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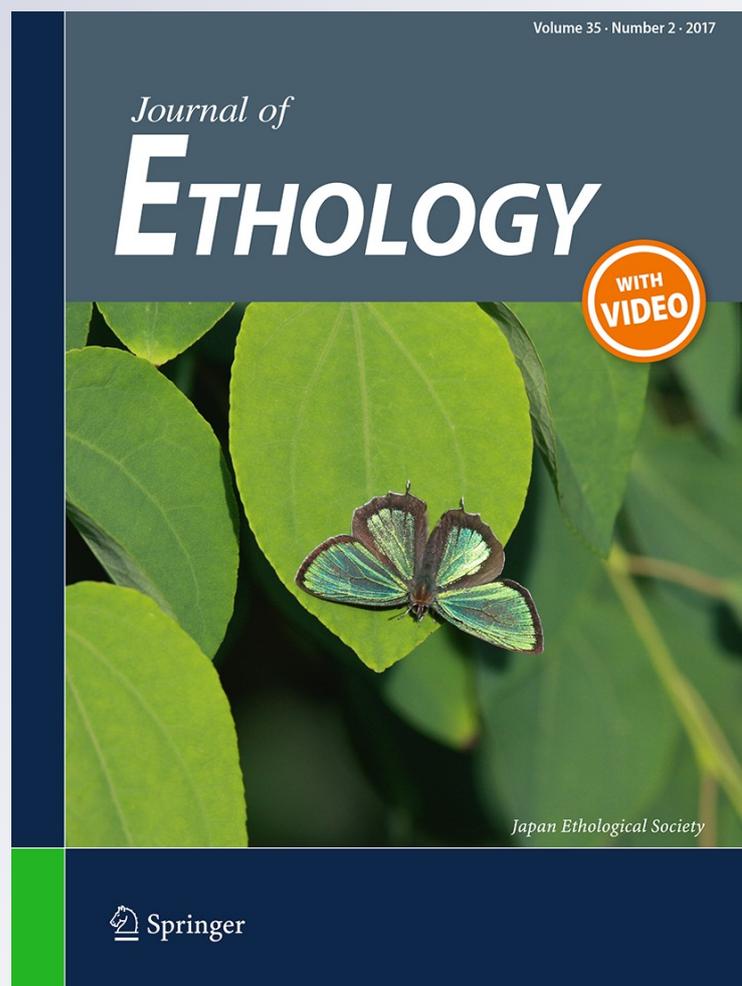
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SHORT COMMUNICATION

Observations on the chemosensory responses of the midget faded rattlesnake (*Crotalus oreganus concolor*): discrimination of envenomated prey in a type II venom species

Anthony J. Saviola[1,2] · Stephen P. Mackessy[1]

Abstract Rattlesnakes use prey chemical cues for ambush site selection and for relocating envenomated (E) prey following a predatory strike. The ability to discriminate between E and non-envenomated (NE) prey cues has been widely studied in rattlesnake species that produce type I venoms, which show high levels of snake venom metalloproteinase (SVMP) activity and low lethal toxicity [lethal dose which kills 50% of test animals (LD_{50}) >1.0 μg/g]. However, E vs. NE prey discrimination studies have not been conducted on rattlesnake species that produce a type II venom that consists of low SVMP activity and high lethal toxicity ($LD_{50} <1.0$ μg/g). In the current study, long-term captive *Crotalus oreganus concolor*, which produce a type II venom, were tested for their ability to discriminate between chemical cues of natural (*Sceloporus undulatus* and *Peromyscus maniculatus*) and non-natural (*Hemidactylus frenatus* and *Mus musculus*) prey cues, as well as for their ability to discriminate between E and NE mouse carcasses, when prey envenomation occurred by a conspecific. Snakes showed significant levels of tongue flicking towards the chemical extracts of *P. maniculatus* and *M. musculus*, suggesting that *C. oreganus concolor* exhibit both innate and experience-based plasticity in response to prey chemical cues. In addition, *C. oreganus concolor* were able to discriminate between E and NE prey sources, when envenomation occurred by a conspecific, indicating that a type II venomous species can also discriminate between E and NE chemical cues.

Introduction

Research on squamate feeding strategies and prey recognition strongly indicates that many snakes and lizards have the ability to discriminate between chemical cues of different prey (Burghardt 1970; Cooper et al. 1990; Clark 2004; Saviola et al. 2012a). This chemosensory response may be innate (Mushinsky and Lotz 1980; Holding et al. 2016) or based on feeding experience (Burghardt et al. 2000), and it often results in fluctuating tongue flick rates towards select prey cues. In squamates, tongue flicking is a stimulus-seeking behavior and the main process for delivering volatile and non-volatile cues to the vomeronasal organ (Halpern 1992; Schwenk 1995). Tongue flicking can be highly variable, and elevated in response to visual-thermal stimuli (Chiszar et al. 1981; Saviola et al. 2011), vibratory cues (Saviola et al. 2012b), or chemical cues (Burghardt 1970; Clark 2004; Saviola et al. 2010; Smith et al. 2015), and it is often correlated with squamate foraging ecology and prey preference (Cooper 1994, 1995, 2008).

Rattlesnakes exhibit multiple sensory modalities during predation, utilizing chemical stimuli to select ambush sites (Roth et al. 1999; Clark 2004) and visual-thermal stimuli to deliver the envenomating strike (Hayes and Duvall 1991). Following the release of prey, rattlesnakes then return to a reliance on chemical cues to relocate the envenomated (E) carcass (Chiszar et al. 1977; Parker and Kardong 2005).

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This ability to relocate E prey and discriminate between E and non-envenomated (NE) prey cues has been thoroughly studied (Dullemeijer 1961; Duvall et al. 1978; Chiszar et al. 1992, 1999, 2008; Greenbaum et al. 2003; Parker and Kardong 2005). Rattlesnakes and other pit vipers (Chiszar et al. 1992; Greenbaum et al. 2003) respond with increased tongue flicking towards E prey when envenomation occurs by a conspecific, a closely related heterospecific (Duvall et al. 1978; Chiszar et al. 2008), or when lyophilized venom is manually injected into a previously euthanized mouse (Chiszar et al. 1999). Interestingly, rattlesnakes are unable to discriminate between E or NE carcasses when venom is painted on the integument of prey, indicating that venom must be injected into tissue for chemical cue discrimination to occur (Chiszar et al. 1992). Further, when testing responses of the western diamondback rattlesnake (*Crotalus atrox*) to mice injected with fractionated venom, snakes were only able to discriminate between E and NE cues when prey was injected with the venom peak containing the crotatroxin disintegrins (Saviola et al. 2013). This work identified these proteins as the "relocator" molecule permitting successful recognition of E prey.

The midget-faded rattlesnake (*Crotalus oreganus concolor*) is a small (adult <700 mm snout-vent length) terrestrial rattlesnake native to extreme southcentral Wyoming, northeastern Utah, and western Colorado. This species feeds primarily on lizards, although small mammals have also been documented in their diet (Parker and Anderson 2007). Unlike many rattlesnake species that undergo an ontogenetic shift in venom composition from higher toxicity and low snake venom metalloproteinase (SVMP) activity in neonates, to decreased toxicity and high SVMP activity in adults [type I venom (Mackessy 2008, 2010)], the venom of *C. oreganus concolor* appears to be paedomorphic (Mackessy et al. 2003), with high toxicity/low SVMP activity carried into adulthood. Adult *C. oreganus concolor*, therefore, produce a type II venom that exhibits low SVMP activity but high toxicity [lethal dose which kills 50% of test animals (LD_{50}) <1.0 μg/g] toward rodents (*Mus musculus*), whereas adults of many rattlesnake species produce type I venoms containing higher SVMP activity with lower toxicities [LD_{50} >1.0 μg/g (Mackessy 2008, 2010)]. To date, the studies that have examined rattlesnake preferences towards E and NE prey cues have utilized only species with type I venoms (Duvall et al. 1978; Chiszar et al. 1992, 1999, 2008; Saviola et al. 2013). However, proteomic analyses of snake venoms [termed "venomics" (Calvete et al. 2007)] continues to identify significant venom variation with regards to general venom composition and overall abundance of specific protein families between and within species. Compositional variation is of interest in E prey discrimination studies because disintegrins, responsible for E vs. NE prey

discrimination (Saviola et al. 2013), appear to be expressed at significantly higher concentrations in venoms of species with type I venoms (see Calvete et al. 2009, 2012). Therefore, the aim of this study was to examine the chemosensory responses of *C. oreganus concolor*, which produces a type II venom, and test whether this species can discriminate between E and NE prey when envenomation occurs by a conspecific.

Materials and methods

Animals

The subjects were five long-term captive adult *C. oreganus concolor*, and all were feeding every 10–14 days on pre-killed NSA mice (*M. musculus*). Mice were culls from the University of Northern Colorado Animal Research Facility and were euthanized by CO_2 asphyxiation and frozen at −20 °C until feeding. Snakes were housed individually in glass aquaria (61.0 × 41.0 × 44.5 cm) containing a paper floor, water bowls, and hide boxes, and maintained on a 12-h:12-h light:dark cycle and provided a thermal range of 26 ± 1 °C.

Chemical cue discrimination

To test the ability of *C. o concolor* to discriminate between chemical cues of different prey, each snake was tested to five scent sources: distilled water (control), and extracts of house gecko (*Hemidactylus frenatus*) and lab mouse (*M. musculus*), non-natural prey for *C. oreganus concolor*, and extracts of prairie lizard (*Sceloporus undulatus*) and deer mouse (*Peromyscus maniculatus*), which represent prey that are naturally encountered by this species (Parker and Anderson 2007). Extracts were prepared by soaking live prey in distilled water at a ratio of 1 mL of distilled water per 1 g of prey source for 10 min (Clark 2004; Saviola et al. 2012a) and were always used within 1 h of preparation. Snakes were fasted for 7–10 days before trials. During trials, extracts were presented to the snakes by soaking a cotton swab in the extract and placing the swab approximately 2.5 cm in front of the snake's nose (Cooper and Burghardt 1990), and the rate of tongue flicking was counted for 60 s. If the snake struck the cotton swab, the trial was terminated, and data were analyzed using the tongue-flick attack score (TFAS) as reviewed in Cooper and Burghardt (1990). Tongue flicking for the 60-s trials was recorded; however, if a snake struck the cotton swab, this indicated maximal response, and TFAS was calculated by rate of TF + (60−latency to strike). All trials were randomized, and due to the small sample size, each snake was tested twice to each chemical extract, with trials

separated by 1 month. Since snakes were observed twice for each treatment, data were averaged over replications and tested for normality using the Kolmogorov–Smirnov (K–S) test, with P to reject normality set at 0.05. The averaged TFAS were compared using a repeated-measures ANOVA followed by Tukey's post hoc test using Prism (version 5.0c). P-values <0.05 were considered as statistically significant.

E vs. NE prey choice

Rattlesnakes were further tested for their responses to E and NE *M. musculus* under conditions where prey envenomation occurred by a conspecific. Previously euthanized mice of similar size and sex were thawed and warmed under heat lamps until skin temperature was 38 ± 1 °C. For all trials, a randomly selected conspecific was allowed to strike and envenomate a mouse carcass presented to the snake on forceps. The carcass was immediately removed from the cage and utilized as the E mouse during behavioral trials. As a control, a second mouse carcass (similar size and sex) received two injections, each containing 50 µL of distilled water, in the thoracic region, dorsal and ventral to the shoulder blade of the mouse. These two injections represented puncture wounds that would naturally occur from the predatory strike, and are in the anatomical region that is most commonly struck during a rattlesnake predatory episode (Kardong 1986). In addition, 100 µL is comparable to the volume of venom injected during a predatory strike (Hayes et al. 1992). Both the E and NE carcasses were placed into a testing apparatus that contained two wire-mesh baskets approximately 5 cm apart (Duvall et al. 1978; Chiszar et al. 1992; 1999; 2008; Saviola et al. 2013; see also Fig. 1). The testing apparatus was thoroughly cleaned with Quatracide disinfectant followed by 95% ethanol, and rinsed with distilled water between trials.

To initiate strike-induced chemosensory searching at the start of the behavioral trial, each *C. oreganus concolor* was allowed to strike a pre-killed mouse suspended on forceps. This carcass was immediately removed without ever touching the bottom or sides of the cage and was discarded. The testing apparatus containing the E and NE carcasses was then placed into the snake's cage (Fig. 1), and following a 2-min acclimation period to account for disturbance, the total number of tongue flicks and total seconds spent investigating either the E or NE carcass were recorded for 10 min. Data were recorded blind to the condition, as the observer was unaware which mouse represented the E or the NE carcass, and all experiments were conducted approximately 10–14 days from the last feeding. Snakes were never test subjects on the day they served as venom donors, and due to the small sample size, experiments were

Fig. 1 The testing apparatus and one of the *Crotalus oreganus concolor* subjects during an envenomated (E) vs. non-envenomated (NE) prey choice trial. The wire mesh baskets held carcasses that were envenomated by a conspecific or were injected with distilled water. All trials were conducted blind so the observer was unaware which was the E or NE mouse. The *arrow* indicates the tongue protruding from the snake's mouth

repeated after 30 days. For each individual, the number of tongue flicks, and the time (seconds) directed towards the E and NE carcasses, were averaged over replications, tested for normality as mentioned above, and compared using a paired t-test (Chiszar et al. 2008; Saviola et al. 2013). All P-values are reported as two-tailed, with $P < 0.05$ considered as statistically significant.

Results

To test for deviations from normality, the K–S test was applied to both the chemical cue discrimination and E vs. NE prey choice data sets. All data sets passed normality ($P > 0.10$), and therefore we cannot reject normality based on the K–S test. When analyzing the tongue flicking response of *C. oreganus concolor*, the ANOVA revealed that there was a significant difference between prey cue extracts ($F_{4,16} = 11.46$, $P < 0.001$). Tukey's post hoc analysis showed that *M. musculus* extract received significantly more tongue flicks when compared to all other extracts ($P < 0.05$) except when compared to *P. maniculatus* ($P > 0.05$). *P. maniculatus* extract also received significantly more tongue flicks when compared to the water control ($P < 0.05$). No other comparisons between prey extracts reached significance ($P > 0.05$; Fig. 2).

To assess if a type II venomous species can discriminate between E and NE prey cues, we tested *C. oreganus concolor* for their responses to E and NE prey items when envenomation occurred by a conspecific. Results showed a strong response to E prey when envenomation occurred by a conspecific, with the mean number of tongue flicks

Fig. 2 Mean number of tongue flicks ± SEM from *C. oreganus concolor* toward extracts of four different prey sources and a water control. *Different letters above bars* indicate significant differences ($P < 0.05$) between responses; *same letters* indicate no significant difference. All trials were 60 s
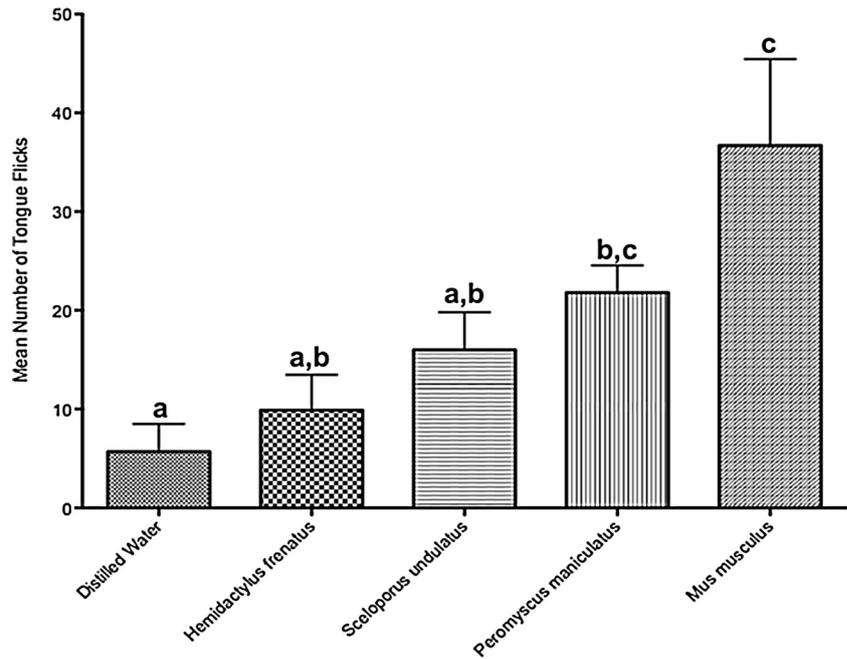
Fig. 3 Mean number of tongue flicks (**a**) and mean number of seconds spent investigating (**b**) ± SEM directed towards E or NE mouse carcasses. For abbreviations, see Fig. 1
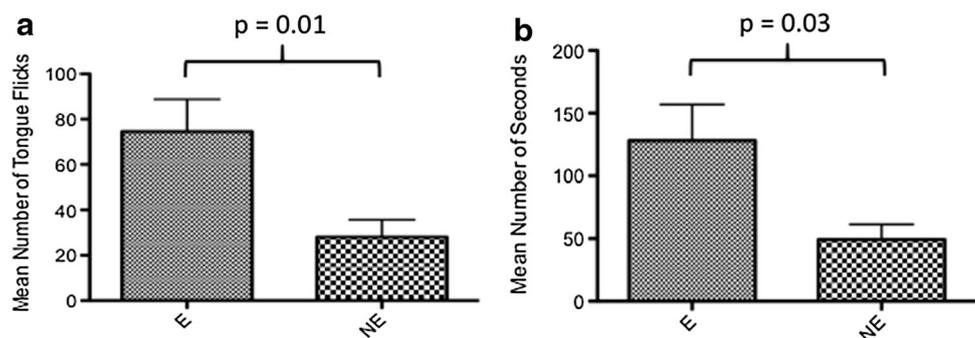
directed towards the E mouse being significantly greater than those toward the NE mouse ($t = 3.871$, $df = 4$, $P < 0.05$; Fig. 3). This significant response was further supported when comparing the mean number of seconds each snake spent investigating the E carcass ($t = 3.131$, $df = 4$, $P < 0.05$; Fig. 3).

Discussion

In the current study, *C. oreganus concolor* showed significantly elevated rates of tongue flicking towards *M. musculus*, a non-natural prey item, and *P. maniculatus* a natural prey item. Although Parker and Anderson (2007) identified both *Sceloporus* sp. and *Peromyscus* sp. as a part of the diet of *C. oreganus concolor*, tongue flicking towards the chemical extract of *Sceloporus undulatus* was not statistically significant when compared to any other treatment, including the water control (Fig. 2). The significant response to rodent cues, but not towards lizard cues,

correlates with an ontogenetic shift in prey preference, and with prey commonly taken by many adult rattlesnakes. For example, analyses of the gut content of the Pacific rattlesnake (*Crotalus oreganus*) identified a diet consisting of more than 50% ectotherms in juvenile snakes, whereas mammal prey were exclusively found in adult snakes (Mackessy 1988); a similar pattern of prey consumption was found for *C. oreganus concolor* (Mackessy et al. 2003). Although *M. musculus* is a non-natural prey item of *C. oreganus concolor*, the strong response to *M. musculus* cues in the present study may be an indication of experience-based plasticity, since these snakes are long-term captives that were fed regularly on *M. musculus*. In addition, two strikes occurred during the behavioral trials, both towards *M. musculus* extract. However, the significant response to *P. maniculatus* extract also indicates an innate component to chemosensory detection. Interestingly, a recent chemical cue discrimination study with dusky pigmy rattlesnakes (*Sistrurus miliarius*) showed that snakes preferred the chemical cues of lizard prey regardless of being

fed rodent prey for a 5-year period (Holding et al. 2016), suggesting that there is a genetic component to prey recognition and that some species lack plasticity in their chemosensory responses (see also Cooper and Secor 2007). Other snake species have been shown to demonstrate variable chemosensory responses that were associated with changed habitat (Burghardt 1993; Burghardt et al. 2000), and prairie rattlesnakes (*Crotalus viridis viridis*) undergo ontogenetic shifts in chemosensory responses to prey extracts that coincide with natural shifts in prey preference (Saviola et al. 2012a). The results presented here demonstrate a chemosensory response that correlates with prey commonly taken at the adult stage of the snake and suggest that there are both innate and learned components to chemosensory detection in *C. oreganus concolor*.

Rattlesnakes, and other pit vipers that strike-and-release prey, must also discriminate between the chemical cues of E prey wandering from the attack site and the chemical cues left by NE conspecific and heterospecific prey. Our results provide evidence that *C. oreganus concolor* can discriminate between E and NE prey cues when prey envenomation occurs by a conspecific. Allowing a conspecific to envenomate prey eliminates the possibility that snakes are responding to prey based on the memory of a specific chemical signature obtained during the predatory strike. In addition, as thermal and visual cues of both the E and NE prey are equal, it can be concluded that chemical cue differences, driven by venom injection, are responsible for snakes showing preference to E prey. The recent finding that disintegrin venom proteins may act as the relocator molecule in venom (Saviola et al. 2013) has prompted additional questions in regards to the pharmacology of disintegrins in relation to the behavior of the animal. Disintegrins are the product of a post-translational modification of the PII class of SVMPs, resulting in the free disintegrin protein in the venom (Calvete et al. 2005). Rattlesnake venoms have been classified into type I (high SVMP activity and low lethal toxicity) and type II (low SVMP activity and high lethal toxicity) (Mackessy 2008, 2010) venoms, and hypothesized that the low SVMP concentration in type II venomous species also leads to low concentrations of disintegrins in venom. This has been confirmed by analyses of these venoms: *Crotalus atrox*, which has high SVMP activity (Mackessy 2008), produces a venom with >6% disintegrin composition (Calvete et al. 2009); conversely, the type II venom of *Crotalus tigris* has very low SVMP activity (Mackessy 2008), and only 0.2% of this species' venom consists of disintegrins (Calvete et al. 2012). An in-depth venomic analysis of *C. oreganus concolor* venom has yet to be completed, but the low SVMP activity observed in this species' venom (Mackessy et al. 2003; Mackessy 2008) suggests significantly lower disintegrin concentrations compared to type I venoms. However, the lower concentration of disintegrins in

a type II venom may be compensated for by the increased toxicity of these venoms towards prey (see Mackessy 2008; Saviola et al. 2013), which should reduce post-strike prey travel distance. Regardless, data here indicate that rattlesnake species producing type II venom can still discriminate between E and NE chemical cues.

Although the current study utilized a relatively small snake sample size, the results suggest that adult *C. oreganus concolor* exhibit chemosensory preference towards rodent cues, and that snakes exhibit innate and adaptive chemosensory responses to prey chemical extracts. The individuals tested in this study were long-term captive snakes that were fed solely on *M. musculus* for years, and a larger sample size of snakes with a more varied diet, as well as chemosensory studies with wild-caught snakes, will provide a deeper understanding of the chemosensory behavior of *C. oreganus concolor*. The ability of *C. oreganus concolor* to distinguish between E and NE prey sources suggests that, regardless of the concentration of specific venom proteins, the sole presence of these compounds in venoms is sufficient to allow discrimination of E and NE prey sources.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical notes All applicable international, national and institutional guidelines for the care and use of animals were followed. All procedures performed in the present study were in accordance with the ethical standards of the Institutional Animal Care and Use Committee of the University of Northern Colorado under Protocol No. 1302D-SM-S-16 (S. P. M.).

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