EVOLUTION OF ENVIRONMENTAL NICHE OF AMPHIBIANS IN WESTERN MEDITERRANEAN AND CONSERVATION IMPLICATIONS.

INTEGRATING MODELLING, ENVIRONMENTAL PHYSIOLOGY AND CONSERVATION TRAITS.

THESIS OF EDUARDO JOSÉ
RODRÍGUEZ-RODRÍGUEZ

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Climatic change is a major engine of evolutionary change (Vrba 1996)

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Evolution of environmental niche of amphibians in Western Mediterranean and conservation implications.

Integrating modelling, environmental physiology and conservation traits.

PhD dissertation:

Eduardo José Rodríguez Rodríguez.

Advisor: Juan Francisco Beltrán Gala

Eduardo

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Index

Resumen general

Los anfibios están entre los organismos más dependientes de las condiciones ambientales, estando limitados por características fisiológicas diferentes según sus estadios de vida (larvario o adulto). Además, nuestra área de estudio, el Mediterráneo Occidental, presenta una interesante historia paleogeográfica y paleo-climática, con la aparición y desaparición de barreras físicas o ambientales, derivadas estas últimas de los relativamente rápidos cambios climáticos producidos durante las glaciaciones. Es importante destacar que los anfibios son el grupo de vertebrados más amenazado a nivel global y que, en el actual escenario de Cambio Global, y más específicamente de Cambio Climático, esperamos cambios bruscos en el hábitat disponible para estas especies.

Por esta razón, el estudio de la evolución del nicho, y sus perspectivas futuras, es una herramienta esencial para la conservación. En este trabajo abordamos este tema mediante una aproximación integrada dividida en dos bloques principales. Un primer bloque centrado en el modelado de nicho ambiental, incluyendo la evolución en la divergencia de este, escenarios futuros y condiciones pasadas que afectan la distribución actual de los anfibios en nuestra área de estudio. Y un segundo bloque que analiza las cuestiones fisiológicas que afectan a ambas fases de vida, e indagando en la respuesta de estos organismos para adaptarse a condiciones ambientales subóptimas. En el resumen gráfico de la página 7 mostramos un esquema de nuestra visión en esta tesis y sus futuras perspectivas.

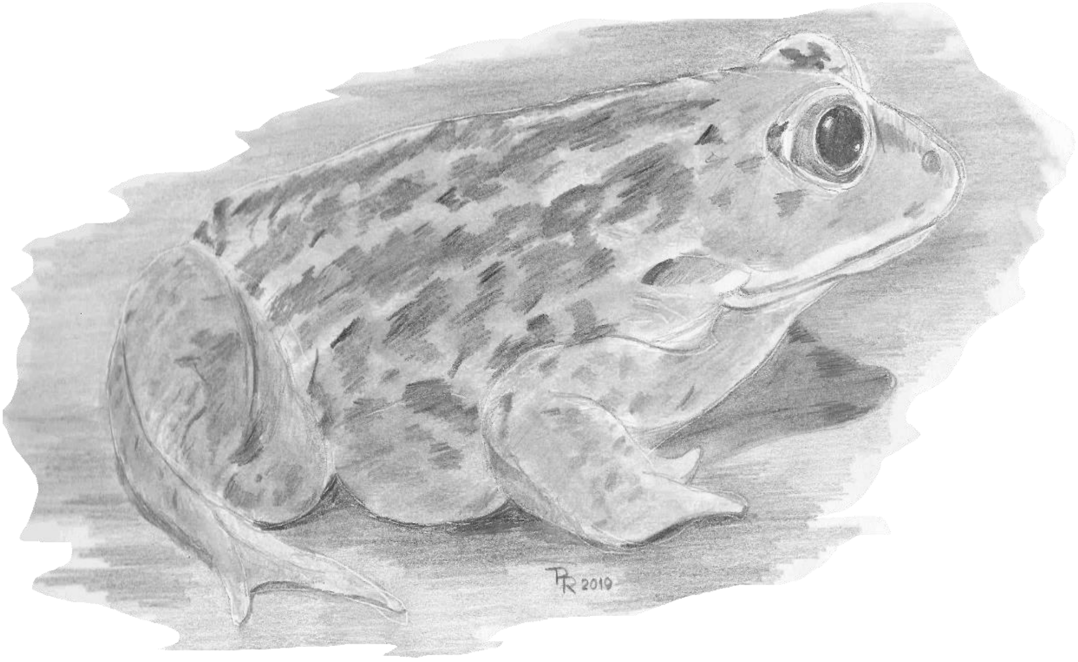
Nuestros resultados muestran que la divergencia de nicho ambiental tiene un papel clave en la evolución, que la estabilidad climática es un promotor de la diversidad en los anfibios del Mediterráneo Occidental y que el calentamiento climático antrópico afectará la disponibilidad de hábitat de estas especies de diferentes maneras, con reducciones en algunas y aumentos en otros. Esperamos que nuestros datos, unidos a futuras investigaciones, proporcionen información útil para la conservación de este grupo tan amenazado.

General abstract

Amphibians are one of the most environment dependent organism, being limited by physiological traits at two main life stages (larvae and adult). In addition, the Western Mediterranean region, our study area, has an old and interesting palaeogeographic and palaeo-climatic history, with the apparition and disappearance of barriers, and relatively fast climate changes between glacial periods. Furthermore, Amphibians are the most threatened group of vertebrates at global scale and, in the actual scenario of Global Change, and specifically, Climate change, we expect severe changes in the habitat suitability for these species.

For this reason, the study of the evolution of the niche, and the future perspectives for it, is an essential tool for the conservation goal. In this work we assess this issue throughout an integrated approach with two main blocks. First, an ecological niche modelling approach, including evolution of niche divergence, future niche scenarios, past environment variables affecting the current distribution of amphibians. And secondly, a physiological analysis block, analysing physiological traits at the two life stages, and inquiring in response of organisms in order to adapt to suboptimal environmental conditions. In the graphical abstract (pg 7) we provide our view about the approach in this Thesis and their future perspectives.

Our results show that divergence of environmental niche have a key role in evolution, that climatic stability is a promoter of diversity in Western Mediterranean amphibians and that anthropogenic climate warming will affect habitat suitability of these species in different way, with reductions in some and increases in others. We expect our data and future research will provide useful information for conservation of this threatened group.



General introduction and objectives.

General Introduction

Ecological niche, evolution hypotheses and research implications

Ecological niche and climatic niche

Ecological niche includes the position and relation of a species with the environment; how it meets its needs for food and shelter, how it survives, and how it reproduces (Schoener, 1989). Ecological niche includes the relation with abiotic and biotic factors. Regarding these factors, and inside those of abiotic nature, we can find the environmental niche, being an important part of it the climatic niche. This includes the relation and possibilities of accomplishing the life cycle of a species respect climatic conditions, like temperature, moisture precipitation, and their changes along the year and it is a key factor in evolution (Bonetti & Wiens, 2014).

The term "ecological niche" probably arrives for the majority of modern ecologist from Theodor Seuss Geisel's writings:

-*"and NUH is the letter I use to spell Nutches, Who live in small caves, known as Niches, for hutches. These Nutches have troubles, the biggest of which is The fact that there are many more Nutches than Niches. Each Nutch in a Nich knows that some other Nutch Would like to move into his Nich very much So each Nutch in a Nich has to watch that small Nich Or Nutches who haven't got Niches will snitch".* -Dr. Seuss, On Beyond Zebra (1955)

However, the study of niche is much older than the own term. It has been mentioned as species' role by most important ecologist as Linnaeus, Darwin or Wallace during the XIX century. Although the tern "niche" was not in used at that moment, pioneering theories of competition and predation by Volterra (e.g., 1926) and Lotka (e.g., 1924) provided much of the foundation for modern explorations into the niches of organisms, being especially evident during the heyday of "niche theory" pioneered by Robert MacArthur (Chase & Leibold, 2003). The father of modern ecology, G. Evelyn Hutchinson, though the niche as an n-dimensional hypervolume (see figure 1), describing all the factors that regulate the existence of species (1965). The modern concept of niche is finally complete with Schoener (1989) definition, mentioned above. More recently, informatics and modern algorithms have provided an immense development in niche research (Araujo & Guisan, 2006).

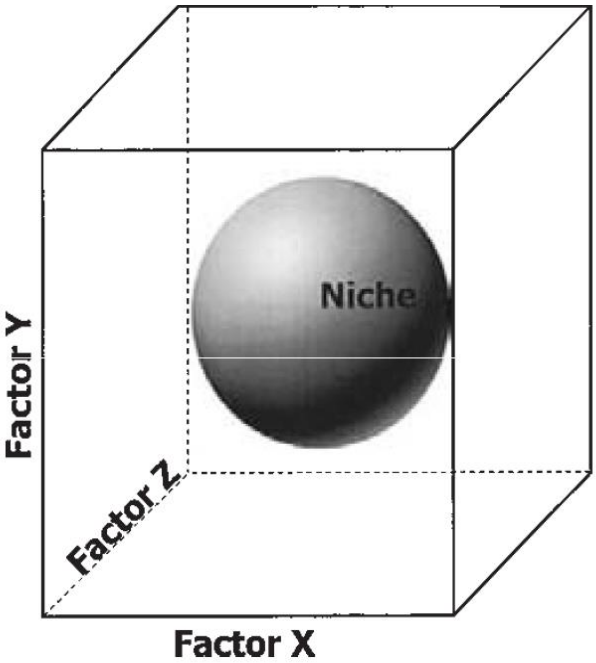


Figure 1: Hypothetical depiction of a three-dimensional volume (three factors) within Hutchison's (1957) *n*-dimensional hypervolume niche. The area within the cube represents the total available amount of each factor, while the area within the sphere represents the amount of each factor needed for a given species to survive; i.e., its niche. Extracted from Chase and Leibold, 2003.

Ecological niche, physiology, intraspecific adaptations and evolution hypotheses

Ecological niche is shaped by physiological traits, including the physiological plasticity, and both arise from genetic adaptations of species and each intraspecific local lineage. It has been proven that intraspecific variation has ecological importance for communities. Bown et al. (2007) proposed that physiological trade-offs at intraspecific level are promoting biodiversity, and Des Roches et al. (2018) found that the removal of some intraspecific elements could have effects in communities comparable with interspecific level. At the same time, the environmental niche of a specie (or lineages) and the environmental variables of landscape are among the main factors shaping the realized distribution. However, current distribution is affected by others important factors as trophic niche, predatory pressure, interspecific competence, current conservation, physical barriers and environmental niche barriers (if the suitable habitat is patched, the species could not to have the capacity of colonize certain suitable areas). In addition, we must consider the past climatic conditions, as they draw a history in the suitable habitat distribution, and the possible apparition of climatic niche barriers.

The ecological niche is not static, and it is subjected to past, current and future evolutionary processes, and amphibians are not an exception (Bonetti & Wiens, 2014). Evolution of niche can match two main hypotheses. The first one is niche conservatism (Peterson et al., 1999; Wiens and Graham, 2005), where phylogenetically related species share similar niche characteristic due to the maintenance of the ancestor niche characteristics (determined by the maintenance of ancestor physiological requirements). The second one is niche divergence (Dickinson and Antonovics, 1973) where the change in its conditions promote the differentiation of separate species. The study of these two evolutive ways are fundamental for understanding of evolutive history and processes of life. Furthermore, we must consider that niche differentiation may occur through the apparition of a barrier and the impossibility or reduction of genetic exchange (allopatric), but niche differentiation in its own is able to differentiate a population without any physical barrier (sympatry) , and Pitteloud et al. (2017) proposes that niche differentiation occurs faster in this second case. For all mentioned reasons, niche evolution is essential to understand evolution history of species.

Classically, it has been described that natural selection can be exercised at difference scales (Owen, 1972). Wiens (1973) recognized different levels or scales of distributional patterns among breeding birds, and identified geographic range, local site and plot patterns in territories, and patterns of utilization. And Johnson (1980) proposed:

-*" A natural ordering of selection processes can be identified. First-order selection can be defined as the selection of physical or geographical range of a species. Within that range, second-order selection determines the home range of an individual or social group. Third-order selection pertains to the usage made of various* *habitat components within the home range. Finally, if third-order selection determines a feeding site, the actual procurement of food items from those available at that site can be termed fourth-order selection.”*-

The niche breadth we can observe in the field is the realized niche, but is not enough to understand the real niche of species, we must consider the entire potential niche in the case of absence of others factors like physical barriers (fundamental niche). Returning to the issue of intraspecific importance, we expect that this hierarchy also works in the case of niche evolution: Entire species, intraspecific levels, local populations and group of individuals. At the same time, we propose that this niche differentiation, promoted by natural selection, will determine the future distribution of species, lineages and groups, generating environmental isolation and promoting speciation.

Modern approaches. Correlative and mechanistic models. Informatics and databases

The characterization of environmental niches is essential to understanding species distributions and patterns of biological diversity, and it has important implications in conservation. Correlative and mechanistic ecological niche modelling (Fielding & Bell, 1997) are proven tools used to approach this characterization (Razgour et al., 2019). Whereas correlative approach relates distribution points with environmental variables, the mechanistic approach uses physiological limits and environmental variables (Kearney & Porter, 2009). Taking account of the available information, it is not surprising that genetic studies combined with ecological niche models have been proven to be powerful tools to study evolutionary processes and resolve biodiversity conservation problems (Razgour et al., 2019). Furthermore, informatic packages and applications allow to relate a wide amount of environmental, genetical, distributional or physiological data (Araujo & Guisan, 2006). All the mentioned data is available nowadays in on-line repositories, like for example the European reptiles and amphibian’s distribution atlas (Sillero et al., 2014), the climatic (Present, past and future) raster database of WorldClim (Fick & Hijmans, 2018), or the sequences repository of GenBank.

Introducing amphibians, our research model.

Origin, diversity and ecological research potential.

The first amphibians arise in the Devonian period from sarcopterygian fishes with lungs and bony-limbed fins (*Sarcopterygii)*. They reached a great diversification during the Carboniferous and Permian periods, being later displaced by emerging new groups of vertebrates, like reptiles (Pough et al., 2015). Modern amphibians (Lissamphibia) are classified in three main orders; Anura, Caudata and Gymnophiona.

Anurans, with around of 6500 species across the world, are the larger group of amphibians and the group with the most diverse ecological requirements (Pough et al., 2015). They also present a high diversity in reproductive and life strategies. They present external fecundation and an oviparous strategy, although the laying eggs strategy is very diverse, including species with paternal care and transport of the laying.

Caudata (urodeles), with around 700 live species, is the order that most resembles the primitive amphibians, with a long body with tail and well-developed limbs, presenting most species internal fecundation and an oviparous strategy, although there are species with direct development.

Though not being present in our study area, Gymnophiona is an order composed by caecilians, with around 200 species in the world. These animals have completely lost limbs, girdles and tail, due to their adaptation to fossorial lifestyle.

Studies of amphibians have played key roles in biological specialities such us developmental biology, ecology, behaviour and medicine. In the case of ecological studies this is due to certain interesting characteristics like reproduction strategies, physiology and the strong relation with environment, being amphibians among the more sensible vertebrate’s species to environmental conditions (Feder & Burggren, 1992). The requirements of these species vary between their two states: Aquatic and terrestrial, being the first phase dependent of water availability and temperature among others, and the second more dependent of moisture and temperature (Navas et al., 2008). Past and present changes in environment may have being determining the evolution of the niche, and in consequence for the evolution of these species. Between the examples we have the higher biodiversity in the glacial refuges of south Europe (Araujo et al., 2008).

Life cycles of amphibians, relation with the environment.

Amphibians, although with differences between species and some exceptions, present complex life cycles with generally two stages, both highly dependent on environment. A larval stage linked to the water and a more terrestrial adult stage. Among these two phases it happens a delicate metamorphosis, also markedly influenced by environmental conditions.

The environmental influence starts with reproduction. Despite the differences among groups (internal fecundation in urodeles, external in anurans for example), the reproduction of amphibians is mediated by climatic conditions, as its success depends on it (woodley, 1995). Induction of reproduction is hormonally mediated, but the cascade starts with the environmental stimuli. The main factors inducing it are rainfall (moisture) and temperature. With the adequate environmental stimuli (Rain, temperature, light…). The hypothalamus produces gonadotropin-releasing hormone (GmRH), which causes the pituitary to release follicle stimulating hormone (FSH) and luteinizing hormone (LH). These substances stimulate the gonads to produce eggs and sperm and to release testosterone and estrogens (Pough et al., 2015).

But the environment dependence does not end here, as the growth and fitness of larvae is strongly affected by temperature, among others, as water column, light…Once the delicate metamorphosis is complete, the survival of juveniles is highly dependent on moisture and temperature. Although mentioned juveniles and adults have certain capacity of manage environmental conditions (throughout active thermoregulation or refuges in case of low moisture or extreme temperatures, among others) their fitness is hopelessly shaped by the environmental tolerance amplitude.

Global change and amphibians

The meaning of Global change, a challenge to amphibians

Global change is a wide term that refers to changes in Earth-systems at planetary scale. It includes changes in climate, oceans, habitats, deep Earth processes, human population, society, economy, transport, communication, health, biodiversity etc.

This is not only a current problem. Global change has been acting since the origin of the planet throughout solar variation, plate tectonics, volcanism, proliferation of life, meteorite impact, changes in earth orbit etc. These changes are responsible for reductions in life diversity, including the five mass extinctions. But now the main cause of global change is the growing human population (and growing demand of resources) and problems derived from this (Some experts described this phenomenon as the Anthropocene). In the last 250 years, human caused alterations have accelerated and caused species extinctions, desertification, pollution, ozone depletion or Climate change, among other large-scale shifts (Borenstein, 2014).

Climatic change, emerging diseases, habitat destruction, among others human promoted or favoured factors can strongly affect the niche of species, and this affection is expected to be more intense in environment high dependant species like amphibians (Wake and Vredenburg, 2008). For example, entire genera, like *Atelopus* sp., are critically endangered due to the chytrid fungus *Batrachochytrium dendrobatidis* (Flechas et al., 2012). Another clear example is the known vulnerability of Southwest Iberian Peninsula amphibians to the introduced red crayfish, *Procambarus clarkii* (Rebelo & Cruz, 2005).

Climate change and others related threats affecting environmental niche

Climate change have been postulated as one of the major threats for biodiversity, and several scenarios have been proposed depending on governments reactions (Fick & Hijmans, 2018). Climate change supposes, among others, changes in the thermal or moisture conditions around the world, and this is affecting obviously the niche of species like amphibians. In addition, there are others proposed problems related with climate change and ectotherms, for example, temperature increase could produce an increase in growth rate. Fast growth and thermal stress can increase oxidative damage to essential biomolecules, accelerating the rate of ageing (Burraco et al., 2020). For this reason, research about environmental niche future perspectives is critical for conservation of this group. Species with strong suitable habitat reductions are expected to be more sensible to extinctions in case of habitat destruction or fragmentation. For this reason, it is important to conserve the remains of suitable habitat for these species in a climate change scenario.

Relating these threats with climatic niche, it has been described that in some groups, like reptiles, the altered thermal niche could drive the diversity loss in numerous areas around the world, as these species cannot evolve quick enough to track climatic change because of constraints arising from the genetic architecture of thermal preference (Sinervo et al., 2010). In fact, amphibians adapt their habits, including refuges, based on thermal and moisture limits (Lange et al., 2020). Furthermore, it has been described that emerging diseases like *Batrachochytrium* sp pathogens or invasive species could increase their incidence in future climate change scenarios, worsening the conservation perspectives (Rahel & Olden, 2008; Xie et al., 2016).

Therefore, all the consequences of anthropogenic climate change, including alterations in thermal or moisture niche availability, expansion of emerging diseases and interspecific and intraspecific erosion of diversity are challenging the conservation of these species, and we must anticipate and try to model habitat suitability responses in order to design effective conservation strategies for Western Mediterranean amphibians.

Western Mediterranean, geographic and climatic history, and data available on amphibians

Western Mediterranean geographic and climatic history

Western Mediterranean has an interesting palaeogeographic and palaeo-climatic history, including physical and marine barriers appearing or disappearing (Ruggieri & Sprovieri, 1976) and glacial periods with refuges in the south, being the last the Würm Glaciation (Feliner, 2011).

Regarding the paleo geographic, the opening of occidental Mediterranean started 26 Ma and around 15 Ma it was connected by a system of straits through the Gibraltar Arc, including two main straits in the Rif (Morocco) region and the South of Antequera (Spain). Later, 6 Ma ago, the pass was closed again, generating the Messininan crisis and opened again around 5 Ma ago (Crespo-Blanc et al., 2016). Recently, Booth Rea et al. (2018) proposed a more complex scenario with the apparition of a Pliocene volcanic archipelago between Cabo de Gata and the eastern Rif coast. These facts draw a scenario of physical barriers and, in consequence, allopatric speciation, followed for connection periods.

Meanwhile, we must remember the interesting past climatic history of the study area. Although four glacial eras have been traditionally considered (Günz, Mindel, Riss & Würm), following the findings of Penck & Brückner (1909), these affirmations have been found partially incorrect. The duration of Pleistocene has been proposed since 2,5Ma to 11.000 years, and in the last 800.000 years at least eight glacial cycles (Glacier and interglacial periods for each cycle) have been described, with an estimated duration of about 100.000 years Milankovitch (1930).

Western Mediterranean and the niche of amphibians. Research potential.

It is expected that climatic conditions have shaped the past and present niche and evolution of species, throughout limiting differently between species considering the different physiological traits of each one. Moreover, in the current scenario of climatic change, these species are challenged, and huge changes in distribution of the niches are expected, adaptation in these species or extinctions (Duarte et al., 2012)

Regarding the amphibians of the western Mediterranean (Anura and Caudata orders only) we have currently great information about distribution thanks to different sources as national atlas (Pleguezuelos e al., 2002), local atlas (Martínez del Mármol et al., 2019) and web repositories (Sillero et al., 2014). This information allows us to model environment requirements. We also have great information about phylogenetics and intraspecific lineages on these species, allowing us the intraspecific approach in our studies. In addition, with already existing and newly produced physiological information in some groups for example, we can infer the traits determining the evolution of the niche in this group. We also have the opportunity of identifying conservation priorities, regarding the predicted available niche loss or gain of species and lineages in a climate change scenario.

Objectives

We aim to provide an overview of climatic niche implications in the amphibians of the Western Mediterranean, including insights in evolution, physiology and conservation. In order to achieve it, we have used a mixed approach integrating ecological niche modelling and physiological data. We have used several species as models to assess our objectives, especially anurans, although also urodeles in the macro-ecologic studies where entire communities are included. Concrete species models are mentioned in each chapter. In chapter 3 we have also implemented a comparison in macroecological aspects with reptiles, for the purpose of understanding differences in the relation with environment of both groups o tetrapods. Specific objectives are:

- To study the implications of niche divergence and niche conservatism in anuran evolution at two levels, inter and intraspecific, determining, in addition, the causes affecting the current evolution of this group.
  Addressed in Chapter 1

- To assess the future perspective (based on predicted future habitat suitability) of Mediterranean anurans in climatic change scenarios, generating valuable information for conservation purposes. **Addressed in Chapter 2**

- To investigate the environmental variables that from the past to now have determined the actual range size and distribution patterns of amphibian's species in Western Europe. In this

chapter we also compare the patterns with a more water independent group of tetrapods, the reptiles.
Addressed in Chapter 3

- To investigate and relate with environment the physiological traits that have determined environmental niche of species at two ontogeny stages, larval (aquatic) and juvenile (terrestrial).
  Addressed in Chapter 4

- To explore physiological mechanism that allow amphibians to adapt to changing environmental conditions.
  Addressed in Chapter 5

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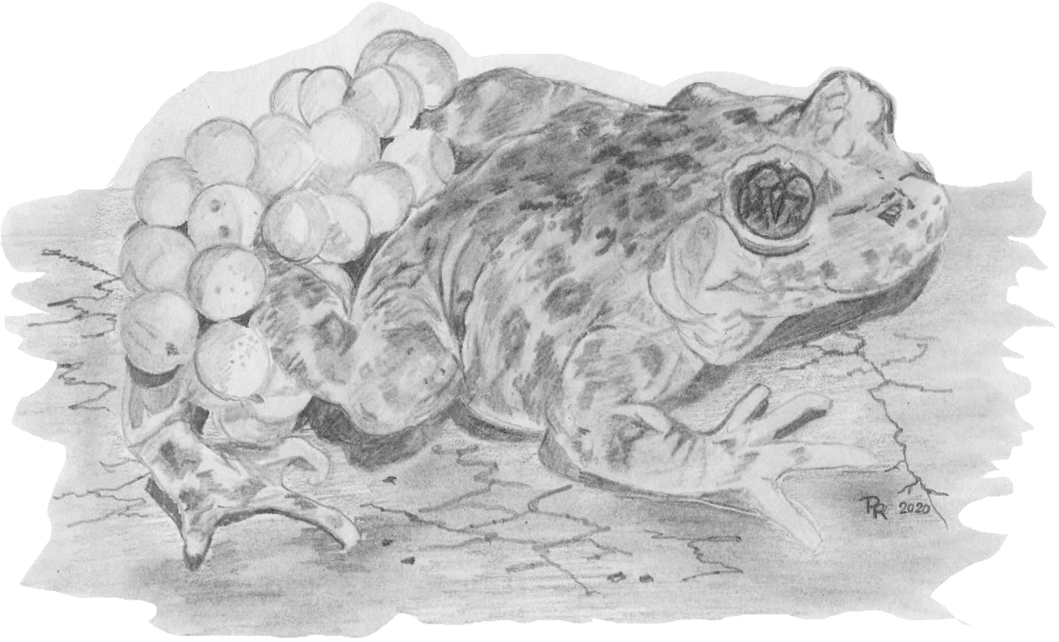


Chapter 1

Chapter 1

Niche models at inter and intraspecific levels reveal hierarchical niche differentiation in midwife toads.

Rodríguez-Rodríguez, E.J.[1], Beltrán, J.F.[1], Tejedo, M.[2], Nicieza, A.G.[3,4], Llusia, D.[5,6], Márquez, R.[7], Aragón, P[8]. Niche models at inter- and intraspecific levels reveal hierarchical niche differentiation in midwife toads. *Sci Rep* 10, 10942 (2020). https://doi.org/10.1038/s41598-020-67992-6

[1]Departamento de Zoología, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain.
[2]Departamento de Ecología Evolutiva, Estación Biológica de Doñana, CSIC, Sevilla, Spain.
[3]Departamento de Biología de Organismos y Sistemas, Universidad de Oviedo, Oviedo, Spain.
[4]Unidad Mixta de Investigación en Biodiversidad (UO-CSIC-PA),Mieres, Spain.
[5]Departamento de Ecología, Facultad de Ciencias. Terrestrial Ecology Group, Universidad Autónoma de Madrid, Madrid, Spain.
[6]Centro de Investigación en Biodiversidad Y Cambio Global (CIBC-UAM),Universidad Autónoma de Madrid, Madrid, Spain.
[7]Fonoteca Zoológica, Departamento de Biodiversidad Y Biología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, Madrid, Spain. [8]Departamento de Biodiversidad, Ecología y Evolución, Universidad Complutense de Madrid, Madrid, Spain.
*email: edurodrodbio@gmail.com

Abstract

Variation and population structure play key roles in the speciation process, but adaptive intraspecific genetic variation is commonly ignored when forecasting species niches. Amphibians serve as excellent models for testing how climate and local adaptations shape species distributions due to physiological and dispersal constraints and long generational times. In this study, we analysed the climatic factors driving the evolution of the genus *Alytes* at inter- and intraspecific levels that may limit realized niches. We tested for both differences among the five recognized species and among intraspecific clades for three of the species (*Alytes obstetricans*, *A. cisternasii*, and *A. dickhilleni*). We employed ecological niche models with an ordination approach to perform niche overlap analyses and test hypotheses of niche conservatism or divergence. Our results showed strong differences in the environmental variables affecting species climatic requirements. At the interspecific level, tests of equivalence and similarity revealed that sister species were non-identical in their environmental niches, although they neither were entirely dissimilar. This pattern was also consistent at the intraspecific level, with the exception of *A. cisternasii,* whose clades appeared to have experienced a lower degree of niche divergence than clades of the other species. In conclusion, our results support that *Alytes*

toads, examined at both the intra- and interspecific levels, tend to occupy similar, if not identical, climatic environments.

Introduction

Climatic factors may act as ecological barriers that can determine the distribution of animal and plant species1.This may explain why incipient speciation processes often can be inferred from the analysis of patterns of niche divergence[2]. Genetic variation, species spatial structure resulting from landscape barriers to gene flow, and intraspecific evolutionary processes (e.g., local adaptation) are the major drivers of speciation. Therefore, it is not surprising that genetic studies combined with ecological niche models have been proven to be powerful tools to study evolutionary processes and resolve biodiversity conservation problems[3]. In addition, the characterization of environmental niches is essential to understanding species distributions and patterns of biological diversity. Correlative ecological niche modelling[33] is a common tool used to approach this characterization3. However, is the species level the most adequate level for this approach? Smith et al.[4] suggest that we must consider local adaptations as evolutionary factors affecting niche requirements, and therefore, inclusion of evolutionary relationships below and above the species level should be considered. For this reason, it is informative to compare niche modes across taxa in a separate way, considering species and local genetic lineages. Amongst vertebrates, amphibians are ideal organisms to analyse this question because their physiology is highly constrained by environmental factors[5,55], and they have low dispersal abilities. This combination of characteristics is expected to promote the evolution of local adaptations to match the spatial complexity of environmental variation[7].

It is well known that vicariant events can drive divergence by limiting genetic exchange among evolutionaryunits8, but currently, there is evidence that environmental factors can also play a key role in biological diversification[9,10,11.] Although habitat suitability models and niche similarity comparisons have been previously conducted at interspecific and intraspecific levels for the midwife toads (*Alytes* sp.), these models have been implemented only in *A. obstetricans*[9].

However, other Mediterranean species, such as *A. cisternasii* and *A. dickhilleni*, have not been studied using nested models (inter- and intraspecific climatic niche divergence schemes) despite the fact that their genetic and phylogenetic discontinuities are well documented[12,13].

Furthermore, an interspecific perspective of climatic niches for all the species in the genus is crucial for a better understanding of climatic determinants and the differentiation processes involved. Recent studies on niche modelling have shown that environmental conditions can drive evolution across geographical ranges and affect patterns of genetic structure[9,14,15]. Moreover, genetic isolation and local adaptation can synergistically influence the fate of species[16], and biogeographic and vicariant events can drive species differentiation (see Martínez-Solano et al.[17]). However, the environmental factors involved in the maintenance of the current differenciation of species and evolutionary units have not been tested as a whole. Tectonic factors, with the formation of a mountain range in the Gibraltar Strait during the Upper Tortonian stage, played an important role in the speciation of the genus *Alytes* [17,18]. In fact, the geographic isolation of *A. muletensis* and *A. maurus* might have strongly affected their environmental niches. Finally, recent work suggests that a more complex geological scenario might have affected the evolutionary history of *Alytes* in its southern range, with a Pliocene volcanic archipelago between Cabo de Gata and the eastern Rif coast[19]. The major aims of this study were (1) to characterize the realized niche differences and environmental factors that promote the differentiation and the observed distribution of *Alytes* species, and (2) to investigate the importance of niche evolution by testing the hypotheses niche conservatism, as the maintenance of ancestral requirements among species with a common ancestor[20], and niche divergence, as the appearance of divergences among these species[2]. Additionally, we tested whether the observed patterns of niche environmental evolution were consistent at two phylogenetic scales: interspecific and intraspecific (i.e., genetic lineages from Dias et al.[12]; Gonçalves et al.[13]; and Maia-Carvalho et al.[9]). We assumed the existence of differentiation at both inter- and intraspecific levels as a consequence of climatic niche differentiation. In this scheme, intra- and interspecific differentiation may be influenced by climate and geographic barriers throughout genetic variation and structure. Finally, we also aimed to determine whether the processes of niche

Methods

Organism presence records and environmental data.

The genus *Alytes* Wagler 1829 currently contains five (six following a recent proposal of Dufrenes et al. [37]) living species. *Alytes obstetricans* (Laurenti, 1768) has the broadest geographical range of all the species in this group. *Alytes cisternasii* Boscá 1879 is endemic to the south-western region of the Iberian Peninsula. *Alytes muletensis* (Sanchiz & Adrover 1979) has a narrower distributional range and is endemic to the northern part of Mallorca Island. *Alytes* (*Baleaphryne*) *dickhilleni* Arntzen & García-París 1995 is endemic to the Betic Region on the south-eastern Iberian Peninsula. Finally, *Alytes maurus* Pasteur & Bons 1962, is a species distributed in some regions of the Rif and Middle Atlas of Morocco. Additionally, four recognized subspecies of *A. obstetricans* have been described: *A. o. almogavarii* Arntzen & García-París (recently proposed as a new species by Dufresnes et al.2019), 1995, from southern France to the Ebro River; *A. o. obstetricans* (Laurenti 1768), from Western Europe to north of the Iberian Peninsula; *A. o. boscai* Lataste 1879, in central and northern Portugal; and finally *A. o. pertinax* García-París & Martínez-Solano, 2001, on the eastern Iberian Peninsula.

The habitats of these five species show a large degree of differentiation. *A. obstetricans* requires areas with high amounts of precipitation and occupy a wide range of habitats, from mountain ranges to crops[21]. *A. muletensis* is only known in a few localities on northern Mallorca Island22, whereas *A. maurus* is restricted to a few localities in the Rif and Middle Atlas Mountains of Morocco and typically occupies humid sites in karst and steep areas[23]. regarding the two Iberian endemics, *A. dickhilleni* is restricted to the Betic region in southeastern Spain[24]. *A. cisternasii* occupies the central-south-western sector of the Iberian Peninsula, usually between 0 and 700 m[25], and, in comparison to the other species of this genus, it is associated with a hotter and drier climate[26].

We used 676 localities to build ecological niche models. A total of 319 *A. dickhilleni* presence points were surveyed by the authors within all their Andalusian distribution areas. We identified 170 local population locations of *A. cisternasii* (including our own collection data and data from Amphibiaweb[27]), 162 of *A. obstetricans* (our data, Amphibiaweb, and in addition, revised data from www.observation.org), 14 of *A. maurus* (our data, Amphibiaweb and Donaire et al.23), and 11 of *A. muletensis* (our data and Amphibiaweb). All populations were separated by at least 200 m. Figure 1 shows the distribution of points selected for all five species.

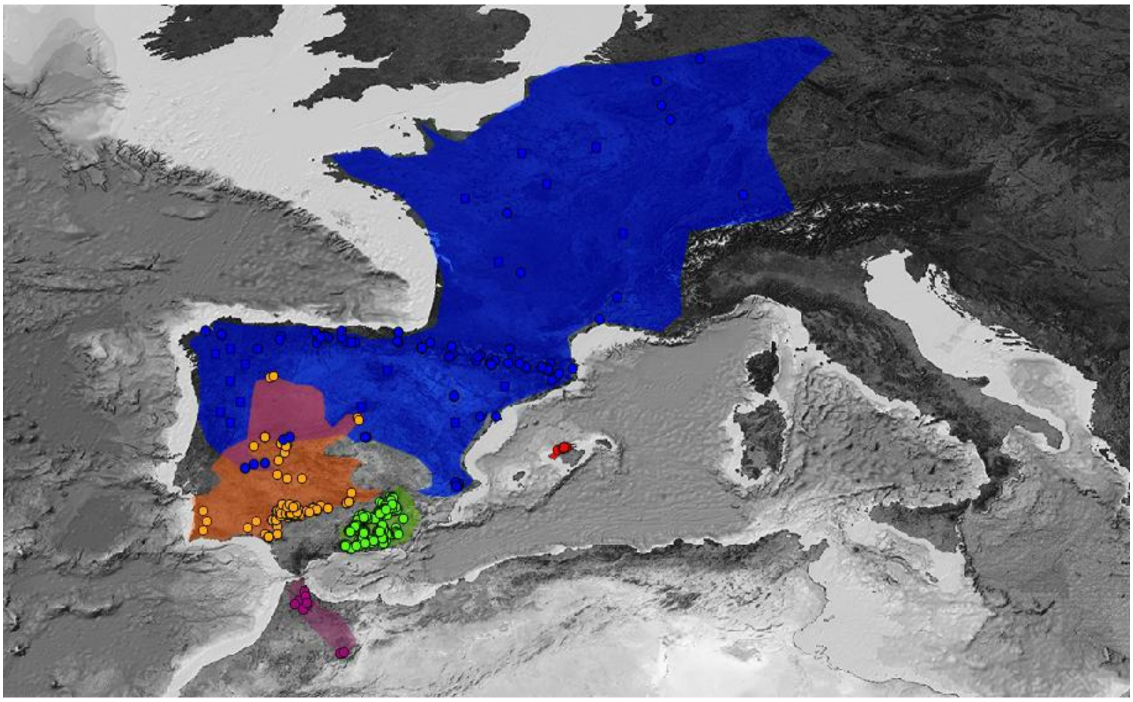


Figure 1. Records of presence included in this study. We considered populations in all the distribution areas, and we only selected populations separated by at least 200 m. A: *Alytes dickhilleni*, B: *Alytes cisternasii*, C: *Alytes maurus*, D*: Alytes obstetricans*, and E: *Alytes muletensis*. Figure created with QGIS Chugiak 2.4.0 (QGIS Development Team. 2018. QGIS Geographic Information System. Open Source Geospatial Foundation Project. https ://qgis.osgeo .org). Background map modified from GEBCO Compilation Group (2019) GEBCO2019 Grid (https ://doi.org/10.5285/836f0 16a-33be-6ddc-e053-6c86a bc078 8e).

To establish intraspecific comparisons among *A. cisternasii*, *A. dickhilleni* and *A. obstetricans*, we used presence data for the genetic lineages reported for these species. Presence data were assigned to lineages according to Gonçalves et al. (*A. cisternasii*)[13], Dias et al. (*A. dickhilleni*)[12], and Maia-Carvalho et al. (*A. obstetricans*)[9] (the central-eastern lineage of *A. obstetricans* was excluded because of the low number of presence points). Some local populations could not be assigned to any of the reported lineages, as these locations are in contact zones our outside the areas surveyed in the cited studies, and therefore, these were removed from the data set. We excluded 42 locations of *A. dickhilleni* and 6 of *A. cisternasii*. For the geographically restricted *A. muletensis* and *A. maurus*, the low number of records and lack of adequate genetic lineage information precluded intraspecific analysis. Even in a well-designed data survey across all of the range of a species, model outputs are sensitive to sampling bias 28. To reduce this bias, we used a sub-sample representing 25% of the populations of each species and lineages included in the study (random selection by MaxEnt). In addition, intraspecific figures show the presence points that were selected for each lineage and then used in the intraspecific correlative model.

We considered climatic and topographic factors to explain the realized distributions and niches of the five *Alytes* species. Climatic variables were obtained from WorldClim version 2 database at 30 s [29], and topographic data were obtained from SRTM (https ://www2.jpl.nasa.gov/srtm/dataprelimdescriptio ns.html). The study area was fixed to the distribution limits of the *Alytes* sp. Genus, and in order to avoid a high correlation and redundancy among the predictors, we performed pairwise Pearson correlations, and for $r > 0.6$, the variable with lower biological relevance was excluded. The final data set included five climatic variables: mean diurnal range, isothermality, minimum temperature of the coldest month, mean temperature of the driest quarter, and precipitation of the wettest month.

Niche model analyses.

We performed two different modelling approaches: ecological niche models (ENMs) and an ordination technique[30]. First, of the different ENMs types, we selected the widely used machine learning method MaxEnt (version 3.4.1) to build the SDMs [31]. The model was fitted using hinge, product, linear, and quadratic features with a maximum of 10,000 background points, 1,000 replicates, and clamping. Models were fitted by using the area under the ROC curve (AUC and ROC represents "receiver operating characteristic" [31]). We used the Cloglog output format. Although this measure has been extensively used to fit models, its usefulness has been criticized, especially for presence/background models such as MaxEnt. Thus, in addition to AUC, we present the values of its components, sensitivity and/or specificity[32]. To obtain these two indices, the continuous MaxEnt output was transformed into a categorical variable (predicted presence/absence). For this transformation, we applied the threshold of the minimum training presence (the lowest suitability scores associated with the populations of each lineage/species) given in the MaxEnt output sheet. We calculated the specificity from the confusion matrix[33]. This is a conservative and realistic threshold since it may include even small suitability scores whenever the lineages/species are present[34]. In this case, a calculation of sensitivity was not necessary since the applied threshold was fixed at the maximum. In addition, we have followed the Raes and Ter Steege validation method[57], including 95% I.C AUC values of null models created with random points of the same size of the presences included in our models. ENM AUC values that are higher than their corresponding 95% CI AUC value of the fitted null model, significantly deviate from what would be expected by chance ($p < 0.05$).

The ordination technique approach was applied to perform niche overlap analyses, either between pairs of the five species or between pairs of the lineages within *A. cisternasii*, *A. dickhilleni and A. obstetricans*. We used the tool Ecospat, which incorporates null hypotheses[35]. For these analyses, we performed the following tests: niche equivalency tests (are niches identical?), similarity tests (are niches more similar than expected by chance?), and niche principal component analysis (PCA). As a measurement of the realized niche overlap, we calculated a Schoener's *D* index through the niche-PCA. This index ranges from 0 to 1 to reflect no overlap to total overlap, respectively [36]. For both the niche equivalency and similarity tests, we used the argument = ”greater” (overlap greater than expected by chance) to test the conservatism hypothesis and the argument = ”lower” (overlap lower than expected by chance) to test the divergence hypothesis[35]. We performed 1,000 permutations for each analysis. Additionally, we integrated phylogenies (inter- and intraspecific levels) using the age-range correlation function of the Phyloclim package[38]. This function is used to test for phylogenetic signals in patterns of niche overlap. Slopes and intercepts derived from a linear model can be used to characterize speciation mode (allopatric versus sympatric) or niche evolution (conservatism versus flexibility) in the clade[39].

Regarding the choice of the geographical extent (and as a consequence, environmental background), we used the software QGIS40 to compile and process environmental data using the extension point sampling tool[41]. Analyses were conducted using R[42]. The study area for the interspecific analysis with MaxEnt and ECOSPAT was adapted to the total distribution of the genus to allow comparisons. At the intraspecific level, we used the same area in each cluster of lineages (the species ranges).

Results

At the interspecific level, the Maxent outputs are shown in Fig. 2. The variables with a relatively higher percentage of contribution in the Maxent models were isothermality (62.5%; *A. obstetricans*), mean temperature of the driest quarter (56.3%; *A. cisternasii*), mean temperature of the driest quarter (40.2%; *A. dickhilleni*), precipitation of the wettest month (90.4%; *A. muletensis*) and, finally, precipitation of the wettest month (73.8%; *A. maurus*). The AUC, 95% I.C. AUC of null model and specificity values were 0.90 ± 0.03, 0.86 and 0.65 (*A. obstetricans*); 0.92 ± 0.10, 0.84 and 0.40 (*A. cisternasii*); 0.94 ± 0.02, 0.90 and 0.67 (*A. dickhilleni*), 0.88 ± 0.16, 0.78 and 0.83 (*A. muletensis*), and 0.98 ± 11, 0.92 and 0.95 (*A. maurus*), respectively. The results of the niche overlap (Schoener's *D*) and similarity and equivalency analyses are shown in Table 1. We found minimal niche overlap with significant *p* values for the equivalency test in the divergence hypothesis, except for the overlap between *A. obstetricans* and *A. maurus*. The magnitude and sign of the variables in the principal component plots of the niche are shown in Fig. 3.

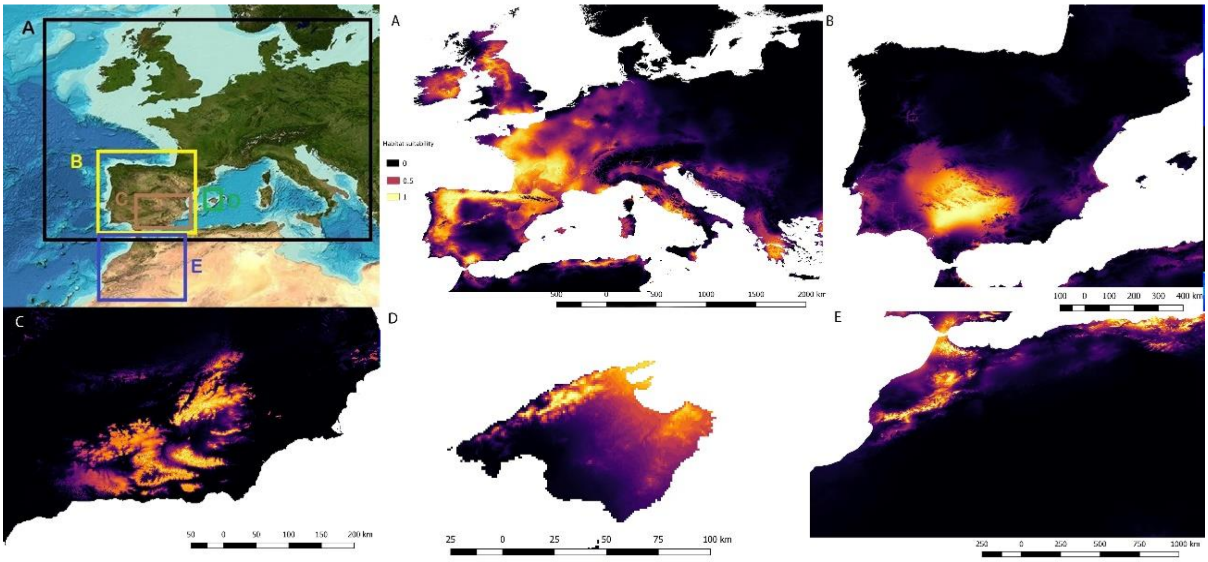


Figure 2. Potential distributions of predicted suitability by Maxent models for *Alytes obstetricans* (**A**), *Alytes cisternasii* (**B**), *Alytes dickhilleni* (**C**), *Alytes muletensis* (**D**) and *Alytes maurus* (**E**). The colour bar is the scale of habitat suitability. Maps created using MaxEnt 3.4.131 and improved with Qgis Chugiak 2.4.0 (QGIS Development Team. 2018. QGIS Geographic Information System. Open Source Geospatial Foundation Project. https ://qgis.osgeo .org).

	A. dickhilleni vs. A. cisternasii	***A. dickhilleni vs. A. obstetricans***	***A. dickhilleni vs. A. muletensis***	***A. dickhilleni vs. A. maurus***	***A. cisternasii vs. A. obstetricans***	***A. cisternasii vs. A. muletensis***	***A. cisternasii vs. A. maurus***	***A. obstetricasn vs. A. muletensis***	***A. obstetrcans vs. A. maurus***	***A. muletensis vs. A. maurus***
Schoener's *D*	0.061	0.106	0	0.013	0.052	0.001	0.018	0.003	0.283	0.014
Equivalency p-values (C/D)	1/**0.009***	1/**0.009***	1/**0.009***	1/**0.009***	1/**0.009***	1/**0.009***	1/**0.009***	1/**0.009***	1/0.217	1/**0.010***
Similarity p-values (C/D)	0.48/0.57	0.52/0.46	1/0.54	0.77/0.2	0.544/0.34	0.26/0.66	0.643/0.31	0.18/0.81	0.69/0.79	0.23/0.81

Table 1. Schoener's *D* and p-values of the five species of *Alytes* (similarity and equivalence tests p-values for niche conservatism (C) and divergence (D) hypotheses). *Significant p-values (boldface).

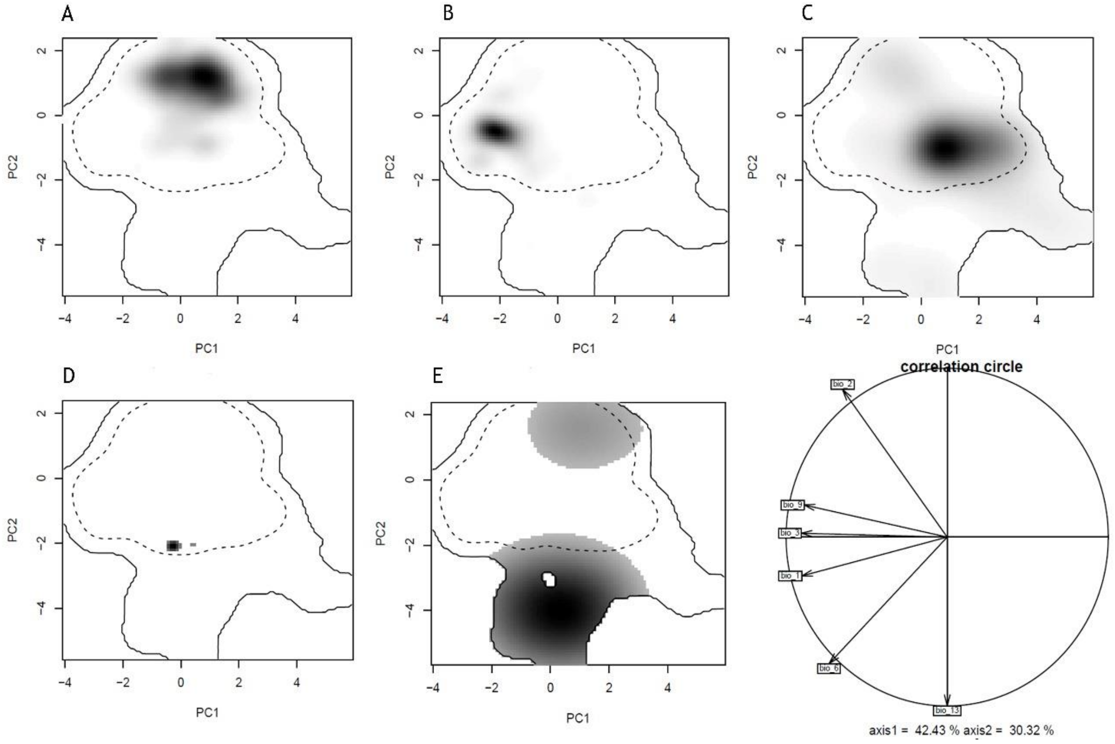
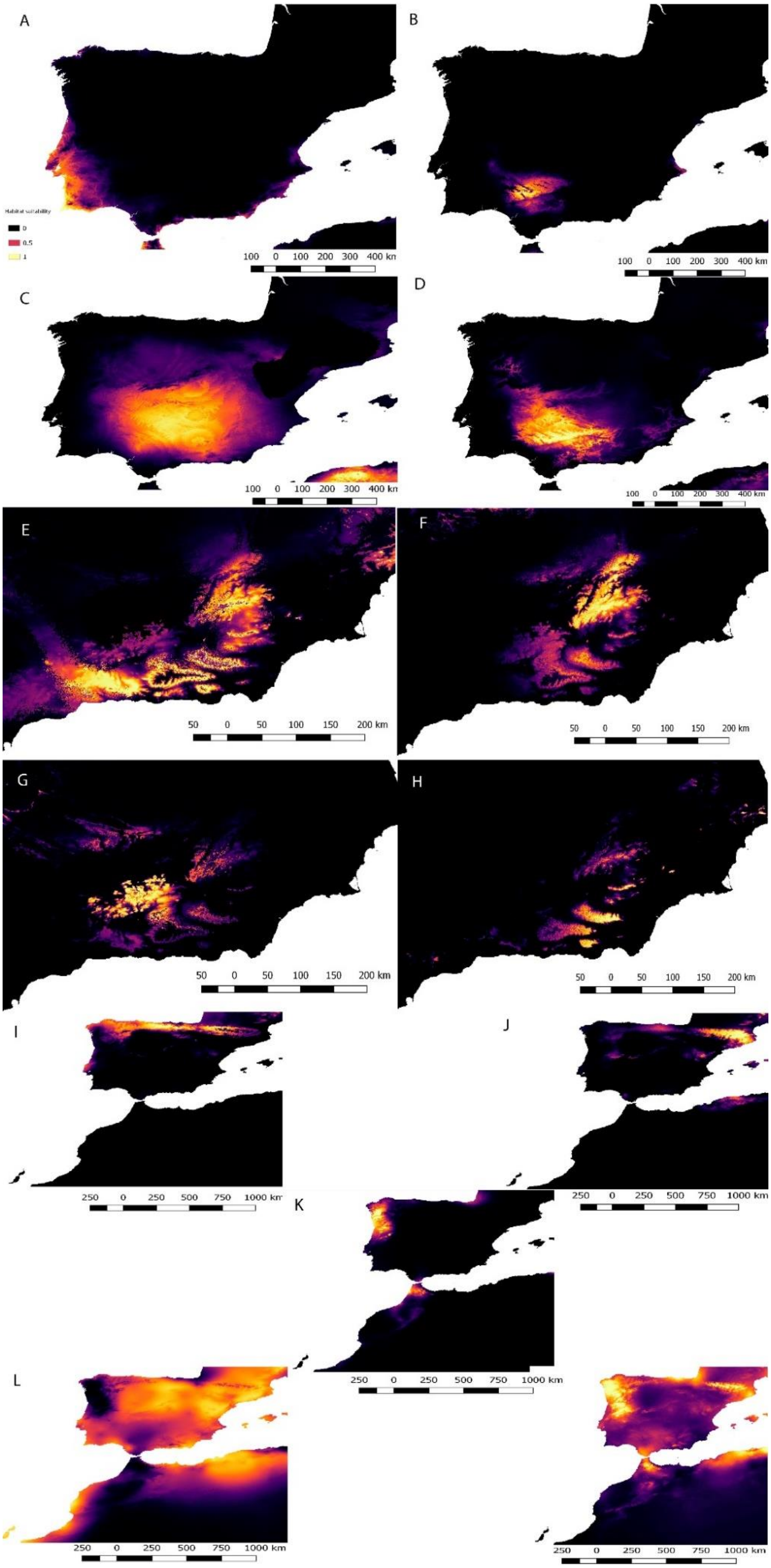


Figure 3. Niches of the five *Alytes* species in the environmental space of the European study area represented along the first principal components (PC) from Ecospat. (**a**) *A. dickhilleni*, (**b**) *A. cisternasii*, (**c**) *A. obstetricans*, (**d**) *A. muletensis* and (**e**) *A. maurus*. (**f**) The contribution of the environmental

variables to the two axes of the PC analysis and the percentage of variation explained by the two axes. Variables: annual mean temperature [Bio_1], mean diurnal range [Bio_2], isothermality [Bio_3], minimum temperature of the coldest month [Bio_6], mean temperature of the driest quarter [Bio_9], and precipitation of the wettest month [Bio_13].

Maxent outputs for intraspecific *A. cisternasii*, *A. dickhilleni* and *A. obstetricans* indicate differences in climatic suitability among lineages. The model outputs are shown in Fig. 4, and the ENM AUC, 95% I.C. AUC of fitted null models and specificity values are shown in Supplementary material 1. The variables with relatively higher contributions in each lineage of *A. cisternasii* were minimum temperature of the coldest month (66.2%) and isothermality (29.7%) for the western lineage, mean diurnal range (47.7%) and precipitation of the wettest month (24.1%) for the southern lineage, mean temperature of the driest quarter (52.3%), isothermality (24.3%) and mean diurnal range (23.4%) for the eastern lineage, and temperature of the driest quarter (45.9%) and mean diurnal range (31.5%) for the northern lineage. For *A. dickhilleni*, the most relevant variables were the mean temperature of the driest quarter (37.8%), precipitation of the wettest month (30%) and isothermality (26.3%) for the southern lineage; annual mean temperature (72.5%) for the northern lineage; precipitation of the wettest month (33.7%), annual mean temperature (29.9%) and isothermality (22.8%) for the western lineage; and annual mean temperature (50.1%) and temperature of the driest quarter (36.6%) for the eastern lineage. Finally, for *A. obstetricans*, the most relevant variables were precipitation of wettest month (90.9%) for the central-western lineage; precipitation of wettest month (36.9%) and annual mean temperature (24%) for the north-eastern lineage, precipitation of wettest month (60.5%) and annual mean temperature (26.7%) for the north-western lineage, mean diurnal range (63.9%) for the south-eastern lineage, and precipitation of wettest month for the south-western lineage.


A
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Habitat suitability
0
0.5
1
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50 0 50 100 150 200 km
250 0 250 500 750 1000 km

Figure 4. Intraspecific Maxent models of Iberian endemics. *A. cisternasii*. **A**: Western lineage, **B**: southern lineage, **C**: eastern lineage, **D**: and northern lineage; *A. dickhilleni*. **E**: Southern lineage, **F**: northern lineage, **G**: western lineage, and **H**: eastern lineage; *A. obstetricans*. **I**: North-western lineage, **J**: north-eastern lineage, **K**: central-western lineage, **L**: south-western lineage, and **M**: south-eastern lineage. The colour bar is the scale of habitat suitability. Maps created using MaxEnt 3.4.131 and improved with Qgis Chugiak 2.4.0 (QGIS Development Team. 2018. QGIS Geographic Information System. Open Source Geospatial Foundation Project. https ://qgis.osgeo .org).

The results of niche overlap (Schoener's *D*) and similarity and equivalency analyses showed low to medium values depending on the pair of lineages compared and the species (see Table 2 for *A. dickhilleni* and *A. cisternasii* and Table 3 for *A. obstetricans*). The south-western lineage of *A. dickhilleni* has lower overlap values than those of the other lineages, and in the equivalency test, we obtained significant results for the divergence hypothesis compared with the niches of the other two lineages (equivalency test, divergence hypothesis, Table 2). There were no significant divergences or convergences between the niches of *A. cisternasii* lineages (Table 2). For *A. obstetricans,* we found significant values in the equivalency test between most lineage comparisons, but we also obtained several significant results for the niche conservatism hypothesis (Table 3). The magnitude and sign of variables in the principal component plots of the niche are provided in Fig. 5. We did not obtain any significance in the age-range correlation test, both at inter- and intraspecific levels (see Supplementary material 2, *p* values of 0.60 [interspecific], 0.28 [*A. cisternasii*], 0.98 [*A. obstetricans*], and 0.64 [*A. dickhilleni*]). In Supplementary material 2 we also provide cluster dendrograms based on niche overlap for both, intraspecific and interspecific levels.

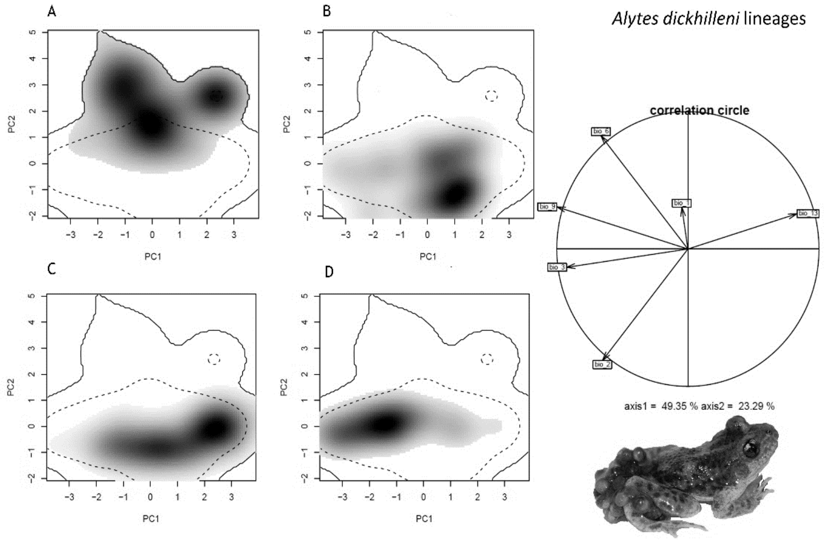
A.dickhilleni	**Southern lineage *vs.* Eastern lineage**	**Southern lineage *vs.* Nortern lineage**	**Southern Lineage *vs.* Western lineage**	**Northern Vs Eastern lineage**	**Northern lineage *vs.* Western lineage**	**Eastern lineage *vs.* Western lineage**
Schoener's *D*	0.087	0.032	0.049	0.461	0.342	0.197
Equivalency p-value (C/D)	1/**0.0099***	1/**0.0099***	1/**0.0099***	1/0.092	1/0.099	1/0.098
Similarity p-value (C/D)	0.722/0.248	0.811/0.214	0.643/0.294	0.168/0.859	0.138/0.815	0.366/0.849

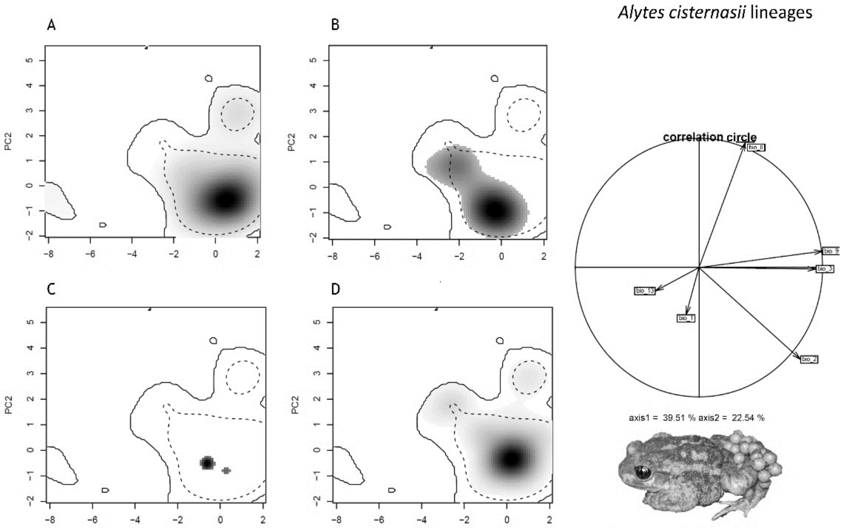
A.cisternasii	**Southern lineage *vs.* eastern lineage**	**Southern lineage *vs.* Western lineage**	**Southern lineage *vs.* Northern lineage**	**Eastern lineage *vs.* Western lineage**	**Eastern lineage *vs.* Northern lineage**	**Western lineage *vs.* Northern lineage**
Schoener's *D*	0.255	0.010	0.481	0.015	0.279	0.016
Equivalency p-value (C/D)	0.818/0.363	1/0.091	1/0.091	1/0.090	0.727/0.363	1/0.090
Similarity p-value(C/D)	0.306/0.683	0.257/0.683	0.267/0.772	0.158/0.881	0.416/0.683	0.257/0.772

Table 2. Schoener's *D* and p-values (similarity and specificity for niche conservatism (C) and niche divergence (D) hypotheses of intraspecific lineages for *A. dickhilleni* and *A. cisternasii.* *Significant p-values (boldface).

Alytes obstetricans	**Schoener's *D***	**Equivalency p-value (C/D)**	**Similarity p-value(C/D)**
North-western *vs*. North-eastern	0.051	1/**0.009***	0.665/0.366
North-western *vs*. South-eastern	0.436	1/0.138	**0.043***/0.971
North-western *vs*. South-western	0.240	0.831/0.099	0.333/0.633
North-western *vs*. Central-western	0.009	1/**0.009***	0.406/0.594
North-eastern *vs*. South-eastern	0.026	1/**0.009***	0.673/0.336
North-eastern *vs*. South-western	0.022	1/**0.009***	0.643/0.287
North-eastern *vs*. Central-western	0.000	1/**0.009***	1/0.386
South-eastern *vs*. South-western	0.165	**0.029***/**0.0398***	0.415/0.613
South-eastern *vs*. Central-western	0.038	**0.009***/**0.009***	0.564/0.673
South-western *vs*. Central-eastern	0.194	0.881/0.128	0.137/0.920

Table 3. Schoener's *D* and p-values (similarity and specificity for niche conservatism (C) and niche divergence (D) hypotheses of intraspecific lineages for *A. obstetricans*. *Significant p-values (boldface).


Alytes dickhilleni lineages


Alytes cisternasii lineages

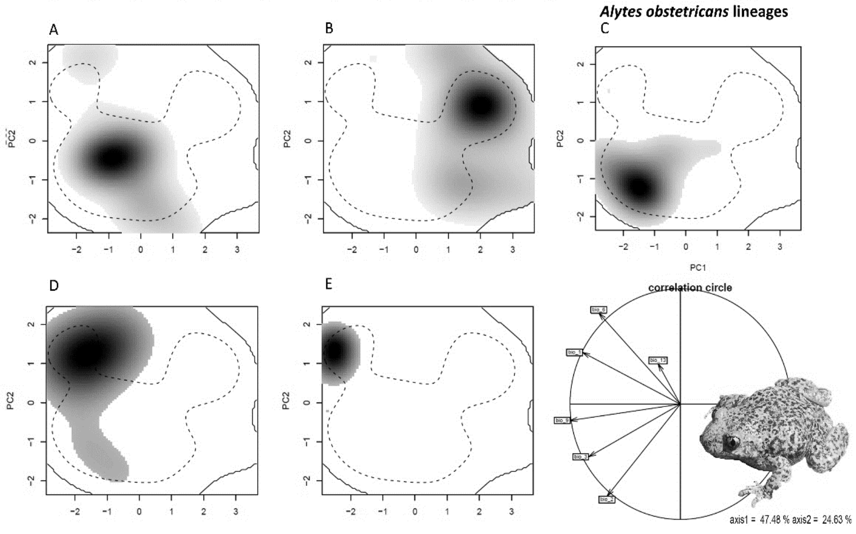

***Alytes obstetricans* lineages**

Figure 5. Niches of the phylogenetic lineages of *Alytes dickhilleni* and *Alytes cisternasii* in the environmental space of theIberian Peninsula and represented along the first principal components axis (PC) from Ecospat under present and climatic change scenarios. For *A. dickhilleni*: (**a**) southern, (**b**) northern, (**c**) eastern, and (**d**) western lineages. For *A. cisternasii*: (**a**) southern, (**b**) eastern, (**c**) western, and (**d**) northern lineages. The contributions of the climatic variables to the two axes of the PC analysis and the percentage of the variation explained by the two axes are also provided. Variables: annual mean temperature [Bio_1], mean diurnal range [Bio_2], isothermality [Bio_3], minimum temperature of the coldest month [Bio_6], mean temperature of the driest quarter [Bio_9], and precipitation of the wettest month [Bio_13]. *A. dickhilleni* and *A. cisternasii* images from the authors and *A. obstetricans* images courtesy of Rafael Carmona González.

Discussion

Our results showed a clear spatial niche segregation when we examined interspecific niche variation in *Alytes* toads. On the Iberian Peninsula, the distribution outputs of the MaxEnt models fit neatly in some places, although they exhibited a slight overlap in certain areas that coincides with the present sympatric distributional area between *A. cisternasii* and *A. obstetricans*. The pattern derived from niche similarity and equivalence tests revealed that each *Alytes* species occupies a non-identical environmental niche since no significant *p* values were found for the hypothesis of complete niche overlap (equivalence test), but instead, highly significant distinctions occurred for the divergence hypothesis (equivalence test) for any pair-species comparisons with the exception of the *A. obstetricans* versus *A. maurus* contrast. This suggests an evolutionary scenario where niches are less equivalent (identical) than expected by chance in relation to different non-exclusive processes, including local adaptation. In addition, we did not find significant results in the similarity test (conservatism hypothesis), rejecting the hypothesis of retained similarity. We also did not find significance in the case of the divergence hypothesis for the similarity test, indicating no greater than expected divergence. However, rejecting the retained similarity, determining that the divergence was not greater than expected was already relatively divergent. *A. cisternasii* has been described as the most phylogenetically distant group within the genus *Alytes*[17]. This is compatible with an evolutionary scenario where the complex formed by *A. obstetricans*, *A. maurus*, *A. muletensis*, and *A. dickhilleni* share a more recent natural history and, consequently, they could also share similar environmental/climatic requirements to a high degree. However, our results did not support this prediction, thus suggesting that the niches of these species evolved in a complex scenario, creating a wide diversity of adaptations. *A. maurus* and *A. obstericans* presented the widest climatic range (see Fig. 2). In comparison to the other species (with the exception of *A. maurus*), *A. obstetricans* inhabits colder areas with higher precipitation. *A. cisternasii* occupies warmer areas with relatively high precipitation (at least in the wettest period); *A. maurus* faces a wider range of temperatures and a high rainfall amount but with the widest variable range. It is important to remark that our models shows two separate areas in the ecological niche models (Ecospat) of *A.maurus*, this being possibly this an artefact due to the lack of knowledge about the distribution or alternatively the isolation of the two extant populations56. In turn, in comparison to *A. cisternasii* and *A. muletensis*, *A. dickhilleni* is present in colder and drier areas. Finally, *A. muletensis* occupies warm and dry areas, although the distribution of this species was much wider in the past[44]; in addition, the current distribution may be restricted to highly isolated populations due to non-climatic factors such as intense predation pressure by the introduced water snake *Natrix maura*[43]. This scenario may bias the output of our correlative model, which relies on distributional and climatic factors. A process to determine the robustness of our approach could be to implement mechanistic physiologically based models and to examine the congruence of both approaches[55].

When examining the degree of environmental niche evolution at the intraspecific level, we found contrasting patterns across and within species. The four clades of *A. cisternasii* tended to exhibit slight niche differentiation differences between the four genetically distinct clades, although correlative models showed different predicted distributions of the four lineages, with the western lineage being the most different, as it is associated with more humid conditions, than those the three other lineages that form a complex with reduced niche differentiation, in agreement with the phylogenetic tree proposed by Gonçalves et al.[13]. However, a contrasting pattern was found in *A. dickhilleni*, whose southern lineage exhibited significant non-equivalence with respect to the other three lineages, reflecting a more separated evolutionary history for this clade. Interestingly, this southern lineage differentiation in climatic requirements, characterized by a reduced diurnal range and mild winters (temperature of the coldest month) whereas the other three lineages exhibited differentiation in their climatic niches, fits well with the observed pattern of genetic divergence, by which the southern clade formed a sister group to a complex containing the other three lineages[12]. Finally, clades of the widespread *Alytes obstetricans* exhibited the highest diversity in the pattern of climatic niche evolution with high niche conservatism. The two southernmost lineages between the north-western and south-eastern lineages (in the similarity test) suggest a diminished importance of niche divergence for the intraspecific lineages described for this species. In addition, we did not find support for the existence of phylogenetic signals in the age-range correlation tests (both at interspecific and intraspecific levels). This allowed us to consider the flexibility in the niche evolution hypothesis as opposed to niche conservatism reported for other groups[36].

Climatic conditions are important factors influencing both the inter- and intraspecific evolution of *Alytes* and consequently, its ecological niche segregation. The evolutionary history of this genus seems to be the result of a combination of vicariant factors influenced by both landscape and geographic factors[17]. Our results for *Alytes* reinforce the idea that intraspecific variability can be one of the major drivers of biodiversity[46]. Our results also match the conclusions of Maia-Carvalho et al.[9] about the ongoing processes of differentiation in *A. obstetricans* but provide a more general, wider view of the generation of diversity. Thus, vicariant and geographic barriers explain the current patterns (inter- and intraspecific) of diversification; environmental and geographical factors can act synergistically to drive differentiation at multiple scales. The intraspecific differences may be explained by the most commonly accepted evolutionary alternatives: (A) niche conservatism that may be the consequences of natural selection[47]. We observed a lower intraspecific niche divergence in *A. cisternasii* than in *A. dickhilleni* and, at the same time, a higher phylogenetic influence (*p* value lower). This may possibly be induced by its specialized thermophilic physiology that may constrain uplift dispersion to mountain ranges[48] (Rodríguez-Rodríguez et al. unpublished data). B) Other selective sources affect relatively lower dispersal ability due to ecomorphological constraints. *A. cisternasii* is the shortest-limbed species in the group [25], and indirectly, this may be associated with a lower dispersal ability than that of the other *Alytes* species[49,50]

Regarding the phylogenetic analysis results, we conclude that no phylogenetic signal was detected at either interspecific or intraspecific levels. This fact is congruent with the conclusions the remaining tests that supported the rejection of retained niche similarity (conservatism), suggesting a nom parallelism of phylogenetic inertia and niche evolution.

Our results support a model of hierarchical niche differentiation in midwife toads. This model helps to understand the evolution of this primitive genus of amphibians, but most importantly, this approach has widespread application in conservation biology. First, it demonstrates the need for a modelling strategy based on targets below the species level. Second, it shows that the identification of a relatively reduced series of bioclimatic variables can enable the identification of the most sensitive taxa or lineages[52]. This emphasizes the importance of evolutionary distinctiveness[53] and the need to connect species range projections with the concept of evolutionarily significant units (ESUs[54]) to prioritize conservation efforts.

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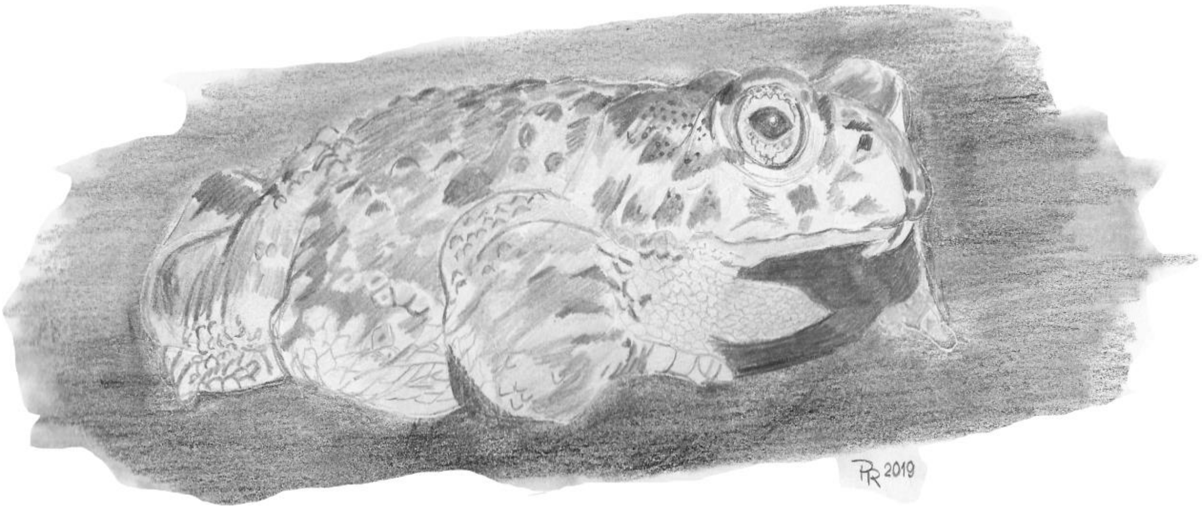

Chapter 2

Chapter 2.

Climate change challenges IUCN conservation priorities. A test with western Mediterranean amphibians.

Rodríguez-Rodríguez, E. J[1]., Beltrán, J. F[1]., El Mouden, E. H[2]., Slimani, T[2]., Márquez, R[3]., & Donaire-Barroso, D[4]. (2020). Climate change challenges IUCN conservation priorities: a test with western Mediterranean amphibians. *SN Applied Sciences*, *2*(2), 1-11.

1. Departamento de Zoología. Facultad de Biología. Universidad de Sevilla, Spain.

2. Cadi Ayyad University, Faculty of Sciences Semlalia., P.O. Box 2390, 40000 Marrakesh, Morocco

3. Fonoteca Zoológica. Dept. de Biodiversidad y Biología Evolutiva. Museo Nacional de Ciencias Naturales-CSIC. Madrid, Spain.

4. Calle Mar Egeo nº7, Cádiz. C.P.11407, Andalusia, Spain.

Abstract

One common approach to assigning conservation priorities during the current biodiversity crisis is to identify and direct efforts to high profile, vulnerable taxa, and important biodiversity areas. We addressed the first issue by assessing the comparison between conservation status and habitat suitability under differing climate change scenarios for sixteen species of five amphibian families (Pelobatidae, Bufonidae, Alytidae, Hylidae and Ranidae) distributed on both sides of the Gibraltar Strait, hoping to identify potentially threatened species under climatic change scenarios which are not considered at risk presently. We have addressed this with an environmental niche modelling (ENM) algorithm (MaxEnt) and projecting the outputs in four future climatic change scenarios. Our results demonstrate that climatic niches of some species may currently match their conservation category, with *Pelobates varaldii* having the narrowest distribution and being the more endangered species, but not all responses to predicted climatic change scenarios are related to conservation status. Some suggest notable changes in potential climatic habitats, with both substantial increase (7 species) and decrease (5 species) represented. Threatened species such as *Pelobates varaldii* could be climatically favored whereas currently more abundant species could maintain, increase, or reduce their habitat distribution. These results have implications for current conservation strategies, and suggest that this approach deserves consideration as part of any species or habitat conservation strategy in the future.

Key Words: *Alytidae*, amphibians, *Bufonidae*, climatic change, conservation, distribution, *Hylidae*, niche modeling, *Pelobatidae*, *Ranidae.*

Introduction

Human-induced climate change has already become a threat to biodiversity, and presents increasing concern regarding further degradation (Sinervo et al., 2010). A fundamental requirement for the conservation of biodiversity is the identification of appropriate priorities, thereby allowing efficient allocation of the limited resources available (Singh 2002). Climate change has already caused, among other aspects, changes in cyclic and seasonal aspects of animal and plant life histories, and distributional patterns of both endothermic and ectothermic vertebrates (Steltzer & Post, 2009). Building a rigorous framework that includes predicting impacts of climate change on biomes and included species is currently one of the main challenges of science (Schwenk et al., 2009)

Amphibians are among those vertebrates with substantial population regression or species extinction reported (Grenyer et al. 2006; Anthony et al. 2008), resulting in a worldwide phenomenon recognized as “amphibian declines”. Among causative factors are emergent diseases (Daszak et al. 1999; Pounds et al. 2006), habitat destruction, and climate change (Wake et al. 2008). Amphibians are especially vulnerable to climate warming (Duarte et al. 2012). As all species rely on the availability of permanent or temporary water for successful breeding, reduction in seasonal precipitation levels, coupled with increasing temperatures, may result in decreased availability of standing water necessary for tadpole development or suitable habitat necessary for the successful maturation of post-metamorphic larvae. Freshwater habitat is also among the most threatened ecosystems due to the synergic effect of overexploitation, contamination and desiccation related to climate change (Abell 2002). Endemic species with restricted distributions or niche conditions may be more vulnerable to anthropic change and its impact, and consequences arising from local and global extinctions (Thomas et al. 2004). Species such as the harlequin group of true toads (genus *Atelopus*) with restricted distributions in Central and South America (La Marca et al. 2005) have suffered dramatic declines or become extinct.

The Western Mediterranean Basin represents one of the planet's 25 "hot spots" for biodiversity (Myers et al. 2000), enforcing the need for identifying conservation priorities and factors representing or determining threats to endemic amphibian species. Understanding habitat suitability, based on demonstrated occupancy by the species, is considered fundamental for assessment of present and future vulnerability. However, current habitat suitability could change under a scenario of climatic and global change, with major contractions in some cases (Della Rocca et al., 2018). Reductions could be more intense independently of taxonomic group in specialist species (Hilpold et al. 2018), therefore the conservation status of specialist species is likely to be modified under future scenarios. Projections of future habitat suitability have been tested in groups as diverse as ants (Nowrouzi et al. 2019) or ticks (Estrada-Pena, 2003), and ecological niche models (ENMs), including climatic models (Melovski et al. 2018), have proved to be efficient for making decisions regarding conservation priorities (Hernández-Quiroz et al. 2018; Ramos-Dorantes et al. 2017). Such models are commonly used to assess impacts of future climatic warming, comparing distributions under current environmental variables with conditions under future projections for these same environmental variables (Pearson & Dawson, 2003). Their interpretations should, however, also consider influences from other factors possibly acting synergistically (Pearson and Dawson 2003), some examples being habitat destruction or physical barriers.

We have selected sixteen amphibian taxa (ten genera; five families [Pelobatidae, Alytidae, Hylidae, Ranidae and Bufonidae]), all of which presently occurring in the Western Mediterranean Basin. Each taxon currently holds a specific conservation designation, and each serve as an example with either a restricted or extensive current distribution range. Using this sample, we assess present and future conservation priorities by comparing climate suitability amplitude and identifying changes in climatic habitat suitability distribution, and amplitude, in predicted climatic change scenarios (We consider conservation priorities not only species in “Threatened” or “Vulnerable” categories, but also Not Threatened species with severe habitat suitability decreases). Our main hypotheses are:

A). Although current threat category may be congruent with the present conservation status of species, this category may not necessarily reflect predicted future habitat suitability changes under a climatic change scenario.

B). Species with a small distribution area are expected to be more vulnerable under a climatic change scenario due to a lower capacity for adaptation to new conditions.

C). Widespread species might suffer important climatic suitability recessions. Without adequate attention, this could derivate in an undetected or lately detected conservation problem. Identifying the limitations of these patterns in future climatic change scenarios will allow us to identify which species will be more vulnerable in the future.

Materials and Methods

Our study area in the western Mediterranean Basin includes Europe's Iberian Peninsula and North Africa's western Maghreb, currently separated by the Gibraltar Strait. The biodiversity of this area has evolved throughout a history of environmental, paleo-geographic and anthropogenic change. Although the Gibraltar Strait is currently a barrier separating Africa from Europe, during the Miocene-Pliocene a variety of terrain elements emerged favoring species dispersion (Booth-Rea et al. 2018; Krijgsman et al. 2018). Additionally, during the Neogene and Pleistocene (including the last glacial periods) barriers to dispersion appeared and disappeared (Ronquist 1997), adding complexity to composition of the biota and precipitating herpetological endemism (Pleguezuelos et al. 2008). We examined the distributions of sixteen amphibian species belonging to the five anuran families represented in the region (the Pelodytidae, present on Iberia is absent from the Maghreb) and summarize these data in Supplementary material 1. These species, represented by a cluster of widespread and narrow species potentially demonstrating similar physiological requirements, are included as a "check" for possible similarities and differences in ecological requirements.

Because numerous cases of absence data could represent false negatives (Pramanik et al. 2018), we consider only known presence data for each species. Presence data have been extracted from: a). unpublished data from the authors, b). Amphibiaweb (https://amphibiaweb.org), c). confirmed (by the authors) photographic evidence from Observation.org (https://observation.org) and the, d). Global Biodiversity Information Facility (GBIF.org [23 January 2020] GBIF Occurrence Download https://doi.org/10.15468/dl.n9vgeb). We have selected, and included in the analyses, only points separated by at least 1 km from among all available points in the distribution areas. Our using a homogeneous distribution along the Iberian Peninsula and North Africa reduces any bias introduced by considering a greater sample from Europe. Our total of 317 "presence locality" data point is inclusive of all species.

Climatic variables for current conditions, at a resolution of 30 arc-seconds (approximately 1 km), were obtained from WorldClim 1.4

(Hijmans et al., 2005). We used data from WorldClim version 1.4 to project habitat suitability in 2050 the Global Circulation Model (GCM) of CCSM4 (http://www.worldclim.org/CMIP5) based on the 5th assessment report of IPCC (Coupled Model Inter-Comparison Project Phase 5; CMIP5). We have considered four carbon emission scenarios: 1). The RCP 2.6 scenario suggests that GHC emissions will peak between 2010-2020, with emissions declining substantially thereafter, it is a globalize world with a fast economic growth with a peak of population around mid-century with a fast introduction of efficient technologies, 2). In RCP 4.5 the emissions peak occurs around 2040, a heterogeneous world with a regionally oriented development and a slower technology development. 3) In RCP 6.0 scenario emissions peak around 2080 (A world with a population peak around mid-century, but with a change toward a service and information economy and the introduction of clean and efficient economy). 4) Finally, in RCP 8.5 scenario emissions continue to rise during the 21st century (This scenario describes a world centered in local solutions to socioeconomic and environmental sustainability). This GCM was selected based on McSweeney et al. (2015) in order to reduce bias for Europe and Africa regions. WorldClim version 2.0 (Fick & Hijmans, 2017) was discarded in this study because this version does not have yet future scenarios projections (Built from the current variables data).

We used Pearson tests for correlations among variables. In the case of correlations over 0.6 one variable was discarded. Selected variables were cropped to match the study area (1.X:-14.9, Y: 26.3 : 2. X:5.9, Y: 44.6) using Qgis (QGIS Development Team 2018). Four variables were selected following tests for correlations: Annual mean temperature, mean diurnal range (average values of monthly maximum temperatures minus minimum temperatures), isothermality (mean diurnal range divided by annual temperature x 100), and mean precipitation during the wettest month. Isothermality, however, was not recommended for future projections by Bedia et al. (2013), and was not included in the analysis of future climatic change scenarios.

The finally selected variables were used to construct habitat suitability models for the present, and for projections of habitat suitability under future carbon emission scenarios, for the sixteen species using MaxEnt (version 3.4.1) software (Phillips et al. 2017). This algorithm estimates the distribution of a species by finding the probability distribution of maximum entropy. We performed 100 repetitions and selected Cloglog as the format for our output. In order to evaluate models, we chose to use area under the curve (AUC), but usefulness of only this accuracy measurement has been criticised when used in presence/background models such as MaxEnt. In addition to AUC, it is recommended to present the value of specificity (real negatives predicted as negative/ total of real negative) and sensitivity (real positives predicted as positive/ total real positives) (Lobo et al. 2008). Specificity was calculated using the Fielding & Bell (1977) method, and the threshold used to convert the MaxEent output (Continuous) in categorical was the Minimum Training Presence (MTP) given by the MaxEnt output sheet, obtaining, therefore, a maximum sensitivity (Value of 1). The true skill statistic (TSS: Sensitivity + Specificity- 1) recommended by Allouche et al. (2006) is equal to specificity in this work. In addition, we used the fractional predicted area value as an indicator of the climatic habitat suitability amplitude of each species (table 2) in the selected geographic area. We also provide the variables with a higher impact in the models (over 20% of importance).

To compare our predictions of future tendencies under differing scenarios with the currently applied conservation status, we have calculated the percentage of climatic suitability change in each species-scenario projection, obtaining a general tendency for each species. The estimation of percentage of change has been calculated using the threshold value of minimum training presence. Species with an estimated climatic suitability change of ±5%, or with opposite results among carbon emission scenarios, were considered stable. Species with Not Threatened conservation status that show a marked decrease are proposed by us as species with underestimated conservation priorities (currently Threatened or Vulnerable species are obviously considered by us as conservation priorities)

Results

Except in the cases of *P. saharicus* and *D. galganoi*, the model output for all current and future climate scenarios presented excellent AUC values (larger than 0.8; Lobo et al. 2008, Shao et al. 2009), and specificity (Table 1). Following Lobo et al., 2008, in the case of *D. galganoi,* AUC is good (0.78), being weaker for *P. saharicus* (0.66).

Table 1

Species	AUC/SD	Specificity	Fractional predicted area	Variables (% of contribution)
Pelobates cultripes	0.869/0.159	0.541	0.459	13(73.7)
Pelobates varaldii	0.989/0.025	0.905	0.095	13(45.5); 3 (29.5)
Bufo spinosus	0.810/0.106	0.498	0.502	13(94)
Bufotes boulengeri	0.892/0.246	0.577	0.423	3(77)
Epidalea calamita	0.826/0.122	0.568	0.432	13(81)
Sclerophrys mauritanica	0.909/0.123	0.592	0.408	3(73.8);13(24.1)
Barbarophryne brongesmai	0.966/0.049	0.893	0.107	3 (81)
Hyla meridionalis	0.856/0.162	0.471	0.529	13 (80.3)
Alytes cisternasii	0.912/0.087	0.721	0.279	13 (74.2)
Alytes obstetricans	0.894/0.120	0.642	0.358	13(58.1); 2 (27.4)
Alytes maurus	0.948/0.091	0.719	0.281	13(80.3)
Alytes dickhilleni	0.979/0.074	0.432	0.568	2 (44.6)
Discoglossus galganoi	0.775/0.168	0.553	0.447	2 (89.6)
Discoglossus scovazzi	0.862/0.158	0.432	0.568	3 (75); 13 (23.2)
Pelophylax perezi	0.850/0.173	0.617	0.383	13 (65.3); 2 (30.2)
Pelophylax saharicus	0.659/0.289	0.210	0.790	3 (6.2); 1 (36.8)

Table 1: AUC, specificity and variables impact for each species model. In variables column: 1: Annual Mean Temperature; 2: Mean Diurnal Range; 3: Isothermality; 13: Precipitation of Wettest Month.

In general, variables with the higher impact were the following two: precipitation during the wettest month, and isothermality (Table 1). Isothermality is correlated with several variables of WorldClim dataset which were excluded from analysis. Annual precipitation also appears highly correlated with precipitation of the wettest month (Pearson coefficient: 0.89). We have included the general tendency of each species under climatic change scenarios (based on results presented below) in order to achieve an overview for predicted tendencies for change and allowing a visual comparison with current conservation status (Table 2).

Considering these variables, the climatic niche of species currently matches their conservation category, with *Pelobates varaldii* having thenarrower distribution, and being the more endangered species. *Pelobates varaldii* is the species with the lower fractional predicted area (0.01, table 2). The second species with a low amplitude of climatic niche range is *Alytes dickhilleni* (0.11) followed by *Barbarophryne brongesmai* (0.12). The rest of the species in our dataset present higher fractional predicted area values, *Pelophylax saharicus* being the species with the higher value (Table 1).

Models under differing scenarios relating to carbon emissions demonstrate variability among species, and scenarios (Table 1, Supp. mat. 3) illustrate either increases or decreases in geographical habitat suitability (see quantitative data in Table 2). Species, including *Pelobates varaldii, P. cultripes, Alytes cisternasii, Discoglossus galganoi, D. scovazzi* and *Pelophylax perezi* show a general (In at least three or more scenarios) climatic habitat suitability expansion (Figure 1, Table 2), whereas *Sclerophrys mauritanica, Barbarophryne brongersmai, Hyla meridionalis, A. obstetricans,* and *A. maurus* show a general climatic habitat suitability decrease (Figure 2, Table 1), and there are differences among scenarios. A pattern of stability was observed in: *Bufo spinosus, Bufotes boulengeri, Epidalea calamita,* and *Pelophylax saharicus*. *Pelobates varaldii*, within the RCP4.5 scenario, had the highest level (+71%) of climatic suitability increase, and *A. obstetricans,* also within the RCP4.5 scenario, had the largest level of decrease (-43%) projected by these future scenarios. Suitability maps of all species and scenarios in Supplementary material 2.

Summarizing, these results indicate that the current IUCN conservation status for these sixteen Mediterranean area species (Table 2) is not always coherent with projected climatic habitat changes; Endangered (*P. varaldii*) or Vulnerable (*A. dickhilleni*) species are predicted to experience climatic suitability increases, while some species of lower concern (*A. obstetricans* and *S. mauritanica*) are predicted to suffer severe decreases (44% of Low Concern species increase climatic suitability, 33% lose potential habitat, and 23% maintain their distributional range).

Species	RCP2.6	RCP4.5	RCP6.0	RCP8.5	Climatic suitable habitat trend	IUCN category
Pelobates cultripes	+9%	+11%	+7%	+10%	Increase	NT
Pelobates varaldii	+44%	+71%	+11%	+11%	Increase	EN
Bufo spinosus	-4%	-7%	+2%	+2%	Stablility	Not evaluated
Bufotes boulengeri	+7%	+6%	-3%	-13%	Stability	LC
Epidalea calamita	-8%	-17%	+1%	+1%	Stability	LC
Sclerophrys mauritanica	-13%	-13%	-27%	-33%	Decrease	LC
Barbarophryne brongesmai	-26%	-24%	-31%	+3%	Decrease	NT
Hyla meridionalis	-18%	-16%	-26%	-28%	Decrease	NT
Alytes cisternasii	+24%	+10%	+5%	+49%	Increase	LC
Alytes obstetricans	-33%	-43%	-5%	-38%	Decrease	LC
Alytes maurus	-18%	-17%	-26%	-35%	Decrease	NT
Alytes dickhilleni	+40%	+49%	+19%	+18%	Increase	VU
Discoglossus galganoi	+22%	+37%	+39%	+2%	Increase	LC
Discoglossus scovazzi	-8%	+%10	+35%	+11%	Increase	LC
Pelophylax perezi	+8%	+10%	+14%	+13%	Increase	LC
Pelophylax saharicus	-5%	-4%	-4%	-3%	Stability	LC

Table 2: Climatic suitability changes (%) under different scenarios in studied species. The IUCN Red List conservation category are also listed.

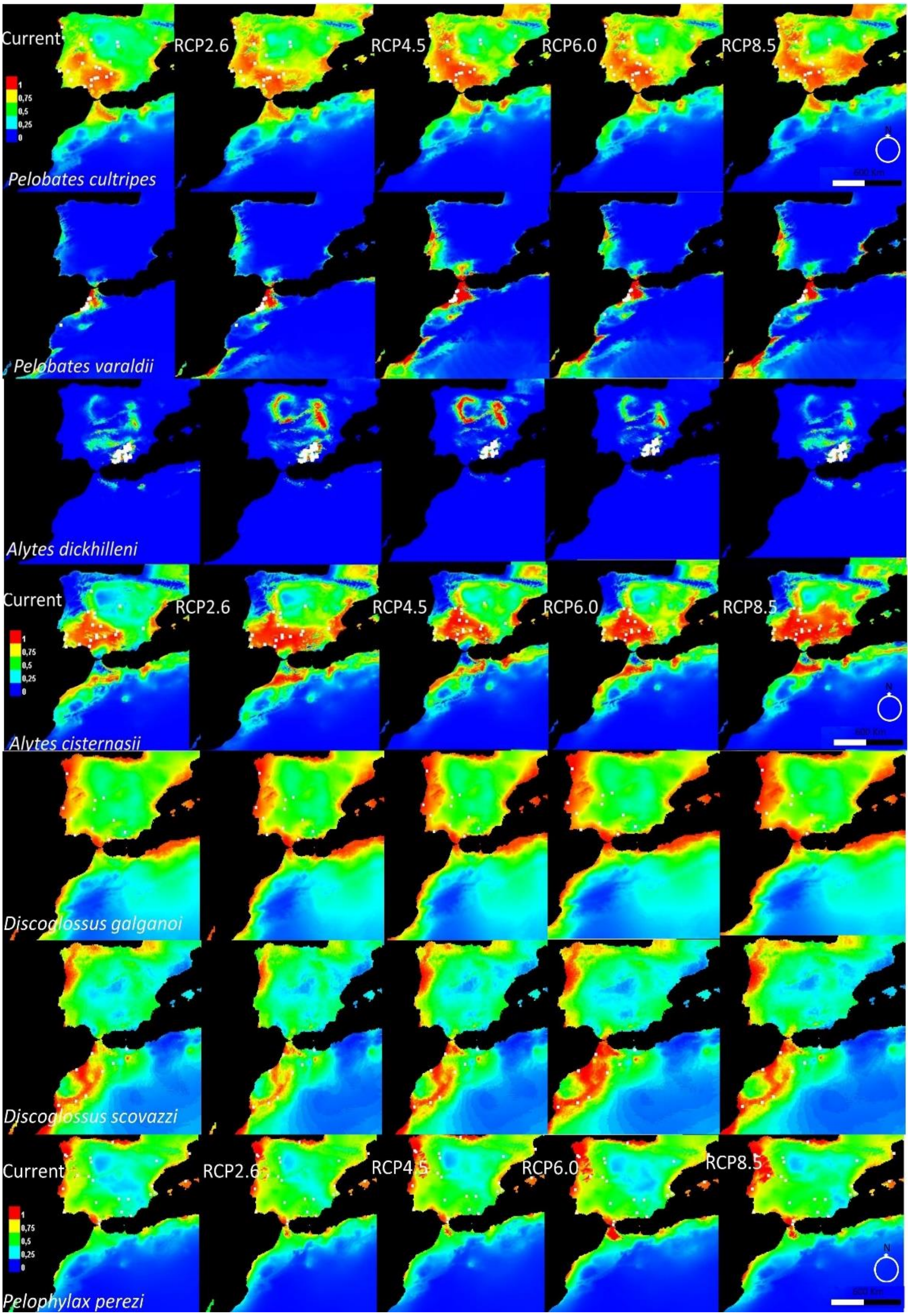

Figure 1: Estimated climatic suitability distribution for the species presenting an important increase (table 2) from current conditions to four predicted climate scenarios for 2050 (IPPC5). Distribution localities (white dots) are also represented.

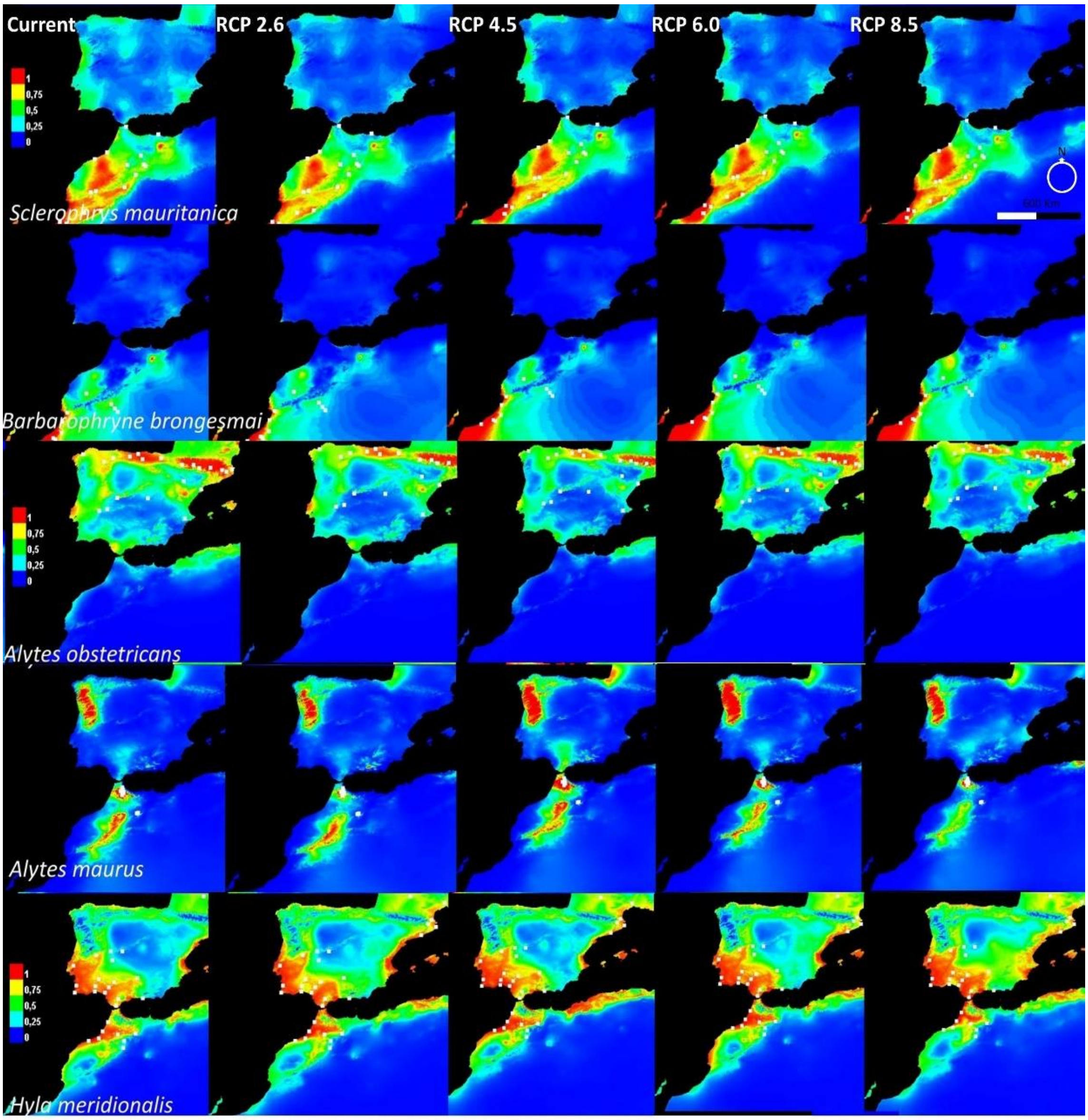

Figure 2: Estimated climatic suitability distribution for the species presenting an important decrease (table 2) from current conditions to four predicted climate scenarios for 2050 (IPPC5). Distribution localities (white dots) are also represented.

Discussion

Endemic, rare or uncommon species are more prone to extinction than more abundant and less restricted species (Hawkins et al. 2000; La Marca et al. 2005). Under a climatic change scenario, these species may be made more vulnerable due to a relatively lower capacity to adapt to new conditions and, consequently demonstrate a lower level of resilience (Hilpold et al. 2018; Solano and Feria 2007).

Our results demonstrate that, currently, *P. varaldii* is the species with the lowest level of suitable climatic habitat within the study area, and, consequently, lives within a niche with the lowest climatic amplitude. This result is concordant with its current distribution (de Pous et al. 2012). Not surprisingly, this species also has the highest IUCN threat category among the selected species (Salvador et al. 2004), and recent surveys suggest its situation may deteriorate further in some areas (Hinckley et al., 2016). The only species catalogued by the IUCN as Vulnerable is *A. dickhilleni* (Bosch et al. 2009b), and the amplitude of its current suitable habitat is, as expected, narrow (0.11 of predicted area).

Surprisingly, our results suggest that *P.varaldii* could clearly increase its potential climatic range under differing climate change scenarios. This result contrasts with the decline in suitable habitat predicted for *A. maurus* (Near Threatened), *A. obstetricans* (Low Concern), *Epidalea calamita* (Near Threatened) and *Bufo spinosus* (Not evaluated in IUCN). *Sclerophrys mauritanica* (Low Concern) presents low (but close to maintenance level) declines in predicted suitable habitat, in addition to differing scenarios of habitat suitability among some areas within its current distribution. Other species, like *Bufotes boulengeri* (Low Concern), maintain habitat suitability across projected climate changes. All Near Threatened species are projected to suffer a loss of habitat suitability, and one Vulnerable species, and one Endangered species is expected to experience an increase in climate suitability. Some species appear to compensate for severe reductions in some areas with incremental increases in others, seeming to be stable under future predictions (Suppl. Mat. 3). These results for amphibians complement those of Enriquez-Urzelai et al. (2019), who suggested climatic niche tracking in response to climate warming.

In relation to the comparative niche amplitude analysis among species, it is important to remark that the selected the study area is separated by the Gibraltar Strait, a biogeographic barrier. The models predict suitable habitat and changes in places where the species could live regarding the climatic presence, but it is not obviously possible due to this barrier.

Araujo et al. (2011) also implemented predictions for all Spanish species, but not for the species of northern Africa not present in Spanish Northern Africa territories. Our results partially agree with these authors in the predicted potential habitat in the future for some of these species (for example, the habitat suitability loss for *H. meridionalis*, is different from the rest of species mostly based in the percentage of change but not in the direction). The differences between our study and Araujo et al. (2011) are likely due to: A) Different environmental current and future predicted data (from projections based on data of the Agencia Estatal de Meteorología (AEMET) of the government of Spain in the case of Araújo et al. 2011; and from WorlClim (CCSM4 global circulation model in our study). B) Study area limits, only Iberian Peninsula in Araujo et al. (2011), and areas of both sides of the Gibraltar Strait in our study. In addition, to reinforce our results we decided to implement models with four future carbon emission scenarios from WorldClim data of the Global Circulation Model CCSM4 (see material and methods) because they are recommended to Africa and Europe (McSweeney et al, 2015). Other work assessing a similar objective is Carvalho et al. (2011). In this work, the responses under climatic change are analyzed in several herpetological species in a different study area that includes some of our tested species in Europe (*A.cisternasii*; *A. dickhilleni* and *P. cultripes*), for which are matching our results. D'Amen et al. (2011) addressed another interesting approach, obtaining the conclusion that natural protected areas will also reduce their efficiency in these scenarios, being this another example of how climatic change are not fully considered in current conservation figures. As we mention in the introduction, further research about geographical conservation priorities inside or study area are of special interest.

Climate change could drive modifications in habitat distribution for species (Pramanik et al. 2018), and in consequence for populations viability (Almpanidou et al., 2016). In numerous cases, narrow species could suffer a reduction of suitable habitat (Qin et al. 2017), and in other cases these endangered species could benefit from change, as our models results suggest. Our data show that future increases and decreases of suitable habitat are not related with current conservation status. This information is useful to take decisions about the species we predict will be more sensible in the incoming future. In a climatic change scenario, increments or reductions in habitat suitability are not related with actual widespread or narrow character of species. Taking into account differences among carbon emission scenarios, we observe how some widespread species will be reduced its habitat suitability in the region while some narrow ones will increment it.

It is important to know that our study only analyzes climatic variables. Another factor like, for example, the substrate can influence the rain infiltration, determining the presence or absence or superficial water bodies which are necessary for amphibians reproduction. Present and future habitat predictions need also consider other environmental factors, such as the impact of future occurrence of diseases (already modelled by Rohr et al. 2011), water bodies availability, habitat fragmentation, land cover, etc. An ensemble forecasting approach could also give more precise results regarding concrete geographical areas (Araújo & New, 2007), but our aim in this work was not to identify small or specific priority areas, we aimed to get an overview of general trends of species under climatic change scenarios. Another possible limitation of this work is the low number of known localities for some species. This fact could eventually induce bias if the narrow distribution is caused by non-climatic factors, although in our case there are available natural landscapes surrounding the range of these species and it is not occupied.

We are assuming that species climatic niche requirements will not change, but climatic change in other cases has been postulated to represent "the principal engine of evolutionary change" (Vrba, 1996). Our results are suggesting that all but one of the analysed species would experiment substantial changes in their distribution, thus providing a mechanism of differentiation, favoured by the short generation time of amphibian species. These results open a window to future studies about species adaptations to climate change, as an evolutionary approach.

Conclusions

One of the essential tasks in conservation biology is to identify the conservation priorities in a limited fund scenario. Environmental conditions and, thus, climate, are basic variables affecting the habitat suitability and, in consequence, the availability of niche for species. Predicted future climatic change cans strongly affect the niche of these species. Our main conclusions are that, for the amphibians of the Western Mediterranean, the current conservation status is concordant with climatic niche availability, but not necessarily in a future climatic change scenario. In addition, the responses to these predicted scenarios are different among species, and it does not depend upon the status of the species. Our prediction suggests that some endemic species, with narrow current distribution could increment their habitat suitability, and some widespread species will reduce it, being essential to take preventive conservation measures. Finally, we would like to propose species conservation priorities for these species. First we obviously confirm the status of the currently Threatened and Vulnerable species (*P. varaldii* and *A.dickhilleni*) but in addition we want to propose several underestimated conservation priorities (*based on our results: S. mauritanica B. brongesmai, H. meridionalis , A. obstetricans, A.maurus*). We consider this information a useful tool in order to plan present and future conservation actions and strategies throughout, for example, the incorporation of these predictions in the UICN conservation status.

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Chapter 3

Chapter 3

Climate stability explains geographic variation in range size in European reptiles and amphibians.

Eduardo José Rodríguez-Rodríguez[1*]; Claudia López-Lafita[1]; Juan Francisco Beltrán[1]; Pedro Abellán[1]. In preparation.

[1]Departamento de Zoología. Universidad de Sevilla, Spain.
Orcid: 0000-0002-1170-0788 (EJRR); 0000-0002-0077-575X (JFB); 0000-0001-5566-5083 (PA)

Biosketch

Authors are interested in the biogeography of vertebrates and invertebrates, and the influence of current and past climatic change, environmental and geographic variables on species distribution and genetic diversity

Abstract

Aim

Species diversity or richness in one area is a relatively common subject of study, but the historical factors determining the current range size of species are usually neglected. Climate variables are assumed to be the main determining factor of the species range. Here, we test two hypotheses: The Climatic Stability Hypothesis (CSH) and the Spatial Heterogeneity Hypothesis (SHH) to explain geographic variation in species range size. Complementarily, we analysed if climatic instability may have induced the colonization of species with wider niches.

Location

Europe was selected as our study area because present a well-documented paleogeographic history, with important changes in species range during the last glacial period.

Taxon

All the amphibians and squamate reptiles of Western Europe.

Methods

Several climatic predictors were tested using the known presence data of amphibians and reptiles in W. Europe. In addition, we modelled the range size patterns of the species and performed randomization tests.

Results

Our results suggest a higher number of small range sizes in both groups in Southern Europe, especially in the Iberian Mediterranean Peninsulas. These patterns were consistent with the climatic stability hypothesis. We have not found a clear influence of spatial heterogeneity.

Main conclusions

The observed patterns of geographical range size are related with climatic stability, being the areas with higher presence of small range size associated to climate refugia during the last glacial period. The range size shows an increment with latitude for both, amphibians and reptiles.

Running title

Range size in European herpetofauna

Key words

Range size, climatic stability, Europe, reptiles, amphibians.

Introduction

Geographic range size is one of the fundamental ecological and evolutionary characteristics of a species, displaying a great variability across species (Willis 1922; Brown et al. 1996). Yet such a diversity in range size is not evenly distributed around the globe, with exceptional numbers of species with relatively small ranges concentrated in some regions of the Earth (Jetz & Rahbek, 2002; Sandel et al., 2011), contributing to create a broad-scale patterns of species endemisms (Lamoreux et al., 2006). Understanding the drivers of geographical variation in range sizes may be key to reveal what shapes species diversity and distributions. Distinct spatial patterns may reflect a variety of contrasting ecological, evolutionary and historical factors via speciation, extinction and range transformations (Gaston 1998). They are also linked to the spatial patterns of species richness and beta diversity (Stevens 1989; Graves & Rahbek, 2005).

Two main hypotheses have been traditionally proposed to explain the geographic variation in range size at large scale: The Climatic Stability Hypothesis (CSH) and the Spatial Heterogeneity Hypothesis (SHH), respectively (e.g. Morueta-Holme et al. 2013; Zagmajster et al. 2014; Zuloaga et al., 2018), which are not mutually exclusive. Climatic stability has been related with narrow distribution ranges, whereas climatic instability has been related with large distribution ranges (Sandel et al., 2011). Short-term climate instability is generally related with the selection of wide range sizes throughout the interannual variability according to the Rapoport rule (Stevens, 1989). This biogeographical rule stablishes that at lower latitudes the range extensions of species are also reduced. The reasoning behind this mechanism is that climatic seasonality increases at high latitudes, selecting species with broad climatic tolerances, which results in species with wider climatic niches, and therefore larger geographical ranges (Addo-Bediako, Chown, & Gaston, 2000). On the other hand, long-term climate stability may imply that the rapid and frequent climatic changes during the Quaternary (Milankvitch oscilations, 10^4-10^6 years) may have selected species with high dispersal abilities, enabling that them to track rapid spatial displacement of climatic conditions, and resulting in species with broader distributions (Dynesius & Jansson, 2000). Furthermore, in climatically instable areas along this climatic cycles, range-restricted species would face greater extinction risks because of the challenges of surviving to stronger climatic variability, thereby reducing paleo-endemisms, as well as limiting speciation and therefore to produce less neo-endemism (Dynesius & Jansson, 2000).

In the other hand, as proposed by the SHH hypothesis, spatial heterogeneity in contemporary climate, topography or habitat may have an important influence in the distribution patterns of species by limiting range expansions or favouring ecological heterogeneity (Ruggiero & Hawkins et al., 2008; Schluter, 2000). Spatial heterogeneity increases habitat diversity, niche space available for niche partitioning in a given region and speciation (Chesson, 2000), and it promotes the persistence, adaptation and diversification of small-ranged species (Ohlemüller et al., 2008). For instance, greater topographic heterogeneity, along with a heterogeneous climate, allows species with diverse evolutionary strategies to coexist at small geographic scales, limiting the habitat suitability for species, and in consequence, affecting the range size (Hawkins & Diniz-Filho, 2006). Moreover, topographic complexity may promote local survival by short-distance dispersal during periods of climate change (Loarie et al., 2009). Similarly, regionally rare climates are more likely to contain many small ranged species, as they may select for species adapted to these rare conditions (Ohlemüller et al., 2008; Sandel et al., 2011). Furthermore, if species can expand their distribution more easily within than across biogeographic boundaries, then those species found in biogeographic provinces or biomes with a large spatial extent should have larger range sizes than species found in small biomes.

European reptiles and amphibians are good models to explore these hypotheses for several reasons. First, they are ectotherms, and therefore more dependent of environmental variables such as temperature and precipitation. Second, they are diverse groups, occurring in a wide variety of habitats and combining a high level of endemism with species widely distributed across the continent (Sillero et al., 2014). Third, both amphibian and reptiles present notable differences in life history (with amphibians more linked to water, and reptiles occurring in dryer areas), dispersion ability, and resilience. As a consequence, their biogeographical history in Europe present both interesting differences and similarities (Sillero et al., 2014). In recent years, numerous studies have assessed the diversity and taxonomy of these groups, with abundant changes and splits based in phylogeographic studies (e.g., Abmadzadeh et al., 2016; Pokrant et al., 2016). The most recent atlas for Europe was published in 2014 (Sillero et al., 2014), although some changes in taxonomy have been reported (more recent taxonomy is the followed by Speybroek et al., 2020); still this atlas provides a great deal of information to investigate the factors affecting the current patterns of distribution of these two groups of vertebrates. Recent studies have focused on assessing the diversity patterns across the continent for both groups (e.g., Araújo et al., 2008; Trakimas, Whittaker, & Borregaard, 2016; Ficetola et al., 2018), but they did not investigate the drivers determining their spatial patterns of range size. The proximity of Africa, and the similar climate around the Mediterranean Sea have favoured also the diversity in the south and east of the continent, with the influence of African Biota (Booth-Rea et al. 2018; Husemann et al., 2014). It is important to consider this fact (some species with wide range are distributed in narrow European niches) to avoid possible bias analysing the environmental factors that determine the extension of distribution range. Studies about environmental and geographic factors affecting these groups may be very useful considering the current conservation crisis of both groups, which are mainly threatened by habitat destruction, climatic change, invasive species, and emergent diseases (Collins, 2010; Pounds et al., 2006 Gibbons et al., 2000).

The study of the drivers of geographic variation in range size at continental to global scales has become an active field of research (e.g. Morueta-Holme et al., 2013; Zagmajster et al. 2014; Zuloaga et al., 2018). However, the relative importance of the hypotheses of climatic stability and spatial heterogeneity in shaping large scale patterns of range size remains to be explored for groups as reptiles and amphibians. Our main goal in this study was to elucidate which factors are determining the continental patterns of range size in both groups. Especifically, we aim to : i) determine the spatial patterns of range size variation in reptiles and amphibians across Europe; and ii) assess the relative role of potential drivers in determining those geographical patterns, and consequently validating or not the hypotheses of climatic stability and spatial heterogeneity.

Material and Methods

Species Data and spatial patterns

Distribution data for native European amphibians and reptiles were obtained from the New Atlas of European Amphibians and Reptiles (NA2RE; Sillero et al., 2014), which covers all the European herpetofauna mapping species distributions on an UTM 50 × 50 km grid. The study area included those grid cells covering the countries of Western Europe, excluding the Portuguese and Spanish islands in the Atlantic Ocean, which are not included in the NA2RE dataset. Coastal cells with less than 10% land were excluded, leaving a total of 2,336 grid cells (Fig. 1). In reptiles, we focused only on Squamata species (lizards and snakes), as they represent a monophyletic lineage. NA2RE data were modified in order to solve two issues: first, to adapt them to the current taxonomy (Speybroek et al., 2020), with the range limits of newly-splitted taxa taken from published references. Species affected included

Bufo spinossus, Natrix astreptophora and *Timon nevadensis* (Artzen et al., 2016; Pokrant et al. 2016; Ahmadzadeh et al., 2016). Second, to reduce possible biases induced by widely distributed African or Asian species with a narrow range in Europe. For this purpose, we excluded species with more than 60% of their range outside the study area. The excluded species were *Chamaeleon chamaeleo, Chalcides ocellatus, Malpolon insignitus, Macroprotodon cucullatis, Macrovipera xanthina and Bufotes boulengeri.* Our final dataset included 66 species of amphibians and 96 of squamate reptiles (see Tables S1 and S2; the spatial patterns of species richness for both groups are shown in Figure S1).

The range size of each species was calculated as the number of grid cells (c. 2,500 Km^2) occupied by the species within the study area. For each cell we calculated the mean range-size as the average of the range size of the species present in that cell (e.g., a cell with two species, one with a range of 40 cells and another with a range of 20 cells, had a value of 30). This variable allowed us to quantify the central tendency of the range sizes in an area. The cluster of range sizes presented in each cell is expected to be determined by evolutionary, dispersive and ecological mechanisms (Morueta-Holme et al., 2013).

In order to identify areas with a mean range size different to the expected from random, we implemented a randomization approach following Moureta-Holme et al. (2013). For every cell, we drew a sample equal to the observed number of species 999 times from the overall range-size frequency distribution and compared this expected distribution to the observed in the cell. Because, independently of the species richness, there is a higher chance of finding a broad-ranged species at any given cell (Gaston, 2003; Zuloaga et al., 2018), we weighted the probability of drawing a particular range size by its own area. This is in fact a bilateral test; thus, the null hypothesis indicates the absence of differences between the mean range sizes expected by random and the observed ones, whereas the alternative hypothesis indicates that the observed mean range size is higher or lower than expected from random. We considered these differences significant when the observed value falls within the percentiles of the 25 lowest and the 25 highest values of the null distribution (Moureta-Holme et al., 2013)

Predictor variables

In order to test the two hypotheses proposed to explain the patterns of spatial variation of mean range size, we included predictors representing climatic stability and habitat area/heterogeneity. Climatic variables were obtained from WorldClim (Hijmans et al., 2005) at a resolution of 10 arc minutes (c. 20 Km resolution). In the case of the topography, we used data from United States Geological Survey (USGS) at 30 arc second resolution (1km resolution). All data were adapted at 2500km^2 resolution used in our grids.

Present intra-annual climatic stability was represented by temperature and precipitation seasonality, whereas the climatic stability at long-term was represented by the temperature and precipitation variation since the Last Glacial Maximum (LGM), being this event one of the most important since the Quaternary (Sandel et al., 2011). This predictor is the result of the difference between the present mean annual temperature and precipitation and in the LGM. Past climatic data were calculated with the average from the Global circulation Models CCSM4 (Gent et al., 2001), MIROC-ESM (Watanabe et al., 2011) and MPI-ESM-P (Jungclaus et al., 2013) available at WordClim database.

Spatial heterogeneity was represented by topographic variability, climate rarity and biome area. For the first variable, we used the standard deviation (SD) of the elevation inside each cell, as a measure of the variability of elevation values within an area. Climate rarity was calculated as the average Euclidian distance in the climatic space between each cell and all neighbouring cells within a radius of 550 km, following Morueta-Holme et al. (2013). First, we merged the variation of the present-day 19 bioclimatic variables from WorldClim in the study area into a principal component analysis (PCA). The first two principal components, describing dominant gradients of variation in the original climate-based variables, accounted for 75% of the variation and were retained as synthetic independent variables for analysis. Then, they were used to calculate the average Euclidian distance in climatic space between each cell and all neighbouring cells within a 550-km radius (high values of climatic rarity indicate rare climatic conditions respect the surrounding area). Finally, we included the variable biome area, following Hawkins et al. (2006). This variable was calculated as the mean size of all biomes found in a cell. The biomes were extracted from biomes delineated by the World Wildlife Fund (http://www.worldwildlife.org/science/ecoregions/biomes.cfm). It is expected that if species were habitat-specific and occurred throughout their preferred biome, range sizes would be larger in cells located in larger biomes than in smaller biomes.

Statistical analyses

We investigated the relationship between the geographical patterns of mean range-size and the different predictors for both taxonomic groups using several approaches. First, we performed univariate ordinary least squares (OLS) linear regressions in order to explore the relationship of the range-size mean and each of the six single predictors (log-transformed where appropriate and standardised). Second, we ran multiple OLS linear regressions using all predictor variables (Tables SX and SX). OLS models left considerable spatial autocorrelation in the residuals (Morans' I > 0.5 in the first 10 distance classes in all models), which can potentially affect significance tests and cause biased parameter estimates (Dormann, 2007). Hence, we used simultaneous autoregressive (SAR) models (Kissling and Carl, 2007) to incorporate spatial autocorrelation using the R package *spatialreg*. The SAR models used a spatial weights matrix with neighbourhoods defined as all cells within 80 km of the focal cell, which significantly decreased the amount of residual spatial autocorrelation with respect to the OLS models (all models had Moran's I < 0.12 in the first 10 distance classes). Model fit (pseudo R^2) of the SAR models was assessed using squared Pearson correlation of predicted and observed values (Kissling and Carl, 2007).

We used a multi-model approach based on Akaike's Information Criterion (AIC) to evaluate the parameter estimates and the relative importance of predictor variables in a likelihood-based framework (Burnham and Anderson 2003). We evaluated all possible subsets of the full SAR model and used AIC values to quantify the support for each model. Then, we obtained the Akaike weight (w) of each model, which can be interpreted as the probability that a specific model is the best in the candidate set for a response variable (Burnham and Anderson 2002). These weights were used to estimate the relative importance of each predictor in explaining the response variable by summing the AIC weights of all models that included this variable in the set of most likely models (ΔAIC < 4; Burnham and Anderson, 2003). All statistical analyses were carried out in R (R Core Team, 2013).

Results

The range size frequency distributions for both groups, amphibians and reptiles showed a lognormal distribution with high number of species (approximately 65% of reptiles and 45% of amphibians) with small range sizes (less than 200 cells) followed by a low number of species with medium and wide sized ranges (Figure S3). In reptiles, the mean of range size acroos species was 183,9 grids, being the species with largerst range size *Natrix natrix* (1645 cells), *Anguis fragilis* (1477 cells), *Zootoca vivipara* (1260 cells) and *Vipera berus* (1108 cells); and the species with smallest range *Dinarolereacerta montenegrina, Eirenis modestus, Iberolacerta martinezricai, Stellagama stellio, Macrovipera schweizeri, Podarcis gaigeae, Podarcis milensis* and *Podarcis pityusensis* (with only one cell). In the case of amphibians, the mean range size across species was 987,08 cells, being the species with biggest range *Bufo bufo* (1818 cells), *Rana temporaria* (1505), *Lissotriton vulgaris* (1359) and *Triturus cristatus* (1101), and the species with the smallest range are *Calotriton arnoldi, Hydromantes ambrosii* and *Hydromantes sarrabusensis* (with only one cell).

We found a clear pattern of smaller range sizes at lower latitudes with the highest frequency of small range sizes in southern Europe. The relation between latitude and mean-range size presented slight differences between amphibians and reptiles. Amphibians appear to have two patterns: a clear tendency to increase until latitude 45, followed by a plateau until latitude 60 where the tendency to increase starts again. Reptiles, however, only presented an increase in range sizes until latitude 50, and then appeared to stabilize (Figure S4). The randomization test showed strong geographical patterns in the distribution of areas departing from random expectation in mean range-size for both reptiles and amphibians (Figures 1 and 2, respectively). For both groups, areas with mean range-sizes significantly lower than expected were concentrated in the southern peninsulas (Iberian, Balkan and Italic) and Mediterranean islands (Corsica, Sardinia, Sicily, Crete and Greek Islands), while areas with significantly larger range sizes were located in northern Europe (with the exception of the northernmost part of Scandinavia, possibly related to the lack of statistical power, as only very few species occur there; see Figures S1 and S2).

Results of SAR models are summarized in Tables 1 and 2. Temperature change since the LGM was the more important predictor of mean range-size for both taxonomic groups in univariate models. This variable alone accounted for 73 and 69% of the variability in the geographical patterns of range size of reptiles and amphibians, respectively, with larger ranges in more climatically unstable areas. For reptiles, summed Akaike weights across SAR models were highest for temperature stability since the LGM, and temperature and precipitation seasonality. Temperature stability since the LGM had also the highest summed Akaike weight in models for amphibians (the best SAR model according to AIC was that with just this variable). Spatial heterogeneity variables (climatic rarity, topographic variability and biome area) showed low coefficients in both univariate and multivariate models, as well as low to moderate importance across SAR models.

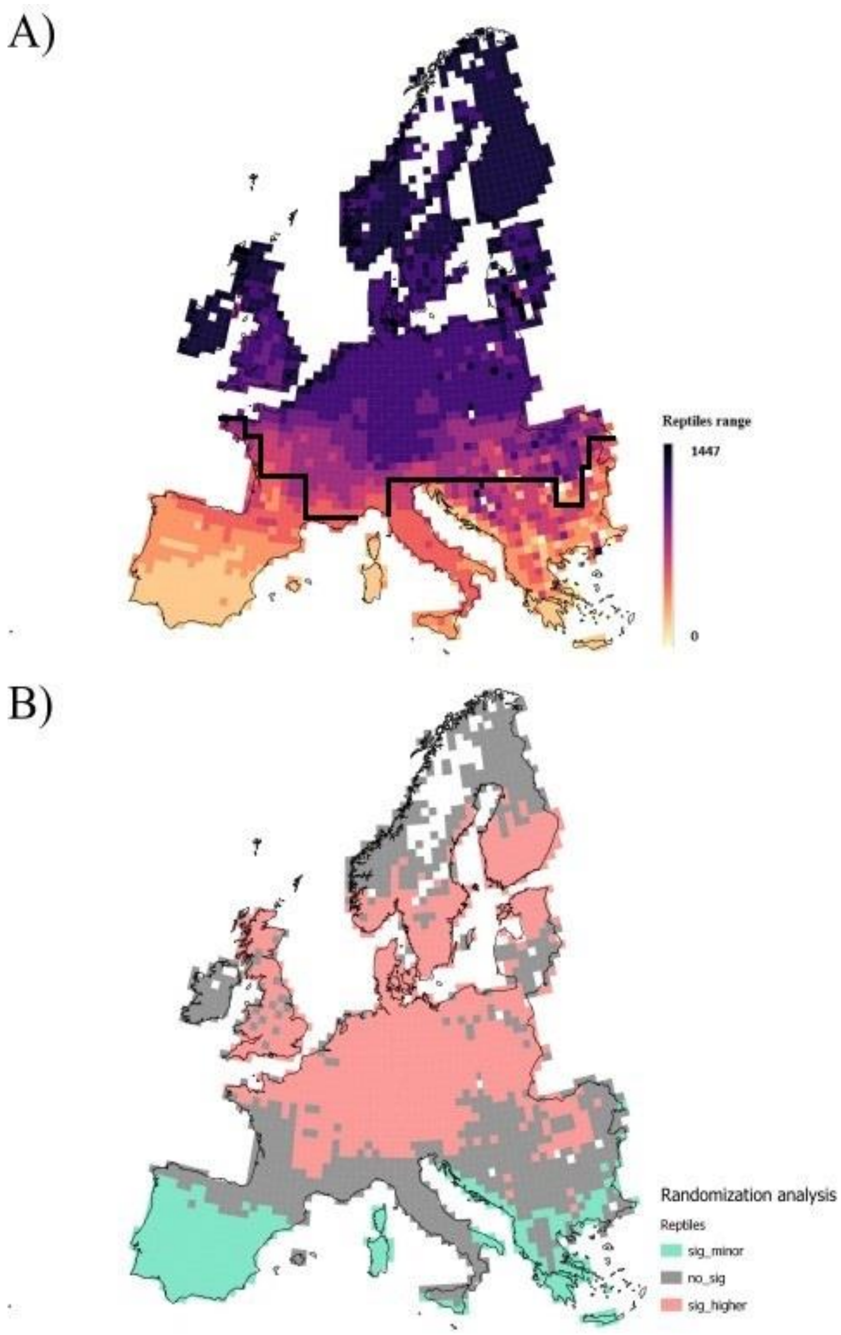

Figure 1. Maps for (a) range-size mean of European squamate reptiles (number of grid cells); and (b) deviations from random expectation. Cells with a value significantly greater or lower than expected given observed species richness are coloured red or green, respectively, while those with a value non-significantly different than expected are coloured grey. Black line marking the 0 ºC of annual average temperature in the las glacial period also provided.

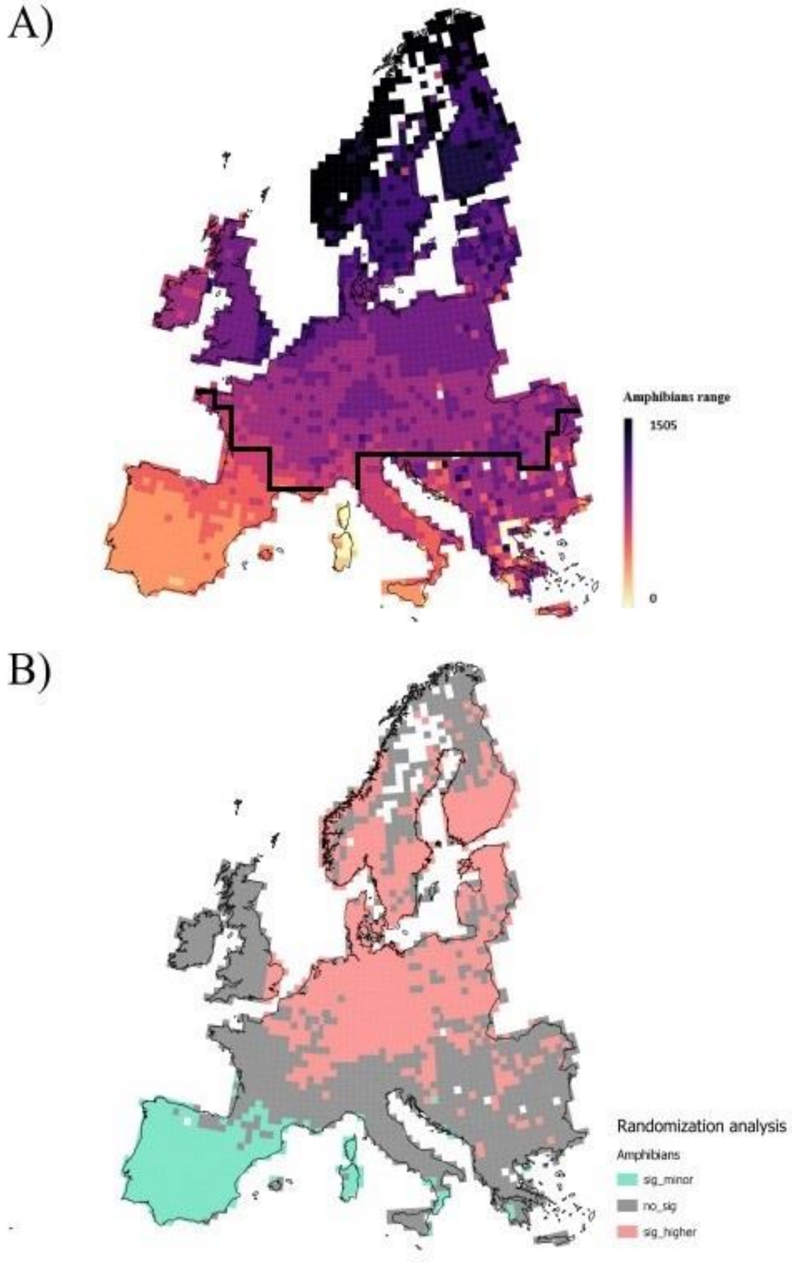

Figure 2. Maps for (a) range-size mean of European amphibians (number of grid cells); and (b) deviations from random expectation. Cells with a value significantly greater or lower than expected given observed species richness are coloured red or green, respectively, while those with a value non-significantly different than expected are coloured grey. Black line marking the 0 ºC of annual average temperature in the las glacial period also provided.

Table 1. Results of simultaneously autoregressive (SAR) models relating mean range-size of European squamate reptiles to different potential predictors.

	Univariate models		Best model		
Variables	**Coefficients**	**R^2**	**Coefficients**	**R^2**	**Σw_{AIC}**
Climatic stability					
Temperature seasonality	-0.033	0.033	-0.154***	0.796	1.00
Precipitation seasonality	-0.159***	0.157	-0.097***		1.00
Temperature difference LGM	0.683***	0.737	0.741***		1.00
Precipitation difference LGM	0.009	0.065			0.37
Spatial heterogeneity					
Topographic variability	0.006	0.183			0.40
Climatic rarity	-0.046**	0.038	-0.029		0.60
Biome area	0.016	0.096			0.51

Standardized regression coefficients and goodness-of-model fit (R^2) are showed for univariate models and for the best multivariate model based on Akaike's Information Criterion. Relative importance of variables (Σw_{AIC}) based on a multi-model information-theory based approach is shown. Significance levels: ** $P \leq 0.01$; *** $P \leq 0.001$.

Table 2. Results of simultaneously autoregressive (SAR) models relating mean range-size of European amphibians to different potential predictors.

	Univariate models		Best model		
Variables	**Coefficients**	**R^2**	**Coefficients**	**R^2**	**Σw_{AIC}**
Climatic stability					
Temperature seasonality	0.131**	0.132			0.32
Precipitation seasonality	-0.030	0.087			0.44
Temperature difference LGM	0.714***	0.692	0.714***	0.692	1.00
Precipitation difference LGM	0.016	0.091			0.38
Spatial heterogeneity					
Topographic variability	-0.002	0.069			0.17
Climatic rarity	0.003	0.003			0.37
Biome area	0.003	0.001			0.25

Standardized regression coefficients and goodness-of-model fit (R^2) are showed for univariate models and for the best multivariate model based on Akaike's Information Criterion. Relative importance of variables (Σw_{AIC}) based on a multi-model information-theory based approach is shown. Significance levels: ** $P \leq 0.01$; *** $P \leq 0.001$.

Discussion

The present study provides an insight into the factors determining the continental patterns in the range size of European reptiles and amphibians. While both the climatic stability and the spatial heterogeneity hypotheses have been proposed to explain the spatial patterns in the geographic range sizes of different taxa, our results show that the spatial distribution of range size for European amphibians and reptiles can be mostly explained by long-term climate stability, with some contribution of present-day climate seasonality, and very low relative importance of factors related to the spatial heterogeneity hypothesis.

Consistent with previous findings for other taxonomic groups (e.g. Aritaet et al., 2005), the range size frequency distribution for the European herpetofauna is strongly right-skewed, with most of species with a relatively small range and only a few with a relatively wide range. Furthermore, the results of our study are congruent with the Rapoport's rule (Stevens, 1989), with an increment of mean range size with latitude for both groups. Although the universality of the Rapoport's rule remains controversial, support for this rule has been found in multiple studies especially across Northern latitudes (e.g., for mammals, Aritaet et al., 2005; damselflies, Swaegers et al., 2014; Canadian freshwater fish, Blanchet et al., 2013; or plants, Morueta-Holme et al., 2013), including amphibians (Whitton et al., 2012) and reptiles (Böhm et al. 2017). As a mechanistic explanation of this pattern, it is often advocated that at higher latitudes seasonal variation in climatic conditions selects for greater tolerance in species to environmental variables, which enables a larger range size (climatic stability/variability hypothesis). Conversely, selection for broader climatic tolerances is weaker in species that inhabit regions with little seasonality (Gaston & Chown, 1999). Our results support that climate stability factors are driving the spatial distribution of range sizes in European amphibians and squamate reptiles, but suggest that these patterns might primarily be driven by climatic variability acting at much longer time-scales than the seasonal scale (Jansson & Dynesius, 2002), as they were mainly explained by the change in temperature since the LGM. Our results are in agreement with previous studies focused in other taxa such as Palaearctic groundwater crustaceans (Zagmajster et al. 2014) and New World plants (Morueta-Holme et al. 2013), for which historical climate stability was found as one of the main drivers of mean range size.

World climate in the Pleistocene was marked by glaciation cycles (Forsström & Punkari, 1997). The last glacial period started 110.000 b.c and ended 10.000 b.c, reaching the ices a great area of the globe, except for areas of latitudes prone to the Ecuador. Europe was not free of these cycles, and during this glaciation ices reached great part of the continent (Aspöck, 2008), leaving as refugia (over 0ºC annual mean temperature) the Mediterranean peninsulas (Iberian, Italic and Balkan), with a more stable climate (Sommer & Nadachowski, 2006; Weis and Ferrand, 2007). Phylogeographical and palaeoecological data have shown that these Mediterranean refugia acted as survival pockets for many temperate animal and plant taxa from which northern areas were later re-colonized (Taberlet et al., 1998; Hewitt, 2004), including multiple examples among Western Palaearctic amphibians and reptiles (see e.g. Martínez-Freiría et al. 2020 with the *Vipera* sp. genus in the Iberian Peninsula; Canestrelli et al., 2012 with *Lissotriton italicus* in the Italic Peninsula and Crnobrnja-Isailovic 2007 in the Balkan Peninsula). The severity of climate in northern Europe would have produced a disproportionate extinction of small-range taxa due to narrow climate tolerance, and a stronger selection for generalism and vagility, so that only those taxa with high dispersal capability and broad environmental tolerance (and consequently larger ranges) would have been able to colonize vacant habitats following climatic recovery from the LGM (Dynesius & Jansson 2000; Sandel et al. 2011).

Climatic stability and the presence of refugia during Quaternary climatic oscillations have been identified as major determinants of present-day endemism at the global scale for a number of taxa (Jansson 2003; Sandel et al. 2011). In Europe, this hypothesis has been also advocated as one of the main factors determining high rates of endemism in the most southern areas for amphibians and reptiles (e.g. Trakimas et al., 2016; Ficetola et al., 2018). Supporting this view, we found that mean range sizes for both groups were higher than the random expectation in northern Europe, while areas where mean range-size assemblages had higher mean than expected coincided with areas of highest climate stability (estimated as the anomaly between LGM and current temperature), such as the Mediterranean peninsulas. Local examples of small range species for these peninsulas are *Alytes dickhilleni*, *Pelodytes ibericus, Timon nevadensis* or *Algyroides marchi* in the Iberian Peninsula, *Salamandrina perspicillata*, *Salamandrina terdigitata* or *Speleomantes* sp. in the Italic Peninsula, and *Algyroides nigropunctatus* or *Podarcis peloponensis* in Balkan Peninsula (additionally, the Mediterranean islands harbour also some species with very small ranges such as *Salamandra Corsica* in Corsica and *Euproctus platycephalus* in Sardinia, although in this case can be determined by the own dispersion limitation). In agreement with our results, Araujo et al. (2008) found that species richness among narrow ranging species of European amphibians and reptiles was markedly constrained by the mean annual freezing conditions in the LGM, whereas widespread species were more constrained by current mean annual freezing conditions. These findings were interpreted as due to limited colonization ability of narrow ranging species that has precluded their occupation of new areas during interglacial periods, while wide-ranging species would have been more successful post-glacial colonizers. In this context, Trakimas et al. (2016) found that amphibians that have successfully recolonized north Europa following deglaciation (and displaying the largest ranges) generally possess traits that indicate the potential for rapid range expansion.

Furthermore, the geographical patterns of range sizes are strongly related with the patterns of species richness for both groups (see Araujo et al., 2008).

We found low support for the role of predictors representing the habitat area/heterogeneity hypothesis. The topographic variability reflects the strength of the climatic gradients and the habitat limitation, restricting the ranges sizes (Ruggiero & Hawkins, 2008). The European continent has a great topographic variability, mainly in central and southern Europe. This is due to the presence of high mountain ranges (such as the Alps and the Pyrenees). These mountain areas interspersed between low areas, not allowing a uniform distribution of herpetofauna (Speybroeck et al., 2017). It could be expected that smaller mid-range sizes will be located in those areas where the standard deviation of the elevation is greater (with greater topographic heterogeneity). But the results do not match what was expected. This may be due to study group is not as dependent on their geography as other groups, at least in Europe.

The climatic rarity in the European continent is generally low and uniform, with the exception of the Iberian Peninsula and northern regions. A greater climatic rarity is observed in those areas where a greater topographic variation is found. In areas with unusual climates, species with smaller ranges would be selected. This is because unusual climates are those that are different from the dominant climatic types of the region and are isolated from similar climates to other places and, therefore, restrict the distribution of species (Ohlemüller et al., 2008). Under this expectation, smallest ranges should be found on the shores of Norway, but this is not the case. The low importance of this predictor may be due to the radio value (550 km) that was used in the computation of climatic rarity, and the low dependence that these groups may have on the geographical heterogeneity of the continent. Overall, the comparatively low explanatory power of habitat area/heterogeneity predictors might indicate that the small range size of many southern species (especially in the case of amphibians) is primarily determined by their poor intrinsic dispersal capacities rather than by the availability and heterogeneity of habitats (see also Zagmajster et al. 2014 for the case of groundwater crustaceans).

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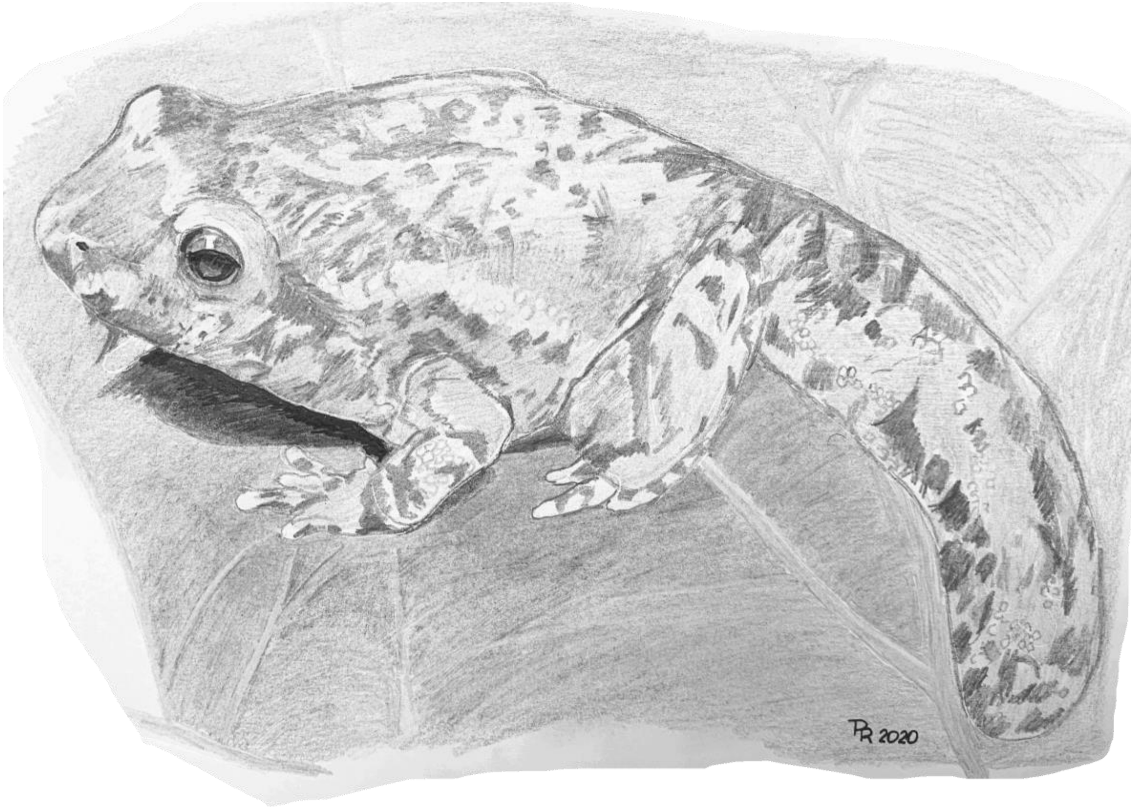

Chapter 4

Chapter 4

Thermal and moisture conditions influencing physiological constraints across different ontogenic stages in midwife toads (*Alytes* sp.)

Rodríguez-Rodríguez, Eduardo José[1]; Beltrán, Juan Francisco[1] ; Márquez, Rafael[2] ; Tejedo, Miguel[3]. In preparation.

[1]Departamento de Zoología, Facultad de Biología. Universidad de Sevilla. Sevilla, Spain.

[2] Departamento de Biogeografía y Cambio Global. Museo Nacional de Ciencias Naturales, CSIC, Madrid. Spain

[3]Estación Biológica de Doñana. CSIC. Sevilla, Spain

Summary

Environmental variables are fundamental in niche selection of the species. In ectotherms, thermal conditions and water availability are main selective factors determining variation of physiological responses that allow species survival. In this work, we aimed to determine how environmental conditions determine different physiological constraints in midwife toads (genus *Alytes)*, at both life stages, aquatic and terrestrial, and therefore with presumable different ecological determinants. At larval stage, temperature is the most important limiting factor for body growth. Tadpoles of *A. cisternasii* showed more thermophilic (2 ºC more for optimal growth respect tested *A. dickhilleni*) in their thermal optimal growth. After leaving the water, the metamorphics of the five species were raised under two experimental moisture scenarios, to determine their growth as well as their locomotor performance and body- shape responses. Our results also show a phylogenetic signal in this response, with the most external species, *Alytes cisternasii* growing at similar rate at both treatments, and thus, showing better adapted to drier conditions than the other species of this genus, which showed a slow growth rate under dry conditions. We also found a strong response in body shape related with the growth rate; nevertheless, no significant differences were observed in the locomotor

performances. We suggest that differences in environmental limiting factors have both phenotypic and fitness consequences which may contribute to define the environmental niche of each species.

Introduction

Environment is a main cause of variation and life history traits of organism (Gebhardt and Stearns, 1993). Interactions of organism with this environment can lead to phenotypic plasticity, with adaptive or not adaptive responses (Gotthard and Nylin, 1995). Reaction norms under environmental conditions can reflect genetic variability and may be subject to selection (Stearns 1989), therefore, these reaction norms can change among different species inside a taxonomic group.

It is known the existence of ontogenic divergence in physiological traits (Kingsolver et al., 2011). Environmental factors also affect differently across the ontogenic history amphibians: with two main stages larvae and adults. These ontogenic stages are living in contrasting environments: The larvae stage is aquatic, thus the main factor is temperature, although this can affect the hydroperiod. In adults, and especially in metamorphic and juveniles, more vulnerable than strict adults, the humidity is also a key factor due to the terrestrial phase, affected at the same time by temperature. Thus, it is interesting to determine the implications from the view of exposure to thermal stress, and organismal sensitivity to these factors at both ontogenic phases. This information is critical, as reliable mechanistic models of species distribution under climatic change scenarios will require precise information of physiological limits and performance constraints stages of organism (Levy, 2017)

There are two main hypotheses explaining the niche evolution of species: Niche conservatism hypothesis (NCH), as the maintenance of ancestral requirements among species with a common ancestor (Wellenreuther et al., 2012), and niche divergence hypothesis (NDH), as the appearance of divergences among these species (Ackerly et al., 2006). Regarding the phylogeny of our study group (Martínez-Solano et al., 2004) and environmental niche modelling studies (Rodríguez-Rodríguez et al., 2020b), we can observe that responses to environmental variables and thus, niche evolution, vary according to the phylogeny of the group, with a sister species of the rest, *A. cisternasii* (Martínez-Solano et al., 2004). Physiological traits of the genus at different ontogenic stages is a solid complement to all this information.

Phenotypic plasticity has been studied in several Mediterranean amphibian species at both ontogenic stages under different environmental conditions (Gómez-Mestre& Tejedo, 2003; Vences et al., 2002; Sinsch et al., 2010; Tejedo & Reques, 1994; Richter-Boix et al., 2011; Richter-Boix et al. 2007). Plasticity response to physiological trait across ontogenic stages is, therefore, an interesting study objective in order to better understand the perspectives in scenarios of climate change.

The *Alytes* genus is a western Palaearctic genus with five species. The distribution of these species is determined by several factors including vicariant events like the Gibraltar Strait dynamics and environmental segregation (Martínez-Solano et al., 2004). These evolutive scenario make this genus a good model to test our main questions:

Our objective is to determine the influence of humidity conditions on the growth, body shape and performance of individuals of midwife toads, evaluating the responses of the different species at metamorphic stage to this factor. In the case of tadpoles (aquatic stage) we aim to evaluate influence of thermal conditions and thermal preferences. Our main hypothesis is that under dry conditions the growth rate could be slowed down, obtaining similar results to body shape responses. We also want to evaluate if there is a phylogenetic signal in this response, with the hypothesis of more similar responses among more closed related species.

1. Are the environmental factors limiting the distribution of species different across the ontogeny of the amphibian genus *Alytes* sp.?

2. Detect the limits or range tolerance of the exposure to thermal and moisture stresses at aquatic and terrestrial stages

3. Provide valuable information for conservation throughout detection organism vulnerability to environmental factors, specially considering the current scenario of climate change.

Material and methods

Study area and species

We selected the genus *Alytes* sp. as research model. This genus is distributed across Occidental Europe and North Africa, with five recognized current species. *Alytes obsteticans* is distributed from Central Europe to the north of the Iberian Peninsula, being present in areas with high pluviometry or in montane areas in the case of the southern populations (Bosch, 2014). Recently, one population of this species has been proposed as a new species, *Alytes almogavarii* (Dufresnes et al., 2019). *Alytes cisternasii* (Márquez, 2017) is a clearly Mediterranean species distributed in the south-west of the Iberian Peninsula. *Alytes dickhilleni* is an endemism of the Betic Mountain range in the south-east of the Iberian Peninsula, occupying mostly montane areas (Salvador, 2015). *Alytes muletensis* is an endemism of the Mallorca Island (Olivier, 2014), and finally *Alytes maurus* is present in some areas of the Rif and the Middle Atlas in Morocco (Donaire et al., 2009). Phylogenetically, *A. cisternasii* is the brother species of the rest of the genus with a divergence time of 16 Ma (Martínez-Solano et al., 2004).

Tadpoles of five species were collected (we have not samples of the recently described *A. almogavarii*): 42 for *A. cisternasii,* 23 for *A. obstetricians,* 29 for *A. maurus,* 56 for *A.dickhilleni,* and 38 for *A.muletensis* (from a captivity program of The Jersey Wildlife Preservation Trust).

Experimental design

Analyses of moisture responses

All individuals were collected as larvae. Once they completed the metamorphosis, individuals were transferred to 1L plastic boxes with 3 cm of vermiculite at in a room with controlled temperature (24^{o} C). This inert substratum was used to simulate the terrestrial environment under different humidity (or dryness) conditions. The absorption capacity and the water potential of vermiculite is well known (Packard et al., 1987). Two levels of humidity factors were established: a humid level with a water potential around 150kPa and a dry level with a potential of 1150 kPa. These two levels have been previously used in natterjack (*Bufo calamita*) metamorphics (Gómez-Mestre & Tejedo, 2004). The experimental plastic containers for the metamorphics were prepared with 30g of vermiculite and 33.8 or 3.9 g of water, for the wet and dry scenarios respectively.

The replicas were distributed in random clusters per species in shelves of the laboratory. All the plastic containers were maintained tightly closed to prevent fluctuations of the humidity level in the vermiculite. To maintain the water potential level of the substratum, the vermiculite substratum was renewed two times per week. This schedule maintained clean the animals' environments and keep the experimental conditions (humidity levels) as constant as possible.

The water-to-land transition experimented by the metamorphosed juveniles in the field is a gradual phenomenon, remaining several days during the tail reabsorption in a strip nearby the aquatic environment. For this reason, to prevent a possible osmotic shock of metamorphic individuals destined to the dry treatment, these individuals were maintained three days at an intermediate water potential of 550 kPa (8.4 g of water/ 30 g vermiculite) before transferred to the definitive dry treatment of 1150 kPa. We considered as start date for experiments one week after the reabsorption of the tail, coinciding with the transfer of individuals from intermediate treatment to the final dry treatment. All individuals were fed *ad libitum* with young (8-10 mm) crickets (*Acheta domesticus*) enriched with vitamins.

Individual body weight was measured at 0.01g with a Sartorius (Entris 64-1S) precision digital analytic balance each 7 days, until complete 10 measures series. Additionally, we implemented jumping performance analysis for individuals of both treatments. To obtain these data we designed a 1 m lane with millimeter paper, and we forced the animals to jump using a stick. We repeated this at the beginning and the final of the growth experiment, obtaining the jumping performance difference under both treatments, relativizing this measure with the weight change, in order to avoid the bias induced by the change of this variable. Finally, we took measures of SVL (Snout-vent length) and HL (hindlimb length) ant both, beginning and the final of the experiment obtaining the difference and relativizing it with weight change again. For this last purpose animals were photographed over millimeter paper, and measures calculated using ImageJ2 software (*Rueden et al., 2017).*

Statistical analysis was performed using Statistica software 8.0 (Weiß, 2007). In order to analyze the affection of each treatment we used a generalized mixed effect model, being the growth the effect and treatment and species the factors. Growth at both treatments was also plotted along the 10 weeks of study using the same software. Same GLM procedure was also implemented in order to analyze the change in the jumping performance, snout-vent length (SVL) and hindlimb length (HL) under both treatments.

Analyses of temperature responses

We estimated thermal sensitivity of tadpoles using thermal performance curves (TPCs). The Thermal Performance Curve (TPC) is a continuous reaction norm in which an organism's performance (i.e., growth, development, locomotor ability) is described as a function of temperature (Huey & Stevenson 1979). TPCs are typically concave and asymmetric curves described by several parameters such as thermal optima (Topt), the temperature at which performance is maximal (Zmax). The thermal performance breadth (Bi), the temperature range over which performance is above some arbitrary level (i.e. 80% of maximal fitness, B80 in our example); and the points at which performance is zero, the critical thermal limits, CTmin and CTmax, which define the tolerance breadth, that once surpassed entail organismal death (Angilletta et al. 2002). We estimated TPCs for two fitness related traits: larval growth and máximum burst locomotion. Tadpole growing performance is a good proxy for fitness, since growth is an integrative process resulting from the interaction among other temperature dependent physiological parameters (Freitas *et al.*, 2010). Locomotor performance has been employed as a good proxy to estimate optimal temperatures in ectotherms since it may correlate with fitness (Jayne and Bennett 1990, Le Galliard et al. 2004, Husak 2006). Additionally, growth and performance thermal sensitivities are

TPCs for larval growth rates were obtained for *A. cisternasii*, *A. obstetricans* and *A. dickhilleni*, by growing a set of 10-15 tadpoles through a range of constant temperatures (9°C, 15ºC, 20ºC, 23.5ºC, 27ºC, 29ºC, 31°C and 33°C) and a photoperiod of 12L:12D, being individually maintained for 10 days in plastic cups with 400 ml of dechlorinated water, aerated with an air pump system and fed *ad libitum*. Water and food were completely renewed four times during the experiment (each 2-3 days) and individuals were checked daily for survival. To keep constant thermal regimes during the experiment, we used portable fluid heaters with regulation adjustment (U201431698). To obtain colder temperature treatments (9 ºC and 15 ºC), we used TECO TK 1000 chillers. During the experiments consigned temperature treatments oscillated in a range of 0.2-0.4 ºC. For two species Adic and Aobs, the 27ºC treatment suffer a thermostat problem during the 8th night and the temperature could not be controlled, and tadpoles, unfortunately died, All the tadpoles were weighed and assigned their stage of development (Gosner, 1960) just before and after the experiment. Then, we estimated relative growth rate (GRr), calculated (following Travis, 1980) as

$$GR = \frac{Wf - Wi}{Wi \; x \; Ndays}$$

*W*i and *W*f were the initial and final weight of the individual respectively and Ndays was the number of days elapsed from the start of the experiment. We considered negative values of relative growth rate as non-growth and therefore analysed as zero (Overgaard *et al.*, 2014). Because anuran larval growth rate decay near metamorphosis (Harris, 1999; Richter-Boix *et al.*, 2011), and in order to obtain only estimates during the linear growth phase, we avoided selecting experimental tadpoles in late developmental stages (> 33 Gosner stages). Sampling tadpoles differing in size and developmental stage were homogeneously distributed across temperature treatments. For each species, we estimated T_{opt}, Zmax and B80, by fitting different models using both population and individual-based approaches, by using a 4th-order polynomial or a modified Gaussian or Beta curve functions that better fits our preliminary data and other larval amphibian studies (Niehaus et al. 2012, Arrighi 2013, Kingsolver et al. 2015).

TPCs for swimming burst performance was measured as the tadpole maximum locomotor performance (burst swimming speed) and was estimated for A. cisternasii and *A. dickhillenii* species. To determine burst swimming speed (i.e. burst speed), we examined 30 and 28 *A. cisternasii* and *A.dickhilleni* tadpoles, respectively, that were placed individually on a portable thermal bath (patent license ES 2372085), which consists of an opened cross section methacrylate tube (100 cm length x 6 cm wide x 3 cm depth) filled with water of the following temperatures: 10°C, 15ºC, 20ºC, 24ºC, 28ºC, and 32°C). We then gently prodded the tadpole with a thin stick to stimulate swimming. Each trial was recorded using a digital camera (30 frames/s) positioned above the tube (JVC Everio GZ-MG505). TPCs were defined using a set of temperatures that were tested in a random order. As species differ in their range of performance, to obtain a complete performance curve, temperature sets were adjusted by adding colder or hotter test temperatures when required. Prior to swimming, tadpoles were submitted for approximately an hour to the test temperature.

Preferred selected temperatures in tadpoles of *Alytes cisternasii* (locality: Almadén de la Plata, Seville. Spain) and *Alytes dickhilleni* (locality: Filabres mountain range, Almeria. Spain) were tested with 15 individuals of each species previously acclimatized to 18 ºC. The experimental design inspired in Drakulić et al., (2017) consisted in a 160 cm channel (10 cm wide and 10cm high, semi-circular section) divided in 16 subsections of 10cm. One extreme was set al 13 ºC using an aquarium cooler and the other at 30 using a resistance. A four-channel probe thermometer (PCE T390 thermometer with TF500 probes) was used to measure the exact temperature in each extremum and each 6 section. With these four temperatures references we were able to estimate the temperature in each sub section. Tadpoles were released in the channel and measures were taken each 5 minutes until accomplish one hour.

Results

Respect the metamorphic stage (moisture experiments), we found significant differences in weight change between both treatments (p-value 0.00238), with a higher increment response for humid treatment in all the species except *Alytes cisternasii,* in which growth rate is similar in both treatments (Moist and dry). We obtained the graphs showed in figure 1.

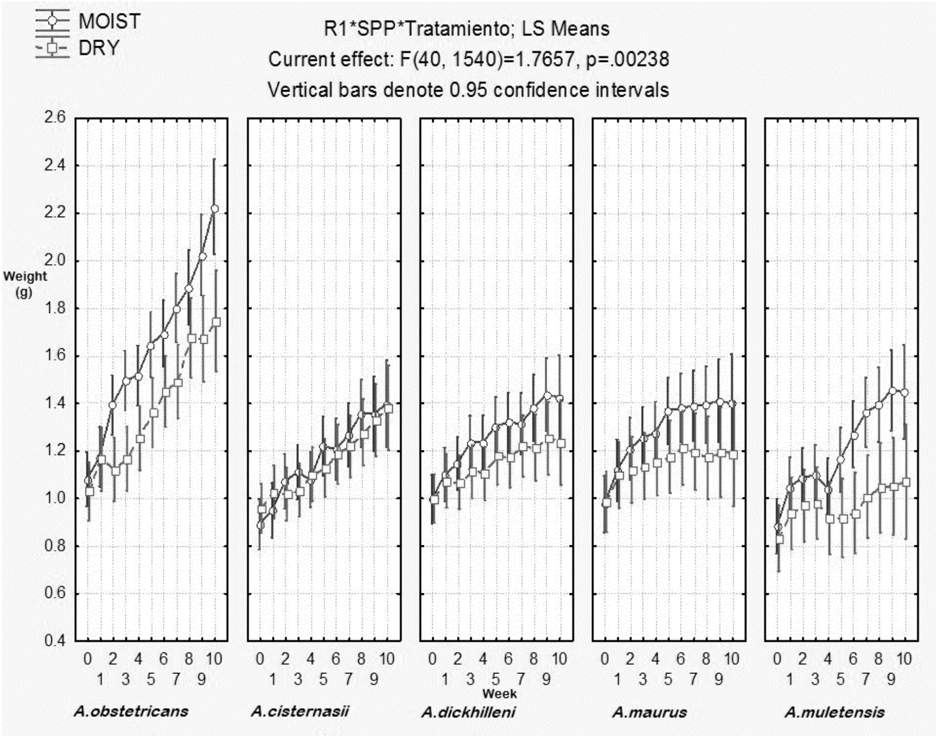

Figure 1. Growth in g of the five species at both tratments. AOBS (*A. obstetricans*), ACIS (*A.cisternasii*), ADIC (*A.dickhilleni*), AMAU (*A.maurus*), AMUL (*A.muletensis*)

Although, we found significative differences in SVL and HLL change under both moisture treatments, we have also detected important correlation with weight change (0.52 in the case of SVL and 0.47 in the case of HL). In the case of jumping performance difference at both treatments we have not obtain significative results and a correlation of 0.23 with weight change). See table 1 for p-values.

	HL increment	**SVL increment**	**Jumping performance variation**
Correlation R (With growth rate)	0.47	0.52	0.23
P-value (Response ~Sp + treatment)	3.09^{e-8}*	4.54^{e-9}*	0.93

Table 1: Correlation R and p-values of GLM analyses of the response variable under both treatments.

Regarding TPC growth and TPC swimming in tadpoles, the functions with better adjustment (table 2, figure 2) were: polynomial grade 3 (*A. cisternasii*), and Gompertz-Gaussian (*A. dickhilleni*) in the case of TPC growth; and polynomial grade 2 (*A. cisternasii* and *A. dickhilleni*) in the case of TPC swimming. Optimal growth for *A. cisternasii* is placed around 25 ºC, and in 23 ºC in the case of *A. dickhilleni.* In the case of swimming, we observe a slight improvement of performance with temperature increase in the case of *A. cisternasii*, with an optimal between 25-30 ºC. Respect *A. dickhilleni* the optimal performance is 24ºC with a reduction of performance at higher temperatures.

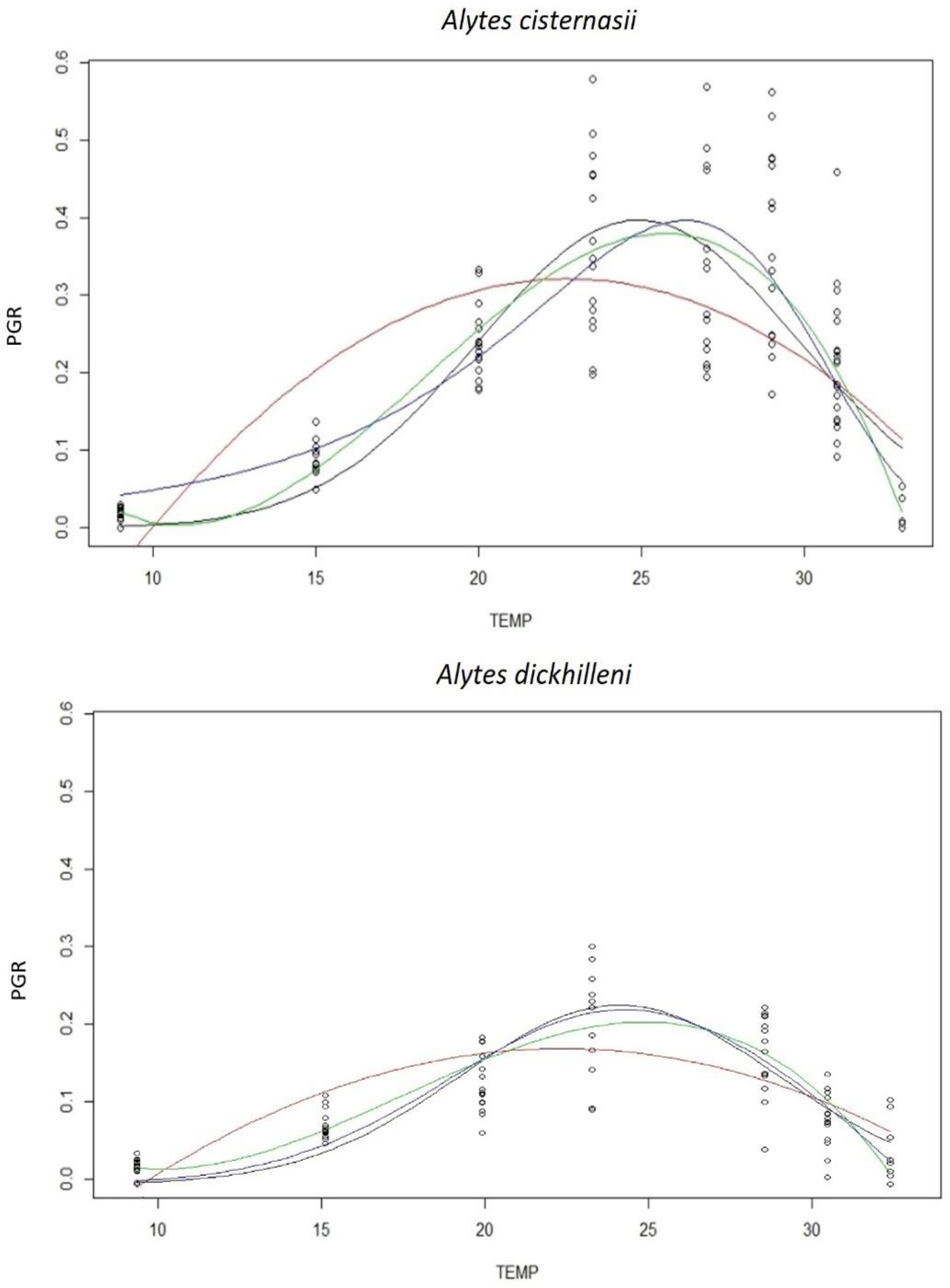

Figure 2. TPC of growth of *A. cisternasii* and *A. dickhilleni*. Black: Gaussian function; Red: Polynomial function grade 2; Green: Polynomial function grade 3; Blue: Gompertz-Gaussian function.

TPC	**Function**	**AIC d**	**Species**
Growth	Gaussian	31.7073/0.4194	*Alytes cisternasii/ Alytes dickhilleni*
	Polynomial function grade 2	76.5983/34.4703	
	Polynomial function grade 3	0.000/4.0296	
	Gompertz-Gaussian function	15.1080/0.0000	
Swimming	Gaussian	0.0064/0.0446	*Alytes cisternasii/Alytes dickhilleni*
	Polynomial function grade 2	0.0000/0.0000	
	Polynomial function grade 3	1.7082/1.3933	
	Gompertz-Gaussian function	Missed/Missed	

Table 2: AIC differences of the different adjustments for both TPC growth and TPC Swimming in A.*cisternasii* and *A.dickhilleni*.

Selected temperatures of tadpoles from the analysed species was 10.91 ±3.22 in *A.dickhilleni* and 13.03±2,97 in *A. cisternasii* (Figure 3)

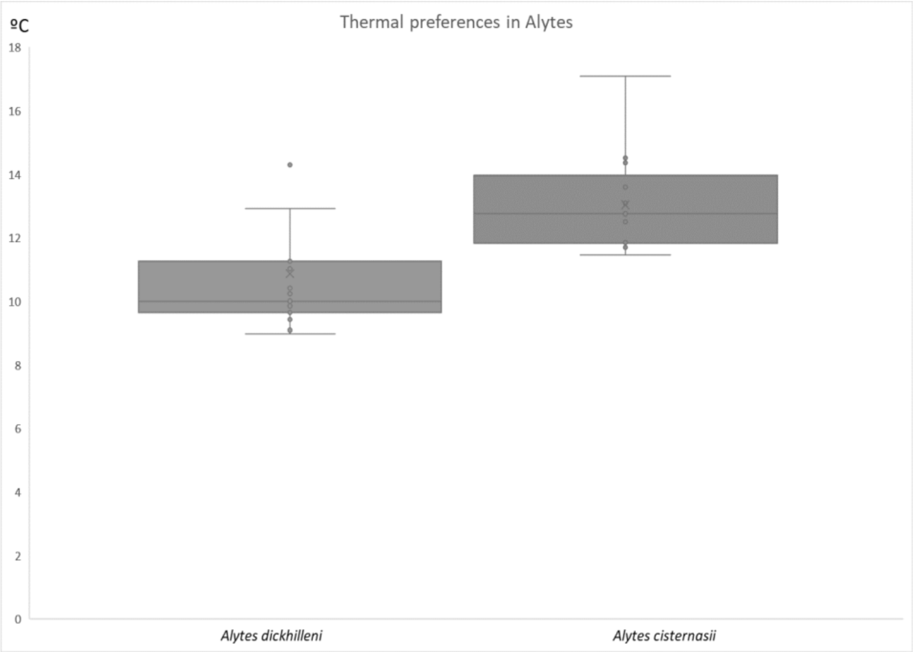

Figure 2. Preferred temperatures of tadpoles of *A. dickhilleni* and *A. cisternasii* acclimatized at 18ºC

Discussion

Our result shows a clear divergence in the physiological traits among the contrasting life stages across ontogeny of *Alytes* sp. This is, therefore, a good example of how environment can limit the survival of a species in a different way among stages of amphibians. This means that, for example, although adults of a species can survive, even reproduce in a concrete scenario, tadpoles could not survive or have a reduced fitness, avoiding the stable reproductive presence of the species in that area. This information is valuable for conservation because knowing physiological limits or preferences we can determine the suitable niche for a species, and the consequences for conservation in a climatic change scenario.

Specifically, growth rate has been postulated as a determinant factor affecting age at maturity, fecundity and survival (Clobert et al., 1998). Growth rate in amphibians has been found to respond to several factors, including tadpole density, pool duration thermal conditions in amphibians (Richter-Boix et al., 2015), but this trait is less studied regarding the moisture in metamorphic (Tejedo & Reques, 1994). At larval stages (aquatic), obviously, humidity is not a limiting factor, obtaining a high importance temperature, also related with pool duration throughout evaporation (Orizaola & Laurila, 2009). In terrestrial stages, thermal conditions are related with humidity level because evaporation increases when temperature increases, suggesting a strong interaction between thermoregulation and water balance in ectotherms (Mautz, 1982), however, little is known about the phenotypic variability of life history traits induced by humidity conditions.

Several studies show that these responses to humidity among the species could also derive in other ecological adaptations as difference between reproductive (Rodrigues da Silva et al., 2010), or activity strategies (Lorenzon et al., 1999). It is important to remark that amphibians are usually less tolerant of desiccation and require higher humidity levels than other terrestrial vertebrates (Ludwig, 1945). Therefore, humidity seems to be a crucial factor affecting the evolution of the old genus *Alytes*, that seems to continue affecting and driving it today.

Our results suggest a better adaptation to dry and thermic environmental conditions in the case of *A. cisternasii*. It is remarkable this is the species of the genus *Alytes* more external phylogenetically (Martinez-Solano et al., 2004). Our results would support that for four out five species of the group (*A. obstetricas*, *A. dickhilleni*, *A. muletensis* and *A. maurus*) low humidity is a limiting factor for growth in juveniles, even under optimal conditions of temperature or feeding. We also find that niche conservatism seems to drive the niche of these last four species respect this variable, being the divergence, the hypothesis explaining the segregation of *A. cisternasii* niche. This fact, consistent with models outputs of (Rodríguez-Rodríguez et al., 2020b), have an important ecological role in the niche selection of the genus, allowing to the most external taxa (*A.cisternasii*) to occupying and survive in Mediterranean areas with long periods of dry conditions. Oppositely, the rest of the genus is more dependent on moisture at least during the first stages of metamorphic.

Alytes cisternasii is the most external group of the genus and it is present in more thermic areas than the rest of the genus (Rodríguez-Rodríguez et al., 2020a; Rodríguez-Rodríguez et al. 2020b). This observation is congruent with the fact that optimal growths, at larval stage (aquatic) occurs at higher temperatures in the first species than in the second, and in addition also congruent with published maximum critical temperature (CTmax; 38.2 in A. cisternasii and 37.6 in A. dickhilleni; Katzenberger, 2014) . Preferred temperatures of tadpoles also show a higher temperature than *A. dickhilleni.* This pattern is less evident in swimming performances. These physiological implications should be severely affecting and shaping the thermal, and meso-Mediterranean distribution of *A.cisternasii.*

Although with differences in the response among species, in the case of the jumping performance change under the two treatments we didn't find significative differences. This indicate that the main affection of the moisture at metamorphic stage is the growth rate, affecting this variable to the performance. This fact is not at surprising result as Mitchell & Bergmann (2016) have already concluded that thermal or moisture habitat preferences do not influence the jumping performance. For SVL and HL we have found significative results but with a considerable correlation with weight change.

Our conclusions are that excluding *A. cisternasii*, all the species of the genus are severely affected in growth, and in consequence in their fitness under dry conditions (at metamorphic stage). In the case of *A. cisternasii*, regarding our results the local adaptations to dry environment are to be an important factor in the species evolution. Jumping performance seems to be not influenced by the moisture during the growth at metamorphic first weeks, being more relate by the own weight. In addition, SVL and HL are strongly correlated with growth rate and affected by both treatments. At larval stage, although with data only in two species we observe also an evident difference in *A. cisternasii* with higher optimal temperatures and CTmax. It is important to remark these results as an example of the different environmental variable affecting the different stages in complex life cycles (Istock, 1967).

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Chapter 5

Chapter 5

Melanophore metachrosis response in amphibian tadpoles: effect of background colour, light and temperature.

Rodríguez-Rodríguez, E[1]., Beltrán, J[1]., & Márquez[2], R. (2020). Melanophore metachrosis response in amphibian tadpoles: effect of background colour, light and temperature, *Amphibia-Reptilia*, Online-first, 1-8.
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[1]Grupo evolución, ecología y conservación de vertebrados. Departamento de Zoología. Facultad de Biología. Universidad de Sevilla.

[2]Fonoteca Zoológica, Departamento de Biodiversidad y Biología Evolutiva, MuseoNacional de Ciencias Naturales-CSIC, Madrid, Spain.

Key words: behavioral, melanosome, melanin, phenotypic plasticity, thermoregulation.

Summary

The developmental and biochemical mechanisms of colour change through chromatophore metachrosis in amphibian tadpoles are relatively well studied, but the environmental factors driving colour change remain unclear. A cryptic response to background colour in order to reduce predation is an intuitively valid explanation, however, other hypotheses need to be explored. In this study, we aimed to investigate the environmental factors driving the melanophore metachrosis process in *Alytes dickhilleni* tadpoles. First, we tested the response to two backgrounds with clearly distinct reflectance: black and white. The proportion of dark tadpoles became significantly higher when they were located on the black background, and pale tadpole proportion was dominant on the white background, as expected from the crypsis hypothesis. Secondly, we added two new factors, temperature and photoperiod, maintaining the background variation. Our results suggest that lower temperatures, and short photoperiods were significantly driving a change to dark colouration in tadpoles, possibly allowing a more efficient thermoregulation, and in consequence, development and growth. Next, we tested whether dark and pale tadpoles selected backgrounds that matched their colouration (black and white background) and found no evidence for behavioral selection. The apparent response in colour change to background appears to be mediated by the background reflectance of light, that there does not seem to be behavioral selection of matching background by the tadpoles, and

therefore it suggests that color change is more likely to be a physiological response with thermoregulatory implications.

Introduction

Information on environmental factors affecting the colouration of amphibian tadpoles is scarce and incomplete. This information is better developed, including genetics and evolution, in several model organisms like birds or fishes. We thus need a better understanding of ontogeny, diversity and functions of colouration in amphibian tadpoles (Thibaudeau & Altig, 2012). Excluding descriptive notes, studies about the functions, including crypsis, thermoregulation, ultraviolet protection, and modification of social interactions of colouration of amphibian larvae, are rare (Caldwell et al., 1982)

Amphibians, unlike other vertebrates (eg. mammals), have melanophores (containing melanin that produce browns and blacks), xanthophores (containing purine and pteridine pigments), and iridophores (containing crystalline purines). These cells arise from some trunk neural-crest cells that migrate dorsolaterally during development (Olsson & Löfberg, 1992). In amphibians, the pigment is located in organelles called melanosomes, pterinosomes, and reflecting platelets, found in each of these cell types respectively (Frost et al., 1984). These organelles have the ability of being rearranged within the cells, allowing metachrosis (i.e., change colour and pattern) (Thibaudeau et al., 2012).

Melanophores are mainly responsible of pigment patterns observed during the first larval stages (Parichy, 1996). A larger quantity of organelles per cell or pigment per organelle results in a higher intensity of black in melanophores. In addition, melanosomes can aggregate within the cell body or disperse radially by means of microtubules (Nascimento et al., 2003) when stimulated by melanocyte stimulating hormone, epinephrine or melatonin (Odenthal et al., 1996).

Excluding studies of biochemistry and cellular mechanism of pigmentation, there is not enough information to understand the influence of colouration on behavioral ecology of tadpoles. Behavioral consequences of ontogenic colour changes are known in only a few cases (Nilsson et al., 2013). Some forms of sociality can often be involved in colouration, promoting the behavioral changes. Several studies show that tadpoles can preferentially recognize and associate with others of their own species, using colour for conspecific recognition (Blaustein, 1998; Foster & McDiarmid, 1982).

Sometimes colour differences among tadpoles of the same species in adjacent ponds are obvious and until recently, these differences were assumed to be caused by light regimes, turbidity and tannin content (Oshima, 2001). The inducible colour response varies among individuals, and variations can also be mediated by the presence of predators and competitors, tadpoles with modified colours proving to have a selective advantage in predatory encounters (McClure & McCune, 2003). Our aim in this study is to assess the effect of some factors: substratum albedo influence (white or black background), light period, temperature, and behavioral selection of background. For this purpose, we used the primitive family Alytidae (Pough et al., 2016) in order to get an example of implications of melanophore metachrosis in basal anuran species.

Our first hypothesis is that albedo of substrate is influencing aggregation of melanosomes of anuran larvae, and thus, influencing the colour of the *Alytes* tadpoles. As for our next hypothesis, we aim to test two points: the role of environmental temperature in colour change, and to evaluate if there is a possible effect of colour in larvae thermoregulation (in case of same environmental temperature) and thus, in larvae thermoregulation. We also test whether photoperiod has a significant role in color change (a longer photoperiod means a longer exposition to radiation). In addition, we tested whether different coloured tadpoles selected different substrate background in relation with their colour.

Methods

The model species selected for this experiment was the Betic midwife toad (*Alytes dickhilleni)*. This is a basal anuran (Pough et al., 2016), with a tadpole that can reach relatively large size (max. 6 cm) and with a prolonged larval period before reaching metamorphosis (from 3 to 16 months depending on temperature)**.** Tadpoles (n=38) were obtained from an artificial pool at Calar Alto (-2.52 W,37.24 N; 1833m; Almeria province, southeastern Spain). Larvae (Stage 30; Gosner 1960) were maintained in two 10 L containers in the same room, with dechlorinated water and the same background (grey). Changes of water was implemented every two days, maintaining it well oxygenated with an air pump. Larvae were fed *ad libitum* once per day with rabbit vegetable pellets (protein: 11.10%; fibre: 18.14%; fat: 7.05%; calcium: 0.25%, Iron: 0.07%), and acclimatized to 18 ºC (Guerrero-Gómez et al., 2019) in order to slow down the metamorphosis, with a 12-12 h photoperiod (elevation of the lab 7 m over sea level).

After a week of acclimatization, larvae were classified to one of two colour morphs: dark or pale. We established a natural, biological marker as criteria for the assignation: Tadpoles with the characteristic "V" of the species visible on the top of the body were classified as pale (see Supplementary material 1), and tadpoles with a colouration that was not pale enough to see the "V" were classified as dark (Supplementary material 1). This colouration classification can be complemented with observation of melanophores with stereomicroscope, so colouration can be easily assigned to one or other stage.

Then, tadpoles were randomly assigned to one of two treatments: One container (n= 19 tadpoles; 10 pale and 9 dark) was located over a black background (albedo=0.18) and the other (n=19 tadpoles; 9 pale and 10 dark) over a white background (albedo=0.75). In the laboratory, tadpole containers were kept 25 cm apart on the same shelf. For one week, the number of pale or dark larvae (see supplementary material 1 for classification criteria) were recorded in each treatment allowing us to obtain the temporal change of colouration each day. At the end of this week pictures of pigment patches in the tail were taken using a Leica Stereo Microscope MZ10 F. In order to confirm our results, as a control, these experiments were repeated inverting the background of the containers after a week, repeating the same procedure (hereinafter, we will call this experiment reversion experiment).

With the aim of understanding the role of tadpole colour on thermoregulation, first, we measured superficial body temperature of each tadpole (using a no-contact Fluke® Precision Infrared Thermometer 572). To avoid the bias of reflectance in infrared measurements we also tested temperature with a cloacal mercury thermometer (Miller & Weber INC NY USA) placed in the back of the body in a sub-sample of the animals, and reflectance was fitted for dark or pale colourations. In addition, in order to ensure we were measuring animal temperature and not the superficial water temperature, the temperature of water in the tanks was verified. Later, we implemented a gradual 6 ºC room temperature reduction (2ºC per day to avoid possible thermal shock) until reaching 12 ºC (final temperature). Temperature of the tadpoles maintained in the same background treatments were recorded as described above. With this final test, we aimed to observe if within the same substratum treatment there were differences in the response under different temperature conditions. The temperature of this last test was selected considering the average day temperature of a natural pond with presence of the species (HOBO Pendant G temperature Data Logger-UA-004-64 placed in natural water bodies of origin).

In order to investigate the role of light in the process of colour change, we implemented a final test dividing each background treatment in two photoperiod treatments, one with 12D—12L h lighting and one with 24 h lighting, resulting in four treatments (white background 12h of light, n= 10; white background 24 h of light n= 9, black background 12 h of light, n= 10; and black background 24 h of light, n= 9).The source of light was artificial (fluorescent tube). Room and water temperature were maintained at 18ºC during all the experiments (water temperature was reset to 18 ºC in the four days preceding this experiment in order to allow acclimatization), and containers under 12 h lighting were covered by panels to avoid influence of any residual light of the 24 h treatment lamp.

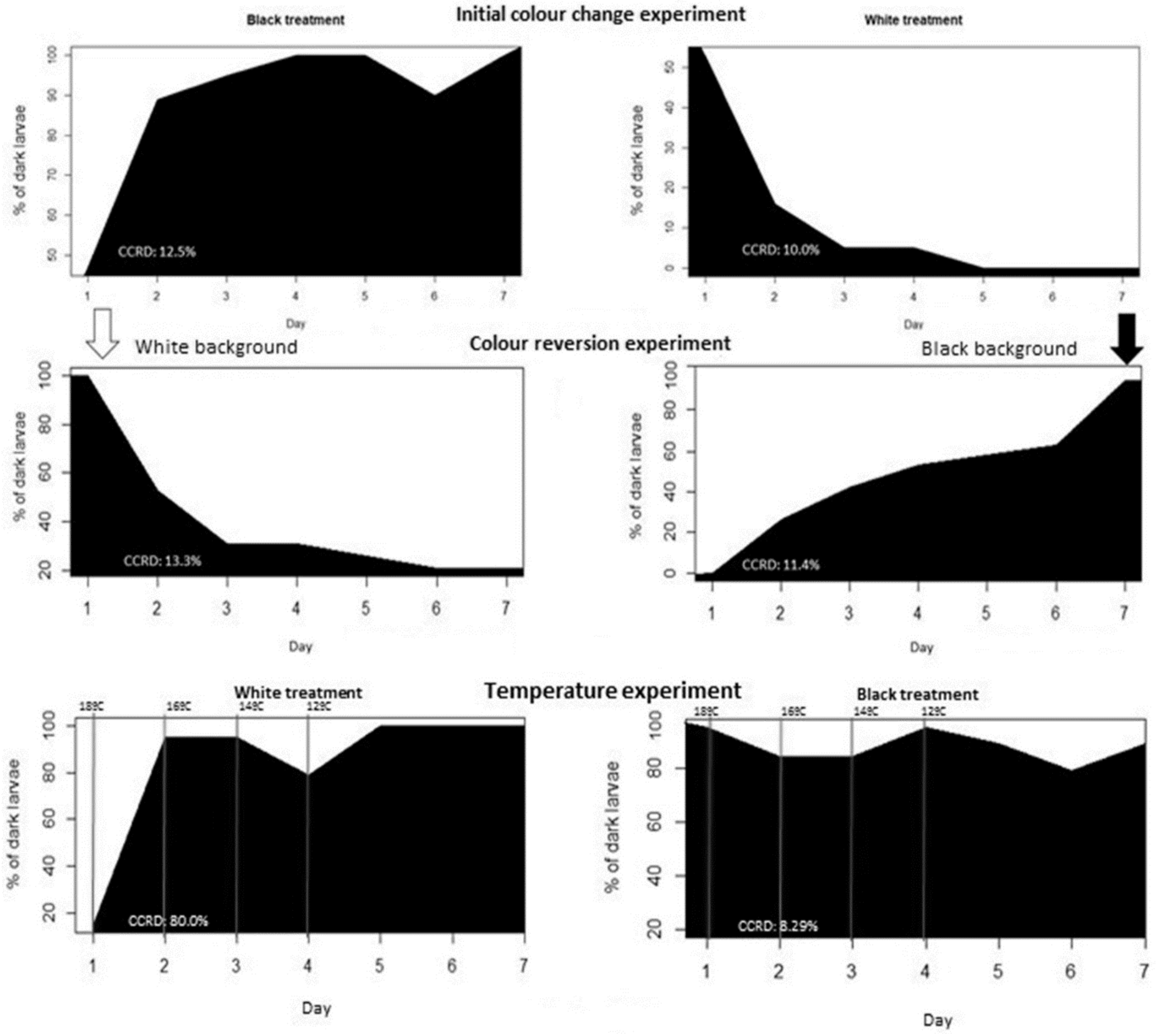
A behavioral test for substratum colour preference was conducted after the initial colour change experiment: We placed the tadpoles in a 10L container with four patches (Two black and two white, see supplementary material 1), and we tested the colour selection of both groups (dark tadpoles, n=19; pale tadpoles, n=19). We tested the response under two scenarios, response of the whole group and individual response. For each group response, we photographed and counted individually the larvae of each treatment (n=19+19= 38) located in black or white patches during one-hour periods until completing 5 observations. In the case of individual response, we selected individual tadpoles of both treatments and released them in the container, recording the data after one-hour periods.

Statistical analyses were implemented using R Statistical software (R Core Team, 2013). McNemar's test (a test used on paired nominal data in order to detect changes in time) was used to test for colouration change, reversion, behavioral group and single behavioral test. For this we constructed the 2x2 contingency matrix with the dichotomic response of body colour and time for each treatment. The Mann-Whitney U test (quantitative response in the case of body temperature) were implemented in order to test this last hypothesis. Dependent variables were Colour (proportion of dark tadpoles vs pale tadpoles) for colour change and photoperiod experiments, and predictor variables being background colour treatment, and background colour treatment + photoperiod respectively. In the case of body temperature experiments the dependent variable was body temperature, and the predictor was tadpole colouration. In the behavioral test the predictor was background colour treatment and the dependent variable was colour selection.

Results

In the case of colour change experiments, results show a significant increment of dark tadpole proportion when they are placed in black background and an increment of pale tadpoles in white background (Figure 1). Results of reversion experiments were also significant under both treatments (change to pale colouration with white background and change to dark colouration with black background). We also observed significant differences in body temperature (0.49 ºC of mean temperature) between tadpoles of different body colour (*p*- value < 0.001), with a higher temperature in dark tadpoles (18.75 ºC ±0.15; pale tadpoles: 18.25±0.21) with the same water temperature (18ºC). Differences for colour change in the decreasing temperature scenario were also significant (Figure 1).

The pictures of tail patches show a clear response in the melanophore patterns (Figure 1). For pale tadpoles we can observe small isolated dots (melanosomes are aggregated at the nucleus), and for dark tadpoles we can observe larger dots (melanosomes are dispersed throughout the cell body and dendritic processes), creating a colour stain (supplementary material 2)


Initial colour change experiment
Black treatment
White treatment
% of dark larvae
50
60
70
80
90
100
CCRD: 12.5%
Day
1
2
3
4
5
6
7
0
10
20
30
40
50
CCRD: 10.0%
White background
Colour reversion experiment
Black background
20
40
60
80
100
CCRD: 13.3%
0
CCRD: 11.4%
Temperature experiment
White treatment
Black treatment
18ºC
16ºC
14ºC
12ºC
CCRD: 80.0%
CCRD: 8.29%

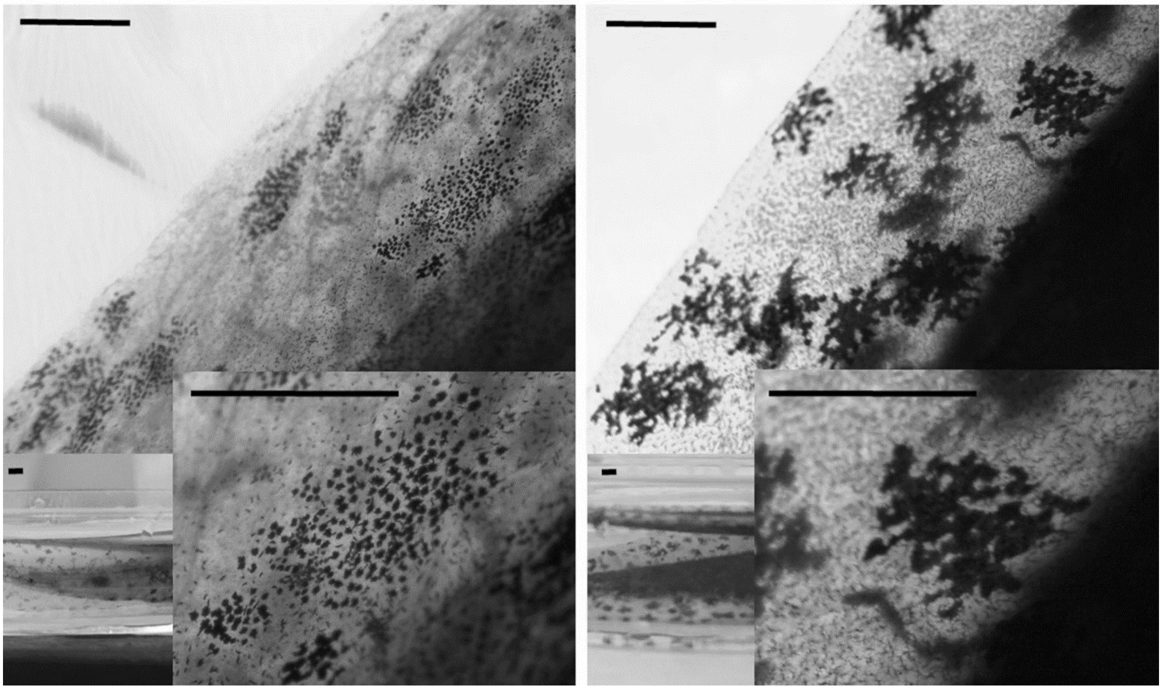


Figure 1. Percentage of dark coloured larvae in each treatment after initial, reversion and temperature experiments. Average colour change rate per day (CCRD) also provided in each experiment. Initial colour change experiment black background (χ^2=10; p-value= 0.005), white background (χ^2=10; p-value= 0.005); Reversion experiment white background (χ^2=15; p-value= 0.005), black background (χ^2=15; p-value= 0.005); temperature experiment (p-value= 0.024). We provide, in addition, section of larval tail crest at white substratum treatment (left) and black substratum treatment (right), with details of black coloured patches. We can observe the effect of melanosome aggregation (left) or dispersion (right) in the melanophore cell. Black bars indicate 1mm.

In the case of the photoperiod influence, we found that the proportion of pale tadpoles increased significantly at longer lighting periods (*p*-value < 0.005). This indicates that the longer photoperiod may cause a shift towards pale colouration in tadpoles at the same temperature and background treatments (Figure 2).

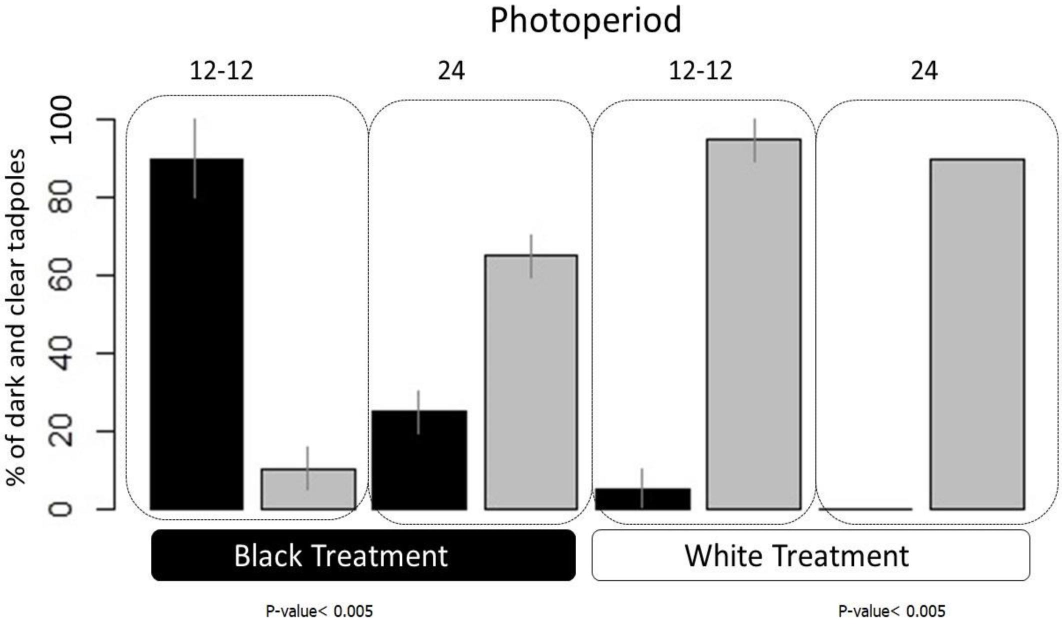


Figure 2. Percentage of colouration response to background and photoperiod treatments at 18ºC. Black bars for dark tadpoles and grey bars for pale tadpoles. P-values < 0.05 for both, white background treatment and lack background treatment.

For both, group and individual behavioural substratum selection test we did not find any significant results, with *p*-values of 0.09 (group test) and 0.51 (individual test).

Discussion

Numerous groups have evolved constitutive colour patterns as adaptation to the habitat, however the fixed pigmentation pattern of some organism might be disadvantageous when they face environmental heterogeneity (Magellan & Swartz, 2013). There are several evolutionary responses to this issue, as a colouration constituting a compromise between the requirements of the differing microhabitats (Merilaita et al., 1999), or the evolution of different morphs, each one adapted to a different part of the environment (Bond, 2007). Finally, another evolutionary possibility is the capacity of colouration plasticity (West-Eberhard, 2007).

Our results show a clear plastic response of *A.dickhilleni* tadpoles to background colouration (mediated throughout albedo). This response occurs within days (Figure 1) and at first sight may be considered an efficient cryptic adaptation. Polo-Cavia & Gomez-Mestre (2016) describe a similar response in the urodele *Lissotriton boscai*, but slower than the observed in our experiments. Polo-Cavia et al. (2016) also found background colouration matching in adults of another species of the same genus, *Alytes obstetricans*, but the environmental factors leading to pigmentation change were not studied. The pictures of tail patches of *A.dickhilleni* also show a clear change in melanophores due to the aggregation or dispersion of melanosomes, a process known as metachrosis (Nascimento et al., 2003). Additionally, in our study we show how temperature, a physical environmental variable, is capable of influence these physiological processes. We have to consider that our experiments were tested in only two aquaria, and although aquaria were located in the same room with the same conditions, it is possible that some uncontrolled factor could affect this non-independent sample. It would be interesting to test these same experiments with one container for each tadpole. In addition, colour change and reversion experiments were conducted immediately after each other, and this implies that there is a small difference in developmental stage of tadpoles between the experiments. However, these differences were of one Gosner stage at most, and thus, never entering in the metamorphosis process.

Thermal conductivity in water is high and tadpoles are completely immersed in it, therefore, heating and cooling rates tend to be very rapid (Wells, 2007). Traditionally, it has been considered that larval stages of amphibians are not capable of any physiological adjustments that result in an alteration of thermoregulation (Bartholomew, 1982). Consequently, it has been accepted that the only way that an aquatic amphibian can alter its body temperature is to move between microhabitats in response to local differences in water temperature. Our results contradict in part this observation because tadpoles of our study species can slightly modify their body temperature throughout melanophore metachrosis and thus, colour change. These subtle differences in temperature (0.4 Cº) may result in significant changes in tadpole growth and development as some studies have concluded (Castano et al., 2010; Sanuy et al., 2008)

Our results also suggest that photoperiod may influence melanophore metachrosis, with a tendency to a paler colouration in the longest period of light. Therefore, we have two main factors affecting this phenomenon of colour change: temperature and photoperiod. Background also is influencing colour change, and we suggest this is a consequence of different light refraction among substrates. Melanophore metachrosis seems to be influencing the thermoregulation of the tadpoles of this species through different light reflection, and in consequence, slight body temperature changes. Large photoperiods (with more radiation received during the day) and increased temperatures induce a pale colouration response and reduces body temperature. Whereas, shorter photoperiods or lower body temperature result in darkening of tadpoles.

Our results suggest that background colour selection cans influence body temperature, not being a behavioural crypsis response, at least not in the absence of predator pressure. However, future studies should include similar observations with and without the presence of predators in order to evaluate behavioural responses in this scenario. Some studies have assessed behavioural physiological and morphological responses under predatory pressure (Zamora-Camacho et al., 2019). Polo-Cavia & Gomez Mestre (2016) have also assessed the influence of predator presence on background colour selection in newt larvae, with a behavioural response detected. Therefore, we consider it as important variable in future studies.

As conclusions, in addition to crypsis, melanophore metachrosis may have important implications in the thermoregulation of this species. At lower temperatures or short photoperiods, the dark colouration increases the retention of radiation and thus, improve heating of tadpoles. Interestingly, we found that tadpoles had no behavioural preference for white or black background. It is important to test these trends in other species of the same genus and tadpoles of other species. The effect of temperature changes associated with change in coloration on metabolism or developmental rate remain to be studied.

Ethical standards.

Authors have all permits for capture, maintenance, and experimentation with these organisms. Permits were provided by Dirección General de Medio Natural, Biodiversidad y Espacios Protegidos (Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible. Junta de Andalucía; Ref: SGYB/AF 12-09-2019).

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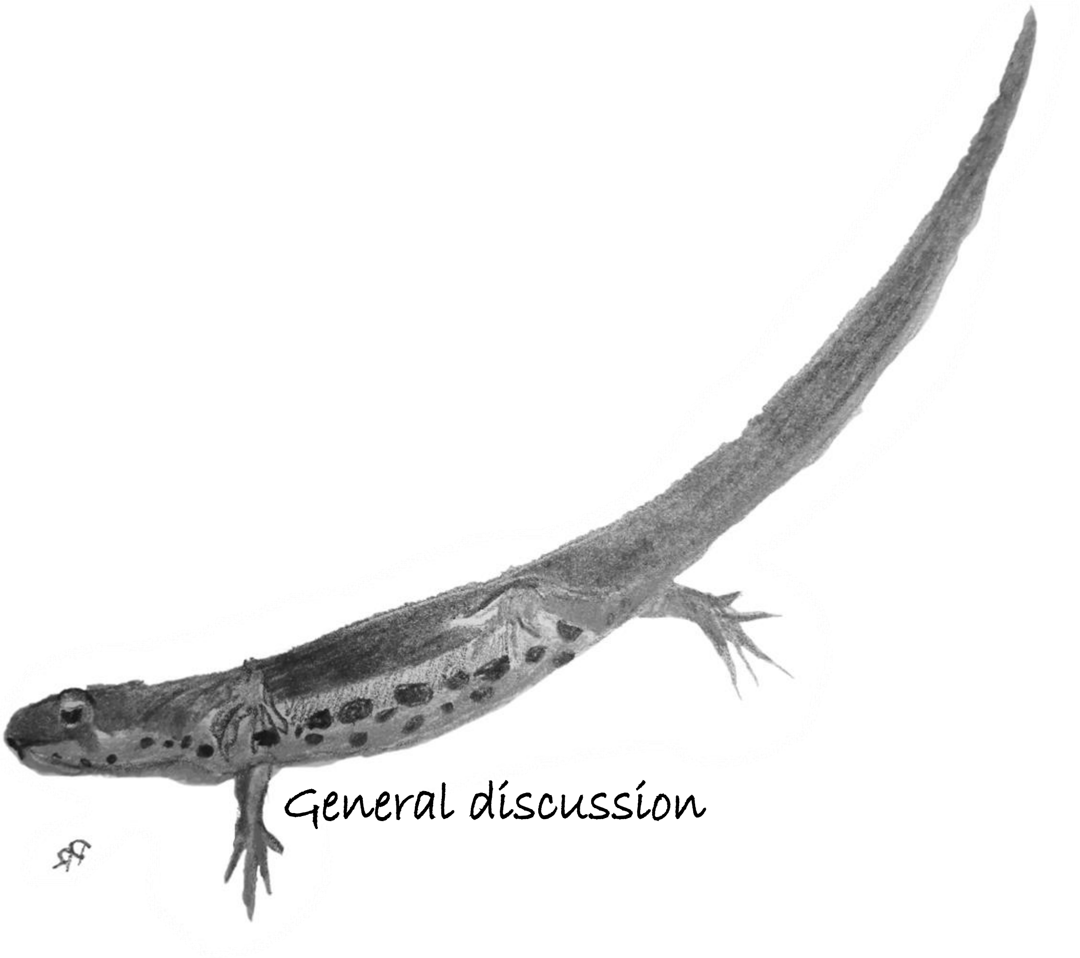

General discussion

General discussion

Our data provide a wide view of the implications of the environmental niche in western Mediterranean amphibians, considering insights in evolution (throughout niche differentiation), conservation (perspectives in a climate change scenario), distribution (Influence of climatic history in distribution range size) and physiology (Physiological adaptations and factor determining the niche). These aspects are discussed below:

Niche of western Mediterranean amphibians in a changing world.

In the first place, this work provides an example of how climatic niche differentiation is implicated in the evolution of the genus. This theme has been studied previously in tropical salamanders (Kozak & Wiens, 2010) but it has been poorly assessed in other amphibians, especially in template or Mediterranean areas. Traditionally, the vicariant speciation has been considered the main cause of speciation in these group around the Occidental Mediterranean (Vences & Wake, 2007; Martínez-Solano, 2004). However, this work provides a clear example how niche differentiation, although sometimes influenced by barriers but not always, is also promoting speciation. This is evident when we consider our results of intraspecific niche differentiation, and the fact that it has been described by some authors that differentiation occurs faster in a sympatric scenario (Pitteloud et al., 2017). These results, and other works assessing other than vicariant factors as bioacoustics ethology (Márquez & Bosch, 1997) draw a complex scenario in the evolution of western Mediterranean amphibians.

Climatic niche is related with the capacity of species to adapt and survive in case of climate change (La Sorte & Jetz, 2012) and it has been described that rates of projected climate change dramatically exceed past rates of climatic niche evolution (Quintero & Wiens, 2013). In addition, it has been described that in some especially environmental dependant groups, like reptiles, the altered thermal niche could drive the diversity loss in numerous areas around the world as these species cannot evolve quick enough to track climatic change because of constraints arising from the genetic architecture of thermal preference (Sinervo et al., 2010). Furthermore, it has been proposed that increases in temperatures can promote an increased oxidative stress in biomolecules of ectotherms, generating an accelerated ageing (Burraco et al., 2020). For these reasons, it is important to detect the most sensible species in multiple scenarios of climate change throughout the variation in available climatic niche. In this work we identify the species with higher projected suitable habitat reductions and relate it with current conservation status. An integrated view of present status and future vulnerability to climate change provides us a real view in conservation priorities.

We also found that climatic stability, linked to glacial refugia, is related with smaller distribution range sizes in Europe. This is probably a consequence of higher biodiversity and endemism rate in these areas (Araujo et al., 2008). The explanation of this is that in areas with no climate stability, and subsequently the impossibility of specific niche evolutions, only generalist species with a wide niche breath can colonize. This fact also supports the idea of a diversity reduction in case of climatic change, including intraspecific genetic lineages (Lourenço-de-Moraes et al., 2019), and the impossibility of adaptation to a fast change in climatic conditions as we have assessed in chapter 2.

Niche of western Mediterranean amphibians and physiological determinants

If we consider the physiological traits that shape the niche of species, in the case of amphibians, we must take in account the complex scenario derived from our results, with different constraints across the different ontogenic stages. Our data test the two main phases, consisting in contrasting environments, aquatic (larvae) and terrestrial. In the last stage we have used juveniles, because they are more sensible to the environment and have an important role in dispersion (Lomnicki, 1988). Obtained data in this work marches with described in other complex life cycle groups (Kingsolver, 2011) and demonstrates the importance of using physiological information of different ontogenic stages for conservation, including complex mechanistic models in an environmental changing scenario like climate change. However, we must not consider the physiological tolerance of a species (environmental niche) as a unit, but a gradient because of at the extremes of the tolerance the species can survive but with pronounced physiological stress, which causes a reduction in fitness (Megía-Palma et al., 2020).

For this reason, we must differentiate among optimal and suboptimal environmental niches.

Finally, we have addressed an example of evolutive physiological response adaptation to the environment. In this case we have used a phylogenetically ancient genus (Pyron & Wiens, 2011), *Alytes* sp. Laurenti 1768, because it implies this adaptation could be of early apparition during evolution, and thus, widely extended around amphibians. We have focused on melanophore metachrosis, and we have found thermal implications, that may influence developmental rate in tadpoles, with the capacity to become darker in poor lighting scenarios, allowing a major retention of radiation. This is important to our general findings in this thesis because it implies certain physiological manoeuvrability, in addition to behavioural, in order to withstand environmental variations. Recently, Martínez-Freiria et al. (2020) have found that an increase in the extent of dark inside the zigzag dorsal pattern of Eurasian vipers is associated to colder environments (Thermal melanism hypothesis). This association was significant in the case of solar radiation, elevation and temperature. These results absolutely match with our results in tadpoles. Although tadpoles have the capacity of colour change throughout melanophore metachrosis, we have found that dark colouration is associated with low temperatures, low radiation exposure and it is related with a thermal benefit. We can say, thus, tadpoles accomplish in some way the thermal melanism hypothesis, but with the possibility of changing colour in order to adapt to environmental conditions.

Relating these findings with our results in chapter 4, we know that the adaptation mechanism to environmental conditions will be different among ontogenic stages because the factors limiting the niche suitability will be also distinct. Our data is an example for aquatic stage (larvae) and temperature as limiting variable. Although adaptation mechanism to environmental temperature are relatively well studied in adults (terrestrial phase), throughout mobility, refuge , thermoregulation, etc (Nowakowski et al., 2011), we have found that moisture is an essential factor affecting this last stage, thus research in this topic is interesting to understand the whole physiological relation of these species with environment.

The general overview and conservation perspectives

Our study has based on the form that the niche concept allows us to describe and evaluate the consequences of tradeoffs of species and how they respond to conditions of the environment. Further, we point out that such trade-offs are important in generating variability among species and in regulating the relative abundances and distributions of species and lineages. In essence, we have generated a simple study framework based on niche requirements and impacts.

Regarding conservation implications, we know that most amphibians have narrow climatic niche widths, especially related to temperature and moisture. This fact implies that this zoological group is especially threatened by anthropogenic climate change (Bonetti & Wiens, 2014), even more so if we consider that it has been described that evolution of ectotherms can't track fast enough the environmental changes (Quintero & Wiens, 2013). In our work we confirm an example of how intraspecific lineages present local climatic niche adaptations. The niche of these is obviously narrower than the whole niche of the specific level, but they also suppose opportunities of survival for the species in case of changes in environmental conditions. For this reason, is essential to preserve each lineage, and we should identify the most risked ones. We also know, considering our results, that species with the widest climatic niche can colonize wider areas after climate change events, and that species with specific niches (narrow suitable habitat) remains in areas with long historic climatic stability. As this fact is also applicable to intraspecific lineages, we should prioritize the surveillance of those with more specific or narrow niches. As all these niche characteristics are determined by physiological traits (including physiological plasticity), and those are determined by genetics, it is crucial the intraspecific genetic information in order to identify the environmental niche conditions of these intraspecific groups.

Conclusions

we provide a general overview from macro (ecological niche modelling, habitat suitability projections under climate change scenarios, and factors affecting the range of species) to micro scale (Physiological constraints and physiological adaptative responses) in the niche evolution of western Mediterranean amphibians. The integration of all this data is a valuable information for conservation, especially in a scenario of climate change. Individual conclusions are:

1-Climatic niche appears to be affecting past and current evolution of Mediterranean amphibians at both levels: Inter and intraspecific.

2-These effects of niche in evolution are currently working, generating a scenario of ongoing intraspecific evolution.

3- The divergence hypothesis seems to have a key role in the evolution of, at least, *Alytes* sp.

4-This evolution is not fast enough to track human caused climate change scenarios.

5-Climate change scenarios could affect differently amphibians species in western Mediterranean. With increases or decreases in suitable climatic habitat among different species. This allows us to identify conservation priorities, both in species an area.

6-Climatic stability have been an engine of increase in endemism rate because only niche generalist species can survive or colonize in a changing scenario. This stability is also related with the smallest distribution range sizes.

7-Physiological characteristics are generating these differences in climatic habitat

8-Physiological limiting factors are not the same at different ontogenic stages

9-Temperature is the main limiting factor of larval growth whereas in the case of juveniles it is moisture.

10-Amphibians have certain capacity of physiological response in order to allow a slight adaptation to a changing or heterogeneous environment.

11-We should prioritize the surveillance of intraspecific lineages with narrow environmental niche in order to avoid the loss of important physiological adaptations to environment, essential for future evolution and resilience of species.

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Future perspectives

In the chapter 1, we have assessed the climatic niche segregation using a correlative approach (distribution points related with environmental variables). It is interesting to implement a comparative with a mechanistic approach (physiological calculated traits and environmental variables) to get a stronger and more comprehensive result. Furthermore, Litvinchuk et al. (2020) use fossil records and past climatic data to model the past niche of species and project it in current climatic raster data, allowing the comparison with the niche contemporaneous species. This is an interesting approach, for example with the genus *Alytes* sp., as it allows to infer the direction of environmental niche evolution.

It would be also interesting to complement the niche availability projections in scenarios of climate change with other niche affections derived from Global change, as example habitat destruction or fragmentation. If we also add projections of suitability for invasive species or emergent diseases, we can obtain a complete overview of niche future perspectives for Mediterranean amphibians.

Regarding the pattern of range size analysed in the chapter 3, addressed only in Europe, it would be interesting to test in the other shore, in the Maghreb region. The problem here is that although the distribution of reptiles and amphibians is well known at macro scale, the information available has more gaps than the European date at local level, inducing possible bias in the results of this area.

The physiological traits analysed in chapter 4 should be repeated in bigger subset of species of the area, in order to have enough data to implement mechanistic models in a wide number of species.

Finally, it is interesting to investigate more physiological adaptations and responses allowing thermal or moisture adaptations as we addressed in chapter 5. All this information combined with our data would generate a good base to anticipate global change and planning future conservation actions, and priority species.

Additionally, and following our results and expertise during this research, we have started a research line exemplified and explained in the Annex.

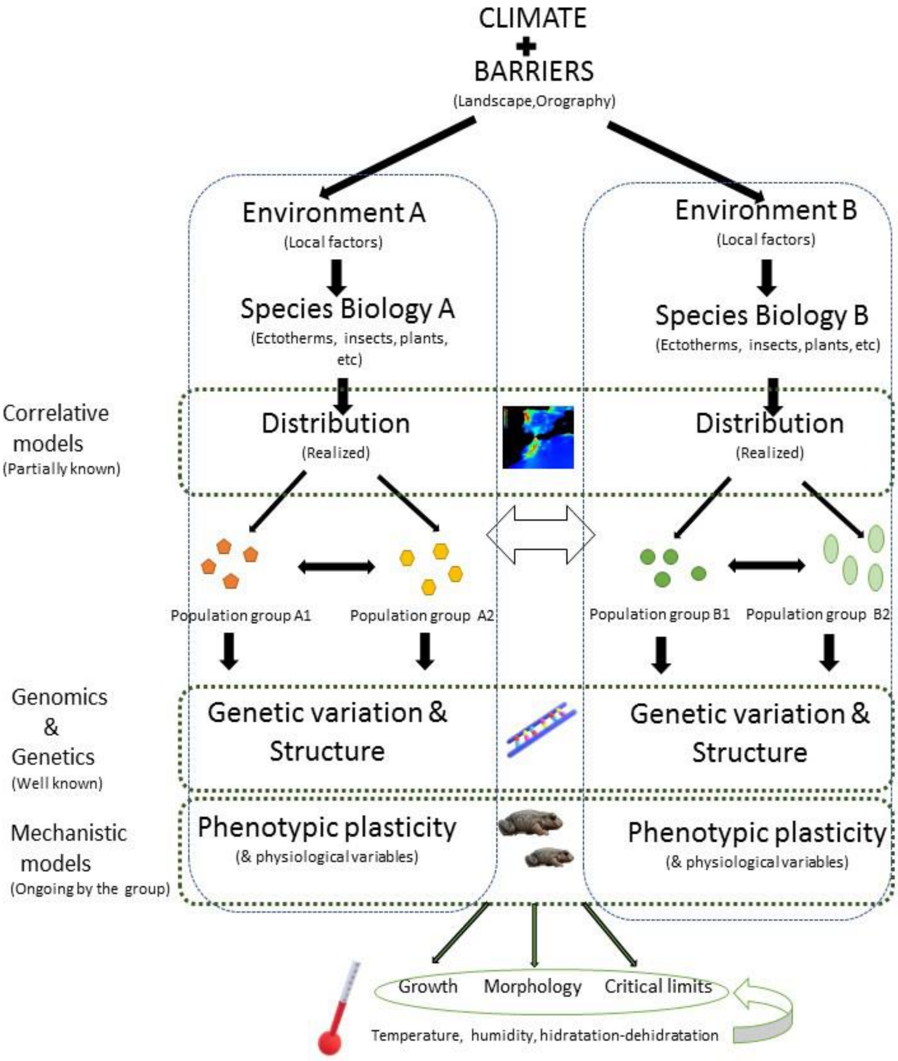

Fig. Graphical abstract of relations between environment and amphibias, including perspectives of research.

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Annex

Can environmental niche models at intraspecific levels reveal ongoing divergence in amphibians?

Evolution is a continuum phenomenon acting in front of us and shaping the biodiversity of our Planet. In most cases, we can only observe and define the products of it, in form of species or other taxonomic units such as subspecies, clades, etc. As a recent approach to explore biodiversity, Integrative taxonomy (IT, see Padial et al. 2010) has pointed out two relevant ideas. The first one is that species will be better delimited if we know what caused their origin and determined their evolutionary trajectories. The second one is that we can incorporate multiple sources of data (morphology, ecology, molecular, etc) of evolutionary independence for species delimitation (Dayrat 2005).

On the other hand, current taxonomy is focused mainly on the species level, and in some cases even subspecies, but it pays few attentions to intraspecific lineages. This lack of interest needs to be re-examined for at least the following two reasons. First, because the studies providing molecular based information on species (their genetic diversity, population structure, connectivity, etc) have increased remarkably, resulting in an enormous amount of detailed information in the databanks. In second place, because this information rarely is used to explore or revise taxonomic status, thus ignoring their value as prompted by IT, and what may be even more interesting their evolutionary momentum.

The midwife toads (*Alytes* sp.) are good examples of integrative molecular and ecological studies with amphibians. Accumulative molecular information gathered on this genus, suggests that the recognized species of this genus may be in fact in an active process of divergence. The common midwife toads (*Alytes obstetricans*, Maia-Carvalho et al., 2018), for instance, shows a current divergence in the environmental niche of several lineages. Not surprisingly, one of them have been recently proposed as candidate to a separate species (Dufresnes et al., 2019). In the same genus, integrative taxonomy incorporating ecological models and mtDNA have been applied to the Moroccan midwife toad, *Alytes maurus,* revealing a low mitochondrial gene structure among populations, what seems explained by a higher climatic suitability during the last glacial period (de Pous et al., 2013). Nevertheless, more insightful studies (e.g. microsatellites) should be carried out to confirm these preliminary results. The case of the endangered Betic midwife toad, *Alytes dickhilleni*, may be another case of ongoing divergence. Dias et al. (2015) found a low mtDNA structure and a marked microsatellite structure in a comprehensive study of the phylogeography of this species and remarked the limited gene flow among the four main genetic lineages (Figure 1). This prompted us to apply ecological niche models and re-evaluate the inter and intraspecific differences in three of the five current described species of midwife toads: *A. obstetricans, A. cisternasii,* and *A. dickhilleni*. In the case of the rest two species, *A. muletensis* and *A. maurus*, there is not available information on microsatellite structure in order to apply the modelling to the intraspecific levels. For the other three, we found that the same processes promoting specific differentiation of ecological niche appear to continue at intraspecific level, i.e. among genetic lineages, therefore acting as divergence mechanism. As result, particularly in the case of *A. dickhilleni*, the niche divergence among these lineages appears more pronounced in one them (i.e. acts in a mosaic way): the one encompassing the Tejeda-Almijara populations.

What are the implications of this for the taxonomy of these species? We think that new methods of data analysis are needed to extract valuable information already available in databanks (molecular, bioclimatic, etc). The Integrative taxonomy approach may be updated incorporating niche models to perform "prospective" taxonomy. This can be done effectively and at cost/results very low. We also suggest that besides species level, to explore intraspecific evolutionary units. Ecological niche models, molecular and morphology analysis could inform us of a current evolutionary process, especially in species of limited mobility as amphibians.

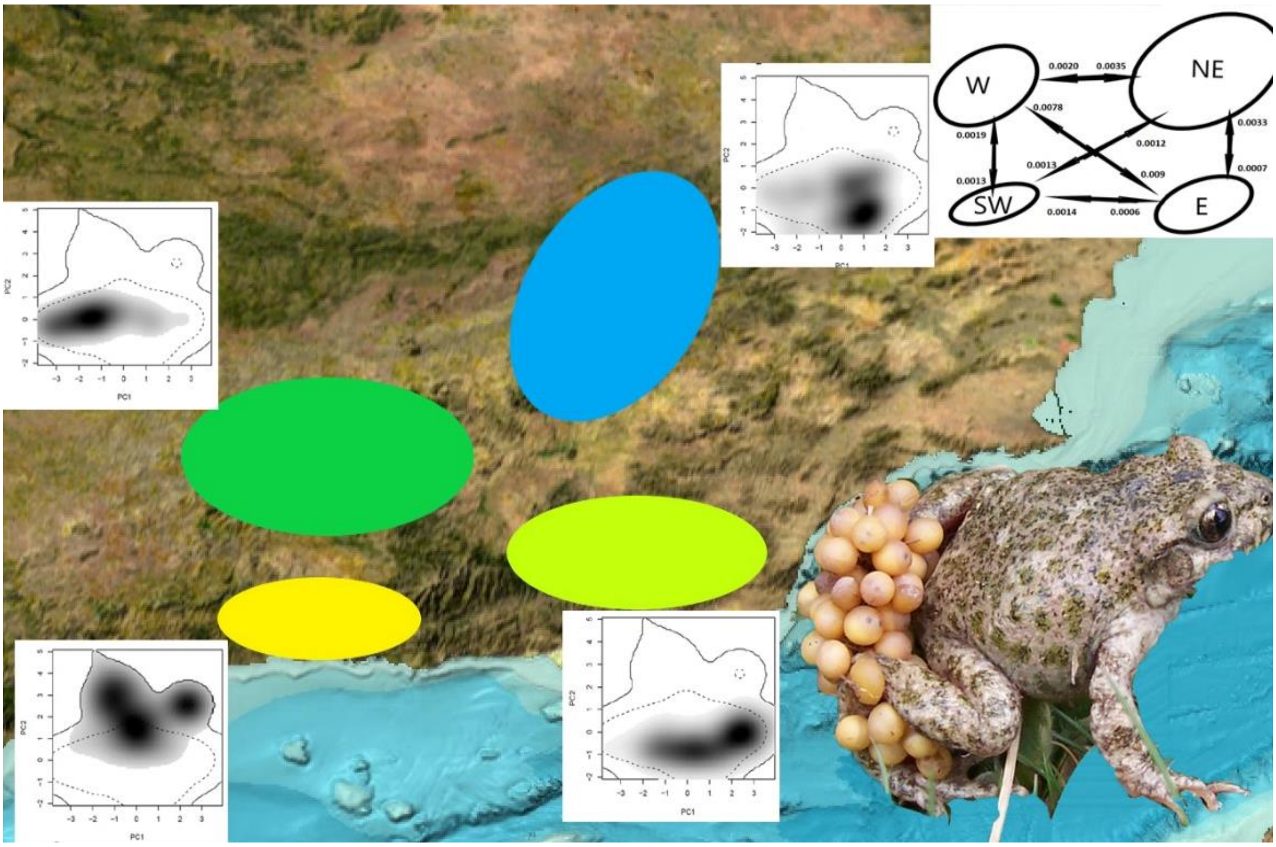

Fig 1. Environmental niche principal components (Bioclimatic variables) extracted from Rodríguez-Rodríguez et al. (2020) based on lineages described by Dias et al. (2015). Colours indicate the approximate geographic distribution of the described lineages along the mountain ranges of SE Spain. Diagrams, modified from Dias et al. (2015), shows Bayesian estimates of migration rates among STRUCTURE detected groups (m values). Note that the most genetic isolated group (SW: Tejeda, Almijara, Alhama, Albuñuelas and West Sierra Nevada mountain ranges) shows also larger niche differentiation. Image of a male *A.dickhilleni* carrying eggs courtesy of Emilio González Miras. Figure created with QGIS Chugiak 2.4.0(QGIS Development Team. 2018. QGIS Geographic Information System. Open Source Geospatial Foundation Project. https ://qgis.osgeo .org). Background map modified from GEBCO Compilation Group (2019) GEBCO 2019 Grid (https ://doi.org/10.5285/836f0 16a-33be-6ddc-e053-6c86a bc078 8e).

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Supplementary material

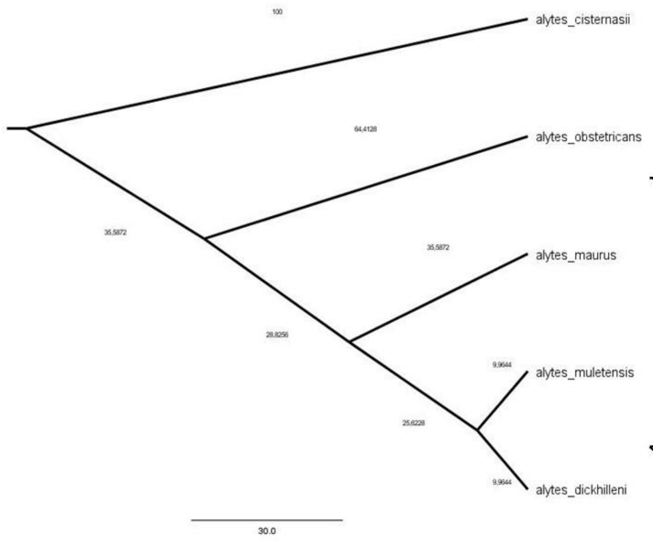
Chapter 1

Supplementary material 1. AUC and specificity values for each intraspecific lineage model.

Species (Lineage)	**AUC**	**95% IC AUC null models**	**Specificity**
***A.cisternasii* (Western)**	0.99±0.10	0.89	0.97
***A.cisternasii* (Southern)**	0.99±0.04	0.91	0.93
***A.cisternasii* (Eastern)**	0.93±0.11	0.84	0.76
***A.cisternasii* (Northern)**	0.93±0.07	0.86	0.67
***A.dickhilleni* (Southern)**	0.95±0.03	0.89	0.75
***A.dickhilleni* (Northern)**	0.97±0.06	0.90	0.81
***A.dickhilleni* (Western)**	0.97±0.08	0.91	0.86
***A.dickhilleni* (Eastern)**	0.99±0.03	0.88	0.88
***A.obstetricans* (North-eastern)**	0.93±0.11	0.86	0.95
***A.obstetricans* (North-western)**	0.98±0.12	0.90	0.82
***A.obstetricans* (Central-western)**	0.99±0.09	0.89	0.99
***A.obstetricans* (South-eastern)**	0.84±0.14	0.81	0.64
***A.obstetricans* (South-western)**	0.87±0.16	0.83	0.68

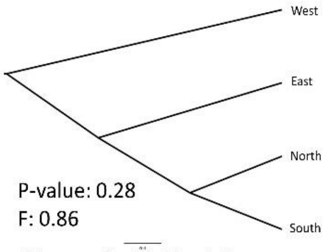
Supplementary material 2. Phyloclim results at intraspecific and interspecific levels; and cluster dendrograms for niche overlap.

Genus *Alytes* sp.



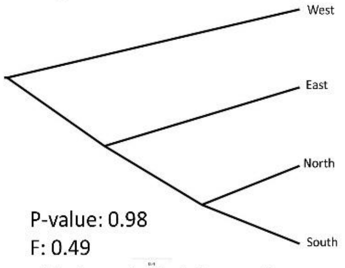
P-value: 0.60
F: 0.70

Alytes cisternasii lineages.



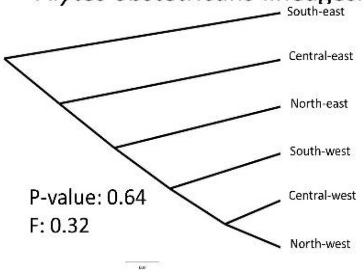
P-value: 0.28
F: 0.86

Alytes dickhilleni lineages.



P-value: 0.98
F: 0.49

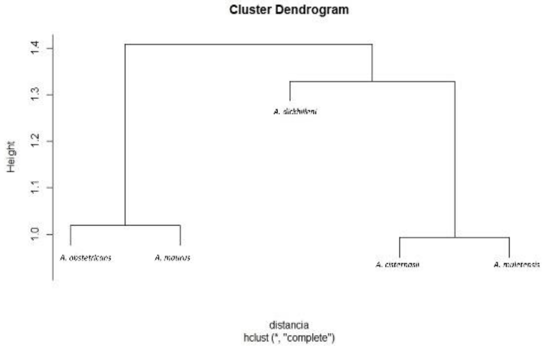
Alytes obstetricans lineages.



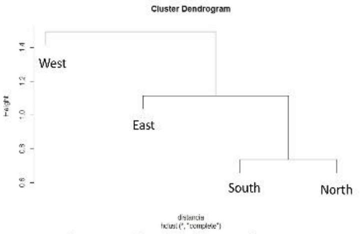
P-value: 0.64
F: 0.32

Niche overlap Dendrograms

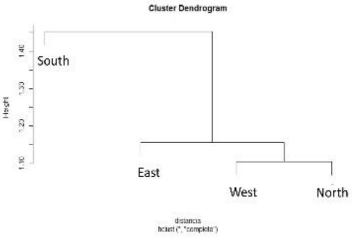
Genus *Alytes* sp.



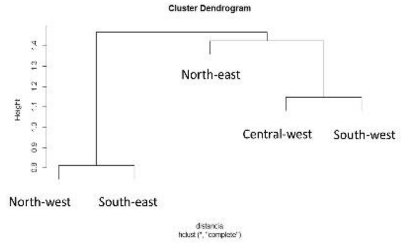
Alytes cisternasii lineages.



Alytes dickhilleni lineages.



Alytes obstetricans lineages.

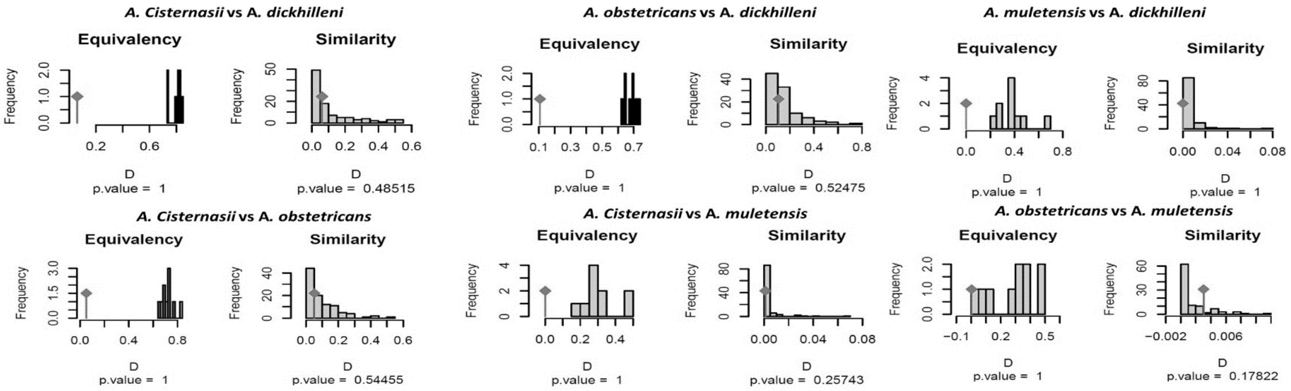


Supplementary material 3.

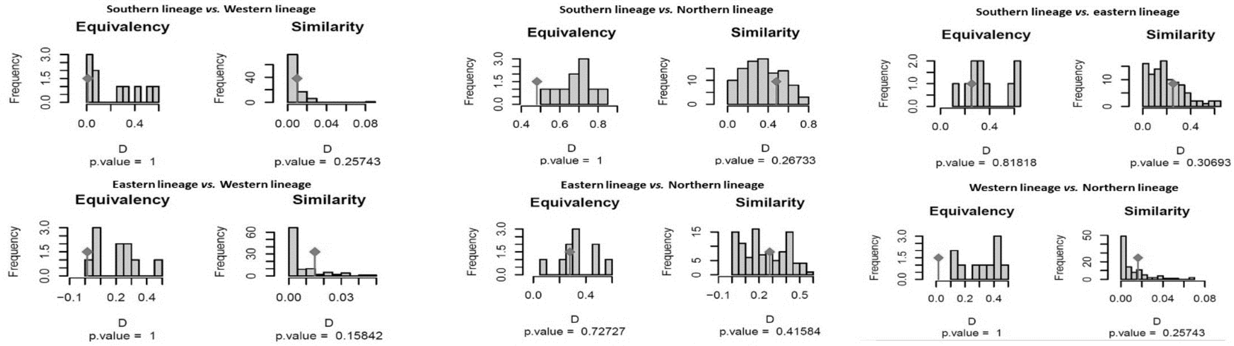
```
##########Script by Rodríguez-Rodríguez, 2020########


######Using Phyloclim niche evolution history (Heibl & Calenge, 2018) ########


#Intalling packages

install.packages(ape)

Install.packages("phyloclim")

#Opening libraries

library("ape")

library("phyloclim")


#Charging phylogeny
```

```
a<-file.choose("alytes.nex")
summary(a)
MyTree<-read.nexus(a)
write.nexus(MyTree)
#Plotting phylogeny
plot(MyTree, type = "phylogram", use.edge.length = TRUE,
    node.pos = NULL, show.tip.label = TRUE, show.node.label = TRUE,
    edge.color = "black", edge.width = 1, edge.lty = 1, font = 3,
    cex = par("cex"), adj = NULL, srt = 0, no.margin = FALSE,
    root.edge = FALSE, label.offset = 0, underscore = FALSE,
    x.lim = NULL, y.lim = NULL, direction = "rightwards",
    lab4ut = NULL, tip.color = "black", plot = TRUE,
    rotate.tree = 0, open.angle = 0, node.depth = 1,
    align.tip.label = FALSE)

#Opening  climatic data. In this case I have imported from excel and named it as CLIM

#Creating niche overlap matrix (Niolap)
niche.overlap(CLIM)
nicheoverlap<-niche.overlap(CLIM)

#Using age range correlation test

age.range.correlation(phy = MyTree, overlap = nicheoverlap, tri = "upper", n= 10
```

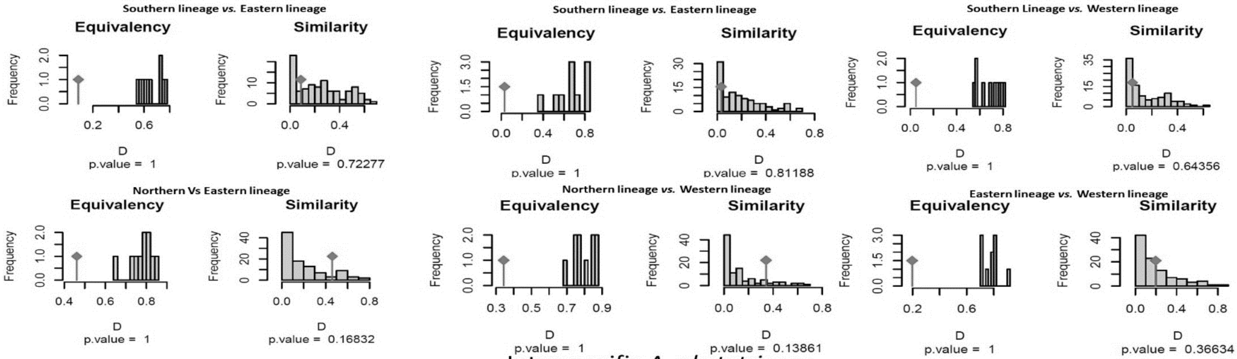
Supplementary material 4. Histogram outputs for Ecospat



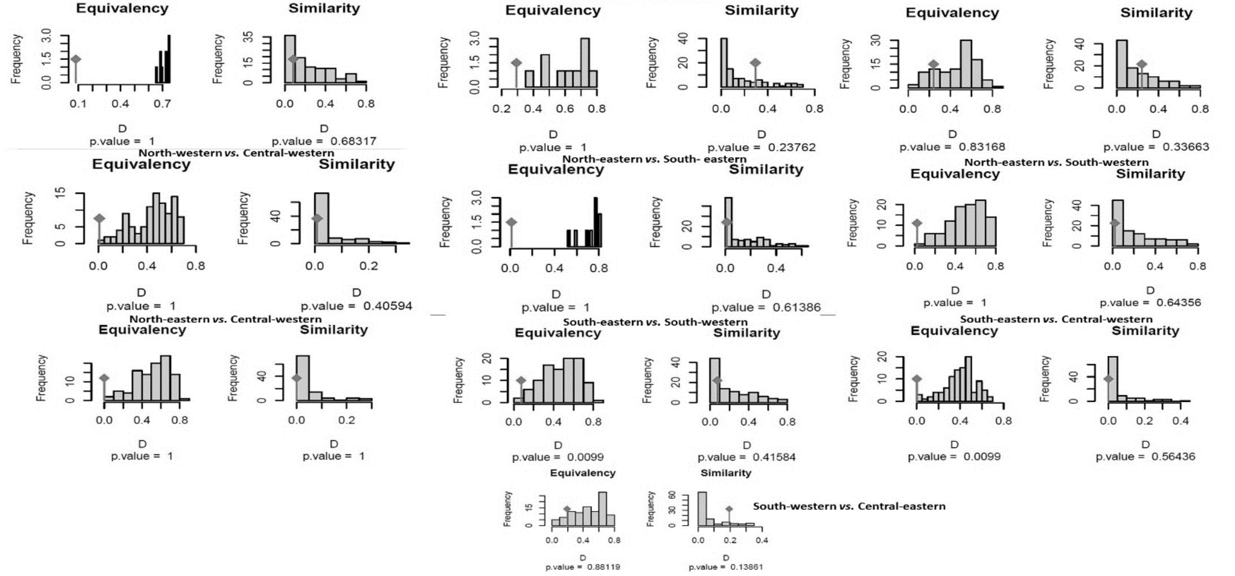
Intraspecific *A. cisternasii*



Intraspecific *A. dickhilleni*



Intraspecific *A. obstetricans*



Chapter 2

Supplementary material 1: Selected species with information on family and distribution.

Family	Species	Distribution
Pelobatidae	*Pelobates cultripes*	Iberian Peninsula and southern France (Recuero 2014)
Pelobatidae	*Pelobates varaldii*	Short coastal area of Morocco from Tanger to Safi (de Pous et al. 2012)
Bufonidae	*Bufo spinosus*	Iberian Peninsula, Northern Africa and Southern France (Ortiz Santaliestra 2014)
Bufonidae	*Bufotes boulengeri*	Widely distributed in the north of Africa from Western Sahara desert to Egypt (Martínez-Solano 2015)
Bufonidae	*Epidalea calamita*	Widely distributed in Europe from the Iberian Peninsula to Belarus and Ukraine, including some islands of United Kingdom (Gómez-Mestre 2014)
Bufonidae	*Sclerophrys mauritanica*	Areas of Morocco, Algeria, Tunisia, Ceuta and Melilla (Donaire-Barroso 2016)
Bufonidae	*Barbarophryne brongesmai*	Restricted to a coastal band and another thin band in the south of the Anti-Atlas, Morocco (Salvador et al. 2006)
Alytidae	*Alytes cisternasii*	The south-occidental part of the Iberian Peninsula (Beja et al. 2009)
Alytidae	*Alytes obstetricans*	Widely distributed in humid areas of Central-south Europe (Bosch et al., 2009a)
Alytidae	*Alytes maurus*	Only known for a few fragmented localities in the Rif and Middle Atlas mountains (Donaire-Barroso et al. 2009a)
Alytidae	*Alytes dickhilleni*	An endemism of oriental Betic region (Bosch et al. 2009b)
Alytidae	*Discoglossus scovazzi*	North Africa
Alytidae	*Discoglossus galganoi*	West and the south of the Iberian Peninsula (Martínex-Solano 2014)
Hylidae	*Hyla meridionalis*	Throughout North Africa, southern half of Iberian Peninsula, and a separated range cluster from North West Spain to South France, with introduced populations in several Islands (Donaire-Barroso et al. 2009b)
Ranidae	*Pelophylax perezi*	Iberian Peninsula
Ranidae	*Pelophylax saharicus*	North Africa

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Beja P, Bosch J, Tejedo M, Lizana M, Martínez-Solano I, Salvador A, García-Paris M, Recuero Gil E, Arntzen JW, Marquez R, Díaz Paniagua C (2009) *Alytes cisternasii*. The IUCN Red List of Threatened Species 2009: e.T55266A11283060. http://dx.doi.org/10.2305/IUCN.UK.2009.RLTS.T55266A11283060.en

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Supplementary material 2 (Distribution data). Geographical coordinates of distribution points used in this work

Species	x	y
Hyla meridionalis	-6.48	37.13
Hyla meridionalis	-7.28	37.22
Hyla meridionalis	-7.24	37.62
Hyla meridionalis	-6.96	38.13
Hyla meridionalis	-8.84	37.13
Hyla meridionalis	-8.72	37.67
Hyla meridionalis	-8.95	39.13
Hyla meridionalis	-7.19	39.93
Hyla meridionalis	-6.20	37.54
Hyla meridionalis	-5.79	37.92
Hyla meridionalis	-3.98	38.23
Hyla meridionalis	-5.64	36.16
Hyla meridionalis	-5.45	36.66
Hyla meridionalis	-3.54	36.73
Hyla meridionalis	-5.94	39.53
Hyla meridionalis	-6.06	40.20
Hyla meridionalis	-5.13	40.20
Hyla meridionalis	2.05	41.41
Hyla meridionalis	3.20	42.00
Hyla meridionalis	2.58	42.24
Hyla meridionalis	2.86	42.54
Hyla meridionalis	3.87	43.79
Hyla meridionalis	1.20	44.26
Hyla meridionalis	-6.25	34.95
Hyla meridionalis	-6.67	34.25
Hyla meridionalis	-6.97	33.73
Hyla meridionalis	-7.76	33.54
Hyla meridionalis	-5.49	34.04
Hyla meridionalis	-5.00	33.55
Hyla meridionalis	-4.11	34.09
Hyla meridionalis	-5.07	33.03
Alytes maurus	-5.24	35.16
Alytes maurus	-5.27	35.10
Alytes maurus	-5.49	35.26
Alytes maurus	-5.36	34.97
Alytes maurus	-5.35	35.52
Alytes maurus	-5.29	35.36
Alytes maurus	-5.25	35.18
Alytes maurus	-4.12	33.67
Alytes maurus	-4.21	33.64
Alytes maurus	-4.21	33.64
Alytes dickhilleni	-2.85	37.37

Alytes dickhilleni	-2.88	36.94
Alytes dickhilleni	-3.39	37.75
Alytes dickhilleni	-2.75	37.93
Alytes dickhilleni	-2.40	37.24
Alytes dickhilleni	-4.07	36.88
Alytes dickhilleni	-2.96	38.18
Alytes dickhilleni	-3.79	37.50
Alytes dickhilleni	-3.51	37.11
Alytes dickhilleni	-2.57	38.36
Alytes cisternasii	-6.30	41.96
Alytes cisternasii	-6.46	37.13
Alytes cisternasii	-3.86	38.48
Alytes cisternasii	-5.80	37.88
Alytes cisternasii	-5.98	40.05
Alytes cisternasii	-3.61	40.65
Alytes cisternasii	-6.59	40.16
Alytes cisternasii	-5.04	37.99
Alytes cisternasii	-6.18	39.01
Alytes cisternasii	-6.36	37.65
Alytes cisternasii	-5.43	38.91
Alytes cisternasii	-1.43	38.70
Alytes cisternasii	-8.68	37.10
Alytes cisternasii	-5.94	39.66
Alytes cisternasii	-8.79	37.73
Alytes cisternasii	-4.00	38.16
Alytes cisternasii	-8.60	38.11
Alytes cisternasii	-5.80	38.12
Alytes cisternasii	-7.43	37.30
Alytes obstetricans	1.43	42.35
Alytes obstetricans	-7.19	39.22
Alytes obstetricans	-8.46	43.33
Alytes obstetricans	2.32	42.18
Alytes obstetricans	-6.83	42.78
Alytes obstetricans	-2.41	43.03
Alytes obstetricans	-3.85	42.97
Alytes obstetricans	0.55	42.32
Alytes obstetricans	-5.80	40.15
Alytes obstetricans	-6.11	43.05
Alytes obstetricans	-4.82	43.15
Alytes obstetricans	0.69	40.77
Alytes obstetricans	-4.18	43.34
Alytes obstetricans	-0.54	38.66
Alytes obstetricans	3.09	42.22
Alytes obstetricans	0.11	42.56
Alytes obstetricans	2.65	42.06
Alytes obstetricans	-3.43	40.13
Alytes obstetricans	-0.78	42.55
Alytes obstetricans	-0.64	41.37
Alytes obstetricans	-6.57	39.35
Alytes obstetricans	3.94	43.68
Alytes obstetricans	4.48	44.34
Alytes obstetricans	1.45	45.07
Alytes obstetricans	1.05	46.85
Pelobates varaldii	-8.79	32.63
Pelobates varaldii	-6.84	34.03
Pelobates varaldii	-6.62	34.13
Pelobates varaldii	-6.32	34.18
Pelobates varaldii	-6.84	34.03
Pelobates varaldii	-6.74	34.00
Pelobates varaldii	-6.30	34.15
Pelobates varaldii	-6.62	34.13
Pelobates varaldii	-6.33	34.12
Pelobates varaldii	-6.43	34.27
Pelobates varaldii	-6.05	35.04
Pelobates varaldii	-6.03	35.04
Pelobates varaldii	-6.22	34.29
Pelobates varaldii	-6.56	34.02
Pelobates varaldii	-6.34	34.54
Pelobates varaldii	-6.42	34.28

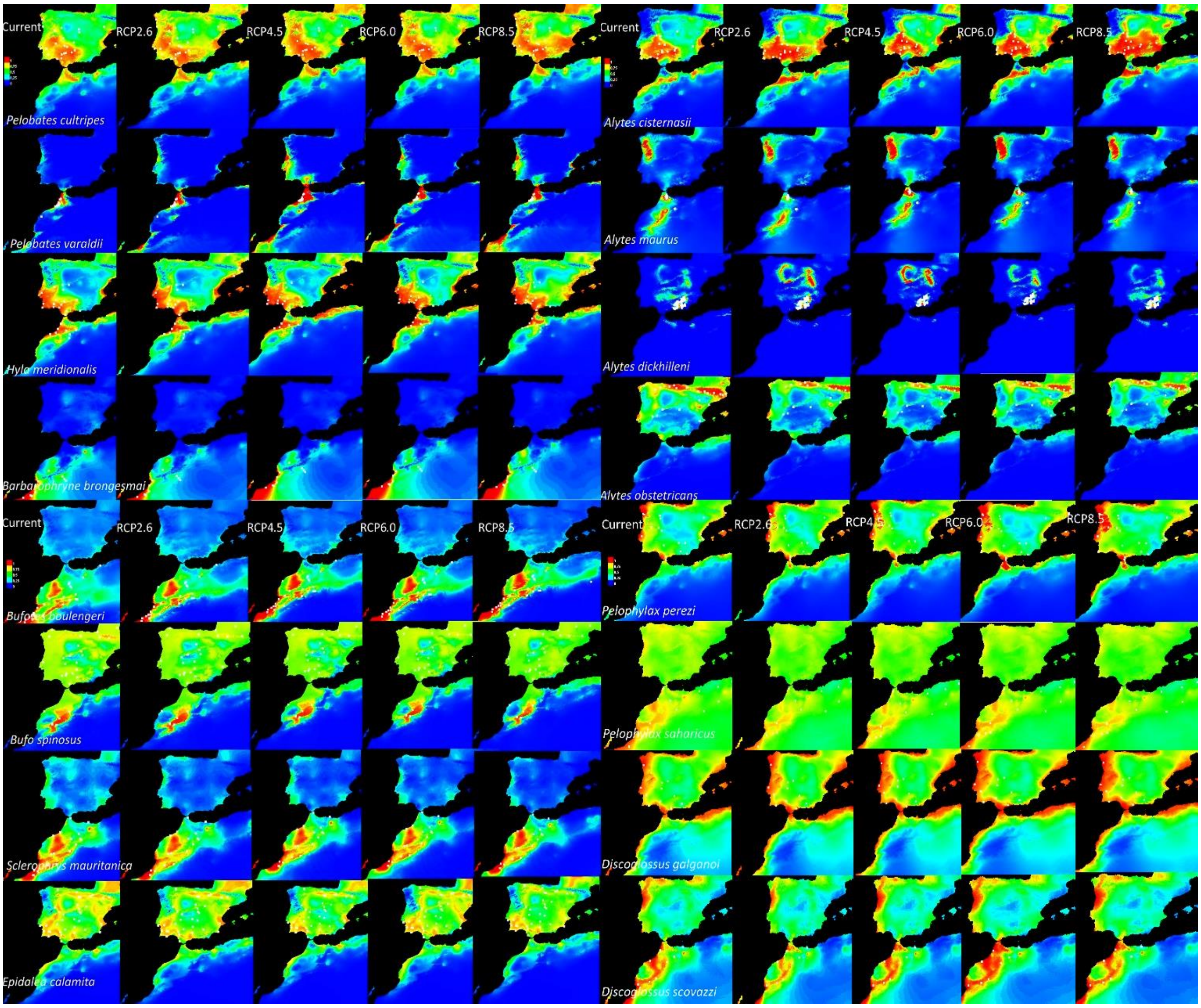
Pelobates varaldii	-6.20	34.94
Sclerophrys mauritanica	-10.49	28.49
Sclerophrys mauritanica	-10.43	28.50
Sclerophrys mauritanica	-8.54	29.73
Sclerophrys mauritanica	-7.68	31.29
Sclerophrys mauritanica	-5.44	35.85
Sclerophrys mauritanica	-3.05	35.30
Sclerophrys mauritanica	-4.35	33.79
Sclerophrys mauritanica	-3.98	33.05
Sclerophrys mauritanica	-4.13	33.18
Sclerophrys mauritanica	-5.34	33.13
Sclerophrys mauritanica	-4.73	32.44
Sclerophrys mauritanica	-5.56	31.54
Sclerophrys mauritanica	-6.84	34.03
Sclerophrys mauritanica	-7.76	33.55
Sclerophrys mauritanica	-7.68	31.29
Sclerophrys mauritanica	-8.05	31.19
Sclerophrys mauritanica	-7.30	29.90
Sclerophrys mauritanica	-9.87	28.98
Sclerophrys mauritanica	-10.34	29.10
Pelobates cultripes	-3.71	41.11
Pelobates cultripes	-6.18	37.23
Pelobates cultripes	-6.16	37.75
Pelobates cultripes	-5.82	37.89
Pelobates cultripes	-5.57	37.93
Pelobates cultripes	-4.74	38.00
Pelobates cultripes	-4.30	38.28
Pelobates cultripes	-8.32	38.49
Pelobates cultripes	-9.14	38.52
Pelobates cultripes	-7.08	40.95
Pelobates cultripes	-8.77	41.50
Pelobates cultripes	-6.18	41.32
Pelobates cultripes	-8.88	42.45
Pelobates cultripes	-0.15	41.48
Pelobates cultripes	-3.66	40.71
Pelobates cultripes	-4.43	39.34
Pelobates cultripes	-5.72	38.89
Pelobates cultripes	-6.99	39.22
Pelobates cultripes	-5.90	39.91
Pelobates cultripes	3.87	43.79
Pelobates cultripes	4.82	43.43
Pelobates cultripes	4.27	44.42
Pelobates cultripes	-5.39	38.75
Pelobates cultripes	-6.76	37.14
Pelobates cultripes	-8.05	37.74
Epidalea calamita	-6.70	43.35
Epidalea calamita	-6.68	43.27
Epidalea calamita	-3.98	43.02
Epidalea calamita	-2.08	42.46
Epidalea calamita	-1.75	42.61
Epidalea calamita	-0.31	42.20
Epidalea calamita	0.55	42.31
Epidalea calamita	-3.46	42.29
Epidalea calamita	-3.71	42.34
Epidalea calamita	-3.91	41.26
Epidalea calamita	-3.66	40.71
Epidalea calamita	-2.61	41.06
Epidalea calamita	-3.59	40.30
Epidalea calamita	-3.43	40.13
Epidalea calamita	-4.36	39.35
Epidalea calamita	-3.66	39.14
Epidalea calamita	-0.54	38.75
Epidalea calamita	-0.74	37.61
Epidalea calamita	-2.82	37.92
Epidalea calamita	-9.15	38.44
Epidalea calamita	-8.32	38.48
Epidalea calamita	-8.61	37.76
Epidalea calamita	-7.96	37.67
Epidalea calamita	-7.74	37.64

Epidalea calamita	-7.98	37.02
Epidalea calamita	-6.34	37.61
Epidalea calamita	-5.61	37.96
Epidalea calamita	-4.30	38.28
Epidalea calamita	-6.19	37.23
Epidalea calamita	-6.15	36.37
Epidalea calamita	-4.37	37.49
Epidalea calamita	-5.98	39.25
Epidalea calamita	-5.84	39.98
Epidalea calamita	-6.48	40.33
Epidalea calamita	-6.75	38.69
Epidalea calamita	-7.09	40.94
Bufo spinosus	-8.80	32.92
Bufo spinosus	-5.34	32.90
Bufo spinosus	-6.72	32.02
Bufo spinosus	-7.72	31.30
Bufo spinosus	-7.70	31.22
Bufo spinosus	-7.14	31.15
Bufo spinosus	-8.10	42.77
Bufo spinosus	-7.73	43.68
Bufo spinosus	-3.78	42.87
Bufo spinosus	-4.12	41.60
Bufo spinosus	-1.19	42.70
Bufo spinosus	-1.47	43.41
Bufo spinosus	0.14	42.59
Bufo spinosus	2.13	42.65
Bufo spinosus	-3.53	40.32
Bufo spinosus	-3.68	40.85
Bufo spinosus	-5.26	40.27
Bufo spinosus	-4.36	39.35
Bufo spinosus	-6.13	39.92
Bufo spinosus	-8.93	39.43
Bufo spinosus	-5.90	39.18
Bufo spinosus	-4.33	38.45
Bufo spinosus	-0.51	38.67
Bufo spinosus	-5.33	36.52
Bufo spinosus	-4.24	37.42
Bufo spinosus	-4.89	37.93
Bufo spinosus	-5.82	37.68
Bufo spinosus	-5.71	37.93
Bufo spinosus	-6.35	37.61
Bufo spinosus	3.30	44.04
Bufo spinosus	1.25	43.92
Barbarophryne brongesmai	-11.53	28.25
Barbarophryne brongesmai	-10.51	28.58
Barbarophryne brongesmai	-10.34	28.50
Barbarophryne brongesmai	-9.87	28.98
Barbarophryne brongesmai	-9.83	29.81
Barbarophryne brongesmai	-9.42	30.47
Barbarophryne brongesmai	-7.94	31.89
Barbarophryne brongesmai	-5.77	30.79
Barbarophryne brongesmai	-5.54	30.53
Barbarophryne brongesmai	-6.04	31.09
Bufotes boulengeri	-3.23	34.14
Bufotes boulengeri	-5.16	33.43
Bufotes boulengeri	-5.25	33.28
Bufotes boulengeri	-5.04	32.92
Bufotes boulengeri	-4.93	32.79
Bufotes boulengeri	-4.28	31.86
Bufotes boulengeri	-4.02	31.21
Bufotes boulengeri	-4.30	31.22
Bufotes boulengeri	-5.16	31.12
Bufotes boulengeri	-5.95	31.52
Bufotes boulengeri	-6.09	30.51
Bufotes boulengeri	-7.97	31.25
Bufotes boulengeri	-9.05	32.73
Bufotes boulengeri	-8.69	30.55
Bufotes boulengeri	-9.66	30.06
Bufotes boulengeri	-8.85	29.06

Bufotes boulengeri	-10.17	29.38
Bufotes boulengeri	-10.11	29.01
Bufotes boulengeri	-10.34	28.50
Bufotes boulengeri	-10.44	28.99
Bufotes boulengeri	-10.79	28.78
Bufotes boulengeri	-11.34	28.49
Bufotes boulengeri	-11.43	28.11
Bufotes boulengeri	-15.72	23.61
Bufotes boulengeri	-13.11	27.14
Bufotes boulengeri	-0.82	35.75
Bufotes boulengeri	4.68	36.00
Bufotes boulengeri	6.62	36.14
Bufotes boulengeri	3.67	32.48
Pelophylax perezi	-5.45	36.428
Pelophylax perezi	-5.487	36.114
Pelophylax perezi	-6.627	36.627
Pelophylax perezi	-2.401	36.832
Pelophylax perezi	-3.317	37.172
Pelophylax perezi	-5.767	37.999
Pelophylax perezi	-2.648	38.301
Pelophylax perezi	-6.086	39.945
Pelophylax perezi	-6.486	40.303
Pelophylax perezi	-6.344	41.957
Pelophylax perezi	-8.811	43. 117
Pelophylax perezi	-4.174	43.329
Pelophylax perezi	3.296	42.323
Pelophylax perezi	2.912	39.877
Pelophylax perezi	-3.577	38.33
Pelophylax perezi	-0.489	38.698
Pelophylax perezi	-8.775	42.198
Pelophylax perezi	-0.349	39.456
Pelophylax perezi	-0.085	38.800
Pelophylax perezi	0.090	41.694
Pelophylax perezi	-8.875	37.204
Pelophylax perezi	-8.988	39.211
Pelophylax perezi	-7.586	40.402
Pelophylax perezi	-8,632	42,091
Pelophylax perezi	-6.257	41.581
Pelophylax saharicus	-5.897	35.556
Pelophylax saharicus	-4.852	34.043
Pelophylax saharicus	-7.854	31.208
Pelophylax saharicus	-3.986	32.931
Pelophylax saharicus	-7.807	30.029
Pelophylax saharicus	-9.531	29.695
Pelophylax saharicus	-10.903	28.280
Pelophylax saharicus	-8.529	29.910
Pelophylax saharicus	-0.005	32.732
Pelophylax saharicus	-0.653	35.244
Pelophylax saharicus	2.033	35.778
Pelophylax saharicus	2,128	33,952
Pelophylax saharicus	5.921	33.973
Pelophylax saharicus	5.903	35.507
Pelophylax saharicus	7.325	36.976
Pelophylax saharicus	4.106	36.456
Discoglossus galganoi	-8.810	42.175
Discoglossus galganoi	-6.087	39.945
Discoglossus galganoi	-5.267	40.144
Discoglossus galganoi	-6.172	39.208
Discoglossus galganoi	-8.392	38.421
Discoglossus galganoi	-9.153	39.310
Discoglossus galganoi	-8.015	37.066
Discoglossus galganoi	-6.344	37.607
Discoglossus galganoi	-4.753	36.681
Discoglossus galganoi	-3.221	37.312
Discoglossus galganoi	-4.297	38.284
Discoglossus scovazzi	-6.287	34.871
Discoglossus scovazzi	-4.272	34.066
Discoglossus scovazzi	-5.317	33.404
Discoglossus scovazzi	-7.761	33.545

Discoglossus scovazzi	-6.882	31.436
Discoglossus scovazzi	-7.832	31.010
Discoglossus scovazzi	-9.48	30.677
Discoglossus scovazzi	-2.988	35.224
Discoglossus scovazzi	-6.949	30.825
Discoglossus scovazzi	-6.287	34.871
Discoglossus scovazzi	-4.163	33.650

Supplementary material 3: Estimated climatic suitability distribution for all the species (table 2) from current conditions to four predicted climate scenarios for 2050 (IPPC5). Distribution localities (white dots) are also represented.



Chapter 3

Table S1. Amphibian species included in this study.

Species	**Authors**
Alytes cisternasii	Boscá, 1879
Alytes dickhilleni	Arntzen & García-Paris, 1995
Alytes muletensis	Sanchiz & Adrover, 1979
Alytes obstetricans	(Laurenti, 1768)
Bombina bombina	(Linnaeus, 1761)
Bombina variegata	(Linnaeus, 1758)
Bufo spinosus	(Daudin, 1803)
Bufo bufo	(Linnaeus, 1758)
Epidalea calamita	Laurenti, 1768
Bufotes viridis	(Laurenti, 1768)
Calotriton arnoldi	Carranza & Amat, 2005
Calotriton asper	(Dugès, 1852)
Chioglossa lusitanica	Bocage, 1864
Discoglossus galganoi	Capula, Nascetti; Lanza, Bullini & Crespo, 1985
Discoglossus montalentii	Capula, Nascetti; Lanza, Bullini & Crespo, 1984
Discoglossus pictus	Otth, 1837
Discoglossus sardus	Tschudi, 1837
Euproctus montanus	(Savi, 1838)
Euproctus platycephalus	(Gravenhorst, 1829)
Hyla arborea complex	Linnaeus, 1758
Hyla intermedia	Boulenger, 1882
Hyla meridionalis	Boettger, 1874
Hyla sarda	(De Betta, 1853)
Ichthyosaura alpestris	(Laurenti, 1768)
Lissotriton boscai	Lataste y Tourneville, 1879
Lissotriton helveticus	(Razoumowsky, 1789)
Lissotriton italicus	(Peracca, 1898)
Lissotriton montandoni	(Boulenger, 1882)
Lissotriton vulgaris	(Linnaeus, 1758)
Pelobates cultripes	Cuvier, 1829
Pelobates fuscus	(Laurenti, 1768)
Pelobates syriacus	Boettger, 1889
Pelodytes ibericus	Sánchez-Herraiz, Barbadillo, Machordom & Sanchíz, 2000
Pelodytes punctatus complex	Daudin, 1802

Pelophylax cretensis	(Beerli, Hotz, Tunner, Heppich & Uzzell, 1994)
Pelophylax epeiroticus	(Schneider, Sofianidou & Kyriakopoulou-Sklavounou, 1984)
Pelophylax perezi	(López-Seoane, 1885)
Pelophylax ridibundus	(Pallas, 1771)
Pelophylax shqipericus	(Hotz, Uzzell, Guenther, Tunner & Heppich, 1987)
Pleurodeles waltl	Michahelles, 1830
Proteus anguinus	Laurenti, 1768
Rana arvalis	Nilsson, 1842
Rana dalmatina	Bonaparte, 1840
Rana graeca	(Boulenger, 1891)
Rana iberica	Boulenger, 1879
Rana italica	(Dubois, 1987)
Rana latastei	(Boulenger, 1879)
Rana pyrenaica	Serra-Cobo, 1993
Rana temporaria	Linnaeus, 1758
Salamandra atra	Laurenti, 1768
Salamandra corsica	Savi, 1838
Salamandra lanzai	Nascetti, Andreone, Capula & Bullini, 1998
Salamandrina perspicillata/terdigitata	(Savi, 1821)/(Bonnaterre, 1789)
Salamandra salamandra	(Linnaeus, 1758)
Speleomantes ambrosii	(Lanza, 1955)
Speleomantes flavus	(Stefani, 1969)
Speleomantes genei	(Temminck & Schlegel, 1838)
Speleomantes imperialis	(Stefani, 1969)
Speleomantes italicus	(Dunn, 1923)
Speleomantes sarrabusesis	Lanza, Leo, Forti, Cimmaruta, Caputo & Nascetti, 2001
Speleomantes strinatii	(Aellen, 1958)
Speleomantes supramontis	(Lanza, Nascetti & Bullini, 1986)
Triturus cristatus complex	Laurenti, 1768
Triturus marmoratus	(Latreille, 1800)
Triturus pygmaeus	(Wolterstorff, 1905)

Table S2. Squamate reptile species included in this study.

Species	**Authors**
Acanthodactylus erythrurus	(Schinz, 1833)
Algyroides fitzingeri	(Wiegmann, 1834)
Algyroides marchi	Valverde, 1958
Algyroides nigropunctatus	(A.M.C. Duméril & Bibron, 1839)
Anguis cephallonica	Werner, 1894
Anguis colchica/fragilis/graeca	Linnaeus, 1758
Archaeolacerta bedriagae	Mertens, 1921
Blanus cinereus/mariae	Vandelli, 1797/ Albert & Fernández, 2009
Chalcides bedriagai	Boscá, 1880
Chalcides chalcides	Linnaeus, 1758
Chalcides ocellatus	Forsskal, 1775
Chalcides striatus	Cuvier, 1829
Chamaeleo chamaeleon	(Linnaeus,1758)
Coronella austriaca	Laurenti, 1768
Coronella girondica	Daudin, 1803
Dalmatolacerta oxycephala	Arnold, Arribas & Carranza, 2007
Darevskia praticola	(Eversmann, 1834)
Dinarolacerta montenegrina	Ljubisavljevic, Arribas & Carranza, 2007
Dinarolacerta mosorensis	(Kolombatovic, 1886)
Dolichophis caspius	(Gmelin, 1789)
Eirenis modestus	(Martin, 1838)
Elaphe quatuorlineata	(Lacépède, 1789)
Elaphe sauromates	(Pallas, 1814)
Eremias arguta	Pallas, 1773
Eryx jaculus	(Linnaeus, 1758)
Euleptes europaea	(Gené, 1839)
Hellenolacerta graeca	Arnold, Arribas & Carranza, 2007
Hemorrhois hippocrepis	(Linnaeus, 1758)
Hemidactylus turcicus	(Linnaeus, 1758)
Hierophis gemonensis	(Laurenti, 1768)
Hierophis viridiflavus	(Lacépède, 1789)
Iberolacerta aranica	(Arribas, 1993)
Iberolacerta aurelioi	(Arribas, 1994)
Iberolacerta bonnali	(Lantz, 1927)
Iberolacerta cyreni	(Müller & Hellmich, 1937)
Iberolacerta galani	Arribas, Carranza & Odierna, 2006

Iberolacerta horvathi	(Méheley, 1904)
Iberolacerta martinezricai	(Arribas, 1996)
Iberolacerta monticola	(Boulenger, 1905)
Lacerta agilis	Linnaeus, 1758
Lacerta bilineata	Daudin, 1802
Lacerta schreiberi	Bedriaga, 1878
Lacerta trilineata	Bedriaga, 1886
Lacerta viridis	(Laurenti, 1768)
Stellagama stellio	(Linnaeus, 1758)
Macroprotodon brevis	Günther, 1862
Macroprotodon cucullatus	Geoffroy Saint-Hilaire, 1827
Macrovipera schweizeri	(F.Werner, 1935)
Malpolon insignitus	(Geoffroy Saint-Hilaire, 1827)
Malpolon monspessulanus	(Hermann, 1804)
Mediodactylus kotschyi	(Steindacher, 1870)
Montivipera xanthina	(Gray, 1849)
Natrix maura	(Linnaeus, 1758)
Natrix astreptophora	(López Seoane, 1884)
Natrix natrix	(Linnaeus, 1758)
Natrix tessellata	(Laurenti, 1768)
Ophiomorus punctatissimus	Bibron & Bory De St. Vincent, 1833
Ophisops elegans	Ménétriés, 1832
Platyceps collaris	(Müller, 1878)
Platyceps najadum	(Eichwald, 1831)
Podarcis bocagei	(Seoane, 1885)
Podarcis carbonelli	Pérez Mellado, 1981
Podarcis cretensis	(Wettstein, 1952)
Podarcis erhardii	(Bedriaga, 1882)
Podarcis gaigeae	(Werner, 1930)
Podarcis hispanica complex	(Steindachner, 1870)
Podarcis lilfordi	(Günther, 1874)
Podarcis melisellensis	(Braun, 1877)
Podarcis milensis	(Bedriaga, 1882)
Podarcis muralis	(Laurenti, 1768)
Podarcis peloponnesiacus	(Bibron & Bory, 1833)
Podarcis pityusensis	(Boscá 1883)
Podarcis sicula	(Rafinesque, 1810)
Podarcis tauricus	(Pallas, 1814)
Podarcis tiliguerta	(Gmelin, 1789)
Podarcis waglerianus	Gistel, 1868

Psammodromus algirus	Linnaeus, 1758
Psammodromus hispanicus complex	Fitzinger, 1826
Pseudopus apodus	(Pallas, 1775)
Tarentola mauritanica	Linnaeus, 1758
Telescopus fallax	(Fleischmann, 1831)
Timon nevadensis	(Buchholz, 1963)
Timon lepidus	(Daudin, 1802)
Typhlops vermicularis	Merren, 1820
Vipera ammodytes	(Linnaeus, 1758)
Vipera aspis	(Linnaeus, 1758)
Vipera berus	(Linnaeus, 1758)
Vipera latastei	Boscá, 1878
Vipera seoanei	Lataste, 1879
Vipera ursinii	(Bonaparte, 1835)
Zamenis longissimus	(Laurenti, 1768)
Zamenis situla	(Linnaeus, 1758)
Zamenis scalaris	(Schinz, 1822)
Zootoca vivipara	Von Jacquin, 1787

Figure S1. Patterns pf species richness for European squamate retiles (A) and amphibians (B). Map projection is Lambert azimuthal equal area (ETRS89).

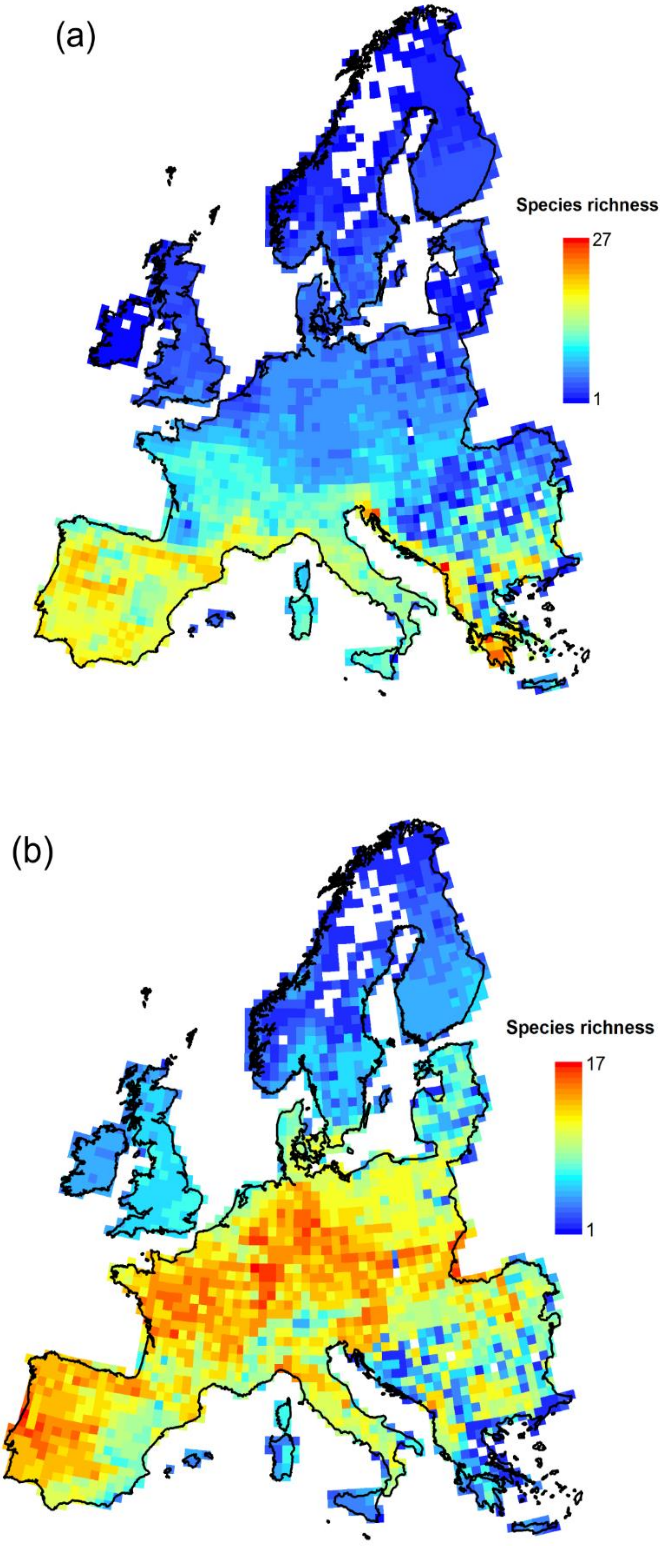
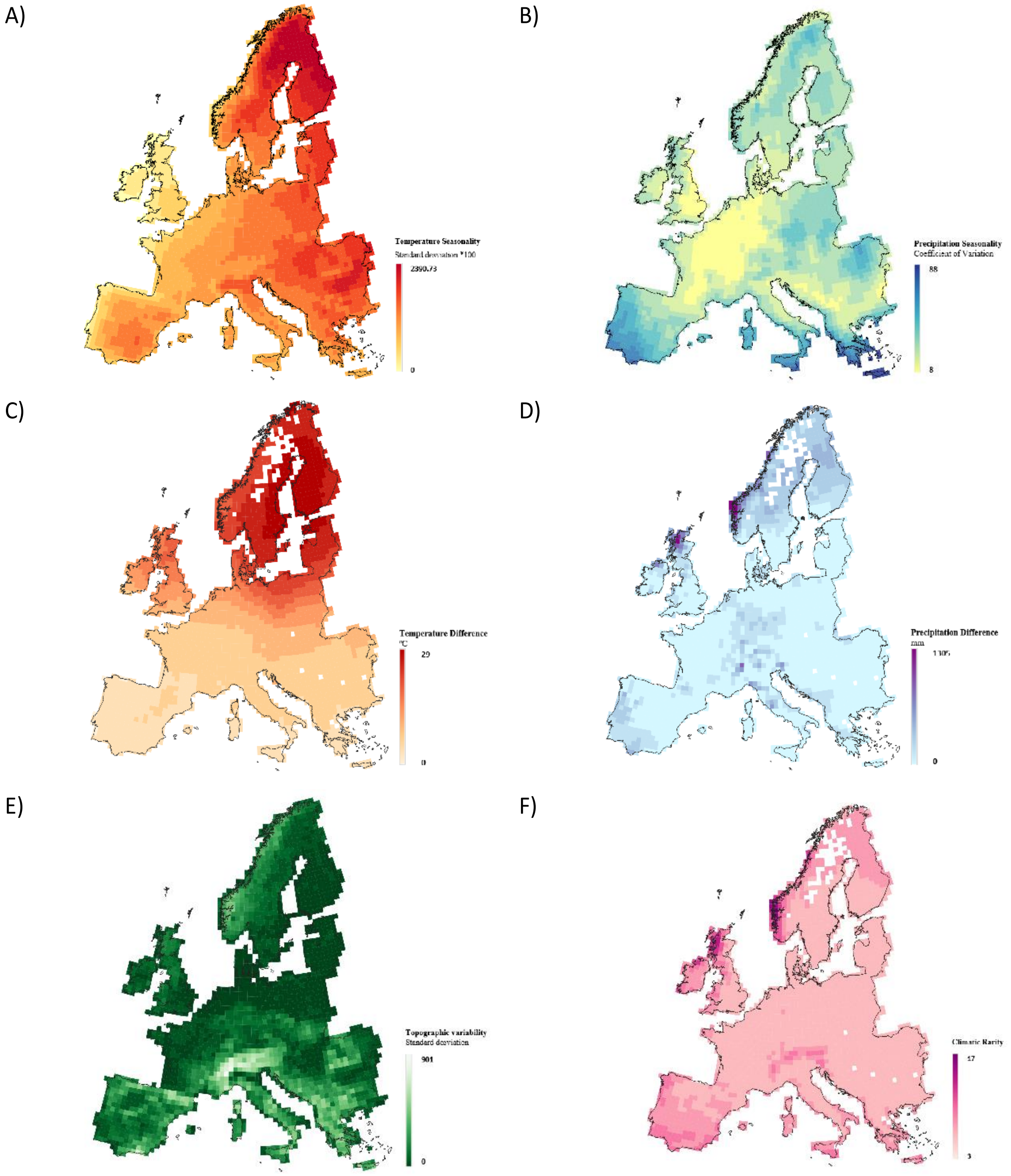


Figure S2. Maps of main potential predictors of mean range-size patterns used in the analysis. A) Temperature seasonality, B) Precipitation seasonality, C) Temperature difference LGM, D) Precipitation difference LGM, E) Topographic variability, F) Climatic rarity, and G) Biome area.



G)

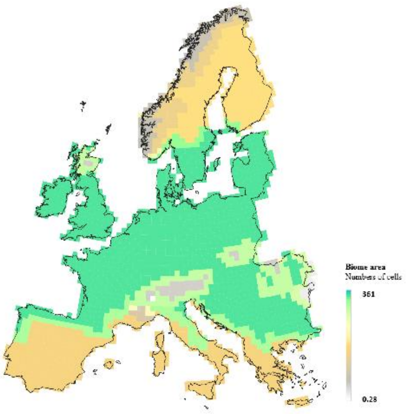
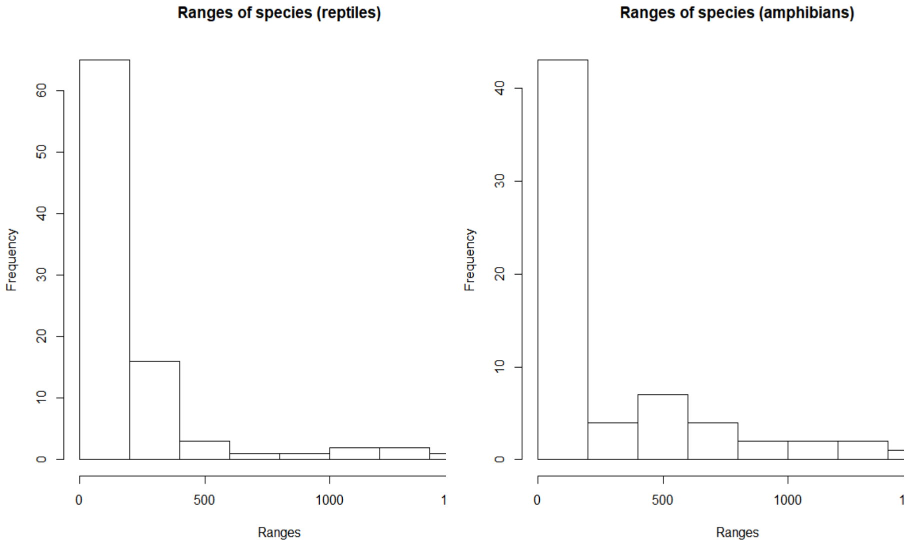


Figure S3. Histograms range size frequency distribution for a) reptiles and b) amphibians.


a)

b)

Figure S4. Relationship between mean range-size and latitude for a) reptiles and b) amphibians.

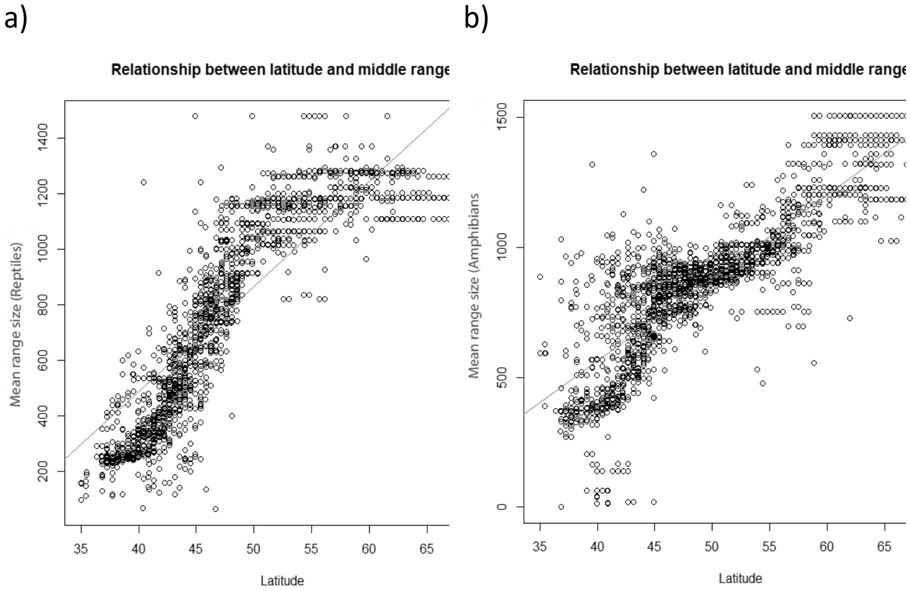


Figure S5. Relationship between mean range-size of reptiles and the six potential predictors used in the analysis. A) Temperature seasonality, B) Precipitation seasonality, C) Temperature difference LGM, D) Precipitation difference LGM, E) Topographic variability, F) Climatic rarity, and G) Biome area.

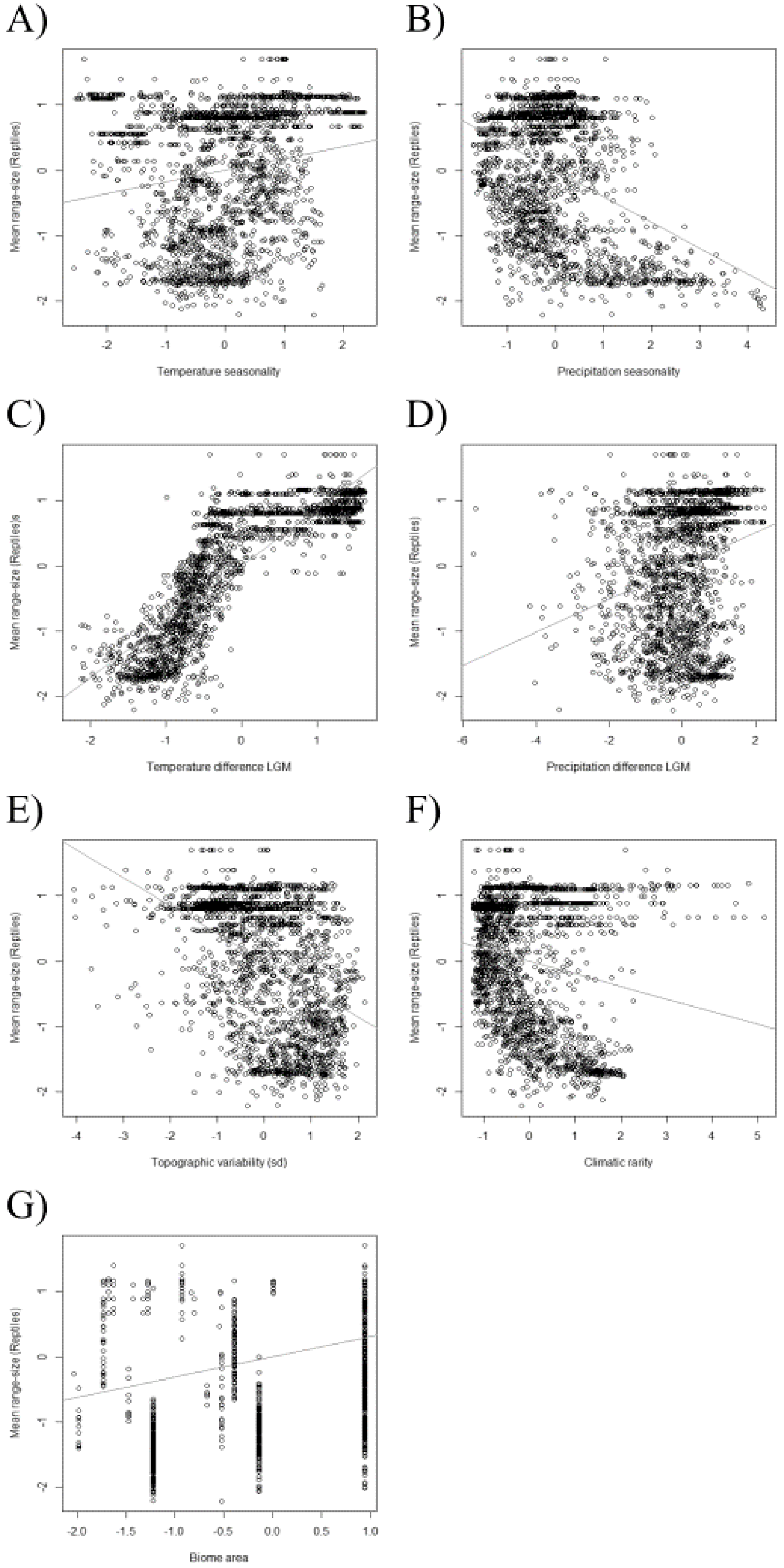
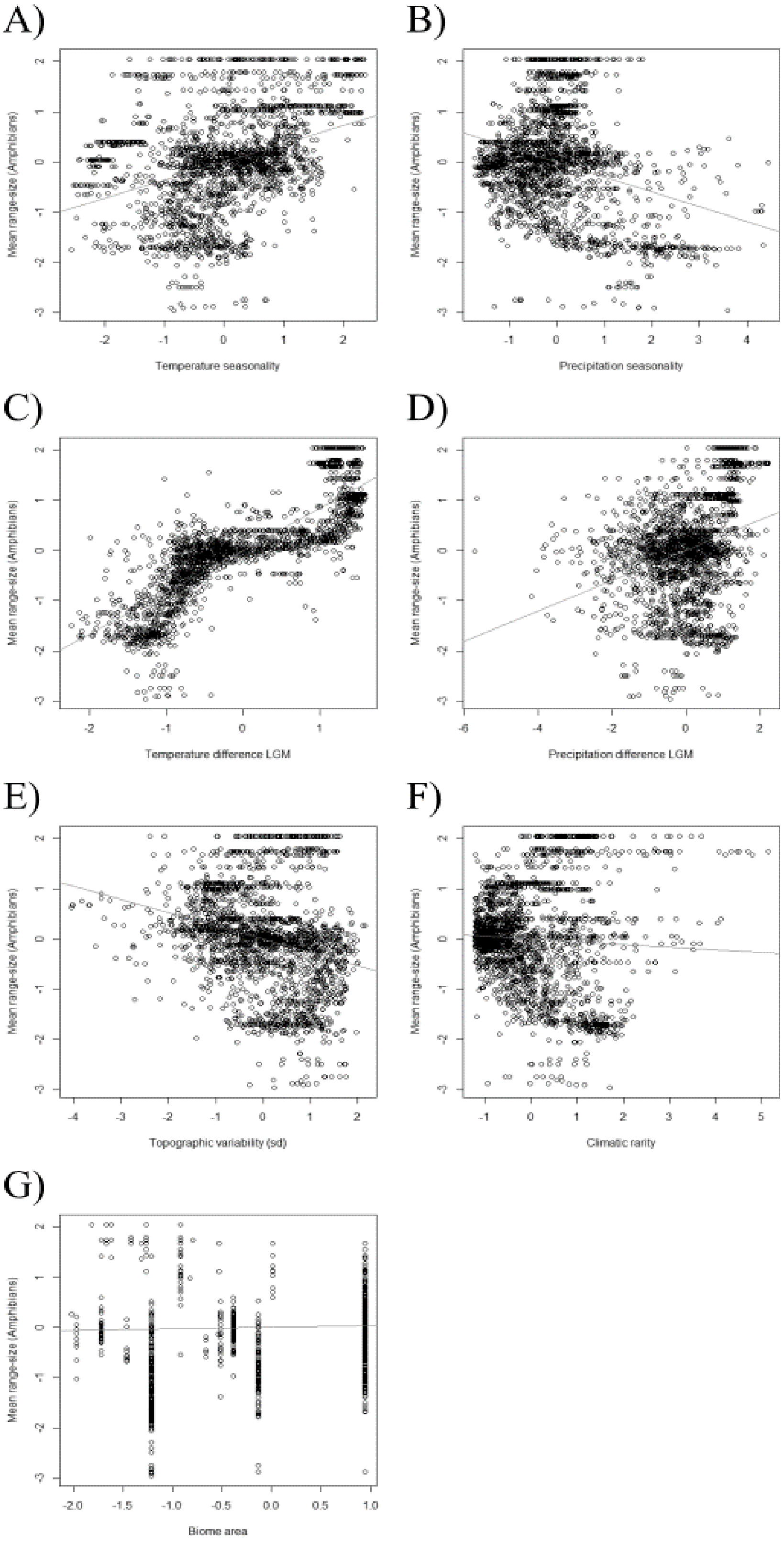
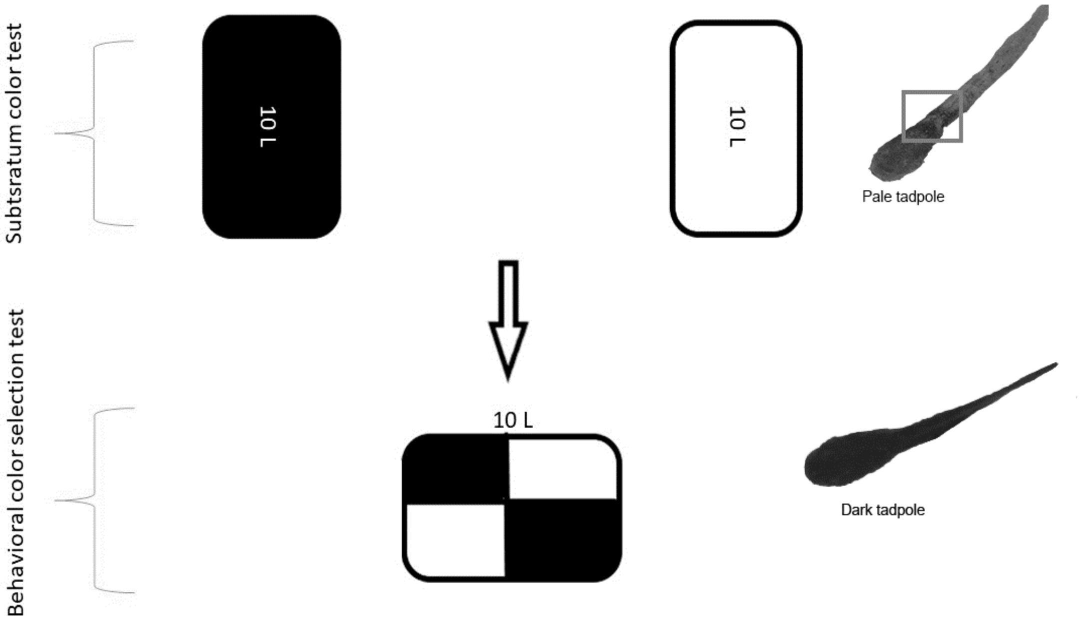


Figure S6. Relationship between mean range-size of amphibians and the six potential predictors used in the analysis. A) Temperature seasonality, B) Precipitation seasonality, C) Temperature difference LGM, D) Precipitation difference LGM, E) Topographic variability, F) Climatic rarity, and G) Biome area.

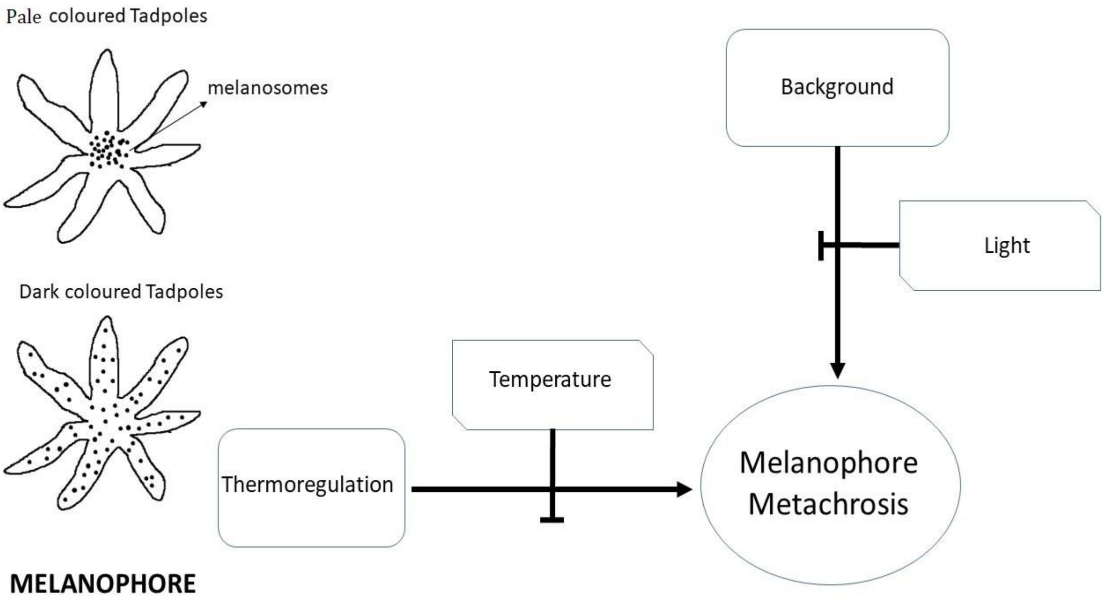


Chapter 5

Supplementary material 1. Experimental design of water container for colour change and colour selection experiments. Criteria to classify larvae under dark or pale coloration is also shown. The characteristic “V” of the species was visible only in tadpoles classified as pale.



Supplementary material 2. Left: Schematized melanophore metachrosis under both treatments. In pale coloured tadpoles, melanosomes are aggregated in melanophore cell body. In dark coloured tadpoles, melanosomes are dispersed radially by means of microtubules. Right: Proposed environmental model explaining the mechanism implicated in melanophore metachrosis observed in amphibian tadpoles



Published works derived from this thesis

-Rodríguez-Rodríguez, E.J., Beltrán, J.F., Tejedo, M., Nicieza, A.G., Llusia, D., Márquez, R., Aragón, P. (2020). Niche models at inter- and intraspecific levels reveal hierarchical niche differentiation in midwife toads. *Sci Rep* 10, 10942. https://doi.org/10.1038/s41598-020-67992-6
Chapter 1

-Rodríguez-Rodríguez, E. J., Beltrán, J. F., El Mouden, E. H., Slimani, T., Márquez, R., & Donaire-Barroso, D. (2020). Climate change challenges IUCN conservation priorities: a test with western Mediterranean amphibians. *SN Applied Sciences*, *2*(2), 1-11. https://doi.org/10.1007/s42452-020-2002-2

Chapter 2

-Rodríguez-Rodríguez, E., Beltrán, J., & Márquez, R. (2020). Melanophore metachrosis response in amphibian tadpoles: effect of background colour, light and temperature, *Amphibia-Reptilia*, Online-first, 1-8. doi: https://doi.org/10.1163/15685381-bja10032

Chapter 5

-Submitted or in preparation, **Chapter 3** and **Chapter 4.**

https://orcid.org/0000-0002-1170-0788

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Collaborating institutions:

Congress comunications derived from this work.

-Rodríguez-Rodríguez, E.J Beltrán, J.F., Tejedo, M., González-Miras, E., Benítez, M., Requez, R. Sapos parteros (*Alytes* spp.), plantas y factores ambientales: ¿Qué nos están diciendo? XV Congreso Luso-Español de Herpetología/ XIX Congreso Español de Herpetología. Biología y Conservación de Herpetos en el Antropoceno. 5-8 septiembre de 2018. Salamanca.

-Rodríguez-Rodríguez, E.J Beltrán, J.F., Márquez, R., Tejedo, M. Phenotypic plasticity of the genus Alytes sp. under different moisture conditions at metamorphic stage. VII congreso bianual de la SESBE- 5-7 febrero de 2020. Sevilla

Additional publications generated or with participation during this work.

-Beltrán, J.F., Rodríguez-Rodríguez, E.J. (2019) New contributions on amphibians and reptile's distribution on the Gran Bilbao Coast, Biscay (Spain) during the decade of 1970-80. (2019). *Boletín de la Asociación Herpetológica Española*. 30 (1).

-Carmona-González, R., Rodríguez-Rodríguez, E.J., Fuentes, I., Beltrán, J. (2020). Necrophiliac behaviour of *Epidalea calamita* (Laurenti, 1768) in Sierra Norte de Sevilla Natural Park, SW Spain. *Boletín de la Asociación Herpetológica Española.*31 (1).

-García-Cardenete, L., Flores, M.A., Yubero, S., Rodríguez-Rodríguez, E.J. (2019). Oued Noun (Morocco): Southwest limit for the genus *Discoglossus*. *Boletín de la Asociación Herpetológica Española.* 30 (2).

-Gross, F., Rodríguez-Rodríguez, E.J. (2019). Nueva cita de *Mauremys leprosa* en el Paraje Natural Brazo del Este (Sevilla), donde se consideraba extinto. *Boletín de la Asociación Herpetológica Española*. 30 (1).

-Rodríguez-Rodríguez, E.J., Carmona-González, R., García-Cardenete, L. (2018). Actualización de la distribución de los reptiles en la provincia de Sevilla. *Boletín de la Asociación Herpetológica Española* 29(2).

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