

# Proximate mechanisms determining size variability in natterjack toads

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## Keywords

amphibia; Anura; Bergmann's rule; life-history traits; age at maturity; longevity; potential reproductive lifespan; skeletochronology.

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Editor: Tim Halliday

Received 15 December 2009; revised 17 February 2010; accepted 17 February 2010

doi:10.1111/j.1469-7998.2010.00702.x

## Introduction

The body size of mammal and bird species often increases from a warm to a cool climate, that is from low to high latitudes and/or altitudes (Bergmann, 1847; Freckleton, Harvey & Pagel, 2003; Meiri & Dayan, 2003). This trend, known as Bergmann's rule, also holds for the intraspecific body size variation in the majority of endotherms (Ashton, 2002a; Meiri & Dayan, 2003). Ray (1960) first proposed that the latitudinal size variation of ectotherms follows the same trend. However, there is considerable controversy about the general presence and direction of latitudinal clines and the underlying mechanisms in amphibians at the intra- and interspecific level (Ashton, 2002b; Krizmanic, Vukov & Kalezic, 2005; Laugen *et al.*, 2005; Olalla-Tarraga & Rodriguez, 2007; Adams & Church, 2008; Cvetkovic *et al.*, 2009). *Plethodon* salamanders exemplify the case: body size was significantly related to the mean annual temperature in only 10 out of 40 species, three negatively and seven positively

## Abstract

In the toad *Bufo calamita*, among-population variation of size follows roughly a converse Bergmann cline, but populations exist that do not fit this pattern. We propose that latitudinal body size variation is a byproduct of adaptive covariation among the life-history traits juvenile growth rate, longevity and lifetime fecundity. We choose five populations (two in Andalusia, two in Catalonia and one in Rhineland-Palatinate) representing a variation of adult size from 39 mm to 95 mm snout-vent length, a latitudinal gradient from 37 to 50° and an altitudinal gradient from sea level to 420 m. Skeletochronology was used to estimate the age-related life-history traits of 313 toads and their lifetime pattern of growth. At southern latitudes, toads matured and reproduced earlier than those at northern latitudes, but had a reduced potential reproductive lifespan due to lower longevity. Age-adjusted adult size depended mainly on the size achieved between metamorphosis and first hibernation or aestivation, which in turn was influenced by local factors. We propose that first-year size corresponds to the duration of the aboveground activity period, temperature during the activity period and the type of shelter sites and hibernacula available in the habitat. After attaining sexual maturity, the growth rates did not differ among populations. Interactions of multiple environmental factors during the first year of life determine age at maturity, adult size and size variation among populations. Local body size and potential reproductive lifespan covary to optimize lifetime fecundity throughout the geographical range. The presence of a small-sized population in southern Spain does not fit the pattern predicted by a converse Bergmann cline, but is compatible with the hypothesis that body size variation among *B. calamita* populations may be the evolutionary byproduct of optimized lifetime fecundity.

(Adams & Church, 2008). Moreover, regional body size clines may be present as in the frog *Rana temporaria* (Elmberg, 1991; Miaud, Guyétant & Elmberg, 1999), but taking into account larger latitudinal gradients, a concave pattern of variation arises as the result of interactions among age effects, phenotypic plasticity and genetic determination (Laugen *et al.*, 2005). Consequently, geographical size variation in amphibian species seems to have multiple causes, which may differ from the ecological, physiological and evolutionary explanations proposed for other tetrapods (e.g. heat balance hypothesis, migration ability hypothesis) and also between the interspecific and intraspecific level (Olalla-Tarraga & Rodriguez, 2007; Adams & Church, 2008; Gaston, 2008). Comprehensive case studies of the proximate causes of body size clines represent a further step deeper in understanding their evolution in amphibians and ultimately in vertebrates (Cvetkovic *et al.*, 2009).

The body size variation of temperate zone bufonid toads is well documented for *Bufo bufo*, *Bufo calamita* and

*B. viridis* in Europe, and for *B. hemiophys* and *B. woodhousii* in northern America (Nevo, 1972; Hemelaar, 1988; Kellner & Green, 1995; Castellano & Giacoma, 1998; Eaton *et al.*, 2005; Leskovar *et al.*, 2006; Cvetkovic *et al.*, 2009). All these case studies agree in that converse latitudinal Bergmann clines covering up to 3000 km characterize intraspecific body size variation. However, there is evidence of at least two populations at the limits of latitudinal distribution range that do not fit the general clinal pattern. In male *B. bufo* living at 64°N (northern range limit), the mean body size was considerably larger than in the more southern populations of Scandinavia (Cvetkovic *et al.*, 2009). Similarly, in the southernmost *B. calamita* population of the Iberian Peninsula, the mean body size was by far smaller than that of more northern populations in Spain (Leskovar *et al.*, 2006; Marangoni, Tejedo & Gomez-Mestre, 2008).

In this paper, we explore the causes of the (near) clinal body size variation in five populations of natterjack toads *Epidalea* (= *Bufo*) *calamita* (Laurenti, 1768) in the framework of the life-history theory. Fecundity increases with size and growth rate with ambient temperature, suggesting a complex interaction among local climate and trade-offs between delayed maturity (= increased body and clutch size) and longevity (Tejedo, 1992; Leskovar *et al.*, 2006; Sinsch, 2008). The populations studied in Spain and Germany represent a variation of adult size from 39 mm to 95 mm (snout–vent length, SVL) and an age range from 2 to 9 years of life, a latitudinal gradient from 37 to 50° and an altitudinal gradient from sea level to 420 m. As skeletochronological age determination also allows for the reconstruction of individual annual bone growth pattern, we were able to estimate the contribution of ontogenetic growth variation to body size at any age (Sinsch, Oromi & Sanuy, 2007b). The aims of this study are (1) to quantify the influence of climate and elevation gradients on the age-adjusted body size variation; (2) to estimate retrospectively the relative contribution of juvenile growth to adult body size; (3) to evaluate the hypothesis that the near-clinal body size variation is a byproduct of covariation between juvenile growth rate, longevity and lifetime fecundity.

## Material and methods

### Study areas

A total of 313 reproductive natterjack toads *E. calamita* were studied at five localities, which include four habitat types, coastal sand dunes (Reserva Biologica de Doñana, Andalusia, Spain), Mediterranean mountains (Navas, Andalusia, Spain), semi-arid arable plains (Balaguer and Mas de Melons, Catalonia, Spain) and flood plains of Central European streams (Urmitz, Rhineland-Palatinate, Germany). The habitat and local climate features, collection dates and numbers of reproductive adults sampled are summarized in Table 1; detailed descriptions of the localities are given in Leskovar *et al.* (2006), Sinsch *et al.* (2007b) and Marangoni *et al.* (2008). Climate data used for modelling are the average of a 30-year period (1971–2000) and refer to the meteorological stations of Reserva Biologica de Doñana (CSIC), and Lleida-Observatorio 2, Spain and Koblenz-Horchheim, Germany.

### Sampling procedure and skeletochronological analysis

Following sunset, toads were collected at the local breeding ponds within 1–4 days of the spring reproduction period (Table 1). At the Urmitz study site, reproductive activity continues until August (Sinsch & Keltch, 2002), and a second toad sample was collected within 12 days in June. The variation in the sex ratio is due to the fact that collecting surveys were not exhaustive and thus, samples represent a random sample of the local breeding assemblage. Toads were released again *in situ* following sex determination, measuring of snout–vent length (SVL, to the nearest mm) and toe-clipping (third toe of the right hind limb). The toes were stored in 70% ethanol at room temperature.

Laboratory protocols followed the standard methods of skeletochronology (e.g. Smirina, 1972). The samples collected in Urmitz, Balaguer and Mas de Melons were embedded in Historesin™ (Jung, Leica Microsystems Nussloch GmbH, Nussloch, Germany) and stained with

**Table 1** Location, climate features and number of toads collected

Locality	Coordinates	Altitude (m a.s.l.)	Air temperature January/July (°C)	Annual precipitation (mm)	Date of collection	Males/females
Reserva Biologica	37°00'44"N 06°30'13"W	24	10.6/24.5	585	14.–15.III. 2002	32/27
Navas	37°47'28"N 06°04'41"W	420	9.6/25.7	640	3.–6.III. 2002	26/28
Mas de Melons	41°30'50"N 00°43'30"E	240	5.3/24.7	369	21.–23.IV. 2004	43/4
Balaguer	41°46'30"N 00°46'50"E	220	5.3/24.7	369	1.IV. 2004	34/14
Urmitz (spring)	50°21'43"N 07°35'48"E	60	2.7/19.5	674	19.–23.IV. 2000	29/25
Urmitz (summer)	50°21'43"N 07°35'48"E	60	2.7/19.5	674	4.–17.VI. 2000	25/26

cresylviolet (details in Sinsch, di Tada & Martino, 2001). Diaphysis was cross sectioned at 10  $\mu\text{m}$  using a Jung RM2055 rotation microtome. The samples collected in Andalusia were embedded in Tissue-Tek (Sakura Finetek, Europe B.V., Alphen aan den Rijn, the Netherlands) and stained with Ehrlich's hematoxylin (details in Tejedo, Reques & Esteban, 1997). Diaphysis was cross sectioned at 16  $\mu\text{m}$  using a cryomicrotome (Microm International GmbH, Walldorf, Germany). A previous study demonstrated that the differences in staining procedure did not interfere with the detectability of LAGs (Sinsch *et al.*, 2007a). Cross sections were examined light microscopically at magnifications of  $\times 200$  using an Olympus BX 50 (Olympus Europa GmbH, Hamburg, Germany) or Leica DME (Leica Microsystems Ventricle GmbH, Wetzlar, Germany). High-resolution cameras Olympus DP20 (Olympus Europa GmbH) and Sony SSC-DC50AP (Sony Deutschland, Köln, Germany) were used to take digital images from those diaphysis sections in which the size of the medullar cavity was at its minimum and that of bone at its maximum.

One digital image per individual was selected for further analysis. Image files were transferred to UTHSCSA ImageTool, version 3.0, and calibrated using a standard micrometer. In a first step of analysis, we recorded the presence/absence of the line of metamorphosis (LM) and of lines of arrested growth (LAGs). The number of LAGs visible in each cross section was assessed by the first author and independently by one or two co-authors. In those toads with no remnant of the LM, we checked for the potential resorption of LAGs using the method of Hemelaar (1985). In a second step, we distinguished annual growth marks (LAGs *sensu stricto*) from non-annual ones (irregular interruptions of the hibernation inactivity periods), using the method described in Sinsch *et al.* (2007b). The age of maturity was defined as the lowest age recorded in a reproductive toad of a given population. Finally, we measured the area of periosteal bone between two neighboring LAGs to the nearest  $\mu\text{m}^2$  in those cross sections in which the bone section was complete, to obtain an objective measure for the annual bone growth. In cases of multiple line formation within one year (common in the samples of Catalonia, Sinsch *et al.*, 2007b), the outermost line of a group was chosen as the final growth mark of a year. The dataset obtained to model the age–size relationship included the categorical variables locality (coded 1–5), spring/summer cohort (coded 1/2), sex (male = 1, female = 2), presence/absence of LM (coded 1/0), presence/absence of non-annual growth marks (coded 1/0) and the quantitative variables age (number of LAGs) and SVL (mm) with  $N = 313$  individual toads. The dataset used to analyze the growth pattern of periosteal bone included additionally the quantitative variables total bone area ( $\mu\text{m}^2$ ) and up to eight areas of yearly bone growth (= growth rate) depending on the age of the toad. The number of observations was lower ( $N = 280$ ) because 33 incomplete bone sections allowed age determination, but not area measurement.

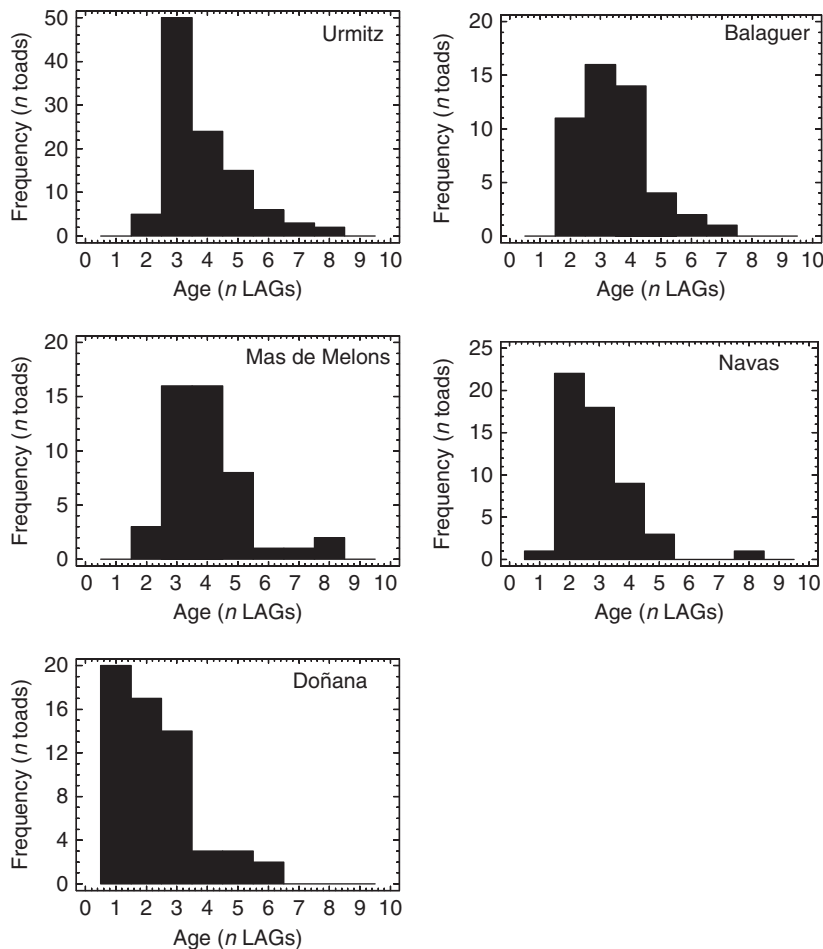
### Statistical analyses

All variables were first tested for normality. Multifactorial analyses of covariance (ANCOVA, type III sums of squares)

were used to assess the effects of age (covariate), cohort and locality on SVL and total bone area, respectively. Multiple comparisons of the least square means were based on 95% Bonferroni confidence intervals. The age–size relationship was modelled using a general linear regression model (GLM). We tested a total of 22 variable transformations to linearize the relationship. The double-reciprocal model provided the best fit (maximum  $R^2$ ). The one-factor regression model of age (1/LAG) and size (1/SVL) was optimized using a Box–Cox transformation and complemented by successively introducing sex and locality (categorical variables). Variables were added to the model, if their corresponding coefficients were significantly different from zero (ANOVA), that is they contributed to the amount of variance explained. Further GLMs were calculated to fit the bone area–size relationship and the age–bone growth rate relationship. All GLM estimates are expressed as mean and corresponding standard error. Significance level was set at  $\alpha = 0.05$ . All calculations were based on the procedures of the program package STATGRAPHICS Centurion, version XV (STATPOINT Inc., Warrenton, VA, USA).

### Results

The area between the outermost LAG and the edge of the bone from the Urmitz population was usually very small in the individuals collected during spring and considerably larger in those collected in June, indicating that toads resume growth at the beginning of the local spring breeding period. The proportion of individuals in which the LM was at least partially visible in the phalange bone sections varied significantly among the localities (ANCOVA,  $F_{4,301} = 66.7$ ,  $P < 0.00001$ ; proportion adjusted for age as covariate,  $F_{1,301} = 3.3$ ,  $P = 0.0714$ ), ranging from 2.3% in Urmitz (least square mean), over 12.9 and 14.4% in Balaguer and Mas de Melons, respectively, 43.3% in Navas and 88.8% in Reserva Biologica de Doñana. Resorption of the inner LAG was detected in only one out of 313 individuals (Urmitz site). The number of individuals showing non-annual growth marks also varied locally. The proportion was low in Navas (13.0%), Urmitz (21.6%) and Reserva Biologica de Doñana (22.1%), suggesting almost uninterrupted hibernation or aestivation, and high in Balaguer (75.0%) and Mas de Melons (80.8%), suggesting short-term growth periods interrupting hibernation (Sinsch *et al.*, 2007b; Orómi, Sanuy & Sinsch, 2010). Local age distributions differed significantly from each other (Kolmogorov–Smirnov test, all paired comparisons  $P < 0.00001$ ; Fig. 1). Minimum age at maturity was one LAG in the Reserva Biologica de Doñana ( $n = 20$ , 33.9% of all individuals) and at Navas ( $n = 1$ , 1.9%), while in all northern populations, maturity was attained at a minimum of two LAGs (Balaguer  $n = 11$ , 22.9%; Mas de Melons  $n = 3$ , 6.4%; Urmitz spring cohort  $n = 2$ , 3.7%; Urmitz summer cohort  $n = 3$ , 5.9%). At Navas, most first breeders pertained to the two LAG class ( $n = 23$ , 40.7%).



**Figure 1** Age distribution of reproductive toads at the five localities studied.

### Local size variation predicted by age and sex

At the Urmitz study site, SVL of adults did not differ between males and females (multifactorial ANCOVA,  $F_{1,104} = 3.0$ ,  $P = 0.0875$ ) and between spring/summer breeding individuals ( $F_{1,104} = 1.7$ ,  $P = 0.1965$ ), but significantly influenced by the covariate age ( $F_{1,104} = 1687$ ,  $P < 0.0001$ ). At the neighboring localities Balaguer and Mas de Melons, SVL of adults was neither influenced by sex (multifactorial ANCOVA,  $F_{1,94} = 3.8$ ,  $P = 0.0553$ ) nor by locality ( $F_{1,94} = 0.01$ ,  $P = 0.9139$ ), but covaried significantly with age ( $F_{1,94} = 5.4$ ,  $P = 0.0224$ ). Consequently, we reduced the six subsets of data (Table 1) to four by pooling those of the spring and summer cohorts at Urmitz and those of Balaguer and Mas de Melons, respectively.

The multifactorial ANCOVA revealed that the age-adjusted least square means of SVL (covariate age:  $F_{1,312} = 90.8$ ,  $P < 0.0001$ ) differed considerably among the localities ( $F_{3,312} = 256.9$ ,  $P < 0.0001$ ), Balaguer/Mas de Melons (79.0 mm), Navas (69.4 mm), Reserva Biologica de Doñana (56.5 mm) and Urmitz (56.5 mm), but not between sexes ( $F_{1,312} = 3.6$ ,  $P = 0.0601$ ). However, multiple range comparison revealed that there were only three size-groups

because the means of the small-sized populations did not vary among each other ( $P < 0.05$ ). Quantitative modelling of size variability by GLM explained 72.7% of SVL variance by the combined effects of locality, sex and age (Table 2A, Fig. 2). Age accounted for only 13.4% of the variance, sex for 0.3%, whereas local differences explained 59.0%. The annual increase of size in adults did not differ significantly among localities (ANOVA,  $P = 0.1081$ ), but the intercepts of the model did (ANOVA,  $P < 0.0001$ ). In summary, the age- and gender-dependent body size variation of adults was low within each locality, and the major source of variation among the localities was the size at which the mostly two LAGs-old individuals became reproductive, that is the size achieved during the juvenile stage and then population divergence in adult size is a function of variation in juvenile growth rates.

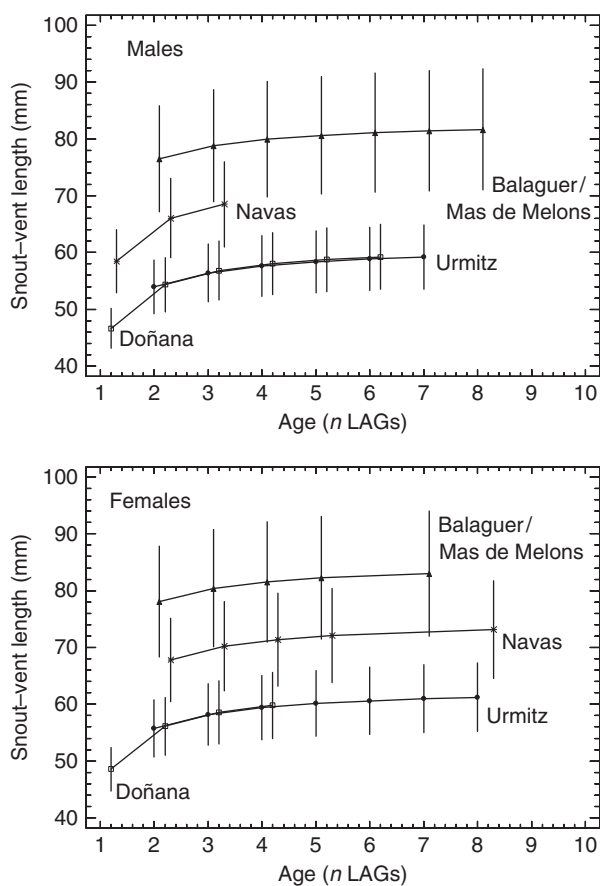
### Patterns of annual bone growth

At the Urmitz study site, the annual bone growth rate of toads that reproduced during spring differed significantly from that of individuals that reproduced during summer at the first (multifactor ANCOVA;  $F_{1,100} = 6.9$ ,  $P = 0.0102$ )

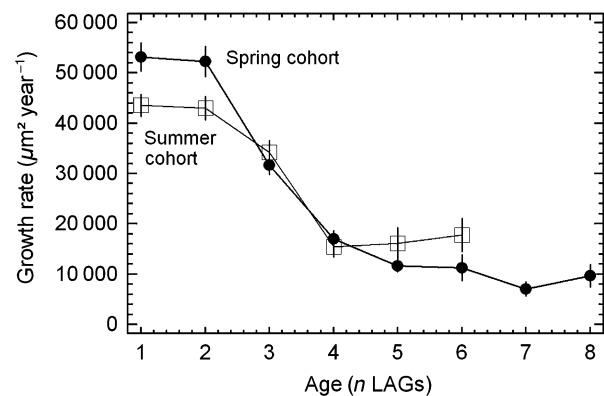
**Table 2** GLM models to predict size (SVL) (A) or by phalange bone size (C) by age and local factors

Model coefficient	Estimate $\pm$ standard error	Wilk's lambda	F-value	Significance	Contribution to $R^2$
<i>(A) Box-Cox transformation applied power: -1.36311</i>					
Constant	0.98698 $\pm$ 0.000234				
1/age	0.003416 $\pm$ 0.000543	0.8858	39.4	$P < 0.00001$	13.4%
Locality					
1/4	0.002129 $\pm$ 0.000157	0.3157	221.8	$P < 0.00001$	59.0%
2/4	-0.003407 $\pm$ 0.0001628				
3/4	-0.00076 $\pm$ 0.000181				
Sex	0.000207 $\pm$ 0.000096	0.9850	4.7	$P = 0.0316$	0.3%
Total $R^2$ (adjusted for degrees of freedom)					72.7%
<i>(B) Box-Cox transformation applied power: 0.193897</i>					
Constant	0.893239 $\pm$ 0.000197				
1/bone area	783.34 $\pm$ 33.10	0.3261	560.2	$P < 0.00001$	80.0%
Region	0.000674 $\pm$ 0.000118	0.8926	32.6	$P < 0.00001$	1.1%
Total $R^2$ (adjusted for degrees of freedom)					81.1%

Best fit (maximum  $R^2$ ): double reciprocal model. Model A differs from B by the introduction of local habitat and climate features as categorical or quantitative variables. Numerical range of variables: age, 1–8 LAGs; sex, male = 1, female = -1; locality, 1 = Urmitz, 2 = Balaguer/Mas de Melons, 3 = Navas, 4 = Reserva Biologica de Doñana; region: 1 = Urmitz/Navas/Reserva Biologica de Doñana, 2 = Balaguer/Mas de Melons.



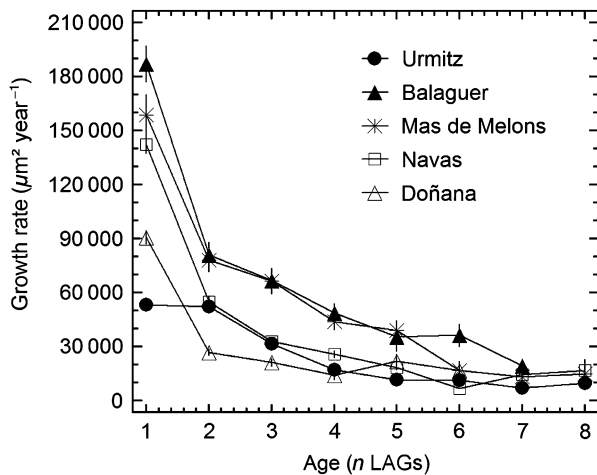
**Figure 2** Age-size relationship for the studied populations (generalized linear regression models). (a) Males; (b) Females. Symbols represent the SVL estimates (least square means) and bars show the corresponding 95% confidence interval.



**Figure 3** Annual growth rate of periosteal bone of toads reproducing in spring ( $N = 52$ ) or in summer ( $N = 49$ ) at the Urmitz study site. Data are given as least square means and the corresponding 95% confidence interval. Statistical details are given in the text.

and second year of life ( $F_{1,100} = 5.2$ ,  $P = 0.0252$ ), respectively, whereas sex did not influence the growth rate in any year ( $F_{1,100}$ ,  $P > 0.05$ ; Fig. 3). Age at the time of capture (multifactor ANCOVA;  $F_{1,100}$ ,  $P > 0.05$ ) did not covary with the annual bone growth rate, suggesting that periosteal bone does not condense with increasing age in natterjack toads. Growth rates were significantly smaller in the summer cohort (least square means: 43 272  $\mu\text{m}^2$  during the first year; 43 362  $\mu\text{m}^2$  during the second year) than in the spring cohort (53 435  $\mu\text{m}^2$  and 51 581  $\mu\text{m}^2$ , respectively). Consequently, we used only the spring cohort of Urmitz for further comparisons with the Spanish populations, which consist exclusively of spring breeders.

At any year of life, the average bone growth rate varied significantly among localities, but not between males and



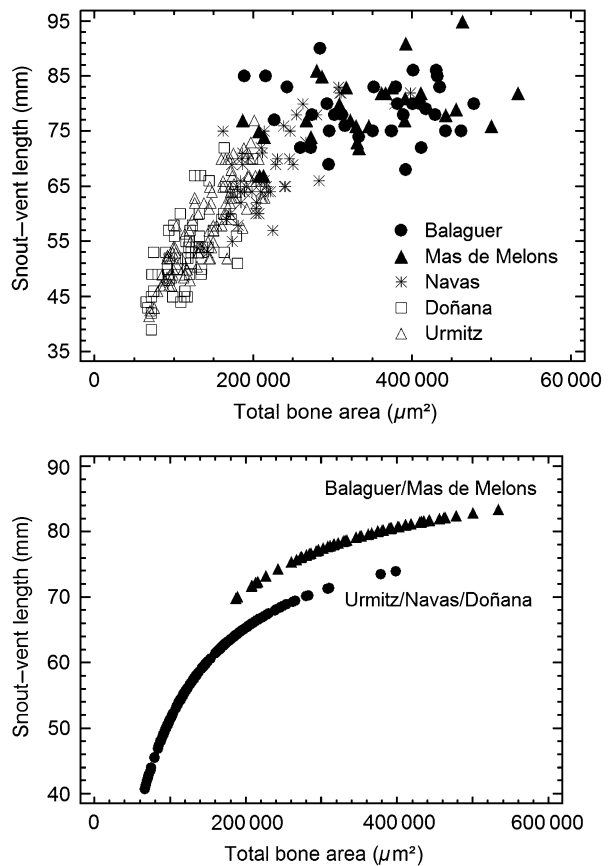
**Figure 4** Least square means and the corresponding 95% confidence interval for the lifetime growth pattern of periosteal bone in five localities. Statistical details are given in the text.

females and independent of age at capture, as revealed by multifactorial ANCOVAs (Fig. 4). During the first year of life (ANCOVA;  $F_{4,230} = 84.2$ ,  $P < 0.0001$ ), bone growth differed significantly among all the localities (multiple range test;  $P < 0.05$ ): Balaguer (least square mean:  $191\,925 \pm 6701 \mu\text{m}^2$ ), Navas ( $138\,456 \pm 5365 \mu\text{m}^2$ ), Reserva Biologica de Doñana ( $83\,117 \pm 5471 \mu\text{m}^2$ ) and Urmitz ( $61\,100 \pm 5749 \mu\text{m}^2$ ). During the second year of life (ANCOVA;  $F_{4,206} = 22.6$ ,  $P < 0.0001$ ), bone grew most in the Catalonian populations forming a homogeneous group (multiple range test;  $P < 0.05$ ), followed by a group formed by the Navas and Urmitz populations, and by the Doñana population. During the subsequent years of life, Urmitz and the Andalusian populations formed a homogeneous group with respect to annual bone growth (multiple range test;  $P < 0.05$ ), whereas the Catalonian population continued to exhibit significantly greater growth rates.

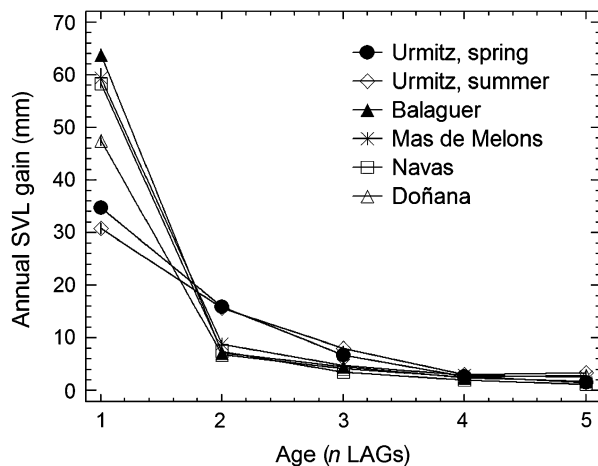
**Retrospective size prediction by bone area**

The GLM of the bone area–adult size relationship explained 81.1% of SVL variance by total bone area as quantitative variable and of region as a categorical variable, which led to a small, but significant contribution due to the difference among the Catalonian populations on the one hand and those of Andalusia and Urmitz on the other (Table 2B, Fig. 5). The variables sex and age did not increase  $R^2$  significantly. The GLM, therefore, allows for a retrospective estimation of SVL at any stage of the lifetime of a natterjack toad calculating the corresponding SVL to the bone area bordered by each LAG.

The translation of bone growth pattern into annual SVL gain revealed significant differences among all the populations with respect to SVL achieved before the formation of the first LAG and between the populations of the Iberian Peninsula and those from Germany for the second and third



**Figure 5** Bone size–SVL relationship. (a) Scatter plot of 280 individuals; (b) GLM model for two groups of localities; coefficients are given in Table 2, and symbols represent individual estimates.



**Figure 6** Least square means of annual SVL gain and the corresponding 95% confidence interval estimated from the bone growth pattern of toads originating from five localities. At the Urmitz site, the toads breeding in spring are distinguished from those breeding during summer.

LAGs (Fig. 6). Except for the first year of life, Spanish populations did not differ in their growth pattern (Fig. 6). While the average first year SVL varied between 47.5 mm (Reserva Biologica de Doñana) and 63.7 mm (Balaguer), second year SVL gain declined sharply at all localities to  $7.4 \pm 1.2$  mm and annual SVL gain remained unchanged at  $2.5 \pm 1.0$  mm from the fourth year onwards (Fig. 6). The first year size at the German locality differed significantly between the members of the spring and summer cohorts and the average SVL was about 14 mm smaller than that of the small-sized Spanish population in Doñana (Table 3). Following the first year of life, the growth pattern of the two cohorts was undistinguishable, but second and third year SVL gain (15.9 mm and 7.4 mm) was significantly greater than in the Spanish populations (ANOVA,  $P < 0.001$ ). From the fourth year onwards, the growth pattern of all populations converged to a homogeneous annual SVL gain of about 2.5 mm.

## Discussion

Size variations among conspecific amphibian populations exposed to environmental gradients are probably driven by different proximate causes than those mentioned to explain latitudinal size clines at the interspecific level in Amphibia (Olalla-Tarraga & Rodriguez, 2007; Gaston, 2008; Cvetkovic *et al.*, 2009). The broad-scale geographical pattern of body size for the European amphibian fauna seems to be compatible with a Bergmann cline, if the data analysis is based on the maximum SVL of each anuran species (Olalla-Tarraga & Rodriguez, 2007). However, the validity of this approach is doubtful because it neglects the intraspecific size variation in species such as *B. bufo*, *B. calamita* and *R. temporaria*, which follows roughly a converse Bergmann cline (Miaud *et al.*, 1999; Laugen *et al.*, 2005; Leskovar *et al.*, 2006; Cvetkovic *et al.*, 2009; this study). Basic to a better understanding of size variation in Amphibia as a group is consequently the analysis of the proximate causes of intraspecific size variation in the framework of the life-history theory (Angilletta *et al.*, 2004; Adams & Church, 2008). The large adult size obtained by delaying sexual maturity provides the benefit of increased clutch size, but may be associated with costs such as increased juvenile and adult mortality. The life-history theory predicts that delayed

maturity and associated size gain should be selected for if lifetime fecundity is increased.

The lifetime growth pattern of European toads includes a short aquatic larval period in which usually less than 20% of the adult size is attained and a terrestrial period with indeterminate growth that declines at sexual maturity (Werner, 1986; Hemelaar, 1988). In *B. calamita*, metamorph size ranges from 4 to 12 mm SVL, covarying with larval density and interspecific competition in the breeding pond (Goater, 1994; Gomez-Mestre & Tejedo, 2002; Sinsch, 2008). The populations studied do not depart from this rule, with the average metamorph size ranging from 6 to 9 mm (Sinsch, Höfer & Keltsch, 1999; N. Oromi, M. Tejedo & F. Marangoni, unpub. obs.). Thus, body size variation is mainly a consequence of the terrestrial growth period. Unlike in *B. viridis*, local genetic adaptations appear to be a negligible source of size variation in *B. calamita* because the populations in Andalusia do not exhibit significant genetic divergence but close to maximum differences in adult size (Castellano & Giacoma, 1998; Marangoni, 2006; this study).

## Environmental causes of phenotypic size plasticity

There are several potential determinants of phenotypic plasticity in the adult body size of amphibians, for example age and associated variation of growth rate, food availability and temperature-dependent limitations of annual growth periods (e.g. Jørgensen, 1992; Gramapurohit, Shanbhag & Saidapur, 2004). The GLM model describing adult growth of natterjacks indicates that the annual growth rates are similar (about  $2.5 \text{ mm year}^{-1}$ ) in all populations and thus independent of latitude. The independence of the growth rate from altitude, however, refers exclusively to the studied lowland range of up to 420 m a.s.l., while natterjacks inhabit altitudes of up to 2540 m a.s.l. in the Sierra Nevada, Spain (Sinsch, 2008). Preliminary data indeed suggest the presence of a converse Bergmann cline in populations of the Sierra de Gredos, Spain (Oromi, study in progress).

In contrast, the intercepts of the GLM, that is the size of the first breeders, do distinguish among the populations, suggesting that the environmental impact on subadult terrestrial growth period is the primary source of size variation among natterjack populations. More precisely, it is the first-year growth that yields the difference, as

**Table 3** Estimated SVL variation at the end of the first terrestrial growth period (one LAG) among the studied toad population

Locality	Average SVL ( $\pm$ s.e.) (mm)	Duration of first year aboveground feeding activity (month)	Duration of first year inactivity period (month)	Feeding/growth during aestivation/hibernation
Reserva Biologica de Doñana	$47.5 \pm 1.0$	4–6	c. 4 (aestivation)	No
Navas	$58.3 \pm 1.0$	4–6	c. 4 (aestivation)	Unknown
Mas de Melons	$59.7 \pm 1.4$	4–6	c. 4 (aestivation + hibernation)	Yes
Balaguer	$63.7 \pm 1.2$	4–6	c. 4 (aestivation + hibernation)	Yes
Urmitz (spring)	$34.8 \pm 1.1$	3–4	c. 6 (hibernation)	No
Urmitz (summer)	$30.8 \pm 1.1$	2–3	c. 6 (hibernation)	No

All SVL estimates differ significantly from each other (ANOVA, multiple-range test;  $P < 0.05$ ).

demonstrated by the retrospective SVL estimation. Within eventual determinants of juvenile growth, food availability does not seem to constrain the terrestrial growth of natterjacks at any site studied so far (review: Sinsch, 2008; Urmitz: Leskovar, Wagner & Sinsch, 2004). Therefore, we first concentrate on the environmental factors modulating and limiting the duration and intensity of the first-year growth period, and then focus on the subsequent geographical variation of life-history traits such as age and size at maturity and their evolutionary significance.

In the temperate zone, the growth rates vary seasonally, determined by the varying environmental temperatures (i.e. duration of activity period) and food supply (Jørgensen, 1992). The growth rates of metamorphs at Urmitz, Germany, provide the test case, as temperature and the non-limiting food supply are identical for the spring and summer cohort, but the latter has a shorter activity period before hibernation, resulting in a *c.* 4 mm lower SVL (Sinsch *et al.*, 1999; Leskovar *et al.*, 2004; Table 3). The subsequent inactivity period of juveniles burrowed in sand does not allow further growth partially due to inherent growth rhythms demonstrated under controlled conditions and the absence of food in the hibernacula (Leskovar & Sinsch, 2001). Thus, the length of the activity period explains entirely the first-year size variation between the members of the spring and summer cohorts in Germany.

Although adults of the Urmitz and the Doñana population are similar-sized, first-year individuals grew about 12 mm larger in Spain, demonstrating different lifetime growth regimes at the two sites. The most likely cause for the faster metamorph growth is ambient temperature, which allows higher metabolic rates and better food utilization in Spain (Jørgensen, 1992). The duration of the aboveground activity period appears to be similar, in Germany ended by hibernation, in Andalusia by aestivation (Table 3). As inactive individuals are burrowed in the sandy soil at both sites, we do not expect the Doñana toads to feed and to grow during aestivation, but this hypothesis remains to be proven. If correct, size variation between first-year individuals of the Urmitz and the Doñana population is a direct temperature effect, which is compensated for adult size by the increased second-year growth of the still immature toads in Urmitz.

The first-year individuals of the remaining three Spanish populations were another 12–15 mm larger than the Doñana toads, although the local climate is similar, but moderately colder during winter. Consequently, the duration of aboveground activity is also similar, but the microhabitats used for aestivation (all localities) and hibernation (Balaguer/Mas de Melons) are rabbit holes, rock fissures and crevices because the absence of sandy soils prevents the toads from actively burrowing as in Urmitz and Doñana (Oromi *et al.*, 2010). Radio-telemetric monitoring of adults at Mas de Melons and several short bone growth periods during winter suggest that the shelter microclimate allows locomotor activity including foraging underground (Sinsch *et al.*, 2007b; Oromi *et al.*, 2010). Similar information is not yet available for the Navas site. Still, it seems reasonable to assume that the different microhabitats used for hibernation

and/or aestivation are the proximate causes for the size difference of juveniles. Cavity systems also provide shelter for invertebrate prey, while the small-volume burrows in sand without connection to the ground surface do not. Thus, the period that toads spend underground can be considered as an extension of the feeding period in Balaguer and Mas de Melons, and additional growth is achieved in comparison with the burrowed soil of the Doñana population. However, field reciprocal transplant experiments on the Spanish spadefoot toad, *Pelobates cultripes*, between Doñana and Navas environments show reduced growth in the sandy environment for all the populations, thus suggesting a role of environmentally induced plasticity in the growth rates across environments that may explain the unexpected lower growth of the Doñana populations (M. Tejedo & F. Marangoni, unpubl. data). Reduced size has also been observed in other amphibian species inhabiting the Doñana region (Diaz-Paniagua, Mateo & Andreu, 1996; Diaz-Paniagua & Mateo, 1999).

In summary, the geographical body size variation in *B. calamita* populations is probably the result of interactions among latitudinal temperature effects, microhabitat choice for hibernation/aestivation and habitat constraints to growth determining a pattern to some extent compatible to a converse Bergmann cline. This is in agreement with the latitudinal trend observed in *B. bufo* (Cvetkovic *et al.*, 2009) and supports the idea that amphibians do not follow Bergmann's rule (Ashton, 2002b; Adams & Church, 2008). The proximate causes identified act mainly on juveniles during their first year of life, which represents the sensitive period for fast growth in anurans (Jørgensen, 1992).

### Fitness consequences of body size variation on lifetime fecundity

The youngest first breeders were 1-year-old individuals (about one-third of the Doñana population), indicating that the threshold size for maturity is about 40 mm SVL in *B. calamita*. This size is reached and often considerably passed in all populations from the Iberian Peninsula during the first year of life, suggesting that natterjacks are sexually mature at the beginning of the second year. The sharp decline of bone growth during the second year is another indicator of previous sexual maturation (Hemelaar, 1988). Still, there was an increasing tendency from south to north to postpone first reproduction to the third (two LAGs) or the fourth year (three LAGs). The benefit is an SVL increase of 7–10 mm on average, allowing for a significantly larger clutch size than in first breeders at the minimum SVL (Tejedo, 1992). In Germany, first-year growth is too low to attain sexual maturity, and therefore, the growth rate remains high during the second year in which individuals pass the threshold size similar to the growth pattern in *B. bufo* (Hemelaar, 1988). Again, most adults postpone reproduction for at least 1 year.

In natterjacks, size at first reproduction and age at maturity are not tightly coupled. Delayed reproduction may be selected for if fitness increases by the SVL-associated



fecundity gain and lifetime fecundity is optimized, that is the product of clutch size and potential reproductive lifespan (longevity – age at first reproduction). The potential reproductive lifespan of females decreases from north to south: 6–5 years (spring/summer cohort) in Urmitz to 3–4 years in Catalonia and Andalusia (Leskovar *et al.*, 2006; this study). Because the average clutch size follows a converse gradient, lifetime fecundity is probably similar in most of the geographical range (Sinsch, 2008). The southernmost Doñana population is the exception from the rule, with small clutches similar to those in Germany and a low potential reproductive lifespan as in the rest of the Iberian Peninsula. Considering the harsh environmental condition at this site and short longevity, selection should favor early reproduction at small SVL (Marangoni, 2006; Marangoni *et al.*, 2008). Applying a life-history perspective, latitudinal body size variation among *B. calamita* populations, and perhaps also that of other amphibians species, is the evolutionary byproduct of optimized lifetime fecundity.

## Acknowledgments

The permissions for field studies and handling of the toads were issued by the Struktur und Genehmigungsbehörde Nord, Koblenz, Germany, the Departament de Medi Ambient, Generalitat de Catalunya and the Consejería de Medio Ambiente de la Junta de Andalucía and the Reserva Biológica de Doñana, Spain. This work was supported by the grant CGL2004-01872/BOS from Dirección General de Investigación Científica y Técnica conceded to M. Tejedo. T.J.C. Beebee and an anonymous reviewer provided useful comments on an earlier draft of the paper.

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