattern of CgV in developmental rates, this result indicates that temporal constraints may impose severe selective pressures on the phenotype of tadpoles regarding development and growth strategies, apparently leading to convergent evolution. Seemingly, not only do populations distributed along environmental clines diverge in the average value of phenotypes, but also in their thermal sensitivity (i.e. plasticity; Hereford, 2009; Benito Garzón et al., 2011; Valladares et al., 2014). Nonetheless, this trend did not mirror in the altitudinal cline, where we found higher potential for plasticity at low altitudes (i.e. warmer environments). Other studies have also reported higher ability for plastic responses in populations from warm compared to cold environments (Kelly et al., 2012; Choda, 2014; Seebacher et al., 2015). The higher plasticity found in lowland populations, could be an adaptive response to avoid the extremely hot temperatures that lowland populations are subjected to, especially towards the end of larval development (Newman, 1989; Reques & Tejedo, 1995). As a none exclusive explanation, the ‘spatial-scale effect’ invoked for geographical patterns of growth rates could also underpin the difference in plasticity recorded along latitudinal and altitudinal clines.

In combination, our results highlight that geographical patterns of phenotypic variation might be synergistically shaped by thermal conditions, temporal constraints, and several other factors (e.g. historical or dispersal factors). Further, these factors may exert divergent selective pressures on different traits (e.g. developmental vs growth rates and the potential for plasticity). As a result of discordant patterns of geographic variation of growth and developmental rates, an interesting pattern of size at metamorphosis emerged at the latitudinal cline: size at metamorphosis peaked in central Sweden populations, followed by northern Sweden, and reaching to the minimum sizes at metamorphosis in southernmost populations. This pattern resembled the concave pattern of size at metamorphosis reported for Fennoscandia populations (Palo et al., 2003; Laugen et al., 2005). According to Laugen et al. (2005), initial differences in body sizes at metamorphosis have lasting effects in latitudinal patterns of body size variation, and they showed that size at metamorphosis and, accordingly adult body size, was maximal at central Sweden, decreasing towards lower and higher latitudes in *R. temporaria*. Most likely, size at metamorphosis is optimized to maximize growth and minimize mortality in both aquatic ant terrestrial habitats (Wilbur & Collins, 1973; Werner, 1986; Enriquez-Urzelai et al., 2013). Thus, we believe that increased size at metamorphosis (mainly due to longer larval periods) reflects that aquatic habitats offer more beneficial conditions for growth than terrestrial habitats with increasing latitude, while terrestrial growth might be more feasible at lower latitudes. At a certain point however (i.e. at intermediate latitudes in Sweden), this tendency gets inverted. Probably, strict temporal constraints at northernmost latitudes make faster development at expenses of lower body sizes at metamorphosis a better strategy to successfully complete the lifecycle. Although body sizes are expected to increase with increasing latitude following Bergmann's rule (Olalla-Tárraga & Rodríguez, 2007; Gouveia & Correia, 2016), we show that complex interactions between thermal conditions, temporal constraints, and historical contingencies might obscure or modify this pattern.

Geographic gradients can impose selective pressures at their extremes, which sometimes lead to predictable, convergent phenotypic evolution (Huey et al., 2000). However, we show that phenotypes do not vary as a linear function along geographic clines in *R. temporaria*, as already shown by previous studies (Palo et al., 2003; Laugen et al., 2005; Lindgren & Laurila, 2009). Further, we demonstrate that altitudinal clines do not replicate exactly the phenotypic consequences of latitudinal clines. Notably, we stress in the potential role of spatial scale to determining the outcome of the selective pressures imposed by latitudinal or altitudinal clines. Thus, we propose that other environmental correlates (i.e. temporal constraints) and idiosyncratic factors (phylogeographic structure or dispersal abilities) may influence microevolutionary processes, ultimately, yielding disparate patterns of phenotypic variation along different environmental clines (Pitchers et al., 2013).

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Supplementary Material for Chapter 2

Table S2.1: Mixed effect ANCOVA for growth rates at each temperature treatment separately (i.e. 13 and 18ºC) including weight (covariate), latitude, and their interaction. *ndf*: numerator's degrees of freedom, *ddf*: denominator's degrees of freedom.

	13ºC		18ºC	
	F (ndf, ddf)	P	F (ndf, ddf)	P
Weight (W)	16.666 (1, 115)	< 0.001	4.195 (1, 164)	0.042
Latitude	3.335 (2, 3)	0.173	19.117 (2, 3)	0.020
W × Latitude	2.099 (2, 115)	0.127	4.012 (2, 164)	0.020

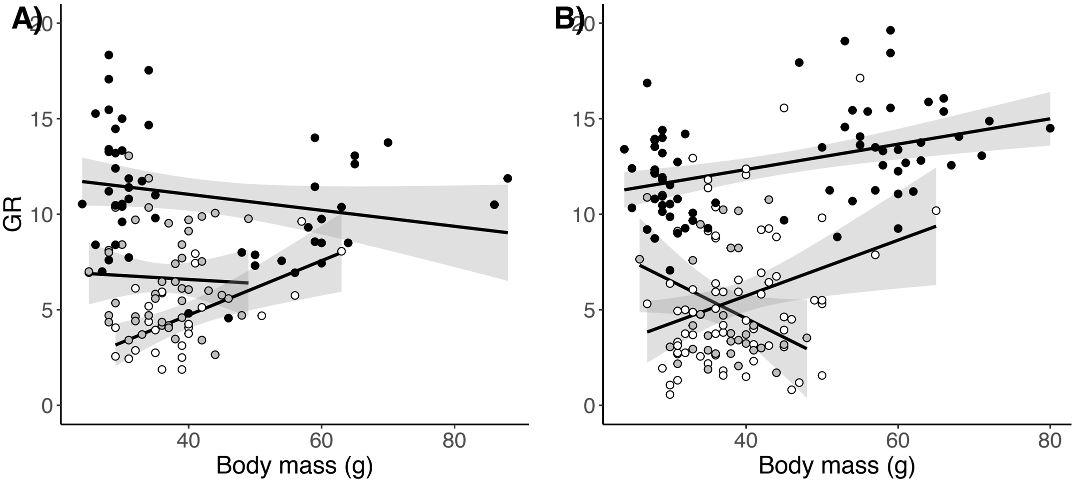


Figure S2.1: Relationship between growth rates and body mass of **(A)** individuals reared at 13ºC and **(B)** 18ºC of populations of *R. temporaria* distributed along a latitudinal cline (*white*: northernmost Sweden populations, *gray*: central Sweden populations, *black*: southernmost populations).

Table S2.2: Mixed effect ANCOVA for larval period at each temperature treatment separately (i.e. 13 and 18ºC) including weight (covariate), latitude, and their interaction. *ndf*: numerator's degrees of freedom, *ddf*: denominator's degrees of freedom.

	13ºC		18ºC	
	F (ndf, ddf)	P	F (ndf, ddf)	P
Weight (W)	6.366 (1, 115)	0.013	9.475 (1, 164)	0.002
Latitude	5.873 (2, 3)	0.092	15.342 (2, 3)	0.027
W × Latitude	1.011 (2, 115)	0.367	0.476 (2, 164)	0.622

Table S2.3: Mixed effect ANCOVA for weight at metamorphosis including weight (covariate), latitude, and temperature (i.e. no-interaction model). *ndf*: numerator's degrees of freedom, *ddf*: denominator's degrees of freedom.

	F (ndf, ddf)	P
Weight (W)	34.26 (1, 289)	< 0.001
Latitude	66.33 (2, 3)	0.003
Temperature	127.94 (2, 289)	< 0.001

Table S2.4: Mixed effect ANCOVA for growth rates at each temperature treatment separately (i.e. 13 and 18ºC) including weight (covariate), altitude, and their interaction. *ndf*: numerator's degrees of freedom, *ddf*: denominator's degrees of freedom.

	13ºC		18ºC	
	F (ndf, ddf)	P	F (ndf, ddf)	P
Weight (W)	23.949 (1, 59)	< 0.001	4.195 (1, 62)	< 0.001
Altitude	2.807 (1, 2)	0.236	19.117 (1, 2)	0.172
W × Altitude	3.478 (1, 59)	0.067	4.012 (1, 62)	0.244

Table S2.5: Mixed effect ANCOVA for larval period at each temperature treatment separately (i.e. 13 and 18ºC) including weight (covariate), altitude, and their interaction. *ndf*: numerator's degrees of freedom, *ddf*: denominator's degrees of freedom.

	13ºC		18ºC	
	F (ndf, ddf)	P	F (ndf, ddf)	P
Weight (W)	0.00 (1, 59)	0.965	29.31 (1, 62)	< 0.001
Altitude	24.17 (1, 2)	0.039	0.28 (1, 2)	0.650
W × Altitude	5.98 (1, 59)	0.017	0.18 (1, 62)	0.671

Table S2.6: Mixed effect ANCOVA for weight at metamorphosis at each temperature treatment separately (i.e. 13 and 18ºC) including weight (covariate), altitude, and their interaction. *ndf*: numerator's degrees of freedom, *ddf*: denominator's degrees of freedom.

	13ºC		18ºC	
	F (ndf, ddf)	P	F (ndf, ddf)	P
Weight (W)	0.033 (1, 59)	0.857	51.7224 (1, 62)	< 0.001
Altitude	0.614 (1, 2)	0.515	7.0871 (1, 2)	0.117
W × Altitude	1.458 (1, 59)	0.232	0.0934 (1, 62)	0.761

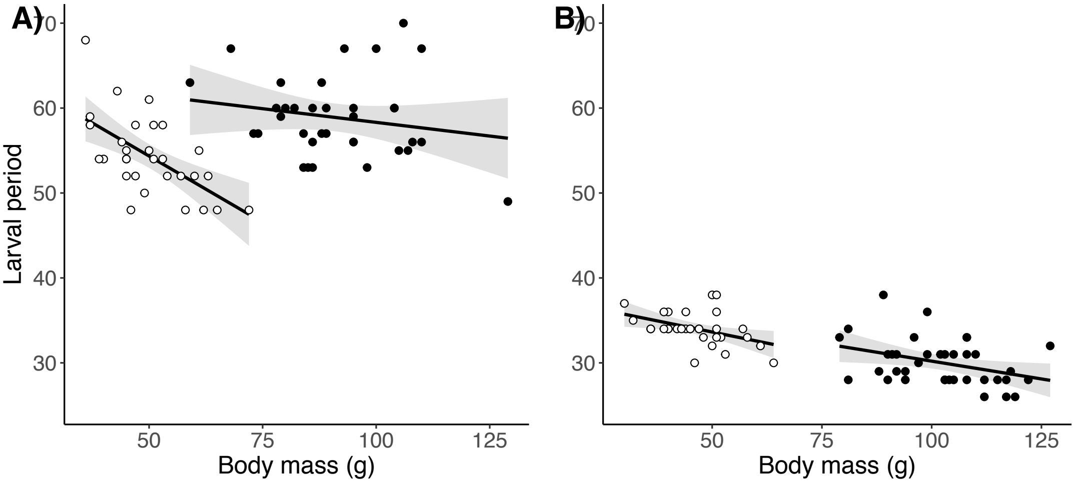
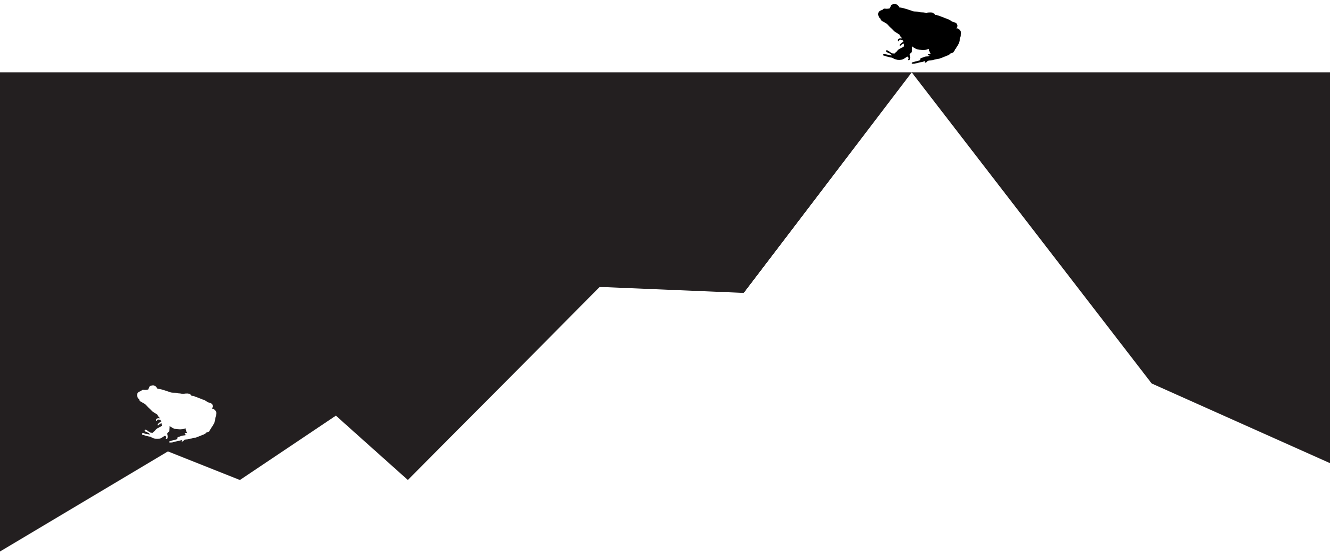


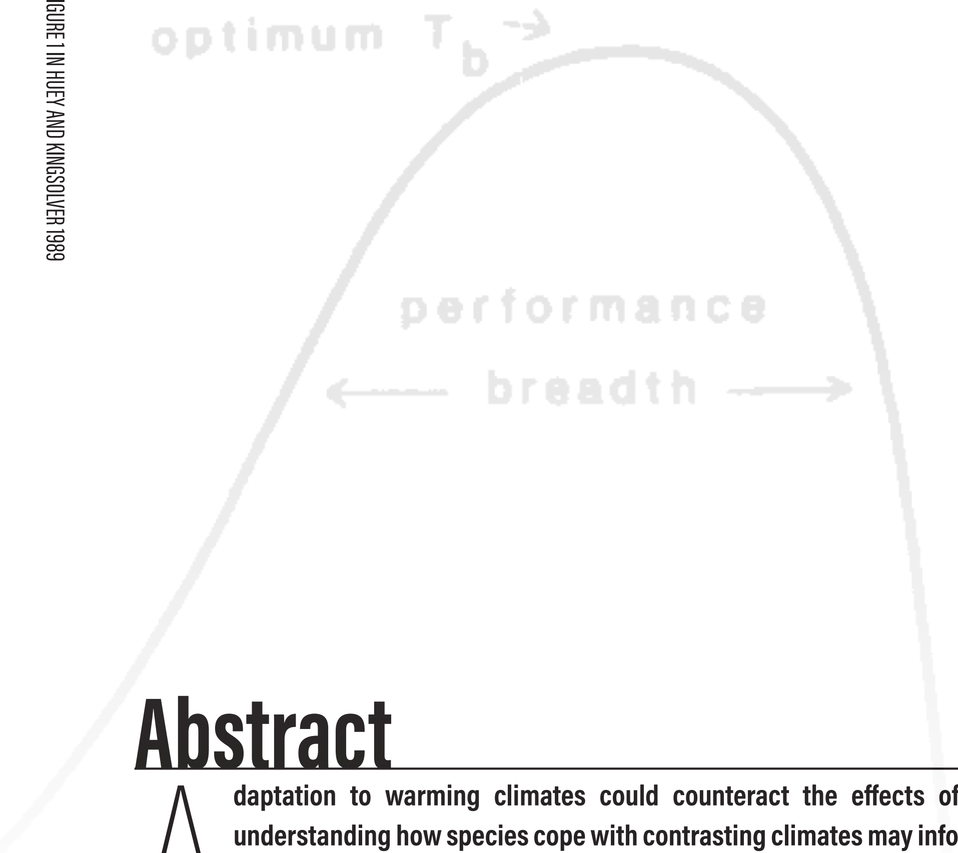
Figure S2.1: Relationship between larval period and body mass of **(A)** individuals reared at 13ºC and **(B)** 18ºC of populations of *R. temporaria* distributed along an altitudinal cline (*white*: high altitude, *black*: low altitude populations).

CHAPTER 3

Hindered and constrained: limited potential for thermal adaptation in metamorphic and adult *Rana temporaria* along altitudinal gradients

URTZI ENRIQUEZ-URZELAI - ANTONIO S. PALACIO - NATALIA MENDEZ - MARTINA SACCO -
ALFREDO G. NICIEZA





SOURCE: FIGURE 1 IN HUEY AND KINGSOLVER 1989

Abstract

Adaptation to warming climates could counteract the effects of global warming. Thus, understanding how species cope with contrasting climates may inform us about the potential for adaptation and the processes that may hamper the divergence of thermal sensitivity (e.g. evolutionary trade-offs, breeding phenology, behavioural thermoregulation). In addition to temperature, time constraints may also exert important selective pressures. Here we compare the thermal sensitivity of locomotor performance of metamorphic and adult European common frogs (*Rana temporaria*) originating from populations along an altitudinal gradient. We employed the template mode of variation (TMV) analysis to decompose the thermal sensitivity of locomotion and explore the existence of trade-offs ('hotter is better' and 'specialist-generalist' trade-off hypotheses) and the degree of local adaptation. To that end, we studied the relationship between TMV parameters and local environmental conditions. Further, we compared preferred temperatures to assess the role of behavioral thermoregulation in limiting thermal adaptation through the 'Bogert effect'. We demonstrate that *R. temporaria* cope with contrasting climates mainly through behavioral thermoregulation, promoting the conservatism of thermal sensitivity. Yet, we observed a trend towards narrower thermal niches shifted towards warmer optimum temperatures at high elevation, conforming to the 'generalist-specialist' trade-off. Apparently, this allows the most specialist, high-altitude populations to exploit resources more effectively than lowland populations during the warmest periods. The limited potential of *R. temporaria* for thermal adaptation suggests that forecasts of global warming should incorporate thermoregulation and explore its potential to buffer species from rising temperatures.

3.1. Introduction

Temperature rules all biological processes and is central to ecology and evolution (Angilletta, 2009; Kingsolver, 2009). In ectothermic animals, body temperatures (T_b) depend largely on ambient temperatures (T_a), which in turn influence the whole-organism performance (**Fig. 3.1**; Huey & Kingsolver, 1989; Ojanguren & Braña, 2000). Since temperature varies geographically, populations across a species range may have evolved independently to compensate environmental pressure through behavioural thermoregulation, physiological acclimation, or local adaptation (Hertz et al., 1983). However, the extent to which the thermal sensitivity can evolve by natural selection is still a matter of debate. The 'labile' and 'conservative' views represent two major alternatives, although they might correspond to the endpoints of a continuum (Hertz et al., 1983; Angilletta et al., 2002; Klepsatel et al., 2013). While the conservative view posits that the evolutionary rate of thermal traits may be insignificant because evolutionary costs exceed benefits, the labile view expects fast evolutionary rates. In the context of anthropogenic global warming, a deeper understanding of geographical patterns of local thermal adaptation may provide useful insights into the evolutionary potential of organisms to cope with the thermal stress associated to climate change (Williams et al., 2008; Valladares et al., 2014; Richter-Boix et al., 2015), and this may be essential to predict future species distributions, and population viabilities.

The co-occurrence of disparate constraints and mechanisms may prevent directional selection and local adaptation of thermal traits. For instance, evolutionary trade-offs may limit the shapes a thermal performance curve (TPC) can attain (see **Fig. 3.1**). The 'hotter is better' and 'specialist-generalist' hypotheses outline two of the major evolutionary trade-offs (Huey & Kingsolver, 1989; Angilletta, 2009). According to the 'hotter is better' hypothesis, individuals from warmer environments should be able to attain higher maximum performance than individuals from colder environments based on the effects of temperature on the mean kinetic energy of molecules and on rates of biochemical reactions (Angilletta et al., 2010). The 'specialist-generalist' hypothesis states that generalist individuals should perform well at a wide range of temperatures at a cost of lower maximum performances compared to specialist individuals. The latter hypothesis stands on the notion that highly efficient enzymes are functional over a narrow range of temperatures (Klepsatel et al., 2013). Although generally accepted, these non-exclusive

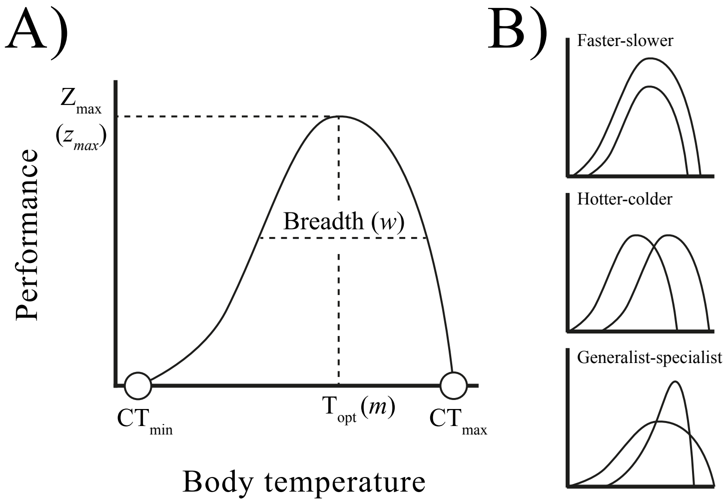


Figure 3.1: The relationship between *Tb* and performance is usually referred to as thermal performance curve (TPC). **(A)** TPCs take a characteristic asymmetric curve shape, bounded within the minimum and maximum temperatures that allow performance (*CTmin* and *CTmax*, respectively). Within those limits, performance increases towards the optimum temperature (*Topt*) at which performance is maximal (*Zmax*), and then falls sharply. Within brackets we show the equivalent parameter from TMV analysis (see Materials and methods). **(B)** The general modes of TPC variation considered in the TMV analysis: vertical shifts (faster-slower) would suggest co- or countergradient variation, horizontal shifts (hotter-colder) adaptation to the most experienced temperatures, and specialist-generalist variation would support the 'specialist-generalist' hypothesis.

hypothesis have received mixed support (Angilletta, 2009). Further, mechanisms such as behavioural thermoregulation, although crucial to buffer the effects of rising temperatures, may weaken directional selection on physiological thermal traits and preclude local adaptation (Kearney et al., 2009; Buckley et al., 2015). This process is generally known as the 'Bogert effect' (Bogert, 1949; Huey et al., 2003). However, the great costs of behavioural thermoregulation (e.g., lost opportunities for energy acquisition, searching for mates) could make the evolution of TPCs crucial for population's long-term persistence (Angilletta et al., 2002).

Time constraints can be another key factor underlying many ecological and evolutionary processes (Dunbar et al., 2009), sometimes tightly intertwined with physical conditions. In fact, the conjoint action of temperature and temporal constraints might shape the optimal thermal strategy, shifting between behavioural thermoregulation and local adaptation of physiological traits. At higher altitudes and latitudes, shorter season lengths reduce the time for activity, and thereby the opportunities for growth, reproduction, and development. These time constraints can be selective forces by themselves, but concurrent with lower temperatures might act as drivers of local adaptation (Conover & Schultz, 1995; Dahl et al. 2012). Consequently, the available time for activity may act as a key selective agent of life-history and thermal traits (Ståhlberg et al., 2001; Levy et al., 2016). For instance, to compensate for a reduced period of activity, individuals may forage more intensively, at the expense of an increased exposure to external factors (Nicieza & Metcalfe, 1997). Also, the thermal performance of key traits may be under natural selection associated with short growth seasons. Many studies have reported increased performance, irrespective of operative temperature, in unfavorable environments (i.e. countergradient variation) as an evolutionary response to shorter season lengths (Nicieza et al., 1994; Conover & Schultz, 1995; Richter-Boix et al., 2010). However, constraints on the evolution of TPCs may prevent the emergence of countergradient variation.

In this study, we explored the geographic variation in thermal sensitivity of locomotor performance associated to an altitudinal gradient in metamorphic and adult European common frogs (*Rana temporaria*, L. 1758). We used the Template Mode of Variation analysis (TMV) to decompose the variation of TPCs into three different components or modes: vertical shift (faster-slower), horizontal shift (hotter-colder), and 'specialist-generalist' trade-off (**Fig. 3.1B**; Izem & Kingsolver, 2005). Considerable amounts of variation accounted by vertical shifts would suggest co- or countergradient variation, horizontal shifts would indicate shifts in optimum temperatures towards the most experienced temperatures, and specialist-generalist variation would indicate a trade-off between maximum performance and width, which would support the 'specialist-generalist' hypothesis.

To explore any potential trade-offs in the evolution of locomotor performance curves and the degree of local adaptation, we analysed the relationships between local environmental conditions and key parameters of TPCs extracted from TMV analyses. It should be pointed that different stages of species with complex life-histories may experience markedly different environments (e.g. while adult *R. temporaria* are mainly nocturnal, metamorphs are almost strictly diurnal; Vences et al., 2000) and therefore these stages may show different thermal adaptations (Kingsolver et al., 2011). Further, time constraints may be more important for stages (e.g. larvae or juveniles) that must attain a certain age or size before the onset of winter (Orizaola et al., 2013). In addition, locomotor performance may influence the ability to escape predators and effectively forage (Wilson, 2001; Hudson et al., 2016). Here, we test the hypothesis that the strength of selection will be higher for juvenile stages, which should show greater degrees of local thermal adaptation of locomotor performance than adults. Finally, we examined the potential role of behavioral thermoregulation in limiting local thermal adaptation via the ‘Bogert’ effect (Buckley et al., 2015). To that end, we analysed the variation in preferred temperatures associated to elevation using thermal gradients.

3.2. Materials and Methods

During 2015 and 2016 we captured adult and newly metamorphosed *R. temporaria* from populations at different elevations in northwestern Spain. We sampled adult males and metamorphs of indeterminate sex from 4 high- and 4 low-elevation populations (12-20 individuals per stage and population) ranging from 251 to 2076 m.a.s.l. (**Fig. 3.2**). Unfortunately, we could not capture metamorphic individuals from one of the low elevation population (MC) due to unexpected human disturbances around the breeding site in spring 2016. On arrival to the facilities of the Research Unit of Biodiversity (University of Oviedo), we placed frogs individually in a temperature and humidity controlled room set at 14ºC for at least a week to acclimate all the experimental animals to common environmental conditions. Animals had free access to dechlorinated tap water and food (big or small *Acheta domesticus* crickets, for adult and metamorphic frogs, respectively), and we kept a constant photoperiod (12L:12D) throughout the acclimation and experimental periods.

To characterize locomotion TPCs, we conducted locomotor trials at six (9, 18, 21, 23, 25, and 28 ºC) and five (9, 18, 23, 25, and 28 ºC) different temperatures in consecutive days, with adults and metamorphs, respectively; the order of these trials was randomized to avoid any temporal bias. Prior to testing, animals were maintained at the test temperature for at least 1h in plastic containers with access to water. For adults, we additionally ensured that T_b matched T_a by checking T_b with an infrared thermometer (± 1.8% precision, IR-750 Amprobe). We measured locomotor performance as the distance moved for 5 minutes in a circular track (1-m diameter) covered with wet soil. We stimulated jumping by tapping animals gently with a wooden stick ten consecutive times. If animals refused to jump, we let them rest for five seconds before stimulating them again. After testing each animal at all temperatures, we photographed and weighed them to the nearest 0.001 g using a Mettler Toledo PL 83-S precision balance. We measured the snout-vent length (SVL) of all animals by using top view photographs and ImageJ 1.49v.

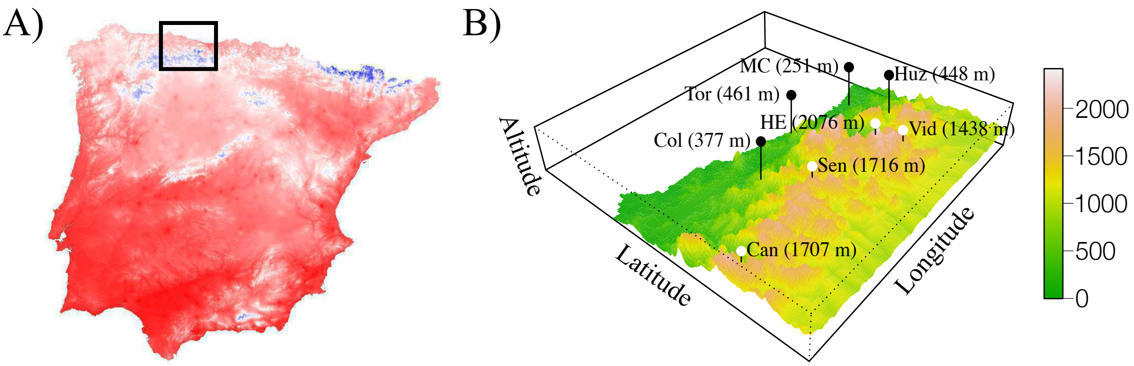


Figure 3.2: **(A)** Study area (blue represents higher altitudes) and **(B)** study populations of *Rana temporaria* distributed along elevation gradients. Populations originate from altitudes ranging from 251 to 2076 m.a.s.l.

We studied the preferred temperature (T_{pref}) of individuals approximately three days after locomotor trials were completed. To measure preferred temperatures, we used eight thermal gradients consisting of 77 × 55 cm aluminum sheets with one of the ends curved downwards. To create a thermal gradient, we lowered the temperature at one of the extremes of each aluminum sheet (to ~7°C) by inserting the curved end in constantly flowing water set to 3°C using two coolers (± 0.1 °C, Teco TC-20). In the other extreme, we used heat tape (50W, Exo Terra) to increase temperature (to ~30°C). We individually placed animals in the center of the thermal gradient and let them acclimate for 30 min. We subsequently obtained T_b (adults) or substrate temperature at the animal's position (metamorphs) with an infrared thermometer (± 1.8% precision, IR-750 Amprobe) every 15 min. Thermal preference experiments lasted for 150 min comprising 30 min for acclimation and measurements during 120 min (total of 9 measurements per individual). After each temperature measurements, we sprayed each thermal gradient with dechlorinated tap water to prevent excessive dehydration of animals.

DATA ANALYSES

Based on the distance moved for 5 minutes and the SVL of each individual, we computed the number of bodies displaced (i.e., a size-corrected surrogate of locomotor performance). Then, we analyzed the TPCs for locomotion using the Template Mode of Variation analysis (TMV; Izem & Kingsolver, 2005). TMV allows to analyze nonlinear reaction norms, for which traditional methods may not be suitable. TMV assumes the existence of a common template shape for TPCs, and models the TPC of each unit of interest (e.g. families or populations) using a three-parameter shape invariant model (Izem & Kingsolver, 2005). The height parameter (*h*) represents the relative height of TPCs, the location parameter (*m*) is equivalent to T_{opt}, and the width parameter (*w*) represents a dimensionless measure of the width of TPCs (Izem & Kingsolver, 2005; Gvoždík & Van Damme, 2008). Since we tested locomotor performance at 5-6 different temperatures, we assumed that the common template corresponded to a fourth-degree polynomial (see Izem & Kingsolver, 2005; Gvoždík & Van Damme, 2008; Richter-Boix et al., 2015). To bound the extremes of TPCs we used observed mean values of CT_{min} and CT_{max} for metamorphic individuals from the same target populations (U. Enriquez-Urzelai et al. unpublished data). We performed TMV analyses for metamorphs and adults separately and extracted the parameters (m, w, and z_{max}) using MATLAB R2017a (The MathWorks Inc. 2017) and code by Izem & Kingsolver (2005).

To characterize the thermal environment of each population we used a high-resolution gridded dataset of daily maximum, minimum, and mean temperatures (E-OBS; Haylock et al., 2008). We extracted the values corresponding to the year 2015 for each population. To account for the effects of differences in breeding phenologies (Gutiérrez-Pesquera, 2016), we calculated the mean of those environmental variables (i.e. mean T_{max}, T_{min}, and T_{mean}) for the observed activity periods of metamorphs and adults in each population (A. G. Nicieza et al., unpublished data). Additionally, we calculated the thermal variation (T_{var}) as the difference between mean T_{max} and T_{min}. The length in days of the activity periods for each ontogenetic stage (time for activity) was used as a surrogate of time constraints. To detect any potential trade-offs and the degree of local adaptation of thermal traits, we performed pairwise Pearson correlation tests between thermal traits (w, m, and z_{max}) and between thermal traits and local conditions (i.e. mean T_{max}, T_{min}, and T_{mean}, T_{var}, altitude, and time for activity). We performed pairwise correlations using the Benjamini-Hochberg (BH) correction for multiple comparisons (Benjamini & Hochberg, 1995), as implemented in the psych R-package (Revelle, 2016).

To estimate preferred temperatures, we calculated mean selected temperatures (V_{mean}) and lower and upper boundaries of the central 50% of selected temperatures ($T_{SEL\text{-}min}$ and $T_{SEL\text{-}max}$ respectively; Hertz et al., 1993; Kearney & Predavec, 2000). We tested for differences in preferred temperatures associated to elevation using mixed effect ANCOVAs. We included body mass as a covariate, population as random (intercept) factor, and elevation ('high' and 'low' altitude) as a fixed factor. We visually assessed the normality of residuals using residual distributions and quantile-quantile plots.

3.3. Results

The three biological modes of variation considered by the TMV analyses accounted for 34.88 and 43.34% of the variation observed in the TPCs of locomotion of metamorphic and adult individuals, respectively. Further, only one of the modes of variation – the generalist-specialist mode – explained a considerable percentage (~30%) of the accounted variation (**Table 3.1**). The thermal traits obtained from the TMV analyses (*w*, *m*, and

Table 3.1: Decomposition of the among-population variation observed in the thermal sensitivity of locomotion for metamorphic and adult individuals of *Rana temporaria*, using the template mode of variation (TMV) analysis.

Mode of variation	(ratio of sum of squares; %)	
	Metamorphs	*Adults*
Generalist-specialist	26.34	34.07
Horizontal shift	8.53	9.24
Vertical shift	1.65×10^{-2}	3.43×10^{-2}
Model total	34.88	43.34

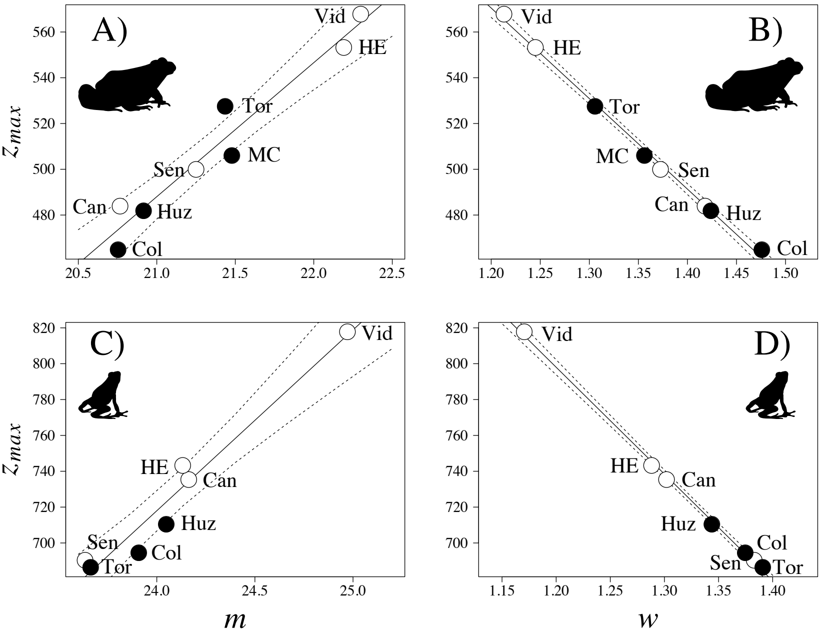


Figure 3.3: Relationship between thermal parameters to explore the validity of **(A and C)** the 'hotter is better' and **(B and D)** the 'specialist-generalist' trade-offs. **(A and B)** panels correspond to adult individuals, and **(C and D)** panels correspond to metamorphic individuals. Note the higher optimum temperatures observed for metamorphic compared to adult individuals. Each data point is labelled with its corresponding population name.

Table 3.2: Correlations between thermal traits (width of the TPC *w*, optimum temperature *m*, and maximum performance z_{max}) and between thermal traits and local conditions (mean *Tmax*, *Tmin*, and *Tmean*, *Tvar*, altitude, and time for activity) for **(A)** adults and **(B)** metamorphic individuals. Values correspond to Pearson's correlation coefficients and values between brackets correspond to the *P*-value after the Benjamini-Hochberg (BH) correction for multiple comparisons ($n = 8$ populations in adults; $n = 7$ populations in metamorphs).

	w	m	zmax	Tmax	Tmin	Tmean	Tvar	Altitude	Time for activity
(A) Adults									
w	1	-0.969 (< 0.001)	-0.998 (< 0.001)	-0.057 (0.919)	0.229 (0.790)	0.162 (0.823)	-0.295 (0.759)	-0.441 (0.529)	0.544 (0.368)
m		1	0.972 (< 0.001)	0.043 (0.919)	-0.185 (0.823)	-0.134 (0.845)	0.237 (0.790)	0.412 (0.558)	-0.547 (0.368)
zmax			1	0.044 (0.919)	-0.241 (0.790)	-0.176 (0.823)	0.301 (0.759)	0.441 (0.529)	-0.547 (0.368)
(B) Metamorphs									
w	1	-0.976 (< 0.001)	-0.999 (< 0.001)	-0.341 (0.539)	0.528 (0.401)	0.062 (0.967)	-0.657 (0.284)	-0.461 (0.469)	0.580 (0.353)
m		1	0.977 (< 0.001)	0.337 (0.539)	-0.408 (0.491)	0.006 (0.990)	0.571 (0.353)	0.279 (0.613)	-0.414 (0.491)
zmax			1	0.354 (0.539)	-0.499 (0.429)	-0.036 (0.974)	0.648 (0.284)	0.448 (0.469)	-0.568 (0.353)

z_{max}) were highly correlated both in adults and metamorphic individuals (**Table 3.2**; **Fig. 3.3**). We found that populations with higher optimum temperatures (i.e. higher *m*), had slightly narrower TPCs (i.e. lower *w*) but higher maximum performances (i.e. higher z_{max}). Apparently, populations from higher altitudes showed slightly narrower TPCs with warmer optimum temperatures. However, this tendency was more accentuated for metamorphic individuals, where only one high altitude population (Sen) clustered with low elevation populations (**Fig. 3.3**). Moreover, regardless of the population of origin, metamorphic individuals showed higher optimum temperatures (*m*) for locomotion than adults.

We did not find any sign of local adaptation of thermal traits. None of the pairwise correlations between thermal traits (*w*, *m*, and z_{max}) and local conditions (mean T_{max}, T_{min}, and T_{mean}, T_{var}, altitude, and time for activity) yielded significant results after correcting for multiple comparisons (**Table 3.2**). Interestingly, individuals from all populations selected similar temperatures (**Fig. 3.4**). Indeed, mixed effect ANCOVAs revealed a lack of divergence in V_{mean}, $T_{SEL\text{-}min}$, or $T_{SEL\text{-}max}$ across altitudes ($P > 0.05$ in all cases).

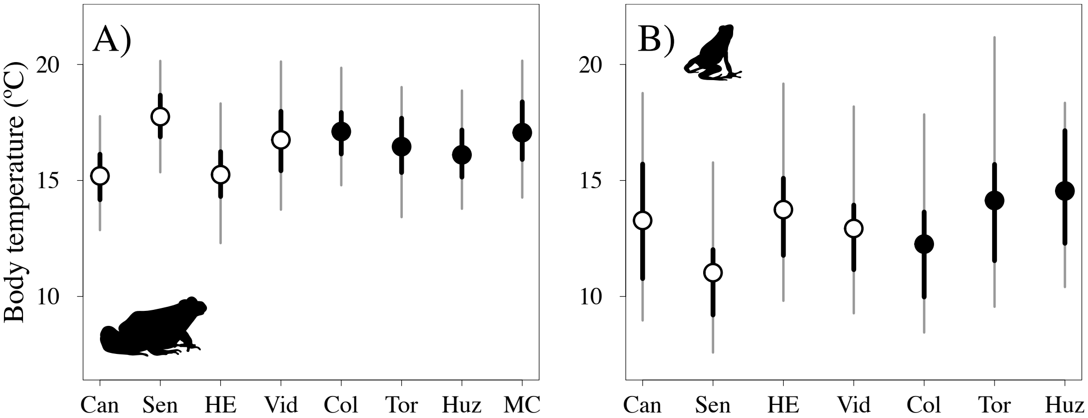


Figure 3.4: Preferred temperatures of **(A)** adult and **(B)** metamorphic individuals from each population. Points represent mean selected temperatures (*Vmean*). Bold black lines represent the central 50% of selected temperatures, and the extremes of those lines correspond to $T_{SEL\text{-}min}$ and $T_{SEL\text{-}max}$ (Hertz et al., 1993; Kearney & Predavec, 2000). Gray lines represent the range of temperatures bounded within the maximum and minimum experienced temperatures.

3.4. Discussion

Understanding how readily the thermal sensitivity evolves is key to foresee the potential of species for evolutionary adaptation to global warming (Williams et al., 2008). Yet, the potential for evolution of thermal sensitivity seems highly taxa- and trait-specific (Angilletta et al., 2002; Muñoz et al., 2014). Moreover, evolutionary trade-offs and the effects of behavioral thermoregulation may hinder thermal adaptation (Huey & Kingsolver, 1989; Huey et al., 2003; Buckley et al., 2015). In our study, small variations in thermal performance curves (TPCs) among populations of *R. temporaria* along an altitudinal gradient supported the 'specialist-generalist' trade-off hypothesis. High-altitude populations seem to be more specialized to warmer temperatures, presumably allowing them to exploit transient food resources. However, behavioral thermoregulation largely constrained the divergence of thermal sensitivity among populations. By avoiding unwanted temperatures, populations may promote the conservation of thermal sensitivity (i.e. the 'Bogert effect'; Bogert, 1949; Huey et al., 2003; Grigg & Buckley, 2013).

TPCs are useful tools to capture the thermal sensitivity of traits in any given species or population, and typically take the form of a left-skewed curve (Huey & Stevenson, 1979; Martin & Huey, 2008). Most likely, interspecific variation in TPCs comprises variations around a common shape (Izem & Kingsolver, 2005). Identifying how thermal sensitivity varies among natural populations can inform us about potential evolutionary constraints and ecological trade-offs (Phillips et al., 2014; Richter-Boix et al., 2015). According to our results, most of the observed variation among populations of *R. temporaria* can be accounted for the 'generalist-specialist' mode (*sensu* Izem & Kingsolver, 2005). This pattern was consistent between metamorphic and adult individuals (**Table 3.1**). Apparently, more generalist populations – i.e. those that maintain a relatively high performance over a broad range of temperatures – attain lower maximum performances than the more specialist populations (Huey & Hertz, 1984; Klepsatel et al., 2013). Moreover, besides slightly higher optima, specialist populations achieved higher maximum performances (**Table 3.2**, **Fig. 3.3**), in accordance with the 'hotter is better' hypothesis (Angilletta et al., 2010). However, horizontal shifts accounted for a very small fraction of the variance in TPCs (< 10 percent for metamorphs and adults; **Table 3.1**). Thus, divergence in optimum temperatures seems to be minor across populations of *R. temporaria*. Other studies have also reported negligible divergences in optimum temperatures despite profound changes in thermal environments, especially at the intraspecific level (Gilchrist et al., 1997; MitchellLampert, 2000; Klepsatel et al., 2013). Presumably, due to the minor divergence in optimum temperatures, the 'specialist-generalist' trade-off is more conspicuous than the 'hotter is better' trade-off among populations of *R. temporaria*.

Fluctuating environments are expected to favor generalist phenotypes that perform well across a wide range of conditions (Lynch & Gabriel, 1987; Condon et al., 2014). However, we found a trend towards narrower thermal niches and slightly higher optimum temperatures with increasing elevation (**Fig. 3.3**). Yet, the pairwise correlations between thermal traits and local conditions did not conform well with this trend. In line with the small variation in optimum temperatures, these results suggest that local adaptation is imperfect in *R. temporaria*. High levels of gene flow between populations at different elevations could impede local adaptation, but that is not the case for this species (Bridle & Vines, 2007; Muir et al., 2014). A plausible, non-mutually exclusive, explanation is that behavioral thermoregulation may limit local adaptation. By being active only when temperatures fall within preferred temperatures, animals could weaken the strength of natural selection on thermal sensitivity (Bogert, 1949; Hertz, 1981; Huey et al., 2003). As previously reported for some lizard (Andrews, 1998; Buckley et al., 2015), we found similar thermal preferences across environmental gradients (**Fig. 3.4**). Seemingly, populations of *R. temporaria* cope with different thermal environments mainly through behavioral thermoregulation and a wide thermal tolerance (Sinsch, 1984; Vences et al., 2002; Gutiérrez-Pesquera et al., 2016). This, may promote the conservatism of the thermal sensitivity in European common frogs across elevations. Similarly, Muñoz et al. (2014) showed that *Anolis* lizards from Hispaniola present conserved critical thermal maxima due to an effective thermoregulation. In addition to temperature, the available time for activity and reproduction may change along with environmental gradients.

Time constraints may play a key role in the evolution of thermal sensitivity (Ståhlberg et al., 2001; Dunbar et al., 2009). Windows for activity may be shorter at high altitudes (colder environments) compared to lower altitudes (warmer environments; Oufiero & Angilletta, 2006; Levy et al., 2016). Therefore, time constraints could promote phenotypes that effectively exploit transient favorable conditions. Further, this pattern may be more evident for ontogenetic stages that must attain a certain threshold (e.g. age or size) before the onset of winter (Orizaola et al., 2013; Walsh et al., 2015). In the case of amphibians in temperate and cold regions, metamorphic individuals have to accumulate reserves during a relatively short time period after coping with the high energetic demands of metamorphosis. Despite behavioral thermoregulation can impede local adaptation, our results show a tendency towards reduced thermal niche breadths and warmer optimum temperatures with increasing elevation, especially for metamorphic individuals. At the metamorphic stage, only one of the studied high-altitude populations (Señales) showed a thermal sensitivity comparable to that of lowland populations (**Fig. 3.3**). It is noteworthy, though, that preferred temperatures are well below optimum temperatures for both metamorphic and adult individuals at all elevations (**Fig. 3.3** and **3.4**). This result is in agreement with current theory, which predicts that preferred temperatures should be centered around sub-optimum temperatures (Martin & Huey, 2008). The theory stands on a property of the asymmetry of TPCs, known as Jensen's inequality; while temperature increases would provoke a sharp decrease in fitness for organisms close to optimum temperatures, at sub-optimum temperatures would increase fitness (Martin & Huey, 2008; Sinclair et al., 2016; Moran et al., 2016). According to our results, at high temperatures high elevation populations would outperform low elevation counterparts. These results suggest that individuals at high altitudes, and particularly metamorphic individuals, may be selected to maximize performance during the warmest periods. Presumably, these warm periods may match resource peaks, as evidenced by peaks in moth abundance at alpine habitats during summer (Alberdi et al., 2012).

Behavioral thermoregulation is a rapid and effective response to avoid unfavorable temperatures, that in turn may hinder the evolution of thermal sensitivity. As temperatures rise due to global warming, the potential to behaviorally compensate for climate warming may decline progressively. Thus, the almost complete lack of local thermal adaptation may confer higher sensitivity in the long term (Kearney et al., 2009; Buckley et al., 2015). Our results suggest that populations of *R. temporaria* at different elevations cope with contrasting thermal environments mainly through behavioral thermoregulation (see also Sinsch, 1984; Vences et al., 2002; Köhler et al., 2011). As a consequence, thermal sensitivity is remarkably conserved among populations, regardless of elevation. Yet, variation in thermal sensitivities supported the 'generalist-specialist' trade-off (Izem & Kingsolver, 2005; Angilletta, 2009). Altogether, our results indicate that high-altitude, more specialist, populations may exploit resources more effectively than low elevation counterparts during the warmest periods, which could be advantageous to face the stronger time constraints on growth and development in mountain areas. Here, it should be noted that the reported differences were small, presumably due to the 'Bogert effect' (Bogert, 1949; Huey et al., 2003). In light of the apparent low potential for evolutionary adaptation (Quintero & Wiens, 2013), we argue that forecasting the response of species to global warming will require the use mechanistic models to incorporate behavioral thermoregulation and explore its potential to buffer species from global warming (Kearney & Porter, 2009).

3.5. References

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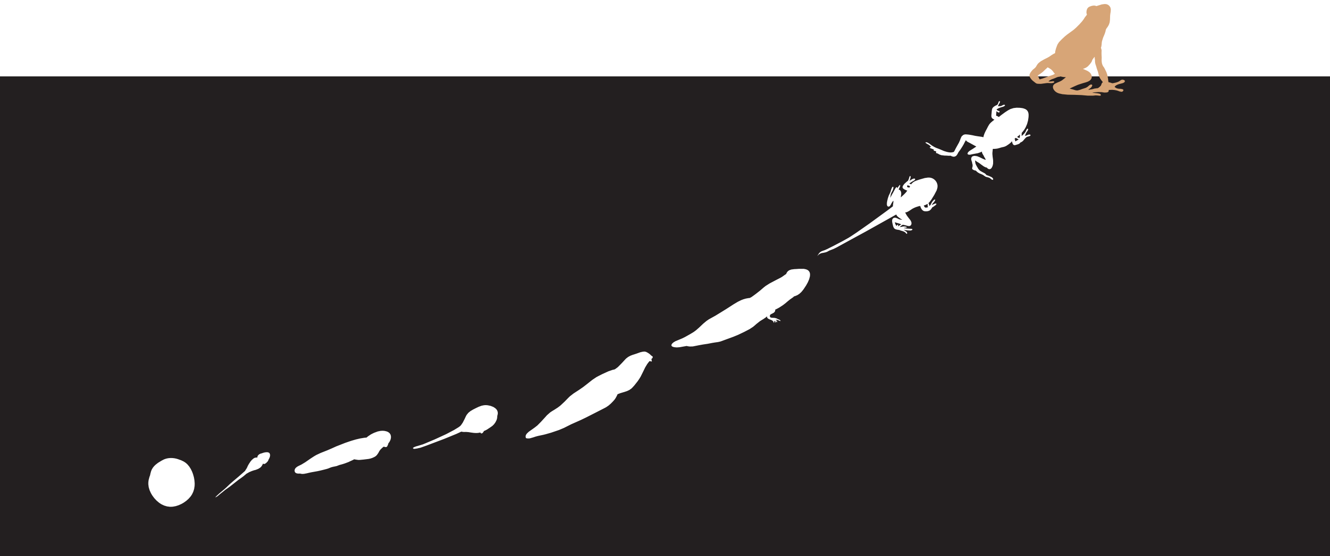
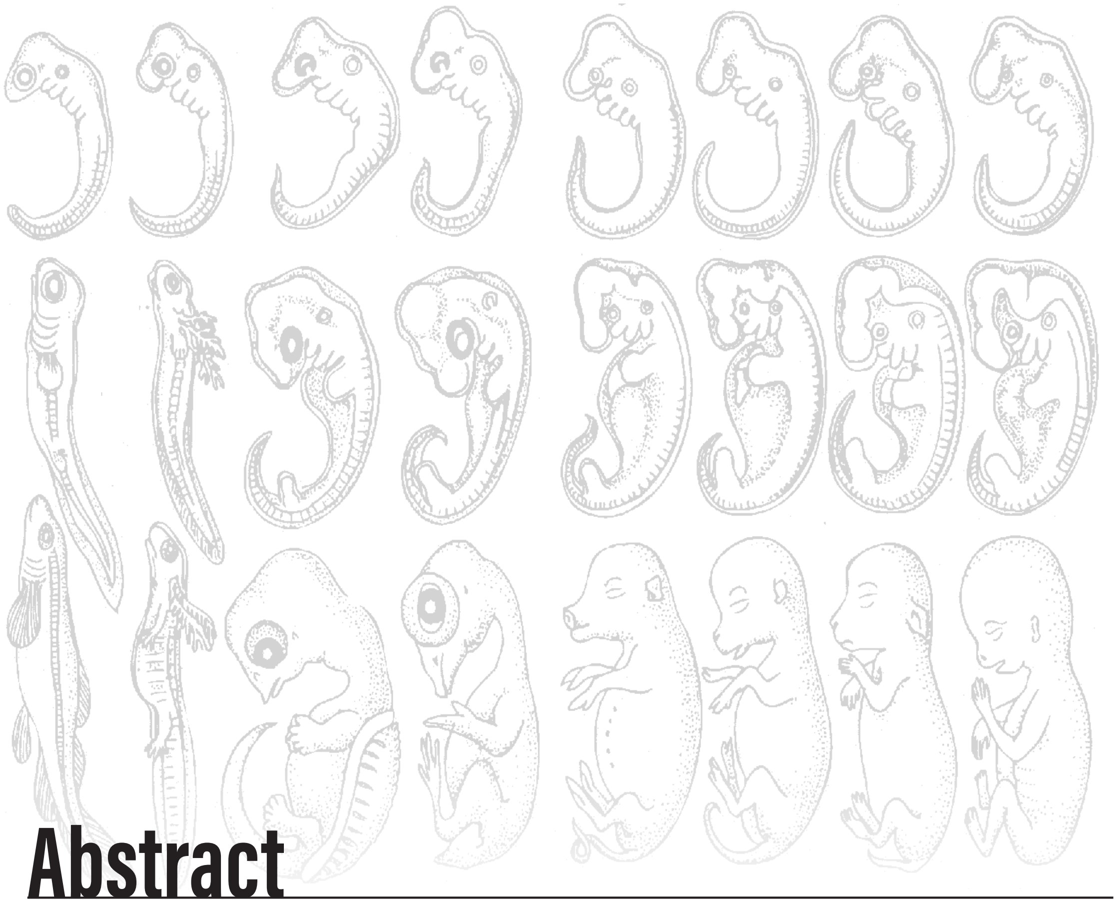
CHAPTER 4

Ontogenetic reduction in thermal tolerance is not alleviated by earlier developmental acclimation in *Rana temporaria*

URTZI ENRIQUEZ-URZELAI - MARTINA SACCO - ANTONIO S. PALACIO - POL PINTANEL - MIGUEL TEJEDO - ALFREDO G. NICIEZA





SOURCE: HAECKEL'S ILLUSTRATION 1874

Abstract

Complex life-histories may promote the evolution of different strategies to allow the optimal matching to the environmental conditions that organisms can encounter in contrasting environments. For ectothermic animals, we need to disentangle the role of stage-specific thermal tolerances and developmental acclimation to predict the effects of climate change on spatial distributions. However, the interplay between these mechanisms has been poorly explored. Here we study whether developmental larval acclimation to rearing temperatures affects the thermal tolerance of subsequent terrestrial stages (metamorphs and juveniles) in common frogs (*Rana temporaria*). Our results show that larval acclimation to warm temperatures enhances larval heat tolerance, but not thermal resistance in later metamorphic and juvenile stages, which does not support the developmental acclimation hypothesis. Further, metamorphic and juvenile individuals exhibit a decline in thermal tolerance, which would confer them a higher sensitivity to extreme temperatures. Because thermal resistance is not enhanced by larval developmental acclimation, these 'risky' stages may be forced to compensate through behavioural thermoregulation and short-term acclimation to face eventual heat peaks in the coming decades.

4.1. Introduction

In species with complex life-histories, transitions between stages involve abrupt changes in size, morphology, physiology, behaviour, and habitat (Wilbur 1980). Most often, different life-history stages occur within particular time windows in well differentiated habitats, and thus they may experience markedly different conditions, especially in seasonally fluctuating environments. Fretwell (1972) stressed the importance of specific periods as key factors determining the dynamics, evolution and thereby the viability of species living in seasonal environments. Similarly, the sensitivity of particular developmental stages might be determinant for the viability of populations in a context of rapid environmental change. Thus, selection could promote stage-specific adaptations, such as specific thermal sensitivities and tolerances. A growing body of evidence points in that direction with stage-specific thermal physiologies adapted to specific microclimates (Coyne et al. 1983; Ragland and Kingsolver 2008; Potter et al. 2010; Kingsolver et al. 2011). Additionally, thermal acclimation allows organisms to adjust thermal tolerances to changing environmental conditions (Hoffmann et al. 2003). In fact, increased heat tolerances triggered by warmer temperatures may constitute an important component of species' ability to face global warming (Williams et al. 2008; Chevin et al. 2010; Ruiz-Aravena et al. 2014; Slotsbo et al. 2016; but see Gunderson and Stillman 2015).

The effects of environmental conditions experienced during a given phase of the cycle can persist and influence further developmental stages within a generation (Álvarez and Nicieza 2002a; Pechenik 2006; Tejedo et al. 2010). Developmental acclimation, opposed to reversible short-term acclimation, is irreversible and represents plastic changes that permeate from early to later stages; theoretically, it might have either positive or negative fitness consequences. If environmental conditions differ considerably among ontogenetic stages, the induced phenotypes could become mismatched to future environments. In contrast, developmental acclimation may be beneficial if the environments of consecutive stages are similar, or environmental conditions during a stage predict reliably future conditions (Beaman et al. 2016; Sgrò et al. 2016). Well developed tadpoles, metamorphs, and early juveniles of most temperate amphibians may experience this last scenario in nature. At temperate zones, temperatures and the risk of pond drying increase as season advances, coinciding with peaks of metamorphosis (Newman 1989; Reques and Tejedo 1995). Accordingly, preferred temperatures increase along larval development (Floyd 1984;

Wollmuth et al. 1987). Since higher temperatures increase rates of growth and development (Álvarez and Nicieza 2002b; Gomez-Mestre et al. 2010), selection may have favoured fast development at later stages due to an increased risk of mortality by pond drying (Newman 1989; Richter-Boix et al. 2011). Finally, acclimation of larvae to high temperatures might enhance post-metamorphic thermal tolerance and, thus, survival during heat pulses.

Increased tolerance to heat could compromise the resistance to cold conditions (Angilletta et al. 2002; Gutiérrez-Pesquera et al. 2016; but see Sørensen et al. 2015) and this coupling may affect the viability of temperate organisms exposed to low temperatures (Stuhldreher et al. 2014). Because developmental acclimation produces irreversible changes (Nyamukondiwa and Terblanche 2010; Slotsbo et al. 2016), this could turn detrimental. Although well developed tadpoles and early post-metamorphic individuals of temperate species share the risk of heat stress, later terrestrial stages may deal with an increased risk of freezing as winter approaches (Vences et al. 2000). Further, under warming climates, the reduced snowpack may increase the risk of reaching extreme cold temperatures underground (Groffman et al. 2001; Sunday et al. 2014), where many amphibians retreat. Consequently, if irreversible (sensu Maynard Smith 1957), heat acclimation in earlier stages could reduce cold tolerance of later stages in ontogeny. Thus, to predict how global warming will affect organisms with complex life cycles, we should consider the link between stage-specific thermal tolerances and the effects of developmental acclimation induced at earlier stages (Terblanche and Chown 2006; Bowler and Terblanche 2008).

Here we studied the acclimation of upper and lower thermal limits of aquatic larvae reared at different temperatures and the potential lasting effects on the thermal tolerance of semiaquatic metamorphs and fully terrestrial juveniles of the European common frog (*Rana temporaria* Linnaeus, 1758). The high specific heat and thermal conductivity of water confer aquatic environments a greater thermal inertia and, thus, more homogeneous temperatures than terrestrial environments (Spotila et al. 1992). Hence, aquatic stages may find limited opportunity for behavioural thermoregulation, which may be especially critical during the process of pond desiccation when water can reach extreme temperatures (Reques and Tejedo 1995; Feder and Hofmann 1999). Terrestrial stages, while subjected to greater diel fluctuations, may be able to regulate body temperatures through behavioural thermoregulation – selecting suitable microclimates (Briscoe et al. 2014) – and evaporative cooling (Bartelt et al. 2010). In this context, we hypothesise that, due to differences in the temperature regimes and the potential for behavioural thermoregulation between aquatic and terrestrial environments, lasting effects of thermal acclimation during larval stages should be decoupled and not affect the thermal tolerance of metamorphic and juvenile frogs, so that stage-specific tolerances prevail. Specifically, we explored these two questions: 1) does the thermal tolerance change through ontogeny? and 2) is thermal tolerance of juvenile frogs affected by the temperature experienced during the larval phase (i.e. developmental acclimation)?

4.2. Materials and methods

We collected *R. temporaria* tadpoles (*n* = 252; Gosner stage 27-28; Gosner 1960) from a population in northern Spain (43°23'N, 4°49'W; 461 m.a.s.l.) on May 2015. On arrival to the facilities of University of Oviedo, we randomly assigned half of the individuals to each acclimation temperature treatment (18 or 25°C). Animals were housed individually in 0.8-L containers, mounted in a rack recirculating-system equipped with biological filtration and temperature control. A constant water-flow renewed water and temperatures were kept constant (± 0.5°C) using heating-cooling units (Teco TC20; Ravenna, Italy). At the start of the experiment, for each temperature acclimation treatment, individuals were randomly assigned to one of four groups corresponding to different ontogenetic stages at which critical thermal limits were examined. Specifically, we conducted tests with individuals at the 'tadpole' stage (Gosner 29-41; hereafter TAD), newly metamorphosed or 'metamorphic' individuals (Gosner 46; MET), 2-week old juveniles (JUV2w), and 4-week old juveniles (JUV4w). Once tadpoles started metamorphosing (stage 42), we weighed, recorded the day of metamorphosis, and placed them individually in containers with access to water and wet substrate in a constant temperature room at 14 ± 1°C. Additionally, we weighed and recorded the date for metamorphic climax (stage 46). Both tadpoles and froglets were fed *ad libitum* with rabbit chow and small-sized *Acheta domestica* crickets, respectively. We set a 12L:12D photoperiod throughout the experiment.

To estimate thermal tolerances (CT_{max} and CT_{min}) we followed Hutchison's dynamic method (Lutterschmidt and Hutchison 1997). We placed individuals in 100-mL plastic containers with dechlorinated water at 20°C, and heated/cooled the water at a rate of 0.25°C min^{-1} using a refrigerated heating bath (HUBER K15-cc-NR; Kältemaschinenbau AG, Germany). We appraised thermal limits when individuals remained unresponsive to external stimuli (10 gently taps with a wooden stick), and recorded water temperature with a quick-recording thermometer (Miller & Weber; Ridgewood, NY, USA) to the nearest 0.1°C. Because of the small body sizes of the experimental animals, we assumed that body temperatures equated water temperature (Lutterschmidt and Hutchison 1997; Gutiérrez-Pesquera et al. 2016). After CT_{max} and CT_{min} tests, we transferred individuals to cold (14°C) or warm water (20°C) respectively, verified survival after 24-h to assure that thermal limits were not surpassed, and weighed them to the nearest 0.001 g.

STATISTICAL ANALYSES

We employed general linear models to assess the effects of acclimation temperature (i.e. rearing temperature) on time until metamorphosis (Gosner stage 42; TM42), body mass at metamorphosis (BM42), and metamorphic climax (BM46). Then, we tested for the effects of acclimation on heat (CT_{max}) and cold tolerance (CT_{min}) and possible carryover effects between ontogenetic stages. Although warm raised tadpoles reached metamorphosis earlier than individuals at 18°C (TM42: $F_{1,140} = 8.27$, $P = 0.005$), we assume it did not affect tolerance limits because our acclimation periods were large enough considering previous research with amphibians showing the stabilization of CT_{max} and CT_{min} after three and four days, respectively (Brattstrom 1968). Moreover, acclimation to different temperatures caused differences in body size (see Results) that may affect tolerance limits (see Ribeiro et al. 2012; Klockmann et al. 2016). Thus, we explored the effects of acclimation, ontogenetic stage, and size (i.e. body mass) on thermal limits with a combination of ANOVAs (without size) and ANCOVAs (including body mass as a covariate). We conducted ANOVAs for both CT_{max} and CT_{min} including acclimation treatment, ontogenetic stage, and their interaction as fixed effects. To explore differences among ontogenetic stages we used Tukey's HSD tests. In the case of the ANCOVAs, to ease interpretation when a third order interaction (i.e. mass × acclimation × ontogenetic stage) was significant, we assessed the effects of mass, acclimation, and their interaction within each ontogenetic stage separately. We tested for the effects of acclimation and ontogenetic stage on size-corrected CT_{max} and CT_{min} only if interactions involving body mass resulted nonsignificant (homogeneity of slopes); in these cases, we dropped the interaction between covariate and factor(s) from models.

Since we measured CT_{max} and CT_{min} on different individuals, we used a randomization approach to test for the effects of acclimation treatment, ontogenetic stage, and their interaction on tolerance ranges (CT_{max} - CT_{min}). Specifically, we randomly selected 14 individuals from each ontogenetic stage and acclimation treatment combination (seven individuals with CT_{max} and seven with CT_{min} values) and computed tolerance ranges. Then, we performed a general linear model with acclimation treatment, ontogenetic stage, and their interaction as fixed effects, and used Tukey's HSD tests to assess which ontogenetic stages differed in thermal tolerance. We repeated the randomization procedure 100 times. All analyses were performed in R 3.3.2 (R Core Team, 2016).

4.3. Results

Tadpoles reared at the warm acclimation temperature reached metamorphosis at smaller sizes than individuals reared at 18 °C (BM42: $F_{1,140} = 4.90$, $P = 0.029$). These differences remained at the metamorphic climax (BM46: $F_{1,140} = 11.91$, $P < 0.001$; **Fig. S4.1**).

CT_{max} values related significantly to acclimation treatment and ontogenetic stage (**Table 4.1a**). Apparently, warmer temperatures led to higher CT_{max}. Individuals at the 'tadpole' stage (TAD) exhibited the highest (Tukey's HSD; $P < 0.001$ in all cases), and 4-week old juveniles (JUV4w) the lowest CT_{max} (Tukey's HSD; $P < 0.05$ in all cases). Metamorphic individuals (MET) and 2-week old juveniles (JUV2w) did not differ in CT_{max} (Tukey's HSD; $P = 0.847$; **Fig. 4.1a**). We found no interaction between acclimation temperature and ontogenetic stage (**Table 4.1a**). Further, the ANCOVA on CT_{max} revealed heterogeneity of slopes (interaction mass × acclimation × ontogenetic stage: $F_{3,74} = 4.009$, $P = 0.011$). Thus, we analysed the effects of mass and acclimation treatment on CT_{max} within

Table 4.1: Analysis of variance (ANOVA) table for **(a)** heat tolerance (*CTmax*) and **(b)** cold tolerance (*CTmin*) including acclimation temperature, ontogenetic stage, and their interaction.

	df	SS	*F*-value	*P*-value
(a) CTmax				
Acclimation	1	2.603	4.941	0.029
Ontogenetic stage	3	87.195	55.165	< 0.001
Acclimation × Ontogenetic stage	3	1.322	0.837	0.478
Residuals	82	43.204		
(b) CTmin				
Acclimation	1	0.764	1.7238	0.193
Ontogenetic stage	3	10.257	7.7163	< 0.001
Acclimation × Ontogenetic stage	3	1.918	1.4425	0.237
Residuals	78	34.562		

Figure 4.1: **(a)** Observed mean heat tolerance (*CTmax*) ± SE, **(b)** and mean cold tolerance (*CTmin*) ± SE during different ontogenetic stages (TAD, MET, JUV2w, and JUV4w) of *Rana temporaria* acclimated to 18 (black) or 25 ºC (white) during the tadpole stage.

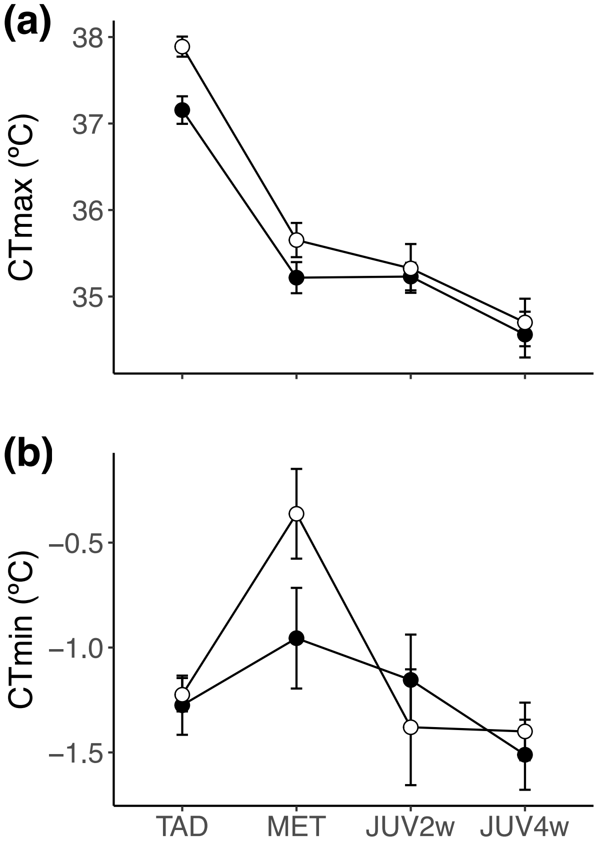


Table 4.2: Analysis of covariance for CTmax of **(a)** tadpoles (TAD), **(b)** metamorphs (MET), and **(c)** 4-week old juveniles (JUV4w), including mass (covariate) and acclimation temperature. Note that the ANCOVA including the interaction between body mass and acclimation revealed significant differences in group slopes for JUV2w, and therefore we did not compare adjusted means at this stage.

	df	SS	*F-value*	*P-value*
(a) TAD				
Mass	1	0.132	0.719	0.410
Acclimation	1	2.318	12.597	0.003
Residuals	15	2.761		
(b) MET				
Mass	1	0.005	0.010	0.923
Acclimation	1	1.540	2.767	0.109
Residuals	25	13.916		
(c) JUV4w				
Mass	1	6.627	20.663	< 0.001
Acclimation	1	0.078	0.244	0.628
Residuals	16	5.132		

Table 4.3: Analysis of covariance (ANCOVA) table for cold tolerance (*CTmin*) including mass (covariate), acclimation temperature, ontogenetic stage, and their interaction.

	df	SS	*F-value*	*P-value*
Mass	1	0.188	0.4262	0.516
Acclimation	1	0.791	1.7919	0.185
Ontogenetic stage	3	10.477	7.9059	< 0.001
Acclimation × Ontogenetic stage	3	2.032	1.5334	0.213
Residuals	77	34.012		

each ontogenetic stage separately (**Table 4.2**). Homogeneity of slopes was met in all stages (all $Ps > 0.21$) except JUV2w (interaction mass × acclimation: $F_{1,21} = 12.56$, $P = 0.002$): larger JUV2w had higher CT_{max} than smaller at 25 °C, while the opposite pattern was found for individuals acclimated to 18 °C (**Fig. S4.2**). ANCOVA indicated that warmer acclimation temperatures lead to higher size-adjusted CT_{max} in TAD but not in MET or JUV4w stages (**Table 4.2**; **Fig. S4.2**).

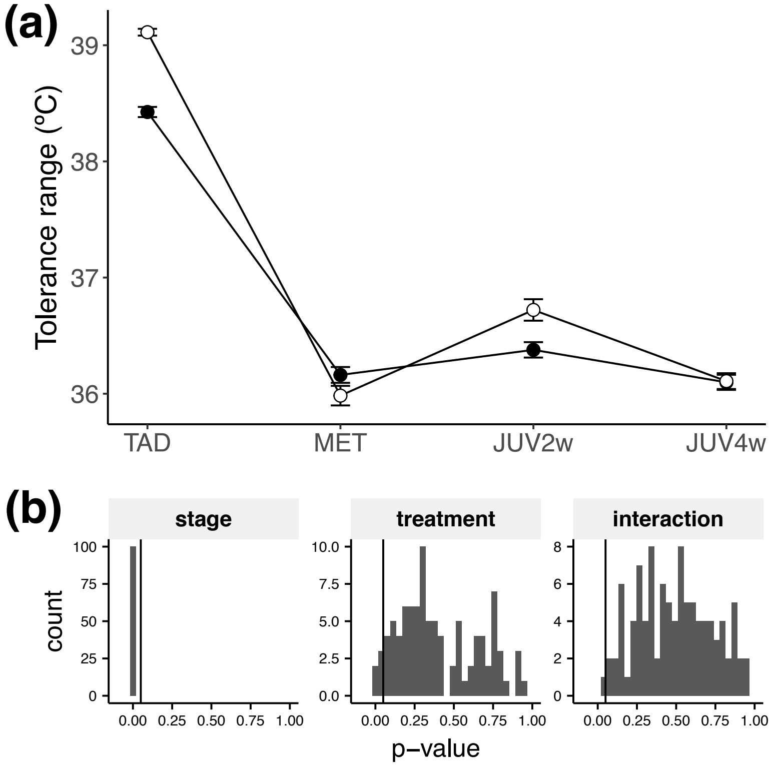
Differences in CT_{min} were exclusively related to ontogenetic stage, irrespective of acclimation temperatures (**Table 4.1b**). Further, we only found lower cold tolerances in warm acclimated individuals at the MET stage (Tukey's HSD; $P < 0.05$, **Fig. 4.1b**), whereas we found indistinguishable CT_{min} for the rest of stages (Tukey's HSD; $P > 0.941$). There was not a significant effect of size on CT_{min} and none of the interactions between size and the rest of the factors resulted significant ($P > 0.069$ in all cases). Consequently, we dropped interactions between covariate and factors. The ANCOVA confirmed strong differences among ontogenetic stages (**Table 4.3**).

Thermal tolerance ranges were associated to ontogenetic stage in all randomization runs (**Fig. 4.2**). Oppositely, acclimation treatment and its interaction with ontogenetic stage resulted non-significant in most runs (**Fig. 4.2b**). Individuals at the TAD stage showed wider tolerance ranges than subsequent stages (Tukey's HSD; $P < 0.001$ in all 100 runs). The following stages did not differ in thermal tolerance range (Tukey's HSD; $P > 0.05$ in all 100 runs).

4.4. Discussion

Understanding how thermal tolerance changes through ontogeny in species with complex life-cycles is key to foresee the impacts of climate change (Levy et al. 2015; MacLean et al. 2016; Levy et al. 2016). Furthermore, the degree to which plastic responses

Figure 4.2: **(a)** Tolerance range ± 95% CI (*CTmax - CTmin*) during different ontogenetic stages (TAD, MET, JUV2w, and JUV4w) of *Rana temporaria* acclimated to 18 (black) or 25 °C (white) during the tadpole stage. **(b)** Significance values (*p-value* distribution) of ontogenetic stage, acclimation treatment, and their interaction obtained from linear models with 100 randomized tolerance datasets (see Materials and methods).



(e.g. developmental acclimation) will protect species from predicted climates remains under debate (Stillman 2003; Donelson et al. 2011; Gunderson et al. 2017). Our results demonstrated that the thermal tolerance of *R. temporaria* changes drastically at life history transitions (e.g. metamorphosis), as reported for other ectotherms (Potter et al. 2010; Kingsolver et al. 2011), including amphibians (Cupp 1980; Floyd 1983). Acclimation to warm temperatures at the larval stage enhanced larval heat tolerance, but did not impact on the thermal tolerance of ongoing terrestrial stages, thus providing no support for the developmental acclimation hypothesis. Apparently, developmental acclimation has negligible effects in some taxa (Terblanche and Chown 2006; Donelson et al. 2011), while it has persistent effects in others (Scott and Johnston 2012; Slotsbo et al. 2016; Kellermann et al. 2017). Besides, a lack of developmental acclimation means that rearing at warm temperatures during the larval stage would not protect earlier terrestrial stages that are exposed to hot late-spring and summer temperatures. Thus, we would expect an increase in juvenile mortality due to predicted extreme heat events under global warming.

Given that rates of projected climate change outpace the potential for evolutionary adaptation and that many species may be unable to track their preferred climatic conditions (Araújo et al. 2006; Chevin et al. 2010; Quintero and Wiens 2013), the degree of phenotypic plasticity and thermal tolerance may be crucial for population viability. Tadpoles can respond plastically to environmental factors by changing developmental and growth rates (Smith-Gill and Berven 1979; Enriquez-Urzelai et al. 2013; Oromí et al. 2015). Temperature is no exception: warmer temperatures lead to higher developmental and growth rates (Tejedo et al. 2010; Mitton and Ferrenberg 2012). Our results show that tadpoles at warmer temperatures metamorphosed faster but with a smaller size, in agreement with previous studies (Álvarez and Nicieza 2002b; Muir et al. 2014; Richter-Boix et al. 2015). Besides, we show that size influences the heat tolerance – although varying among ontogenetic stages –, but not the cold tolerance of *R. temporaria*. Variation in heat resistance with size has already been observed in invertebrates (Peck et al. 2009; Ribeiro et al. 2012; Klockmann et al. 2016), but, remarkably, not in vertebrates (see Ospina and Mora 2004; Duarte et al. 2012). However, the effect of size varied among ontogenetic stages. Heat tolerance was unrelated to size in some ontogenetic stages (TAD and MET), but positively related in others (JUV4w). Notably, in early juveniles (JUV2w) the influence of size on heat tolerance changed with acclimation temperature: we found a positive effect at high temperatures and a negative relationship at low temperatures. Global warming could cause smaller sizes at metamorphosis impacting on fitness (e.g. reduced fecundity at maturity; Berven 1981; Smith 1987), and as we show, reducing the resistance of smaller juvenile *R. temporaria* to heat extremes. Yet, the effect of body size on thermal tolerance is lower than the variance explained by ontogeny.

Most studies on ectotherms' vulnerability to global warming focus on adults (Araújo et al. 2006; Sunday et al. 2014) or larvae (Gerick et al. 2014; Gutiérrez-Pesquera et al. 2016). However, our results show that post-metamorphic individuals may be particularly susceptible to extreme temperatures due to a reduction in heat tolerance (Cupp 1980; Floyd 1983; L. M. Gutiérrez-Pesquera, P. Pintanel, A. López-Rosero, A. Merino-Viteri and M. Tejedo, unpublished data; **Figs. 4.1**, **S4.2**). As a consequence, the thermal tolerance range is notably reduced during metamorphosis and thereafter remains similar for the rest of terrestrial ontogenetic stages (MET, JUV2w, JUV4w; **Fig. 4.2**). This is a relevant result because widely applied approaches often ignore the variation in thermal tolerance among life-history stages. However, analysing the resistance of the weakest element (i.e. the stage with the lowest tolerance) may help us detect the sites and populations more prone to local extinction (Pincebourde and Casas 2015).

Different ontogenetic stages not only may encounter different thermal conditions, but also different opportunities for behavioural thermoregulation (Kingsolver et al. 2011; Beaman et al. 2016; Sinclair et al. 2016). Air temperatures fluctuate more profoundly than water temperatures (Feder and Hofmann 1999). Hence, intuitively, terrestrial stages are more likely to encounter more extreme temperatures than aquatic larvae, but they have also a greater opportunity for behavioural thermoregulation. This may allow individuals to escape unwanted temperatures (Kearney and Porter 2009), but also may weaken directional selection on thermal traits, through a process known as the 'Bogert effect' (Huey et al. 2003; Buckley et al. 2015). Oppositely, in water, the potential of behavioural thermoregulation to buffer heat or cold waves may be more limited. Furthermore, amphibian terrestrial stages may benefit from short-term acclimation (Rutledge et al. 1987). This complex scenario, could favour the maintenance of stage-specific tolerance ranges.

Acclimation temperatures can also modify thermal tolerances (Overgaard et al. 2008) and protect populations from reaching detrimental temperatures, although its absolute impact on thermal resistance seems smaller than that found across ontogenetic stages. The interplay between these factors, however, has received less attention (MacLean et al. 2016; Sgrò et al. 2016; Slotsbo et al. 2016). Under a warming climate, developmental acclimation to warmer temperatures could be advantageous for earlier terrestrial stages (e.g. metamorphs) to confront heat extremes, but it could be detrimental for later terrestrial stages (e.g. older juveniles) if it reduces cold resistance (Stuhldreher et al. 2014; Beaman et al. 2016). Altogether, our results suggest that developmental acclimation to warmer temperatures may not benefit post-metamorphic stages. Contrarily, exposure to warm temperatures at the larval stage seems to carryover onto metamorphic individuals, in the form of reduced cold tolerance (**Fig. 4.1b**). Although metamorphic individuals of *R temporaria* emerge during the hottest months, even during summer, temperatures can drop sharply at high altitudes where the species is present. According to our results, increasing temperatures at the larval stage can have a negative influence on population growth: metamorphs will not benefit from developmental acclimation, but rather, they would be more sensitive to cold temperatures.

In conclusion, different ontogenetic stages of any given species may encounter different challenges and opportunities. *Rana temporaria* faces limited scope for behavioural thermoregulation and the risk of overheating at the aquatic larval stage (Feder and Hofmann 1999). In contrast, metamorphic and juvenile stages can thermoregulate to a higher extent than tadpoles. However, they confront an increasing risk of freezing as winter approaches (Vences et al. 2000). Although most forecast of the consequences of global warming for biodiversity conservation ignore how thermal tolerance varies between life stages, the viability of the weakest link could restrict the future distribution of a species (Briscoe et al. 2012; Pincebourde and Casas 2015). Further studies that combine stage-specific thermal sensitivities and tolerances with available microclimates for thermoregulation may shed further light on the actual vulnerability of species, and how complex life-cycles may be disrupted as a result of anthropogenic global warming (Kearney and Porter 2009; Levy et al. 2015).

4.5. References

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Supplementary Material for Chapter 4

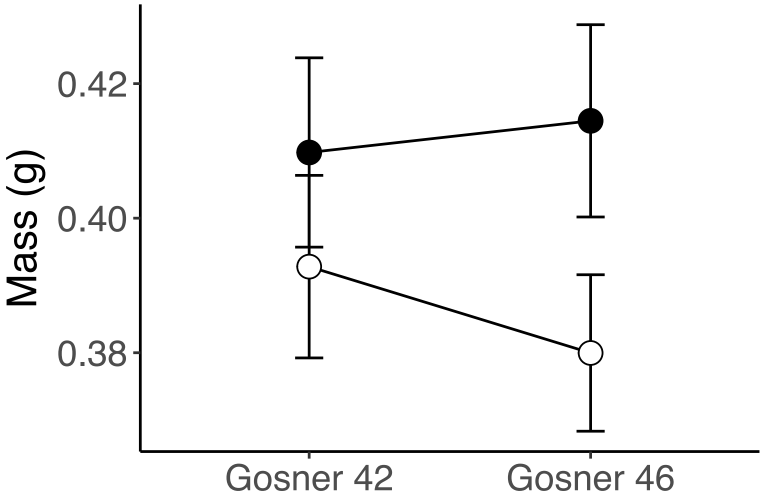


Figure S4.1: Body mass in grams ± SE of individuals acclimated to 18 (*black*) or 25 ºC (*white*) at metamorphosis (Gosner stage 42; BM42) and metamorphic climax (Gosner stage 46; BM46).

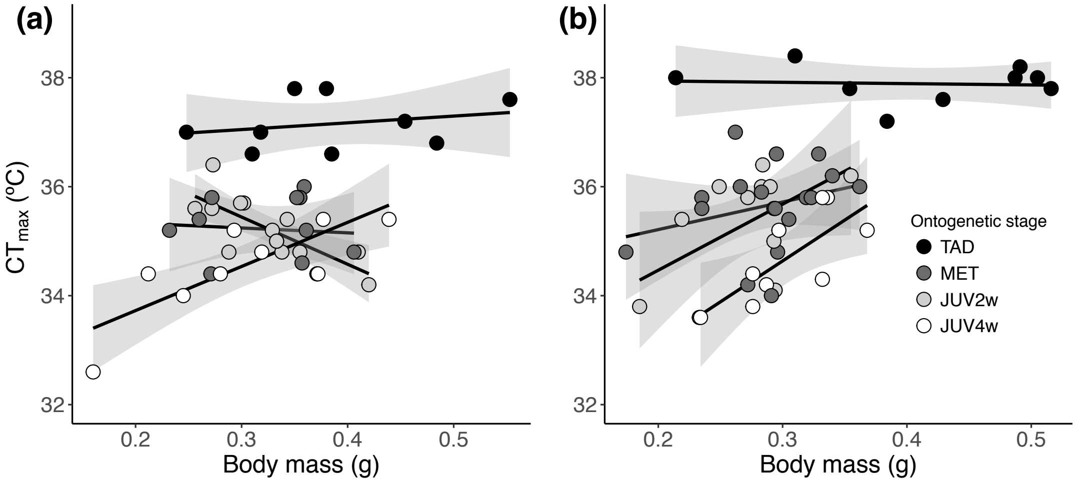
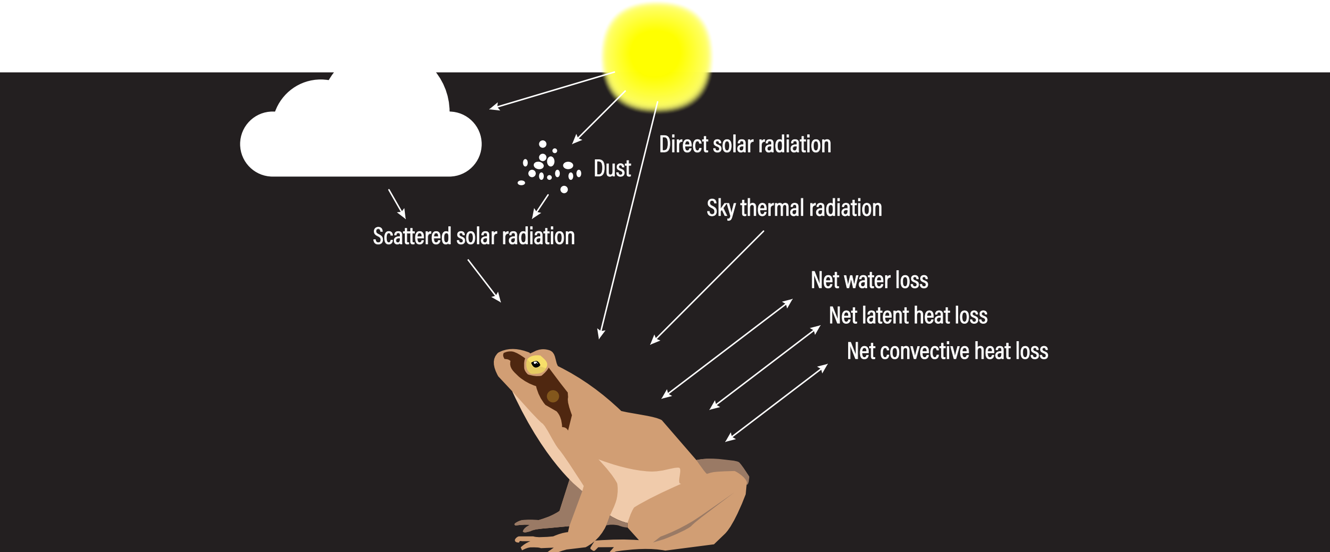


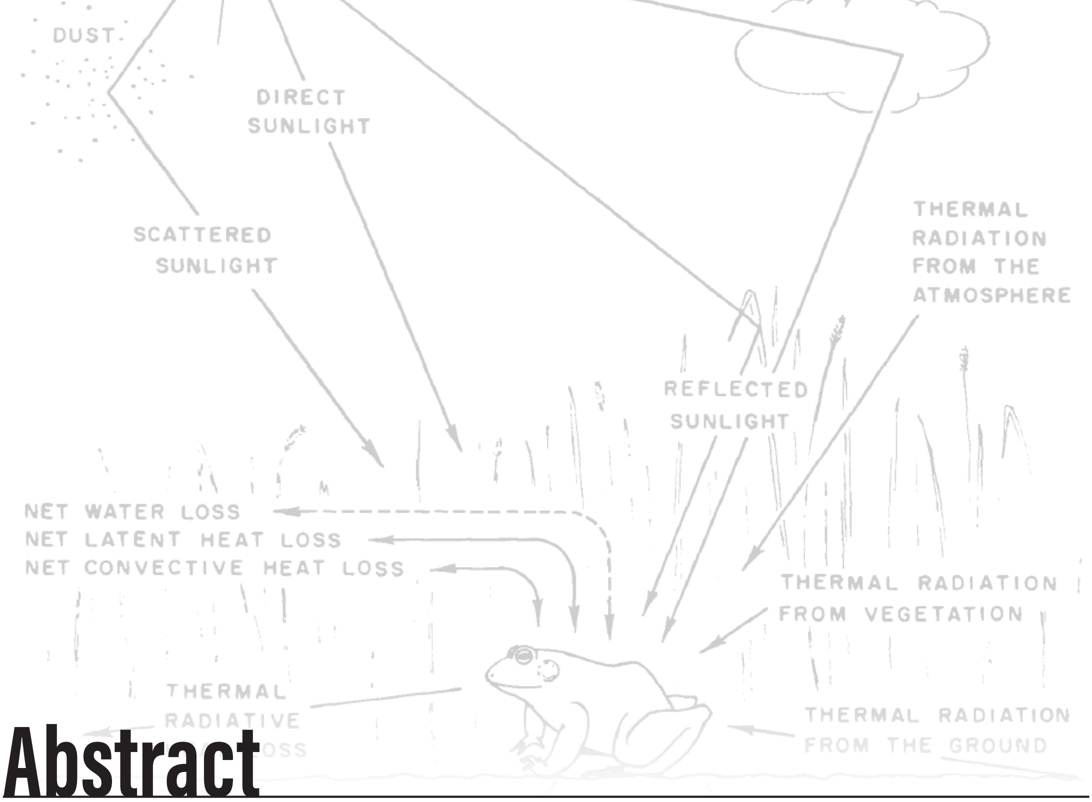
Figure S4.2: Relationship between size (i.e. body mass) and heat tolerance (*CTmax*) of **(a)** individuals acclimated to 18 ºC and **(b)** individuals acclimated to 25 ºC, corresponding to different ontogenetic stages. TAD: *black*; MET: *dark gray*; JUV2w: *light gray*; JUV4w: *white*.

CHAPTER 5

Mechanistic niche modelling reveals similar vulnerability to climate change across altitudinal gradients in juvenile *Rana temporaria*

URTZI ENRIQUEZ-URZELAI - REID TINGLEY - MICHAEL R. KEARNEY - MARTINA SACCO -
ANTONIO S. PALACIO - MIGUEL TEJEDO - ALFREDO G. NICIEZA





SOURCE: FIGURE 3 IN TRACY 1976

Abstract

Geographic phenotypic variation, acclimation capacity, and behavioral thermoregulation jointly influence the intrinsic vulnerability of species to climate change. The importance of interactions between these factors, however, remains poorly understood. We demonstrate how advances in mechanistic niche modelling can be used to integrate and assess the influence of these sources of uncertainty in forecasts of climate change impacts. We explored geographic variation in thermal tolerance (i.e. maximum and minimum critical thermal limits) and its potential for acclimation in juvenile European common frogs (*Rana temporaria*) along altitudinal gradients. Further, we employed a mechanistic niche modelling framework (NicheMapR) to assess the relative contributions of geographic phenotypic variation, acclimation capacity, and behavioral thermoregulation in determining the potential impacts of climate change on thermal safety margins and activity windows. High altitude populations had slightly wider tolerance ranges driven by slight increases in heat tolerance, but showed lower potential for acclimation. Plausibly, wider thermal fluctuations in high altitude environments favor more tolerant but less plastic phenotypes, thus reducing the risk of encountering stressful temperatures during unpredictable extreme events. Biophysical models of thermal exposure indicated that observed phenotypic and plastic differences provide limited potential to protect juvenile frogs from changing climates. Indeed, the risk of reaching body temperatures beyond the species' thermal tolerance range was similar across altitudes. In contrast, behavioral thermoregulation – in particular an ability to seek cooler retreat sites – played an essential role in buffering populations from thermal extremes predicted under climate change scenarios. Predicted changes also altered current activity windows, but high altitude populations were predicted to remain more temporally constrained than lowland conspecifics. Our results demonstrate that altitudinal variation in thermal tolerances and acclimation capacity are likely to be insufficient to buffer juvenile *R. temporaria* from predicted climate change. Instead, our findings suggest that behavioral thermoregulation will be the only effective mechanism for juvenile *R. temporaria* to avoid thermal stress under future climates.

5.1. Introduction

Anthropogenic climate change is a major threat to global biodiversity, stimulating numerous attempts to predict the vulnerability of populations, species, and ecosystems (Thomas et al. 2004; Williams, Jackson & Kutzbacht 2007; McCain & Colwell 2011; Buckley & Kingsolver 2012; Orizaola & Laurila 2016). Projected rates of climate change may hinder species' abilities to adapt to novel conditions or to track their climatic requirements through dispersal (Araújo, Thuiller & Pearson 2006; Quintero & Wiens 2013). Thus, intraspecific phenotypic variation, acclimation capacity, and behavioral thermoregulation are critical for species' persistence in a warming climate (Kearney, Shine & Porter 2009; Richter-Boix et al. 2015).

Macrophysiological studies on the vulnerability of ectotherms to climate change have flourished in recent years, largely due to an increasing availability of physiological data and fine-resolution climate layers. Many of these studies have suggested that species occupying warmer and more stable environments will be disproportionately vulnerable to warming (i.e. towards lower altitudes and latitudes; Deutsch et al. 2008; Tewksbury, Huey & Deutsch 2008; Duarte et al. 2012; but see Overgaard et al. 2011; Overgaard, Kearney & Hoffmann 2014). A number of recent investigations have challenged this view, however, demonstrating that thermal safety margins (i.e. the difference between experienced maximum temperatures and heat tolerance) and acclimation potential of species from high altitudes and latitudes may not be as high as previously thought (Gerick et al. 2014; Sunday et al. 2014; Gunderson & Stillman 2015; Gunderson, Dillon & Stillman 2017). In addition, intraspecific variation in environmental tolerances, acclimation capacity, and the potential for behavioral thermoregulation to buffer species from thermal extremes have largely been overlooked in forecasts of species' responses to climate change (Valladares et al. 2014; Buckley, Ehrenberger & Angilletta 2015).

Environmentally-driven phenotypic and genetic variation among populations is widespread (Conover & Schultz 1995; Linhart & Grant 1996). Yet, compromises between local adaptation, acclimation, and behavioral thermoregulation are emerging as key determinants of vulnerability to climate change. Behavioral thermoregulation may weaken selection on thermal tolerance and the potential for thermal acclimation – a process known as the 'Bogert effect' (Bogert 1949; Huey & Kingsolver 1993; Huey, Hertz & Sinervo 2003; Muñoz & Losos 2017). Further, local adaptation to warmer environments may reduce acclimation potential (Stillman 2003; but see Calosi, Bilton & Spicer 2008). Reduced potential for local adaptation and acclimation of thermal performance may, in turn, increase the vulnerability of populations to environmental change (Buckley et al. 2015; Gunderson & Stillman 2015). Thus, we need to develop a deeper understanding of geographic variation in thermal tolerances, acclimation potential, and behavioral thermoregulation, as well as an increased appreciation of their potential interconnections.

In ectotherms, daily and seasonal thermal fluctuations exert strong selective pressures on thermal traits (Richter-Boix et al. 2015; Gutiérrez-Pesquera et al. 2016). This could partly explain the observed wider thermal tolerance breadths towards higher altitudes and latitudes (Addo-Bediako, Chown & Gaston 2000; Gutiérrez-Pesquera et al. 2016). Shorter growing season lengths at high altitudes and latitudes also restrict the time available for growth and development (Dahl et al. 2012). Consequently, individuals may need to expose themselves to large daily thermal fluctuations to exploit transient food resources. In doing so, however, organismal body temperatures could approach critical thermal limits. To accurately predict these risks for terrestrial species requires an understanding of the processes of heat exchange (i.e. convection, radiation, conduction, evaporation, and metabolism; Heath 1964; Tracy 1976; Kearney & Porter 2009) and behavioral thermoregulation; air temperatures alone may be uninformative of these events. Moreover, the choice of appropriate spatial and temporal scales is crucial to capture effective thermal environments and extreme temperatures that, however short, may be more important than the daily average in constraining a species' or a population's long-term persistence (Kearney, Matzelle & Helmuth 2012; Gerick et al. 2014).

Here, we study geographic variation in thermal tolerance and the effects of thermal acclimation on those limits, in juvenile (~2 weeks after metamorphosis) European common frogs (*Rana temporaria*) from populations distributed along altitudinal gradients. Most studies on the impacts of climate change on amphibians have focused on the larval (Gerick et al. 2014; Gutiérrez-Pesquera et al. 2016) or adult stage (Sunday et al. 2014). However, newly metamorphosed individuals must rapidly gain reserves prior to the onset of winter, which can approach rapidly, especially at high altitudes within the species' range. In addition, *R. temporaria* juveniles are predominantly diurnal (Vences et al. 2000) and are thus more likely to encounter highly stressful temperatures than are the nocturnal adult or aquatic larval life stages.

To realistically capture the potential thermal extremes experienced by *R. temporaria* juveniles, we apply a thermodynamically-grounded mechanistic niche model (NicheMapR; Porter et al. 1973; Kearney & Porter 2016) in combination with daily gridded weather data for Europe (Haylock et al. 2008; Brinckmann, Krähenmann & Bissolli 2016). NicheMapR integrates a microclimate model of the conditions above- and below-ground for a certain level of shade, with an animal biophysical model that solves coupled heat- and mass-balance equations to predict constraints on body temperatures given an individual's behavior, morphology, and available microclimates. Specifically, we address the following questions: 1) Do juvenile *R. temporaria* differ in thermal tolerance and acclimation potential along altitudinal gradients? 2) How does geographic variation in phenotype, acclimation potential, and behavioral thermoregulation influence the species' vulnerability to climate change? 3) How will exposure to thermal stress and resulting activity restrictions change with climate change at different elevations?

5.2. Materials and Methods

STUDY SYSTEM

Rana temporaria is widespread across Europe from northern Spain to northern Scandinavia and from sea-level to a maximum of 3000 m.a.s.l. (García-París, Montori & Herrero 2004). Thus, it encounters markedly different thermal environments across its geographic range. *R. temporaria* is thought to have originated in the Iberian Peninsula, which also acted as one of the main glacial refugia (Veith et al. 2012; Vences et al. 2013). As a consequence of multiple refugia within the Iberian Peninsula (i.e. refugia within refugia; Gómez & Lunt 2007) multiple lineages diverged during climatic oscillations (Veith et al. 2012). During 2015 (August-October) and 2016 (June-August) we sampled juvenile individuals (~2 weeks after metamorphosis) from two replicate altitudinal gradients corresponding to two different lineages of *R. temporaria* (from central and eastern Cantabrian Mountains, thereafter '*central*' and '*eastern*'; Choda 2014). We sampled between 65 and 95 individuals from a total of 7 populations (**Table 5.1**).

GEOGRAPHIC VARIATION IN THERMAL TOLERANCE AND PLASTICITY

Upon arrival at the facilities of the Research Unit of Biodiversity (University of Oviedo), juveniles were randomly assigned to one of two acclimation temperature

Table 5.1: Lineage, sample size (*n*), longitude, latitude, altitude, and percentage of canopy cover (i.e. tree and shrub cover; extracted from the Spanish Forest Map at a resolution of 1:50,000; www.mapama.gob.es) for each population.

Population	Lineage	*n*	Longitude	Latitude	Altitude	Canopy cover (%)
Candioches (Can)	central	95	-5.92123	42.99991	1707 **(H)**	0
Señales (Sen)	central	89	-5.24043	43.07440	1716 **(H)**	0
Color (Col)	central	80	-5.27671	43.29492	377 **(L)**	85
Tornería (Tor)	central	78	-4.82462	43.38735	461 **(L)**	0
Hoyo Empedrado (He)	eastern	80	-4.75022	43.02275	2076 **(H)**	0
Vidrieros (Vid)	eastern	77	-4.60121	42.95523	1438 **(H)**	0
Huzmeana (Huz)	eastern	65	-4.23107	43.15771	448 **(L)**	80

treatments (14 or 24 °C). We placed each juvenile in a different plastic container with access to dechlorinated tap water to prevent dehydration. We placed plastic containers in two different weather-rooms set at 14 ± 1 and 24 ± 1 °C, respectively, with a photoperiod of 12L:12D, and let juveniles acclimate for 3-5 days. That acclimation period represents the time required to stabilize critical thermal limits (Gutiérrez-Pesquera et al. 2016). We supplied juveniles with small *Acheta domestica* crickets *ad libitum*.

After the acclimation period, we estimated thermal tolerances (upper and lower critical thermal limits, CT_{max} and CT_{min}, respectively). For each population and acclimation temperature, juveniles were assigned to either CT_{max} or CT_{min} experiments. To estimate thermal tolerances, we followed Hutchison's dynamic method (Lutterschmidt & Hutchison 1997). We placed individuals in 100-mL plastic containers with dechlorinated tap water at 20°C, and heated or cooled the water at a constant rate of 0.25°C per minute using a refrigerated heating bath (HUBER K15-cc-NR; Kältemaschinenbau AG, Germany), for CT_{max} and CT_{min}, respectively. We considered that thermal limits had been reached when individuals remained unresponsive to external stimuli (10 gentle taps with a wooden stick). At that point, we recorded water temperature with a quick-recording thermometer (Miller & Weber; Ridgewood, NY, USA) to the nearest 0.1 °C. Because of the small size of the individuals, we assumed that body temperatures equated water temperature (Lutterschmidt & Hutchison 1997; Gutiérrez-Pesquera et al. 2016). After CT_{max} and CT_{min} tests, we transferred individuals to cold (14 °C) or warm water (20 °C) respectively, and we verified survival after 24h to ensure that thermal limits were not surpassed. Finally, we weighed each individual to the nearest 0.0001 g.

We used mixed effect ANCOVAs to test for effects of body mass, elevation, lineage, and acclimation temperature treatment. Body mass (M) was used as a covariate, and population was treated as a random effect. Elevation (H = high and L = low, **Table 5.1**), lineage ('*central*' and '*eastern*'), acclimation treatment (14 and 24 °C), and their interactions were treated as fixed factors. If interactions involving body mass resulted nonsignificant (homogeneity of slopes), we dropped the interaction between covariate and factors from models. We visually assessed the normality of residuals using residual distribution and quantile-quantile plots.

BIOPHYSICAL MODELLING

We developed mechanistic niche models using NicheMapR – an R implementation of the biophysical models developed by Porter and colleagues (Porter et al. 1973; Kearney & Porter 2016). NicheMapR includes programs that solve heat and mass budgets for both microclimates and animals given terrain and weather conditions, and the animal's morphology, behavior, and physiology. In this way it can estimate hourly operative temperatures (T_e) – the steady-state temperatures that the animal could achieve in a given habitat. The microclimate model uses maximum and minimum air temperatures, precipitation, cloud cover, relative humidity, and wind speed values to reconstruct available microclimates. We obtained daily values for these parameters from the ECA&D (Haylock et al. 2008) and DecReg/MiKlip (Brinckmann et al. 2016) projects for each studied population during 2014 and 2015 (see **Supplementary Material**). To estimate available microclimates under a climate warming scenario, we examined projections for two time periods (2050 and 2070) using three global circulation models (GCMs: CCSM4, HadGEM2-CC, and GFDL-CM3) and two emission scenarios (low, RCP 4.5, and high, RCP 8.5). We computed monthly differences between current and projected climates (maximum and minimum temperatures, and precipitation) using WorldClim layers – IPCC5 – at a spatial resolution of 30 arc-seconds (Hijmans et al. 2005). We extracted projected monthly anomalies for each population, interpolated those to daily data, and added them to the observed values from ECA&D (see **Supplementary Material**). We used weather data corresponding to the first modelled year (i.e. 2014) as the spin-up period (e.g. for snowpack); we only present results for 2015.

We modelled a medium-sized early juvenile (0.3 g), with its midpoint at 0.5 cm; a shape equivalent to that of the leopard frog (*Lithobates pipiens*); and assumed that 90% of the skin acted as a free water surface when individuals were active (see Kearney et al. (2008) for more information). Since juveniles are predominantly diurnal, we allowed only diurnal activity, bounded within the maximum (VT_{max} = 18.5 °C) and minimum (VT_{min} = 9.5 °C) temperatures selected in a thermal gradient experiment conducted on ~16 juveniles of each of the studied populations (U. Enriquez-Urzelai et al. unpublished data). When T_e fell outside of these temperatures, we assumed that animals burrowed underground to the depth with the closest temperature to their preferred temperature (T_{pref} = 13.1 °C), obtained from the same thermal gradient experiment (U. Enriquez-Urzelai et al. unpublished data). See **Supplementary Material** for detailed figures showing T_e traces and behaviour of example simulations.

To quantify exposure to stressful temperatures, we modelled the T_e of non-thermoregulating (above ground) or thermoregulating individuals (burrowing up to 30 cm underground) in full sun (0% shade) or deep (90%) shade. *Rana temporaria* juveniles remain at the edge of water bodies, where they forage on invertebrates (Vences et al. 2000; García-París et al. 2004), and retreat to small cracks and crevices in the soil during the hottest months of the year (U. Enriquez-Urzelai & A. G. Nicieza, personal observation). However, like other similar species (Lamoureux & Madison 1999; Roznik & Johnson 2009; Qi et al. 2011), radio-tracked adult *R. temporaria* also use small mammal burrows during summer (U. Enriquez-Urzelai, A. Gandara, A. G. Nicieza unpublished data). Thus, we allowed thermoregulating juveniles to burrow up to 30 cm underground to examine any potential shortcoming of behavioural thermoregulation (see **Results**). Subsequently, we computed the thermal safety margins (*TSM*: $CT_{max} - T_{e,max}$ and $T_{e,min} - CT_{min}$; *sensu* Sunday et al. 2014) of individuals acclimated to 14 and 24 °C. Positive *TSM* indicate that critical thermal limits exceed experienced thermal extremes, while negative *TSM* suggest exposure to temperatures outside the tolerance range. To explore the impacts of climate change on activity windows (hours year^{-1}) for populations at different altitudes, we modelled thermoregulating juveniles – using underground burrows up to 30 cm deep – both for current and future climates, and with full sun or deep (90%) shade.

5.3 Results

GEOGRAPHIC VARIATION IN THERMAL TOLERANCE AND PLASTICITY

Homogeneity of slopes was met for CT_{max} (**Table S5.1**). We found differences in CT_{max} due to mass, acclimation treatment, and altitude. Further, we found differences in the acclimation potential due to altitude (significant acclimation treatment × altitude interaction; **Table 5.2**). Heavier juveniles showed slightly higher CT_{max} values, and populations from high altitudes showed higher CT_{max} but lower acclimation potential than lowland populations (**Fig. 5.1A**). Lineage did not affect CT_{max}.

CT_{min} varied with mass and acclimation treatment. We also found differences in acclimation potential of CT_{min} due to mass (mass × acclimation treatment), altitude (altitude × acclimation treatment), and mass within lineage (mass × lineage × acclimation

Table 5.2: Analysis of covariance for heat tolerance – upper thermal limit or *CTmax* –, including mass (covariate; *M*) and altitude (*alt*), lineage (*lin*), acclimation treatment (*acc*), and their interactions. Population was included as random factor. '*df*' stands for degrees of freedom.

	F-value	df	P-value
Mass (*M*)	18.62	1, 272	< 0.0001
Altitude (*alt*)	13.78	1, 3	0.034
Lineage (*lin*)	0.22	1, 3	0.672
Acclimation treatment (*acc*)	37.98	1, 272	< 0.0001
alt* × *lin	0.12	1, 3	0.750
alt* × *acc	6.82	1, 272	0.010
lin* × *acc	0.66	1, 272	0.418
alt* × *lin* × *acc	0.00	1, 272	0.964

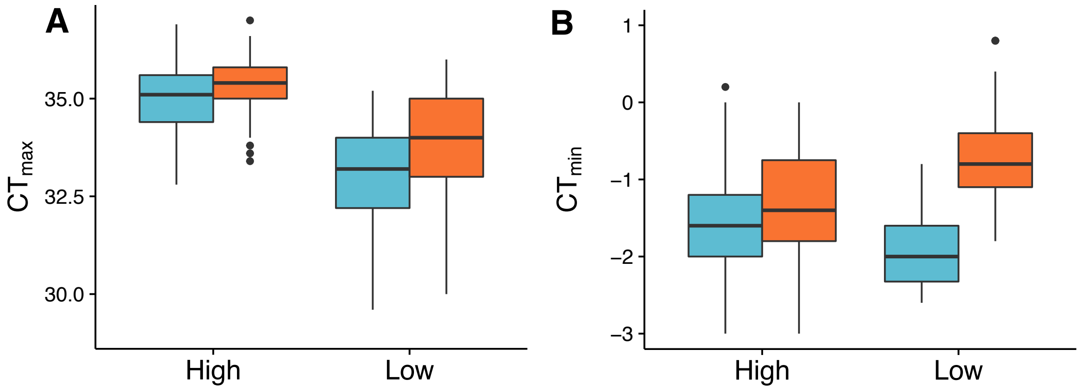


Figure 5.1: **(A)** Heat tolerance – upper thermal limit or *CTmax* – and **(B)** cold tolerance – lower thermal limit or *CTmin* – of *Rana temporaria* juveniles from high and low elevations, acclimated to 14 (*blue*) or 24 ºC (*orange*).

Table 5.3: Analysis of covariance for cold tolerance – lower thermal limit or *CTmin* –, including mass (covariate; *M*) and altitude (*alt*), lineage (*lin*), acclimation treatment (*acc*), and their interactions. Population was included as random factor. '*df*' stands for degrees of freedom.

	F-value	df	P-value
Mass (M)	9.38	1, 261	0.002
Altitude (alt)	0.00	1, 3	0.982
Lineage (lin)	5.13	1, 3	0.109
Acclimation treatment (acc)	95.68	1, 261	< 0.0001
M* × *alt	0.83	1, 261	0.362
M* × *lin	0.34	1, 261	0.560
alt* × *lin	2.87	1, 3	0.189
M* × *acc	22.88	1, 261	< 0.0001
alt* × *acc	26.76	1, 261	< 0.0001
lin* × *acc	0.70	1, 261	0.404
M* × *alt* × *lin	0.11	1, 261	0.742
M* × *alt* × *acc	3.05	1, 261	0.082
M* × *lin* × *acc	4.31	1, 261	0.039
alt* × *lin* × *acc	0.01	1, 261	0.920
M* × *alt* × *lin* × *acc	0.50	1, 261	0.480

treatment; **Table 5.3**). Smaller juveniles showed higher acclimation potential, and this effect was more evident in the 'central' lineage. Similar to CT_{max}, heavier juveniles showed higher CT_{min} values, and acclimation potential was lower in high-altitude populations compared to lowland conspecifics (altitude × acclimation treatment; **Fig. 5.1B**).

EXPOSURE TO THERMAL EXTREMES

PHYSIOLOGICAL THERMOCONFORMITY

Niche modelling simulations suggested that, under the current climate and full sun conditions, non-thermoregulating juveniles from both high and low altitudes had CT_{max} values that exceeded operative temperatures (i.e., positive *TSM*), due to effects of evaporative cooling. The higher acclimation potential of CT_{max} observed in lowland populations resulted in remarkably similar *TSM* among different altitudes when individuals were acclimated to high temperatures (**Fig. 5.2A-B**). Conversely, all high altitude populations and one low altitude (Huz) population showed negative *TSM* to cold extremes. This pattern was also evident when individuals were acclimated to low temperatures (**Fig. 5.2C-D**).

Our simulations further suggest that, by 2050, evaporative cooling may be insufficient to buffer low altitude populations exposed to full sun from extreme heat. By

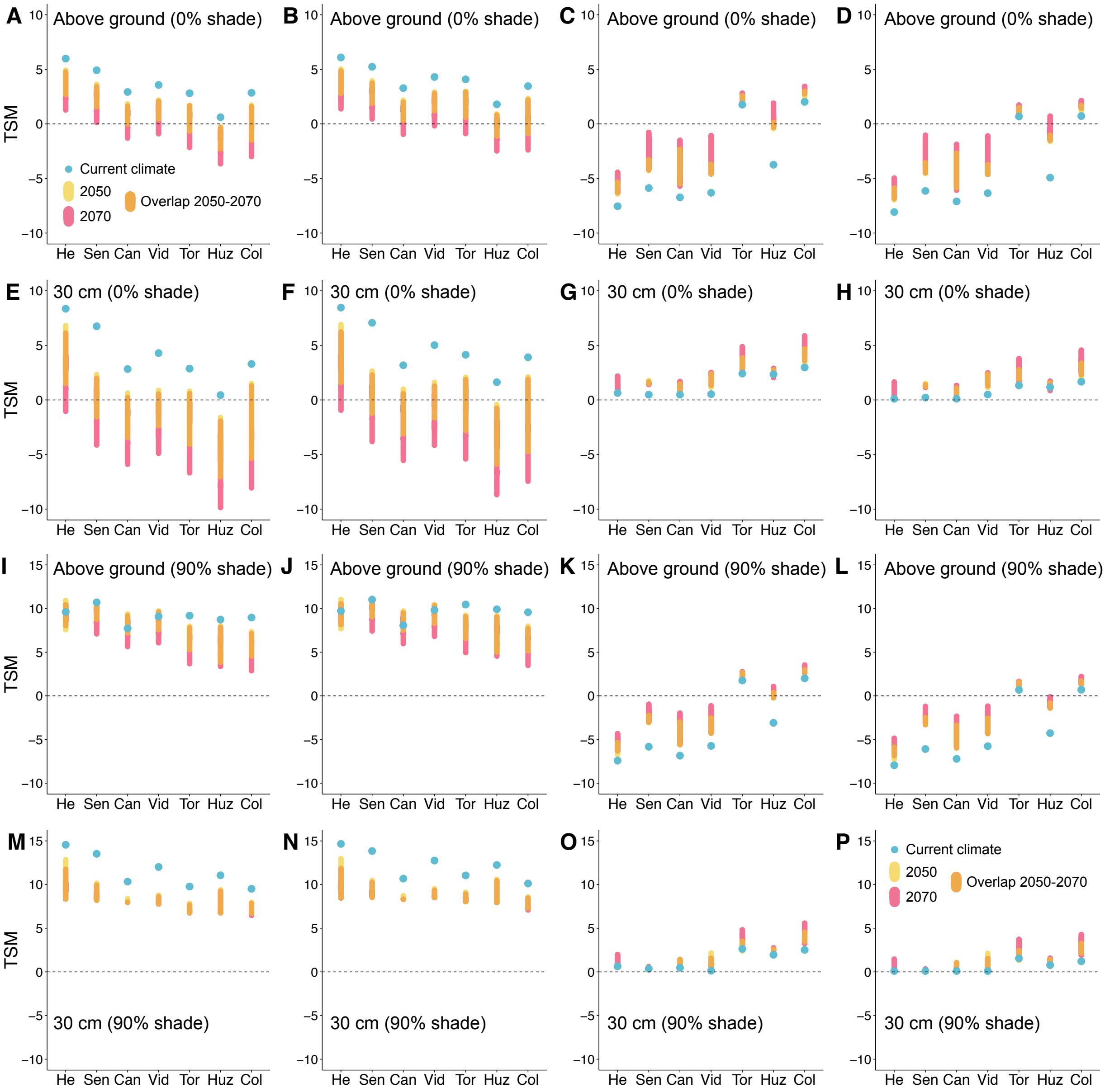


Figure 5.2: Thermal safety margins (TSM) of **(A-D)** nonthermoregulating and **(E-H)** thermoregulating juveniles using burrows of up to 30 cm depth in full sun (0% shade), and **(I-L)** nonthermoregulating and **(M-P)** thermoregulating juveniles using burrows of up to 30 cm depth in deep (90%) shade. **(A, E, I, M)** show TSM to extreme heat of individuals acclimated to 14ºC, **(B, F, J, N)** TSM to extreme heat of individuals acclimated to 24ºC, **(C, G, K, O)** TSM to extreme cold of individuals acclimated to 14ºC, and **(D, H, L, P)** TSM to extreme cold of individuals acclimated to 24ºC. *Blue dots*: under the current climate; *yellow bars*: under projected climates for 2050; *red bars*: under projected climates for 2070; *orange*: overlap between simulations for 2050 and 2070.

2070, evaporative cooling may also become insufficient for two high altitude populations (Can and Vid) under full sun conditions; the other two highland populations (Sen and He) will experience T_e close to their CT_{max} (**Fig. 5.2A**-**B**). Acclimation to warm temperatures had limited effects on an individuals' capacity to buffer heat stress under future climate regimes (**Fig. 5.2**; compare panel **A** with **B**). Deep shade, by contrast, may protect individuals from overheating under projected climates (**Fig. 5.2I**-**J**). Regardless of shading level, by 2070, only the lowland population that currently experiences negative *TSM* to cold extremes (Huz) will be able to tolerate the lowest temperatures during the year, and this was evident only when individuals were acclimated to low temperatures (**Fig. 5.2C**-**D**, **K**-**L**).

ACCOUNTING FOR BEHAVIORAL THERMOREGULATION

Under current climatic conditions and both shading levels, retreating to burrows as deep as 30 cm allowed juveniles to maintain positive *TSM* under both hot and cold extremes; however, by 2050, individuals from both high and low altitudes may need to seek deeper burrows to avoid exceeding their CT_{max} when exposed to full sun (**Fig. 5.2E**-**F**). Even individuals from the population at the highest altitude (He) may need to burrow deeper than 30 cm by 2070 to escape heat stress. Acclimation to warm temperatures had limited impacts on these projections (**Fig. 5.2**; compare panel **E** with **F**, and **M** with **N**). Burrows in deep shade, however, are predicted to remain thermally suitable under projected climates (**Fig. 5.2M**-**N**).

ACTIVITY WINDOWS

Niche modelling simulations revealed that high altitude populations have reduced opportunities for activity compared to lowland conspecifics. Interestingly, while deep shade (90%) enabled individuals from lowland populations to be active for longer periods, it reduced activity windows in high altitude populations (**Fig. 5.3**). Shade allowed juveniles from all populations to be active during the hottest hours during summer, but prevented high altitude populations from achieving the temperatures required for activity during the coldest seasons.

Most of the studied populations were predicted to show a decline in activity under climate change scenarios when individuals were restricted to full sun. The only exception was the population at the highest altitude (He), for which individuals were predicted to have similar activity windows under current and future climates (**Fig. 5.3A**). Opportunities for activity of high altitude populations are predicted to decline most in summer, and increase in spring, autumn, and winter. Lowland populations will experience reductions

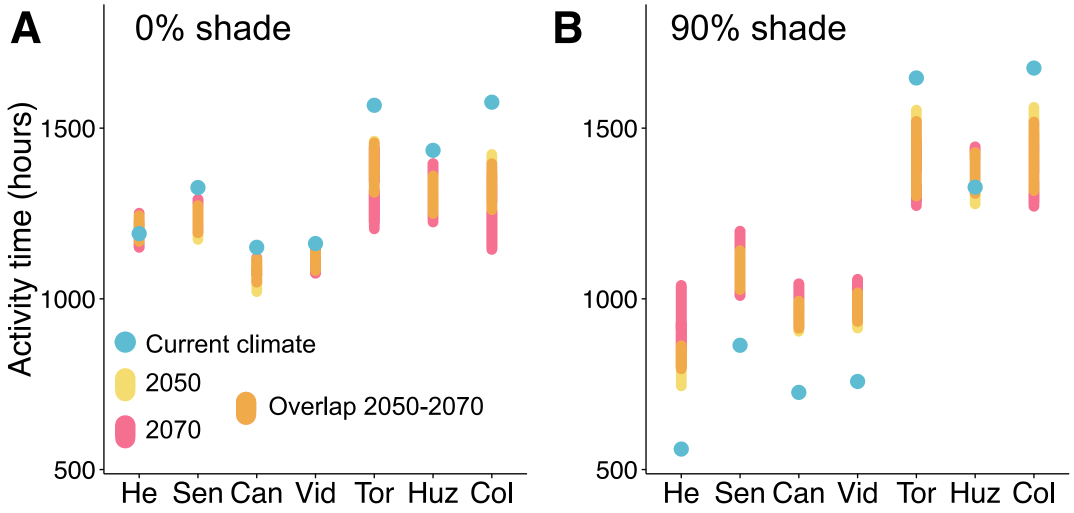


Figure 5.3: Activity time in hours under **(A)** full sun (0% shade) and **(B)** deep shade (90% shade). *Blue dots*: under the current climate; *yellow bars*: under projected climates for 2050; *red bars*: under projected climates for 2070; *orange*: overlap between simulations for 2050 and 2070.

in activity during spring and autumn, and increases during winter (**Fig. 5.4A, C, E, G**). Altogether, reductions in activity hours will exceed increases in full sun conditions under projected future climates (**Fig. 5.3A**).

When simulated to experience deep shade, activity times for high altitude populations under climate change are predicted to decrease in summer, but increase in spring and autumn (**Fig. 5.4B, D, F, H**). Overall, activity times in deep shade are predicted

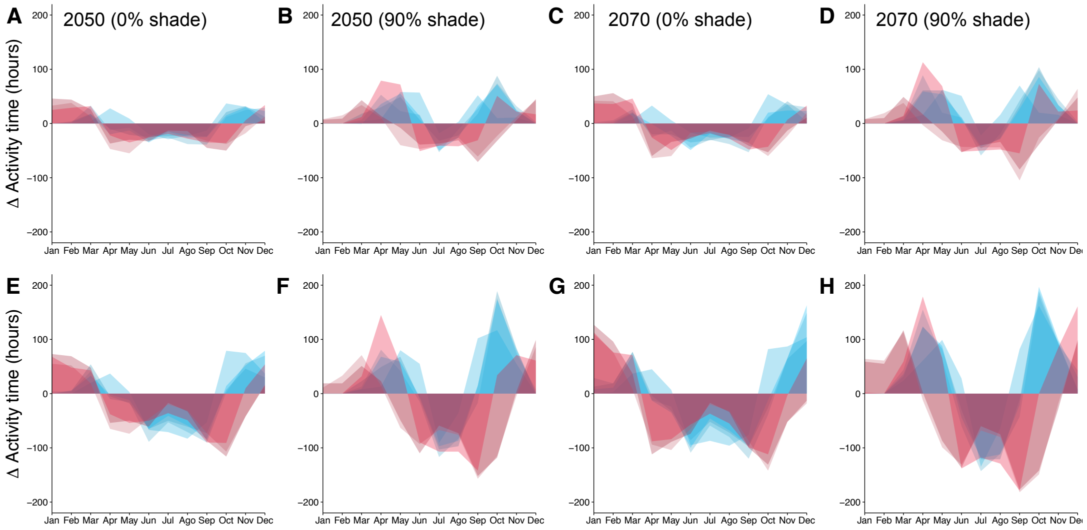


Figure 5.4: Differences in activity time relative to current climates under the specified shading levels for **(A-D)** a low emission scenario (i.e. RCP 4.5) and the most benign GCM (CCSM4), and for **(E-H)** a high emission scenario (i.e. RCP 8.5) and the GCM with the highest projected temperature increases (GFDL-CM3). *Blue polygons*: high elevation populations; *Red polygons*: low elevation populations.

to increase under climate change, but were, nevertheless, predicted to be shorter than when individuals were restricted to full sun. Lowland populations were predicted to experience a marked reduction in activity times during spring, summer, and autumn, and an increase during winter (**Fig. 5.4B, D, F, H**). It should be noted, however, that although high altitude populations show an increase in activity times under future climates in deep shade, they will remain more temporally constrained than their lowland counterparts under the same conditions (**Fig. 5.3B**).

5.4. Discussion

Most species constitute an array of locally adapted and unequally plastic populations (Hereford 2009; Benito Garzón et al. 2011; Orizaola & Laurila 2016). Accordingly, thermal traits and their plasticity frequently vary among populations of the same species, typically paralleling changes in the thermal environment (Freidenburg & Skelly 2004; Sinclair, Williams & Terblanche 2012; Sinclair et al. 2016). Ultimately, geographic phenotypic variation may result in an uneven sensitivity to thermal extremes across the range of a species, and consequently influence forecasts of climate change impacts (Pearson, Lago-Leston & Mota 2009; Valladares et al. 2014). Behavioral thermoregulation, a mechanism by which animals can buffer themselves against extreme temperatures, will also be decisive for terrestrial ectotherms under changing climates (Kearney et al. 2009; Sunday et al. 2014). Yet, we are just starting to understand the interplay between environmental tolerances, plasticity, and behavioral thermoregulation, and how this interplay will influence climate change impacts (Williams et al. 2008; Chevin, Lande & Mace 2010; Huey et al. 2012).

Our results show that while heat tolerance (CT_{max}) of juvenile R. temporaria increases slightly with altitude, cold tolerance (CT_{min}) does not. This contrasts with previous interspecific studies which revealed higher geographic variation in CT_{min} compared to CT_{max} associated to latitudinal and altitudinal clines (Sørensen, Dahlgaard & Loeschcke 2001; Sunday, Bates & Dulvy 2011; von May et al. 2017). It is noteworthy, however, that the cold tolerance of this species is beyond the freezing point of water, regardless of altitude (**Fig. 5.1**). This result suggests that individuals may be at risk of freezing both at high and low altitudes, as already reported for this species (Muir, Biek & Mable 2014). The extreme cold tolerance of *R. temporaria* juveniles, together with the lack of cryoprotectors (e.g. glucose or urea) found for this species (Ludwig, Sinsch

& Pelster 2015; J. M. Carvajalino-Fernández, U. Enriquez-Urzelai, A. G. Nicieza & M. Tejedo, unpublished data) suggest that this species tolerates the freezing of some portion of extracellular water (Costanzo et al. 2013). Previous studies have similarly reported increased thermal tolerance ranges towards higher latitudes and altitudes (Gaston & Chown 1999; Araújo et al. 2013; Gutiérrez-Pesquera et al. 2016). However, we found that wider thermal tolerance in high altitude populations was driven by small shifts toward higher heat tolerance.

Additionally, we found that juvenile *R. temporaria* originating from high altitude populations showed lower acclimation potential (i.e. plasticity), not only in heat tolerance, but also in cold tolerance. Recent studies have shown that acclimation potential decreases with increasing latitude (Seebacher, White & Franklin 2015; Gunderson & Stillman 2015). Although plastic thermal tolerances may be adaptive (Sultan & Spencer 2002), the wider thermal fluctuations and shorter growing season lengths associated with high elevations, plausibly, favor more tolerant and less plastic phenotypes, which avoid paying the costs of plasticity (Dewitt, Sih & Wilson 1998).

Projected rates of climate change exceed the estimated pace of historic niche evolution (Quintero & Wiens 2013). The high similarity in thermal tolerance observed here between phylogenetic lineages of *R. temporaria*, coupled with only slight differences in heat tolerance between populations from different altitudes, suggest that *R. temporaria* may not be capable of adapting to novel conditions through niche evolution. Additionally, our mechanistic niche models revealed that acclimation provides limited potential to buffer individuals from heat stress under changing climates, in agreement with recent macrophysiological studies (Gunderson & Stillman 2015; Gunderson et al. 2017). Acclimation to warm or cold temperatures had negligible effects on estimates of current and future thermal safety margins, even in the most plastic lowland populations (**Fig. 5.2**). Indeed, the negligible degree of local adaptation, together with the higher potential for acclimation of lowland populations, led to a striking similarity in thermal safety margins across altitudes. Thus, although we expect biodiversity to shift towards higher altitudes (McCain & Colwell 2011), our results show that the risk of overheating will be similar at different altitudes (see Overgaard et al. (2014) for a similar result across latitudes).

Taken together, our results suggest that altitudinal variation in thermal tolerances and acclimation capacity are largely insufficient to buffer juvenile *R. temporaria* from thermal extremes under current and future climates. Instead, mechanistic niche models revealed that behavioral thermoregulation is a key mechanism to escape extreme cold temperatures in all high altitude populations and in one lowland population (**Fig. 5.2**). The use of behavioral thermoregulation to avoid extreme temperatures could partly account for the observed low degree of local adaptation, through the 'Bogert effect' (Bogert 1949; Buckley et al. 2015; Muñoz & Losos 2017). In addition, our models suggest that behavioral thermoregulation will be key to compensating the loss of effectiveness of evaporative cooling under future climates.

Evaporative cooling is a highly efficient mechanism that allows wet skinned ectotherms, such as amphibians, to dissipate excessive heat (Tracy 1976; Kearney & Porter 2009). Our results show that, due to evaporative cooling, juvenile *R. temporaria* avoid overheating at all altitudes, even in full sun, as pointed out by Sunday et al. (2014). Notwithstanding, by 2050, lowland populations are predicted to be incapable of buffering themselves from extreme heat exclusively through evaporative cooling. Remarkably, by 2070 evaporative cooling may not protect even some high altitude populations (Can and Vid) from overheating, and others (Sen and He) will experience body temperatures close to their maximum heat tolerance (**Fig. 5.2**). Thus, although evaporative cooling might represent an effective mechanism to avoid overheating under the current climate, regardless of altitude, behavioral thermoregulation might become crucial for juveniles to buffer heat stress in the future (Kearney & Porter 2009; Sunday et al. 2014; Ruiz-Aravena et al. 2014). Indeed, our simulations suggest that juvenile *R. temporaria* could potentially avoid high temperatures by seeking deep retreat sites, or by restricting their activity to shaded microhabitats. However, typical burrow depths (30 cm) may become insufficient to buffer rising temperatures at all altitudes. Additionally, canopy cover is minimal at the studied sites, especially those at high altitudes (**Table 5.1**). Thus, at high altitudes, current shade levels and currently-occupied burrows are unlikely to protect juvenile frogs from extreme temperatures.

Our models correctly captured the observation that, under the current climate, *R. temporaria* populations from high altitudes are more temporally constrained than lowland conspecifics (Miaud, Guyetant & Elmberg 1999; **Fig. 5.3**). However, our simulations also uncovered important interactions between activity restrictions, altitude, and the available amount of shade. Under the current climate, deep shade allowed populations at all altitudes to increase activity times during the hottest season, but prevented high altitude populations from attaining body temperatures required for activity during the coldest seasons. As a consequence, while shading widened activity windows at low altitudes, it narrowed activity windows of high altitude populations. Thus, the thermal challenge of ectotherms might be to stay cool in warm environments and warm in cold environments (Kearney et al. 2009).

Under a warmer climate, populations from colder environments (e.g. the tops of mountains) could benefit from wider activity windows (Levy et al. 2016). However, we demonstrate that warming could narrow the window for activity of juvenile *R. temporaria* at all altitudes. In general, decreases in activity hours during warmer months exceeded increases during colder months (**Fig. 5.4**). Only high altitude populations in deep shade had wider activity windows under climate change. Regardless of the available amount of shade, activity windows will remain narrower with increasing altitude.

Geographic phenotypic variation, the potential for plastic responses, behavioral thermoregulation, and their interactions may mediate the impacts of climate change on the extinction risk of populations throughout a species' distribution (Kolbe, Kearney & Shine 2010; Valladares et al. 2014; Buckley et al. 2015; Gunderson et al. 2017). We show that mechanistic niche models offer a flexible means with which to integrate and assess the influence of these sources of uncertainty in climate change forecasts (Kearney & Porter 2009; Moran, Hartig & Bell 2016). Applying this modelling framework to juvenile *R. temporaria* suggests that the primary source of forecast uncertainty is the role of behavioral thermoregulation, in particular, the species' ability to seek deeper retreats and shaded microhabitats. Despite phenotypic variation in juvenile *R. temporaria* associated with altitudinal gradients, we found that the risk of reaching detrimental body temperatures under changing climates was predicted to be similar across altitudes. Furthermore, although we expect populations from colder environment to benefit from wider activity windows under climate change (Levy et al. 2016), juvenile *R. temporaria* at high altitudes were predicted to be more temporally constrained than lowland counterparts under both current and future predicted climates. Hence, climate change vulnerability may be quite similar across altitudes in *R. temporaria*. Further work integrating other sources of uncertainty (e.g. complex life histories) may help advance the reliability and robustness of climate change predictions.

5.5 References

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Supplementary Material for Chapter 5

CLIMATE DATA

To estimate available microclimates, NicheMapR (Porter et al. 1973; Kearney & Porter 2016) uses maximum (T_{max}) and minimum (T_{min}) air temperatures, precipitation (*Prec*), cloud cover (*CC*), humidity (*RH*), and wind speed (*WS*) values. We obtained gridded daily T_{max}, T_{min}, T_{mean} and *Prec* for Europe at a resolution of 0.25º (ECA&D; Haylock et al. 2008) and gridded daily *WS* for Europe at a resolution of 0.044º (DecReg/MiKlip; Brinckmann, Krähenmann & Bissolli 2016). With these datasets, we could extract daily T_{max}, T_{min}, T_{mean}, *Prec*, and *WS* for all the studied populations. To estimate daily values of *CC* and *RH* in all the study sites we also obtained Spanish weather stations measurements for T_{max}, T_{min}, T_{mean}, *Prec*, *CC*, and *RH* from ECA&D, and monthly maximum cloud cover (CC_{New}) and maximum relative humidity (RH_{New}) from a global dataset (New et al. 2002). As a first step, we determined the statistical relationship between environmental variables in a station-specific fashion. Specifically, we developed general linear models (GLMs) to predict daily *CC* and *RH* measured by each weather station. To predict daily *CC*, we transformed *CC* to proportion data and logit-transformed it before fitting GLMs with the structure shown in **eq. 1**.

$$\text{logit}(CC) \sim Prec + T_{max} + T_{min} + CC_{New} \quad \textbf{(eq.1)}$$

To predict daily *RH*, first we transformed *RH* to vapour pressure (*VP*) based on T_{mean} using the *VAPPRS()* function implemented in NicheMapR. Then we fitted GLMs with the structure shown in **eq. 2**.

$$VP \sim Prec + Prec^2 + T_{max} + T_{min} + T_{min}^{\;2} + RH_{New} \quad \textbf{(eq.2)}$$

Then, we extracted the coefficients of the intercept and coefficients of each explanatory variable from each weather station-specific GLM and interpolated these model parameters across Spain using each weather station's latitude, longitude, and altitude as covariates. For the interpolation we used thin plate spline regressions as implemented in the *Tps()* and *interpolate()* functions of the fields and raster R-packages respectively. As a result, we obtained five interpolated layers of GLM parameters with which to predict *CC* (interpolated *intercept*, *Prec*, T_{max}, T_{min} and CC_{New} coefficients) and seven interpolated layers with which to predict *VP* (interpolated *intercept*, *Prec*, $Prec^2$, T_{max}, T_{min}, $T_{min}{}^2$ and RH_{New} coefficients). Predicted *VP* values were back-transformed to *RH* using the *VAPPRS()* function.

We compared our predictions for *CC* and *RH* with data from weather stations close to our study populations, obtained from the Spanish weather bureau (Aemet; **Fig. S5.1**). For each Aemet station we extracted daily T_{max}, T_{min}, and *Prec* from ECA&D gridded layers, and New's *CC* and New's RH_{max} from New et al. (New et al. 2002) dataset, and the interpolated coefficient values. With these values, we predicted *CC* and *RH*, and performed linear regressions and computed the mean square error (MSE) between observed and predicted values (**Fig. S5.2-S5.5**).

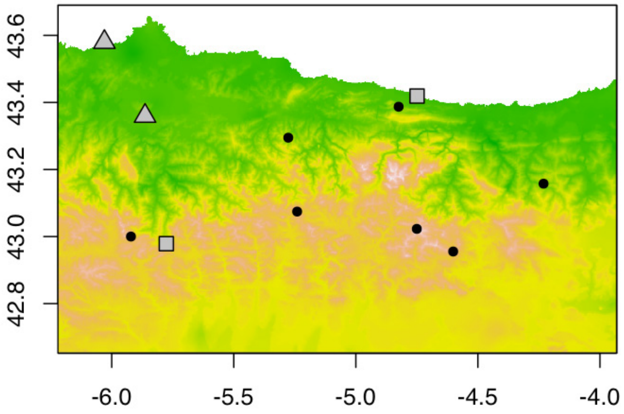

Figure S5.1: Grey squares represent weather stations obtained from Aemet with observed values of *RH*, grey triangles weather stations with observed *CC*, and black points our study populations.

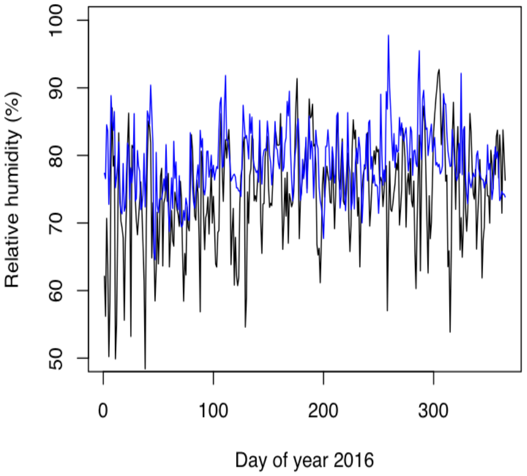

Figure S5.2: Observed (black line) and predicted (blue line) *RH* for the coastal weather station. MSE: 8.939. Linear regression: $F_{1,363} = 37.27$, $P < 0.001$, $R^2 = 0.091$.

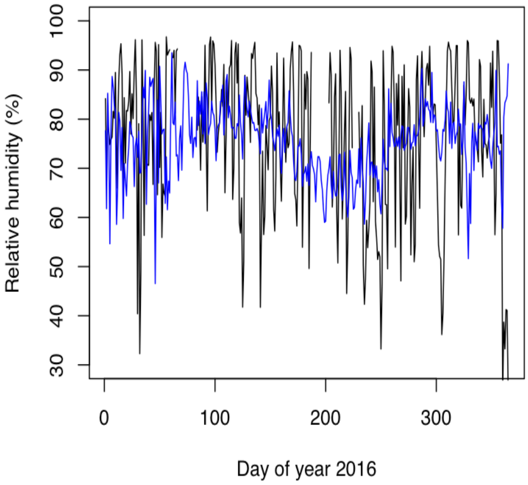

Figure S5.3: Observed (black line) and predicted (blue line) *RH* for the mountainous weather station. MSE: 16.092. Linear regression: $F_{1,324} = 7.618$, $P = 0.006$, $R^2 = 0.020$.

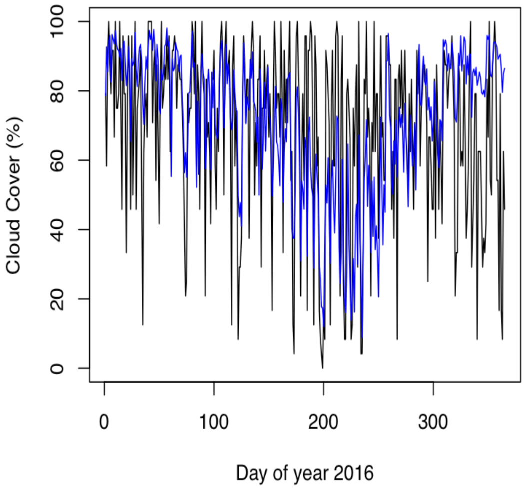

Figure S5.4: Observed (black line) and predicted (blue line) *CC* for the coastal weather station. MSE: 22.450. Linear regression: $F_{1,363} = 146.9$, $P < 0.001$, $R^2 = 0.286$.

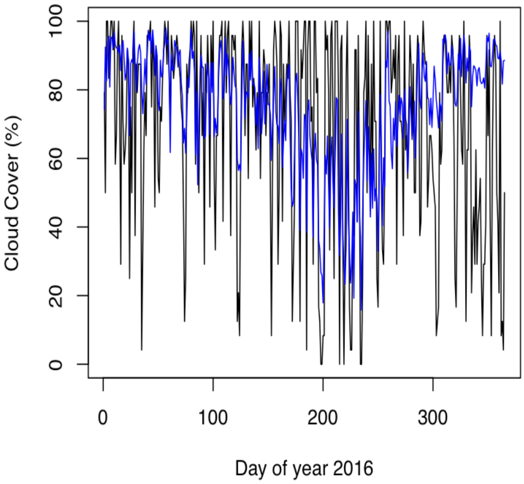

Figure S5.5: Observed (black line) and predicted (blue line) *CC* for the inland weather station. MSE: 25.096. Linear regression: $F_{1,363} = 138.4$, $P < 0.001$, $R^2 = 0.274$.

FUTURE CLIMATES

To make climate change projections, we obtained current and future T_{max}, T_{min}, and *Prec* for three different global circulation models or GCMs (CCSM4, HadGEM2-CC, and GFDL-CM3) and two emission scenarios (RCP 4.5 and 8.5) from WorldClim (Hijmans et al. 2005). For each GCM and emission scenario combination we computed the monthly difference with the baseline (i.e. current climate). To estimate future T_{max} and T_{min}, we interpolated (spline) monthly T_{max} and T_{min} anomalies to daily values and added them to the values extracted from ECA&D gridded data (**Fig. S5.6**). For T_{mean} we added the mean between T_{max} and T_{min} anomalies to the extracted T_{mean} values from ECA&D gridded data. For future precipitation regimes, we divided the estimated monthly change in precipitation by the number of rainy days in each month obtained from the ECA&D dataset. Subsequently, we subtracted that value from the precipitation of each rainy day of the corresponding month. All the rainy days that resulted in negative precipitation were converted to no-rain days (*precipitation*=0), and we subtracted that difference from the heaviest precipitation event of that month (**Fig. S5.7**).

EXAMPLE NICHEMAPR SIMULATIONS

We modelled *R. temporaria* juvenile (0.3 g) allowing only diurnal activity, bounded within the maximum (VT_{max} = 18.5 °C) and minimum (VT_{min} = 9.5 °C) temperatures selected in a thermal gradient experiment (U. Enriquez-Urzelai et al. unpublished data).

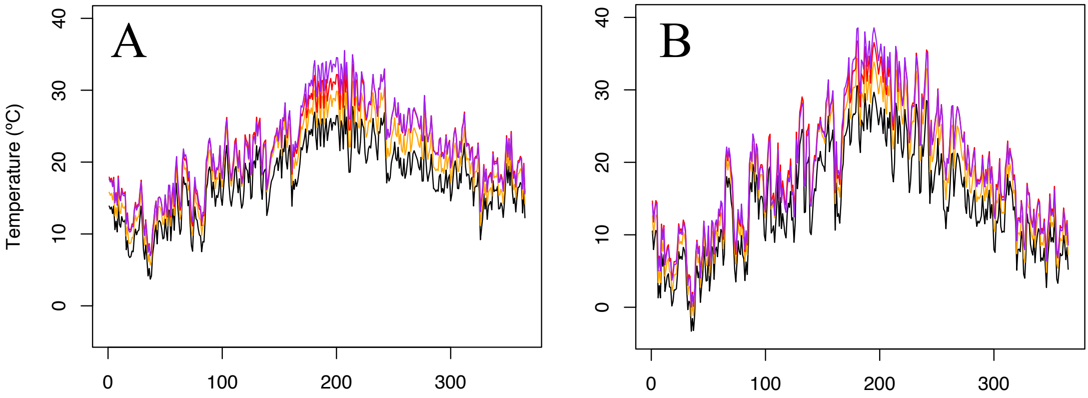

Figure S5.6: Maximum temperature (*Tmax*) change **(A)** in a lowland population and **(B)** in a highland population. *Black*: current *Tmax*; *Orange*: *Tmax* in 2070 under RCP 8.5 and CCSM4; *Red*: RCP 8.5 and HadGEM2-CC; *Orange*: RCP 8.5 and GFDL-CM3.

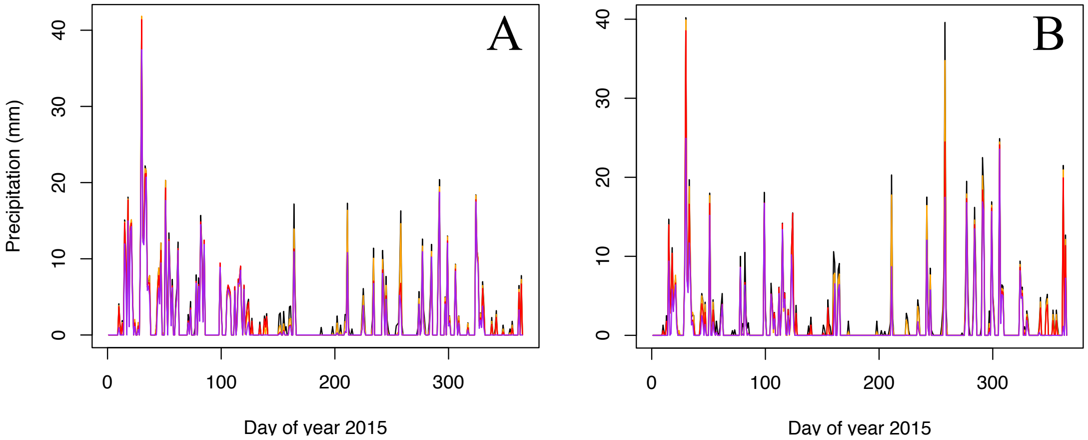

Figure S5.7: Precipitation change **(A)** in a lowland population and **(B)** in a highland population. *Black*: current precipitation; *Orange*: precipitation in 2070 under RCP 8.5 and CCSM4; *Red*: RCP 8.5 and HadGEM2-CC; *Orange*: RCP 8.5 and GFDL-CM3.

We modelled non-thermoregulating (above ground) or thermoregulating individuals (burrowing up to 30 cm underground) under full sun (0% shade) or deep (90%) shade conditions. For the sake of simplicity, we only show example simulations under full sun conditions (**Fig. S5.8** and **S5.9**). In the case of thermoregulating individuals, when T_e fell outside VT_{max} or VT_{min}, animals burrowed underground to the depth with the closest temperature to their preferred temperature (T_{pref} = 13.1 °C). We show T_e traces and behaviour (i.e. active, non-active, and depth if burrowed underground) of example simulations for a high (Candioches) and a low (Color) elevation population: under current and predicted climates for 2070, and either static above ground or burrowing underground to thermoregulate.

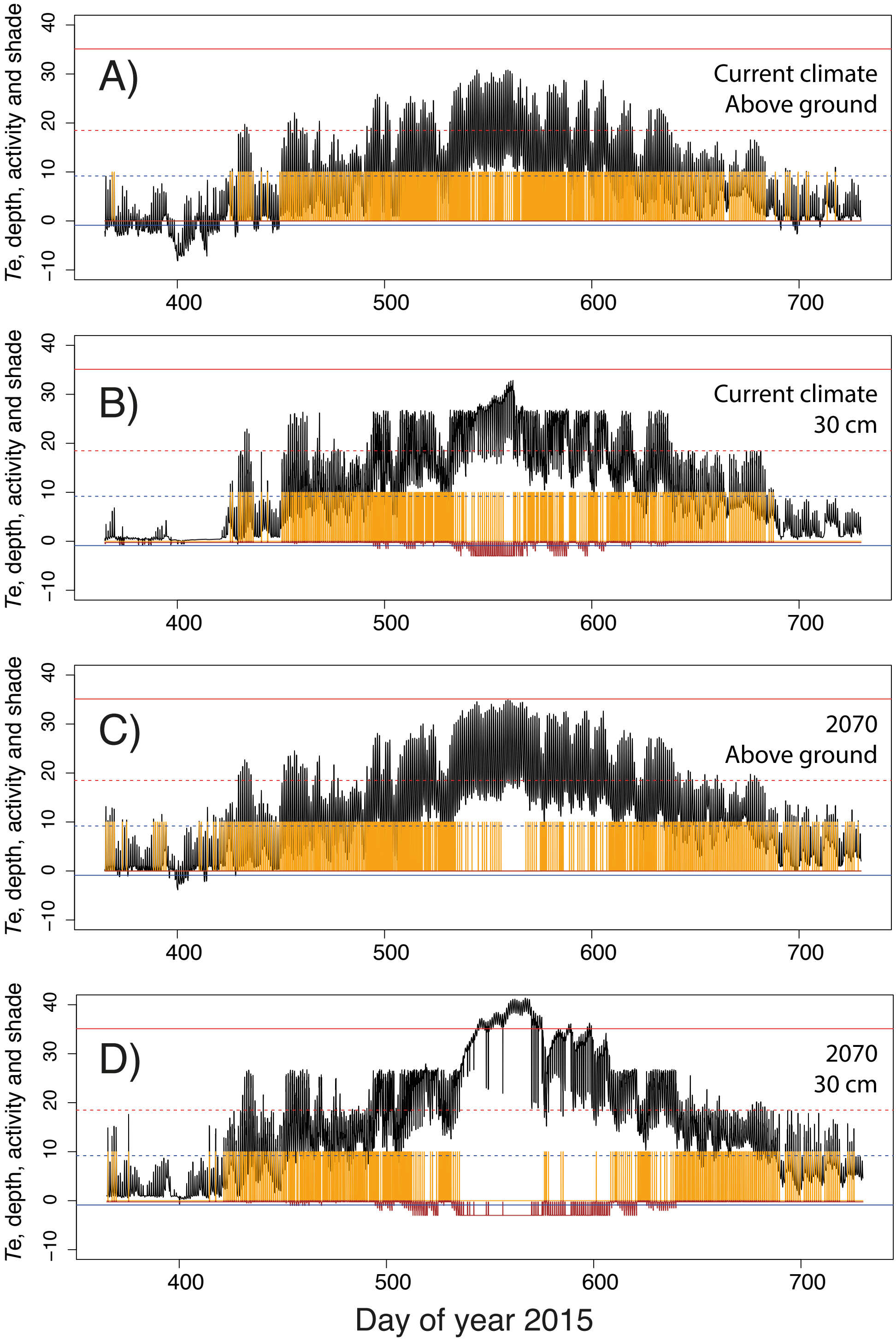

Figure S5.8: Four example simulations for a high elevation population (Candioches). **(A)** A non-thermoregulating metamorph under the current climate, **(B)** a thermoregulating (up to 30 cm) metamorph under the current climate, **(C)** a non-thermoregulating metamorph under projected climates for 2070, and **(D)** a thermoregulating (up to 30 cm) metamorph under projected climates for 2070. Example simulations for 2070 correspond to a high emission scenario (i.e. RCP 8.5) and the GCM with the highest projected temperature increases (GFDL-CM3). *Black lines*: *Te* trace; *Orange lines*: non-active (value = 0) or active (value = 10); *Brown lines*: depth of burrows (depth in cm / 10) used to avoid extreme temperatures; *Dashed red lines*: *VTmax*; *Dashed blue lines*: *VTmin*; *Red lines*: *CTmax*; *Blue lines*: *CTmin*.

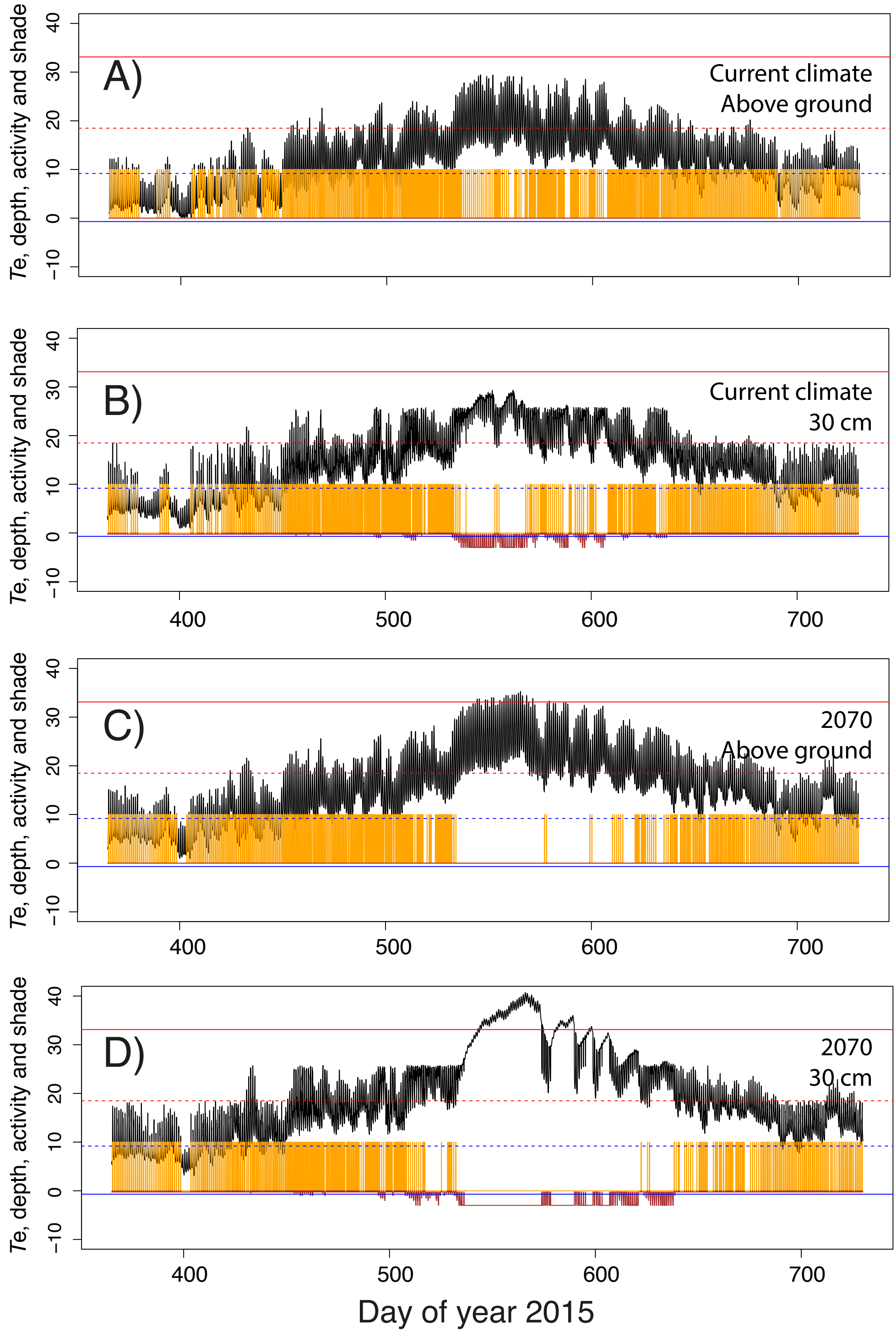

Figure S5.9: Four example simulations for a low elevation population (Color). **(A)** A non-thermoregulating metamorph under the current climate, **(B)** a thermoregulating (up to 30 cm) metamorph under the current climate, **(C)** a non-thermoregulating metamorph under projected climates for 2070, and **(D)** a thermoregulating (up to 30 cm) metamorph under projected climates for 2070. Example simulations for 2070 correspond to a high emission scenario (i.e. RCP 8.5) and the GCM with the highest projected temperature increases (GFDL-CM3). *Black lines*: *Te* trace; *Orange lines*: non-active (value = 0) or active (value = 10); *Brown lines*: depth of burrows (depth in cm / 10) used to avoid extreme temperatures; *Dashed red lines*: *VTmax*; *Dashed blue lines*: *VTmin*; *Red lines*: *CTmax*; *Blue lines*: *CTmin*.

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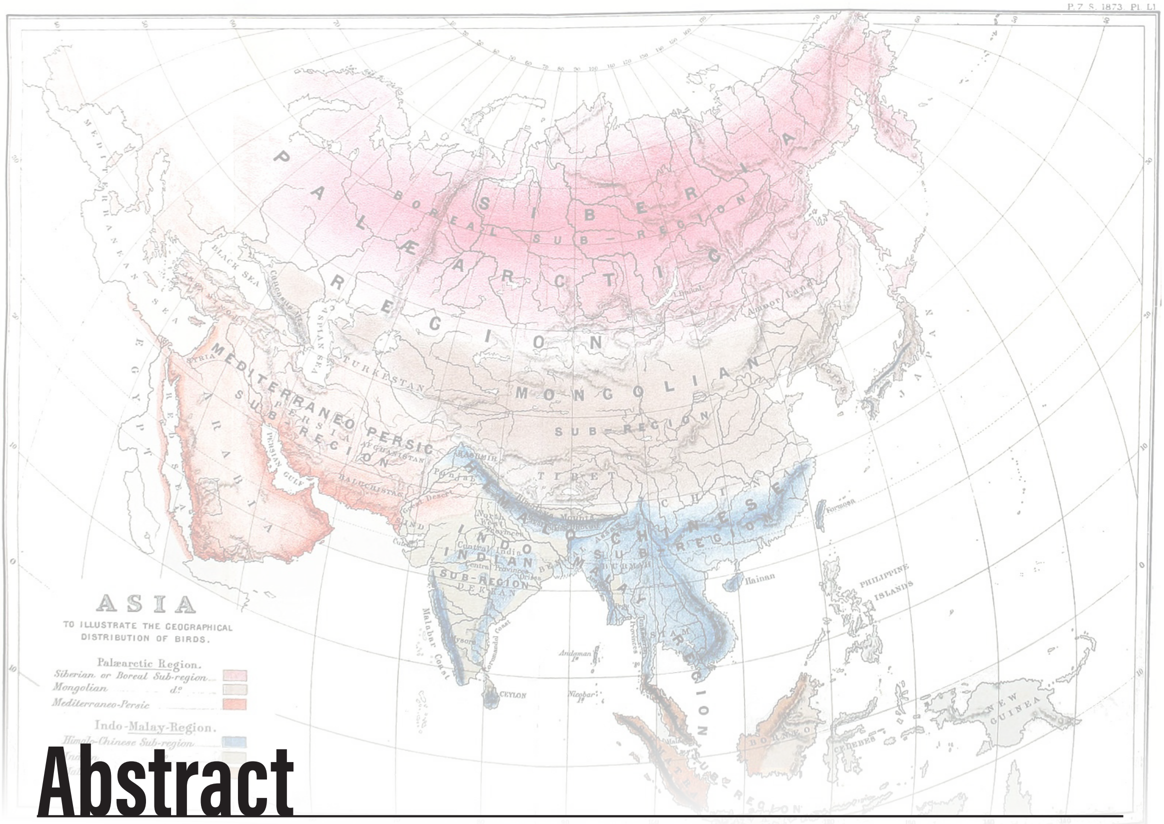
CHAPTER 6

Integrating mechanistic and correlative SDMs to identify drivers of distributional shifts: range contractions in an amphibian are associated to pond temperatures

URTZI ENRIQUEZ-URZELAI - MICHAEL R. KEARNEY - ALFREDO G. NICIEZA - REID TINGLEY

SOURCE: MODIS, NASA



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Abstract

Predicting how species will shift their distributions in response to climate change is complex. Insights into the causal mechanisms that limit species distributions are likely to improve our ability to anticipate species responses. For species with complex life-histories, such as amphibians, a mechanistic understanding of how climate change impacts different lifecycle stages may be crucial. The vulnerability of each stage of a species' lifecycle to climate change will depend on stage-specific environmental tolerances, the extent of change in stage-specific microclimates, and the potential of a given stage to buffer limiting conditions through behavior. Here we use mechanistic niche modelling (NicheMapR) to derive 'proximate', process-based variables for tadpole, juvenile, and adult R. temporaria, and use these variables in a correlative species distribution model (Maxent) to investigate the potential effects of climate change on R. temporaria across Europe. We modelled the duration and maximum and minimum temperatures of shallow (30 cm) ponds where tadpole R. temporaria typically develop, as well as activity windows for juveniles and adults. Further, we compared the importance of these mechanistic variables with commonly used macroclimatic 'distal' layers (i.e. bioclimatic layers from WorldClim) in determining the species' contemporary distribution. Permutation-based tests of variable importance revealed that maximum temperature of the warmest month (a distal variable) and maximum pond temperatures (a proximal variable) were the most important range-limiting factors; this was consistent with the observed upper thermal tolerance limit of R. temporaria tadpoles. We find that range shift forecasts in central Europe are far more pessimistic based on 'distal' macroclimatic, compared to projections based on 'proximate' mechanistic variables. Models identified mountainous regions and high latitudes as important climate refugia for R. temporaria, but predicted extensive decreases in climatic suitability in southern Europe, which harbors a highly significant fraction of the genetic diversity of the species. Our predictions can help in directing actions, for instance, to ensure thermally suitable water bodies for tadpoles.

6.1. Introduction

Many organisms are shifting their geographic distributions tracking changes in preferred climatic conditions (Parmesan, 2006; Lenoir & Svenning, 2015; Pecl et al., 2017). Species distribution models, or SDMs, have become one of the primary tools with which to explore associations between environments and species' distributions, and to forecast distribution shifts in changing climates (Pearson & Dawson, 2003; Elith & Leathwick, 2009). Amongst SDMs, a plethora of approaches exist that vary in the degree to which processes are explicitly incorporated. Correlative and mechanistic SDMs can be viewed as two endpoints of that continuum (Dormann et al., 2012; but see Peterson et al., 2016). Correlative SDMs predict distributions by statistically linking current distributions (presences or abundances) to environmental layers (Guisan & Thuiller, 2005; Elith et al., 2006). These models may implicitly capture biotic and abiotic processes that limit species ranges; due to the availability of the required data, correlative SDMs have been widely applied to climate change forecasting (reviewed in Elith et al., 2006; 2010; Pacifici et al., 2015). In contrast, mechanistic SDMs explicitly model range-limiting processes, often from heat and mass balance principles (Kearney & Porter, 2009). Despite the detailed species' information required to fit mechanistic SDMs, they are progressively becoming more widely applied (Kearney, 2012; Levy et al., 2015; Briscoe et al., 2016).

Not surprisingly, both approaches to model species' distributions have strengths and limitations. Correlative SDMs are a powerful tool to predict current distributions, but may be unreliable when predicting into novel environments (e.g., in time or space; Elith et al., 2010). Mechanistic SDMs, on the other hand, do not require extrapolation, because they directly integrate species' functional traits with available microclimates. Thus, we should strongly consider the use of these models to predict species' distributions in changing climates (Kearney & Porter, 2009; Buckley et al., 2010; Sears et al., 2011). Furthermore, mechanistic models can offer invaluable insights into the proximate constraints that underpin species' range limits (Kearney & Porter, 2009). Mechanistic SDMs will, however, only be useful if the proper limiting processes can be unambiguously identified (Elith et al., 2010).

A critical step in modelling species' distributions is to include relevant predictor variables (Araújo & Guisan, 2006; Bucklin et al., 2014). Most attempts to predict species distributions rely on macroclimatic environmental layers, which, at best, indirectly capture the microclimatic conditions experienced by animals (Potter et al., 2013; Sunday et al., 2014; but see Bennie et al., 2014). Further, most SDMs focus on a single lifecycle stage (e.g. adults), or combine life stages (Gerick et al., 2014; Riddell et al., 2017). Yet, most species possess complex life-histories, which poses a further challenge for predicting the biotic consequences of climate change (Levy et al., 2015; 2016). Different lifecycle stages may experience different microclimates (and thus possess different scopes for thermoregulation), or have different thermal physiologies (resulting in different inherent sensitivities to climate change) (Kingsolver et al., 2011; Briscoe et al., 2012; Pincebourde & Casas, 2015; Sinclair et al., 2016)(Radchuk et al., 2012).

On way to potentially overcome these limitations is to integrate correlative and mechanistic SDMs (e.g., applied in tandem) (Meineri et al., 2015; Mathewson et al., 2017). For instance, macroclimatic variables and stage-specific traits could be bridged using mechanistic SDMs, and converted into more proximate 'environmental' variables (*mechanistic variables* hereafter) for use as inputs in correlative SDMs. Furthermore, to project SDMs into warming climates, mechanistic variables could be derived using future macroclimatic conditions without risk of extrapolation (Elith et al., 2010; Mathewson et al., 2017). Additionally, variable importance procedures developed for correlative SDMs (Thuiller et al., 2009) can allow us to generate hypotheses about which lifecycle stage or modelled process constrains species' current distributions. Using both approaches can enable robust and useful insights into '*why*' and '*where*' species will persist or perish under climate change (Kearney et al., 2010; Briscoe et al., 2016).

Here, we modeled the distribution of the European common frog (*Rana temporaria*) under current and future climates, using a widely applied correlative SDM method (i.e. Maxent; Phillips et al., 2017), and both macroclimatic (i.e. WorldClim; Hijmans et al., 2005) and mechanistic variables. In *R. temporaria* – as in most temperate amphibians – embryos and larvae are aquatic, whereas juveniles and adults are terrestrial. Due to the high specific heat and thermal conductivity of water, aquatic stages are confronted with a limited scope for behavioural thermoregulation compared to terrestrial stages (Feder & Hofmann, 1999; Sears et al., 2011; Duarte et al., 2012; Kearney et al., 2014). Moreover, juvenile are diurnal, whereas adults can be active both at day and night time as long as environmental conditions are suitable (Sinsch, 1984; Vences et al., 2000; 2002). Consequently, the different lifecycle stages of *R. temporaria* have unique thermal physiologies (Enriquez-Urzelai et al., unpublished data).

In order to depict stage-specific microclimates and physiologies, we derived proximate mechanistic layers using NicheMapR (Kearney & Porter, 2016), a framework for mechanistic niche modelling consisting of a microclimate model and an animal model (Porter et al., 1973; Kearney & Porter, 2009). Due to the dependence of aquatic stages on water, we modelled pond duration and maximum and minimum pond temperatures using the microclimate model alone. To model activity windows for juveniles and adults, we integrated the microclimate model with the animal model, parameterized with specific traits for each stage (Enriquez-Urzelai et al., unpublished data). We employed permutation-based variable importance procedures to generate hypotheses regarding the lifecycle stage(s) or process(es) that limit the distribution of *R. temporaria* across Europe. Finally, to evaluate the consequences of considering stage-specific microclimates and physiologies, we compared current and future predicted distributions of R. temporaria using macroclimatic or mechanistic environmental variables.

6.2. Materials and Methods

PRESENCE DATA

We downloaded data on the distribution of *Rana temporaria* from the GBIF portal (accessed 20/04/2017; http://www.gbif.org/). Points from GBIF represent museum specimens, fossils, observations, and other kinds of georeferenced localities. We retained only presence records corresponding to non-duplicated observations (n = 6707). To reduce spatial-autocorrelation and sampling bias (Phillips et al., 2017), we thinned the dataset to guarantee a minimum distance of 20 km between adjacent presences (n = 3423), as implemented in the *thin()* function of the spThin R-package.

MACROCLIMATIC VARIABLES

To capture the macroclimatic conditions encountered by *R. temporaria* throughout its distribution, we downloaded six variables from the WorldClim dataset (Hijmans et al., 2005; http://www.worldclim.org/) at a spatial resolution of 10 arc-minutes (~19 km^2): annual mean temperature (Bio1), maximum temperature of the warmest month (Bio5), minimum temperature of the coldest month (Bio6), annual mean precipitation (Bio12), precipitation of the wettest month (Bio13), and precipitation of the driest month (Bio14).

We chose these variables because they reflect temperature/precipitation averages and extremes expected to limit species' distributions (Carey & Alexander, 2003; Araújo et al., 2006; Quintero & Wiens, 2013).

We obtained bioclimatic layers for current conditions, as well as for future (2070) climates. To account for uncertainty in future climate predictions (Wiens et al., 2009), we considered two global circulation models (GCMs: CCSM4, and GFDL-CM3) and two emission scenarios (low, RCP 4.5, and high, RCP 8.5). CCSM4 predicts moderate-to-low changes in environmental conditions, while GFDL-CM3 projects higher environmental changes.

MECHANISTIC VARIABLES

We developed more proximate, mechanistic variables using NicheMapR – an R implementation of the heat- and mass-balance models developed by Porter and colleagues (Porter et al., 1973; Kearney & Porter, 2016). NicheMapR includes a microclimate model and an animal model. The microclimate model comprises a set of FORTRAN routines that reconstruct available microclimates to an animal, given terrain (e.g., slope, aspect, elevation, soil properties, available shade) and climate conditions (i.e., maximum and minimum air temperatures, precipitation, cloud cover, relative humidity, wind speed). We obtained climate conditions from a global dataset of monthly means (1961-1990) at a resolution of 10 arc-minutes (New et al., 2002). We derived slope and aspect layers from a digital elevation model (GTOPO30; https://lta.cr.usgs.gov/GTOPO30/), resampled to a resolution of 10 arc-minutes with the *resample()* function. With these data, the microclimate model provides hourly estimates of solar and infrared radiation, above-ground air temperature, wind velocity, and relative humidity at the animal's height, and soil temperature profiles at ten user-specified soil nodes (i.e. depths; see Kearney et al., 2014; Kearney & Porter, 2016 for more details). To capture extreme conditions, which could impose distribution constraints, we only modelled microclimates in full sun conditions (0% shade).

The outputs of the microclimate model can be used directly (e.g., as predictor variables in a correlative SDM), or as inputs for the animal model (Kearney & Porter, 2016). We used the microclimate model alone to explore putative limiting factors at the aquatic larval stage (**Fig. 6.1**). Specifically, based upon radiation exchange, convection, conduction,

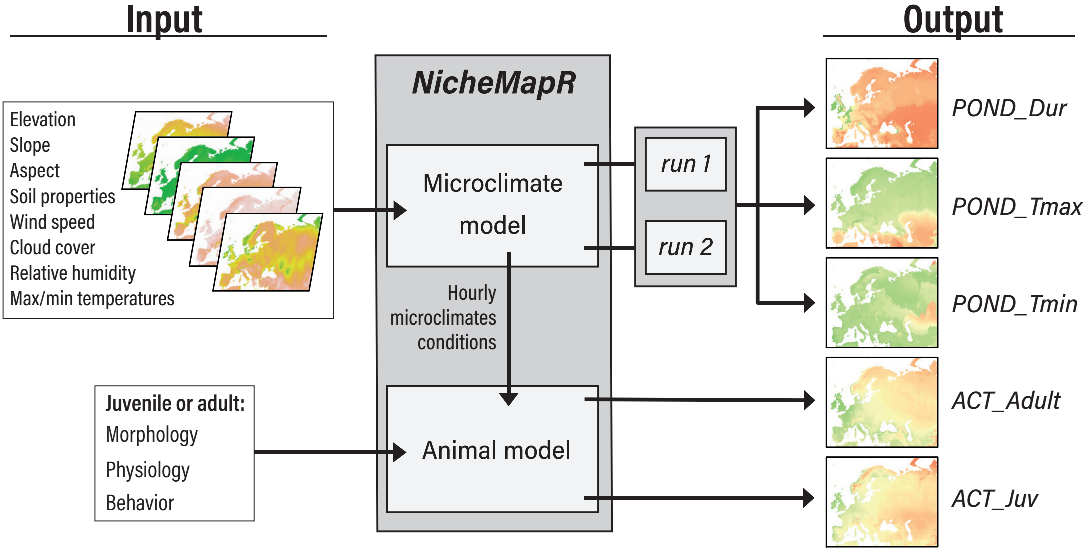

Figure 6.1: Graphical illustration of the framework to generate mechanistic 'proximate' variables. We used climate and terrain data as input for the microclimate model within NicheMapR (Kearney & Porter, 2016). Using different parametrizations of the model (*run1* and *run2*, see Materials and Methods and Supplementary Material) we computed pond duration (*POND_Dur*), and maximum (*POND_Tmax*) and minimum pond temperatures (*POND_Tmin*) across Europe. Additionally, we generated layers of the activity windows of juvenile and adult *R. temporaria*, using stage-specific morphology (e.g. size), physiology, and behavior as input for the animal model of NicheMapR.

and evaporation, we modelled maximum pond duration and maximum and minimum pond temperatures (Kearney et al., 2009). To that end, we ran the microclimate model twice. In the first run (*run1*), we modelled a pond with a maximum depth of 30 cm (similar to breeding ponds most often used by the species). We assumed that all 10 arc-minute grid cells contained some fraction with soil made primarily of clay (90% in our simulations), which could retain water after reaching saturation. We computed the hydrolic properties of the soil with the *soil_hydro()* function provided by M. R. Kearney. Additionally, to impose water catchment by the pond, we increased the rain multiplier parameter (*rainmult* = 2.8) and decreased the proportion of rain that comes in the first rainy day of each month (*rainfrac* = 0.1). In the second run (*run2*), we modelled snowfall and snowpack using the microclimate model with default parameters and the moisture and snow subroutines selected, as implemented in the *micro_global()* function of NicheMapR (Kearney & Porter, 2016). With both runs, we computed the maximum duration that the pond remained filled and uncovered by snow (*POND_Dur*, in days; **Fig. S6.4**). Further, we computed maximum (*POND_Tmax*, in °C; **Fig. S6.5**) and minimum water temperatures when uncovered by snow (*POND_Tmin*, in °C; **Fig. S6.6**). We modelled water temperature as the temperature of saturated soil (obtained in *run1*), two nodes below each hourly estimate of pond depth. These modelling choices provided rough estimates of pond temperatures recorded by dataloggers deployed in ponds in northern Spain and central Sweden (see **Supplementary Material**).

We coupled the output of the microclimate model with the animal model to explore spatial patterns of potential activity times of juvenile and adult R. temporaria (**Fig. 6.1**). We parameterized the microclimate model with default parameters (as in *run2*), and we supplied the output to the animal model (*ectotherm()* function of NicheMapR). The animal model computes hourly operative temperatures (T_e) iteratively, given available microclimates (output of microclimate model) and the animal's morphology, behavior, and physiology. We derived layers for activity potential of juveniles (*ACT_Juv*, in h year^{-1}; **Fig. S6.7**) and adults (*ACT_Adult*, in h year^{-1}; **Fig. S6.8**) by modelling a medium-sized juvenile and adult (0.3 and 30 g, respectively). We set their midpoints at 0.5 cm and 2 cm respectively, and assumed a shape equivalent to that of the leopard frog (*Lithobates pipiens*). Likewise, we assumed that 90% of the skin acted as a free water surface when individuals were active (see Kearney et al., 2008 for more information). Since juveniles are predominantly diurnal, we allowed only diurnal activity, bounded within the maximum (VT_{max} = 18.5 °C) and minimum (VT_{min} = 9.5 °C) temperatures selected in a thermal gradient experiment conducted on juveniles from 7 populations from the Iberian Peninsula (U. Enriquez-Urzelai et al. unpublished data). For adults, we allowed activity during all hours of the day. Maximum temperature for activity was set as the maximum temperature (VT_{max} = 19.4 °C) selected in thermal gradient experiments with adults (U. Enriquez-Urzelai et al. unpublished data), but minimum temperatures were set to 0 (VT_{min} = 0 °C), since *R. temporaria* is known to be active at this temperature (Ludwig et al., 2015; A. G. Nicieza and À. Richter-Boix, personal communication).

When T_e of juveniles or adults fell outside of these temperatures for activity, animals were simulated to burrow underground to the depth with the closest temperature to their preferred temperature (juveniles: T_{pref} = 13.1 °C; adults: T_{pref} = 16.6 °C), obtained from thermal gradient experiments (U. Enriquez-Urzelai et al. unpublished data). Furthermore, we included the water-balance between frogs and soil, with a simplified version of the model developed by Tracy (1976). When frogs were not able to gain water from the substrate – i.e. when microclimate model estimates of soil water potential were below the water potential reported for *L. pipiens*, -72.5 J kg^{-1} (Tracy, 1976) – and they lost more than 20% of body mass as water, we forced them to retreat and rehydrate. We allowed frogs to be active the next hour.

We also derived all five mechanistic variables (i.e., *POND_Dur*, *POND_Tmax*, *POND_Tmin*, *ACT_Juv*, and *ACT_Adult*) for 2070 climates. To simulate future climates, we downloaded current and 2070 maximum and minimum temperature and precipitation layers from the WorldClim dataset (Hijmans et al., 2005), and computed predicted monthly differences (i.e. offsets). As for bioclimatic variables, we considered two global circulation models (CCSM4, and GFDL-CM3) and two emission scenarios (RCP 4.5 and RCP 8.5). We added the offsets to the baseline maximum and minimum temperatures and precipitation values (New et al., 2002), and reran NicheMapR microclimate (and when necessary) animal models (**Fig. 6.1**). Due to the high computational demand of generating mechanistic variables – involving six NicheMapR models per grid cell, 81,664 grid cells, and 5 climate scenarios (2,449,920 models in total; **Fig. 6.1**) – these were generated using a Scientific Modelling Cluster (Xeon 3.60 GHz cores; Cluster de Modelización Científica UNOV05-23-009, Scientific-Technical Services from the University of Oviedo, Mieres, Spain).

MAXENT MODELS

We fit correlative SDMs using Maxent, and both macroclimatic and mechanistic predictor variable sets for current climates. We fitted models as implemented in the maxnet R-package – an R implementation of the new release of Maxent (v. 3.4.1) – based on point processes (i.e. inhomogeneous Poisson processes; Phillips et al., 2017). Maxent is a presence-only modelling approach; instead of true absences, it uses a large random sample of the environmental space (i.e. background), representative of all available environments. Due to the wide distribution of *R. temporaria*, we used all of Europe as available background. We used hinge features only with the regularization parameter set to 2.5. These settings produce smoother response curves that reduce overfitting, which is especially relevant when projecting to novel conditions (Elith et al., 2010; Briscoe et al., 2016).

To produce maps of current and 2070 climatic suitability, we projected Maxent models onto current and 2070 macroclimatic and mechanistic layers using the '*cloglog*' transformation, implemented in the maxnet R-package. To identify the extent to which

Maxent models extrapolated, we built multivariate environmental similarity surfaces (*MESS*; Elith et al., 2010) as implemented in the dismo R-package (Hijmans et al., 2016; Fig. S9). For presentation purposes, we averaged climatic suitability scores across all different GCM and emission scenarios (see **Fig. 6.3**). Nevertheless, for southern Europe, the area of the distribution range expected to undergo the greatest changes over the next decades, we present the prediction of each GCM and emission scenario combination to assess their influence in future distributions. To visually compare predictions based on macroclimatic or mechanistic variables, we identified suitable areas (suitability > 0.3) predicted using each variable set, and the areas of model congruence (as in Kearney et al., 2010).

MODEL EVALUATION

We assessed the performance of Maxent models with 10-fold cross-validation. In each repetition, we fitted a model using 70% of the data and computed the area under the ROC curve (AUC) with the remaining 30%. We calculated AUCs by comparing the predicted suitability scores of the held-out 30% and the background, using the dismo R-package (Hijmans et al., 2016). Finally, we averaged the AUCs of the 10 repetitions.

We estimated variable importance to determine the roles of macroclimatic and mechanistic variables in explaining the current distribution of *R. temporaria*. To estimate variable importance, we used the approach proposed by Thuiller et al. (2009), as implemented in the *ecospat.maxentvarimport()* function of the ecospat R-package (Broennimann et al., 2015). The method consists of a randomization procedure followed by Pearson's correlations. In each repetition, two models are fit: one with all environmental variables 'untouched' and the other with the variable under investigation randomly permutated. Variable importance is then estimated as 1 – r, the Pearson's correlation between predictors. High correlation values mean that predictions do not differ markedly. In that cases, 1 – r would be a small number, and the variable would be considered unimportant. We repeated this randomization procedure 10 times and averaged the importance values for each variable. Additionally, we visually inspected response curves (derived using the clog-log transformation) to further explore relationships between variables and climatic suitability.

6.3. Results

Maxent models performed well regardless of the underlying predictor set. The model with macroclimatic variables showed slightly higher AUC values (mean AUC = 0.899, 95% CI = 0.895 - 0.903) than the model built with mechanistic variables (mean AUC = 0.834, 95% CI = 0.826 - 0.842). Results from variable importance tests suggest that maximum temperatures constrain the geographic distribution of *R. temporaria*. Amongst macroclimatic variables, Bio5 (maximum temperature of the warmest month) had, by far, the highest variable importance value (0.689). Bio14 (precipitation of the driest month), and Bio1 (annual mean temperature) had much lower impacts on predictions (0.097 and 0.049, respectively). Bio6, Bio12, and Bio13 had negligible influence (< 0.002 in all cases). In the case of the model built with mechanistic variables, *POND_Tmax* was the most influential predictor (0.860). *POND_Dur* and *ACT_Adult* had relatively small influences (0.044 and

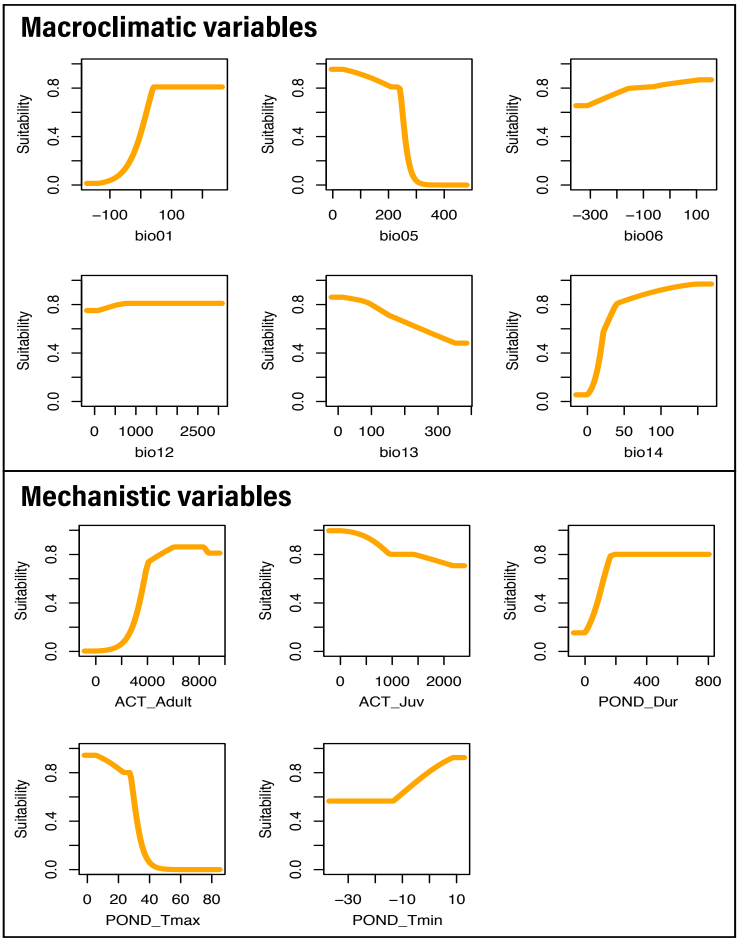

Figure 6.2: Climatic suitability changes at the range of values of each predictor variable (response curves). Curves where obtained with the '*cloglog*' transformation of Maxent output.

0.041, respectively); *POND_Tmin* and *ACT_Juv* had negligible impacts (0.004 and 0.003, respectively).

Climatic suitability decreased sharply at maximum temperatures (Bio5) above ~25 °C (**Fig. 6.2**), and in very dry (low precipitation at the driest month; Bio14) or very cold regions (low mean temperatures; Bio1). Suitability also decreased markedly at maximum pond temperatures (*POND_Tmax*) of ~40 °C (**Fig. 6.2**). On the other hand, climatic suitability increased with increasing pond duration (*POND_Dur*) or increasing activity windows for adults (*ACT_Adult*).

We found a high congruence between climatic suitability predictions obtained using macroclimatic vs mechanistic variables for current, but not future (2070) climates (**Fig. 6.3**). *MESS* maps suggest that none of the models had to extrapolate for current or 2070 climates (**Fig. S6.9**). The Maxent model based on macroclimatic variables predicted extensive range contraction in central Europe for 2070 (see **Fig. 6.3A**, **D**). The model based

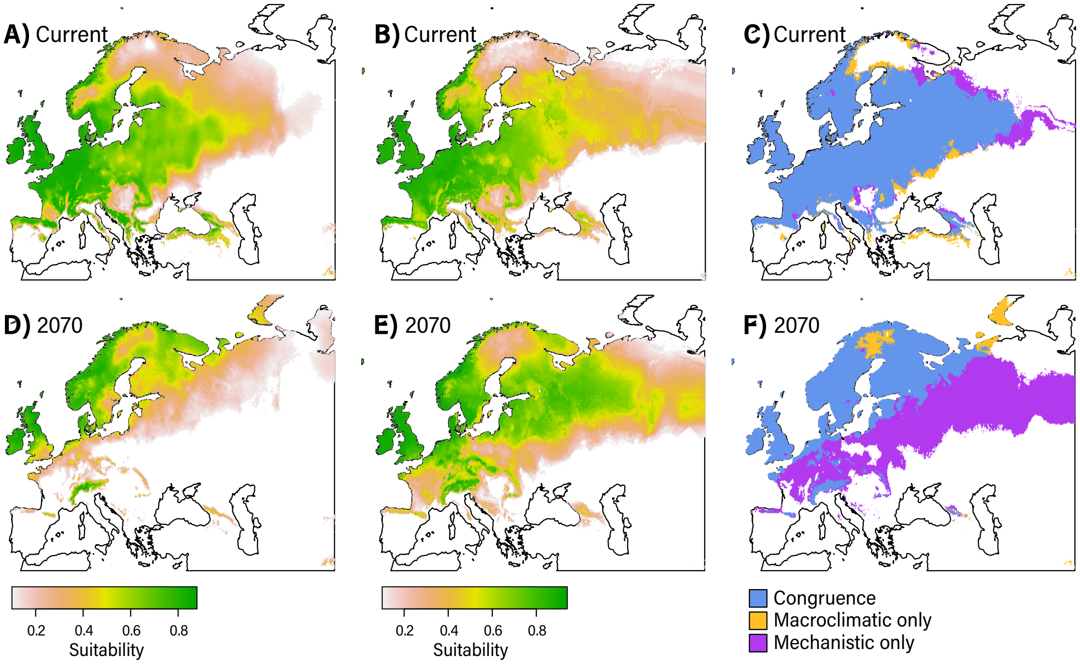

Figure 6.3: Predicted climatic suitability for *R. temporaria* under (**A-C**; *first row*) the current climate and (**D-F**; *second row*) future, 2070, climates (averaged for all 4 climate change scenarios). In **A** and **D** panels (*first column*) we made the projections using the Maxent model built with macroclimatic variables. Alternatively, in **B** and **E** panels (*second column*), we employed models built with mechanistic variables for projections. (**C** and **F**) Areas of congruence (i.e. agreement; *blue*), and areas only predicted with macroclimatic (*yellow*) or mechanistic variables (*purple*).

on mechanistic variables predicted a lower suitability decrease in central Europe (**Fig. 6.3B**, **E-F**). Both models similarly identified the coldest regions as suitable areas under 2070 climates (i.e. high mountain ranges such as the Alps or the Pyrenees, and northern Europe). Furthermore, both models forecasted a marked decrease in climatic suitability at the current southern range margin of *R. temporaria* (**Fig. 6.3C**, **F**; **Fig. 6.4**). The model based on mechanistic variables, however, predicted slightly lower suitability decreases in southern Europe, especially in the case of the most benign circulation model (CCSM4) and a low emission scenario (RCP 4.5; **Fig. 6.4A-B**). In contrast, both models predict that most of the southern range of *R. temporaria* will become unsuitable (**Fig. 6.4A**, **C-E**) under a more extreme climate change scenario (GFDL-CM3 and RCP 8.5).

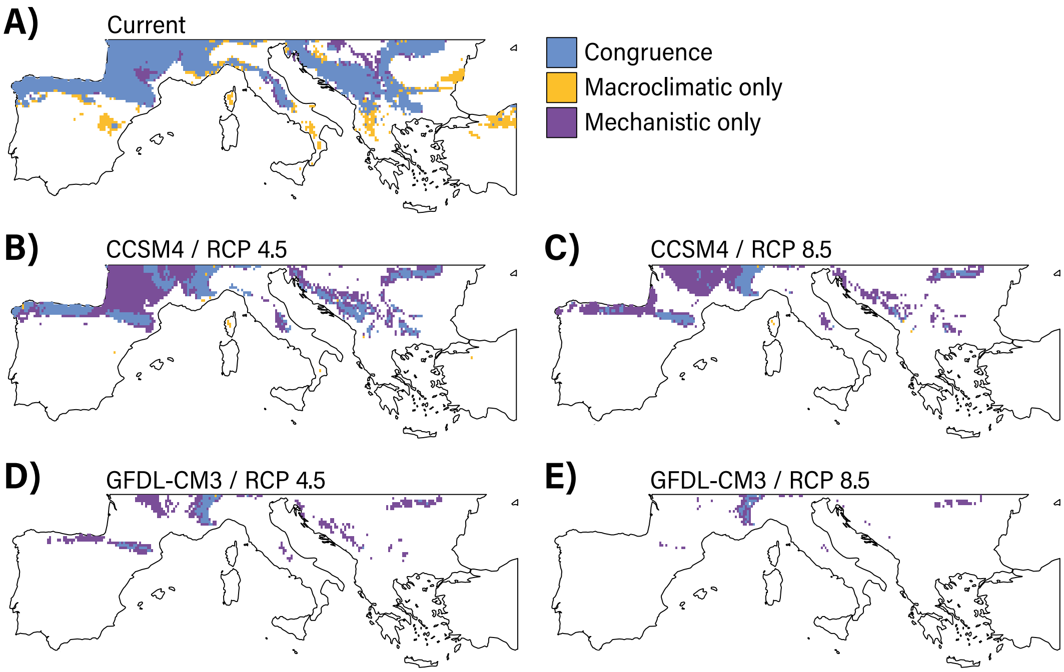

Figure 6.4: Areas of congruence between models (i.e. agreement; *blue*), and areas only predicted with macroclimatic (*yellow*) or mechanistic variables (*purple*) at southern Europe. We made projections for **(A)** current climates, and **(B-E)** each climate change scenario.

6.4. Discussion

Disentangling the processes and lifecycle stages that constrain species' distributions may be crucial to improving predictions of species contemporary distributions, as well as species range shifts in response to climate change (Helmuth et al., 2005; Kearney & Porter, 2009; Radchuk et al., 2012; Moritz & Agudo, 2013; Evans et al., 2015). Efforts that combine or integrate different approaches (e.g. correlative and mechanistic SDMs) are particularly promising, since they offer robust and independent lines of evidence (Kearney et al., 2010; Briscoe et al., 2016; Bonebrake et al., 2017). Here we show how mechanistic niche modelling can be used to derive 'proximate' process-based variables (**Fig. 6.1**, **S6.4**-**S6.8**), which in combination with correlative SDM methods, can help us identify range-limiting factors. Our results suggest that maximum temperatures underpin the contemporary geographic distribution of *Rana temporaria*, most likely due to their impact on aquatic tadpoles (**Fig. 6.2**), which have limited scope to behaviorally avoid maximum pond temperatures. Furthermore, we show that climate-induced range forecasts for *R. temporaria* in central Europe are far more pessimistic based on 'distal' macroclimatic variables (i.e. WorldClim), compared to projections based on 'proximate' mechanistic variables (**Fig. 6.3**). However, both types of models predicted extensive range contractions in southern Europe, and showed that cold regions (mountainous regions and high latitudes) will be important climate refugia for *R. temporaria* under climate change (**Fig. 6.4**).

Correlative SDMs have been criticized for their inability to detect causal relationships between environmental conditions and species distributions (Dormann et al., 2012; Cabral et al., 2017). However, Searcy & Shaffer (2016) demonstrated that permutation tests on Maxent models built using best-practice procedures accurately identified range-limiting environmental factors for a North American salamander. Here, we extended this idea to mechanistically-derived 'proximate' variables, which directly reflect a potentially limiting process (i.e. activity windows for juveniles and adults, maximum and minimum pond temperatures, and pond durations).

Activity windows can impose strong constraints on species distributions (Gunderson & Leal, 2015; Levy et al., 2016; but see Kearney, 2013). To persist, individuals must remain active long enough to forage sufficiently to fulfill the minimum requirements for growth, storage, and, in the case of adults, breed (Angilletta et al., 2002; Sinervo et al., 2010). Activity restrictions under climate change may also underpin reported extinction events (Sinervo et al., 2010; Mathewson et al., 2017). Our simulations show that activity windows for juvenile and adult frogs vary markedly across Europe, as well across time (see **Fig. S6.7, S6.8**). Nonetheless, we found that spatial heterogeneity in activity potentials of adult and juvenile *R. temporaria* had only weak impacts on the species' current distribution. Our mechanistic simulations also predicted that thermoregulation would allow juvenile and adult *R. temporaria* to maintain similar maximum body temperatures across Europe (e.g., by retreating to burrows to avoid stressful temperatures), and thus, we did not consider those layers as potential range-limiting factors in Maxent models.

We did find, however, that maximum pond temperatures were an important driver of the distribution of *R. temporaria* in Europe. Interestingly, models built with 'distal' macroclimatic and 'proximate' mechanistic variables both highlighted the importance of maximum temperature (i.e. Bio5 and *POND_Tmax* respectively). Many species' range limits coincide with thermal isotherms (Salisbury, 1926; Root, 1988), possibly mediated by critical thermal limits (Calosi et al., 2010; Bozinovic et al., 2011; Kellermann et al., 2012). Our results suggest that climatic suitability of *R. temporaria* decreases at locations where ponds reach temperatures > 40 °C (**Fig. 6.2**). This temperature threshold is remarkably similar to the critical thermal maxima of *R. temporaria* tadpoles estimated in laboratory experiments (~38 °C; Gutiérrez-Pesquera et al., 2016; Enriquez-Urzelai et al., unpublished data). In the shallow ponds in which *R. temporaria* commonly breeds, thermal conditions are quite homogeneous relative to deeper ponds and terrestrial habitats (Feder & Hofmann, 1999; Livingstone et al., 1999), and tadpoles temperatures often equal water temperatures because of their small size (Lutterschmidt & Hutchison, 1997). With very limited scope for thermoregulation, tadpoles may be subjected to the actual thermal fluctuations of ponds, and thus extreme pond temperatures may set the limits of the distribution of pond-breeding amphibians (Duarte et al., 2012; Gouveia et al., 2013; Gerick et al., 2014; Gutiérrez-Pesquera et al., 2016).

Climate change may impact species' distributions by altering the microclimates they use through their lifecycle (Radchuk et al., 2012; Bozinovic & Pörtner, 2015; Pincebourde & Casas, 2015). The vulnerability of different lifecycle stages will depend on their specific environmental tolerances, the change in stage-specific microclimates, and the potential to buffer limiting conditions via behavior (Kingsolver et al., 2011; Briscoe et al., 2012; Sunday et al., 2014; Buckley et al., 2015). Air temperature rises and more frequent extreme events (e.g. heat waves) due to climate change will imply coincidental changes in pond temperatures. Species could respond *in situ* by increasing upper thermal limits through genetic adaptation or phenotypic plasticity (Chevin et al., 2010; Kelly et al., 2016). However, the potential for adaptation and plastic responses to shift thermal tolerances seems limited in ectotherms, relative to predicted environmental changes (Kellermann et al., 2009; Gunderson & Stillman, 2015; van Heerwaarden et al., 2016; Enriquez-Urzelai et al. unpublished data). Thus, species or populations living closer to their maximum thermal tolerance (e.g. southernmost populations of *R. temporaria*) might be especially vulnerable to climate change (Duarte et al., 2012; Gerick et al., 2014; Gutiérrez-Pesquera et al., 2016).

In line with that expectation, Maxent models built either with 'distal' or 'proximate' variables predicted a marked northward shift of the southern range limit of *R. temporaria*. However, the magnitude of that shift differed depending on the predictor set used (**Fig. 6.3C, F**). While models based on macroclimatic variables predicted that most of continental Europe will become unsuitable for *R. temporaria*, models based on mechanistic 'proximate' variables predicted a more moderate, but nonetheless significant, decline in climatic suitability. Other studies have similarly found that forecasts can disagree considerably between mechanistic and correlative SDMs (Gritti et al., 2013; Serra-Diaz et al., 2013; Muhling et al., 2017; but see Kearney et al., 2010; Briscoe et al., 2016). Diamond et al. (2012) showed that thermal tolerances predicted the response of ant species to experimental climate warming better than correlative SDMs at their southern limit. In our study, the Maxent model built with mechanistic variables, and largely driven by maximum pond temperatures, reflects the limits of the thermal niche of *R. temporaria* tadpoles and therefore could be more reliable (Martínez et al., 2014). Future studies could usefully validate that prediction using empirical data on *R. temporaria* range shifts.

Congruent forecasts of climate change impacts driven by independent lines of evidence may be more reliable (Gritti et al., 2013; Briscoe et al., 2016); this was the case for southern Europe. We found that models based on proximal and distal variables predicted alarming decreases in climatic suitability for the southernmost populations of *R. temporaria* (**Fig. 6.4**). Further, both types of models identified similar regions as important climate refugia under climate change. These include the highest latitudes of Europe, as well as mountainous areas, such as the Alps, the Pyrenees, and the Cantabrian Mountains in the northern Iberian Peninsula, which could act as important climate refugia for southern populations. However, under the most extreme climate change scenario, most mountain ranges will also become unsuitable by 2070, excluding the Alps (**Fig. 6.4C-E**). Considering that most of the genetic and phylogenetic diversity of *R. temporaria* is concentrated in the northern Iberian Peninsula (Vences et al., 2017), climate change could cause extensive loss of distinctive genetic diversity (i.e. cryptic biodiversity loss; Bálint et al., 2011; Moritz & Agudo, 2013) and likely the extinction of evolutionary significant units.

Designing more effective conservation actions for biodiversity under climate change will require understanding the processes that cause local extinctions (Mitchell et al., 2013; 2016);(Evans et al., 2015; Cabral et al., 2017). By identifying potential causes of extinctions, practitioners could implement directed mitigating measures. Here we identified maximum pond temperature as an important range-limiting factor for *R. temporaria* and predicted alarming decreases in climatic suitability in genetic 'hotspots' of the species (e.g. northern Iberian Peninsula). To prevent ponds from reaching lethal temperatures, conservation actions could be aimed, for instance, at increasing vegetation cover around ponds. Furthermore, ecophysiologists and modellers could test these range-limiting factors, and even explore whether they hold true for other species (e.g. maximum pond temperatures via tadpoles' critical thermal maxima in amphibians; Duarte et al., 2012; Gouveia et al., 2013; Gutiérrez-Pesquera et al., 2016), to perform physiologically informed forecasts of species responses to climate change (Evans et al., 2015).

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Supplementary Material for Chapter 6

VALIDATION OF POND DURATION AND POND TEMPERATURES

We modelled pond duration and temperature as explained in the Materials and Methods section of the main text. To explore whether our 'pond model' accurately captured the conditions of the ponds that the European common frog (*Rana temporaria*) commonly uses, we visually inspected predicted pond duration (and the length of time the ponds would be covered by snow). We also compared predicted temperatures with pond temperatures recorded by dataloggers deployed in ponds in northern Spain and central Sweden (**Fig. S6.1**). The monitored ponds in northern Spain correspond to important breeding points for a high-altitude population (Candioches/Cubilla; 1707 m.a.s.l.) and a low-altitude population (Color; 377 m.a.s.l.); data were provided by Alfredo G. Nicieza. Àlex Richter-Boix kindly provided datalogger data from central Sweden corresponding to seven closely distributed ponds from a pond system in which *R. temporaria* breeds. Due to the proximity of ponds in Sweden, we only used three ponds for calibration.

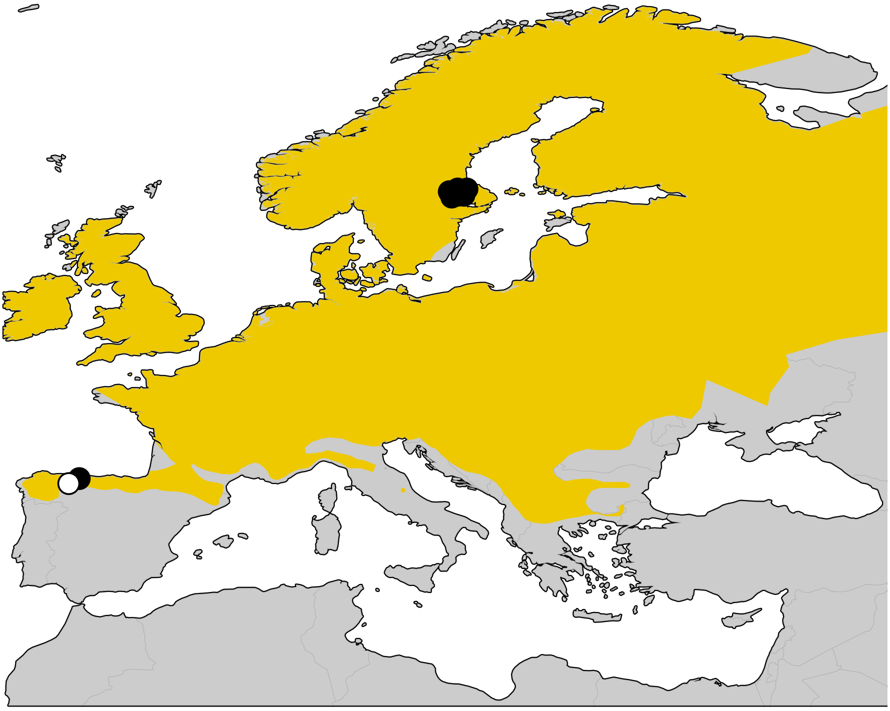

Figure S6.1: Geographical coordinates of the ponds used to validate the 'pond model'. The white point corresponds to the high-altitude pond in northern Spain. The yellow polygon depicts the distribution of *R. temporaria* obtained from the IUCN (available at http://www.iucnredlist.org).

Predicted pond durations matched the observations of our research group in northern Spain (Alfredo G. Nicieza, personal observation), and the periods reported for Swedish populations. We predict that shallow ponds (i.e. 30 cm) at high altitudes located in the southern limit of the distribution of *R. temporaria* would remain filled and free of snow for 135 days, and that they would dry several times during the summer (in July; **Fig. S6.2A**). In comparison, ponds in northern Spain and at low elevations are predicted to remain filled during almost the entire year (333 days), and dry out only during summer (in August; **Fig. S6.2B**). Yet, they may remain free of snow during the whole year. These predictions agree with our observations in the field. For all three ponds in central Sweden (latitude ~59), we predicted maximum pond durations of 153 days (**Fig. S6.2C**; Note that we only present predictions for one of the Swedish populations due to high similarities between them). For southern (latitude ~55) and northern (latitude ~68) Swedish populations, growth season length spans for ~230 and ~100 days, respectively (Orizaola et al., 2013). Thus, we believe that predicted pond durations reflect the period available for larval development.

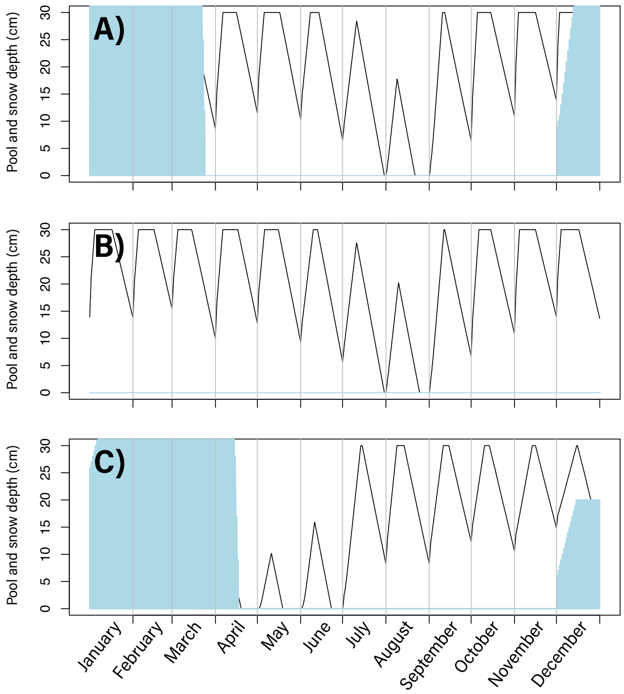

Figure S6.2: Predicted pool depth (*lines*) and snow depth (*blue polygons*) during the whole year in **(A)** a pond at a high altitude southern population (Candioches), **(B)** a pond at a low altitude southern population (Color), and **(C)** a pond at central Sweden. By adding the number of days that ponds remained filled continuously and uncovered by snow, we computed maximum pond duration.

Predicted temperatures resembled registered pond temperatures using dataloggers in northern Spain and central Sweden (**Fig. S6.3**). It is noteworthy, however, that at some locations, our model overestimated (**Fig. S6.3B**) or underestimated (**Fig. S6.3E**) thermal variation. In the case of the lowland southern population, the breeding water body is a small pond at a shaded roadside, where water flows constantly. Additionally, ponds in Sweden possess different shading levels (À. Richter-Boix, personal communication). Due to computational limitations, we only modelled ponds with still water and at full sun (0% shade). A high rate of water renewal (i.e. flow) or different shading levels could cause the differences between predictions and observations.

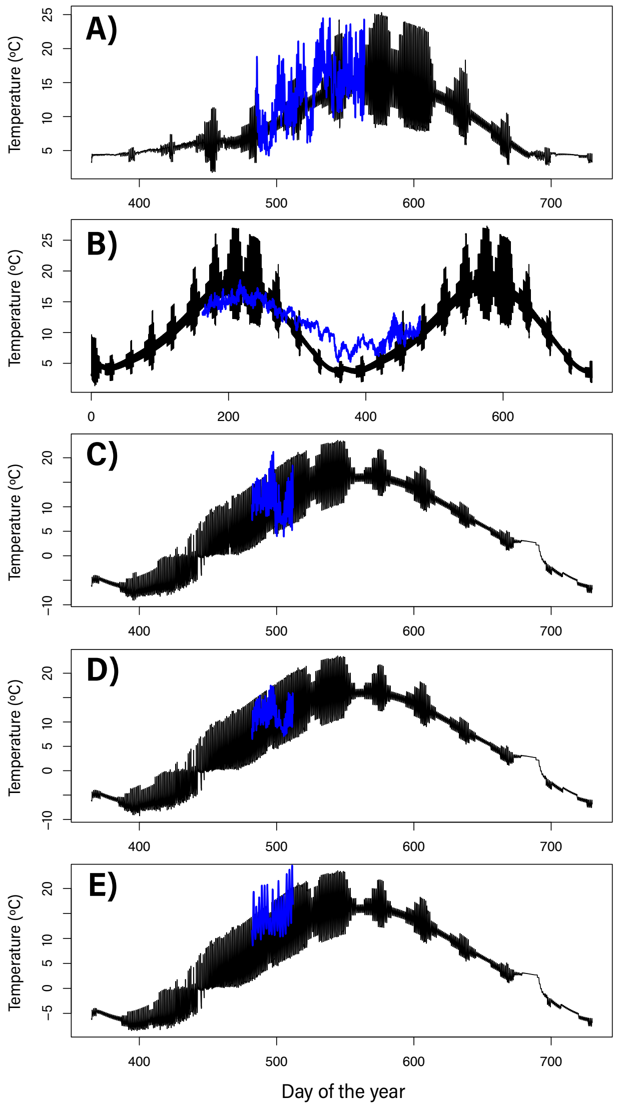

Figure S6.3: Predicted (black) and observed (blue) pond temperatures at **(A)** a high altitude southern population, **(B)** a low altitude southern population, and **(C, D and E)** at central Sweden.

MECHANISTIC 'PROXIMATE' LAYERS

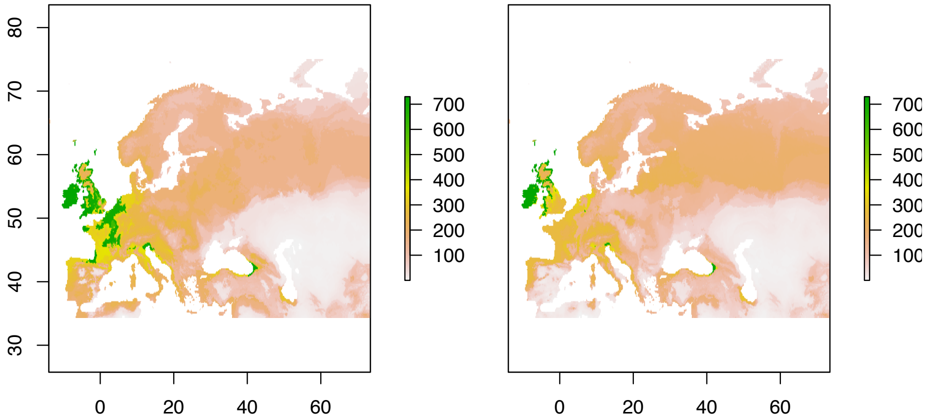

Figure S6.4: Pond duration (*POND_Dur*) in days during two years for (*left* panel) current climates and (*right* panel) a climate change scenario (GFDL-CM3 and RCP 8.5).

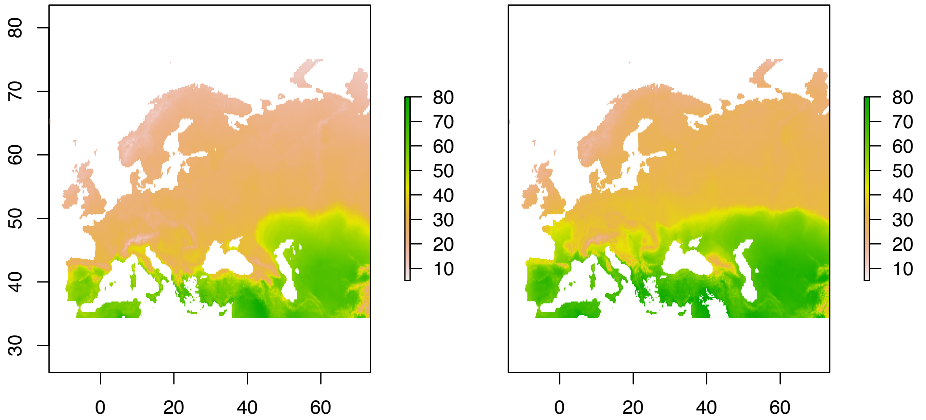

Figure S6.5: Maximum pond temperature (*POND_Tmax*) under (*left* panel) current climates and (*right* panel) a climate change scenario (GFDL-CM3 and RCP 8.5).

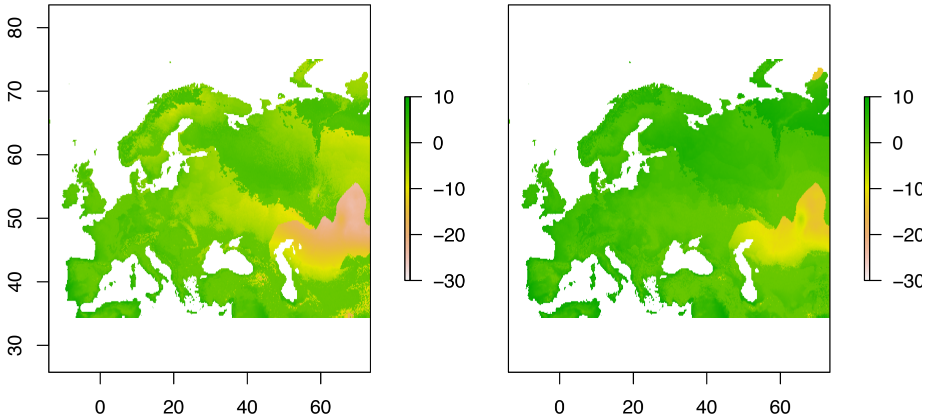

Figure S6.6: Minimum pond temperature (*POND_Tmin*) under (*left* panel) current climates and (*right* panel) a climate change scenario (GFDL-CM3 and RCP 8.5).

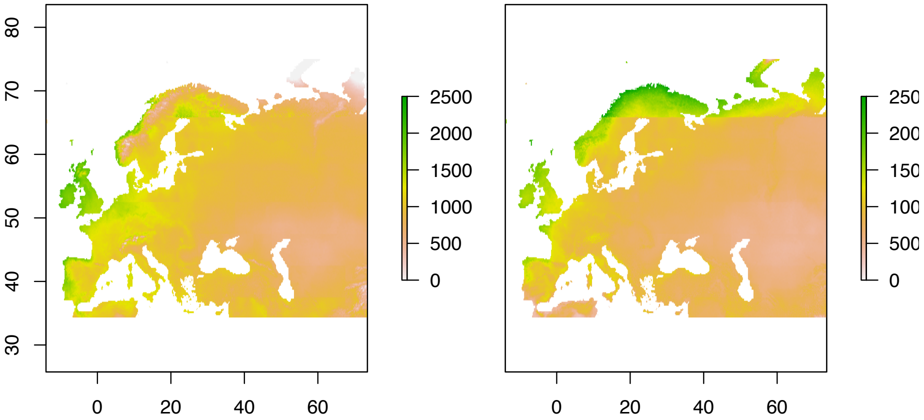

Figure S6.7: Activity windows for juveniles (*ACT_Juv*) under (*left* panel) current climates and (*right* panel) a climate change scenario (GFDL-CM3 and RCP 8.5).

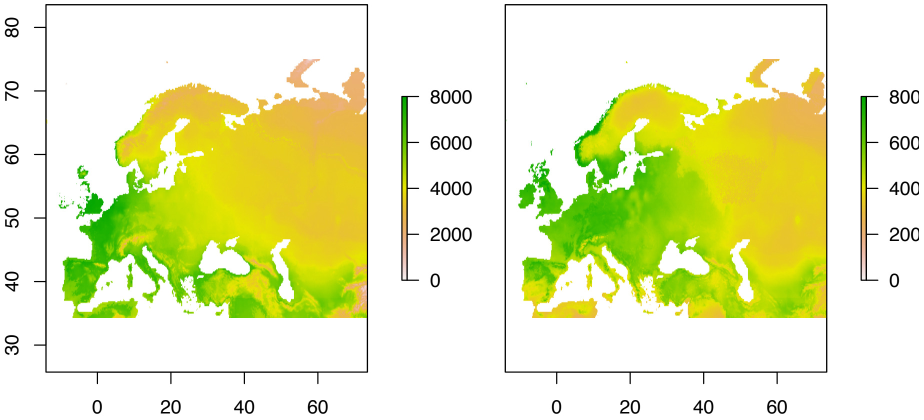

Figure S6.8: Activity windows for adults (*ACT_Adult*) under (*left* panel) current climates and (*right* panel) a climate change scenario (GFDL-CM3 and RCP 8.5).

MULTIVARIATE ENVIRONMENTAL SIMILARITY SURFACE (MESS) MAPS

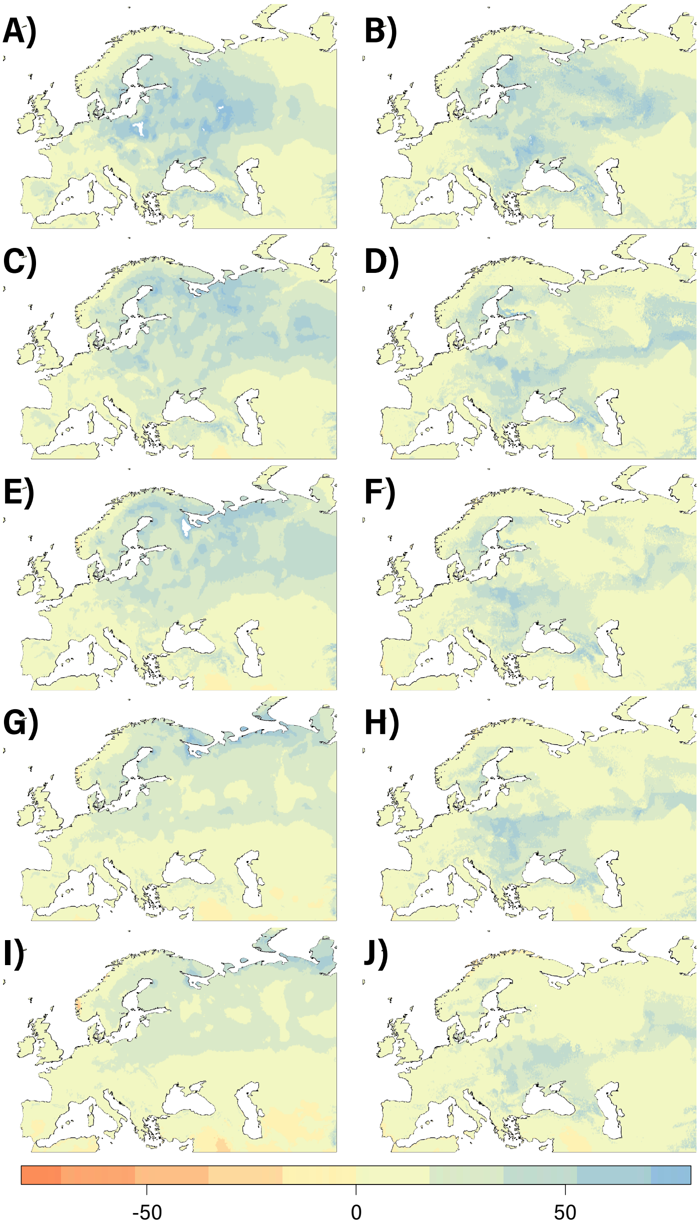

Figure S6.9: MESS maps for models built with (*first column*) macroclimatic WorldClim layers or (*second column*) mechanistic variables. The first row **(A, B)** correspond to current climates, and the rest to climate change scenarios. **(C, D)** CCSM4 global circulation model and a low emission scenario, RCP 4.5, **(E, F)** CCSM4 and RCP 8.5, **(G, H)** GFDL-CM3 and RCP 4.5, and **(I, J)** GFDL-CM3 and RCP 4.5. All maps are depicted with the same scale. Cold colors (*blue*) denote interpolation, while hot colors (*red*) denote extrapolation.

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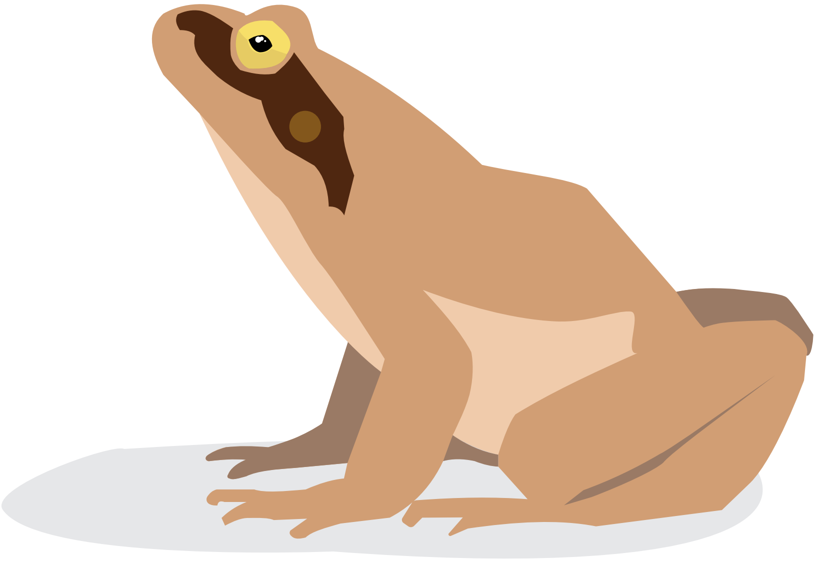
GENERAL DISCUSSION



Anticipating the impacts of anthropogenic climate change on biodiversity is challenging. This requires from gaining insights into the actual exposure to environmental change at the microclimatic scale, the intrinsic sensitivity of individuals, the likely mechanisms with which they may respond to that change, and how these aspects change through ontogeny (Williams et al., 2008; Huey et al., 2012; Maino et al., 2016). Organisms may respond to changes in their microclimates through mechanisms that occur at different time scales. These include genetic adaptation (e.g. niche evolution), which involves several generations, and plastic responses (i.e. developmental acclimation, 'short term' acclimation, and behavioral adjustments) that occur within the same generation or lifecycle stage (Gvoždík & Castilla, 2001; Chevin et al., 2010; Seebacher & Franklin, 2012). If these mechanisms fall short and individuals are not able to disperse to locations with suitable climatic conditions, populations would go extinct. In this thesis I have combined methods from a variety of fields (e.g., biogeography, thermal biology, and species distribution modelling) to shed further light into different pieces of this puzzle and provide a mechanistic forecast of the impacts of climate change on the European common frog, *Rana temporaria*.

Adaptation via niche evolution might be crucial for many species to overcome climate change (Skelly et al., 2007; Wiens et al., 2010; Quintero & Wiens, 2013; Schwallier et al., 2015). However, the extent to which species' niches evolve is still a matter of debate (Angilletta et al., 2002; Pearman et al., 2008; Angilletta, 2009; Holt, 2009). According to the 'phylogenetic niche conservatism' hypothesis (PNC), species may have a tendency to retain ancestral ecological traits, including the niche (Wiens et al., 2010). European amphibian species showed signals of both conservatism and divergence in macroclimatic niches, but we observed that several endemic species (e.g. *Rana iberica* and *R. graeca*) possess highly conserved niches, which could make them especially vulnerable to climate change. Yet, niche evolution appeared common at the species level. Oppositely, common garden experiments performed on different lifecycle stages of *Rana temporaria* from populations along environmental clines revealed that, although the thermal sensitivity of tadpoles varies geographically, the thermal niche of juveniles and adults are considerably conserved at the population level. Moreover, although we observed signs of thermal niche evolution in tadpole *R. temporaria*, a study involving neighboring populations demonstrated that the thermal tolerance variation associated to altitudinal clines is minimal in tadpoles of this species (Gutiérrez-Pesquera, 2016). Most likely, slow rates of niche evolution have led to mixed signals of niche conservatism and divergence at the species level, but widespread niche conservatism at the population level, as also concluded by Peterson (2011) in a review on PNC. Taken together, all the evidence suggests that the pace of niche evolution in *R. temporaria* (and plausibly other vertebrates; e.g. Baer & Travis, 2000) may not be fast enough to counteract climate change.

Plastic responses could provide a more immediate way to coping with rapid climate change (Stillman, 2003; Gvozdik, 2012). Individuals exposed to warm temperatures could plastically acclimate to those conditions 'in the short term' and, additionally, this physiological response could carry-over to later stages. The latter phenomenon is usually known as developmental acclimation and, together with 'short term' acclimation, could result beneficial under changing climates (Beaman et al., 2016). We demonstrated that tadpole and juvenile *R. temporaria* exposed to warm temperatures acclimated by enhancing their heat tolerance at expenses of cold tolerance. However, the degree of phenotypic change accomplished via 'short term' acclimation turned out moderate and had a very limited impact on vulnerability assessments conducted for juveniles. Furthermore, heat tolerance decreased sharply at metamorphosis (as already reported for other amphibians; Cupp, 1980; Floyd, 1983), and the acclimation expressed at the tadpole stage did not carry-over to juveniles. On the contrary, warm larval rearing temperatures led to smaller metamorphs and juveniles, which could have a reduced ability to forage and escape predators (Levy et al., 2016; Carlo et al., 2017). Thus, acclimation – either in the form of 'short term' or developmental acclimation – may be largely insufficient to buffer expected temperature rises (Gunderson & Stillman, 2015; Gunderson et al., 2017). Rather, early exposure to warm temperatures could have negative impacts on adult recruitment and, ultimately, the long-term persistence of populations (Carlo et al., 2017).

Given enough microclimatic heterogeneity and the ability to move, animals could also behaviorally control the environmental conditions they encounter and, consequently, their physiological state (e.g. temperature, hydration level, and metabolism) (Huey, 1991; Huey et al., 2012). This mechanism to cope with environmental conditions could weaken selective pressures, allowing populations to persist in locations with challenging environments without altering their physiology (Bogert, 1949; Huey et al., 2003; Weatherhead et al., 2012). The lack of divergence in thermal niches observed in juvenile and adult *R. temporaria* suggests that thermoregulation represents an important mechanism to occupy areas with contrasting climates, as already demonstrated for lizards (Gvoždík & Castilla, 2001; Gvoždík, 2002; Huey et al., 2003; Muñoz et al., 2016; Muñoz & Losos, 2018). Possibly, a limited scope for thermoregulation in ponds makes the evolution of thermal niches a better strategy for tadpoles to deal with distinct environmental conditions. This could partly explain the slightly higher degree of thermal niche divergence observed at the tadpole stage compared with terrestrial stages (i.e. juveniles and adults). Plausibly, different opportunity for behavioral thermoregulation between ontogenetic stages of *R. temporaria* has led to disparate patterns of thermal adaptation, as reported for other ectotherms (Kingsolver et al., 2011; Lockwood et al., 2018).

Intertwined, stage-specific thermal tolerances and behavioral options could confer divergent vulnerability to climate change among different lifecycle stages, and should be considered in vulnerability assessments (Huey et al., 2012; Buckley & Huey, 2016; Hodgson et al., 2016; Sinclair et al., 2016). Mechanistic niche models showed that under climate change, thermoregulation will be crucial for terrestrial stages of *R. temporaria* to avoid reaching body temperatures outside their tolerance range. However, air temperature rises will deteriorate the thermal quality of retreats. Hence, the vulnerability of terrestrial stages may partly depend on their ability to find cooler microhabitats (i.e. deeper retreats and shaded microhabitats) (Kearney et al., 2009; Sunday et al., 2014; Ortega et al., 2016). Oppositely, although aquatic tadpoles have somewhat wider thermal tolerance ranges, they are ‘trapped’ in ponds and subjected to the thermal fluctuations of water. Accordingly, tadpoles, often present when pond temperatures increase (Reques & Tejedo, 1995), could represent the most climatically sensitive lifecycle stage in *R. temporaria* (*sensu* Lawrence et al., 2015). In line with this guess, we found that maximum pond temperatures of > 40 ºC – which closely resemble the heat tolerance of tadpoles, ~38 ºC – limit the contemporary geographic distribution of *R. temporaria*. Alarmingly, our models predicted that pond temperature increases due to climate change would cause extensive range contractions in the southernmost distribution limit of this frog. As a consequence, a considerable fraction of the intraspecific diversity of *R. temporaria* would be irreversibly lost, including the oldest mitochondrial lineages (Vences et al., 2017).

The erosion of biodiversity projected for the next decades has brought to light the need to move towards a more mechanistic understanding of the links between species, climates, and distributions (Carvalho et al., 2010; Sinervo et al., 2010; Angilletta & Sears, 2011; Dawson et al., 2011). Following the same logic, in addition to where species might be prone to extinction, we need to evaluate the processes that may drive (or prevent!) population collapses (Watson et al., 2013), accounting for the vulnerability of different segments of the population (e.g. different lifecycle stages, sex or size classes, reproductive status). Thus, integrative research programs should be preferred; combining observations, experiments, and modelling, we can contribute new insights into the causal mechanisms that will compel range contractions. By identifying the most vulnerable segments of populations (tadpoles in the case of *R. temporaria*, and possibly most pond breeding amphibians; Duarte et al., 2012; Gouveia et al., 2013), the most likely range-limiting processes (maximum pond temperatures), and the most effective mechanisms to cope with environmental change (behavioral adjustments), we have contributed new knowledge that could be of interest for physiological ecologists, conservation biologists, and practitioners. On the one hand, we have highlighted the thermal tolerance of tadpoles as a decisive functional trait that confers sensitivity to climate change. Consequently, to evaluate the probability of extinction, physiological ecologists should be encouraged to measure this trait for as much species as possible (Tejedo et al., 2012; Gerick et al., 2014; Gutiérrez-Pesquera, 2016; Gutiérrez-Pesquera et al., 2016). On the other hand, our results imply that ensuring thermally suited water bodies could minimize the impacts of temperature rises as climate changes. This could lead to biologically sound, evidence-based mitigating strategies (Cuddington et al., 2013).

As pointed out by George E. P. Box, “all models are wrong, but some are useful” (Box, 1976). Hopefully, the models presented in this thesis will be useful inasmuch as they reflect the thermal limitations of key lifecycle stages of *R. temporaria*, which are vital to foresee the impacts of climate change (Kingsolver et al., 2011; Huey et al., 2012). However, it is important to honestly address the assumptions and limitations. Perhaps one of the greatest assumptions that comes to mind is that our mechanistic models accurately reflect how individuals and microclimates interact. Future work could involve intensive programs of microclimate monitoring at large-scales to better calibrate mechanistic models (e.g. deploying dataloggers in ponds, terrestrial shelters, and other important microenvironments), combined with field studies to explore individuals’ behavioral choices and their physiological consequences (e.g. through biotelemetry; Cooke et al., 2004).

Additionally, the dispersal capacity of species will be decisive under rapid climate change. The thermal sensitivity of the locomotor function – characterized for some populations in this thesis – and other key traits associated to dispersal ability (e.g. emigration distance of individuals from natal sites, life history traits; Estrada et al., 2016) could be bridged with biophysical, dispersal, and demographic models to increase biological realism.

It is also noteworthy that during this thesis I have solely focused on the thermal biology of *R. temporaria*: invasive species, pollutants, habitat loss, emergent diseases (e.g. devastating quitrid fungus), and altered biotic interactions (e.g. with predators) are also important threats to biodiversity (Egea-Serrano et al., 2012; Li et al., 2016; Pecl et al., 2017; Stegen et al., 2017). The impacts of some of these factors will also be mediated by the thermal physiology of species. For instance, habitat modification alters thermal landscapes and the response of species may depend on their thermal biology (Nowakowski et al., 2018). Likewise, the sensitivity to the quitrid fungus varies according to the degree of overlap between the thermal tolerance of hosts (i.e. amphibians) and the quitrid itself (Greenspan et al., 2017a,b). Due to the pervasive effects of temperature, the models developed in this thesis could represent the backbone for further models that would increase the reliability and would incorporate other threats to biodiversity.

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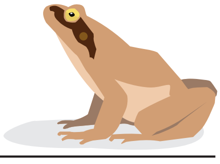
CONCLUSIONS



1 A mosaic of niche evolution and conservatism drove the formation of the European amphibian assemblage. Major geologic events and climatic oscillations promoted allopatric and parapatric speciation, triggering climatic niche diversification. However, many species – including several endemisms – show conserved niches, which possibly sets limits to the extent of their contemporary distributions and confers sensitivity to ongoing climate change (Chapter 1).

2 At the population level, the degree of divergence in thermal niches between populations of the European common frog (*Rana temporaria*) facing disparate climates varies among lifecycle stages. This suggests that different stages perceive and interact differently with the environment and highlights the need to include lifecycle stage considerations into assessments of vulnerability to climate change (Chapters 2, 3, 4 and 5).

3 *Rana temporaria*, at the tadpole stage, is an eurythermal organism and thermal niches, measured as the thermal sensitivity of growth and development, vary across latitudinal and altitudinal clines. Furthermore, at both extremes of the latitudinal gradient, plasticity is maximal. Thermal niche divergence at the tadpole stage is not a simple linear function of thermal conditions along environmental clines, but rather, the result of complex interactions between thermal and temporal constraints, and other historical or phylogeographical factors (Chapter 2).

4 Juvenile and adult *Rana temporaria* are cold adapted, eurythermal organisms and show negligible geographic variation in thermal niches, revealing that terrestrial stages do not cope with contrasting climates through thermal adaptation and supporting the ‘conservative’ view of thermal niche evolution (Chapters 3 and 5).

5 Mixed signals of niche evolution and conservatism at the species level and generalized conservatism in thermal niches at the population level (notably among terrestrial lifecycle stages) indicate that the pace of niche evolution in *Rana temporaria* might be too slow to compensate climate change (Chapters 1, 2, 3, and 5).

6 Acclimation to prevailing temperatures modifies the thermal niches of aquatic and terrestrial lifecycle stages of *Rana temporaria* but provides little protection against climate change. Warm-acclimated tadpoles and juveniles are able to increase their heat tolerance in the short term. Achieved changes, however, lag behind predicted temperature rises. Furthermore, increased heat tolerance comes at expenses of cold tolerance. If climate change was to increase thermal fluctuations, individuals acclimated to warm temperatures could reach lethally cold body temperatures during quick temperature drops (Chapters 4 and 5).

7 Thermal acclimation expressed during the tadpole stage does not transcend metamorphosis in *Rana temporaria*, providing no support for the developmental acclimation hypothesis. Rather than increasing the thermal tolerance of juveniles, early exposure to warm conditions at the tadpole stage compromises the cold tolerance of juveniles and leads to smaller sizes at metamorphosis compared to siblings raised in colder conditions. Hence, warm temperatures during the larval stage, instead of protecting early terrestrial stages from extreme climatic events, will reduce juveniles' ability to forage and escape predators (Chapter 4).

8 Behavioral thermoregulation represents the primary mechanism that allows terrestrial stages of *Rana temporaria* to persist in localities with different environmental conditions. By behaviorally selecting the microclimates that fall within their thermal niche, individuals avoid reaching harmful temperatures without altering their thermal physiology, adhering to the 'Bogert effect'. This effect underlies the lack of thermal adaptation observed at the population level (Chapters 3, 5, and 6).

9 Evaporative cooling is an effective mechanism for wet-skinned ectotherms to dissipate excessive heat, but, as climate changes, it will turn insufficient and terrestrial stages of *Rana temporaria* will be forced to thermoregulate in order to prevent exceeding thermal limits. To that end, juvenile and adult frogs will have to seek deeper retreats or shaded microhabitats. This pattern is congruent across elevations (Chapter 5).

10 Stage-specific thermal niches and microclimate availability for thermoregulation confers differential vulnerability to climate change among lifecycle stages of *Rana temporaria*. Tadpoles, although more resistant to heat than terrestrial stages, face limited ability for behavioral adjustments in shallow ponds and thus, they represent the most climatically sensitive stage. As such, the thermal tolerance of tadpoles together with maximum pond temperatures imposes limits to the geographic distribution of *Rana temporaria* (Chapters 4 and 6).

11 Our models predict that pond temperature increases due to climate change, will result in extensive habitat suitability declines in the southern margin of the distribution of *Rana temporaria* and will push its southern limit northwards. As a consequence, most genetic diversity within *Rana temporaria* – harbored in the Iberian Peninsula – would be in jeopardy.

12 Integrative research programs headed towards a more mechanistic understanding of species' distributions offer causal and robust insights into the effects of climate change. Combining observations, ecophysiological experiments, and correlative and mechanistic modelling, we identified tadpoles as the most vulnerable stage, behavioral thermoregulation as the main coping mechanism, and maximum pond temperatures as the likely process that will compel range contractions in *Rana temporaria*. Thus, we contribute new knowledge to anticipate the impacts of climate change on this amphibian, which could also serve to inform evidence-based conservation strategies.

RESUMEN Y CONCLUSIONES

INTRODUCCIÓN

El clima de nuestro planeta está cambiando a una tasa sin precedentes y amenaza la biodiversidad, el funcionamiento de los ecosistemas e incluso el bienestar humano (Pecl et al., 2017). Los gases invernadero emitidos como consecuencia directa o indirecta de toda actividad humana, evitan que la radiación solar salga de nuestra atmosfera. Esto está ocasionando el calentamiento de la atmosfera y los océanos, y con ello el aumento del nivel del mar, y la contracción de las masas de hielo y nieve (IPCC5). Todas las formas de vida del planeta están respondiendo al cambio climático en curso y ya se han registrado cambios en fenología (esto es, cambios en la fecha de floración, reproducción o migración), en interacciones entre especies y, principalmente, en las distribuciones (Parmesan, 2006). En concreto, las especies se están desplazando hacia zonas más frías: hacia los polos del planeta y las cumbres de las montañas, y a mayores profundidades en los océanos. Por tanto, para anticipar los impactos del cambio climático es de vital importancia desvelar los factores evolutivos, ecológicos y fisiológicos que subyacen a las distribuciones de las especies.

El 'nicho' de las especies es un concepto clave en ese sentido. G. Evelyn Hutchinson vislumbró el nicho como un rasgo propio de las especies y definió el nicho fundamental o fisiológico como un hipervolumen n-dimensional en el espacio ambiental multidimensional – en otras palabras, la combinación de las tolerancias a todos los factores ambientales (p.ej. temperatura, humedad) de una especie (Hutchinson, 1957). Todas las localidades que muestren condición ambientales que caen dentro de este hipervolumen podrían sustentar poblaciones estables (Kearney & Porter, 2009). Sin embargo, los organismos no son capaces de colonizar todos esos lugares debido a barrares a la dispersión o porque otras especies (p.ej. depredadores, competidores o parásitos) los excluyen. La combinación de condiciones ambientales favorables (esto es, nicho fundamental) que finalmente ocupan las especies se conoce como nicho realizado, y espacialmente, representa su distribución geográfica. Las distribuciones geográficas, aunque en muchas ocasiones a escala fina están moldeadas por interacciones bióticas, a gran escala reflejan las limitaciones fisiológicas de las especies (Soberón & Nakamura, 2009). Se podría decir, por tanto, que la fisiología de las especies va a mediar entre el cambio climático y los desplazamientos de las distribuciones de especies (Seebacher & Franklin, 2012).

Pero la fisiología, así como otros muchos rasgos incluyendo el hábitat, cambian a lo largo del ciclo de vida de la mayoría de especies. Esto es especialmente intuitivo en especies con ciclos de vida complejos, como los anfibios, en los que el cambio de nicho a lo largo del ciclo de vida es evidente, por ejemplo, durante la metamorfosis (Wilbur, 1980). La vulnerabilidad o susceptibilidad a la extinción de una población o especie, dependerá de la sensibilidad fisiológica de cada estadio de su ciclo de vida, del nivel de exposición al cambio en los (micro)ambientes que los distintos estadios ocupan y de su capacidad de respuesta (Williams et al., 2008). Frente al cambio ambiental, los distintos estadios podrían responder in situ mediante adaptación genética a las nuevas condiciones, plasticidad fenotípica y respuestas comportamentales como la termorregulación. Además, alguno de los estadios del ciclo de vida podría resultar particularmente sensible a cambios en las condiciones ambientales y limitar la distribución actual y futura (Radchuk et al., 2012).

Existen muchos métodos para cuantificar el nicho y todos tienes fortalezas y debilidades. Para explorar el nicho de las especies, la mayor parte de los estudios emplean datos de distribución de especies y capas climáticas globales (p.ej. Araújo et al., 2006; Quintero & Wiens, 2013). Esta aproximación ha sido especialmente prolífica para entender la evolución del nicho a nivel de especie, y cómo esto ha influido sobre los patrones actuales de biodiversidad. Sin embargo, esta manera de cuantificar el nicho lleva a una simplificación excesiva del nicho y puede no ser muy informativa de los mecanismos que limitan las distribuciones de las especies (Kearney & Porter, 2009). Por otro lado, el nicho de las especies (y de cada uno de los estadios del ciclo de vida; Kingsolver et al., 2011) también se puede cuantificar de forma más realista mediante experimentación. Esta aproximación resulta mucho más costosa y podría no ser factible para un gran número de especies o rasgos fisiológicos. Sin embargo, integrando parámetros fisiológicos específicos de cada estadio de una especie obtenidos a través de experimentos con modelos de distribución mecanicista, podemos identificar los mecanismos o estadios que limitan la distribución de especies. De esta manera podemos generando hipótesis sobre las potenciales causas de extinciones asociadas al cambio climático, así como proponer medidas de conservación para minimizar sus impactos.

PLANTEAMIENTO DE ESTA TESIS DOCTORAL

En esta tesis, adopto una aproximación integrativa para anticipar el impacto del cambio climático sobre la rana bermeja (*Rana temporaria*). Gracias al concepto de nicho, ahora sabemos que podemos obtener información sobre la distribución de las especies midiendo rasgos directamente sobre ellas (p.ej. su fisiología; Kearney et al., 2008) y que, a su vez, es posible aproximar las tolerancias fisiológicas de las especies a partir de sus distribuciones geográficas (Sillero, 2011). Por todo ello, a lo largo de esta tesis, empleo información sobre la distribución de especies y la combino con experimentación sobre distintos estadios del ciclo de vida de la rana bermeja (renacuajos, juveniles y adultos). Junto con esto, utilizo modelos de distribución correlativos y modelos de nicho mecanicistas para predecir los efectos de los cambios ambientales. De esta manera es posible estimar la vulnerabilidad de los distintos estadios y, por consiguiente, de la rana bermeja frente al cambio climático.

En el **capítulo 1** comparamos los nichos climáticos de los anfibios europeos basándonos en sus distribuciones para estimar su potencial evolutivo. Recopilamos datos sobre la distribución geográfica de 41 especies de anfibios europeos (Sillero et al., 2014), capas climáticas para todo el rango de su distribución (extraídas de WorldClim y relacionadas con condiciones medias y extremas de temperatura y precipitación; Hijmans et al., 2005), y 100 hipótesis filogenéticas (Roquet et al., 2014). Con estos datos, cuantificamos el nicho climático de las especies y los comparamos mediante métodos filogenéticos comparados (señal filogenética y ajuste de modelos evolutivos), así como métodos basados en solapamiento de nicho (análisis de equivalencia y similitud de nichos; Broennimann et al., 2012). Además, utilizamos la relación entre solapamiento geográfico y el tiempo de divergencia entre especies para las interrelaciones entre la evolución/conservación de nicho, el modo de especiación (i.e. alopátrico, simpátrico, o parapátrico), y desplazamientos pasados de los rangos de distribución.

En capítulos subsiguientes, medimos experimentalmente distintos aspectos del nicho fundamental de *R. temporaria*, aunque limitado al nicho térmico (esto es, el eje del nicho fundamental que corresponde a la temperatura). Más específicamente, utilizamos experimentos de ambiente común para estudiar cómo varía el nicho térmico de renacuajos, juveniles y adultos de la rana bermeja a lo largo de gradientes latitudinales y altitudinales, donde las poblaciones están sujetas a distintas condiciones climáticas.

Por un lado, estudiamos la variación geográfica del nicho térmico de renacuajos de *R. temporaria*, definido como la sensibilidad térmica del crecimiento y el desarrollo (**capítulo 2**). Para ello, obtuvimos puestas de *R. temporaria* de poblaciones a lo largo de un gradiente altitudinal (2 poblaciones de baja altitud y 2 de alta montaña del norte de la Península Ibérica) y uno latitudinal (2 poblaciones de la Península ibérica, 2 del centro de Suecia y 2 del norte de Suecia) y comparamos las tasas de desarrollo y crecimiento de renacuajos expuestos a dos temperaturas distintas (13 y 18ºC). De esta manera, testamos hipótesis de convergencia evolutiva a lo largo de gradientes climáticos.

Por otro lado, exploramos los mecanismos que permiten a las fases terrestres (juveniles y adultos) de la rana bermeja sobrevivir bajo distintas condiciones climáticas (**capítulo 3**). Con ese fin, estudiamos la sensibilidad térmica de la locomoción y las temperaturas preferidas de juveniles y adultos a lo largo de un gradiente altitudinal en el norte de la Península Ibérica (4 poblaciones altas y 4 bajas). Más concretamente, medimos la distancia total recorrida de cada individuo a 9, 18, 21, 23, 25 y 28ºC durante 5 minutos en una pista circular. Con esto caracterizamos y comparamos las curvas de desempeño térmico (TPCs) para la locomoción. Además, medimos las temperaturas preferidas de los mismos individuos en un gradiente térmico, para comprender si las poblaciones de ambientes fríos prefieren temperaturas más frías que las poblaciones de ambientes cálidos.

En el **capítulo 4**, exploramos posibles efectos arrastrados entre estadios del ciclo de vida en una población de *R. temporaria*. Para ello, expusimos renacuajos a dos temperaturas (18 y 25ºC), y medimos las tolerancias térmicas (límites críticos térmicos o CTs) de renacuajos, individuos recién metamorfoseados, juveniles son 2 y 4 semanas de cada uno de los tratamientos. Este diseño experimental nos permite medir el efecto que tiene la exposición temprana a temperaturas cálidas sobre los animales expuestos (renacuajos) y en fases subsiguientes (individuos recién metamorfoseados y juveniles).

Los modelos mecanicistas ofrecen un marco flexible para integrar distintos mecanismos que podrían limitar o permitir la persistencia de una especie en una localidad. En el **capítulo 5**, realizamos un experimento para determinar los CTs y su potencial de aclimatación en juveniles de rana bermeja de 3 poblaciones a baja altitud y 4 de alta montaña. Obtuvimos juveniles de cada población y los aclimatamos a dos temperaturas distintas (14 y 24ºC). Así, además de la variación geográfica en tolerancia térmica, obtuvimos el potencial para incrementar la resistencia a través de la aclimatación. Después, integramos las tolerancias térmicas y potencial de aclimatación obtenido en este capítulo, con las temperaturas preferidas para juveniles presentadas en el capítulo 3 y modelos mecanicistas de nicho (NicheMapR; Porter et al., 1973; Kearney & Porter, 2016).

En breve, NicheMapR permite reconstruir los microclimas disponibles para un organismo, así como su comportamiento partiendo de su nicho térmico. De esta manera, exploramos la exposición de juveniles a temperaturas extremas a distintas altitudes, su potencial de mitigarlas mediante aclimatación, y el rol de la termorregulación comportamental.

Finalmente, en el **capítulo 6** combinamos los parámetros ecofisiológicos medidos en los capítulos anteriores, y generamos varias capas que reflejan mecanismos que podrían limitar la distribución de *R. temporaria* a escala europea usando NicheMapR. Específicamente, basándonos en las constricciones térmicas de adultos y juveniles, modelamos el tiempo de actividad para cada uno de ellos. Además, modelamos las temperaturas máximas y mínimas, así como el hidroperiodo de una charca típica utilizada por *R. temporaria* (~30 cm de profundidad), y que podrían suponer constricciones a los renacuajos. Todas estas capas las integramos con modelos correlativos para desvelar los mecanismos tras la distribución de la rana bermeja y anticipar cambios en su distribución por el cambio climático.

CONCLUSIONES

1 Un mosaico de evolución y conservación de nicho impulsó la formación del conjunto de anfibios europeos. Los principales eventos geológicos y oscilaciones climáticas facilitaron la especiación en alopátrica o parapátrica, desencadenando la diversificación de sus nichos climáticos. Sin embargo, muchas especies – incluyendo numerosos endemismos – mostraron signos de nichos conservados, lo que podría limitar la extensión de su distribución actual y conferirles una mayor sensibilidad ante el cambio climático (Capítulo 1).

2 A nivel poblacional, el nivel de divergencia del nicho térmico entre poblaciones de la rana bermeja (*Rana temporaria*) que encuentran distintos climas varía entre los distintos estadios del ciclo de vida. Esto sugiere que cada estadio percibe e interactúa de forma distinta con su entorno y resalta la necesidad de incluir consideraciones sobre estadios del ciclo de vida en evaluaciones sobre la vulnerabilidad frente al cambio climático (Capítulos 2, 3, 4 y 5).

3 *Rana temporaria*, durante la fase de renacuajo, es un organismo euritermo cuyo nicho térmico, medido como la sensibilidad térmica del crecimiento y desarrollo, varía a lo largo de gradientes latitudinal y altitudinales. Además, en ambos extremos del gradiente latitudinal, la plasticidad es máxima. La divergencia del nicho térmico durante el estadio de renacuajo no es una simple función lineal de las condiciones térmicas a lo largo de clinas ambientales, sino el resultado de complejas interacciones entre limitaciones térmicas y temporales, así como otros factores históricos o filogeográficos (Capítulo 2).

4 Los juveniles y adultos de *Rana temporaria* son organismos euritermos adaptados al frío que apenas muestran variación geográfica en nichos térmicos, revelando que los estadios terrestres de esta especie no afrontan distintas condiciones climáticas mediante adaptación térmica. Esto apoya la posición 'conservadora' de la evolución del nicho térmico (Capítulos 3 y 5).

5 Señales mixtas de evolución y conservación de nicho a nivel de especie y conservación generalizada a nivel poblacional (especialmente entre los estadios terrestres del ciclo de vida) indican que el ritmo de evolución del nicho en *Rana temporaria* podría ser demasiado lento para compensar el cambio climático (Capítulos 1, 2, 3 y 5).

6 La aclimatación a las temperaturas predominantes modifica el nicho térmico de estadios acuáticos y terrestres del ciclo de vida de *Rana temporaria*, pero ofrecen una protección muy limitada ante el cambio climático. Los renacuajos y juveniles son capaces de aclimatarse a temperaturas cálidas incrementando en el corto plazo su tolerancia al calor. Los cambios producidos, sin embargo, se quedan cortos comparados con los incrementos de temperatura esperados. Además, el incremento en tolerancia al calor se da a expensas de la tolerancia al frío. Si el cambio climático incrementa las fluctuaciones térmicas, individuos aclimatados a temperaturas cálidas podrían alcanzar temperaturas letalmente frías durante rápidos desplomes de las temperaturas (Capítulos 4 y 5).

7 La aclimatación térmica expresada durante la fase de renacuajo no transciende la metamorfosis en *Rana temporaria*, no apoyando la hipótesis de la aclimatación del desarrollo. En vez de incrementar la tolerancia térmica de los juveniles, la exposición

temprana a temperaturas cálidas compromete la tolerancia al frío de los juveniles, que además alcanzan menores tamaños en metamorfosis que los individuos desarrollados en temperaturas más frías. Por tanto, temperaturas cálidas durante el periodo larvario, en lugar de proteger a los estadios terrestres tempranos de eventos climáticos extremos, reducirá la capacidad de los juveniles para forrajear y escapar de depredadores (Capítulo 4).

8 La termorregulación comportamental representa el principal mecanismo que permite a los estadios terrestres de *Rana temporaria* persistir en localidades con distintas condiciones climáticas. Mediante la selección de los microclimas que caen dentro de su nicho térmico, evitan alcanzar temperaturas nocivas sin alterar su fisiología térmica, adhiriéndose al 'efecto Bogert'. Este efecto subyace a la ausencia de adaptación térmica observada a nivel poblacional (Capítulos 3, 5 y 6).

9 El enfriamiento por evaporación es un mecanismo efectivo que ayuda a disipar el calor excesivo a ectotermos de piel húmeda, pero a medida que el clima cambie, perderá efectividad y los estadios terrestres de *Rana temporaria* se verán obligados a termorregular para evitar exceder sus tolerancias térmicas. Para ello, tendrán que refugiarse en microambientes más profundos o sombreados. Este patrón se mantiene a lo largo del gradiente altitudinal (Capítulo 5).

10 Nichos térmicos específicos de cada estadio del ciclo de vida y una diferente disponibilidad de microclimas para la termorregulación, confieren una vulnerabilidad diferencial a los distintos estadios de *Rana temporaria*. Los renacuajos, aunque son más resistentes al calor que las fases terrestres, se enfrentan a un limitado potencial para ajustes comportamentales en las charcas someras y, por ello, representan el estadio climáticamente más sensible. Como tal, la tolerancia térmica de los renacuajos en combinación con las temperaturas máximas de las charcas, establecen limites a la distribución geográfica de *Rana temporaria* (Capítulos 4 y 6).

11 Nuestros modelos predicen que, a consecuencia del cambio climático, los aumentos de temperatura en las charcas reducirán la favorabilidad del hábitat en gran parte

del margen sur de la distribución de *Rana temporaria*, empujando el límite sur de su distribución hacia el norte. De esta manera, la mayor parte de la diversidad genética de *Rana temporaria* – albergada en la Península Ibérica – se vería amenazada.

12 Programas de investigación integrativos dirigidos hacia una compresión mecanicista de las distribuciones de las especies, ofrecen puntos de vista causales y robustos de los efectos del cambio climático. Combinando observaciones, experimentos ecofisiológicos y modelado correlativo y mecanicista, hemos identificado a los renacuajos como el estadio más vulnerable, la termorregulación comportamental como el principal mecanismo de respuesta, y las temperaturas máximas de las charcas como potencial proceso que provocará la contracción del rango de distribución de *Rana temporaria*. Por todo ello, contribuimos conocimiento nuevo para anticipar el impacto del cambio climático en este anfibio, lo que también podría servir para proponer estrategias de conservación basadas en evidencias.

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