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The Green Bamboo Pit Viper, *Trimeresurus stejnegeri*, Discriminates Chemical Stimuli Among Anuran Species

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Abstract: The green bamboo pit viper, *Trimeresurus stejnegeri*, is a sit-and-wait predator that forages mainly on frogs in Taiwan. We predicted that *T. stejnegeri* is able to identify prey at the species level to locate appropriate ambushing sites. We conducted a chemical preference test among frog species and compared its result with the natural diet of *T. stejnegeri*. The test suggested that *T. stejnegeri* can discriminate prey species even within the same family. The three highest tongue flick ambush scores (TFAM) were obtained in *Odorrana swinhoana*, *Polypedates braueri*, and *Zhangixalus moltrechti*, which corresponded to the dominant prey items found in stomach contents. Some frog species, however, did not show coincidence between TFAM and the snake's natural diet. Abundance, active period, and defensive mechanisms of frogs are potential factors affecting the realized diet of *T. stejnegeri* in the field. We also propose that ambushing behavior exhibited during the chemical preference test should be considered an important indicator of interest in potential prey for sit-and-wait species of snakes.

Key words: Ambush behavior; Chemical stimulus; Green bamboo pit viper; Stomach contents; Tongue flick ambush score

Introduction

Chemical stimuli originating from prey allow predators to detect the presence of prey by olfactory senses (e.g., Mathis, 2003; Nevitt et al., 2008; Togunov et al., 2017). One advantage of the olfactory system is that it enables predators to gain prey information when the prey is not visible, as in a turbid or dark environment (Cunningham et al., 2009; Lunt and Smee, 2015). In addition, the olfactory system may enable predators to identify the exact species they encounter (Mathis, 2003; Saidapur et al., 2009; Ferrari et al., 2010).

Snakes largely depend on the chemosensory system to detect the presence of prey by the vomeronasal organ. They deliver chemicals in the environment with the forked tongue to the vomeronasal organ (Schwenk, 1995). Many studies have shown that snakes discriminate chemical stimuli between different prey animals (Burghardt, 1967; Clark, 2004; Cooper, 2007; Goetz et al., 2018). Chemical stimulus tests based on tongue-flick frequency are a common method to determine prey preference in snakes (Weldon and

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Schell, 1984; Clark, 2004; Tsairi and Bouskila, 2004; Cooper, 2007). Behavioral experiments have shown that snakes can chemically discriminate between species belonging to different higher taxa (e.g., Aves, Mammalia, Anurans, Squamata, or Insecta) and that chemically preferred animals coincide with the natural diet (e.g., Burghardt, 1967; Cooper, 2007).

Discriminating prey species among family, genus, or even species levels is important for snakes. This should be especially true for snakes that use sit-and-wait foraging tactics because sit-and-wait snakes usually search for suitable ambushing sites based on the concentration of prey scent (Tsairi and Bouskila, 2004; Bevelander et al., 2006; Du et al., 2009). To determine an effective ambushing site, snakes should be able to identify prey scent to the species level because potential prey animals would differ in abundance, availability, aggregation, agility, and palatability, even among congeneric species (Weldon and Schell, 1984; Kidera and Ota, 2008; Wittenberg, 2012), which would affect capture success by snakes.

The green bamboo pit viper, *Trimeresurus stejnegeri*, is a common snake distributed throughout Taiwan (Tsai and Tu, 2000). Frogs, especially rhacophorid and ranid species, comprise most of its diet (Creer et al., 2002). Thus, *T. stejnegeri* is frequently observed ambushing close to the aquatic habitats that frogs use as breeding sites. Considering the ecological and behavioral variation among frogs, the ability to identify frog species is expected to be crucial in order for *T. stejnegeri* to locate suitable ambushing sites. Here, we conducted a chemical preference test to examine whether the snake discriminates frog species with which it is sympatric. We also examined stomach contents of *T. stejnegeri* to determine the actual prey species consumed at our study sites. Then, we compared the result of the chemical preference test to the realized diet. In addition, we focused on behaviors, such as head movement and initiation of ambushing posture, during the chemical preference test, because previous studies have demonstrated that snakes alter their behavior when a preferred scent is present during chemical stimulus tests (Halpern and Frumin, 1979; Weldon and Schell, 1984).

Materials and Methods

Seventeen *T. stejnegeri* (3 females and 14 males) were captured in three different locations; Chongguang village in Hualien County (23°54'11.4" N, 121°29'43.4" E, n=3), San-fu Leisure Farm (24°37'02.4" N, 121°45'14.7" E, n=6), and Shuanglianpi (24°45'00.9" N, 121°38'25.3" E, n=8) in Ilan County, from April to November, 2018. Eight anuran species that were used as chemical stimuli in the experiment (see below) were abundant at these sites (Gong and Yang, 2017). Snout-vent length (SVL) of *T. stejnegeri* was 488±55 mm (mean±SD). Captured snakes were kept in the Amphibian Conservation Laboratory, National Dong Hwa University, Taiwan during the experiment. Each individual was individually maintained in a plastic box (17×28×18 cm). The four walls and ceiling had several drilled holes to provide ventilation. A water pipe cut in half longitudinally was provided for shelter. A bowl filled with distilled water and a branch were set in the plastic box. Fluorescent lighting was provided for 12 h per day to simulate the daylight cycle in the field. Snakes were fasted for 7 days before the experiment. All individuals were tested within two weeks after being brought to the laboratory.

The experiment was conducted between 18:00 and 0:00 h, the time during which *T. stejnegeri* is most active in the field (Creer et al., 2002; Tsai, 2007). Ambient temperature was maintained at 24–26°C during the experiment. Odors of eight species for anurans, from three families, were used as chemical stimuli: *Bufo bankorensis* (Bufonidae); *Babina adenopleura*, *Hylarana latouchii*, and *Odorrana swinhoana* (Ranidae); *Buergeria otai*, *Buergeria robusta*,

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Polypedates braueri, and *Zhangixalus moltrechti* (Rhacophoridae). Distilled water was used as control. Each snake was tested with the eight anuran species' odors and with distilled water. Based on the location of each species' breeding site, *Bf. bankorensis* and *H. latouchii* were classified as ground-dwelling species which could be found in various water habitat; *O. swinhoana*, *Br. otai*, and *Br. robusta* were classified as lotic-dwelling species; and *Bb. adenopleura*, *P. braueri*, and *Z. moltrechti* were classified as lentic-dwelling species.

Before the experiment, the lid of the plastic box was opened halfway for 10 min. If the snake was hidden inside the shelter, the shelter was gently removed in advance to enable the snake to detect the chemical stimulus more effectively. A single individual of each tested prey species was collected to provide an extract of the species' chemical stimulus. Each frog or toad was soaked in 20 ml of distilled water for more than 10 min. To reduce any potential body size effect, we used frogs of which SVL was 30 mm to 50 mm. Paper towel (30×10 cm) was folded twice into a square shape (10×10 cm) with the aid of forceps (length: 250 mm). Ten ml of the aqueous surface extract of each frog/toad was absorbed on the paper towel (Clark, 2004). We used forceps to pick up the paper towel and hold it in front of the test individual. During the test, paper towel was maintained at a horizontal angle, to reduce the visible area of the paper towel seen by the snake. The paper towel was held less than 5 cm from the snout of the snake. We avoided touching the tip of the forceps and paper towel with our hands during the entire process, to minimize contamination with human odor. Forceps were washed with distilled water between trials, to remove odor remaining from the prior test. The interval between trials of each chemical stimulus was more than 30 min, to reduce the effects of the preceding stimulus.

Each trial was conducted for 1 min, during which the numbers of tongue flicks and head movements were counted. We considered that head movement occurred if the anterior part of the body slowly moved toward the stimulus while the posterior part of the body remained stationary in S-shape. In addition to those two counts, the duration of ambush behavior was recorded if the snake exhibited an ambushing posture. We considered ambush behavior to occur if the snake suddenly curled its body into a tight S-shape posture, recording its duration, in seconds, until the snake loosened the S-shape posture. Based on Tsai (2007), *T. stejnegeri* is well adapted to artificial environments and feeds readily on prey held by forceps. Thus, the S-shaped posture observed during the experiment can be interpreted as an indication of predatory interest by the snake in the stimulus, rather than as a defensive response.

To examine whether snakes decrease tongue flicks after initiating ambush posture, we counted the number of tongue flicks separately before and after ambushing posture was initiated and maintained. If no tongue flick was observed within 30 s after the stimulus was presented, we touched the snake's snout gently three times with the paper towel. If no tongue flick occurred after an additional 30 s, the trial was terminated. The sequence of tested stimuli was randomized among individuals, to avoid order effects. The number of tongue flicks and head movements were counted by the same observer. Each trial was recorded with a video camera (Sony, CX370V) to determine the exact time when the individual initiated and terminated ambush behavior.

We used a Generalized Linear Mixed Model (GLMM) to examine the response of tongue flicks to different chemical stimuli (Danaisawadi et al., 2015). Species nested within anuran family was used as fixed factor. The individual snake was included in the model as a random factor. If main effects of anuran families or species were detected, multiple comparisons were conducted using an estimated marginal means test with Bonferroni correction (Tourmente et al.,

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2019). Paired comparisons were made among anuran families and distilled water, to determine whether snakes discriminate anuran scents from the control and whether snakes discriminate among anuran families. Paired comparisons were also conducted among species within a family (Rhacophoridae and Ranidae) to determine whether snakes differentiate confamilial species.

Compared to active foragers, maintaining crypsis is more important for sit-and-wait predators in order to increase foraging success. This leads to the prediction that ambushing snakes might reduce tongue flicks to avoid being detected by prey through the movement of the tongue (Cooper, 2007). To test this prediction, chemical stimuli for which *T. stejnegeri* showed a high level of interest were examined using two-tailed Wilcoxon-signed rank tests. If a snake exhibited the ambushing posture during a trial, we compared the number of tongue flicks before and after ambushing behavior was initiated.

In addition, assuming that *T. stejnegeri* shows a shift of behavior from frequent tongue flicks to an ambush posture with reduced tongue flicks when it recognizes a chemical stimulus of interest, we evaluated the response of snakes by combining tongue flicks and ambushing behavior. To quantify this responsiveness, we modified the tongue flick ambush score (TFAM) used by Clark (2004) as below:

TFAM=TF+Duration of ambush behavior

TF indicates the number of tongue flicks recorded during 60 s. Duration of ambush behavior was the total duration, in seconds, that the snake exhibited ambush behavior. We used a GLMM to examine the effects of the chemical stimulus on TFAM (Danaisawadi et al., 2015). Species was nested within family as fixed factor, and the individual snake was included in the model as a random factor. If main effects of anuran families or species were detected, multiple comparisons were conducted using an estimated marginal means test with Bonferroni correction (Tourmente et al., 2019). Paired comparisons were made among anuran families and distilled water, to determine whether snakes discriminate anuran scents from the control and whether snakes discriminate among anuran families. Paired comparisons were also conducted among species within a family (Rhacophoridae and Ranidae) to examine whether snakes differentiate confamilial species. All analyses were conducted using SPSS 22.

To examine whether the prey preferences observed in the tongue flick experiment reflect the diet in the wild, stomach contents of *T. stejnegeri* were investigated between April 2017 and June 2019 in Chinan Forest Recreation Area, Hualien County, Taiwan (23°55'06.5" N, 121°30'06.3" E), as well as in the three locations where the snakes were collected for the above experiment. The eight anuran species used as the chemical stimuli were also present in Chinan Forest Recreation Area. Stomach contents of *T. stejnegeri* were examined by palpation and forced regurgitation. Prey remains were identified to the species level whenever possible. Snakes were released at the exact capture site after examination of stomach contents, except for individuals that retained for use in the tongue flick experiment.

Results

Although the test subjects demonstrated different numbers of tongue flicks, most individuals showed similar trends in their responses to the different chemical stimuli. Nonetheless, a statistically significant effect of chemical stimulus was not detected in the number of tongue flicks (GLMM, $F=1.253$, $df=1, 8$, $P=0.277$). The estimated marginal mean (±SE) of tongue flick among families, from highest to lowest, was: Rhacophoridae (17.3±2.4), Ranidae (11.9±2.7), Bufonidae (9.4±4.7), and distilled water (5.3±4.7). The estimated marginal mean (±SE) of each stimulus, from highest to lowest, was: *Z. moltrechti* (21.1±7.4), *P. braueri* (20.5±6.9), *O. swinhoana* (13.9±3.9), *Br. robusta* (13.1±4.7), *Br. otai* (12.9±3.2), *H. latouchii*

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(11.3±4.0), *Ba. adenopleura* (10.4±2.6), *Bf. bankorensis* (9.5±2.9), and distilled water (5.2±2.3).

Ambush behavior was observed in only 12 trials. The number of snakes that showed ambush behavior was highest in response to the stimuli from Rhacophoridae (n=7/68), followed by Ranidae (n=3/51), Bufonidae (n=1/17), and water (n=1/17). The longest mean ambush duration was observed in response to the stimuli from Rhacophoridae (mean±SE=40.1±5.3 s), followed by Ranidae (11.3±2.0 s), water (28 s), and Bufonidae (22 s). Once the snake started ambush behavior, ambush posture of all individuals did not change after it began. The mean (±SE) number of tongue flicks per second before and after ambush behavior were 0.378 (±0.16) and 0.310 (±0.18), respectively (Fig. 1). The mean number of tongue flicks per second before ambush behavior tended to be higher in response to cues from Rhacophoridae (0.57) than from Ranidae (0.10), Bufonidae (0.21), and distilled water (0.06). In 10 out of the 12 trials snakes initiated tongue flicks before ambush behavior occurred. For the preferred stimuli (*H. latouchii*, *O. swinhoana*, *Z. moltrechti*, and *P. braueri*), the number of tongue flicks per second did not show significant differences between before and after ambushing (Z=–0.169, n=7, P=0.866). Snakes did not frequently exhibit head movements during the trials. Overall, head movement was recorded in 37 trials. More than half of the head movements were recorded in trials with Rhacophoridae (n=21/68), followed by trials with Ranidae (n=14/51). Few individuals showed head movements in trials with Bufonidae (n=2/17). No head movement was observed during trial with water. The highest number of head movements was observed in trials with *Z. moltrechti* (n=7/17).

Significant effects of chemical stimuli were detected in TFAM (GLMM, F=3.287, df=1, 8, P=0.019), in which significant differences were detected in responses by family (F=6.198, df=3, 17, P=0.005) and by species

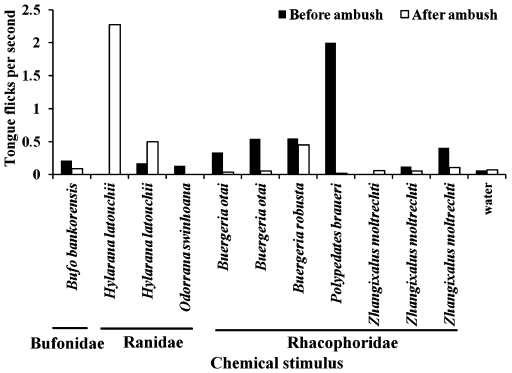


FIG. 1. The number of tongue flicks per second observed before and after ambush behavior occurred in each trial where *Trimeresurus stejnegeri* exhibited ambush posture. Ambush behavior was exhibited in a total of 12 trials, involving eight individual snakes.

(F=3.287, df=8, 17, P=0.019) (Fig. 2). The estimated marginal mean of TFAM of each family, from highest to lowest, was: Rhacophoridae (19.6±0.5), Ranidae (17.2±4.1), Bufonidae (9.8±2.7), and distilled water (6.6±2.0). Distilled water showed significant differences from Rhacophoridae (P=0.005) and Ranidae (P=0.027). Rhacophoridae also showed a significant difference from Bufonidae (P=0.001). The estimated marginal mean of each stimulus, from highest to lowest, was: *Z. moltrechti* (28.8±9.4), *P. braueri* (21.8±7.4), *O. swinhoana* (21.4±8.4), *H. latouchii* (20.7±8.9), *Br. otai* (16.9±4.3), *Br. robusta* (10.8±4.2), *B. bankorensis* (9.8±2.7), *Ba. adenopleura* (9.5±1.8), and distilled water (6.6±2.0). Among Rhacophoridae, significant species differences were detected only between *Z. moltrechti* and *Br. robusta* (P=0.041). No significant differences were observed between species in the paired comparisons among Ranidae.

A total of 418 individuals of *T. stejnegeri* were captured in the field, and 46 of those individuals contained stomach contents. Thirty-four stomach contents were identified as frog species (Table 1); 16 items were rhacophorids, 11 were ranids, four were microhyl-

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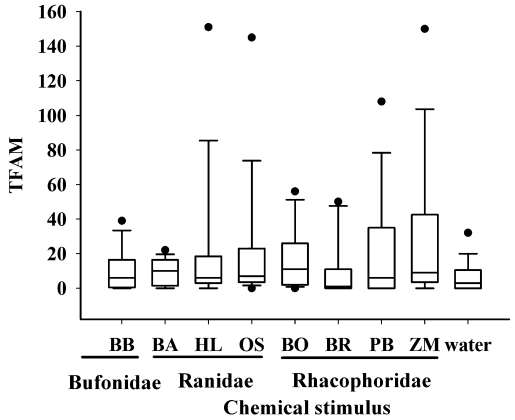


FIG. 2. Tongue flick ambush score (TFAM) of *Trimeresurus stejnegeri* in the chemical stimulus test. Solid horizontal lines within boxes represent the median. Edges of the boxes represent 25% and 75% quartiles. Whiskers represent 10th and 90th percentiles. Black dots represent outliers. BB, *Bufo bankorensis*; BA, *Babina adenopleura*; HL, *Hylarana latouchii*; OS, *Odorrana swinhoana*; BO, *Buergeria otai*; BR, *Buergeria robusta*; PB, *Polypedates braueri*; ZM, *Zhangixalus moltrechti*.

ids, and one was a dicroglossid species. Nine items were identified as reptiles, including *Diploderma swinhonis*, *Gekko hokouensis*, and *Hemidactylus bowringii*. The remaining three items could not be identified because only pieces of tissue were recovered. The two most common prey species were *O. swinhoana* (n=8) and *P. braueri* (n=7).

DISCUSSION

Trimeresurus stejnegeri discriminated among anuran families based on chemical stimuli. *Trimeresurus stejnegeri* did not prefer Bufonidae, which was represented by the chemical stimulus of *Bf. bankorensis* in our study. This may be due to the toxic secretions of Bufonidae (Garg et al., 2007). Stomach contents of *T. stejnegeri* also showed no evidence of bufonid species. These results suggest that the toxic secretion of toads could drive *T. stejnegeri* to avoid selecting toads as diet.

Although *T. stejnegeri* generally showed interest in rhacophorid and ranid species, the different level of chemical preference among the species in those two families indicated that the snake recognized the difference among those confamilial species. Three of the five chemically preferred anuran species, (*Z. moltrechti*, *P. braueri*, and *O. swinhoana*) showed levels of chemical preference consistent with their occurrence in the natural diet (Table 2). The breeding period of *P. braueri* and *O. swinhoana* occurs from spring to fall, when *T. stejnegeri* is active. Their high rates of occurrence in the natural diet indicate that both those species are important prey of *T. stejnegeri*. Diet selection based on prey abundance is a common foraging tactic of predators to increase foraging success (Riechert, 1991).

Zhangixalus moltrechti was less frequently observed in the natural diet of *T. stejnegeri* than were *P. braueri* and *O. swinhoana*. This may reflect the fact that the breeding season of *Z. moltrechti* is restricted to winter (Chang et al., 2014), when we did not conduct the field survey. Given that *Z. moltrechti* appeared in the stomach contents of *T. stejnegeri* even in the non-breeding period, we consider that *Z. moltrechti* is a potentially preferred dietary item of *T. stejnegeri*.

The present result suggests that *T. stejnegeri* is able to discriminate congeneric species. *Buergeria robusta* and *Br. otai* are both lotic-dwelling species (Huang et al., 2001), but they yielded inconsistent results in the TFAM and in the natural diet. Not many behavioral or ecological differences can be found between these species. We suggest that body size could be a factor underlying the different results between them, because adult *Br. otai* are smaller than *Br. robusta* (Wang et al., 2017). Further experiments should be conducted to test this presumption.

For *H. latouchii*, *T. stejnegeri* showed high interest in TFAM, but only one individual was found among the stomach contents. Although *H. latouchii* is a common species at our study sites, the low predation rate suggests that this

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Table 1. Stomach contents of *Trimeresurus stejnegeri* captured in Hualien and Ilan Counties, Taiwan during 2017 to 2019. Stomach contents other than frogs (nine reptile species and three unidentified items) were excluded.

Order	Family	Species	n
Anura	Microhylidae	*Microhyla fissipes*	4
	Dicroglossidae	*Fejervarya kawamurai*	1
	Ranidae	*Babina adenopleura*	1
		Hylarana guentheri	1
		Hylarana latouchii	1
		Odorrana swinhoana	8
	Rhacophoridae	*Buergeria otai*	3
		Kurixalus idiootocus	2
		Polypedates braueri	7
		Zhangixalus moltrechti	4
		Unable to identify	2

Table 2. Diet preference of *Trimeresurus stejnegeri*, compared between chemical stimulus tests (tongue flick ambush [TFAM] score) and stomach contents collected in the field. Estimated marginal means tests (EMM) were made between the control stimulus (distilled water) and each anuran stimulus. Preference was categorized as little interest (L: EMM<10), medium interest (M: 10<EMM<20), and high interest (H: EMM>20). Numerals listed for the natural diet indicate the number of individuals found in stomach contents. BB, *Bufo bankorensis*; BA, *Babina adenopleura*; HL, *Hylarana latouchii*; OS, *Odorrana swinhoana*; BO, *Buergeria otai*; BR, *Buergeria robusta*; PB, *Polypedates braueri*; ZM, *Zhangixalus moltrechti*.

	Bufonidae	Ranidae			Rhacophoridae			
	BB	BA	HL	OS	BO	BR	PB	ZM
TFAM	L	L	H	H	M	M	H	H
Natural diet	0	1	1	8	3	0	7	4

species possesses effective antipredator traits that lower the chance of predation by *T. stejnegeri*. Because both the snake and the frog are nocturnal, cryptic body coloration of the frogs would have little effect on predator avoidance. We suggest that locomotor ability of the frog enhances successful escape from the snake's attack and may be a key factor behind the inconsistent results between TFAM and the stomach contents. Staged encounter experiments could be performed to test this hypothesis.

Because of the long breeding period (Cui et al., 2016) and high abundance of *Ba. adenopleura*, we expected that *T. stejnegeri* would show a high level of interest in this frog. However, *T. stejnegeri* showed low interest in *Ba. adenopleura*, based on TFAM, and only one *T. stejnegeri* contained *Ba. adenopleura* in its stomach contents. In addition, no previous research has reported *Ba. adenopleura* in the snake's diet. It is likely that *Ba. adenopleura* possesses unpalatable skin secretions that defend it against predators.

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We did not conduct chemical preference tests with four frog species, *M. fissipes*, *F. kawamurai*, *H. guentheri*, and *K. idiootocus*, which were found among the stomach contents of *T. stejnegeri*. This was because these four species have not been recorded as common frogs at our study sites and each species has been reported from only one or two sites. However, the occurrence of multiple individuals of *M. fissipes* as the stomach contents of *T. stejnegeri* suggests the importance of this frog as a potential dietary component of the snake, which warrants further chemical preference tests in the future.

Frequent tongue flicks by snakes could increase the likelihood of detection by prey (Chiszar and Radcliffe, 1976). In the present study, no significant difference in tongue flick frequency in response to preferred stimuli was detected between before and after ambushing behavior. However, the overall tendency suggests that *T. stejnegeri* may decrease tongue flicks after initiating ambushing behavior. Cessation of tongue flicking immediately after initiating an ambush posture suggests that the purpose is to decrease the possibility of detection by the prey. Previous studies have shown that the final decision to initiate striking prey depends on a combination of senses, such as visual and tactile ones (Catania, 2010; Chen et al., 2017). This indicates that snakes do not require tongue flicks to locate prey and launch a strike within a close range. We suggest that exhibiting ambush posture during the experiment is an important indicator of prey preference in ambushing snake species. Nevertheless, *T. stejnegeri* emitted more tongue flicks after ambushing in two trials with *H. latouchii*, suggesting that tongue flicking may serve other functions when in ambush posture.

In summary, we demonstrated that *T. stejnegeri* showed different levels of preference for chemical stimuli among prey species. In sit-and-wait predators, selecting ambush sites with high abundance of prey would be an ideal tactic to decrease search time for prey and enhance foraging rate (Wittenberg, 2012). However, strong chemical prey preference does not necessarily coincide with the actual diet in the wild. Defensive mechanisms of prey species could affect their abundance in the realized diet. Future research on defensive mechanisms among various prey species, such as locomotor ability and chemical defenses, and ambush site selection by *T. stejnegeri* are necessary to clarify the discrepancy between observed chemical preferences and actual diet.

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