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**BEHAVIORAL ASPECTS OF CHEMORECEPTION IN JUVENILE
COTTONMOUTHS (*AGKISTRODON PISCIVORUS*)**

A Master's Thesis

Presented to

The Graduate College of
Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree
Master of Science, Biology

By

Chelsea E. Martin

December 2019

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BEHAVIORAL ASPECTS OF CHEMORECEPTION IN JUVENILE COTTONMOUTHS

(*AGKISTRODON PISCIVORUS*)

Biology

Missouri State University, December 2019

Master of Science

Chelsea E. Martin

ABSTRACT

For snakes, chemical recognition of predators, prey, and conspecifics has important ecological consequences. For example, detection of predator cues can reduce predation risk. Similarly, scent trailing of conspecifics to communal hibernacula can improve overwinter survival for neonates. I used y-maze choice trials to examine scent-trailing ability of 32 captive-born juvenile cottonmouths (*Agkistrodon piscivorus*) in two separate experiments. In conspecific trailing tests, subjects preferred to follow cues from their own mothers over a blank control cue, but also preferred to trail cues from unrelated adult females compared to cues from their own mothers. My results are consistent with previous reports and suggest that juvenile cottonmouths also trail conspecifics to hibernacula. However, the preference for trailing non-maternal cues, given the occurrence of post-partum mother-offspring affiliations in cottonmouths, is not easily explained. In predator avoidance trials, test subjects showed no preference for the blank control versus the king snake cue arm, or non-predator control (crayfish) and kingsnake arm. Indifference to kingsnake cues is inconsistent with results from tests with colubrids where kingsnake cues were clearly avoided. Although kingsnakes are known predators of venomous snakes, it is possible that cottonmouths may not avoid chemical cues without visual confirmation of a threat. My results support a growing awareness that pitviper behaviors are more complex than currently appreciated.

KEYWORDS: chemical cues, scent trailing, predator avoidance, pitvipers, *Agkistrodon piscivorus*

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December 2019

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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OVERVIEW

Chemoreception is a dominant sensory modality in snakes and pervasively influences their ecology and behavior (Ford and Burghardt, 1993). The importance of chemosensation in snakes (and the clade of lizards to which they belong) has long been recognized by the presence of a forked tongue that delivers chemical cues to the vomeronasal system (Schwenk, 1995). Because vomerolfaction typically involves nonvolatile chemicals, many chemical ecology questions pertaining to snakes may be appropriately assessed via manipulations of substrate cues. The ability of snakes to sense chemical cues, makes tongue flick rate (TFR) an important response variable that reflects interest in a particular cue (Cooper, 1998).

Chemical cues have been shown to mediate many aspects of snake behavior. Female pheromones are essential in identification and mate location (Ford and O'Bleness, 1986; LeMaster and Mason, 2001), and can be used by males, to discern the recent mating history of females (O'Donnell et al., 2004; Thomas, 2011). In foraging contexts, chemical cues are used to identify and track prey (Burghardt, 1967; Webb and Shine, 1992; Cooper and Secor, 2007). In crotalids, the trail of envenomated prey is located through chemical cues (Chiszar et al. 1986). Chemical cues are also frequently important in predator recognition and provide a means of lowering predation risk by avoiding locations where predator cues have been deposited (Burger, 1989; Burger, 1990).

Emerging information on pitviper communication suggests that their social behavior may be more complex than previously thought and is largely mediated through chemical senses. For example, Clark (2007) reported that timber rattlesnakes (*Crotalus horridus*) use chemical information from conspecifics to determine if they had feed recently and therefore, infer the

quality of foraging habitat. A variety of snakes have recently been shown to recognize relatives through chemical discrimination (Clark, 2004; Pernetta et al., 2009; Clark et al., 2014). Also, maternal attendance of litters by females is apparently widespread in pit vipers (Greene et al., 2002) and is influenced by chemical cues emitted by their neonatal offspring (Hoss et al., 2014). Maternal–offspring associations also likely underlie the propensity for neonatal rattlesnakes to trail conspecifics to communal hibernacula (Brown and Maclean, 1983; Hileman et al., 2015). The “cryptic sociality” of pit vipers is consistent with experimental evidence of the secretions of unique musk glands of snakes being used as an alarm cue—a social signal that alerts individuals of predatory threat (Meinders, unpublished). Although the existing literature on snake chemical ecology provides a reasonable understanding of chemical perception in snakes, this information is based on relatively few taxa. Therefore, the purpose of my thesis was to fill in a phylogenetic gap in the literature by contributing information on the chemical ecology of cottonmouths, a locally abundant semi-aquatic pitviper.

The first chapter of my thesis examines the scent trailing behavior of juvenile cottonmouths. Individuals were studied to determine if they preferred to trail their mothers over that of an adult conspecific or a blank control. Ontogeny of this choice was also examined by testing individuals at 3–4 months and 14–15 months of age.

The second chapter of my thesis focuses on the avoidance behavior of juvenile cottonmouths when exposed to cues from kingsnakes (*Lampropeltis floridana*) a known predator (Weldon and Burghardt, 1979). Individuals were tested against a blank and animal control in dichotomous choice tests. Threat sensitivity was also examined by exposing juveniles to predator cues when in the presence of conspecific musk.

**CONSPECIFIC SCENT TRAILING IN JUVENILE COTTONMOUTH (*AGKISTRODON*
PISCIVORUS)**

Abstract

In temperate regions where winter is harsh and suitable hibernacula are rare, there are considerable survival advantages for neonate snakes to locate suitable hibernacula. In neonatal rattlesnakes, orientation and navigation to hibernacula has been suggested to occur via scent trailing of conspecific chemical cues. I examined the ability of captive-born juvenile cottonmouths (*Agkistrodon piscivorus*) to trail cutaneous cues from maternal and non-maternal conspecifics in y-maze trials. Juveniles preferred the arm containing the maternal cue compared to a blank control, but also preferred to trail cues from unrelated adult females compared to cues from their own mothers. My results are consistent with reports of scent trailing in neonate rattlesnakes and suggest that juvenile cottonmouths trail conspecifics to hibernacula. However, the apparent preference for non-maternal cues has not, to our knowledge, been reported in snakes. Given the occurrence of post-partum mother-offspring affiliations in cottonmouths, an attraction to non-maternal cues is not easily explained. Future research on this topic is needed to explain a possible cause.

Introduction

In places where winter weather poses a survival risk, snakes hibernate in subterranean cavities below the frostline. Many snakes in northern temperate latitudes overwinter in communal hibernacula at permanent structures that provide suitable refuges from severe winter conditions (Gregory 1984). When appropriate hibernacula are low in abundance, or spatially separated from other essential resources, snakes may migrate considerable distances to discrete communal dens (Gregory 1977; Duvall et al. 1985). In communally hibernating pit vipers, orientation and subsequent navigation to dens by neonates has been suggested to occur via scent trailing of conspecific chemical cues (Brown and MacLean 1983; Hileman et al. 2015). Chemical trailing of conspecific cues to dens may confer a significant survival advantage to naïve neonates and thus represent an adaptive strategy (Graves et al. 1986).

Radiotelemetry studies have provided considerable insight into the spatial biology and social behavior of rattlesnakes. In locations with cold climates, snakes typically migrate between communal hibernacula and summer foraging sites (e.g. Duvall et al. 1985). However, pregnant females within these populations often remain relatively close to dens in thermally favorable “rookery” habitats (Macartney and Gregory 1988; Reinert and Zappalorti 1988; Graves and Duvall 1993). Postnatal attendance of litters by mothers is common and usually occurs until postnatal ecdysis, followed by departure of the female to a hibernaculum (Greene et al. 2002). Telemetry studies have reported that neonates appear to track their mothers toward den locations (Reinert and Zappalorti 1988; Cobb et al. 2005; Jellen and Kowalski 2007; Muellman et al. 2018) and experimental studies have confirmed trailing of adult females by neonates in y-maze tests (Brown and MacLean 1983; Hileman et al. 2015). However, neonatal massasaugas (*Sistrurus catenatus*) were equally likely to trail unrelated adult females as their own mothers in

y-maze trials (Hileman et al. 2015). This result is expected given that neonates could benefit from following any conspecific to a suitable hibernaculum upon which the entire population would be converging. Because winter severity is thought to create a selective pressure for neonatal trailing behavior, rattlesnake populations in milder climates, where suitable hibernacula would likely be more abundant or unnecessary, may lack a trailing response. This expectation is consistent with reports of field studies from southern locations (Figueroa et al. 2008; Howze et al. 2012). Determining the prevalence of neonatal trailing behavior in crotalid snakes, including its ecological correlates and phylogenetic scope, will require testing of additional species across different climatic circumstances.

Northern Cottonmouths (*Agkistrodon piscivorus*) are semi-aquatic pit vipers associated with aquatic habitats of the southeastern United States. Cottonmouth populations hibernate communally throughout the northern portions of their range and migrate between hibernacula and foraging areas (Smith 1961; Anderson 1965; Blem and Blem 1995). Pregnant female cottonmouths appear to give birth close to hibernacula as a consequence of using rookery habitats in close proximity to hibernacula (Crane and Greene 2008), or by migrating to hibernacula just prior to parturition (Delisle et al. 2019). Post-partum females attend their litters (Hoss et al. 2015; Delisle et al. 2019), but there is currently no information concerning the propensity of neonates to trail conspecifics to dens in communally hibernating populations.

I used y-maze tests to study conspecific trailing behavior of juvenile cottonmouths from a population near the species' northwestern range limit in Southwest Missouri where winters are harsh. The objectives of my study were (1) to determine if juvenile cottonmouths would trail cutaneous chemical cues deposited by adult females during a time period that was appropriate for locating a hibernaculum, (2) to determine if the same juveniles would preferentially follow cues

derived from their own mothers over those from an unrelated adult female, and (3) to determine if there is ontogeny in conspecific trailing behaviors. I predicted that young juveniles would scent trail conspecific cues over a blank control and that cues from maternal and unrelated source individuals would be equally attractive in y-maze tests. Because older – and more experienced – juveniles may not require conspecific scent trails to locate hibernacula, I predicted that 14–15 month old cottonmouths would not exhibit a trailing response.

Materials and Methods

Experimental subjects and stimulus animals. Test subjects were 48 juvenile cottonmouths from 18 wild-caught gravid females (10 in 2016 and 8 in 2017) that were collected from a single population in Stone County, Missouri and held until parturition. The eight females (SVL range 52.5–62.0 cm, mass range 206–305 g) that produced litters in 2017 were retained and used as stimulus donors but the 2016 females were released in September 2016.

Cottonmouths were collected under permits issued by the Missouri Department of Conservation, and research procedures were approved by the Missouri State University IACUC (October 2017, Protocol 18-010).

Housing and maintenance. Cottonmouths were maintained in a vivarium at Missouri State University at 25 °C with a 12:12 light:dark cycle. Snakes were housed individually in clear plastic cages (33L × 18W × 9H cm) with a water dish, PVC pipe shelter, and newspaper substrate. Shelves used to store snake cages were heated along one edge to create a thermal gradient within each cage where a maximum body temperature of approximately 30 °C could be attained. Snakes were fed an assortment of commercial bait minnows approximately every 10 days, and water was available ad libitum.

Stimulus collection. I collected cutaneous cues from snakes by rubbing a cotton ball along the dorsal, ventral, and lateral surfaces of stimulus donors (cottonmouth: n=8, 50–60 cm, SVL). Because musk may act as an alarm substance (Graves and Duvall 1986), I attempted to prevent contamination by collecting cues outside the room where trials occurred and covering the cloaca and tail region of donor snakes when collecting cutaneous cues. New cues were collected during each day of testing to ensure that the integrity of cues was uniform across trials.

Testing apparatus. I conducted trailing experiments in a y-maze apparatus similar to those used in previous studies of conspecific trailing by neonatal rattlesnakes (Brown and Maclean 1983; Hileman et al. 2015). The maze design used in my study consisted of a 63-cm long introduction arm connected to two 95-cm long test arms. The walls of each arm were 15-cm high and the distal end of each test arm was blocked with a 15 × 15 cm wall. All portions of the maze were constructed out of 1-cm thick plywood connected to a wooden panel base.

Procedure. For testing, snakes were transferred to a separate temperature controlled room. To encourage subjects to explore the apparatus, I conducted all trials under dimly lighted conditions and within a time frame (1500–1800 hrs) when I observed the captive snakes to be most active. I tested juvenile cottonmouths in y-maze dichotomous choice tests in all scent trailing experiments. During each trial I covered the floor of the apparatus with plain butcher paper. The scents used in each trial (control vs. maternal or conspecific vs. maternal) were randomly assigned to each arm of the maze. I applied cues to butcher paper substrate by placing a cotton ball containing the desired treatment on the floor at the proximal end of the test arm and rolling it continuously to the distal end of that arm. The cotton ball used to apply a treatment remained at the distal end of the arm during the trial. I replaced the butcher paper after each

trial. A curtain divided the room so that simultaneous trials could be run without influencing trials in adjacent mazes.

Because juvenile cottonmouths are easily disturbed, I attempted to minimize handling stress by transferring each test subject from its cage to the y-maze enclosed within a portable transport shelter. Shelters consisted of a 13.5×7 cm halved piece of PVC pipe with the concave surface oriented downward, which was attached to a clear plastic bottom (Fig. 1). One end of the shelter was closed with a plastic end cap while the open end had a 3×7 cm clear plastic door attached to the adjacent front section of the shelter floor with a strip of masking tape. The tape connection served as a hinge that allowed closure of the door once the snake was inside. Several days prior to testing, home shelters were replaced with transport shelters in each snake's cage. Most snakes were concealed within shelters prior to testing, allowing the door to be closed and taped shut before transport to the y-maze apparatus. Others were coaxed into the shelter and entrapped inside before moving to the y-maze.

Each trial began with a shelter containing a snake positioned at the opening of the introduction arm with the door facing into the maze. Snakes were allowed a 5 min acclimation period, after which the door of the shelter was opened. If after 10 min the snake did not emerge, I tilted the shelter until the snake gently slid into the maze. During trials I stood stationary behind the apparatus and recorded the snake's behavior as it moved forward through the maze. Response variables recorded in each trial included: time spent in each arm, time within the y-junction, and the number of tongue flicks in each experimental arm. Trials lasted a maximum of 60 min but ended when a final choice was made. A choice was recorded when an individual reached the end of an experimental arm and made contact with the cotton ball.

Statistical analyses. I used Chi-Square Goodness-of-Fit tests to compare response frequencies for the experimental and control scents, and independent samples t-tests to assess mean differences in tongue flick rates between choice arms. Parametric assumptions were met. Means \pm 1SD are reported. Significance was assessed at $\alpha < 0.05$. All analyses were done in RStudio version 1.1.414.

Data collection. I randomly tested 32 juvenile cottonmouths, in two experiments, using y-maze dichotomous choice trials to determine if these snakes exhibited trailing behavior when exposed to chemical cues of adult conspecifics. In the first experiment, I tested 16 snakes offered the choice between chemical cues from their own mothers and a blank control. Because a previous study of another species found that neonates would preferentially trail cues from their own mothers over those of other conspecifics (Hileman et al. 2015), I performed a second experiment to address whether relatedness of cue donor influenced trailing behavior. To determine if maternal cues are preferred over other conspecific cues, 16 additional snakes were tested under conditions where each arm of the y-maze was treated with either maternal scent or the scent of a presumed unrelated adult female. The non-relative conspecific cues were collected from one of the seven other adult females in the lab. Juveniles were randomly assigned to one of the two experiments and the order of testing was randomized. In all trailing experiments the location of maternal cues was randomly determined for each trial. Tests were conducted during December 2017–January 2018 at 1500–1800 when individuals were 3–4 months old and were still approximately neonate-sized (18–27.7 cm, SVL; 9.3–25.92 g).

To determine if preferences change with age I also tested snakes 14–15 months of age ($n=16$, 26.8–31.9 cm, SVL; 28.26–39.97 g) with chemical cues from presumably unrelated (all stimulus donors were from the same population as test subjects but were not their mothers)

conspecific adult females paired with a blank control using the same methods described for younger snakes. For this experiment, testing occurred during March–April 2017 at 1500–1800.

Results

On average juveniles took 19.03 ± 13.7 min to complete a trial, with 100% ($n=32$) of individuals making a final choice. Juveniles showed a preference for the maternal experimental arm (observed = 13; expected = 8; Fig. 2) over that of the blank control (observed = 3; expected = 8; $n = 16$, $X^2 = 6.25$, $df=1$, $P=0.01$). On average juveniles spent 4.23 ± 1.32 min in the maternal arm and had a TFR of 12.32 ± 6.62 flicks/min. Individuals spent 3.03 ± 2.7 min and had a TFR of 17.04 ± 6.31 flicks/min while in the control arm. There was no difference in the time spent ($P=0.533$), or TFR ($P=0.378$) between the two experimental arms.

However, juveniles also showed a preference for the conspecific experimental arm (observed = 12; expected = 8; Fig. 2) over that of the maternal (observed = 4; expected = 8; $n=16$, $X^2 = 4$, $df = 1$, $P=0.04$). Juveniles spent 3.73 ± 0.9 min in the conspecific arm and 6.6 ± 3.37 min in the maternal arm and there was no difference in time spent between the two experimental arms ($P=0.191$). There was also no difference in TFR between the experimental arms (conspecific: 20.52 ± 5.95 ; maternal: 13.01 ± 5.59 ; $P=0.071$). Combining both experiments, individuals that made the minority decisions ($n=7$, 12.72 ± 5.51 flicks/min) had lower TFR's than individuals that made the majority decision ($n=25$, 18.71 ± 6.27 flicks/min, $P = 0.03$; Fig. 3).

The 14–15 month old individuals took on average 12.18 ± 17.25 min to complete trials, with 100% ($n=16$) of individuals making a final choice. Individuals showed no preference for conspecific cues (observed = 10; expected = 8; Fig. 4) over control cues (observed = 6; expected

= 8; $n = 16$, $X^2 = 1$, $df = 1$, $P = 0.317$), and there was no difference in the amount of time spent in each experimental arm (conspecific: 2.23 ± 0.65 min; control: 1.22 ± 1.21 min). TFR were very similar for individuals that chose the conspecific experimental arm ($n = 10$, 37.18 ± 6.08 flicks/min) compared to those who chose the control arm ($n = 6$, 34.81 ± 6.9 flicks/min; $P = 0.504$).

Discussion

My results are consistent with previous experimental and observational studies reporting scent trailing of conspecifics by newborn snakes during the fall. This phenomenon has been especially well studied in communally hibernating rattlesnakes where the behavior of pregnant females is thought to facilitate den location by offspring. Pregnant females typically occupy home ranges centered on rookery sites that are closer to hibernacula than other members of the population (Graves et al., 1986). Following parturition, females typically associate with their offspring (Greene et al., 2002) before dispersing to hibernacula (Reinert and Zappalorti, 1988; Cobb et al., 2005; Jellen and Kowalski, 2007; Muellman et al., 2018). Female cottonmouths exhibit parallel behaviors including occupying rookery habitats near hibernacula (Crane and Greene, 2008) or migration from wetlands to parturition sites at or close to hibernacula (Delisle et al., 2019). Following parturition, females associate with offspring (Hoss and Clark, 2014; Hoss et al., 2014). Delisle et al. (2019) reported that telemetered neonates shared hibernacula with their mothers and other cottonmouths. My study is the first to experimentally demonstrate that cottonmouth newborns also scent trail conspecifics.

Cottonmouth offspring discriminated between maternal and unrelated conspecific females and preferred to trail cues from the latter. Neonates of several rattlesnake species have been reported to aggregate with their mothers following parturition (Graves et al., 1986; Reinert

and Zappalorti, 1988; Greene et al., 2002) and trail females that have left their litters (Cobb et al., 2005; Jellen and Kowalski, 2007; Muellman et al., 2018). Although these observations have often been interpreted to suggest that offspring might prefer to follow trails derived from maternal cues over other individuals, it is possible that neonates were simply following the nearest available conspecific trail. However, neonatal massasaugas trailed cues from both maternal and unrelated conspecific donors and exhibited no preference between them in y-maze trials (Hileman et al., 2015). In communally hibernating species, it may be advantageous for neonates to follow cues of any adult conspecific because their trails may reveal information about the location of an appropriate den (Burger, 1989). The lack of preference for maternal cues may also be understandable if parturition and hibernation are sufficiently separated in time that offspring exhibit extended surface activity following parturition, eliminating an urgent need to locate hibernacula. In southwestern Missouri, cottonmouth litters are typically born in late August to early September, do not enter hibernation until mid-October, and have been observed to contain stomach contents during this time (B. Greene, personal observation). While this temporal separation may explain an offspring's equal attraction to cues derived from relatives and non-relatives, it is unclear why an individual would prefer to trail a non-relative over their own mothers.

Individuals who made the majority choice in y-maze trials exhibited higher TFR and spent less time in their choice arm than individuals that made the minority decision. As suggested by Hileman et al. (2015), it appears that individuals exhibiting higher TFR's collected more chemical information about their environment, allowing them to locate trails more accurately. Snakes making the minority decision had lower TFR's and also spent consistently more time in the control arm, indicating that snakes locating a chemical trail completed trials

more quickly. Brown and Maclean (1983) reported that newborn timber rattlesnakes (*Crotalus horridus*) exhibited both a “tail contraction response” and an elevated TFR when they encountered a chemical trail. Snakes that failed to perform these behaviors usually made incorrect choices in y-maze tests. Of related interest in Brown and Maclean’s study was a negative correlation of newborn trailing success with size of the donor snake used to create a trail in the maze. Collectively, these observations indicate a strong correlation between trailing performance and vomeronasal involvement and suggest that the inclusion of TFR and other measures of chemosensory responses improve interpretation of results.

I also examined the ontogeny of scent trailing and found that one year old juveniles did not exhibit a preference between the control and the conspecific cue, indicating that their propensity to scent trail was absent. To my knowledge, no previous studies have examined the ontogeny of scent trailing behavior in juvenile pit vipers. However, it is likely that selective pressure for scent trailing of conspecifics would be most prevalent in naïve neonates where overwinter survival is strongly tied to locating suitable den sites (Brown and Maclean, 1983; Reinert and Zappalorti, 1988; Cobb et al., 2005). Older experienced juveniles may not benefit from trailing adults because they would presumably have learned the location of hibernaculum after their first winter. Chemical cues may be most important to neonates for locating communal hibernacula and used in combination with additional cues by older individuals (Mason, 1992). My individuals were tested during April–May and had not undergone a previous overwinter period, indicating that they are not ‘experienced individuals’. Additional testing needs to be done during an ecologically relevant time to determine if my results are consistent.

Overall, my data suggest that young cottonmouths trail conspecifics during the fall of their birth, supporting the hypothesis that this behavior facilitates location of hibernacula. I

recommend that future studies, using larger sample sizes, investigate the consistency of the apparent preference to track unrelated females over their own mothers. Cottonmouths occur in regions where winter severity ranges from harsh to mild ,and surface activity may occur all year, because of this they may offer an excellent opportunity to examine the influence of climate on neonate trailing behavior. Future studies investigating whether scent trailing behavior varies across latitudes would be of great interest.

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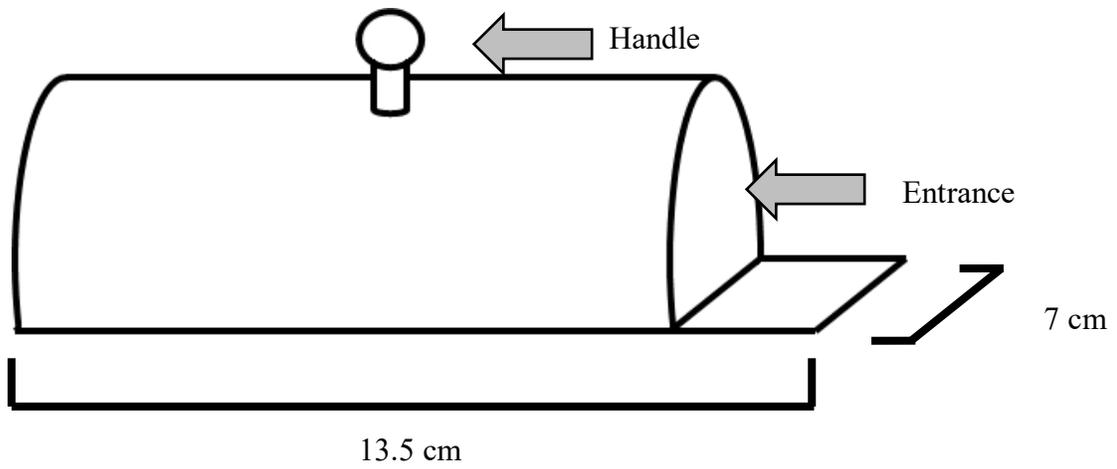


Fig. 1. Mobile shelter used to transfer snakes to a y-maze apparatus. One side of the shelter was open to allow snakes to enter. The door was then taped shut so that the shelter could be moved without handling the snake. A central metal eye bolt creates a handle to allow safe manipulation of the shelter with a snake hook.

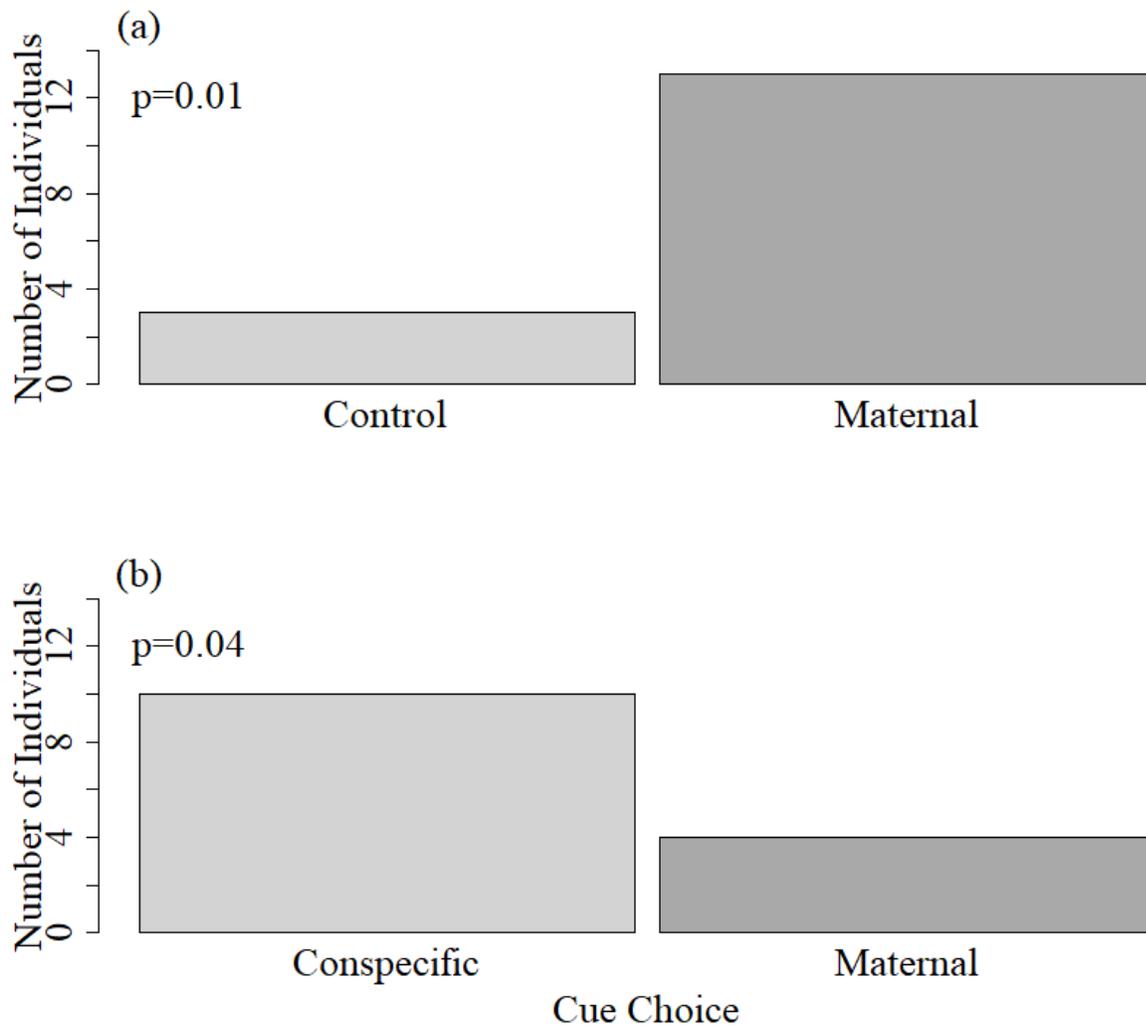


Fig. 2. The number of three month-old juvenile cottonmouths tested in y-maze trials that chose (a) the maternal cue arm vs. the control and (b) the conspecific cue arm vs. the maternal cue arm.

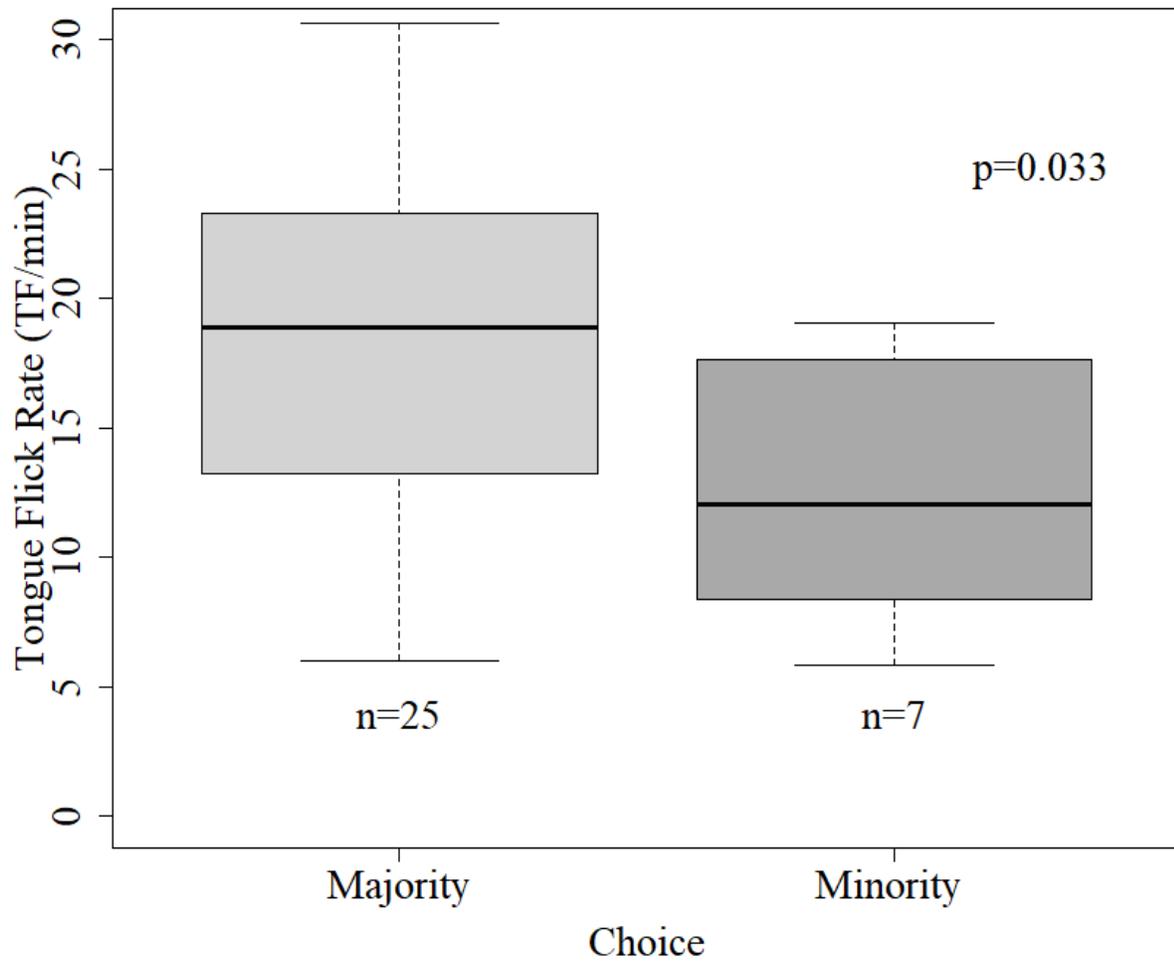


Fig. 3. Tongue flick rates of individual cottonmouths that made the majority decision (n=25) compared to individuals that made the minority decision (n=7) in y-maze dichotomous choice tests.

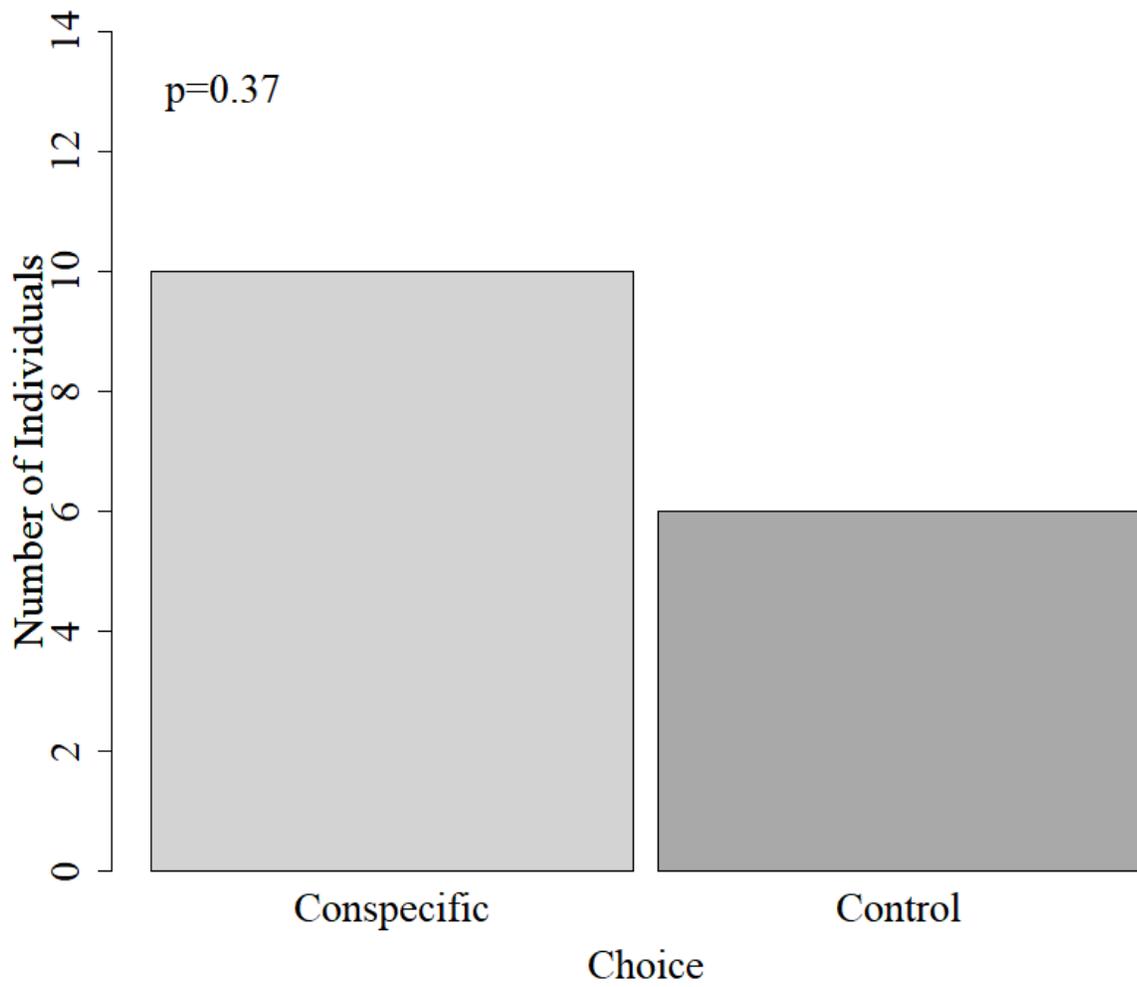


Fig. 4. The number of 14–15 month old cottonmouths that chose experimental arms containing either the conspecific cue or the control cue in y-maze trials.

AVOIDANCE OF KINGSSNAKE CHEMICAL CUES IN JUVENILE COTTONMOUTHS
(*AGKISTRODON PISCIVORUS*)

Abstract

Chemical recognition of cues deposited by predators allows early detection and avoidance, which can increase survival rates for prey. Response to predatory chemical cues has been well documented in lizards; however, few studies have looked at the avoidance response of cues from predators in snakes. In my study I used y-maze choice trials to examine avoidance of kingsnake (*Lampropeltus floridana*) chemical cues in captive-born juvenile cottonmouths (*Agkistrodon piscivorus*). Test subjects showed no preference between the blank control and kingsnake cue arm, or the non-predator control (crayfish) and kingsnake arm. When in the presence of musk, a supposed alarm cue, subjects also had no preference between a blank control and kingsnake cue. Indifference to kingsnake cues is inconsistent with results from similar tests in colubrids where kingsnake cues were clearly avoided. Although kingsnakes are known predators of venomous snakes, it is possible that cottonmouths may not exhibit an avoidance response to chemical cues without visual confirmation of a threat. Additional experiments are needed to determine if a change in the testing apparatus or an addition of a visual cue would illicit an avoidance response.

Introduction

Predation constitutes a significant survival threat to most animals, resulting in strong selection pressure for recognition and avoidance of predators (Lima and Dill, 1990).

Chemosensation is a valuable means of predator detection because chemical cues can be perceived under low illumination or in complex habitat structure where predators may be concealed. Additionally, residual cues left by predators improve predation risk assessment by indicating where predators have recently been. Evidence for the importance of chemical information in predator/prey interactions is exhibited by the diverse array of prey defenses induced by predator chemical cues, including changes in morphology, physiology, life history and behavior of prey (Kats and Dill, 1997). Behavioral responses to predator chemical cues can include reduced activity (Davis et al., 2012), alarm signaling (Mathis 2009), aggregation and defensive posturing (Soltz Herman and Valone, 2000; Cabido et al., 2004; Landová et al., 2016), and escape and/or avoidance (Cupp, 1994; Monclus et al., 2005; Durand et al., 2012).

Interactions between lizards and their snake predators have offered excellent model systems to study the coevolution of predator-prey relationships. In areas where lizards and snakes interact within the same habitat space, and under circumstances where visual perception is likely to be limited, selection for chemical recognition of predators may be high (e.g. Downes and Shine, 1998; Cabido et al., 2004). Behavioral evidence for recognition of snake scents include reduced activity, freezing, and increased tongue flick rates (TFR; Cooper, 1990; Downes, 2002; Landová et al., 2016), and avoidance of predator-scented shelters (Head et al., 2002; Cisterne et al., 2014; Kabes and Clark, 2016). Variation in responses to heterospecific chemical cues is consistent with selection for antipredator behavior to match the level of threat posed by a particular predator. Thus, many lizard species exhibit antipredator responses to saurophagous

snakes but not sympatric snakes that do not feed on lizards (Thoen et al., 1986; Stapley, 2003; Cabido et al., 2004). The importance of predation pressure in determining the level of response is supported by clear differences between island populations of a lizard species in sympatry or allopatry with a particular snake predator (Mori and Hasegawa, 1999; Durand et al., 2012). Further evidence of evolved responses is indicated by ontogenetic differences, with juveniles responding more strongly than adults to snakes that are a threat only to young lizards (Head et al., 2002; Stapley, 2003; Landová et al., 2016). However, some lizards lack threat sensitivity and exhibit generalized antipredator responses to all sympatric snakes (Webb et al., 2009; Webb et al., 2010; Landová et al., 2016). A generalized antipredator response may also be adaptive for avoiding dangerous species included within an ecologically similar group of predators.

Snake and lizards belong to the same order (Squamata) however, relatively little is known about antipredator responses of snakes to predator chemical cues compared to lizards. Much of the existing literature entails tests of responses to chemical cues of snakes in the genus *Lampropeltis* (kingsnakes), which are known for their ophiophagous tendencies. Exposure to cutaneous chemicals from ophiophages typically elicits elevated TFR in test subjects relative to control cues (Weldon, 1982; Placyk and Burghardt, 2011). Antipredator behaviors reported in response to ophiophage chemical cues in test subjects exploring open environments have included increased movement rates (Placyk and Burghardt, 2011) and avoidance in y-maze tests (Burger, 1989; Himes, 2003).

North American crotalid snakes exhibit a collection of antipredator behaviors (the “ophiophage defensive response”, ODR) in response to chemical cues from various snake predators (Bogert, 1941). The ODR has been particularly well documented in response to kingsnakes (Carpenter and Gillingham, 1975), which are important snake predators that are

immune to North American snake venoms. The ODR is characterized by head hiding, thrashing of the body, and a defensive posture known as body bridging, which involves the elevation of the body in vertical loops (Weldon and Burghardt, 1979; Gutzke et al., 1993). The recognition of kingsnake predators appears to be a primitive crotaline trait, occurring in rattlesnake populations that are not sympatric with kingsnakes (Bogert, 1941), and is apparently an innate behavior, as it can be elicited without prior experience in some species (Weldon and Burghardt, 1979). Other responses to kingsnake chemical cues include increased heart rate (Cowles and Phelan, 1958). The ODR is elicited by chemical cues from kingsnakes and apparently cannot be triggered by visual cues (Miller and Gutzke, 1999).

Defensive behaviors of Northern Cottonmouths (*Agkistrodon piscivorus*) have been well-characterized due to the presence of a suite of easily quantified stereotypical responses to predatory threat. In response to human aggressors in simulated predation events, Gibbons and Dorcas (2002) reported that cottonmouths would typically attempt to escape or use defensive displays rather than bite. Some of the variation in defensive tactics may be explained by ontogeny as juvenile cottonmouths exhibit more vigorous defensive responses than adults (Roth and Johnson, 2004) and do not habituate across successive trials (Glaudas et al., 2006). Although experiments using human predators have been useful in elucidating patterns of defensive behavior in cottonmouths, antipredator responses to natural predators are less understood. Adult cottonmouths exposed to kingsnakes may exhibit an ODR (Gutzke et al., 1993) but only when chemical cues are present (Miller and Gutzke, 1999). When exploring a novel environments differing only in chemical treatment, cottonmouths executed reduced activity, via lower TFR, in response to kingsnake chemical cues than to blank controls, but did not exhibit any other antipredator behaviors (Chiszar et al., 1978).

The purpose of this study was to assess whether kingsnake chemical cues alone would elicit an avoidance response in juvenile cottonmouths. I also examined the avoidance response of juveniles to kingsnake chemical cues when in the presence of an alarm cue. Kingsnakes are known predators of cottonmouths (Burkett, 1966; Ernst, 1992), are sympatric with them throughout their range, and have been repeatedly shown to elicit antipredator behaviors in adult cottonmouths (Carpenter and Gillingham, 1975; Gutzke et al., 1993). I tested only small juvenile cottonmouths, which should experience the highest predation risk and therefore were predicted to be highly responsive to predator cues, and exposed them to chemical cues from kingsnakes and controls in discrete choice experiments. Given the clear threat posed by kingsnakes, I predicted that cottonmouths would prefer to explore an experimental arm of a y-maze containing a control cue rather than one containing kingsnake chemical cues. Although avoidance of kingsnake cues during y-maze trials has been shown in colubrid snakes, it has yet to be demonstrated for crotalines.

Materials and Methods

Experimental subjects and stimulus animals. Test subjects were 48 juvenile cottonmouths from 18 wild-caught gravid females (10 in 2016 and 8 in 2017) that were collected from a single population in Stone County, Missouri and held until parturition. The eight females (SVL range 52.5–62.0 cm, mass range 206–305 g) that produced litters in 2017 were retained and used as stimulus donors but the 2016 females were released in September 2016. I used three captive-born Florida Kingsnakes (*Lampropeltis floridana*), including one juvenile (56 cm SVL, 85 g) and two adults (SVL 96.2 and 101 cm, mass 510 and 591g) as predator cue donors. Kingsnakes are specialized snake predators that readily consume pit vipers. Eight wild caught

Ringed Crayfish (*Faxonius neglectus*) collected from local streams were used as non-predator cue donors. I selected crayfish as a non-predator control because they are abundant in aquatic systems but are neither predators nor prey of cottonmouths. Cottonmouths were collected under permits issued by the Missouri Department of Conservation, and research procedures were approved by the Missouri State University IACUC (October 2017, Protocol 18-010).

Housing and maintenance. Cottonmouths were maintained in vivarium at Missouri State University at 25 °C with a 12:12 light:dark cycle. Snakes were housed individually in clear plastic cages (33L x 18W x 9H cm) with a water dish, PVC pipe shelter, and newspaper substrate. Shelves used to store snake cages were heated along one edge to create a thermal gradient (25–30 °C) within each cage. Snakes were fed a multispecies selection of commercial bait minnows approximately every 10 days, and water was available ad libitum. To avoid habituation of test subjects to predator cues (e.g. Downes 2002), kingsnakes and cottonmouths were maintained in separate rooms. Kingsnakes were maintained as described for cottonmouths but in larger cages and fed thawed mice weekly. Crayfish were housed in 9.5 liters tanks fitted with an aerator and a cover object and fed algae disks bi-weekly.

Stimulus collection. I collected cutaneous cues from snakes by rubbing a cotton ball along the dorsal, ventral, and lateral surfaces of stimulus donors (kingsnake: n=3, 39–93.2 cm, SVL; 30–300 g). Because musk may act as an alarm substance (Graves and Duvall 1986), I attempted to prevent contamination by collecting cues outside the room where trials occurred and covering the cloaca and tail region of donor snakes when collecting cutaneous cues. I collected musk cues by covering the cloaca with a paper towel and then agitating the stimulus donor until musking occurred. The musk-soaked pieces of paper towel were combined with 10 mL of water to create a musk solution for use in trials. At least two stimulus donors were used in

preparing each musk solution. Crayfish cues (n=8) were collected by first drying the crayfish and then rubbing a cotton ball along the dorsal surface of the stimulus donor. New cues were collected during each day of testing to ensure that the integrity of cues was uniform across trials.

Testing apparatus. I conducted trailing experiments in a y-maze apparatus similar to those used in previous studies of conspecific trailing by neonatal rattlesnakes (Brown and Maclean 1983; Hileman et al. 2015). The maze design used in my study consisted of a 63-cm long introduction arm connected to two 95-cm long test arms. The walls of each arm were 15-cm high and the distal end of each test arm was blocked with a 15 × 15 cm wall. All portions of the maze were constructed out of 1-cm thick plywood connected to a wooden panel base.

Procedure. For testing, snakes were transferred to an adjacent room. To encourage subjects to explore the apparatus, I conducted all trials under dimly lighted condition and within a time frame (1500–1800 hrs) when I observed captive snakes were the most active. I tested juvenile cottonmouths in y-maze dichotomous choice tests in predator avoidance experiments. During each trial I covered the floor of the apparatus with plain butcher paper. The scents used in each trial were randomly assigned to each arm of the maze. I applied cues to butcher paper substrate by placing a cotton ball containing the desired treatment on the floor at the proximal end of the test arm and rolling it continuously to the distal end of that arm. The cotton ball used to apply a treatment remained at the distal end of the arm during the trial. I replaced the butcher paper after each trial. A curtain divided the room so that simultaneous trials could be run without disrupting adjacent trials.

Because juvenile cottonmouths are easily disturbed, I attempted to minimize handling stress by transferring each test subject from its cage to the y-maze enclosed within a portable transport shelter. Shelters consisted of a 13.5 × 7 cm halved piece of PVC pipe with the concave

surface oriented downward, attached to a clear plastic bottom (Fig. 1). One end of the shelter was closed with a plastic end cap while the open end had a 3×7 cm clear plastic door attached to the adjacent front section of the shelter floor with a strip of masking tape. The tape connection served as a hinge that allowed closure of the door once the snake was inside. Several days prior to testing, normal shelters were replaced with transport shelters in each snake's cage. Most snakes were concealed within shelters prior to testing, allowing the door to be closed and taped shut before transport to the y-maze apparatus.

Each trial began with a shelter containing a snake positioned at the opening of the introduction arm with the door facing into the maze. Snakes were allowed a 5-min acclimation period, after which the door of the shelter was opened. If after 10-min the snake did not emerge I tilted the shelter until the snake gently slid into the maze. During trials I stood stationary behind the apparatus and recorded the snake's behavior as it moved forward through the maze. Response variables recorded in each trial included: time spent in each arm, time within the y-junction, and the number of tongue flicks in each experimental arm. Trials lasted a maximum of 60 min but ended when a final choice was made. A choice was recorded when an individual reached the end of an experimental arm and made contact with the cotton ball.

Statistical analyses. I used Chi-Square Goodness-of-Fit Tests to compare response frequencies for the experimental and control scents, and independent samples t-tests to assess mean differences in TFR between choice arms. Parametric assumptions were met. Means are reported ± 1 SD. Significance was assessed at $\alpha < 0.05$. All analyses were done in RStudio version 1.1.414.

Data collection. In this experiment, I compared juvenile cottonmouths' use of space in y-maze tests where each test arm contained either kingsnake chemical cues or scents from crayfish

(non-predator) or a blank control. To control for experience-related order effects, I initially tested each snake once where kingsnake cues were either paired with a blank control (n=19), or with crayfish cues (n=13). The arm containing kingsnake cues was randomly determined for each trial. Following the kingsnake cue trials, I retested 16 snakes used in the previous trials to evaluate responses when given a choice between crayfish cues and a blank control. All tests were conducted during February–March 2018 at 1500–1800 when individuals were 5–6 months old.

Because cottonmouths unexpectedly did not exhibit avoidance of kingsnake chemical cues, I conducted additional experiments using cottonmouth musk as a cue. Responses to musk secretions are consistent with its function as a chemical alarm cue in rattlesnakes (Graves and Duvall, 1986) and cottonmouths (Meinders, unpublished). Therefore, I expected that including musk in the testing environment would increase threat sensitivity of test subjects. To determine if musk alone would elicit an avoidance response, I first tested 15 juvenile cottonmouths in y-maze experiments where the experimental arms contained either musk solution or a blank control. I then used these snakes in an additional y-maze experiment where kingsnake chemical cues were paired with a blank control, but with a cotton ball soaked with musk solution in the y-junction. Test subjects for both experiments were 14–15 month old. After musk solutions were prepared as described earlier (see general procedures), I applied 10 drops to a cotton ball, which was either rolled down one arm of the maze or placed in the y-junction. All tests were conducted at 1500–1800 during October–November of 2018.

Results

Individuals took an average of 13.5 ± 14.17 min to complete kingsnake trials. There was no difference in the number of snakes that selected the blank control (observed= 7, expected= 9.5) or the kingsnake experimental arms (observed=12, expected= 9.5, $X^2 = 1.32$, $df=1$, $n=19$, $P=0.251$, Fig. 2), and there was no difference in the average time spent in choice arms (control: 3.8 ± 4.45 min; kingsnake: 2.85 ± 0.93 min; $P=0.353$). There was also no preference between the crayfish (observed = 6; expected = 6) and kingsnake experimental arms (observed=6; expected = 6; $X^2 = 0$, $df=1$, $n=12$, $P=1.0$), with one individual failing to make a choice within 60 min. Again, there was no difference in the average time spent in choice arms (crayfish: 8.63 ± 11.13 min; kingsnake: 3.38 ± 1.41 min; $P=0.303$). Snakes selected the blank control arm (observed= 11; expected = 7) significantly more often than the experimental arm containing crayfish cues (observed = 3, expected = 7; $n= 14$, $X^2 = 4.57$, $df=1$, $P=0.033$, Fig. 2), and there was no difference in the amount of time spent between the two experimental arms (control: 3.12 ± 4 min; crayfish: 3.54 ± 1.15 min; $P=0.791$). The mean trial length for individuals that chose an experimental arm was 15.84 ± 19.4 min; with two individuals failing to make a choice within 60 min. No difference in TFR between choices in any of the trails was observed (control vs. kingsnake: control: $n= 7$, 24.16 ± 15.34 flick/min; kingsnake: $n=12$, 28.15 ± 9.94 flicks/min; $P = 0.553$; control vs. crayfish: control: $n=11$, 21.01 ± 84 flicks/min; crayfish: $n=3$, 18.08 ± 8.91 ; $P = 0.644$; crayfish vs. kingsnake: crayfish: $n=6$, 19.43 ± 6.63 flicks/min; kingsnake: $n=6$, 22.61 ± 9.01 flicks/min; $P = 0.505$).

It took 15 individuals on average 9.45 ± 11.75 min to complete musk trails, with individuals having no preference for either the control (observed = 6; expected= 7.5;) or the musk cue (observed = 9, expected = 7.5; $X^2 = 0.6$, $df=1$, $n=15$, $P=0.439$, Fig. 3) with no

difference between the amount of time spent in the experimental arm (control: $1.67 \pm \text{min}$; musk: $2.03 \pm \text{min}$). There was also no preference between kingsnake (observed = 9, expected = 7) and the control arms when in the presence of musk (observed = 5, expected = 7; $\chi^2 = 1.14$, $df=1$, $n=14$, $P=0.29$, Fig. 4) On average individuals took 10.2 ± 11.5 min to complete trials, with no difference in the amount of time spent in the two experimental arms (kingsnake: $1.43 \pm \text{min}$; control: $2.11 \pm \text{min}$). No difference in TFR was observed in either experiment (control vs. musk: control: $n= 6$, 26.86 ± 6.79 flick/min; musk: $n=9$, 25.53 ± 3.89 flicks/min; $P = 0.677$; control vs. kingsnake – with musk : control: $n=5$, 24.88 ± 4.38 flicks/min; kingsnake: $n=9$, 27.07 ± 5.80 flicks/min; $P = 0.445$).

Discussion

Y-maze experiments provide an ecologically relevant situation for testing avoidance of predator chemical cues in snakes. Surprisingly, my test subjects neither avoided kingsnake chemical cues in favor of a blank control nor decreased their TFR in the presence of kingsnake cues, as cottonmouths have been reported to do in exploratory trials (Chiszar et al., 1978). This outcome contradicts previous studies of hatchling pine snakes (*Pituophis melanoleucus*) that avoided kingsnake chemical cues in Y-maze experiments (Burger, 1989; 1990). Consistent with increased vigilance, Burger (1989) also reported that juvenile pinesnakes emitted higher TFR and moved more slowly when exploring the maze arm with kingsnake cues compared to a control arm.

The failure of cottonmouths in my study to exhibit avoidance of kingsnake cues is paradoxical. The innate recognition of kingsnake chemical cues in many crotalid snakes, including cottonmouths, is well established (Cowles, 1938; Carpenter and Gillingham, 1975;

Weldon and Burghart, 1979). Exposure to kingsnakes has long been known to elicit a suite of unique defensive behaviors (including body bridging, head hiding, body inflation, and avoidance) in crotalids (Weldon and Burghart, 1979). However, this response is not necessarily exhibited by all members of a population and appears to vary inversely with size of the test subject and test experience (Gutzke et al., 1993). Because my study involved a reasonable sample size derived from a single cohort of young snakes with no prior exposure to kingsnake cues, it seems unlikely that I would not have observed any antipredator responses among my test subjects. Juvenile cottonmouths do not habituate to predatory threats therefore reinforcement to retain this innate response is also unlikely (Glaudas et al., 2006). The ODR has typically been reported when subjects are confronted with an actual kingsnake, or chemical cues from a kingsnake during handling disturbance. It is possible that the lack of disturbance in my testing design explains the apparent lack of choice by cottonmouths. Because residual chemical cues can be perceived long after a predator has left the area, they may not constitute a sufficient threat to promote avoidance of the stimulus (e.g. Head et al., 2002). Despite the ecological relevance of y-maze testing designs for evaluating predator avoidance, my study is one of very few to do so. The contrasting results between my data and the clear avoidance of kingsnakes reported for pine snakes by Burger (1989) could simply be a consequence of phylogenetic differences in responses of crotalids and colubrids. Additional trailing studies may be very useful for clarifying responses to snakes posing different threats (Weldon, 1990).

When testing the animal control cue I found that snakes had a preference for the control arm over that of the crayfish arm indicating that cottonmouths were avoiding crayfish cues. Crayfish were potentially stressed during cue collection which could have elicited an alarm cue and explain why cottonmouths avoid crayfish cues. Phylogenetically dissimilar species have

been reported to respond to alarm cues from organisms that share habitats when both may be exposed to similar predation threats. For example Ozark zigzag salamanders (*Plethodon angusticlavius*) respond to alarm cues from earthworms, an organism that shares the same microhabitat (Crane et al., 2013). Rainbow darters (*Etheostoma caeruleum*) also respond to alarm cues from aquatic Oklahoma Salamanders (*Eurycea tyrnerensis*; Anderson and Mathis, 2016). Because cottonmouths live primarily in association with aquatic habitats, where crayfish are abundant, responding to crayfish alarm cues could be beneficial in avoiding predators. Additional explanations for avoidance to crayfish cues include juveniles experiencing an antipredator response as juvenile cottonmouths are small and could be preyed upon by larger crayfish. Snakes could have also been experiencing neophobia as all individuals were naive to crayfish cues prior to trails (Brown et al., 2013).

For my second experiment I tested the behavior of juvenile cottonmouths to kingsnake chemical cues while in the presence of musk. Musk gland secretions have been implicated as alarm pheromones in other crotalids (Graves and Duvall, 1988) and behaviors of juvenile cottonmouths in defensive and foraging contexts change in a manner consistent with alarm pheromone predictions in the presence of musk (Meinders and Greene, unpublished). The intent of this experiment was to determine if the addition of an apparent alarm substance (musk) would promote avoidance behavior in the presence of kingsnake cues by elevating test subjects' threat sensitivity. However, I found that snakes still did not avoid kingsnake chemical cues even when in the presence of musk, an alarm cue. Although individuals did not respond with avoidance, it is possible that a subtle response could have occurred. Chiszar et al. (1978), found that cottonmouths reduced TFR when exposed to kingsnake chemical cues during exploratory trails

compared to a control cue. Due to the design of my trials I was unable to accurately record changes in TFR between cues or changes in activity levels.

In conclusion, I found no evidence that juvenile cottonmouths avoid chemical cues of kingsnakes. Despite the threat posed by kingsnakes, I propose that cottonmouths do not perceive a sufficient threat level from chemical cues alone to illicit an avoidance response. In the future, more studies need to examine the response of crotaline snakes to chemical cues from Ophiophagus predators, to gain a better understanding of this innate response.

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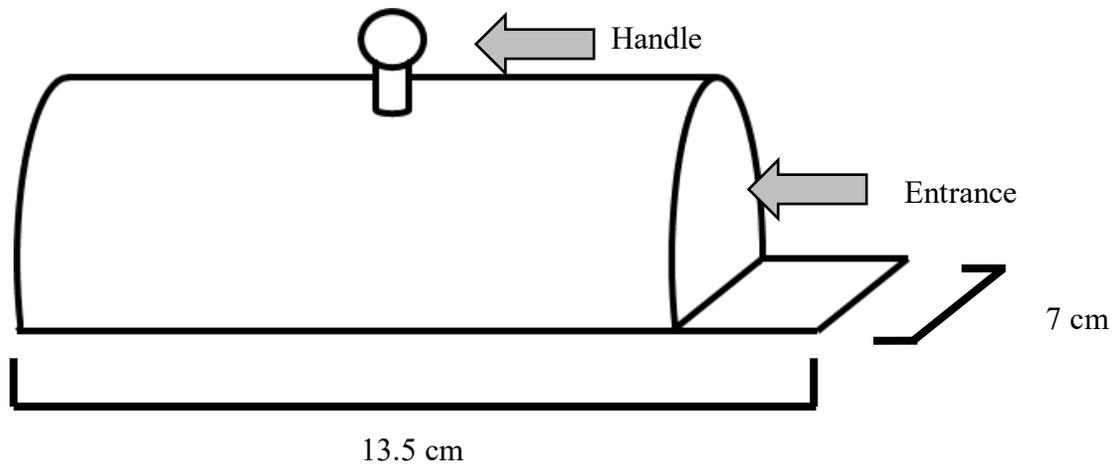


Fig. 1. Mobile shelter used to transfer snakes to a y-maze apparatus. One side of the shelter is open to allow snakes to enter. The door is then taped shut so that the shelter can then be moved without handling the snake. A central metal eye bolt creates a handle to allow safe manipulation of the shelter with a snake hook.

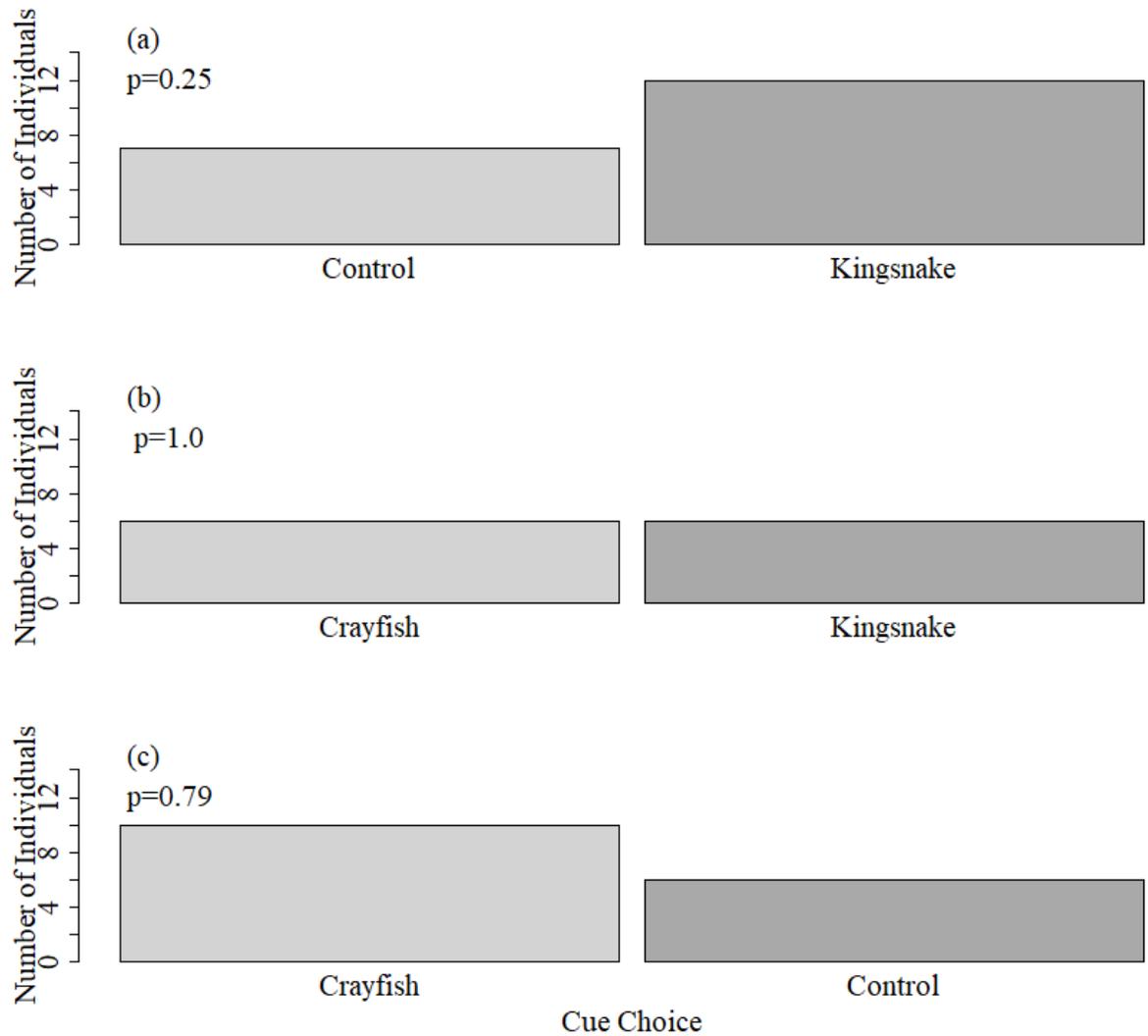


Fig. 2. The number of juvenile cottonmouths ($n=16$ in each trial) that chose (a) the kingsnake cue arm vs. the control arm, (b) the crayfish cue arm vs. the kingsnake arm, (c) and the crayfish cue arm over that of the control arm.

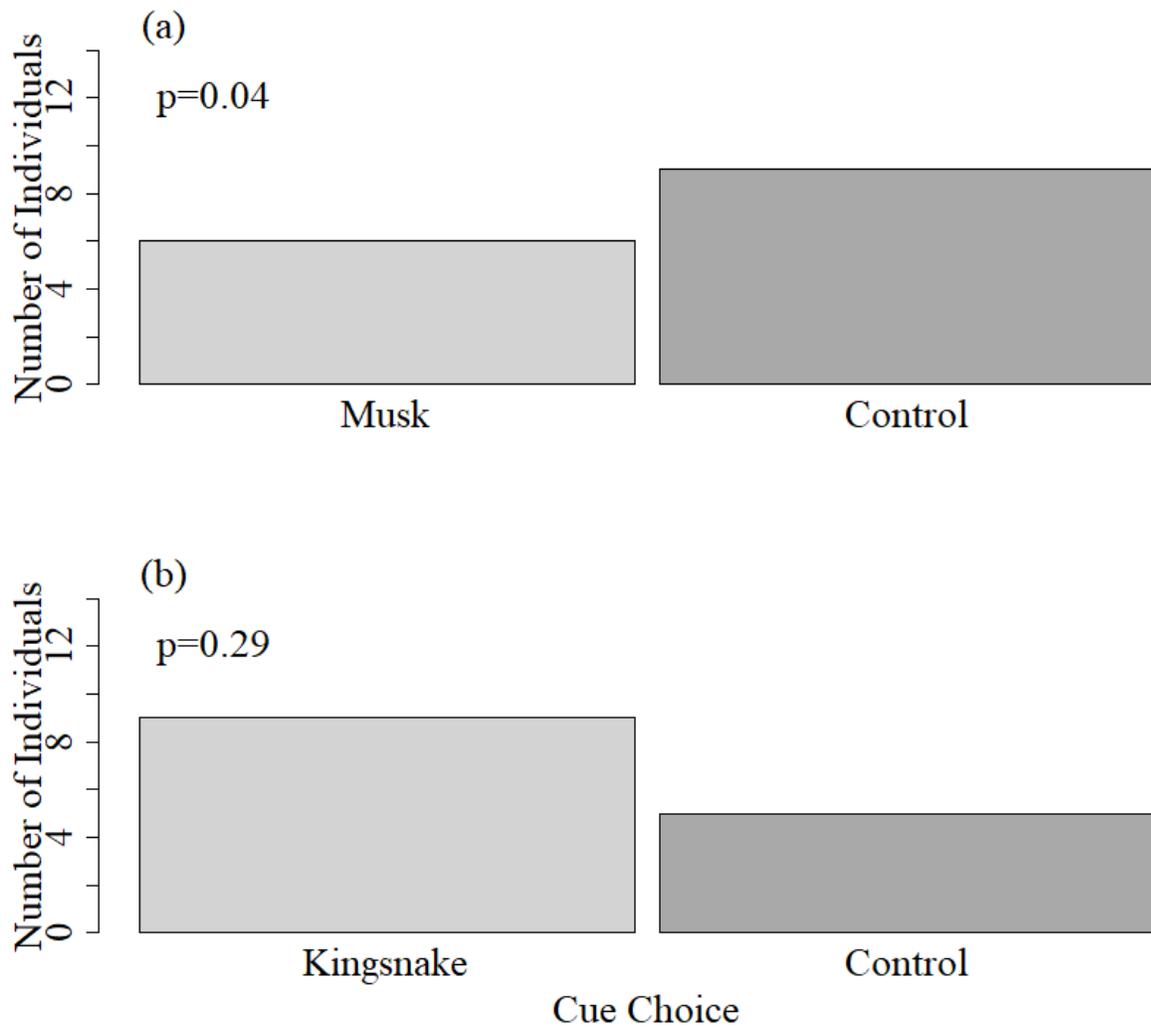


Fig. 3. (a) The number of juvenile cottonmouths (n=15) that chose the musk arm vs control (b) the number of individuals (n=14) that chose the kingsnake over the control when in the presence of musk.

SUMMARY

Few studies have explored behavioral response to chemical cues in snakes outside of a foraging or mating context. As our understanding of their complex behavior grows it will be imperative that we continue to explore how their behavior is influenced by chemoreception. My thesis research is novel in that it examined the response of cottonmouths to two ecologically relevant cues.

Similarly to other crotalines, juvenile cottonmouths scent trail conspecifics to aid in location of hibernacula. Juveniles can discriminate between their mother and non-related conspecifics however, it is unclear why they had a preference to trail conspecifics. Lower tongue flick rates during trails indicate that individuals may not be taking in enough information to successfully scent trail.

Juvenile cottonmouths are unique in that they do not respond with avoidance behavior when kingsnake chemical cues are detected. Threat levels were raised when snakes encountered kingsnake cues in the presence of musk, however no response was observed. Cottonmouths may need a visual confirmation of a threat to illicit an avoidance response.

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