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Effects of temperature and food level on plasticity of metamorphic traits in *Bufo gargarizans gargarizans* larvae

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Abstract. Many environmental factors such as temperature or food level may influence growth and mortality risks of ectothermic vertebrates in both aquatic and terrestrial habitats. In this study, plasticity in growth rates, survival, larval period, and size at metamorphosis were examined in *Bufo gargarizans gargarizans* larvae under different combinations of temperature and food level. Our results showed that larvae metamorphosed at an older age when reared at 17.3°C. A significant interaction between food level and temperature revealed that the food level has obviously affected length of larval period when tadpoles raised at 17.3 °C, but not at 27.3 or 31.3 °C. Also, we found clear evidence that growth rates are influenced by both temperature and food level. Interestingly, tadpoles reared at 17.3 °C had larger size at metamorphosis than those reared at other temperatures, suggesting that *B. g. gargarizans* larvae reared at cold temperatures have a longer developmental period but they are also larger as metamorphs than conspecifics reared at warmer temperatures. Therefore, the global climate change or local manipulations of the environment may promote growth and development of *B. g. gargarizans* larvae, but not large size at metamorphosis.

Keywords. Amphibia, Anura, Bufonidae, *Bufo gargarizans gargarizans*, larval period, metamorphic size, phenotypic plasticity, China.

In animals with complex life cycles, such as amphibians, metamorphic size and timing are important fitness components (Arnold and Wassersug, 1978; Wilbur, 1980). Many environmental factors such as temperature or food availability may influence size and age at metamorphosis (reviewed by Álvarez and Nicieza, 2002). Generally, low temperatures resulted in a longer developmental periods, but larger metamorphic size than conspecifics reared at warmer temperatures because of differential effects on growth and differentiation (Smith-Gill and Berven, 1979; Álvarez and Nicieza, 2002). However, previous studies indicated that the temperature variation of the reaction norms among species and phylogenetic groups was a puzzling problem, suggesting an interplay between phylogeny and adaptation to specific habitats (aquatic and terrestrial) (e.g., Blouin, 1992; Morand et al., 1997; Joly et al., 2005).

A steady food supply generally elicits a younger age and larger size at metamorphosis (Werner, 1986). This also correlates with younger age and larger size at first reproduction, and thus potentially higher fecundity (Harris, 1999; Werner, 1986; Semlitsch et al., 1988). This variation in metamorphic traits may have strong effects on later fitness as early metamorphosis, and large size at metamorphosis are favoured because of their positive effects on juvenile survival and adult fecundity (Wells, 2007).

Temperature and food availability have been studied in numerous anurans (see review by Angilletta and Dunham, 2003), but little is known about how the interaction between temperature and food availability can affect tadpole growth and development (but see: Laugen et al., 2005; Castano et al., 2010; Courtney Jones et al., 2015; Yu et al., 2015). In this study, we examined the potential

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interactive effects of food level, and rearing temperature on the plasticity of metamorphic traits of *Bufo gargarizans gargarizans*, including the length of larval period, survival, the size at metamorphosis, and growth rate. As model species we used *Bufo gargarizans gargarizans*, a widely distributed toad, breeding in different aquatic habitats (Yu and Guo, 2013), which may offer a large variation in both temperature and resource availability during larval development.

Bufo gargarizans gargarizans is sexually dimorphic and is widely distributed in East Asia. Female toads are the larger sex, and clutch size is positively correlated with female body size (means = 9325 ± 279.05, range = 3275–15880; Yu and Sharma, 2012). It is an explosive breeder with typical breeding habitats concentrated along the vegetated edges of large still water bodies and a relatively short breeding season (6-14 days; Wells, 2007; Yu and Sharma, 2012). The tadpoles hatch after two weeks in the breeding ponds. The timing of larval development in natural ponds is about 50-55 days, when water temperature varies from 6 (at night of early–March) to 29 °C (at noon of mid–April, mean temperature less than 25 °C, Yu, personal observations). Rich *Spirogyra* and pondweed grow in pond, and provide food for *B. g. gargarizans* tadpoles in the larval period (Wei et al., 2011).

During the peak period of breeding activity, we collected a total of 25 amplectant pairs in one population in Shihe County (32°08'N, 114°01'E; datum = WGS84), Henan, the central plains of China, in mid–February 2012. Those animals were transported to laboratories close to spawning sites. We kept pairs separately in plastic cask (20 L) filled with approximately 12-15 cm of pond water until the eggs were deposited. Once oviposition was completed, we collected 40 fresh eggs from each of the egg masses from 20 *B. g. gargarizans* females because five female toads did not lay eggs. On the same day, we put all fresh eggs into two 80 L plastic containers with an automatic aerator, and we left them there until hatching. A total of 360 tadpoles (Gosner stage 25; Gosner, 1960) were randomly allocated to six experimental treatments (n = 60). Larvae were fed with commercial fish food (Bieyanghong, Biological Co. Ltd., Hangzhou, China, protein content, PC; protein >45%, lipids >12%, algae >12%, fiber >4%, ash <10%). Tadpoles were exposed to a 13L:11D photoperiod throughout the study period and the water in the containers was changed weekly.

We used a 2×3 factorial design to examine the effects of food level and rearing temperature on larval growth rates and post-metamorphic performance. To evaluate the effects of food level, half of the tadpoles in each temperature treatment were placed at low mass-specific food level (25 mg food/g tadpole per day) and a half on a high food regimen (50 mg food/g tadpole per day) throughout the experiment. These food levels were chosen to be consistent with the previous study on this species by Zhang et al. (2007). For each food regimen, three different temperature levels were kept across the rearing period: low (room) temperature (17.3 ± 1.33 °C; mean ± SD); middle temperature (27.3 ± 1.42 °C); high temperature (31.3 ± 0.56 °C). The "low" and "middle" temperature were chosen because they fall within the range this species experiences in the field, while "high" temperature approached the avoidance temperature (Ma and Long, 2005). Aquarium heaters (Minjiang, BaolaiHD–200, Guangzhou, China) were used to raise the water temperature in the "middle" and "high" treatments. For each temperature treatment, 120 individual vessels with foam board (60 for each diet treatment), each of which is 300 ml, were randomly placed in two rectangular tanks (110 × 90 × 60 cm; L × W × H). To further minimize the possible effects of such heterogeneity, the positions of the 120 tadpole containers within a given temperature were reassigned at random every three days.

After the first metamorph (defined as the emergence of at least one forelimb, Gosner stage 42) was discovered, the six large tanks were checked daily and all metamorphs found were collected and kept individually in plastic vials (8 cm diameter) with sand and 1 mm of water until tail re-sorption was completed (Gosner stage 46). Four variables were measured: (1) age at complete metamorphosis (number of days from the beginning of the experiment until complete metamorphosis, Gosner stage 46); (2) SVL (snout-to-vent length) at complete metamorphosis (SVL was measured with digital calipers to the nearest 0.01 mm; (3) growth rate was measured as the SVL at complete metamorphosis divided by the age; (4) the percentage of surviving tadpoles that metamorphose.

We analysed the length of larval period, SVL at metamorphosis, and growth rate by using a generalized linear model (GLM) with type III mean squares and temperature, food level, and their interaction as fixed factors. We used log-linear model to test survival. If the overall GLM or log-linear model results were significant, the data were analysed with ANOVAs by using post-hoc multiple comparisons (Fisher's LSD) or a Chi-square test to evaluate differences between food levels or between temperatures (SPSS 13.0, SPSS Inc., 2004, Chicago, IL, USA). All given P-values are two-tailed, with values presented as means ± standard error.

The effects of rearing temperature or food level on the length of larval period were significant (temperature, $F_{2, 183} = 266.50$, $P < 0.001$; food level, $F_{1, 183} = 38.61$, $P < 0.001$), as well as their interaction ($F_{2, 183} = 10.08$, P

< 0.001). Tadpoles raised at 17.3 °C took longer to metamorphose than those raised at 27.3 and 31.3 °C (all P < 0.05, Fig 1), while no difference was found between the latter temperature treatments. Tadpoles feeding on high food level reached metamorphosis earlier than those raised at low food level (P < 0.001). A significant interaction revealed that tadpoles raised at 17.3 °C, high food level reached metamorphosis earlier than those raised at low food level (post-hoc tests: P < 0.001), but there were not different at 27.3 or 31.3 °C (both P > 0.05; Fig. 1).

The rearing temperature significantly affected SVL at metamorphosis ($F_{2, 183}$ = 3.68, P = 0.027, Fig. 1), but food level, as well as temperature × food level interaction did not (food level: $F_{1, 183}$ = 2.06, P = 0.153; interaction: $F_{2, 183}$ = 1.37, P = 0.257). Tadpoles reared at 17.3 °C had significantly larger SVL at metamorphosis than those at 31.3 °C (P = 0.016), or marginal significantly than those at 27.3 °C (P = 0.051).

The rearing temperature or food level positively influenced growth rate (temperature, $F_{2, 183}$ = 113.89, P < 0.001; food level, $F_{1, 183}$ = 25.72, P < 0.001, Fig. 1), but the interaction between rearing temperature and food level was not significant ($F_{2,183}$ = 0.37, P = 0.694). Tadpoles feeding on a high food level had a greater growth rate than those raised at low food level (P < 0.05). Tadpoles reared at 17.3 °C had a lower growth rate than those raised at 27.3 and 31.3 °C (both P < 0.001), but there was no difference between the latter two temperature treatments (P = 0.350).

Survival at metamorphosis was affected by food level (Z = -2.65, P = 0.008): tadpoles feeding on a high food level had a higher survival rate than those raised at low food level. In contrast, nor the rearing temperature, nor the interaction between temperature and food level were significant (temperature: Z = -0.81, P = 0.416; interaction: Z =1.52, P = 0.129; Fig. 1).

In anuran amphibians, age and size at metamorphosis may be reduced with increasing temperature (Harkey and Semlitsch, 1988; Newman, 1998; Beck and Congdon, 2000; Merila et al., 2000; Laugen et al., 2003; Palo et al., 2003; Liess et al., 2013; Courtney Jones et al., 2015; Yu et al., 2015). Our results confirmed that *B. g. gargarizans* metamorphosed at a younger age when reared at higher temperature. However, tadpoles reared at low temperature had larger SVL at metamorphosis than those reared at other temperatures, which are consistent with previous studies (Atkinson, 1994, 1996; Angilletta and Dunham, 2003; Arendt, 2011; Yu et al., 2016).

Temperature can affect metamorphic size in two ways. First, temperature is a major proximal factor determining growth and development, which cause large differences in size at metamorphosis (Smith–Gill and Berven, 1979). This is the expected outcome if growth rates are more responsive to temperature than differentiation rates. Second, temperature may influence the extent to which food level can affect growth and development, which in turn influence size at metamorphosis. In our study, food level had no effect on SVL independently from the temperature treatment. However, cold-reared individuals had slow growth rates compared to tadpoles in warmer temperatures. This was also reflected in the age at metamorphosis, which was significantly older at low temperatures compared to higher temperatures. Therefore, we suggested that *B. g. gargarizans* larvae reared at cold temperatures have a longer developmental period but they may also become larger as metamorphs than conspecifics reared at warmer temperatures, independently from the available food level.

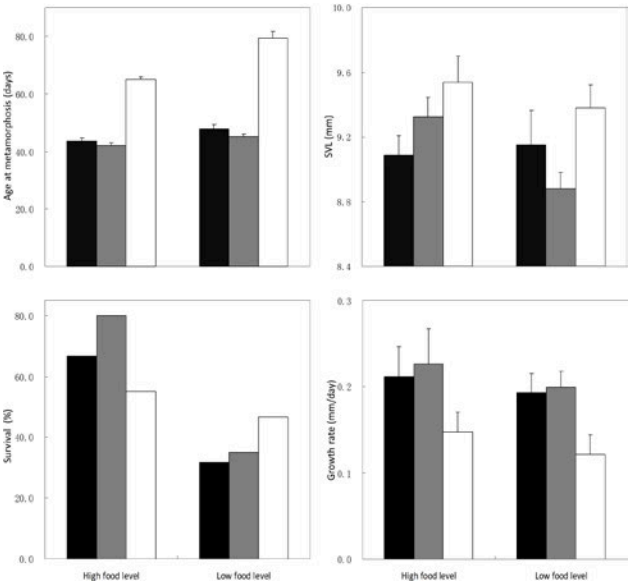

Fig. 1. Influences of temperature and food level on age, SVL, growth rate and survival at complete metamorphosis of *B. g. gargarizans* (Gosner stage 46; black columns = high temperature; grey columns = middle temperature; open columns = low temperature).

The food availability during the larval stage has important effects on timing of metamorphosis (Leips and Travis, 1994). Several experimental studies have demonstrated that high food availability with a large proportion of protein can produce a two-fold effect, accelerating both growth and developmental rates (Nathan and James, 1972; Steinwascher and Travis, 1983; Pandian and Marian, 1985). Laugen et al. (2003) suggested the effect of high food availability for maximum growth and development rates were larger in the warm temperature treatments. In this study, at low temperature, tadpoles raised with high food level reached metamorphosis earlier than

those raised at low food level, but they were not different at middle and high temperature. Thus, the effects of food availability on larval growth were partially dependent on developmental temperature.

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