Current Herpetology 40(1): 103–106, February 2021 doi 10.5358/hsj.40.103

The Japanese Common Toad, *Bufo japonicus formosus*, Contains Toxin in the Egg Stage

Hisanori OKAMIYA[1]*, Kotaro TAKAI[2], and Osamu KISHIDA[2]

[1]*Department of Biological Sciences, Graduate School of Sciences, Tokyo Metropolitan University, Hachioji, Tokyo 192–0397, JAPAN*
[2]*Tomakomai Experimental Forest, Field Science Center for Northern Biosphere, Hokkaido University, Tomakomai, Hokkaido 053–0035, JAPAN*

Abstract: Bufonid toads generally possess cardiotoxic steroids called bufadienolides as defensive chemicals. Although knowledge of the life stages at which the toad species possess the poison is important for our understanding of diversity of toxicity among bufonid toads, this knowledge is limited. In the present study, we revealed that the Japanese common toad, *Bufo japonicus formosus*, possesses toxins at the unfertilized egg stage by conducting a bioassay experiment. Recent studies documented that hatchlings of *B. j. formosus* have lethal toxic effects on native frog tadpoles (*Rana pirica*) in the invasive area of the toad (Hokkaido). In our bioassay experiment using *R. pirica* tadpole as a predator, no tadpoles died when they did not consume any prey item during two-days experimental period. However, approximately 90% of *R. pirica* tadpoles immediately died when they consumed an unfertilized egg of *B. j. formosus*. These results suggest that the toxin at the early life stages of *B. j. formosus* is, at least partly, provided from female parent.

Key words: Bioassay; *Bufo japonicus formosus*; Maternal provisioning; Toad toxin

Introduction

Bufonid toads rely on noxious secretions for protection against predators (Toledo and Jared, 1995). Their main toxins are cardiotoxic steroids called bufadienolides (Flier et al., 1980). These compounds are synthesized by toads de novo (Chen and Osuch, 1969; Porto and Gros, 1971; Porto et al., 1972) and are present in their tissues from a very early developmental stage (Hayes et al., 2009; Üveges et al., 2017). In the cane toad (*Rhinella marina*), the diversity and amount of bufadienolides are highest in eggs and gradually decrease until developmental stage 25 (Gosner, 1960; Hayes et al., 2009). This suggests that cane toad tadpoles produce no bufadienolides, relying instead on maternal provisioning of these toxins. High concentrations of bufadienolides in the ovary of female toads under reproductive condition and high toxicity of toad eggs have been reported in the gulf coast toad (*Incilius valliceps*) (Licht, 1968). In contrast, the majority of hatchlings

* Corresponding author.
E-mail address: h.okamiya@gmail.com

contain no bufadienolides in the common toad (*Bufo bufo*). Their toxins accumulate quickly in the young tadpoles, and after reaching a peak in the mid-aged larvae, decrease to lower quantities as metamorphosis approaches (Üveges et al., 2017). Therefore, the common toad tadpoles synthesize their toxins de novo, and the maternal provisioning of toxins appears to be limited in this species. These findings indicate that toads have different developmental stages at which they acquire toxins among different species.

The Japanese common toad, *B. japonicus formosus* is a species of toad native to the main island (Honshu) of Japan that has invaded Hokkaido Island that lacks a native toad (Matsui and Maeda, 2018). Recently, Kazila and Kishida (2019) experimentally revealed that *B. j. formosus* hatchlings in Hokkaido have lethal toxic effects on native frog tadpoles (*Rana pirica*). The early hatchlings of *B. j. formosus* (developmental stage 19) contain lethal venom (Oyake et al., 2020) and therefore are presumed to already contain toxins at the egg stage as a product of maternal provisioning. However, this premise is not supported by empirical evidence. In this study, we experimentally examined toxicity of unfertilized eggs of *B. j. formosus* using a predatory *R. pirica* tadpole.

Materials and Methods

Obtaining unfertilized eggs of Bufo japonicus formosus

In the breeding season of *B. j. formosus* (25 and 29 April 2020), we collected two preoviposition female toads in the breeding ponds in Sapporo City (43°00'24" N, 141°18'12" E), Hokkaido Prefecture. We obtained two clutches of unfertilized egg clusters (Clutch-1 and Clutch-2) from the female toads. Ovulation was induced by keeping only females in tanks containing natural water (i.e., drawn from an unpolluted clear river) at 20°C. We successfully collected egg clusters from two female toads. The unfertilized egg clusters were kept in 4 l tanks filled with natural water at 5°C for several hours (i.e., until the start of the experiment) in an experimental room at the Tomakomai Experimental Forest, Hokkaido University. Just before the start of the experiment, unfertilized eggs were carefully separated from their jelly coat by manual operation. The removal of the external jerry is required for the experiment because the jelly coats prevent predatory *R. pirica* tadpoles from consuming the eggs (Okamiya, personal observation).

Egg collection and rearing of Rana pirica *for bioassay*

Several egg masses of *R. pirica* were collected from two regions (Chitose [42°29'00" N, 141°20'36" E] and Erimo City [42°06'28" N, 143°15'48" E]) in Hokkaido on 29 March and 5 April 2020, respectively. We kept the eggs and hatchlings in 4 l tanks (200 individuals per tank) filled with 2 l of natural water in a natural temperature condition in the experimental room until the start of the experiment. Each frog tadpole was fed daily rabbit chow pellets. The amount of the food was adjusted according to tadpole size so as to avoid water deterioration from excess food.

Toxicity evaluation by bioassay

In the experiment, we used *R. pirica* tadpoles at developmental stage 25 (Gosner, 1960). The snout-vent length (mean±SD) of the frog tadpoles was 9.17 mm±0.57 and 8.64 mm±0.67 in Chitose and Erimo, respectively (N=20). The diameter of the toad eggs was 2.04 mm±0.32 and 2.56 mm±0.30 in Clutch-1 and Clutch-2, respectively (N=20). Frog tadpoles were assigned to either a toad treatment, in which a toad egg was presented as a prospective prey item to a frog tadpole, or a control treatment, in which the tadpole was not given a toad egg (i.e., starved treatment). The toxicity of the toad eggs was assessed using the mortality rate of frog tadpoles that consumed an egg (Kazila and Kishida, 2019). We performed all experiments at 18°C in the experimental room. We used a polypropylene container (7.5×4.8×3.8 cm

high) filled with 100 ml of natural water as the experimental unit.

In the experiment (started 26 and 29 April 2020), we haphazardly selected 50 frog tadpoles from each region (i.e., Chitose and Erimo) and placed them individually into each of 25 containers for the toad treatment, and 25 containers for the control treatment. The tadpoles were allowed to habituate for 15 min, and then we placed one toad egg into each container of the toad treatment. We defined the start of the experiment as the time when the eggs were placed in the experimental containers of the toad treatment. Forty-eight hours after the start of the experiment, we checked egg consumption and frog tadpole survival. We judged that consumption had occurred if most of the egg had disappeared. For each frog population, we calculated the mortality rate of the frog tadpoles in each toad treatment (Clutch-1 and Clutch-2) as the percentage of dead individuals relative to the number of individuals that consumed an egg. To obtain the consumption tendency, we calculated the percentage of individuals that consumed an egg relative to the number of the initial number of individuals used in the treatment. We also checked all surviving tadpoles for abnormal behavior such as impaired swimming ability.

Results and Discussion

No tadpoles died in the control treatment. In contrast, in Clutch-1 of the toad treatment, 100% and 80% of *R. pirica* tadpoles that consumed a toad egg died in Chitose and Erimo, respectively (consumption tendencies were 96% and 80%, respectively). In Clutch-2, all *R. pirica* tadpoles that consumed a toad egg died in both Chitose and Erimo (i.e., mortality rates were 100%; consumption tendencies were 88% and 64%, respectively).

Past studies showed that all *R. pirica* tadpoles that preyed on a toad hatchling died without exception (Kazila and Kishida, 2019; Oyake et al., 2020). In this study, however, a few tadpoles that preyed on a toad egg survived until the end of the experiment (20% of Erimo tadpoles that consumed a toad egg). Unfertilized eggs of toads are quite fragile and often rupture as soon as they are bitten by tadpoles and their contents begin to dissolve in environmental water. On this account, perhaps the surviving tadpoles were unable to ingest a lethal dose of toxins. Because all tadpoles that consumed a toad egg were overtly affected by exposure to toad poison (abnormal behavior and impaired swimming ability), all the toad eggs using the experiment likely contained toxins. On the whole, our experimental study demonstrated that unfertilized eggs of *B. j. formosus* already contain the toxins, and therefore, suggests that the bufadienolides of *B. j. formosus* are, at least partly, provided as a maternal production.

Accumulation timing of bufadienolides in life history may greatly vary among bufonid toads. While the maternal provisioning of bufadienolides has been reported in the cane toad (*R. marina*) (Hayes et al., 2009) and the gulf coast toad (*I. valliceps*) (Licht, 1968), the contrasting pattern is found in the common toad (*B. bufo*) (Üveges et al., 2017). Üveges et al. (2017) showed that most of hatchlings of the common toad did not contain bufadienolides at a detectable amount while their toxins accumulate rapidly as the tadpoles grow. Because the common toads are phylogenetically closer to the *B. j. formosus* than the cane toad and the gulf coast toad, this knowledge and our results allow us to expect that accumulation timing of bufadienolides exhibit a characteristic independent of the phylogenetic relationship of bufonid toads. Further investigations on accumulation timing of bufadienolides of other toad species are required to deepen our understanding of diversity of bufadienolides possession in bufonids.

Acknowledgments

We thank Akira Mori for providing rele-

vant information and literatures on biosynthesis of the bufadienolides. We also thank Tatsuhiro Tokuda for his field assistance and Yoshihiro Inoue for his assistance in the experiment. This work was partly supported by a Grant-in-Aid for JSPS Fellows (Research Project Number: 19J10846) from the Japan Society for the Promotion of Science to H.O. and Asahi glass foundation and The Mitsui & Co. Environment Fund to O.K.

Literature Cited

Chen, C. and Osuch, M. V. 1969. Biosynthesis of bufadienolides—3β-hydroxycholanates as precursors in *Bufo marinus* bufadienolides synthesis. *Biochemical Pharmacology* 18: 1797–1802.

Flier, J., Edwards, M. W., Daly, J. W., and Myers, C. W. 1980. Widespread occurrence in frogs and toads of skin compounds interacting with the ouabain site of Na+, K+-ATPase. *Science* 208: 503–505.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183–190.

Hayes, R. A., Crossland, M. R., Hagman, M., Capon, R. J., and Shine, R. 2009. Ontogenetic variation in the chemical defenses of cane toads (*Bufo marinus*): Toxin profiles and effects on predators. *Journal of Chemical Ecology* 35: 391–399.

Kazila, E. and Kishida, O. 2019. Foraging traits of native predators determine their vulnerability to a toxic alien prey. *Freshwater Biology* 64: 56–70.

Licht, L. E. 1968. Unpalatability and toxicity of toad eggs. *Herpetologica* 24: 93–98.

Matsui, M. and Maeda, N. 2018. *Encyclopaedia of Japanese Frogs.* Bun-ichi Sogo Shuppan, Tokyo.

Oyake, N., Sasaki, N., Yamaguchi, A., Fujita, H., Tagami, M., Ikeya, K., Takagi, M., Kobayashi, M., Abe, H., and Kishida, O. 2020. Comparison of susceptibility to a toxic alien toad (*Bufo japonicus formosus*) between predators in its native and invaded ranges. *Freshwater Biology* 65: 240–252.

Porto, A. M., Baralle, F. E., and Gros, E. G. 1972. Biosynthesis of bufadienolides in toads: III —Experiments with [2-^{14}C] mevalonic acid, [20-^{14}C] 3β-hydroxy-5-pregnen-20-one and [20-^{14}C] cholesterol. *Journal of Steroid Biochemistry* 3: 11–17.

Porto, A. M. and Gros, E. G. 1971. Biosynthesis of the bufadienolide marinobufagin in toads *Bufo paracnemis* from cholesterol-20-^{14}C. *Experientia* 27: 506–506.

Toledo, R. D. and Jared, C. 1995. Cutaneous granular glands and amphibian venoms. *Comparative Biochemistry and Physiology Part A: Physiology* 111: 1–29.

Üveges, B., Fera, G., Móricz, Á. M., Krüzselyi, D., Bókony, V., and Hettyey, A. 2017. Age- and environment-dependent changes in chemical defences of larval and post-metamorphic toads. *BMC Evolutionary Biology* 17: 137.

Accepted: 28 August 2020