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Source: Current Herpetology, 43(1) : 22-30

Published By: The Herpetological Society of Japan

URL: https://doi.org/10.5358/hsj.43.22

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*Current Herpetology* 43(1): 22–30, February 2024
doi 10.5358/hsj.43.22

# An Investigation of Chemical Prey Discrimination by *Rhabdophis subminiatus* Hints the Source of Nuchal Gland Toxins

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**Abstract: The nuchal glands are organs of chemical defense embedded under the skin of several species of *Rhabdophis* and contain toxins sequestered from consumed prey. *Rhabdophis subminiatus* possesses nuchal glands, but not much information is available on its toxin source and prey preference, particularly based on its chemosensory response. We hypothesized that the response of *R. subminiatus* toward toads would be higher than other potential prey because the snake needs to sequester toxins contained in toad skin. We also examined the snake preference toward several bufonid species to test a hypothesis that the preference toward *Duttaphrynus melanostictus* is higher than other toad species due to its high availability in the habitat of the snake. To test the hypotheses, we conducted chemical response tests and compared the tongue-flick attack score (TFAS) of the snake toward prey chemical stimuli. The first experiment used only one toad species (*D. melanostictus*) and various potential prey animals for the source of chemical stimuli. The second experiment used chemical stimuli from several species of frogs and toads. In the first experiment, snakes showed the highest response toward *D. melanostictus*. However, in the second experiment, snakes showed the highest response toward a toad, *Ingerophrynus biporcatus*, followed by *D. melanostictus*, and a lower response toward another toad, *Phrynoidis asper*. These results indicate that the snake has a high preference toward toads, but its preference varies among toad species. Taken together, this study suggests that *R. subminiatus* discriminates different potential prey chemical cues and probably consumes toads to sequester their toxins.**

Key words: Bufadienolide; Bufonidae, Prey preference; Tongue flicks; Toxin sequestration

## Introduction

The nuchal glands are organs of chemical defense embedded under the dorsal skin of

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several species of *Rhabdophis* and contain toxins sequestered from consumed prey (Hutchinson et al., 2012; Mori et al., 2012). The red-necked keelback snake, *Rhabdophis subminiatus*, is one of the species that possesses nuchal glands (Smith, 1938). A previous study showed that *R. subminiatus* exhibits antipredator behavior related to the nuchal glands and that smaller snakes may depend more on the nuchal glands to deter predators (Anita et al., 2022). This finding implies that the acquisition of toxins for the nuchal glands to function properly as a defensive strategy is essential. However, not much information is available on the toxin acquisition process for the nuchal glands of *R. subminiatus*. Studying the prey selection and feeding behavior of this species is necessary to obtain information on the possible source of the toxins.

Records on feeding observations of *R. subminiatus* outside the Indonesia region have reported that the species consumes a toad, *Duttaphrynus melanostictus* (Mohammadi and Hill, 2012), and various frogs such as *Uperodon globulosus* (Shihan and Kabir, 2015), *Fejervarya* sp. (Rahman et al., 2012), *Hylarana taipehensis* (Shalauddin et al., 2019), *Polypedates leucomystax* (Harman and Master, 2019), and *Odorrana mawphlangensis* (Saikia et al., 2021). Among several toad species on Java island, toads that have been documented to be consumed by *R. subminiatus* are *D. melanostictus* and *Ingerophrynus biporcatus* (De Lang, 2017). An Asian common toad, *D. melanostictus*, is native to Southeast Asia and the Sundaic islands of Java and Sumatra, but it has recently invaded other islands, specifically in the eastern part of Indonesia (Church, 1960; Reilly et al., 2017). This widespread and habitat-generalist species is known to be tolerant to disturbances and resource limitations when compared to other amphibians on the island of Java (Iskandar, 1998). The crested toad, *I. biporcatus*, is an endemic species with a limited distribution in Sumatra, Java, and the lesser Sunda islands of Indonesia. *Ingerophrynus biporcartus* is considered to be vulnerable to competition with *D. melanostictus* (Church, 1960), and in Java, the former tends to be restricted to more forested regions (Iskandar, 1998).

In experimental studies of squamate reptiles, the frequency of tongue flicking has been used as a measure of prey chemical discrimination, detection of predator chemicals, and response to pheromones (Cooper, 1998). Many studies have shown that the presentation of chemical stimuli aids in investigating snake prey preference (Burghardt, 1967; Tanaka et al., 2001; Aubret et al., 2006; Fukuda et al., 2021). These experimental snakes responded by flicking their tongue and also biting a cotton swab applicator soaked in prey chemical stimuli, especially if it carried the chemical stimuli of their preferred prey (Cooper and Burghardt, 1990). An attack or bite is a strong indicator of a predatory response, and the integration of the tongue flick and attack variables resulted in an index known as tongue flick attack score (TFAS), developed by Burghardt (1970).

*Rhabdophis subminiatus* is a common snake species on Java Island, and is frequently encountered in gardens below 900 m asl (De Lang, 2017). However, its feeding behavior has rarely been documented, and publications investigating its prey preference based on chemosensory response are not currently available. Such investigation would provide data on the potential prey preference of the snake and reveal the possible toxin source of its nuchal gland fluids. Previous studies have shown that their congeneric species, *R. tigrinus*, preys on toads and sequesters the toad toxins as the source of the nuchal gland toxin (Hutchinson et al., 2007, 2012). Based on this finding and several records on the diet of *R. subminiatus*, we predict that *R. subminiatus* would prey on toads to acquire the toxin. There are at least four genera of toads distributed in Java: *Phrynoidis*, *Duttaphrynus*, *Ingerophrynus*, and *Leptophryne*. Two species of *Leptophryne*, *L. borbonica* and *L. cruentata*, are found in Java, but they are distributed in primary rainforest which is not the habitat of *R. subminiatus*. Toad species that could be found around *R. subminiatus* habitat belong to three of the four genera, namely *P. asper*, *D. melanostictus*, and *I. biporcatus*. Thus, we focused on these three toad species. We first hypothesized that the response of *R. subminiatus* from Java Island toward toad chemicals would be higher than other potential prey animals. Then, to test the hypothesis that its preference toward *D. melanostictus* is higher because the toad is more available in the habitat of *R. subminiatus*, we examined the snake preference toward several other bufonid species distributed in the habitat. We conducted chemical response tests and compared tongue flick attack score toward different chemical stimuli to test these hypotheses.

## MATERIALS AND METHODS

### *Subjects*

Adults of *R. subminiatus* were used for experiments 1 and 2. All snakes were collected from West Java (Karawang, Jatinangor) and Central Java (Cilacap), Indonesia. In experiment 1, 16 snakes (seven males and nine females) with mean snout-vent length (SVL) and mean body mass of 422.4 mm (345–512 mm) and 29.23 g (16.50–50.70 g), respectively, were used. Eight snakes (three males and five females) were used in experiment 2, with mean SVL of 385.8 mm (360–539 mm) and mean body mass of 28.56 g (16.00–40.10 g). During captivity, all snakes were individually housed in modified transparent plastic containers (ca. 490×330×270 mm) with a water dish and paper as the substrate. The enclosure had a 12:12 h light:dark cycle and ambient temperature ranged from 25 to 34°C. The snakes were fed with two individuals of *Fejervarya* spp. twice a week. The subjects had been captive for approximately two weeks to four months before experiment 1 and approximately two to fourteen months before experiment 2.

### *Chemical stimuli*

The animals used for the chemical stimuli in experiment 1 were selected because these species or their close relatives and *R. subminiatus* are sympatric. All animals were collected from the same habitat as *R. subminiatus*, specifically from West Java and Central Java. In experiment 1, one toad species, *D. melanostictus*, which is common prey and abundant in the habitat of *R. subminiatus*, was used. In total, eight types of odors were used in experiment 1: distilled water, cologne, Nile tilapia fish (*Oreochromis niloticus*), rice field frogs (*Fejervarya* sp.), green puddle frogs (*Occidozyga lima*), common house geckos native to South and Southeast Asia (*Hemidactylus frenatus*), house mice (*Mus musculus*), and Asian common toads (*D. melanostictus*). All animals were alive except for the fish, which were removed from the water shortly before the experiment started. Immediately before each trial, the tip of sterile cotton swabs were rubbed on the wet skin surface of animals at least three times to collect the odors. Distilled water was used as odorless control, while cologne served as pungency control for a biologically irrelevant odor. The cologne used in the experiment was the Casablanca brand of PT Priskila Prima Makmur that was diluted to 10% with distilled water (Takeuchi and Mori, 2012). Both odorless and pungency controls were prepared by dipping the cotton swab into the fluid shortly before each trial.

In experiment 2, the prey stimuli were limited to several species of frogs and toads distributed in the same habitat as *R. subminiatus*. Rice field frogs (*Fejervarya limnocharis*), fanged river frogs (*Limnonectes macrodon*), and three bufonids, *I. biporcatus*, *P. asper*, and *D. melanostictus*, were used in this experiment. Water was used as a control odor by dipping the cotton swab into the fluid shortly before each trial. All animals were swabbed alive, except for *I. biporcatus*, which had been frozen and was thawed shortly before the experiment. The protocol for experiment 2 is similar to experiment 1: immediately before each trial, sterile cotton swabs were rubbed on the skin surface of animals to collect the odors.

### *Chemical response test*

The experiment was conducted from 0900 to

1400 h in a closed room. Ambient temperature was maintained at 25–26°C during the experiment. Approximately 16 hours before the experiment, the cages were moved to the experimental room. Water dishes and paper substrate were subsequently removed from the cages. The snakes were left undisturbed for a 16-hour acclimation prior to the experiment. Each cage was separated by a cardboard to eliminate the visual effects of snakes in adjacent cages. The procedure was based on a well-established test of chemical discrimination by the vomeronasal organs in Squamata (Burghardt, 1970; Cooper, 1998).

Cotton swabs bearing the chemical stimuli were presented to each snake in a randomized and counterbalanced order. The trial began by gently removing the cage cover, and then the tip of a cotton swab was presented approximately 1–2 cm in front of the snake's snout. The number of tongue flicks directed to the swab was counted for 60 seconds after the first tongue flick was observed. If a snake did not show any tongue flick for 30 seconds after the presentation of the swab, the tip of the swab was touched gently to the snout of the snake. If a snake bit or struck the swab, the trial was stopped, and the remaining time was recorded to calculate attack latency. If a snake did not flick its tongue after 60 seconds of stimulus presentation, the trial was terminated, and afterwards, a trial with the same stimulus was repeated after all stimuli were tested with the snake. A minimum of 15-minute interval was allowed between trials for the same individual snake. Snakes were considered to have more preference for the stimuli when they attempted to bite or strike the cotton swabs. The tongue flick attack score (TFAS) (Burghardt, 1970; Cooper and Burghardt, 1990), was calculated as follows.

$$\text{TFAS} = \text{TFmax} + (\text{Test duration} - \text{Attack latency}),$$

where TFmax is the maximum number of tongue flicks for an individual from any trial, test duration is the length of trial, which was 60 seconds, and attack latency is the time (in seconds) from the first tongue flick to striking or biting. Attack latency is equal to the test duration if there are no strikes or bites.

### *Statistical analysis*

To determine the effect of chemical stimuli on the TFAS, the Friedman test was utilized, followed by pairwise multiple comparisons using Wilcoxon signed rank test. In multiple comparisons, the Bonferroni correction was not used due to the conservative nature of this correction (Perneger 1998; Moran 2003; Nakagawa 2004). Instead, results with $P<0.05$, $P<0.01$, or $P<0.001$ levels were presented for multiple comparisons. To evaluated the effect of captive time on snake responses, we performed correlation analysis using Spearman's coefficient. We examined the effects of captive length (days) on TFAS particularly to the species of *Fejervarya* because we used this genus as captive diet and thus effects of captive time, if any, is expected to be detected most clearly in them. All statistical analyses were conducted using R statistical analysis (R Core Team, 2022).

## RESULTS

In experiment 1, snakes showed the highest mean TFAS toward the chemical stimuli of *D. melanostictus*. The mean TFAS (±SE) toward each chemical stimulus, from highest to lowest, were: 46.6±6.1 for *D. melanostictus*, 36.7±6.06 for *O. lima*, 35.1±5.28 for cologne, 26.3±4.97 for *Fejervarya* sp., 23.9±4.77 for *H. frenatus*, 21.4±3.57 for *O. niloticus*, 20.3±2.87 for *M. musculus*, and 18.3±3.16 for water. There were significant effects of chemical stimuli on the TFAS in experiment 1 ($\chi^2=17.6$, $P<0.05$; Fig. 1). Bites to the cotton swab were observed in two individual snakes toward the frog stimulus of *O. lima* and *Fejervarya* sp. Multiple comparisons (Table 1) revealed that the TFAS toward *D. melanostictus* was higher than that toward *Fejervarya* sp. and *H. frenatus* ($P<0.05$). The TFAS toward *D. melanostictus* was significantly higher than that toward *O. niloticus*, *M. musculus*, and water ($P<0.01$). The TFAS toward cologne was higher than that

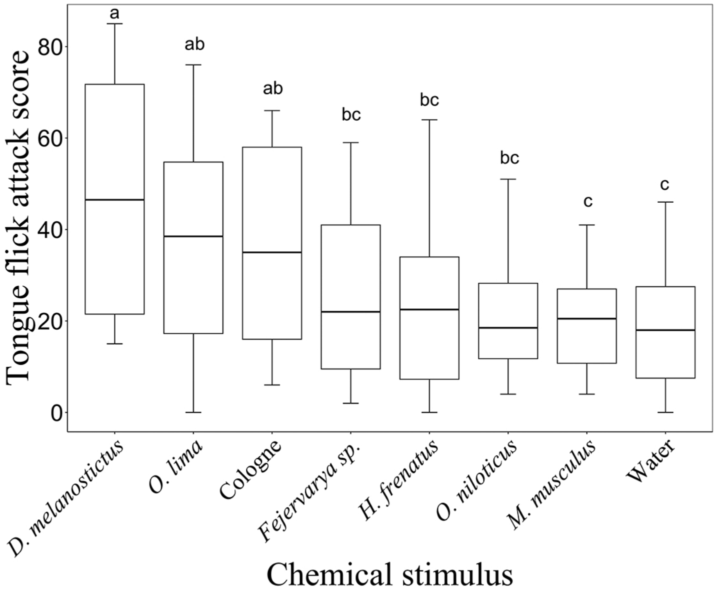

FIG. 1. Tongue flick attack scores of adult *Rhabdophis subminiatus* in experiment 1. The median is represented by the middle horizontal line in the box plot. Edges of the boxes represent 25% and 75% quartiles, and range is represented by whiskers. The chemical stimuli were toads (*Duttaphrynus melanostictus*), frogs (*Occidozyga lima* and *Fejervarya* sp.), lizards (*Hemidactylus frenatus*), fish (*Oreochromis niloticus*), mammals (*Mus musculus*), cologne, and water. Boxplots sharing the same lowercase letters do not differ significantly (P>0.05).

TABLE 1. The comparison of tongue flick attack scores in *Rhabdophis subminiatus* (n=16) for each pair of eight chemical stimuli in experiment 1. P-values obtained by Wilcoxon signed-rank tests are shown.

| Stimulus | *D. melanostictus* | *O. lima* | Cologne | *Fejervarya* sp. | *H. frenatus* | *O. niloticus* | *M. musculus* |
|---|---|---|---|---|---|---|---|
| *O. lima* | 0.231 | | | | | | |
| Cologne | 0. 222 | 0.910 | | | | | |
| *Fejervarya* sp. | 0.016* | 0.070 | 0.244 | | | | |
| *H. frenatus* | 0.012* | 0.140 | 0.115 | 0.691 | | | |
| *O. niloticus* | 0.005** | 0.059 | 0.112 | 0.518 | 0.691 | | |
| *M. musculus* | 0.003** | 0.014* | 0.032* | 0.320 | 0.569 | 0.897 | |
| Water | 0.003** | 0.021* | 0.030* | 0.196 | 0.597 | 0.485 | 0.518 |

* P<0.05, ** P<0.01.

toward *M. musculus* and water (P<0.05). TFAS toward *O. lima* was significantly higher than that toward *M. musculus* and water (P<0.05). There was no significant difference among *D. melanostictus*, cologne, and *O. lima*. There was no pair that showed significant differences at P<0.001 level. We found no significant correlation between captive time and the TFAS toward *Fejervarya* sp. (ρ=–0.042, P=0.876).

In experiment 2, snakes showed the highest mean TFAS toward *I. biporcatus*, followed by *D. melanostictus*. The mean of TFAS (±SE) toward each chemical stimulus, from highest to lowest, was: 24.4±6.57 for *I. biporcatus*, 9.5±3.9 for *L. macrodon*, 9.25±2.86 for *D. melanostictus*, 5.12±2.41 for *P. asper*, 3.5±1.52

for *F. limnocharis*, and 3.25±1.69 for water. There were significant effects among chemical stimuli and the TFAS ($\chi^2$=19.6, P<0.01; Fig. 2). Multiple comparisons (Table 2) revealed that the TFAS toward *I. biporcatus* was significantly higher than all chemical stimuli except for *D. melanostictus* (P<0.05). The TFAS toward *D. melanostictus* was significantly higher than *F. limnocharis* and water (P<0.05). Between frogs, the TFAS toward *L. macrodon* was significantly higher than toward *F. limnocharis* (P<0.05). The mean TFAS toward *P. asper* was lower compared to the other two toad species. There was no pair that showed significant differences at the P<0.01 or P<0.001 levels. We found no significant correlation between captive time and TFAS toward *F. limnocharis* (ρ=–0.292, P=0.483).

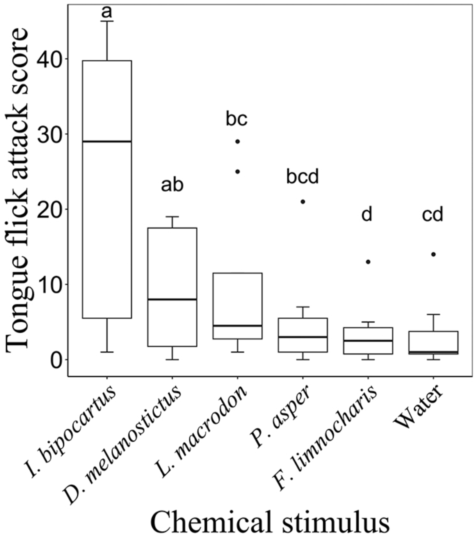

FIG. 2. Tongue flick attack scores of adult *Rhabdophis subminiatus* toward chemical stimuli of several species of toads (*Ingerophrynus biporcatus*, *Phrynoidis asper* and *Duttaphrynus melanostictus*) and frogs (*Fejervarya limnocharis* and *Limnonectes macrodon*) in experiment 2. The median is represented by the middle horizontal line in the box plot. Edges of the boxes represent 25% and 75% quartiles, range is represented by whiskers, and dots are extreme values. Boxplots sharing the same lowercase letters do not differ significantly (P>0.05).

## DISCUSSION

The TFAS patterns in both experiments agree to the natural diet of *R. subminiatus*, which consists of toads and frogs. These results suggest that *R. subminiatus* discriminates different potential prey chemical cues and also imply that they would consume toads as the toxin source for the nuchal glands. In experiment 1, however, the TFAS toward cologne was relatively high, and no significant differences were found in the TFAS between toads and cologne. The high response to cologne may be due to intensity of some chemicals in the perfume that stimulates snakes to explore it. The TFAS in response to cologne differ significantly from the response toward the odorless control (water), which indicates that snakes can detect the scent. On the other hand, overall responses were generally lower in experiment 2 than in experiment 1. One possible reason is that the longer captive time of the snakes in the former might have reduced

TABLE 2. The comparison of tongue flick attack scores in *Rhabdophis subminiatus* (n=8) for each pair of six chemical stimuli in experiment 2. P-values obtained by Wilcoxon signed-rank tests are shown.

| Stimulus | *I. biporcatus* | *L. macrodon* | *D. melanostictus* | *P. asper* | *F. limnocharis* |
|---|---|---|---|---|---|
| *L. macrodon* | 0.025* | | | | |
| *D. melanostictus* | 0.076 | 0.833 | | | |
| *P. asper* | 0.039* | 0.235 | 0.288 | | |
| *F. limnocharis* | 0.022* | 0.012* | 0.034* | 1.000 | |
| Water | 0.014* | 0.059 | 0.049* | 0.916 | 1.000 |

* P<0.05

their responses, but we found no correlation between captive time and the TFAS to the species of *Fejervarya* within each experiment. Therefore, it is unclear what caused the general differences in the TFAS between the two experiments.

Experiment 2 indicates that the TFAS toward two of the toad species, *I. biporcatus* and *D. melanostictus*, is not significantly different. This result suggests that the snake has a high preference for toads, and this preference is not specific to only *D. melanostictus*. Although there is a record of *I. biporcatus* consumed by the snake, this toad is now rarely found in snake habitats. The population of *I. biporcatus* has been reported to be decreasing due to the loss or modification of habitat (Church, 1960; Iskandar and Mumpuni, 2004). This suggests that the probability of *R. subminiatus* encountering *I. biporcatus* is lower than with *D. melanostictus*. Even so, the result of this study indicates that the preference of the snake toward *I. biporcatus* is still preserved. To our knowledge, there are no detailed studies about the chemical constituent of the parotoid gland secretions of *I. biporcatus* from Java and its congeneric, but a previous study has shown that gland fluids of *D. melanostictus* contain several forms of bufadienolides (Gao et al., 2010). The toad population dynamics and its toxin component deserve further investigation from a conservation perspective as well as the effect on snake preference. Previous studies have shown that the availability of prey can modify the innate preferences of predators or shift their preference ontogenetically because this may have adaptive value in response to environmental challenges (Waters and Burghardt, 2013; Goetz et al., 2018).

Further investigation to determine how snakes can discriminate between toxic and non-toxic prey so as to select a particular toad over another may be worth doing, as one of our results showed that the TFAS to an Asian giant toad, *P. asper*, is significantly low. A study on skin secretions of *P. asper* from Thailand showed that the secretions contain bufotalin as the major bufadienolide, with a lesser component of resibufogenin, and minor amounts of other bufadienolides and bufotoxins (Daly et al., 2004). The probability that *R. subminiatus* might encounter *P. asper* is relatively high, as these species occupy similar habitats. *Phrynoidis asper* is found in primary and secondary rainforests and lives along the banks of small to medium-sized streams and large rivers (Inger et al., 1974). In addition, *P. asper* in Java can also be found in gardens, near human settlements, and relatively far from rivers or water bodies. Thus, the low TFAS toward this toad is not explained by habitat differences between the snake and the toad. A possible factor that may contribute to the low preference is the pungent smell of the skin secretion of *P. asper*. The unpleasant smell of skin secretions of some anurans has been considered one of the defensive strategies to ward off predators. Compared to *D. melanostictus*, the skin secretion of *P. asper* was copious and its smell was more pungent, at least to the human olfactory system (S. Anita, personal observation). Thus, the pungent smell of *P. asper* skin secretions may have a powerful effect to deter predation by *R. subminiatus* which is physiologically tolerant to bufadienolides. However, this conjecture needs to be investigated further.

Another noteworthy result is the different TFAS toward different frog species. In the present experiments, significant and high TFAS's were observed toward *O. lima* and *L. macrodon*. *Occidozyga lima* can be found in flooded lowland grassland, rivers, swamps, freshwater lakes, ponds, and irrigated agricultural lands. *Limnonectes macrodon* is an endemic species to Sumatra and Java, and mainly lives in forested areas near streams. This indicates that *O. lima* and *L. macrodon* tend to be more aquatic and common in water bodies which are also the common habitat of natricine snakes. This overlapped habitat increases the possibility for snakes to encounter both frog species and may explain the high preference toward them. On the other hand, snakes had a lower preference for *Fejervarya* sp. in experiment 1 and *F. limnocharis* in experiment 2. Frogs belonging to this genus were the only prey items provided to the snakes during captivity which lasted from two weeks to fourteen months before the experiments. We did not find any correlation between captive time and the TFAS toward *Fejervarya* spp. in experiments 1 and 2. This indicates that the treatment in captivity did not elevate their preference toward the frogs. A study in rattlesnakes also showed that a long-term exclusive diet did not elevate the preference for chemical cues toward the exclusive prey (Holding et al., 2016). Taken together, these preferences toward frogs suggest that even though the snakes had been captive for a relatively long time, and their feeding was restricted to only certain frogs, they may retain the preference to the scents of particular prey rather than change the preference to recently fed prey.

## ACKNOWLEDGMENTS

We thank Syaripudin for helping with the experimental facility and snake maintenance. We are grateful to the Indonesia Endowment Fund for Education (LPDP) for granting a Ph.D. scholarship and research fund to SA. This study was supported in part by JSPS Grants-in-Aid for Scientific Research (17H03719, 21H02551). We also thank Alan Savitzky, Koshiro Eto, Taku Okamoto, and Naoki Mori for valuable comments on the early manuscript. The collection, care and experimental procedures were approved by the Committee of Ethical Clearance for Research of the Indonesian Institute of Sciences (Letter number: 9/klirens/XI/2020).

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*Accepted: 27 September 2023*