PHYSIOLOGICAL ECOLOGY - ORIGINAL PAPER

Irreversibility of a bad start: early exposure to osmotic stress limits growth and adaptive developmental plasticity

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Abstract Harsh environments experienced early in development have immediate effects and potentially long-lasting consequences throughout ontogeny. We examined how salinity fluctuations affected survival, growth and development of *Fejervarya limnocharis* tadpoles. Specifically, we tested whether initial salinity effects on growth and rates of development were reversible and whether they affected the tadpoles' ability to adaptively accelerate development in response to deteriorating conditions later in development. Tadpoles were initially assigned to either low or high salinity, and then some were switched between salinity levels upon reaching either Gosner stage 30 (early switch) or 38 (late switch). All tadpoles initially experiencing low salinity survived whereas those initially experiencing high salinity had poor survival, even if switched to low salinity. Growth and developmental rates of tadpoles initially assigned to high salinity did not increase after osmotic stress release. Initial low salinity conditions allowed tadpoles to attain a fast pace of development even if exposed to high salinity afterwards. Tadpoles experiencing high salinity only late in development metamorphosed faster and at a smaller size, indicating an adaptive acceleration of development to avoid osmotic stress. Nonetheless, early exposure to high salinity precluded adaptive acceleration of development, always causing delayed metamorphosis relative to those in initially low salinity. Our results thus show that stressful environments experienced early in development can critically impact life history traits, having long-lasting or irreversible effects, and restricting their ability to produce adaptive plastic responses.

Introduction

Harsh environmental conditions can have different consequences for survival and life history traits of organisms depending on the ontogenetic time point at which they are exposed to them. Thus, harsh environments experienced at an early developmental stage not only have immediate effects on the organism but can also have long lasting consequences throughout ontogeny (Metcalfe and Monaghan 2001). In organisms with complex life cycles, the consequences of altering growth and developmental trajectories early in ontogeny can even persist after metamorphosis, affecting their size, morphology and degree of maturation at later stages (Pechenik 2006; Gomez-Mestre et al. 2010). Exposure to stressful conditions early in development can also restrict the ability to produce adaptive plastic responses later in life (Alvarez and Nicieza 2002; De Block and Stoks 2005; Kishida et al. 2010), hence reducing their ability to cope with changing

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environments. Some species, however, can attenuate the negative effects of poor initial conditions by achieving compensatory growth if the environmental conditions improve (Metcalfe and Monaghan 2001; Ali et al. 2003). It is essential to understand how organisms become more or less sensitive or responsive to environmental changes over the course of ontogeny if we want to predict how environmental factors will influence their survival and evolution (Hensley 1993).

The environments experienced by amphibian larvae are usually heterogeneous and unpredictable, as they are often at risk from pond drying, food shortages, extreme temperatures, predator attacks, water acidity or salinity (Newman 1992; Alford 1999; Rose 2005; Wu and Kam 2009). Development of amphibian larvae is very plastic, varying their time to and size at metamorphosis either adaptively in response to specific environmental cues or non-adaptively due to environmental fluctuations themselves (Newman 1992; Rose 2005; Rudolf and Rödel 2007). Tadpoles should grow as much as possible while conditions are benign in their larval environment (i.e. risk of mortality is low) but accelerate development and achieve an early metamorphosis if the environment becomes inhospitable (Werner 1986; Benard 2004; Vonesh and Warkentin 2006). However, the effect of any given environmental factor on tadpole development is often highly dependent on the stage and condition of the animal. Thus, the same environmental factor may decrease survivorship, growth and developmental rates if experienced during premetamorphic stages, but accelerate development and induce early metamorphosis if experienced during prometamorphic ones (Denver 1997; Denver et al. 2002). Accelerating larval development may come at the cost of smaller and less mature juveniles (Newman 1992), but so does continued development under stressful conditions, in addition to reducing larval survival. This is relevant because small size at metamorphosis reduces post-metamorphic survival (Smith 1987; Berven 1990; Gomez-Mestre and Tejedo 2003; Chelgren et al. 2006), delays time to sexual maturity (Semlitsch et al. 1988; Berven 1990), and reduces the size at maturity (Semlitsch et al. 1988), overall reducing population recruitment (Smith 1987).

Salinity is an important stressor for most amphibians due to their highly permeable skin and inability to concentrate urine (Balinsky 1981; Duellman and Trueb 1994). All tadpoles are sensitive to salinity, and moderate salinity (2–9 parts per thousand; ppt) decreases tadpole survival, growth, and development (Christy and Dickman 2002; Gomez-Mestre et al. 2004; Chinathamby et al. 2006; Wu and Kam 2009). In most systems, pond salinity is lowest at the beginning of the amphibian reproductive period, as ponds fill up, and gradually increases over time as ponds evaporate and are maximal just prior to pond desiccation.

Surprisingly, no studies to date have addressed how salinity fluctuations can affect amphibian larval development. However, a few species breed in coastal pools with strong marine influence where salinity can fluctuate considerably (Wu and Kam 2009; Sillero and Ribeiro 2010). Tadpoles of the Indian rice frog (*Fejervarya limnocharis*) living in the brackish water of coastal rock puddles and pools formed by uplifted coral on the tropical Green Island of Taiwan provide an excellent model system to study the effects of salinity-induced stress over larval ontogeny on growth, development and adaptive developmental plasticity (Wu and Kam 2009). We examined how changes in salinity affected survival, growth and development of *F. limnocharis* tadpoles. Specifically, we tested whether salinity effects early in development were reversible and whether they affected tadpoles' ability to adaptively accelerate development.

Materials and methods

Study animals

Fejervarya limnocharis is a medium-sized frog (30–60 mm) distributed throughout east, southeast, and south Asia, including many small, isolated islands (Sumida et al. 2007). In Taiwan, *F. limnocharis* is widely distributed up to elevations of 1,000 m on the main island and on offshore islands (Alexander et al. 1979; Yang 1998). *Fejervarya limnocharis* usually breeds from February to September, with breeding correlated with rainfall and irrigation and restricted by low temperatures (Alexander et al. 1979). This species usually breeds in temporary freshwater pools such as rice pools and roadside puddles, but some populations breed in the brackish water of rock pools in coastal areas (Wu and Kam 2009). Mean clutch size varies greatly, with a range of about 450–1,800, and the larval period is less than 2 months (Alexander et al. 1979).
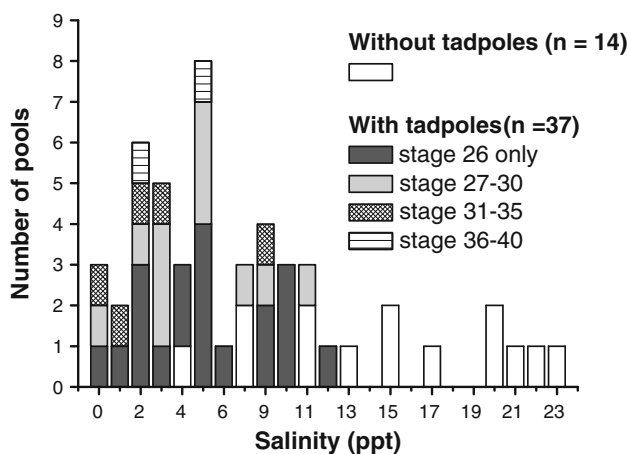
Study site

Green Island (121.28°E, 22.35°N) is a small tropical island off the southeastern coast of Taiwan, with a total area of 15 km^2. The climate on this island is characterized by high air temperature and abundant annual rainfall, with no distinct dry season (Ni 2003). Typhoons are frequent between June and September, and usually bring abundant rains. The monthly average air temperature was 23.5°C and the annual rainfall was 2,575 mm (1998–2006; data from the Central Weather Bureau, Taiwan). During the breeding season of *F. limnocharis* (February–September), the most abundant rains occurred mainly between July and

September (average monthly rainfall about 290–320 mm). *Fejervarya limnocharis* usually breeds in rock pools just above the high tide line. Pools with tadpoles varied greatly in size and shape (range of maximum diameter 11–534 cm), and were relatively shallow in depth (range of maximum depth 5–22 cm) (Wu and Kam 2009). These rock pools are uncommon breeding sites for amphibians because their small size, seawater spills and strong rainfalls during the monsoon season cause frequent and large salinity fluctuations. Salinity in these pools varies between 0 and 23 ppt, although no tadpoles have been found in pools with salinity over 12 ppt (Wu and Kam 2009). Our field surveys showed tadpoles could be exposed to different salinities (0–12 ppt) during ontogeny, especially at an early developmental stage (Gosner stage 26; Gosner 1960) (Fig. 1).

Experimental design

We collected tadpoles of *F. limnocharis* at Gosner stage 26 (Gosner 1960) from six pools (salinity was 0, 1, 1, 2, 3 and 3 ppt) on Green Island in June 2006. Tadpoles from each pool were mixed together and then randomly assigned to one of six treatments. Tadpoles were initially assigned to either low or high salinities (3 and 9 ppt, respectively). We established experimental salinity switches at specific developmental stages, an early salinity switch at Gosner stage 30, and a late switch at Gosner stage 38. The reason we chose stage 30 and stage 38 for switch points is because the former is within the premetamorphic period, when tadpoles grow with few morphological changes and low plasma thyroid hormone (TH), and the latter is within the

prometamorphic period, when tadpoles grow with obvious morphological changes and increasing plasma TH (Denver 2009). Hence, we established a total of six treatments as follows: constant low salinity (LLL), low salinity with early switch to high salinity (LHH), low salinity with late switch to high salinity (LLH), constant high salinity (HHH), high salinity with early switch to low salinity (HLL), and high salinity with late switch to low salinity (HHL). Because higher salinity slows down the rate of tadpole development (Gomez-Mestre et al. 2004; Wu and Kam 2009), tadpoles reached the specific developmental stages at different time intervals. Because the time required for animals to develop from one stage to the next is not uniform, but depends on the specific stages and environmental conditions, the timing of salinity switches varied across treatments and were determined by average developmental stage. Salinity switches occurred on days 6.8 ± 0.2 (mean $\pm$ SE, $n = 20$), 20.3 ± 0.2 ($n = 20$), 12.2 ± 0.6 ($n = 20$), and 27.3 ± 2.7 ($n = 9$), for LHH, LLH, HLL and HHL, respectively. Likewise, the average tadpole weight at switch points was 114 ± 2.5, 343.1 ± 6.8, 94.6 ± 4.3, and 329.8 ± 19.1 mg, for LHH, LLH, HLL and HHL, respectively. Each treatment was replicated 20 times, each replicate consisting of individual tadpoles raised in plastic containers (length $\times$ width $\times$ height $= 10.5 \times 7.5 \times 4.5$ cm) holding 100 ml of water. Containers were kept covered with a transparent perforated lid to reduce evaporation. We obtained the different saline solutions by dissolving Coralife scientific-grade marine salt (Energy Savers, Carson, CA, USA) in distilled water, and the level of salinity was checked at 26°C with a salinity refractometer. We fed the tadpoles boiled vegetables ad libitum and changed the water every third day. Tadpoles were kept in incubators at 26°C under a 12:12 h light:dark cycle. We monitored tadpole survival and water salinity daily, adjusting salinity when necessary. We measured the growth and development of tadpoles once a week until metamorphosis by staging and weighing each tadpole to the nearest 0.0001 g. Before weighing, we gently blotted each tadpole to remove excess water. Tadpoles were considered to have reached metamorphosis at Gosner stage 42, upon forelimb emergence (Gosner 1960). At this time, we recorded the date and measured tadpole body size.

Data analyses

We used the Kaplan–Meier survival analysis to compare the curves of tadpoles surviving to metamorphosis in different treatments (Kaplan and Meier 1958). All individuals were weighed and staged weekly to determine growth trajectories in each treatment. We tested for differences in growth and development among treatments by using a repeated measures ANOVA (Potvin et al. 1990). This

Fig. 1 Salinity of pools with and without Indian rice frog (*Fejervarya limnocharis*) tadpoles. Tadpoles appeared only in pools with salinities under 12 ppt, and they could experience different salinities throughout development. We categorized four developmental period of tadpoles according to the oldest tadpoles we surveyed in each pool. We surveyed a total of 51 pools
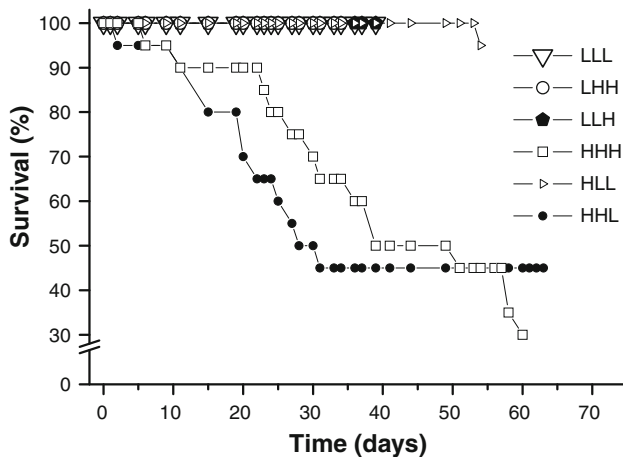
analysis could only be conducted over four time points because after 3 weeks tadpoles in some treatments had metamorphosed. We tested the assumption of Type H covariance (Huynh and Feldt 1970) in our repeated measures ANOVAs, applying sphericity tests (Anderson 1958). As the data did not satisfy such an assumption, we adjusted the significance levels for the F tests according to an epsilon correction factor of their degrees of freedom (Huynh and Feldt 1976).

Larval period and size at metamorphosis were heteroscedastic, and therefore we ranked these variables prior to conducting ANOVA to test for differences among treatments, followed by Fisher LSD post hoc tests. Data analysis was conducted with SPSS 11.0 (SPSS, Chicago, IL, USA) and SAS 9.2 (SAS Institute, Cary, NC, USA).

Results

Survival and metamorphosis

All tadpoles initially assigned to low salinity (LLL, LLH, and LHH treatments) survived to metamorphosis (Fig. 2). Survivorship was decreased in tadpoles initially exposed to high salinity in accordance with the duration of exposure to salinity, survival being 95, 45 and 30% in HLL, HHL and HHH, respectively. The survival of tadpoles significantly differed among the initially high salinity treatments (log rank test, $\chi^2 = 11.78$, $P = 0.0028$, $df = 2$). Tadpole survival in HLL was significantly higher than that at either constant high salinity (HHH) ($\chi^2 = 8.77$, $P = 0.0030$, $df = 1$) or HHL ($\chi^2 = 11.18$, $P = 0.0010$, $df = 1$), whereas HHH and HHL did not differ from one another ($\chi^2 = 1.71$, $P = 0.6790$, $df = 1$; Fig. 2). We did not find

any morphological abnormalities during ontogeny in tadpoles in different salinities.

Growth and development

Growth and development of tadpoles differed significantly during the first 3 weeks among treatments (repeated measures ANOVA, treatment × time: $F_{15,318} = 30.63$, $P < 0.0001$ and $F_{15,315} = 18.14$, $P < 0.0001$, respectively; Fig. 3). Early exposure to high salinity had a strong effect on tadpole growth and development. Tadpoles exposed to high salinity early in development grew and developed more slowly than those whose early development occurred in low salinity, regardless of whether they were exposed to low salinity later in life or not (i.e. Lxx vs. Hxx, treatment × time: $F_{3,330} = 72.75$, $P < 0.0001$ and $F_{3,327} = 68.30$, $P < 0.0001$, respectively; Fig. 3). On average, tadpoles in initially high salinity (Hxx) gained 41% as much mass during the first 3 weeks as those in initially low salinity (Lxx). After those 3 weeks, tadpoles in treatments with early exposure to high salinity were in stages 34–35, before complete toe separation, whereas tadpoles in treatments exposed to low salinity early in development were in

Fig. 3 Growth (a) and development (b) of *F. limnocharis* tadpoles in different salinity treatments. The last datum point for each *line* represents the time at which the first tadpole metamorphosed. Sample sizes during the first three weeks were 20 per treatment, except for HHH and HHL ($n = 18$ and $n = 14$, respectively). Sample size in the fourth week was 12, 13, and 9 for HHH, HLL and HHL, respectively. *Bars* ±SE
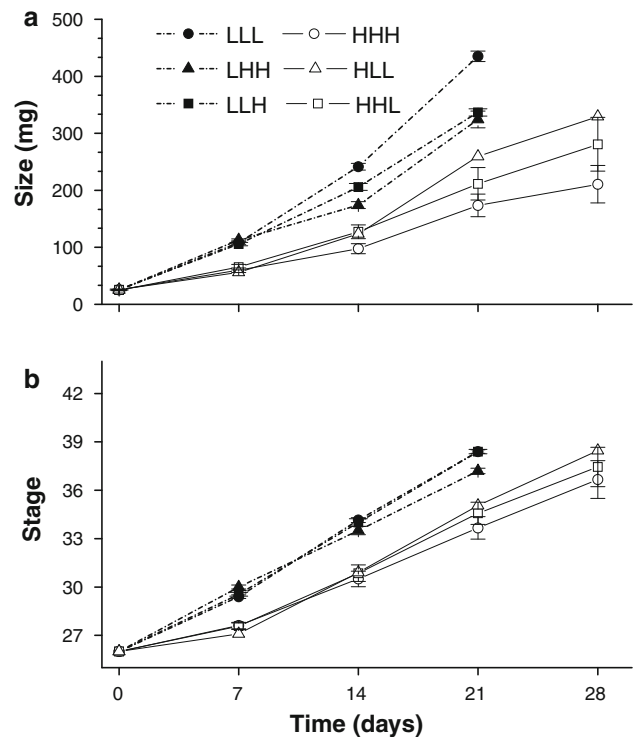

Fig. 2 Survival (%) of *F. limnocharis* tadpoles reared in different salinity regimes. The last datum point for each treatment reflects the time at which the last tadpole metamorphosed

stages 38–39, much closer to metamorphosis (Gosner 1960). Nonetheless, switching to high salinity significantly reduced growth and slowed down development during the first 3 weeks in tadpoles initially in low salinity (among Lxx treatments, treatment $\times$ time: $F_{6,171} = 28.75$, $P < 0.0001$ and $F_{6,171} = 12.10$, $P < 0.0001$, respectively). Extended exposure to salinity further contributed to reduced growth (among Hxx treatments, treatment $\times$ time: $F_{6,147} = 5.42$, $P = 0.004$) but not to influence development (treatment $\times$ time: $F_{6,144} = 1.98$, $P = 0.1240$) in tadpoles initially in high salinity.

Metamorphic traits

The time to and size at metamorphosis of tadpoles significantly differed among treatments (ANOVA, $F_{5,88} = 14.61$, $P < 0.0001$ and $F_{5,88} = 11.30$, $P < 0.0001$, respectively). In initially low salinity treatments (LLL, LHH, and LLH), post hoc comparisons showed that time to metamorphosis of tadpoles switched to high salinity at Gosner stage 30 (LHH) was statistically similar to that at a constant low salinity (LLL), whereas time to metamorphosis of tadpoles switched to high salinity at Gosner stage 38 (LLH) was significantly shorter than that at a constant low salinity, indicating a developmental acceleration in response to late increase in salinity (Fig. 4a). The average developmental acceleration was 2.4 days, approximately a 9% shorter larval period than tadpoles in constant low salinity. Size at metamorphosis of juveniles from LLL was on average 32% greater than that of LHH individuals, and 30% greater than those in LLH (Fig. 4b), whereas LHH and LLH did not differ from one another (Fig. 4b).

Time to metamorphosis did not vary among treatments with initial high salinity (HHH, HLL, and HHL; Fig. 4a). Size at metamorphosis of juveniles from HHH, however, was on average 46% lower than juveniles from HLL, and 31% smaller than juveniles from HHL (Fig. 4b).

Discussion

As expected, higher salinity reduced survival, growth and development of tadpoles when exposure occurred early in development (premetamorphosis). Some species seem to avoid breeding in pools with high salinity (Gordon et al. 1961; Haramura 2008), but even if *F. limnocharis* could discriminate, their offspring would still be at high risk of experiencing harmful salinities as a result of high evaporation rates in the small coastal rocky pools in which they breed. Salinity stress experienced early in development markedly reduced growth and development of *F. limnocharis* tadpoles, seemingly incapable of compensatory growth upon release from salinity stress. Consequently,
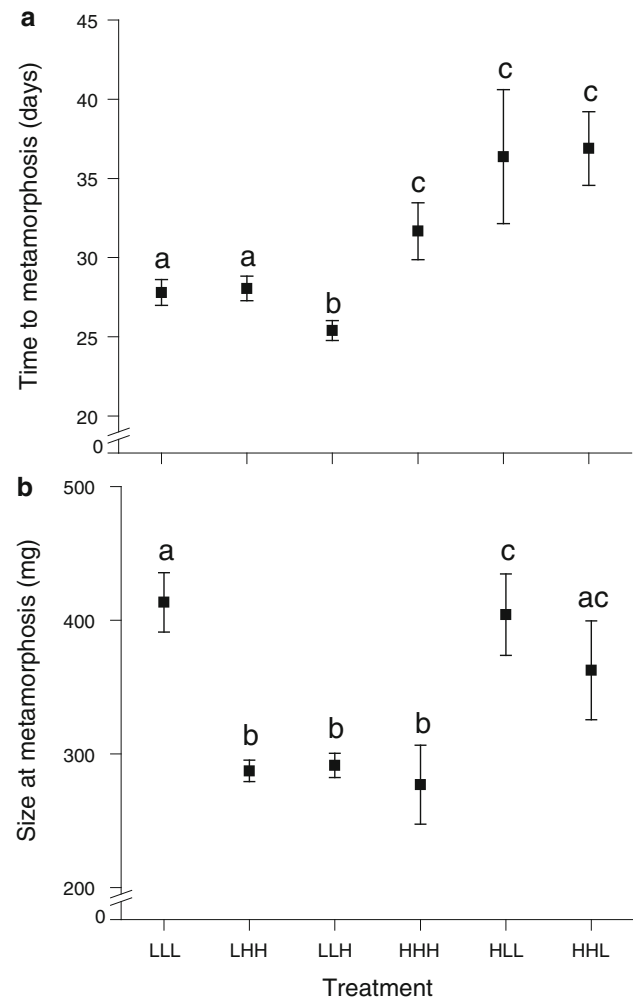
Fig. 4 Time to (**a**) and size at metamorphosis (**b**) for *F. limnocharis* tadpoles in different salinity regimes. Identical *letters* above *bars* indicate no significant difference between treatments. Sample sizes were 20 per treatment, except for treatments HHH, HLL, and HHL ($n = 6$, $n = 19$ and $n = 9$, respectively). *Bars* ±SE

even if *F. limnocharis* tadpoles survive initial exposures to osmotic stress, they are likely to suffer increased vulnerability to other risks, as reduced growth and extended development exposes them to aquatic predators for a longer time, puts them at risk of pond desiccation, reduces their odds of post-metamorphic juvenile survival (Newman 1992; Alford 1999), and impacts population dynamics (Karraker et al. 2008).

Lack of compensatory growth to overcome early osmotic stress

The longer tadpoles stayed in high salinity, the stronger were the adverse effects, but with a strong preeminence of the conditions experienced early in development. Even if released from osmotic stress, tadpoles exposed to high salinity early in development could not increase growth and

developmental rates enough to match LLL, and metamorphosed later and often at a smaller size (Fig. 3a, b). However, tadpoles released from osmotic stress early (HLL) managed to reach similar size at metamorphosis than tadpoles maintained in low salinity throughout development, although it took longer to reach metamorphosis, indicating their ability for partial growth compensation (Ali et al. 2003; Squires et al. 2010). Tadpoles exposed to high salinity early in development (Hxx), or for a long period of time (LHH), may have been forced to allocate more energy to osmoregulation (Gomez-Mestre et al. 2004), and consequently it may have taken longer to offset this increased energy consumption and attain the minimum size for metamorphosis. Wilbur and Collins (1973) hypothesized that tadpoles must attain the minimum (threshold) size before metamorphosis. We hypothesize that tadpoles of *F. limnocharis* at Gosner stage 38 have attained the threshold size for metamorphosis. Morey and Reznick (2000) demonstrated that the threshold size exists and that it differs among species, with a range between Gosner stages 35 and 39, and the difference is probably related to adaptation to different regimes of habitat ephemerality. During the period of Gosner stages 36–41 (prometamorphosis), the endocrine system of tadpoles is sufficiently developed to adjust the developmental rate in response to environmental variation (reviewed by Denver 2009). In contrast, tadpoles at Gosner stage 30 (premetamorphosis) could not initiate metamorphosis after switching from low to high salinity (LHH), and showed decreased growth and development rates, consistent with previous studies (Gomez-Mestre et al. 2004; Chinathamby et al. 2006; Wu and Kam 2009).

Previous studies have shown compensatory growth in tadpoles following release from food deprivation (Alford and Harris 1988; Beachy et al. 1999; Capellan and Nicieza 2007), pond desiccation (Denver et al. 1998) and salinity (Squires et al. 2010). The capacity for compensatory growth may vary among species or depend on the type of environmental stress encountered (Capellan and Nicieza 2007). The response to water refilling after habitat desiccation in tadpoles of *Scaphiopus hammondii* is partially reversible, restoring the body size and retarding metamorphosis, but the reversibility depends on the developmental stage of tadpoles (Denver et al. 1998).

Several lines of evidence suggest that the development of internal gills is critical in enhanced salinity tolerance. Tadpoles experiencing low salinity in early development (before Gosner stage 38) had much higher survival rates (100%) than tadpoles exposed to high salinity during the same period, even if they were later switched to low salinity. Gills are the main organs responsible for ion and water balance in tadpoles (Dietz and Alvarado 1974; Uchiyama and Yoshizawa 1992; Ultsch et al. 1999), and tadpoles may lack sufficient salt-excreting ability to cope with high osmotic

stress until internal gills develop (Chinathamby et al. 2006). Uchiyama and Yoshizawa (1992) suggested that the euryhaline tadpoles of *Fejervarya cancrivora* (*Rana cancrivora*) tolerated higher salinity once they had internal gills than those still relying on external ones because the former are more abundant in mitochondria-rich cells (MR cells), thought key in ion excretion. Concordantly, MR cells only appeared in *Rana dalmatina* tadpoles once internal gills had developed, but were absent in their external gills (Brunelli et al. 2004). Shifts to high salinity did not influence tadpole survival initially assigned to low salinity, maybe because their gills were already well developed. Therefore, the timing of internal gill development relative to salinity exposure may determine tadpole survival.

Early exposure to salinity precluded adaptive developmental plasticity

In our system, initial low salinity conditions seemed to allow tadpoles to build enough capacity to maintain normal development even if exposed to high salinity later in development, although this may have been energetically very costly as it came at the expense of reduced size at metamorphosis (LLL vs. LHH, Fig. 4). Priming fast development at the expense of reduced size at transformation is concordant with an adaptive acceleration of development to avoid stressful conditions (Newman 1992; Denver 1997). Such developmental acceleration was even more marked in the LLH treatment, in which tadpoles reared in low salinity throughout much of their development were exposed to osmotic stress at a late stage. Tadpoles in LLH showed the shortest time to metamorphosis, even shorter than those continuously in low salinity, evidencing developmental acceleration again at the cost of reduced size at metamorphosis. Tadpoles growing in low salinity seemed to have attained the threshold size for metamorphosis at Gosner 38, and precipitated metamorphosis either as a neuroendocrine direct response to increased salinity, or indirectly when growth was adversely impacted by high salinity (Werner 1986). Such shifts in metamorphic timing may provide a means for tadpoles to adapt to the unpredictable nature of rock pools, which frequently experience large and fast fluctuations in salinity, due to wave spray, evaporation, or rainfall (Wu and Kam 2009). Nonetheless, early exposure to high salinity precluded adaptive acceleration of development, always causing delayed metamorphosis relative to those in low salinity early in development. This result highlights the fact that stress experienced early in ontogeny may condition growth and developmental trajectories to the point of limiting the potential for adaptive plastic responses in later stages.

In conclusion, our results indicate that the salinity experienced in the early stages was important for survival,

growth and development of *F. limnocharis* tadpoles. Furthermore, the effects of osmotic stress on growth were partially reversible but those on development were irreversible even after release from stress. However, *F. limnocharis* tadpoles can accelerate development in response to increased water salinity if exposed during prometamorphosis. If low salinity allows adequate growth early in development, tadpoles can quickly attain a minimum size for metamorphosis, which would enable them to accelerate development if conditions deteriorated (e.g., salinity increased). Conversely, early exposure to osmotic stress reduces growth and developmental rates so that tadpoles can no longer build up energetic reserves required to accelerate metamorphosis. We hypothesize that osmoregulation under osmotic stress may take an important metabolic toll that impairs the accumulation of body fat required to undergo accelerated metamorphosis. This study highlights the importance of the environmental conditions experienced early in development, and how they can critically impact life history traits.

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