Anti-predator defences of a bombardier beetle: is bombing essential for successful escape from frogs? (#25710)

First submission

Editor guidance

Please submit by **29 Mar 2018** for the benefit of the authors (and your $200 publishing discount).

Structure and Criteria
Please read the 'Structure and Criteria' page for general guidance.

Custom checks
Make sure you include the custom checks shown below, in your review.

Author notes
Have you read the author notes on the guidance page?

Raw data check
Review the raw data. Download from the location described by the author.

Image check
Check that figures and images have not been inappropriately manipulated.

Privacy reminder: If uploading an annotated PDF, remove identifiable information to remain anonymous.

Files

Download and review all files from the materials page.

3 Figure file(s)
2 Table file(s)
5 Video file(s)
1 Other file(s)

Custom checks

Vertebrate animal usage checks

- Have you checked the authors ethical approval statement?
- Were the experiments necessary and ethical?
- Have you checked our animal research policies?

Structure and Criteria

Structure your review

The review form is divided into 5 sections.
Please consider these when composing your review:

1. **BASIC REPORTING**
2. **EXPERIMENTAL DESIGN**
3. **VALIDITY OF THE FINDINGS**
4. General comments
5. Confidential notes to the editor

You can also annotate this PDF and upload it as part of your review

When ready submit online.

Editorial Criteria

Use these criteria points to structure your review. The full detailed editorial criteria is on your guidance page.

BASIC REPORTING

- Clear, unambiguous, professional English language used throughout.
- Intro & background to show context. Literature well referenced & relevant.
- Structure conforms to PeerJ standards, discipline norm, or improved for clarity.
- Figures are relevant, high quality, well labelled & described.
- Raw data supplied (see PeerJ policy).

EXPERIMENTAL DESIGN

- Original primary research within Scope of the journal.
- Research question well defined, relevant & meaningful. It is stated how the research fills an identified knowledge gap.
- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- Impact and novelty not assessed. Negative/inconclusive results accepted. *Meaningful* replication encouraged where rationale & benefit to literature is clearly stated.
- Data is robust, statistically sound, & controlled.
- Conclusions are well stated, linked to original research question & limited to supporting results.
- Speculation is welcome, but should be identified as such.

Standout reviewing tips

The best reviewers use these techniques

Tip	***Example***
Support criticisms with evidence from the text or from other sources	*Smith et al (J of Methodology, 2005, V3, pp 123) have shown that the analysis you use in Lines 241-250 is not the most appropriate for this situation. Please explain why you used this method.*
Give specific suggestions on how to improve the manuscript	*Your introduction needs more detail. I suggest that you improve the description at lines 57- 86 to provide more justification for your study (specifically, you should expand upon the knowledge gap being filled).*
Comment on language and grammar issues	*The English language should be improved to ensure that an international audience can clearly understand your text. Some examples where the language could be improved include lines 23, 77, 121, 128 – the current phrasing makes comprehension difficult.*
Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

Anti-predator defences of a bombardier beetle: is bombing essential for successful escape from frogs?

Shinji Sugiura Corresp.

Corresponding Author: Shinji Sugiura
Email address: ssugiura@people.kobe-u.ac.jp

Some animals, such as the bombardier beetles (Coleoptera: Carabidae: Brachinini), have evolved chemical defences against predators. When attacked, bombardier beetles can discharge noxious chemicals at temperatures of approximately 100°C from the tip of their abdomens, “bombing” their attackers. Although many studies to date have investigated how bombardier beetles discharge defensive chemicals against predators, relatively little research has examined how predators modify their attacks on bombardier beetles to avoid being bombed. In this study, I observed the black-spotted pond frog *Pelophylax nigromaculatus* (Anura: Ranidae) attacking the bombardier beetle *Pheropsophus jessoensis* under laboratory conditions. In Japan, *Pe. nigromaculatus* is a common generalist predator in grasslands where the bombardier beetle also frequently occurs. Almost all the frogs (92.3%) observed rejected live bombardier beetles; 65.4% stopped their attacks once their tongues touched the beetles, and 26.9% spat out the beetles immediately after taking the beetles into their mouths. Only 7.7% of the frogs swallowed live bombardier beetles. When dead beetles were provided instead, 85.0% of the frogs rejected the dead beetles, 65.0% stopped their attacks after their tongues touched the beetles, and 20.0% spat out the beetles. Only 15.0% of the frogs swallowed the dead beetles. Rejection rates between live and dead beetles were not significantly different. Thus, the results suggest that the frogs tended to stop their predatory attack before receiving a bombing response from the beetles. Therefore, bombing was not essential for the beetles to successfully defend against the frogs. Using its tongue, *Pe. nigromaculatus* may be able to rapidly detect a deterrent chemical or physical characteristics of its potential prey *Ph. jessoensis* and thus avoid injury by stopping its predatory attack before the beetle bombs it.

Anti-predator defences of a bombardier beetle: is bombing essential for successful escape from frogs?

Shinji Sugiura

Graduate School of Agricultural Science, Kobe University, Kobe, Japan

Corresponding author: S. Sugiura, ssugiura@people.kobe-u.ac.jp, sugiura.shinji@gmail.com

Running title: Bombardier beetle defences against frogs

ABSTRACT

Some animals, such as the bombardier beetles (Coleoptera: Carabidae: Brachinini), have evolved chemical defences against predators. When attacked, bombardier beetles can discharge noxious chemicals at temperatures of approximately 100°C from the tip of their abdomens, “bombing” their attackers. Although many studies to date have investigated how bombardier beetles discharge defensive chemicals against predators, relatively little research has examined how predators modify their attacks on bombardier beetles to avoid being bombed. In this study, I observed the black-spotted pond frog *Pelophylax nigromaculatus* (Anura: Ranidae) attacking the bombardier beetle *Pheropsophus jessoensis* under laboratory conditions. In Japan, *Pe. nigromaculatus* is a common generalist predator in grasslands where the bombardier beetle also frequently occurs. Almost all the frogs (92.3%) observed rejected live bombardier beetles; 65.4% stopped their attacks once their tongues touched the beetles, and 26.9% spat out the beetles immediately after taking the beetles into their mouths. Only 7.7% of the frogs swallowed live bombardier beetles. When dead beetles were provided instead, 85.0% of the frogs rejected the dead beetles, 65.0% stopped their attacks after their tongues touched the beetles, and 20.0% spat out the beetles. Only 15.0% of the frogs swallowed the dead beetles. Rejection rates between live and dead beetles were not significantly different. Thus, the results suggest that the frogs tended to stop their predatory attack before receiving a bombing response from the beetles. Therefore, bombing was not essential for the beetles to successfully defend against the frogs. Using its tongue, *Pe. nigromaculatus* may be able to rapidly detect a deterrent chemical or physical characteristics of its potential prey *Ph. jessoensis* and thus avoid injury by stopping its predatory attack before the beetle bombs it.

Subjects: Animal Behaviour, Ecology, Entomology, Evolutionary Studies, Zoology

Key words: Carabidae, chemical defence, predator, prey,

INTRODUCTION

Physical and chemical defences have evolved in many organisms to protect against natural enemies (Edmunds, 1974; Eisner, Eisner & Siegler, 2005). For example, some plant and animal species have developed physical deterrents such as thorns and spines (Edmunds, 1974; Cooper & Owen-Smith, 1986; Eisner, 2003; Sugiura & Yamazaki, 2014; Sugiura, 2016; Ito, Taniguchi & Billen, 2016), while other species produce defensive chemicals, including toxic substances, to prevent themselves from being eaten (Eisner, 2003; Eisner, Eisner & Siegler, 2005; Derby, 2007; Mithöfer & Boland, 2012). Organisms whose defence mechanisms can cause severe injury to their natural enemies have also evolved warning signals, such as conspicuous body colouration or particular sounds (Lev-Yadun, 2001; Ruxton, Sherratt & Speed, 2004; Inbar & Lev-Yadun, 2005; Bonacci et al., 2008; Lev-Yadun, 2009; Bura et al., 2016; Sugiura & Takanashi, 2018). In response, predators have evolved specific abilities to avoid such well-defended prey by recognising warning colouration or detecting chemical signals (Edmunds, 1974; Ruxton, Sherratt & Speed, 2004; Skelhorn & Rowe, 2006; Williams et al., 2010).

Adult bombardier beetles (Coleoptera: Carabidae: Brachinini) bomb, i.e. discharge noxious chemicals from the tip of their abdomens at temperatures of approximately 100°C, when they are disturbed or attacked (Aneshansley et al., 1969; Dean, 1979; Eisner, 2003; Eisner, Eisner & Siegler, 2005; Arndt et. al., 2015). Previous studies have investigated how bombardier beetles successfully defend against predators (Eisner, 1958; Eisner & Meinwald, 1966; Eisner & Dean, 1976; Dean, 1980a; Eisner, Eisner & Aneshansley, 2005; Eisner et al., 2006). Bombardier beetles can aim their abdominal discharge in virtually any direction, spraying various parts of their own bodies (e.g., legs and dorsal surface) with the toxic chemicals (Eisner & Aneshansley, 1999). Dean (1980b) reported that predators displayed intense responses to the unheated chemical discharges of bombardier beetles in experiments. This suggests that the cooled chemicals coating the beetles' body surfaces function as the primary defence against predators, although more research is needed to clarify the relative importance of chemical toxicity and heat for overall successful anti-predatory defence.

Frogs and toads are important predators of carabid beetles (Larochelle, 1974a,b). However, bombardier beetles have rarely been found in the gut contents and faeces of frogs and toads (Larochelle, 1974a,b; Sarashina, Yoshihisa & Yoshida, 2011; except Mori, 2008), suggesting that bombardier beetles are effective at defending themselves against these predators (Eisner & Meinwald, 1966; Dean, 1980a; Esiner, 2003; Sugiura & Sato, 2018). Still, only a few studies have investigated the factors that cause anuran predators to stop preying on bombardier beetles (Dean, 1980b). Elucidating these ecological factors would contribute to a better understanding of the evolution of anti-predatory defences in insects.

This study aims to investigate the responses of the black-spotted pond frog *Pelophylax nigromaculatus* (Hallowell) (Anura: Ranidae) to the defensive behaviour of the bombardier beetle *Pheropsophus jessoensis* (Morawitz). In July 2017, I found both *Ph. jessoensis* and *Pe. nigromaculatus* co-occurring in the same grassland habitats in Kato City, Hyogo Prefecture, Japan. *Pelophylax nigromaculatus* is a generalist predator that has been reported to prey on carabid beetles (Maeda & Matsui, 1999; Hirai & Matsui, 1999; Sano & Shinohara, 2011; Sarashina, Yoshihisa & Yoshida, 2011), indicating that this frog species is a potential predator of adult *Ph. jessoensis*. Because *Ph. jessoensis* can spray their predators with hot and toxic chemicals, it was hypothesised that (1) *Pe. nigromaculatus* has evolved a high tolerance to heat and toxins to prey on *Ph. jessoensis*, or (2) *Pe. nigromaculatus* has evolved specific behaviours in response to warning signals to avoid attacking *Ph. jessoensis*. To test these hypotheses, I observed *Pe. nigromaculatus* attacking *Ph. jessoensis* under laboratory conditions using a digital video camera ~~to record the frogs attacking the bombardier beetles~~. Acceptance or rejection of prey was investigated using slow-motion videos. Furthermore, both dead and live beetles were used to test whether bombing is essential for successful defence against predatory attacks by *Pe. nigromaculatus*.

MATERIALS AND METHODS

Study species

Pheropsophus jessoensis is a species of bombardier beetles found in East Asia (Ueno et al., 1985; Jung et al., 2012) that commonly inhabits farmland, grassland, and forest edges in Japan (Habu & Sadanaga, 1965; Ueno et al., 1985; Yahiro et al., 1992; Ishitani & Yano, 1994; Fujisawa et al., 2012; Ohwaki et al., 2015; Sugiura & Sato, 2018). Adult *Ph. jessoensis* can eject toxic chemicals (1,4-benzoquinone and 2-methyl 1,4-benzoquinone) at a temperature of approximately 100°C from their rear ends when disturbed (Video S1; Kanehisa and Murase, 1977; Kanehisa, 1996). ~~For the experiments~~, adult *Ph. jessoensis* were collected from grasslands and forest edges in Kato (34°54′N, 135°02′E, 120 m above sea level), Hyogo Prefecture, central Japan, from May to August in 2016 and 2017 (Sugiura & Sato, 2018). The mean body weight (± SE) of the collected beetles was 213.6 ± 7.0 (range: 110.3–294.0) mg. Study individuals were maintained separately in plastic cases (diameter: 85 mm; height: 25 mm) with wet tissue paper in the laboratory at 25°C. Dead larvae of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) were provided as food (Sugiura & Sato, 2018). Beetles were not used repeatedly in different feeding experiments.

Pelophylax nigromaculatus is a species of true frogs that inhabits wetlands and farmlands in East Asia (Liu et al., 2010; Tsuji et al., 2011; Komaki et al., 2015), and is one of the most abundant frog species found in traditional agricultural landscapes including farmlands, grasslands, and forest edges in Japan (Hirai, 2002; Honma, Oku & Nishida, 2006; Tsuji et al., 2011; Matsuhashi & Okuyama, 2015). Using its tongue, *Pe. nigromaculatus* can easily catch prey and swallow prey smaller than itself (Video S2; Honma, Oku & Nishida, 2006). ~~For this study,~~ individuals of *Pe. nigromaculatus* were collected from wetlands and forest edges in Takarazuka-shi (34°53′N, 135°17′E, 230 m above sea level), Sanda-shi (34°57′N, 135°11′E, 180 m above sea level), and Sayo-cho (35°02′N, 134°20′E, 180 m above sea level), Hyogo Prefecture, central Japan, from May to August in 2016 and 2017. Although *Pe. nigromaculatus* has recently been classified as near threatened (NT) in the Japanese Red Data List (Ministry of the Environment of Japan, 2017), this species was abundant at the collection sites. Both juveniles and adults were collected, with a mean body weight (± SE) of 9.8 ± 1.0 (range: 2.7–26.7) g. Small and large frogs were maintained separately in small (120 × 85 × 130 mm, length × width × height) and large plastic cages (120 × 185 × 130 mm, length × width × height), respectively, in the laboratory at 25°C. Live larvae of *S. litura*, *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae), and *Zophobas atratus* Fabricius (Coleoptera: Tenebrionidae) were provided as food. Frogs were starved for 24 h before the feeding experiments to standardise their hunger level (cf. Honma, Oku & Nishida, 2006). In total, 46 frogs were used in the experiments. As with the beetles, individual frogs were not used repeatedly. The frogs were released after the experiments were completed.

Feeding experiments

Feeding experiments were all conducted at 25°C. To start, a frog was placed in a transparent plastic container (120 × 85 × 130 mm, length × width × height). Then, a transparent glass petri dish (45 mm in diameter, 15 mm in height) containing a live bombardier beetle was placed outside the plastic container where the frog could see it. Frogs that did not try to attack the beetle were not used for the feeding experiments. If a frog displayed attacking behaviour, a live beetle was then placed in the container with the frog. The resulting behaviours were recorded on video using a digital camera (iPhone 6 plus, Apple) at 240 frames per second. If a frog swallowed the beetle, I also observed whether it then vomited the beetle (cf. Sugiura & Sato, 2018). Vomited beetles were checked to see whether they were still alive. Frogs that did not vomit after swallowing were considered to have digested the beetle. Frog faeces were examined after the experiment to confirm whether the beetles were digested. In total, 26 frogs and 26 bombardier beetles were used for the experiment with live beetles.

A second set of frogs were presented with dead adult beetles to test whether the bombing response is essential for deterring a predatory attack. *Pelophylax nigromaculatus* usually does not attack motionless prey. However, in a pilot test, an individual of *Pe. nigromaculatus* attacked and ingested a dead caterpillar (*S. litura*) when forceps were used to move the caterpillar within the frog's field of view. For this experiment, the bombardier beetles were killed in a freezer at −15°C. First, a dead beetle was placed in the plastic container (120 × 85 × 130 mm, length × width × height) within the frog's field of view. If the frog did not initially respond to the beetle, forceps were used to move the dead beetle within the frog's field of view again. Frogs that did not attack the dead beetles were not used in these experiments. The predatory behaviours of the frogs were recorded using the same digital video camera. Twenty frogs and 20 dead beetles were used in this experiment.

The videos of frog behaviour were played back using QuickTime Player version 10.4 (Apple, Inc.). Frog responses to the bombardier beetles were grouped into four categories (cf. Ito, Taniguchi & Billen, 2016; Matsubara & Sugiura, 2017; Sugiura & Sato, 2018): (1) frogs that contacted the beetles but did not take the beetles into their mouths; (2) frogs that spat out the beetles after taking them into the mouth; (3) frogs that swallowed beetles but vomited them afterward; and (4) frogs that swallowed and digested the beetles.

The experiments were undertaken in accordance with the Kobe University Animal Experimentation Regulations (Kobe University's Animal Care and Use Committee, H28, H29).

Data analysis

Fisher's exact tests were used to compare the rates of swallowing and digestion by the frogs between live and dead beetles. A significantly higher rate of swallowing or digesting dead beetles compared to live beetles would indicate that the beetles' bombing response is important for successful defence against frog predation. I also used *t*-tests to compare the mean weights of live and dead beetles and the mean weights of the frogs that attacked live and dead beetles. In addition, generalised linear models (GLMs) with a binomial error distribution and a logit link were used to identify factors that contributed to frogs' successful swallowing and digestion of the bombardier beetles. The success or failure (1/0) of frogs' swallowing and digesting beetles was used as the response variable. Frog weight, beetle weight, and beetle condition (live or dead) were treated as fixed factors. To test the fit using binomial distributions, I also considered whether residual deviance was larger (overdispersion) or smaller (underdispersion) than the residual degrees of freedom (Crawley, 2005). All analyses were performed using R ver. 3.3.2 (R Development Core Team 2016).

RESULTS

In the experiment using live adult bombardier beetles (n = 26), almost all of the frogs (92.3%) rejected the beetles without swallowing them (Fig. 1); 65.4% stopped attacking the beetles immediately after touching the beetles with their tongues (Fig. 2; Video S3), and 26.9% spat out the beetles after taking the beetles into their mouths (Fig. 3; Video S4). In some cases, the beetles could be heard bombing the frogs just before the frogs spat out the beetles (Video S4). Only two frogs (7.7%) were observed to swallow the bombardier beetles; one of the frogs successfully digested the beetle, but the other frog vomited the beetle 18 min after swallowing it (Table 1). The vomited beetle was still alive. Of the frogs that took the beetles into their mouths, 88.9% initially stopped attacking the beetles when their tongues first touched the beetles, but resumed their predatory attack soon thereafter (Fig. 3; Video S4).

When dead beetles were used (n = 20), 85.0% of the frogs rejected the dead beetles without swallowing them (Fig. 1); 65.0% stopped attacking the beetles after their tongues touched the dead beetles (Video S5), and 20.0 % spat out the beetles after taking the beetles into their mouths (Fig. 1). Only 15.0% of the frogs swallowed the dead beetles. Similar to the experiment using live beetles, 85.7% of the frogs that took beetles into their mouths were initially deterred when their tongues first touched the beetles, but continued with their predatory behaviour soon afterwards.

No significant differences were found between the proportion of dead and live beetles that were swallowed (15.0% vs. 7.7%, respectively, p = 0.64, Fisher's exact test) or digested (15.0% vs. 3.8%, respectively, p = 0.30, Fisher's exact test) by the frogs. The mean body weights of the dead and live beetles used were 216.3 ± 9.4 mg (n = 20) and 211.5 ± 10.3 mg (n = 26), respectively, which were not significantly different (*t*-test, $t = -0.35$, $P = 0.73$). Similarly, the mean body weight of the frogs that attacked dead beetles (8.5 ± 1.4 g, $n = 20$) was not significantly different (*t*-test, $t = 1.1$, $P = 0.26$) from the mean weight of the frogs that attacked live beetles (10.8 ± 1.4 g, $n = 26$).

Whether beetles were swallowed was associated with both frog and beetle size (GLM; Table 1). Beetles were more likely to be swallowed with increasing frog size and decreasing beetle size (Table 1). Digestion rates were positively correlated with frog size, but not with beetle size (GLM; Table 2). However, in both analyses, the residual deviance was smaller than the residual degrees of freedom, indicating underdispersion (Tables 1 and 2).

DISUSSION

Some arthropods, anurans, and birds are able to successfully prey on bombardier beetles despite their bombing defences (Eisner & Dean, 1976; Conner & Eisner, 1983; Nowicki & Eisner, 1983; Dean, 1980a; Eisner, Eisner & Aneshansley, 2005). In some instances, though, bombardier beetles can defend themselves effectively against predatory arthropods and anurans (Eisner, 1958; Eisner & Meinwald, 1966; Eisner & Dean, 1976; Dean, 1980a; Esiner, 2003; Eisner et al., 2006). Results from this study demonstrate the successful defence by the bombardier beetle *Ph. jessoensis* against the pond frog *Pe. nigromaculatus* (Fig. 1). Almost In this study we found all individuals of *Pe. nigromaculatus* used in this study rejected individuals of *Ph. jessoensis* without attempting to swallow the beetles (Fig. 1). In contrast, two toad species, *Bufo japonicus* Temminck & Schlegel and *B. torrenticola* Matsui (Bufonidae), both with a much larger body size than *Pe. nigromaculatus*, were reported to easily swallow individuals of *Ph. jessoensis*, although 43% of the toads eventually vomited the beetles (Sugiura & Sato, 2018). Therefore, the success of the defence mechanism of *Ph. jessoensis* depends on the predator species.

When the frogs took live beetles into their mouths, bombing could be heard on video, followed in several cases by the frogs spitting out the beetles (Fig. 3; Video S4). However, no bombing was heard when the frogs stopped their predatory attack before taking live beetles into their mouths (Video S3). In addition, in the experiment using dead beetles, only 15.0% of the frogs swallowed the beetles (Fig. 1). The proportions of beetles swallowed and digested by the frogs were also not significantly different between live and dead beetles (Fig. 1). ~~These results~~ indicate that the high-speed release of hot and noxious chemicals, or bombing, was not essential for *Ph. jessoensis* to evade predation by *Pe. nigromaculatus*. Which factors, then, stopped the frogs from attacking? Three potential reasons can be considered: (1) the frogs recognised the warning colouration of the beetles; (2) the body size of the beetles was too large for the frog to accommodate; and (3) the frogs reflexively avoided the beetles after detecting toxic substances or other deterrent characteristics on the beetles' body surfaces.

The bombardier beetle *Ph. jessoensis* does have a striking yellow and black pattern on its body that could serve as warning colouration (Fig. 1), although this has not been empirically demonstrated. Anuran predators can avoid toxic prey by recognising certain colours or other morphological characteristics and then ignoring those prey (Brower, Brower & Westcott, 1960; Brower & Brower, 1962; Dean, 1980a; Taniguchi et al., 2005; Ito, Taniguchi & Billen, 2016). Therefore, *Pe. nigromaculatus* may recognise the body patterns and shape of *Ph. jessoensis* as warning signals before attacking it. However, the frogs that did not try to attack individuals of *Ph. jessoensis* were not used for the feeding experiments.

Small individuals of *Pe. nigromaculatus* have been reported to spit out large prey that they were unable to swallow after taking the prey into their mouths (Honma, 2004; Honma, Oku & Nishida, 2006). However, 65.4% of the frogs in the experiment with live beetles and 65.0% of the frogs in the experiment with dead beetles stopped their predatory attack before taking the beetles into their mouths (Fig. 1, 2; Video 3, 5). Although the GLM analysis indicated that both frog size and beetle size were correlated with the frequency of beetles' being swallowed by the frogs, the residual deviance was much smaller than the residual degrees of freedom (underdispersion), indicating that there was less variation in the data than expected (Tables 1). Thus, these results do not provide strong evidence that the frogs were deterred by the large body size of the beetles.

The rapid responses of the frogs' tongues to contact with the bombardier beetles (Fig. 2) could be considered a reflex action (cf. Kumai, 1981). Frogs are known to use their tongues as a chemical detector (Dean, 1980b; Kumai, 1981; Barlow, 1998) as well as a prey-catching tool (Noel et al., 2017). For example, chemical or electrical stimulation of the tongue can generate reflex responses in *Pe. nigromaculatus* (Kumai, 1981; Takeuchi, Satou & Ueda, 1986). Because *Pe. nigromaculatus* is a generalist predator that can attack a variety of arthropods within its field of view (Hirai & Matsui, 1999; Honma, 2004; Honma, Oku & Nishida, 2006; Sano & Shinohara, 2011; Sarashina, Yoshihisa & Yoshida, 2011), *Pe. nigromaculatus* may have evolved specific responses to toxic prey to avoid being injured by trying to eat them. The results of this study suggest that the tongues of *Pe. nigromaculatus* may be able to rapidly detect toxic substances or other characteristics on the body surface of the bombardier beetles, and the frogs subsequently avoid the beetles to prevent themselves from being bombed and injured.

CONCLUSIONS

In one study, the chemicals produced by bombardier beetles' bombing did not stimulate toads' tongues less intensely than did the heat from the chemical reaction (Dean, 1980b). Other than this study, the relative importance of the toxic chemicals and heat produced by bombing for the successful escape of bombardier beetles from predators has been largely unexplored. Results here suggest that the cooled toxic chemicals covering the beetles' bodies alone could stop the frogs from attacking. Frogs may be able to detect toxic substances or other deterrent characteristics on unsuitable prey with their tongues, and thus reject the prey to avoid injury. Previous studies have been focused on how frogs and toads use their tongues to catch prey (Ewert, 1970; Nishikawa & Gans, 1996; Monroy & Nishikawa, 2010; Noel et al., 2017), but there is little research on how frogs and toads use their tongues to detect toxins in potential prey (but see Dean, 1980b). Further studies are needed to clarify the mechanisms of frogs' recognising and avoiding toxic prey.

ACKNOWLEDGEMENTS

I am grateful to K. Sakagami, K. Uchida, and A. Ushimaru for providing valuable information about the collection sites. I thank K. Sakagami, W. Higashikawa, and M. Ito for helping to collect the study animals, and S. Matsubara and Y. Maeda for helping to maintain them. T. Takanashi provided valuable advice on this research.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This study was partly supported by the Fujiwara Natural History Foundation.

Grant Disclosures

The following grant information was disclosed by the author:

Fujiwara Natural History Foundation.

Competing Interests

The author declares there are no competing interests.

Author Contributions

Shinji Sugiura conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The experiments were undertaken in accordance with the Kobe University Animal Experimentation Regulations (Kobe University's Animal Care and Use Committee, H28, H29). No frogs were seriously injured or killed during the feeding experiments. My study also complies with the current laws of Japan.

Data Availability

The following information was supplied regarding data availability:

Raw data will be available from the Figshare Digital Repository.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/

REFERENCES

Aneshansley DT, Eisner T, Widom JM, Widom B. 1969. Biochemistry at 100°C: explosive secretory discharge of bombardier beetles (*Brachinus*). *Science* **165**:61–63. DOI 10.1126/science.165.3888.61.

Arndt EM, Moore W, Lee WK, Ortiz C. 2015. Mechanistic origins of bombardier beetle (Brachinini) explosion-induced defensive spray pulsation. *Science* **348**:563–567. DOI 10.1126/science.1261166.

Bonacci T, Aloise G, Brandmayr P, Brandmayr TZ, Capula M. 2008. Testing the predatory behaviour of *Podarcis sicula* (Reptilia: Lacertidae) towards aposematic and non-aposematic preys. *Amphibia-Reptilia* **29**: 449–453. DOI 10.1163/156853808785111986.

Barlow LA. 1998. The biology of amphibian taste. In: Heatwole H, Dawley E, eds. *Amphibian biology, volume 3: sensory perception*. Chipping Norton, Australia: Surrey Beatty & Sons, 743–782.

Brower JVZ, Brower LP. 1962. Experimental studies of mimicry. 6. The reaction of toads (*Bufo terrestris*) to honeybees (*Apis mellifera*) and their dronefly mimics (*Eristalis vinetorum*). *The American Naturalist* **96**:297–307. DOI 10.1086/282237.

Brower LP, Brower JVZ, Westcott PW. 1960. Experimental studies of mimicry. 5. The reactions of toads (*Bufo terrestris*) to bumblebees (*Bombus americanorum*) and their robberfly mimics (*Mallophora bomboides*), with a discussion of aggressive mimicry. *The American Naturalist* **94**:343–355. DOI 10.1086/282137.

Bura BL, Kawahara A, Yack J. 2016. A comparative analysis of sonic defences in Bombycoidea caterpillars. *Scientific Reports* **6**:31469. DOI 10.1038/srep31469.

Cooper SM, Owen-Smith N. 1986. Effects of plant spinescence on large mammalian herbivores.

Oecologia **68**:446–455. DOI 10.1007/BF01036753.

Conner J, Eisner T. 1983. Capture of bombardier beetles by ant lion larvae. *Psyche* 90:175–178. DOI 10.1155/1983/25010.

Crawley MJ. 2005. *Statistics, an introduction using R*. New York: John Wiley.

Dean J. 1979. Defensive reaction time of bombardier beetles: an investigation of the speed of a chemical defense. *Journal of Chemical Ecology* **5**:691–701. DOI 10.1007/BF00986554.

Dean J. 1980a. Encounters between bombardier beetles and two species of toads (*Bufo americanus*, *B*. *marinus*): speed of prey-capture does not determine success. *Journal of Comparative Physiology* **133**:41–50. DOI 10.1007/BF00660180.

Dean J. 1980b. Effects of thermal and chemical components of bombardier beetle chemical defense: glossopharyngeal response in two species of toads (*Bufo americanus*, *B*. *marinus*). *Journal of Comparative Physiology* **133**:51–59. DOI 10.1007/BF00660181.

Derby CD. 2007. Escape by inking and secreting: marine molluscs avoid predators through a rich array of chemicals and mechanisms. *Biological Bulletin* **213**:274–289. DOI 10.2307/25066645.

Edmunds M. 1974. *Defense in animals*. Harlow: Longman.

Eisner T. 1958. The protective role of the spray mechanism of the bombardier beetle, *Brachynus ballistarius* Lec. *Journal of Insect Physiology* **2**:215–220.

Eisner T. 2003. *For love of insects*. Cambridge: The Belknap Press of the Harvard University Press.

Eisner T, Aneshansley DJ. 1999. Spray aiming in the bombardier beetle: Photographic evidence. *Proceeding of the National Academy of Sciences USA* **96**:9705–9709. DOI 10.1073/pnas.96.17.9705.

Eisner T, Dean J. 1976. Ploy and counterploy in predator–prey interactions: Orb-weaving spiders versus bombardier beetles. *Proceeding of the National Academy of Sciences USA* **73**:1365–1367. DOI 10.1073/pnas.96.17.9705.

Eisner T, Meinwald J. 1966. Defensive secretions of arthropods. *Science* 153:1341–1350. DOI 10.1126/science.153.3742.1341.

Eisner T, Eisner M, Aneshansley DJ. 2005. Pre-ingestive treatment of bombardier beetles by jays: food preparation by “anting” and “sand-wiping”. *Chemoecology* **15**:227–233. DOI 10.1007/s00049-005-0316-6.

Eisner T, Eisner M, Siegler M. 2005. *Secret weapons: defenses of insects, spiders, scorpions, and other many-legged creatures*. Cambridge: The Belknap Press of the Harvard University Press.

Eisner T, Aneshansley DJ, del Campo ML, Eisner M, Frank JH, Deyrup M. 2006. Effect of bombardier beetle spray on a wolf spider: repellency and leg autotomy. *Chemoecology* **16**:185–189. DOI 10.1007/s00049-006-0346-8.

Ewert JP. 1970. Neural mechanisms of prey-catching and avoidance behaviour in the toad (*Bufo bufo* L.). *Brain, Behavior and Evolution* **3**:36–56. DOI 10.1159/000125462.

Fujisawa T, Lee CM, Ishii M. 2012. Species diversity of ground beetle assemblages in the distinctive landscapes of the Yodo River flowing through northern Osaka Prefecture, central Japan. *Japanese Journal of Environmental Entomology and Zoology* **23**:89–100. DOI 10.11257/jjeez.23.89.

Habu A, Sadanaga K. 1965. Illustrations for identification of larvae of the Carabidae found in cultivated fields and paddy-fields (III). *Bulletin of the National Institute of Agricultural Sciences, Series C: Plant Pathology and Entomology* **19**:81–216 (in Japanese).

Hirai T. 2002. Ontogenetic change in the diet of the pond frog, *Rana nigromaculata*. *Ecological Research* **17**:639–644. DOI 10.1046/j.1440-1703.2002.00521.x.

Hirai T, Matsui M. 1999. Feeding habits of the pond frog, *Rana nigromaculata*, inhabiting rice fields in Kyoto, Japan. *Copeia* 1999:940–947. DOI 10.2307/1447969.

Honma A. 2004. MOMO VideoArchives: momo040902cj01b. http://movspec.mus-nh.city.osaka.jp/ethol/showdetail.php?movieid=momo040902cj01b. Accessed: 28 January 2018.

Honma A, Oku S, Nishida T. 2006. Adaptive significance of death feigning posture as a specialized inducible defence against gape-limited predators. *Proceedings of the Royal Society B: Biological Science* **273**:1631–1636. DOI 10.1098/rspb.2006.3501.

Inbar M, Lev-Yadun S. 2005. Conspicuous and aposematic spines in the animal kingdom. *Naturwissenschaften* **92**:170–172. DOI 10.1007/s00114-005-0608-2.

Ishitani M, Yano K. 1994. Species composition and seasonal activities of ground beetles (Coleoptera) in a fig orchard. *Japanese Journal of Entomology* **62**:201–210.

Ito F, Taniguchi K, Billen J. 2016. Defensive function of petiole spines in queens and workers of the formicine ant *Polyrhachis lamellidens* (Hymenoptera: Formicidae) against an ant predator, the Japanese tree frog *Hyla japonica*. *Asian Myrmecology* **8**:1–6. DOI 10.20362/am.008014.

Jung JK, Kim ST, Lee SY, Park CK, Lee EH, Lee JH. 2012. Ground beetle (Coleoptera: Carabidae) assemblage in the urban landscape, Korea. *Journal of Ecology and Field Biology* **35**:79–89. DOI 10.5141/JEFB.2012.012.

Kanehisa K. 1996. Secretion of defensive substance by Carabidae and Brachinidae. *Bulletin of the Research Institute for Bioresources, Okayama University* **4**:9–23 (in Japanese with English summary).

Kanehisa K, Murase M. 1977. Comparative study of the pygidial defensive systems of carabid beetles. *Applied Entomology and Zoology* **12**:225–235. DOI 10.1303/aez.12.225.

Komaki S, Igawa T, Lin SM, Tojo K, Min MS, Sumida M. 2015. Robust molecular phylogeny and palaeodistribution modelling resolve a complex evolutionary history: glacial cycling drove recurrent mtDNA introgression among *Pelophylax* frogs in East Asia. *Journal of Biogeography* **42**:2159–2171. DOI 10.1111/jbi.12584.

Kumai T. 1981. Reflex response of the hypoglossal nerve induced by chemical stimulation of the tongue and electrical stimulation of the glossopharyngeal nerve in the frog. *Japanese Journal of Physiology* **31**:625–637. DOI 10.2170/jjphysiol.31.625.

Larochelle A. 1974a. The American toad as champion carabid beetle collector. *The Pan-Pacic Entomologist* **50**:203–204.

Larochelle A. 1974b. Carabid beetles (Coleoptera: Carabidae) as prey of North American frogs. *The Great Lakes Entomolosit* **7**:147–148.

Lev-Yadun S. 2001. Aposematic (warning) coloration associated with thorns in higher plants. *Journal of Theoretical Biology* **210**:385–388 DOI 10.1006/jtbi.2001.2315.

Lev-Yadun S. 2009. Aposematic (warning) coloration in plants. In: Baluska F, ed. *Plant-environment interactions. From sensory plant biology to active plant behavior*. Berlin: Springer-Verlag, 167–202 DOI 10.1007/978-3-540-89230-4_10.

Liu K, Wang F, Chen W, Tu L, Min MS, Bi K, Fu J. 2010. Rampant historical mitochondrial genome introgression between two species of green pond frogs, *Pelophylax nigromaculatus* and *P. plancyi*. *BMC Evolutionary Biology* **10**:201. DOI 10.1186/1471-2148-10-201.

Maeda N, Matsui M. 1999. *Frogs and toads of Japan: revised edition*. Tokyo: Bun-ichi Sogo Shuppan.

Matsubara S, Sugiura S. 2017. Chemical defence of turnip sawfly larvae against Japanese tree frogs. *Journal of Asia-Pacific Entomology* **20**:225–227. DOI 10.1016/j.aspen.2017.01.001.

Matsuhashi T, Okuyama F. 2015. *Frogs and toads of Japan*. Tokyo, Japan: Yamakei (in Japanese).

Monroy JA, Nishikawa K. 2010. Prey capture in frogs: alternative strategies, biomechanical trade-offs, and hierarchical decision making. *Journal of Experimental Zoology* **315**:61–71. DOI 10.1002/jez.601

Mori I. 2008. Predation by introduced bullfrog Rana catesbeiana on a breeding male of Rhacophorus schlegelii and the other animals. *Bulletin of the Okayama Prefecture Nature Conservation Center* **16**:61–62 (in Japanese).

Nishikawa KC, Gans C. 1996. Mechanisms of tongue protraction and narial closure in the marine toad *Bufo marinus*. *Journal of Experimental Biology* **199**:2511–2529.

Nowicki S, Eisner T. 1983. Predatory capture of bombardier beetles by a tabanid fly larvae. *Psyche* **90**:119–122. DOI 10.1155/1983/54806.

Mithöfer A, Boland W. 2012. Plant defense against herbivores: chemical aspects. *Annual Review of Plant Biology* **63**:431–450. DOI 10.1146/annurev-arplant-042110-103854.

Noel AC, Guo HY, Mandica M, Hu DL. 2017. Frogs use a viscoelastic tongue and non-Newtonian saliva to catch prey. Journal of the Royal Society Interface **14**: 20160764. DOI 10.1098/rsif.2016.0764.

Ohwaki A, Kaneko Y, Ikeda H. 2015. Seasonal variability in the response of ground beetles (Coleoptera: Carabidae) to a forest edge in a heterogeneous agricultural landscape in Japan. *European Journal of Entomology* **112**:135–144. DOI 10.14411/eje.2015.022.

R Development Core Team. 2016. *R, a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Ruxton GD, Sherratt TN, Speed MP. 2004. *Avoiding attack: the evolutionary ecology of crypsis, aposematism, and mimicry*. Oxford: Oxford University Press.

Sano M, Shinohara M. 2011. Species comparison of frogs food habits during mating seasons in Uenohara, Yamanashi Pref., Japan. *Bulletin of Teikyo University of Science and Technology* **8**:101–111 (in Japanese with English summary).

Sarashina M, Yoshihisa Y, Yoshida T. 2011. Stomach contents of invasive Black-spotted Pond frog (*Rana nigromaculata*) in urban landscape of Sapporo City. *Journal of Rakuno Gakuen University* **36**:81–86 (in Japanese with English summary).

Skelhorn J, Rowe C. 2006. Taste-rejection by predators and the evolution of unpalatability in prey. *Behavioral Ecology and Sociobiology* **60**:550–555. DOI 10.1007/s00265-006-0199-8.

Sugiura S. 2016. Bagworm bags as portable armour against invertebrate predators. *PeerJ* **4**:e1686 DOI 10.7717/peerj.1686.

Sugiura S, Sato T. 2018. Successful escape of bombardier beetles from predator digestive systems. *Biology Letters* **14**:20170647. DOI 10.1098/rsbl.2017.0647.

Sugiura S, Takanashi T. 2018. Hornworm counterattacks: Defensive strikes and sound production in response to invertebrate attackers. *Biological Journal of the Linnean Society*. DOI 10.1093/biolinnean/blx156.

Sugiura S, Yamazaki K. 2014. Caterpillar hair as a physical barrier against invertebrate predators. *Behavioral Ecology* **25**:975–983. DOI 10.1093/beheco/aru080.

Takeuchi H, Satou M, Ueda K. 1986. EMG analysis of head muscle during naturally-occurring and electrically- evoked snapping, rejecting and avoiding behavior in the Japanese toad. *Zoological Science* **3**:992.

Taniguchi K, Maruyama M, Ichikawa T, Ito F. 2005. A case of Batesian mimicry between myrmecophilous staphylinid beetle, *Pella comes*, and its host ant, *Lasius* (*Dendrolasius*) *spathepus*: an experiment using the Japanese tree frog *Hyla japonica* as a real predator. *Insectes Sociaux* **52**:320–322. DOI 10.1007/s00040-005-0813-1.

Tsuji M, Ushimaru A, Osawa T, Mitsuhashi H. 2011. Paddy-associated frog declines via urbanization: A test of the dispersal-dependent-decline hypothesis. *Landscape and Urban Planning* **103**:318–325. DOI 10.1016/j.landurbplan.2011.08.005.

Ueno S, Kurosawa Y, Sato M (eds). 1985. *The Coleoptera of Japan in color vol. II*. Osaka, Japan: Hoikusha (in Japanese).

Williams BL, Hanifin CT, Brodie ED Jr, Brodie ED III. 2010. Tetrodotoxin affects survival probability of rough-skinned newts (*Taricha granulosa*) faced with TTX-resistant garter snake predators (*Thamnophis sirtalis*). *Chemoecology* **20**:285–290. DOI 10.1007/s00049-010-0057-z.

Yahiro K, Fujimoto T, Tokuda M, Yano K. 1992. Species composition and seasonal abundance of ground beetles (Coleoptera) in paddy fields. *Japanese Journal of Entomology* **60**:805–813.

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/4DLLp7

Figure legend

Figure 1 Behavioural responses of the black-spotted pond frog *Pelophylax nigromaculatus* to live and dead adult individuals of the bombardier beetle *Pheropsophus jessoensis*. 'Stop attack': the frogs stopped their attacks after their tongues touched the beetles. 'Spit out': the frogs spat out the beetles immediately after taking the beetles into their mouths. 'Swallow': the frogs successfully swallowed the dead beetles.

Figure 2 Temporal sequence of the frog *Pelophylax nigromaculatus* rejecting a live adult *Pheropsophus jessoensis* without taking the beetle into its mouth. The frog stopped the attack immediately after its tongue touched the beetle (see Video S3).

Figure 3 Temporal sequence of the frog *Pelophylax nigromaculatus* spitting out a live adult *Pheropsophus jessoensis* after taking the beetle into its mouth. Bombing by the beetle was audible just before the frog spat out the beetle (1675–1800 ms; see Video S4).

Supplemental Information

Video S1 An adult *Pheropsophus jessoensis* bombing. The beetle discharged toxic chemicals when its eggs were pinched with a pair of forceps.

Video S2 The frog *Pelophylax nigromaculatus* preying on a carabid beetle.

Video S3 The frog *Pelophylax nigromaculatus* rejecting a live *Pheropsophus jessoensis*. The frog stopped the attack immediately after its tongue touched the beetle.

Video S4 The frog *Pelophylax nigromaculatus* spitting out a live *Pheropsophus jessoensis*. The frog took the beetle into its mouth but immediately spat out the beetle.

Video S5 The frog *Pelophylax nigromaculatus* rejecting a dead *Pheropsophus jessoensis*. The frog stopped its attack immediately after its tongue touched the dead beetle.

Table 1 (on next page)

Table 1. Results of a generalised linear model (GLM) testing potential factors influencing whether the frog *Pelophylax nigromaculatus* successfully swallowed the bombardier beetle *Pheropsophus jessoensis* in feeding experiments.

Table 1. Results of a generalised linear model (GLM) testing potential factors influencing whether the frog *Pelophylax nigromaculatus* successfully swallowed the bombardier beetle *Pheropsophus jessoensis* in feeding experiments.

Response variable	Explanatory variable (fixed effect)	Coefficient estimate	SE	z value	p value
Swallowing success[1)]	Intercept	0.75455	2.33617	0.323	0.7467
	Frog weight	0.17883	0.09121	1.961	0.0499
	Beetle weight	−0.03247	0.01613	−2.012	0.0442
	Beetle treatment [2)]	2.21476	1.40849	1.572	0.1159

[1)] The binomial error distribution was used. Residual deviance: 21.645 on 42 degrees of freedom

[2)] Live beetles were used as a reference.

Table 2(on next page)

Table 2. Results of a generalised linear model (GLM) testing potential factors influencing whether the frog *Pelophylax nigromaculatus* successfully digested the bombardier beetle *Pheropsophus jessoensis* in feeding experiments.

Table 2. Results of a generalised linear model (GLM) testing potential factors influencing whether the frog *Pelophylax nigromaculatus* successfully digested the bombardier beetle *Pheropsophus jessoensis* in feeding experiments.

Response variable	Explanatory variable (fixed effect)	Coefficient estimate	SE	z value	p value
Digestion success [1)]	Intercept	−1.81388	2.689	−0.675	0.5
	Frog weight	0.19522	0.09722	2.008	0.0446
	Beetle weight	−0.02403	0.01654	−1.453	0.1462
	Beetle treatment [2)]	2.94459	1.64319	1.792	0.0731

[1)] The binomial error distribution was used. Residual deviance: 18.786 on 42 degrees of freedom

[2)] Live beetles were used as a reference.

Figure 1

Figure 1 Behavioural responses of the black-spotted pond frog *Pelophylax nigromaculatus* to live and dead adult individuals of the bombardier beetle *Pheropsophus jessoensis*.

'Stop attack': the frogs stopped their attacks after their tongues touched the beetles. 'Spit out': the frogs spat out the beetles immediately after taking the beetles into their mouths. 'Swallow': the frogs successfully swallowed the dead beetles.

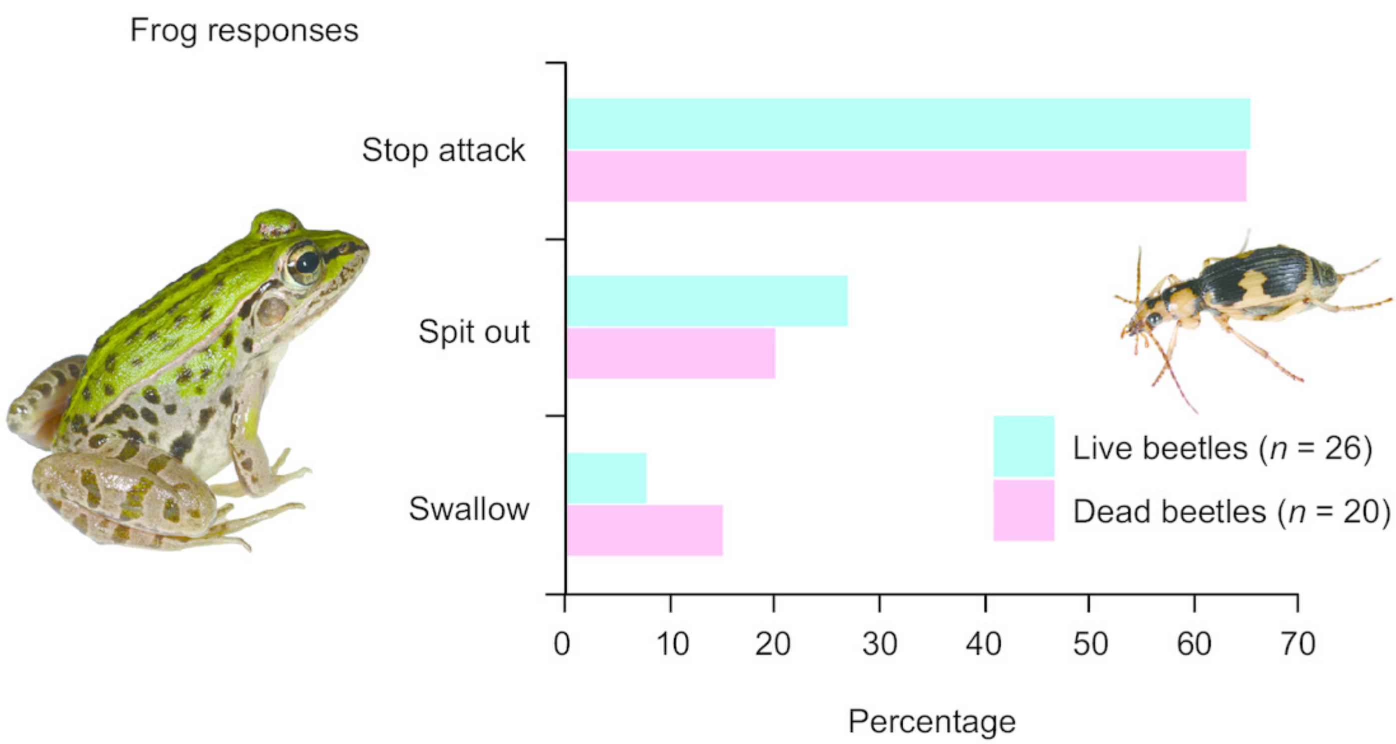


Figure 1

Figure 2

Figure 2 Temporal sequence of the frog *Pelophylax nigromaculatus* rejecting a live adult *Pheropsophus jessoensis* without taking the beetle into its mouth.

The frog stopped the attack immediately after its tongue touched the beetle (see Video S3).

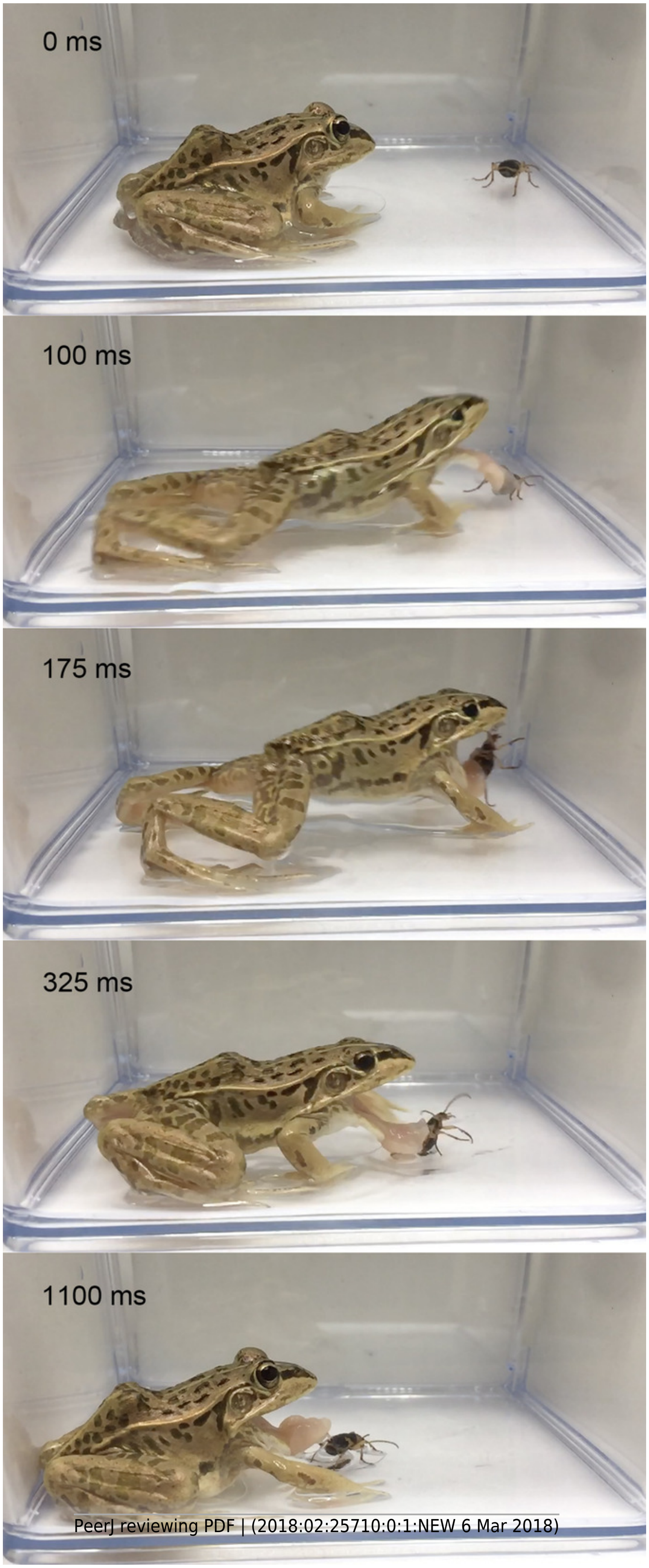
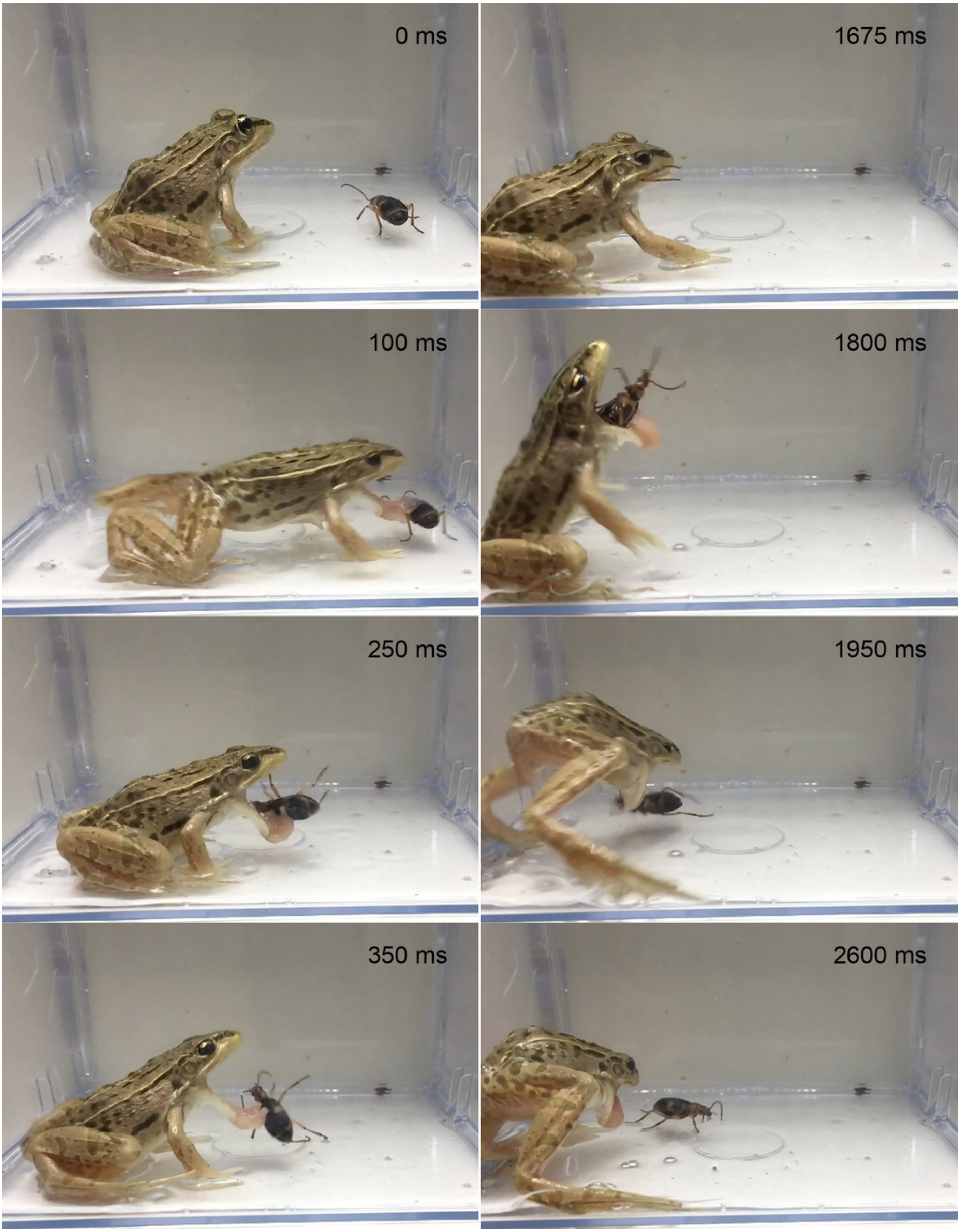

0 ms
100 ms
175 ms
325 ms
1100 ms

Figure 3

Figure 3 Temporal sequence of the frog *Pelophylax nigromaculatus* spitting out a live adult *Pheropsophus jessoensis* after taking the beetle into its mouth.

Bombing by the beetle was audible just before the frog spat out the beetle (1675–1800 ms; see Video S4).


0 ms
1675 ms
100 ms
1800 ms
250 ms
1950 ms
350 ms
2600 ms