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**Are toxic effects of alien species affected by their prey? Evaluation by
bioassay with captive-bred toad embryos and a vulnerable predator**

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Abstract Toads of the Family Bufonidae possess neurotoxins (bufadienolides) which are generally considered to be synthesized *de novo*. Thus, invasive toad species pose a threat to native predators via toxic effects. However, the influence of diet on toad toxicity is poorly understood. We evaluated the effect of diet on toxicity of embryos of the invasive toad *Bufo japonicus formosus* in Japan, using native tadpoles (*Rana pirica*) as a bioassay. Specifically, we compared the toxicity of embryos spawned by captive toads reared entirely on non-toxic prey versus embryos spawned by wild toads. Embryos of captive-reared toads and wild toads were comparable in terms of toxic effects: all tadpoles that consumed a toad embryo died, irrespective of origin of embryos. Embryo toxicity appears to be via parental (likely maternal) provisioning. Our study indicates that adult *B. j. formosus* produce toxins (likely bufadienolides) in amounts sufficient for embryos to be fatal to native predator tadpoles irrespective of whether the adult diet contains toxins. Thus, the toxic effects of invasive *B. j. formosus* on *R. pirica*, and possibly on other sensitive native predator species, are likely to be widespread and occur regardless of variation in prey biota in invaded areas in Japan.

Keywords Bioassay, Captive-bred, Prey item, Toad toxicity

Introduction

Toxic chemicals are widespread in nature from unicellular organisms to plants and animals. These chemicals fulfill multiple ecological roles, acting as a defense against predators, competitors, parasites and pathogens. They can also be used for killing prey (Brodie 2009). Some species produce toxic chemicals *de novo*, but other species cannot

45 biosynthesize these components (Shimizu and Kobayashi 1983; Daly et al. 1997; Kudo
46 et al. 2015). For example, cultured pufferfish are well known to be non-toxic (Matsui et
47 al. 1982). In nature, the toxin is biosynthesized by bacteria and later accumulated in
48 pufferfish through the food chain (Yotsu-Yamashita 2001; Noguchi and Arakawa 2008).
49 Other organisms known to acquire toxins from dietary food items include molluscs
50 (Winters et al. 2018), insects (Kowalski et al. 2020; Whitaker and Salzman 2020),
51 amphibians (Savitzky et al. 2012), reptiles (Mebs 2001; Hutchinson et al. 2007) and birds
52 (Dumbacher et al. 2004). These examples demonstrate that the toxicity of an organism
53 can be strongly affected by the prey items it consumes.

54 Anuran toads belonging to the Family Bufonidae are well known to contain neurotoxin
55 for protection against predators (Toledo and Jared 1995). One of the main toxic
56 compounds present in the secretion of these toads are steroids called bufadienolides (Flier
57 et al. 1980), which exert a cardiotoxic effect by inhibiting membrane-bound heart tissue
58 Na^+/K^+ adenosine triphosphatase (ATPase; Kamboj et al. 2013; Toledo and Jared 1995).
59 Bufadienolides are not restricted to bufonid toads, being also found in some bacteria,
60 fungi, plants and fireflies (Gao et al. 2011; Smedley et al. 2017). Previous studies have
61 provided physiological evidence that bufadienolides are synthesized *de novo* in adult
62 bufonids *Rhinella marina* Linnaeus (= *Bufo marinus*) and *Rhinella paracnemis* Lutz (= *Bufo paracnemis*)
63 (Chen and Osuch 1969; Porto and Gros 1971; Porto et al. 1972). In
64 early life-history stages, there can be variation among toad species regarding the timing
65 of toxin production. Ovarian eggs and unfertilized eggs of *Incilius valliceps* Wiegmann
66 (= *Bufo valliceps*) are highly toxic (Licht 1968), and a high concentration of
67 bufadienolides has been recorded in recently deposited eggs of *R. marina* (Hayes et al.
68 2009). For *R. marina*, the diversity and amount of bufadienolides are highest in eggs and

decrease as tadpoles develop (Hayes et al. 2009), indicating maternal provisioning of toxins. In contrast, hatchlings of *Bufo bufo* Linnaeus do not contain bufadienolides, but these toxins accumulate quickly in young tadpoles (Üveges et al. 2017). Thus, it is the tadpole stage of *B. bufo* that synthesizes toxins *de novo*, as confirmed by histological studies that demonstrate the presence of underlying secretory cells and glands during larval life (Delfino et al. 1995).

While members of other anuran families (dendrobatids, mantellids, myobatrachids, eleutherodactylids) acquire and sequester toxins such as alkaloids from arthropod prey (Saporito et al. 2011; Savitzky et al. 2012), the existence of a synthetic pathway for production of bufadienolides in bufonid anurans (Chen and Osuch 1969; Porto and Gros 1971; Porto et al. 1972) suggests these toads have the ability to produce toxins without relying on specific prey for toxin acquisition. However, the influence of variation in diet on toad toxicity has not been directly investigated. Interestingly, dietary alkaloid sequestration has been documented for two bufonid toad species of the genus *Melanophryniscus* (Daly et al. 2007), demonstrating the potential for toxicity of bufonids to be influenced by prey diet.

For bufonid toad species that become invasive, one significant ecological impact on native biota is poisoning of native predators (Shine 2010). This toxic impact occurs because native predator species lack tolerance to toad toxin due to their short interaction history within the invaded region (Crossland and Alford 1998; Crossland 2000; Crossland et al. 2008; Letnic et al. 2008; Crossland and Shine 2010). The Japanese common toad, *Bufo japonicus formosus* Boulenger, is a member of the Family Bufonidae that is native to the main Island (Honshu) of the Japanese archipelago, but has become invasive in the island of Hokkaido (Matsui and Maeda 2018). Like other bufonid species discussed above,

93 this toad possesses bufadienolides (Inoue et al. 2020), and recent experimental studies
94 demonstrated that invasive *B. j. formosus* embryos and hatchlings have lethal toxic effects
95 on predatory native frog tadpoles (*Rana pirica* Matsui) and salamander larvae (*Hynobius*
96 *retardatus* Dunn) in Hokkaido (Kazila and Kishida 2019; Oyake et al. 2020). The toxic
97 effects on *R. pirica* tadpoles are particularly strong due to their low toxin resistance
98 (Oyake et al. 2020); in natural ponds in Hokkaido, where both *R. pirica* and invasive *B.*
99 *j. formosus* breed, *R. pirica* tadpoles frequently experience mass mortality shortly
100 following hatching of *B. j. formosus* eggs (Kishida and Yamaguchi unpublished data).
101 These impacts are similar to those of invasive cane toads *R. marina* on native predatory
102 frog tadpoles in Australia, where native tadpoles are highly sensitive to cane toad toxins
103 (Crossland and Alford 1998; Crossland 2000; Crossland and Shine 2010) and tadpole
104 populations in natural waterbodies experience significant mortality when exposed to eggs
105 and hatchlings of *R. marina* (Crossland et al. 2008).

106 If the toxicity of an alien species is affected by the prey it consumes, then the ecological
107 impact of that invasive species on native predators may vary over space or time depending
108 on spatial or temporal variation in prey items. For *B. j. formosus*, the bufadienolide toxin
109 profile is known to vary among geographic areas within the native range in Japan (Inoue
110 et al. 2020), and the potential exists for at least some of this variation to be driven by
111 spatial variation in prey items. In this study, we investigated whether variation in diet
112 alters the toxicity of invasive *B. j. formosus* to native aquatic predators in Hokkaido.
113 Specifically, we compared the behavioral and toxicity responses of native *R. pirica*
114 tadpoles to *B. j. formosus* embryos spawned by wild toads (i.e., adult toads that consumed
115 wild diet) versus embryos spawned by captive toads fed non-toxic prey items through
116 their entire life. Our aims were to determine (1) does tendency of native tadpoles to

consume toad embryos differ according to adult toad diet?, and (2) does toxicity of toad embryos to native tadpoles differ according to adult toad diet?

Materials and Methods

Egg collection and rearing of captive toads

On 29 May 2017, part of an egg cluster of *B. j. formosus* was collected from Hida City, Gifu Prefecture. The embryos and hatched tadpoles were kept at 18 °C in the animal-rearing room at Gifu World Freshwater Aquarium. The tadpoles were fed tropical fish food (Tetra Vegetable, Tetra®) and raised until metamorphosis. Metamorphosed toads were raised on a diet of farmed crickets (*Gryllus bimaculatus* De Geer) and giant mealworms (*Zophobas atratus* Fabricius) in natural temperature conditions until sexual maturity. These diet items do not contain bufadienolides, or to our knowledge, any toxic compounds that could be sequestered by captive-reared toads. On 29 November 2019, nine individuals (five males and four females) were moved to an outdoor cage for winter hibernation to increase the likelihood of breeding success in the following spring. The outdoor cage (110 × 60 × 80 cm) made of wooden boards with mesh screens (mesh size 1 × 1 cm) was set on concrete pavement. We placed a tank (46 × 33 × 16 cm) with 20 L of water in the cage as a breeding site for toads. We did not observe any potential prey items such as invertebrates in the cage while toads were hibernating. Even if potential prey animals did enter the cage during this time, the toads were unlikely to consume them due to their propensity to not feed during the winter period and early-spring breeding period. Breeding activity started on 28 March 2020 and we found a spawned egg cluster

on 3 April 2020. The clutch size of this cluster was 717 eggs. The egg cluster was transferred to a laboratory at the Tomakomai Experimental Forest of Hokkaido University for experiments. Embryos were kept in a 4 L tank filled with 2 L of aged tap water in natural temperature (13-15 °C) and light regime (L13:D11) conditions in the experimental room.

Egg collection and rearing of wild toads

For comparison of toxicity, four egg clusters of *B. j. formosus* from two wild populations were collected at Ibigawa Town, Gifu Prefecture ($N = 1$) and Tokigawa City, Saitama Prefecture ($N = 3$) in April 2020. The eggs were transferred to the experimental room at the Tomakomai Experimental Forest, and kept in a tank ($28.9 \times 16.2 \times 9.5$ cm) filled with 2 L of aged tap water at ambient temperature (13-15 °C) and light regime (L13:D11) conditions. We do not know the diet of the individual wild toads that produced these egg clusters. Toads in general are considered to be indiscriminate feeders, and in natural habitats of both native and invaded regions, *B. japonicus* feed on earthworms, snails and a diverse array of arthropods (Hirai and Matsui 2002; Sarashina and Yoshida 2015).

Egg collection and rearing of *Rana pirica*

To assess the toxicity of the toad embryos, we used predatory *R. pirica* frog tadpoles as a bioassay in the experiment. Several egg masses of *R. pirica* were collected from two regions (Chitose and Erimo City) in Hokkaido on 29 March and 5 April 2020, respectively.

The eggs were transferred to the experimental room at the Tomakomai Experimental Forest. We kept the eggs and hatchlings in tanks ($28.9 \times 16.2 \times 9.5$ cm) filled with 2 L of aged tap water at natural temperature conditions in the experimental room until the start of the experiment. Every two days, we replaced rearing water and added rabbit chow pellets *ad libitum* as food for *R. pirica* tadpoles.

Toxicity evaluation bioassay

In the experiment, we used *R. pirica* tadpoles at developmental stage 25 (Gosner 1960) and *B. j. formosus* embryos at stage 17-18 (near hatching time). The snout-vent lengths of frog tadpoles (mean $\pm$ SD) were $9.33 \text{ mm} \pm 0.94$ and $8.10 \text{ mm} \pm 0.54$ for Chitose and Erimo regions, respectively ($N = 20$). The total lengths of toad embryos were $4.95 \text{ mm} \pm 0.27$, $4.91 \text{ mm} \pm 0.32$, $4.26 \text{ mm} \pm 0.17$, $3.86 \text{ mm} \pm 0.30$, and $4.27 \text{ mm} \pm 0.19$ for captive-bred, Gifu, Saitama-1, Saitama-2 and Saitama-3, respectively ($N = 20$). Frog tadpoles were assigned to either a toad treatment, in which one toad embryo was presented as a prospective prey item to a frog tadpole, or a control treatment, in which the tadpole was not given a toad embryo (i.e., starved conditions). Toxicity of *B. j. formosus* was assessed using the mortality rate of frog tadpoles that consumed a toad embryo according to previous knowledge (Kazila and Kishida 2019). We performed all experiments at 20°C in the experimental room, using a polypropylene container ($7.5 \times 4.8 \times 3.8$ cm) filled with 100 ml of aged tap water as the experimental unit.

In the experiment (beginning 15 April 2020), we haphazardly selected 200 *R. pirica* frog tadpoles from each region (i.e., Chitose and Erimo) and placed them individually into each of the following combinations: (1) 50 containers for the captive-bred toad

189 treatment, (2) 25 containers for each wild toad population (Gifu, Saitama-1, Saitama-2
190 and Saitama-3), and (3) 50 containers for the control treatment (Fig. S1). The sample size
191 of the wild toad population treatment (2) was half that of the other treatments (1 and 3)
192 to prevent excessive use of animals. This unbalanced experimental design was acceptable
193 because previous studies have consistently shown that native *R. pirica* tadpoles die after
194 consuming wild toad embryos (Kazila and Kishida 2019; Oyake et al. 2020). The tadpoles
195 were allowed to habituate for 15 min, then we placed one toad embryo into each container
196 of the toad treatment. We defined the start of the experiment as the time when toad
197 embryos were placed in experimental containers. Forty-eight hours later, we checked
198 embryo consumption and frog tadpole survival. We determined consumption tendency by
199 calculating the percentage of tadpoles that consumed a toad embryo relative to the initial
200 number of tadpoles in the treatment. For each frog population, we calculated the mortality
201 rate of tadpoles in each toad treatment as the percentage of dead individuals relative to
202 the number of individuals that consumed a toad embryo. Since no tadpoles died in the
203 control treatment, mortality comparisons were made only between toad treatments. We
204 used Fisher's exact tests to compare the proportional data among captive-bred treatments,
205 among wild population treatments, and between captive-bred and wild population
206 treatments. We used the Bonferroni method to correct P-values for multiple testing.

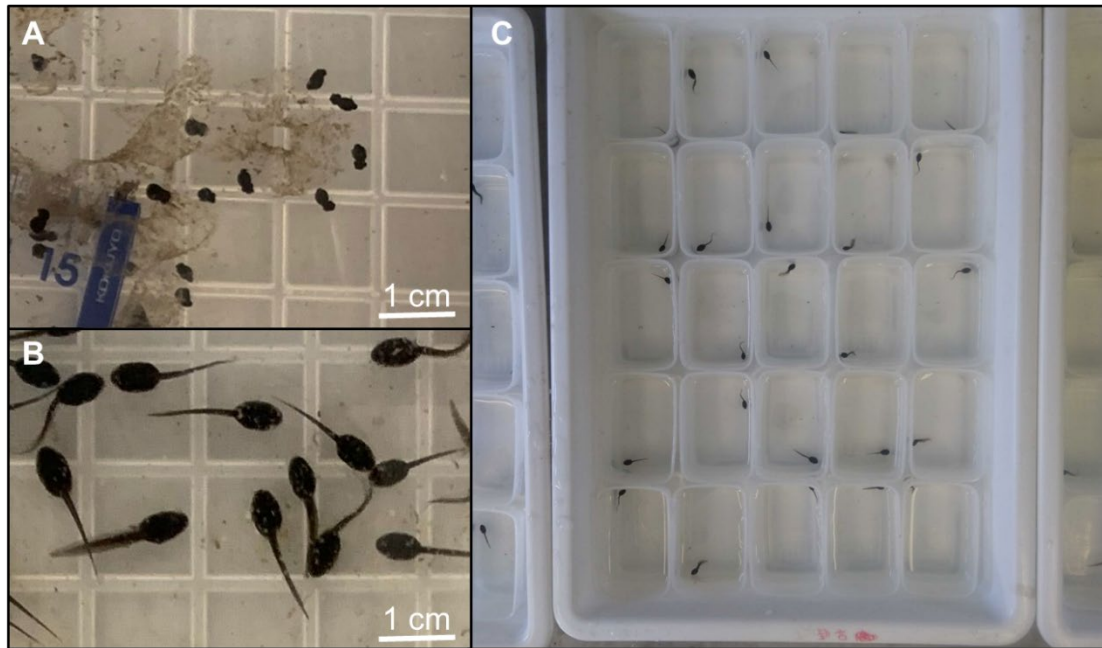


Figure S1 Photographs of the study organisms (embryos of *Bufo japonicus formosus* (A) and tadpoles of *Rana pirica* (B)) and the bioassay experiment (C)

Results

After Bonferroni correction, we found no significant variation in the consumption tendency of tadpoles from different populations (Chitose vs Erimo) in either captive-bred or wild population treatments (Fisher's exact test, $P > 0.05$; Fig. 1a). Therefore, we pooled tadpole source data from captive-bred and wild population treatments respectively, and compared consumption between these treatments. The result showed that the tendency of tadpoles to consume toad embryos from the captive-reared treatment was significantly (but slightly) higher than for embryos from the wild population treatment (Fisher's exact test, $P = 0.047$; Fig. 1a).

There was no significant treatment effect on toxicity of toad embryos to native tadpoles: all *R. pirica* tadpoles that consumed a toad embryo died, regardless of treatment

(Fig. 1b).

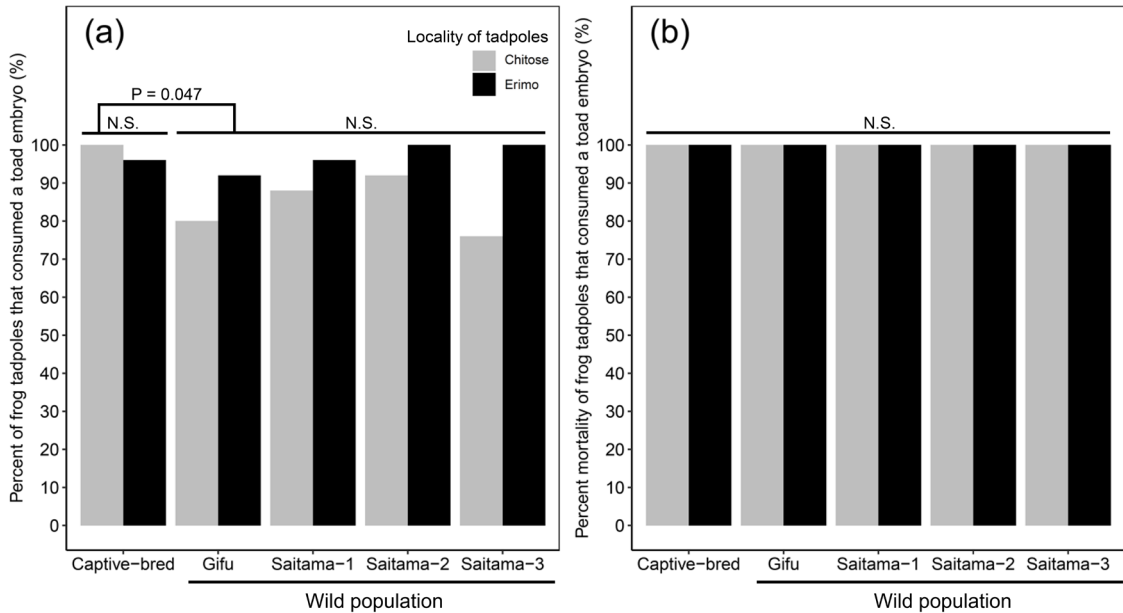


Fig. 1 Percent of *Rana pirica* tadpoles that consumed a toad embryo (a) and percent mortality of *R. pirica* tadpoles that consumed a toad embryo (b) from captive-bred and wild population toad treatments, over the 48-hour experiment duration. Grey and black bar indicates Chitose and Erimo *R. pirica* tadpoles, respectively. N.S.: not significant by Fisher's exact test

Discussion

Our results showed that embryos of captive-reared toads were slightly more likely to be eaten by native tadpoles than embryos of wild toads. However, embryos of captive-reared and wild toads were equally toxic to native tadpoles: all tadpoles that consumed toad embryos died regardless of adult diet treatment.

The result that native tadpoles had a slightly, but significantly ($p=0.047$), increased tendency to consume embryos of captive-reared toads than wild toads may be due to the

240 difference in embryo developmental stage between treatments. The captive-bred embryos
241 were slightly more developed than wild-source embryos at the start of the experiment,
242 with many embryos in the captive treatment having hatched from the jelly coat. This
243 likely allowed *R. pirica* tadpoles to more easily feed on these embryos. In the wild
244 population treatment, tadpoles could not easily eat embryos due to the protection of the
245 jelly coat, and consequently fewer tadpoles consumed embryos than those in the captive-
246 reared treatment.

247 Our results also show that toxicity of *B. j. formosus* embryos to native tadpoles did not
248 vary with the prey diet of adult toads that produced these embryos. Toad embryos were
249 highly toxic to native tadpoles (100% mortality if ingested) regardless of whether
250 embryos were spawned by toads reared on a wild diet versus a captive diet known to be
251 devoid of toxic substances. Nonetheless, we note that our experiment assessed toxicity
252 using native tadpoles as a bioassay; we did not measure diversity or concentration of toxin
253 chemicals in toads *per se*. Toxin production in toads is energetically costly
254 (Blennerhassett et al. 2019). Thus, it may be that diet can affect the diversity or
255 concentration of toxin chemicals in toads (i.e., a low energy diet may result in lower
256 diversity or concentration of toxins than a high energy diet, based solely on the energetic
257 requirements of toxin production). However, if such effects do occur, they may be
258 inconsequential in terms of the ecological impact of invasive *B. j. formosus* on native
259 predators if low diversity / concentration of toxins produced under low energy diet
260 conditions still exceeds the critical threshold for sensitivity of native predators to these
261 toxins. Future studies could clarify this issue.

262 Like many bufonid toad species, *B. j. formosus* possess toxic bufadienolide chemicals
263 (Inoue et al. 2020). Data from other studies suggest the toxic effects of embryos we

observed were likely the result of maternal provisioning of toxins in embryos. High toxicity of ovarian eggs and unfertilized eggs, and high concentrations of bufadienolides in recently deposited eggs, have been reported for other bufonid species (Licht 1968; Hayes et al. 2009). In the cane toad, *R. marina*, the diversity and amount of bufadienolides are highest in eggs and decrease during tadpole life (Hayes et al. 2009). Thus, toxins in *R. marina* eggs and tadpoles occur via maternal provisioning. However, in the common toad, *B. bufo*, bufadienolides are absent in hatchlings but occur in young tadpoles (Üveges et al. 2017). This indicates that *B. bufo* tadpoles synthesize their toxins *de novo*, as confirmed by histological studies (Delfino et al. 1995). Because unfertilized eggs and fertilized embryos of *B. j. formosus* contain toxin (Okamiya et al. 2021; present study), the toxin present in eggs and embryos of this species is probably due to maternal provisioning. In this context, our study provides insight into the synthesis of toxicity in *B. j. formosus*, as we showed that embryos spawned by a captive-reared female toad exhibit high toxicity. This captive-reared *B. j. formosus* female had been raised on prey items (i.e., tropical fish food, crickets and giant mealworms) that are commonly used for captive animals (e.g., fish, amphibians and reptiles). These prey items do not contain bufadienolides, and are not known to contain any other toxins. Our study therefore suggests that adult females of *B. j. formosus* synthesize toxins through a physiological pathway that does not rely on acquiring toxic chemicals from prey items. Given that adult *B. j. formosus* possess bufadienolides and are not known to possess any other class of toxin (Inoue et al. 2020), we suggest the toxic effects we observed are due to maternal provisioning of bufadienolides in eggs (as occurs in another bufonid toad, *R. marina*: Hayes et al. 2009). We are unaware of any published data that demonstrates bufadienolide toxins are sequestered from prey by bufonid toads, or indeed by any species of anuran.

288 The spawning of a captive-reared toad gave us the unique opportunity to examine the
289 production of toxins, using toxin-sensitive native tadpoles as a bioassay for toxicity.
290 Because breeding of captive-reared toads is very difficult to achieve, our experimental
291 result provides valuable evidence of the biosynthesis of toxin in *B. j. formosus*.
292 Nonetheless, it does raise a question as to whether our result represents a specific response
293 of the captive-reared individual for toxin production rather than a common response of
294 the species in general. However, we consider it unlikely that the individual captive female
295 toad was uniquely capable of biosynthesizing toxin from non-toxic prey. So far, we have
296 confirmed that wild *B. j. formosus* embryos collected from various geographic regions
297 exhibit significant toxicity to *R. pirica* tadpoles without exception (Kazila and Kishida
298 2019; Oyake et al. 2020; Okamiya et al. 2021). Furthermore, the toxicity of embryos of
299 the captive-reared toad to *R. pirica* in our experiment was comparable to the toxicity of
300 wild toad embryos. Therefore, it is reasonable to assume that the captive-reared toad had
301 the same biosynthetic pathway of toxin production as wild toads, although future tests
302 using embryos from other captive-reared toads are needed to confirm our argument.

303 The captive *B. j. formosus* fed non-toxic prey items in our experiment produced
304 embryos with toxins sufficiently potent to kill all *R. pirica* frog tadpoles that consumed
305 them. This result strongly suggests that *B. j. formosus* will exert consistent negative
306 effects on the survival of *R. pirica* tadpoles on Hokkaido, regardless of variation in the
307 prey biota in invaded areas. *B. j. formosus* has steadily expanded its range on Hokkaido
308 since its initial introduction (Naito and Shiga 2016). Based on our results, we believe this
309 toad may have adverse effects on *R. pirica* frogs throughout the entire invaded area.

310 The embryos from the captive-reared toad used in this study were obtained by the
311 skilled techniques of an aquarium keeper. It is usually difficult to obtain breeding

individuals of non-model organisms, especially vertebrates with long generation times. We emphasize that interdisciplinary collaboration with professionals skilled in reproductive techniques and husbandry can be an effective approach to solving difficult-to-verify ecological challenges.

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Conflicts of interest/Competing interests The authors declare that they have no conflict of interest.

Ethics approval Our work has been performed in accordance with the guidelines for proper conduct of animal experiments in Japan and was approved by the committee for animal experiments in FSC of Hokkaido University (ID2 - 5).

Consent to participate All authors fully consent to participate of this study.

Consent for publication All authors fully consent to the contents and submission of this

manuscript.

Availability of data and material All raw data is available upon request from the corresponding author.

Authors' contributions HO and OK conceived the study concept. MT collected samples and conducted breeding. HO and OK designed and performed the experiment and statistical analyses. All authors contributed to writing the manuscript.

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