RH: Causes of amphibian dwarfism

Dwarfism in close continental amphibian populations despite lack of genetic isolation

Lee Hyeun-Ji¹, Juli Broggi^{1,2}, Gregorio Sánchez-Montes^{1,3}, Carmen Díaz-Paniagua¹ and Ivan Gomez-Mestre^{1*}

1. Ecology, Evolution, and Development Group, Department of Wetland Ecology, Doñana Biological Station, Consejo Superior de Investigaciones Científicas, 41092 Seville, Spain

2. Department of Biology, Section of Evolutionary Ecology, University of Lund, S-223 62 Lund, Sweden

3. Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas, 28006 Madrid, Spain

*Corresponding author: igmestre@ebd.csic.es

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

28 L. Hyeun-Ji was given permits to handle animals by the Andalusian state government
29 (certificate nr. EXP-000337).

30 **DATA ACCESIBILITY**

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

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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. By studying six populations divergent in body size, we have tested for genetic isolation of the dwarf populations using multilocus genotypes (16 microsatellites), and explored whether they 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. Moreover, juveniles upon metamorphosis did not show the size pattern of the populations in their adult stage. 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 studies focusing on these early life stages are called to unravel the relative roles of the environment and adaptive genetic variation on this ecological 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 following patterns according to altitude and latitude associated with temperature, photoperiod, or precipitation (Hoffmann 1978, Ashton 2002, Rodríguez et al. 2008). For instance, some taxonomic groups increase in size with latitude (Bergmann's rule), as in colder climates selection 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 far from universal since ectotherms do not necessarily 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

of predators or competitors, or resource limitation (Lomolino 2005), island populations can evolve gigantic or dwarf versions of their mainland counterparts (Keogh et al. 2005). A general pattern is island dwarfism in large species driven by resource limitation and island gigantism in small species 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 depending on the size of the prey items of each of the islands they inhabited (Keogh et al. 2005). Remarkably, snakes residing on islands with prey items of similar size than in the mainland also were similar in size to their mainland conspecifics, stressing the importance of ecological resources as main factor shaping the body size of this system (Keogh et al. 2005). Island dwarfism and gigantism not only implicate mere size differences but are also related to alterations in gonadal maturation rate, size at sexual maturation, and degree of sexual display (Raia et al. 2010).

Large size is considered beneficial in general (Roff 1992, Andersson 1994), it has been shown to be positively correlated with fitness increase in various empirical studies (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 (Kingsolver and Pfennig 2004). Moreover, large size may come at the expense of long developmental periods, accelerated growth, reduced mobility, and heat stress (Blanckenhorn 2000, Teuschl et al. 2007). Indeed, optimal body size has been shown to be limited by resource availability, foraging capacity, and food-processing capacity (Lundberg and Persson 1993). For instance, snakes have been shown to converge to an optimal body size when inhabiting islands (Boback et al. 2003). Further, body size is largely heritable, and h^2 values of body size heritability between 0.58 and 0.69 have been

documented in flycatchers (Merilä 1997), as well as for a wide array of phenotypic traits across diverse taxa (Ritland and Ritland 1996, DiBattista et al. 2009). Hence, it can be inferred that intraspecific size variation is highly influenced by the environment as well as heritable (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, continental systems lacking major geographical barriers require large (> 1000 km) geographical distances, or steep altitudinal gradients to find differences in body size (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). 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 (Doñana National Park), however, we have found an up to 35 % decrease in body size among continental populations over a geographical distance of 60 km in the absence of an efficient geographical barrier. These include amphibians like 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). No evident geographical barrier separates Doñana National Park from

neighbouring populations, making this remarkable phenomenon a unique study-system to understand factors contributing to sharp differentiation in body size.

We focused on the local dwarfism of continental populations of natterjack toads *Epidalea calamita* (Laurenti 1768) without apparent geographical barriers preventing contact among neighbouring populations (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 of the two above stated factors. Hence, small size might either be selected for or be a side effect of the different environmental conditions of the localities. We applied a multifaceted approach aimed at explaining the sharp body size differentiation among six *E. calamita* populations and the potential causes and reasons explaining its persistence. 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.

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 2017). Natterjacks vary substantially in size across their extensive distribution range (ca. 38% over > 3,300 km), while 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 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 but rather sharp cline in body 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 km². 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 calcareic cambisol and calcareic 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 H₂O, 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 coefficient 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 *adeget* (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 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ determination. All samples were combusted at 1,020 °C 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 ($\delta^{13}\text{C}$) and atmospheric N₂ ($\delta^{15}\text{N}$). Replicate assays of standards routinely inserted within the sampling sequence indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, 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 *E. calamita* actively calling (thus sexually mature) *E. calamita* males 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 individuals were not the identical individuals that we took samples from for the

genetics analyses. 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 °C. 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 CO₂-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 (mLO₂/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 (btw 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 while undergoing metamorphosis and hence have not been feed before measurement. Upon tail resorption, toadlets were weighed on an electronic scale and 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 H₂O, heated up to 60 °C 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 °C. 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 °C 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 μ L containing 10 μ L of LightCycler 480 SYBR Green I Master (Roche Molecular Systems, Inc., USA) and 1 μ L of DNA at 20 ng/ μ L 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 μ L) 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 $\pm$ 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 for approx. 10 call recordings of 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, since in nature anurans initiate mating with the male whose

call they find most attractive by taking such measures (Gerhardt and Huber 2002).

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 Hothorn 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 $\delta^{13}\text{C}$ data, we first normalized the values in order to control for variation in lipid content in the samples using the equation $\delta^{13}\text{C}_{\text{normalized}} = \delta^{13}\text{C}_{\text{untreated}} - 1.11 + 0.37\text{C:N}$, 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).

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; 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).

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).

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 0.00305 g in body mass and 1.57 mm in SVL, corresponding to 35 % and 14.84 % of the maximum observed values of body mass and SVL, respectively. However, those size differences did not mirror patterns of variation in adult body size as one of the dwarf 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).

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 an amphibian species 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. Hence, the dwarf Doñana populations are the only populations that are up to a 35.6 % smaller in body size than populations merely 60 km away. We have found that i) there is no evidence for 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 inbreeding, in concordance with values observed in other Iberian populations (Sánchez-Montes et al. 2017a), and iv) effective population sizes at both sites in Doñana are comparable to those of large-bodied populations. The above conclusions are in accordance with previous genetic analyses of natterjack populations (Gomez-Mestre and Tejedo 2004; Rowe et al. 2006), 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 higher neutral differentiation than the rest, which could be an indicator of some adaptive differentiation in this locality. However, this pattern was not consistent in the other dwarf population (Navazo del Toro). Moreover, the observed differentiation was so meagre that Bayesian clustering methods failed in detecting any evidence of genetic structure (Figure S5 in Supplementary Material).

Furthermore, our results showed that most of the differentiation was driven by the loci departing from HWP (see Table 1 for F_{IS} and Table 2 for G_{ST} , Jost's D). Future genomic studies may be able to shed light on the adaptive genetic differentiation of the dwarf populations. At this point however, we are limited to conclude that the size difference was not explained by the genetic differentiation observed across the populations.

Dwarfism in natterjack toads from Doñana is likely a plastic response to environmental factors, with no evidence to date of it being adaptive. Reduced body size in the Doñana populations results among other things 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. These results are limited because we did not test whether preference for small males calling with higher dominant frequencies in the Doñana females is driving the size difference.

We found substantial differences across localities in key climatic and habitat variables, even at such small spatial scale. 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 *E. calamita* populations from Doñana did not show reduced size at metamorphosis compared to the other study localities. Nevertheless, the inverse relationship between ambient 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

513 taxa (Sheridan and Bickford 2011, Caruso et al. 2015). The Doñana population is exposed to
514 a warmer and drier climate than the other populations (Figure 2a,b), which in combination
515 with sandy soil with poor water retention, cause severe droughts over extended periods of
516 time. This undoubtedly affects the growth of post-metamorphic juveniles, since amphibians
517 have permeable skin, and thus dry substrates induce juveniles to increase the time spent
518 buried, compromise their ability to capture prey, and constrains their growth (Gomez-Mestre
519 and Tejedo 2005). Growth over the juvenile period in amphibians, and natterjack toads in
520 particular, is critical and largely determines the differences in adult size (Sinsch et al. 2010).
521 Hence, hampered juvenile growth due to drier conditions in Doñana, and not size at
522 metamorphosis *per se*, seems to be a strong determinant of reduction in their adult size.

523 Throughout its range, delayed age at maturity usually correlates with larger body size
524 in natterjack toads (Sinsch et al 2010), and delayed maturation is compensated with extended
525 lifetime fecundity due to increased longevity (Leskovar et al. 2006, Oromi et al. 2012).
526 Within our study area, growth patterns were similar across populations, except that the dwarf
527 Doñana populations showed a downward shift in their curves, as they matured at a smaller
528 size than the rest of the populations (Figure 4a). We initially hypothesized that toads from
529 dwarf populations could be maturing at an earlier age and reducing their growth afterwards.
530 One of the dwarf populations indeed had its age structure shifted towards younger individuals
531 (El Puntal; Figure 4b). However, the other dwarf population (Navazo del Toro) did not,
532 possibly due to the lack of young individuals, because scarce rainfall in the previous year
533 limited availability of breeding sites, and hence reproduction of this population. Meanwhile,
534 one of the Sierra populations also had an age structure biased towards younger individuals
535 and yet their average size was normal (Figure 4b). Hence, since both the size at
536 metamorphosis and the age at sexual maturity are not determinants of adult body size and
537 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.

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 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, 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 $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ is parallel to the pattern we observed for SMR (Figure 3a,b). Lower $\delta^{15}\text{N}$ in Doñana than in the northern populations can therefore also be partly due to higher SMR causing $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ 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 limited juvenile growth is the main reason for local dwarfism. Consistently, size at metamorphosis of individuals that metamorphosed in the laboratory do not follow the same pattern as in mature adults, stressing that it is indeed the growth during the terrestrial phase that determines the size pattern. 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. We can therefore show that size reduction in this system is environmentally driven and associated with reduced metabolic rate and lower nitrogen isotopic values. Once the genome of the species is sequenced, future studies will be able to address whether dwarfism in Doñana is a by-product of the environment or an adaptation to the local environment.

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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 proportions (HWP, out of 16 markers), and effective population size estimates with 95% CI (N_e). Estimates of F_{IS} are shown both including and excluding loci out of HWP.

Population	N	AR	H_O	H_E	F_{IS} (all loci)	F_{IS} (excluding loci out of HWP)	HWP	N_e
Navazo del Toro	27	10.36	0.70	0.83	0.1543 (0.110-0.195)	0.040 (-0.024-0.094)	4	117 (69-253)
El Puntal	33	10.58	0.73	0.82	0.1162 (0.074-0.154)	0.055 (0.006-0.105)	4	81 (52-144)
Aznalcazar	30	10.14	0.71	0.81	0.1274 (0.092-0.161)	0.085 (0.033-0.129)	6	193 (103-730)
Gerena	30	10.12	0.71	0.82	0.1240 (0.067-0.172)	0.084 (0.020-0.146)	4	73 (47-133)
El Pedroso	31	10.08	0.71	0.82	0.1258 (0.091-0.161)	0.072 (0.005-0.138)	4	124 (76-254)
Constantina	29	9.99	0.73	0.82	0.1001 (0.056-0.139)	0.065 (-0.007-0.134)	4	81 (46-146)

Table 2. Genetic differentiation (without 7 loci out of HWP)

Square matrix showing pairwise genetic differentiation among the six populations after excluding 7 loci out of HWP. Upper triangular: Hedrick's G_{ST} , lower triangular: Jost's D, with 1000 bootstrapped 95% confidence intervals (95% CI). Estimates significantly different from zero (i.e., with 95% CI not including zero) are marked in bold.

	Navazo del Toro	El Puntal	Aznalcazar	Gerena	El Pedroso	Constantina
Navazo del Toro	-	0.006 (0-0.064)	0.038 (0-0.102)	0.059 (0-0.143)	0.031 (0-0.099)	0.041 (0-0.105)
El Puntal	0 (0-0.054)	-	0.065 (0.005-0.136)	0.079 (0.013-0.155)	0.051 (0-0.118)	0.068 (0.014-0.132)
Aznalcazar	0.017 (0-0.070)	0.032 (0-0.094)	-	0.018 (0-0.092)	0.031 (0-0.081)	0.036 (0-0.095)
Gerena	0.026 (0-0.090)	0.028 (0-0.090)	0.005 (0-0.063)	-	0.051 (0-0.127)	0.030 (0-0.106)
El Pedroso	0.007 (0-0.067)	0.007 (0-0.064)	0.011 (0-0.055)	0.021 (0-0.081)	-	0.012 (0-0.064)
Constantina	0.006 (0-0.059)	0.029 (0-0.080)	0.014 (0-0.063)	0.011 (0-0.069)	0.001 (0-0.044)	-

Figure legends

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.

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.

Figure 3. (a) Natterjack toad populations varied in their isotopic signature roughly following a geographical pattern of increased $\delta^{15}\text{N}$ and decreased $\delta^{13}\text{C}$ 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 $\pm$ 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 ($N=30\pm 3$ per population). Doñana individuals are between 20 and 30% lighter than those from the other populations. Whiskers indicate $\pm$ standard deviations (c, d)

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.

Supplementary material

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: 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}
<i>Ecal4.6</i>	27	9	90	8.05	0.59	0.83	0.144	0.288	0.068	0.515
<i>Ecal4.14</i>	26	11	78.57	9.99	0.69	0.88	0.069	0.213	0.02	0.417
<i>Ecal3.29</i>	27	6	75	5.17	0.22	0.38	0.054	0.422	-0.052	0.804
<i>Ecal4.2</i>	27	17	35.42	13.87	0.48	0.91	<0.001	0.468	0.258	0.675
<i>Ecal3.19</i>	21	8	72.73	7.2	0.38	0.84	0.002	0.545	0.282	0.793
<i>Ecal4.26</i>	27	26	48.15	19.98	0.74	0.94	<0.001	0.214	0.049	0.398
<i>Ecal4.29</i>	27	12	70.59	10.86	0.96	0.88	0.889	- 0.093	-0.176	0.017
<i>Ecal4.3</i>	25	12	75	10.98	0.96	0.89	1	-0.08	-0.147	0.011
<i>Ecal4.16</i>	27	5	100	4.63	0.63	0.69	0.424	0.093	-0.183	0.359
<i>Ecal3.26</i>	27	15	44.12	12.58	0.52	0.88	<0.001	0.408	0.203	0.617
<i>Ecal4.8</i>	24	17	47.22	13.47	0.71	0.89	<0.001	0.202	0.001	0.418
<i>Ecal4.21</i>	26	9	75	7.97	0.62	0.82	0.227	0.248	0.037	0.472
<i>Ecal3.4</i>	27	7	50	6.73	0.89	0.76	1	- 0.166	-0.317	0.002
<i>Ecal4.20</i>	27	19	79.17	15.82	0.96	0.92	0.956	-0.05	-0.105	0.032
<i>Ecal4.18</i>	26	9	81.82	8.43	0.88	0.83	1	- 0.068	-0.216	0.097
<i>Ecal4.24</i>	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}
<i>Ecal4.6</i>	33	8	80	7.37	0.73	0.83	0.075	0.129	-0.046	0.314

<i>Ecal4.14</i>	33	10	71.43	9.25	0.79	0.87	0.408	0.094	-0.054	0.255
<i>Ecal3.29</i>	33	6	75	5.26	0.33	0.39	0.455	0.144	-0.093	0.408
<i>Ecal4.2</i>	33	23	47.92	17.06	0.7	0.93	<0.001	0.251	0.084	0.423
<i>Ecal3.19</i>	22	9	81.82	8	0.32	0.86	0.005	0.629	0.387	0.851
<i>Ecal4.26</i>	29	25	46.3	18.98	0.97	0.94	<0.001	- 0.025	-0.08	0.053
<i>Ecal4.29</i>	33	12	70.59	10.83	0.88	0.89	0.999	0.008	-0.109	0.147
<i>Ecal4.3</i>	32	12	75	10.57	0.88	0.86	0.998	-0.02	-0.144	0.125
<i>Ecal4.16</i>	33	4	80	3.98	0.48	0.66	0.014	0.265	-0.013	0.531
<i>Ecal3.26</i>	33	19	55.88	14.66	0.7	0.89	<0.001	0.22	0.056	0.39
<i>Ecal4.8</i>	31	22	61.11	16.43	0.9	0.91	<0.001	0.008	-0.104	0.132
<i>Ecal4.21</i>	31	9	75	7.35	0.61	0.73	0.185	0.159	-0.04	0.368
<i>Ecal3.4</i>	33	8	57.14	7.47	0.88	0.81	0.698	- 0.084	-0.21	0.067
<i>Ecal4.20</i>	33	16	66.67	12.68	0.76	0.88	0.062	0.134	-0.004	0.289
<i>Ecal4.18</i>	33	9	81.82	8.6	0.79	0.85	1	0.069	-0.087	0.243
<i>Ecal4.24</i>	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
<i>Ecal4.6</i>	30	7	70	6.46	0.5	0.76	0.015	0.344	0.092	0.595
<i>Ecal4.14</i>	27	12	85.71	9.92	0.56	0.87	<0.001	0.364	0.155	0.578
<i>Ecal3.29</i>	29	7	87.5	5.68	0.48	0.55	0.156	0.127	-0.116	0.396
<i>Ecal4.2</i>	30	27	56.25	19.66	0.9	0.91	<0.001	0.014	-0.091	0.136
<i>Ecal3.19</i>	27	7	63.64	6.46	0.52	0.81	0.086	0.363	0.124	0.598
<i>Ecal4.26</i>	29	24	44.44	17.92	0.72	0.93	<0.001	0.217	0.052	0.393
<i>Ecal4.29</i>	30	13	76.47	11.21	0.93	0.87	0.668	-0.07	-0.17	0.041
<i>Ecal4.3</i>	30	13	81.25	11.2	0.93	0.85	0.999	- 0.094	-0.183	0.013
<i>Ecal4.16</i>	30	4	80	3.83	0.5	0.58	0.958	0.135	-0.122	0.4
<i>Ecal3.26</i>	30	15	44.12	12.22	0.7	0.87	<0.001	0.196	0.029	0.375
<i>Ecal4.8</i>	28	16	44.44	12.31	0.68	0.86	<0.001	0.208	0.024	0.406
<i>Ecal4.21</i>	30	10	83.33	7.86	0.43	0.79	<0.001	0.449	0.236	0.661
<i>Ecal3.4</i>	30	8	57.14	7.33	0.77	0.77	0.964	0.005	-0.158	0.184
<i>Ecal4.20</i>	30	15	62.5	13.05	1	0.9	0.721	- 0.116	-0.155	-0.087
<i>Ecal4.18</i>	30	9	81.82	7.97	0.83	0.84	0.929	0.005	-0.149	0.178
<i>Ecal4.24</i>	30	10	66.67	9.23	0.9	0.86	0.997	-	-0.173	0.091

0.051

Gerena

	N	A	%	AR	H_O	H_E	HWE	F_{IS}	F_{IS_Low}	F_{IS_High}
<i>Ecal4.6</i>	30	8	80	7.17	0.67	0.78	0.415	0.148	-0.072	0.37
<i>Ecal4.14</i>	29	11	78.57	10.08	0.69	0.84	0.105	0.183	0.013	0.37
<i>Ecal3.29</i>	30	7	87.5	6.28	0.5	0.68	0.127	0.266	-0.009	0.532
<i>Ecal4.2</i>	30	19	39.58	14.98	0.87	0.9	<0.001	0.036	-0.071	0.158
<i>Ecal3.19</i>	27	11	100	9.38	0.56	0.86	0.007	0.355	0.13	0.577
<i>Ecal4.26</i>	29	28	51.85	20.58	0.83	0.95	<0.001	0.125	-0.008	0.273
<i>Ecal4.29</i>	30	11	64.71	9.92	0.83	0.86	0.083	0.036	-0.103	0.187
<i>Ecal4.3</i>	29	13	81.25	11.22	0.86	0.84	0.953	- 0.021	-0.149	0.123
<i>Ecal4.16</i>	30	4	80	3.83	0.4	0.66	0.001	0.392	0.085	0.667
<i>Ecal3.26</i>	28	20	58.82	15.2	0.57	0.91	<0.001	0.375	0.188	0.566
<i>Ecal4.8</i>	30	13	36.11	10.34	0.6	0.68	0.057	0.112	-0.086	0.323
<i>Ecal4.21</i>	29	9	75	7.82	0.59	0.78	0.16	0.253	0.032	0.478
<i>Ecal3.4</i>	30	6	42.86	5.23	0.8	0.71	0.532	-0.13	-0.303	0.066
<i>Ecal4.20</i>	30	13	54.17	11.3	0.87	0.86	<0.001	- 0.003	-0.117	0.129
<i>Ecal4.18</i>	30	9	81.82	8.17	0.93	0.85	0.802	- 0.093	-0.184	0.021
<i>Ecal4.24</i>	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}
<i>Ecal4.6</i>	31	7	70	6.78	0.45	0.79	0.031	0.431	0.206	0.651
<i>Ecal4.14</i>	30	8	57.14	7.33	0.43	0.81	0.001	0.464	0.228	0.689
<i>Ecal3.29</i>	30	6	75	5.12	0.37	0.49	0.294	0.258	-0.07	0.585
<i>Ecal4.2</i>	31	23	47.92	18.23	0.84	0.93	0.427	0.093	-0.024	0.228
<i>Ecal3.19</i>	26	9	81.82	7.66	0.35	0.82	<0.001	0.579	0.342	0.8
<i>Ecal4.26</i>	26	26	48.15	18.43	0.92	0.93	<0.001	0.008	-0.092	0.132
<i>Ecal4.29</i>	31	10	58.82	8.56	0.74	0.84	0.003	0.118	-0.058	0.305
<i>Ecal4.3</i>	31	13	81.25	11.15	0.87	0.86	0.351	- 0.017	-0.13	0.112
<i>Ecal4.16</i>	31	4	80	3.93	0.48	0.57	0.5	0.146	-0.127	0.428

<i>Ecal3.26</i>	31	16	47.06	13.87	0.84	0.92	1	0.088	-0.051	0.237
<i>Ecal4.8</i>	31	16	44.44	13.08	0.74	0.87	<0.001	0.147	-0.03	0.335
<i>Ecal4.21</i>	31	10	83.33	8.49	0.77	0.84	0.249	0.073	-0.096	0.259
<i>Ecal3.4</i>	31	9	64.29	7.46	0.84	0.79	0.001	-0.065	-0.22	0.106
<i>Ecal4.20</i>	31	17	70.83	13.84	0.9	0.9	<0.001	-0.008	-0.128	0.125
<i>Ecal4.18</i>	31	8	72.73	7.98	0.87	0.87	1	-0.006	-0.132	0.132
<i>Ecal4.24</i>	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}
<i>Ecal4.6</i>	29	8	80	7.69	0.66	0.77	0.67	0.145	-0.066	0.373
<i>Ecal4.14</i>	28	10	71.43	9.28	0.64	0.87	0.958	0.259	0.064	0.471
<i>Ecal3.29</i>	29	5	62.5	4.81	0.48	0.57	0.133	0.158	-0.15	0.463
<i>Ecal4.2</i>	29	21	43.75	16.79	0.83	0.93	<0.001	0.109	-0.027	0.26
<i>Ecal3.19</i>	23	10	90.91	8.58	0.48	0.85	0.013	0.435	0.191	0.678
<i>Ecal4.26</i>	26	23	42.59	17.43	0.77	0.93	<0.001	0.171	-0.002	0.357
<i>Ecal4.29</i>	29	8	47.06	7.41	0.76	0.82	0.973	0.069	-0.117	0.266
<i>Ecal4.3</i>	28	10	62.5	9.63	0.79	0.85	1	0.079	-0.079	0.26
<i>Ecal4.16</i>	29	5	100	4.5	0.45	0.6	0.115	0.252	-0.024	0.537
<i>Ecal3.26</i>	27	14	41.18	11.49	0.85	0.85	<0.001	-0.004	-0.148	0.16
<i>Ecal4.8</i>	29	17	47.22	13.68	0.83	0.85	0.005	0.029	-0.115	0.187
<i>Ecal4.21</i>	29	7	58.33	6.46	0.62	0.74	0.561	0.166	-0.056	0.389
<i>Ecal3.4</i>	29	8	57.14	7.12	0.72	0.78	0.081	0.077	-0.112	0.277
<i>Ecal4.20</i>	29	21	87.5	16.85	0.93	0.92	<0.001	-0.01	-0.094	0.1
<i>Ecal4.18</i>	29	8	72.73	7.44	1	0.83	0.2	-0.203	-0.259	-0.167
<i>Ecal4.24</i>	29	12	80	10.68	0.93	0.88	0.437	-0.057	-0.147	0.057

S5. Genetic differentiation (showing all 16 loci)

Square matrix showing pairwise genetic differentiation among the six populations using all 16 loci. Upper triangular: Hedrick's G_{ST} , lower triangular: Jost's D, with 1000 bootstrapped 95% confidence intervals (95% CI). Estimates significantly different from zero (i.e., with 95% CI not including zero) are marked in bold.

	Navazo del Toro	El Puntal	Aznalcazar	Gerena	El Pedroso	Constantina
Navazo del Toro		0.031 (0-0.089)	0.066 (0.013- 0.126)	0.097 (0.038- 0.173)	0.057 (0.005- 0.124)	0.044 (0-0.110)
El Puntal	0.006 (0-0.058)		0.082 (0.028- 0.148)	0.130 (0.073- 0.195)	0.081 (0.029- 0.148)	0.108 (0.052- 0.170)
Aznalcazar	0.039 (0-0.102)	0.058 (0.007- 0.118)		0.072 (0.017- 0.141)	0.069 (0.021- 0.132)	0.066 (0.015- 0.127)
Gerena	0.052 (0.002- 0.113)	0.069 (0.019- 0.132)	0.036 (0-0.095)		0.077 (0.024- 0.148)	0.076 (0.020- 0.149)
El Pedroso	0.022 (0-0.084)	0.044 (0-0.101)	0.041 (0-0.098)	0.044 (0-0.106)		0.048 (0-0.104)
Constantina	0.011 (0-0.070)	0.068 (0.018- 0.122)	0.036 (0-0.088)	0.045 (0-0.109)	0.023 (0-0.074)	

S6: Bayesian clustering analysis results, showing that there is no genetic clustering among populations regardless of K

Major modes for the uploaded data:

K=1

K=2

K=3

K=4

K=5

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

S7: Snout-to-Vent length and body mass of metamorphs

Population	SVL (mm)	Body mass (g)
Constantina	9.01	0.00564
El Puntal	9.62	0.00673
El Pedroso	9.73	0.00784
Gerena	9.99	0.00893
Aznalcazar	10.12	0.00817
Navazo del Toro	10.58	0.00870

S8: 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.

S9: 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)