

Effects of phenotypic plasticity on post-metamorphic traits during pre-metamorphic stages in the anuran *Pelodytes punctatus*

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ABSTRACT

Question: In organisms with a complex life cycle, are stages phenotypically coupled or does metamorphosis break all developmental links?

Hypothesis: For those organisms with developmental phenotypic plasticity, if metamorphosis does not break all developmental links, then changes in juvenile performance will occur as a cost of phenotypic plasticity.

Organism: The anuran *Pelodytes punctatus* from the north-east Iberian Peninsula.

Methods: Two experimental treatments: (1) constant water level and (2) drying treatment. Larvae phenotypic plasticity was measured using morphological and life-history (time to and mass at metamorphosis) traits. After metamorphosis, toadlet morphology and its jump capacity were measured in both treatments.

Results: Tadpoles in the drying treatment accelerated metamorphosis and reached this stage with a lower body mass. They also showed a reduced tail fin during the larval phase. Toadlets in the drying treatment showed shorter and less muscular hind limbs and a reduced jump capacity compared with individuals in the constant water treatment independently of time of development.

Keywords: habitat desiccation, metamorphosis, morphology, phenotypic plasticity, tadpoles.

INTRODUCTION

Amphibians have a complex life-history strategy (Wilbur, 1980). Organisms with a series of discrete, free-living states might be expected to possess more morphological adaptations than taxa with simple life histories, particularly when successive stages occur in radically distinct environments (Hanken, 1992). For anurans, a change in selective environment during the life cycle is accompanied by metamorphosis (Wilbur, 1980). This process is commonly thought to be beneficial because it breaks the genetic and developmental relationships between traits expressed at distinct stages (Ebenman, 1992; Hanken, 1992; Moran, 1994). Thus, the

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adaptive decoupling hypothesis (Moran, 1994) may allow traits designed for a given function to evolve independently in different life stages (Moran, 1994), thereby allowing pre- and post-metamorphic stages to adapt independently to their respective environments. This hypothesis is supported by experimental studies on developmental compartmentalization (Alberch, 1987; Parichy, 1998), which show that adult structures may arise *de novo* from embryonic cells that remain undifferentiated until metamorphosis and not as modifications of pre-existing larval structures. However, many post-metamorphic features arise from the re-patterning of larval precursors (Alley, 1989; Alley and Omerza, 1999).

In a recent study, Watkins (2001) examined genetic correlations between equivalent larval and adult characters (locomotor performance) in the Pacific tree frog *Hyla regilla*, and found greater phenotypic correlations between than within phases. These results do not provide support for the adaptive decoupling hypothesis for the traits measured (Watkins, 2001). In addition, correlations between larval tail phenotype and adult limb morphology have been reported (Van Buskirk and Saxer, 2001). Other trade-offs between phases can occur between functionally unrelated traits as a result of developmental or physiological constraints (Álvarez and Nicieza, 2002). For example, accelerated or retarded differentiation from anuran larvae as a consequence of water temperature or food availability affects juvenile morphology (Emerson, 1986; Emerson *et al.*, 1988; Tejedo *et al.*, 2000; M. Tejedo, M.J. Sánchez-Herráiz and C. Pertoldi, unpublished), energy reserves and jump performance (Tejedo *et al.*, 2000; Álvarez and Nicieza, 2002). Studies of other organisms with complex life cycles (barnacles, copepods and insects) show similar effects of larval history on later life stages (reviewed by Pechenik *et al.*, 1998). The correlations between phases indicate that the adaptive larval phenotype is constrained so as to preserve juvenile functionality but has a low impact on adult fitness. This fact has important implications for the study of adaptation in organisms with complex life cycles (Deban and Marks, 2002).

Here we studied ontogenetic trade-offs in the development of anuran plasticity in response to drying ponds. In temperate areas, anuran reproduction frequently occurs in unpredictable freshwater habitats that vary in duration. An unpredictable habitat, and the fact that larvae cannot move to an alternative environment, provide the cues for plasticity during this stage (Doughty and Reznick, 2004).

In response to a drying pond, the larvae of some species exhibit accelerated developmental rates at the expense of growth in order to metamorphose before the water is lost (e.g. Newman, 1989; Denver, 1997; Morey and Reznick, 2004). The benefit of accelerated development under these conditions is clear, but there must also be a trade-off, otherwise natural selection would presumably quickly render the larval phase redundant (Lane and Mahony, 2002). Although anuran plasticity has been addressed in several studies, few have focused on the potential costs of plastic responses because after several years in the adult form costs cannot be detected. Fitness reversal is normally interpreted based on indirect support from other studies (Doughty and Reznick, 2004). Smaller metamorphs that result from accelerated development show reduced survivorship to maturity (Goater, 1994; Newman and Dunham, 1994) and smaller size at maturity (Smith, 1987). The direct costs of larval plasticity on tadpole growth rate (Van Buskirk, 2000; Relyea, 2002), and on froglet morphology and mortality in their first terrestrial period after metamorphosis, have been studied previously (e.g. Van Buskirk, 2000; Van Buskirk and Saxer, 2001; Lane and Mahony, 2002; Relyea and Hoverman, 2003).

Because anuran larval plasticity is expressed in many other forms (behavioural plasticity and morphological modifications) and these changes could improve performance at metamorphosis (Tejedo *et al.*, 2000; Van Buskirk and Saxer, 2001; Álvarez and Nicieza, 2002), it is of interest to examine the links between larval response to the environment and their possible effects

on froglet morphology. Morphology related to locomotor performance and the level of energy reserves are probably crucial for the determination of froglet survival and early growth (Pfennig, 1992; Álvarez and Nicieza, 2002).

We studied the plasticity response of *Pelodytes punctatus* (Daudin 1803) to habitat dehydration and the most direct cost after metamorphosis. We designed a laboratory experiment to evaluate whether pond drying promotes phenotypic plasticity in larval morphology and in life-history traits. We also analysed the effect of pond desiccation on juvenile morphology and jumping performance to establish whether there is a link between larval response to treatments and metamorphic consequences after metamorphosis.

MATERIALS AND METHODS

Experimental procedures

We used three clutches of *Pelodytes* collected from an ephemeral rain pond in Garraf (30 km south of Barcelona, Spain) on the same day in March 2002. Egg masses hatched in outdoor buckets and all experiments were started when tadpoles reached Gosner's stage 25. The effect of pond drying was analysed in two treatments: a constant treatment and a drying treatment. The former simulated a permanent pond without changes in water volume during tadpole development, and had a larval density of three individuals (one individual from each clutch) per two liters [a density similar to that observed in natural ponds (A. Richter-Boix, unpublished data)]. In contrast, the drying treatment simulated a temporal pond by reducing water volume during larvae development. The reduction in water level followed the curve $D_j = 1 - (j/t)^a P$ defined by Wilbur (1987), where D_j is the desired depth on day j , t is the target day for depth = 0 (110 in our case, the mean of temporary pond duration in our area of study), a is a shape parameter (0.4 in our treatment), and P is the depth at the start of the experiment. The two treatments were replicated 50 times, with a total of 100 experimental units (300 tadpoles) arranged in a random fashion.

Experimental units consisted of plastic boxes filled with 2 litres of dechlorinated tap water. To reduce the probability of infection and fouling, the water was changed every 12 days. In the drying treatment, we adjusted the water level every 4 days following the planned drying curves. The two treatments were performed under the same natural light and photoperiod, without thermal control, in laboratories at the University of Barcelona. Tadpoles were fed periodically (approximately every 2 days) with a mixture (4:1) of rabbit chow and fish food *ad libitum*.

Estimating the response of larvae to drying

Larval phenotypic plasticity was measured at two levels: morphological and life history. Twelve randomly selected experimental units were taken from each treatment on day 25 and all tadpoles were killed and preserved in 4% formalin for later morphological measurements. All the tadpoles in these selected experimental tubs were weighed to a precision of 0.001 g and their Gosner development stage was determined. We also made a total of six linear measures of traits that exhibit plasticity in other ecological contexts (e.g. Van Buskirk and Saxer, 2001; Relyea, 2002): two related to the body (body length and body depth), two to the tail musculature (tail musculature length and tail musculature depth) and two to the tail fin (tail fin length and tail fin depth) (Fig. 1A).

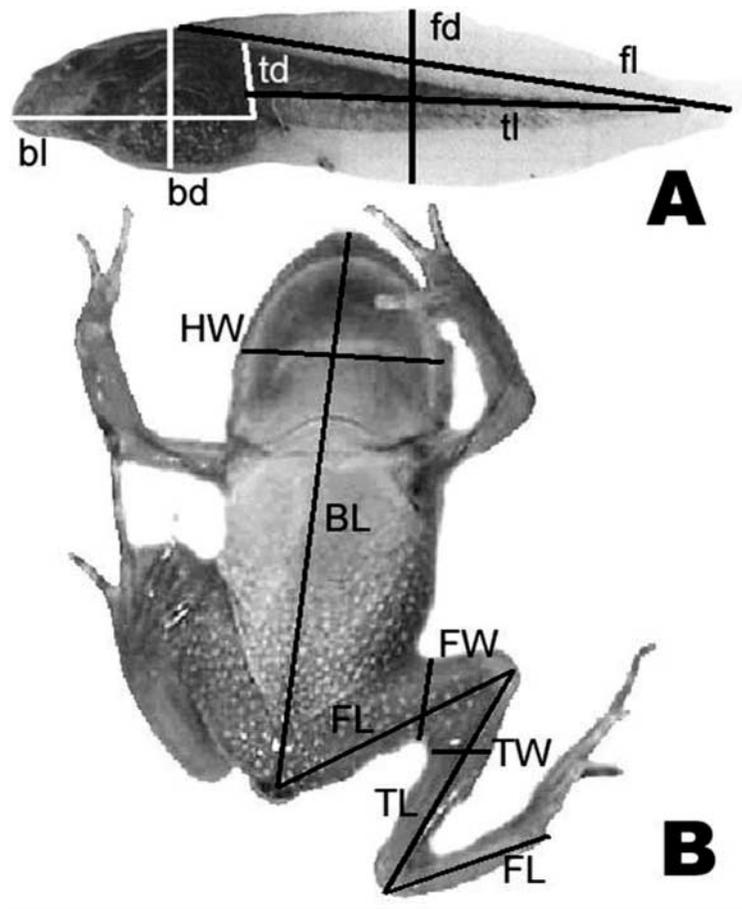


Fig. 1. Tadpole (A) and toadlet (B) morphological measures. The six traits for tadpoles are: bl = body length; bd = body depth; tl = musculature tail length; td = musculature tail depth; fl = tail fin length; fd = tail fin depth. The seven traits for toadlets are: BL = body length; HW = head body width; FL = femur length; FW = muscular femur width; TL = tibia-fibula length; TW = tibia-fibula width; FL = foot length.

The remaining experimental units (38 per treatment) were maintained until individuals completed metamorphosis. We measured mass at metamorphosis, time to metamorphosis and survival to metamorphosis. Time to metamorphosis was measured at Gosner's stage 42 at the time of collection. Survival was expressed as the proportion of larvae per tub that completed development.

Estimating metamorph response to drying

When metamorphosing tails were fully resorbed (stage 45), tadpoles were weighed to a precision of 0.001 g. We made seven measurements on the body and hind legs (Fig. 1B). Measures related to the legs were taken on both sides and the average was used for analysis.

All the limb measurements are associated with jumping performance (Peters, 1994), and thus may affect metamorphic survival.

Finally, we measured the hopping performance of toadlets one day after they reached stage 46. Toadlets were placed at the centre of a table (0.8×0.6 m) and allowed to hop repeatedly until they showed signs of fatigue. Generally, they jumped without any stimulation but when necessary they were prodded to induce an escape response. For each toadlet, we marked successive landing positions and later measured the distances between pairs of landmarks. We analysed total distance covered (sum of all distances) and length of the longest jump.

Statistical analyses

We studied the effects of the treatments on life-history parameters, such as larval period, mass at metamorphosis and survival, by a multivariate analysis of variance, with subsequent separate univariate analyses of variance for each parameter. Measures of larval period, development stage and mass at metamorphosis were log-transformed to improve normality and homogeneity of variances. As survival was expressed as a proportion, these data were arcsine-transformed before analysis. In all analyses, we used the box mean values to avoid pseudoreplication.

Before morphological analysis, tadpole and toadlet measures were corrected for variation in body size. To generate size-corrected measures, we used the residuals of the morphological measures of log-transformed traits after regressions against body size. Total tadpole length is not a good measure of body size because it is mostly a measure of tail length (Van Buskirk, 2002). We therefore used centroid size, obtained from landmarks (Loy *et al.*, 1993). Coordinates of these landmarks were collected using the TPSDIG computer program, version 1.30 (Rohlf, 2001). The centroid size, the square root of the sum of squared distances of a set of landmarks from their centroid (Bookstein, 1991), was calculated for each specimen and used to represent size. After performing this correction, tadpole morphology was assessed first with multivariate analysis for all traits together and then with a univariate analysis for each variable.

In the case of toadlets, we worked with the residuals of all morphometric measures with respect to their log-transformed individual body mass at metamorphosis. As the morphological traits of toadlets could be influenced by development time, we used time to metamorphosis as the covariate in all analyses. After multivariate analysis of covariance, individual analyses of covariance for individual traits were performed. Given our focus on absolute and size-independent hopping performance, this trait was analysed twice. One analysis was done with residuals with respect to body mass condition and the other without.

At the end of the experiment, we detected unequal replication because of 100% mortality in some experimental units. We therefore used type III sum of squares in all analyses of variance and covariance.

RESULTS

Response of larvae to drying

The multivariate responses of developmental variables differed ($\lambda = 0.7563$; $F_{3,60} = 6.442$, $P = 0.00745$). Drying had a significant effect on two metamorphic responses: larval period

and mass at metamorphosis ($F_{1,63} = 5.57$, $P = 0.0213$ and $F_{1,62} = 13.926$, $P = 0.0004$ respectively). Individuals in the drying treatment had shorter larval periods and smaller sizes at metamorphosis than those in the constant treatment. However, the treatments did not differ in tadpole survival (61.12 ± 23.08 vs. 71.04 ± 19.19 for the constant and drying treatment, respectively) ($F_{1,74} = 2.243$, $P = 0.1380$). Correlation analysis between mass at metamorphosis and larval period showed a low but positive and significant relationship for the constant treatment ($F_{1,36} = 4.8781$, $P = 0.03365$; $R^2 = 0.1193$), whereas it was non-significant for the drying treatment ($F_{1,24} = 0.0027$, $P = 0.9871$; $R^2 = 0.00001$) (Fig. 2).

Tadpoles measured at day 25 did not differ in their development stage ($Z_{22} = 1.433$, $U = 47$, $P = 0.1489$; Mann-Whitney U -test), but differences were detected in their mass ($t_{22} = -2.6817$, $P = 0.0136$). Larvae in the constant treatment were larger than those in the drying treatment. In addition, the size-independent morphological traits of tadpoles differed in the two treatments (Table 1). The greatest changes were in the tail fin parameters. Tadpoles in the drying treatment showed shorter and shallower tail fins than controls, but no differences in measures related to tail musculature were detected between the two groups (Fig. 3).

Metamorph morphology and hopping performance

The morphological features of the toadlets that metamorphosed in the constant and drying treatments differed independently of larval period, with no effect of time to metamorphosis on toadlet morphology (Table 2). Analyses of covariance showed that proximal leg traits (femur and tibia-fibula) were shorter in individuals in the drying treatment and that these had a relative lower width (Table 2, Fig. 4). The distal leg trait (foot) did not show

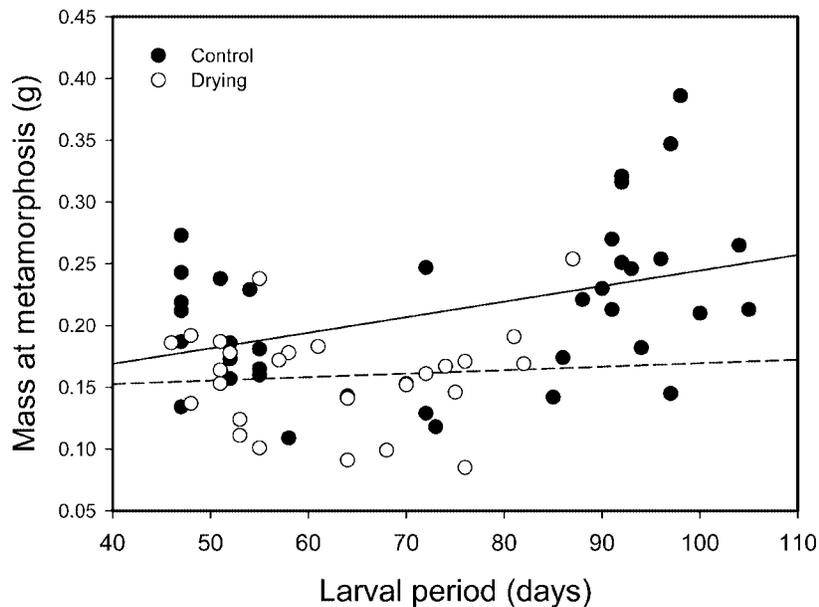


Fig. 2. Relationship between larval period and mass at metamorphosis for the two experimental treatments.

Table 1. Results of multivariate (MANOVA) and univariate (ANOVA) analyses of variance for morphological traits of tadpoles at day 25 of the experiment

Analysis	Dependent variable	Wilks' λ	d.f.	MS effect	F	P
MANOVA		0.0187	6, 17		148.6842	<0.0001
ANOVA	Body length		1, 22	0.00032	6.0860	0.02188
	Body depth		1, 22	0.00052	2.5312	0.12588
	Tail length		1, 22	0.00001	0.3968	0.53520
	Tail depth		1, 22	0.00007	2.1230	0.15922
	Tail fin length		1, 22	0.0130	136.9564	<0.0001
	Tail fin depth		1, 22	0.0921	475.328	<0.0001

Note: All variables were log transformed and regressed to their centroid size as a body size measure before analysis.

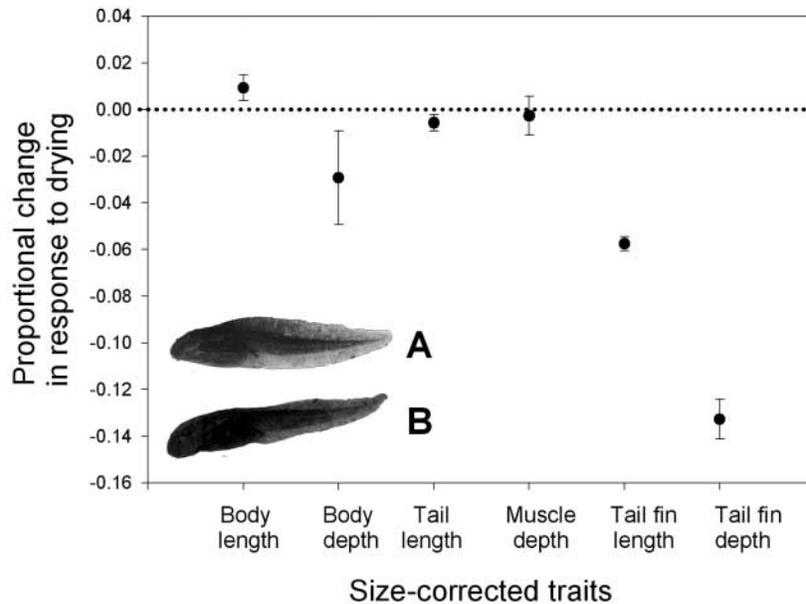


Fig. 3. Proportional change in shape of tadpoles reared in the dry treatment, relative to tadpoles in the control treatment. (A) Constant treatment tadpole and (B) drying treatment tadpole. The dashed line shows when the trait was identical to the control. All traits are residuals after regression against centroid size. Bars show standard error of the mean.

differences between treatments. The magnitude of these differences was 0.90% for the femur and 1.12% for the tibia-fibula. In addition, toadlets in the drying treatment had greater head widths than those in the constant treatment.

There were small but significant differences in absolute jumping capacity between treatments ($\lambda = 0.9015$, $F_{2,62} = 3.384$, $P = 0.0403$). Toadlets in the constant treatment jumped further (6%) and covered greater distances (22.5%) than those in the drying treatment. This was because the former were larger. These differences disappeared after correction for body mass ($\lambda = 0.9735$, $F_{2,62} = 0.8281$, $P = 0.4416$).

Table 2. Results of multivariate (MANCOVA) and univariate (ANCOVA) analyses of covariance for morphological traits of toadlets at metamorphosis

Analysis	Factor	Dependent variable	Wilks' λ	d.f.	MS effect	<i>F</i>	<i>P</i>
MANCOVA	Treatment		0.2828	7, 54		19.5552	<0.0001
	Covariate		0.8214	7, 54		1.6768	0.1343
ANCOVA		Body length		1, 61	0.0176	3.8246	0.0551
		Head width		1, 61	0.1724	50.8041	<0.0001
		Femur length		1, 61	0.1443	26.6134	<0.0001
		Femur width		1, 61	0.2911	78.0455	<0.0001
		Tibia-fibula length		1, 61	0.0524	13.3775	<0.0005
		Tibia-fibula width		1, 61	0.3147	48.6931	<0.0001
		Foot length		1, 61	0.0129	1.1953	0.2786

Note: Time to metamorphosis was used as the covariate in all cases. All variables were log transformed and regressed to body mass at metamorphosis before analysis.

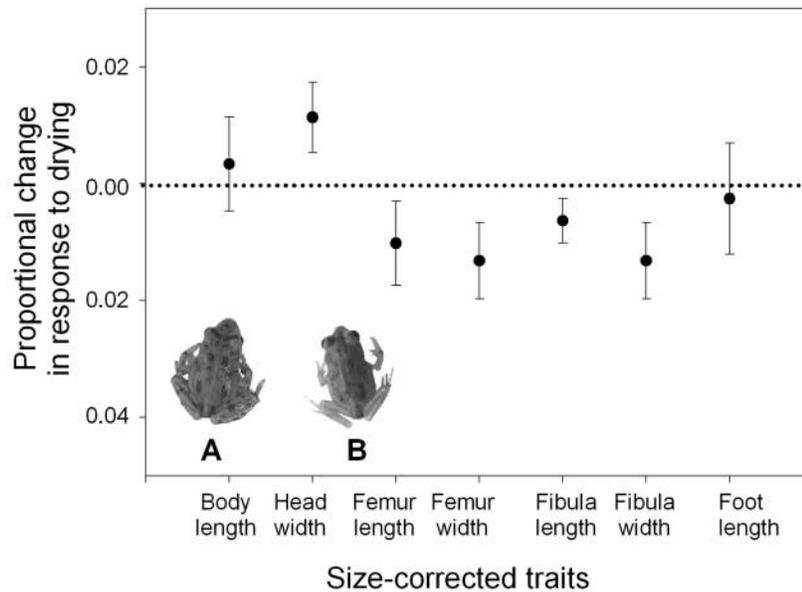


Fig. 4. Proportional change in morphology of toadlets reared in the drying treatment, relative to toadlets in the control treatment. (A) Constant treatment toadlet and (B) drying treatment toadlet. The dashed line shows when the trait was identical to the control. All traits are residuals after regression against mass at metamorphosis. Bars show standard error of the mean.

DISCUSSION

Our results indicate that *Pelodytes punctatus* shows a plasticity response to drying conditions that affects toadlet morphology. However, few delayed consequences of these reactions were detected just after metamorphosis.

Life-history phenotypic plasticity related to drying ponds is well documented (e.g. Newman, 1992; Denver, 1997; Denver *et al.*, 1998). Several amphibian species accelerate metamorphosis in

response to habitat desiccation (Wilbur, 1987; Crump, 1989; Newman, 1989; Denver, 1997; Laurila and Kujasalo, 1999; Morey and Reznick, 2004). This accelerated development in dry conditions could be considered an adaptive trait that allows tadpoles to escape from a degrading environment and avoid mortality caused by water scarcity (Doughty and Reznick, 2004). Our results show that tadpole growth rate decreases and development rate increases as ponds dry, thereby resulting in smaller size at metamorphosis, as reported for other species (Newman, 1989; Denver, 1997).

However, although the life-history response to a drying environment appears to be a frequent event, to our knowledge ours is the first study to address the morphological plasticity of tadpoles in response to habitat desiccation. A field study with *Rana temporaria* showed that tadpoles in small temporary ponds had relatively smaller tail fins and narrower bodies than those in large ponds (Vences *et al.*, 2002). In our case, the tadpoles in the drying treatment showed a particular phenotype, which is similar and in line with this previous study. The morphological differences between treatments, independently of developmental rate, indicate that these traits may be the result of drought stress, and not a consequence of a high development rate.

We propose that the tail fin becomes obsolete for tadpoles that maintain a position in the mid-water column in shallow waters, like *Pelodytes* (Díaz-Paniagua, 1987; Richter-Boix *et al.*, 2004). In these organisms, the tail fin contributes most to maintaining anterior stability in the mid-water column, which disappears in drying ponds (Wassersug and Hoff, 1985; Hoff and Wassersug, 1986). Tadpoles of diverse species living in ephemeral pools show a similar morphology, with an elongated and finless tail and a depressed body (Altig and McDiarmid, 1999).

An alternative explanation for the adaptive significance of this morphology is that a tail without a fin can be resorbed faster by tadpoles during metamorphosis, as proposed by Van Buskirk and Saxer (2001). Downie *et al.* (2004) reported a significant correlation between metamorphic duration and tail length for several frog species. The time to metamorphosis is likely to be dependent on the amount of tissue to be transformed (Van Buskirk and Saxer, 2001).

We detected morphological variations induced by drought not only during the larval phase but also as a carry-over effect in metamorphs. Differences in growth rate can produce changes in head width and leg length at metamorphosis (Emerson, 1986; Newman, 1989; Blouin and Brown, 2000; M. Tejedo, M.J. Sánchez-Herráiz, C. Pertoldi, A. Richter-Boix, A. Nicieza and I. Gómez-Mestre, unpublished). Newman (1989) reported toadlets with shorter legs in conditions of short pond duration compared with individuals in less ephemeral habitats. Correlations between shorter legs, a low development rate and several stress factors during larval development (density effects, food availability, predation risk, pond duration) were observed in another study (M. Tejedo, M.J. Sánchez-Herráiz and C. Pertoldi, unpublished). In that study, Tejedo *et al.* showed that *Pelodytes punctatus*, which shows a faster developmental rate, produces toadlets with longer legs. A faster developmental rate was seen in animals fed *ad libitum* compared with those subjected to restricted food availability. In both studies, *Pelodytes* raised under stress resulted in toadlets with shorter legs. However, we observed differences in toadlet morphology as a function of treatment but not larval period. Therefore, we hypothesize that toadlet constitution at metamorphosis depends directly, in part, on the tadpole morphology induced by the drying treatment.

This argument was offered by Van Buskirk and Saxer (2001) in a study in which tadpole predator-induced morphology resulted in froglets with distinct morphological traits. Tadpoles exposed to predators showed deeper and longer tail fins, a common tadpole response to predation (e.g. Van Buskirk, 2002), and more muscular legs. Similarly, *Rana sylvatica* froglets exposed to a predator had longer legs (Relyea, 2001). In our case, shorter and shallower

finned tadpoles gave rise to individuals with legs that were shorter and had a low muscular mass. Part of the mass accumulated in the tail when resorbed during metamorphosis is reinvested in new structures and tissues (Hourdry and Beaumont, 1985), possibly leg tissues. Similarly, Watkins (2001) showed larger phenotypic correlations for locomotor traits between phases (tadpole/froget) than within phases. These studies demonstrate that anuran metamorphosis does not break the developmental relationship between the traits evaluated at distinct stages, and that larval and adult traits are not entirely independent of evolution (Pechenik *et al.*, 1998). Thus, juvenile morphology may be partially constrained by tadpole morphology (Van Buskirk and Saxer, 2001) and vice versa. Larval phenotypic plasticity may be constrained to originate a functional juvenile.

We observed low costs associated with jump performance, which is consistent with the findings of previous studies (Tejedo *et al.*, 2000; Van Buskirk and Saxer, 2001). Changes in the length of the hind limb are not sufficient to generate measurable consequences (Emerson, 1978). Small morphological differences lead to differences in absolute jumping performance, which could affect escape performance from predators. However, these distinct morphologies probably disappear over a few weeks during the juvenile stage due to compensatory growth, a phenomenon observed in *Pelobates cultripes*, *Pelodytes punctatus* and *P. ibericus* (M. Tejedo, I. Gómez-Mestre and F. Marangoni, unpublished).

Our results show low direct costs associated with the plastic response of tadpoles to pond desiccation. Fitness costs may appear later in the terrestrial stage and may be associated with juvenile size and survival. Accelerated metamorphosis or permanence in the larval habitat is a trade-off between size and shape at metamorphosis and the risk of mortality in the terrestrial habitat, especially when size at transformation is positively correlated with toadlet and adult fitness (Smith, 1987; Goater, 1994; Lane and Mahony, 2002; Relyea and Hoverman, 2003). The assumption that it is beneficial to remain in the aquatic habitat for as long as possible was proposed by Wilbur (1980), although it was questioned by Werner (1986). However, if growth rate is similar in the terrestrial and aquatic habitats, then accelerated metamorphosis does not compromise fitness, because the small early metamorphs stay longer in the terrestrial habitat and reach a similar size to later metamorphs (Loman and Claesson, 2003).

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