JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2011.02370.x

Evolutionary reduction of developmental plasticity in desert spadefoot toads

S. S. KULKARNI*, I. GOMEZ-MESTRE†, C. L. MOSKALIK*, B. L. STORZ‡ & D. R. BUCHHOLZ*

*Department of Biological Sciences, University of Cincinnati, Cincinnati, OH, USA
†Department of Wetland Ecology, Doñana Biological Station, CSIC, Spain
‡Department of Biology, Centre College, Danville, KY, USA

Keywords:

adaptation;
amphibians;
directional selection;
metamorphosis;
phenotypic plasticity.

Abstract

Organisms vary their rates of growth and development in response to environmental inputs. Such developmental plasticity may be adaptive and positively correlate with environmental heterogeneity. However, the evolution of developmental plasticity among closely related taxa is not well understood. To determine the evolutionary pattern of plasticity, we compared plasticity in time to and size at metamorphosis in response to water desiccation in tadpoles among spadefoot species that differ in breeding pond and larval period durations. Like most tadpoles, spadefoot tadpoles possess the remarkable ability to accelerate development in response to pond drying to avoid desiccation. Here, we hypothesize that desert spadefoot tadpoles have evolved reduced plasticity to avoid desiccation in ephemeral desert pools compared to their nondesert relatives that breed in long-duration ponds. We recorded time to and size at metamorphosis following experimental manipulation of water levels and found that desert-adapted species had much less plasticity in larval period and size at metamorphosis than nondesert species, which retain the hypothetical ancestral state of plasticity. Furthermore, we observed a correlation between degree of plasticity and fat body content that may provide mechanistic insights into the evolution of developmental plasticity in amphibians.

Introduction

Developmental plasticity is the ability of a given genotype to give rise to different phenotypes when reared in different environmental conditions (Pigliucci, 1996, 2001; Callahan *et al.*, 1997; Schlichting & Pigliucci, 1998; West-Eberhard, 2003). The degree of developmental plasticity is expected to correlate positively with environmental heterogeneity experienced by a population, providing increased survivorship in variable environments (Bradshaw, 1965; Moran, 1992; Schlichting & Pigliucci, 1998; West-Eberhard, 2003). Indeed, such correlations have been found when comparing degree of plasticity among species or populations (Van Tienderen, 1997; Pigliucci *et al.*, 1999; Merilä *et al.*, 2000a,b, 2004; Richter-Boix *et al.*, 2006; Lind & Johansson, 2007; De Block *et al.*, 2008). Lack of association between environmental heterogeneity and degree of plasticity is often attributed to costs and limits of plasticity, unreliability of environmental cues and/or lack of genetic variation for plasticity (Moran, 1992; Newman, 1992; Gavrilets & Scheiner, 1993; DeWitt, 1998; Schlichting & Pigliucci, 1998; Relyea, 2002; Merilä *et al.*, 2004). In certain cases, however, changes in degree of plasticity may be merely a consequence of the developmental changes occurring in response to selection on trait values within a single environment (Brady & Griffiths, 2000; Pigliucci & Murren, 2003; Pigliucci *et al.*, 2006). More empirical work is needed to elucidate how species differences in degree of plasticity evolve.

Correspondence: Daniel R. Buchholz, Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221, USA.
Tel.: +1 513 556 9725; fax: +1 513 556 5299;
e-mail: buchhodr@ucmail.uc.edu

To examine the evolution of developmental plasticity, we chose to compare the ability to accelerate development among spadefoot toad species in response to water level reduction. Amphibian larvae vary their developmental rate in response to changes in environmental conditions, consequently affecting time to and/or size at metamorphosis (Newman, 1987, 1988, 1989, 1994; Audo *et al.*, 1995; Relyea & Werner, 2000; Relyea, 2001; Álvarez & Nicieza, 2002; Altwegg & Reyer, 2003; Benard, 2004; Gomez-Mestre *et al.*, 2010). Water availability ranks among the most important environmental factors affecting amphibian development. Indeed, tadpoles respond to pond drying as a significant threat by accelerating development, thereby escaping death by desiccation but incurring smaller sizes that can have profound fitness consequences (Newman, 1988; Denver *et al.*, 1998; Loman, 1999; Altwegg & Reyer, 2003; Loman & Claesson, 2003).

The response of tadpoles of the New World spadefoot toad *Scaphiopus couchii* to pond drying has been used as a model for understanding phenotypic plasticity (Newman, 1987, 1988, 1989, 1994, 1998). *Scaphiopus couchii* shows developmental acceleration in response to decreasing water levels. *Scaphiopus couchii* is believed to be plastic in metamorphic timing due to great desiccation risk in deserts. However, no comparative studies in plasticity to pond desiccation have been performed in spadefoot toads to understand directionality of its evolution. Related studies suggest that *S. couchii* may actually have less plasticity in time to and size at metamorphosis compared to other spadefoot species. The first indication of reduced plasticity to pond duration in *S. couchii* came from showing lower plasticity in *Scaphiopus* compared to *Spea* in response to altered food levels, a potential cue for pond drying (Morey & Reznick, 2000, 2004). In addition, a comparative analysis using data from natural ponds to sketch the pattern of evolution of developmental plasticity suggested that *S. couchii* has the least plasticity in larval period (Gomez-Mestre & Buchholz, 2006).

Scaphiopus couchii is also known for having the shortest larval period among frogs; metamorphosis occurs in as little as 8 days (Newman, 1987). The evolution of short larval periods was likely an adaptation to avoid desiccation in short-lived desert pools (Buchholz & Hayes, 2002). The Old World spadefoot toad, *Pelobates cultripes*, breeds in long-lasting ponds, and its larval period in nature can last from 4 to 6 months to overwintering (Busack & Zug, 1976; Buchholz & Hayes, 2002). The larval periods and breeding pond durations for *Spea multiplicata* are intermediate between *S. couchii* and *P. cultripes* (Buchholz & Hayes, 2000, 2002; Morey & Reznick, 2004). Even though the split between *Scaphiopodidae* (*Scaphiopus* and *Spea*) and *Pelobatidae* (Old World genus, *Pelobates*) likely occurred > 80 Ma in the Cretaceous due to formation of the Atlantic Ocean (Sage *et al.*, 1982; Marjanovic & Laurin, 2007), the evolution of the dramatic differences in larval period among spadefoot species likely coincided with aridification of southwestern North America ~23 Ma, because selective pressure for reduced larval periods was likely lacking earlier, though larval period divergence prior to desert formation is not ruled out.

Phylogenetic analysis suggested that the ancestral level of developmental plasticity in spadefoot toads was likely to have been similar to that of extant *Pelobates* (Gomez-Mestre & Buchholz, 2006). Thus, Gomez-Mestre & Buchholz (2006) proposed that developmental acceleration in *Scaphiopus* was genetically accommodated from ancestral plasticity, i.e. ancestral plasticity might have enabled survival as the deserts gradually formed allowing subsequent selection on genotypes more likely to be capable of short larval periods. However, such genetic accommodation does not necessarily lead to an evolutionary reduction in the degree of plasticity, which may depend on the extent of environmental heterogeneity experienced by the descendant lineages, the existence of costs of plasticity and the existence of developmental constraints arising from the response to directional selection for trait values within the extreme environment (Pigliucci & Murren, 2003; West-Eberhard, 2003; Pigliucci *et al.*, 2006; Crispo, 2007).

Given the divergent larval periods among spadefoot toad species and the potential for independent evolution of larval period plasticity in the face of potential differences in pond duration variability experienced along ancestor/descendent lineages, we compared the ability to accelerate development in response to water reduction stress among spadefoot species. We hypothesized that New World species (*S. multiplicata* and *S. couchii*) would exhibit reduced plasticity in time to and size at metamorphosis compared to Old World species (*P. cultripes*), as they breed in consistently shorter lasting ponds. If so, such reduced plasticity would be a derived trait, relative to the predicted ancestral state of plasticity in the group resembled by *Pelobates* (Gomez-Mestre & Buchholz, 2006). To compare plasticity across species, we subjected New and Old World spadefoot toad tadpoles to two water treatments (constant high and constant low water levels) in identical laboratory conditions and measured time to and size at forelimb emergence (FLE, Gosner stage 42). Water treatments were conducted at two temperatures starting at three Gosner stages (Gosner, 1960).

In addition, we analysed variation in the size of fat bodies among and within species in response to water reduction to begin to address the physiological basis of altered plasticity across species. Previous studies have shown that tadpoles reduce food intake when exposed to low water stress (Denver *et al.*, 1998). Because reduced food intake in the low water treatment may result in smaller fat bodies and because shorter larval periods reduce the time for fat acquisition, we expected a reduction in fat bodies in response to low water conditions and we expected faster-developing species to have smaller fat bodies compared to slower-developing species.

Materials and methods

Collection, animal care and breeding of adults

Adults of *S. couchii* and *S. multiplicata* were collected from SE Arizona and SW New Mexico in the summer of 2007. Six clutches of *P. cultripes* were collected from Doñana National Park, Spain, in November 2007 and shipped as prefeeding tadpoles to the University of Cincinnati. Adult spadefoot toads were maintained in screen-covered plastic boxes in 15-cm-deep soil, fed *ad libitum* vitamin-dusted crickets and sprinkled with water once a week. Adults were hormonally stimulated to breed as described in the study of Buchholz & Hayes (2002). Two days after fertilization, tadpoles were transferred to large stock tanks with aeration and fed *ad libitum* finely powdered rabbit chow twice a day. Tadpoles were reared at 28 °C with a 12L/12D cycle until they reached Gosner stages 32, 35 or 38. These stages were reached by 5, 7 and 9 days after hatching for *S. couchii*, 8, 12 and 16 days for *S. multiplicata* and 27, 32 and 38 days for *P. cultripes*, respectively.

Experimental set-up and design

Because the species studied in this report live in very different natural habitats, we used multiple conditions (two temperatures × three beginning developmental stages) within each species to minimize the possibility that any given species was being favoured by the specific experimental set-up when comparing effects of water reduction among species. In particular, to account for potentially different thermal preferences and the possibility that one temperature may be closer to the optimal for one species than another, we conducted the water reduction experiment at two temperatures (24 and 28 °C) experienced by these species in their natural habitats (Pomeroy, 1981; Newman, 1989; Buchholz & Hayes, 2002). Furthermore, previous studies used mid-prometamorphic stages (Gosner 36–38) to examine plasticity to water availability (Denver *et al.*, 1998), but the developmental timing of environmental input during the larval period is potentially very different across species. Indeed, different spadefoot species vary in the stage at which food deprivation allows a plastic response (Morey & Reznick, 2000). Thus, to further attempt a more robust and reliable comparison across species, we made the comparisons of the effect of water level treatment across three developmental stages: Gosner stage 32 (limb buds paddle-shaped, early-prometamorphic), Gosner stage 35 (mid-prometamorphic) and Gosner stage 38 (maximal tadpole size, late prometamorphic). All tadpoles from a particular species and beginning stage were obtained and exposed to high and low water treatments at the two temperatures on the same day, and this process was performed on three different days (1 day per beginning stage) for each species. The high water treatment was 4 L per tank (100 mm water depth in 12 × 12 × 30 cm tanks) for each species, stage and temperature. The low water treatment (4–7 mm for *S. couchii*, 8–14 mm for *S. multiplicata* and 13–19 mm for *P. cultripes*) in the same size tanks was determined by the volume of water required to just submerge the tadpole, and thus, depths varied with species-specific tadpole size. Tadpoles were reared individually and fed *ad libitum* finely ground rabbit chow twice daily, and water was changed daily, as per previous studies which showed these rearing conditions were most favourable across species (Buchholz & Hayes, 2000, 2002). The sample sizes were 10 for *S. couchii* and *S. multiplicata* and 8 for *P. cultripes*, for a total of 120 or 96 tadpoles per species, respectively. We used one breeding pair of *S. couchii* and *S. multiplicata* and a mixture of six clutches of *P. cultripes*. In addition, a preliminary experiment with one clutch of *S. couchii* from a different breeding pair had been conducted to confirm that we could observe the expected effect of high vs. low water treatments at 28 °C beginning at Gosner stages 32, 35 and 38 with a sample size of 10 for a total of 60 additional tadpoles for *S. couchii*. This amount of within-species sampling for across-species comparisons was sufficient in our case as the large magnitude of plasticity differences observed among species exceeded previously documented within-species variation in plasticity (Newman, 1988; Morey & Reznick, 2000, 2004). Furthermore, we found that the two sibships of *S. couchii* showed nearly identical reaction norms and therefore, only one clutch is used for further analysis of plasticity comparisons across three species (Appendix S1). We also found that the degree of variation within the mixture of *P. cultripes* clutches is not higher than would be expected from that of a single clutch. Nevertheless, it is possible that species measurements taken in a study may not necessarily characterize all individuals or populations throughout the distributional range of the species, and this caution is implicit when making comparisons across species and assessing the generality or applicability of the results.

To examine variation across species in a tadpole's ability to accumulate fat, we treated a subset of *S. couchii* and *S. multiplicata* tadpoles (20 tadpoles/tank, 6-L tanks) with 1 mM methimazole, which blocks thyroid hormone synthesis and thus development (Buckbinder & Brown, 1993), for 3 and 6 months, respectively, beginning 2 days after egg laying. Tadpoles were fixed in neutral buffered formalin until fat body dissection. *Spea multiplicata* did not advance beyond Gosner stage 34, and *S. couchii* did not advance beyond Gosner stage 37. Water and methimazole were replaced every 2–3 days. Tadpoles were fed *ad libitum* finely ground rabbit chow twice daily. Methimazole did not appear to affect feeding in these species.

Measurements

We measured snout-to-vent lengths (SVL) to the nearest 1 mm with digital callipers and weights to the nearest 0.01 g at the beginning of the treatment and at FLE. Time in days from beginning of water reduction to FLE was also recorded. We randomly selected three individuals from the tadpoles at tail resorption (Gosner 46) from the high and low water treatments and weighed fat bodies dissected from individuals after fixation in neutral buffered formalin (4% formaldehyde in phosphate buffered saline) using an analytical balance to the nearest 0.1 mg. Quantitative assessment of fat in the methimazole experiment was not possible because *S. couchii* from the control and methimazole treatments and *S. multiplicata* from control treatment possessed either very small fat bodies or no fat at all.

Statistical analysis

To examine the effect of water treatment on time to and size at FLE within each temperature and beginning stage among the three spadefoot toad species (with the pilot study from *S. couchii* excluded), we used general linear models using species and water treatments (high and low) as factors. Given significant species by treatment interactions, we conducted Tukey–Kramer *post hoc* tests to determine significant differences in time to and size at FLE between water treatments within species. To compare the degree of plasticity across species, we carried out a sequential series of linear models excluding one species at a time to detect significant species by treatment interaction effects (i.e. degree of plasticity) between pairs of species. To reduce type I error due to multiple testing, the Dunn–Sidak sequential correction of the significance level was applied with the alpha value for pairwise testing set to 0.0169 for (k = 3), 0.0253 for (k = 2) and 0.05 for (k = 1) and 'k' is a number of comparisons being made. We did not statistically analyse *within-species* differences in larval period plasticity due to variation in temperature or developmental stage because the aim of this study was to compare plasticity *across species* in response to water availability. To compare fat bodies across species, we used relative fat weight (weight of fat/weight of animal, both blotted dry after fixation) for statistical comparisons. Wilcoxon test was used to test for significant differences in relative fat weight among species. Likewise, we used the Wilcoxon test to test for significant differences in SVL between methimazole and control animals in each species.

Results

Larval period comparisons across species

Effect of water treatment on time to forelimb emergence
Larval development proceeded at different rates in different species, being fastest in *S. couchii*, slower in *S. multiplicata* and slowest in *P. cultripes* in nine of 12 conditions (three stages, two temperatures, two water levels) (Fig. 1a, Appendix S1). Decreased water level had a strong effect on larval period in *P. cultripes* and *S. multiplicata*, such that the low water level significantly reduced the time to FLE in four of six comparisons per species (Fig. 1a, Appendix S1). In contrast, the effect of water level on *S. couchii* was significant in only one of six comparisons. Species-by-treatment interactions were significant in all six conditions (three beginning stages and two temperatures), indicating species differences in their ability to respond to the water reduction treatment (Table 1a). When further analysed with sequential exclusion of species, *P. cultripes* and *S. multiplicata* differed significantly in three, *S. multiplicata* and *S. couchii* differed significantly in four, and *P. cultripes* and *S. couchii* differed significantly in four of six conditions (Table 1b, Fig. 1a). In most cases, *P. cultripes* showed the greatest ability to respond to water reduction stress (as determined by magnitude of difference between high and low water conditions), *S. multiplicata* was intermediate, and *S. couchii* was least plastic (and mostly not plastic at all).

Effect of water treatment on size at forelimb emergence
Snout-to-vent lengths and weight at FLE were smallest in *S. couchii*, intermediate in *S. multiplicata*, and largest in *P. cultripes* for 12 of 12 conditions (three stages, two temperatures, two water levels) for each measure of size (Fig. 1b,c, Appendix S1). In general, water level treatment had almost no effect on SVL and weight in *S. couchii*, and a pronounced effect of reduced size in *S. multiplicata* and *P. cultripes* (Figs 1b,c and 2a, Appendix S1). Low water resulted in significantly smaller tadpoles in *P. cultripes* in 11 of 12 comparisons across stages and temperatures and in *S. multiplicata* in 10 of 12 comparisons (Fig. 1b,c). In contrast, *S. couchii* tadpoles had only 1 of 12 significant differences due to water level treatment. Species by treatment interactions were significant in all 12 conditions (SVL and weight at FLE for three beginning stages and two temperatures) indicating any two or all three species differ in their ability to respond to the water reduction treatment (Table 1a). When further analysed with pairwise testing, *P. cultripes* and *S. multiplicata* differed significantly in 10, *S. multiplicata* and *S. couchii* differed significantly in 11 and *P. cultripes* and *S. couchii* differed significantly in 11 of 12 conditions (Table 1b, Fig. 1b,c). In all cases, *P. cultripes* showed the greatest ability to respond to water reduction stress (as determined by steepness of the slope between high and low water conditions), *S. multiplicata* was intermediate, and *S. couchii* was least plastic (and mostly not plastic at all).

Fat content comparisons across species

Size of fat bodies varied across species, as well as within species, in response to water level. *Pelobates cultripes* from the high water treatment had much larger fat bodies than

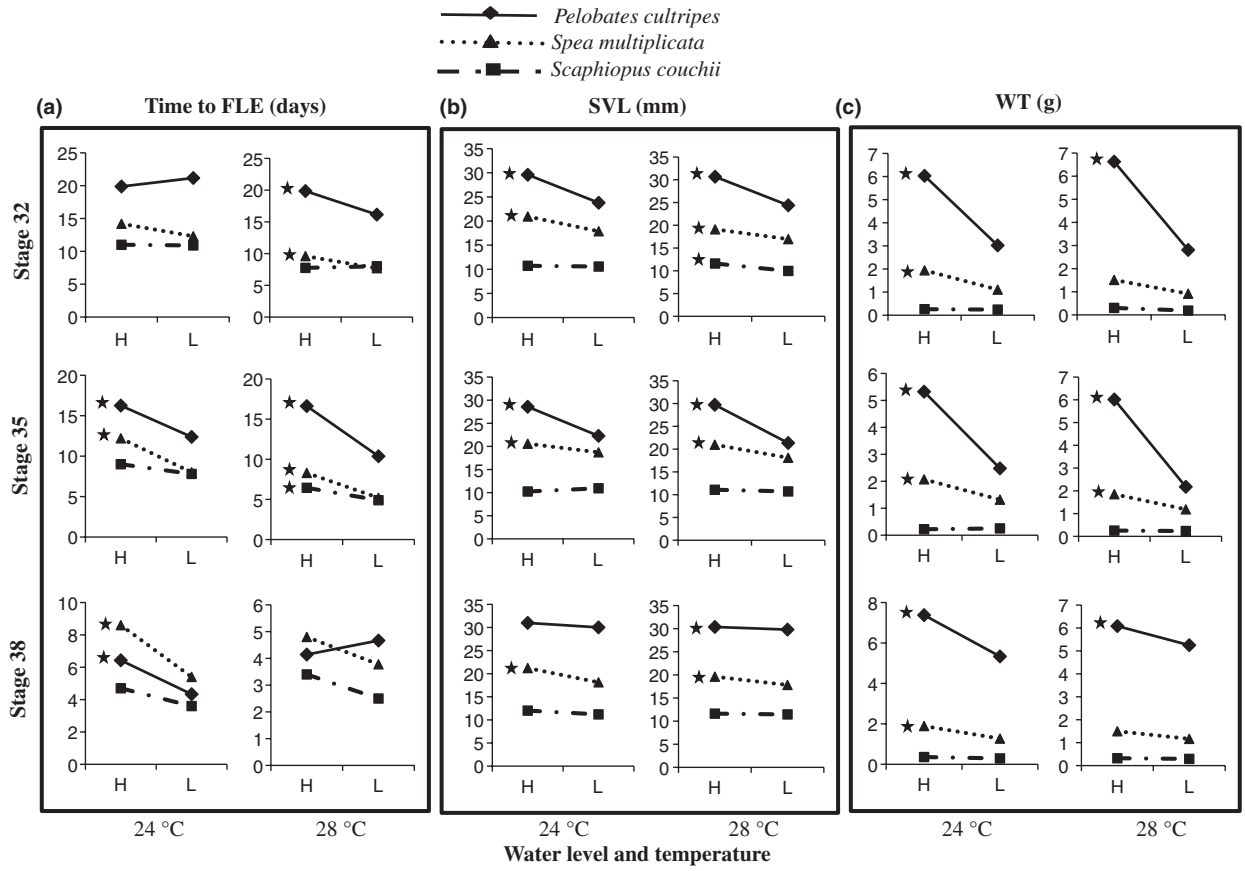

Fig. 1 Reaction norms for time to metamorphosis [forelimb emergence, (FLE)] (a), snout-to-vent length (SVL) at FLE (b) and weight (WT) at FLE (c) for three species of spadefoot toad tadpoles in response to high (H) and low (L) water treatments. Reaction norms are plotted by joining the means of the high and low water treatments in each condition. Reaction norm elevation indicates the trait mean value, whereas the slope indicates plasticity. Significance due to treatment within temperature and stage for time to and size at FLE was determined using Tukey–Kramer *post hoc* test. ★$P < 0.05$.

in the low water treatment (high: 30.67 ± 8.75 mg/mg body weight and low: 1.41 ± 0.39, $P < 0.05$) (Fig. 2b). Similarly, *S. multiplicata* from the high water treatment had larger fat bodies compared to the low water treatment (high : 31.74 ± 3.46 mg/mg body weight and low: 11.96 ± 4.79, $P < 0.05$). In contrast, *S. couchii* fat bodies were absent at tail resorption in both high and low water treatments. *Spea multiplicata* tadpoles treated with methimazole grew to abnormally large larval sizes (control: 15.83 ± 0.27 mm and methimazole: 25.9 ± 1.08, $P < 0.05$) because the drug blocked thyroid hormone production and prevented metamorphosis for the duration of treatment (Fig. 3a). On the other hand, there was no difference in the size of *S. couchii* tadpoles (control: 11.23 ± 0.38 mm and methimazole: 10.30 ± 0.66, $P > 0.05$). As expected, *S. multiplicata* tadpoles treated with methimazole had larger fat bodies compared to stage-matched but much younger controls (Fig. 3b). However, *S. couchii* tadpoles treated with methimazole had minimal fat bodies even with abundant food for months (Fig. 3b).

Discussion

Our results indicate that *S. couchii* has evolved a markedly reduced degree of plasticity (resulting in a flatter reaction norm) in larval period and size at FLE in response to water reduction. The plasticity in larval period and size of the other desert species *S. multiplicata* was intermediate between *S. couchii* and the nondesert species *P. cultripes*. Our results complement the two other studies comparing spadefoot species, where reduced plasticity was observed in *S. couchii* compared to *Spea hammondii* and *Spea intermontana* in response to different food regimes (Morey & Reznick, 2000, 2004). Combined with previous comparisons of larval period duration across all spadefoot species (Buchholz & Hayes, 2000, 2002), these results reveal an association between short larval period and low phenotypic plasticity. Interestingly, this among-species pattern was also observed within sibships of *S. couchii* (Newman, 1988). Newman found that the ability of *S. couchii* tadpoles to accelerate development at the

Table 1 Results from general linear models testing for differences in time, size [snout-to-vent length (SVL)] and weight (WT) to metamorphosis [forelimb emergence (FLE)], across species, water level treatment, and their interaction, according to initial stage (Stage) and temperature (Temp). (a) Least-squares mean analysis with spadefoot species and water treatment as factors detected significant interaction effects (bold numbers) for each beginning stage and temperature at $\alpha = 0.05$. (b) Least-squares mean analysis detected significant interaction effects (bold numbers) between *species* (two species at a time) and water treatment as factors at each beginning stage (Stage) and temperature (Temp) at $\alpha = 0.0169$ for ($k = 3$), 0.0253 for ($k = 2$) and 0.05 for ($k = 1$) after Dunn–Sidak sequential correction, where 'k' is the number of comparisons being made. Only interaction effects are shown.

(a) Response	Stage	Temp.	Effect	d.f.	*F*	*P* value
Time to FLE	32	24	Species	2,46	151.28	< 0.0001
	32	24	Treat.	1,46	0.28	0.5982
	32	24	Species × Treat.	2,46	4.32	**0.0191**
	35	24	Species	2,48	66.04	< 0.0001
	35	24	Treat.	1,48	47.55	< 0.0001
	35	24	Species × Treat.	2,48	6.34	**0.0036**
	38	24	Species	2,47	41.37	< 0.0001
	38	24	Treat.	1,47	58.45	< 0.0001
	38	24	Species × Treat.	2,47	5.59	**0.0066**
	32	28	Species	2,44	272.81	< 0.0001
	32	28	Treat.	1,44	38.53	< 0.0001
	32	28	Species × Treat.	2,44	16.22	**< 0.0001**
	35	28	Species	2,49	247.30	< 0.0001
	35	28	Treat.	1,49	145.23	< 0.0001
	35	28	Species × Treat.	2,49	19.88	**< 0.0001**
	38	28	Species	2,46	19.30	< 0.0001
	38	28	Treat.	1,46	4.42	0.0410
	38	28	Species × Treat.	2,46	4.38	**0.0182**
SVL at FLE	32	24	Species	2,45	15.12	< 0.0001
	32	24	Treat.	1,45	71.51	< 0.0001
	32	24	Species × Treat.	2,45	18.13	**< 0.0001**
	35	24	Species	2,48	3.32	0.0446
	35	24	Treat.	1,48	54.05	< 0.0001
	35	24	Species × Treat.	2,48	31.92	**< 0.0001**
	38	24	Species	2,46	33.18	< 0.0001
	38	24	Treat.	1,46	30.78	< 0.0001
	38	24	Species × Treat.	2,46	12.47	**< 0.0001**
	32	28	Species	2,43	15.10	< 0.0001
	32	28	Treat.	1,43	135.62	< 0.0001
	32	28	Species × Treat.	2,43	23.68	**< 0.0001**
	35	28	Species	2,48	10.66	0.0001
	35	28	Treat.	1,48	229.57	< 0.0001
	35	28	Species × Treat.	2,48	74.67	**< 0.0001**
	38	28	Species	2,45	22.54	< 0.0001
	38	28	Treat.	1,45	28.89	< 0.0001
	38	28	Species × Treat.	2,45	7.08	**0.0021**
Wt at FLE	32	24	Species	2,45	6.94	0.0023
	32	24	Treat.	1,45	54.16	< 0.0001
	32	24	Species × Treat.	2,45	21.63	**< 0.0001**
	35	24	Species	2,48	12.15	< 0.0001
	35	24	Treat.	1,48	100.86	< 0.0001
	35	24	Species × Treat.	2,48	49.38	**< 0.0001**
	38	24	Species	2,46	14.21	< 0.0001
	38	24	Treat.	1,46	116.37	< 0.0001
	38	24	Species × Treat.	2,46	35.15	**< 0.0001**
	32	28	Species	2,43	9.36	0.0004
	32	28	Treat.	1,43	103.06	< 0.0001
	32	28	Species × Treat.	2,43	51.85	**< 0.0001**
	35	28	Species	2,48	57.43	< 0.0001
	35	28	Treat.	1,48	302.13	< 0.0001
	35	28	Species × Treat.	2,48	168.13	**< 0.0001**
	38	28	Species	2,45	2.78	0.0724
	38	28	Treat.	1,45	43.92	< 0.0001
	38	28	Species × Treat.	2,45	18.31	**< 0.0001**

(b) *S. couchii*					*S. multiplicata*				
Temp.	Stage	d.f.	*F*	*P* value	Temp.	Stage	d.f.	*F*	*P*
Larval period									
S. multiplicata									
24	32	1,34	4.75	0.0363					
24	35	1,34	23.32	**< 0.0001**					
24	38	1,36	11.92	**0.0014**					
28	32	1,33	13.45	**0.0008**					
28	35	1,35	19.04	**0.0001**					
28	38	1,35	0.06	0.8026					
P. cultripes									
24	32	1,28	1.09	0.3061	24	32	1,30	8.03	**0.0081**
24	35	1,30	6.61	**0.0153**	24	35	1,32	0.07	0.7945
24	38	1,29	2.62	0.1165	24	38	1,29	1.84	0.1859
28	32	1,26	22.86	**< 0.0001**	28	32	1,29	10.08	**0.0035**
28	35	1,31	27.13	**< 0.0001**	28	35	1,32	12.72	**0.0011**
28	38	1,29	8.81	**0.0059**	28	38	1,28	5.51	0.0262
SVL at FLE									
S. multiplicata									
24	32	1,33	27.45	**< 0.0001**					
24	35	1,34	24	**< 0.0001**					
24	38	1,35	33.49	**< 0.0001**					
28	32	1,32	0.73	0.3995					
28	35	1,34	24.93	**< 0.0001**					
28	38	1,34	21.55	**< 0.0001**					
P. cultripes									
24	32	1,27	27.55	**< 0.0001**	24	32	1,29	3.31	0.0793
24	35	1,30	52.1	**< 0.0001**	24	35	1,31	17.08	**0.0002**
24	38	1,28	0.31	0.5848	24	38	1,28	13.33	**0.0010**
28	32	1,25	34.6	**< 0.0001**	28	32	1,28	26.64	**< 0.0001**
28	35	1,30	133.78	**< 0.0001**	28	35	1,31	52.94	**< 0.0001**
28	38	1,28	7.28	**0.0116**	28	38	1,27	0.11	0.7395
Weight at FLE									
S. multiplicata									
24	32	1,33	73.95	**< 0.0001**					
24	35	1,34	127.65	**< 0.0001**					
24	38	1,35	38.66	**< 0.0001**					
28	32	1,32	34.21	**< 0.0001**					
28	35	1,34	82.99	**< 0.0001**					
28	38	1,34	64.44	**< 0.0001**					
P. cultripes									
24	32	1,27	26.29	**< 0.0001**	24	32	1,27	0.11	0.7395
24	35	1,30	62.69	**< 0.0001**	24	35	1,29	14.38	**0.0007**
24	38	1,28	56.69	**< 0.0001**	24	38	1,31	33.64	**< 0.0001**
28	32	1,25	57.88	**< 0.0001**	28	32	1,28	16.36	**0.0003**

Table 1 (*Continued*)

(b) *S. couchii*					*S. multiplicata*				
Temp.	Stage	d.f.	*F*	*P* value	Temp.	Stage	d.f.	*F*	*P*
28	35	1,30	209.28	**< 0.0001**	28	35	1,28	48.81	**< 0.0001**
28	38	1,28	22.89	**< 0.0001**	28	38	1,31	137.86	**< 0.0001**

'*P*' value < 0.05 for 'Species' represents significant difference in elevation of reaction norms (mean trait value) among species. '*P*' value < 0.05 for 'Treat.' represents significant difference in particular response variable between high and low water combining all species together. '*P*' value < 0.05 for 'Species × Treat' represents significant difference in steepness of reaction norms for three species or can also be interpreted as either any two or all the three species that differ significantly in their ability to respond to water treatments.
d.f., degrees of freedom.

expense of size at metamorphosis in response to water availability varied across five sibships and that the sibships with the fastest average developmental rate were associated with the least overall plasticity in growth and development. Our results enable direct comparisons of plasticity across species and are consistent with our previous phylogenetic hypothesis of the evolution of plasticity (Gomez-Mestre & Buchholz, 2006). Thus, we conclude that the low level of plasticity in *S. couchii*, together with its overall fast developmental rate, is derived from ancestors with greater levels of plasticity, closely resembled by *P. cultripes*.

Studies using *Rana temporaria* yielded results consistent with this relationship between larval period and plasticity. Northern populations of *R. temporaria* had

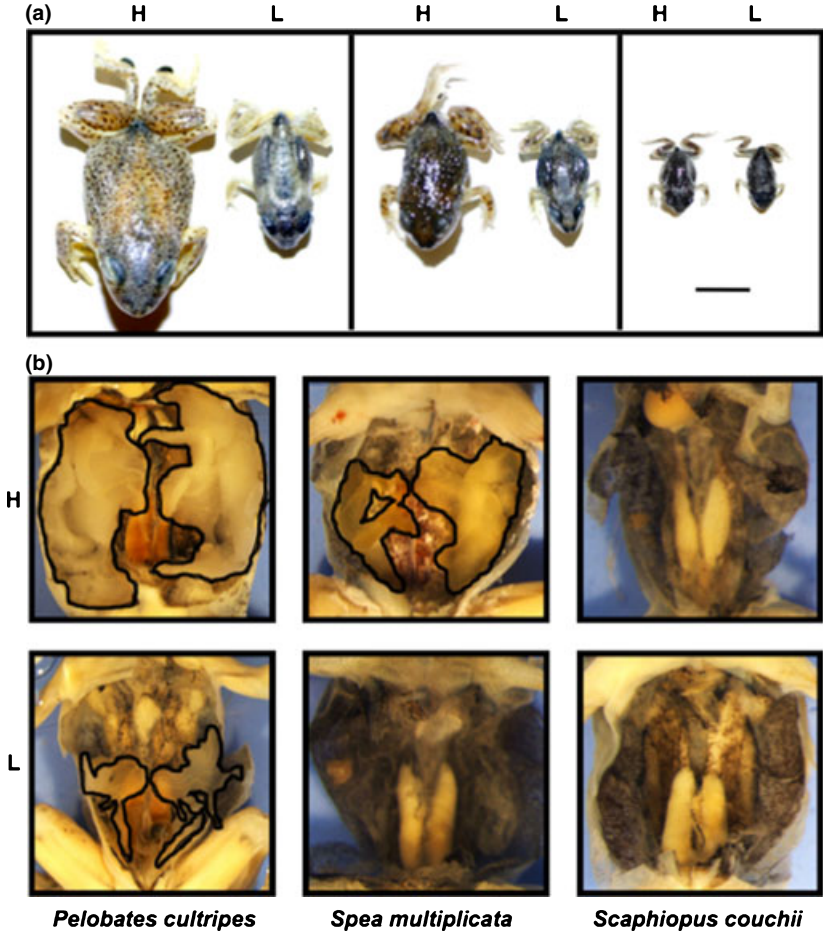

Fig. 2 Morphological comparisons of size and fat body content for three species of spadefoot toad tadpoles in response to high (H) and low (L) water treatment at 28 °C starting at Gosner stage 35. (a) Exemplar metamorphs from high and low water treatments show differences in size at tail resorption in response to water treatment within and between species. All images are to scale. Scale bar = 10 mm. (b) Exemplar metamorphs from high and low water treatments show differences in fat bodies (outlined in black) at tail resorption within and between species. Photographs are adjusted to equalize snout vent lengths for within- and between-species comparisons. The same individuals from panel a are shown. Shown are representatives from three individuals examined per species per water treatment per stage.

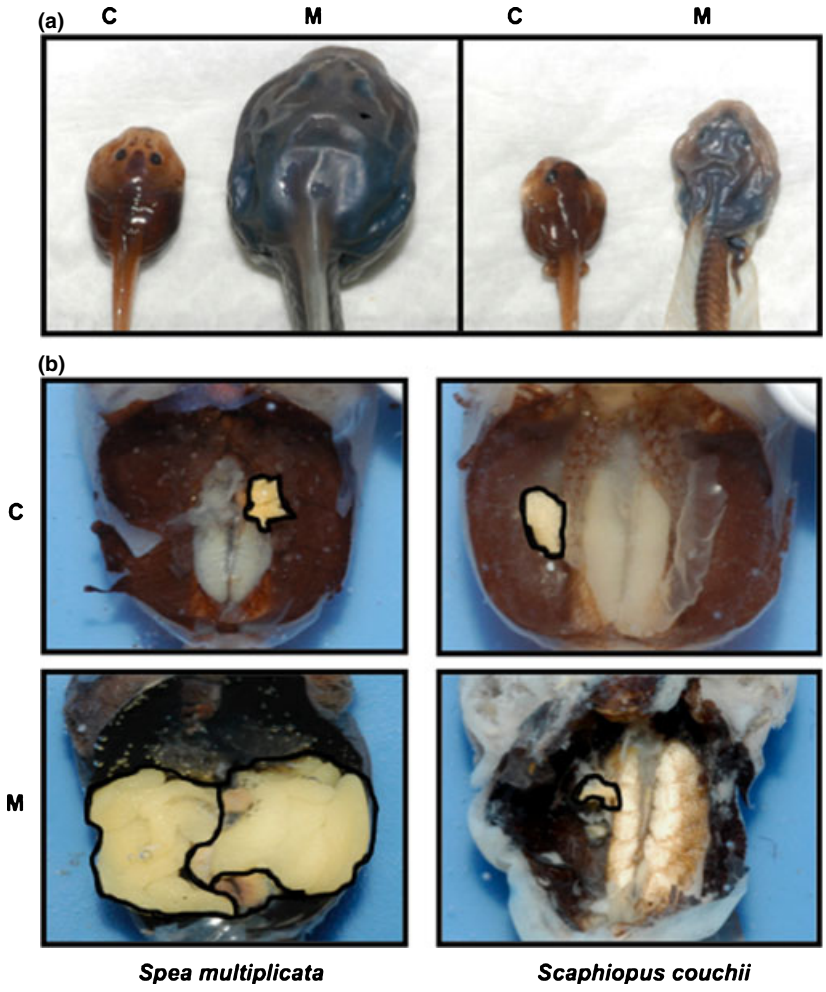

Fig. 3 Morphological comparisons of size and fat body content for tadpoles of *Scaphiopus couchii* and *S. multiplicata* reared in methimazole (M) for 3 and 6 months, respectively. Tadpoles of *S. multiplicata* reached Gosner stage 34–35, whereas *S. couchii* tadpoles reached Gosner stage 37–38. Control (C) tadpoles are stage-matched. (a) Exemplar tadpoles show increased size of methimazole-treated tadpoles compared to controls. All images are to scale. Scale bar = 10 mm. (b) *Scaphiopus multiplicata* from methimazole and control treatments show differences in fat bodies (outlined in black), whereas *S. couchii* do not show any difference in fat bodies between treatments. Photographs are adjusted to equalize snout–vent lengths for within -and between- species comparisons. The same individuals from panel a are shown. Shown is a representative sample from three individuals examined per species per treatment.

faster development and were less plastic in time to metamorphosis in response to pond-drying risk compared to southern populations (Laurila *et al.*, 2002; Merilä *et al.*, 2004). The authors concluded that selection favoured rapid development due to a shorter growing season in the northern part of Sweden at the expense of plasticity, suggesting that reduced plasticity was a side effect of selection for a shorter larval period in the north. Interestingly, the tadpoles from northern populations were larger and less plastic in size compared to the southern more plastic but smaller tadpoles. Our results with *S. couchii* showed reduced plasticity associated with shorter larval periods and smaller tadpoles, and thus, the physiological basis of relationship between size and the evolution of plasticity in spadefoot toads may be different from that in *R. temporaria*.

At least two possibilities explain the evolution of reduced plasticity in *S. couchii*: (i) adaptive plasticity and/or (ii) indirect consequence of selection for rapid metamorphosis. Adaptive plasticity is predicted to evolve by natural selection to better cope with environmental heterogeneity, such that degree of plasticity should correlate with environmental variability, all else being equal (Bradshaw, 1965; West-Eberhard, 1989, 2003). Increased aridification of south-western North America likely caused breeding pond durations to be consistently short and hence less variable compared to the pond durations experienced in the Old World and presumably too by their last common ancestor in North America. The

duration of ponds used by *S. couchii* ranges from 7 to 19 days (Morey & Reznick, 2004) or 3 to < 30 days (Newman, 1989), whereas ponds for *P. cultripes* last on average 152 days and have a range of 30–360 days (Gomez-Rodriguez, 2009). *Spea multiplicata* ponds last between 36 and 127 days (Morey & Reznick, 2004). As a result, New World spadefoot toads have experienced directional selection for shorter larval periods and may have also experienced selection for reduced larval period plasticity to match the consistently short and less variable pond durations.

However, there is no direct evidence to support that selection directly favoured reduced developmental plasticity in desert spadefoot toads. Theory predicts that selection can favour reduced plasticity to reduce costs incurred in maintaining the ability to be plastic if organisms experience consistently homogeneous environmental conditions (Pigliucci & Murren, 2003; Auld *et al.*, 2010). Plasticity can be costly due to maintenance costs of the sensory and regulatory mechanisms that produce the plastic response: costs associated with flexibility in production of phenotypes inducible by environmental signals in excess of costs paid by fixed genotypes to produce the same phenotype; information acquisition costs obtained during environmental sampling; developmental instability costs that result if plastic development is more variable than fixed development; and genetic costs such as linkage of plasticity genes with genes conferring low fitness (DeWitt, 1998; Auld *et al.*, 2010). Further analysis of the mechanistic basis for plasticity is required to shed light on what, if any, costs are associated with the ability to accelerate development in response to reduced water level in spadefoot toads. Nonetheless, *S. couchii* is still capable of accelerating development to some extent (i.e. there is loss of plasticity compared to ancestral state but not complete phenotypic canalization), suggesting that they may still be paying (i) whatever costs are associated with maintenance of the sensory and regulatory mechanisms enabling the plastic response, (ii) information acquisition costs incurred during environmental sampling and/or (iii) genetic costs such as linkage of genes enabling plasticity with genes conferring low fitness.

Reduced developmental plasticity, however, may also have evolved as an indirect consequence of developmental/endocrine changes incurred in response to directional selection for fast mean development in *S. couchii*, rather than selection for reduced plasticity *per se*. Indeed, other aspects of *S. couchii* biology are believed to be a secondary consequence of the evolution of accelerated metamorphosis, such as altered timing of gonad differentiation and shorter relative limb length (Buchholz & Hayes, 2005; Gomez-Mestre & Buchholz, 2006). A known endocrine mechanism underlying short larval periods in *S. couchii* is increased tissue content of thyroid hormone and tissue sensitivity and responsivity to thyroid hormone (Buchholz & Hayes, 2005). A potential evolutionary change to achieve higher thyroid hormone levels is an increase in the production of corticotropin-releasing hormone (CRH) in the hypothalamus, which would lead to increased thyroid hormone production and rate of morphological change (Denver, 2009). CRH also regulates production of stress hormones in tadpoles, particularly corticosterone, which increases the sensitivity and responsivity of a tissue to thyroid hormone. Importantly, CRH mediates larval period plasticity in tadpoles (Denver, 2009). Specifically, tadpoles respond to desiccation stress by up-regulating CRH, leading to increased thyroid hormone and corticosterone, which together increase the rate of development. Thus, *S. couchii* may have evolved constitutively high CRH levels to achieve higher basal thyroid and corticosterone levels, promoting its faster development. As a consequence, constitutively high CRH levels in *S. couchii* may preclude its increase in the stressful low water conditions and thereby curtail a stress-mediated plastic response to low water.

The reduced size at metamorphosis in *S. couchii* may have been a direct consequence of initiating metamorphosis at an early age, even under ideal rearing conditions, constitutively sacrificing time required to grow larger. Ontogenetic switch points such as metamorphosis are likely to exhibit clear limits to plasticity, because they often require a minimum stage and/or size before which they are simply incapable of triggering the transition (Gomez-Mestre *et al.*, 2008). Consistent with its small size and lack of plasticity, the path taken by *S. couchii* to achieve short larval periods seems to have been to canalize the timing of metamorphic initiation to an early time point virtually devoid of environmental input, perhaps related to altered regulation of CRH physiology.

Lack of fat bodies in *S. couchii* is consistent with our hypothesis of high basal levels of stress hormones. *Pelobates cultripes* and *S. multiplicata* have reduced fat bodies in the low water treatment. Such a reduction in fat bodies is not seen in *S. couchii*, whose tadpoles do not gain any fat during larval development, regardless of water availability. Even when allowed enough time and food to store fat via the treatment with methimazole, tadpoles of *S. couchii* are poor at fat storage (Fig. 3b). Because stress hormones promote increased lipolysis (Schreck, 1993), the hypothesized increase in basal stress hormone levels may explain lack of fat bodies in the tadpoles of *S. couchii* even in high water and methimazole treatments.

Acknowledgments

We thank Arizona Game and Fish Commission (SP565423) and New Mexico Department of Game and Fish (3364) for issuing collecting permits for *Scaphiopus couchii* and *Spea multiplicata*. We also thank the Consejería de Medio Ambiente from Junta de Andalucía for issuing collecting permits for *Pelobates cultripes*.

References

Altwegg, R. & Reyer, H. 2003. Patterns of natural selection on size at metamorphosis in water frogs. *Evolution* **57**: 872–882.

Álvarez, D. & Nicieza, A.G. 2002. Effects of temperature and food quality on anuran larval growth and metamorphosis. *Funct. Ecol.* **16**: 640–648.

Audo, M.C., Mann, T.M., Polk, T.L., Loudenslager, C.M., Diehl, W.J. & Altig, R. 1995. Food deprivation during different periods of tadpole (*Hyla chrysoscelis*) ontogeny affects metamorphic performance differently. *Oecologia* **103**: 518–522.

Auld, J.R., Agrawal, A.A. & Relyea, R.A. 2010. Re-evaluating the costs and limits of adaptive phenotypic plasticity. *Proc. R. Soc. Lond. B* **277**: 503–511.

Benard, M.F. 2004. Predator-induced phenotypic plasticity in organisms with complex life histories. *Annu. Rev. Ecol. Syst.* **35**: 651–673.

Bradshaw, A.D. 1965. Evolutionary significance of phenotypic plasticity in plants. *Adv. Genet.* **13**: 115–155.

Brady, L.D. & Griffiths, R.A. 2000. Developmental responses to pond desiccation in tadpoles of the british anuran amphibians (*Bufo bufo, B. calamita* and *Rana temporaria*). *J. Zool.* **252**: 61–69.

Buchholz, D.R. & Hayes, T.B. 2000. Larval period comparison for the spadefoot toads *Scaphiopus couchii* and *Spea multiplicata* (Pelobatidae: Anura). *Herpetologica* **56**: 455–468.

Buchholz, D.R. & Hayes, T.B. 2002. Evolutionary patterns of diversity in spadefoot toad metamorphosis (Anura: Pelobatidae). *Copeia* **2002**: 180–189.

Buchholz, D.R. & Hayes, T.B. 2005. Variation in thyroid hormone action and tissue content underlies species differences in the timing of metamorphosis in desert frogs. *Evol. Dev.* **7**: 458–467.

Buckbinder, L. & Brown, D.D. 1993. Expression of the *Xenopus laevis* prolactin and thyrotropin genes during metamorphosis. *Proc. Natl. Acad. Sci. USA* **90**: 3820–3824.

Busack, S.D. & Zug, G.R. 1976. Observations on the tadpoles of *Pelobates cultripes* from southern Spain. *Herpetologica* **32**: 130–137.

Callahan, H.S., Pigliucci, M. & Schlichting, C.D. 1997. Developmental phenotypic plasticity: where ecology and evolution meet molecular biology. *Bioessays* **19**: 519–525.

Crispo, E. 2007. The Baldwin effect and genetic assimilation: revisiting two mechanisms of evolutionary change mediated by phenotypic plasticity. *Evolution* **61**: 2469–2479.

De Block, M., McPeek, M.A. & Stoks, R. 2008. Life-history evolution when lestes damselflies invaded vernal ponds. *Evolution* **62**: 485–493.

Denver, R.J. 2009. Stress hormones mediate environment-genotype interactions during amphibian development. *Gen. Comp. Endocrinol.* **164**: 20–31.

Denver, R.J., Mirhadi, N. & Phillips, M. 1998. Adaptive plasticity in amphibian metamorphosis: response of *Scaphiopus hammondii* tadpoles to habitat desiccation. *Ecology* **79**: 1859–1872.

DeWitt, T.J. 1998. Costs and limits of phenotypic plasticity: tests with predator-induced morphology and life history in a freshwater snail. *J. Evol. Biol.* **11**: 465–480.

Gavrilets, S. & Scheiner, S.M. 1993. The genetics of phenotypic plasticity. VI. theoretical predictions for directional selection. *J. Evol. Biol.* **6**: 49–68.

Gomez-Mestre, I. & Buchholz, D.R. 2006. Developmental plasticity mirrors differences among taxa in spadefoot toads linking plasticity and diversity. *Proc. Natl. Acad. Sci. USA* **103**: 19021–19026.

Gomez-Mestre, I., Touchon, J.C., Saccoccio, V.L. & Warkentin, K.M. 2008. Genetic variation in pathogen-induced early hatching of toad embryos. *J. Evol. Biol.* **21**: 791–800.

Gomez-Mestre, I., Saccoccio, V.L., Iijima, T., Collins, E.M., Rosenthal, G.G. & Warkentin, K.M. 2010. The shape of things to come: linking developmental plasticity to post-metamorphic morphology in anurans. *J. Evol. Biol.* **23**: 1364–1373.

Gomez-Rodriguez, C. 2009. Condicionantes Ecológicos De La Distribución De Anfibios En El Parque Nacional De Doñana. Universidad de Salamanca.

Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**: 183–190.

Laurila, A., Karttunen, S. & Merilä, J. 2002. Adaptive phenotypic plasticity and genetics of larval life histories in two *Rana temporaria* populations. *Evolution* **56**: 617–627.

Lind, M.I. & Johansson, F. 2007. The degree of adaptive phenotypic plasticity is correlated with the spatial environmental heterogeneity experienced by island populations of *Rana temporaria*. *J. Evol. Biol.* **20**: 1288–1297.

Loman, J. 1999. Early metamorphosis in common frog *Rana temporaria* tadpoles at risk of drying: An experimental demonstration. *Amphibia-Reptilia* **20**: 421–430.

Loman, J. & Claesson, D. 2003. Plastic response to pond drying in tadpoles *Rana temporaria*: tests of cost models. *Evol. Ecol. Res.* **5**: 179–194.

Marjanovic, D. & Laurin, M. 2007. Fossils, molecules, divergence times, and the origin of lissamphibians. *Syst. Bio.* **56**: 369–388.

Merilä, J., Laurila, A., Pahkala, M., Räsänen, K. & Timenes Laugen, A. 2000a. Adaptive phenotypic plasticity in timing of metamorphosis in the common frog. *Rana temporaria*. *Ecoscience* **7**: 18–24.

Merilä, J., Laurila, A., Timenes Laugen, A., Räsänen, K. & Pahkala, M. 2000b. Plasticity in age and size at metamorphosis in *Rana temporaria* – comparison of high and low latitude populations. *Ecography* **23**: 457–465.

Merilä, J., Laurila, A. & Lindgren, B. 2004. Variation in the degree and costs of adaptive phenotypic plasticity among *Rana temporaria* populations. *J. Evol. Biol.* **17**: 1132–1140.

Moran, N.A. 1992. The evolutionary maintenance of alternative phenotypes. *Am. Nat.* **139**: 971–989.

Morey, S. & Reznick, D. 2000. A comparative analysis of plasticity in larval development in three species of spadefoot toads. *Ecology* **81**: 1736–1749.

Morey, S.R. & Reznick, D.N. 2004. The relationship between habitat permanence and larval development in California spadefoot toads: field and laboratory comparisons of developmental plasticity. *Oikos* **104**: 172–190.

Newman, R.A. 1987. Effects of density and predation on *Scaphiopus couchii* tadpoles in desert ponds. *Oecologia* **71**: 301–307.

Newman, R.A. 1988. Adaptive plasticity in development of *Scaphiopus couchii* tadpoles in desert ponds. *Evolution* **42**: 774–783.

Newman, R.A. 1989. Developmental plasticity of *Scaphiopus couchii* tadpoles in an unpredictable environment. *Ecology* **70**: 1775–1787.

Newman, R.A. 1992. Adaptive plasticity in amphibian metamorphosis. *Bioscience* **42**: 671–678.

Newman, R.A. 1994. Effects of changing density and food level on metamorphosis of a desert amphibian, *Scaphiopus couchii*. *Ecology* **75**: 1085–1096.
Newman, R.A. 1998. Ecological constraints on amphibian metamorphosis: interactions of temperature and larval density with responses to changing food level. *Oecologia* **115**: 9–16.
Pigliucci, M. 1996. How organisms respond to environmental changes: from phenotypes to molecules (and vice versa). *Trends Ecol. Evol.* **11**: 168–173.
Pigliucci, M. 2001. *Phenotypic Plasticity: Beyond Nature and Nurture*. The Johns Hopkins University Press, Baltimore.
Pigliucci, M. & Murren, C.J. 2003. Perspective: genetic assimilation and a possible evolutionary paradox: can macroevolution sometimes be so fast as to pass us by? *Evolution* **57**: 1455–1464.
Pigliucci, M., Cammell, K. & Schmitt, J. 1999. Evolution of phenotypic plasticity a comparative approach in the phylogenetic neighbourhood of *Arabidopsis thaliana*. *J. Evol. Biol.* **12**: 779–791.
Pigliucci, M., Murren, C.J. & Schlichting, C.D. 2006. Phenotypic plasticity and evolution by genetic assimilation. *J. Exp. Biol.* **209**: 2362–2367.
Pomeroy, L.V. 1981. Developmental polymorphism in the tadpoles of the spadefoot toad *Scaphiopus multiplicatus*. PhD Dissertation, Univ of California, Riverside.
Relyea, R.A. 2001. Morphological and behavioral plasticity of larval anurans in response to different predators. *Ecology* **82**: 523–540.
Relyea, R.A. 2002. Costs of phenotypic plasticity. *Am. Nat.* **159**: 272–282.
Relyea, R.A. & Werner, E.E. 2000. Morphological plasticity in four larval anurans distributed along an environmental gradient. *Copeia* **2000**: 178–190.
Richter-Boix, A., Llorente, G.A. & Montori, A. 2006. A comparative analysis of the adaptive developmental plasticity hypothesis in six mediterranean anuran species along a pond permanency gradient. *Evol. Ecol. Res.* **8**: 1139–1154.
Sage, R.D., Prager, E.M. & Wake, D.B. 1982. A cretaceous divergence time between pelobatid frogs (*Pelobates* and *Scaphiopus*): Immunological studies of serum albumin. *J. Zool. (Lond.)* **198**: 481–494.
Schlichting, C.D. & Pigliucci, M. 1998. Phenotypic evolution: a reaction norm perspective.
Schreck, C.B. 1993. Glucocorticoids: metabolism, growth and development. In: *The Endocrinology of Growth, Development, and Metabolism in Vertebrates* (M.P. Schriebman, C.G. Scanes & P.K.T. Pang, eds), pp. 367–392. Academic Press, London.
Van Tienderen, P.H. 1997. Generalists, specialists, and the evolution of phenotypic plasticity in sympatric populations of distinct species. *Evolution* **51**: 1372–1380.
West-Eberhard, M.J. 1989. Phenotypic plasticity and the origins of diversity. *Annu. Rev. Ecol. Syst.* **20**: 249–278.
West-Eberhard, M.J. 2003. *Developmental Plasticity and Evolution*. Oxford University Press, Oxford.

Supporting information

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

Appendix S1 Summary data table for the effect of water level treatment on time to and size at FLE in *Pelobates cultripes*, *Spea multiplicata*, and *Scaphiopus couchii*.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

Received 30 March 2011; revised 19 July 2011; accepted 20 July 2011