

# Adaptation or exaptation? An experimental test of hypotheses on the origin of salinity tolerance in *Bufo calamita*

I. GOMEZ-MESTRE & M. TEJEDO

Departamento de Biología Evolutiva, Estación Biológica de Doñana – CSIC, Sevilla, Spain

## Keywords:

adaptation;  
*Bufo calamita*;  
drought tolerance;  
exaptation;  
metamorph;  
osmotic stress;  
salinity tolerance.

## Abstract

The natterjack toad (*Bufo calamita*) shows variation in embryonic and larval salinity tolerance across populations in southern Spain. However, its aquatic/terrestrial biphasic life cycle, together with remarkable differences in salinity tolerance between Spanish and UK freshwater populations suggest an alternative hypothesis to local adaptation. Drought resistance during the terrestrial phase and salinity tolerance during the aquatic phase are both related to osmotic stress tolerance, and if there were an association between them, one could have evolved as an exaptation from the other. To test such an association, we reared *B. calamita* juveniles from three populations known to differ genetically in their salinity tolerance, under either dry or humid conditions. Drought decreased growth rate, enhanced burying behaviour, and decreased foraging activity and efficiency. No significant population  $\times$  treatment interaction was found for any variable, i.e. populations were equally affected by drought. These results do not support the hypothesis of a genetic association between salinity and drought tolerance.

## Introduction

Development is a highly compartmentalized process (Alberch, 1987; Carroll *et al.*, 2001) and, consequently, organisms with complex life cycles such as amphibians often show certain degree of decoupling between larval and adult traits (Wassersug, 1975; Shaffer *et al.*, 1991; Ebenman, 1992; Moran, 1994; Phillips, 1998). However, trait decoupling across the metamorphic boundary that separates life stages in organisms with complex life cycles is not universal for all traits and organisms (Babcock & Blais, 2001; Watkins, 2001) and sometimes a persistence of a trait in a given stage may be because of its developmental linkage to other trait(s) selected for in a different stage (Cheverud *et al.*, 1983; Cheverud, 1984). Some traits may share a developmental history in spite of metamorphosis, and postmetamorphic traits may be affected by the environment experienced during pre-metamorphic stages (Goater, 1994; Tejedo *et al.*, 2000; Relyea, 2001; Relyea & Hoverman, 2003; M. Tejedo, M. J. Sánchez-Herráiz & C. Pertoldi, unpublished). This

fact has important implications for the study of adaptation in organisms with complex life cycles, because variation in any particular trait under certain environmental conditions in one life stage could be affected by selection acting on an associated trait from another life stage experiencing a different environment (Deban & Marks, 2002).

One important consequence of the 1960 s and 1970 s critiques against the adaptationist programme (Williams, 1966; Gould & Lewontin, 1979) has been the incorporation of a historical concept of adaptation to mainstream evolutionary biology (Amundson, 1996). Gould & Vrba (1982) proposed a restriction of the concept of adaptation to a trait evolved through the direct action of natural selection for its current function. Conversely, they defined exaptation as a trait evolved for other usages (or no function at all), and later co-opted for its current function. This distinction, seldom challenged (but see Endler & McLellan, 1988; Reeve & Sherman, 1993), has gained general theoretical acceptance, but has rarely been tested empirically, due to practical difficulties in determining the causal origins of traits. As Amundson (1996, p. 46) puts it, 'the question of trait origin is loaded against the ecologist', and the fact that character evolution may often comprise a mixture of exaptations and

Correspondence: Ivan Gomez-Mestre, Department of Biology, Boston University, 5 Cummington St., Boston, MA 02215, USA.  
Tel.: (617)358 2384; fax: (617)353 6340; e-mail: igmestre@bu.edu

secondary adaptations in a nested hierarchy (Larson & Losos, 1996; Andrews *et al.*, 2002) is both intriguing and discouraging. Two main approaches may be followed in the study of trait origins. First, a phylogenetically based comparative method (Baum & Larson, 1991; Harvey & Pagel, 1991; Armbruster, 1993, 1994, 1997; Pellmyr, 1997; Kardon, 1998) is used. Here, the evolutionary sequence of character states and environmental shifts are plotted onto a phylogeny of the taxa studied in order to sort out the historical order of appearance of trait/function relationships. Secondly, a more mechanistic approach consists of analysing trait correlations and the presumed historical selective regimes that shaped them (Roff, 1989; Price & Langen, 1992; Lauder, 1996; Ketterson & Nolan, 1999; Owings *et al.*, 2002).

Gomez-Mestre & Tejedo (2003, 2004) presented a case of local adaptation of the embryonic and larval stages of some natterjack toad (*Bufo calamita*) populations to brackish water in southern Spain. Significant differences in water salinity tolerance were found between native populations and populations transplanted to brackish water ponds. Salinity tolerance seemed linked to fitness and there was an apparent genetic basis underlying the trait. Taken together, these results fulfil the criteria for a nonhistorical definition of adaptation (Endler, 1986; Sinervo & Basolo, 1996), or more appropriately, of an adaptation (Gould & Vrba, 1982; Gould, 2002). This salinity tolerance in embryonic and larval *B. calamita* from brackish pond populations may have originated historically either by the direct action of selection on spontaneous genotypic variants with different salinity tolerances (in which case the trait should be considered an adaptation) or by a correlated response to selection acting on some other trait (in which case tolerance to water salinity would have evolved as an exaptation).

Although mean population performance under brackish conditions differed markedly among populations, there was still substantial variation in salinity tolerance within populations (i.e. across sibships; Gomez-Mestre & Tejedo, 2003). Salinity-tolerant populations have not been geographically isolated from freshwater populations and some degree of gene flow among them might have occurred (Gomez-Mestre & Tejedo, 2004). Thus, gene flow could explain the persistence of within-population variation in salinity tolerance, because resistant individuals are not counter-selected in freshwater environments. In addition, environmental heterogeneity fostered by the presence of a mosaic of fresh and brackish water ponds and fluctuations in the levels of salinity could relax selection in the brackish environments enough to allow for the temporary permanence of less resistant individuals. At a broader scale, all five southern Spain populations studied so far (even freshwater populations) have shown higher embryonic salinity tolerance than any of the UK populations tested (Beebee, 1985). *Bufo calamita* shows a steep South-to-North decreasing gradient in genetic diversity at microsatellite

loci, most likely as a consequence of use of Pleistocene refugia in the Iberian Peninsula and later northwards expansion (Beebee & Rowe, 2000). That could also be reflected in genetic diversity for quantitative traits. If that is the case, salinity tolerance in the UK populations might not have evolved to the same extent as in southern Spain due to lack of genetic variation. This scenario would be consistent with an adaptive explanation of the origin of salinity tolerance.

However, also running North-to-South in western Europe is a steep gradient of decreasing rainfall, increasing potential evapotranspiration, and increasing summer drought (Estrela *et al.*, 1996). If embryonic and larval water salinity tolerance in *B. calamita* were correlated with drought tolerance during the terrestrial phases (juvenile and adult), any one of these traits could have evolved as an additive exaptation (Arnold, 1994) from the other. Considering that freshwater is the standard larval environment for most *B. calamita* populations (and the vast majority of amphibians; Balinsky, 1981), while terrestrial drought is a common selective pressure to amphibians, and in particular to those in southern Europe, it seems reasonable to assume that, if these two traits related to osmotic stress tolerance were associated, the polarity of the co-option would have been from drought tolerance to a secondary adaptation to water salinity tolerance. This would explain overall differences in salinity tolerance between Spanish and British populations, as well as the presence of salinity tolerant individuals in Spanish freshwater populations.

This hypothesis relies on the existence of an association between salinity tolerance in the early, aquatic stages, and drought tolerance of the terrestrial juvenile and adult stages. In this paper, we present an experiment designed to test the existence of such an association, as a first step in the study of the historical origin of salinity tolerance. We exposed *B. calamita* juveniles (because they are more prone to desiccation than adults are) to either humid or dry conditions, and monitored their survival, growth and behaviour for 5 weeks. The individuals used came from either freshwater or brackish water populations proved to differ in their degree of salinity tolerance (Gomez-Mestre & Tejedo, 2004).

## Materials and methods

### Populations, husbandry and experimental design

Between late March and early April 2001, *B. calamita* tadpoles between Gosner stages 31 and 35 (Gosner, 1960) were sampled from three populations in south-western Spain. Two of these populations breed in freshwater environments, either in shallow ponds on sandy soil surrounded by pine woods at the Parque Natural del Entorno de Doñana (Huelva province), or in ponds on granitic soil in the Sierra Norte de Sevilla (Sevilla province). They will be referred to as 'Doñana'

and 'Pedroso', respectively. The third population included in the study, 'Jarales', breeds in a brackish lagoon on clay and haline sediments. However, in very rainy years, a freshwater pond is formed only 200 m away from the brackish lagoon and the greater part of the toad breeding takes place in that pond instead. In the spring of 2001 the Jarales population had largely bred in this smaller freshwater pond, providing tadpoles from a population locally adapted to water salinity (Gomez-Mestre & Tejedo, 2003) whose tadpoles had been naturally growing in fresh water. Thus, freshwater-reared tadpoles from two freshwater populations and one brackish water population were brought to the laboratory, where they were kept at similar low densities in 5 L plastic trays with dechlorinated tap water and fed *ad libitum*. As the tadpoles reached metamorphosis, they were transferred to individual Petri dishes with wet moss until complete tail resorption (36-stage Gosner), and then weighted to the nearest 0.1 mg with a Mettler Toledo AG245 scale (Mettler Toledo, Greifensee, Switzerland). Larvae from each population had been growing in different ponds for 2–3 weeks before we collected them, and this could potentially have affected their performance during the experiment, confounding environmental and genetic effects on their tolerance to drought. However, rearing them in the laboratory under low density and *ad libitum* food supply for some time seemed to normalize possible initial differences among the larvae. For instance, mass at metamorphosis did not vary significantly among populations ( $0.119 \pm 0.003$ ,  $0.118 \pm 0.007$ ,  $0.110 \pm 0.005$ , mean mass (g)  $\pm$  SE for Jarales, Doñana and Pedroso populations respectively).

Toadlets from each population were moved individually to 500 mL lidded plastic cups filled with a vermiculite layer and randomly assigned to either level of the experimental humidity factor. Vermiculite is an inert substrate with a high capacity for water absorption that is well described in terms of its hydric potential (Packard *et al.*, 1987). Two hydric potentials were chosen to simulate 'humid' and 'dry' soils, after Packard *et al.* (1987) and Packard (1991). The humid treatment had an approximate hydric potential of  $-150$  kPa and was achieved adding 16.9 mL of dechlorinated tap water to 15 g of constant-weight dried vermiculite. For the dry treatment, 1.95 mL of water were added to 15 g of dried vermiculite, attaining an approximate hydric potential of  $-1150$  kPa. To avoid the osmotic shock of a sudden change from wet moss to a  $-1150$  kPa level of humidity, the newly metamorphosed toadlets assigned to the dry treatment were first allowed to acclimate at an intermediate humidity level ( $-550$  kPa; 4.2 mL of water added to 15 g of vermiculite) for a week before being transferred to the definitive dry treatment. Therefore, for each metamorph, the experiment began 1 week after complete tail resorption. The design was factorial with three populations (two from fresh and one from brackish environments)  $\times$  two humidity levels, with 12 and 15

replicates per population at the wet and dry levels, respectively, and distributed in randomized blocks on shelves in the laboratory. The vermiculite in the cups was renewed twice a week, ensuring that the substrate remained clean and the humidity levels were restored often. Toadlets were fed pin-head crickets every third day, and the crickets were dusted with a vitamin complex once a week. The toadlets spent extended periods of time buried in the substrate, while the crickets remained at the surface of the vermiculite, so the time spent buried was one of inactivity and nonfeeding for the toadlets. To test whether the different levels of humidity affected the level of activity of the toadlets, we recorded whether they were buried or on the surface during each daily census, alternating these between morning and evening to avoid possible circadian effects. The experiment lasted 5 weeks and the toadlets were censused daily, weighted weekly, and morphological measurements were taken at the end of the experiment. Once the experiment was over, we performed trials of foraging efficiency with the toadlets, as a measure of general condition. The toadlets were kept at the same humidity level experienced during the main experiment, and were starved for 3 days. On the morning of the fourth day, each toadlet was individually offered crickets *ad libitum*. The total number of attempts at prey capture and their relative success were recorded for 4 min. *Bufo calamita* juveniles capture small prey items by quickly projecting and retracting the tongue in a very conspicuous manner, so it was unambiguous to determine when an attempt had been made, and whether it had been successful. All toadlets tried to forage during the trials. All toadlets were released back in their original field sites.

### Statistical procedures

All the analyses were performed with SAS statistical package (SAS Institute, 1999). The main response variables studied were survival, body weight, morphology and toadlet activity. Survival was analysed by fitting a Cox regression model using PROC PHREG (Allison, 1995), using 'population of origin', 'humidity level', and weight at metamorphosis as covariates.

Growth trajectories for each population at each humidity level were analysed by fitting a repeated measures analysis of variance to the weekly body weight data with PROC GLM using 'population of origin' and 'humidity level' as main factors in the model, and weight at metamorphosis as a covariable. However, the structure of the covariance matrix did not satisfy the Huynh-Feldt condition (as indicated by a sphericity test; Anderson, 1958; SAS Institute, 1999) and the degrees of freedom of the *F*-tests, as well as the significance levels had to be corrected accordingly (Huynh & Feldt, 1976; SAS Institute, 1999).

Toadlet activity was coded as a binary variable, number of censuses found buried or on the surface was analysed with a generalized linear model assuming an underlying

binomial distribution of the residuals and using a logit link function fitted with the SAS macro GLIMMIX. The main factors included in this analysis were 'population of origin' and 'humidity', plus weight at metamorphosis as covariable.

Environmental conditions experienced during growth phases often affect not only the overall body size but also the body shape of amphibians (Emerson *et al.*, 1988; Blouin & Brown, 2000; Relyea, 2001, Tejedo *et al.*, unpublished). To test whether humidity affected the body shape of the toadlets, we measured their snout-to-vent length (SVL), right hind limb length (HLL), and head width (HW), the first two measures taken over millimetred paper and the latter with a caliper, all to the nearest 0.5 mm. None of the three variables showed deviations from the normal expectancy, but were log-transformed to improve linearity. To obtain a measure of relative, size-independent morphology, SVL, HLL and HW were regressed against body mass and the residuals were saved and used as response variables in subsequent analyses (Relyea, 2002). A general linear model was fitted with PROC MIXED to analyse the effect of humidity and population of origin on relative SVL, HLL and HW.

Finally, data from the prey-capture trials was used to test if humidity or population of origin affected the number of attempts (motivation), and/or the number of crickets preyed upon over the number of attempts made (accuracy). The first question was tested with a generalized linear model assuming an underlying Poisson distribution of the residuals and using a log link function with the macro GLIMMIX. To test for differences in accuracy we coded each attempt as either failed or successful, and assuming a binomial distribution of the residuals, fitted a model using a logit link function with the same SAS macro. 'Population of origin' and 'humidity' were considered random and fixed factors, respectively, throughout the analyses.

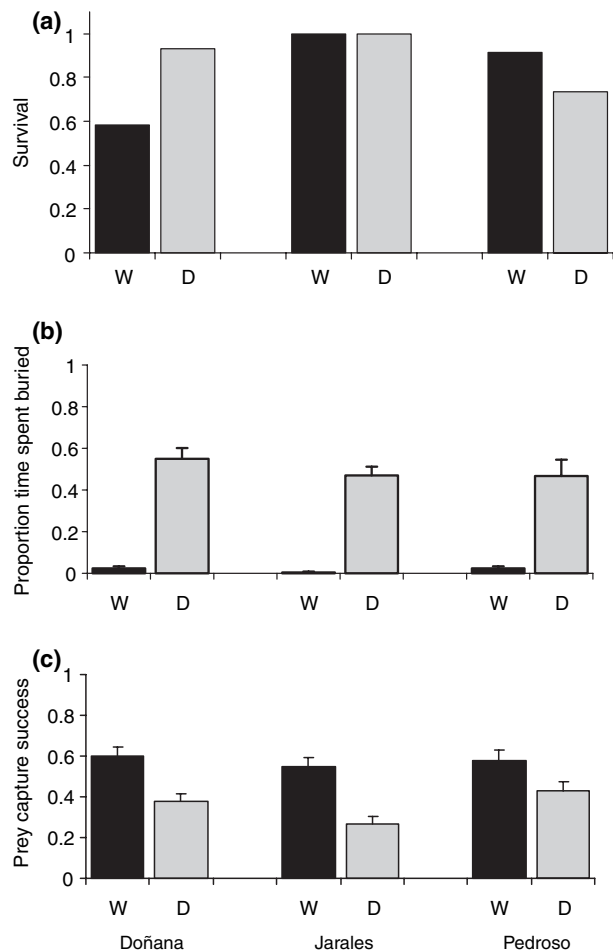
## Results

### Survival

Survival was high throughout the experiment (86.4% of the initial metamorphs). Weight at metamorphosis significantly affected survival ( $\chi^2 = 11.64$ ,  $P < 0.001$ ,  $n = 80$ ; logistic coefficient  $56.21 \pm 16.32$  SE), while neither population of origin ( $\chi^2 = 0.92$ , n.s.) nor humidity ( $\chi^2 = 0.37$ , n.s.) significantly affected survival during the 5 weeks that the experiment lasted ( $83 \pm 6$  and  $88.9 \pm 5$  % average survival  $\pm$  SE in humid and dry conditions, respectively; Fig. 1a).

### Growth

The toadlets grew considerably during the experiment, from  $155.9 \pm 4.7$  mg (mean  $\pm$  SE,  $N = 80$ ) to  $562.4 \pm 20.1$  mg ( $N = 70$ ), an average 2.6-fold increase. However

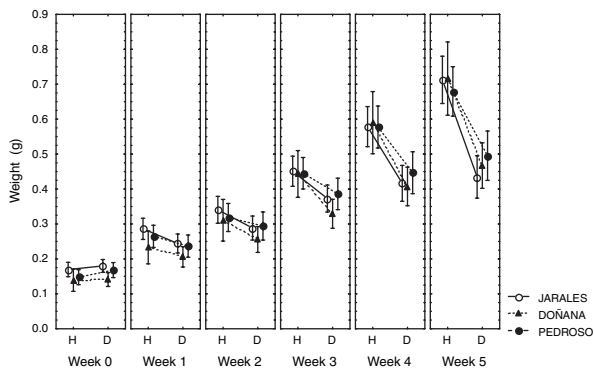


**Fig. 1** (a) Overall toadlet survival across populations and treatments. (b) Mean proportion of toadlets of each population in each treatment found buried in the substrate over 21 censuses. Error bars indicate SE. (c) Average prey capture success ( $\pm$ SE) for each population and treatment, measured as the number of crickets eaten over the number of attempts made during four minute trials.

growth was not the same across treatments. The dry environment significantly reduced growth rate (Table 1, Figs 2 and 3c); toadlets in the humid treatment weighted  $690 \pm 24$  mg ( $N = 30$ ) on average while those in the dry treatment weighted only  $461 \pm 18$  mg ( $N = 40$ ) by the end of the experiment. However, neither population of origin nor its interaction with humidity affected growth rate. The significant 'humidity  $\times$  time' interaction indicates that the growth trajectory was different between humidity levels (Fig. 2). Time also interacted significantly with weight at metamorphosis, suggesting that individuals with different initial weights experienced different growth trajectories. SVL was also significantly smaller in the dry treatment than in the humid treatment by the end of the experiment ( $20.13 \pm 0.27$  and  $17.13 \pm 0.29$  mm for the humid and dry treatments respectively;  $F_{1,64} = 47.90$ ,  $P < 0.001$ ).

**Table 1** Summary statistics for the repeated measures ANOVA on body mass. The experimental time frame was 5 weeks, with weekly measurements. Humidity levels clearly affected body mass and there were significant differences between humid and dry toadlets in their growth trajectory, as indicated by the 'humidity × time' term. Neither population of origin nor its interaction with time had a significant effect on body mass.

Source	d.f.	F	P
Population	2, 60	2.22	0.117
Humidity treatment	1, 60	44.01	<0.001
Time (weeks)	5, 305	745.2	<0.001
Population × humidity	2, 60	0.004	0.996
Population × time	10, 305	1.51	0.134
Humidity × time	5, 305	60.08	<0.001



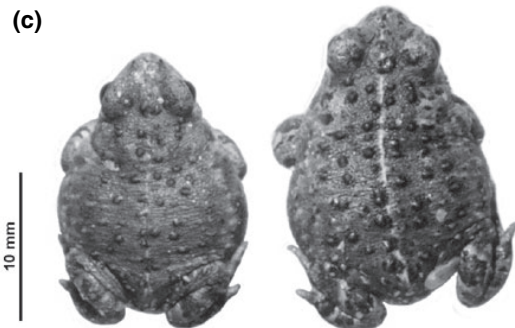
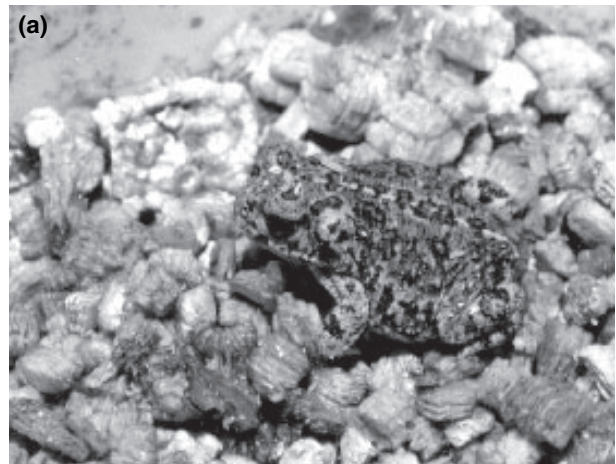
**Fig. 2** Reaction norms of mean body weight ( $\pm$ SE) for each population across humid (H) and dry (D) conditions. The dry treatment imposed a slower growth rate than the humid treatment, but the slope of the reaction norms of the three populations did not differ significantly, indicating a lack of genotype × environment interaction.

### Toadlet burying

The *B. calamita* juveniles were buried, on average, in 28% of the censuses made. Humidity had a very significant effect on proportion of time spent buried ( $\chi^2 = 63.56$ ,  $P < 0.001$ ), and toadlets under dry conditions spent more time buried (Figs 1b and 3 a,b). Neither population of origin nor its interaction with humidity had a significant effect on this variable. Weight at metamorphosis affected time spent buried ( $\chi^2 = 8.94$ ,  $P < 0.05$ ) so as that bigger toadlets were found buried more often ( $r^2 = 0.32$ ,  $P < 0.05$ ; Pearson product-moment correlation,  $N = 80$ ).

### Morphological changes

Body length relative to body mass did not vary significantly between humidity treatments, suggesting that the size differences found were caused by differential growth, and not merely by water loss. The analysis of relative HW and HLL showed that shape, and not only overall body



**Fig. 3** *Bufo calamita* juveniles in the humid (a) and dry (b) treatments. Toadlets in the dry treatment spent significantly more time buried in the substrate, and attained a smaller size than toadlets in the humid treatment (c: left, dry; right, humid).

size, differed between humidity treatments ( $F_{1,68} = 14.55$ ,  $P < 0.05$ ;  $F_{1,68} = 11.95$ ,  $P < 0.05$ ; for HW and HLL respectively). Toadlets in the dry treatment had, on average, 6.2% narrower relative HWs and 6.5% shorter relative hind limbs than those in the humid treatment. Population of origin did not affect relative changes in morphology.

### Prey capture success

Toadlets from the humid treatment made more attempts at prey capture than those from the dry treatment ( $\chi^2 = 39.45$ ,  $P < 0.001$ ). The efficiency of predation attempts (no. successes/no. attempts) was also significantly higher for the toadlets in the humid treatment ( $\chi^2 = 31.95$ ,  $P < 0.001$ ; Fig. 1c).

### Discussion

Evolution is inherently a historical process, and consequently evolutionary explanations of biological diversity should take into account the history of organisms when inferring the processes that shaped such diversity (Arnold, 1994; Gould, 2002). Two brackish water populations of *Bufo calamita* from Southern Spain have higher salinity tolerance than freshwater populations (Gomez-Mestre & Tejedo, 2003, 2004; M. Tejedo, I. Gomez-Mestre & R. Reques, unpublished). However, drought is a more ubiquitous stress than water salinity throughout the distribution of *B. calamita*, so if salinity tolerance in the aquatic phase was linked to drought tolerance during the terrestrial phase, salinity tolerance could have evolved as an additive exaptation from drought tolerance in these populations. In this study, we tested whether toadlets from a salinity-tolerant brackish water population (Jarales) had also a higher drought tolerance than toadlets from two freshwater populations (Doñana and Pedroso).

### Effect of terrestrial osmotic stress on juveniles

The low mortality rates and substantial growth of the juveniles throughout the study indicate that the experimental conditions imposed were not too extreme. The main factor determining survival of the toadlets was weight at metamorphosis, a common result in amphibian studies (Berven & Gill, 1983; Pough & Kamel, 1984; Goater, 1994; Newman & Dunham, 1994; Reques & Tejedo, 1997; Gomez-Mestre & Tejedo, 2003). However, even if the dry treatment did not increase mortality, it considerably decreased toadlets growth rate. The toadlets in dry conditions weighed on average 33% less than those in humid conditions after only 5 weeks (Fig. 2). Water loss could have been a confounding factor in assessing size differences between treatments because weight was the only size measure taken repeatedly during the experiment, but differences in SVL confirmed that overall size, and not just weight, differed across treatments (Fig. 3c). Decreased growth rates could have been the consequence of physiological adjustments, if toadlets were forced to allocate energy to osmoregulation that would have contributed to growth under more benign conditions. Alternatively, it could have been a consequence of shifts in behaviour, because toadlets in the dry treatment spent about half the time buried

(Fig. 1b) and hence not feeding. Furthermore, predation trials showed that even during their active periods, eagerness and accuracy in prey capture was lower in toadlets from the dry treatment (Fig. 1c). Likewise, moderately high water salinity has been found to interfere with the control and accuracy of tongue protraction in other bufonids (Dole *et al.*, 1985, 1994), suggesting that this may be a general consequence of osmotic stress. With this experimental design it was not possible to distinguish cause from effect between decreased growth and increased burying behaviour, although both were likely caused by physiological changes induced by osmotic stress. However, their eventual consequences were a smaller size and a reduced ability to capture prey. In addition to weight and SVL, toadlets differed in body shape across treatments, with dry-reared animals having relatively shorter hind limbs and narrower heads. This implies that the smaller toadlets in the dry environment did not scale isometrically with the bigger ones from the humid treatment. However, because morphological measurements were only taken at the end of the experiment, it cannot be concluded that this change in shape was caused directly by drought: it may be that toadlets in dry conditions only went through a portion of an allometric growth trajectory (Blouin & Loeb, 1991). Relyea & Hoverman (2003) found that *Hyla versicolor* froglets reared under high juvenile competition conditions showed relatively narrower bodies, but contrary to our results, relatively longer hind limbs. So far only Relyea & Hoverman (2003) and this study report alterations in the relative shape of juvenile anurans due to alterations in their growing conditions, but the body parts affected and the direction of change is likely to depend on the species studied, the persistence of carry over effects from the larval environment, and their ability for compensatory growth (M. Tejedo & I. Gomez-Mestre, unpublished).

### Lack of association between drought and salinity tolerance

No significant interaction between humidity and population of origin was detected for any of the variables studied. In fact, the reaction norms for body size of the three populations across environments were remarkably parallel (Fig. 2), indicating a high similarity in drought tolerance among populations within the humidity range used. These results do not support the hypothesis of a coupling between embryonic and larval salinity tolerance and juvenile drought tolerance. Instead, this species seems to experience a decoupling between pre-metamorphic and postmetamorphic osmotic stress tolerance.

Three lines of argumentation can be used to explain the lack of support to the coupling hypothesis. First, the level of drought used in the experiment may not have been stressful enough as to point out potential differences in mean population tolerance, as survival was high

in both treatments. If any genetic coupling exists between parallel tolerance to aquatic and terrestrial osmotic stress, this may be expressed only at the hardest conditions (i.e. Schlichting & Pigliucci, 1998). Secondly, because populations adapted to brackish water are not isolated from freshwater populations (Gomez-Mestre & Tejedo, 2004), the results could be biased if, by chance alone, we had sampled genotypes less tolerant than average in the brackish population, and genotypes more tolerant than average in both freshwater populations. Even in spite of the moderate sample size of the experiment, the probability of this scenario is very low.

Finally, different pathways for the aquatic and terrestrial osmoregulatory physiology may simply, however, dismiss the coupling hypothesis. Most terrestrial amphibians stop voiding urine and overstimulate the synthesis and accumulation of urea as a means of increasing their internal osmolality to cope with osmotic stress (Balinsky *et al.*, 1972; Katz & Hoffman, 1990; Sinsch *et al.*, 1992; Hoffman & Katz, 1997). Unfortunately, the information available on tadpole osmoregulation is scarce. *Bufo calamita* tadpoles are capable of producing urea (Gomez-Mestre *et al.*, 2004), but do not seem to accumulate it even under osmotic stress, and instead they experience a (most likely passive) rise in electrolytes, namely sodium and chloride (Gomez-Mestre *et al.*, 2004). Although we still ignore the specific mechanism conferring salinity tolerance in populations adapted to brackish water, it seems that aquatic and terrestrial stages of *B. calamita* may have fundamentally different physiological responses to osmotic stress, each evolving independently of the other.

The results from this study suggest that salinity tolerance in the aquatic phase of *B. calamita* is more likely to have evolved in these populations as an adaptation, rather than an exaptation from drought tolerance, although further research will be needed in order to obtain a formal rejection of the exaptation hypothesis. In particular, a common garden approach to evaluate the differences between a larger number of Iberian and UK populations could be combined with a comparison of salinity tolerance among populations with different mean drought tolerance. In addition, a better understanding of larval osmoregulatory physiology together with a stringent experimental design that includes harder conditions both in the water and terrestrial environment, may help to consider osmotic tolerances at the larval and terrestrial phase as either a suite of coupling traits or, alternatively, being the result of independent evolution via decoupling of physiological abilities at the different stages.

## Acknowledgments

We thank A. Marco and C. Diaz-Paniagua for their advice on the experimental levels of hydric potential and for providing the vermiculite. K.M. Warkentin, J. Touchon,

C.J. Schneider, R. Wassersug, R.A. Relyea and an anonymous referee made helpful comments that greatly improved the manuscript. This work was supported by grant PB96-0861 from Dirección General de Investigación Científica y Técnica conceded to M. Tejedo and predoctoral grant AP97 05421411 from the Dirección General de Enseñanza Superior e Investigación Científica to I. Gomez-Mestre. Thanks also to Consejería de Medio Ambiente de la Junta de Andalucía, and especially to B. Moreno and the staff of the Reservas Naturales de las Zonas Húmedas del Sur de Córdoba, and the Reserva Biológica de Doñana, for providing the corresponding permits and facilities.

## References

- Alberch, P. 1987. Evolution of the developmental process: irreversibility and redundancy in amphibian metamorphosis. In: *Development as an Evolutionary Process* (R. Raff & E. Raff, eds), pp. 23–46. Liss, New York.
- Allison, P.D. 1995. *Survival Analysis Using the SAS System. A Practical Guide*. SAS Institute, Cary, NC.
- Amundson, R. 1996. Historical development of adaptation. In: *Adaptation* (M. R. Rose & G. V. Lauder, eds), pp. 11–53. Academic Press, San Diego, CA.
- Anderson, T.W. 1958. *An Introduction to Multivariate Statistical Analysis*. John Wiley & Sons, New York.
- Andrews, P.W., Gangestad, S.W. & Matthews, D. 2002. Adaptationism – how to carry out an exaptationist program. *Behav. Brain Sci.* **25**: 489–553.
- Armbruster, W.S. 1993. Evolution of plant pollination systems: hypotheses and tests with the neotropical vine *Dalechampia*. *Evolution* **47**: 1480–1505.
- Armbruster, W.S. 1994. Early evolution of *Dalechampia* (Euphorbiaceae): insights from phylogeny, biogeography, and comparative ecology. *Ann. Mo. Bot. Gard.* **81**: 302–316.
- Armbruster, W.S. 1997. Exaptations link evolution of plant-herbivore and plant-pollinator interactions: a phylogenetic inquiry. *Ecology* **78**: 1661–1672.
- Arnold, E.N. 1994. Investigating the origins of performance advantage: adaptation, exaptation and lineage effects. In: *Phylogenetics and Ecology* (P. Eggleton & R. Vane-Wright, eds), pp. 123–168. The Linnean Society of London, Academic Press, London.
- Babcock, S.K. & Blais, J.L. 2001. Caudal vertebral development and morphology in three salamanders with complex life cycles (*Ambystoma jeffersonianum*, *Hemidactylium scutatum*, and *Desmognathus ocoee*). *J. Morphol.* **247**: 142–159.
- Balinsky, J. 1981. Adaptation of nitrogen metabolism to hypertonic environment in Amphibia. *J. Exp. Zool.* **215**: 335–350.
- Balinsky, J.B., Dicker, S.E. & Elliott, A.B. 1972. The effect of long-term adaptation to different levels of salinity on urea synthesis and tissue amino acid concentrations in *Rana cancrivora*. *Comp. Biochem. Physiol.* **43B**: 71–82.
- Baum, D.A. & Larson, A. 1991. Adaptation reviewed: a phylogenetic methodology for studying character macroevolution. *Syst. Zool.* **40**: 1–18.
- Beebee, T.J.C. 1985. Salt tolerances of natterjack toad (*Bufo calamita*) eggs and larvae from coastal and inland populations in Britain. *Herpetol. J.* **1**: 14–16.

- Beebee, T.J.C. & Rowe, G. 2000. Microsatellite analysis of natterjack toad *Bufo calamita* Laurenti populations: consequences of dispersal from a Pleistocene refugium. *Biol. J. Linn. Soc.* **69**: 367–381.
- Berven, K.A. & Gill, D.E. 1983. Interpreting geographic variation in life history traits. *Am. Zool.* **23**: 85–97.
- Blouin, M. & Brown, S.T. 2000. Effects of temperature-induced variation in anuran larval growth rate on head width and leg length at metamorphosis. *Oecologia* **125**: 358–361.
- Blouin, M.S. & Loeb, L.G. 1991. Effects of environmentally induced development-rate variation on head and limb morphology in the green tree frog, *Hyla cinerea*. *Am. Nat.* **138**: 717–728.
- Carroll, S.B., Grenier, J.K. & Weatherbee, S.D. 2001. *From DNA to Diversity*. Blackwell Science, London.
- Cheverud, J.M. 1984. Quantitative genetics and developmental constraints on evolution by selection. *J. Theor. Biol.* **110**: 155–171.
- Cheverud, J.M., Rutledge, J.J. & Atchley, W.R. 1983. Quantitative genetics of development: genetic correlations among age-specific trait values and the evolution of ontogeny. *Evolution* **37**: 895–905.
- Deban, S.M. & Marks, S.B. 2002. Metamorphosis and evolution of feeding behaviour in salamanders of the family *Plethodontidae*. *Zool. J. Linn. Soc.* **134**: 375–400.
- Dole, J.W., Rose, B.B. & Baxter, C.F. 1985. Hyperosmotic saline environment alters feeding behavior in the Western toad, *Bufo boreas*. *Copeia* **1985**: 645–648.
- Dole, J.W., Palmer, B.D. & Rose, B.B. 1994. The effect of hyperosmotic stress on tongue extension in the Western toad, *Bufo boreas*. *J. Herpetol.* **28**: 261–264.
- Ebenman, B. 1992. Evolution in organisms that change their niches during their life cycle. *Am. Nat.* **139**: 990–1021.
- Emerson, S.B., Travis, J. & Blouin, M. 1988. Evaluating a hypothesis about heterochrony: larval life-history traits and juvenile hind-limb morphology in *Hyla crucifer*. *Evolution* **42**: 68–78.
- Endler, J.A. 1986. *Natural Selection in the Wild*. Princeton University Press, Princeton, NJ.
- Endler, J.A. & McLellan, T. 1988. The process of evolution: towards a newer synthesis. *Ann. Rev. Ecol. Syst.* **19**: 395–421.
- Estrela, T., Marcuello, C. & Iglesias, A. 1996. *Water resources problems in Southern Europe*. European Environment Agency, Copenhagen, Denmark. Topic Report No 15/1996.
- Goater, C.P. 1994. Growth and survival of postmetamorphic toads: interactions among larval history, density and parasitism. *Ecology* **75**: 2264–2274.
- Gomez-Mestre, I. & Tejedo, M. 2003. Local adaptation of an anuran amphibian to osmotically stressful environments. *Evolution* **57**: 1889–1899.
- Gomez-Mestre, I. & Tejedo, M. 2004. Contrasting patterns of quantitative and neutral genetic variation in locally adapted populations of the natterjack toad, *Bufo calamita*. *Evolution* **58**: 2343–2352.
- Gomez-Mestre, I., Tejedo, M., Ramayo, E. & Estepa, J. 2004. Developmental alterations and osmoregulatory physiology of a larval anuran under osmotic stress. *Physiol. Biochem. Zool.* **77**: 267–274.
- Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**: 183–190.
- Gould, S.J. 2002. *The Structure of Evolutionary Theory*. The Belknap Press of Harvard University Press, Cambridge, MA.
- Gould, S.J. & Lewontin, R.C. 1979. The spandrels of San Marco and the Panglossian Paradigm: a critique of the adaptationist programme. *Proc. R. Soc. Lond.* **205**: 581–598.
- Gould, S.J. & Vrba, E.S. 1982. Exaptation: a missing term in the science of form. *Paleobiology* **8**: 4–15.
- Harvey, P.H. & Pagel, M. 1991. *The Comparative Method in Evolutionary Biology*. Oxford University Press, Oxford.
- Hoffman, J. & Katz, U. 1997. Salt and water balance in the toad *Bufo viridis* during recovery from two different osmotically stressful conditions. *Comp. Biochem. Physiol.* **117A**: 147–154.
- Huynh, H. & Feldt, L.S. 1976. Estimation of the box correction for degrees of freedom from sample data in the randomized block and split plot designs. *J. Educ. Stat.* **1**: 69–82.
- Kardon, G. 1998. Evidence from the fossil record of an antipredatory exaptation: conchiolin layers in corbulid bivalves. *Evolution* **52**: 68–79.
- Katz, U. & Hoffman, J. 1990. Changing plasma osmolality – a strategy of adaptation of anuran Amphibia to water scarcity under burrowing conditions. In: *Biology and Physiology of Amphibians* (W. Hanke, ed.), pp. 351–356. G. Fischer Verlag, Stuttgart.
- Ketterson, E.D. & Nolan, V. 1999. Adaptation, exaptation, and constraint: a hormonal perspective. *Am. Nat.* **154**: S4–S25.
- Larson, A. & Losos, J.B. 1996. Phylogenetic systematics of adaptation. In: *Adaptation* (M. R. Rose & G. V. Lauder, eds), pp. 187–220. Academic Press, San Diego, CA.
- Lauder, G.V. 1996. The argument from design. In: *Adaptation* (M. R. Rose & G. V. Lauder, eds), pp. 55–91. Academic Press, San Diego, CA.
- Moran, N.A. 1994. Adaptation and constraint in the complex life cycles of animals. *Ann. Rev. Ecol. Syst.* **25**: 573–600.
- Newman, R.A. & Dunham, A.E. 1994. Size at metamorphosis and water loss in a desert anuran (*Scaphiopus couchii*). *Copeia* **2**: 372–381.
- Owings, D.H., Rowe, M.P. & Rundus, A.S. 2002. The rattling sound of rattlesnakes (*Crotalus viridis*) as a communicative resource of ground squirrels (*Spermophilus beecheyi*) and burrowing owls (*Athene cunicularia*). *J. Comp. Psychol.* **116**: 197–205.
- Packard, G.C. 1991. Physiological and ecological importance of water to embryos of oviparous reptiles. In: *Egg Incubation. Its Effects on Embryonic Development in Birds and Reptiles* (D. C. Deeming & M. W. J. Ferguson, eds), pp. 213–228. Cambridge University Press, Cambridge.
- Packard, G.C., Packard, M.J., Miller, K. & Boardman, T.J. 1987. Influence of moisture, temperature, and substrate on snapping turtle eggs and embryos. *Ecology* **68**: 983–993.
- Pellmyr, O. 1997. Pollinating seed eaters: why is active pollination so rare? *Ecology* **78**: 1655–1660.
- Phillips, P.C. 1998. Genetic constraints at the metamorphic boundary: morphological development in the wood frog, *Rana sylvatica*. *J. Evol. Biol.* **11**: 453–463.
- Pough, F.H. & Kamel, S. 1984. Post-metamorphic change in activity metabolism of anurans in relation to life history. *Oecologia* **65**: 138–144.
- Price, T. & Langen, T. 1992. Evolution of correlated characters. *Trends Ecol. Evol.* **7**: 307–310.
- Reeve, H.K. & Sherman, P.W. 1993. Adaptation and the goals of evolutionary research. *Q. Rev. Biol.* **68**: 1–32.
- Relyea, R.A. 2001. The lasting effects of adaptive plasticity: predator-induced tadpoles become long-legged frogs. *Ecology* **82**: 1947–1955.



- Relyea, R.A. 2002. Competitor-induced plasticity in tadpoles: consequences, cues, and connections to predator-induced plasticity. *Ecol. Monogr.* **72**: 523–540.
- Relyea, R.A. & Hoverman, J.T. 2003. The impact of larval predators and competitors on the morphology and fitness of juvenile treefrogs. *Oecologia* **134**: 596–604.
- Reques, R. & Tejedo, M. 1997. Reaction norms for metamorphic traits in natterjack toads to larval density and pond duration. *J. Evol. Biol.* **10**: 829–851.
- Roff, D.A. 1989. Exaptation and the evolution of dealation in insects. *J. Evol. Biol.* **2**: 109–123.
- SAS Institute 1999. *SAS/STAT Software User's Guide*. Release 8.00. SAS Institute, Cary, NC.
- Schlichting, C.D. & Pigliucci, M. 1998. *Phenotypic Evolution. A Reaction Norm Perspective*. Sinauer Associates, Sunderland, MA.
- Shaffer, H.B., Austin, C.C. & Huey, R.B. 1991. The consequences of metamorphosis on salamander (*Ambystoma*) locomotor performance. *Physiol. Zool.* **64**: 212–231.
- Sinervo, B. & Basolo, A.L. 1996. Testing adaptation using phenotypic manipulations. In: *Adaptation* (M. R. Rose & G. V. Lauder, eds), pp. 149–185. Academic Press, San Diego, CA.
- Sinsch, U., Seine, R. & Sherif, N. 1992. Seasonal changes in the tolerance of osmotic stress in natterjack toads (*Bufo calamita*). *Comp. Biochem. Physiol.* **101A**: 353–360.
- Tejedo, M., Semlitsch, R.D. & Hotz, H. 2000. Covariation of morphology and jumping performance in newly metamorphosed water frogs: effects of larval growth history. *Copeia* **2000**: 448–458.
- Wassersug, R.J. 1975. The adaptive significance of the tadpole stage with comments on the maintenance of complex life cycles in amphibians. *Am. Zool.* **15**: 405–417.
- Watkins, T.B. 2001. A quantitative genetic test of adaptive decoupling across metamorphosis for locomotor and life-history traits in the Pacific tree frog, *Hyla regilla*. *Evolution* **55**: 1668–1677.
- Williams, G.C. 1966. *Adaptation and Natural Selection*. Princeton University Press, Princeton, NJ.

Received 14 September 2004; revised 19 November 2004; accepted 21 November 2004