Eco-evolutionary dynamics and the evolution of phenotypic plasticity

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Eco-evolutionary dynamics and the evolution of phenotypic plasticity

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Department of Wetland Ecology
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"Eco-evolutionary dynamics and the evolution of phenotypic plasticity"

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CERTIFICAN:

Que los trabajos de investigación desarollados en la Memoria de Tesis Doctoral **"Eco-evolutionary dynamics and the evolution of phenotypic plasticity"** son aptos para ser presentados por la Licenciada Lee, Hyeun-Ji ante el Tribunal que en su día se designe, para aspirar al grado de Doctora por la Universidad de Sevilla.

Y para que así conste, y en cumplimiento de las disposiciones legales vigentes, firman el presente documento en Sevilla, a 26 de Noviembre de 2020.

Director

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To my dear sister, Hyeun-Ju.
현주에게.

Table of contents

- p. 15 - Summary (English)
- p. 18 - Resumen (Español)
- p. 22 - General Introduction

- p. 38 - Chapter 1: Dwarfism in close continental amphibian populations despite lack of genetic isolation

- p. 99 - Chapter 2: Population divergence of developmental plasticity in the spadefoot toad *Pelobates cultripes,* using RNA-seq

- p. 163 - Chapter 3: Shifts in the developmental rate of spadefoot toad larvae cause decreased complexity of pigmentation patterns post metamorphosis

- p. 195 - Chapter 4: Demographic consequences of predator-induced life history shifts in the waterflea *Daphnia magna*

- p. 227 - General Discussion
- p. 235 - Conclusions
- p. 240 - Acknowledgments

Summary

Throughout this dissertation, I strive to understand how the interaction between the environment and the genotype can shape phenotypic variation within a species. Variation in phenotypic expression can either result from differential gene expression in response to environmental variability (environmentally-sensitive genes) and/or genetic adaptation to the varying environment along a geographical gradient. I aim to answer how the interplay of ecological factors and underlying genetic mechanisms allow organisms to express the adequate phenotype in its current environment, and I incorporate an array of study questions and systems in my dissertation.

In my first chapter, I investigate an intriguing case of continental dwarfism in amphibians, focusing on the natterjack toad *Epidalea calamita* in southern Spain. The dwarf populations in Doñana are about 30% smaller than conspecific populations only about 60 km North, while lacking an efficient geographical barrier. To fully understand the various factors that are driving the intraspecific size variation in this species, I incorporate standard metabolic rate analysis, stable isotope analysis, skeletochronology, male advertisement call description, female behavioral assays, and population genetics using neutral markers. Moreover, I have analyzed and described the climatic variables of each of the regions. I found that the observed cline in size is present in the absence of

genetic differentiation across populations, while there are ecological differences across the studied populations.

From my second to third chapter, I am using the spadefoot toad *Pelobates cultripes* as model species to study the evolution of phenotypic plasticity. For my second chapter I raise tadpoles of *P. cultripes* and conduct water drop experiments on 10 populations of *P. cultripes* tadpoles. Half of those populations originate from long lasting ponds and half from short lasting ponds. These populations are predicted to have evolved a different capacity to respond to pond desiccation and accelerate development, thus the phenotypic as well as transcriptomic reaction norm are expected to vary across populations. Further, since developmental acceleration is achieved through increased stress hormone levels, corticosterone levels are expected to vary across individuals raised in different experimental treatments.

In continuation of this experiment, for my third chapter, I have taken photographs of the dorsal pigmentation pattern of the *P. cultripes* individuals originating from my experiment at metamorphosis. The photographs were used to test whether the dorsal pigmentation of juvenile toads differed according to the environmental stress experienced across life stages. I have found that individuals originating from the low water treatment (i.e. experienced environmental stress) metamorphosed with a darker dorsum with more continuous blotches, whereas individuals raised under benign conditions metamorphosed with a lighter dorsum and more segregated blotches. The ecological significance of dorsal pigmentation in spadefoot toads requires further study. My dissertation is one of the few studies in literature that has explored the

dorsal pigmentation of amphibians across different life stages, in the context of phenotypic plasticity.

In my fourth chapter, I use the waterflea *Daphnia magna*, a study system that is readily manipulated in lab conditions due to its fast reproductive cycle. This allowed me to conduct large-scale experiments comprising several generations and populations, a process which was more complicated with amphibians. I conducted an experiment on five genotypes originating from two subpopulations of *Daphnia*, of which only half of the treatment received a predator cue. Subsequently, I described how the shifts in life-history traits experienced through phenotypically plastic responses of *D. magna* have altered the population dynamics of this species.

Resumen

A lo largo de esta tesis me he propuesto comprender cómo las interacciones entre el genotipo y el ambiente pueden moldear la variación fenotípica intraespecífica. La variación fenotípica dentro de una especie puede ser debida a una expresión génica diferencial en respuesta distintos estímulos ambientales, o bien estar causada por divergencias genéticas (adaptativas o no). Mi objetivo es conocer cómo la interacción entre factores ecológicos externos y los mecanismos genéticos subyacentes permiten a los organismos expresar un fenotipo adecuado a las condiciones ambientales imperantes. Esta cuestión es apasionante pero compleja, y para abordarla he planteado una serie de preguntas más concretas y he buscado distintos sistemas de estudio apropiados para responderlas.

En mi primer capítulo investigo un intrigante caso de marcada variación fenotípica en un carácter de enorme importancia en todos los organismos: el tamaño corporal. En concreto me he enfocado en comprender las causas de un marcado gradiente de tamaño en poblaciones continentales de anfibios, usando como modelo de estudio al sapo corredor, *Epidalea calamita*, en el suroeste de España. Las poblaciones enanas del Parque Nacional de Doñana son

aproximadamente un 30% menores que poblaciones a tan sólo 60 km de distancia, en ausencia de barrera biogeográfica alguna. Para entender la contribución de distintos factores al establecimiento de este escarpado gradiente intraespecífico de tamaño en una escala geográfica tan pequeña, he combinado análisis de tasa metabólica, análisis de isótopos estables, esqueletocronología, análisis bioacústico de la variación en el canto, análisis comportamentales, y análisis genético-poblacionales usando marcadores neutrales. Además, he analizado y descrito en detalle la variación entre las distintas poblaciones en las principales variables bioclimáticas. He encontrado que la clina de tamaño observada tiene lugar en ausencia de una diferenciación genética entre poblaciones, y sin un aislamiento genético aparente, si bien existen diferencias ecológicas entre las poblaciones estudiadas y todo indica que diferencias en humedad y temperatura durante la etapa de crecimiento juvenil post-metamórfico pueda ser decisiva en la formación de este patrón.

En los siguientes dos capítulos, uso el sapo de espuelas, *Pelobates cultripes*, como especie modelo para estudiar la evolución de la interacción entre el genotipo y el fenotipo, es decir la evolución de la plasticidad fenotípica. Para mi segundo capítulo he realizado un análisis comparado de la capacidad de respuesta al riesgo de desecación del medio acuático en larvas de 10 poblaciones de sapo de espuelas, pertenecientes a dos regiones (entorno de Doñana y sierra de Madrid). Para cada una de estas dos regiones, escogí las poblaciones de modo que la mitad provenieran de medios acuáticos de largo hidroperiodo y la mitad de medios de corto hidroperiodo. Para este estudio colecté puestas de las distintas poblaciones y crié las larvas resultantes en condiciones estándar de laboratorio, exponiendo la mitad de ellas a simulación de desecación de la charca

reduciendo el nivel de agua. Mi hipótesis inicial fue que estas poblaciones diferirían en su capacidad de respuesta a la desecación del medio mediante aceleración de su desarrollo larvario, y esperaba que hubiesen por tanto divergido en sus normas de reacción fenotípicas y transcriptómicas. Además, puesto que la aceleración en el desarrollo es detonada por el incremento en la secreción de glucocorticoides, esperaba que los niveles corticosterona variaran entre individuos conforme al tratamiento experimental y su población de origen.

Como continuación de este experimento, para mi tercer capítulo tomé fotografías del patrón de pigmentación dorsal de individuos de *P. cultripes* que fueron metamorfoseando. Mediante estas fotografías cuantifiqué la variación en el patrón dorsal de pigmentación para examinar la idea de que este patrón variara conforme a la población de origen y el grado de estrés ambiental experimentado durante el desarrollo larvario. Encontré que los individuos provenientes del tratamiento de bajo nivel de agua, que habían sido inducidos a acelerar su desarrollo (i.e. habían experimentado mayor estrés ambiental), metamorfosearon con un dorso más oscuro y con manchas más continuas, mientras que individuos que habían experimentado condiciones más benignas metamorfosearon con un dorso más claro y manchas más segregadas. El significado ecológico de la pigmentación dorsal en sapos de espuela y otros anfibios debe ser estudiado con más detenimiento en el futuro. Este capítulo es uno de los pocos ejemplos en la literatura que ha estudiado variaciones en la pigmentación dorsal a través de distintas fases ontogenéticas, y he mostrado que se trata de un carácter plástico.

En mi cuarto capítulo, me planteo la pregunta de cuáles pueden ser las consecuencias demográficas poblacionales de la plasticidad fenotípica individual.

Abordar esta cuestión con anfibios era inviable y recurrí a otro sistema de estudio clásico en la literatura de plasticidad, el de defensas inducidas por depredador en pulgas de agua, *Daphnia magna*. Este organismo tiene un ciclo reproductivo muy rápido y es fácil de manipular en el laboratorio, lo que me permitió lanzar un experimento a gran escala que comprendiera varias generaciones y poblaciones en un tiempo razonable. Llevé a cabo un experimento con cinco genotipos originados a partir de dos subpoblaciones de *Daphnia*, en el que la mitad de las réplicas fueron expuestos a pistas de depredador. A continuación, describí cómo los cambios inducidos por la presencia de depredadores en los caracteres de historia de vida de los distintos clones de *D. magna* alteraron la dinámica demográfica, acortando el tiempo de generación y alcanzando antes la capacidad de carga del sistema.

General Introduction

In the ever-changing environment in which we live in, populations are globally exposed to environmental fluctuations, and such environmental variation induces phenotypic changes in the individuals conforming those populations. Organisms must express an adequate phenotype to survive and reproduce in the environment they experience, and such fine-tuning of the phenotype to the environment is mainly achieved by a genomic response to ecological input modulated through the neuroendocrine system. The extent to which organisms can alter their phenotype to increase their fitness to match the reigning environmental conditions will depend on its genetic background, their capacity to assess environmental cues, cue reliability, and the magnitude of environmental changes in relation to their potential for phenotypic change. Such interactions may undergo selection and result in local adaptation (Kawecki and Ebert 2004, Hereford 2009, Sanford and Kelly 2011). However, the extent to which intraspecific phenotypic variation is adaptive will depend on the equilibrium among genetic variation, gene flow, and selection (Savolainen *et al*. 2007).

In highly variable environments, environmentally-sensitive genes may be differentially expressed in response to environmental variability and give rise to "phenotypic plasticity", which can be defined as the ability of a single genotype to produce various phenotypes depending on the environmental conditions (West-Eberhard 1989, West-Eberhard 2003). The varying degrees of the environmental heterogeneity may steer the evolution of phenotypic plasticity in different ways; phenotypic plasticity may persist if favored by environmental heterogeneity and hence is adaptive (Lind and Johansson 2007, Lázaro-Nogal *et al*. 2015), or alternatively, if the environment becomes homogeneous selection may favor the adequate phenotype in that environment and undergo canalization, a process known as genetic assimilation (Grether 2005, Pigliucci *et al*. 2006). Hence, phenotypic plasticity is not only subject to evolution, but may actively act as leader of the course of evolution (Price *et al*. 2003, Schwander and Leimar 2011).

In this dissertation, I do not limit myself to a single species or system, but employ various study systems to strive to understand the correlation between nature and nurture and the evolution of phenotypic plasticity, choosing study systems in accord to the scientific question posed. To do so, I start with i) addressing a case of steep variation in body size across a short geographical scale in the natterjack toad (*Epidalea calamita*) in southwestern Spain, and describe various ecological factors contributing to this phenomenon, as well as the genetic structure of the studied populations. I then go on to ii) explore the phenotypic plasticity of spadefoot toads (*Pelobates cultripes*) by conducting a common garden experiment and analyzing both phenotypic reaction norms as well as transcriptomic reaction norms, and continue to iii) ask whether environmental stress experienced during the larval phase of *P. cultripes* may

have an influence on the dorsal pigmentation pattern of this species, and if so, to what extent and what the ecological consequences would be. Finally, I iv) expand my research onto the population level to test the demographic consequences of environmentally-induced individual shifts in phenotype, conducting an intergenerational experiment with the waterflea *Daphnia magna.*

A case of extreme dwarfism in the natterjack toad

Size variation across the animal kingdom, ranging from some minute ants to gigantic blue whales, has long intrigued biologists. Size not only varies among species but also within a species. This is particularly informative because it reflects how the combination of genotype and varying environments can shape different phenotypes within a species. The main external determinants of intraspecific size variation include abiotic factors such as temperature, photoperiod, altitude, and latitude (Hoffmann 1978, Ashton 2002, Rodríguez *et al*. 2008), or selection forces such as competition or predation (Lomolino 1985). Remarkable differences in body size within species over short geographical distances have usually been reported for islands or island-like systems, where effective geographical barriers have led populations to evolve independently from each other (Lomolino 2005, Keogh *et al*. 2005, Jaffe *et al*. 2011). Meanwhile, in continental systems, comparatively greater distances have to be travelled to be able to observe similar degrees in size variation (Brumfield and Remsem 1996, Cruz *et al*. 2005).

However, in Doñana National Park (southwestern Spain), amphibians are remarkably smaller in comparison to other populations in the vicinity while

apparently lacking an efficient geographical barrier such as a big mountain chain or a river. In this dissertation I focus on the natterjack toad (*Epidalea calamita*) as example of an amphibian species showing such local reduction in body size. Natterjack toad populations from Doñana experience a 2.1 fold change in body mass in comparison to populations only as little as 37 km northwards from the National Park (Sierra Norte de Sevilla). For this study, I studied six populations: two dwarf sized populations of Doñana National Park (Navazo del Toro and El Puntal), two geographically intermediate populations, whose individuals were already as large as those of the northern populations (Aznalcazar and Gerena), and finally two large sized populations of the Sierra Norte de Sevilla (Constantina and El Pedroso).

To explore whether the observed size differences were due to genetic differentiation or ecological factors, I tested for genetic isolation of the dwarf populations using neutral markers; whether they differ in their trophic status analyzing stable isotope traces; whether their metabolism differs, comparing their standard metabolic rate; whether populations differ in age structure and age at sexual maturity employing skeletochronology and telomere length. Moreover, I have also quantified variation in advertisement calls across populations and experimentally tested for behavioral reinforcement of the body size gap through female preferences. Last, but not least, I have described the climatic variables of the regions to be able to connect the toads' ecology with the local climate. I found this phenomenon of local dwarfism to occur in the absence of genetic isolation and with similar age structures and effective population sizes. Dwarf populations, however, differed in trophic status, were found to have lower metabolic rate, and exposed to drier and warmer climatic conditions,

suggesting that size differences are driven by local climatic conditions, particularly influential during the post-metamorphic stage.

Developmental plasticity in the spadefoot toad

My second chapter focuses on understanding the evolution of phenotypic plasticity and how genetic accommodation occurs over the course of adaptive evolution across populations within a species. The Western spadefoot toad (*Pelobates cultripes*) is a developmentally plastic organism, as it can adjust its developmental rate according to the environmental conditions experienced. This allows tadpoles of *P. cultripes* to grow large without significant advancements in development under benign conditions or accelerate its development when conditions worsen, as when ponds begin to dry up. Producing such adaptive responses to rapid environmental changes is key to survive perturbations such as the current climate change. Developmental acceleration is achieved through increased corticosterone and thyroid hormone (Kulkarni *et al*. 2017), but comes at the expense of a high metabolic cost, increased oxidative stress, and metamorphosing at a smaller size (Gomez-Mestre *et al*. 2013, Kulkarni *et al*. 2017).

Phenotypically plastic responses in divergent environments can be subject to divergent selection and if environmental variation decreases, can even evolve into canalized alternative phenotypes. This process is known as genetic accommodation (West-Eberhard 2003, Ehrenreich and Pfennig 2015). Spadefoot toads conform an ancient group of species whose most common recent ancestor showed similar levels of developmental plasticity as present day's *P. cultripes*.

Meanwhile, *Spea multiplicata* and *Scaphiopus couchii* adapted to breed in short lasting ponds, and while *S. multiplicata* retained an intermediate degree of plasticity, development in *S. couchii* became rather canalized into an accelerated version of the ancestral state, while constitutively expressing high levels of stress hormones (Kulkarni *et al*. 2017).

To understand how the observed pattern of variation in developmental rate among species originates, we first need to understand the mechanisms causing adaptive divergence in developmental responsiveness among populations within species. Thus, to study genetic accommodation among populations I focused on populations of *P. cultripes* originating from both short- and long-lasting ponds, across two main geographical areas. I conducted common garden experiments to explore how individuals originating from populations with varying regimes of pond flooding (hydroperiod) differ in their ability to respond to pond drying by accelerating their development. I sampled 3-5 egg clutches from each of six populations in Madrid and Segovia provinces (central Spain) and four populations from Huelva province (southern Spain). Within each region, half of the populations chosen breed in long-lasting ponds and the other half in short-lasting ponds. That way, I explored potential differences in adaptive plasticity according to the varying hydroperiods of their pond of origin. All tadpoles were raised under identical conditions, except that the water level of half the tadpoles from each population was dropped to induce developmental acceleration. The tadpoles exposed to risk of pond drying (low water treatment simulating desiccation) were expected to accelerate their development and metamorphose earlier. To see how individuals originating from different populations with varying hydroperiods were able to accelerate

their development when confronted with pond drying, I recorded duration of larval period and size at metamorphosis. In addition, I measured their snout length, hindlimb length, and snout-to-vent length to explore what consequences developmental acceleration bore on their morphology upon metamorphosis.

One week after exposure to low-water treatment, three replicates of each egg clutch for each treatment were sacrificed to obtain tail tissue for corticosterone assays and liver tissue for RNA-seq analysis. Since developmental acceleration is achieved through increased levels of stress hormone and enhanced metabolic activity (Kulkarni et al. 2017), I was interested in looking at the degree of stress hormone levels across populations originating from short-or long-lasting ponds. Individuals that accelerated their development were expected to have a higher level of stress hormones, whereas individuals originating from short-lasting ponds were predicted to constitutively express high levels of stress hormones, as they may have evolved constitutive fast development in their ponds of origin. To explore these hypotheses, I conducted RNA-seq analyses and mapped the reads onto a high quality draft genome of the species available to my research group and in preparation for publication. In addition to differential gene expression, I also looked at standing genetic variation across populations by employing genome-wide-association-studies (GWAS) looking for among-population variation in single nucleotide aq<polymorphisms (SNPs) in the same set of Illumina reads used for RNA-Seq.

Pigmentation pattern in the context of phenotypic plasticity

Metamorphosis was thought to represent a clean slate, a big anatomical and functional divide between subsequent life stages that offers the opportunity for relatively independent trait evolution (Moran 1994, Watkins 2001). However, it has been well documented that variation in growth and development during one life stage affects the phenotype of subsequent stages (Pechenik 2006, Moore and Martin 2019). Such carry-over effects have been well studied in amphibians, regarding the consequences of altered larval growth on post-metamorphic size, weight, and shape (Gomez-Mestre *et al*. 2010, Van Allen *et al*. 2010, Boes and Benard 2013). However, little is known about how developmental conditions experienced during the larval stages of species with complex life cycles can affect the pigmentation pattern of post-metamorphic individuals. Much empirical work on pigmentation has rather focused on its role as honest signals indicating individual quality (Hill *et al*. 2006, Weiss *et al*. 2011, Galvan *et al*. 2018) or condition dependent signals showing less attractive traits if individuals experienced less favorable conditions during their development (Tibbetts 2010, Sheehan and Tibbetts 2011, Hill 2011). Fewer empirical studies have focused on describing features of animal pigmentation *pattern* (e.g. complexity, heterogeneity, lacunarity), and its relationship to body condition or stress endured. Nonetheless, Pérez-Rodríguez *et al*. (2013) have shown that fractal dimensions are an adequate way of assessing individual quality of red-legged partridge; animals exposed to low food conditions showed lower fractal dimension.

To our knowledge, no attempt has been made to quantify general features of pigmentation patterns in amphibians before. Amphibian larvae can decouple growth and differentiation to adjust their development to local environmental

conditions (Gomez-Mestre *et al*. 2010, Touchon *et al*. 2015), constituting an ideal system to test for consequences of plastic alterations of development on pigmentation. As more elaborately outlined for the previous chapter, spadefoot toads accelerate their development in response to pond desiccation to shorten their larval period and metamorphose earlier by means of increased corticosterone levels and metabolic rate, which leads to allometric changes in body shape (Gomez-Mestre and Buchholz 2006, Gomez-Mestre *et al*. 2013). In the third chapter of my dissertation, I test whether the dorsal pigmentation pattern of juvenile spadefoot toads (*P. cultripes*) is affected by developmental alterations during the larval phase, thereby linking developmental plasticity, truncated growth, increased corticosterone, and potential effects on pigmentation. I used digital imaging to estimate parameters describing different aspects of the texture and complexity of the dorsal pattern of juvenile spadefoot toads, such as entropy and fractal dimension (Pérez-Rodríguez *et al*. 2017), and tested the impact of environmentally induced alterations on larval development on the dorsal pigmentation of the terrestrial phase of *P. cultripes*.

The weight of phenotypic plasticity on population demography

In my last and fourth chapter, I finally move on to population-level consequences of phenotypically plastic responses. Organisms constantly experience environmental stresses throughout their lives, and environmental perturbations capable of altering the phenotype of the individuals exposed can have an impact on population dynamics (Rudolf and Singh 2013, Van Allen and Rudolf 2013). This is possible by either the direct changes in the number of

individuals, which pass from the first life stage to the next (e.g. recruitment), or carry-over effects, that might lead to individual variation (e.g. rate of maturation, survival, or fecundity). Quantitative and qualitative effects of the phenotype across life stages are linked interdependently; plasticity might improve the quality of an organism while lowering its long-term survival (Vonesh and Osenberg 2003, Gomez-Mestre *et al*. 2013, Burraco *et al*. 2017). Environmental stress experienced during ontogeny has been shown to influence the mean individual phenotype across life stages (Beckerman *et al*. 2002). For instance, exposure to mercury during the larval stage led to a one-year-delayed maturity and to a reduction of the number of eggs produced each year. Subsequently, this led to the reduction of larval competition, and thus had a positive influence on population growth (Willson *et al*. 2012). In contrast, it has also been suggested that a 3-year delayed maturity due to density-dependent reduction in size influenced the population dynamics of the marbled salamander negatively (Taylor *et al*. 2006). Hence, to fully assess the viability and structure of a population, it is essential to take an organismal approach and take into consideration the individual fecundity and life history of each individual comprising the population.

This last chapter was carried out during a short research stay in Leuven, Belgium, where I aimed to explore the plastic responses to predator cues, and how this subsequently influences population dynamics, using the waterflea *Daphnia magna* as model organism. The host lab in Leuven had *D. magna* clones with strong plastic responses to the presence of fish, while each genotype differed in their capacity to plastically respond to predator cues (clones selected from Stoks *et al*. 2016). *D. magna* clones are an ideal study system for plasticity,

since all the phenotypic variation observed is plastic, it has a short generation time and relatively large populations can be kept under laboratory conditions. This was of huge advantage for my dissertation, as it was realistically not feasible to run experiments on several generations and various populations with amphibians. The experimental design included 5 clones for each of two subpopulations, all originating from a single population, and half of those were exposed to predator cues (i.e. fish kairomone) and the other half was not. Aquaria were inoculated with the same water inoculum. I expected faster development and higher abundance of eggs to be produced by clones exposed to predator cues, and I expected these changes to have a strong impact on the population demography of this species. I explored how alternative environmentally induced phenotypes contributed to population decline or growth by estimating population size, extinction risk and population growth rate.

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Chapter 1

Dwarfism in close continental amphibian populations despite lack of genetic isolation

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RH: Causes of amphibian dwarfism

Dwarfism in close continental amphibian populations despite lack of genetic isolation

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Abstract

Ample variation in body size is common in vertebrates over extensive geographical distances, or in isolated populations, where effective geographical barriers may cause dwarfism or gigantism. Here we study potential causes of extreme size reduction in continental populations of amphibians within a short geographical distance and in the absence of geographical barriers. Natterjack toads (*Epidalea calamita*) in Doñana National Park (Spain) experience up to 2.1-fold difference in body mass in as little as 37 km. Studying six populations divergent in body size, we tested for genetic isolation of the dwarf populations using multilocus genotypes (16 microsatellites), and explored whether populations differ in trophic status (through stable isotope analysis), standard

metabolic rate, and growth pattern, senescence and age structure (conducting telomere length assays and skeletochronology). We also recorded advertisement calls across populations and experimentally tested for behavioural reinforcement of the body size variation through female preferences. Local dwarfism in these populations occurs in the absence of genetic isolation and while maintaining relatively high effective population sizes. Dwarf populations, however, are exposed to drier and warmer climatic conditions, have different trophic status, show lower mass-specific metabolic rate, and male advertisement calls with a higher dominant frequency. Juvenile growth differed among populations, reaching the adult stage at different body sizes. Altogether, our results suggest a significant influence of environmental conditions on the physiology and ecology of the Doñana *E. calamita* populations, mainly affecting toads between metamorphosis and sexual maturity. Further experimental and genomic studies focusing on these early life stages are necessary to dissect the relative roles of the environment and adaptive genetic differentiation on this phenomenon.

Keywords: intraspecific size variation, continental dwarfism, body size, anurans, *Epidalea calamita*

Introduction

Body size is one of the most important features of organisms because it largely influences their life history, generation time, metabolism, and ecology (Martin and Palumbi 1993, Woodward et al. 2005). Intraspecific body size variation over broad geographical distances within species is partly shaped by abiotic factors

according to altitude and latitude associated with temperature, photoperiod, or precipitation (Hoffmann 1978, Ashton 2002, Rodríguez et al. 2008). For instance, some taxonomic groups grow to larger sizes in higher altitudes (Bergmann's rule), as selection in colder climates favours a lower surface to volume ratio to minimize heat loss (Ashton 2002, Meiri et al. 2005). While seemingly valid for endotherms (Meiri and Dayan 2003), this pattern is not universal since ectotherms do not always follow Bergmann´s rule (Mousseau 1997, Adams and Church 2008, Pincheira-Donoso et al. 2008; but see Cruz et al. 2005). Further, ecological interactions (i.e. competition, predation, resource limitation) are also important selection forces giving rise to intraspecific size variation (Lomolino 1985).

Remarkable differences in body size within species over much shorter geographical distances (as short as 30 km) than in continental systems have been reported for islands or island-like systems (Lomolino 2005, Keogh et al. 2005, Jaffe et al. 2011). When islands are formed, colonizing organisms adapt to local resources, competitors and predators or lack thereof. Depending on the relative importance of intraspecific competition, ecological release from predators or competitors, or resource limitation (Lomolino 2005), island populations can evolve gigantic or dwarf versions of their mainland counterparts (Keogh et al. 2005). Generally, island dwarfism in large species is driven by resource limitation and island gigantism in small species is due to competitive release (Lomolino 1985). Large mammals (i.e. mammoths, elephants, and ungulates (Roth 1990, Raia and Meiri 2006, Herridge and Lister 2012)) are well-known examples of island dwarfism, while chelonians (Jaffe et al. 2011) and lizards (Runemark et al. 2015) exhibit island gigantism. Tiger snakes evolved

both gigantism and dwarfism within only 30 km of distance according to the prey size in the islands they inhabited (Keogh et al. 2005). Remarkably, snakes residing on islands with prey items of similar size to those in the mainland were also similar in size to their mainland conspecifics, stressing the importance of ecological resources in shaping the body size of this system (Keogh et al. 2005). Island dwarfism and gigantism syndromes not only implicate mere size differences but involve important life history trait alterations in gonadal maturation rate, size at sexual maturation, and degree of sexual display (Raia et al. 2010).

In general, large size is considered beneficial (Roff 1992, Andersson 1994), and numerous studies show it is positively correlated with fitness (Semlitsch et al. 1988, Wiklund and Kaitala 1995, Taylor et al 1998). However, Kingsolver and Pfennig (2004) pointed out that the benefits of large size would only persist if size is subject to directional selection. Moreover, large size may come at the cost of long developmental periods, accelerated growth, reduced mobility, and heat stress (Blanckenhorn 2000, Teuschl et al. 2007). In fact, optimal body size has been shown to be limited by resource availability, foraging capacity, and food-processing capacity (Lundberg and Persson 1993), or affected by island size (Boback et al. 2003). Further, body size is largely heritable, and values of body size heritability (h^2) between 0.58 and 0.69 have been documented in a wide array of taxa (Merilä 1997, Réale et al. 1999, DiBattista et al. 2009). Intraspecific size variation is therefore both heritable and highly influenced by the environment (Mousseau and Roff 1987), and subject to strong selection (Kingsolver and Pfennig 2004, Meiri et al. 2005).

While size differences are observed in mainland-island systems within short distances, equivalent differences in body size in continental systems lacking major geographical barriers typically require large (> 1000 km) geographical distances, or steep altitudinal gradients (e.g. Chilean Fox; Fuentes and Jaksic 1979*, Cinnycerthia* wrens; Brumfield and Remsen 1996, *Liolaemus* lizards; Cruz et al. 2005). In amphibians, intraspecific differences in life-history traits such as body size, phenology, and age of sexual maturity are frequently observed along broad geographical clines (Duellman and Trueb 1986, Morrison & Hero 2003; Olalla-Tárraga and Rodríguez 2007). Altitudinal gradients also lead to body size variation in amphibians (Miaud et al. 2000a, Gül et al. 2011), with individuals from high elevations being on average 8.7% larger than those counterparts from low elevations given an average altitudinal range of 1664.9 m between the lowland and highland populations (Morrison & Hero 2003).

In the southwest of the Iberian peninsula, we have found a decrease in body size of up to 35 % at Doñana National Park (Huelva province) compared to continental populations of vertebrates at the Sierra Norte of Seville (Sevilla province). The two regions are a short geographical distance (60 km) away from each other and lack an efficient geographical barrier. Amphibian species showing this local phenomenon of steep size reduction include small marbled newts (*Triturus pygmaeus*), bosca's newts (*Lissotriton boscai*), spadefoot toads (*Pelobates cultripes*), and natterjack toads (*Epidalea calamita*) (Diaz-Paniagua et al. 1996, Díaz-Paniagua and Mateo 1999, Marangoni et al. 2008). The pattern, however, is also present in other land vertebrates like deer, boar, and badgers (Revilla et al. 1999).

We focused on the local dwarfism of continental populations of natterjack toads *Epidalea calamita* comparing the small bodied populations of Doñana with other populations in the apparent absence of geographical barriers (Figure 1). Local dwarfism could have multiple mutually non-exclusive causes: genetic differentiation (adaptive divergence or neutral drift following genetic isolation), environmental factors (e.g. exposure to different climatic conditions or resource availability, both potentially promoting differences in metabolism, growth, and time to or size at maturation), and the interaction between environmental and genetic variation, i.e., G x E. Hence, the small body size observed in the Doñana area may be the result of either genetic differentiation, selection or the phenotypic consequence of experiencing different local environmental conditions. We applied a multifaceted approach aimed at explaining observed sharp body size differences among six *E. calamita* populations. First, we analysed environmental variation (climate, soil and vegetation) among populations that could drive the observed size pattern. Second, we studied the genetic structure across populations to determine the extent to which dwarf populations are genetically isolated, have low effective population sizes and/or have suffered inbreeding. Third, we examined possible differences in trophic status and energy expenditure by comparing isotopic profiles and standard metabolism among populations. Fourth, we studied age structure, growth patterns, and senescence by means of skeletochronology and telomere length assays to test whether dwarf populations show a distinct ontogenetic growth pattern and reach sexual maturity at an earlier age following a prematurely reduced growth rate. Finally, we also studied average female

preferences to divergent male advertisement calls, and whether they are related to male size.

Figure 1. Geographical location of the six natterjack toad (*Epidalea calamita*) populations studied. All populations are north of the Guadalquivir river and no major geographical barrier separates them. Toad pictures in both panels are scaled to the mean snout-to-vent length of each population. Populations marked in yellow are within Doñana National Park and their individuals are between 20 and 30% shorter in snout-to-vent length than those from the other populations.

MATERIAL & METHODS

STUDY SITES AND MODEL SPECIES

The natterjack toad is a common species in Europe, distributed from the southern Iberian Peninsula to eastern Estonia (AmphibiaWeb 2019). Natterjacks vary substantially in size across their extensive distribution range (ca. 34% over > 2,300 km, (Sinsch et al. 2010)), whereas they show an equivalent reduction in size (35.6%) between Doñana populations and those merely 60 km away (Marangoni et al. 2008). We sampled two populations within Doñana National Park (El Puntal 36°57'34.89"N, 6°26'46.41"W; and Navazo del Toro 37°0'44.09"N, 6°30'14.93"W), two populations of large sized individuals of Sierra Norte (Constantina 37°53'15.75"N, 5°36'13.49"W, El Pedroso 37°50'5.17"N, 5°46'23.49"W), and two additional inland populations consisting of large individuals with a geographically intermediate position (Aznalcazar 37°16'38.24"N, 6°14'2.83"W, Gerena 37°32'20.96"N, 6°10'23.33"W). Body size across these populations does not follow a gradual cline but rather discontinuous differences in body size where the Doñana populations are small and the rest are similar in size (Figure 1). Doñana is a sandy area close to the Atlantic Ocean, in which dominant vegetation is Mediterranean heath. Vegetation in Aznalcazar consists of vast pinewood close to wide marshes, whereas in Gerena, Constantina, and El Pedroso mainly sparse oak forests and Mediterranean heath are found.

CLIMATE, SOIL TYPE AND VEGETATION DATA

To characterize the local environment around each of the localities studied, we chose four additional points within a radius of 5 km from each focal locality in addition to the central point of the locality (i.e. 30 data points in total). For each of the 30 points we extracted average monthly climate data for temperature and precipitation (minimum, mean, and maximum) during the period of 1960-1990 and derived bioclimatic variables using Worldclim Version 1 (Hijmans et al. 2005). We also extracted average monthly climate data for temperature, precipitation, wind speed, and water vapour pressure (minimum, mean, and maximum) during the period of 1970-2000 and derived bioclimatic variables using Worldclim Version 2 (Fick and Hijmans 2017). Both versions are based on a spatial resolution of 1 km^2. In addition, we extracted average monthly climate data for temperature and precipitation (minimum, mean, and maximum each) during the period of 1950-1999 from the Digital Climatic Atlas of the Iberian Peninsula (Ninyerola et al. 2000), which uses a spatial resolution of 200 x 200 m. There was a smooth altitudinal cline across the studied populations, from 5 to 601 m asl, but we did not explicitly include altitudinal data in our models as we were more interested in directly modelling the potential climatic variation associated with such mild altitudinal variation. We extracted soil type data from the European Soil Database (ESDB) using the ESDBv2 raster library (Panagos et al. 2012), and from the Spanish Digital Geological Map (GEODE, Roldán et al. 2018) which is based on a scale of 1:50,000. Final data points were extracted using the ENVI software (ENVI v.4.8).

The soil of the Doñana area was mostly arenic luvisol, both in Navazo del Toro and El Puntal. Luvisol was in general a well-aerated soil with well-developed layers of clay. However, in case of arenic luvisol, there was a

significant layer (from ≤50 cm up to ≤100 cm) of sand above the clay, thus the area was very dry and poor in nutrients (World reference base for soil resources 2014). Soils in Aznalcazar were calcaric cambisol and calcaric fluvisol, with a little bit of eutric planosol. These are fairly developed soil types with clay layers that can retain water and nutrients. At Gerena, soil consisted mostly of eutric regosol, with partial presence of chromic vertisol. Regosol was a very underdeveloped soil, with almost no layers of clay and mostly rocky. However, eutric regosol refers to soil that has a rich clay layer 20–100 cm deep above the rocky layer. Chromic vertisol has well developed clay layers and is very rich in nutrients. Constantina and Pedroso consisted largely of eutric regosol. Soil types have been characterised according to the World reference base for soil resources (2014).

We also obtained Normalized Difference Vegetation Indices (NDVI) from satellite images through the Modis Vegetation index MOD13Q1 (Didan 2015). The best available pixel values from 16-day acquisition periods each were chosen under a resolution of 250 x 250 m and under criteria such as low clouds, low view angle, and the highest NDVI values. The data were acquired on a 16-day basis from 18/02/2000 to 02/02/2016.

POPULATION GENETICS – MICROSATELLITE ANALYSES

Between 20/01/2015 and 23/03/2015 we collected a total of 180 *E. calamita* adult males across all populations (Table 1). All individuals were captured at night while calling, to ensure including only sexually mature males. We measured snout-to-vent length (SVL) on graph paper to the nearest 0.5 mm, and

body mass on a transportable scale to the nearest 0.1 g. We then clipped the fourth toe of the right hindlimb, storing the samples in 100% ethanol at -20 °C. We used these toe samples for both skeletochronology and DNA extractions (to be used for microsatellite genotyping and telomere analyses). All laboratory procedures were conducted at Estación Biológica de Doñana (hereafter, EBD).

Genomic DNA was extracted from toe clips using an extraction robot (Freedom EVO 100; Tecan). All DNA samples were genotyped at 16 microsatellite loci (Sánchez-Montes et al. 2017a) with PCR multiplex cycles consisting of initial denaturation (95 °C, 5 min), 30 cycles of denaturation (95 °C, 30 s), annealing (60 °C, 90 s), and extension (72 °C, 30 s), followed by a final extension step (60 °C, 30 min). All reactions were run in a total volume of 15 μL, containing 7.5 μL of Type-it Master Mix (Qiagen), 1.2 μL of primer mix, 5.3 μL of H_2O, and 1 μL of DNA. Genotyping was performed on an ABI PRISM 3130 sequencer with the GeneScan 500 LIZ standard (Applied Biosystems). Allele peaks were assigned manually in GeneMapper v.4.0 (Applied Biosystems).

We calculated basic genetic diversity indexes (mean allelic richness, observed and expected heterozygosity, inbreeding coeficient F_{IS}) for each population and estimated pairwise population differentiation by Hedrick's G_{ST} (Hedrick 2005) and Jost's D (Jost 2008) using the package *diveRsity* (Keenan et al. 2013) in R (R Core Team 2015). We used GENEPOP v4.0 (Rousset 2008) to test for departures from Hardy-Weinberg proportions (HWP) and linkage disequilibrium, and then re-estimated F_{IS} and both genetic differentiation indices (G_{ST} and D) after excluding loci out of HWP. We then tested for isolation by distance (IBD) across populations via Mantel tests with the package *adegenet*

(Jombart 2008). Geographic distances between all population pairs were calculated based on the package *Imap* (Wallace 2012).

We estimated the effective size (N_e) of each population by the sibship frequency method implemented in COLONY v2.0.6.4 (Jones and Wang 2010). We used all available genotypes of each population as offspring, without specifying any candidate fathers or mothers, set weak paternal and maternal sibship size priors = 1, and genotype error rates of 0.05 for every marker (Sánchez-Montes et al. 2017a,b). We conservatively assumed the possibility of polygamy in both sexes and performed two runs per population with "very long" run length and "very high" precision (Wang 2016). Finally, we tested for genetic structure across populations by means of unsupervised Bayesian clustering analyses in Structure (Pritchard et al. 2000). We ran 10 analyses for each value of K (the number of clusters) from one to ten using an admixture model with correlated allele frequencies (Falush et al. 2003), with 1,000,000 burn-in and 1,000,000 post burn-in iteration steps. Ten additional analyses were run under the same settings after removing loci out of HWP from the dataset.

STABLE ISOTOPES ANALYSIS

Small portions of 52 *E. calamita* egg clutches (ca. 150 eggs each) were collected from all 6 populations (7 from Navazo, 4 from El Puntal, 8 from Gerena, 12 from Aznalcazar, 11 from Constantina, and 10 from Pedroso) between 20/01/2015 and 23/03/2015. We brought the eggs from each clutch to a walk-in climatic chamber at EBD and kept them in activated carbon-dechlorinated tap water at 20 °C, and 12:12 L:D photoperiod until hatching. Hatchlings were collected at

stage 23 Gosner (Gosner 1960) before they reached the free-feeding stage. This allowed direct assessment of the maternal diet, in particular during ovulation. Hatchlings were euthanized with MS-222, dried to constant dry mass in the oven at 60 °C, and ground to a fine powder using mortar and pestle. We weighed samples to the nearest 0.1 mg and placed them in tin capsules for $\delta^{13}C$ and $\delta^{15}N$ determination. All samples were combusted at 1,020 ºC using a continuous flow isotope-ratio mass spectrometry system by means of Flash HT Plus elemental analyzer coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany). The isotopic composition is reported in the conventional delta (δ) per mil notation (‰), relative to Vienna Pee Dee Belemnite ($\delta^{13}C$) and atmospheric N2 ($\delta^{15}N$). Replicate assays of standards routinely inserted within the sampling sequence indicated analytical measurement errors of ±0.1‰ and ±0.2‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. The standards used were: EBD-23 (cow horn, internal standard), LIE-BB (whale baleen, internal standard) and LIE-PA (Razorbill feather, internal standard). These laboratory standards were previously calibrated with international standards provided by the International Atomic Energy Agency (IAEA, Vienna).

STANDARD METABOLIC RATE

We collected 67 sexually mature natterjack male toads from our six study populations (16 from Navazo, 10 from El Puntal, 11 from Gerena, 10 from Aznalcazar, 10 from Constantina, and 10 from Pedroso) between 20/01/2015 and 23/03/2015. These were different individuals from those sampled for

genetics analyses, albeit being captured at the same location. Individuals were captured after dusk and placed individually in plastic containers. Males were then brought to the laboratory (EBD) for overnight respirometry measurements. Toads were individually placed in an air-sealed chamber (0.5 L) inside an insulated Styrofoam container, each chamber contained a piece of cloth soaked in water to maintain air-humidity. Toads were allowed an acclimatization time of approx. 5 hours, to ensure measuring Standard metabolic rate (hereafter, SMR) at a postabsorptive stage of the individuals. SMR was determined as the average minimal oxygen consumption throughout the night in an open circuit respirometer, at a constant temperature of 25 ºC. The respirometer consisted of two independent modules consisting of 4 and 8 channels respectively. Both modules had the same components except for the number of channels. Outdoor air was pushed towards each chamber through independent mass-flow controllers (Flow-bar-8), adjusted to 80 mL/min. A multiplexer (RM4-8) valve system conducted outcoming air in 10 min cycles from each chamber towards the CO_2-Oxygen analyzer (FOXBOX-C field gas analyzer, Sable Systems Int., USA) before being released. Drierite was used to reabsorb air humidity before gas analyses. SMR is considered the lowest rate of metabolism, measured at a particular temperature, in a resting and post-absorptive ectotherm according to the Glossary of terms for thermal physiology (International Union for Physiological Sciences 2001). Hence, the values of oxygen consumption (mLO_2/min) were averaged for the most stable 10 minutes of each measurement session (approx. 60 min), calculated following Hill (1972). All animals were returned unharmed to the field at dawn after the experiment.

METAMORPHIC SIZE AND ADULT AGE DETERMINATION

Size at metamorphosis is a highly plastic trait in amphibians, sensitive to key factors such as temperature, food availability, or larval density, among many others. We compared size at metamorphosis across populations by collecting individuals in their late stages of larval development (Gosner stage 40-42) at their sites of origin; these were brought to the laboratory and kept in climatic chambers under identical conditions (20 °C and 80 % air humidity) until they completed reabsorbing the tail and underwent metamorphosis. Sample sizes ranged from 20 to 25 individuals per population. Once the forelimbs emerged, metamorphs were housed in 10 L lidded plastic containers with moist paper towels to provide humidity and refugium while reabsorbing their tails. Amphibians do not feed during the last metamorphic stages and hence metamorphs were not fed before they were measured. Upon tail resorption, toadlets were weighed on an electronic scale and their snout-vent-length measured with callipers.

To estimate the age of adult individuals from each population, we obtained cross sections of the phalanx of 180 clipped toe samples (see POPULATION GENETICS – MICROSATELLITE ANALYSES and Table 1). Glass slides were immersed in a solution of 1 g of glicerol and 0.1 g of chromium potassium sulphate dissolved in 200 mL of H_2O, heated up to 60 ºC to facilitate adhesion of the bone sections. After five minutes of bathing in the solution, slides were dried in the oven for 24 h at 60 ºC. Phalanxes were decalcified in 3% nitric acid overnight, and subsequently cleaned with distilled water for 2 h with an agitator. We then embedded samples in OCT (Optimal Cutting Temperature, Agar

Scientific Ltd., UK), froze them at -20 ºC in a cryostat (MikroM, Germany) and cut them at a thickness of 12 μm. Samples were then directly adhered to treated slides, and stained with hematoxylin for 1 h. Finally, the slides were washed with tap water to remove hematoxylin excess and mounted with a cover glass using Aquatex (Sigma-Aldrich, USA). We estimated the age of the toads by counting the lines of arrested growth (LAG) observed on the sections under a microscope (Sinsch 2015). Since all samples originated from sexually mature males, all individuals had undergone a minimum of one resting period. Thus, we expected all samples to have at least one LAG.

TELOMERE LENGTH ASSESSMENT

Relative telomere length was estimated by q-PCR following Criscuolo et al. (2009). The single copy gene glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as control to adjust the quantity of telomere sequences to the amount of DNA in the q-PCR reaction. The final PCR volume was 20 μL containing 10 μL of LightCycler 480 SYBR Green I Master (Roche Molecular Systems, Inc., USA) and 1 μL of DNA at 20 ng/μL of DNA. The reactions for telomeres and GADPH were done in different plates due to differing PCR conditions. Telomere PCR conditions consisted of denaturation (95°C, 10 min) followed by 40 cycles of annealing (56°C, 10 s) and extension (72°C, 40 s). GADPH PCR started with denaturation (95°C, 10 min) followed by 50 cycles of annealing (56°C, 15 s) and extension (95°C, 60 s). Both were performed in a LightCycler 480 RT-PCR System (Roche Molecular Systems, Inc., USA). The primer concentration was 5mM for GADPH and 10mM for telomeres. Each

sample was run in duplicate and samples with a coefficient of variation greater than 5 % were removed from analyses. Each 96-well plate included serial dilutions of DNA (120 ng, 40 ng, 10 ng, 2.5 ng, 0.66 ng of DNA per µL) from a reference pool (the internal control) run in triplicate, which were used to generate the standard curves, plus a blank control. Quantification cycle values (Ct) were transformed into normalized relative quantiles (NRQs) following the procedure of Hellemans et al. (2007), to control for the amplifying efficiency of each qPCR. The amplification efficiency for telomere products ranged between 1.873 and 2.092 and, for the GADPH products, between 1.872 and 2.021. The slope of the calibration curve ranged between -3.669 and -3.120 for the telomere product and between -3.671 and -3.273 for the GADPH product. CV calculated for a reference sample run in all plates was 1,5% for telomere RT-PCR and 1,2% for the GADPH RT-PCR. The melting curves of q-PCR products confirmed no evidence of primer dimer or non-specific amplifications.

CALL RECORDINGS AND FEMALE MATING PREFERENCE ASSAYS

We recorded male advertisement calls between dusk and midnight with a shotgun microphone (Sennheiser ME64 with K6 powering modules; frequency response ± 2.5 dB from 40 to 20,000 Hz; Hanover, Germany). The microphone was connected to a flash memory recorder (Zoom H1, Japan) with a sampling rate of 44.1 kHz and a resolution of 16 bit. Unlike call characteristics such as note repetition rate and note duration, which vary to a large extent according to daily fluctuations of rainfall and temperature, dominant frequency is an honest signal of body size of male anurans (Gerhardt and Huber 2002). Hence, dominant

frequency (Hz) of each note was analysed by plotting the spectrograms, and averaged ~ 10 call recordings from 10 individuals for each population. We conducted these analyses using Raven Pro version 1.4 (Cornell lab of Ornithology, 2011).

To test for female preferences based on call recordings, we collected 25 females from large sized populations while in amplexus to ensure sexual receptivity (10 females from Aznalcazar, 5 from Constantina, and 10 from Pedroso). Females were brought to the laboratory that very night and released at dawn where captured previously after the experiment. Preference tests were conducted in a 150 x 100 x 25 cm fiberglass arena with a wireless loud speaker (UE Boom, Ultimate Ears, Irvine, USA) in each of the two far ends. We placed each female in random sequence in the centre of the arena, covered by a meshed box for five minutes to allow them to acclimate. In each trial we always played back calls from northern populations on one speaker and calls from either one of the Doñana populations on the other, while randomly alternating among our recorded calls. We then gently removed the box covering the female and gave her ten minutes to make a choice. We considered that a female showed preference for one speaker when she approached it close, touched it or circled around it.

STATISTICAL ANALYSES

All statistical analyses were conducted in R v.3.2.1 (R core team, 2015). Climate data was first analysed by means of a principal component analysis including all 19 bioclimatic variables obtained from Worldclim (S1 in Supplementary

Material) using the function 'princomp'. We then extracted the scores from the two main components and conducted general linear models to determine their association with body size variation across populations. We checked these and all other linear models in the study for parametric assumptions using the package *lmtest* (Zeileis and Horthorn 2002). We used linear models to test for differences among populations in body size (SVL, in mm), dominant frequency of advertisement calls (Hz), isotopic values and telomere length. In the case of $\delta^{13}C$ data, we first normalized the values in order to control for variation in lipid content in the samples using the equation $\delta^{13}C_{normalized} = \delta^{13}C_{untreated} - 1.11 + 0.37C{:}N$, following Caut et al. (2013). The linear model to test for differences in metabolic rate (VO_2, mLO_2/min) across populations included body mass as a covariate. Differences in age structure across populations were assessed by using two-sample Kolmogorov-Smirnov tests (*ks.test* function), and subsequently adjusting the significance level for multiple comparisons using the false discovery rate approach (FDR). Growth curves across populations were compared by fitting Von Bertalanffy curves for each population (Von Bertalanffy 1938, Pujol-Buxó et al. 2012), using the 'vbfr' function of the R package *fishmethods* (Nelson 2014). We obtained the parameters L_{inf} (mean asymptotic SVL), K (growth coefficient), t_0 (hypothetical age at size 0). Subsequently, residual analyses were done to test our model fits. For the female preference tests, we excluded data from unresponsive females, and fit a binomial model for 12 trials on responsive subjects. The model was fit with an alternative hypothesis that the true probability of success was greater than 0.5 (representing a random choice) and a confidence level of 0.95.

Results

VARIATION IN CLIMATE, SOIL, AND VEGETATION ACROSS SITES

The first principal component extracted explained 77% of the total variance and represented variation in rainfall and temperature, as "annual precipitation (Bio 12)", "precipitation of coldest quarter (Bio 19)", "precipitation of wettest quarter (Bio 16)", "precipitation of wettest month (Bio 13)", "precipitation of driest quarter (Bio 17)", "precipitation of driest month (Bio 14)", "max temperature of coldest month (Bio 6)", and "mean temperature of coldest quarter (Bio 11)" all showed high factor loadings on PC1. PC2 explained 19% of the total variance and represented variation in the higher temperature range as "mean temperature of driest quarter (Bio 9)" and "mean temperature of warmest quarter (Bio 10)" showed the highest loadings for this component (Table S2 in Supplementary Material). The two Doñana localities were characterized by lower winter rainfall and moderate temperatures, clearly differentiated from the other localities in the PC1 *vs*. PC2 plot (Figure 2b). Linear models fitted on PC loadings showed significant differences across study sites for PC1 ($F_{5,24} = 200.1$, $p < 0.001$) and for PC2 ($F_{5,24} = 42.03$, $p < 0.001$). NDVI variation showed that vegetation density significantly differs across populations ($F_{5,24} = 6.48$, $p = 0.0006$) and is significantly less dense in Doñana than in the Sierra Norte area ($F_{1,28} = 11.59$, $p = 0.002$) (Figure S3 in Supplementary Material).

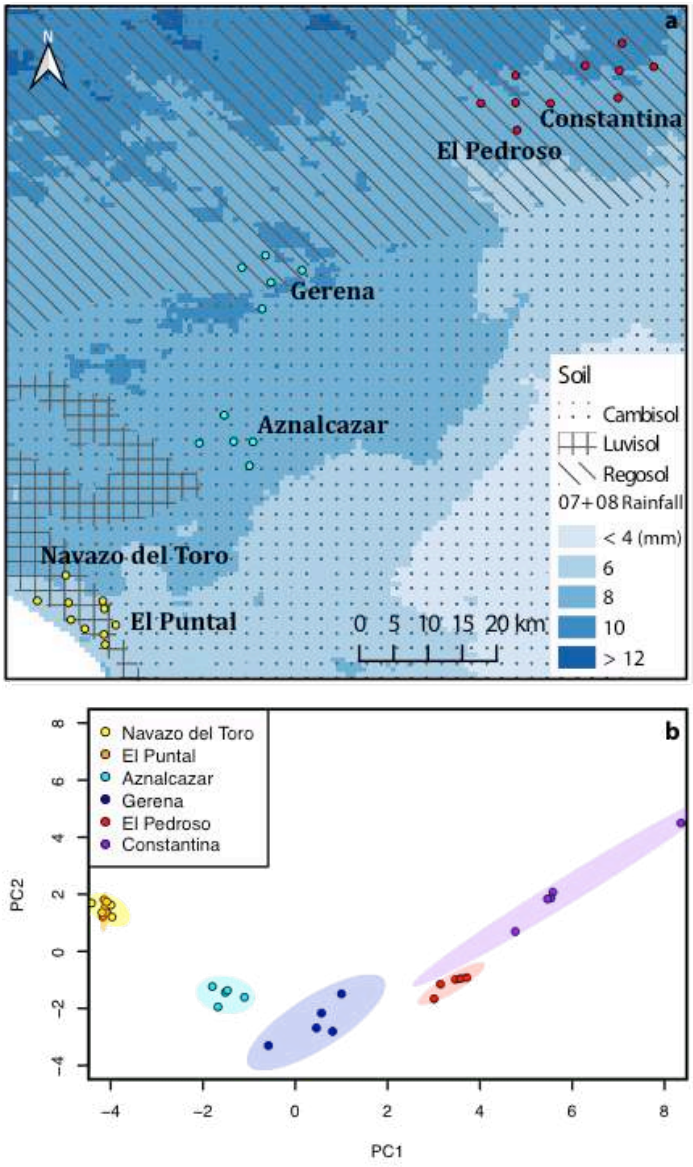

Figure 2. **(a)** Environmental conditions varied conspicuously among populations. Dwarf Doñana populations experience warmer and drier conditions than the other populations, here visualized plotting rainfall (in mm) over the months of July and August, which constitute the harshest months for amphibians in the region. Soils are also different, being sandy luvisol in Doñana, in which water percolates quickly. **(b)** Principal component analysis results showing how climate variables differ markedly across the geographical cline. PC1 represents mostly variation in rainfall and winter temperature, whereas PC2 represents variation in summer temperature.

LACK OF GENETIC STRUCTURE OR ISOLATION BY DISTANCE

The six populations showed similar levels of genetic diversity, with high average allelic richness and heterozygosity and medium-large estimated effective population sizes (Table 1). We found high inbreeding rates in all populations, but these results were heavily affected by loci out of HWP, and consequently all F_{IS} values were markedly reduced after excluding these markers (Table 1). In total, seven loci (*Ecal4.14*, *Ecal4.2*, *Ecal3.19*, *Ecal4.26*, *Ecal3.26*, *Ecal4.8* and *Ecal4.20*) showed significant departures from HWP in at least one population (Table S4 in Supplementary Material), and these markers also affected genetic differentiation estimates (Table 2, Table S5 in supplementary material). After excluding them, overall differentiation among populations was low, except for one dwarf population (El Puntal, Table 2). We did not find any evidence of isolation by distance (Mantel test with G_{ST}: R = 0.261, p = 0.153; with D: R = 0.262, p = 0.153) or genetic structure across the six populations, neither including nor excluding loci out of HWP (Figure S6 in Supplementary Material).

Table 1. Genetic diversity across six natterjack toad populations in southern Spain widely divergent in body size. Estimates were derived from multilocus genotypes over 16 microsatellite loci. Sample size (N), mean allelic richness (AR), observed (H_O) and expected (H_E) heterozygosity, inbreeding coefficient (F_{IS}), number of loci with significant (Bonferroni-corrected) departures from Hardy-Weinberg equilibrium (HWE, out of 16 markers), and effective population size estimates with 95% CI (N_e).

	N	AR	H_O	H_E	F_{IS}	HWE	N_e
Navazo del Toro	27	10.36	0.70	0.83	0.1543	4	117 (69-253)
El Puntal	33	10.58	0.73	0.82	0.1162	4	81 (52-144)
Aznalcazar	30	10.14	0.71	0.81	0.1274	6	193 (103-730)
Gerena	30	10.12	0.71	0.82	0.1240	4	73 (47-133)
El Pedroso	31	10.08	0.71	0.82	0.1258	4	124 (76-254)
Constantina	29	9.99	0.73	0.82	0.1001	4	81 (46-146)

Table 2. Genetic differentiation

Square matrix showing pairwise genetic differentiation among the six populations. Upper triangular: Hedrick's G_{ST}, lower triangular: Jost's D.

	Navazo del Toro	El Puntal	Aznalcazar	Gerena	El Pedroso	Constantina
Navazo del Toro		0.034	0.083	0.105	0.071	0.090
El Puntal	0.028		0.082	0.119	0.140	0.075
Aznalcazar	0.060	0.076		0.065	0.063	0.072
Gerena	0.089	0.061	0.078		0.048	0.116
El Pedroso	0.052	0.074	0.087	0.070		0.052
Constantina	0.040	0.099	0.060	0.069	0.043	

ASSESSMENT OF TROPHIC STATUS VIA STABLE ISOTOPES

The isotopic signature for nitrogen significantly differed across localities ($F_{5,93}$ = 33.15, $p < 0.001$), and the same was true for carbon ($F_{5,93}$ = 25.76, $p < 0.001$). Both isotopes showed a pattern of geographical cline across localities, with increasing $\delta^{15}N$ and decreasing $\delta^{13}C$ values northwards (Figure 3a).

POPULATION DIFFERENCES IN STANDARD METABOLIC RATE

Standard metabolic rate varied greatly across localities even after accounting for body size ($F_{6,60}$ = 12.12, $p < 0.001$). Dwarf Doñana populations showed the lowest metabolic rates, whether size-adjusted or not (Figure 3b).

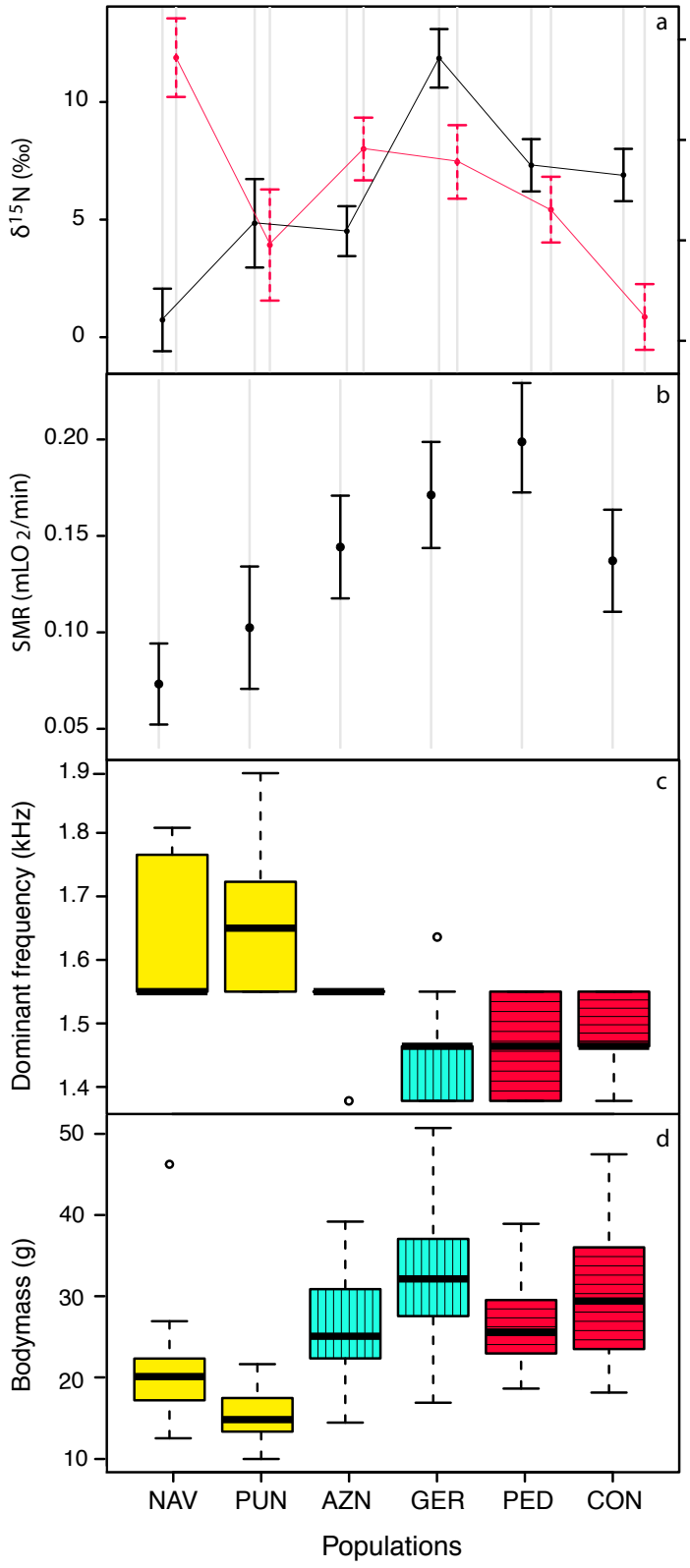

Figure 3. **(a)** Natterjack toad populations varied in their isotopic signature roughly following a geographical pattern of increased $\delta^{15}N$ and decreased $\delta^{13}C$ in a southwest to northeast cline. **(b)** Adjusted means for standard metabolic rate across populations corrected for body mass. Dwarf Doñana populations showed significantly lower standard metabolic rates than larger bodied populations. Whiskers indicate +/- standard errors (a, b). **(c)** Dominant

frequency (kHz) shows a sharp geographical cline across populations in accordance with body size; higher (1636-1655 kHz) dominant frequency in the dwarf populations and lower (1441-1550 kHz) dominant frequency in the larger sized populations. **(d)** Population variation in male body mass (N=30±3 per population). Doñana individuals are between 20 and 30% lighter than those from the other populations. Whiskers indicate +/- standard deviations (c, d)

AGE STRUCTURE, GROWTH PATTERNS AND TELOMERE LENGTH

Size at metamorphosis varied across populations ($F_{5,122}$ = 39.67, p < 0.0001), with maximum differences across populations of 0.305 mg in body mass and 1.57 mm in SVL, corresponding to 35 % and 14.84 % of the maximum observed values for body mass and SVL, respectively. However, those size differences did not mirror patterns of variation in adult body size as one of the Doñana populations (Navazo del Toro) had the largest metamorphs whereas one of the Sierra populations (Constantina) had the smallest metamorphs (Figure 4a, Table S7 in Supplementary Material).

Kolmogorov-Smirnov tests showed that age structure differed significantly across populations (Table S8 in Supplementary Material), with one dwarf population from Doñana (El Puntal) and one large sized population from Sierra Norte (Constantina) showing a greater proportion of young adults (Figure 4b). Growth trajectories were similar in shape across populations since the growth coefficient K was homogeneous across them (Figure 4a). Nevertheless, the maximum size at infinity (L_{inf}) was smaller for both Doñana populations than the rest, indicating that their growth curves plateaued at smaller size than the rest (Figure 4a). Details of maximum size at infinity L_{inf}, growth coefficient K, hypothetical age at size 0 t_0 provided in Table S9 in Supplementary Material.

Telomere length showed a significant negative relationship with body size ($F_{1,179}$ = 4.465, p = 0.035) and age ($F_{1,156}$ = 9.991, p = 0.0018) across individuals from all populations pooled together (Figure 5). We also found significant differences among populations in their average telomere length, although it did not match population variation in body size as both a large bodied and a dwarf population showed longer telomeres than the rest ($F_{5,175}$ = 5.265, p = 0.0001). However, the populations with the highest mean telomere length were the two populations with the highest proportion of young individuals (El Puntal and Constantina).

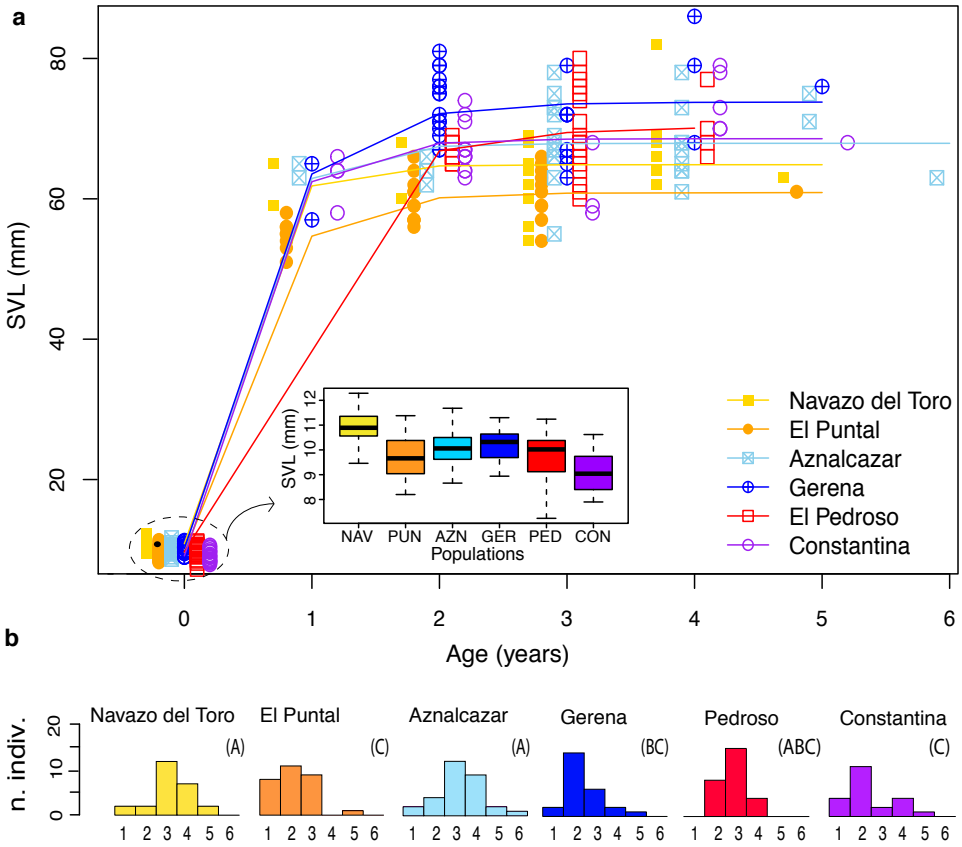

Figure 4. **(a)** Von Bertalanffy growth curves for each population from metamorphosis (age 0) onwards. The shape of the growth curves is homogeneous (no major differences in growth coefficient K), except that the average asymptotic size L_{inf} is smaller for the two Doñana populations, indicating that natterjack toads from Doñana reach the plateau at a smaller size than the rest. Small boxplot shows the average size of metamorphs of each population, showing that one of the dwarf populations (NAV) has the largest metamorphs and one of the large sized populations (CON) has the smallest metamorphs. Differences among populations in size at metamorphosis therefore do not predict observed differences in adult body size, indicating that broad adult size differences become apparent largely during post-metamorphic growth in this system. **(b)** Age structure for each population as estimated through skeletochronology. Populations with different letters in brackets significantly differ in their age distribution.

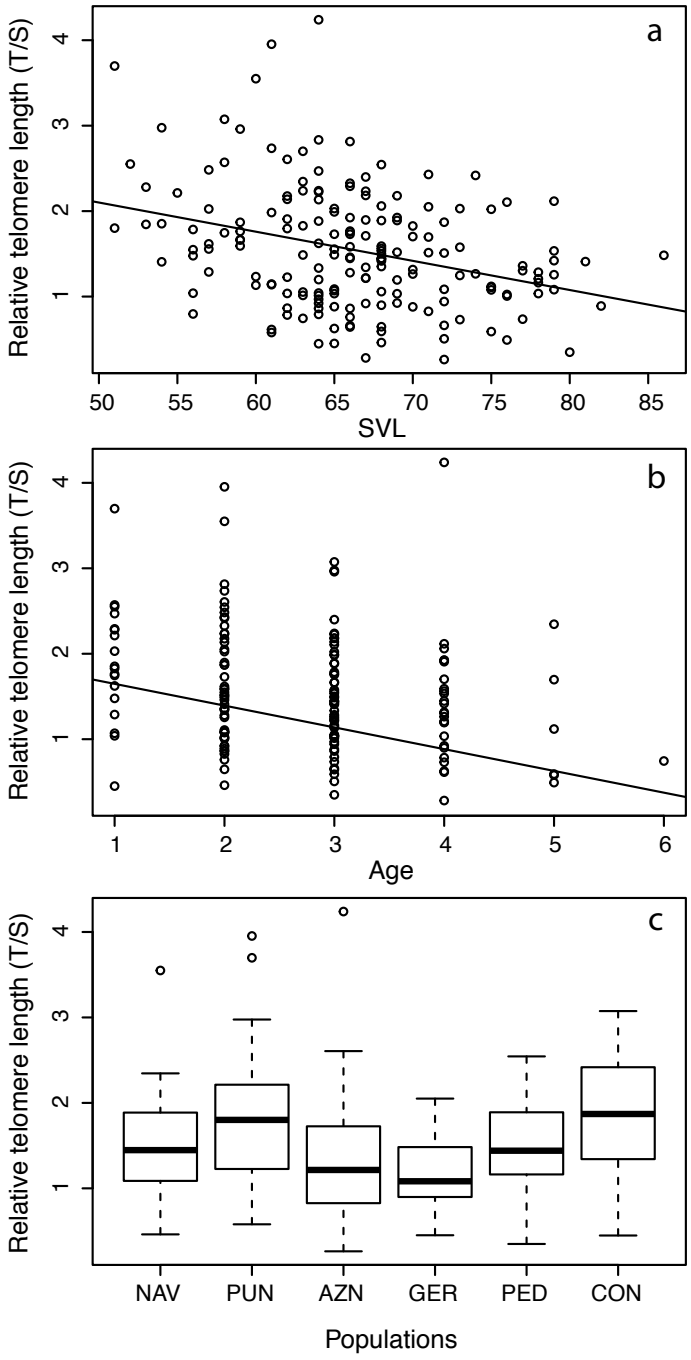

Figure 5. Relative telomere length (telomere / single copy gene ratio) varied negatively with body length **(a)** and age **(b)**. We also observed significant differences among populations in mean telomere length **(c)**. The two populations with a higher proportion of young adults (PUN and CON) showed significantly longer telomeres.

CALL VARIATION AND FEMALE MATING PREFERENCE

The average dominant frequency of the calls showed significant differences among populations ($F_{5,64}$ = 15, p < 0.001, Figure 3c), in accordance with the observed size differences. Smaller males from Doñana (El Puntal and Navazo) called on average with 11% higher dominant frequency than the other populations. During mate choice tests 13 females showed no preference towards either speaker, instead staying still or trying to escape. Of the 12 responsive females, 11 out of 12 showed a strong preference towards males from large bodied populations, hence discriminating against smaller Doñana males (binomial test p = 0.003). The probability of females choosing calls of males from larger populations was 92%.

Discussion

Here we present evidence for dwarfism in continental populations of natterjack toads in the absence of genetic isolation and without any major barrier to gene flow among populations. Amphibians in Doñana National Park are conspicuously smaller than those from neighbouring populations (Diaz-Paniagua et al 1996, Marangoni et al. 2008), and toads of geographically intermediate populations are as large as those of the northern populations. We have found that i) there is little genetic structure across our sampled populations, ii) the dwarf Doñana populations are therefore not isolated, iii) all six populations have similarly high levels of genetic variation and low inbreeding, in concordance with values observed in other Iberian populations (Oromi et al. 2012; Sánchez-Montes et al. 2017a), and iv) effective population sizes at both sites in Doñana are comparable to those of large-bodied populations. The four above conclusions are in

accordance with previous genetic analyses of natterjack populations (Gomez-Mestre and Tejedo 2004; Rowe et al. 2006; Oromi et al. 2012), especially given the absence of barriers to gene flow and the fact that natterjack toads are capable of migrating over long distances (Sinsch 1997, Miaud et al. 2000b). Note that one dwarf population (El Puntal) showed a somewhat higher neutral differentiation than the rest, but not the other dwarf population from Doñana (Navazo del Toro). Moreover, the observed differentiation was so modest that Bayesian clustering methods failed to detect any evidence of genetic structure (Figure S5 in Supplementary Material). Furthermore, our results showed that most of the differentiation observed was driven by loci departing from HWP, and if removed from the analysis population differentiation was even lower (see Table 1 for F_{IS} and Table 2 for G_{ST}, Jost´s D). This lack of genetic structure indicated by our microsatellite analysis does not preclude the possibility that selection could have resulted in population divergence in loci directly associated with body size. Future genomic studies combining neutral and non-neutral markers will shed light on the potential adaptive genetic differentiation of the dwarf populations. At this point however, we can conclude that the size differences observed are not the result of genetic isolation and drift.

Dwarfism in natterjack toads from Doñana seems so far to be a consequence of different growing environments, with no evidence to date of it being adaptive. We found substantial differences among localities in key climatic and habitat variables. The Doñana area is drier and warmer than the other localities and this is accentuated in summer, harshening the aestivation conditions for natterjack toads. Increased temperatures often accelerate larval development resulting in smaller body sizes at metamorphosis (Kingsolver and

Huey 2008), but our natterjack toad populations from Doñana did not show reduced size at metamorphosis compared to the other study localities. A biogeographical analyses of body size variation across 265 European and North American amphibian species showed that potential evapotranspiration (PET) and mean annual temperature (AET) are the two main drivers of body size in these species, with anurans decreasing in size with increased PET and AET (Olalla-Tárraga and Rodríguez 2007). This inverse relationship between ambient humidity and temperature and body size is commonly observed across taxa and holds true for natterjack toads throughout their evolutionary history (Martínez-Monzón et al. 2017). This common phenomenon in line with the current global warming is already causing reductions in body size worldwide in diverse taxa (Sheridan and Bickford 2011, Caruso et al. 2015).

The Doñana populations experience a warmer and drier climate than the other populations (Figure 2a,b), which in combination with sandy soil with poor water retention, causes severe droughts over extended periods of time. This undoubtedly affects the growth of post-metamorphic juveniles, since amphibians have permeable skin. A dry substrate constrains juvenile growth in natterjack toads by 21.5% in terms of body mass and by 19.3% in body length over just five weeks (Gomez-Mestre and Tejedo 2005). Interestingly, juveniles from Doñana suffered the same reduction in body size than other populations, including Pedroso, included also in this study. Juvenile growth in amphibians, and in particular natterjack toads, is critical and largely determines adult size differences (Sinsch et al. 2010). Hence, hampered juvenile growth due to drier conditions in Doñana, and not size at metamorphosis *per se*, seems to be a strong determinant for the reduction in adult size.

Throughout its range, delayed age at maturity usually correlates with larger body size in natterjack toads (Sinsch et al 2010), and delayed maturation is compensated with extended lifetime fecundity due to increased longevity (Leskovar et al. 2006, Oromi et al. 2012). Within our study area, growth patterns were similar across populations, except that the dwarf Doñana populations showed a downward shift in their curves, as they matured at a smaller size than the rest of the populations (Figure 4a). We initially hypothesized that toads from dwarf populations could be maturing at an earlier age and reducing their growth afterwards. One of the dwarf populations indeed had its age structure shifted towards younger individuals (El Puntal; Figure 4b). However, the other dwarf population (Navazo del Toro) did not, possibly due to the lack of young individuals, because scarce rainfall in the previous year limited availability of breeding sites, and hence reproduction of this population. Meanwhile, one of the Sierra populations also had an age structure biased towards younger individuals and yet their average size was normal (Figure 4b). Since size at metamorphosis and age at sexual maturity are not determinants of adult body size and post-maturation growth patterns are similar across populations, constrained growth during the juvenile phase seems to be the strongest determinant of reduced body size in Doñana populations, which in turn seems associated with a warmer and drier environment. Reduced body size also resulted in an average higher dominant frequency of the mating calls emitted by males. Female preference for lower dominant frequency is common in anurans (Gerhardt and Huber 2002), and we observed that the differences in dominant frequency across the studied populations were large enough to bias the preference of large females from neighbouring populations against calls of small Doñana males. However,

whether small Doñana females also prefer lower dominant frequencies emitted by larger males remains to be tested.

Steep differences in growth during early ontogenetic stages may result in differential erosion of telomeric chromosomal regions (Lee et al. 2012, Burraco et al. 2017), which in turn can translate into differences in longevity (Monaghan et al. 2008). We observe a very clear negative association between telomere length and age and size (Figure 5). Moreover, differences in telomere length among populations matched their age structure as the two populations with a greater proportion of young adults sampled also showed significantly longer telomeres. Slow juvenile growth could have resulted in milder telomere shortening, but only one of the dwarf Doñana populations showed longer telomere length, as did also one of the large bodied populations with presumably faster juvenile growth. Dynamics of telomere shortening across these populations are therefore likely affected by additional factors apart from differences in growth rate.

Metabolic rate is intrinsically related to body size (White et al. 2006). However, even accounting for differences in body mass we found a geographical cline implying substantial physiological differences among those populations. Dwarf Doñana populations presented lowest mass-specific SMR (metabolic intensity) as opposed to highest values found in the Sierra Norte populations (Figure 3b). Increased aridity causing prolonged water deprivation in Doñana may induce longer periods of inactivity for toads, resulting in reduced metabolic intensity but it may also cause reduced metabolic rate to decrease their energetic expenditure, since aestivation results in depressed metabolic rate (Withers

1993) and dehydration results in reduced oxygen uptake during exercise (Gatten 1987). Differences in temperature and aridity may thus be driving the observed metabolic differences among populations, as the dwarf populations experience prolonged periods of drought that could demand a reduction in the cost of living. Whether these adjustments result from physiological constraints or adaptive energy saving mechanisms remains to be studied.

We also observed marked differences in the isotopic signature among the populations studied (Figure 3a). Populations varied in both $\delta^{13}C$ and $\delta^{15}N$, indicating that either toads at the different localities feed on different carbon sources and consume preys at different trophic levels, or consume similar prey but the baseline isotopic signature differs across localities. The differences observed for $\delta^{15}N$ are wide enough that toads in the different populations would be considered to be at rather different trophic levels (Kupfer et al 2006). Natterjack toads, however, are all carnivores and feed on locally available arthropods (García-París et al 2004). The observed variation in climate, soil, and vegetation could be biasing the guild of available prey to toads in the different populations or altering their isotopic values. It would be worth investigating if invertebrate prey from the oligotrophic soils of Doñana are responsible for these differences in isotopic values among natterjack populations, and whether such isotopic variation is associated with reduced nutritious values of prey. Bioaccumulation of ^{15}N occurs because absorption is higher than elimination (Karasov and Martinez del Rio 2007). Some amino acids appear to retain approximately the same nitrogen isotopic composition of food, whereas others become enriched in ^{15}N by the animal's metabolism (Martinez del Rio et al. 2009). Interestingly, the pattern we observe for $\delta^{15}N$ is parallel to the pattern we

observed for SMR (Figure 3a,b). Lower $\delta^{15}N$ in Doñana than in the northern populations can therefore also be partly due to higher SMR causing $\delta^{15}N$ enrichment in the northern populations.

Conclusions

Dwarfism in natterjack toads in southern Spain occurs over an extremely short geographical range and in the absence of any major biogeographical barrier. This dwarfism persists despite lack of population isolation or genetic differentiation. Reduced body size results in mating calls of higher dominant frequency in dwarf Doñana males, which are discriminated against by females from larger bodied populations. We observe population variation in their isotopic signatures, with dwarf populations from Doñana showing markedly lower $\delta^{15}N$ values, indicating that they are either feeding on different prey items or on similar prey with lower baseline nitrogen isotopic signature. Earliest age of maturation is similar, but age structure varies significantly across populations with those having a greater proportion of young adults showing longer telomeres. Post-maturation growth patterns are similar across populations, but populations differ considerably in male size at maturity, indicating that growth during the terrestrial phase largely determines the pattern of local dwarfism. Consistently, size at metamorphosis of individuals that metamorphosed in the laboratory do not follow the same pattern as in mature adults, indicating that it is growth during the terrestrial phase what largely determines the pattern of variation in size. Juvenile growth is limited by the drier and warmer local climate in combination with soils with little water retention. Such drier conditions are also likely responsible for the

lower SMR observed in the dwarf populations. Our results suggest that size reduction in this system is largely environmentally driven and associated with reduced metabolic rate and lower nitrogen isotopic values. Experimental approaches such as common garden experiments in combination with detailed genomic analyses once genomic resources become available for this species, will definitely determine if there is any adaptive component to this steep local reduction in body size.

Declarations

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Author contributions

L. Hyeun-Ji conducted the experiments, analysed the data, and wrote the manuscript. J. Broggi provided crucial help for the standard metabolic rate experiment, G. Sánchez-Montes for the population genetics analysis, and C. Díaz Paniagua for skeletochronology and stable isotope analysis. I. Gomez-Mestre designed the experiments and supervised the whole process of preparing the manuscript.

Permits

Permits to conduct this research were granted by Consejería de Medio Ambiente from Junta de Andalucía, and all procedures were approved by IACUC at Estación Biológica de Doñana (permit #17_01 and #17_08).

Data accessibility

Data are archived in an institutional public repository (http://digital.csic.es/handle/10261/171767).

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Supplementary materials

S1: Worldclim bioclimatic variables

BIO1 = Annual mean temperature

BIO2 = Mean diurnal range (mean of monthly (max temp – min temp))

BIO3 = Isothermality (BIO2/BIO7)(*100)

BIO4 = Temperature seasonality (standard deviation*100)

BIO5 = Max temperature of warmest month

BIO6 = Max temperature of coldest month

BIO7 = Temperature annual range (BIO5 - BIO6)

BIO8 = Mean temperature of wettest quarter

BIO9 = Mean temperature of driest quarter

BIO10 = Mean temperature of warmest quarter

BIO11 = Mean temperature of coldest quarter

BIO12 = Annual precipitation

BIO13 = Precipitation of wettest month

BIO14 = Precipitation of driest month

BIO15 = Precipitation seasonality (coefficient of variation)

BIO16 = Precipitation of wettest quarter

BIO17 = Precipitation of driest quarter

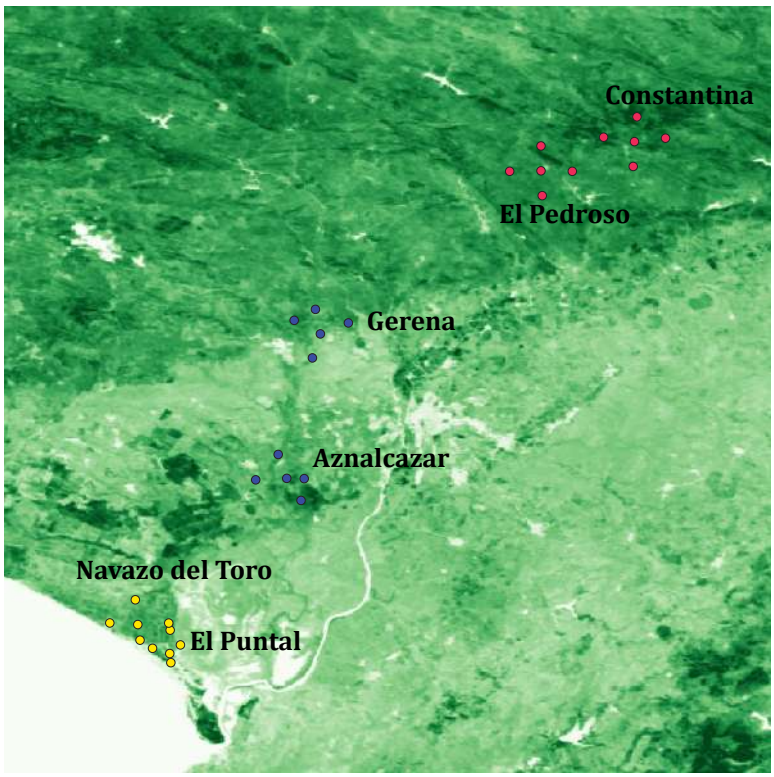
BIO18 = Precipitation of warmest quarter

BIO19 = Precipitation of coldest quarter

S2: Factor loadings of Principal Component Analysis (PCA)

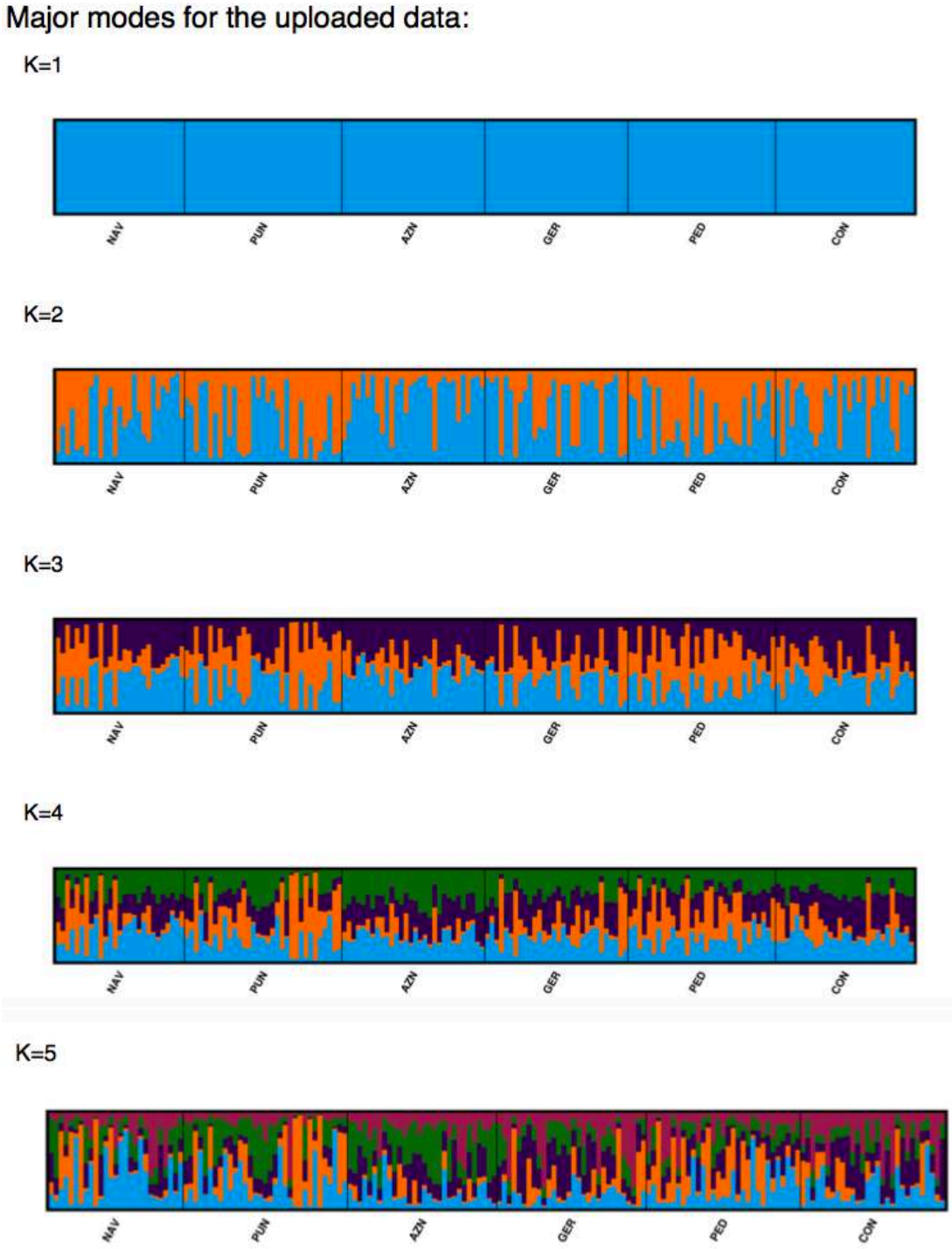
	PC1	PC2
Bio_12 (Annual precipitation)	**0.99**	0.00
Bio_19 (Precipitation of coldest quarter)	**0.99**	-0.01
Bio_16 (Precipitation of wettest quarter)	**0.99**	-0.07
Bio_11 (Mean temperature of coldest quarter)	**-0.99**	-0.03
Bio_13 (Precipitation of wettest month)	**0.99**	-0.05
Bio_17 (Precipitation of driest quarter)	**0.98**	0.12
Bio_6 (Max temperature of coldest month)	**-0.98**	-0.14
Bio_14 (Precipitation of driest month)	**0.95**	-0.07
Bio_1 (Annual mean temperature)	-0.94	0.30
Bio_15 (Precipitation seasonality (coefficient of variation))	-0.94	-0.24
Bio_8 (Mean temperature of wettest quarter)	-0.91	-0.30
Bio_4 (Temperature seasonality (standard deviation*100))	0.89	0.44
Bio_7 (Temperature annual range (BIO5 - BIO6))	0.87	0.48
Bio_18 (Precipitation of warmest quarter)	0.86	0.03
Bio_2 (Mean diurnal range (mean of monthly (max temp – min temp)))	0.84	0.52
Bio_9 (Mean temperature of driest quarter)	-0.13	**0.99**
Bio_10 (Mean temperature of warmest quarter)	-0.27	**0.96**
Bio_3 (Isothermality (BIO2/BIO7)(*100))	0.44	0.78
Bio_5 (Max temperature of warmest month)	0.66	0.74
Proportion of variation explained	**0.74**	**0.21**

S3: Mean NDVI values across study populations

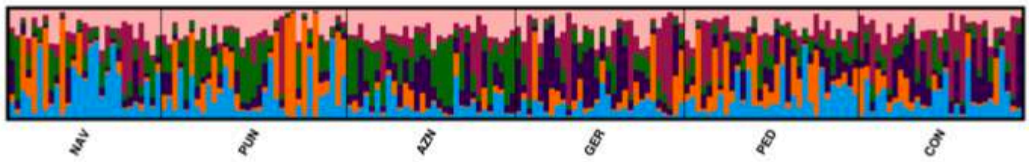


S3: Figure visualizing NDVI across study populations, showing that on average the Doñana area is less densely vegetated than the Sierra Norte area.

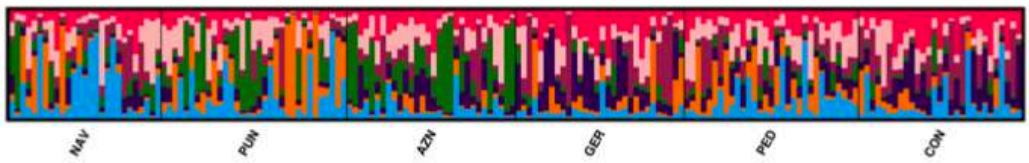
S4: Bayesian clustering analysis results, showing that there is no genetic clustering among populations regardless of K



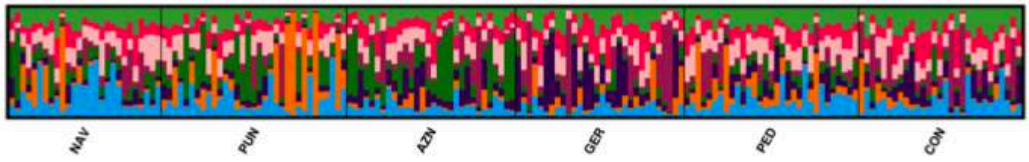
K=6



K=7

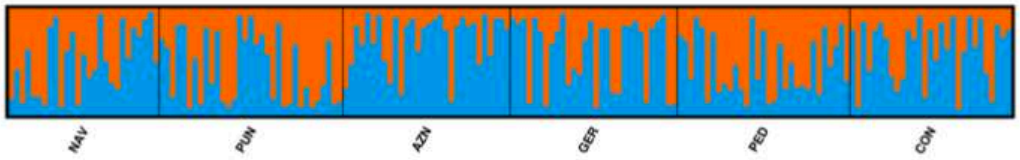


K=8

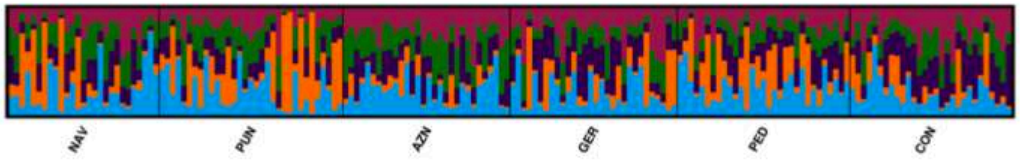


Minor modes for the uploaded data:

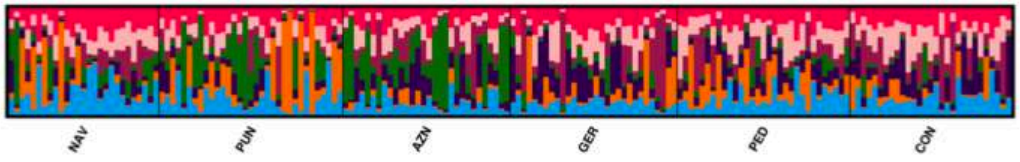
K=2 MinorCluster1



K=5 MinorCluster1



K=7 MinorCluster1



Division of runs by mode:

K=1	5/5
K=2	3/5, 2/5
K=3	5/5
K=4	5/5
K=5	3/5, 2/5
K=6	5/5
K=7	3/5, 2/5
K=8	5/5

S5: Loci showing significant departures from Hardy-Weinberg equilibrium (HWE)

Genetic diversity estimates detailed by locus and population. *N*: sample size, A: number of alleles observed, %: percentage of the total number of alleles observed at a specific locus that were present in the population, AR: allelic richness, H_O and H_E: observed and expected heterozygosity, F_{IS}_Low and F_{IS}_High: lower and upper bounds of the 95% CI for F_{IS}. Only loci with p<0.001 (marked in bold) showed significant departures from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction.

Navazo del Toro

	N	A	%	AR	H_O	H_E	HWE	F_{IS}	F_{IS} _Low	F_{IS} _High
Ecal4.6	27	9	90	8.05	0.59	0.83	0.144	0.288	0.068	0.515
Ecal4.14	26	11	78.57	9.99	0.69	0.88	0.069	0.213	0.02	0.417
Ecal3.29	27	6	75	5.17	0.22	0.38	0.054	0.422	-0.052	0.804
Ecal4.2	27	17	35.42	13.87	0.48	0.91	**<0.001**	0.468	0.258	0.675
Ecal3.19	21	8	72.73	7.2	0.38	0.84	0.002	0.545	0.282	0.793
Ecal4.26	27	26	48.15	19.98	0.74	0.94	**<0.001**	0.214	0.049	0.398
Ecal4.29	27	12	70.59	10.86	0.96	0.88	0.889	-0.093	-0.176	0.017
Ecal4.3	25	12	75	10.98	0.96	0.89	1	-0.08	-0.147	0.011
Ecal4.16	27	5	100	4.63	0.63	0.69	0.424	0.093	-0.183	0.359
Ecal3.26	27	15	44.12	12.58	0.52	0.88	**<0.001**	0.408	0.203	0.617
Ecal4.8	24	17	47.22	13.47	0.71	0.89	**<0.001**	0.202	0.001	0.418
Ecal4.21	26	9	75	7.97	0.62	0.82	0.227	0.248	0.037	0.472
Ecal3.4	27	7	50	6.73	0.89	0.76	1	-0.166	-0.317	0.002
Ecal4.20	27	19	79.17	15.82	0.96	0.92	0.956	-0.05	-0.105	0.032
Ecal4.18	26	9	81.82	8.43	0.88	0.83	1	-0.068	-0.216	0.097
Ecal4.24	27	11	73.33	9.96	0.93	0.87	1	-0.067	-0.159	0.053

El Puntal

	N	A	%	AR	H_O	H_E	HWE	F_{IS}	F_{IS} _Low	F_{IS} _High
Ecal4.6	33	8	80	7.37	0.73	0.83	0.075	0.129	-0.046	0.314
Ecal4.14	33	10	71.43	9.25	0.79	0.87	0.408	0.094	-0.054	0.255
Ecal3.29	33	6	75	5.26	0.33	0.39	0.455	0.144	-0.093	0.408
Ecal4.2	33	23	47.92	17.06	0.7	0.93	**<0.001**	0.251	0.084	0.423
Ecal3.19	22	9	81.82	8	0.32	0.86	0.005	0.629	0.387	0.851
Ecal4.26	29	25	46.3	18.98	0.97	0.94	**<0.001**	-0.025	-0.08	0.053
Ecal4.29	33	12	70.59	10.83	0.88	0.89	0.999	0.008	-0.109	0.147
Ecal4.3	32	12	75	10.57	0.88	0.86	0.998	-0.02	-0.144	0.125
Ecal4.16	33	4	80	3.98	0.48	0.66	0.014	0.265	-0.013	0.531
Ecal3.26	33	19	55.88	14.66	0.7	0.89	**<0.001**	0.22	0.056	0.39
Ecal4.8	31	22	61.11	16.43	0.9	0.91	**<0.001**	0.008	-0.104	0.132
Ecal4.21	31	9	75	7.35	0.61	0.73	0.185	0.159	-0.04	0.368
Ecal3.4	33	8	57.14	7.47	0.88	0.81	0.698	-0.084	-0.21	0.067
Ecal4.20	33	16	66.67	12.68	0.76	0.88	0.062	0.134	-0.004	0.289
Ecal4.18	33	9	81.82	8.6	0.79	0.85	1	0.069	-0.087	0.243
Ecal4.24	33	12	80	10.86	0.94	0.88	1	-0.064	-0.139	0.034

Aznalcazar

	N	A	%	AR	H_O	H_E	HWE	F_{IS}	F_{IS} _Low	F_{IS} _High
Ecal4.6	30	7	70	6.46	0.5	0.76	0.015	0.344	0.092	0.595
Ecal4.14	27	12	85.71	9.92	0.56	0.87	**<0.001**	0.364	0.155	0.578
Ecal3.29	29	7	87.5	5.68	0.48	0.55	0.156	0.127	-0.116	0.396
Ecal4.2	30	27	56.25	19.66	0.9	0.91	**<0.001**	0.014	-0.091	0.136

Ecal3.19	27	7	63.64	6.46	0.52	0.81	0.086	0.363	0.124	0.598
Ecal4.26	29	24	44.44	17.92	0.72	0.93	**<0.001**	0.217	0.052	0.393
Ecal4.29	30	13	76.47	11.21	0.93	0.87	0.668	-0.07	-0.17	0.041
Ecal4.3	30	13	81.25	11.2	0.93	0.85	0.999	-0.094	-0.183	0.013
Ecal4.16	30	4	80	3.83	0.5	0.58	0.958	0.135	-0.122	0.4
Ecal3.26	30	15	44.12	12.22	0.7	0.87	**<0.001**	0.196	0.029	0.375
Ecal4.8	28	16	44.44	12.31	0.68	0.86	**<0.001**	0.208	0.024	0.406
Ecal4.21	30	10	83.33	7.86	0.43	0.79	**<0.001**	0.449	0.236	0.661
Ecal3.4	30	8	57.14	7.33	0.77	0.77	0.964	0.005	-0.158	0.184
Ecal4.20	30	15	62.5	13.05	1	0.9	0.721	-0.116	-0.155	-0.087
Ecal4.18	30	9	81.82	7.97	0.83	0.84	0.929	0.005	-0.149	0.178
Ecal4.24	30	10	66.67	9.23	0.9	0.86	0.997	-0.051	-0.173	0.091

Gerena

	N	A	%	AR	H_O	H_E	HWE	F_{IS}	F_{IS} _Low	F_{IS} _High
Ecal4.6	30	8	80	7.17	0.67	0.78	0.415	0.148	-0.072	0.37
Ecal4.14	29	11	78.57	10.08	0.69	0.84	0.105	0.183	0.013	0.37
Ecal3.29	30	7	87.5	6.28	0.5	0.68	0.127	0.266	-0.009	0.532
Ecal4.2	30	19	39.58	14.98	0.87	0.9	**<0.001**	0.036	-0.071	0.158
Ecal3.19	27	11	100	9.38	0.56	0.86	0.007	0.355	0.13	0.577
Ecal4.26	29	28	51.85	20.58	0.83	0.95	**<0.001**	0.125	-0.008	0.273
Ecal4.29	30	11	64.71	9.92	0.83	0.86	0.083	0.036	-0.103	0.187
Ecal4.3	29	13	81.25	11.22	0.86	0.84	0.953	-0.021	-0.149	0.123
Ecal4.16	30	4	80	3.83	0.4	0.66	0.001	0.392	0.085	0.667
Ecal3.26	28	20	58.82	15.2	0.57	0.91	**<0.001**	0.375	0.188	0.566
Ecal4.8	30	13	36.11	10.34	0.6	0.68	0.057	0.112	-0.086	0.323
Ecal4.21	29	9	75	7.82	0.59	0.78	0.16	0.253	0.032	0.478

Ecal3.4	30	6	42.86	5.23	0.8	0.71	0.532	-0.13	-0.303	0.066
Ecal4.20	30	13	54.17	11.3	0.87	0.86	**<0.001**	-0.003	-0.117	0.129
Ecal4.18	30	9	81.82	8.17	0.93	0.85	0.802	-0.093	-0.184	0.021
Ecal4.24	30	12	80	10.47	0.87	0.86	0.765	-0.005	-0.128	0.136

El Pedroso

	N	A	%	AR	H_O	H_E	HWE	F_{IS}	F_{IS} _Low	F_{IS} _High
Ecal4.6	31	7	70	6.78	0.45	0.79	0.031	0.431	0.206	0.651
Ecal4.14	30	8	57.14	7.33	0.43	0.81	0.001	0.464	0.228	0.689
Ecal3.29	30	6	75	5.12	0.37	0.49	0.294	0.258	-0.07	0.585
Ecal4.2	31	23	47.92	18.23	0.84	0.93	0.427	0.093	-0.024	0.228
Ecal3.19	26	9	81.82	7.66	0.35	0.82	**<0.001**	0.579	0.342	0.8
Ecal4.26	26	26	48.15	18.43	0.92	0.93	**<0.001**	0.008	-0.092	0.132
Ecal4.29	31	10	58.82	8.56	0.74	0.84	0.003	0.118	-0.058	0.305
Ecal4.3	31	13	81.25	11.15	0.87	0.86	0.351	-0.017	-0.13	0.112
Ecal4.16	31	4	80	3.93	0.48	0.57	0.5	0.146	-0.127	0.428
Ecal3.26	31	16	47.06	13.87	0.84	0.92	1	0.088	-0.051	0.237
Ecal4.8	31	16	44.44	13.08	0.74	0.87	**<0.001**	0.147	-0.03	0.335
Ecal4.21	31	10	83.33	8.49	0.77	0.84	0.249	0.073	-0.096	0.259
Ecal3.4	31	9	64.29	7.46	0.84	0.79	0.001	-0.065	-0.22	0.106
Ecal4.20	31	17	70.83	13.84	0.9	0.9	**<0.001**	-0.008	-0.128	0.125
Ecal4.18	31	8	72.73	7.98	0.87	0.87	1	-0.006	-0.132	0.132
Ecal4.24	31	10	66.67	9.31	1	0.86	1	-0.168	-0.21	-0.138

Constantina

	N	A	%	AR	H_O	H_E	HWE	F_{IS}	F_{IS} _Low	F_{IS} _High
Ecal4.6	29	8	80	7.69	0.66	0.77	0.67	0.145	-0.066	0.373
Ecal4.14	28	10	71.43	9.28	0.64	0.87	0.958	0.259	0.064	0.471
Ecal3.29	29	5	62.5	4.81	0.48	0.57	0.133	0.158	-0.15	0.463
Ecal4.2	29	21	43.75	16.79	0.83	0.93	**<0.001**	0.109	-0.027	0.26
Ecal3.19	23	10	90.91	8.58	0.48	0.85	0.013	0.435	0.191	0.678
Ecal4.26	26	23	42.59	17.43	0.77	0.93	**<0.001**	0.171	-0.002	0.357
Ecal4.29	29	8	47.06	7.41	0.76	0.82	0.973	0.069	-0.117	0.266
Ecal4.3	28	10	62.5	9.63	0.79	0.85	1	0.079	-0.079	0.26
Ecal4.16	29	5	100	4.5	0.45	0.6	0.115	0.252	-0.024	0.537
Ecal3.26	27	14	41.18	11.49	0.85	0.85	**<0.001**	-0.004	-0.148	0.16
Ecal4.8	29	17	47.22	13.68	0.83	0.85	0.005	0.029	-0.115	0.187
Ecal4.21	29	7	58.33	6.46	0.62	0.74	0.561	0.166	-0.056	0.389
Ecal3.4	29	8	57.14	7.12	0.72	0.78	0.081	0.077	-0.112	0.277
Ecal4.20	29	21	87.5	16.85	0.93	0.92	**<0.001**	-0.01	-0.094	0.1
Ecal4.18	29	8	72.73	7.44	1	0.83	0.2	-0.203	-0.259	-0.167
Ecal4.24	29	12	80	10.68	0.93	0.88	0.437	-0.057	-0.147	0.057

S6: Snout-to-Vent length and body mass of metamorphs

Population	SVL (mm)	Body mass (g)
Constantina	9.01	0.564
El Puntal	9.62	0.673
El Pedroso	9.73	0.784
Gerena	9.99	0.893
Aznalcazar	10.12	0.817
Navazo del Toro	10.58	0.870

S7: Kolmogorov-Smirnov test results

	NAV	PUN	AZN	GER	PED	CON
NAV		0.002	1	0.006	0.6	0.003
PUN	0.002		0.004	0.6	0.05	0.7
AZN	1	0.004		0.01	0.3	0.005
GER	0.006	0.6	0.01		0.09	0.9
PED	0.6	0.05	0.3	0.09		0.05
CON	0.003	0.7	0.005	0.9	0.05	

*each value indicates the p-value of the Kolmogorov-Smirnov test, indicating whether two populations differ from each other in age structure.

S8: Von Bertalanffy growth curves parameters

	L_{inf}	K	t_0
Navazo del Toro	64.86	2.88	-0.06
El Puntal	60.90	2.11	-0.08
Aznalcazar	67.91	2.45	-0.07
Gerena	73.79	1.83	-0.08
Pedroso	70.26	1.43	-0.10
Constantina	68.57	2.27	-0.06

(L_{inf} = mean asymptotic SVL, K = growth coefficient, t_0 = hypothetical age at size 0)

Chapter 2

Population divergence of developmental plasticity in the spadefoot toad *Pelobates cultripes,* using RNA-seq

In prep.

RH: Developmental plasticity of spadefoot toads

Population divergence of developmental plasticity in the spadefoot toad, *Pelobates cultripes*

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Abstract

Environmental heterogeneity is pervasive in nature and favours the evolution of phenotypic plasticity when no single trait value can maximize fitness across environments. Phenotypic plasticity is therefore a common characteristic of living organisms and is based on the differential activation of neuroendocrine pathways in response to reliable environmental cues that can ultimately also result in differential gene expression. Solid experimental and theoretical work indicates that adaptive phenotypic plasticity can foster rapid evolutionary divergence through genetic accommodation, However, although the pattern resulting from genetic accommodation can sometimes be quite apparent when comparing taxa that have been independently evolving in contrasting environments for a long time, we still lack a good sense of how the process is initiated and its underlying mechanisms. In that sense, the evolution of divergent larval developmental rates among spadefoot toad species is highly congruent

with the process of genetic accommodation, but we need to understand how such divergence is initiated. To address this question, we have conducted a common garden experiment on ten *Pelobates cultripes* populations from two distinct regions within the Iberian Peninsula and locally associated with ponds of different duration. We have observed marked differences across these populations in their capacity to accelerate development in response to decreased water levels, and have subsequently conducted an RNA-Seq analysis to determine which genes were being differentially expressed in their liver, given the considerable metabolic effort required for accelerating development. Our results show that differences in both climate and hydroperiod heterogeneity may have been responsible for observed regional divergence in adaptive plasticity, whereas no local adaptive divergence was observed associated with pond type within each of those two regions. Genes differentially expressed in individuals subjected to reduced water levels were associated with thyroid hormone, glucocorticoids, and the urea cycle, all processes known to be tightly linked to the regulation of metamorphosis.

Keywords: phenotypic plasticity, development, genetic accommodation, differential gene expression, transcriptomics, RNA-seq, clima, hydroperiod, *Pelobates cultripes*

Introduction

Adaptive phenotypic plasticity is the capacity of a genotype to assess and respond to heterogeneous environments expressing appropriate phenotypes to the local conditions, hence enhancing the survival and fitness of organisms (West-Eberhard 1989). Plasticity will evolve given sufficient environmental

heterogeneity and/or gene flow among populations exposed to different selection pressures (Sultan and Spencer 2002). This ability to adjust the phenotype to the local environmental conditions can give rise to rapid adaptive divergences, especially when colonizing novel environments or facing rapid environmental changes such as the current global change (Le Rouzic and Carlborg 2008, Charmantier *et al*. 2008, Lande 2009; Pfennig *et al*. 2010).

Traditionally, biologists were convinced that genetic changes must proceed for evolution of phenotypes to happen, however, recent studies show that phenotypes may play a crucial role in leading the course of evolution (Pfennig *et al*. 2010, Casasa and Moczek 2018). For instance, it has been shown in North American spadefoot toads that selection acting on ancestral phenotypic plasticity resulted in morphological divergence among species (Levis *et al*. 2018). Induced phenotypes can be subject to divergent selection depending on various selection regimes, for instance the degree of environmental heterogeneity. The loss of environmental variability may result in one alternative phenotype being favoured by selection, and subsequently canalized into that phenotype (Pigliucci *et al*. 2006, Lande 2009). Phenotypic plasticity has strong evolutionary potential since induced plasticity lead ancestral states to diverge into a wide array of reaction norms, giving rise to diversified populations, and ultimately, species (Pigliucci and Murren 2003). Such a process where plasticity undergoes adaptive genetic changes fuelled by selection is known as genetic accommodation (West-Eberhard 2003; Pfennig *et al*. 2010). Genetic accommodation has been empirically demonstrated across various taxa, for instance in wing morphology of fruitflies exposed to heat shocks (Waddington 1953), or in color morphs of tobacco hawk moth larvae also in response to heat

shocks (Suzuki and Nijhout 2006). However, the underlying genetic mechanisms for this phenomenon still remain a vast field to be explored, although recent transcriptomic approaches are beginning to shed light on it (Casasa *et al*. 2020).

The advent of the –omics era is boosting studies on genome-wide changes and differential gene expression using non-model organisms (Minelli and Fusco 2010), as modern next-generation-sequencing techniques can identify the genes under selection as well as transcriptional plasticity (Jones *et al*. 2017, Jones and Robinson 2018). Genome-wide changes can be achieved by signal transduction via the neuroendocrine system through changes in hormone level changes, enzymes, or epigenetic signals that in turn are mediated by mechanisms such as DNA methylation, histone modification, or interference by small RNAs (Gilbert 2005, Gilbert and Epel 2009). In developmentally plastic organisms, however, genomes can be differentially expressed (hereafter, DE) according to the varying environmental conditions, without actual changes in the sequence (Aubin-Horth and Renn 2009). Taking a transcriptomic approach to developmental plasticity can help identify which DE genes are responsible for the adaptive plastic responses induced by the environment, and assess the extent to which organisms can respond to environmental pressures at a molecular level (Aubin-Horth and Renn 2009, Johansson *et al*. 2013).

Species groups wherein certain phenotypic traits have evolved independently under divergent selection regimes are excellent model systems to explore the process of genetic accommodation (Gomez-Mestre and Buchholz 2006). One such system is the evolution of developmental rate across spadefoot toad species, which is highly congruent with the idea of evolutionary divergence through genetic accommodation of ancestral plasticity.

Amphibian larvae often have the ability to accelerate their development to metamorphose faster and escape desiccation, given rapid pond drying (Denver 1998, Richter-Boix *et al*. 2006). Spadefoot toads comprise an ancient group of species that have evolved markedly divergent developmental rates (Buchholz and Hayes 2002), of which the ancestral state of development showed similar levels of developmental plasticity as today´s Western spadefoot toad, *Pelobates cultripes* (Gomez-Mestre and Buchholz 2006). The spadefoot toad *P. cultripes* is a developmentally plastic species that adjusts its developmental rate to the environmental conditions experienced: it grows large without significant advancements in development under benign conditions, whereas it accelerates its development and metamorphoses earlier to escape desiccation when ponds begin to dry up quickly. Meanwhile, the spadefoot toads *Spea multiplicata* and *Scaphiopus couchii* in North America adapted to breed in much more ephemeral ponds (Levis *et al*. 2018). While *S. multiplicata* retained a rather intermediate degree of plasticity, development in *S. couchii* became highly canalized into an accelerated version of ancestral state and has a very short larval period of approx. 7-10 days (Gomez-Mestre and Buchholz 2006). Developmental acceleration is achieved through increased corticosterone and thyroid hormone levels, and overexpression of thyroid hormone receptors (Gomez-Mestre *et al*. 2013, Kulkarni *et al*. 2017). This comes at the expense of a high metabolic cost, increased oxidative stress, and morphometric consequences (i.e. metamorphosing with a lower body mass, shorter snout-to-vent-, hindlimb-, and snout length; Gomez-Mestre *et al*. 2013). It has been shown that *P. cultripes* substantially increases its corticosterone and thyroid hormone levels if exposed to desiccation, while *S. multiplicata* has an intermediate level of hormone

expression and *S. couchii*, being canalized into a fast developmental rate, constitutively expresses high levels of stress hormones and a much higher metabolic rate (Kulkarni *et al*. 2017).

Although the pattern across spadefoot toad species seems clear, it is the result of a long period of independent evolution (> 100 my), which has resulted in various other changes, like a major divergence in their genome sizes (Zeng *et al*. 2014). Therefore, it is critical that we study the mechanisms leading to genetic accommodation at a finer scale, both geographically and temporally, as when populations within species adaptively diverge in their developmental responsiveness. In that spirit, here we study the potential divergence in adaptive developmental plasticity among *P. cultripes* populations from two different regions within the Iberian Peninsula, and within those regions, comparing populations breeding in either short- or long-lasting ponds.

We conducted a common garden experiment to test for population differences in their ability to accelerate development and achieve an early metamorphosis in response to decreased water level. We predicted that increased heterogeneity in pond hydroperiod would have favoured the evolution of plasticity, resulting in steeper reaction norms, and consequently genotypes from ponds with consistently shorter hydroperiods would be more canalized into constitutive fast development. In addition to duration of the larval period, we also recorded data on size and morphology at metamorphosis, which are known to be affected by changes in developmental rate (Gomez-Mestre and Buchholz 2006; Gomez-Mestre et al. 2010). Moreover, we combined the common garden experiment with standardized sampling of liver tissue for RNA-seq analysis of differential gene expression for the three most plastic and the three

most canalized populations, in order to compare their transcriptomic reaction norms. Further, we also used the reads obtained from the RNA-seq analysis to identify single nucleotide polymorphisms (SNPs) to use in determining the genetic structure of our study populations both within and between regions. By thoroughly studying this system from a multifaceted angle, also employing geographic information system (GIS) techniques to infer recent variation in pond hydroperiod, determine the degree of developmental plasticity of all populations, and evaluate the transcriptomic changes associated with such phenotypic plasticity, we aim to better understand the mechanisms that underlie the divergence in developmental plasticity across spadefoot toad populations.

Material and methods

STUDY POPULATIONS

We selected 4 populations in southern Spain (Doñana National Park, Huelva province) and 6 populations in central Spain (Madrid and Segovia province) (Table 1) to account for regional variation.

Table 1. Study populations

Region	Populations	N	W
Southern Spain	Llano	36°49'28.17"	6°21'39.61"
Southern Spain	Espajosas	37°05'0.774"	6°29'18.56"
Southern Spain	Jimenez	36°59'24.4"	6°27'21.8"
Southern Spain	Jabata	37°02'14.1"	6°26'53.4"
Central Spain	Roblellano	40º 51' 27.7"	3º 37' 42.6"
Central Spain	Canencia	40º 52' 17.1"	3º 45' 24.7"
Central Spain	Santo Tome	41º 11' 58.0"	3º 35' 17.9"
Central Spain	Turrubuelo	41º 19' 19.2"	3º 35' 17.5"
Central Spain	Buitrago	40º 58' 27.8"	3º 38' 39.2"
Central Spain	Valdemanco	40º 51' 11"	3º 38' 41"

To account for potential variation in developmental plasticity due to varying hydroperiod regimes, we carefully chose half of our populations to be short-lasting ponds and the other half to be long-lasting ponds (Figure 1) (for detailed methods on how average hydroperiods were estimated, see section "HYDROPERIOD").

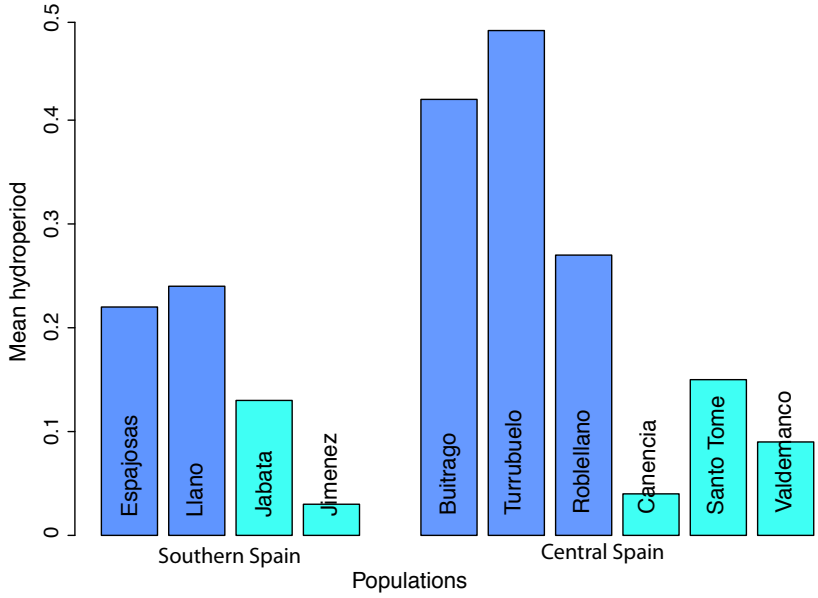

Figure 1. Mean pond duration (hydroperiod) over the last 25 years for the different study populations, based on GIS inference of the inundation period and expressed as a proportion of the year that ponds remained flooded. We refer to Espajosas, Llano as long-lasting/ Jabata, Jimenez as short-lasting in southern Spain, and Buitrago, Turrubuelo, Roblellano as long lasting/ Canencia, Santo Tome, Valdemanco as short-lasting in central Spain.

SAMPLE COLLECTION AND EXPERIMENTAL SETUP

We collected parts of *P. cultripes* egg clutches (40-50 eggs) from six populations in central Spain (Madrid and Segovia province) and four populations in southern Spain, within Doñana National Park (Huelva province). Egg clutches

of *P. cultripes* range from approx. 2000-2500 eggs (Lizana *et al*. 1994, Diaz-Paniagua *et al*. 2005), hence our sampling was not detrimental to the populations. Our sample size consisted of 660 tadpoles of central Spain (2016: 6 populations x 3 families x 2 treatments x 15 tadpoles = 540, 2017: 4 populations x 1 family x 2 treatments x 15 tadpoles = 120) and 280 tadpoles of southern Spain (2017: 4 populations x 5 families x 2 treatments x 7 tadpoles = 280).

The portions of egg clutches collected were brought to climatic chambers at Doñana Biological Station and kept under identical conditions (20 °C, 12L:12D cycle) until hatching. As hatchlings reached the free-feeding stage (Gosner stage 25; Gosner 1960), we haphazardly individualized them into 3.8 L containers with 3.5 L of water resulting in a 15 cm high water column. Tadpole containers were cleaned every third day and tadpoles were fed *ad libitum* with a mixture of ground rabbit chow and lightly boiled spinach. At Gosner stage 35 (Gosner 1960), when the digits of the hindlimbs became distinguishable, we reduced the water in the containers of half of the tadpoles from each sibship to 650 mL, only 4.5 cm deep to induce developmental acceleration (Kulkarni *et al*. 2011). One week after exposure to low water treatment, 3 tadpoles of each sibship/population and of both treatments were euthanized by immersion in a lethal concentration of buffered MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma). Those tadpoles were dissected to obtain liver tissue for RNA extraction and gene expression analysis. Liver samples were snap frozen in liquid nitrogen and stored at -80 °C until RNA was extracted.

LARVAL PERIOD AND MORPHOLOGICAL MEASUREMENTS

Larval period was estimated from the onset of the experiment until each individual reached Gosner stage 42 (Gosner 1960). After forelimb emergence, metamorphs were left only with 2 cm of water and provided with soaked tissue paper for shelter and to perch on as metamorphosis progressed. Upon complete tail resorption (Gosner stage 46), we weighed each toadlet on a digital scale to the nearest 0.1 mg, and measured snout length, hindlimb length, and snout-to-vent length (SVL). Snout length was measured perpendicularly from the tip of the eye to the end of the snout. Hindlimb length was the sum of foot (from the tarsal-metatarsal articulation to the tip of the longest toe), tarsal, fibiotibula, and femur length. Snout-to-vent length (SVL) was measured from the tip of the snout to the tip of the vent. All lengths were measured on a grid paper to the nearest 0.5 mm, pressing the animal slightly against the grid paper to minimize error.

CLIMATE DATA

For each of our 10 study sites (central Spain: Roblellano, Buitrago, Canencia, Valdemanco, Turrubuelo, Santo Tome/ southern Spain: Jimenez, Llano, Espajosas, Jabata), we extracted average monthly climate data for temperature and precipitation (minimum, mean, and maximum) during the period of 1970-2000 and obtained bioclimatic variables from Worldclim Version 2.1 (Fick and Hijmans 2017). The data was based on a spatial resolution of 1 km^2 (30 seconds). Bioclimatic variables (specifically, the 19 bioclimatic variables were used in this study, as outlined in Supplementary material S1) are derived from the monthly temperature and rainfall values in order to generate more biologically meaningful variables. The bioclimatic variables represent annual trends (e.g., mean annual temperature, annual precipitation) seasonality (e.g., annual range

in temperature and precipitation) and extreme or limiting environmental factors (e.g., temperature of the coldest and warmest month, and precipitation of the wet and dry quarters).

ESTIMATION OF HYDROPERIOD USING REMOTE SENSING

To test for local adaptation to different larval growth seasons, we aimed to chose populations originating from short-lasting ponds as well as long-lasting ponds (Figure 1).

Figure 1 Our study sites are not permanent water bodies, but dry up completely in the summer. There are short-lasting ponds that dry up earlier (left) and long-lasting ponds that may stay for a longer time period (right), allowing the tadpoles more time to develop.

In continuation, we aimed to get a realistic approximation of the suitable period for larval development of *P. cultripes* by determining the heterogeneity of the hydroperiod of each of the ponds of origin, while taking into account that the actual suitable growth period would be limited by temperature thresholds even when ponds are filled up. Pond durations of our 6 populations of central Spain

were determined by calculating monthly variation in NDWI (Normalized Difference Water Index; Gao 1996). Subsequently the proportion of number of photos with inundated areas in comparison to the total number of photos per year was determined (data availability from 1994 to 2019). Additionally, Sentinel-2 (data availability from 2016 to 2020) has been used to verify the effect of pixel sizes. Images with a cloud concentration higher than 10 % have been excluded from the analyses (Google Earth Engine). Pond durations of our 4 populations of the Doñana area (southern Spain) have been determined by counting the number of pixels of the area that was filled with water in each photo, based on photos obtained by Landsat with data availability from 1974 to 2014, using images extracted from the sensors Multi Spectral Scanner (MSS), Tematic Mapper (TM), Enhanced Tematic Mapper plus (ETM+), and Operational Land Imager (OLI) (Díaz-Delgado *et al*. 2016). This data was converted from a 0 – 365 scale to a 0 – 100 scale to make it comparable with data of the central populations. Both databases were based on a spatial resolution of 30 m^2 and temporal resolution of 16 days. Finally, the coefficient of variation in hydroperiod was calculated as a proxy for heterogeneity in pond duration across locations in the last two decades.

STATISTICAL ANALYSES OF PLASTICITY AND ENVIRONMENTAL VARIABLES

All statistical analyses were conducted in R v.3.2.1 (R core team, 2015). The effect of water treatment on larval period and morphometric consequences has been tested using the package lmtest v.0.9-38 (Zeileis and Horthorn 2002), by building generalized linear models with an underlying gamma error distribution. Water treatment, pond type, population, and region have been added as fixed

effects and clutch nested within population as random effect. The significance of each term was verified by running likelihood ratio tests specifying a chi-squared test on each model in comparison to another model excluding that term. Analyses of variation in snout and hindlimb length were corrected for variation in body length by including SVL as covariate in the models.

A principal component analysis was conducted on climatic data including all 19 bioclimatic variables obtained from Worldclim using the function 'prcomp' to explore differences in climate across localities. When clustering by region was confirmed, we further regressed variables of interest (annual precipitation, precipitation seasonality, annual mean temperature, temperature seasonality) against plasticity achieved in each population (standardized as the difference of larval period across water treatments).

TRANSCRIPTOMICS - SAMPLE PREPARATION

Based on the observed developmental responses (see results section), we chose the three most plastic populations (Llano, Jabata, Espajosas; all from southern Spain) and the three least plastic ones (Buitrago, Canencia, Turrubuelo; all from central Spain) and conducted RNA-Seq analyses on preserved liver tissue of their experimental tadpoles. We haphazardly chose one tadpole from each of four sibships per population as biological replicates, resulting in 48 samples (4 replicates x 6 populations x 2 treatments). In preparation for RNA extraction, each cryopreserved liver sample was homogenized in 1 mL of TRIzol reagent (Sigma-Aldrich Canada Ltd.), and RNA was extracted using Qiagen RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The quantity and quality of the extractions were assayed using a Bioanalyzer 2100 (Agilent

Technologies, Waldbronn, Germany). Library preparation and sequencing was done by BGI (http://www.bgi.com/global/) on a DNBSeq™ platform with PE100. Each sequenced library had a minimum of 20 million pair-end reads.

SEQUENCING QUALITY CONTROL

Raw sequencing reads were cleaned in two steps with the script *clean_pe_rna.sh* (see Supplementary material S2 for full script), which is a wrapper around BBTools v.38.86. The first step consists of trimming remaining DNBSEQ adaptor sequences and poly-A stretches longer than 3 bp from both ends of the reads. The second step is aimed at removing low-quality sequences, including those from the phiX viral genome (a common spiking sequence used in NGS) and sequencing artifacts. Also, we trimmed low-quality bases (PHRED score < 10) from the ends of the reads. Finally, reads with an average PHRED score < 12 after the trimming process were removed as well.

READ MAPPING

We used the mapping-based mode of the *salmon* software v.1.2.1 (Patro *et al*. 2017) to quantify RNAseq reads. For this, we used reference transcripts derived from the genome assembly PECUL23, a draft genome of *P. cultripes* available to our research group, in preparation for publication. To improve the mapping performance, we included a decoy that consisted of non-coding regions of the genome as well as the mitochondrial genome (mtDNA onward) and a consensus of the nuclear ribosomal cistron (nrDNA onward). The mitogenome was not assembled for PECUL23 and so was assembled by mapping the clean reads of one of the biological replicates of our experiment (Bui1L9) against a published *P.*

cultripes mitochondrial genome (Genbank accession number: NC_008144.1; Gissi *et al*. 2006). The nrDNA was assembled following the structure: 5'ETS--18S_rRNA--ITS1--5.8S_rRNA--ITS2--26S_rRNA--3'ETS, using MEGAHIT v.1.2.9 (Li *et al*. 2015). Since the nrDNA represents a consensus of a tandem repeat it can be represented as a circular sequence. Both, mtDNA and nrDNA, are usually over-represented in RNAseq experiments (despite poly-A enrichment or rRNA depletion), therefore they had to be integrated in the decoy reference to avoid forced mapping artifacts.

DIFFERENTIAL GENE EXPRESSION ANALYSIS

The raw counts quantified by *salmon* across all libraries were combined into a single counts matrix using the R package *tximport* v.1.101 (Soneson *et al*. 2016). Raw count data at the transcript level were used for Differentially Expressed Genes (hereafter, DEG) analysis. The DEG analysis was run using the R package DESeq2 v.1.22.2 (Love *et al*. 2019: updated version of Love *et al*. 2014). The count data was transformed using Variance Stabilizing Transformation (VST) in order to normalize the data and for removal of the variance's dependency on the mean. The transformed data was used for counts exploration, employing a principal component analysis. As strong clustering across localities (Central vs. South) was observed that may obscure treatment effects, we proceeded to run the DEG models on the counts of each population independently, setting water level as the treatment contrast for the analyses.

By default, the log fold change (LFC) calculation uses a normal prior distribution, centred on zero with a scale that is fit to the data (Love *et al*. 2014), and applies a strong filter threshold excluding genes with low *p*-value LFC. We

used an alternative shrinkage estimator using the R package apeglm (Zhu *et al*. 2018). This allowed shrinking low counts and contrasting them by effect size ranking *p* values associated with a null hypothesis, keeping genes with a small *p*-value and low variation in expression. To test for significant differences in expression between the two treatments (i.e. low/high water level), we estimated fold changes for each gene, which estimates the effect size, arbitrarily treating tadpoles originating from the high water treatment as control, using default settings of the DESeq2 pipeline. A diagnostic check of the model fit was done using the function "plotDispEsts" of the DESeq2 package, comparing the final shrunk estimates from gene-wise assessments towards the fitted approximations. We considered any gene with a false discovery rate < 0.05 to be DE between the two water level treatments.

FUNCTIONAL ENRICHMENT ANALYSIS

To test whether particular ontology terms or functional pathways were significantly enriched, all DEG that had available *Xenopus tropicalis* annotations (blastx hits of the *P. cultripes* genome transcripts against the *X. tropicalis* proteome v.9.1 of *Ensembl* release 100), were further analyzed using the R package *gprofiler2* v.0.2.0 (Reimand *et al*. 2019). This list of DEG (pooling up and down regulated genes) was compared to a custom background consisting of annotations of all *X. tropicalis* annotations of the *P. cultripes* genome. Three Gene Ontology groups (Molecular Function, Biological Process, and Cellular Component), KEGG (Encyclopedia of Genes and Genomes), and Reactome classification systems were used as domains for searching functional

enrichment. A threshold of $p < 0.05$ was set as cut-off for significantly enriched pathways.

VARIANT CALLING USING RNAseq READS

We aimed to identify single nucleotide polymorphisms (SNPs) present in our RNA sequences, to verify genetic clustering across populations. In order to do so, the reads were mapped to a reference that included the genome assembly, the mtDNA, and the nrDNA (Pcu23.ss.mt.nr.fa). We used two splice-aware mappers, GMAP v.2019.09.12 (Wu. *et al*. 2010) and STAR v.2.7.5a (Dobin *et al*. 2013). Then we proceeded to do variant calling on the resulting BAM files using two commonly used variant callers, *freebayes* v.1.3.2 (Garrison and Marth arXiv) and *bcftools* (part of *samtools* v.1.9) (Li 2011). Finally, SNP counts across method combinations (gsnap + bcftools, gsnap + freebayes, star + bcftools, and star + freebayes) were compared.

The resulting VCF files were converted into phylogenetic matrices using *vcf2phylip* v.2.3 (Ortiz 2019). The matrices were analyzed in IQ-TREE v.2 (Minh *et al*. 2020) to obtain phylogenetic trees using the model GTR+ASC (general time reversible nucleotide substitution model with ascertainment bias correction to account for only including variable sites in the alignments) and support was derived from 5000 ultrafast bootstraps (Hoang *et al*. 2018).

Results

DEVELOPMENTAL ACCELERATION AND MORPHOLOGICAL CONSEQUENCES

There was a significant region effect on larval period (χ^2 = 6.84, p = 0.008, Table 1), and also a highly significant interaction of water treatment x region (χ^2 = 44.2, p < 0.001). On the contrary, pond type (short-lasting/long-lasting) had no significant effect on larval period (χ^2 = 0.02, p = 0.89), and neither did the interaction of water treatment x pond type effect (χ^2 = 0.07, p = 0.78). Thus, we proceeded with exploring the effects of water treatment on developmental acceleration and its morphological consequences within each region.

Table 1. Plasticity achieved of each population, obtained by standardized differences of larval period across water treatments. Positive plasticity values indicate the number of days accelerated when exposed to low water treatment, whereas negative values indicate that some populations had a longer larval period in control than in the low water treatment.

Region	**Population**	**Plasticity**
Southern Spain	Espajosas	56.11
Southern Spain	Llano	42.69
Southern Spain	Jabata	55.25
Southern Spain	Jimenez	16.42
Central Spain	Canencia	6.81
Central Spain	Buitrago	-5.69
Central Spain	Turrubuelo	-12.97
Central Spain	Santo Tome	-19.77
Central Spain	Roblellano	3.27
Central Spain	Valdemanco	10

For the central Spain populations, reduced water level did not cause a general shortening of larval period (χ^2 = 0.04, p = 0.85). Nevertheless, a significant water treatment x population interaction was found (χ^2 = 20.99, p = 0.0008), showing that not all populations responded the same way to decreased water levels. (Figure 3b, Supplementary material S3). Decreased water level decreased body mass upon metamorphosis by 21% on average (χ^2 = 212.58, p < 0.001), and body mass also showed a significant water treatment x population interaction (χ^2 = 12.17, p = 0.03), showing population differences in growth under varying water levels (Figure 4b). Body length (SVL) of the metamorphosing toadlets, and size-corrected snout and hindlimb lengths decreased in individuals exposed to reduced water level (SVL: χ^2 = 183.31, p < 0.001, 8.67 % decrease (Figure 4d), snout: χ^2 = 3.74, p = 0.05, 4.76 % decrease, hindlimb: χ^2 = 14.19, p = 0.0001, 10.55 % decrease (Figure 5b,d)). However, these morphological features were not affected by a water x population effect (SVL: χ^2 = 7.24, p = 0.2, snout: χ^2 = 7.61, p = 0.18, hindlimb: χ^2 = 2.12, p = 0.83).

The southern populations instead significantly accelerated their development in response to decreased water level (χ^2 = 45.36, p < 0.001), achieving metamorphosis on average 22.8 % earlier (Fig. 3). We observed no significant water x population effect on time to metamorphosis (χ^2 = 3.5, p = 0.32) (Figure 3a, Supplementary material S4). Decreased water level significantly decreased body mass (χ^2 = 121.34, p < 0.001, 36.8 % decrease) and body length (SVL) upon metamorphosis (χ^2 = 104.46, p < 0.001, 12 % decrease) (Figure 4a,c). The interaction of water treatment and population had a significant effect on body length (SVL) (χ^2 = 9.45, p = 0.02), while it had a

marginally significant effect on body mass upon metamorphosis (χ^2 = 7.52, p = 0.05). Neither snout (χ^2 = 0.01, p = 0.91) nor hindlimb (χ^2 = 0.99, p = 0.32) lengths were significantly decreased when exposed to reduced water levels in these southern populations, and there was no significant water x population effect on either snout (χ^2 = 1.16, p = 0.76) or hindlimb lengths (χ^2 = 1.93, p = 0.59) of individuals of both water treatments upon metamorphosis (Figure 5a,c).

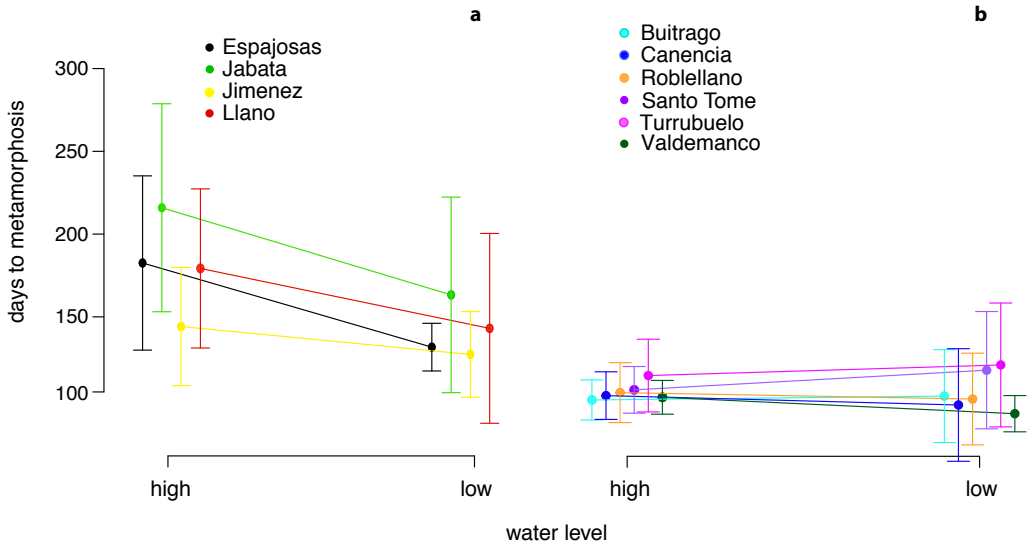

Figure 3 Southern populations (a) accelerated their development significantly shortening their larval period when exposed to the low water treatment, whereas the central populations (b) did not readily do so. Further, it is noteworthy that the southern populations had a broader range of larval period whereas the central populations were much more canalized into fast development.

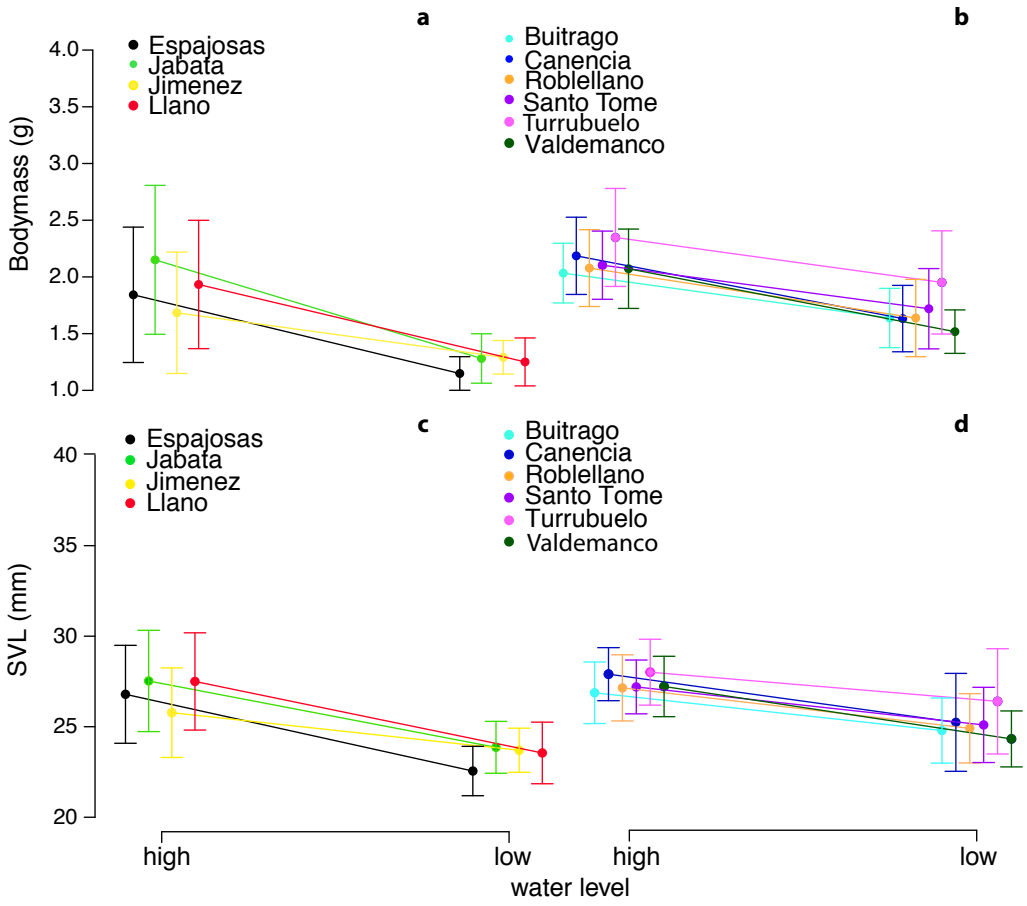

Figure 4 Individuals of both the southern (a, c) and central (b, d) populations metamorphosed with a lower body mass and shorter snout-to-vent (SVL) length when exposed to low water treatment. This was true for all populations regardless of whether they accelerated their development or not.

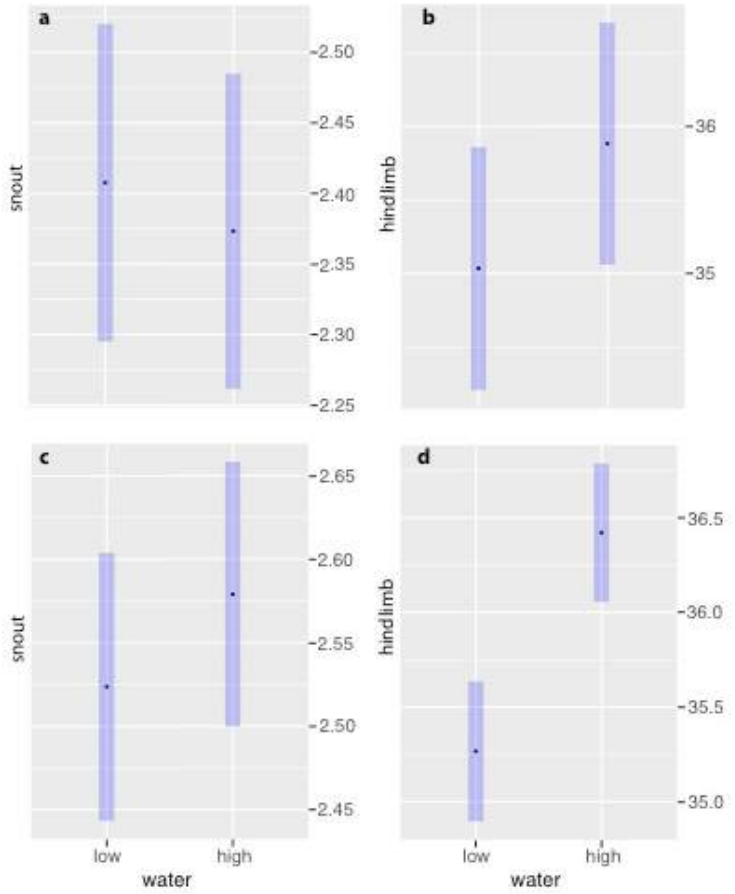

Figure 5 Adjusted means of snout and hindlimb lengths corrected for body size in southern (a, b) and central (c, d) populations. Individuals from southern populations did not metamorphose with a shorter snout when exposed to low water treatment and the effect on the hindlimb was mild and not significant. However, individuals from the central populations exposed to low water, metamorphosed with blunter snouts and proportionally shorter hindlimbs than control individuals developing in constant high water.

CLIMATE DATA

The first principal component extracted explained 77% of the total variance and represented variation in rainfall and temperature, as most variables (Bio_1 (annual mean temperature), Bio_4 (temperature seasonality), Bio_6 (max temperature of coldest month), Bio_8 (mean temperature of wettest quarter), Bio_9 (mean temperature of driest quarter), Bio_10 (mean temperature of warmest quarter), Bio_11 (mean temperature of coldest quarter), Bio_14 (precipitation of driest month), Bio_15 (precipitation seasonality (coefficient of variation)), Bio_17 (precipitation of driest quarter), Bio_18 (precipitation of warmest quarter)) loaded heavily on PC1, and all to a similar degree. The second principal component explained 19% of the total variance and represented variation in Bio_12 (annual precipitation) and Bio_2 (mean diurnal range (mean of monthly (max temp – min temp))) (Supplementary material S5, S6). The local climates of the populations from central and southern Spain were clearly clustered, divided by PC1 (Figure 6).

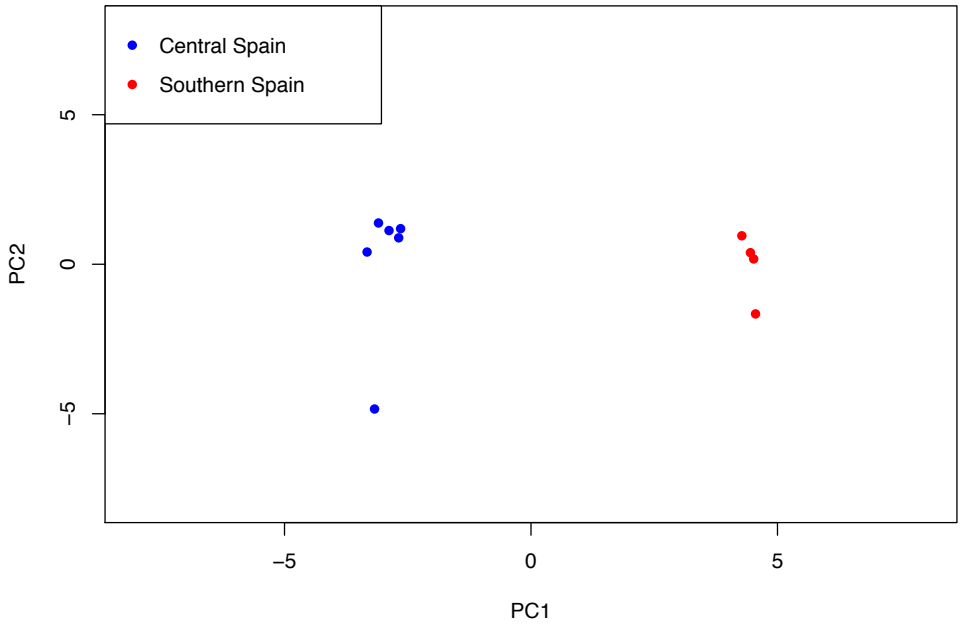

Figure 6 PCA plot showing that localities were clearly differentiated in climate. Variation along the PC1 axis shows regional differences in annual mean temperature, temperature seasonality, max temperature of coldest month, mean temperature of wettest quarter, mean temperature of driest quarter, mean temperature of warmest quarter, mean temperature of coldest quarter, precipitation of driest month, precipitation seasonality, precipitation of driest quarter, and precipitation of warmest quarter, which can largely be summarized into variation in temperature and precipitation across the two regions.

Plasticity (standardized difference of larval period across water treatments) was significantly explained by Bio_01 (annual mean temperature, $F_{1,8} = 20.46$, $p = 0.001$), Bio_04 (temperature seasonality, $F_{1,8} = 17.28$, $p = 0.003$), Bio_15 (precipitation seasonality, $F_{1,8} = 24.48$, $p = 0.001$), but not Bio_12 (annual precipitation, $F_{1,8} = 0.25$, $p = 0.6$).

HYDROPERIOD

Although the average inundated period of ponds across localities was similar ($F_{1,8} = 0.69$, $p = 0.4$, Figure 7a), their heterogeneity (as determined by the coefficient of variation of hydroperiod) differed across localities, although

marginally non-significant due to low sample size ($F_{1,8}$ = 5.21, p = 0.05, Figure 7b). The same pattern was observed regarding regional differences in plasticity (standardized difference of larval period across water treatments) ($F_{1,8}$ = 23.28, p = 0.001, Figure 7c).

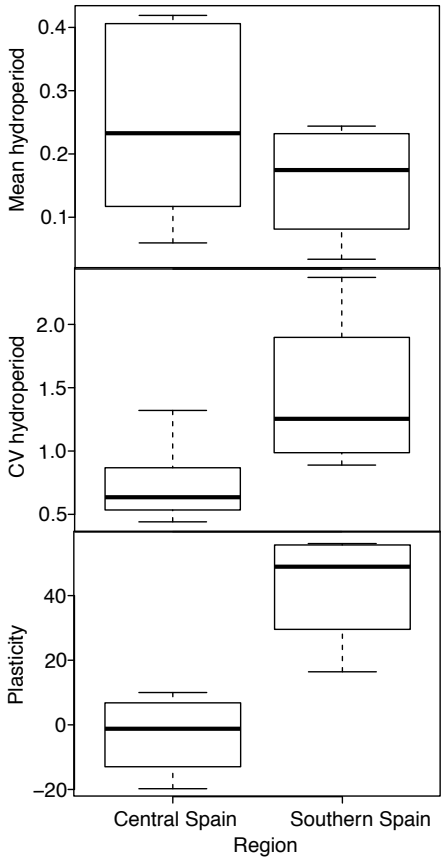

Figure 7 The mean hydroperiod was similar across central and southern Spain (a) while the coefficient of variation varied, showing higher environmental heterogeneity in the southern populations (b). Consistently, the southern populations showed a higher degree of plasticity achieved than the central populations (c), i.e. a greater capacity to accelerate development and achieve an early metamorphosis.

DIFFERENTIAL GENE EXPRESSION

The PCA on vst-transformed count data (RNA-seq reads mapped to each transcript in the transcriptome per library) showed clear clustering by region (southern VS central). PC1 explained 33% of the variance in count data and PC2 explained an additional 16%, with the two regions clearly differentiated by PC2

(Figure 8). The region effect is masking the treatment effect resulting in unequal variances across populations and so the DEG analysis was conducted for each population independently.

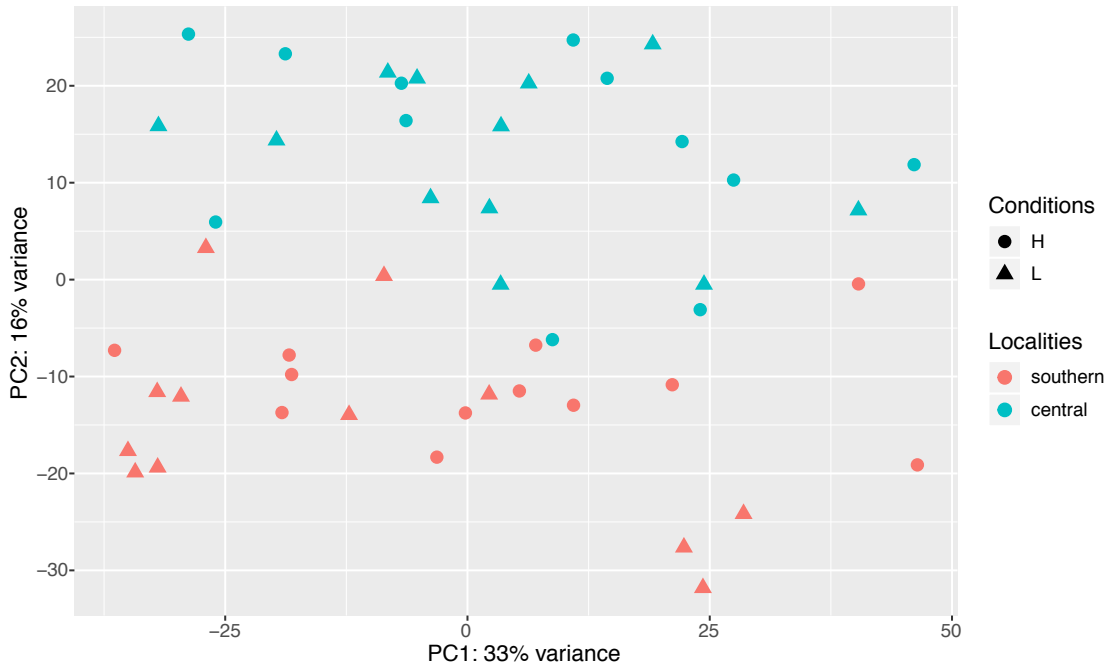

Figure 8 PCA plot showing clustering by localities (southern and central Spain) divided along the PC1 axis, while there is no clear differentiation by experimental conditions (H: high, L: low water treatment). PC1 explained 33% of the variance and PC2 another 16%.

Our DEG analyses for each population showed that the number of DEG in response to water level was similar across populations and regions (Table 2), with the exception of Llano (plastic southern population), which had substantially more DEG in comparison to all other populations.

Table 2. Summary of DEG analysis across populations. Total nonzero read counts after filtering out transcripts with zero or low (<2) counts. Also shown are significantly DE transcripts (false discovery rate *adjp* < 0.05), and within those, the number of up-regulated (LFC > 0) and down-regulated (LFC < 0) transcripts.

	Llano	Espajosas	Jabata	Buitrago	Canencia	Turrubuelo
Total	38,601	38,784	38,887	38,403	37,509	38,316
low count <2	10,938 (28 %)	8,272 (21 %)	8,294 (21 %)	5,212 (14 %)	8,000 (21 %)	9,637 (25 %)
Outliers	2,553 (6.6 %)	2,545 (6.6 %)	2,751 (7.1 %)	2,193 (5.7 %)	1,993 (5.3 %)	2,100 (5.5 %)
DE transcripts *adjp* < 0.05	692	216	113	123	213	199
LFC > 0	335 (0.87 %)	117 (0.3 %)	55 (0.14 %)	70 (0.18 %)	101 (0.27 %)	115 (0.3 %)
LFC < 0	357 (0.92 %)	99 (0.26 %)	58 (0.15 %)	53 (0.14 %)	112 (0.3 %)	84 (0.22 %)

To compare how many transcripts were consistently up- or down-regulated across populations, we used the R package *upset* to plot the number of DE transcripts of each population and the intersects between populations (Figure 9). By a large margin, most DEGs were population specific, with comparably fewer shared DEGs across populations. Nonetheless, we found 24 up and 5 down regulated genes shared by at least two of the three plastic (southern) populations compared to 3 up and 4 down regulated genes in less-plastic (central) populations. We also found 17 genes that are up regulated and 14 down regulated genes that were shared between at least one plastic and one less plastic population. Based on the available genome annotations for *X. tropicalis* and *P. cultripes*, we identified 4 genes of interest that were up-regulated in two of the highly plastic southern populations (Llano and

Espajosas): XB-GENE-997273 related to amine oxidase, XB-GENE-5754317 associated to adipocyte plasma membrane associated protein (APMAP), XB-GENE-482624 associated to arginase 2, and XB-GENE-977784 associated to argininosuccinate synthase 1. Also, two genes of interest directly linked to developmental acceleration have been identified among the up regulated genes from the Llano population: XB-GENE-484679, the thyroid hormone induced bZip protein and XB-GENE-6071044, a thyroid hormone receptor interactor 1.

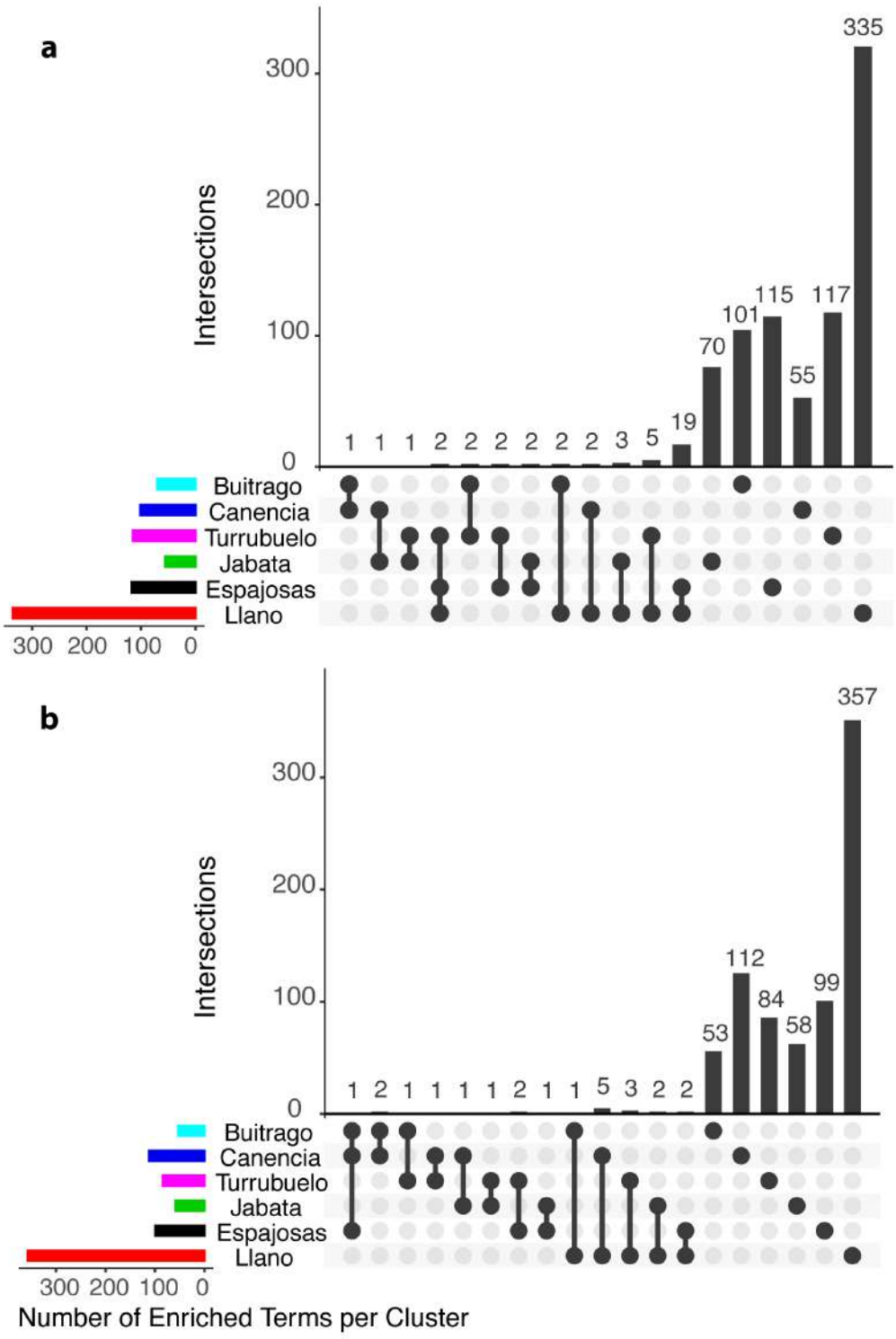

Figure 9 Number of up-regulated transcripts in each population (a) and number of down-regulated transcripts in each population (b) in response to low water conditions and following developmental acceleration, and corresponding intersects across populations for each (the bars connected by dots show how many transcripts each population group has in common, for instance, Llano and Espajosas have 19 up regulated transcripts in common).

Further, we explored the association between the total number of DE transcripts in each population with the level of plasticity (standardized difference of larval period across water treatments) shown in each population (Figure 10). No significant relationship between number of DE transcripts and plasticity was found ($F_{1,4} = 0.32$, $p = 0.6$).

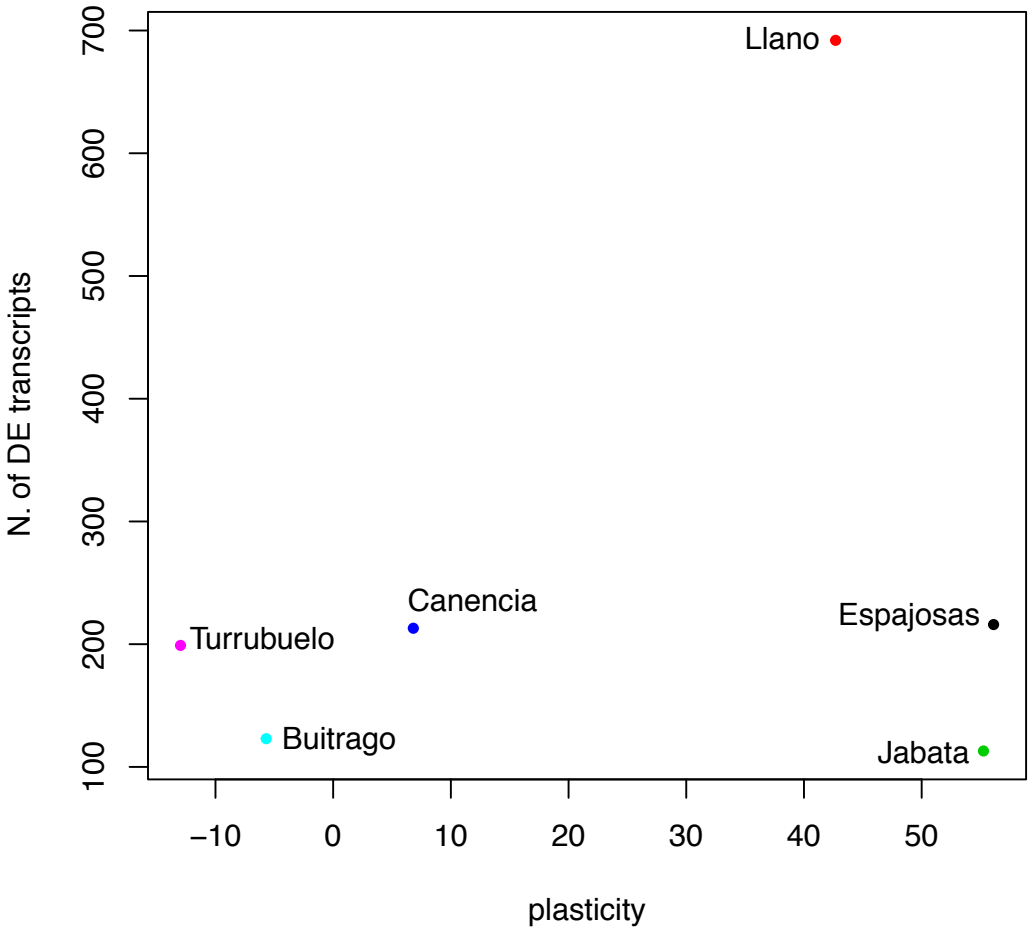

Figure 10 Lack of relationship between number of DE transcripts (sum of up and down regulated transcripts) and plasticity achieved (the slope of larval period across water treatments). The three southern populations (Espajosas, Llano, Jabata) are more plastic than the central populations (Canencia, Buitrago, Turrubuelo). However, the number of DE transcripts was similar across most populations of the two localities except for Llano, which has an exceptionally high number of DE transcripts.

FUNCTIONAL ENRICHMENT

The enrichment analysis identified 34 enriched terms in the Llano population (Figure 11), of which the majority were involved in the metamorphic process and developmental acceleration, 1 enriched term was identified in Espajosas (urea cycle), and 2 in Buitrago (adipocytokine signalling pathway, regulation of macromolecule metabolic process). No functionally enriched pathways were identified in the other three populations Jabata, Canencia, and Turrubuelo.

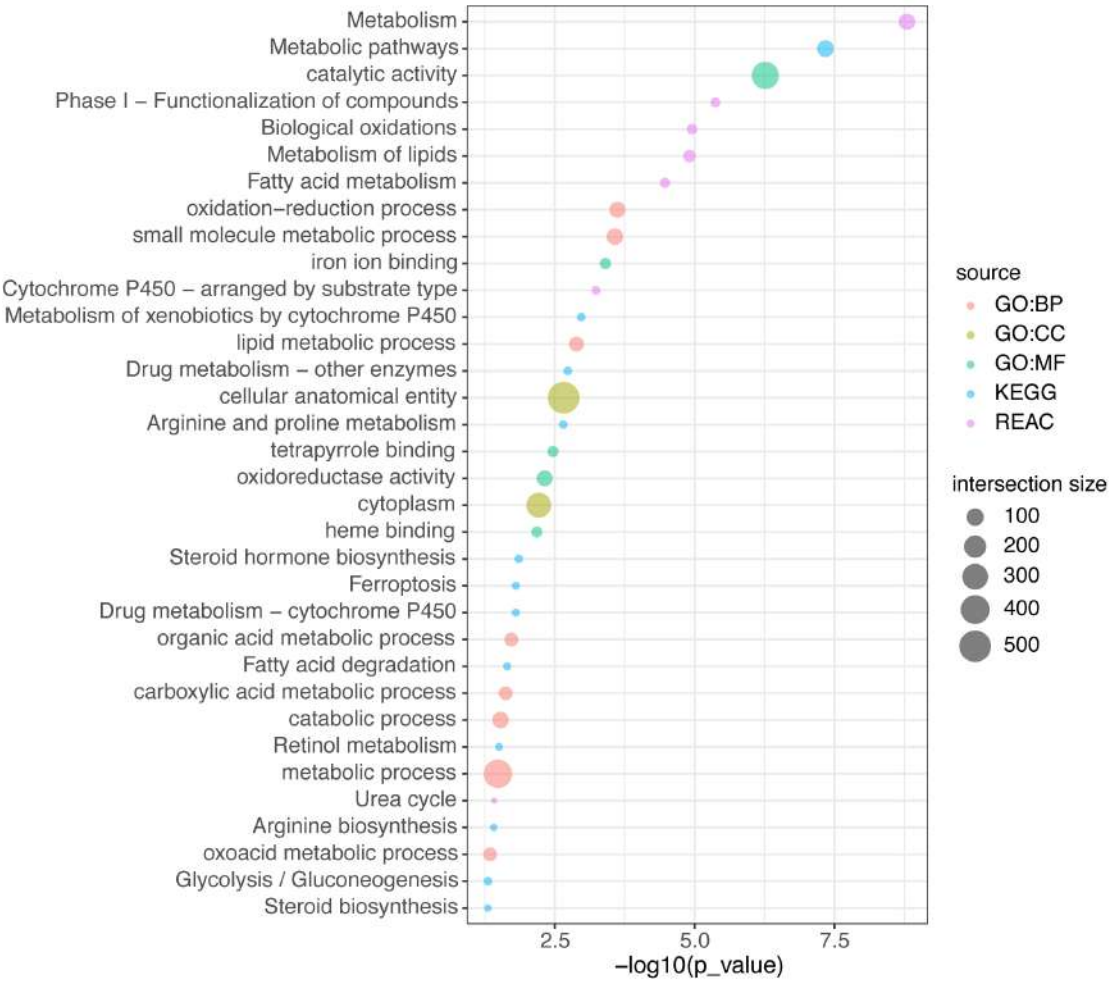

Figure 11 Bubbleplot, showing the list of significantly enriched functional pathways and corresponding intersection sizes of the Llano population.

VARIANT CALLING USING RNA-seq READS

Different numbers of SNPs have been quantified using combinations of different methods (Table 3)

Table 3. Final tally of SNPs across methods

Method	Min. 4 samples per SNP	Min. 24 samples per SNP	Min. 24 variable sites
gsnap+ bcftools	2,116,637	1,118,245	507,094
gsnap+freebayes	847,805	543,132	373,388
star + bcftools	1,161,453	773,116	470,613
star + freebayes	788,763	522,771	364,351

Preliminary phylogenetic analyses showed that while the topology of the clades Turrubuelo, Buitrago, and Canencia (central Spain) were similar and showed high support across all methods, the clades Espajosas, Llano, and Jabata southern Spain) were more intermixed across methods. Nevertheless, there was a consistent differentiation across the central and southern populations across all methods. Combining gsnap and freebayes showed the best support (Figure 12; trees resulting from other methods shown in Supplementary material S7).

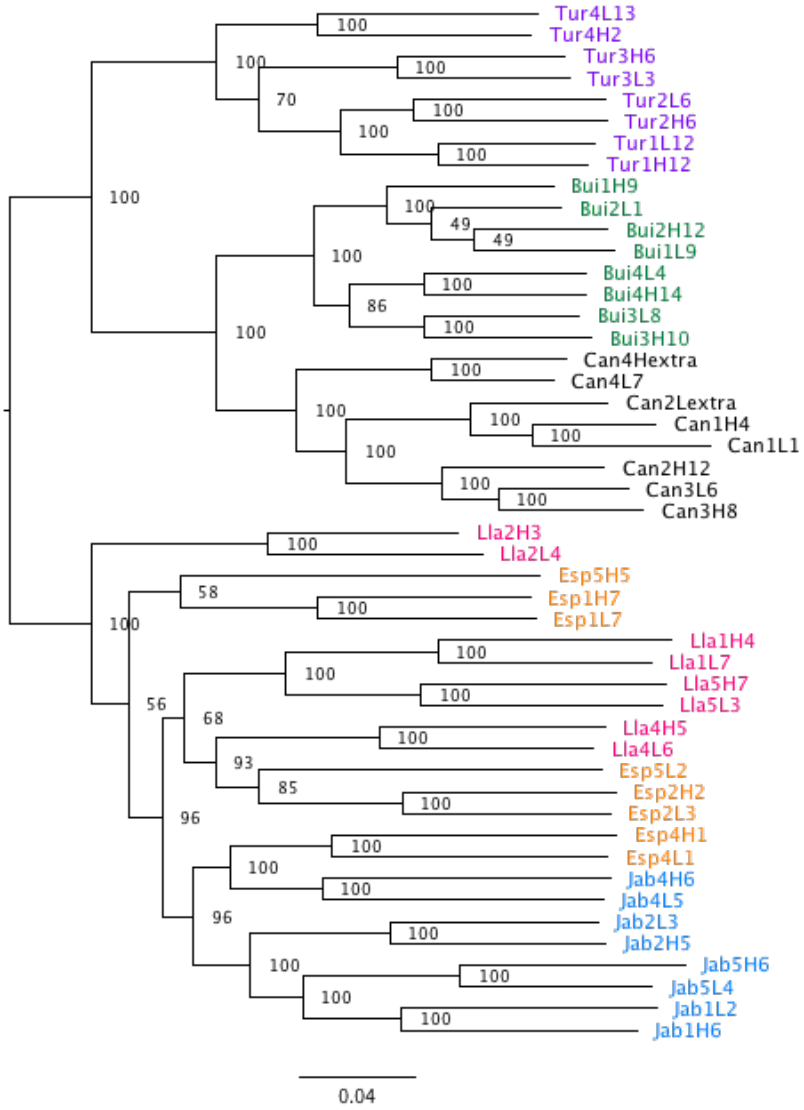

Figure 12 Phylogenetic tree resulting from combining the methods *gsnap* and *freebayes*, showing clear differentiation across central and southern populations. The central populations are clearly differentiated while the southern populations are somewhat intermixed.

Discussion

We observed marked population differences in their ability to accelerate their response when facing reduced water levels indicative of pond drying. However, pond type (short-lasting or long-lasting) was not a determining factor of population divergence in developmental plasticity in spadefoot toads, as populations breeding in either type of pond showed a similar capacity for developmental acceleration in response to reduced water level (Figure 3). This lack of fine-scale adaptive divergence within regions according to pond duration is not entirely surprising given the broad interannual variation in hydroperiod and pond characteristics typical of Mediterranean temporary ponds (Gómez-Rodríguez *et al*. 2010). Such environmental heterogeneity selects for increased phenotypic plasticity (Pigliucci *et al.* 1999; Lind and Johansson 2007). Also, gene flow among populations may have impeded local divergences, as it would have homogenized the reaction norms of the different populations within each region, especially for the southern populations. These southern populations were geographically not too distant from each other and there were no major geographical barriers among them. Consequently, gene flow among these populations was apparent as genetic variation in SNPs showed lack of structure and a high level of intermixture.

Instead, we found marked regional differences in developmental plasticity. The populations from central Spain showed consistently faster developmental rates than the southern ones, and a reduced capacity for developmental acceleration in response to pond drying (Figure 3). The two regions are clearly differentiated in their climate, both in terms of temperature and precipitation (Figure 6), and their divergence in the degree of developmental

plasticity was significantly correlated with such climatic divergence. In Doñana National Park, where the temperature is benign in winter and there is no risk of freezing, spadefoot toads may start breeding as early as October or postpone breeding until late March or early April, as it is entirely dependent upon rainfall stochasticity (Díaz-Paniagua 1986; Díaz-Paniagua *et al*. 2005). Regardless of the timing of breeding, southern spadefoot toad tadpoles can continue to grow until ponds dry up, which usually takes place sometime in June. The breeding period of the central populations (Madrid and Segovia) is more constrained in time due to the colder temperatures. Even if ponds fill up earlier in the season, spadefoot toads will not start breeding until temperatures start to rise and the risk of freezing is lower, by mid- or late February (Salvador *et al*. 1986; Salvador and Carrascal 1990; Lizana *et al*. 1994). Hence, the southern populations dispose of a longer window for adults to breed and for larvae to grow (~7 *vs*. 3 months), which has allowed for longer larval periods to evolve compared to the central populations. Moreover, remote sensing-based estimates of hydroperiod fluctuations for the last >20 years show that there is higher heterogeneity in the hydroperiod regimes of the southern populations (Figure 7). Taken together, longer duration of the growing season and greater heterogeneity in pond duration explain the adaptive divergence observed between regions so that the southern ones experience longer larval periods, but with a greater capacity for developmental acceleration when facing risk of pond drying.

Under constant water conditions, the central populations attained similar sizes at metamorphosis than the southern ones, but over a shorter larval period (Figure 3, 4), hence showing an overall faster growth rate. Interestingly, however, regardless of whether larval period was effectively shortened or not in

response to pond drying, individuals from both regions experienced similar consequences from exposure to low water. Developmental acceleration comes at the expense of reduced body mass, body length and allometric changes in head shape and limb length (Gomez-Mestre and Buchholz 2006; Gomez-Mestre et al. 2010). However, the central populations experienced such detrimental consequences of exposure to risk of pond drying and to a similar degree than the more plastic southern populations but without the adaptive reward of accelerated development (Figure 4, 5).

Phenotypic plasticity is ultimately the end product of differential gene expression. Indeed, it has been defined as the "re-programming of the genome in response to the environment" (Aubin-Horth and Renn 2009). Recent innovations such as transcriptomic approaches and genome-wide-association-studies (GWAS) serve as powerful tools to identify the genes of interest responsible for the observed phenotypic expressions under given environmental pressures (Lafuente and Beldade 2019). The main advantage of transcriptomic approaches is that it strives to identify a series of up and down-regulated genes, that are likely to add up to explain the biological phenotype observed (Aubin-Horth and Renn 2009), which can be subsequently explored to understand the biological functions of the genes and pathways involved.

Contrary to our prediction that the more plastic southern populations would have a higher number of DEG involved in developmental acceleration in comparison to the more canalized central populations, we found that the number of DEG was within a similar range across populations (Table 2) with the exception of Llano. In general, the degree of DEG is expected to reflect the capacity of the organism to respond to environmental pressures (Aubin-Horth

and Renn 2009), as has been demonstrated for instance in the correlation between beetle morphology and DEG count in response to different nutrition scenarios (Casasa *et al*. 2020). Nevertheless, during the process of genetic accommodation, environmentally induced changes in gene expression may differ across genes and populations, maintaining similar degrees of environmental sensitivity even though trait responsiveness is reduced or lost (Renn and Schumer2013). The differences in developmental plasticity observed in our system while the extent of differential gene expression is similar across populations (with the exception of Llano) may be driven by standing genetic variation across localities, or changes in the responsiveness of different sets of genes. Further weighted gene co-expression network analysis (WGCNA) would help elucidate whether specific clusters of genes explain the divergence in gene expression responsible for the observed region-by-environment interaction. Moreover, genome wide association studies (GWAS) aimed at identifying patterns of divergent standing genetic variants across populations in SNPs associated with developmental rate would be critical, although this would require a larger sample size.

Differentially expressed genes associated with developmental responses

Two transcripts involved in thyroid hormone expression have been identified based on the annotations of *X. tropicalis* in the Llano population (XB-GENE-484679, the thyroid hormone induced bZip protein and XB-GENE-6071044, a thyroid hormone receptor interactor 1). These changes are congruent with key mechanisms known to mediate developmental acceleration in *P. cultripes*: elevated thyroid hormone secretion and overexpression of the

thyroid hormone receptor β (TRβ; Gomez-Mestre et al. 2013; Kulkarni *et al*. 2017). Further, we detected four up-regulated genes in two of the highly plastic southern populations (Llano and Espajosas). These genes were: XB-GENE-5754317, linked to adipocyte plasma membrane associated protein (APMAP); XB-GENE-997273, related to amine oxidase; XB-GENE-482624 associated to arginase 2; and XB-GENE-977784 associated to argininosuccinate synthase 1. These genes are involved in lipid metabolism and the urea cycle, which is activated by the thyroid hormone during metamorphosis (Patterton and Shi 1994, Callery and Elinson 1996). The adipocyte plasma membrane associated protein (APMAP) is linked to adipocyte differentiation, and can also catalyze antioxidative detoxification reactions (Ilhan *et al*. 2008). Amine oxidase is an enzyme that deaminates amino acids, generating ammonia, which then is dealt with through the urea cycle. The latter two, arginase 2 and argininosuccinate synthase 1 are both enzymes involved in the urea cycle, which is of particular importance because amphibians switch to ureotelism from ammoniotelism during metamorphosis. Aquatic larvae excrete ammonia, which is highly toxic and thus needs to be washed off with abundant water, which is easily achieved in an aquatic medium. During metamorphosis, amphibians switch to a metabolically more expensive but less toxic production of urea. The activation of the urea cycle is dependent on thyroid hormone, so it normally takes place as thyroid hormone increases as they approach metamorphic climax (Balinsky *et al*. 1972, Wright and Wright 1996, Gomez-Mestre *et al*. 2004). Since *P. cultripes* larvae are elevating thyroid hormone levels in response to decreased water levels (Kulkarni *et al*. 2017), it is reasonable that the urea cycle is also becoming activated, as the increased expression of arginase and argininosuccinate

synthase indicate. It has been shown that terrestrial embryos of some semi-terrestrial species have evolved an early switch to ureotelism (Shoemaker and McClanahan 1973, Alcocer *et al*. 1992, Grafe *et al*. 2005). Further, *Scaphiopus couchii* larvae, which have evolved a highly canalized and fast developmental rate, have been shown to be ureotelic from an earlier stage onwards (Jones 1980), suggesting a heterochronic shift in the activation of the urea cycle with respect to *P. cultripes*. Our finding that *P. cultripes* tadpoles induced to accelerate their development show increased expression of genes involved in the urea cycle is congruent with a general pattern of plastic responses in *P. cultripes* being canalized in the derived development of *S. couchii*.

We have identified 34 enriched pathways in the Llano population related to metabolism and important hormones triggering the metamorphic process. Enriched pathways involved in the metabolism of lipids ('metabolism of lipids', 'fatty acid metabolism', 'lipid metabolic process', 'fatty acid degradation') are of importance because *P. cultripes* tadpoles are known to require a substantial metabolic effort to achieve accelerated development, actively using up their fat bodies in the process. Consequently, terms involved in lipid metabolism are expected to be enriched in accelerating individuals. The fast developing close relative *S. couchii,* has been shown to be incapable of accumulating fat bodies, even when treated with a chemical blocker of metamorphosis and therefore experiencing a prolonged larval period (Kulkarni *et al*. 2011). Further enriched pathways involved in the urea cycle ('arginine and proline metabolism', 'urea cycle', 'arginine biosynthesis') show that our *P. cultripes* tadpoles increased thyroid hormones in response to low water treatment, which subsequently triggered changes in the urea cycle to switch to ureotelism and precipitate a

precocious metamorphosis (Patterton and Shi 1994, Callery and Elinson 1996). Metamorphosis in *P. cultripes* tadpoles is achieved by increased thyroid hormone as well as corticosterone (Kulkarni et al. 2017), and accordingly, two pathways ('steroid hormone biosynthesis', 'steroid biosynthesis') involved in the production of corticosterone have been found to be functionally enriched. In Espajosas, one functionally enriched pathway involved in 'urea cycle' has been identified. This is particularly interesting as Llano and Espajosas both have this pathway enriched and share four transcripts involved in the switch from ammoniotelism to ureotelism during the metamorphic process. We have also found two enriched pathways each involved in 'adipocytokine signalling pathway' and 'regulation of macromolecule metabolic process' in Buitrago, one of the canalized populations. Adipocytokines are cell signalling proteins secreted by the adipose tissue, like leptin, and mRNA for these accumulate in fat tissue before metamorphosis (Bender *et al*. 2018), inducing a cessation in food uptake (Crespi and Denver 2006). Together with the fact that the plastic populations and canalized populations had a similar number of DEG count, it shows that the canalized populations are also able to respond to decreased water level, even if not at a high degree as the plastic southern populations.

The role of genetic assimilation and genetic accommodation in evolution remains controversial among biologists (de Jong and Crozier 2003, de Jong 2005, Orr 1999; Futuyma 2017). However, not always does genetic change have to precede phenotypic variation. Given sufficient plasticity, the expression of adaptive phenotypes may be induced environmentally, further revealing cryptic genetic variation (Braendle and Flatt 2006). Suzuki and Nijhout (2006) demonstrated this by showing that experimental lines of *Manduca sexta* selected

for increased or decreased environmental sensitivity rapidly evolved divergent reaction norms for color polyphenism. Alternatively, phenotypic plasticity may actively trigger evolution by allowing survival under novel selection regimes (Schlichting and Wund 2014). This aspect is of particular interest in the context of global climate change, in which species are exposed to rapid environmental changes. Unfortunately, it remains a difficult task to experimentally demonstrate evolutionary change in sensitivity to the environment, even in systems in which genetic variation accumulated by climate-driven evolution is reported (Franks 2011), mainly due to the difficulties of running common garden experiments over different time series (Kelly 2019).

In this study we have demonstrated that populations originating from different hydroperiod regimes differed in their capacity of developmental acceleration in response to pond desiccation, raising the importance of environmental variation over large temporal and spatial scales in the context of phenotypic plasticity (Gómez-Rodríguez *et al*. 2010, Kelly 2019). Adaptive plasticity is considered to contribute to the maintenance of genetic variation in the face of rapid environmental changes (Charmantier *et al*. 2008, Gomez-Mestre and Jovani 2013). However, phenotypic plasticity may not always result in adaptive population divergence because it can potentially 'shield' genes from selection (Fox *et al*. 2019). The potential of phenotypic plasticity being adaptive in the face of drastic changes such as the global climate change may depend on the degree of genetic variation of the populations and the strength of selective forces in each environment (Charmantier and Gienapp 2014).

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Supplementary materials

S1. Worldclim bioclimatic variables

BIO1 = Annual mean temperature

BIO2 = Mean diurnal range (mean of monthly (max temp – min temp))

BIO3 = Isothermality (BIO2/BIO7)(*100)

BIO4 = Temperature seasonality (standard deviation*100)

BIO5 = Max temperature of warmest month

BIO6 = Max temperature of coldest month

BIO7 = Temperature annual range (BIO5 - BIO6)

BIO8 = Mean temperature of wettest quarter

BIO9 = Mean temperature of driest quarter

BIO10 = Mean temperature of warmest quarter

BIO11 = Mean temperature of coldest quarter

BIO12 = Annual precipitation

BIO13 = Precipitation of wettest month

BIO14 = Precipitation of driest month

BIO15 = Precipitation seasonality (coefficient of variation)

BIO16 = Precipitation of wettest quarter

BIO17 = Precipitation of driest quarter

BIO18 = Precipitation of warmest quarter

BIO19 = Precipitation of coldest quarter

S2. clean_pe_rna.sh full script used for RNA read cleaning

```
echo "Usage:"
echo ""
echo "bash clean_pe.sh raw_data_foldername R1_ending"
echo ""
echo "* raw paired end data has no uniform ending for filenames R1_ending may
be _R1.fq.gz, _R1_.fastq.gz, _1.fastq.gz, etc"
echo ""
echo "First, adapters are removed with bbduk"
echo "Second, PhiX and sequencing artifacts are filtered out, then quality is
trimmed using bbduk.sh"
echo "bbduk.sh uses PHRED algorithm, filtering based on error probability
instead of raw quality score"
echo ""
echo ""
echo "#########################################"
echo "# STEP 1: Remove adapters with bbduk.sh #"
echo "#########################################"
echo ""

# Create folder for decontaminated reads
mkdir 01_adapters_removed

# Switch to input folder given by first argument $1
cd $1

# R1 ending pattern is given in $2
patternI1=$2
read1="_R1.fq.gz"
readH="_R#.fq.gz"
readS="_SE.fq.gz"

# RAM for bbduk.sh
RAM="-Xmx32g"

# Remove adapters with bbduk, two consecutive rounds
for file in *$patternI1; do
        echo "Removing adapters: "${file//$patternI1/}
        time ( bbduk.sh $RAM in=${file//$patternI1/$readH} out=stdout.fq int=f
\
        ref=$HOME/software/bbmap/resources/adapters.fa,$HOME/software/b
bmap/resources/polyA.fa.gz \
        ktrim=r k=21 mink=11 hdist=2 tpe tbo minlength=21 trimpolya=4 \
        stats=../01_adapters_removed/${file//$patternI1/.bbduk.adapters_round
1.stats.txt} \
        2>../01_adapters_removed/${file//$patternI1/.bbduk.adapters_round1.l
og.txt} \
```

```
        | bbduk.sh $RAM in=stdin.fq int=f
out=../01_adapters_removed/${file//$patternI1/$readH} \
        ref=$HOME/software/bbmap/resources/adapters.fa,$HOME/software/b
bmap/resources/polyA.fa.gz \
        ktrim=r k=19 mink=9 hdist=1 tpe tbo minlength=21 trimpolya=4 \
        stats=../01_adapters_removed/${file//$patternI1/.bbduk.adapters_round
2.stats.txt} \
        2>../01_adapters_removed/${file//$patternI1/.bbduk.adapters_round2.l
og.txt} )
        echo ""
        echo "After first round:"
        grep "Result:"
../01_adapters_removed/${file//$patternI1/.bbduk.adapters_round1.log.txt} |
column -ts $'\t'
        echo ""
        grep -A4 "#Name"
../01_adapters_removed/${file//$patternI1/.bbduk.adapters_round1.stats.txt} |
column -ts $'\t'
        echo ""
        echo "After second round:"
        grep "Result:"
../01_adapters_removed/${file//$patternI1/.bbduk.adapters_round2.log.txt} |
column -ts $'\t'
        echo ""
        grep -A4 "#Name"
../01_adapters_removed/${file//$patternI1/.bbduk.adapters_round2.stats.txt} |
column -ts $'\t'
        echo ""
        echo ""
done

# # Remove adapters with bbduk, single round
# for file in *$patternI1; do
#       echo "Removing adapters: "${file//$patternI1/}
#       time bbduk.sh $RAM in=${file//$patternI1/$readH}
out=../01_adapters_removed/${file//$patternI1/$readH} \
#       ref=$HOME/software/bbmap/resources/adapters.fa \
#       ktrim=r k=21 mink=11 hdist=2 tpe tbo minlength=21 \
#
        stats=../01_adapters_removed/${file//$patternI1/.bbduk.adapters.stats.t
xt} \
#       2>../01_adapters_removed/${file//$patternI1/.bbduk.adapters.log.txt}
#       echo ""
#       grep "Result:"
../01_adapters_removed/${file//$patternI1/.bbduk.adapters.log.txt}
#       echo ""
#       grep -A4 "#Name"
../01_adapters_removed/${file//$patternI1/.bbduk.adapters.stats.txt}
#       echo ""
```

```
#       echo ""
# done

echo
"#####################################################
##############"
echo "# STEP 2: Remove contaminants and quality filter/trim with bbduk.sh #"
echo
"#####################################################
##############"

# Create folder for clean reads
cd ..
mkdir 02_clean
cd 01_adapters_removed

# Remove contaminants with bbduk.sh
for file in *$read1; do
        echo "Cleaning: "${file//$read1/}
        time bbduk.sh $RAM in1=${file//$read1/$readH}
out=../02_clean/${file//$read1/$readH} \
        outs=../02_clean/${file//$read1/$readS} \
        ref=$HOME/software/bbmap/resources/phix174_ill.ref.fa.gz,$HOME/sof
tware/bbmap/resources/sequencing_artifacts.fa.gz \
        k=31 hdist=1 qtrim=lr trimq=10 maq=12 minlength=21 maxns=5
ziplevel=5 \
        stats=../02_clean/${file//$read1/.contaminants.stats.txt} \
        &>../02_clean/${file//$read1/.cleaning.log.txt}
        echo ""
        grep "Result:" ../02_clean/${file//$read1/.cleaning.log.txt} | column -ts
$'\t'
        echo ""
        grep -A2 "#Name" ../02_clean/${file//$read1/.contaminants.stats.txt} |
column -ts $'\t'
        echo ""
        echo ""
done

echo "#######################################"
echo "# STEP 3: Quality checks with fastqc #"
echo "#######################################"

cd ../02_clean

# Run fastqc
mkdir 00_fastqc_before 01_fastqc_after
cd ../$1
fastqc -o ../02_clean/00_fastqc_before --nogroup -t 6 *q.gz
```

```
cd ../02_clean
fastqc -o 01_fastqc_after --nogroup -t 6 *_R[12].fq.gz

cd ..

# Perform cleanup of intermediate .fq.gz files (uncomment)
# rm 01_adapters_removed/*q.gz

echo "Done."
```

S3. Duration of larval period across water level treatments in populations and sibships of central Spain (Madrid and Segovia province). While the central populations did not accelerate their development as drastically as the southern populations, certain sibships (Valdemanco 1, 2, Canencia 1, 3, 4, and Roblellano 1) accelerated their developmental period substantially in the low water treatment and few (Turrubuelo 1, 3, Buitrago 2, 3, and Roblellano 2) did so slightly. Hence, there is a large variation in developmental plasticity among populations and genetic families.

Population	Sibship	Total average ± std	Low average ± std	High average ± std
Valdemanco	1	95.22 ± 10.77	87.91 ± 6.96	101.92 ± 9.24
Valdemanco	2	101.17 ± 12.45	93.17 ± 11.31	109.17 ± 7.53
Valdemanco	3	93.83 ± 10.66	93.83 ± 13.30	93.83 ± 7.78
Turrubuelo	1	110.64 ± 26.43	108.4 ± 32.61	112.5 ± 21.34
Turrubuelo	2	140.41 ± 35.79	155.55 ± 37.83	125.27 ± 27.43
Turrubuelo	3	103.88 ± 11.93	100.33 ± 9.82	107.42 ± 13.18
Turrubuelo	4	169.70 ± 34.80	186.82 ± 42.21	154 ± 15.48
Santo Tome	1	114.05 ± 15.72	113.80 ± 20.65	114.25 ± 11.09
Santo Tome	2	104.30 ± 20.54	104.36 ± 25.46	104.25 ± 15.96
Santo Tome	3	117.45 ± 38.70	135.55 ± 47.94	99.36 ± 11.21
Santo Tome	4	164.05 ± 39.97	184.64 ± 36.96	141.40 ± 20.75
Buitrago	1	104.41 ± 23.11	109 ± 31.53	100.58 ± 13.07
Buitrago	2	98.17 ± 19.02	96.83 ± 24.82	99.5 ± 11.69
Buitrago	3	103.21 ± 15.63	99.08 ± 13.63	107.33 ± 16.97
Buitrago	4	135.22 ± 36.31	149.25 ± 47.21	124 ± 21.04
Canencia	1	90.13 ± 8.37	85 ± 4.39	95.25 ± 8.37
Canencia	2	112.48 ± 41.96	118 ± 58.87	107.42 ± 17.66
Canencia	3	97.48 ± 12.70	91.2 ± 7.74	105.33 ± 13.57
Canencia	4	130.04 ± 11.30	123.33 ± 7.10	136.75 ± 10.87
Roblellano	1	90.65 ± 5.90	87 ± 4.36	95.11 ± 4.26

Roblellano	2	93.57 ± 10.63	90.25 ± 10.90	97.18 ± 9.50
Roblellano	3	120.79 ± 29.98	123.42 ± 36.94	118.17 ± 22.36

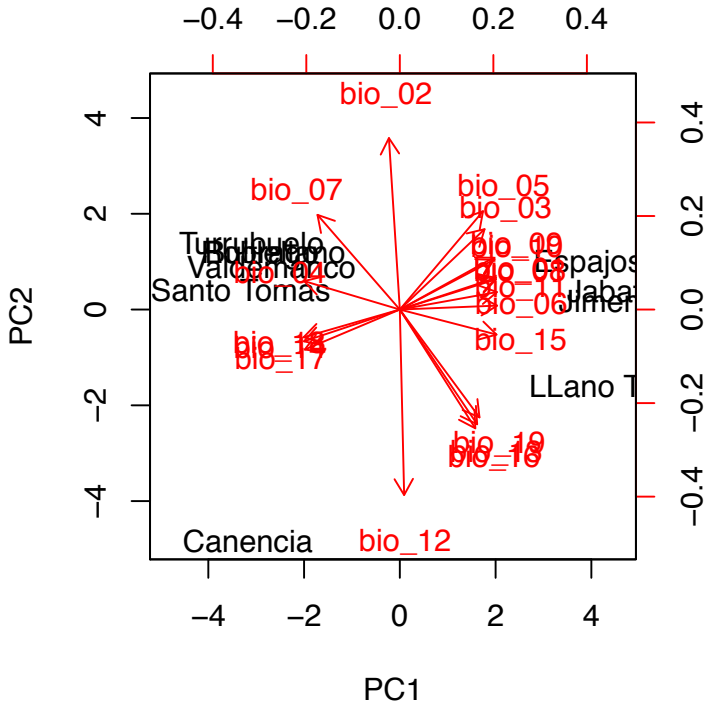
S4. Duration of larval period across water level treatments in populations and sibships of southern Spain (Doñana National Park: Huelva province). The developmental acceleration in the southern populations was much more drastic and consistent across populations and sibships in comparison to the central populations, while very few sibships (3 and 4 of Jimenez) did not accelerate their larval period in response to low water treatment.

Population	Sibship	Total average ± std	Low average ± std	High average ± std
Jabata	1	219 ± 78.30	179.25 ± 96.02	258.75 ± 29.5
Jabata	2	151.13 ± 67.31	124.5 ± 20.42	177.75 ± 90.91
Jabata	3	144.57 ± 30.7	135.5 ± 22.83	156.67 ± 40.77
Jabata	4	155.43 ± 29.81	128.33 ± 15.5	175.75 ± 18.23
Jabata	5	238 ± 50.15	214.67 ± 63.07	255.5 ± 37.75
Espajosas	1	155.75 ± 62.9	132.25 ± 19.52	179.25 ± 85.9
Espajosas	2	121.63 ±17.86	112 ± 8.08	131.25 ± 20.79
Espajosas	3	136.86 ± 22.62	126.5 ± 1	150.67 ± 32.13
Espajosas	4	167 ± 50.24	122.75 ± 14.97	211.25 ± 21.08
Espajosas	5	162.14 ± 52.11	122.33 ± 20.53	192 ± 21.08
Jimenez	1	143.11 ± 38	116.25 ± 11.64	175.75 ± 33.97
Jimenez	2	123.13 ± 17.85	113.25 ± 12.18	133 ± 18.31
Jimenez	3	131.5 ± 29.68	136.25 ± 5.38	126.75 ± 5.38
Jimenez	4	98.75 ± 15.92	101.75 ± 17.78	95.75 ± 15.84
Jimenez	5	139.14 ± 36.59	128.67 ± 33.17	147 ± 41.85
Llano	1	153.25 ± 35.5	126.5 ± 12.87	180 ± 29.44
Llano	2	141.88 ± 37.77	110.75 ± 4.86	173 ± 26.87
Llano	3	115.13 ± 14.17	110 ± 8.6	120.25 ± 18
Llano	4	133 ± 25.95	117.25 ± 18.93	148.75 ± 23.49
Llano	5	220 ± 78.35	208.25 ± 104.5	231.75 ± 55.08

S5. Factor loadings of Principal Component Analysis (PCA)

	PC1	PC2
Bio_1 (Annual mean temperature)	**0.26**	0.08
Bio_2 (Mean diurnal range (mean of monthly (max temp – min temp)))	-0.03	**0.45**
Bio_3 (Isothermality (BIO2/BIO7)(*100))	0.23	0.21
Bio_4 (Temperature seasonality (standard deviation*100))	**-0.26**	0.07
Bio_5 (Max temperature of warmest month)	0.22	0.26
Bio_6 (Max temperature of coldest month)	**0.26**	0.01
Bio_7 (Temperature annual range (BIO5 - BIO6))	-0.22	0.25
Bio_8 (Mean temperature of wettest quarter)	**0.26**	0.08
Bio_9 (Mean temperature of driest quarter)	**0.25**	0.13
Bio_10 (Mean temperature of warmest quarter)	**0.25**	0.13
Bio_11 (Mean temperature of coldest quarter)	**0.26**	0.04
Bio_12 (Annual precipitation)	0.01	**-0.50**
Bio_13 (Precipitation of wettest month)	0.21	-0.31
Bio_14 (Precipitation of driest month)	**-0.26**	-0.09
Bio_15 (Precipitation seasonality (coefficient of variation))	**0.26**	-0.07
Bio_16 (Precipitation of wettest quarter)	0.20	-0.32
Bio_17 (Precipitation of driest quarter)	**-0.25**	-0.11
Bio_18 (Precipitation of warmest quarter)	**-0.26**	-0.07
Bio_19 (Precipitation of coldest quarter)	0.21	-0.29
Proportion of variation explained	**0.77**	**0.19**

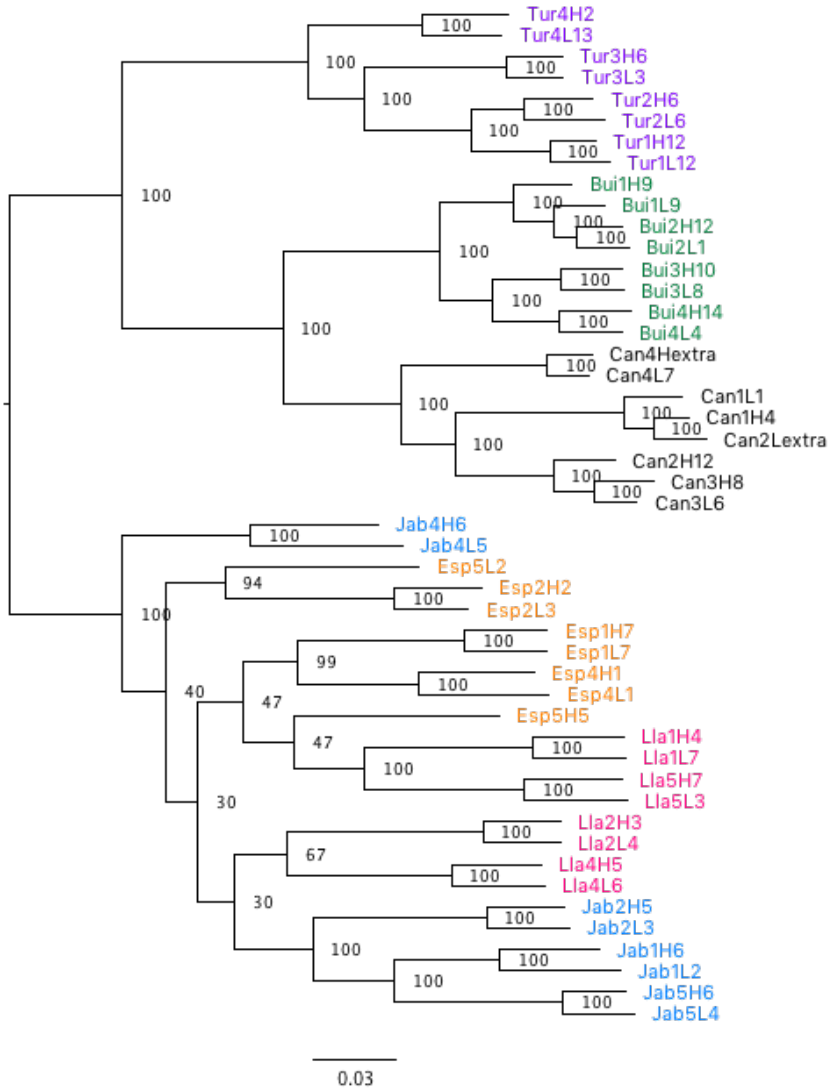
S6. Biplot of the principal component score vectors



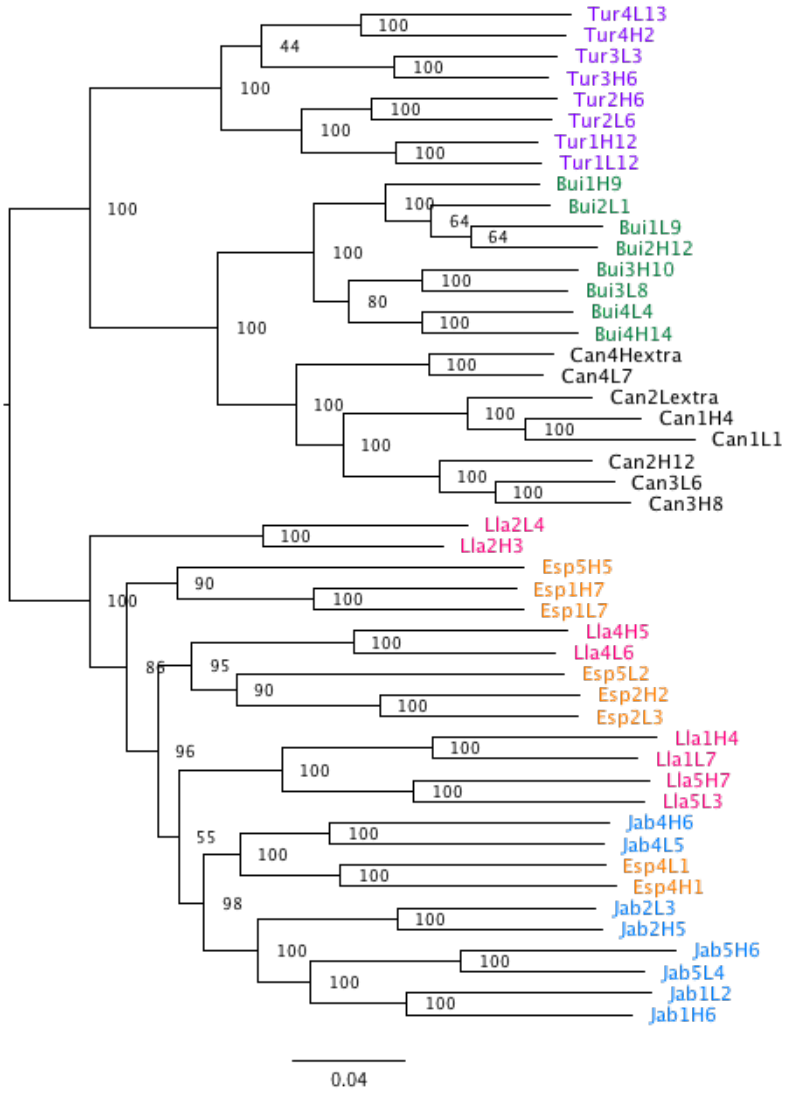
Biplot: Bio_1 (Annual mean temperature), Bio_4 (Temperature seasonality (standard deviation*100)), Bio_6 (Max temperature of coldest month), Bio_8 (Mean temperature of wettest quarter), Bio_9 (Mean temperature of driest quarter), Bio_10 (Mean temperature of warmest quarter), Bio_11 (Mean temperature of coldest quarter), Bio_14 (Precipitation of driest month), Bio_15 (Precipitation seasonality (coefficient of variation)), Bio_17 (Precipitation of driest quarter), Bio_18 (Precipitation of warmest quarter)) loaded heavily on PC1. Bio_2 (Mean diurnal range (mean of monthly (max temp – min temp)) and Bio_12 (Annual precipitation) explained most of the variation of PC2.

S7. Phylogenetic trees resulting from SNP count data

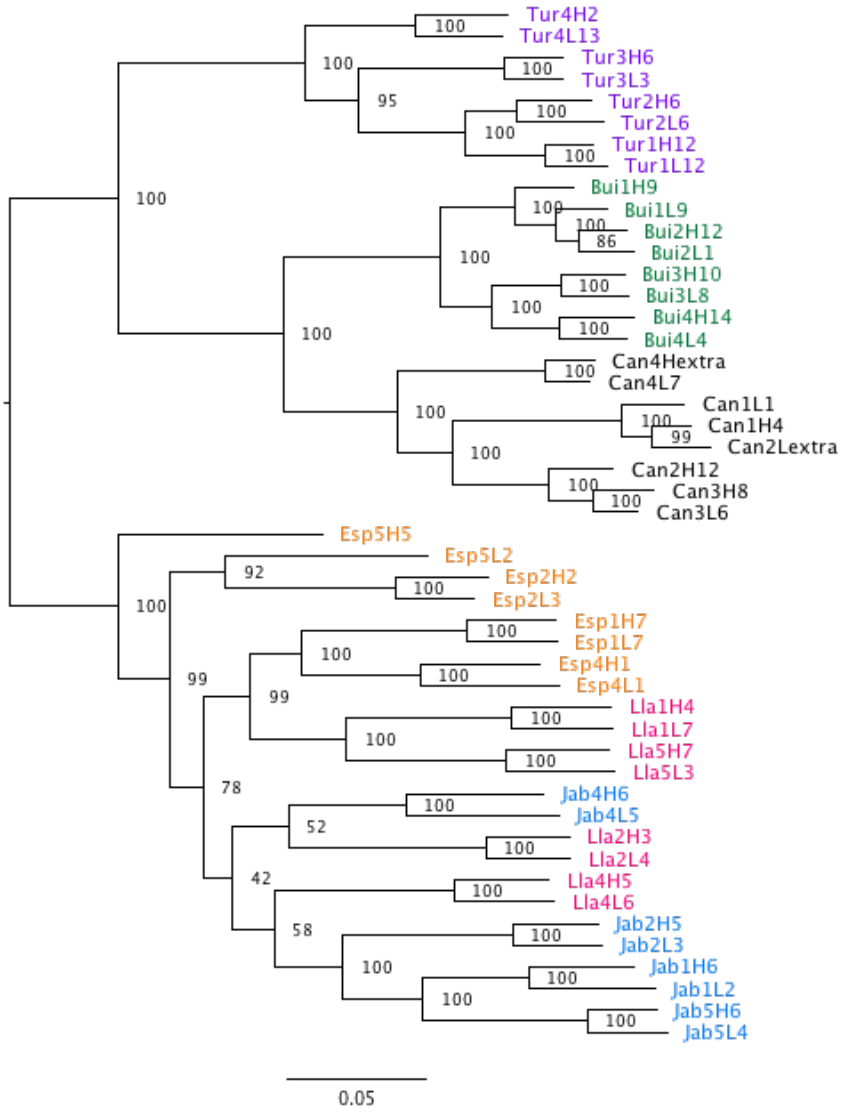
1) gsnap + bcftools



2): star + freebayes



3) star + bcftools



Chapter 3

Shifts in the developmental rate of spadefoot toad larvae cause decreased complexity of post-metamorphic pigmentation patterns

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RH: Dorsal pigmentation affected by pre-metamorphic stress

Shifts in the developmental rate of spadefoot toad larvae cause decreased complexity of post-metamorphic pigmentation patterns

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Abstract

Amphibian larvae are plastic organisms that can adjust their growth and developmental rates to local environmental conditions. The consequences of such developmental alterations have been studied in detail, both at the phenotypic and physiological levels. While largely unknown, it is of great importance to assess how developmental alterations affect the pigmentation pattern of the resulting metamorphs, because pigmentation is relevant for communication, mate choice, and camouflage and hence influences the overall fitness of the toads. Here we quantify the variation in several aspects of the pigmentation pattern of juvenile spadefoot toads experimentally induced to accelerate their larval development in response to decreased water level. It is known that induced developmental acceleration comes at the cost of reduced size at metamorphosis, higher metabolic rate, and increased oxidative stress. In

this study, we show that spadefoot toads undergoing developmental acceleration metamorphosed with a less complex, more homogeneous, darker dorsal pattern consisting of continuous blotches, compared to the more contrasted pattern with segregated blotches and higher fractal dimension in normally developing individuals, and at a smaller size. We also observed a marked effect of population of origin in the complexity of the pigmentation pattern. Complexity of the post-metamorphic dorsal pigmentation could therefore be linked to pre-metamorphic larval growth and development.

Keywords: amphibian, spadefoot toad, *Pelobates cultripes*, phenotypic plasticity, pigmentation pattern, dorsal pigmentation, environmental stress, carry-over effect, complex life cycle

Introduction

The evolution of colour and pigmentation as a means of signalling is a common phenomenon across the tree of life (Protas and Patel 2008). This signalling is important for species recognition (Robertson and Greene 2017), social interactions within species such as mate choice and intra-sexual competition, interactions between species such as aposematism, mimicry or invitations for pollination and seed dispersal, and for camouflage to avoid detection. Much empirical work has focused on pigmentation as honest signals indicating individual quality (Hill *et al*. 2006). Classic examples of this are colour patches in birds that can convey honest information about parasite and pathogen resistance (Hamilton and Zuk 1982, Lindström and Lundström 1982). Yet fewer empirical studies have focused on describing features of animal pigmentation *pattern* (e.g. complexity, heterogeneity, lacunarity), and its relationship to body

condition or stress endured (Pérez-Rodríguez et al 2017). This is especially relevant for camouflage, where patterning is just as important as the pigmentation itself (Stevens *et al*. 2006, Stevens *et al*. 2009; Allen *et al*. 2010, Kelley *et al*. 2013).

When colouration is not uniform, the arrangement of different coloured features can generally be defined as spots, stripes or polygons (Kondo and Shirota 2009). These three arrangements have evolved many times independently in the animal kingdom and play an important role in communication (e.g. egg spots in cichlids (Theis *et al*. 2012), splotches in cuttlefish (Palmer *et al.* 2006), barred plumage in birds (Gluckman and Cardoso)) and crypsis (e.g. disruptive contrast (Stevens *et al.* 2006), outline- and surface disruption (Stevens *et al*. 2009), and counter shading (Rowland *et al*. 2007)). This two- and sometimes three-dimensional attribute of colouration can vary not only between species, but also between individuals within species (Singh and Nüsslein-Volhard 2015) and often times serve as signal of an individual's quality for mate choice. Both colour and its patterning are highly evolvable and the correct formation of patterning can have direct fitness consequences, especially in the context of camouflage (Ruxton *et al*. 2004, Manriquez *et al.* 2008) and mimicry (Nishikawa *et al*. 2013, Stevens and Ruxton 2019).

In the same way that fluctuating asymmetry resulting from disruption to development can be used as a signal of individual quality, the correct formation of patterning is contingent on developmental homoeostasis (Pérez-Rodríguez *et al.* 2017). Patterning has a strong genetic component (Singh and Nüsslein-Volhard 2015, Wittkopp *et al.* 2003) although the formation of complex patterns can result from developmental alterations (Kondo and Shirota 2009), possibly

influenced by environmental stressors (Hiyama *et al.* 2012). Indeed, a higher pattern complexity in the black bib of red-legged partridges has been shown to be an indication of higher individual quality (Pérez-Rodríguez *et al.* 2013). Likewise, ornament symmetry in little bustards (Jiguet and Bretagnolle 2014) and facial patterning of paper wasps (Tibbetts and Curtis 2007) also reflect the developmental conditions experienced.

While much of the research on colour patterning in vertebrates has focused on birds and teleost fish (Protas and Patel 2008), amphibians have been an important system in this topic as well (Robertson and Greene 2017, Park *et al.* 2010, Rudh and Qvarnström 2013, Rabbani *et al.* 2015). A characteristic feature of many amphibians is their complex life cycle, which most typically consists of an aquatic larval stage and a terrestrial adult phase (Gomez-Mestre *et al.* 2012). In fact, amphibian larvae and adults differ physiologically, anatomically, and ecologically (Wells 2007) to the extent that they are at times conceptually treated as separated units (Haas 2003, Wollenberg Valero *et al.* 2017). Nonetheless, multiple studies show that changes in the developmental trajectory of larvae have direct effects on adults (Van Allen *et al.* 2010, Touchon *et al.* 2013, Gomez-Mestre *et al.* 2010). This is particularly relevant for pigmentation, as the adult patterning forms during late stages of the larval development and metamorphosis (Thibaudeau and Altig 2012). Hence, if development of pigmentation is altered during this critical period, the adult phenotype will be directly affected. This has been shown in zebrafish, which undergo similar complex life cycles, where environmental stress at the larval stage affects adult pigmentation (Parichy and Turner 2003). Amphibian larvae can decouple growth and differentiation to adjust their development to local environmental

conditions (Gomez-Mestre *et al.* 2010, Touchon *et al.* 2015), constituting an ideal system to test for consequences of plastic alterations of development on pigmentation. Understanding the evolution of colour patterning in adult frogs, regardless of its function in signalling or camouflage, is therefore contingent on understanding how larval development affects the correct formation of patterns.

Here we test whether the dorsal pigmentation pattern of juvenile spadefoot toads (*Pelobates cultripes*) is affected by developmental alterations during the larval phase. Adults are semi-fossorial and nocturnal, typically living in sandy substrate where they can bury themselves easily. Their dorsal colour patterning can be described as darkly mottled with uneven blotches on a light uniform background and likely serves camouflage by background matching. Its larvae can grow for extended periods of time, reaching large sizes at metamorphosis under benign conditions, but can accelerate development and precipitate metamorphosis if at risk of desiccation from pond drying (Gomez-Mestre *et al.* 2013). Developmental acceleration is in part achieved through increased corticosterone levels and metabolic rate and although critical for evading drying ponds, it comes at the cost of smaller size at metamorphosis, higher oxidative stress, reduced immunocompetence and allometric changes in body shape (Gomez-Mestre *et al.* 2013, Gomez-Mestre *et al.* 2006, Gervasi and Foufopoulos 2008). To test whether post-metamorphic pigmentation patterning is impacted by environmentally induced acceleration of pre-metamorphic larval development in spadefoot toads, we reared individualized tadpoles from multiple egg clutches and from four populations in standardized conditions, and experimentally induced accelerated development in half of them by reducing their water level. Upon metamorphosis, we compared body mass, snout-to-vent

length, and dorsal patterning between accelerated and non-accelerated individuals. Patterning was quantified using digital imaging to estimate parameters describing different aspects of the texture and complexity of pigmentation.

Material & Methods

STUDY ANIMALS AND PREPARATION OF DORSAL PIGMENTATION PHOTOS

We collected portions of five egg clutches of four *P. cultripes* populations from southern Spain (Huelva province) and reared them in climatic chambers of Estación Biológica de Doñana, CSIC (Seville). Upon reaching the free-feeding stage, 14 tadpoles per sibship (clutch) were individualized in 4 L round plastic containers. They were kept in identical conditions (20°C, carbon filtered dechlorinated tap water) and fed a mixture of ground up rabbit chow (alfalfa) and spinach. At Gosner stage 35 (i.e. stage at which digits are identifiable on the hindlimb bud (Gosner 1960)), we dropped the water volume to 650 mL in half the containers to simulate risk of pond drying. Gosner stage 35 is the optimal developmental stage at which spadefoot toad larvae are able to perform the most acceleration in comparison to other developmental stages (Kulkarni *et al*. 2011). The experimental procedures were approved by CSIC's IACUC committee (authorization #560-2017), and all experiments were performed in accordance with relevant guidelines and regulations.

IMAGE ACQUISITION AND PREPROCESSING

We recorded the duration of the larval period of each toad, marked by the completion of metamorphosis (full tail resorption, Gosner stage 46) (Gosner 1960) at which stage we also measured snout-to-vent length (to the nearest 0.1 mm) and body mass (to the nearest 0.1 mg). A dorsal photograph of each toad placed over laminated grid paper was taken with a Nikon D50 camera. Since photos were taken from slightly different distances, images were downscaled to the same resolution (13.2 px/mm) by bicubic interpolation. We focused our analysis of pigmentation patterns on the torso and head (except eyeballs), excluding the limbs to avoid noise from variation in posture. Regions of Interest (ROI) on the toad's dorsal surface were manually outlined.

The resulting ROI images were transformed to grayscale 8-bit images (256 intensity values). Intensity values of images were obtained from the image histogram, in which the reflectance of each pixel ranged from 0 to 255. Uneven illumination was corrected using a pseudo-flat field correction tool (blurring radius: 40 pixels). Contrast was enhanced by a histrogram stretching to a full 8-bit range of gray (0.1 % saturated pixels). The unsharp masking filter tool of imageJ (weight value of 0.5 and blur radius of 1.0) was applied to improve sharpness. Due to artificial light sources, wet dorsal skin of toads showed specular reflection, which may potentially have resulted in errors in image analyses. We therefore applied threshold criteria to determine areas affected by specular reflection, and removed them from the ROIs (intensity values > 190).

EXTRACTION OF TEXTURAL FEATURES

Image texture can be defined as the spatial arrangement of intensities (Tuceryan and Jain 1998, Di Cataldo and Ficarra 2017). Skin texture was characterized by

measuring the likelihood of observing an intensity value at randomly selected pixels in the images (i.e. first-order statistics) and the spatial relationships between them (i.e. second-order statistics) (Tuceryan and Jain 1998, Di Cataldo and Ficarra 2017). From the frequency distribution of gray scales in the images, the following first-order statistics were calculated: mean (μ), variance (σ^2), skewness (S), and kurtosis (K). Second-order textural features were extracted using the Gray Level Co-occurrence Matrix (GLCM) (Di Cataldo and Ficarra 2017, Haralick *et al.* 1973, Haralick 1979, Conners *et al.* 1984). GLCM is a matrix representation of an image in which the gray tone of each pixel is quantized to a set (G) of N_θ levels. Information on texture is specified by the matrix of relative frequencies $P_{(i,j|d,\theta)}$ in which two neighboring pixels separated by distance d and angle θ occur on an image, one with gray tone i and another with j (Haralick et al 1973). GLCMs of irregular ROIs were calculated from a distance between pixel (d) = 1 at four directions (θ) = 0°, 45°, 90°, and 135°, and then averaged for each image. In order to characterize the textural features of the dorsal images of the toads, we obtained the following five GLCM measurements:

i) Angular second moment (ASM): measure of homogeneity, high values indicate very few dominant gray-scale transitions (i.e. homogeneous images).

$$ASM = \sum_{i=0}^{G-1}\sum_{j=0}^{G-1} P\{(i,j)\}^2$$

ii) Contrast (CON): measure of local intensity variation between a continuous set of pixels.

$$CON = \sum_{n=0}^{G-1} n^2 \left\{ \sum_{i=0}^{G-1}\sum_{j=0}^{G-1} P(i,j) \right\}, |i-j| = n$$

iii) Inverse difference moment (*IDM*): weighted by the inverse of the contrast (i ≠ j) which is influenced by homogeneity, high values indicate homogeneous textures:

$$IDM = \sum_{i=0}^{G-1}\sum_{j=0}^{G-1}\frac{P(i,j)}{1+(i-j)^2}$$

iv) Entropy (*ENT*): statistical measure of information content estimating the randomness of intensity distribution of textures (i.e. Images presenting low entropy are homogeneous):

$$ENT = -\sum_{i=0}^{G-1}\sum_{j=0}^{G-1}P(i,j)\times\log\big(P(i,j)\big)$$

v) Correlation (*COR*): measure of linear dependency between gray scales on neighboring pixels at the specified positions. High correlation values indicate images with regions presenting similar intensity:

$$COR = \sum_{i=0}^{G-1}\sum_{j=0}^{G-1}\frac{\{i\times j\}\times P(i,j)-\{\mu_i\times\mu_j\}}{\sigma_i\times\sigma_j}$$

(μ stands for the mean and σ for the standard deviation of brightness)

The plug-in tool GLCM Texture v.0.008 (Cabrera 2005, Albregtsen 2008) of the software ImageJ was used.

SPATIAL HETEROGENEITY (FRACTAL DIMENSION AND LACUNARITY) ANALYSIS

Fractal dimension of gray-scale images (Sarkar and Chaudhuri 1994) was measured on ROIs applying differential box-counting methods using the plug-in FracLac in ImageJ (Karperien 1999-2013). A series of boxes (samples) of decreasing scale (ε) were projected over a gray-scale image with an intensity $I_{(i,j)}$

scaled to a range of 0 to 255 for each pixel (*i,j*). For each box, the difference between the maximum and minimum values of pixel intensity ($\delta I_{i,j,\varepsilon}$) was calculated and the slope of the log-log regression between box size and the sum of all the intensity differences ($I\varepsilon = \sum [1 + \delta I_{i,j,\varepsilon}]$) was used to calculate the gray fractal dimension (D_B). Fractal dimension constitutes a measure of spatial complexity (Mandelbrot 1982, Smith *et al.* 1996, Lopes and Betrouni 2009):

$$D_B = \lim_{n\to\infty} \ln(I_\varepsilon) \Big/ \ln\left(1/\varepsilon\right)$$

Lacunarity (λ) is a measure of spatial heterogeneity that complements fractal dimension; whereas D_B measures *how much* space is filled, lacunarity describes the spatial size of gaps and measures *how* space is filled (Tolle *et al.* 2003). Thus, a fractal with large and heterogeneous gaps has high lacunarity values and vice versa:

$$\lambda = CV_{\varepsilon,g}^2 = \left(\sigma/\mu\right)^2$$

where σ stands for the standard deviation, μ for the mean difference in intensity for pixels per box at the scale ε and orientation g.

STATISTICAL ANALYSES

Variables were normalized using the *bestNormalizer* (Peterson *et al.* 2019) package in R (version 3.6.2). Fractal dimension (D_B) and entropy required no transformation, whereas mean (μ), variance (σ^2), contrast, and lacunarity (λ) were log-transformed. A Box Cox transformation was applied to angular second moment (*ASM*) and correlation (*COR*), and a square root to inverse difference moment (*IDM*). We then standardized each variable to a mean of 0 and standard deviation of 1. We then performed a principal component analysis (PCA, R

version 3.6.2.) on the transformed variables, using factor loadings to assess the relative contribution of each variable to the variation observed in each of the principal components. Some of these variables were complementary to each other and showed high collinearity. Thus, multiple variables provided similar information regarding the frequency distribution of grey scales (*mean*, *variance*, *skewness*, *kurtosis*) whereas others described homogeneity in the pattern (*ASM*, *IDM*, *entropy*, *contrast*, *correlation*) and yet others spatial complexity (*lacunarity*, *fractal dimension*). We visualized the pairwise relationship among these variables using the *ggpair* function of the R package GGally (Schloerke 2020), and selected three variables of interest with low collinearity among them and loading on different principal components: *mean*, *angular second moment,* and *fractal dimension*. We then fitted multivariate Bayesian hierarchical linear models on these three variables using the R package *brms* (Buerkner 2016). We began with comparing model performance including and excluding sibship and population as random (group) effects, and included those in further analyses as models performed better with random effects. We compared model performance of fitting either on i) only water treatment, ii) water treatment and larval period, or iii) water treatment and body mass upon metamorphosis as fixed (population) effects, including sibship nested within population as random (group) effect for each model. All three response variables in the models were fitted within a gaussian distribution with default prior settings, with 152 observations (i.e. metamorphs) and 4 group levels (i.e. populations) for each model. Two sampling chains ran for 2000 iterations with a warm-up period of 1000 iterations for each model, hence yielding 2000 samples for each parameter. As diagnostic checks, chain convergence, autocorrelations and posterior predictive distributions were

visually inspected and effective sample sizes calculated. We also checked for low pareto k heritage (< 0.7) for each observation within each model. To explore how much variation in the response variables were explained by our models, we employed a Bayesian generalization of the R^2 coefficient. Finally, the best model fit (i.e. the model with the highest predictive accuracy based on the expected log pointwise predictive density [ELPD]) was evaluated across models by estimating the out-of-sample predictive fit using the leave-one-out cross-validation (loo) and widely applicable information criterion (waic) criteria.

Results

EFFECTS OF WATER LEVEL ON GROWTH AND DEVELOPMENT

Experimentally decreasing water level led tadpoles to accelerate their development by 21 % on average ($F_{1,150}$ = 24.8, p < 0.001), reducing the age when Gosner stage 46 was reached from 171±55 days to 132±44 days. Larvae subjected to reduced water levels metamorphosed at lower body mass and shorter body length: body mass was decreased from 1.84±0.97g to 1.24±0.31g, representing 34% decrease ($F_{1,150}$ = 86.36, p < 0.001), and snout-to-vent length was decreased from 26.62±12.13mm to 23.39±4.79mm, representing a 14% decrease ($F_{1,150}$ = 101.4, p < 0.001).

PRINCIPAL COMPONENT ANALYSIS OF PIGMENTATION PATTERN CHARACTERISTICS

11 variables have been calculated to describe 3 features: frequency distribution of grey scales (*mean, variance, skew,* and *kurtosis*), homogeneity (*angular second*

moment, inverse difference moment, entropy, contrast, and *correlation*), and complexity (*lacunarity* and *fractal dimension*). We observed substantial variation in pigmentation patterns in the metamorphosed toads emerging from our experiment. A principal component analysis showed that most of the calculated pattern descriptors loaded considerably on the first principal component, with *entropy*, *mean, variance, and contrast* of the grayscale distribution of the pattern showing similar magnitudes but opposite directions to *kurtosis*, *correlation* and *inverse difference moment* (S1 in Supplementary material). *Fractal dimension* and *lacunarity* loaded heavily on the second principal component while loading in opposite directions, *angular second moment* loaded on the third principal component, and *skewness* on the fourth component. The numerous variables showing similar loadings on PC1 indicated high collinearity among variables, which we further visualized using the *ggpair* function of the R package GGally (Schloerke 2020) (S2 in Supplementary material). Principal components 1, 2, and 3 together explained over 89% of the variance in the pigmentation patterns across toads (S1 in Supplementary material). The PC scores largely overlapped across populations and across treatments (Figure 1).

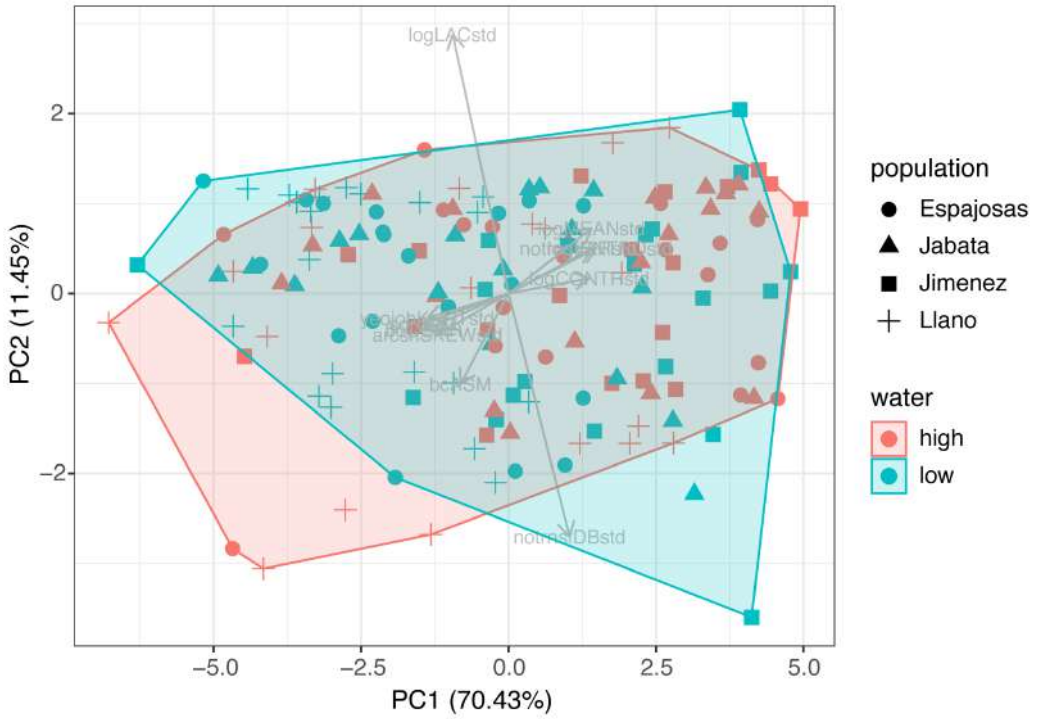

Figure 1. Principal component analysis: Most variables that loaded heavily on the first principal component (*entropy*, *mean gray value, gray value variance,* and *contrast* pointing to opposite directions than *kurtosis*, *correlation* and *inverse difference moment, while all loaded at a similar magnitude*) explained 70.43 % of the variation. The second principal component explained 11.45 % of the variation and was heavily loaded by *fractal dimension* and *lacunarity*.

EFFECTS OF DEVELOPMENTAL ACCELERATION, POPULATION, AND SIBSHIP ON PIGMENTATION PATTERN

We used Bayesian hierarchical linear models to test for the effects of experimentally decreasing water level on multivariate pigmentation pattern, specifically on the *mean* of the grayscale distribution, *angular second moment,* and *fractal dimension* chosen based on their high loading on different principal components and low collinearity among each other (Figure 2; see Material and Methods section for more detail).

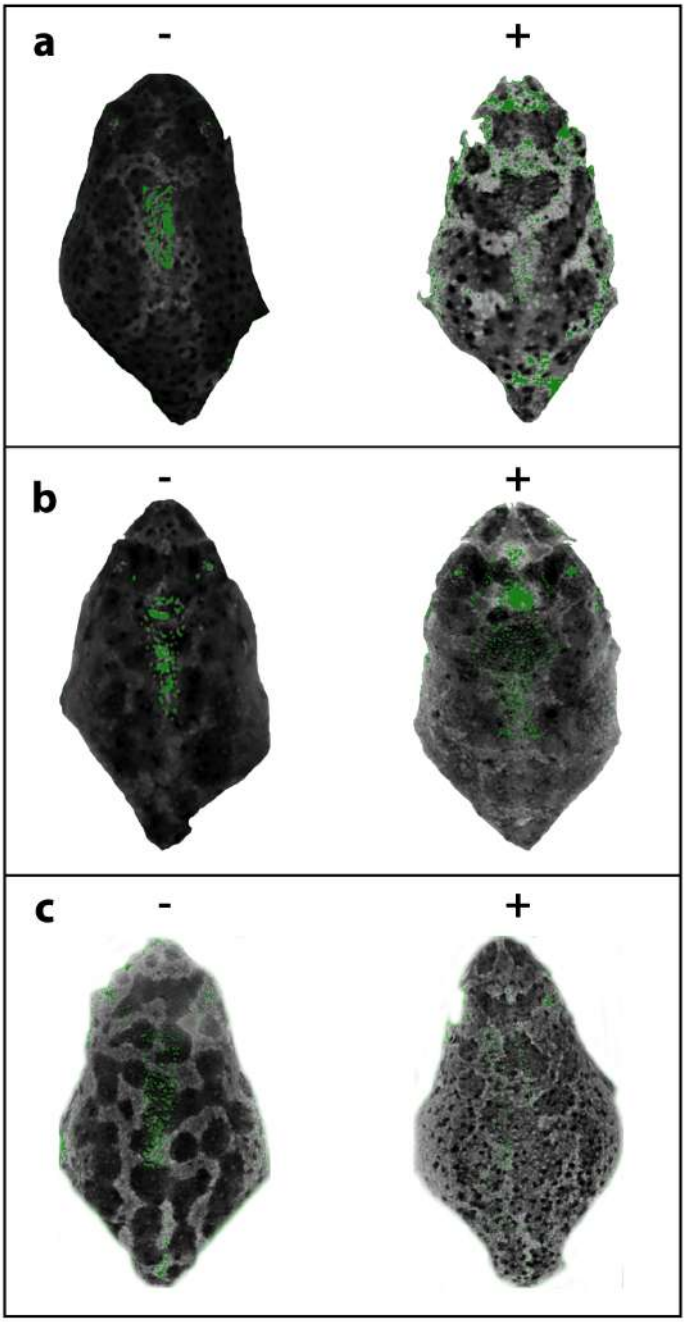

Figure 2. Photographic representation of dorsal pigmentation pattern differences as described by the three most informative patterning statistics a) mean grey value, b) fractal dimension and c) angular second moment. The left panel (-) shows exemplary individuals with low extremes and the right pane (+) shows exemplary individuals with high extremes for each descriptor. When raised under environmentally stressful (i.e. low water level treatment) conditions, toads metamorphose with an overall darker dorsal pattern (i.e. lower mean grey value), with decreased heterogeneity (i.e. lower fractal dimension) and with a more homogeneous dorsal pattern (i.e. higher angular second moment).

Our model comparisons based on measures of the prediction accuracy by expected pointwise predictive density (ELPD) showed that the model including sibship nested within population as random (group) effect was the better fit than the one excluding it: the predictive ability was improved by adding the random effect according to both the loo (ΔELPD: fit1 = 0.0, fit0 = -42.8; SE: fit1 = 0.0, fit0 = 15.5) and waic criteria (ΔELPD: fit1 = 0.0, fit0 = -43.3; SE: fit1 = 0.0, fit0 = 15.6), where fit1 is the model including random effects and fit0 is the model excluding random effects, respectively. Therefore, it could be inferred that sibship and population effect explained a considerable fraction of the variance of each of these variables and we included *population* and *sibship* (clutch) as random effects in the subsequent models. Since aspects of the pigmentation pattern seemed to be attributable to sibship, we estimated broad sense heritability (H^2) of these three variables (Table 1) using the *sommer* (Covarrubias-Pazaran 2016) package in R.

The Bayesian generalized coefficients of the first model including only water treatment as fixed effect (fit1) showed that the experimental treatment (i.e. water level) explained a considerable fraction of the variance of the variables, while more so in *mean* and *angular second moment* than in *fractal dimension* (mean: R^2 = 0.29, fractal dimensions: R^2 = 0.17, angular second moment: R^2 = 0.23). Such proportion of variance explained increased when larval period was added as an additional fixed effect (fit2), and again, *mean* and *angular second moment* were better explained than fractal dimension (mean: R^2 = 0.36, fractal dimensions: R^2 = 0.20, angular second moment: R^2 = 0.31). Consistently, including body mass at metamorphosis as additional fixed effect improved the model fit (fit3), and mean and angular second moment were better explained

than fractal dimension (mean: R^2 = 0.37, fractal dimension: R^2 = 0.19, angular second moment: R^2 = 0.31). In all models, 100 % of the observations had a lower pareto *k* value than 0.7, indicating optimal effective sample size.

Our model comparisons based on measures of the prediction accuracy by expected pointwise predictive density (ELPD) showed that the model including both experimental treatment (i.e. water level) and larval period was the best fit (fit2). This model had the highest predictive ability according to both the loo (ΔELPD: fit2 = 0.0, fit3 = -1.1, fit1 = -5.3; SE: fit2 = 0.0, fit3 = 3.6, fit1 = 5.2) and waic criteria (ΔELPD: fit2 = 0.0, fit3 = -1.0, fit1 = -4.7; SE: fit2 = 0.0, fit3 = 3.5, fit1 = 5.6).

The conditional effects of this best fitting model (Figure 3) showed that decreased water levels led to lower mean of the grayscale distribution, lower fractal dimension and higher angular second moment. Similarly, the duration of the larval period was positively associated with mean grayscale and fractal dimension and negatively with angular second moment.

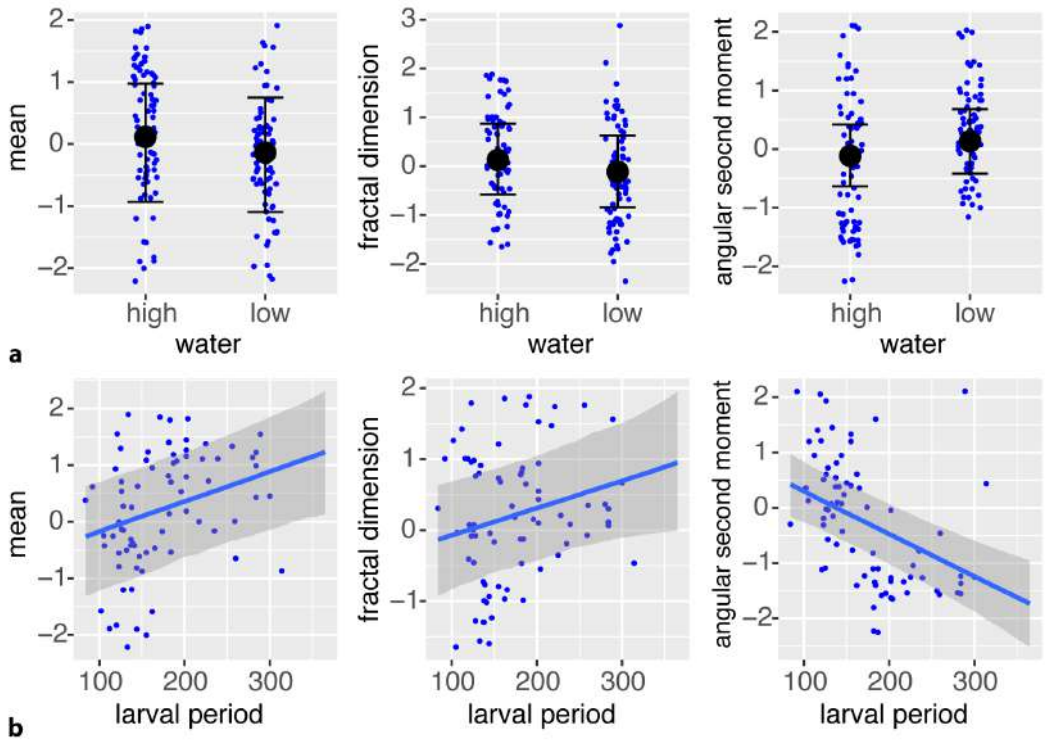

Figure 3. Conditional effects of the best fitting Bayesian model including water level treatment and larval period as fixed effects showed that decreased water levels led to lower greyscale mean and fractal dimension and higher angular second moment values. Note that the big black dots visualize the model estimates (predicted values of the response), the upper and lower bounds stand for the uncertainty intervals of the response, and the blue dots represent the original data points. Furthermore, the duration of the larval period was positively correlated with mean and fractal dimension while negatively with angular second moment.

Discussion

The dorsal pigmentation pattern of *P. cultripes* metamorphs varied in texture and complexity across populations and sibships, but also in response to altered developmental rate. The among-sibship and among-population variation is intriguing because it suggests a heritable component. Our estimated broad-sense heritability for brightness, homogeneity and complexity aspects of the pigmentation patterns ranged between 0.15-0.30, although our full sib design prevented us from disentangling additive genetic from non-additive and

maternal components (Lynch and Walsh 1998) and should only be considered a general indication of a hereditary component of the pattern.

As expected, spadefoot toad tadpoles accelerated their development in response to reduced water level, resulting in tadpoles metamorphosing earlier but at a smaller size. Interestingly, this developmental acceleration of the larvae also caused alterations in different aspects of the dorsal pigmentation patterns of the emerging toads. Toads that had accelerated their larval development (i.e. experienced stressful conditions) showed a less contrasted and more homogenous pattern (less random in its intensity, as interpreted based on low fractal dimensions and high angular second moment values; Figure 2, 3) compared to control toads (i.e. benign conditions), with the blotches being darker and more highly connected amongst each other. Toads induced to accelerate their development also showed a lower complexity of their pigmentation pattern and lower fractal dimension, or "gappiness" (Pérez-Rodríguez *et al.* 2017).

Developmental acceleration in response to pond drying is known to take a considerable physiological toll on amphibian larvae (Gervasi and Foufopoulos 2008, Burraco *et al*. 2017). In order to achieve such acceleration, spadefoot toads increase the corticosterone and thyroid hormone secretion and their metabolic rate (Gomez-Mestre *et al*. 2013, Kulkarni *et al*. 2017). The formation of the adult pigmentation pattern in the epidermis of amphibians is induced during metamorphosis by the melanocyte-stimulating hormone (MSH), in interaction with thyroid hormone (Bagnara and Fernandez 1993), to the extent that hypophysectomy causes bleaching of tadpole skin (Frieden and Just 2012). For instance, in oriental fire-bellied toad tadpoles (*Bombina orientalis*), experimental

inhibition of thyroid hormone led to a marked reduction in the size of melanophores on their dorsal skin (Park *et al*. 2010). We thus hypothesize that enhanced pituitary activity in response to perceived risk of predation may trigger over-melanization of tadpole skin as they approach metamorphosis. Sexual hormones are also known to affect the development of pigmentation pattern in amphibians, and disruption of these hormones cause profound changes to the pigmentation of post-metamorphic amphibians (Noriega and Hayes 2000, Hayes *et al*. 2002, Bagnara and Matsumoto 2006), although it is still unclear how induced developmental acceleration may affect the secretion of these hormones. Pigments such as carotenoids are known to prevent oxidative stress (Schanz *et al*. 1999) and enhance the coloration of metamorphosing frogs (Cabrera-Guzmán *et al*. 2020), and their availability is reduced in birds when raised under stressful conditions (Isaksson *et al.* 2005, Schanz *et al*. 1999). In amphibians, however, a direct link between pigmentation and oxidative stress is not yet clearly established as not enough studies have explored amphibian pigmentation in the context of environmental stress experienced during their larval period.

Rather, past studies have focused on pigmentation as aposematism (Summers and Clough 2001, Vences *et al.* 2003) or camouflage (Sköld *et al.* 2012). Interestingly, deformed individuals originating from stressful conditions showed larger pigment spots despite being smaller in size in comparison to individuals originating from benign conditions in northern leopard frogs (*Rana pipiens*) (Gallant and Teather 2001). Newts have been shown to be capable of background matching (Garcia and Sih 2003, Polo-Cavia and Gomez-Mestre 2017), and newts that expressed more pigments have been shown to have a

higher metabolic rate, suggesting a metabolic cost entailed in pigment expression (Polo-Cavia and Gomez-Mestre 2017), but it is still unknown whether such increased metabolism has carry-over consequences.

While we find that *P. cultripes* metamorphs originating from stressful conditions (i.e. accelerated development) show darker and more continuous blotches, at this stage it is hard to tease apart whether this was to compensate for smaller size resulting from limited time of growth or rather a side effect of developmental acceleration and insufficient time for growth. Although variation in coloration can often play a role in thermoregulation, UV protection, predator avoidance, or sexual signaling (Summers *et al.* 1999, Blaustein and Belden 2003, Rudh and Qvarnström 2013) we do not yet know whether variation in the pigmentation pattern of this burrowing, nocturnal species with aquatic acoustic communication is of any adaptive value. Adaptive coloration in amphibians remains a vast field to be explored, as for instance, subterranean caecilians have evolved bright coloration (Wollenberg and Measey 2009). *Pelobates cultripes* is mostly an amphibian of nocturnal habits (although males may stay by the clutch during daytime on the first day post-oviposition) and its dorsal pigmentation likely serves as camouflage rather than communication (Stevens and Merilaita 2011). However, whether the observed variation in pigmentation and complexity across populations is an evolved response to different levels of predation pressure and whether developmental alterations of such patterns increase their risk of predation remain open questions. For now, our finding that tadpoles induced to accelerate their development emerged with a more homogeneous and darker pigmentation pattern (Figs. 2, 3) suggests two hypotheses. First, the development of lighter, well-contrasted, complex dorsal

patterns in normally developing individuals may be disrupted due to physiological stress, indicating deterioration of body condition that carry over to the juvenile phase. Second, the darker and more homogenous pigmentation of accelerating individuals may simply reflect heterokairic effects (i.e. environmentally induced shifts in the timing of developmental events; Spicer and Burggren 2003, Rundle and Spicer 2016), wherein skin development did not proceed at the same pace as that of other organs in accelerating individuals. Individuals subject to developmental acceleration may not have had enough time and/or resources to allocate for the optimal expression of pigmentation, which is what might have led to their darker and less complex patterns.

Amphibians show remarkable variation in pigmentation patterns, both within and among species. Here we show that patterns can be altered due to plastic alterations of development. Future studies would have to determine whether the texture and complexity of the pattern are heritable, the relative importance of parental effects, whether it is static or changes ontogenetically, and whether it shows geographic variation or phylogenetic signals.

Declarations

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Author contributions

LHJ executed the experiment, LHJ, MAR, and HCL analyzed the data, and LHJ, HCL and IGM wrote the manuscript. IGM conceived and supervised the study.

Competing interest

The authors declare no competing interests.

Data accessibility

Data are archived in an institutional public repository (Digital CSIC: http://hdl.handle.net/10261/219661).

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Chapter 4

Demographic consequences of predator-induced life history shifts in the waterflea *Daphnia magna*

In prep.

Demographic consequences of predator-induced life history shifts in the waterflea *Daphnia magna*.

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Abstract

Prey may often respond to the presence of predators by altering its behavior, morphology, and/or life-history traits. Empirical studies on the ecology and evolution of predator-induced defenses in waterfleas are generally focused on life-history traits at the individual level and have not quantified the consequences of phenotypic shifts at the population level. In this study, we assess the demographic consequences of predator-induced changes in life history traits in *D. magna*, across multiple genotypes (clones) originating from periods of divergent selection regimes within a single population. Waterfleas exposed to predator cues reached maturity faster and at a smaller size, and these changes in life history scale-up to a faster population growth, reaching their carrying capacity earlier than predator-free populations. This pattern was consistent across most replicated populations, although to a varying degree

across genotypes, while there was no clear difference across subpopulations originating from varying fish predation pressures. Hence, we show that plastic phenotypic responses can determine population demography, altering their eco-evolutionary dynamics.

Keywords: Phenotypic plasticity, *Daphnia magna*, waterflea, life history traits, predator-induced defenses, population demography

Introduction

The mere sensing of chemical predator cues can elicit anti-predatory behavior in various organisms and further enhance the survival of adequately responding organisms; hence predator-induced defenses are widespread and serve as a key example of adaptive phenotypic plasticity (Karban and Myers 1989, Trussell 1996, Tollrian and Harvell 1999, Chen 2008). Nevertheless, expressing predator-induced defenses may come at the cost of sub-lethal fitness consequences, such as reduced growth (Peckarsky *et al*. 1993), changes in their trophic niche (Arribas *et al*. 2018), shifts in timing of reproduction (Stibor and Lüning 1994), or carry-over effects spilling over subsequent life stages (Benard and Fordyce 2003, Van Allen *et al*. 2010, Touchon *et al*. 2013). To avoid potential costs of inducing anti-predatory traits in the absence of predators, predator-induced defenses have evolved as plastic responses that are only triggered at the presence of predators (Tollrian and Harvell 1999).

Many species across various taxa alter their phenotypes in response to perceived risk of predation; predator-induced defenses may be expressed by morphological changes (Harvell 1984, McCollum and Leimberger 1997, Van Buskirk 2009, Auld and Relyea 2011), or life-history shifts comprising

alterations in activity- and growth rate (Werner and Anholt 1996, Laurila *et al*. 1998), growth rate and timing of reproduction (Crowl and Covich 1990, Stibor 1992), timing of egg hatching (Blaustein 1997) or resource allocation (Rinke *et al*. 2008). Such predator-induced defenses are achieved by altering their metabolism (Barry and Syal 2013, Burraco *et al*. 2013), and/or other behavioral and physiological mechanisms (Slos and Stoks 2008, Steiner and Van Buskirk 2009). In general, such predator-induced defenses convey critical benefits in terms of survival in the face of predation pressure (Tollrian and Harvell 1999), and critical shifts in life-history traits induced by the presence of predators ultimately influence the community dynamics of the plastically responding species in an indirect way (Peckarsky *et al*. 2008, Bestion *et al*. 2015). Therefore, to understand and quantify the impact of predator-induced defenses and evolutionary changes on predator-induced plasticity responses, we need to link life-history trait changes to population-level consequences.

Genetically identical organisms have been shown to differ in their degree of predator-induced defenses when partitioned into distinct populations that experience different environmental conditions (i.e. inhabit distinct microhabitats) and hence are exposed to different predation and competition pressures (Mathis *et al*. 1993, Blázquez *et al*. 1997). Such local population differences in morphological and behavioral pre-adaptations may trigger the evolution of different phenotypically plastic responses (Gross and MacMillan 1980, Relyea 2002), and even act further as selective force leading to divergent evolution across populations (Vervust *et al*. 2007, Bell *et al*. 2010). Varying degrees of induced anti-predator mechanisms across genetically identical populations is an interesting aspect to approach in the context of global climate

change, since organisms will gradually be forced to survive harsher environments and populations able to respond plastically will be able to enhance their survival (Reusch 2013).

The waterflea *Daphnia magna* is known to show plastic responses to the presence of water-borne fish cues (e.g. fish kairomone) in morphological, life history, and behavioral traits (Stibor and Lüning 1994, Tollrian 1995, Boersma *et al*. 1998). In *D. magna,* populations originating from habitats with high fish predation pressure have been shown to respond to predator cues with more negative phototactic behavior than populations from habitats lacking fish (De Meester 1993). Together with this finding and the fact that this species has a relatively short generation time, *D. magna* is an excellent system to study the influence of predator-induced phenotypic plasticity on population dynamics. A recent resurrection ecology study on *D. magna* (Stoks *et al*. 2016) has revealed fast evolution of life history traits and their plasticity in the presence of predator kairomones in response to changes in fish predation pressure, and the existence of ample variation in life history responses to predator kairomones across clones even within a single population (Cousyn *et al*. 2001, Stoks *et al*. 2016).

The combination of predator-induced plasticity and genetic variation may affect key aspects of the life history traits of individuals, which eventually may add up to alter the population demography. Meanwhile, it is not straightforward to predict demographic consequences of life history shifts that were measured on single individuals, because key features such as individual growth, clutch size and mortality depend on population density as well. To disentangle these effects, we focused on both i) demographic changes by monitoring the population size

and ii) individual responses by measuring life history traits such as survival, body size, brood size, and number of offspring.

When exposed to predator cues, *D. magna* tend to mature earlier and at a smaller size, and produce more but smaller offspring (Boersma *et al.* 1998, Stoks *et al*. 2016). Hence, we would expect that populations exposed to predator cues would show a higher population growth rate, reaching carrying capacity more rapidly. Further, due to the smaller body size induced by predator cues, we would also expect that exposed populations would reach higher peak densities (c.f. higher densities translate in the same biomass), as has been shown that body mass and density are negatively correlated across various animal taxa (Peters and Wassenberg 1983) and especially in zooplankton (Cyr *et al*. 1997). We further predicted that populations exposed to predator cues would be more prone to declines because of reduced starvation resistance in smaller animals (Gergs and Jager 2014) and because populations were bound to reach maximum carrying capacity (Hayward *et al*. 2007, Rintala and Tiainen 2008). To test whether predator-induced phenotypic plasticity resulted in the predicted shifts in population demography across different genotypes, we designed an experiment in which we compared population dynamics of the same *D. magna* genotypes cultured in the presence or absence of predator kairomones. We performed this experiment on five different genotypes originating from two subpopulations (total: 10 genotypes) known to differ in their life history responses to non-consumptive predatory effects.

Material and Methods

ORIGIN OF CLONES AND SELECTION OF CLONES

The *D. magna* clones used in this study are a subset of the clones used in a previous resurrection ecology study (Stoks *et al*. 2016). The clones originate from pond sediments of a manmade pond used for fish stocking in Oud-Heverlee, Leuven, Belgium (50°50´N – 4°39´E). Three depths of sediment layers, each corresponding to a period of different fish stocking, were selected and classified into three subpopulations (Cousyn *et al*. 2001): 1) the bottom subpopulation corresponding to clones collected from sediment from a period when there was low fish predation pressure (1970-1972); 2) the middle subpopulation corresponding to clones taken from the middle sediment layer, belonging to a period when fish stocking increased and thus fish predation pressure was stronger (1973-1987); 3) the "Top" subpopulation when fish stocking decreased until it completely ceased and thus represents a period of relaxed fish predation pressure (1988-1990).

To increase our capacity to gain insight into population-level consequences of phenotypic plasticity, we selected clones divergent in their degree of responsiveness to predator kairomones. For our experiment we chose clones from the two extreme subpopulations: the bottom ("low fish") subpopulation that occurred in the absence of fish, from which we expected to find low capacity to plastically respond to predator cues (i.e. fish kairomones); and the middle ("high fish") subpopulation, which occurred during a period of intensive fish stocking, from which we therefore expected higher capacity to plastically respond to predator cues. Evidence for evolved accumulated plasticity in a given environment has been shown by De Meester (1993), in which clones

originating from high fish pressure conditions had a more negative phototactic behavior than clones originating from low fish pressure conditions.

We chose our clones of interest based on the estimates of plasticity presented in Stoks *et al*. (2016), which showed slopes of reaction norms of a variety of plastic life history traits in response to predator cues: age at maturity, alertness, fecundity at early life stage, fecundity at late life stage, horizontal migration, intrinsic growth rate, phototactic behaviour, size at maturity, size of neonates, offspring size at early life stage, offspring size at late life stage, somatic growth, spine size of adults, spine size of neonates, respectively. Among those, we selected five traits showing strong responses to the presence of fish kairomones: age at maturity, clutch size, phototactic behaviour, size at maturity, and somatic growth rate (Stoks *et al*. 2016). We summed up their standardized plasticity values to identify clones that differed in overall amplitude of predator-induced plasticity. We then selected five highly responsive clones of the 'high fish' subpopulation (i.e. the potentially most plastic subpopulation) and five little responsive clones of the 'low fish' subpopulation (i.e. the potentially least plastic subpopulation) to predator kairomones. We conducted the experiment on clones 1, 4, 5, 7, and 8 for the middle ("high fish predation") subpopulation, and clones 5, 6, 9, 10, and 11 for the bottom ("low fish predation") subpopulation (Figure 1).

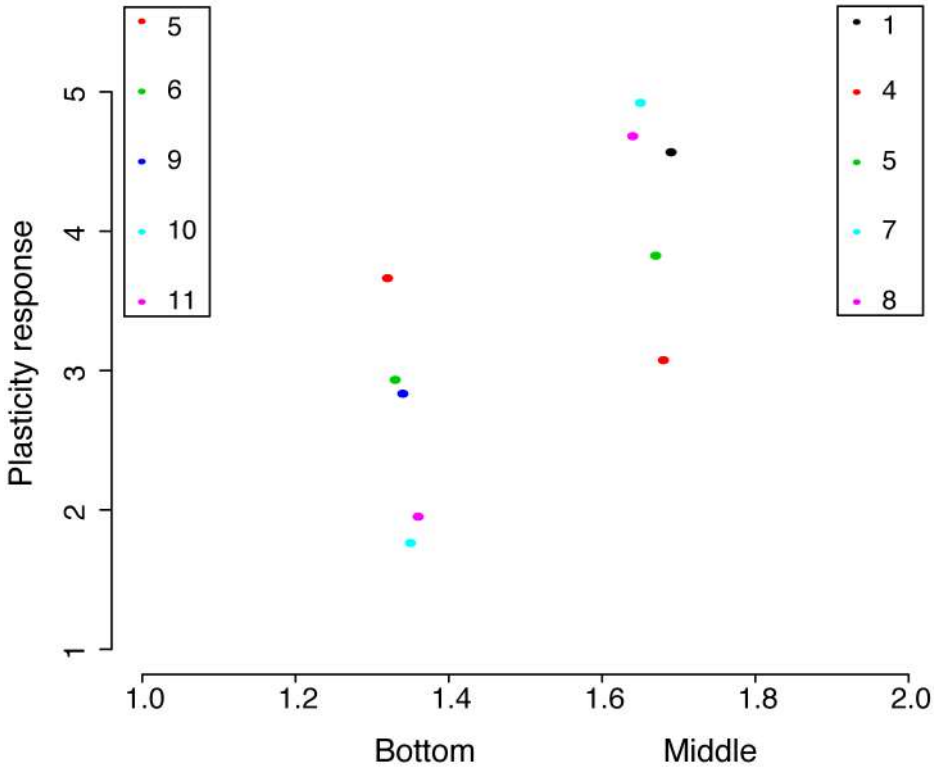

Figure 2 Visualization of the sum of standardized phenotypic plasticity values of age at maturity, clutch size, phototactic behavior, size at maturity, and somatic growth rate, respectively, indicating the sum for each of the clones selected in each subpopulation.

EXPERIMENTAL CONDITIONS

Animals were kept in the laboratoy of aquatic ecology, evolution, and conservation in Leuven, Belgium, under standardized temperature (20 ± 1°C) and photoperiod (14 : 10 L : D), and were fed *ad libitum* daily (100,000 cells/mL) with fresh green algae (*Acutodesmus obliquus*).

For each clone, we set up three replicate lines for each of two treatments, one where daphnia were exposed to fish kairomones and one control without predator cues. Thus, the experiment consisted of 2 subpopulations x 5 clones x 2 treatments x 3 replicates, adding up to 60 aquaria in total. Prior to the onset of the experiment, three individuals were selected from stock jars of each clone and raised in 200 mL jars for three consecutive generations, to eliminate potential

maternal effects generated in the stock jars conditions. When the third generation was released, we randomly selected seven juveniles, and gradually increased water volume until we finally transferred them to 3 L aquaria. From this moment onwards, the predator cue treatment started receiving fish kairomone on a 24-hour basis. All replicates were treated identically.

We prepared fish kairomone by keeping three pumpkinseed sunfish (*Lepomis gibbosus*) in 20 L of water. To standardize the concentration of fish kairomone in the fish predation cue treatment, the medium in the aquarium with fish was changed every 24 hours and the medium was added to aquaria with *D. magna*, after filtering over 0.2 μm filter using a high-pressure filter at 10 psi. We added 400 mL of filtered medium to the 2 L aquaria daily, which translated into a fish kairomone concentration of approx. 3 fish per 100 L of water. We renewed the medium in all aquaria on a daily basis. Aquaria assigned to the predator cue treatment received 400 mL of filtered fish water and 1.6 L of dechlorinated water, whereas the no predator cue treatment received 2 L of dechlorinated water daily.

MEASURING POPULATION DEMOGRAPHY

We first determined population size after 14 days of first adding the fish kairomone, because given the life cycle of the species, this was the minimum time necessary to be able to start observing sharp differences between aquaria that received predator cues and aquaria that did not. From that time onwards, we estimated population size for each population every 4 days by taking a short video of approximately 7-8 s using a Canon EOS 700D + 18/55 IS STM camera mounted on a setup shielded from light and connected to a computer to use the

corresponding EOS utility software. To reduce overlapping individuals in the water column and hence reduce counting error, the aquaria were emptied in an oven dish, shallower and larger than the aquaria. We analyzed the resulting video files using the R package *trackDem* (Bruijning *et al*. 2018), which converts movie files into stacked image sequences, and subsequently tracks and counts all moving objects.

ASSESSING INDIVIDUAL LIFE HISTORY TRAITS

To obtain estimates of survival, reproduction, growth, and offspring number and size of exposed and non-exposed individuals from the different clones and subpopulations, we randomly isolated two individuals from each aquarium at every given time *t* in PVC tubes with holes drilled in them, wrapped in very fine meshes. The PVC tubes were carefully placed inside the aquaria, so the isolated individuals could still receive the same medium as the rest of the individuals. To account for the different growth curves and reproductive potentials in the juvenile and adult phases, one juvenile and one adult were haphazardly chosen. This allowed monitoring those individuals separately while they were living in the same medium with the rest of the population. At every given time *t+1,* in 4 days increments, those same individuals were re-measured to record 1) survival, 2) whether they reproduced, and if yes, how many eggs they produced, 3) growth, 4) whether they released offspring, and if yes, 5) how many, 6) offspring size, and 7) the sex of the offspring. We subsequently removed these individuals from the experiment and new individuals were selected and measured.

STATISTICAL ANALYSES

We fitted generalized linear mixed models on the individual measurements to test the effect of exposure to predator cues on life-history traits. Specifically, we quantified growth, reproduction (split into whether carrying eggs or not/ actual egg count), offspring (split into whether released offspring or not/ actual offspring count), and offspring size, to explore whether these traits differed across treatments and clones. We fitted models with the different life-history traits as response variables and initial body size, clone, treatment, and interactions as independent variables. To test for demographic consequences, we used population size as dependent variable in additional models, using the same independent variables. We tested the contribution of each independent variable fitting nested models either including or excluding each term and comparing the change in deviance between consecutive models. Models on egg count and offspring count were run using the R package *glmmTMB* specifying a negative binomial error distribution (Brooks et al. 2017). To assess the slope of population growth, estimate the inflection point when carrying capacity was reached, and calculate the slope of population decrease after peak density was reached, we fitted segmented regressions using the *segmented* package in R (Muggeo 2008). All statistical analyses were conducted using R v.3.2.1 (R core team, 2015).

Results

EFFECT OF PREDATOR CUES ON GROWTH

The predator cue treatment had a significant effect on individual growth (χ^2 = 0.116, p = 0.02), and individuals of the non-predator treatment grew to be on

average 11% larger (Figure 2). Population size had a highly significant effect on individual growth as well, since daphnia grew less as populations reached high density (χ^2 = -2.19, p < 0.001). Clones did not differ significantly in their growth across predator cue treatment and control (χ^2 = -0.19, p = 0.14).

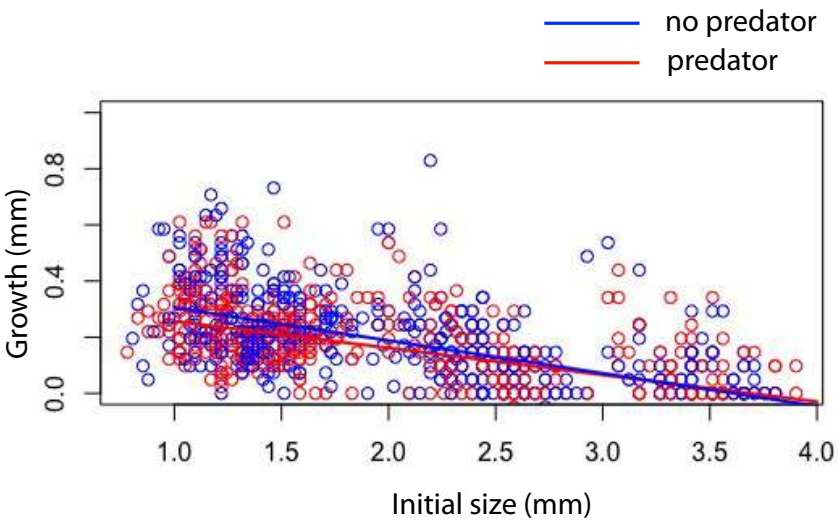

Figure 3 Predator cue negatively influenced the growth of *D. magna*, and individuals of the no predator treatment grew to be larger.

EFFECT OF PREDATOR CUES ON EGG CARRYING PROBABILITY AND EGG COUNT

The predator cue treatment had a significant effect on egg carrying probability (χ^2 = 10.72, p = 0.001): individuals in the predator cue had a greater chance of carrying eggs, and did so at a smaller size (Figure 3). We also observed a significant effect of treatment and population density interaction, and fewer *D. magna* individuals carried eggs once populations had already reached high density (χ^2 = 5.29, p = 0.02). Further, there was significant clonal variation in their likelihood of carrying eggs (df = -9, χ^2 = 29.5, p = 0.0005).

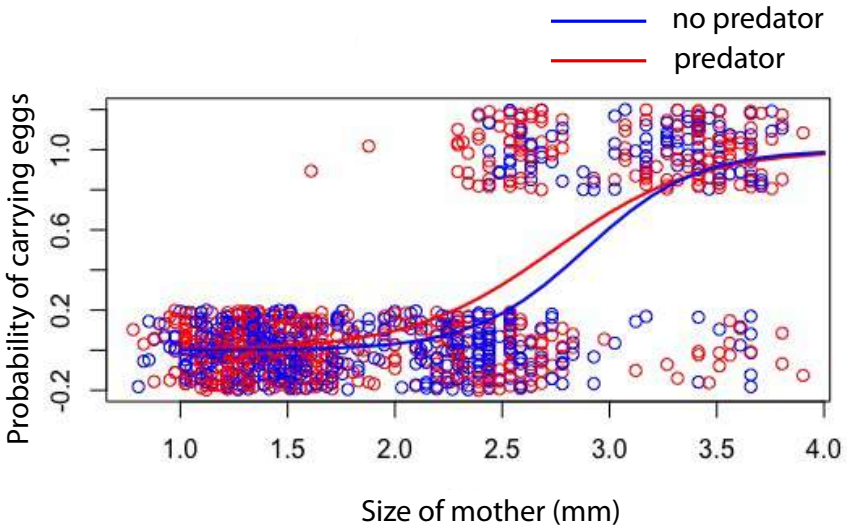

Figure 4 *Daphnia magna* individuals of the predator treatment started carrying eggs at higher frequencies and starting at a smaller size

Individuals in the predator cue treatment showed a 17 % increase in the number of eggs produced in comparison to individuals from the control treatment (χ^2 = 4.08, p = 0.04). Individuals carried significantly fewer eggs when populations already reached their carrying capacity (χ^2 = 116.12, p < 0.001). Also, there was a significant clonal variation on egg count (df = 9, χ^2 = 21.79, p = 0.009), showing that not all clones responded the same way.

EFFECT OF PREDATOR CUES ON OFFSPRING RELEASING PROBABILITY, OFFSPRING COUNT AND SIZE

The interaction of treatment and clone had a significant effect on offspring release (df = 9, χ^2 = 76.9, p < 0.001) (Figure 4). Significantly fewer *D. magna* individuals released offspring when populations already reached high density (χ^2 = -453.8, p < 0.001).

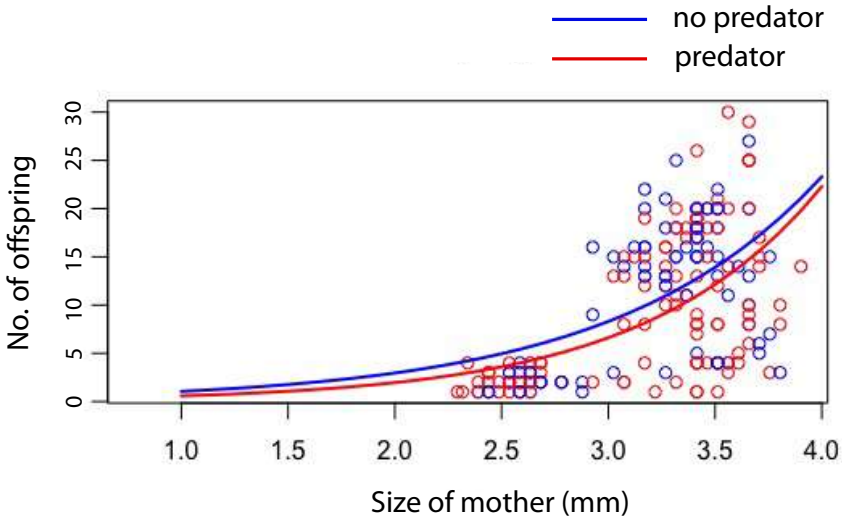

Figure 5 Individuals of the predator treatment started producing offspring at a smaller size

Offspring count significantly differed across clones (df = 9, χ^2 = 18.16, p = 0.03). Clutch size significantly decreased once populations already reached carrying capacity (χ^2 = 74.29, p < 0.001)

Mothers of the non-predator treatment produced significantly larger offspring (3 % increase in length) compared to mothers from the predator treatment (χ^2 = -0.06, p = 0.003) (Figure 5). Population density had a significant effect on offspring size (χ^2 = -0.19, p < 0.001) and there was a significant clonal variation in offspring size (df = -9, χ^2 = -0.29, p < 0.001), showing differences across clones.

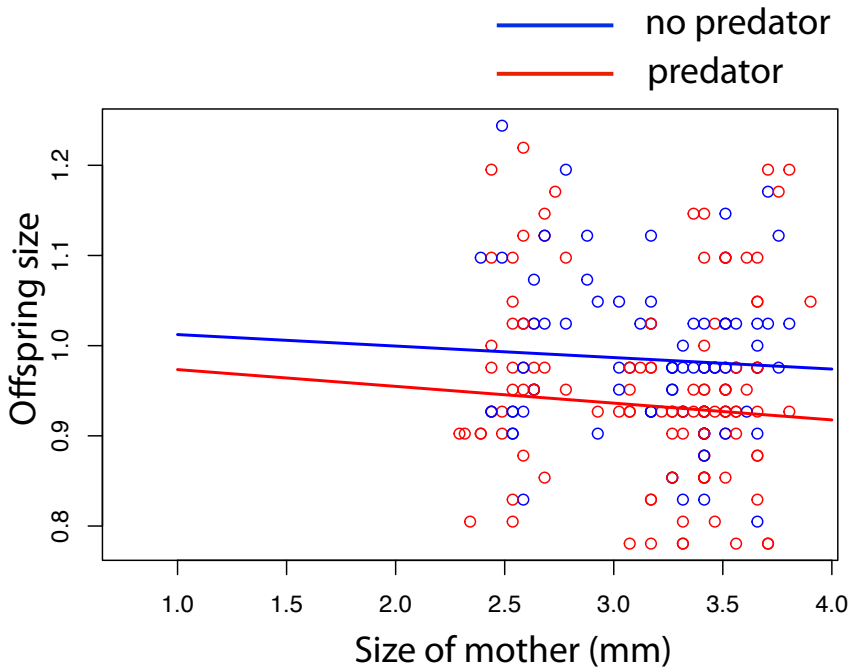

Figure 6 *D. magna* individuals produce smaller offspring when exposed to predator cues

PREDATOR INDUCED CHANGES IN POPULATION DEMOGRAPHY

Generally, aquaria exposed to predator cues tended to reach their carrying capacity sooner (Figure 6). Most populations increased in density up to a certain point when carrying capacity was reached, and then density started to drop. Clones differed as to when carrying capacity was reached, since they reached carrying capacity at different densities (Table 1a,b). While there was clonal variation, there was no clear-cut difference across the middle ("high fish") and bottom ("low fish") subpopulations.

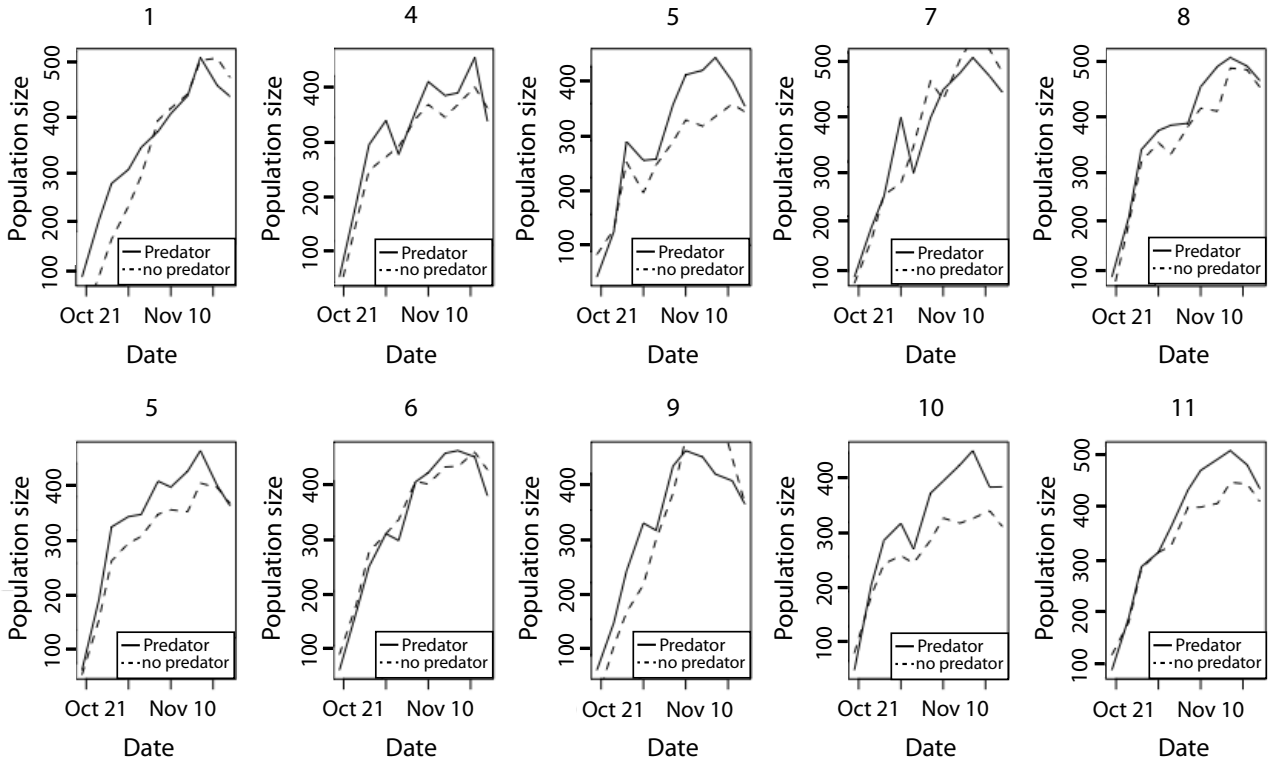

Figure 7 Population density tends to increase faster when exposed to predator cues.

Table 1a. Slopes of population growth and decrease, estimated breakpoint, carrying capacity (middle subpopulation)

Sub population : Middle	Genotype	Treatment	Slope of increase	Slope of decrease	Estimated breakpoint	Carrying capacity	Post carrying capacity
	1	A	12.19	-14.05	322.135 (18/11/17)	504	415.8
	1	B	16.46	-7.36	322.036 (18/11/17)	553	471.7
	4	A	12.19	-14.08	322.14 (18/11/17)	343	307
	4	B	21.09	2.07	301.803 (29/10/17)	246*	298.9*
	5	A	10.69	-8.99	319.782 (16/11/17)	419	365.7
	5	B	8.75	-0.38	313.855 (10/11/17)	352	325
	7	A	14.38	-16.43	321.609 (18/11/17)	480	405
	7	B	17.24	-7.67	319.783 (16/11/17)	466*	488*
	8	A	11.73	-7.44	320.024 (16/11/17)	463	424
	8	B	29.61	4.02	301.018 (28/10/17)	305*	374*

Table 1b. Slopes of population growth and decrease, estimated breakpoint, carrying capacity (bottom subpopulation)

Sub population : Bottom	Genotype	Treatment	Slope of increase	Slope of decrease	Estimated breakpoint	Carrying capacity	Post carrying capacity
	5	A	12.28	-8.33	316.412 (12/11/17)	390	360.8
	5	B	16.40	-0.58	308.708 (05/11/17)	350	339.7
	6	A	16.95	-8.33	316.994 (13/11/17)	466	418.3
	6	B	17.44	-0.67	310.997 (07/11/17)	431	401.9
	9	A	14.63	-5.99	313.237 (09/11/17)	358	328.3
	9	B	16.38	-9.67	317.517 (14/11/17)	498	357.7
	10	A	9.97	-8.06	319.36 (15/11/17)	419	348.2
	10	B	19.89	2.12	300.991 (28/10/17)	255*	287.8*
	11	A	15.89	-12.24	319.844 (16/11/17)	493	452.5
	11	B	10.96	-14.7	322.807 (19/11/17)	477	401.6

Note: Values of estimated breakpoints correspond to dates, such as for example: January 1st = 1, December 31st = 365, hence, 320, for instance, is November 16th. Carrying capacity refers to the population size at break point. Post carrying capacity refers to the average population density after carrying capacity has been reached already. Note that for some populations (marked with asterisks*), the average density from carrying capacity onwards is actually higher than the density at carrying capacity. This is due to the fact that there is large clonal variation, some clones may actually reach a second carrying capacity, but this is outside of the scope of this study as the experiment was terminated prior to that point.

Discussion

Phenotypically plastic responses have been shown to have the potential to alter population density (Rodd *et al*. 1997, Creighton 2005). The relationship between phenotype and population density is dynamic, as shifts in phenotype can have demographic consequences, and high population densities can in turn trigger phenotypic switches. Thus, high densities can sometimes trigger the expression of polyphenisms, a special form of plasticity in which drastic shifts in phenotype are expressed in response to environmental cues (Stockton *et al*. 2020). Aphids, for instance, can markedly alter their phenotype to the point of exhibiting wing polymorphisms (i.e. winged or wingless morphs; Brisson 2010), body-color polymorphisms (in response to symbiotic bacterium; Fukatsu 2010, Tsuchida *et al*. 2010), and reproductive mode polyphenism (i.e. sexual reproduction or parthenogenesis; Ogawa and Miura 2014). Polyphenic switches in their reproductive mode affect the demography of this species, because when aphids reproduce parthenogenetically, they show a nesting structure in which multiple generations develop simultaneously (Ogawa and Miura 2014). Such transgenerational transfer of maternal signals may allow aphids to asexually produce large numbers of offspring given favorable conditions, hence experiencing fast population growth (Ogawa and Miura 2014). In a pierid butterfly (*Pieris napi*), there is a clear-cut seasonal polyphenism between spring- and summer generation characterized by a resource allocation tradeoff between flight and reproduction. While summer generation butterflies have greater dispersal ability, the spring generation butterflies produce significantly more eggs, which increases the population density of the spring generation (Karlsson and Johansson 2008). The desert locust *Schistocerca gregaria* also exhibits an

interesting case of phase polyphenism, changing between a solitary phase and a more active, gregarious phase (Pener 1991). Locust phase traits serve as defense mechanisms against predation; the conspicuous black and yellow pattern expressed during the gregarious phase signal toxicity to potential predators (Sword *et al*. 2000), while these locusts wear cryptic coloration during the more vulnerable, solitary phase (Despland and Simpson 2005). Such differences in coloration across phases achieved by phase polyphenism is an excellent mechanism to avoid detection by predators, and enhances the survival of these locusts, subsequently impacting the population growth of this species (Sword *et al*. 2000). While phenotypic plasticity may act as a selective force altering population dynamics, population density is also a regulating factor of phenotypic plasticity itself. This may be actively regulated by the organism: the desert locust *S. gregaria* has been shown to readily manipulate their egg size and number in response to the varying population densities across gregarious and solitary phases (Maeno *et al*. 2020). Alternatively, population density may indirectly affect the physiology of the organism and hence alter the magnitude of phenotypic plasticity achieved (Nussey *et al*. 2005).

In this study, we show that *D. magna* individuals tended to grow less, matured at smaller sizes, and produced larger egg clutches that hatched into smaller offspring in response to predator cues. Moreover, we found that clones significantly differed in their propensity to produce eggs, the number of eggs produced, and the size of the offspring, suggesting that genotypes have intrinsic differences in reproduction. While we have found that not all genotypes responded in the same way to the presence of predator cues, the difference across clones originating from the low fish abundance and the high fish

abundance subpopulations was often times not clear cut. It can be inferred that whether the subpopulations of origin have been exposed to low- or high fish predation pressure, *D. magna* individuals responded to fish kairomones to a similar degree. Interestingly, the hatching likelihood of the eggs was determined by the interaction with the environment. We found that there was a 23 % increase in the number of eggs hatched in the predator cue treatment in comparison to the control. Consequently, there was a 47 % increase in the number of ephippia produced in the no-predator cue treatment compared to the predator cue treatment. When exposed to harsh environmental conditions (mostly characterized by low food conditions), *D. magna* individuals reproduce sexually, producing sexual eggs covered by a protective membrane (i.e. ephippia) that may endure long periods of time before hatching (Carvalho and Hughes 1983, Antunes *et al*. 2003). While it may seem contradictory that our results found higher production of ephippia in the no-predator cue treatment, this may be because our ephippia count comes mostly from data before the aquaria reached their carrying capacity. The individuals exposed to predator-cue treatment released smaller offspring, lowering food stress in the growing phase of the population.

Most importantly however, here we have shown that non-consumptive effects of predators on the life history traits of individual *D. magna* scale up to alter the population demography of populations, and increased population density eventually acts as a barrier to predator-induced defenses. Population density increased faster when daphnia were exposed to predator cues because individuals matured at a smaller size and produced larger egg clutches. Consequently, they also reached their carrying capacity earlier, resulting in

slowed down individual growth, production of smaller egg clutches, and fewer offspring released. This had further consequences on the phenotypic plasticity achieved; while individuals exposed to predator cues produced larger egg clutches at low population density, they were not longer capable of doing so when carrying capacity was already reached. These results show that individual life history traits and population demography are tightly linked to each other in an eco-evolutionary dynamic way, since the ecological consequences of the induced phenotypes determine a new selective scenario for the plastic genotypes to evolve in. Further, if individuals continue to mature early and produce large egg clutches in response to predator cues even after reaching carrying capacity, eventually the predator-induced defenses would become counterproductive, as animals would end up suffering starvation. This is of great importance in organisms such as *D. magna* that inhabit confined water spaces, and do not have the ability to disperse into other ponds. Hence, responding to increasing population density and lowering their predator-induced defenses enables *D. magna* to survive, and vice versa. In an evolutionary long-term scenario, *D. magna* persist by maintaining the equilibrium between predator-induced defenses and responding to changes in population density, especially as they approach the carrying capacity of the system.

A future step to be taken would be to assess the population demography of our study populations by implementing the integral projection modeling (IPM) approach. This will aid in documenting the population level consequences of predator induced life history shifts and its underlying genetic variation. A classic matrix projection model divides populations into groups without considering individuals classified according to continuous variables such as body

size, and considers 'separate' classes (Ellner and Rees 2006). Unlike this classic approach, IPMs deal with continuous distributions in discrete time to qualitatively assess individual variation based on demographic transitions (Ellner and Rees 2006, Ozgul *et al*. 2010). Hence, the IPM approach aids in putting ecology in a demographic context and is ideal to assess how life history features of individuals in distinct stages within a population influence population growth (Coulson *et al.* 2011). To calculate the population growth rate of a given population, one has to consider four life history traits: survival, reproduction (i.e. presence of eggs, if yes, how many, and when hatched, what size did the offspring achieve), and growth (comparing growth of a certain individual at time t versus at time t+1) (Ellner and Rees 2006). The data in our study has been collected considering this, and while outside of the scope of this dissertation, it would be an essential future step to take to be able to understand more thoroughly the ecological consequences of phenotypic plasticity on population demography.

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General Discussion

Throughout this dissertation, I have explored the variation of phenotypic characters across different environmental selection regimes. In chapter 1, I investigated dwarf populations of the natterjack toad *Epidalea calamita* in southern Spain in which the environment potentially acted as a limiting factor of growth. In chapter 2, I studied the developmental plasticity of the spadefoot toad *Pelobates cultripes*, teasing apart the phenotypic reaction norms and the differential gene expression level across various populations of spadefoot toads originating from two locations. In chapter 3, I went on to show that environmental stress (i.e. low water conditions) experienced in the larval stage of spadefoot toads carries over to influence the dorsal pigmentation pattern upon metamorphosis of this species with a complex life cycle. In chapter 4, I took one step further and linked phenotypically plastic responses to population level consequences; exploring how varying life-history traits resulting from predator-induced defenses may influence the population dynamics of the waterflea *Daphnia magna*.

A case of extreme dwarfism in the natterjack toad

Dwarfism in Doñana National Park (southern Spain) that occurs over an extremely short geographical range and in the absence of any major biogeographical barrier has been reported not only in amphibians (Díaz-Paniagua and Mateo 1999, Marangoni and Tejedo 2008, Marangoni *et al*. 2008), but also in mammals (Revilla et al. 1999). In this dissertation, we have investigated a particular case of extreme dwarfism in the natterjack toad (*Epidalea calamita*) that experiences an up to 2.1 fold change in body mass in as little as 37 km distance. While our 6 study populations were distributed across a geographical gradient, the body size did not continuously increase along the geographical gradient: the two geographically intermediate populations consisted of individuals as large as those of the large sized northern populations, starkly contrasting the dwarf sized southern populations.

Further, we have found that the observed dwarfism persists despite lack of population isolation or genetic differentiation. It is worthwhile to note that *cline* was a term proposed by Huxley (1938) to refer to a geographical gradient of phenotypic characters. A cohort is considered to follow a *continuous cline*, when it consists of an inter-breeding unit (i.e. no genetic differentiation) and is hence a geographical, as well as a biological reality (Huxley 1938). Further, early geneticists have shown that isolation by distance can act like actual geographic barriers to give rise to geographical divergence within congeners (Wright 1943, Endler 1977). The dwarfism of the natterjack toads of Doñana National Park studied in this dissertation may be considered a unique case, because it persists while the phenotypic character (body size) observed does not strictly follow a geographical cline, but is rather confined to a certain location and there is no marked genetic differentiation across the populations.

The dwarfism of the natterjack toads in Doñana National Park was found to be associated to ecological parameters; populations varied in their trophic status and metabolic rate. The skeletochronology data showed similar growth curves across populations, only that dwarf Doñana populations plateaued at a smaller size, while age structure varied significantly across populations. Further, our metamorph size data indicated that growth during the terrestrial phase largely determined the pattern of local dwarfism. Hence, the drier and warmer local climate in combination with soils with low water retention (i.e. sandy substrates of Doñana National Park) may act as a limiting factor of juvenile growth of the small sized Doñana populations.

Developmental plasticity in the spadefoot toad

Early models have shown that amphibian larvae are able to respond to density stress and resource limitation experienced during the larval period by altering their developmental rate and further alter their growth rate, which results in differences in growth curves and size at metamorphosis (Wilbur and Collins 1973, Travis 1984). Consistently, many empirical studies have shown that amphibian larvae followed different growth patterns under distinct ecological scenarios (Petranka and Sih 1986, Berven 1990, Arribas *et al*. 2014). Spadefoot toad larvae have the capacity of accelerating their development when they sense pond desiccation (i.e. low water treatment), which is followed by shortened larval periods and smaller size upon metamorphosis (Gomez-Mestre *et al*. 2013). In this dissertation, we have explored the phenotypic reaction norms and differential gene expression levels in response to low water

treatment of ten populations of spadefoot toads originating from two geographical regions in Spain (southern and central Spain).

Contrary to our predictions that duration of inundated period of pond of origin would be a determining factor of varying degrees of plasticity across our study populations, populations breeding in ponds with different hydroperiods within each region had similar capacity of developmental acceleration in response to low water treatments. A clearer difference, however, was observed across regions (i.e. southern *vs*. central populations), with Doñana (southern) populations having considerably higher plasticity than populations from Madrid and Segovia (central). Our climate and hydroperiod data showed that the southern populations and central populations experience different climatic conditions (i.e. temperature, precipitation) but have similar average hydroperiods. Rather, differences in developmental plasticity between regions seems to be explained by climatic differences as well as divergent heterogeneity of the hydroperiod, where southern populations experience greater environmental heterogeneity. Regardless of whether they accelerated their development or not, all individuals exposed to low water treatment underwent morphological consequences, suggesting an incomplete form of canalization in the central populations.

Data on differentially expressed genes in the liver of tadpoles did not reflect the phenotypic reaction norm pattern in that more plastic populations did not generally show a greater number of differentially expressed genes than the non-plastic populations (except for Llano). The rest of the populations all responded to decreased water level, but with similar magnitudes of DEG despite the varying degrees of phenotypic responses. While contradictory at first glance,

gene expression underlying phenotypic plasticity may be regulated to a similar degree in both plastic (ancestral) and non-plastic (canalized) genotypes (Renn and Schumer 2013). This occurs in a special case of genetic accommodation when the environmental sensitivity in canalized genotypes is not lost, but the differences in plasticity are rather observed due to alterations in gene responsiveness (Renn and Schumer 2013). This raises the importance of future genome wide association studies (GWAS) and weighted gene co-expression network analysis (WGCNA).

Pigmentation pattern in the context of phenotypic plasticity

In continuation of the water drop experiment conducted in chapter 2 to determine varying degrees of developmental plasticity across populations and its underlying differential gene expression levels, I went on to explore whether environmental stress (i.e. low water conditions) experienced during the larval phase carried over to influence the dorsal pigmentation pattern of the terrestrial stage of spadefoot toads. Toads that had accelerated their larval development (i.e. experienced stressful conditions) showed a less contrasted and more homogenous pattern (less random in its intensity, as interpreted based on low fractal dimensions and high angular second moment values compared to control toads experiencing benign conditions), with the blotches being darker and more highly connected amongst each other. Toads induced to accelerate their development also showed a lower complexity of their pigmentation pattern and lower fractal dimension, or "gappiness" (Pérez-Rodríguez *et al*. 2017).

Spadefoot toad larvae achieve developmental acceleration by increasing their levels of corticosterone and thyroid hormone secretion as well as their

metabolic rate (Gomez-Mestre *et al*. 2013, Kulkarni *et al*. 2017). The formation of the adult pigmentation pattern in the epidermis of amphibians is induced during metamorphosis by the melanocyte-stimulating hormone (MSH), in interaction with the thyroid hormone (Bagnara and Fernandez 1993). Empirical studies have shown that experimentally manipulating thyroid hormone or sexual hormone levels can alter the pigmentation of post-metamorphic amphibians (Noriega and Hayes 2000, Hayes *et al*. 2002, Park *et al*. 2010). The exact mechanisms of how such hormonal changes trigger differences in pigmentation patterns remains yet to be understood. Nevertheless, this dissertation contributes to the still scarce literature that have explored post-metamorphic pigmentation of amphibians in the context of environmental stress experienced during the larval period, and opens up doors for many future questions to be answered.

The weight of phenotypic plasticity on population demography

The waterflea *D. magna* has been shown to respond plastically to predator cues; they grew less, carried eggs at a smaller size, produced larger egg clutches, but smaller offspring when exposed to fish kairomone. Genotypes were found to have intrinsic differences in reproduction, however, the genotypes originating of the subpopulation with higher fish predation pressure did not necessarily have stronger plastic responses to fish kairomones in comparison to the subpopulation originating from a low fish predation period (Cousyn *et al*. 2001). Changes in life-history traits induced by predator presence had a significant impact on the population demography of this species. Aquaria exposed to fish kairomones reached carrying capacity earlier due to the

increased number of offspring, and hence started dropping in their population density earlier, and vice versa.

Phenotypically plastic responses that potentially alter the population density of the plastically responding species has been reported in insects as well as birds (Rodd *et al*. 1997, Creighton 2005). In polyphenism, a special form of phenotypic plasticity in which the organism drastically changes its phenotype according to the environment, plasticity strongly influences population density (Sword *et al*. 2000, Ogawa and Miura 2014). Remarkably, population density is not only affected by phenotypic plasticity, but also does readily influence phenotypic plasticity, both in a direct (i.e. the organism responds to population density, regulating its plastic responses) (Maeno *et al*. 2020) as well as indirect (i.e. density triggers changes in physiology, which in turn influences phenotypically plastic responses) way (Nussey *et al*. 2005). Most importantly, it has been shown in this study that individual life history traits and population demography are highly intertwined; the ecological consequences of the induced phenotypes alter the population demography, which in turn leads phenotypes to respond under a different environmental pressure.

Synthesis and future directions

In chapter 1, we have shown that dwarfism in Doñana National Park is mainly determined by ecological factors in the absence of genetic isolation. A next step would be to conduct a common garden experiment, to explore whether size differences across populations would persist if raised in the opposite environment, or would be cancelled out eventually. Further, once genomic resources become available for this species, analyses on the genome level will

aid in determining if there is an adaptive component to this steep local reduction in body size that went undetected by neutral markers.

In chapter 2, we have experimentally demonstrated the different phenotypic reaction norms of the ancestral and canalized genotype of *P. cultripes* originating from two different localities that differed in climatic and hydroperiod regimes. Our differential gene expression analysis did not reflect the pattern observed in the phenotypic reaction norms, suggesting that the genes of the canalized group still retain environmental sensitivity while they lost responsiveness. Employing weighted gene co-expression network analysis (WGCNA) may identify the frequency of key genes regulating this system.

In chapter 3, we have shown that environmental stress experienced in the larval phase of spadefoot toads carryovers to determine the dorsal pigmentation pattern upon metamorphosis. This finding gives rise to a vast field of research for the future: whether changes in pigmentation pattern are ontogenetic, whether the texture and complexity of the pattern are heritable, and if so, what are the relative importance of parental effects, and whether it shows geographic variation or phylogenetic signals are important questions that remain to be addressed.

In chapter 4, we have shown that the waterflea *D. magna* responds plastically to predator presence and these predator-induced shifts in life history traits impact their population demography. A future task to be tackled is to implement an integral projection modeling (IPM) approach to allow documenting the population level consequences of predator induced life history shifts and its underlying genetic variation.

Conclusions

1. Amphibians in Doñana National Park (southwestern Spain) are remarkably small. In particular, natterjack toads (*Epidalea calamita*) experience an up to 2.1-fold difference in body mass in as little as 37 km and in the absence of apparent geographical barriers.

2. Local dwarfism in these populations occurs in the absence of genetic isolation and with similar age structure and effective population sizes. Dwarf populations, however, are exposed to drier and warmer climatic conditions, have different trophic status congruent with a more oligotrophic environment, show lower mass-adjusted metabolic rate, and their males call with a higher dominant frequency. Size differences seem to be environmentally induced with no indication of genetic differentiation, but are tightly linked to the toads' physiology and ecology.

3. Spadefoot toad larvae are capable of accelerating their development and metamorphose earlier to escape pond drying when exposed to low water conditions, which is followed by morphometric consequences.

4. Individuals from central Spain did not accelerate their development as sharply as the individuals from southern Spain in response to low water. However, individuals of central Spain underwent changes in morphology when exposed to low water treatment (shorter snout and hindlimb length upon metamorphosis) while the highly plastic southern populations did not suffer such alterations.

5. Our differential gene expression analysis identified a high number of DEG count in the Llano (southern Spain) population while the rest of the populations had a similar DEG count. This suggests that the genes of the canalized group of

populations still retain environmental sensitivity while the responsiveness of the trait is lost.

6. Toads that experienced low water conditions and accelerated their larval development showed a less contrasted and more homogenous pigmentation pattern, as characterized by low fractal dimensions and high angular second moment values. In comparison to control toads, they metamorphosed with darker blotches that were connected amongst each other, while toads that experienced benign conditions metamorphosed with lighter, segregated blotches.

8. The waterflea *Daphnia magna* shifts its life history traits when induced by predator presence: it grows faster, matures at a smaller size, produces larger sizes of egg clutches, while the offspring hatch at a smaller size. Such predator-induced defenses lead into fast increment of population density. When carrying capacity is already reached, *D. magna* individuals slow down their growth and produce smaller egg clutches. Phenotypic plasticity and population density are highly intertwined in this species and maintaining equilibrium across the two is crucial to enhance survival.

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I shall end by quoting the ending of 'Peter Camenzind' by Hermann Hesse:

„.....denn ich muss bekennen, dass Fortgang und Vollendung desselben auf schwachen Beinen stehen. Vielleicht kommt noch einmal die Zeit, daß ich von neuem beginne, fortfahre und vollende; dann hat meine Jugendsehnsucht recht gehabt, und ich bin doch ein Dichter gewesen. Das wäre mir soviel oder mehr als der Gemeinderat und als die Steindämme wert. Das Vergangene und doch Unverlorene meines Lebens aber, samt allen den lieben Menschenbildern, von der schlanken Rösi Girtanner bis auf den armen Boppi, wöge es mir nicht auf."

My translation & interpretations:

„.....I must acknowledge, that 'leaving' and 'finishing' both stand on weak legs (*strength lies in continuing to strive*). Perhaps there will come another time, in which I start from the beginning, continue, and finish; then my passion of my youth had been right, and I indeed have been a poet (*a scientist*). That would be as much as, or more worthy to me than the municipal council and the stone dams (*everything that matters to me*). However, it could never make up for the bygone things of my life that are not yet lost, including all the lovely human beings, from the thin Rösi Girtanner to the poor Boppi, (*everyone mentioned in this text*) I have met on my path."