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The shape of things to come: linking developmental plasticity to post-metamorphic morphology in anurans

I. GOMEZ-MESTRE*†‡, V. L. SACCOCCIO*, T. IIJIMA*, E. M. COLLINS*, G. G. ROSENTHAL§ & K. M. WARKENTIN*

*Department of Biology, Boston University, Boston, MA, USA
†Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas, Avda. Américo Vespucio s/n, Sevilla, Spain
‡Instituto Cantábrico de Biodiversidad, Universidad de Oviedo, Oviedo, Spain
§Department of Biology, Texas A & M University, College Station, TX, USA

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Abstract

Development consists of growth and differentiation, which can be partially decoupled and can be affected by environmental factors to different extents. In amphibians, variation in the larval environment influences development and causes changes in post-metamorphic shape. We examined post-metamorphic consequences, both morphological and locomotory, of alterations in growth and development. We reared tadpoles of two phylogenetically and ecologically distant frog species (the red-eyed treefrog *Agalychnis callidryas* and the African clawed frog *Xenopus laevis*) under different temperatures with *ad libitum* food supply and under different food levels at a constant temperature. Low temperature and low food levels both resulted in similarly extended larval periods. However, low temperature yielded relatively long-legged frogs with a lower degree of ossification than warm temperature, whereas low food yielded relatively short-legged frogs with a higher degree of ossification than high food levels. Such allometric differences had no effect on locomotor performance of juveniles. Our results provide a basis for understanding the relationship between growth, differentiation and post-metamorphic shape in anurans and help explain many of the discrepancies reported in previous studies.

Introduction

Most organisms have complex life cycles, with one or more abrupt ontogenetic changes in their morphology, physiology and behaviour, often associated with a change in habitat (Wilbur, 1980; Werner, 1988). The timing of such ontogenetic switch points (e.g. hatching, metamorphosis) is often highly dependent upon the environment (Newman, 1992). Thus, environmental conditions experienced during the larval stage often affect development in ways that transcend the metamorphic boundary causing carry-over or latent effects on post-metamorphic phenotypes (Beckerman *et al.*, 2002; Gimenez, 2006; Pechenik, 2006). Such latent effects have been demonstrated in at least six different animal phyla (Pechenik, 2006).

Correspondence: Ivan Gomez-Mestre, Instituto Cantábrico de Biodiversidad, Universidad de Oviedo, Oviedo E-33006, Spain. Tel.: +34 985 104831; fax: +34 985 104866; e-mail: igmestre@ebd.csic.es

Individual plastic changes in the timing of ontogenetic switch points, i.e. *heterokairic* changes (Spicer & Burggren, 2003), can be caused by alterations in either growth rate, differentiation rate or both (Smith-Gill & Berven, 1979; Alberch & Alberch, 1981; McCoy *et al.*, 2007). Although growth and differentiation are highly interrelated processes of development, they can nevertheless be decoupled to a large extent (Smith-Gill & Berven, 1979). This is particularly true in amphibians, whose larvae can often develop quickly with little overall change in size or grow over extensive periods of time with minor or no changes in developmental stage. Some changes in developmental trajectories are adaptive responses to environmental conditions; others are not (Smith-Gill, 1983). Thus, some amphibian species are capable of accelerating metamorphosis to escape desiccating ponds or evade predators, and some are also capable of delaying metamorphosis to avoid juvenile predators (Newman, 1992; Benard, 2004; Vonesh & Warkentin, 2006). Non-adaptive developmental alterations include extended

larval development owing to food limitation, crowding or harsh abiotic conditions (Alford, 1999; Gomez-Mestre & Tejedo, 2003).

Altering metamorphic timing, however, affects juvenile shape in addition to size, in part because of allometric differences in growth between body and limbs (Emerson, 1986; Emerson *et al.*, 1988). Previous studies have documented delayed effects of the larval environment on juvenile morphology, but incongruent effects have been reported for different species and environments. Thus high larval density, low food availability, low temperature and the presence of predators can all delay metamorphosis; however, their effects on juvenile morphology vary not only in magnitude but in direction as well. For instance, increased larval density or low food availability always results in long larval periods and relatively short-legged juveniles (Emerson, 1986; Tejedo *et al.*, 2000a; Relyea & Hoverman, 2003), whereas low temperatures result in long larval periods and relatively long-legged frogs (Gomez-Mestre & Buchholz, 2006), and short pond durations result in short larval periods and relatively short-legged frogs (Richter-Boix *et al.*, 2006; Márquez-García, 2009). Even the same environmental factor, exposure to caged predators, can result in either long-legged or short-legged juveniles in different species, even if metamorphosis is similarly delayed (Relyea, 2001; Van Buskirk & Saxer, 2001; Capellan & Nicieza, 2007). Thus, under some conditions, long larval periods result in long-legged juveniles, whereas under other conditions, they result in short-legged juveniles.

In view of these previous studies, it was clear that larval period is an insufficient parameter to understand the carry-over effects that environmentally induced developmental alterations have on post-metamorphic individuals. Environmental factors with a strong effect on growth rate (i.e. increase in size), such as food level or larval density, may alter larval period via different mechanisms than do factors with a relatively stronger effect on differentiation (i.e. maturation or specialization of cell types, tissues or organs), such as temperature or pond desiccation. Such mechanistically different changes in development may result in phenotypically different animals, despite similar effects on time to metamorphosis. Thus, similarly long larval periods could result in opposite changes in post-metamorphic morphology, if growth and differentiation were affected differently in the different environments. To test this idea, we conducted two experiments on two widely divergent frog species, the red-eyed treefrog (*Agalychnis callidryas*; Anura: Hylidae) and the African clawed frog (*Xenopus laevis*; Anura: Pipidae).

Food and temperature seemingly affect growth and differentiation in fundamentally different ways: low food slows growth rate more than differentiation rate, whereas low temperature slows differentiation rate more than growth rate (Smith-Gill & Berven, 1979; Blouin & Brown, 2000). Hence, for each species, we reared tadpoles under (a) different temperature conditions with *ad libitum* food supply (hence affecting differentiation more strongly than growth) and (b) different food levels and constant temperature (hence affecting growth more than differentiation). We then compared the timing of metamorphosis, relative changes in morphology, level of ossification and locomotor performance of individuals from each larval environment. The two species studied are phylogenetically very distantly related (Frost *et al.*, 2006; Wiens, 2007). They also differ widely in their ecology: *A. callidryas* is a terrestrial Mesoamerican treefrog of arboreal habits, whereas *X. laevis* is a strictly aquatic African frog, so the selective pressures acting on their morphology have probably been very different over a long evolutionary span. Congruence in their developmental response to common environmental inputs strongly suggests the generality of the pattern across anurans.

Methods and materials

Experimental design and animal husbandry

Agalychnis embryos were obtained from a breeding colony maintained at Boston University. Breeding was stimulated by exposing pairs of adult frogs to cycles of simulated rain in custom-built breeding chambers (Fenolio, 1996). We collected clutches from five pairs and kept them moist at 25 °C until all eggs hatched. Hatchlings were randomly assigned to experimental treatments within 24 h of hatching. *Xenopus* hatchlings were obtained from a commercial distributor (Nasco, Fort Atkinson, WI, USA) and randomly assigned to experimental treatments. No information on the number of breeding pairs contributing eggs could be obtained. All tadpoles were reared individually in 4-L plastic containers filled with carbon-filtered tap water arrayed in randomized blocks across shelves (see Table 1 for sample sizes). Temperature and food treatments were chosen based on previous experience and thermal requirements of each species. Water was renewed weekly. For both species, we recorded duration of the larval period (in days) and weight at metamorphosis (in mg). We considered metamorphic climax (end of the larval period) to be Gosner stage 42 in *Agalychnis* [forelimb emergence (Gosner, 1960)] and Nieuwkoop–Faber stage 62 in *Xenopus* [short, straight tentacles, but with the head still broader than the cranial part of the trunk (Nieuwkoop & Faber, 1994)]. Survival was high (over 82%) in all treatments (see Table 1).

Rearing of *Agalychnis callidryas*

Agalychnis tadpoles were fed alfalfa powder (Now Foods, Bloomingdale, IL, USA). For the food manipulation experiment, we reared *Agalychnis* tadpoles at a constant temperature of 25 °C (range 24.6–25.4 °C) in a walk-in

Table 1 Independent manipulations of both temperature and food level during larval development affected the larval period, size at metamorphosis and juvenile shape in red-eyed treefrogs (*Agalychnis callidryas*) and African clawed frogs (*Xenopus laevis*). We present the average trait values per treatment (and SE) for each species. Body and hindlimb growth curves for a subset of these animals are presented in Fig. 2.

	Agalychnis				*Xenopus*			
	Temperature		Food		Temperature		Food	
Treatment conditions and sample size	High 29 °C $N = 21$	Low 21 °C $N = 15$	High 600 mg week^{-1} $N = 28$	Low 150 mg week^{-1} $N = 17$	High 24 °C $N = 26$	Low 18 °C $N = 27$	High 600 mg week^{-1} $N = 30$	Low 150 mg week^{-1} $N = 29$
Larval period (days)	29.21 (0.72)	49.53 (0.81)	36.39 (1.09)	60.93 (1.35)	29.58 (0.74)	53.63 (0.73)	40.43 (0.60)	50.03 (0.61)
Mass (mg)	75.99 (2.16)	70.11 (2.43)	87.56 (4.20)	45.10 (5.20)	67.75 (3.46)	63.15 (3.26)	120.29 (3.09)	41.54 (3.15)
Snout–vent length (mm)	20.86 (0.32)	19.91 (0.38)	21.42 (0.29)	15.41 (0.37)	18.49 (0.32)	18.51 (0.33)	22.54 (0.31)	16.42 (0.31)
Hindlimb length (mm)	34.21 (0.60)	34.07 (0.71)	35.62 (0.55)	24.64 (0.71)	25.88 (0.51)	26.6 (0.51)	32.68 (0.50)	22.15 (0.51)
Head width (mm)	6.89 (0.14)	7.00 (0.16)	7.26 (0.07)	5.87 (0.09)	5.66 (0.11)	5.55 (0.11)	6.72 (0.10)	5.10 (0.10)
Snout length (mm)	2.70 (0.07)	2.41 (0.09)	2.85 (0.06)	1.86 (0.08)	3.27 (0.07)	3.24 (0.07)	3.90 (0.10)	3.11 (0.10)
Survival (%)	86	100	93	82	88	96	96	100

environmental chamber. Tadpoles were photographed and then randomly assigned to either low or high (i.e. *ad libitum*) food level treatments (150 and 600 mg week^{-1}, respectively). For the temperature manipulation experiment, we reared *Agalychnis* tadpoles at two different temperatures (21 and 29 °C) under *ad libitum* food conditions in incubators. Upon metamorphic climax, water was removed and replaced by soaked *Sphagnum* to provide a wet substrate during tail resorption.

Rearing of *Xenopus laevis*

Xenopus tadpoles were fed commercial frog brittle (Nasco). In the food manipulation experiment, we used the same two levels of food used for *Agalychnis* and kept the tadpoles at constant 21 °C. In the temperature manipulation experiment, we fed tadpoles *ad libitum* and reared them at either 18 °C or 24 °C. Being an aquatic species, *Xenopus* tadpoles completed metamorphosis in the water.

Locomotion trials

Upon complete tail resorption, we conducted videotaped trials of jumping performance of *Agalychnis* and swimming performance of *Xenopus*. *Agalychnis* jumping trials were conducted at 25°C and videotaped from above. We placed juvenile frogs individually under a plastic cup in the centre of a plastic arena (60 × 70 cm) and allowed them to acclimate for 5 min. Then we removed the cup and prodded the froglet gently with the blunt end of forceps to make it jump. We stimulated froglets to jump repeatedly until they showed signs of exhaustion, counting the number of jumps. To estimate the maximum jump distance achieved by each froglet, we reviewed the trials, chose the two longest jumps, captured and superimposed the frames with the take off and landing of the frog using the software Image J (NIH, Bethesda, MD, USA) and measured the linear distance between the positions of the frog's vent in each frame (Van Buskirk & McCollum, 2000). *Xenopus* swimming trials were conducted at 21 °C in a hexagonal plexiglas arena (60 cm in diameter) filled with water. The arena was suspended above ground, and the trials were filmed from beneath to avoid the distorting effect of waves at the water surface (Van Buskirk & McCollum, 2000). Each froglet was allowed to acclimate to the arena undisturbed for 5 min, and then it was stimulated to swim through gently prodding. Most of the *Xenopus* froglets tested showed no signs of exhaustion even after repeated swim bursts, and we therefore extended the trials until they swam rapidly at least three times. We videotaped at 30 frames s^{-1} and digitized the 15 first frames of each swim burst, thus calculating sprint distance over the first 0.5 s. We used linear regressions to test the effects of size and size-corrected (using body length as covariate in analyses of covariance) hindlimb length on the number of jumps and maximum distance jumped in *Agalychnis* and the maximum sprint distance in *Xenopus*.

Morphology and ossification

To examine the effect of rearing conditions on the allometric relationship between body and hindlimb growth rate during larval development, we took weekly measurements of a subset of tadpoles (10–15) of each species from each treatment. Tadpoles were placed in a small water-filled plexiglas container with a scaled sliding wall that was shifted until it nearly touched the tadpole. We then took lateral digital pictures of the tadpoles with little parallax error and without having to anaesthetize them repeatedly. We measured body length (snout-to-vent, SVL) and hindlimb length from the pictures using Image J. We used repeated measures ANOVAS to test for differences in growth rates between treatments within species, after log-transforming the variables to improve linearity.

We examined the effects of larval-rearing conditions on post-metamorphic morphology in all individuals. Immediately after the locomotion trials, we blotted dry and weighed the juveniles to the nearest 0.1 mg using an electronic scale. We then killed the froglets by immersion in a lethal dose of the anaesthetic tricaine methane sulphonate (MS-222) and preserved them in 10% neutral buffered formalin for 4 days. We used a calliper to take four linear external morphological measurements (to the nearest 0.1 mm): snout length (distance between front inter-ocular line and the tip of the snout, SN), head width (linear distance between both mouth corners, HW), complete hindlimb length (distance from vent to the tip of the longest toe, HL) and snout-to-vent length (from tip of the snout to the vent, SVL). A few animals died before complete tail resorption; for these, we have SVL but not mass or locomotor performance data. We analysed changes in body shape using analyses of covariance (ANCOVA) including SVL as a covariate in the models. To estimate the error incurred in our morphological measurements, we measured each individual twice in a subset of twelve specimens per species, balanced across treatments and calculated our repeatability through variance component estimation (Lessells & Boag, 1987). Repeatability estimates for *A. callidryas* were: 0.996 (SVL), 0.973 (HW), 0.999 (HL) and 0.911 (SN). Repeatability estimates for *X. laevis* were: 0.996 (SVL), 0.974 (HW), 0.998 (HL) and 0.927 (SN).

To obtain a quantitative marker of differentiation, we assessed the level of ossification of the complete skeleton in a random subset of juveniles from different larval environments as they completed tail resorption (Gosner stage 46). We skinned, eviscerated, cleared and differentially stained the specimens for cartilage (with Alcian blue) and bone (with Alizarin red) following Hanken and Wassersug (1981). We then took whole-skeleton digital pictures of the stained specimens under standardized light conditions. Because bone (stained with Alizarin red) has a different colour signature than cartilage (stained with Alcian blue), we used an algorithm in MATLAB 9.0 with Image Processing plug-in (The MathWorks, Natick, MA, USA) to automatically quantify bone and cartilage (code available upon request). Pixels from digital images had RGB (red, green, blue) values ranging from 0 to 255. We operationally defined 'red' pixels as having a $(R + 1)/(G + 1)$ ratio greater than 1 and 'blue' pixels as having a $(2 \times B + 1)/(R+G + 1)$ ratio greater than 1 and quantified the red and blue pixels in the image. These thresholds included bone and cartilage and excluded background elements. We analysed differences between larval environments in the degree of ossification through analysis of covariance, testing for differences in the number of red pixels (bone) while controlling for the number of blue pixels (cartilage). To estimate effect sizes of differences between treatments in larval period, mass at metamorphosis, morphological traits and level of ossification, we calculated Cohen's *d* (Cohen, 1988; Rosenthal *et al.*, 2000).

Results

Effects of larval environment on time to and size at metamorphosis

Low temperatures resulted in significantly longer larval periods than high temperatures in both species (*Agalychnis*: $F_{1,34} = 386.48$, $P < 0.0001$, $d = 6.34$; *Xenopus*: $F_{1,51} = 537.82$, $P < 0.0001$, $d = 6.41$; Fig. 1). Likewise, low food levels resulted in longer times to metamorphosis than high food levels (*Agalychnis*: $F_{1,43} = 277.63$, $P < 0.0001$, $d = 4.97$; *Xenopus*: $F_{1,57} = 125.30$, $P < 0.0001$, $d = 2.91$; Fig. 1). Mass at metamorphosis (after tail resorption) was significantly lower in juveniles from the low food treatment in both species (*Agalychnis*: $F_{1,36} = 40.42$, $P < 0.0001$, $d = -2.19$; *Xenopus*: $F_{1,57} = 318.28$, $P < 0.0001$, $d = -4.65$), and so was SVL (*Agalychnis*: $F_{1,43} = 382.32$, $P < 0.0001$, $d = -5.072$; *Xenopus*: $F_{1,57} = 553.05$, $P < 0.0001$, $d = -3.63$). Differences in temperature, however, did not significantly affect post-metamorphic mass (*Agalychnis*: $F_{1,34} = 3.27$, $P = 0.08$, $d = -0.64$; *Xenopus*: $F_{1,49} = 0.93$, $P = 0.339$, $d = -0.27$) or SVL (*Agalychnis*: $F_{1,34} = 3.61$, $P = 0.07$, $d = -0.67$; *Xenopus*: $F_{1,52} = 0.003$, $P = 0.957$, $d = 0.01$), although *Agalychnis* showed a trend towards higher mass at high temperature (Table 1).

Induced shifts of growth curves, juvenile shape and ossification

Body growth rates differed significantly between food levels in both species ('treatment × time' factor in repeated measures ANOVA on SVL; *Agalychnis*: $F_{8,144} = 6.97$, $P < 0.0001$; *Xenopus*: $F_{8,224} = 28.25$, $P < 0.0001$; Fig. 2), and so did limb growth rates (*Agalychnis*: $F_{7,126} = 9.07$, $P < 0.0001$; *Xenopus*: $F_{7,196} = 7.65$, $P < 0.0001$; Fig. 2). Differences in temperature also significantly altered body (*Agalychnis*: $F_{4,72} = 19.27$, $P < 0.0001$; *Xenopus*: $F_{8,216} = 94.67$, $P < 0.0001$) and limb growth rates (*Agalychnis*: $F_{3,54} = 20.33$, $P < 0.0001$; *Xenopus*: $F_{7,189} = 32.97$, $P < 0.0001$; Fig. 2).

Analyses of covariance indicated that developing at low temperature resulted in relatively longer hindlimbs in both species (*Agalychnis*: $F_{1,33} = 8.92$, $P < 0.01$, $d = 1.03$; *Xenopus*: $F_{1,51} = 6.66$, $P = 0.013$, $d = 0.70$; see Table 1). In *Agalychnis*, low temperatures also resulted in greater head width ($F_{1,33} = 6.66$, $P = 0.01$, $d = 0.89$) but had no effect on snout ($F_{1,33} = 1.61$, $P = 0.213$), whereas heads were not affected in *Xenopus* (head width: $F_{1,51} = 1.85$, $P = 0.18$; snout length: $F_{1,51} = 0.10$, $P = 0.751$). Lack of differences across treatments in snout length could be partly attributable to greater measurement error, judging from repeatability estimates. Low food, in contrast, resulted in relatively shorter hindlimbs

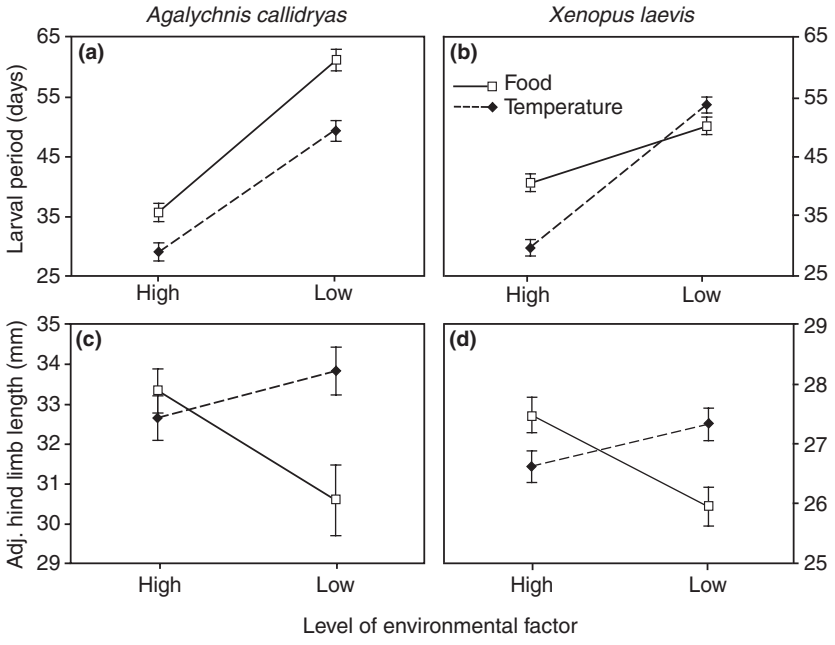

Fig. 1 Effects of environmental conditions on larval period and juvenile shape in the frogs *Agalychnis callidryas* (a, c) and *Xenopus laevis* (b, d). Metamorphosis was delayed in both species by both low temperature and low food availability (a, b). Nonetheless, these factors had opposite effects on relative hindlimb length, controlling for differences in snout–vent length through analysis of covariance (c, d). Data are means (a, b) and least square means, adjusted for snout-to-vent length (c, d) ± SE.

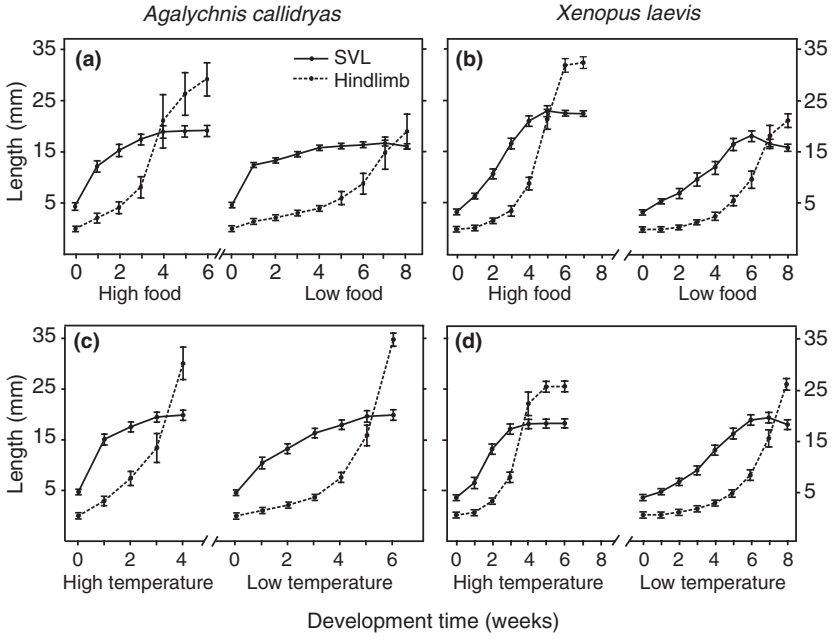

Fig. 2 Effects of environmental conditions on the allometric relationships between body (SVL) and hindlimb growth curves in the frogs *Agalychnis callidryas* (a, c) and *Xenopus laevis* (b, d). Growth curves represent weekly measurements of individual tadpoles (mean ± SE, $N = 10$ *Agalychnis* and 15 *Xenopus* per treatment). Body and hindlimb growth trajectories flattened under constrained food availability, yielding smaller body sizes and shorter legs at metamorphosis (last data point). In contrast, low temperatures resulted in a similar size at metamorphosis as did high temperatures, but in longer legged juveniles. Data were log-transformed to improve linearity prior to analysis by repeated measures ANOVA.

in both species (*Agalychnis*: $F_{1,42} = 6.47$, $P = 0.015$, $d = -0.99$; *Xenopus*: $F_{1,56} = 4.98$, $P = 0.030$, $d = -0.74$). Low food also resulted in relatively narrower heads in *Agalychnis* ($F_{1,42} = 8.44$, $P = 0.006$, $d = -1.12$) but not in *Xenopus* ($F_{1,56} = 0.23$, $P = 0.635$). Larval-rearing conditions also affected the degree of ossification observed in juveniles. Froglets emerging from low temperature treatments showed a lower level of ossification than those at high temperature (*Agalychnis*: $F_{1,18} = 21.11$, $P < 0.0001$, $d = -2.33$; *Xenopus*: $F_{1,50} = 17.56$, $P = 0.0001$, $d = -0.68$; Fig. 3). Conversely, tadpoles reared under low food levels were more ossified after metamorphosis than those reared at higher food levels (*Agalychnis*: $F_{1,12} = 5.65$, $P = 0.035$, $d = 1.44$; *Xenopus*: $F_{1,57} = 7.22$, $P = 0.009$, $d = 0.67$; Fig. 3). Growth rate, estimated as the ratio of mass at metamorphosis over larval period, was reduced at both low temperature and low food level, but it was more strongly affected by low food than by low temperature treatments in both species (*Agalychnis*: food, $F_{1,34} = 54.16$, $P < 0.0001$, $d = -3.0$;

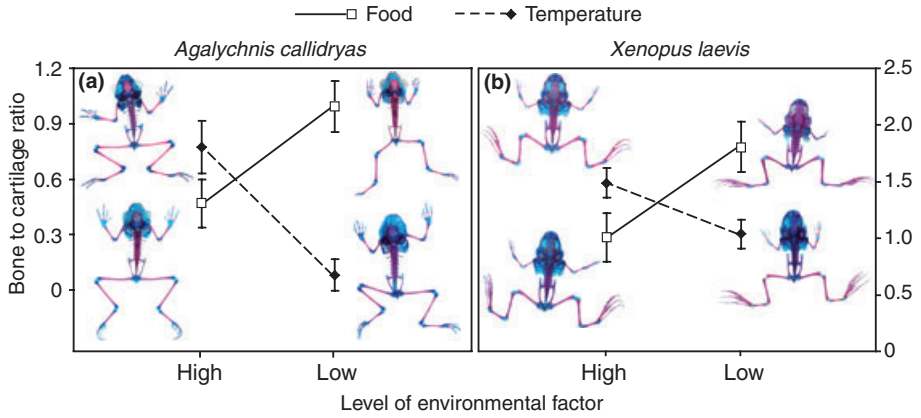

Fig. 3 Effects of environmental conditions on ossification of juvenile frogs, *Agalychnis callidryas* (a) and *Xenopus laevis* (b). Skeletal ossification, a quantitative marker of differentiation, was measured from digital pictures of cleared and stained specimens (inset images) as the amount of red pixels (bone, stained with Alizarin red) controlling for blue pixels (cartilage, stained with Alcian blue) using ANCOVA. Despite their longer larval periods, animals reared at low temperature had a smaller fraction of their skeleton ossified at tail resorption than did those reared at high temperature, indicating thermal constraints on skeletal differentiation. Animals reared on limited food were more ossified at tail resorption than were well-fed animals, indicating that any dietary constraints on skeletal differentiation were more than compensated by their longer larval period.

temperature, $F_{1,30} = 46.96$, $P < 0.0001$, $d = -2.22$; *Xenopus*: food, $F_{1,56} = 307.85$, $P < 0.0001$, $d = -4.09$; temperature, $F_{1,48} = 47.68$, $P = 0.001$, $d = -2.15$).

Effect of juvenile shape on locomotor performance

Larger *Agalychnis* frogs were capable of a greater number of jumps ($N = 40$, $r = 0.71$, $P < 0.0001$) and of jumping greater distances than smaller frogs ($N = 40$, $r = 0.78$, $P < 0.0001$). Similarly, larger *Xenopus* frogs were capable of sprinting greater distances in 0.5 s ($N = 104$, $r = 0.65$, $P = 0.03$). However, relative hindlimb length (i.e. corrected for SVL through ANCOVA) had no significant effect on either *Agalychnis* maximum jump distance ($N = 40$, $r = 0.17$, $P = 0.09$) or *Xenopus* sprint distance ($N = 104$, $r = 0.04$, $P = 0.87$).

Discussion

Similar shifts in larval period but opposite changes in shape

It was suggested that metamorphosis entailed such a thorough developmental remodelling that it offered a fresh start in the next life stage (Moran, 1994). This idea is supported by the fact that some adult structures do not originate from larval ones but from quiescent cells (Alberch, 1987; Fritzsch, 1990; Parichy, 1998), and some larval traits show no phenotypic or genetic correlation with their post-metamorphic counterparts (Watkins, 1997, 2001; Gomez-Mestre & Tejedo, 2005). However, most cell lineages give rise to both larval and adult structures (Hanken, 1992), and the decoupling between larval and post-metamorphic traits is far from complete. It is now clear that plasticity in the timing of ontogenetic switch points is widespread in organisms with complex life cycles and often has continued effects on subsequent life stages (Marshall, 2003; Pechenik, 2006). In reviewing the existence of latent effects in animals with complex life cycles, Pechenik (2006) concluded that not all species respond to pre-metamorphic conditions in the same way and that different stressors seem to have different effects within species. Despite such disparity, some common patterns may be found via experimental manipulation of the environment experienced by developing organisms.

Temperature and food levels in our experiments affected tadpole growth and differentiation rates, resulting in changes in larval period, size at metamorphosis, post-metamorphic hindlimb length and level of ossification in both species. Both species experienced similar heterokairic changes in development that transcended metamorphosis and carried over to the juvenile phase. Whereas both low temperature and low food level prolonged the larval period to a similar extent, they had opposite effects on other traits, so that late-metamorphosing animals could be long-legged or short-legged, and well or poorly ossified, depending on the environmental factor, and presumably the developmental mechanisms, that altered their phenotype. Probably, no natural environmental factors can affect differentiation entirely without affecting growth, and *vice versa*, because growth and differentiation are partially correlated aspects of development. However, they can be decoupled to a large extent (Smith-Gill & Berven, 1979; Blouin & Brown, 2000; McCoy *et al.*, 2007), and it is the relative impact of the larval environment on either growth or differentiation that results in the observed changes in juvenile shape. For instance, both low

temperature and low food levels caused reduced growth rates, but low food availability did so to a greater extent than low temperature.

Low temperature in unconstrained growth conditions (i.e. high food availability) delayed metamorphosis but resulted in metamorphs of similar mass as those reared at high temperature (Figs 1 and 2; Table 1). Despite their similar size, the animals reared at low temperature were less ossified, indicating a lower degree of differentiation. At low temperature, the exponential phase of hindlimb growth was somewhat delayed but also extended (Fig. 2), resulting in relatively long-legged juveniles. Reduced food availability also caused delayed metamorphosis and, unsurprisingly, reduced mass at metamorphosis. However, lowering food levels, unlike lowering temperature, caused a marked flattening of growth curves, particularly that of the hindlimb (Fig. 2), and resulted in short-legged juveniles. Food-limited individuals, however, were more ossified than juveniles fed *ad libitum*, indicating that low food constrained mass at metamorphosis without similarly limiting differentiation.

The last common ancestor of the two species studied likely existed over 180 million years ago (Wiens, 2007), and they are highly divergent ecologically. The similarity in their developmental responses to basic environmental manipulations, such as food availability and temperature, suggests that these are likely common features of anuran development. Factors affecting larval development can clearly exert opposite effects on morphology and/or performance in subsequent life stages, even if they result in similar alterations of the larval period.

Patterns of larval period plasticity and juvenile shape

Emerson (1986) first showed a link between the timing of metamorphosis and juvenile morphology. Since then, reports have accumulated of both positive and negative allometries between larval period and relative limb length and head length and width. Our results explain the apparent discrepancies in the previous literature and indicate a clear link between developmental plasticity and post-metamorphic shape, based on the relative impact of the environment on growth and differentiation. We summarize the expected consequences of developmental plasticity on metamorphosis and post-metamorphic morphology in Fig. 4, depicting four extreme scenarios of the continuum between optimal and impaired development. If growth is enhanced (Fig. 4a), for instance via high food availability or low density, tadpoles will metamorphose quickly and juveniles will attain large sizes, show long hindlimbs and will be relatively less ossified than animals developing under less favourable growth conditions. On the other hand, if differentiation is enhanced, as in response to reduced water volume or owing to high temperature, tadpoles will also metamorphose quickly, but attain a smaller size (or not, because differentiation has less bearing on size

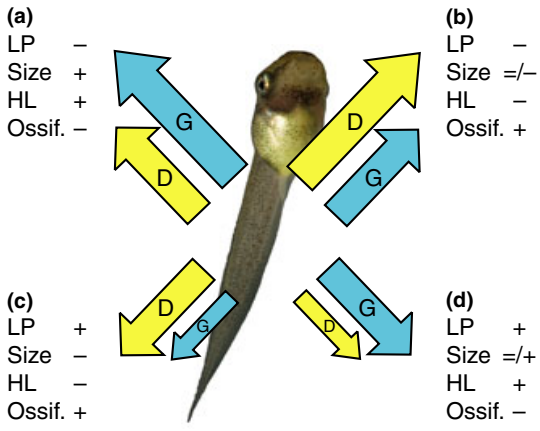

Fig. 4 Environmental conditions experienced during the larval phase can affect growth and differentiation to different extents, causing not only shifts in time to and size at metamorphosis, but also alterations in the developmental trajectories of various anatomical features. Here, we summarize how such developmental modifications result in post-metamorphic changes in morphology and degree of ossification. Yellow/light grey arrows, marked '*D*', represent differentiation, whereas blue/dark grey arrows ('*G*') stand for growth. Long wide arrows indicate that the corresponding component of development is enhanced (a, b), whereas short thin ones indicate that it is constrained (c, d). (a) Enhancing growth (e.g. by increasing food level or reducing larval density) results in a short larval period and/or increased size at metamorphosis, proportionately long hindlimbs and a low level of ossification. (b) Enhancing differentiation (e.g. by increasing temperature or decreasing water volume) results in a short larval period, equal or smaller size at metamorphosis, proportionately shorter limbs and high degree of ossification. (c) Constraining growth (e.g. by reducing food level or increasing larval density) results in a long larval period, reduced size at metamorphosis, proportionately short limbs and high level of ossification. (d) Constraining differentiation (e.g. by low temperature) results in a long larval period, equal or larger size at metamorphosis, proportionately longer hindlimbs and a low level of ossification.

than growth does), with relatively shorter limbs (potentially caused by shortened limb elongation phases; Gomez-Mestre & Buchholz, 2006) and higher degree of ossification (Fig. 4b). In turn, if growth is constrained (e.g. at low food levels or high larval density), tadpoles will metamorphose late and juveniles will be small and short-limbed, but more ossified (Fig. 4c). Conversely, if differentiation is constrained, as in low temperature, tadpoles will also take longer to metamorphose but will do so at equal or bigger size and will show longer hindlimbs but be less ossified (Fig. 4d).

This simple model of the relative weight of altered growth and differentiation fits the vast majority of published reports. When food level or larval density was manipulated, tadpoles in growth-constraining conditions invariably metamorphosed later, at a smaller size, and with proportionately shorter limbs (Emerson, 1986; Tejedo *et al.*, 2000a; Relyea & Hoverman, 2003). Also in

agreement with our data, most studies manipulating temperature or pond duration (i.e. having a greater impact on differentiation than on growth) showed that long larval periods result in proportionately longer limbs, often without significant change in body size (Newman, 1989; Gomez-Mestre & Buchholz, 2006; Richter-Boix *et al.*, 2006; Márquez-García, 2009). Exposing tadpoles to the nonlethal presence of predators, however, has yielded mixed results in terms of juvenile shape. Predator presence induced longer larval periods in all these studies but resulted in either proportionately longer hindlimbs (Relyea, 2001), shorter hindlimbs (Nicieza *et al.*, 2006; Capellan & Nicieza, 2007) or had no effect on hindlimb length (Van Buskirk & Saxer, 2001). However, shorter hindlimbs resulted only when size at metamorphosis was also reduced (Nicieza *et al.*, 2006; Capellan & Nicieza, 2007), whereas longer hindlimbs occurred when no differences in size between exposed and unexposed tadpoles were detected (Relyea, 2001). This suggests that growth may have been limited in the first two studies but not the third. Some antipredator defences of tadpoles may limit growth more than others (e.g. reduced foraging activity vs. induced morphology), and the developmental stage at which environmental induction occurs is also likely to affect the extent to which developmental trajectories are altered. The sole study that does not fit the predictions of our model is Blouin & Brown, 2000; where *Rana cascadae* froglets reared at 25 °C were reported to have on average 2.6% longer tibiofibulae than those reared at 18 °C. A positive genetic correlation between larval period and relative hindlimb length in unconstrained growth conditions has been found in two frog species (Emerson *et al.*, 1988; Blouin & Loeb, 1991; Ficetola & De Bernardi, 2006). Unless *R. cascadae* is an exception to such genetic correlation, one would expect *ad libitum*–fed, fast-developing tadpoles to become short-legged juveniles and *vice versa*, as found for five other species (Gomez-Mestre & Buchholz, 2006; Richter-Boix *et al.*, 2006). A reanalysis of Blouin and Brown's data (from their Fig. 2a) suggests that their result was heavily dependent upon a single observation that constitutes an outlier within the warm-reared tadpoles, as evidenced by the extreme studentized deviate or ESD method ($Z = 2.72$, $P < 0.05$). Further studies on this species should help clarify the degree of concordance with the model here proposed.

Larval period is an insufficient parameter to understand carry-over effects of larval environments, because it subsumes a variety of different developmental processes, which can modify larval period to a similar extent and yet result in very different size, shape and state of differentiation of post-metamorphic individuals.

Mechanistic explanations for divergent patterns

Depletion of energy storage and subsequent growth limitation is likely to be a key determinant of developmental trajectories across taxa with complex life cycles, but it is not the only one (Pechenik, 2006). In this study, we found that different environmental factors have congruent effects on one aspect of development (metamorphic timing) and opposite effects on other aspects of development (hindlimb development, ossification); these therefore must be regulated through different processes. Adaptive accelerations of metamorphosis are mediated by an increase in thyroid hormone (TH) levels and tissue sensitivity to TH (Denver, 1998; Denver *et al.*, 1998; Buchholz & Hayes, 2005). Nevertheless, accelerating metamorphosis does not equally accelerate other aspects of development, such as sexual differentiation (Buchholz & Hayes, 2005; McCoy *et al.*, 2007) or building of the immune response (Gervasi & Foufopoulos, 2008), and emerging froglets vary substantially in these traits.

Under unconstrained growth conditions, heterokairic shifts in the timing of the TH-dependent metamorphic climax truncate or extend the larval limb elongation phase (Gomez-Mestre & Buchholz, 2006) so that longer larval periods yield proportionately longer hindlimbs at metamorphosis. The longest larval periods in amphibians are found in species with overwintering tadpoles, which take 1 year or longer to reach metamorphosis. Preliminary data suggest that overwintering tadpoles metamorphose into longer legged frogs than do conspecifics metamorphosing within one breeding season (Y.C. Kam, personal communication).

Under growth-constraining conditions, however, long larval periods result in relatively shorter hindlimbs, regardless of a postponed TH surge. Crespi and Denver (Crespi & Denver, 2006) identified *Xenopus'* leptin and its receptor. Leptin is a type-I cytokine hormone secreted by adipose tissue that induces growth and development of the hindlimb during early development in tadpoles (Crespi & Denver, 2006). Because of its positive correlation with fat content (Delavaud *et al.*, 2000; Sagawa *et al.*, 2002), leptin is a strong candidate regulator to explain reduced hindlimb growth under food deprivation. In contrast, leptin levels are not expected to vary substantially in response to changes in temperature.

Little effect of juvenile shape on locomotor performance

Locomotor performance (sprint distance in *Xenopus* and maximum jump distance in *Agalychnis*) was highly dependent on size, but not on differences in relative hindlimb length. This result agrees with most published studies on the effect of juvenile morphology on locomotor ability (Emerson *et al.*, 1988; Alvarez & Nicieza, 2002; Nicieza *et al.*, 2006, but see Ficetola & De Bernardi, 2006; Tejedo *et al.*, 2000b). Slight differences in shape (≤10% changes in relative hindlimb length) seem to have little or no effect on locomotor performance; it is therefore unlikely that they are under strong selection. Such morphological variation may then easily evolve as a

correlated by-product of selection acting on larval period (Gomez-Mestre & Buchholz, 2006).

Conclusions

The timing of transitions between adjacent life stages in organisms with complex life cycles is affected by environmental conditions through whole-body or tissue-specific alterations of growth and differentiation. Such heterokairic shifts in metamorphic timing can cause allometric changes in post-metamorphic shape and degree of maturation, indicating that metamorphosis does not entirely reset the effects of the larval environment. Nevertheless, identical shifts in time to metamorphosis induced in response to different environmental factors may have very different post-metamorphic phenotypic consequences depending on how growth and differentiation are affected relative to each other.

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