

Behavioral Responses of a Dietary Specialist, the Queen Snake (*Regina septemvittata*), to Potential Chemoattractants Released by Its Prey

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ABSTRACT.—Queen Snakes (*Regina septemvittata*) are extreme dietary specialists, feeding nearly exclusively on freshly molted crayfish. To elucidate specific cues that mediate prey detection and foraging behavior in *R. septemvittata*, we examined the response of *R. septemvittata* and the dietary generalist *Nerodia sipedon sipedon*, to chemical compounds produced by crayfish during the molting cycle. Ingestively naive neonate snakes were presented with 20-hydroxyecdysone, ecdysone, methyl farnesoate, ponasterone A, xanthurenic acid, and distilled water. Their response was measured using a modification to the tongue-flick attack score. *Regina septemvittata* exhibited an elevated tongue-flicking response to ecdysone, whereas *N. s. sipedon* exhibited no differential responses to these same arthropod molting chemicals. In a second experiment, *R. septemvittata* showed a two-fold higher tongue-flicking response to intermolt crayfish extract that contained ecdysone than to intermolt crayfish extract alone, whereas *N. s. sipedon* demonstrated no differential response between these extracts. In a third experiment consisting of feeding trials, *R. septemvittata* was offered live intermolt crayfish with and without ecdysone. The presence of ecdysone increased the feeding scores of *R. septemvittata*. When ecdysone was present, crayfish were ingested in five of the seven trials, but when ecdysone was absent crayfish were not ingested in any trial. These results suggest that ecdysone may play a critical role in the interaction between *R. septemvittata* and their prey. With numerous stream contaminants known to imitate ecdysone and disrupt the arthropod molting cycle, this finding may have important implications for the conservation of *R. septemvittata*.

Many snake species are known for their extreme or unique dietary specializations. For example, snakes of the genus *Dasyeltis* are egg-eating specialists that can engulf hard-shelled bird eggs in their entirety (Gartner and Greene, 2008), and snakes of the genus *Dipsas* extract snails from their shells by using specialized mandibular teeth (Savitzky, 1983). Given the limitations of evolutionary modification to the basic body form of snakes, it is not surprising that this group exhibits extensive evolutionary modification of the structure of the skull, teeth, and cranial musculature associated with prey capture and ingestion (Cundall and Greene, 2000). Of all North American snakes, the members of the genus *Regina* are among the most stenophagic. To varying degrees, all four species belonging to this genus (*R. alleni*, *R. grahamii*, *R. rigida*, and *R. septemvittata*) specialize at locating and consuming crayfish (Gibbons and Dorcas, 2004). *Regina alleni* and *R. rigida* have unique, hinged palatine teeth modified for grasping the hard exoskeleton of their preferred prey, a characteristic not shared with *R. grahamii* and *R. septemvittata* (Rossman, 1985).

The most strictly monophagous member of the genus *Regina* is the Queen Snake, *R. septemvittata*. Studies consistently indicate that crayfish comprise nearly 100% of its diet (e.g., Raney and Roecker, 1947; Branson and Baker, 1974; Gibbons and Dorcas, 2004). Of particular interest, however, is the finding that this species specializes on primarily capturing and devouring freshly molted crayfish (Wood, 1949; Brown, 1979). These crayfish have a soft exoskeleton, are relatively defenseless, and more easily handled by the snakes (Wood, 1949; Godley, 1980). Freshly molted crayfish are also more nutritious than intermolt crayfish due to increased amounts of soluble proteins and lipids extracted from the shell and conserved in the body immediately before ecdysis and the loss of a large proportion of nondigestible material (Godley, 1980). However, adult crayfish molt an average of only twice per year (St. John, 1976), and the exoskeleton of a crayfish hardens within hours of ecdysis (Stevenson, 1968). Because of their vulnerability during this brief period, crayfish typically remain hidden until the integument hardens. This leaves foraging *R. septemvittata* an exceptionally limited time to find and consume cryptic prey that occur at low density. A specialized and highly directed

foraging strategy is required to make such an ephemeral food source energetically profitable.

Regina septemvittata has been observed actively foraging during the daytime among the rocks in stream bottoms (Ernst, 2003; Ernst and Ernst, 2003). Although both appearance and behavioral cues may play a role in the ability of *R. septemvittata* to discern between freshly molted and intermolt crayfish, visual foraging cues alone are probably insufficient to locate an adequate number of such inconspicuous prey dispersed at low density in a complex stream-bottom environment. Underwater foraging activity is reportedly accompanied by frequent tongue flicking (Pinder, 1966), suggesting that chemical cues may play an important role in prey location.

Ingestively naïve neonate *R. septemvittata* show a greater response to chemical extracts prepared from freshly molted crayfish than to extracts prepared from intermolt crayfish (Burghardt, 1968; Waters and Burghardt, 2005), indicating that *R. septemvittata* has an innate ability to exploit the hormonally controlled molting cycle of crustaceans to enhance their foraging success. These observations suggest that the vomeronasal organ in *R. septemvittata* probably detects at least one of the chemicals involved in the crayfish molting cycle. Studies of gartersnakes (*Thamnophis* spp.) have isolated chemoattractants from their prey (earthworms), identified binding sites for these particular molecules in the vomeronasal organ (Luo et al., 1994), and contributed to our understanding of the importance of the vomeronasal organ for prey detection in snakes (Halpern and Frumin, 1979; Halpern and Kubie, 1979). In this work, we compared the response of the dietary specialist *R. septemvittata* and the dietary generalist *Nerodia sipedon sipedon* to a variety of chemical compounds associated with the molting cycle of crayfish to elucidate the specific cues that may mediate prey detection and foraging behavior in *R. septemvittata*.

MATERIALS AND METHODS

We used seven neonate *R. septemvittata* born in captivity on 2 September, 2008 and eight neonate *N. s. sipedon* born in captivity on 19 September, 2008. Both litters were born from wild-caught females from Berks County, Pennsylvania. Snakes were housed communally by species and supplied water ad libitum in a room maintained at 28°C with a 12 : 12 (L : D) photoperiod. All the experiments were completed within 12 weeks after birth, and the snakes were not fed until the

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feeding trials conducted as part of experiment 3. Appalachian Brook Crayfish (*Cambarus bartonii bartonii*) were captured from streams in southeastern Pennsylvania and maintained in the laboratory at 12°C.

Experiment 1.—Five chemicals were chosen for examination because of their production by and presence in arthropods during molting: 20-hydroxyecdysone (β -ecdysone, Krishnakumar and Schneiderman, 1970; Rao et al., 1972), ecdysone (α -ecdysone, Stevenson and Tschantz, 1973; Chang and O'Connor, 1977), methyl farnesoate (Chang et al., 1993), ponasterone A (Kobayashi et al., 1967), and xanthurenic acid (Naya et al., 1989). We purchased all compounds except methyl farnesoate from VWR International, LLC (Radnor, PA). Methyl farnesoate was purchased from Echelon Biosciences, Inc. (Salt Lake City, UT). We diluted all compounds with distilled water to concentrations of 1 μ g/mL and added small quantities (<0.11%) of nondenatured ethanol for dissolution. Distilled water containing 0.11% ethanol served as a control.

Regina septemvittata and *N. s. sipedon* were housed individually in small plastic tanks (15 \times 13 \times 11 cm) in a darkened room during testing. Fluorescent lighting was positioned to illuminate the tanks but not the experimenter. Snakes were acclimated to the experimental tanks a minimum of 1 h before the first trial and allowed 30 min of rest between trials. All trials were completed between 1300 and 1900 h (EST). For each set of trials each tank was assigned randomly one of six chemical treatments: 20-hydroxyecdysone, ecdysone, methyl farnesoate, ponasterone A, xanthurenic acid, and the distilled water control. Snakes were assigned randomly to each tank at the beginning of each trial. A cotton-tipped applicator saturated with the assigned chemical solution was slowly positioned 1–2 cm anterior to the snout of the snake. One-minute trials began after the first tongue flick. Trials were omitted if the snake failed to tongue flick within 1 min. Several *R. septemvittata* touched their snout to the applicator and repeatedly rubbed and pushed against the applicator. This rubbing behavior seemed analogous to observations of this species natural foraging behavior of pushing their snouts into stream beds to move stones that may be concealing crayfish (Ernst, 2003; Ernst and Ernst, 2003). On other occasions, we also have observed captive *R. septemvittata* rub their nose against a crayfish before striking. Although no strikes occurred during any of the chemical trials (experiments 1–2), the nose rubbing behavior was substituted for striking in the traditional tongue-flick attack score (TFAS) because of its suspected relationship to prey recognition in this species. The trials were continued for 1 min regardless of whether this behavior took place. The total number of tongue flicks as well as the time elapsed before the first rubbing behavior was recorded to calculate a modified TFAS (Burghardt, 1967; Cooper and Burghardt, 1990).

Experiment 2.—Based on the results of experiment 1, the same snakes were tested for their response to crayfish extract with and without added ecdysone. Crayfish extract was prepared by stirring a live intermolt *C. b. bartonii* in water heated to 50°C for 1 min in the proportion of 10 mL of water/1.5 g of body mass, following the methods of Burghardt (1968). Ecdysone was added to a sample of the cooled crayfish extract to a concentration of 1 μ g/mL. These two crayfish extract solutions were used to determine whether the presence of ecdysone elicited a greater response than crayfish odors alone. Data for experiment 2 were collected in the same manner as for experiment 1.

Experiment 3.—The third experiment on feeding response was conducted only on neonate *R. septemvittata*. Snakes were placed individually in a plastic tank (15 \times 13 \times 11 cm) containing 80 mL of water. A small, live, intermolt crayfish (mean length = 7.4 mm) was introduced for 5 min. The crayfish was then removed, allowed to swim in a dish of ecdysone solution (1 μ g/mL) for 1 min, and returned to the tank for a

second 5-min trial. During each trial, the number of strikes and time elapsed until the snake grasped and eventually swallowed the crayfish were recorded. If the snake exhibited predatory behavior during the trial, the snake was observed for an additional 15 min. During these extended observation periods, we recorded the time until the snake grasped and eventually ingested the crayfish, but we ignored any additional strikes. We calculated “feeding scores” as a modification of the TFAS of Burghardt (1967) according to the following equation:

$$\text{no. of strikes} + (1,200 - \text{seconds elapsed until grasp}).$$

This equation differs from the TFAS of Burghardt (1967) by the replacement of the number of tongue flicks, the 60-sec trial, and seconds elapsed until strike with the number of strikes, a 1,200-sec trial, and seconds elapsed until the snake grasped the crayfish in its jaws, respectively. Higher feeding scores corresponded to more rapid predatory responses.

Repeated measures analysis of variance (model I, randomized block design) followed by Tukey's a posteriori tests were used to distinguish among mean modified TFAS and mean feeding scores (Sokal and Rohlf, 1995) in all three experiments. Before these analyses, the equality of group variances was confirmed with Brown and Forsythe's modification of Levene's test (Brown and Forsythe, 1974), and approximate normality was confirmed with g_1 and g_2 statistics and normal probability plots (Sokal and Rohlf, 1995). A one-tailed McNemar's test with exact binomial probability calculations (Zar, 1996) was used to determine whether the presence of ecdysone significantly increased the ingestion rate of crayfish by snakes during experiment 3. McNemar's test was performed using VassarStats (<http://faculty.vassar.edu/lowry/VassarStats.html>). All other statistical analyses were performed with Statistica (version 8.0, StatSoft, Tulsa, OK). We required a $P < 0.05$ (Type I error) for the rejection of null hypotheses.

RESULTS

Experiment 1.—Ingestively naïve *R. septemvittata* exhibited a greatly elevated mean modified TFAS response to ecdysone over all other tested chemicals (analysis of variance [ANOVA] $F_{5,35} = 3.69$, $P = 0.009$; Tukey's test: ecdysone/20-hydroxyecdysone, $P = 0.003$; ecdysone/methyl farnesoate, $P = 0.022$; ecdysone/ponasterone A, $P = 0.012$; ecdysone/xanthurenic acid, $P = 0.009$; ecdysone/water, $P = 0.022$). The snakes demonstrated a nearly three-fold increase in the TFAS when exposed to ecdysone (Fig. 1A). Comparatively, neonate *N. s. sipedon* exhibited no elevation in TFAS in response to any of the tested chemicals (Fig. 1B) and no differential response among the chemical compounds ($F_{5,40} = 0.24$, $P = 0.94$).

Experiment 2.—*Regina septemvittata* also showed two-fold higher mean modified TFAS response to intermolt crayfish extract that contained ecdysone than to the intermolt crayfish extract alone ($F_{1,6} = 21.79$, $P = 0.003$). *Nerodia s. sipedon* demonstrated no differential response to the intermolt crayfish extract with and without added ecdysone ($F_{1,6} = 1.12$, $P = 0.33$). *Regina septemvittata* and *N. s. sipedon* responded similarly to the intermolt crayfish extract without ecdysone, but the addition of ecdysone resulted in a strongly elevated response by *R. septemvittata* (Fig. 2).

Experiment 3.—In feeding trials, the presence of ecdysone caused an increase in the feeding scores of *R. septemvittata* ($F_{1,12} = 9.48$, $P = 0.009$). The mean feeding score during the trials with ecdysone was 558 (SE = 181.05, $N = 7$), whereas the mean feeding score during the trials without ecdysone was 0.4 (SE = 0.43, $N = 7$). When ecdysone was present, *R. septemvittata* struck and eventually ingested the crayfish in five of the seven trials. In the two ecdysone trials during which *R. septemvittata* did not strike, there was still a notable increase in foraging

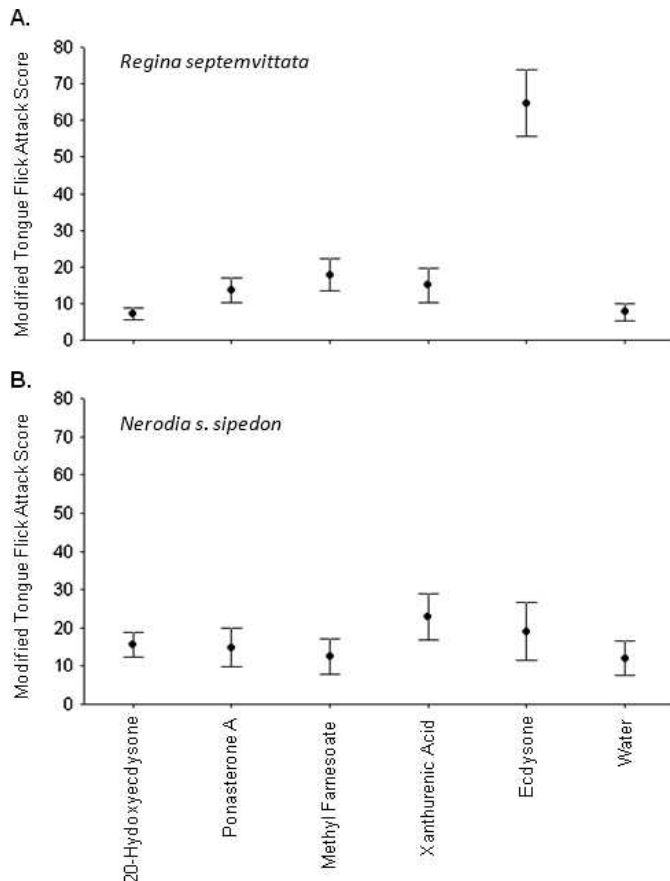


FIG. 1. Comparison of mean modified tongue-flick attack scores (\pm SE) for 7 neonate *Regina septemvittata* (A) and 8 neonate *Nerodia sipedon sipedon* (B) in response to chemicals produced by crayfish during molting.

behavior. There was a marked change in the behavior of both snakes from minimal movement during the trials without ecdysone to active searching during the trials with ecdysone. Only one *R. septemvittata* struck at a crayfish when no ecdysone was present. However, these strikes seemed to be defensive instead of predatory because they occurred in response to the crayfish repeatedly pinching the head of the snake. The presence of ecdysone significantly increased the frequency of ingestion of crayfish by *R. septemvittata* in these feeding trials (McNemar's test, $P = 0.03$).

DISCUSSION

Our results suggest that ecdysone may play a critical role in the interaction between *R. septemvittata* and its primary prey, crayfish. The lack of a differential response by *N. s. sipedon* to the same chemicals further suggests that these two snake species probably rely on different cues for prey recognition. Crayfish are not a commonly reported dietary item for *N. sipedon* (Gibbons and Dorcas, 2004). The response of *R. septemvittata* to ecdysone correlates well with the monophagous behavior of the species. An analysis of food consumption of *R. septemvittata* found that all crayfish were consumed when soft, during the A₁ and early A₂ molt stages (Godley et al., 1984).

If *R. septemvittata* depend on ecdysone as a means to locate recently molted crayfish, the excretion of ecdysone into the environment would need to be specific to these postmolt stages. Ecdysone is secreted by the crustacean Y-organ into the hemolymph, where it is rapidly converted into the active molting hormone 20-hydroxyecdysone (Chang and O'Connor,

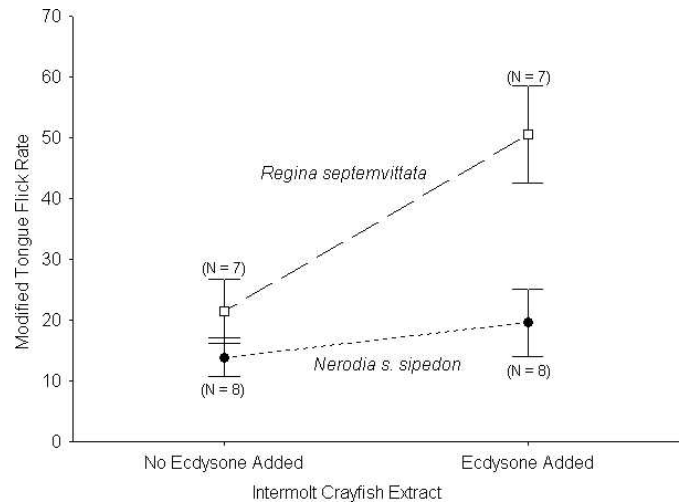


FIG. 2. Comparison of mean modified tongue-flick attack scores (\pm SE) for neonate *Regina septemvittata* ($N = 7$) and *Nerodia sipedon sipedon* ($N = 8$) in response to intermolt crayfish (*Cambarus bartonii bartonii*) extracts prepared with and without added ecdysone.

1977). This hormone has been shown to both trigger the crustacean molting process and accelerate each successive premolt stage (Stevenson and Tschantz, 1973). Ecdysteroid concentration in the hemolymph increases as the molting process proceeds, peaks approximately 6 days before molting and then decreases rapidly until molting occurs (Nakatsuji et al., 2000). American Lobsters (*Homarus americanus*) excrete ecdysone into the surrounding water at the highest concentrations during the molt stage D₂ (immediately before molting) and also at molt stage C immediately after molting (Snyder and Chang, 1991). Thus, ecdysone evidently serves its role and peaks in concentration in the body of lobsters during the premolt, but it is not excreted from the body into the environment until molting occurs. Assuming a similar process in freshwater crayfish, the release of ecdysone into stream water could facilitate the location by *R. septemvittata* of the ephemeral, highly cryptic, soft-bodied crayfish prey that this species is known to prefer.

Ecdysone also has been identified as the secretory product of the insect prothoracic gland (King et al., 1974), and the ubiquitous nature of ecdysteroids in arthropods may explain the occasional ingestion by *R. septemvittata* of other invertebrates such as fairy shrimp (Adler and Tilley, 1960) and dragonfly larvae (Raney and Roecker, 1947). However, if ecdysone were solely responsible for the identification of suitable prey by *R. septemvittata*, it is unlikely that this species would be so strongly monophagous because many other aquatic arthropods may excrete ecdysone during molting. The combination of ecdysone and crayfish odor (as tested in this study) may be necessary to elicit a predatory response. Or some other unique characteristic of crayfish (e.g., movement behavior) may serve to ultimately trigger striking and feeding behavior. Predatory strikes occurred only during the feeding trials with live crayfish, and it seemed that the movement of the prey contributed in stimulating strike responses during these trials.

Understanding the role of ecdysone in the foraging behavior of *R. septemvittata* may have important conservation implications. Ecdysone receptors in arthropods do not tightly bind their ligands and are therefore quite sensitive to interference from other molecules. Multiple water contaminants (e.g., heavy metals, estrogenic compounds, and pesticides) have been shown to inhibit molting in arthropods by interfering with proper molecular binding (Zou and Fingermann, 1997; Rodriguez Moreno et al., 2003; Yang et al., 2008). Curiously,

populations of *R. septemvittata* seem to be declining over portions of their native range despite the presence of abundant crayfish populations (Trauth, 1991; Johnson, 1992). *Regina septemvittata* is currently of conservation concern (considered extinct, endangered, threatened, imperiled, or vulnerable) in eight states and the District of Columbia (Gibbons and Dorcas, 2004; <http://www.state.nj.us/dep/fgw/tandespp.htm>; <http://www.naturalheritage.state.pa.us/vertebrates.aspx>). Due to the rarity of this species in Pennsylvania and New Jersey, our results were garnered from a relatively small number of snakes all from the same litter. Consequently, litter and sex effects could not be tested. Although this potential short-coming should be noted, the extreme dietary specialization consistently observed across age classes and throughout the geographic range of *R. septemvittata* suggests that these effects are probably minimal.

Chemosensory impairment by chemical contaminants has been noted in other species of conservation concern, such as salmon, where exposure to a realistic concentration of a pesticide mixture replicated from a British Columbian river reduced response by salmon to a behaviorally relevant amino acid odorant (Tierney et al., 2008). In view of our findings, continued studies of the delicate foraging strategy of crayfish snakes could determine whether a subtle, chemically mediated disruption of olfactory capabilities could reduce foraging success and contribute to the reported declines in *R. septemvittata* populations.

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