Effects of hydroperiod duration on developmental plasticity in tiger frog (*Hoplobatrachus chinensis*) tadpoles

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Abstract: While developmental plasticity can facilitate evolutionary diversification of organisms, the effects of water levels as an environmental pressure on tiger frogs remains unclear. This study clarifies the relationship by studying the responses of tiger frog (*Hoplobatrachus chinensis*) tadpoles to simulated hydroperiods (i.e., constant low water levels, constant high water levels, increasing water levels, decreasing water levels, rapid changes in water levels and gradual fluctuations in water levels) in a laboratory setting. ANOVA analysis showed that none of the water level treatments had any significant effect on the total length, body mass, or developmental stages of *H. chinensis* tadpoles half way through development (11 days old). Tadpoles raised in rapidly fluctuating water levels had protracted metamorphosis, whereas tadpoles raised under low and gradually fluctuating water levels had shortened metamorphosis. None of the water level treatments had a significant effect on the snout-vent length (SVL) or body mass of *H. chinensis* tadpoles at Gosner stage 42, or on the body mass of tadpoles at Gosner stage 45. However, the tadpoles raised in high levels and rapidly fluctuating water levels, significantly larger SVL at Gosner stage 45, while ones under gradually fluctuating water levels had smaller SVL than the other groups. Time to metamorphosis was positively correlated with body size (SVL) at metamorphosis in *H. chinensis* tadpoles. *H. chinensis* tadpoles under constant low water level had the highest mortality rate among all the treatments (*G*-test). Moreover, ANOVA and ACNOVA (with body length as the covariate) indicated that water levels had no significant effect on either the morphology (i.e. head length, head width, forelimb length, hindlimb length and body width) or the jumping ability of juvenile *H. chinensis.* These results suggest that the observed accelerated metamorphosis and high mortality of *H. chinensis* tadpoles under decreasing water level treatment was driven by density-induced physical interactions among increasing conspecifics.

Keywords: *Hoplobatrachus chinensis*; Tadpole; Water level; Metamorphosis; Developmental plasticity; Jumping ability

Developmental plasticity is the ability of a given genotype to give rise to different phenotypes when reared in different environmental conditions, and it can increase fitness of certain populations in variable environments (Fusco & Minelli, 2010; West-Eberhard, 2003). The intensity developmental plasticity is correlated with both biological factors, such as food availability (Enriquez-Urzelai et al, 2013), pressure from predators (Hossie & Murray, 2012; Relyea, 2002) and population densities (Browne et al, 2003; Newman, 1994; Semlitsch & Caldwell, 1982; Tejedo & Reques, 1994), and non-biological factors, such as water temperature (Newman 1989), hydrological periods (Amburgey et al, 2012; Loman, 2002; Row & Dunson, 1995; Ryan & Winne, 2001) and the hydroperiod duration (Johansson et al, 2005; Newman, 1992; Tejedo & Reques, 1994).

One example of developmental plasticity is the adaptation of tadpoles to temporary changes in their environment (Newman, 1992). Amphibians usually choose to reproduce in temporary water bodies such as puddles and ditches, which are likely to dry up. Amphibian larvae must metamorphose and grow quickly in order to migrate out of ponds before they evaporate (Altwegg & Reyer, 2003; Johansson et al, 2005). Some

Received: 07 August 2013; Accepted: 10 October 2013

Foundation items: This study was supported by the National Natural Science Foundation of China (31270443, 30970435), Technology Plan Project of Lishui (20110426) and the College Students Technology Innovation Project in Zhejiang Province (2011R429008).

tadpoles respond to changes in their aquatic environment by either expediting development when puddles are drying or protract it when ponds are replenished (*Hyla pseudopuma,* Crump, 1989; *Rana temporaria*, Loman, 1999; *Scaphiopus couchii,* Newman, 1989; *Pelodytes punctatus*, Richter-Boix et al, 2006a; *Pelobates syriacus*, Székely et al, 2010; *Rana esculenta*, Semlitsch & Reyer, 1992; *Rhinella spinulosa*, Márquez-García et al, 2009). However, expedited development may result in a high tadpole mortality rate (Laurila & Kujasalo, 1999; Loman & Claesson, 2003; Rudolf & Rödel, 2007), comparatively smaller body sizes (Altwegg & Reyer, 2003; Reques & Tejedo, 1997) and decreased jumping ability (Richter-Boix et al, 2006b).

The tiger frog (*Hoplobatrachus chinensis*) is a Class II National Protected Species and uses temporary ponds, such as those found on farmland and the surrounding ditches, to propagate (Fan et al, 2012; Lin & Ji, 2005). In Lishui, the breeding season of tiger frog is from May to September, the first half of which is the raining season (May to June), followed by the hottest time of year (July to September). However, rainfall is often unpredictable, being low in frequency and high in precipitation. As a result, water levels during the tiger frog's breeding season are both uni-directional with either gradient increase or decrease, and bi-directional with fast or slow fluctuations.

This study measured the developmental phenotypic plasticity of tiger frog tadpoles in response to six different changes in water level under laboratory settings. Three data points were measured： (1) time taken for tadpoles to metamorphose and body size at time of metamorphosis under different water levels, (2) time of phenotypic plasticity, based on the length of time for morphology and the developmental stages of tadpoles, and (3) impact of water levels on mortality and jumping ability of juvenile frogs.

MATERIALS AND METHODS

Experimental animals

Experimental adult tiger frogs were obtained from the Laboratory of Herpetology, Lishui University. One hundred and eighty eggs were randomly selected from five clutches laid on the same day and were incubated in a plastic container (70 cm × 50 cm × 40 cm, length × width × height) with water (20 cm), until individuals reached Gosner stages 26−27 (Gosner, 1960). The environmental conditions in the laboratory were (14L:10D) photoperiod with a water temperature of 28±1℃.

Experimental design

In the wild, temperature, precipitation and size of water body organically affect each pond's hydroperiod, so six different water level treatments were devised to simulate the natural environment. In treatment 1 (constant low) and treatment 2 (constant high), water levels were maintained at 30 mm and 100 mm during the experiment, respectively. In treatment 3 (increase), the water level was originally set at 30 mm, and then increased by 5 mm every 48 hours until a maximum volume of 100 mm was reached. In treatment 4 (reduction), the water level was originally set at 100 mm, and then reduced by 5 mm every 48 hours until a minimum volume of 30 mm was reached. In treatment 5 (fast fluctuation), the water level was originally set at 100 mm, and then was adjusted repeatedly (during each cycle, water level was reduced to 30 mm after 48 hours, and then increased to 100 mm after another 48 hours). In treatment 6 (slow fluctuation), water level was originally set at 100 mm, and then adjusted repeatedly (during each cycle, the water levels were reduced to 30 mm after 120 hours, and then increased to 100 mm after another 120 hours). Tadpoles were randomly assigned to each treatment with 5 replicates each (one tail-nipped replicate of each treatment was reserved to replace the dead individuals) (6 treatments×5 replicates×8 animals/ container=240 tadpoles total). Tadpoles were fed with bullfrog pellets (protein) once daily.

During the experiment (approximately 50 days), the outdoor area was equipped with a transparent weather shed and bug net, and the evaporation loss of every container was compensated twice daily. Every 48 hours, water was completely replaced with de-chlorinated tap water. Moreover, the containers were also rearranged to minimize temperature differences. HOBO temperature data loggers (USB, U12-006) were read hourly to track the water temperatures of the six treatments. Tadpoles were checked daily and dead ones were replaced with similar sized, tail-nipped ones from the reserved replicate of each treatment (tail-nipped individuals were not included in statistical analysis). Time of metamorphosis was defined as both forelimbs emerged (Gosner stage 42). After morphological measurements, the metamorphosed individuals were housed in plastic bowl (opening diameter=150 mm) with 1mm mesh covering until tails were reabsorbed (Gosner stage 45).

Morphological measurements

Prior to the experiment, 15 tadpoles (Gosner stage 26−27) were randomly selected. Their initial body mass and length (snout to tip of the tail) were 20.93±0.89 (16.00−26.00) mg and 13.20±0.21 (11.86−14.55) mm. respectively. Individuals body mass, body length and development stage were determined on the days of 11th age (August 13), the forelimbs emergence (Gosner stage 42), and the tail reabsorbed (Gosner stage 45). For tadpoles, the total length was measured (TL), whereas juvenile frogs at Gosner stage 42−45 the length from snout to vent (SVL) was measured. Tadpoles (including individuals at Gosner stage 42) were placed on a Petri dish with size standard on the bottom, and photographed by digital camera (Sony DSC-T100) from above, and then their morphology was analyzed by ImageJ 1.44p software (±0.01 mm). Developmental stages were determined with anatomical microscope (Nikon XTS30) following the table proposed by Gosner. Digital caliper was read for the head width, head length, body width, SVL, forelimb length and hindlimb length of juvenile frogs (Gosner stage 45) (Lin & Ji, 2005). Tadpoles were placed on dry surface water with blotting paper and then weighed by electronic scale (Sartorius, ±0.001 g). Time of metamorphosis was defined as the days from the beginning of experiment to the time the forelimbs emerged (Gosner stage 42). Survival rate was defined as the percentage of individuals that survived over the total sample number.

Leaping distance measurements

Ten to 15 minutes were allowed for the juvenile frog to acclimate the indoor environment (25±1℃). Then individuals with limbs soaked with 1% green edible pigment solution were motivated to jump on the white skip resistant paper by the gentle touch of a twig. At least 10 jumps were required for each individual. Jumping distance was defined as the distance between two successive hindlimb marks, and the longest distance was measured using a ruler (± 0.01 cm) (Köhler et al, 2011).

Statistical analysis

All statistical analysis was conducted in Statistica 5.0. All variables were tested for normality and homogeneity using the Kolmogorov-Smirnov test and F-max test, respectively. Linear regression, one-way ANOVA, one-way ANCOVA and Turkey's *post hoc test* were used to evaluate the differences in the variables and slope homogeneity among different treatments. Nonparametric analysis was conducted using a *G*-test. All results were expressed as mean±*SE*, with α=0.05 taken as statistically significant.

RESULTS

Development at 11 days old

ANOVA analysis indicated that water levels had no significant effect on total length (TL) ($F_{5,194}$=0.47, P=0.798), body mass ($F_{5,194}$=0.88, P=0.497), or developmental stages ($F_{5,194}$=2.03, P=0.076) of 11-day-old tadpoles (Figure 1).

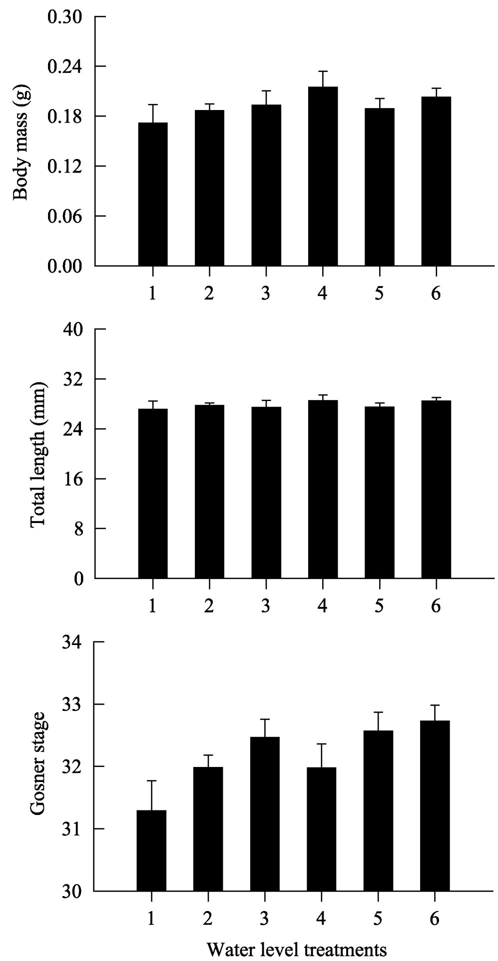

Figure 1 Effect of water levels on the total length, body mass and Gosner stage of *H. chinensis* tadpoles on the 11th day of age

1: Constant low; 2: Constant high; 3: Increasing; 4: Reduction; 5: Fast fluctuation; 6: Slow fluctuation.

Survival rate, time to metamorphosis and size at metamorphosis

G-test results indicated that water levels had a significant effect on the survival rate of individuals at Gosner stage 42 (G=12.87, df=5, P<0.05), but not on the ones at Gosner stage 45 (G=6.54, df=5, P>0.20). The survival rate of individuals at Gosner stage 42 (G=83.77, df=1, P<0.001) and Gosner stage 45 (G=70.23, df=1, P<0.001) in constant low water level (treatment 1) were both significantly lower than the other treatments (Figure 2).

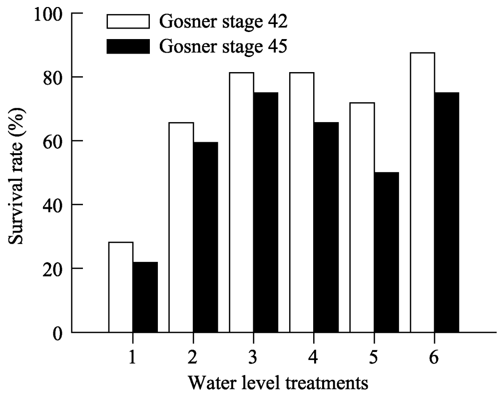

Figure 2 Effect of water levels on the survival rate of *H. chinensis* tadpoles at Gosner stage 42 and 45

1: Constant low; 2: Constant high; 3: Increasing; 4: Reduction; 5: Fast fluctuation; 6: Slow fluctuation.

One-way ANOVA analysis indicated that water level had a significant effect on time to metamorphosis ($F_{5,127}$=3.02, P<0.013; 1^{b}, 2^{ab}, 3^{ab}, 4^{ab}, 5^{a}, 6^{b}). For individuals in the fast fluctuation treatment, time to metamorphosis was accelerated, whereas for individuals under constant low and slow fluctuation treatment, time to metamorphosis was shortened (Figure 3).

ANOVA analysis showed that water levels had significant effect on the SVL, but not on the body mass of juvenile frogs at Gosner stage 45 (SVL: $F_{5,105}$=3.25, P<0.009; 1^{ab}, 2^{a}, 3^{a}, 4^{ab}, 5^{ab}, 6^{b}; body mass: $F_{5,105}$=2.18, P=0.062), nor on the SVL nor body mass of ones at Gosner stage 42 (SVL: $F_{5,124}$=0.90, P=0.483; body mass: $F_{5,127}$=1.11, P=0.359) (Figure 4). When treatments 1 to 6 were taken as variables, the time to metamorphosis and size at metamorphosis (SVL) were significantly positively correlated (r^2=0.86, $F_{1,4}$=25.10, P<0.008) (Figure 5).

Morphological features and jumping ability of juvenile frogs

ANCOVA analysis with SVL as covariant showed that there was no significant effect of water levels on juvenile frogs' (Gosner stage 45) head length ($F_{5,104}$=1.34, P=0.263), head width ($F_{5,104}$=1.40, P=0.229),

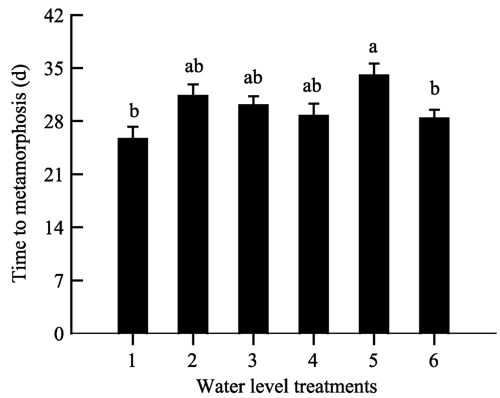

Figure 3 Effect of water levels on the time to metamorphosis of *H. chinensis* tadpoles

1: Constant low; 2: Constant high; 3: Increasing; 4: Reduction; 5: Fast fluctuation; 6: Slow fluctuation; Different superscripts indicate significant difference (Tukey's test, α=0.05, a>b).

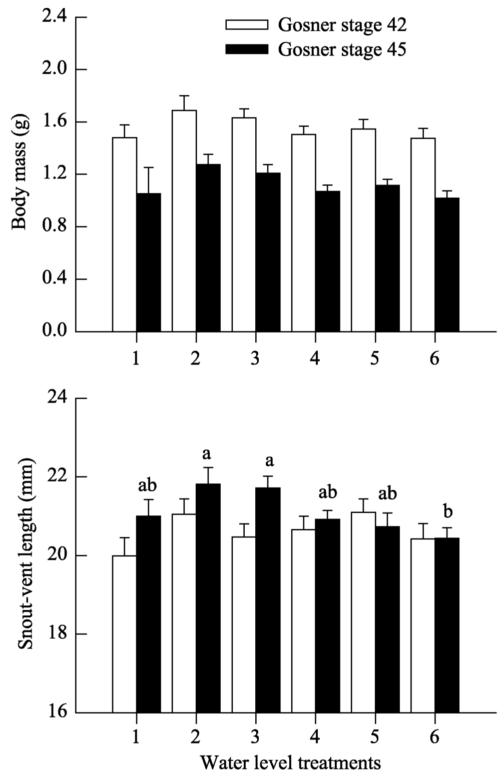

Figure 4 Effect of water levels on the snout-vent length (SVL) and body mass of juvenile *H. chinensis* at Gosner stage 42 and 45

1: Constant low; 2: Constant high; 3: Increasing; 4: Reduction; 5: Fast fluctuation; 6: Slow fluctuation; Different superscripts indicate significant difference (Tukey's test, α=0.05, a>b).

forelimb length ($F_{5,104}$=1.24, P=0.297), hindlimb length ($F_{5,104}$=0.44, P=0.818) or body width ($F_{5,104}$=0.44, P=0.818). ANOVA analysis ($F_{5,102}$=0.88, P=0.499) and

ANCOVA analysis with SVL as covariant ($F_{5,101}$=0.66, P=0.658) indicated that water levels had no significant effect on the jumping abilities of juvenile frogs (Gosner stage 45) (Figure 6).

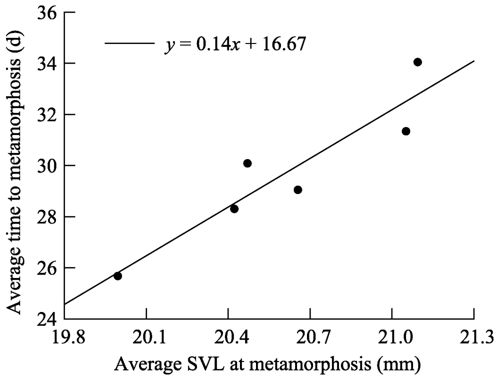

Figure 5 Correlation between average time and snout-vent length (SVL) at metamorphosis with different water levels

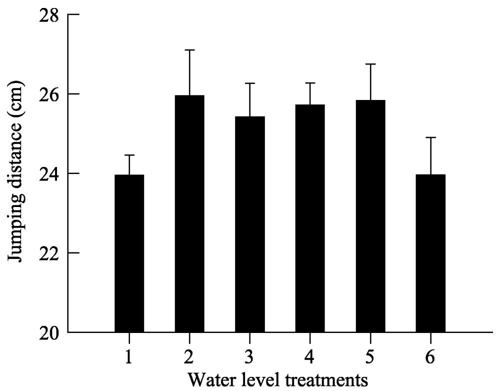

Figure 6 Effect of water levels on the jumping abilities of juvenile *H. chinensis* at Gosner stage 45

1: Constant low; 2: Constant high; 3: Increasing; 4: Reduction; 5: Fast fluctuation; 6: Slow fluctuation.

DISCUSSION

Environmental heterogeneity plays a pivotal role in the evolution of biological phenotypic plasticity (Sultan & Spencer, 2002). Natural selection favors animals with the most suitable phenotype for interacting with their environment, and variations in phenotype, and the corresponding genotype, exist to adapt to a specific environment (Via & Lande, 1985; Whitlock, 1996). Indeed, the temporary ponds many amphibians choose to spawn or tadpoles use to grow are typical examples of environmental heterogeneity.

Time to metamorphosis and size at metamorphosis

Amphibians are characterized by a phenotypic plasticity both in time to metamorphosis and in size at metamorphosis, especially for species' that breed and develop in temporary ponds (*H. pseudopuma*, Crump, 1989; *Scaphiopus hammondii*, Denver et al, 1998; *R. spinulosa*, Márquez-García et al, 2009; *S. couchii*, Newman, 1989; Richter-Boix et al, 2011; *Bufo americanus,* Wilbur, 1987). The present study indicated that water levels can significantly influence the time to metamorphosis of tiger frog tadpoles. The shortest metamorphosis time was recorded for tadpoles under constant low and gradually fluctuating treatments, which supports previous research (Denver et al, 1998; Loman, 1999; Newman, 1989; Richter-Boix et al, 2006b; Spieler, 2000; Wilbur, 1987). However, different phenomena were observed in *H. pseudopuma*, *Pleurodema dipolister*, and *Rhinella granulose* tadpoles (Crump, 1989; Maciel & Juncá, 2009). Rapidly fluctuating water levels treatment is the closest approximation to the natural environment of tiger frog tadpoles, and the longest metamorphosis period was recorded under this treatment. The researchers believe that this is a result of long-term population adaptation.

The size at metamorphosis in amphibians could be affected by the development rate (Newman, 1992; Wilbur & Collins, 1973). Our results showed that although water level had no effect on the size of tadpoles at Gosner stage 42, relatively smaller and bigger body sizes were recorded under constant low and constant high water levels, respectively. For tadpoles at Gosner stage 45, individuals under constant high and increasing water levels had relatively larger SVL, whereas individuals under gradually fluctuated water level treatment were comparatively small (Figure 4). Under different water levels, the time to metamorphosis and size at metamorphosis were significantly positively correlated. These findings are consistent with earlier reports. For example, *S. hammondii* tadpoles that live in permanent ponds can protract metamorphosis and are larger than those in temporary ponds (Morey & Reznick, 2000). Moreover, tadpoles of many species of *Pelobates* can accelerate their development migrate before a pond dries, but they have a smaller body size (Denver et al, 1998; Newman, 1989; Pfennig et al, 1991).

A series of studies show that the developmental responses of amphibian tadpoles to drying habitats were induced by changes to a series of environmental factors, such as an increase in water temperature (Gotthard & Nylin, 1995; Newman, 1992; Tejedo & Reques, 1994; Wilbur, 1990), alterations of substance concentrations in water (Gerlanc & Kaufman, 2005; Morey & Reznick, 2004), increased density of tadpoles (e.g. food limitation, recrement accumulation and interspecies physical contact) (Brady & Griffiths, 2000; Enriquez-Urzelai et al, 2013; Leips et al, 2000; Loman, 1999; Newman, 1989; Wilbur & Collins, 1973), pressure from predators (Hossie & Murray, 2012) and the decrease of available swimming area (Denver et al, 1998), etc. In this study, the average water temperature of the six different treatments was comparable (ANOVA, P>0.05), and water was completely changed every 48 hours to minimize the influence of PH, salinity (e.g. ammonium salt) (Gerlanc & Kaufman, 2005), waste products (e.g. faeces and CO_2) and dissolved oxygen (Morey & Reznick, 2004), etc. These components cannot not be used to explain the accelerated metamorphosis of tadpoles in decreasing water levels. Moreover, the tadpoles in the present study were fed with abundant food (low water level did not interfere with individual swimming or food intake) and were well protected, so food and predator are irrelevant to the shortened metamorphosis of tadpoles in reduced water levels.

The density of tadpoles increased alongside water level reduction, and was accompanied by more physical interaction among conspecifics. In most amphibians, tadpole aggregation hinders development and growth (Semlitsch & Caldwell, 1982). Denver et al (1998) reported that the decrease of swimming volume was directly responsible for the shortened metamorphosis and small body size of *S. hammondii* tadpoles. So, when the concentrations of soluble substances cannot explain the responses of tadpoles to water level reduction, then increased physical interaction among conspecifics might be considered. In our study, tiger frog tadpoles under constant low water level treatment had the highest mortality rate and the shortest metamorphosis time of all groups (Figure 2 and 3). Therefore, increased physical contact among conspecifics induced by the increased in population density that accompanies a decrease in water level could be an important factor in the tiger frog tadpoles' accelerated metamorphosis.

Developmental plasticity and mortality of tadpoles, as well as heterogenic growth and jumping ability of juvenile frogs

In this experiment, the six different water levels had no effect on the growth (body length and mass) or developmental stage of the tiger frog tadpoles 11 days of age. These phenomena indicate that tiger frog tadpoles demonstrate low plasticity in repose to chances in water levels during the early stages of development. In the wild, adult tiger frogs prefer to spawn after it rains. As a result, tadpoles usually will not detect water evaporation until after the initial development stages only by then will accelerated growth be triggered (Newman, 1992). These results are supported by the observation of tadpole plasticity during the mid-phrase of development (prior to metamorphosis, Gosner stage 36−38), who responded to water volume variations in laboratory (Denver et al, 1998; Kulkarni et al, 2011).

The survival rate of tiger frog tadpoles at metamorphosis (Gosner stage 45) by treatment type, from high to low, is gradual fluctuation, increasing, decreasing, constant high, fast fluctuation, and constant low (22%) (Figure 2), The extremely high mortality is likely due to intensified competition causes by high population density, also called the density-dependent effect (Alford et al, 1999; Loman, 1999; Reques & Tejedo, 1997). Moreover, cannibalism exists among tiger frog tadpoles, so mortality could increase with density (unpublished data). The relatively high mortality among tadpoles in the rapidly fluctuating water levels also indicates that tiger frog tadpoles are sensitive to dramatic decreases of water, and that inter-individual competition increases alongside density.

Field survey data showed that habitats that are drying out were negatively correlated with the head morphology and hindlimb length of amphibians, which are vital to an individual's fitness and to adapting to environmental changes (Johansson & Richter-Boix, 2013; Newman 1989; Márquez-García et al, 2009; Richter-Boix et al, 2006b; Tejedo et al, 2010). In the present study, however, no significant differences were found in the head length, head width, body width, forelimb length, hindlimb length and jumping ability of juvenile tiger frogs (Gosner stage 45) under different water levels. These might be due to interspecific differences and the directional developmental differentiation in amphibians responding to drying signals.

In summary, water levels could significantly impact the time to metamorphosis and size at metamorphosis of tiger frog tadpoles, but not the morphology or jumping ability of juvenile frogs. Time to metamorphosis and size at metamorphosis were positively correlated, whereas high mortality rate and small body size were positively correlated with water level reduction. The phenotypical plasticity was more obvious during the post phase of tadpole development, and more frequent physical interaction among conspecifics induced by increased population density may be the main reason why tiger frog tadpoles accelerate their metamorphosis in drying habitats.

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