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ORIGINAL ARTICLE

Foraging Responses of the Larvae of Invasive Bullfrogs (*Lithobates catesbeianus*): Possible Implications for Bullfrog Control and Ecological Impact in China

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Abstract The predatory behavior of invasive species can affect their ecological impact, and offer opportunities for targeted control. In Australia, tadpoles of invasive cane toads (*Rhinella marina*) do not consume eggs of native anurans, but are strongly attracted to (and consume) newly-laid eggs of conspecifics; chemical cues from such eggs (or adult secretions) thus can be used to attract toad tadpoles to traps. Do other invasive anurans show similar selectivity? Our laboratory trials on a Chinese population of invasive American bullfrogs (*Lithobates catesbeianus*) revealed similar behaviors as exhibited by Australian cane toads. Bullfrog tadpoles rarely consumed the eggs of native anurans, but were attracted to both bullfrog eggs and bullfrog skin secretions. Although the attraction response was less intense in bullfrogs than in cane toads, it might nonetheless enable selective removal of bullfrog tadpoles from invaded sites.

Keywords alien species, biocontrol, *Bufo marinus*, pheromone, *Rhinella marina*, *Lithobates catesbeianus*, tadpole ecology

1. Introduction

Invasive species are an increasing ecological problem, and have caused the extinction of many native taxa (Clavero and Garcia-Berthou, 2005). Invaders come from a wide variety of phylogenetic lineages, from viruses to mammals (Elton, 1958; Torchin and Mitchell, 2004), and can affect the invaded ecosystems via a diverse range of processes (e.g., parasitism, competition, predation: Blackburn *et al*., 2004; Gaertner *et al*., 2009). Several species of anuran amphibians (frogs and toads) have been identified as ecologically damaging invaders (Kraus, 2009). Two of the most ecologically destructive invasive anurans are the cane toad (*Rhinella marina*) and the American bullfrog (*Lithobates catesbeianus*). Although they come from highly divergent phylogenetic lineages within the Anura (Bufonidae versus Ranidae, which diverged at least 15million years ago: Roelants *et al*., 2007), these two high-profile invasive species share several common traits. Notably, they are both native to the Americas, they both rank among the largest anurans, they both are toxic to predators in the larval stage (Crossland *et al*., 2008; Szuroczki *et al*., 2011), and both have been widely translocated from their native ranges. The cane toad was introduced to about 40 countries for use in controlling insect pests, whereas the bullfrog was introduced over 40 countries for human consumption (Ficetola *et al*., 2007; Lettoof *et al*., 2017; Li *et al*., 2011b). The primary ecological impact of invasive cane toads has been to fatally poison predators (Shine, 2010) whereas the primary impact of the bullfrog has been to consume native taxa (Wang *et al*., 2008; Wu *et al*., 2005).

In the current paper, we explore the possibility

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that research on one of these species may help us to understand (and perhaps, control) the other. Notably, recent studies on cane toads in Australia have revealed that the tadpoles of this species are selective predators on the eggs of anurans. Larval cane toads are not attracted to the eggs of native anurans, and do not consume them; thus, predation by toad tadpoles does not imperil the eggs of native anuran species. In striking contrast, however, toad larvae are strongly cannibalistic. They are attracted to chemical cues released by the eggs of conspecifics, and follow those chemical gradients to locate and consume the eggs. That response is so strong that traps baited with the appropriate chemicals (toxins obtained from the skin glands of adult toads) can be used to trap toad tadpoles, eradicating the alien species from spawning ponds (Crossland *et al.*, 2011b). Given broad ecological similarities between cane toads and American bullfrogs (see above), we conducted laboratory trials to ask the following questions: 1) Within their invasive range in China, do American bullfrog larvae consume the eggs of native anurans? 2) Are bullfrog larvae attracted to the eggs or skin secretions of conspecifics? The answers to these two questions may clarify the ecological impact of invasive bullfrogs; and also, may identify opportunities for targeted control.

2. Materials and Methods

2.1 Study species The American bullfrog, *Lithobates catesbeianus* (= *Rana catesbeiana*), is native to eastern North America (Frost, 2007; Shaw, 1802) but has been introduced to Europe, South America (Stumpel, 1992) and Asia (Bai *et al.*, 2012b; IUCN, 2003), as well as other parts of the world (Ficetola *et al.*, 2007; Laufer *et al.*, 2008). It is ecologically destructive (Kraus, 2009; Lowe *et al.*, 2000), and appears to have caused declines of native amphibians (Bury and Luckenbach, 1976; Fisher and Shaffer, 1996; Kiesecker and Blaustein, 1997; Moyle, 1973) via competition, predation, breeding interference and transmission of diseases (Bai *et al.*, 2010, 2012a; Blaustein and Kiesecker, 2002; Kats and Ferrer, 2003; Kiesecker and Blaustein, 1998; Kupferberg, 1997a; Laufer *et al.*, 2008; Li *et al.*, 2011a, 2011 b; Liu *et al.*, 2015; Pearl *et al.*, 2004, 2005; Werner *et al.*, 1995; Zhu *et al.*, 2014a, 2014b).

Bullfrog tadpoles may compete with native anurans for cover and food (Boone *et al.*, 2004; Kiesecker and Blaustein, 1998; Kupferberg, 1997a; Lawler *et al.*, 1999; Wu *et al.*, 2005), but direct predation may be a more important mechanism of impact (e.g., Hirai, 2004; Hothem *et al.*, 2009; Pearl *et al.*, 2004; Wang *et al.*, 2007; Wang and Li, 2009; Werner *et al.*, 1995).

The bullfrog was introduced to Taiwan, china in 1924 and to mainland China in 1959, and commercial breeding has expanded since the 1980s, resulting in occasional releases or escapes (Wu *et al.*, 2004; Bai *et al.*, 2012b). The bullfrog has now established populations in several Chinese provinces (Li *et al.*, 2006; Wang and Xie, 2004; Wu *et al.*, 2004).

In this study, we used ten native anuran species that are common and distributed in various parts of China. We collected *Fejervarya limnocharis*, *Pelophylax nigromaculatus*, and *Microhyla ornata* from Taohua Island. These three species are common in the Zhoushan Archipelago where the invasion of bullfrogsis considered a serious threat (Li *et al.*, 2006). Additional native taxa (*Bufo gargarizans*, *B. raddei*, *Rana chensinensis*, *Bombina orientalis*) were collected from Haidian, Beijing, northern China; these species are common in Beijng, where is a potential invasion zone by bullfrogs (Ficetola *et al.*, 2007). *R. amurensis* and *R. dybowskii* were collected from Baishan, Jilin, located in the northeast of China with a high latitude and cold winter, is yet to be invaded by bullfrogs but is a potential invasion zone (Ficetola *et al.*, 2007). *R. kukunoris* was collected from Nuoergai, Sichuan. This species is endemic to the plateau region of western China. It has been reported that bullfrogs have invaded the Qinghai-Tibet plateau, so bullfrogs may also affect the survival of this unique species (Mimawangdui, 2004). Given the high potential for ecological impact, it is of great significance to study the effect of bullfrogs on native anuran species in China.

2.2 Study Site Taohua Island (122°13′–122°19′ E and 29°46′–29°52′ N) lies within the Zhoushan Archipelago (29°31′–31°04′ N and 121°30′–123°25′ E) east of mainland China. We collected invasive *L. catesbeianus* from this site. Adults of the native species were collected from the source areas, taken to Taohua Island, and released in artificial ponds, where they spawned. We used these eggs to study predation by invasive bullfrog tadpoles.

2.3 Collection of eggs and tadpoles Every day at 0500 h in April of 2013 and 2014, we collected newly-laid eggs by hand and transported them to the laboratory in plastic containers with pond water. All containers were maintained in the laboratory at ambient temperature (20 ± 0.5 °C).

Bullfrog tadpoles (stage 28–32: Gosner 1960) were collected by netting themat night from permanent ponds on Taohua Island in April of 2013 and 2014. They were then transported to the laboratory, where they were kept at low densities and fed algal pellets *ad libitum* at ambient temperature (20 ± 0.5 °C).

2.4 Bullfrog tadpoles as egg predators–intrinsic predation rates To determine if bullfrog tadpoles will consumeanuran eggs, we placed eggs and tadpoles (Gosner stage 28–32) in 190 × 140 mm^2 plastic containers filled to a depth of 50 mm with water. We placed ten eggs in one corner of each container and one tadpole in the opposite corner. Each experiment included the eggs of only a single species of anuran. For each species, we repeated the trial eight times and recorded the number of eggs eaten after 72 hours. Eggs of the following native anurans were tested: *B. gargarizans*, *B. raddei*, *B. orientalis*, *P. nigromaculatus*, *F. limnocharis*, *M. ornata*, *R. amurensis*, *R. chensinensis*, *R. kukunoris and R. dybowskii*. Additionally (i.e., separately), we ran the same experiments testing bullfrog tadpoles as predators of conspecific eggs.

2.5 Bullfrog tadpoles as egg predators–effects of alternative food To determine whether or not the presence of algal pellets reduces predationon eggs (i.e., because alternative food is available) or increases predation rate (i.e., the scent of algae pellets induces feeding behavior), we exposed bullfrog tadpoles to 10 eggs of native amphibians (*B. gargarizans*, *F. limnocharis*, *P. nigromaculatus*) in the presence and absence of homobaric algae pellets (one tadpole per container). Each trial included the eggs of only a single species of anuran. We also ran experiments testing predation by bullfrog tadpoles on conspecific eggs in the presence and absence of algae pellets. Eggs and tadpoles (Gosner stage 28–32) were placed in 190 × 140 mm^2 plastic containers filled to a depth of 50 mm with water. For every species, we repeated the trial eight times and recorded the number eaten after 72 hours.

2.6 Attraction of bullfrog tadpoles to eggs and skin secretions This experiment investigated the effects of embryonic developmental stage and adult skin secretions on the attraction of tadpoles (Gosner stage 28–32) to the eggs of bullfrogs (*L. catesbeianus*) and native amphibians (*B. gargarizans*, *B. raddei*, *F. limnocharis and P. nigromaculatus*). We tested attraction to eggs < stage 12, eggs > stage 16 and skin secretions for the first three species, and eggs > stage 16 for the last two species.

Preparation of eggs cues: Every day at 0500 h, we collected newly-laid eggs as described above. Then we placed 350 mm sections of egg strings in the experimental containers (see below) to allow any pheromone to be released into the water.

Preparation of skin secretions: We obtained skin secretions of *B. gargarizans* and *B. raddei* for attraction experiments by gently squeezing the shoulder (parotoid) glands to expel 0.05 g toxin (from three toads of each species) onto the underside of a glass microscope slide. Because bullfrogs have no skin glands, we used a glass slide to scrape the skin to obtain 0.05 g of secretions (from three frogs). All samples were stored at 4 °C prior to the experiment.

After preparation of egg cues and skin secretions, we placed the sections of egg string, glass slides smeared with 0.05 g skin secretions or algal pellets (of the same mass as the egg string) into 750 ml plastic containers with 250 ml of water for 24 hours. Containers of water without eggs, parotoid secretion or algae pellets served as controls. All containers were left at ambient temperature (20 ± 0.5 °C) and selected haphazardly for use in the experiment as described below. Immediately prior to their addition to the experimental trays, the water in these holding containers was carefully separated from the eggs, skin secretion or algal pellets (i.e., tadpoles were only exposed to water that had contained these materials, not to the materials themselves).

Twenty bullfrog tadpoles (Gosner stage 28–32; captured in the field) were placed in each of 24 plastic trays (700 × 450 × 90 mm^3) filled with 12 L of water. A mesh container (150 × 100 × 70 mm^3; mesh size 1.5 mm) was placed in one corner of each tray as a point source for the addition of experimental water, and the trays were visually divided into quarters using a string placed above the water line. After 10 min of acclimation, the tadpoles were released in the center of the container. The initial allocation of tadpoles to treatments was randomized.

We scored the responses of tadpoles in two ways: the number of tadpoles present in the tray segment where water was added, and the number of tadpoles exhibiting active predation behavior (i.e., oriented with the mouth to the substrate or mesh container wall, with rapid side-to-side head movements; tadpoles exhibiting this behavior were only seen in the quarter of the tray where water was added). Both responses were measured 5, 10, 15, 20, 30 and 60 min after water was added to the experimental trays. Each treatment was replicated four times.

2.7 Statistical analyses We used logistic regression (Warton and Hui, 2011) to compare the proportion of eggs of each species eaten by bullfrog tadpoles,and the proportion of bullfrog tadpoles attracted to water-borne cues of eggs and adult skin secretion.For analysis of the attraction experiments, we initially used Treatment, Time and Treatment*Time as fixed effects, and Tray as a random effect. In all but one instance, the Treatment*Time interaction was non-significant. In these cases, we removed the interaction term and ran the model again to obtain Treatment estimates. Time effects were non-significant. For *P. nigromaculatus* eggs $>$ stage 16, the interaction term was significant. However, inspection of the interaction plot showed this was solely due to tadpole responses at the final 60-minute time period (i.e. the estimate of Treatment effect is still meaningful for the majority of time intervals). All analyses were based on the quasi-binomial distribution to account for over-dispersion of data.

3. Results

3.1 Bullfrog tadpoles as egg predators–intrinsic predation rates We did not formally analyse rates of predation on the eggs of *B. raddei*, *B. orientalis and R. kukunoris*, because no eggs of these species were eaten by bullfrog tadpoles. We used predation on *P. nigromaculatus* eggs (low predation rate: 1 of 80 eggs eaten) as the reference group to assess predation by bullfrog tadpoles on eggs of other native anuran species.

Predation rates on eggs of *B. gargarizans* ($p = 0.603$), *F. limnocharis* ($p = 0.381$) and *R. dybowskii* ($p = 0.939$) were as low as predation on *P. nigromaculatus* eggs (i.e. very few eggs eaten; Figure 1). However, predation rates were significantly higher for *M. ornata* ($p = 0.032$), *R. amurensis* ($p = 0.04$), *R. chensinensis* ($p = 0.01$) and conspecific eggs ($p = 0.014$; Figure 1). On average, bullfrog tadpoles were 11 times more likely to eat *R. amurensis* eggs than the reference *P. nigromaculatus* eggs, 13 times more likely to eat *M. ornata*, 21 times more likely to eat *R. chensinensis* eggs and 18 times more likely to eat conspecific *L. catesbeianus* eggs (Figure 1). Nonetheless, egg predation rates for these species were still relatively low (number of eggs eaten: 12 of 80 *R. amurensis* eggs, 11 of 80 *M. ornata* eggs, 29 of 80 *R. chensinensis* eggs, 15 of 80 conspecific eggs).

3.2 Bullfrog tadpoles as egg predators–effects of alternative food For the experiment on impacts of alternative food on rates of predation on anuran eggs, we used predation rates on eggs in the absence of alternative food as the reference group. The addition of algal pellets had no significant effect on egg predation rates by bullfrog tadpoles for any species (*B. gargarizans* $p = 0.566$, *F. limnocharis* $p = 1.00$, *P. nigromaculatus* $p = 0.566$, *L. catesbeianus* $p = 0.84$; Figure 2).

3.3 Attraction of bullfrog tadpoles to eggs and skin secretions Bullfrog tadpoles showed no significant attraction response (relative to the Control treatment) for most of the cues tested (algal pellets $p = 0.208$, *B. gargarizans* eggs $<$ stage 12 $p = 0.334$, *B. gargarizans*

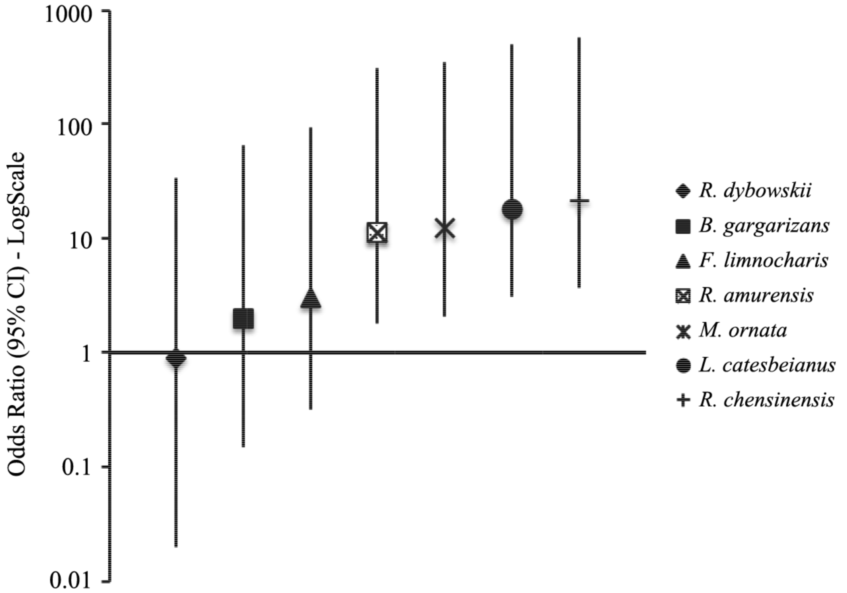

Figure 1 Odds ratio for predation by *Lithobates catesbeianus* tadpoles on anuran eggs, plotted on a log scale. Vertical lines represent 95% confidence intervals. The horizontal red line at an odds ratio of 1.0 represents the reference group (predation on *P. nigromaculatus* eggs). Predation rate is not significantly different from the reference group if the 95% confidence interval line overlaps the red line at an odds ratio of 1.0.

skin secretion $p = 0.266$, *B. raddei* eggs < stage 12 $p = 0.401$, *B. raddei* eggs > stage 16 $p = 0.454$, *B. raddei* skin secretion $p = 0.924$, *F. limnocharis* eggs > stage 16 $p = 0.230$; Figure 3). However, bullfrog tadpoles were significantly attracted to conspecific eggs < stage 12 ($p = 0.019$), conspecific adult skin secretions ($p = 0.009$) and *B. gargarizans* eggs > stage 16 ($p = 0.037$; Figure 3). In contrast, bullfrog tadpoles showed significant aversion to *P. nigromaculatus* eggs > stage 16 as well as conspecific eggs > stage 16 (in both instances, $p < 0.001$; Figure 3).

We did not formally analyse differences in feeding behavior among treatments because so few tadpoles showed this response (overall: 80 instances of such behavior in 6120 tadpoles observations = 1.3% response rate). For the treatments identified above as having significant attraction, there was low feeding behaviour response (*L. catesbeianus* eggs < stage 12 and adult skin secretion cues: 24 instances of such behavior in

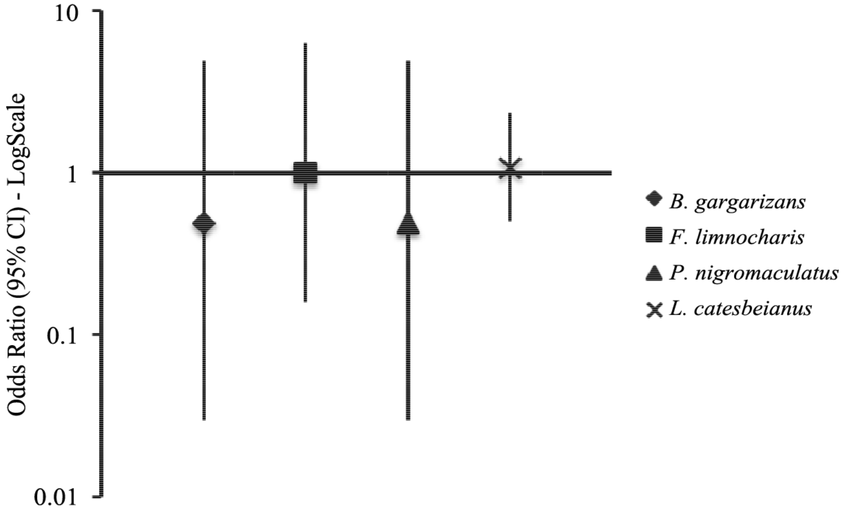

Figure 2 Odds ratio for effect of presence of alternate food (algae pellets) on egg predation by *Lithobates catesbeianus* tadpoles plotted on a log scale. Vertical lines represent 95% confidence intervals. The horizontal red line at an odds ratio of 1.0 represents the reference group (predation on eggs in the absence of alternate food). Predation rate is not significantly different from the reference group if the 95% confidence interval line overlaps the red line at an odds ratio of 1.0.

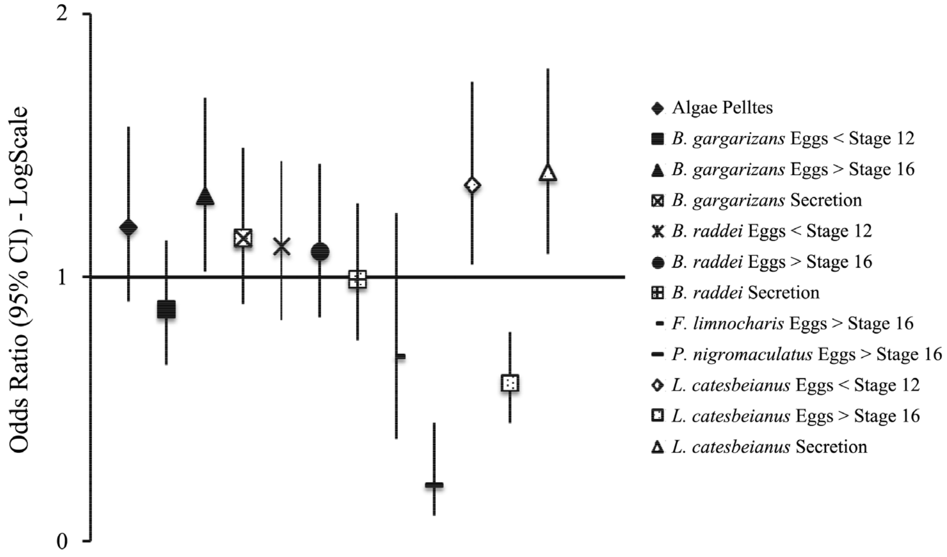

Figure 3 Odds ratio for attraction responses to egg cues and adult skin secretion by *Lithobates catesbeianus* tadpoles. Vertical lines represent 95% confidence intervals. The horizontal red line at an odds ratio of 1.0 represents the reference group (control water). Attraction is not significantly different from the reference group if the 95% confidence interval line overlaps the red line at an odds ratio of 1.0. Significant responses at an odds ratio > 1 represent attraction, while significant responses at an odds ratio < 1 represent repulsion.

960 tadpoles observations = 2.5% response rate; *B. gargarizans* eggs > stage 16: 6 instances in 480 tadpoles = 1.25% response rate). Thus, even when a significant attraction response was observed in response to *L. catesbeianus* eggs < stage 12 and adult skin secretion cues, and *B. gargarizans* eggs > stage 16, it was not strongly associated with overt feeding behavior.

4. Discussion

Studies on cane toads in Australia have reported that the larvae of this species do not consume the eggs of native anurans, even though they consume conspecific eggs (Crossland, 1998; Crossland *et al.*, 2011a, 2011b). Our studies showed low predation rates on the eggs of native Chinese anurans by American bullfrog tadpoles also, despite the fact that these larvae are far larger than are those of cane toads (maximum masses of approximately 20 *vs* 0.1g: Hagman and Shine, 2009; Kupferberg, 1997b) and hence, might be expected to be more effective predators. The native eggs most often consumed by bullfrog tadpoles were those of *M. ornata*, *R. amurensis* and *R. chensinensis*. Two of these taxa (*M. ornata* and *R. amurensis*) are included in the Red List of Threatened Species by the World Conservation Union (IUCN). However, the risk posed by egg predation is likely to be low (especially, compared to the risk posed by predatory adult bullfrogs). For example, *M. ornata* breeds in temporary pools that form in the rainy season whereas bullfrogs breed in larger permanent ponds, reducing rates of encounter in nature.In our study, bullfrog tadpoles never ate eggs of *B. raddei*, *B. orientalis* or *R. kukunoris*, and rarely ate eggs of other native species (*B. gargarizans*, *F. limnocharis*, *P. nigromaculatus*, *R. dybowskii*). Thus, as has been concluded for cane toads in Australia, invasive bullfrog tadpoles likely do not pose a substantial risk to the eggs of native anurans in China.

Providing alternative food (algal pellets) had no significant effect on rates of predation on eggs of native anurans or conspecifics by bullfrog tadpoles. Any reduction in predation rate would have been difficult to detect, given the already-low rates of predation on eggs observed in our experiments. Nonetheless, the addition of alternative food clearly did not stimulate increased predation on eggs. The absence of a response for the three native species tested might be a consequence of a lack of recent common evolutionary history between these species and the American bullfrog. In this study, we were unable to collect eggs of other native species to assess broader trends. Obtaining such data would help determine whether the predatory behavior of bullfrog tadpoles on native anuran eggs is selective or random. The continued predation on conspecific eggs, despite the presence of alternative food, hints that reduction of future competition (rather than immediate nutritional input) might have favoured the evolution of cannibalism in bullfrog tadpoles. That interpretation has been strongly supported in studies of invasive cane toads (Crossland *et al.*, 2011a).

Interestingly, bullfrog tadpoles were attracted to cues from conspecific eggs and conspecific skin secretions, but were not attracted to cues from the eggs or skin secretions of mostnative anurans (the exception being *B. gargarizans* eggs > stage 16). Indeed, some developmental stages of eggs of one native anuran species (*P. nigromaculatus*) repulsed bullfrog tadpoles. We were unable to obtain data for attraction responses of bullfrog tadpoles to eggs or skin secretions of *M. ornata*, *R. amurensis*, *R. dybowskii* or *R. chensinensis* because they were not collected from Zhoushan. Nonetheless, the general lack of attractionfor native anurans is consistent with the low tendency for bullfrog tadpoles to consume the eggs of native anurans.

In contrast, bullfrog tadpoles showed significant attraction to chemical cues from conspecific eggs (at early developmental stages, < stage 12) as well as skin secretions collected from conspecific adults. This behavior provides a striking parallel to that seen in invasive cane toads in Australia (Crossland and Shine, 2011b). In the case of cane toads, the attraction to conspecific eggs has the same chemical basis as the attraction to skin secretions: toxins present in both substancesare the active ingredients that elicit larval attraction (and cannibalism) (Crossland *et al.*, 2010, 2011a, 2011b). Hence, the bullfrog tadpoles in China as well as those of cane toads in Australia appear to use chemicals present in skin secretions to locate conspecific eggs, which they then consume. Given that no other anurans have been reported to utilise this mechanism, the behavior appears to have evolved convergently in these two large invasive anurans —one a bufonid, the other a ranid.

However, the magnitude of attraction by bullfrog tadpoles in China (odds ratio effect size for < stage 12 eggs = 1.35, adult skin secretion = 1.40) is much less than that for cane toads in Australia (e.g. overall odds ratio effect size for stage 16–24 embryos = 4.70, effect size forembryos stage 20–23 = 18.20; reanalysis of data in Crossland and Shine, 2011b). Thus, although there is potential for the trapping methodology used for cane toad tadpoles in Australia (Crossland *et al.*, 2012) to be applied to bullfrog tadpoles in China, that method is likely to be relatively ineffective in the latter situation. Future research should seek to determine the substance responsible for attraction, the concentration that attracts bullfrog tadpoles effectively, and how to amplify the effect to efficiently remove large numbers of tadpoles. Research could also explore the possibility that conspecific attraction (sociality) also is favoured at the invasion front in tadpoles, as it is in adults of another invasive anuran, the cane toad (Gruber *et al.*, 2017).

In summary, our studies on bullfrog tadpoles in China, stimulated by previous research on cane toads in Australia, have discovered both similarities and differences between these two systems. Broadly, the toxic tadpoles of both of these invasive anuran species exhibit low rates of predation on the eggs of native anurans, but are attracted to (and consume) newly-laid eggs of their own species. In both cases, the proximate cue eliciting approach by the cannibalistic tadpoles involves chemicals that are also present on the skin of the adult animals.A cautionary note is always required when attempting to extrapolate laboratory results to the field: such linkages are not always strong (Brower and Zar, 1984; Dodds, 2002; Perotti, 2016; but see Crossland and Shine 2011b, and Crossland *et al*., 2012 for an example of strong linkages). Thus, although the usefulness of this response in targeted control of bullfrogs has yet to be demonstrated, our study provides an example of the potential value of looking for generalities in behavior and ecology among invasive species from disparate phylogenetic lineages.

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