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Chemosensory responses to chemical and visual stimuli in five species of colubrid snakes

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Abstract. Snakes utilize chemical and visual stimuli during predation, however the emphasis on these cues and which cues are used to initiate predation varies among species. For example, rattlesnakes using the ambush strategy rely on chemical cues to locate an ambush station, then visual and thermal cues to initiate envenomating strikes, then chemical cues again to track prey. By contrast, many natricine snakes use chemical cues to initiate predation, increasing the rate of tongue flicking regardless of whether visual cues are present. The present study examined the individual and interactive effects of chemical and visual stimuli of prey on the predatory behavior of five snake taxa representing three feeding guilds. Bull snakes (*Pituophis catenifer*), Eastern Corn snakes (*Pantherophis guttatus*), and Midland Rat snakes (*Scotophis spiloides*) have a diet primarily consisting of mammals; Western Fox snakes (*Mintonius vulpina*) prey primarily on bird eggs; and Common Kingsnakes (*Lampropeltis getula*) prey equally on mammals and reptiles. Three patterns of response to chemical and visual stimuli of the test prey (*Mus musculus*) were observed. Mammal specialists responded to chemical cues. Fox snakes responded to visual cues, but not to chemical cues. Kingsnakes exhibited increased rates of tongue flicking in response to both chemical and visual stimuli. This study suggests correlations between the evolution of prey preference, foraging ecology and the utilization of chemical or visual stimuli by snakes.

Keywords. Chemical cues, predatory behavior, tongue flicking, visual cues, vomeronasal chemoreception

INTRODUCTION

Most squamates have three chemosensory systems: the nasal olfactory system, the vomeronasal system, and taste buds (Schwenk, 1995). Chemoreception in snakes is known

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to mediate numerous behaviors, including mate selection, exploratory behavior, predator identification, prey choice, and prey location (Kubie et al., 1978; Weldon and Burghardt, 1979; Chiszar and Scudder, 1980; Clark 2004; Saviola et al., in press). Snakes typically recognize potential prey either by chemical cues produced by exocrine glands and other sources (Chiszar et al., 1990; Duvall et al., 1990), or by thermal and visual cues (Herzog and Burghardt, 1974; Chiszar et al., 1981; Hennessy and Owings, 1988). Natricine snakes, for example, respond to visual stimuli with orientation and investigation, however further behaviors such as increased rates of tongue flicking, trailing, and striking are initiated by the presence of olfactory cues (Burghardt, 1969, 1970; Chiszar et al., 1981). Midland Rat snakes (*Scotophis spiloides*) utilize the behavior of avian provisioning as a visual stimulus for locating bird nests in a natural setting (Eichholz and Koenig, 1992; Neal et al., 1993; Mullin and Cooper, 1998). However, Crotalid species utilize chemical cues in ambush site selection, yet visual-thermal cues initiate the strike, then chemical cues are again used to relocate the envenomated prey (Dullemeijer, 1961; Chiszar et al., 1992; Kardong, 1992; Clark, 2004). Broad headed snakes (*Hoplocephalus bungaroides*) select ambush sites based on both visual and chemical stimuli (Du et al., 2009). Clearly, the pattern of response to stimuli arising from prey varies among snake taxa.

Tongue flicking in snakes is a stimulus-seeking behavior, and is the main process for delivering volatile and non-volatile cues to the vomeronasal organs (Halpern, 1992; Cooper, 1994), which mediates definitive analysis of chemical information (Cowles and Phelan, 1958; Halpern, 1992). Tongue flicking is activated by detection of volatile chemical cues by the nasal olfactory system, and by visual or thermal stimulation (Burghardt 1970; Chiszar et al., 1981; Burghardt and Denny, 1983; Saviola et al., 2011); therefore, the rate of tongue flicking can be used as a convenient measure of a snake's response to any or all of these stimuli.

Chiszar et al. (1981) showed, during presentation of prey, that Garter snakes responded with an increased rate of tongue flicking to chemical cues irrespective of whether visual stimuli were present or absent, whereas Rattlesnakes did not respond significantly to chemical stimuli and showed a greater rate of tongue flicking to visual-thermal stimuli relative to Garter snakes. Further, Burghardt and Denny (1983) showed that Garter snakes selected moving prey when chemical cues were present compared to moving prey without chemical cues. Feeding experience may alter chemosensory responses in snakes (Burghardt, 1993), and Cooper (2008) showed that evolutionary changes in chemosensory responsiveness to chemical cues from prey is correlated with dietary change. Relatively few studies examining chemosensory responses in snakes combine chemical stimuli with the presence of visual stimuli, or examine chemosensory responses derived from visual cues alone. In addition, interactions between these two stimuli are infrequently reported, however there is accumulating information demonstrating the interactive effects of chemical and visual cues stimulating vomeronasal chemoreception in a few species of snakes (Terrick et al. 1995; Shivik, 1998). The goal of the present study was to test the hypothesis that snake species with different diets will show different responses to prey-derived cues, favoring those most likely to allow detection and capture of preferred prey. We tested responses to chemical and visual stimuli in snake taxa that feed primarily on either mammals (Eastern Corn snake, *Pantherophis guttatus*, Midland Rat snake, *Scotophis spiloides*, and Bull snake, *Pituophis catenifer*), bird eggs (Western Fox snake, *Mintonius vulpina*), or equally on several different prey types (Common Kingsnake, *Lampropeltis getula*) (Rodríguez-Robles and DeJesús-Escobar, 1999; nomenclature from Collins and Taggart, 2009). We measured the rate of tongue flicking as well as time spent investigating the stimuli as indicators of predatory behavior.

MATERIALS AND METHODS

Six *L. getula*, nine *P. guttatus*, five *S. spiloides,* four *M. vulpina,* and seven *P. catenifer* were subjects. The snakes were all wild-caught adults, and had been in captivity for at least two years, housed individually in glass terraria (62x32x32 cm) containing paper floor covers, hide boxes, water bowls, and rocks to assist with shedding. The laboratory was maintained at 26-30 °C and photoperiod was automatically controlled on a 12:12 Light:Dark cycle. Prey used for the behavioral experiments were mice (*Mus musculus*) provided by the Department of Molecular, Cellular, and Developmental Biology, University of Colorado at Boulder. Snakes were fed euthanized laboratory mice weekly prior to and during the study period, and feeding during the experiment occurred several hours after tests. Successive tests on the same snakes were always separated by at least one week. All snakes always attacked prey immediately during feeding sessions, hence we considered them to be equally responsive to prey.

Stimuli were presented to snakes using plexiglass boxes (10x10x10 cm) of four types: clear without perforations, permitting only visual cues to be presented to the snake; clear with perforations on each side, allowing presentation of both visual and chemical cues; black with no perforations, preventing both visual and chemical cues, and black with perforations, allowing presentation of chemical but not visual cues (Chiszar et al., 1981, 2009; Saviola et al., 2011). All perforations were circular, two mm in diameter, therefore large enough to permit passage of volatile chemicals, but not large enough to permit visual examination of the interior of the box. All boxes were washed using Quatricide-PV®, a commercial disinfectant and deodorant to remove any traces of chemical cues between trials. Each snake was tested with each of the four boxes, and a single adult male mouse, 17-20 grams, was placed inside the boxes. As an additional control, we also observed each snake with a black, unperforated box without a mouse.

The five conditions were offered to each snake in random order, and the length of each trial was 10 min. A box was placed into the snake's terrarium, and all tongue flicks emitted and seconds spent investigating the stimulus box were recorded every minute for the duration of the trial. We also recorded a third operationally distinct dependent variable, the number of tongue flicks aimed at the stimulus box. Correlations between all pairs of dependent variables were calculated with the result that the third variable was highly related to those already mentioned (r = 0.75 and r = 0.88, respectively). Not surprisingly, the outcomes of inferential test (see below) were not different for this dependent variable relative to the outcomes for the other two, especially time spent investigating the stimulus boxes. Hence, we omit further treatment of the third dependent variable. Incidentally, the correlation between the first and second dependent variables was r = 0.65. Though significant, this correlation was low enough to permit different, inferential outcomes, and this in fact occurred in a few cases. Since it was obvious which condition was being tested (i.e. clear box vs. a black box, etc), we were unable to record the dependent variables blind to the conditions.

Data for each species were analyzed first by 5 (species) x 5 (conditions) x 10 (minutes) ANOVAs completed on total tongue flicks and number of seconds at the box. Then data for each species were analyzed separately using analyses of variance (ANOVA) and Newman-Kuels post-hoc tests. Two types of ANOVAs were used. First, a one-way ANOVA with repeated measures was applied to each of the five conditions (four conditions containing a live mouse and the additional control of the black, unperforated box without a mouse) for each species. Second, 2x2 ANOVAs treated presence vs. absence of chemical and visual cues as orthogonal factors, using the four relevant conditions. In the 2x2 ANOVAs, all interactions involving subjects were pooled, giving rise to a composite error term with greater power than each of the individual interactions (Hicks, 1964).

RESULTS

Tongue Flicks

Mean number of tongue flicks (± SEM) emitted during the 10 min trials are shown in Figure 1. For all conditions and species, a 5 (species) x 5 (conditions) x 10 (min) ANOVA treated species as a between-subjects factor and conditions and minutes as repeated-measures factors. All main effects were significant (species: $F = 7.69$, df = 4,26, $p < 0.01$; conditions: $F = 8.05$, df = 4, 104, $p < 0.01$; minutes: $F = 7.32$, df = 9, 234, $p < 0.01$), but no interaction was significant. However, the species x conditions interaction was close ($F = 2.00$ df = 16, 104, $p < 0.05$), but then corrected by the Greenhouse and Geisser (Greenhouse and Geisser, 1959; see also Winer, 1971) method, the df were reduced to 4, 26, and the F ratio was then only marginal ($0.10 > p > 0.05$). No other interaction came close to being significant before or after the Greenhouse and Geisser (1959) correction. This is particularly interesting in the case of the robust main effect of minutes. The mean number of tongue flicks declined over minutes starting at 96.8 on min 1 (across all species and conditions) and ending at 65.5 on minute 10, but the magnitude of the decline did not vary with species or conditions. In other words, the rate of habituation (or sensory adaptation) was essentially constant, regardless of the starting level. It must be kept in mind, however, that the rate on minute 10 was rather high, indicating that the snakes were still interested in the stimuli. Whether, the rate of tongue flicking after minute 10 would continue to decline at a constant rate is conjectural.

Data from each species were subjected to additional ANOVAs in order to follow up the 5x5x10 ANOVA. First, the data from each species were subjected to one-way repeated measures ANOVAs to verify the conditions main effect. Second, the data from each species were subjected to 2x2 repeated-measures ANOVAs to assess the effects of absence vs. presence of visual and chemical cues. All *F* ratios from these ANOVAs are shown in Table 1. Results of Newman-Keuls post-hoc comparisons are shown by superscripts in Fig. 1.

Four one-way ANOVAs revealed significant main effect of conditions; for Bull snakes the *F* ratio for the conditions main effect fell slightly short such that $0.10 > p > 0.05$. Thus, one or more significant differences probably existed among conditions within each species (see Fig. 1). For all species except Kingsnakes the mean for the additional control (empty unperforated black box) was not significantly different from the mean for the condition where a mouse was in the unperforated black box, implying that all snakes except Kingsnakes failed to detect the mouse in the latter condition. Because Kingsnakes appeared to detect the mouse when no chemical or visual cues were explicitly provided, we executed the 2x2 ANOVA on the Kingsnake data twice, first we used the data from the control in which a mouse was present in the unperforated black box, then we substituted data from the additional control in a second 2x2 ANOVA. The purpose of this procedure was to remove all possible prey-derived cues, in case Kingsnakes had heightened sensitivity (especially to chemical cues) that other species lack. If such was the case, then the addi-

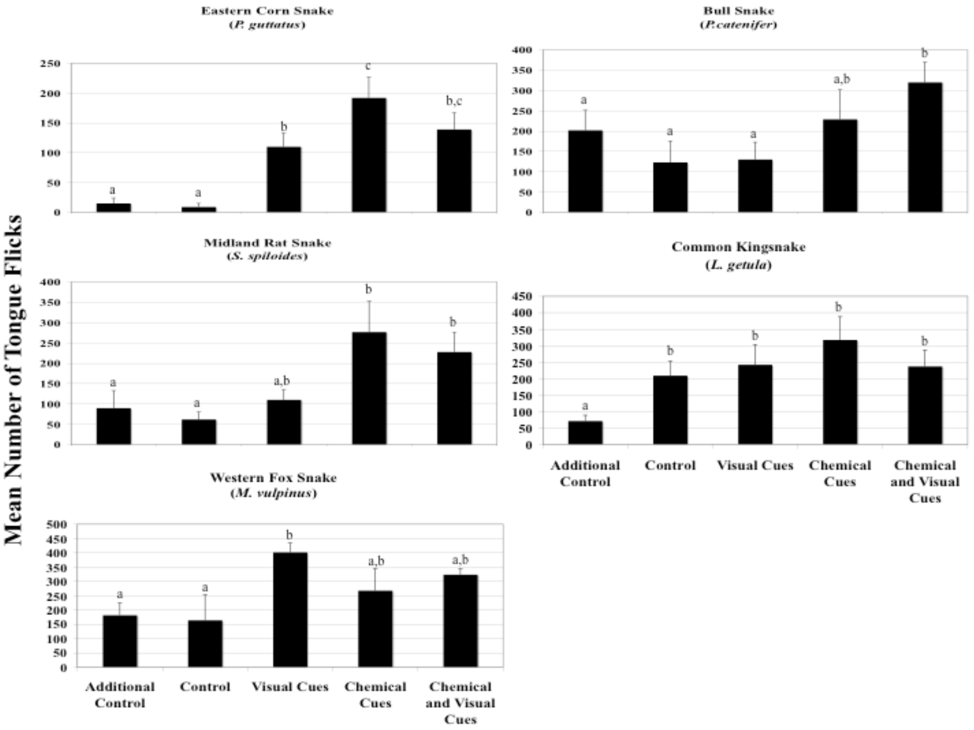

Fig. 1. Mean number of tongue flicks (those directed at the stimulus as well as any others) for the duration of the trial (± SEM) from the five species tested. Dissimilar letters above histogram bars indicate significant differences between responses; same letters indicate no significant differences. The additional control refers to the treatment with an unperforated empty black box.

Table 1. ANOVA outcomes for each species for mean numbers of tongue flicks shown in Figure 1. The second 2x2 ANOVA results for *L. getula* substitutes the additional control (unperforated empty black box) for the standard control (unperforated black box containing a mouse).

Species	One-Way ANOVA		2x2 ANOVA			
			Chemical cues	Visual cues	Interaction	
	F	df	F	F	F	df
P. guttatus	13.69*	(4, 32)	20.43**	1.03	10.79**	(1, 24)
P. catenifer	2.67^{+}	(4, 24)	3.44*	0.00	2.80	(1, 18)
M. vulpinus	4.03*	(4, 12)	0.08	9.18*	3.48^{+}	(1, 9)
S. spiloides	3.17*	(4, 16)	13.76**	0.00	1.16	(1, 12)
L. getula	3.38*	(4, 20)	0.19	0.90	1.11	(1, 15)
			6.07*	0.84	6.64*	(1, 15)

$+\ .10 > p > .05$; $^{*}p < 0.05$; $^{**}p < 0.01$

tional control would be more appropriate for Kingsnakes than the standard control. Outcomes of both 2x2 ANOVAs are shown in Table 1.

Two patters of response are visible in Table 1. Corn snakes, Bull snakes, Rat snakes, and Kingsnakes, (second ANOVA) exhibited significant effects of chemical cues, no effects of visual cues, and Corn and Kingsnakes also exhibited a significant interaction. These four species responded to visual cues slightly more when chemical cues were absent but not when chemical cues were present. For Corn snakes and Kingsnakes, this circumstance was quite obvious and it was also clear the presence of chemical cues generated a stronger response than visual cues. Hence, for these four species, chemical cues were of primary importance and visual cues were at most secondary. Fox snakes, by contrast, revealed a significant effect of visual cues, no effect of chemical cues and a marginal interaction, reflecting use of chemical cues when visual cues were absent but not when visual cues were present.

Number of Seconds Spent Investigating Stimuli

Figure 2 shows the mean number of seconds spent investigating the stimulus boxes for all species and conditions. A 5 (species) x 5 (conditions) x 10 (min) ANOVA revealed a strong main effect of conditions ($F = 18.24$, $df = 4, 104$, $p < 0.01$) but no main effect of species or minutes. One interaction survived the Greenhouse and Geisser (1959) correction, species x condition ($F = 3.40$, $df = 16, 104$, $p < 0.01$; when corrected, the df drops to 4, 26, and this caused a drop in $p < 0.05$).

One-way ANOVAs (Table 2) revealed significant main effects of conditions in all species, except Rat snakes where the effect was marginal ($0.10 > p > 0.05$). Hence, there likely were significant differences among treatment means in all species. Subsequently 2x2 ANOVAs verified significant effects of chemical cues in Corn snakes, Rat snakes, Bull snakes, and Kingsnakes (second ANOVA). Fox snakes did not respond to chemical cues but exhibited a strong effect of visual cues. Bull snakes also showed a significant effect of visual cues, and Kingsnakes showed a marginal effect (second ANOVA). Corn snakes also showed a marginal interaction. Bull snakes, Corn snakes, Rat snakes, and Kingsnakes showed strong effects of chemical cues and a small effect of visual cues when chemical cues were not present. Hence, chemical cues were of primary importance for these four taxa and visual cues were, again, at most secondary. For Fox snakes, on the other hand, visual cues were of primary importance and chemical cues added little or nothing to the response.

The species x conditions interactions detected by the 5x5x10 ANOVAs (marginal for tongue flicking, significant for time spent investigating the boxes) are explained by the differential responses shown by Fox snakes versus the other four taxa.

The fact that the two dependent variables gave different ANOVA outcomes is important. It tells us that tongue flicking is readily elicited and runs its course more-or-less independently of other factors. Number of seconds spent investigating the boxes appears to be a more discriminating dependent variable that reveals whether or not the stimulus is strong enough to command attention. That is, a snake may be tongue flicking (searching) but this does not necessarily imply that the snake is aware of the presence of a mouse. A lot of time spent at the box, on the other hand, may imply that the snake is aware of the prey.

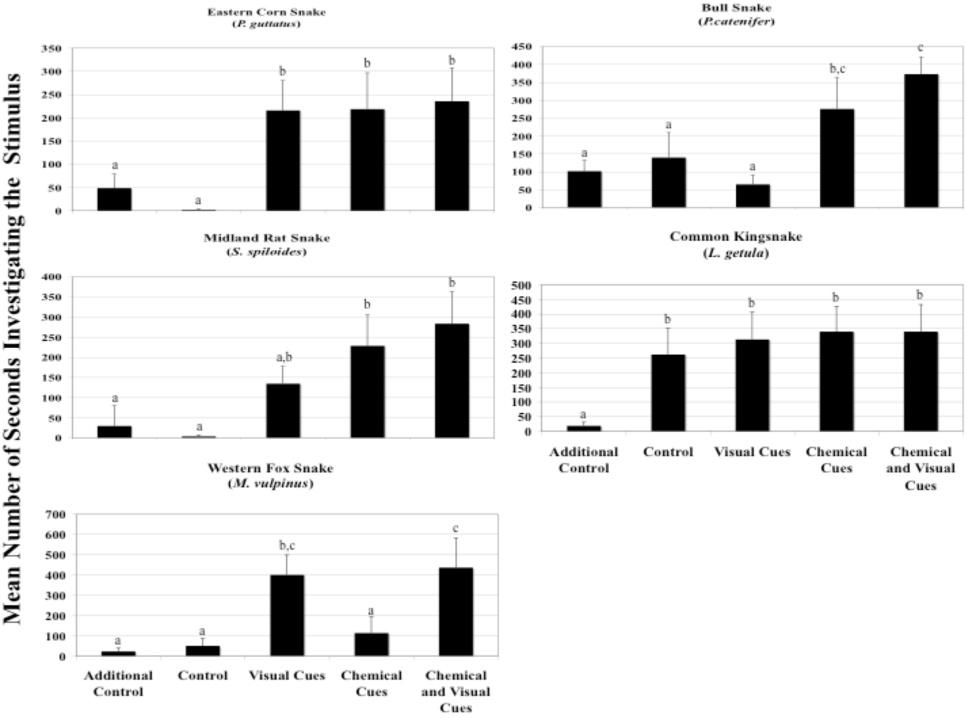

Fig. 2. Mean number of seconds spent investigating the stimulus boxes (± SEM) by the five species tested. Dissimilar letters above histogram bars indicate significant differences between responses; same letters indicate no significant differences.

Table 2. ANOVA outcomes for each species for means shown in Figure 2.

Species	One-Way ANOVA		2x2 ANOVA			
			Chemical cues	Visual cues	Interaction	
	F	df	F	F	F	df
P. guttatus	4.16^{**}	(4, 32)	4.67^{*}	4.40^{*}	3.22^{+}	(1, 24)
S. spiloides	2.58^{+}	(4, 16)	8.95^{*}	2.20	0.37	(1, 12)
M. vulpinus	5.39^{*}	(4, 12)	0.29	13.93^{**}	0.02	(1, 9)
P. catenifer	4.45^{**}	(4, 24)	11.06^{**}	0.02	1.66	(1, 18)
L. getula	3.37^{*}	(4, 20)	0.44	0.10	0.10	(1, 15)
			5.06^{*}	3.57^{+}	1.60	(1, 15)

+ .10 > p > .05; *p < 0.05; **p < 0.01

DISCUSSION

An unexpected outcome of this study was the finding that Kingsnakes detected the mouse inside the unperforated black box whereas none of the other taxa appeared to do so. This could have been based on greater sensitivity by Kingsnakes to tactile or thermal cues. It is also possible that Kingsnakes were able to detect small traces of chemical cues in the mouse-present control condition whereas the other taxa lacked this keener sensitivity. Whatever the reason, it appears that Kingsnakes have a sensory ability that the other snakes lack.

Aside from this aspect of the results, we saw two patterns of behavior. For all taxa except Fox snakes, chemical cues were utilized and visual cues played only a secondary role, if any in our study. The second pattern was observed in Fox snakes where visual cues invariably exerted a significant main effect on both dependent variables, with chemical cues having no significance.

Natural selection can be expected to influence snakes to respond to those stimuli that are most likely to lead to capture of preferred prey (Tinbergen, 1951; Cooper, 2008). In other words, the evolution of prey-type preferences and foraging ecology could also determine differential responses to chemical and visual stimuli. Bull snakes, Corn Snakes, and Rat snakes emphasize mammals in their diet, Fox snakes primarily utilize birds eggs, and Kingsnakes equally take several types of prey, ranging from mammals and snakes to lizards and squamate eggs (Mullin, 1998; Rodríguez-Robles and DeJesús-Escobar, 1999). Therefore, our study encompasses two or three distinct snake feeding guilds.

A strong response from snakes to rodent chemical cues may arise due to the numerous exocrine glands of mammals associated with their heavy reliance upon pheromonal cues during social and reproductive behaviors (Mateo and Johnston, 2000). Therefore, snakes specializing on rodent prey may have taken advantage of the sensory channel most valuable to their prey and, hence, of stimuli most available to the predators. While chemical cues are critical for prey recognition in some snakes, visual cues are key to capturing prey in other snake species (Ford and Burghardt, 1993; Saviola et al., 2011). Consistent with the results from the present study, Burghardt and Abeshaheen (1971) found that neonate Fox snakes did not strike at cotton-swabs soaked in prey extract, but did respond with strikes to a wide variety of visual stimuli. The greater rate of tongue flicking in the presence of visual cues observed here is also consistent with the fact that Fox snakes prey on ground-nesting birds (Rodríguez-Robles and DeJesús-Escobar, 1999), and that foraging snakes may locate nests by responding to the visual cues of provisioning birds (Porter and Czaplicki, 1977). Although rodents leave odor trails that can be utilized by predators (Chiszar et al., 1992; Clark, 2004), birds flying to and from nesting sites leave little, if any, chemical trails on the ground. Distant visual cues associated with arboreal nest cavities have been shown to be crucial in the foraging behavior of Gray Rat snakes (*Pantherophis obsoleta*) (Mullin and Cooper, 1998), although chemical cues appear to be more important as the snake gets close to the prey. The role of visual cues during predation has apparently increased in several *Thamnophis* species that respond to aquatic prey outside of tongue flicking range (Drummond, 1985). While chemical cues may be used when the snake is relatively close to the source, initial predation responses and the activity of searching are likely based primarily on visual cues from the prey.

Lampropeltis getula appears to be a generalist preying on ectotherm and endotherm prey with similar frequency (Rodríguez-Robles and DeJesús-Escobar, 1999). Such a highly varied diet may be the reason why these latter snakes showed strong rates of response to all conditions (original control box containing a live mouse as well as visual and chemical cues from prey). Perhaps these snakes attend to any disturbance or to all stimuli available to them, with no preference for one modality over another. Thus, all stimulus conditions always elicited investigation, which would make sense if the highly varied diet results in equal attention to all sensory modalities. The apparent uniformity of response across stimulus dimensions in Kingsnakes is consistent with the results from several earlier studies with this species. Neonatal Kingsnakes did not respond differentially when offered various prey extracts (Brock and Meyers, 1979), and Williams and Brisbin (1978) found that extracts of mouse, snake, chick, and amphibians did not elicit significantly different rates of tongue flicking in Kingsnakes fed exclusively on mice for 2-10 years.

Foraging behavior and diel activity patterns of prey will affect the foraging behavior of predators. Active tongue flicking to detect prey is thought to be absent in ambush foragers (except during prior location of a favorable station), as this behavior may cause the predator to be detected by the prey (Cooper, 1994, 1995). In contrast, tongue flicking (to detect volatile and non-volatile cues) in active foragers would cause little if any problems since the predators are already engaged in locomotion and other obvious movements (Cooper, 1995). Foraging underground or during the night, on the other hand, will provide few if any visual stimuli, therefore blind, fossorial, crepuscular or nocturnal snakes rely on chemical cues, whereas diurnal species foraging above ground may tend to utilize visual cues. For instance, several studies have indicated that blind snakes follow trails of commonly consumed ant prey, however ignore trails of non-consumed prey (Watkins et al., 1969; Gehlbach et al., 1971; Webb and Shine, 1992), further emphasizing the importance of chemical stimuli in predation. Although primarily diurnal, Bull snakes capture their prey by actively searching underground tunnels and other retreats of prey, or by pursuing small mammals while they rest at night (Rodríguez-Robles, 2002).

The snakes in the present study were all tested with laboratory mice (*Mus musculus*), and thus the responses to chemical stimuli by these snakes could be specific to this prey species (known to be particularly odoriferous by comparison with deer mice, *Peromyscus maniculatus*) (Duvall et al., 1990). However, Clark (2004) found that *Crotalus horridus,* born and raised in captivity and never having encountered natural prey items, still showed a greater response to chemical cues from their natural prey than to chemical cues from closely related species (see also Burghardt, 1969). Hence odoriferousness of *M. musculus* may not have biased results for snakes involved in the present study.

Several studies have examined the correlation between diet and chemosensory responses of squamates to chemical cues (e.g. Burghardt, 1967; Gove and Burghardt, 1975; Cooper et al., 2000; Cooper, 1995, 2008), and such responses have evolved to coincide with dietary shifts (Cooper, 1997, 2008; Saviola et al., in press). Although some studies have demonstrated the importance of visual cues in initiating vomeronasal chemoreception (Ford and Burghardt, 1993; Drummond 1985; Saviola et al., 2011), others fail to emphasize the combination. Recently, working with Mangrove Saltmarsh Snakes (*Nerodia clarkii compressicauda*), Hansknecht and Burghardt (2010) showed that that there was decreased lingual luring and predatory attacks in the presence of a single prey cue, however the combination of chemical and visual cues increased both of these behaviors to a greater extent. Our results indicate that even in the presence of visual stimuli, 4 of the 5 species tested demonstrated significant rates of tongue flicking to chemical cues, and visual cues were of secondary importance in our study. Therefore, it may be suggested that even in the presence of visual stimuli, for many snakes, chemical cues play primary importance in initiating predation.

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