Sugiura S, Sato T. 2018 Successful escape of bombardier beetles from predator digestive systems. *Biol. Lett.* (doi: 10.1098/rsbl.2017.0647)

Electronic supplementary material

Material and methods

(a) Study species

The bombardier beetle *Pheropsophus jessoensis* is common in farmland, grasslands, and forest edges in East Asia [1–7]. Although it produces 1,4-benzoquinone and 2-methyl-1,4-benzoquinone [8], the effectiveness of the defensive behaviour remains unclear. Adult *P. jessoensis* and other carabid species were collected from a forest edge in Kato (34°54′N, 135°02′E, 120 m above sea level) and Kobe (34°43′N, 135°14′E, 120 m above sea level), Hyogo Prefecture, central Japan in May–August, 2016 and 2017. Adults were maintained separately in plastic cases (diameter, 85 mm; height, 25 mm) with wet tissue papers and were provided dead larvae of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) as food under laboratory conditions (25°C). Individual *P. jessoensis* were each used in only a single feeding experiment.

Bufo japonicus is common in various habitats in Japan, including forests, grasslands, and farmlands, while *B. torrenticola* is found only in forests surrounding mountain streams in central Japan [9–11]. Toads were collected from forest edges in Aridagawa-cho, Wakayama Prefecture, central Japan (34°03′N, 135°27′E, 300–900 m above sea level) in May–August, 2016 and 2017. Small and large toads were maintained separately in plastic cages (180 × 110 × 140 mm, length × width × height) and plastic containers (320 × 220 × 140 mm, length × width × height) under laboratory conditions (25°C), respectively. Carabid beetles were frequently found in the faeces of the collected toads, which indicates that both toad species successfully attacked and digested the carabids. Larvae of *S. litura*, *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae), and *Zophobas atratus* Fabricius (Coleoptera: Tenebrionidae) were provided to the toads as food. In total, 81 *B. japonicus* and 50 *B. torrenticola* were used in the experiments. The same toads were not used in different experiments.

(b) Experiments

To clarify how *P. jessoensis* can escape from inside a toad, we conducted the following feeding experiments (25°C). A toad and a beetle were placed in a glass container (300 × 150 × 230 mm, length × width × height). We observed how the toads attacked the beetles. The behaviours of several toads were recorded using the movie function of a digital camera (iPhone 6 plus, Apple) at 240 frames per second. When the toads swallowed the beetles, we investigated whether they vomited the beetles in plastic cages or plastic containers. If the toad did not vomit within 330 min of swallowing a beetle, we deemed that the toad had successfully digested the beetle. This assumption was also tested by investigating the faeces of toads. When toads vomited, we recorded the time from swallowing to vomiting to the nearest minute and checked whether the vomited beetles were alive. Live beetles were maintained separately in plastic cases. The vomiting behaviour of several toads was also filmed using video cameras (Handycam, HDR-PJ790V and HDR-CX630V, Sony, Japan). This experiment used 37 *P. jessoensis*, 23 *B. japonicus*, and 14 *B. torrenticola*.

We measured the amount of defensive chemicals in *P. jessoensis*. First, we weighed 104 *P. jessoensis*. Then, we repeatedly prodded the beetles with forceps; the simulated attacks forced them to exhaust their chemicals (thereafter, 'treated' beetles). We calculated the amount of defensive chemicals by reweighing the treated beetles.

To clarify whether the hot chemicals discharged by *P. jessoensis* can induce toads to vomit, we provided toads with treated beetles that were unable to eject a chemical spray. The toads that swallowed the treated beetles were observed in plastic cages or plastic containers for 330 min after swallowing them. This experiment used 37 treated *P. jessoensis*, 23 *B. japonicus*, and 14 *B. torrenticola*.

To clarify the tolerance of *P. jessoensis* for toad digestive juices, we investigated the survival of *P. jessoensis* in toad stomachs. First, we used forceps to open the toad's mouth. Then, we stuffed an adult beetle (*P. jessoensis* or other carabid species) into the distal toad oesophagus using forceps. Treated *P. jessoensis* that were unable to discharge defensive chemicals were used in this experiment. Adults of 14 carabid species were also used as reference species (electronic supplementary material, table S3). We used the stomach-flushing method [12] to remove the *P. jessoensis* and other carabid species from the toad stomachs 20 min after stuffing them into the toads. A plastic tube (diameter 9.0 or 2.0 mm) connected to a tap was inserted into the opened mouth of each toad; water flowing from the tap through the tube flushed out the beetles. The activity and survival of the beetles taken from the toad stomachs were checked. Even when the beetles removed from the toad stomachs were initially motionless, some beetles revived within 330 min. Therefore, we considered that the beetles could survive the toad stomachs when carabid beetles could revive within 330 min after being taken from toad stomachs. This experiment used 28 treated *P. jessoensis*, 29 other carabid beetles, 35 *B. japonicus*, and 22 *B. torrenticola* (electronic supplementary material, table S3). No toads died as a result of this experiment.

(c) Data analysis

All the analyses were performed using R ver. 3.3.2 [13]. The Pearson correlation coefficient was used to investigate the relationship between body weight and the amount of chemicals for ejection in *P. jessoensis*. A generalised linear model (GLM) with a binomial error distribution and a logit link was used to clarify which factors determined the successful escape of *P. jessoensis* from inside a toad. Escape success or failure (1/0) of *P. jessoensis* was used as the response variable. Toad species, toad weight, *P. jessoensis* weight, and *P. jessoensis* condition (control or treated beetles) were treated as fixed factors. The same model was also used to compare the survival rate inside toad stomachs between *P. jessoensis* and other carabids. Survival or non-survival (1/0) of *P. jessoensis* and other carabids was used as the response variable. Toad species, toad weight, carabid species (*P. jessoensis* or other carabids), and carabid weight were treated as fixed factors. A GLM with a Poisson error distribution and a log link was used to clarify what factors determined the time required from swallowing to vomiting in toads. The time required from swallowing to vomiting was used as the response variable. Toad species, toad weight, and *P. jessoensis* weight were treated as fixed factors. When the residual deviance was 1.5 times larger than the residual degrees of freedom (i.e., overdispersion), a quasi-binomial or quasi-Poisson error distribution was used instead of a binomial or Poisson error distribution, respectively.

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Supplementary videos

Video S1. A bombardier beetle *Pheropsophus jessoensis* spraying. The beetles ejected defensive chemicals when their legs were pinched with forceps. Slow-motion footage was recorded at 960 frames per second (Cyber-shot DSC, RX10-III, Sony, Japan).

Video S2. Predation and emetic behaviour of a juvenile *Bufo japonicus*. The toad easily swallowed an adult *Pheropsophus jessoensis*. However, the toad vomited the beetle up 88 min after swallowing it; that is, the beetle escaped from the toad’s mouth 88 min after being swallowed.

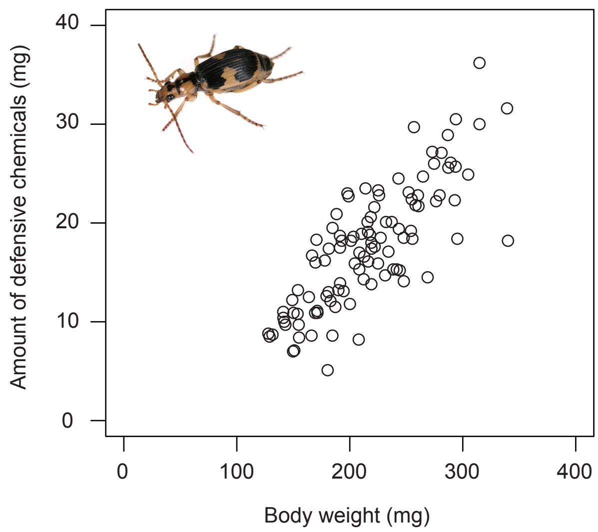

Figure S1. Relationship between body weight and the amount of defensive chemicals in the bombardier beetle *Pheropsophus jessoensis* (Pearson correlation coefficient, $r = 0.74$, $N = 104$, $p < 0.0001$).

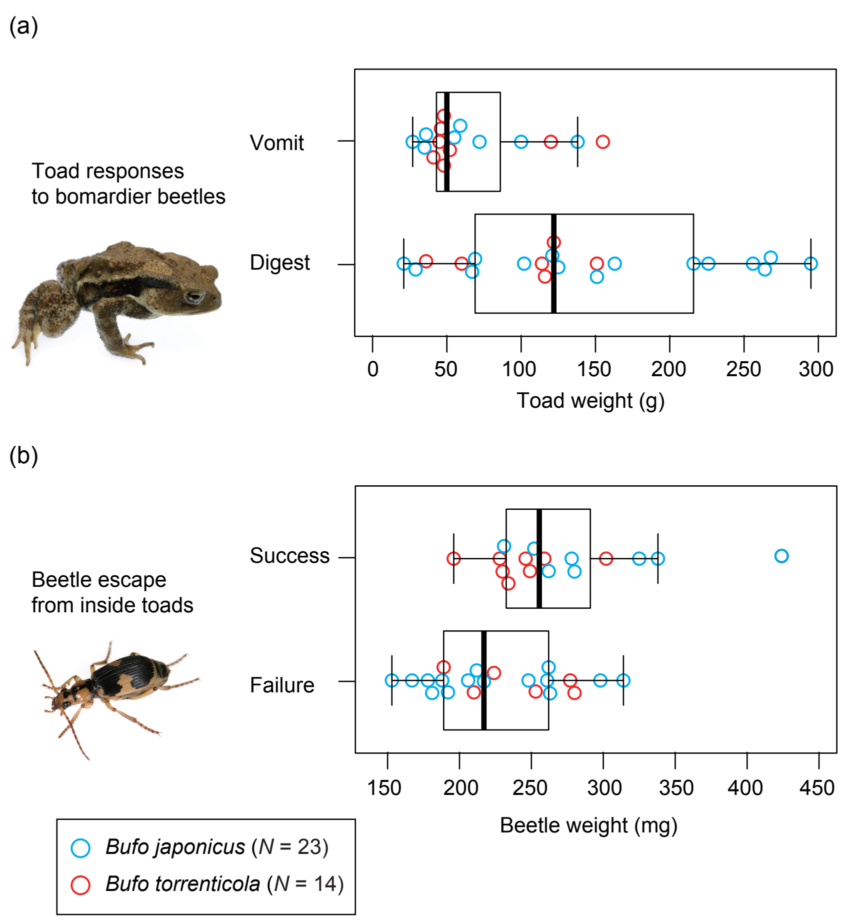

Figure S2. Boxplots of the relationships between body size and the behavioural responses of toads and bombardier beetles. (a) Relationship between toad responses (digest or vomit) and weight. (b) Relationship between *Pheropsophus jessoensis* escape success/failure and weight. The thick vertical lines within the boxes show median values. The right and left boxes show the 75th and 25th percentiles, respectively. Whiskers show values within the 1.5 interquartile range. Blue and red circles indicate individuals of *Bufo japonicus* and *B. torrenticola*, respectively.

Table S1. Results of a generalised linear model for the time required from swallowing to vomiting in toads.

Response variable	Explanatory variable (fixed effect)	Coefficient estimate	SE	*z* value	*p* value
Time from swallow -ing to vomiting [1)]	Intercept	2.936275	1.181925	2.501	0.0279
	Toad species [2)]	−0.019216	0.470558	−0.041	0.9681
	Toad weight	0.004039	0.005058	0.799	0.4401
	Beetle weight	0.002068	0.004085	0.506	0.6219

[1)] A quasi-Poisson error distribution was used because the residual deviance was much larger than the residual degrees of freedom (i.e., overdispersion).

[2)] *Bufo japonicus* was used as a reference.

Table S2. Results of a generalised linear model of factors influencing the successful escape of *Pheropsophus jessoensis* from inside a toad.

Response variable	Explanatory variable (fixed effect)	Coefficient estimate	SE	*z* value	*p* value
Successful escape [1)]	Intercept	−3.482874	2.363478	−1.474	0.140583
	Toad species [2)]	1.725159	0.909867	1.896	0.057953
	Toad weight	−0.028379	0.011459	−2.477	0.013263
	Beetle treatment [3)]	−3.078998	0.92141	−3.342	0.000833
	Beetle weight	0.019363	0.009362	2.068	0.038614

[1)] The binomial error distribution was used.

[2)] *Bufo japonicus* was used as a reference.

[3)] Control beetles were used as a reference.

Table S3. The survival rates of *Pheropsophus jessoensis* and other carabid beetles in the toad stomachs.

		Survival rate % (*N*)		
Species	Subfamily	*Bufo japonicus*	*B. torrenticola*	Total
Pheropsophus jessoensis Morawitz [1)]	Brachininae	82.4 (14/17)	72.7 (8/11)	78.6 (22/28)
Other carabid species		66.7 (12/18)	18.2 (2/11)	48.3 (14/29)
Brachinus scotomedes Redtenbacher [1)]	Brachininae	100.0 (1/1)	100.0 (1/1)	100.0 (2/2)
Carabus yaconinus Bates	Carabinae	50.0 (1/2)	0 (0/1)	33.3 (1/3)
Chlaenius abstersus Bates	Harpalinae	50.0 (1/2)	0 (0/1)	33.3 (1/3)
Chlaenius micans (Fabricius)	Harpalinae	−	0 (0/1)	0 (0/1)
Chlaenius naeviger Morawitz	Harpalinae	100.0 (1/1)	0 (0/1)	50.0 (1/2)
Chlaenius posticalis Motschulsky	Harpalinae	100.0 (1/1)	−	100.0 (1/1)
Chlaenius variicornis Morawitz	Harpalinae	−	100.0 (1/1)	100.0 (1/1)
Haplochlaenius costiger (Chaudoir)	Harpalinae	0 (0/2)	0 (0/1)	0 (0/3)
Galerita orientalis Schmidt-Göbel	Harpalinae	100.0 (1/1)	−	100.0 (1/1)
Planetes puncticeps Andrewes	Lebiinae	100.0 (2/2)	0 (0/1)	66.7 (2/3)
Dolichus halensis (Schaller)	Pterostichinae	100.0 (2/2)	0 (0/1)	66.7 (2/3)
Lesticus magnus (Motschulsky)	Pterostichinae	100.0 (2/2)	0 (0/1)	66.7 (2/3)
Trigonotoma lewisii Bates	Pterostichinae	0 (0/1)	−	0 (0/1)
Archipatrobus flavipes Motschulsky	Trechinae	0 (0/1)	0 (0/1)	0 (0/2)
Total		74.3 (26/35)	45.5 (10/22)	63.2 (36/57)

[1)] The bombardier beetles were repeatedly attacked using forceps; the simulated attacks forced the beetles to exhaust their chemicals before the feeding experiment.

Table S4. Results of a generalised linear model of factors influencing the survival of *Pheropsophus jessoensis* and other beetle species in toad stomachs.

Response variable	Explanatory variable (fixed effect)	Coefficient estimate	SE	*z* value	*p* value
Beetle survival [1)]	Intercept	1.839799	0.98728	1.864	0.0624
	Toad species [2)]	−1.351476	0.656943	−2.057	0.0397
	Toad weight	0.00304	0.004074	0.746	0.4555
	Beetle species [3)]	−1.536647	0.649149	−2.367	0.0179
	Beetle weight	−0.001503	0.001809	−0.831	0.4062

[1)] The binomial error distribution was used.

[2)] *Bufo japonicus* was used as a reference.

[3)] *Pheropsophus jessoensis* was used as a reference.