Original Article

Sex-dependent implications of primary productivity and conspecific density on geographic body size variation in a newt: disentangling local, large scale and genetic factors

Miguel Peñalver-Alcázar¹, Iñigo Martínez-Solano^{2,3,4}, Fernando Sequeira², Pedro Aragón¹

1. Department of Biogeography and Global Change, Museo Nacional de Ciencias Naturales, CSIC (MNCN-CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain.

2. CIBIO-InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, Universidade do Porto, 4485-661 Vairão, Portugal.

3. Ecology, Evolution and Development Group, Department of Wetland Ecology, Estación Biológica de Doñana (EBD-CSIC), Avenida Américo Vespucio, s/n, 41092 Sevilla, Spain.

4. Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales, CSIC (MNCN-CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain

Correspondence: Miguel Peñalver-Alcázar, Department of Biogeography and Global Change, Museo Nacional de Ciencias Naturales, CSIC (MNCN-CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain. E-mail: miguel.penalver@mncn.csic.es

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ABSTRACT

Aim

Geographic body size variation has interested biologists since the 19th century. However, there is no consensus regarding the mechanisms involved, especially in amphibians. At the intra-specific level, different approximations and hypotheses have addressed this problem, but no study has integrated information at different spatio-temporal ecological and (phylo)genetic scales while considering differences between sexes. We investigated the mechanisms involved in geographic body size variation in the Iberian Newt (*Lissotriton boscai*), accounting for the main hypotheses potentially explaining variation in this phenotypic trait in amphibians.

Location

Iberian Peninsula.

Methods

We used a novel multivariate technique (partial least squares regression) that accounts for interdependence among variables while allowing for the inclusion of multiple local and macroscale predictors. We considered intra-specific genetic differentiation including information from the mitochondrial and nuclear genomes in populations representative of the species' distribution. We addressed causal hypotheses of heat conservation, temperature-related development, water and food availability, seasonality, competition and predation.

Results

Annual primary productivity and intra-specific competition were of greatest relevance to explain body size variation in females, while seasonality of primary productivity was more important in males. Differences between sexes are interpreted based on previous experiments on the behavioural ecology of this species.

51 **Main conclusions**

52 This study shows that sex-dependent implications of competition for food drive
53 geographic body size variation in an urodele amphibian, supporting the density-resource
54 hypothesis. Our results suggest that food availability, conspecific density and
55 competition are important in explaining intra-specific body size variation in amphibians.
56 This study highlights how selecting model species with an experimental background
57 can help interpret geographic variation in phenotypic traits. We emphasize the value of
58 simultaneously considering alternative hypotheses of phenotypic variation at different
59 ecological and (phylo)genetic spatio-temporal scales in revealing potentially hidden
60 relationships.

61

62 Keywords: Amphibians, body size variation, ectotherm, genetic variation, intra-specific
63 competition, local scale, macroscale, partial least squares analysis, primary productivity

64

65 INTRODUCTION

66 Body size is a functional trait strongly linked with species ecology and evolution (Roff,
 67 1992) and its geographic variation has interested biologists since the seminal work of
 68 Karl Bergmann (Bergmann, 1847). Bergmann's rule states that body size increases at
 69 higher latitudes or altitudes (where temperatures are cooler), as an adaptive mechanism
 70 of heat conservation. It was originally proposed at the inter-specific level and extended
 71 later to the intra-specific level by Rensch (1938) and Mayr (1956). While several
 72 studies have shown support for Bergmann's rule (e.g. Meiri & Dayan, 2003), the
 73 generality of this pattern and its underlying mechanism(s) have been criticized in
 74 endotherms (e.g. McNab, 1971; Geist, 1987) and even more so in ectotherms.
 75 Particularly amphibians are considered a puzzling group (Ashton, 2002; Olalla-Tárraga
 76 & Rodríguez, 2007; Adams & Church, 2008). Alternative mechanistic hypotheses have
 77 been proposed to explain geographical body size variation, which consider different
 78 physiological and life history traits, including seasonality (Boyce, 1979), food
 79 availability (Meiri *et al.*, 2007; Huston & Wolverton, 2011) and competition
 80 (Desdevises *et al.*, 2003; Olson *et al.*, 2009). Additional hypotheses are taxon-specific,
 81 such as desiccation tolerance in amphibians (Ashton, 2002 but see Gouveia & Correia,
 82 2016 for a recent reformulation of this hypothesis).

83   Geographical body size variation studies face several challenges. First,
 84 environmental factors are often correlated and share spatial variation with genetic
 85 factors. Consequently, some authors have suggested performing phylogenetic and
 86 phylogeographic analyses, both at the inter-specific (Lomolino *et al.*, 2006) and intra-
 87 specific (Ashton, 2004) levels. Second, mechanistic hypotheses are not necessarily
 88 exclusive and may have synergistic or counteracting effects (Mayr, 1956; Lomolino *et*
 89 *al.*, 2006). Third, the rapid increase in the availability of digitised macroscale

environmental factors has led to a bias against local drivers of phenotypic diversification. Finally, the observational nature of these studies and the multiple non-exclusive hypotheses imply working with large multivariate datasets. Principal component analysis, classical multiple linear regressions, and less frequently, information-theoretic approaches, have been used alone or combined to deal with this (Ficetola *et al.*, 2010; Aragón & Fítze, 2014). However, these techniques have several drawbacks, including non-independence among predictors, loss of degrees of freedom or failure to maximize the variance explained in the response variable (Legendre & Legendre, 1998). These limitations are addressed using partial least squares regression (PLSR), a promising technique recently introduced in ecological studies (Mevik & Wehrens, 2007; Carrascal *et al.*, 2009).

Here we describe range-wide patterns of body size variation in an urodele amphibian, the Iberian newt (*Lissotriton boscai*), and unravel the underlying mechanisms using an integrative, multiscale approach considering historical factors. We built models for each sex, using local and macroscale predictors while accounting for population genetic structure. We selected *L. boscai* because previous studies have reconstructed its evolutionary history (Martínez-Solano *et al.*, 2006, Teixeira *et al.*, 2015), and background information exists on local factors potentially affecting body size, including water-chemistry characteristics (Ortiz-Santaliestra *et al.*, 2011) and intra-specific interactions (Aragón, 2009a). Moreover, in the context of the global amphibian declines (Collins & Crump, 2009), body size variation within species can reveal factors (natural *versus* anthropogenic) affecting population health. We sampled 23 populations representative of the species' environmental, phylogenetic and geographic variation. Body size and genetic samples were taken and the newts' environment was characterized at the micro and macroscale. We tested eight mechanistic hypotheses

potentially involved in geographic body size variation: (1) Bergmann's rule or heat conservation: larger body size allows reducing heat loss in colder environments (Bergmann, 1847). According to Olalla-Tárraga and Rodríguez (2007), in amphibians this mechanism might rely on the species thermoregulatory behaviour; (2) Water availability: larger body size favours higher desiccation tolerance due to a small surface to volume ratio (Ashton, 2002); (3) Converse water availability: larger body size is related to higher availability of water resources (Ficetola *et al.*, 2010); (4) Primary productivity: higher availability of food resources leads to an increase in body size (Huston & Wolverson, 2011); (5) Endurance: larger body size is related to higher body fat reserves in colder and highly seasonal locations, increasing survivorship during adverse conditions (Boyce, 1979; Ashton, 2002); (6) Seasonality: individuals living in populations with longer favourable activity seasons are larger than those living in populations with shorter and fluctuating activity seasons (Boyce, 1979); (7) Competition and/or predation: competition and predation cause species to deviate from their optimal body size (Damuth, 1993). Specifically, recent studies in amphibians suggest that a higher number of conspecific competitors can lead to a smaller body size (Green & Middleton, 2013) and that a higher number of inter-specific competitors and/or predators can result in smaller body size (Ousterhout *et al.*, 2015); (8) Temperature size rule: development and growth rates are lower in colder environments, leading to longer embryonic development and growth periods, which result in delayed maturity and larger adult body size (Angilletta & Dunham, 2003).

We used PLSR to assess the relationship and relative importance of environmental predictors related to these hypotheses in *L. boscai*, considering relevant (phylo)genetic predictors. This study entails important advances: 1) we applied a novel multivariate technique (PLSR) that allows including a large set of variables while

accounting for their interdependence, 2) we used multiple local and macroscale predictors to encompass several mechanistic hypotheses, and 3) we accounted for intra-specific genetic structure using molecular markers in the mitochondrial and nuclear genomes. Mitochondrial DNA sequences reveal deeper historical processes whereas nuclear microsatellites inform about more recent population events (Freeland *et al.*, 2011). To our knowledge this is the first study integrating local and macroscale abiotic and biotic factors for both sexes and accounting for deep and recent genetic structure, while explicitly dealing with multicollinearity and/or redundancy.

MATERIALS AND METHODS

Study species

The Iberian newt, *Lissotriton boscai* (Lataste 1879), is endemic to the western Iberian Peninsula, ranging from sea level to 1870 m a.s.l. (Fig. 1). Densities are greater in central and northern populations than in southern and easternmost ones. It inhabits diverse aquatic environments including shallow streams, rivers, natural ponds, cattle troughs and water springs (García-París *et al.*, 2004). Females are larger than males, and seasonal activity varies among populations and between sexes (Caetano & LeClair, 1999; Díaz-Paniagua & Mateo, 1999). At the beginning of the breeding season, males arrive to the aquatic habitat first, followed by a gradual arrival of females (Faria, 1995; Caetano & LeClair, 1999). Neither sex shows territorial behaviour. Females compete amongst themselves for food, resulting in agonistic interactions characterized by single quick and usually harmless bites. Males compete with other males during courtship, displaying agonistic behaviours including chases, pushes and bites (Faria, 1995; Aragón, 2009a, b). Inter-specific interactions with other newt species (*Triturus marmoratus* and *Triturus pygmaeus*) exist and known predators include reptiles (*Natrix*

natrrix, *Natrix maura* and *Vipera seoanei*) and amphibians (*T. marmoratus*, *Salamandra salamandra*; García-París *et al.*, 2004). Phylogeographic analyses revealed two major geographically-structured lineages, each containing three sub-clades (Martínez-Solano *et al.*, 2006; Teixeira *et al.*, 2015).

Data collection

From March to June 2013, we sampled 23 populations of the Iberian newt. Adult newts were caught in the aquatic phase and measured *in situ* for body size (snout-to-vent length; SVL). Tail tips were collected for genetic analysis, preserved in 1 ml tubes with 100% ethanol and stored at -20°C. Sampling time was recorded to calculate capture rate as a proxy for the relative population density. We obtained SVL measures for 457 individuals (255 females and 202 males) and 87 genetic samples. Sampled populations included small watering troughs, small natural and artificial ponds, shallow streams and rivers. The populations' small dimensions allowed us to cover the whole habitat multiple times to capture as many newts as possible. Vegetated margins were also screened for newts. Samples were taken from mid-late morning to late evening, as adult newts in the aquatic phase are mainly active during daylight and twilight (García-París *et al.*, 2004).

Microhabitat variables were measured at each population, including nitrate concentration (using a nitrate photometer), conductivity, pH and water temperature (using a multi-parametric device). We also took two 250 ml water samples to quantify chlorophyll a concentration, a proxy for primary productivity, kept them at -20 °C in a portable freezer and transferred them to the laboratory for spectrophotometric analyses (American Public Health Association, 1989). These parameters are biologically relevant for amphibians and standard measures of water quality and conservation status (Dodd,

2010). Water temperature is related to embryonic development, metamorphosis and growth rates in amphibians (Morrison & Hero, 2003) and may affect and inform on activity of individuals in this species (Caetano & LeClair, 1999; Díaz-Paniagua & Mateo, 1999). Higher nitrogen concentration and acidification at breeding sites have been linked to global amphibian declines (Collins & Crump, 2009). In *L. boscai*, nitrogen excess and low pH cause higher mortality and delayed growth rates in embryos and larvae, and physiological and behavioural alterations in adults (Ortiz-Santaliestra *et al.*, 2011). Once measurements and tissue samples were taken, individuals were released at their capture point.

We obtained temperature and precipitation variables with a resolution of 1 km from the WorldClim database (Hijmans *et al.*, 2005) for macroscale characterization of each population. We selected those variables related to our main hypotheses. Annual mean temperature was used to test Bergmann's and temperature-size rules; temperature and precipitation seasonality were selected to test the endurance and seasonality hypotheses; and annual precipitation was used to test the water and converse-water availability hypotheses. We calculated annual potential evapotranspiration following Thornthwaite (1948). We computed year-average monthly values of enhanced vegetation indexes (EVI) for 2012-2013 using satellite images at a 1-km resolution from MODIS (<https://modis.gsfc.nasa.gov>) as a proxy for primary productivity in sampling sites. We also calculated primary productivity seasonality as $100 * (\text{std. dev. of mean monthly EVI values} / \text{mean of the mean monthly EVI values})$. To account for potential effects of known predators and competitors of *L. boscai*, we derived predator and competitor richness from the atlases of the Asociación Herpetológica Española (www.herpetologica.com) and Societas Europaea Herpetologica (<http://www.seh-herpetology.org/>) at 1-km and 10-km resolution, respectively. Finally, we recorded the

capture date, as the day number within the year, of each population in order to measure and control for temporal autocorrelation.

Genetic sequencing and phylogenetic factor extraction

Genetic analyses aimed at: 1) verifying that sampled populations were representative of previously described haplotype clades by comparing Bayesian phylogenies from our sampling with those in Martínez-Solano *et al.* (2006) (Appendix S2 in Supporting Information), and 2) accounting for (phylo)genetic variation in final combined models by including relevant phylogenetic factors as predictors (Diniz-Filho *et al.*, 1998; Aragón & Fitze, 2014).

These analyses were performed with two different molecular markers: one mitochondrial DNA gene (*nad4*) and seven polymorphic microsatellite loci. We sequenced and genotyped samples using the protocols described in Martínez-Solano *et al.* (2006) and Sequeira *et al.* (2012), respectively (Appendices S1 and S2). We calculated a pairwise genetic distance matrix at the population level for the mitochondrial marker (Table S2.1, Appendix S2) in MEGA 5.0 using the Tamura-Nei model with gamma-distributed substitution rates among sites (Tamura *et al.*, 2011). For microsatellite markers, we estimated standard genetic diversity indices (average sample size over loci, number of alleles, allelic richness, observed heterozygosity, and expected heterozygosity) and tested for linkage disequilibrium (LD) and departures from Hardy-Weinberg equilibrium (HWE) among the loci and populations (details in Appendix S3). We also estimated genetic differentiation between all populations pairs using pairwise F_{ST} values (Weir & Cockerham, 1984) and identified values significantly > 0 through 999 permutations (Table S3.2, Appendix S3) using GENODIVE 2.0.b (Meirmans & Van Tienderen, 2004). F_{ST} estimates the genetic differentiation among populations regarding

their inherent genetic structure, which may express the degree of evolutionary independence of populations (Freeland *et al.*, 2011). Then, to account for phylogenetic inertia and inter-population genetic variability in statistical models, pairwise genetic distance matrices for mitochondrial and microsatellite markers were used to derive independent (phylo)genetic eigenvectors (PVs), following Diniz-Filho *et al.* (1998) and Aragón and Fitze (2014). We performed principal coordinate analyses (PCoA) based on a double-centred pairwise genetic distances matrix among populations to extract independent PVs for mitochondrial and microsatellites distance matrices. Similarly to principal components analysis (PCA), PCoA yields orthogonal PVs, with coefficients that are informative of variation and relationships among samples in a reduced multivariate space of (phylo)genetic relatedness. PCoA provides a set of mitochondrial and microsatellite PVs. These (phylo)genetic predictors may reflect either evolutionary relationships at different temporal scales (mtDNA distance matrix) or some degree of gene flow among spatially close populations (pairwise F_{ST} matrix).

Ecological and combined model building

We first calculated the mean, minimum and maximum SVL for each population and sex. We then performed Pearson's correlations between pairs of body size variables (mean, minimum and maximum) to test the degree of collinearity between variables and assess the suitability of performing different analyses with each variable. We found a strong and significant correlation between the three pairs of variables for both sexes (Pearson's $r > 0.83$, $P < 0.0001$ in all cases), indicating they are likely to generate similar results. Therefore, we only used mean SVL in subsequent analyses. Mean SVL is commonly used in studies on geographical body size variation of urodele amphibians at the intra-specific level (e.g. Ficetola *et al.*, 2010; Romano & Ficetola, 2010), and

265 hence results are comparable with previous findings. We also performed Pearson's
 266 correlations between mean SVL of females and males and assessed the sexual
 267 dimorphism in body size by fitting a linear mixed model with mean SVL as dependent
 268 variable, sex as a fixed factor, and population as a random factor. Finally, we used mean
 269 SVL in PLSR to disentangle potential mechanisms involved in geographical body size
 270 variation. PLSR combines features of multiple linear regression and PCA and has been
 271 used in econometrics and chemometrics and more recently in ecological studies (Wold
 272 *et al.*, 2001; Carrascal *et al.*, 2009). PLSR overcomes limitations of classical
 273 multivariate techniques, providing robust results in situations with many interrelated
 274 predictors (Mevik & Wehrens, 2007; Carrascal *et al.*, 2009). PLSR relies on the
 275 extraction of orthogonal latent-factors (PLSR components), which result from the linear
 276 combination of predictors and response variables. PLSR components are extracted
 277 iteratively and determined from the residual variation not explained by the preceding
 278 latent-factor. They account for a successively lower percentage of the original variance
 279 (explanatory capacity, R^2) of the response variable while maximising the information
 280 explained by the predictors. Most of the variance in the explanatory variables is
 281 concentrated in the first few PLSR components and less important PLSR components
 282 are discarded (e.g. via cross-validation), reducing the multidimensionality. The PLSR
 283 technique balances information in predictors and response variable, decreasing the
 284 potential effect of higher but irrelevant predictor variations. Thus, PLSR overcomes
 285 intercorrelation issues among variables by deriving orthogonal PLSR components as in
 286 PCA. However, PLSR has an advantage over PCA by including the response variable(s)
 287 in components calculation. PLSR components can be described using the predictor
 288 weights within each component, which indicate the direction and strength of the
 289 relationship between predictors and the dependent variable. The contribution of each

predictor (i.e. explained information) within each component can be assessed through their squared weights (Wold *et al.*, 2001; Carrascal *et al.*, 2009) and the variable importance for projection (VIP). Predictors with $VIP > 1$ are the most relevant for explaining the dependent variable (Wold *et al.*, 2001).

The PLSR model building was performed using mean SVL as the response variable and different sets of predictors. We followed this with two subsequent steps: 1) ecological models (only ecological predictors), and 2) combined models (ecological + PVs predictors from PCoA). Selecting and including the correct number of relevant PVs is a crucial step because too many or too few can lead to overestimation or underestimation, respectively (Desdevises *et al.*, 2003; Diniz-Filho *et al.*, 2012). Among the different existing approaches to deal with this issue, we followed the selection criterion based on the significance of linear regressions on the relationship between PVs and the residuals of the ecological models. This approach is better for selecting relevant PVs than using either the first few PVs (based on a broken-stick criterion) or the whole set of PVs derived from a PCoA (Desdevises *et al.*, 2003; Diniz-Filho *et al.*, 2012). Including only the first PVs with the highest eigenvalues (higher phylogenetic distances and thus ancient phylogenetic relationships) would neglect PVs with lower eigenvalues (lower phylogenetic distances and thus more recent phylogenetic relationships). However, if all PVs were included in the models, phylogenetic relationships would explain all variation in the studied trait, which would not be realistic for a single phenotypic trait. This method has been successfully applied at inter-specific (Diniz-Filho *et al.*, 2012) and intra-specific levels (Aragón & Fitze, 2014).

PLSR models were performed separately for females and males due to the existence of sexual size dimorphism, and because each sex can be affected differently by ecological, social and evolutionary factors (Shine, 1989), as reported in this species

(Aragón, 2009a, b, 2011). We fitted PLSR models with the orthogonal scores algorithm (NIPALS) and selected components that remained significant after a cross-validation procedure (default setting ‘CV’ in ‘pls’ package; Mevik & Wehrens, 2007), which divides the data set into 10 randomly selected subsets. The importance of PLSR components was interpreted using their R^2 , considering predictors of greater relevance when $VIP > 1$ and explained information (i.e. square of predictor weights*100) $> 10\%$ within at least one component. Interactions among predictors were not considered because it is not feasible to test them when the number of predictors is as high as in our study. Finally, we used the Akaike information criterion (AIC) (Burnham & Anderson, 2004) for model selection to corroborate the results of PLSR models (Galván & Rey Benayas, 2011), and provide results comparable to previous studies (e.g. Ficetola *et al.*, 2010; Romano & Ficetola, 2010). We ran generalised linear models (GLM) with predictors with $VIP > 1$ and fitted all possible models, and calculated their second-order AIC (AICc) and weight AIC (AICw) scores. We selected models with a $\Delta AICc < 2$ and calculated the relative variable importance of each predictor as the sum of the AICw of all selected models (Burnham & Anderson, 2004). Before model building, predictors were standardized (Quinn & Keough, 2002).

Finally, we explored the potential existence of spatial autocorrelation. Moran’s I and spatial correlograms were calculated based on the residuals of the final combined models (Appendix S1). PLSR and model selection analyses were performed in R 3.0.2 (R Core Team, 2013), using ‘pls 2.4-3’ and ‘MuMIn 1.9.11’ (Bartón, 2013), and spatial autocorrelation analyses in SAM 4.0 (Rangel *et al.*, 2010).

RESULTS

General results

We found body size variation among the 457 individuals sampled across the 23 study populations (Fig. 1). Mean SVL ranged from 34.6 to 43.3 mm in females, and from 29 to 34.3 mm in males. While there is little overlap in the size distributions between sexes, in some populations the largest males were larger than the smallest females. Females were significantly larger than males across all populations ($F_{1,21} = 257.30$, $P < 0.0001$; mean $\pm$ SE, Females: 37.4 ± 0.40 , $N = 255$; Males: 31.9 ± 0.40 , $N = 202$). Variation in the sexual dimorphism index [(mean length of larger sex/mean length of smaller sex) – 1] among populations ranged from 0.10 to 0.26. The correlation between mean SVL of females and males was significant and positive (Pearson's $r > 0.76$, $P < 0.0001$).

Phylogenetic analyses were concordant with previous studies (Martínez-Solano *et al.*, 2006, Teixeira *et al.*, 2015, Fig. S2.1, Appendix S2), confirming that sampled populations are representative of intra-specific haplotype clades. Standard genetic diversity measures for microsatellite loci are shown in Table S3.1, Appendix S3. For all populations and across all loci, there were no significant deviations from Hardy-Weinberg or linkage equilibrium after Bonferroni correction (Table S3.1, Appendix S3). While most pairwise F_{ST} comparisons were significantly different from zero, some produced non-significant results, indicating gene flow between spatially close populations (Table S3.2, Appendix S3). Therefore, the incorporation of PVs derived from the pairwise F_{ST} matrix into combined models allowed to consider potential non-independence among several populations.

Final Combined Models

PCoA analyses provided 23 and 22 PVs for males and females, respectively. For female SVL, residuals of ecological models were significantly correlated with mitochondrial

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nad4-PVs 6 and 14, and *F_{ST}*-PVs2, 4 and 22 (Pearson's $r > |0.43|$, $P < 0.05$ in all cases).

For male SVL, residuals of ecological models were significantly correlated with the *F_{ST}*-PV11 (Pearson's $r = -0.45$, $P = 0.03$). Therefore, these PVs were included in the final combined models. This procedure was not trivial since three PVs (*nad4*-PV6, *F_{ST}*-PV2 and *F_{ST}*-PV11) had a VIP > 1 or explained information $> 10\%$ in the PLSR components (Tables 1 and 2). Moran's *I* correlograms showed that there was no spatial structure in the residuals of final combined models (Fig S1.1, Appendix S1).

When accounting for genetic variation among populations in combined models of mean SVL, we found differences and similarities between males and females regarding the number of retained factors and the relationship and relevance of ecological predictors. The PLSR models provided three significant components explaining 89.6% of the original variance for females (PLRS-1: $r = 0.79$, $P < 0.0001$; PLRS-2: $r = 0.75$, $P < 0.0001$; PLRS-3: $r = 0.6$, $P < 0.05$; Table 1), and one significant component explaining 48.1% of the original variance for males (PLSR-1: $r = 0.706$, $P < 0.001$; Table. 2). The relationship of most predictors had the same direction for both sexes in PLRS-1 (14 out of 17, Tables 1 and 2), verifying the robustness of our models. The three predictors with different directions were not considered relevant according to VIP < 0.7 in all cases) and information explained in PLRS-1 ($< 2.5\%$ in all cases).

Primary productivity-related variables were relevant for both sexes (according to VIPs and explained information in at least one PLRS component, and relative variable importance in AIC models), but sex-specific difference existed. Mean annual primary productivity at the macroscale was more relevant for females (mean EVI, Table 1; Fig. 2a, c), whereas its seasonal fluctuation was more relevant for males (EVI seasonality, Table 2; Fig. 2d). Furthermore, primary productivity at the microhabitat level (chlorophyll a concentration) was slightly more relevant for females (VIP = 1.48,

explained information in PLSR-1 = 11.6%, Table 1) than for males (VIP = 1.18, explained information in PLSR-1 = 7.8%, Table 2). Similarly, annual mean temperature and potential evapotranspiration were more relevant in males than in females (Tables 1 and 2; Fig. 2), and this difference was stronger in potential evapotranspiration (females: VIP = 0.53, explained information in PLSR-1 = 7.8%; males: VIP = 1.72, explained information in PLSR-1 = 16.8%). Finally, a striking difference between sexes was that relative density was a highly relevant predictor of female SVL (VIP = 1.27, explained information in PLSR-1 = 10.24%; Fig. 2a), but not of male SVL (VIP = 0.18, explained information in PLSR-1 = 0.16%). Other predictors showed a moderate influence, since at least one of the selection criteria was not met (i.e. their VIP < 1 or explained information < 10% in PLRS, or the predictors were not selected by AIC models). These were temperature seasonality, conductivity and water temperature for females (Table 1), and chlorophyll a concentration, mean EVI, precipitation seasonality and annual precipitation for males (Table 2). The remaining predictors were less important in PLRS models for both sexes (i.e. both VIP < 1 and explained information < 10%).

Overall, individuals in populations with higher primary productivity and lower annual temperature, or smaller primary production fluctuations at the macroscale present larger body sizes. The ecological factor showing the strongest sex-bias was the local conspecific density, which was negatively associated with body size in both sexes. However, for females this predictor was among the three of greatest relevance, whereas for males it was among the three of lowest relevance (Tables 1 and 2).

DISCUSSION

The complex interdependence among ecological factors and other selective forces along the evolutionary history of species suggests that ecogeographical rules result from

different mechanisms acting individually or synergistically (Mayr, 1956; Lomolino *et al.*, 2006). Therefore, the study of patterns and processes of body size variation has led to a prolific but chaotic research field (Huston & Wolverton, 2011). Here we considered local and macroscale predictors linked to alternative non-exclusive hypotheses explaining geographical body size variation. We found similarities and differences in the response of female compared to male Iberian newts to environmental and climatic predictors, after accounting for genetic structure. The differences existed in the magnitude rather than the direction of the relationships. For females, our results suggest that conspecific density, competition interference and resource availability influence body size variation, supporting the hypothesis of density-dependent resource availability. For males, primary production seasonality was one of the most relevant predictors of body size variation. Additionally, the direction of predictors with moderate contribution points to alternative non-exclusive hypotheses, such as heat conservation, temperature-size rule or seasonality. Overall, by combining multiple hypotheses-related predictors, using PLSR and information-theoretic approaches, our study provides new insights into the complexity of geographical body size variation.

The effect of ecological, climatic and social factors may be sex dependent (Shine, 1989). Our findings show striking differences between the sexes with regard to the implications of conspecific density and food availability. Female body size was negatively and positively associated with relative density and mean primary productivity (a proxy of food availability) respectively, while only the seasonality of primary productivity exerted a negative effect on male body size. In amphibians, it is well established that density-dependent mechanisms affect growth in larval and/or adult phases (e.g. Petranksa & Sih, 1986; Denton & Beebee, 1993). Recent studies suggest that body size variation may result from reduced foraging efficiency under higher intra

440 and/or inter-specific competition interference for acquiring food resources (Green &
 441 Middleton, 2013; Ousterhout *et al.*, 2015). Green and Middleton (2013) showed that
 442 density-dependent competition interference in adult Fowler's toads led to smaller body
 443 size at the intra-specific level over a temporal scale of 23 years in one population.
 444 Ousterhout *et al.* (2015) found that density-dependent competition interference, at least
 445 at the inter-specific level, in three *Ambystoma* salamander species might reduce
 446 foraging efficiency and lead to small larval body sizes over a portion of the species
 447 ranges. Here we provide novel evidence of a sex-specific density-dependent
 448 competition effect on adult body size variation at the intra-specific level over a species'
 449 entire geographic range. Our findings at a geographical scale (encompassing the
 450 species' range) are in line with the hypothesis of density-dependent resource availability
 451 proposed by Green and Middleton (2013) at a temporal dimension for a single
 452 population. Differences between females and males may result from sex-specific
 453 foraging strategies and efficiency. Specifically, sexes in this species use information
 454 differently to obtain food. Females of *L. boscai* use the foraging behaviour of
 455 conspecific females as a cue to detect food, leading to increased competition among
 456 females foraging together. However, males use trial-error tactics to detect food
 457 (regardless of the feeding activities of conspecific males), leading to a lower degree of
 458 direct intra-specific competition (Aragón, 2009b). In agreement with this, another
 459 experiment with this species has shown that changes in the social environment
 460 uncoupled behavioural syndromes in females but not in males (Aragón, 2011).
 461 Moreover, when same-sex individuals were experimentally paired, females exhibited
 462 stronger agonistic interactions than males during feeding activities (Aragón, 2009b),
 463 whereas males only showed interference behaviour during courtship (Faria, 1995;
 464 Aragón, 2009a). Finally, seasonality of productivity at the macroscale was of greatest

relevance in explaining male size. This is consistent with adult males arriving at the aquatic habitat earlier than females (Faria, 1995; Caetano & LeClair, 1999), and hence being subjected to differences in the temporal availability of food. Precipitation seasonality (i.e. ephemeral ponds) and temperature seasonality (i.e. fluctuating thermal suitability) had similar although lesser effects.

Although chlorophyll concentration, temperature, and seasonality of temperature and precipitation differed between sexes in the magnitude of their relationship, all were associated with a small body size in both sexes (Tables 1 and 2). The negative relationship between primary productivity at the local scale (i.e. chlorophyll a concentration) and body size in females and males might be explained by local eutrophication, causing reduced growth rates (Camargo & Alonso, 2006). Interestingly, the effect of chlorophyll concentration differed in direction from PLSR-1 to PLSR-2 components in females. Since the PLSR-2 component works on the residual variation not explained by the first one, a positive effect of chlorophyll on body size (as expected from the primary productivity hypothesis) may arise after removing the negative effect of chlorophyll in the PLSR-1 component. We also found a negative relationship between temperature and body size, in line with the heat conservation hypothesis and with observations in other urodele species, where individuals from populations in colder areas exhibit larger body sizes (Ashton, 2002; Ficetola *et al.*, 2010). However, the compliance of this pattern with the heat conservation mechanism in urodeles remains questionable. For instance, according to the heat-balance hypothesis proposed by Olalla-Tárraga and Rodríguez (2007), an active thermoregulation explanation would not hold for urodeles (mostly thermoconformers). This negative relationship is also consistent with the temperature-size rule. A previous study showed that despite individuals of *L. boscai* from northern populations being older and larger, body size was

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not correlated with age in any population (Díaz-Paniagua & Mateo, 1999). Moreover, these authors found that smaller sizes can be observed in older individuals, which might be due to the influences of many other overriding factors. Therefore, although demographic structure may play a role in shaping life-history traits in amphibian populations, it is unlikely that only longevity explains differences in body size, and, indeed, previous evidence shows that ecological and climatic predictors are of greater relevance (Ficetola *et al.*, 2010; Green & Middleton, 2013).

Few geographical body size variation studies have addressed local and macroscale predictors simultaneously (e.g. Meiri *et al.*, 2007; Romano & Ficetola, 2010). Local predictors can provide information about key physiological, behavioural and ecological traits. Meiri *et al.* (2007) suggested that improving autoecological knowledge of species is crucial to obtain better understanding of patterns and processes underlying body size variation. Moreover, these authors highlighted that resource availability predictors might be better descriptors than other commonly used ecological and climatic predictors. While food availability and presence of competitors have been considered chief predictors in body size clines (McNab, 1971; Olson *et al.*, 2009), population density has rarely been considered in geographical body size variation studies. The influences of food availability, density and competition might be more important for amphibian intra and inter-specific body size variation than other ecological or climatic parameters. This could explain the contradictory body size relationships in this group (Ashton, 2002; Adams & Church, 2008) and why climatic parameters poorly predict body size in amphibians (e.g. Green & Middleton, 2013).

Body size variation in response to changing ecological or climatic conditions can be mediated through genetic and/or non-genetic factors (Aragón & Fitze, 2014; Caruso *et al.*, 2014; Ficetola *et al.*, 2016). Specifically, density-dependent body size

515 changes related to resource availability in the Iberian Newt may be (partially) explained
 516 by phenotypic plasticity. Therefore, we considered inter-population genetic structure by
 517 using two different and complementary molecular markers, which provide information
 518 at two evolutionary temporal scales. Mitochondrial DNA sequences are useful to infer
 519 past evolutionary events in *L. boscai*, reflected in geographically structured
 520 phylogenetic inertia arising from old, multiple refugia (Martínez-Solano *et al.*, 2006).
 521 Microsatellite loci are more useful to identify recent gene flow, as a result of their
 522 highly polymorphic nature and fast mutation rate (Freeland *et al.*, 2011 and references
 523 therein). Similarly, phenotypic plasticity may be responsible for rapid temporal body
 524 size variation in response to density and food availability in adult Fowler's toads (Green
 525 & Middleton, 2013). However, we cannot discard other processes, such as phylogenetic
 526 inertia or local adaptation. At least one mitochondrial phylogenetic factor was relevant
 527 in our PLSR, suggesting a partial role of phylogenetic inertia in body size variation in *L.*
 528 *boscai*. Interestingly, this mitochondrial phylogenetic inertia was expressed by a PLRS
 529 component different from the PLSR component associated to relevant ecological
 530 factors. Given that PLSR components are orthogonal, the phylogenetic inertia acting
 531 here is likely independent of contemporary ecological factors. This agrees with a
 532 previous study in a vertebrate ectotherm from the same glacial refugia, where variation
 533 partitioning revealed that mitochondrial phylogenetic inertia affected body size
 534 independently of ecological factors (Aragón & Fitze, 2014). However, the relevance of
 535 two genetic factors from microsatellites in PLSR is compatible with shorter-term
 536 evolutionary processes, and it might indirectly reflect local adaptation (Kawecki &
 537 Ebert, 2004) or a genetic-based adaptive phenotypic plasticity varying at the inter-
 538 population level (Agrawal, 2001). For instance, important larval life-history traits show
 539 adaptive genetic differentiation between amphibian populations along altitudinal or

latitudinal gradients, allowing faster larval development and growth to face seasonal constraints but often resulting in a smaller size at metamorphosis and adulthood (Laugen *et al.*, 2003; Luquet *et al.*, 2015).

To conclude, key variables associated with geographic body size variation in an urodele amphibian are conspecific density and primary productivity, supporting the density-resource availability hypothesis. These relationships were sex-specific and in line with experimental evidence of sex-dependent use of social information on conspecific feeding activities. Future experiments should consider explicitly the interaction of inter-population variation of competition with other relevant environmental parameters. More generally, we advocate for geographic phenotypic variation studies encompassing different (phylo)genetic and ecological spatio-temporal scales, with analytical designs addressing the interdependence among predictors. Finally, correlative studies will benefit from selecting model species with a well-established experimental background to make better inferences about evolutionary and ecological processes.

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740

741 **SUPPORTING INFORMATION**

742 Additional Supporting Information may be found in the online version of this article

743 **Appendix S1** Spatial autocorrelation in models

744 **Appendix S2** mtDNA marker sequencing and phylogenetic analyses

745 **Appendix S3** Microsatellites, genetic diversity and population differentiation

746

747 BIOSKETCH

748 **Miguel Peñalver-Alcázar** is interested in the study of patterns and processes along
749 geographical and environmental gradients using amphibians and reptiles as model
750 species.

751

752 Author contributions: P.A and M.P.A designed the study; M.P.A performed the field
753 sampling, collected the data and performed all the analyses; F.S. performed mtDNA
754 sequencing and microsatellite genotyping; I.M.S and F.S. guided and contributed to
755 discuss phylogeographic and microsatellite analyses. M.P.A and P.A led the writing and
756 I.M.S and F.S reviewed and commented the paper.

757 Editor: Len N. Gillman

758

Tables

Table 1. Predictor weights for selected components of partial least squares regression (wPLSR) of female mean snout-to-vent length (SVL) of Iberian newts and their variable importance for projection (VIP), and relative variable importance (Relative VarImportance) in Akaike information criterion models. R^2 is the proportion of the original variance of female mean SVL explained by each PLSR component. Bold lettering denotes predictors with explained information (square of predictor weights*100) > 10% or VIP > 1.

Predictor variable	VIP	w PLSR-1	w PLSR-2	w PLSR-3	Relative VarImportance
<i>Ecological predictors</i>					
Chlorophyll a concentration	1.48	-0.34	0.27	0.25	0.40
Mean EVI	1.35	0.32	-0.09	0.34	1
Relative density	1.27	-0.32	-0.30	0.12	1
Conductivity	1.27	-0.29	0.15	0.34	
Temperature seasonality	1.07	-0.06	0.26	-0.26	0.68
Precipitation seasonality	0.95	-0.23	0.11	0.10	
EVI seasonality	0.91	-0.23	-0.11	-0.18	
Annual precipitation	0.88	0.21	-0.12	0.30	
Annual mean temperature	0.86	-0.30	-0.03	0.13	
Water temperature	0.82	-0.35	-0.02	0.05	
Capture date	0.79	-0.11	-0.18	0.18	
Competitor richness	0.73	-0.08	0.27	-0.18	
Predator richness	0.72	-0.02	0.24	-0.17	
Altitude	0.68	0.04	0.26	-0.24	
Potential Evapotranspiration	0.53	-0.28	0.10	-0.02	
NO ₃ concentration	0.34	-0.10	0.01	0.14	
pH	0.14	-0.06	0.03	-0.02	
<i>(Phylo)genetic predictors</i>					
<i>nad4</i> -PV6	1.73	-0.01	0.39	0.36	0.09
F _{ST} -PV22	1.18	-0.27	-0.20	-0.14	0.53
F _{ST} -PV4	0.92	0.10	0.13	0.33	
<i>nad4</i> -PV14	0.76	0.16	0.17	0.16	
F _{ST} -PV2	0.60	-0.06	-0.47	0.05	
R ² by the PLSR component		0.626	0.212	0.057	

Table 2. Predictor weights for selected components of partial least squares regression (w PLSR) of male mean snout-to-vent length (SVL) of Iberian newts and their variable importance for projection (VIP), and relative variable importance (Relative VarImportance) in Akaike information criterion models. R^2 is the proportion of the original variance of male mean SVL explained by each PLSR component. Bold lettering denotes predictors with explained information (square of predictor weights*100) > 10% or VIP > 1.

Predictor variable	VIP	w PLSR-1	Relative VarImportance
<i>Ecological predictors</i>			
Potential Evapotranspiration	1.72	-0.41	0.28
Annual mean temperature	1.62	-0.38	0.60
EVI seasonality	1.41	-0.33	0.43
Chlorophyll a concentration	1.18	-0.28	
Mean EVI	1.04	0.24	0.08
Precipitation seasonality	1.03	-0.24	0.08
Annual precipitation	1.02	0.24	0.40
Capture date	0.65	0.15	
Temperature seasonality	0.65	-0.15	
pH	0.62	0.15	
NO ₃ concentration	0.59	-0.14	
Conductivity	0.57	-0.13	
Water temperature	0.54	-0.13	
Competitor richness	0.39	-0.09	
Relative density	0.18	-0.04	
Predator richness	0.10	0.02	
Altitude	0.02	0.00	
<i>(Phylo)genetic predictors</i>			
F _{ST} -PV11	1.87	-0.44	0.81
R^2 by the PLSR component		0.498	

Figure legends

Figure 1. Distribution of the 23 sampled populations (coloured circles) within the geographical range of *Lissotriton boscai* (depicted by the dashed line) in the Iberian Peninsula. Background map shows the elevation, where higher intensity in the grey scale represents higher altitude. The width of the circles is proportional to mean snout-to-vent length (SVL) values and colours represent the different clades (Martínez-Solano et al. 2006; Fig. S2.1, Appendix S2). Population names correspond to population codes in Table S3.1, Appendix S3.

Figure 2. Relationships between mean female snout-to-vent length (SVL) and the first (a), second (b) and third (c) relevant partial least squares regression (PLSR) component; and the relationship between mean male SVL and the first (d) relevant PLSR component. Predictors with explained information > 10% in each PLSR component and variable importance for projection (VIP) > 1 are shown in the x-axes. Bold lettering denotes predictors with higher relative variable importance in Akaike information criterion models (see Tables 1 and 2). Sample sizes are 23 and 22 populations for females and males, respectively, across the distributional range of *Lissotriton boscai*.