Herpetology Notes, volume 6: 183-185 (2013) (published online on 20 April 2013)

Overwintered *Hynobius retardatus* salamander larvae can induce defensive bulgy morph in *Rana pirica* tadpoles

Osamu Kishida[1*] and Ayumi Tezuka[1]

Rana pirica frog tadpoles are excellent organisms for study of inducible morphological defenses (reviewed by Kishida et al., 2010). They exhibit the induced defensive phenotype in the presence of predatory salamander larvae (*Hynobius retardatus*), which is called “bulgy phenotype” (Kishida and Nishimura, 2004). Because *H. retardatus* salamander larvae swallow the tadpoles whole, their predation success highly depends on balance between gape width of the salamander and body size of the prey item (Kishida and Nishimura, 2004). So, the bulgy phenotype is highly effective to prevent the tadpoles from being swallowed by the gape-limited predators (Kishida and Nishimura, 2004; Takatsu and Kishida, 2013).

Previous studies about bulgy phenotype have focused only on Age-0 young-of-the-year larvae of *H. retardatus* salamanders (hereafter, YOY salamander larvae) as agents inducing bulgy phenotype of YOY of *R. pirica* frog tadpoles (hereafter, we describe just frog tadpoles because they do not overwinter as larvae), because YOY salamander larvae are one of the primary predators of the frog tadpoles from late May to August (e.g., Kishida and Nishimura, 2004, 2006; Kishida et al., 2006, 2007, 2009; Takatsu and Kishida, 2013). While YOY salamander larvae are well-recognized agents inducing bulgy phenotype of the frog tadpoles, overwintered (hereafter, OW) salamander larvae, which are defined as individuals hatched in previous year (i.e., 1-2 ages) and have not metamorphosed within the hatch year (Michimae, 2011), can be prospective agents inducing the phenotype. Although density of OW *H. retardatus* salamander larvae is fewer than that of YOY salamander larvae (i.e., fewer than 10 overwintered larvae/m^2), OW *H. retardatus* salamander larvae can be the primary predators for *R. pirica* tadpole hatchlings in some permanent ponds during their early developing period. However, previous studies have not documented whether OW salamander larvae induce bulgy phenotype of the frog tadpoles. Here, we conducted the following experiment to confirm whether OW salamander have capable to induce the defensive bulgy phenotype of tadpole hatchlings.

We collected 5 OW salamander larvae from several ponds along Yamadori-no-sawa River in Teshio Experimental Forest of Hokkaido University. We collected ten egg masses of *R. pirica* from a pond in Teshio Experimental Forest of Hokkaido University on mid-May, 2012. There are many ponds along Yamadori-no-sawa River, in some of which frog tadpoles coexist with OW and YOY salamander larvae. Eggs were placed into tanks having aged tap water and maintained at 17 ºC in the laboratory of Teshio Experimental Forest of Hokkaido University. One week after hatching of most of eggs of *R. pirica* frog (May 31, 2012), we randomly selected 100 *R. pirica* hatchlings and assigned them into each of 10 plastic tanks as experimental units (44 L x 32.5 W x 16 D cm) filled with 4-L of aged tap water. We also assigned one OW salamander larva into half of the 10 tanks (i.e., OW treatment), but did not assign OW salamander into the remaining half (i.e., No-OW treatment). Mean ± 1 SD of Snout-Vent length of OW salamander larvae and frog tadpoles were 30.60 ± 4.77 (N=5) and 6.30 ± 0.82 (N=21), respectively. The experiment was conducted in the laboratory at 17 ºC with a natural day:night (14:10) cycle. Throughout of the experiment, all of the water in each experimental unit was changed and 0.13g (dry weight) of rabbit chow was added for feeding tadpoles on Monday, Wednesday and Friday. At that time, we counted surviving tadpoles in all tanks to confirm how many tadpoles were consumed, and then we removed certain number of tadpoles from tanks of OW treatment to keep density of the both treatments were uniform (i.e., number of tadpoles in all tanks were adjusted to the least number of tadpoles among the tanks). 8 days after beginning of the experiment, we terminated the experiment. We

[1]Teshio Experimental Forest, Field Science Center for Northern Biosphere, Hokkaido University, Toikanbetsu, Horonobe, Hokkaido, 098-2943, Japan
[*]Corresponding author. E-mail: bulgy_tadpoles@hotmail.com

Table 1. Results of nested ANCOVA on body width of *R. pirica* tadpoles.

	SS	*d.f.*	*MS*	*F*	*P*
Treatment	44.49	1	44.49	169.47	<0.001
Tank	2.11	8	0.26	1.33	0.230
Body length	43.17	1	43.17	218.79	<0.001
Treatment*Body length	1.24	1	1.24	6.28	0.013
Error	32.16	163	0.20		

randomly selected 20 tadpoles from each tank and scanned them from ventral aspect. We listed the body width and body length (i.e., snout-vent length) of the frog tadpoles by measuring their scanned images on a computer screen.

Because the bulgy phenotype is characterized as large body width relative to body length, we need to consider effects of body length to evaluate expression of bulgy phenotype. To determine whether OW salamander larvae induced bulgy phenotype of *R. pirica* frog tadpoles, a nested-ANCOVA on body width was conducted, in which we considered presence of OW salamander as a fixed effect, tank as a random effect, body length as covariate, and interactive effects between OW salamander presence and body length. Before these analyses, we selectively removed the data of 25% of tadpoles having smallest body width from each tank of No-OW treatment (i.e., 5 small individuals were removed from each tank of No-OW treatment.). The reason of such data correction is to prevent overestimation of bulgy phenotypes due to possible selective predation in OW treatment. While only 1% of frog tadpoles were dead in the No-OW treatment during the experiment, 22% of *R. pirica* tadpoles had been dead in the OW treatment (i.e., average ± 1 standard deviation of number of tadpoles reduced in No-OW and OW treatments during the experiment were 1 ± 0.63 and 22 ± 8.46, respectively). This result implies that most of 22% of mortality were caused by consumption by the OW salamander larvae. This consumption of frog tadpoles possibly results in larger body width of surviving tadpoles in OW treatment than those in the No-OW treatment, because the salamander larvae consume small tadpoles selectively (Kishida and Nishimura, 2004; Takatsu and Kishida, 2013). If we compare the morphology of the tadpoles between the treatments without data correction in order to evaluate induction of bulgy phenotype in the OW salamander treatment, the selective predation effects may overestimate extent of bulgy phenotype in OW treatment. Thus, our data correction ensure conservative test for our hypothesis.

The Nested-ANCOVA revealed that there are significant effects of treatment (i.e., OW presence or absence), covariate (i.e., body length), and their interaction (Table 1). While the interaction between treatment and covariate is significant, we found strong significant effect of presence of OW salamander larvae. *R. pirica* tadpoles in OW treatments had larger body width than those in No-OW treatments (Fig. 1). This result indicates that OW salamander larvae have capability to induce bulgy phenotype of *R. pirica* frog tadpoles. Indeed, most of *R. pirica* tadpoles have thickened epithelium tissue over their body, which is a visible characteristic of bulgy phenotype (Kishida and Nishimura, 2004; see also physiological study [Mori et al., 2012]).

Does expression of bulgy phenotype of frog tadpoles in the presence of OW salamander larvae improve their survivorship? Survival advantage by being bulgy phenotype may be attained through the following two hypothetical processes. First is process via direct

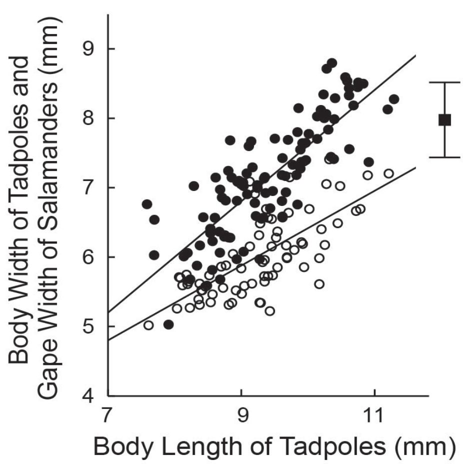

Figure 1. Body length and width (mm) of *R. pirica* tadpoles in the presence and absence of overwintered *H. retardatus* salamander, and gape width (mm) of the overwintered salamander larvae. Black and white circles represent morphology of *R. pirica* tadpoles in the presence and absence of overwintered salamander larvae, respectively. Regression lines representing shape of *R. pirica* tadpoles in the presence ($Y = 0.8013X - 0.407$, $R^2 = 0.6753$) and absence ($Y = 0.5382X + 1.0338$, $R^2 = 0.5001$) of overwintered salamander larvae are obtained by method of least squares. A black square and error bars indicate average ± 95% confidence interval of gape width of overwintered *H. retardatus* salamander larvae used in the experiment (N = 5).

defense against OW salamander larvae. Although density of OW salamander larvae is relatively low in natural pond habitats (0-6 individuals/m^2, Kishida, O. *unpublished data*); their predation pressure on tadpole hatchlings is sometimes very high because of large size differences between OW salamander larvae and tadpole hatchlings (i.e., size of hatchlings of *R. pirica* tadpoles is very small) (Kishida, O. *unpublished manuscript*). If frog tadpoles rapidly enlarge their body by exhibiting bulgy phenotype and thus enter into size-refuge on OW salamander larvae, bulgy phenotype reduces vulnerability of frog tadpoles. Indeed, some proportions of tadpoles with bulgy phenotype in OW treatment had body width as large as gape width of OW salamander larvae used in the experiment only for 8 days induction period (Fig. 1.). On the contrary, all tadpoles in the No-OW treatment had smaller body width than gape width of OW salamander larvae. Because the salamander larvae cannot swallow the tadpoles of which body size is larger than gape size of the salamander (Takatsu and Kishida, 2013), some individual tadpoles in OW treatment were in size-refuge. Therefore, *R. pirica* tadpoles are likely to gain fitness advantage in the presence of OW salamander larvae by exhibiting bulgy phenotype.

Second is a process via indirect defense against YOY salamander larvae which will be possible primary predators in the later period. In early spring (April to early May), adults of *R. pirica* frogs and *H. retardatus* salamanders lay their eggs into small pond just after melting snow. Since, in general, eggs of *H. retardatus* salamander hatch 1-4 weeks later than those of *R. pirica* tadpoles (Kishida, O. *personal observation*), YOY salamander larvae are not actual predators for frog tadpoles in their early developing period. However, as the YOY salamander larvae grow after hatch, they become dangerous predator because of their rapid growth and plastic induction of offensive phenotype (i.e., YOY salamander larvae enlarge their gape in the presence of *R. pirica* tadpoles (Michimae and Wakahara, 2002; Kishida et al., 2009)). Since bulgy phenotype of frog tadpoles is extremely effective defense against such dangerous predators (Takatsu and Kishida, 2013), by exhibiting bulgy phenotype in response to the presence of OW salamander larvae in advance, frog tadpoles may lower likelihood of being consumed by YOY salamander larvae in later period.

Acknowledgements. We are very grateful to Tomoko Sato, Kunio Takatsu, Zacharia Costa and all of members in Teshio Experimental Forest of Hokkaido University for their contribution to the experimental study. This study was supported by JSPS KAKENHI 24370004, 23247004, 23570036.

References

Kishida, O., Mizuta, Y., Nishimura, K. (2006): Reciprocal phenotypic plasticity in a predator-prey interaction between larval amphibians. *Ecology* **87**: 1599-1604.

Kishida, O., Nishimura, K. (2004): Bulgy tadpoles: inducible defense morph. *Oecologia* **140**: 414-421.

Kishida, O., Nishimura, K. (2005): Multiple inducible defenses against multiple predators in anuran tadpoles (*Rana pirica*). *Evolutionary Ecology Research* **7**: 619-631.

Kishida, O., Nishimura, K. (2006): Flexible architecture of inducible morphological plasticity. *Journal of Animal Ecology* **75:** 705-712.

Kishida, O., Trussell, G.C., Nishimura K. (2007): Geographic variation in a predator-induced defense and its genetic basis. *Ecology* **88**: 1948-1954.

Kishida, O., Trussell, G.C., Nishimura, K. (2009): Top-down effects on antagonistic inducible defense and offense. *Ecology* **90**: 1217-1226.

Kishida, O., Trussell, G.C., Mougi, A., Nishimura, K. (2010): Evolutionary ecology of inducible morphological plasticity in predator-prey interaction: toward the practical links with population ecology. *Population Ecology* **52**: 37-46.

Michimae, H., Wakahara, M. (2002): A tadpole-induced polyphenism in the salamander *Hynobius retardatus*. *Evolution* **56**: 2029-2038.

Michimae, H. (2011). Plasticity in the timing of a major life-history transition and resulting changes in the age structure of populations of the salamander *Hynobius retardatus*. *Biological Journal of the Linnean Society* **102**: 100-114.

Mori, T., Kitani, Y., Ogihara, J., Sugiyama, M., Yamamoto, G., Kishida, O., Nishimura, K. (2012): Histological and MS spectrometric analyses of the modified tissue of bulgy form tadpoles induced by salamander predation. *Biology Open* **1**: 308-317.

Takatsu, K., Kishida, O. (2013): An offensive predator phenotype selects for an amplified defensive phenotype in its prey. *Evolutionary Ecology* **27**:1-11.

Accepted by Zoltán T. Nagy