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Plasticity of metamorphic traits of tadpoles of *Rana chensinensis* (Anura: Ranidae): interactive effects of food level and water exchange

Wen H. Shi, Hai Y. Li, Xiao Q. Shen, and Tong L. Yu
College of Life Science, Xinyang Normal University. SD 464000, China. E-mail: yutonglei_00000@163.com.

Abstract

Plasticity of metamorphic traits of tadpoles of *Rana chensinensis* (Anura: Ranidae): interactive effects of food level and water exchange. In nature, ambient conditions may strongly affect morphological variation, especially in animals with complex life cycles, such as amphibians. Although food level and water exchange have a strong effect on the metamorphic traits of larvae, little is known about effects of interaction between both factors on length of larval period and size at metamorphosis. In this study, we evaluated plasticity of metamorphic traits of the Chinese brown frog (*Rana chensinensis*) under different combinations of food levels and water exchange. Age and mass at metamorphosis were susceptible to food level and varied with water exchange. High food levels could lead to shorter larval periods except for weekly water changes. Further, at low food levels, tadpoles with frequent water exchange (renewed every 2 days) attained a larger size than those at other treatments of water changes. The effects of water changes were dependent on food level. Our results also demonstrated that frequent water exchange and high food levels have positive effects on survival of tadpoles of Chinese brown frogs. We found a significant interaction between food level and water exchange, suggesting that this novel mechanism is selected for when frequent water exchange is likely to prove profitable when food is insufficient or environmental stresses are present.

Keywords: Amphibians, Chinese Brown Frog, Food availability, Growth rate, Mass at metamorphosis, Water exchange period.

Resumo

Plasticidade das características metamórficas dos girinos de *Rana chensinensis* (Anura: Ranidae): efeitos interativos do nível de alimento e das trocas de água. Na natureza, as condições ambientais podem afetar fortemente a variação morfológica, especialmente em animais com ciclos de vida complexos, como os anfíbios. Embora o nível de alimento e a troca de água tenham um forte efeito nas características metamórficas das larvas, pouco se sabe sobre os efeitos da interação entre

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ambos os fatores na duração do período larval e no tamanho na metamorfose. Neste estudo, avaliamos a plasticidade das características metamórficas da rã-castanha-chinesa (*Rana chensinensis*) sob diferentes combinações de níveis de alimento e troca de água. A idade e a massa na metamorfose foram susceptíveis ao nível de alimento e variaram com a troca de água. Níveis elevados de alimento podem levar a períodos larvais mais curtos, exceto com trocas de água semanais. Além disso, com níveis baixos de alimento, os girinos com trocas frequentes de água (renovada cada 2 dias) atingiram um tamanho maior do que os girinos com outros tratamentos de trocas de água. Os efeitos das mudanças de água dependeram do nível de alimento. Nossos resultados também demonstraram que a troca frequente de água e níveis elevados de alimento têm efeitos positivos sobre a sobrevivência dos girinos da rã-castanha-chinesa. Encontramos uma interação significativa entre o nível de alimento e a troca de água, o que sugere que este novo mecanismo é selecionado quando a troca frequente de água é suscetível de ser proveitosa quando o alimento é insuficiente ou quando há estresse ambiental.

Palavras-chave: Anfíbios, Disponibilidade de alimento, Massa na metamorfose, Período de troca de água, Rã-castanha-chinesa, Taxa de crescimento.

Introduction

In nature, length of larval period and size at metamorphosis are important fitness components (Arnold and Wassersug 1978, Wilbur 1980), especially for animals with complex life cycles, such as amphibians. Larval amphibians are more likely to experience variation in food availability owing to the variety of aquatic spawning sites (Morey and Reznick 2004, Skelly 2004). Besides energy absorption, food supply has been regarded as an essential proximal cause of plasticity of metamorphic traits (Newman 1998, reviewed by Álvarez and Nicieza 2002, Castano *et al.* 2010).

Generally, adequate food supply enhances growth and development of larvae, thus allowing them either to maximize size at metamorphosis or minimize larval period (Pandian and Marian 1985, Arendt and Hoang 2005, Yu *et al.* 2015, 2016a, b, c, Yu and Han 2020). In contrast, low food levels, high larval density, or both in combination usually result in food limitation, thus negatively weighing on metamorphosis. For example, tadpoles prolong larval period to meet the minimum threshold of size at metamorphosis because growth rate is low when food limitation leads to poor conditions (reviewed by Wilbur and Collins 1973). Burraco *et al.* (2021) confirmed that when conditions are favorable (such as warm temperature and abundant food), larvae completely offset the delay in hatching without any negative impact on their body mass. When conditions are unfavorable (such as cold temperature and limited food), these compensatory responses are hindered, and if the hatching delay is prolonged in such adverse conditions, it completely disrupts the ability to compensate. Beyts *et al.* (2023) found that under familiar conditions, there was an increase in individual variance in plasticity and predictability in the high food treatment, while in an unfamiliar context, there was an increase in individual variance in personality only in the low food treatment.

Other factors can also affect metamorphic time and growth rate (Rose 2005). Intraspecific competition is a widespread phenomenon in nature, acting through interference and exploitative mechanisms (Steinwascher 1978). For example, some anuran larvae can employ chemical cues to inhibit the growth of small individuals in intraspecific competition (e.g., Rose and Rose 1961, Stepanova 1974, Rot-Nikcevic *et al.* 2005, 2006). In particular, interference mechanisms embrace direct interactions between individuals of the same species (Hettyey *et al.* 2014, Zewe and Booth 2014), usually occurring at low food levels

(Richards 1962). Carbon dioxide, oxygen, nitrite, and heavy metals present in water have an impact on the growth and development of aquatic organisms (e.g., Ishimatsu *et al.* 2004, Hong *et al.* 2020, Verberk *et al.* 2020, Edwards *et al.* 2023). For example, higher oxygen tension or decreasing carbon dioxide tension seems to prevail in frequent water exchange (Adolph 1931). It is generally known that low population density and frequent water change accelerate growth of aquatic organisms (Richards 1958, Hailey *et al.* 2006).

Few studies have investigated how water exchange and food level are interrelated to influence metamorphic traits of anuran larvae. In this study, we evaluated whether food level and water exchange affect metamorphic time and size at metamorphosis of the Chinese brown frog (*Rana chensinensis* David, 1875). Specifically, we hypothesized that tadpoles fed on high food levels and reared at frequent water changes should have higher growth and survival rates. We also hypothesized that tadpoles should have larger size at metamorphosis when they were reared with frequent water changes because of lower chemical waste.

Materials and Methods

Study Species

Female frogs are the larger sex and are widely distributed in regions north of the Yangtze River in China (Yu *et al.* 2015). These frogs are explosive breeders because they have a relatively short breeding season (8–16 days; Wells 2007, Yu *et al.* 2015). The frogs prefer to select small and medium quiet water bodies as spawning habitats and lay eggs along edges of ponds. The beginning of the breeding period for *R. chensinensis* tends to be later at higher latitudes. Tadpoles have a longer development time to reach metamorphosis (80–90 days) in natural ponds because water temperature is colder during the larval stage (mean temperature less than 20°C; Yu pers. obs.). The oral structure of tadpoles is highly unique, characterized by semi-circular grinding teeth that can crush both animal and plant food into a paste, enabling them to be classified as scraper species (Feng *et al.* 2003). Rich *Spirogyra* Link and Nees and *Potamogeton crispus* L. are considered as the natural food of tadpoles of *R. chensinensis* (Cao *et al.* 2002).

Field and Laboratory Procedures

From 11 to 19 February 2021, ten fresh egg masses of *R. chensinensis* were collected in Xinyang (114°06' E, 32°12' N; 22–100 m a.s.l.), Henan, China. We selected 100 eggs from each of the egg masses and put them into a 2–l plastic container filled with a depth of approximately 15 cm of fresh water, where they were allowed to hatch. The experiment commenced on 28 February 2021 and concluded on 24 June 2021, spanning a duration of 116 days. We conducted this research in the laboratory located 0.5 km from the spawning site, thus avoiding confounding environmental effects and predation pressure.

Experiment Design

We used a 2 × 4 factorial design to evaluate the effects of food level and water exchange on metamorphic traits of tadpoles of *R. chensinensis* (Figure 1). A total of 320 tadpoles were randomly allocated into eight experimental treatments. To estimate the effects of food level, half of the tadpoles in all treatments were placed at low mass-specific food level (6% of tadpole mass per day, LFL) and half on a high food regimen (12% of tadpole mass per day, HFL) based on previous studies on this species (Zhang *et al.* 2007). For each food regimen, four treatments of water exchange were used during the growing period: once every two days, once every four days, once a week, and once every two weeks. In this experiment, an opaque round plastic bowl with dimensions of 15 cm upper diameter, 10.5 cm lower diameter, and 7.3 cm height, each of which is 0.5 l, was used to house two tadpoles; thus, 20 bowls (40 tadpoles) were used in each experimental treatment. During the water exchange process, a large bucket with a capacity of 100 L was filled with tap water and exposed to chlorine for three days prior to use. Subsequently, all water in the containers was replaced simultaneously.

Tadpoles were carefully chosen before being used in each treatment. Initially, any large or small tadpoles were visually identified and removed. A total of 30 tadpoles, similar in size, was placed in a circular basin measuring 40 cm in diameter and filled with 2 cm of clean water. A caliper was positioned at the center, and photographs were taken. The *tpsdig2* computer

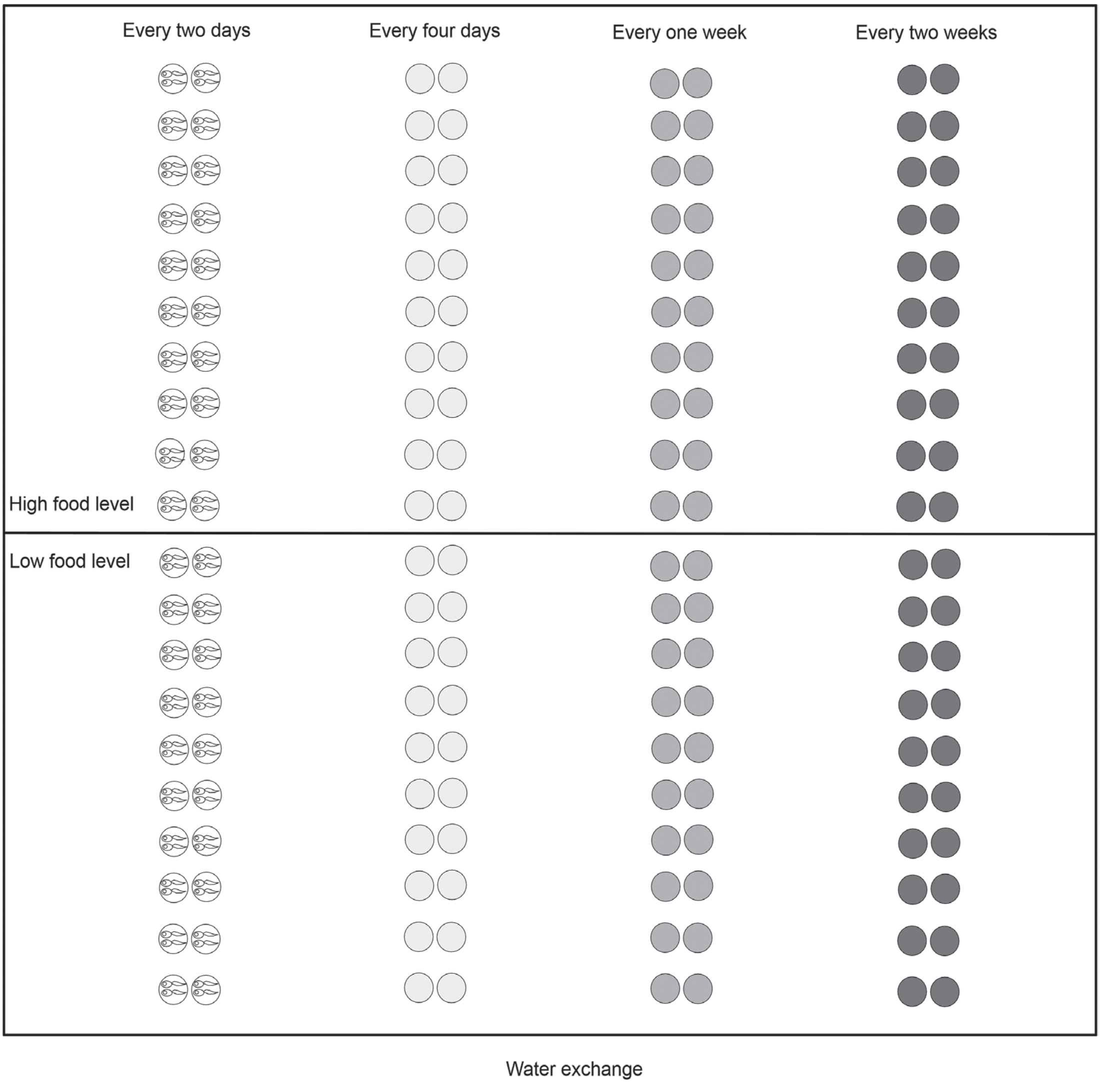

Figure 1. Experimental set-up (above the horizontal line, high food level; below the horizontal line, low food level).

software was used to calculate the average body length. Throughout the experiment, tadpoles with a body length exceeding or falling below the mean by 1 mm were excluded, guaranteeing that individuals of the same size were used at the beginning of an experiment. We randomly chose one tadpole from each clutch to put into each treatment so that the same number of tadpoles from each family was used in the experiment, thus avoiding parental or genetic effects and intraspecific competition. At 10:00 h every morning, a designated individual fed the tadpoles with commercial fish food (30% protein, 10% lipids, 18% algae, 10% fiber, 15% ash, 10% moisture) or changed the water. Tadpoles were reared at ambient temperature (17.3 ± 1.33°C) and a photoperiod of 13L:11D.

Once the first metamorph (29 April 2021, defined as the emergence of at least one forelimb, Gosner Stage 42) was discovered, we surveyed 160 bowls at least once a day until all metamorphs were found (24 June 2021). Four variables were measured: (1) length of larval period was calculated as number of days from hatching until metamorphosis; (2) body mass was measured using an electric balance (to the nearest 0.001 g); (3) growth rate was calculated as the mass at metamorphosis divided by the larval period (Laurila 2000); and (4) survivorship was classified on a three-point scale based on measurements of tadpoles in a plastic bowl: 100% = two tadpoles survived until metamorphosis; 50% = one of two tadpoles survived until metamorphosis; 0 = no tadpoles survived until metamorphosis.

Data Analysis

We used a generalized linear model (GLM) with type III mean squares to analyze the effects of water exchange, food level, and their interaction on the length of larval period, mass at metamorphosis, growth rate, and survivorship. If the overall GLM results were significant, we used ANOVAs with post-hoc multiple comparisons (Fisher's LSD) to assess differences between food levels or between water exchanges. All the analyses were done with SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA).

Results

Effects of Food Level and Water Exchange on Length of Larval Period and Mass at Metamorphosis

The effect of food level on length of the larval period was significant ($F_{1, 208} = 25.522$, $p < 0.001$, Table 1, Figure 2A). Frequent water exchanges tended to reduce the larval period but was not significant ($F_{3, 208} = 2.148$, $p = 0.095$). A significant interaction between food level and water exchange ($F_{3, 208} = 3.038$, $p = 0.030$) revealed that high food availability resulted in faster growth, leading to shorter larval periods when tadpoles were reared at three treatments of water exchange (all $p < 0.05$), after once a week ($p = 0.529$).

Mass at metamorphosis was affected by food level ($F_{1, 209} = 136.043$; Table 1, Figure 2B), but the effect of water exchange was not statistically significant ($F_{3, 209} = 1.841$, $p = 0.141$). The interaction between food level and water exchange was significant ($F_{3, 209} = 3.970$, $p = 0.009$), revealing that LFL tadpoles reared at water exchange once every two days were larger than those reared at water exchanges once a week and once every two weeks (both $p < 0.020$), but the latter was similar ($p = 0.869$). HFL tadpoles had a larger body mass than LFL tadpoles independent of water exchange (all $p < 0.001$).

Effects of Food Level and Water Exchange on Growth Rate and Survivorship

The effect of food level on growth rate was significant ($F_{1, 208} = 30.272$, $p < 0.001$), while water exchange had no effect on growth rate ($F_{3, 208} = 2.425$, $p = 0.067$; Table 1, Figure 2C). The interaction between food level and water exchange was not significant ($F_{3, 208} = 2.438$, *p* = 0.066); however, LFL tadpoles reared at frequent water exchange (once every two days) were larger than those reared at other treatments of water exchange (once every four days or two weeks, both $p < 0.042$), but there was no difference between once every two days and once a week ($p = 0.088$). HFL tadpoles had faster growth than LFL tadpoles independent of water exchange (all $p < 0.001$).

The effect of food level on survivorship was significant ($F_{1, 208} = 119.434$, $p < 0.001$, Table 1, Figure 2D), indicative of high survival with high food quantity. Water exchange had an effect on survivorship ($F_{3, 151} = 2.958$, $p = 0.034$), revealing that tadpoles reared at frequent water exchange have higher survivorship to metamorphosis than those reared at other treatments of water exchange (both $p < 0.034$), but water exchange

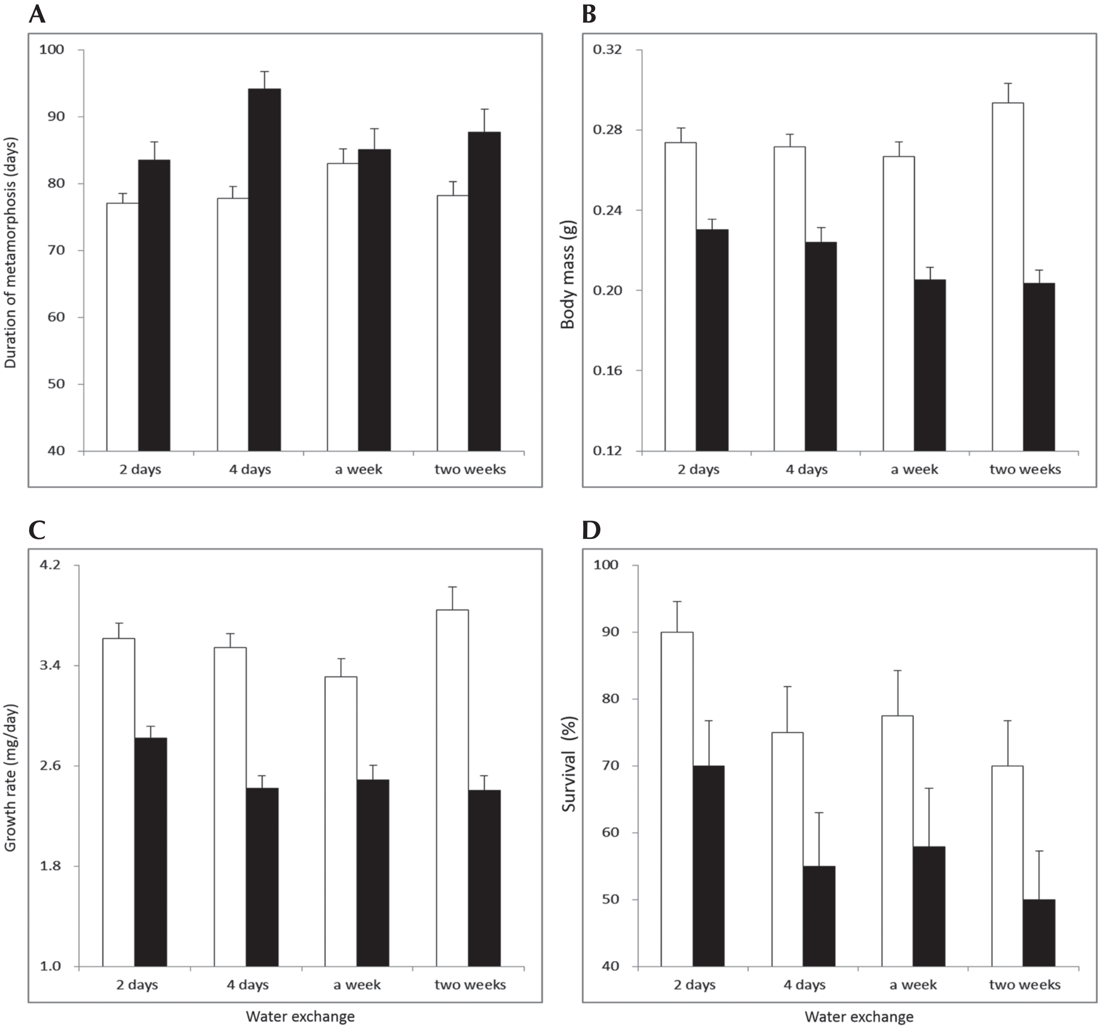

Figure 2. Influence of water exchange and food level on age at metamorphosis (**A**), body mass (**B**), growth rate (**C**), and survival (**D**) of the Chinese brown frog, *Rana chensinensis* at forelimb emergence (Gosner stage 42; open columns, high food level; black columns, low food level).

Table 1. The generalized linear model for the effects of water changes and food level on metamorphic traits in a population of *Rana chensinensis.*

Response variable	Source of variation	*df*	MS	*F*-value	*p*-value
Length of larval period	Water exchanges	3	323.015	2.148	0.095
	Food level	1	3837.183	25.522	< 0.001
	Water exchanges × Food level	3	456.764	3.038	0.030
	Error	208	150.348		
Body mass	Water exchanges	3	0.003	1.841	0.141
	Food level	1	0.193	136.043	< 0.001
	Water exchanges × Food level	3	0.006	3.970	0.009
	Error	209	0.001		
Growth rate	Water exchanges	3	1.152	2.425	0.067
	Food level	1	56.746	119.434	< 0.001
	Water exchanges × Food level	3	1.159	2.438	0.066
	Error	208	0.475		
Survival	Water exchanges	3	0.289	2.958	0.034
	Food level	1	1.574	16.092	< 0.001
	Water exchanges × Food level	3	0.00004	< 0.001	1.000
	Error	151	0.098		

once every two days and once a week was similar ($p = 0.089$). The interaction of food level and water exchange was not significant ($F_{3,\,151} < 0.001$, $p = 1.000$).

Discussion

Many environmental factors, especially temperature, food source, and predation pressure, may affect metamorphic traits of larval amphibians (reviewed by Laurila *et al.* 2001, Pacheco *et al.* 2019, Borah *et al.* 2022, Grott *et al.* 2022). In most cases, high quality environmental conditions often lead to faster development (reviewed by Álvarez and Nicieza 2002). Several experimental studies have demonstrated that high food level with a large proportion of protein can lead to double effects, accelerating both growth and development (Nathan and James 1972, Steinwascher and Travis 1983, Pandian and Marian 1985, Leips and Travis 1994). Our results revealed that food level affects metamorphic time, mass at metamorphosis, growth rate, and survivorship, suggesting that high food availability plays an important part in accelerating both growth and development of tadpoles of Chinese brown frogs.

Bilski (1921) first found that frequent water exchange or crowding would retard the growth rate of tadpoles of *Bufo* Garsault, 1764 and *Pelophylax lessonae* (Camerano, 1882). In this case, frequent water exchange was considered to be an interference, which stimulated a decrease of body fat stores and resulted in inhibition of growth (Meier *et al.* 1973). Further analysis found that interference must reach a certain kind and amount before growth can be stopped. Previous studies confirmed that flowing water and the severity and amount of agitation could inhibit growth. For example, tadpoles were put into unfolded cheese cloth bags, which were lifted up and down at rates of 6 to 12 strokes per minute in beakers of water (Adolph 1931). Our results indicate positive effects of frequent water exchange on the growth of tadpoles. Results of Hailey *et al.* (2006) were consistent with ours, indicating that changing water (once every 2–3 days) has a positive effect on growth and development of tadpoles of *Engystomops pustulosus* (Cope, 1864) compared to the control (once every 7 days). Frequent water exchange (e.g., once every two days) in our study not only failed to inhibit growth of tadpoles of Chinese brown frogs but also promoted their growth.

This finding may have two possible explanations. First, frequent water exchange seems to reduce intraspecific competition. When food is scarce, a significant interaction between food level and water exchange revealed that LFL tadpoles reared at frequent water exchange were larger at metamorphosis than those reared at middle and low water exchange. Previous studies have demonstrated that growth-inhibiting alga or chemicals were detected in the faeces or old medium of anuran larvae (Griffiths *et al.* 1993, Bardsley and Beebee 2001). Intraspecific competition can be mediated by the production of chemical waste or growth-inhibiting cells released into the spawning sites by anuran larvae (Schoener 1983, Griffiths 1991, Griffiths *et al.* 1991). Morin and Johnson (1988) found that growth inhibitors operate in natural ponds, suggesting that competition mechanisms based on food limitation are probably most important (Petranka 1989). Frequent water exchange can minimize the accumulation of chemical waste or inhibitory cells that have impacts on growth. Second, in the current study, a single individual had a surface water area of 36.2 sq. cm per liter, which was much lower than that of an optimal surface water area (133 sq. cm) for growth at the same rate (Adolph 1931). In this case, frequent water exchange is beneficial to growth because this increases oxygen tension or decreases carbon dioxide tension. Additionally, water exchanges occurring early in the daily photoperiod stimulated larval growth, whereas water exchanges in the middle of the daily photoperiod stimulated metamorphosis in Bullfrog tadpoles, *Aquarana catesbeiana* (Shaw, 1802) (Horseman *et al.* 1976). Our results indicated that frequent water exchanges have positive effects on tadpole growth.

We found a significant interaction between food level and water exchange, indicating that LHL tadpoles with frequent water exchange (once every two days) had significantly faster growth than those in any other treatment of water exchange. The underlying mechanism for this result may be the effect of water exchange on social behavior (Griffiths and Foster 1998). Tadpoles in low food treatments could grow faster from frequent water exchange because it helps to minimize intraspecific competition and increase oxygen tension, which may result in elevating metabolic rate (reviewed by Beck and Congdon 2000).

In conclusion, we found evidence that both frequent water exchange and high food level have positive effects on growth and development of tadpoles of Chinese brown frogs. Moreover, we found a significant interaction between food level and water exchange, suggesting that this novel mechanism is selected for when frequent water exchange is likely to prove profitable when food is insufficient or the environment is stressful.

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