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Spatial Learning of Shelter Locations and Associative Learning of a Foraging Task in the Cottonmouth, (Agkistrodon Piscivorus)

Rhyan Robert Friesen

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SPATIAL LEARNING OF SHELTER LOCATIONS AND ASSOCIATIVE

LEARNING OF A FORAGING TASK IN THE COTTONMOUTH,

(AGKISTRODON PISCIVORUS)

A Masters Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Rhyan Friesen

May 2017

SPATIAL LEARNING OF SHELTER LOCATIONS AND ASSOCIATIVE

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(AGKISTRODON PISCIVORUS)

Biology

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Master of Science

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ABSTRACT

Learning processes can have significant adaptive value in mediating behaviors with important fitness consequences. For snakes, learning may ensure the localization of key resources including food and shelter. Snakes are proportionately underrepresented in reptile learning studies, and the majority of research subjects are within the family Colubridae. I presented captive cottonmouth snakes with two types of learning challenges. In a spatial learning experiment, snakes learned the location of a cover object in a circular laboratory arena, as demonstrated by a 56% reduction of time and 57% reduction of distance travelled to a shelter across a series of training trials. In a foraging experiment, snakes were trained to feed on live minnows in water bowls associated with a landmark. When given a dichotomous choice between two water bowls containing only fish cues, trained snakes were significantly more likely to set up ambush postures at the landmark bowl rather than the control. Untrained snakes presented with the same challenge showed no preference for either option.

KEYWORDS: cottonmouth, *Agkistrodon piscivorus*, pitviper, snake, associative learning, spatial memory, conditioning, visual cues, chemosensory cues

This abstract is approved as to form and content

_____

Brian Greene, PhD
Chairperson, Advisory Committee
Missouri State University

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Approved:

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Brian Greene, PhD

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I thank the following people for their support during the course of my graduate studies: Dr. Brian Greene, Dr. Alicia Mathis, and Dr. Day Ligon for their expertise, lab space, and resources.

I dedicate this thesis to my lovely wife and daughters.

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INTRODUCTION

The ability to locate resources, such as food, water, and shelter, has important

survival consequences for animals. Learning allows animals to modify their responses

through experience to more efficiently exploit these resources. For example, spatial

learning should enhance the ability to locate shelters and feeding areas in complex habitat

structure (Reinert 1993). Associative learning may also improve foraging performance

by allowing animals to recognize cues that indicate prey abundance or proximity

(Kuszewska et al. 2016). Because learning ability may have both ecological and

phylogenetic components, it is important to conduct comparative studies across a range

of taxa (Bingman 1992; Stone et al. 2000; Kuszewska et al. 2016).

Reptiles have historically been thought to have limited learning abilities compared

to other terrestrial vertebrates (Brattstrom 1974; Burghardt 1977). Initial explanations for

the apparent impoverishment of reptile learning ability included the assumption that

instinctive behavior patterns were not subject to experiential modification, and that many

early studies were methodologically inappropriate for reptiles (Suboski 1992).

Eventually, subsequent studies revealed that reptiles are capable of a wide range of

learned responses. For example, reptiles exhibit learned discrimination using a variety of

sensory modalities, including taste (Paradis & Cabanac 2004), vision (Schroeder & Loop

1976; Gaalema 2011; Clark et al. 2014), and hearing (Lenhardt 1981). Studies of

monitor lizards (*Varanus griseus*) have found a capacity for classical conditioning

(Sollertinskaya 1967) and problem solving (Manrod et al. 2008).   Despite being

generally less social than birds and mammals, reptiles have recently been shown to learn

tasks through imitation of conspecifics. Such social learning has been demonstrated in turtles (Wilkinson et al. 2010; Davis & Burghardt 2011) and lizards (Noble et al. 2014, Kis et al. 2014), importantly demonstrating that these reptiles possess some cognitive abilities that are similar in scope to those of mammals and birds.

Snakes are underrepresented in the learning literature relative to other reptiles. The paucity of learning studies in snakes is likely a consequence of assumed cognitive deficiencies and a historical use of study designs that are inappropriate for snakes (Abramson & Place 2008; Emer et al. 2015). The available studies of learning in snakes have focused mainly on ecologically relevant topics. For example, habituation of defensive responses is widespread in snakes, occurring in natricine (Hampton & Gillingham 1989; Herzog et al. 1989), and xenodontine colubrids (Burghardt 1977), and crotalines (Glaudas 2004; Place & Abramson 2008). Operant conditioning has been demonstrated in at least two snake species. In one study, indigo snakes, *Drymarchon corais,* were conditioned to flip a contact relay for a reward of water (Kleinginna 1970). In another study, large Burmese pythons, *Python molurus bivitattus,* were able to learn to push an illuminated button to receive access to a food item (Emer et al. 2015). There have been no studies on conditioning in pitvipers of any species.

Spatial learning has particularly important fitness consequences in snakes that must find and remember the locations of essential resources in structurally complex habitats (Burghardt 1977). Studies of spatial learning ability in snakes have involved two species, each of which demonstrated an ability to learn the location of a shelter in a novel environment using visual cues. Young corn snakes, *Elaphe guttata guttata*, learned to identify escape locations, demonstrated by non-random directional movement and

decreased latencies to the goal after training in a novel environment (Holtzman 1998; Holtzman et al. 1999). In a parallel study, juvenile spotted pythons, *Anteresia maculosus*, also showed a decrease in distance travelled. The study also provided data on male and female performance, indicating that males travelled longer distances (Stone et al. 2000). Collectively, these studies provide a standard spatial learning study design that may be adapted to other snake taxa across a variety of ecological circumstances. To date, spatial learning abilities of pitvipers have not been experimentally evaluated.

Pitvipers, especially rattlesnakes, have received considerable research attention relative to most other lineages of snakes, leading to their characterization as model organisms (Beaupre & Duvall 1998). Despite their biology being thoroughly described overall, very few formal studies of learning in rattlesnakes, or other viperid taxa, have been conducted. The lack of attention to learning processes in these snakes may be explained in part by challenges inherent in working with the organisms themselves. In addition to their venom creating difficulties for researchers, most viperids have low energetic demands and feed relatively infrequently, resulting in food being a poor motivator in conditioning studies (Abramson & Place 2008). Pitvipers have a long history of behavioral study, particularly regarding prey discrimination and other aspects of foraging. Although many authors reported that test subjects' behavior changed over time, these studies were not designed to test hypotheses about learning and lacked the appropriate controls to evaluate learning mechanisms or distinguish between innate and learned behaviors (reviewed in Abramson & Place 2008).

The foraging behavior of rattlesnakes is well characterized. These sit-and-wait strategists typically establish coiled ambush postures in locations that are selected in

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association with prey chemical cues (Duvall et al. 1990; Roth et al. 1999). However, structural features also play a role in foraging microhabitat selection, as demonstrated by adoption of ambush postures in association with vegetation structure typically used by prey species (Reinert 1993; Theodoratus & Chiszar 2000). It is unknown whether such selection patterns are innate or subject to experiential modification. Timber rattlesnakes have been reported to modify their foraging behavior due to experience with chemical cues (Clark 2004a). Given that snakes are known to respond to visual stimuli associated with foraging success (e.g. Mullin & Cooper 1998), it is reasonable to expect that snakes might also adjust foraging behavior based on experience with such cues.

The cottonmouth snake (*Agkistrodon piscivorus*) is a common pitviper associated with aquatic habitats in the southeastern United States. Many aspects of cottonmouth behavior have been experimentally studied, including foraging (Savitsky 1992; Lillywhite et al. 2015), social interactions (Roth & Lutterschmidt 2011; Hoss & Clark 2014; Hoss et al. 2015), and defense (Gibbons & Dorcas 2002; Roth & Johnson 2004; Glaudas & Winne 2007). Studies of learning in cottonmouths are entirely limited to habituation of defensive responses (Glaudas 2004; Glaudas et al. 2006). I chose to study learning to add a new facet to the existing research on cottonmouth behavior and address questions that have been largely unexplored in viperid snakes.

I conducted three experiments to evaluate learning abilities of cottonmouths in spatial and foraging contexts. The first experiment was intended to compare spatial learning in cottonmouths to previous studies in which corn snakes, *Elaphe guttata guttata* (Holtzman et al. 1999), and spotted pythons, *Anteresia maculosus* (Stone et al. 2000), placed in an unfamiliar area, learned the location of a shelter using a visual cue. No

similar studies have been conducted in pitvipers. Because cottonmouths frequently hide in habitat structure, I predicted they would also learn to locate shelter by demonstrating a decrease in latency and distance travelled to cover over time. In addition, I predicted that male cottonmouths would engage in more exploratory behavior because they maintain substantially larger home ranges and apparently have greater spatial processing abilities than females (Roth et al. 2006).

The other two experiments addressed foraging-related questions. The first tested whether cottonmouths conditioned to feed on minnows in water bowls would exhibit foraging postures in association with fish chemical cues. Despite successful experimental induction of foraging postures in a variety of rattlesnakes in both natural and captive settings (Roth et al. 1999; Theodoratus & Chiszar 2000; Clark 2004a), and its suggested utility as a behavioral assay for prey preference experiments in viperids (Clark 2004a), it was necessary to validate this method in captive cottonmouths before assuming its efficacy as a response variable for learning experiments. I predicted that foraging cottonmouths would preferentially set up ambush postures at sites with prey chemical cues relative to control sites.

A final experiment was designed to evaluate whether cottonmouths can use experience with visual cues to select ambush sites. Timber rattlesnakes (*Crotalus horridus*) have been shown to use experience with chemical cues to make foraging site selections (Clark 2004b). However, the extent to which pitvipers can utilize experience with visual cues in foraging contexts is unknown. I hypothesized that cottonmouths would be able to associate a visual cue with successful foraging experience.

MATERIALS AND METHODS

Test Animals

I conducted experiments on 33 captive-raised juvenile cottonmouths (*Agkistrodon piscivorus*) (45-51 cm, SVL; 8-15 g) born in the lab to four pregnant females collected in southwestern Missouri over two consecutive years. All snakes were either 12 months old (n=18) or 24 months old (n=15) at the time of testing. Snakes were individually housed in rectangular 20 L plastic containers (25 x 45 x 15 cm) with newspaper bedding and a halved 5 x 15 cm section of PVC pipe for cover, and maintained at a room temperature of 23 to 27°C with a 12:12 light-dark cycle. I fed the snakes live bait minnows, a mixture of *Pimephales*, *Promelas,* and Various shiners (*Cyprinella sp.),* 3-4 cm in length, every 11-14 days from a clear plastic water bowl, which also provided water *ad libitum* between feeding events. The adult snakes were collected under permits issued by the Missouri Department of Conservation, and experimental procedures were approved by the Missouri State University IACUC (June 2012, Protocol 12-042).

Experiment One: Spatial Learning

Using a study design similar to Holtzman et al. (1999), I conducted trials in a circular plastic arena, 1 m in diameter with 25 cm walls. The interior surfaces of the arena were coated with black textured paint to facilitate locomotion. The arena contained four 5 x 15 x 5 cm sections of halved PVC pipe, identical to cover objects found in the snake cages. The cover objects were oriented lengthwise at 90° angles from the center

and 15 cm away from the edge of the arena (F1). Three of the four cover objects were sealed at both ends with plastic stoppers to prevent snakes from entering.

One of the four cover objects had a single open end, oriented toward the periphery of the arena that was not visible from the snake's starting point. This arrangement required the snake to explore behind each of the cover objects to find the entrance to the shelter. The initial location of the useable cover object was randomized for each snake, but then remained constant for that snake in successive trials. A 30 x 50 cm white plastic card was in a fixed position along the western periphery of the arena, 20 cm above the arena floor, to provide the snakes with a large visual cue. The position of the cue card remained constant for each snake throughout the experiment. During trials, the arena was surrounded by a blue plastic curtain to minimize confounding effects of visual cues outside the test arena. A single light source was suspended above the arena to create an illuminated open area that would encourage the snakes to seek cover, and an HD video camera (Canon ts400) recorded the events of each trial.

For this experiment, I used 14 of the 15 two-year-old juvenile cottonmouths, eight males and six females. One individual was not included, due to a spinal deformity. The decision was made not to use the one-year-old snakes (n=18) in this test. In preliminary trials, the younger snakes would remain stationary and engage in defensive behavior. Training sessions were conducted from 0900-1400 to control for time of day effects between day and evening time periods. The training sessions consisted of four trials per day and continued for four consecutive days. To minimize effects of recent feeding history on mobility, trials were conducted 5 d after each snake's previous meal. The

testing order for each snake was randomized for each day, and snakes were rested in their home enclosures for a minimum of 30 min between trials.

Each trial began with the snake being placed under an inverted opaque plastic container in the center of the arena. The snake was allowed to acclimate for 30 s, after which the container was lifted. Snakes were considered to have located the cover object when any part of the snake's head, including its tongue, broke the plane of the hidden opening. Any snake that had not located the cover object within 15 min, for any trial, was directed, using snake tongs, toward the cover object. Once the snake entered the cover object, it was allowed to remain in cover for 1 min. The snake was then removed and placed back into its home enclosure. Following each trial, the arena was cleaned with alcohol and the cover objects were rinsed in bleach solution and then tap water to eliminate chemical cues from previous trials.

Following the 4 d of training sessions, video files were downloaded and evaluated to determine the duration of each trial. I also determined the distance travelled by each snake from the video files using Venier's Logger Pro 3.8.6 software to trace the path of each snake as it moved through the arena.

Experiment Two: Foraging Responses to Chemical Cues

Free-ranging pitvipers are known to establish foraging postures according to the location of prey chemical cues (Roth et al. 1999; Theodoratus & Chiszar 2000). This behavior has also been observed in captive timber rattlesnakes (*Crotalus horridus*), suggesting that foraging postures can be used as a bioassay for assessing responses to prey chemical cues in pitvipers (Clark 2004c). I wanted to verify that captive

cottonmouths also exhibit this behavior before using it to evaluate conditioning in captive snakes.

The pre-conditioning of snakes to feed from their water bowls created a convenient way to evaluate foraging responses to chemical cues. I conducted a series of three dichotomous mock foraging trials for each snake in a repeated measures design. Each trial involved two water bowls being placed on opposite, randomly determined sides of the cage. Each bowl contained either a solution containing one of three possible cues or tap water. Two different prey chemical cues (fish and rat) and one novel cue (mint extract) were used as chemical cues with the order of cue presentation randomized for each snake. Fish chemical cues were derived from 1000 ml of aquarium water containing 60 minnows. The chemical cue solution was prepared by thawing a frozen laboratory rat pup (*Rattus norvegicus*), in 1000 ml of water. I used 1.0 ml of mint extract in 1000 ml of tap water to prepare the novel stimulus for each trial. During each trial, 50 ml of tap water was added to each bowl. In addition, 2.0 ml of cue solution was added to one of the water bowls and to the other bowl a comparable volume of untreated tap water.

I tested 33 juvenile cottonmouths from four litters. Individuals were 12 or 24 months old. All trials were conducted 11-14 days after the previous feeding to standardize hunger levels and promote strong feeding responses. To minimize disturbance, trials were conducted in each snake's home enclosure. Prior to each trial, the snake was transferred to a holding tank while the experimental conditions were set up. The enclosure floor was covered in brown paper with a line bisecting the width of the enclosure to facilitate scoring of snake locations. Two water bowls were placed on opposite ends of the enclosure, equal distance from the center line (F2). One bowl

contained either a prey species chemical cue or mint extract solution and the other contained tap water. There was no cover object in the enclosure. Once the enclosure was adjusted and the stimulus added, I placed a snake in the middle of the container, closed the lid and turned off the overhead lights to the lab. A small LED lamp was placed in the corner of the room to provide dim ambient light to simulate crepuscular light levels. Trials were started in late afternoon and each snake's location was evaluated two hours later, when cottonmouths normally forage. Site selection was determined by recording in which half of the enclosure the snake was residing. If the snake's body straddled the midline, the residing side was determined by the location of the head.

Experiment Three: Conditioning Using Visual Stimuli

I used 32 of the 33 juvenile cottonmouths (excluding one individual with a spinal deformity) from the previous experiment to test whether snakes could learn to associate a visual landmark with a successful foraging site. The enclosure setup and snake transfer protocol were identical to experiment two. To train the snakes to associate a visual landmark with a prey item (minnow), two bowls filled with 50 ml tap water were placed on opposite halves of the enclosure. In the treatment group (n=16), one of the bowls contained a live minnow; this bowl was also paired with a red plastic card (13 x 18 cm) which was fastened to the side of the enclosure above the bowl to serve as a visual cue (F2). The control group snakes (n=16) were also fed a live minnow from one bowl, but with the plastic card location randomly assigned rather than paired with the minnow location. Conditioning trials were set up in the late afternoon and then snakes were observed two hours later in the early evening to record if the prey had been consumed.

The enclosures were restored to the normal maintenance setup the following morning. Conditioning trials occurred every 11-14 days, and continued for eight feeding cycles.

Fourteen days after the final conditioning trial I tested snakes for associative learning in their next feeding cycle. Each snake was placed into the enclosure set up identical to conditioning trials, but with both bowls containing only aquarium water with fish chemical cues, no actual fish. The plastic cue card from the training trials was placed in a randomly-selected side of the cage. After two hours, the location of each snake was noted with snake choice scored as in experiment two. Following the test for associative learning, the snakes resumed normal feeding cycles without the red card and were retested after two additional feeding cycles (spanning 25 days) to determine if learned patterns were retained without reinforcement.

Data Analysis

The data from experiment one were evaluated for adherence to assumptions of ANOVA and transformed when necessary to improve normality and heterogeneity of variances prior to testing. Differences in time elapsed and distance travelled across trials were evaluated using a two-way ANOVA; with repeated measures (RMANOVA), with day and trial as fixed factors. Additional RMANOVA's were performed to evaluate male and female performance. Significant results were followed by multiple comparison procedures (Tukey Test) to evaluate differences among individual means. I calculated effect size using eta squared η^2 (Small=0.01, Medium=0.06, Large=0.14) (Nakagawa & Cuthill 2007). For experiment two, frequencies of snake responses were evaluated using chi-square tests of independence. I calculated effect sizes using Phi φ for the 2 x 2 tables

(Small=0.10, Medium=0.30, Large=0.50) and Cramer's V for the larger tables

(Small=0.07, Medium=0.21, Large=0.35) (Nakagawa & Cuthill 2007). Results are

reported as mean ±1SE. I used the stat. package SigmaPlot® Version 11 for all three

experiments and alpha was established as 0.05.
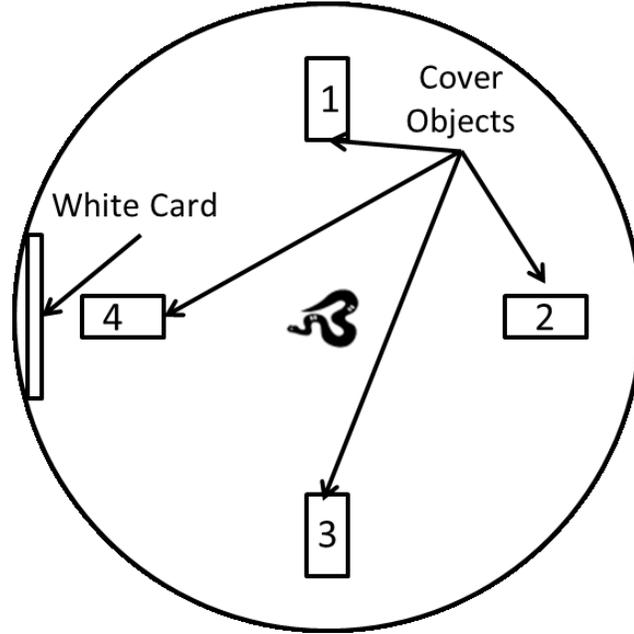
Figure 1. Arena layout used in the spatial learning experiment. The arena was 100 cm in diameter with an outer edge 25 cm high. Four cover objects (semi-circular sections of halved PVC pipe) were placed perpendicular to, and 15 cm from, the edge of the arena. One of the four contained an opening facing the edge of the arena. Both ends of the other three shelters were closed. The cue card provided a constant visual cue.

Figure 2. Testing arena setup for (A) Experiment Two: foraging responses to chemical cues and (B) Experiment Three: conditioning trials.

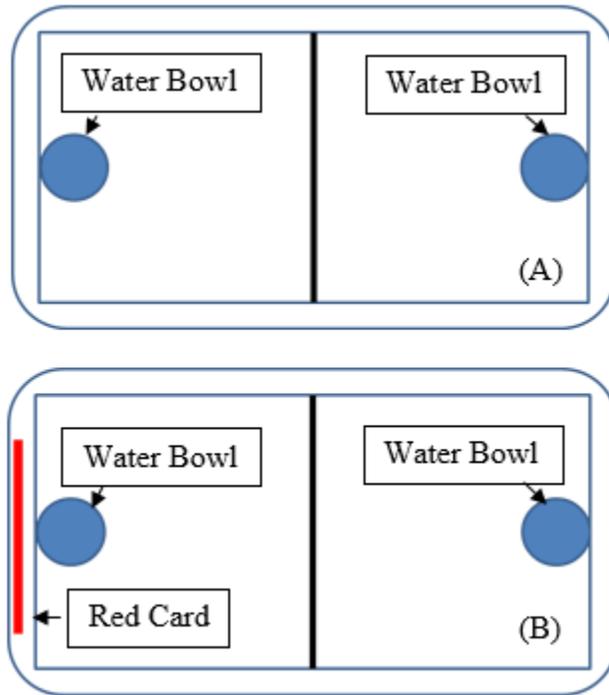**RESULTS**

Experiment One: Spatial Learning

Latencies. The mean time for snakes to locate cover objects declined significantly over successive days from 466.09 ± 69.37 s on day one to 207.29 ± 23.86 s on day three (RMANOVA $F_{3, 13}$=9.952, P<0.001, η^2=0.166; F3). This difference was due to mean differences between day one and day two (Tukey Test, P<0.005) with no significant decline after day two (Tukey Test, P=0.499-0.893).

There was a significant interaction between day and trial ($F_{9, 208}$=2.918, P=0.004, η^2=0.065; F3). Mean latencies for day one decreased significantly across successive trials with trial one significantly exceeding values for trials three (Tukey Test, P<0.001) and four (Tukey Test, P=0.004; F3). However, declines in trial latencies plateaued after day one and were not significantly different among other days (Tukey Test, P=0.189-1.000), suggesting that snakes learned shelter locations on the first day of testing.

Distances Travelled. There was a 57% reduction in mean distance travelled (RMANOVA $F_{3, 13}$=16.929, P<0.001, η^2=0.188; F4) over four consecutive days of trials from a maximum of 457.71 ± 54.26 cm on day one to 198.03 ± 13.25 cm on day four. The greatest reduction in distance occurred from day one to day two (Tukey Test, P<0.001) with no significant differences among later days (Tukey Test, P=0.187-0.636).

The interaction between day and trial was also non-significant (RMANOVA $F_{9, 208}$=1.707, P=0.095, η^2=0.056; F4). Mean distances travelled declined significantly across day one trials with trial four significantly lower than trials one and two (Tukey

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Test, $P<0.001$-0.004; F4). Trials within the remaining days showed no significant decreases among trials (Tukey Test, $P=0.635$-1.000).

Sexual Differences. Mean daily latencies to enter shelters decreased significantly for males over the four-day period (RMANOVA $F_{3,\ 18}=16.452$, $n=8$, $P<0.001$, $\eta^2=0.343$; F5), and this was entirely due to the latency for day one significantly exceeding values for all other days (Tukey Test, all $P<0.001$). Mean latencies for days two through four were not significantly different (Tukey Test, $P=0.737$-0.993). There was a significant day x trial interaction (RMANOVA $F_{9,\ 208}=2.823$, $P=0.007$, $\eta^2=0.107$). Analysis of pairwise comparisons revealed that, within day one, trial one had a significantly greater latency than trials three and four (Tukey Test, $P<0.001$-0.047); no other comparisons were significant (Tukey Test, $P=0.061$-1.000).

In contrast to males, latencies for females were not significantly different among days (RMANOVA $F_{3,\ 18}=1.306$, $P=0.309$, $\eta^2=0.037$; F5). However, there was a significant day x trial interaction (RMANOVA $F_{9,\ 208}=2.308$, $n=6$, $P=0.031$, $\eta^2=0.134$). Pairwise comparisons revealed that, within day one, trial two had a significantly larger latency than trial four (Tukey Test, $P=0.037$), but no other means were significantly different (Tukey Test, $P=0.07$-0.99). Comparisons of trials within all other days were non-significant (Tukey Test, $P=0.065$-1.000).

Males took significantly longer to enter shelters than females across days (RMANOVA $F_{3,\ 18}=8.750$, $P<0.001$, $\eta^2=0.083$; F5). However, this difference was only apparent on day one (Tukey Test, $P<0.001$) when mean latencies for males were 2.5-fold greater than mean latencies for females; mean daily latencies for each sex were not significantly different on subsequent days (Tukey Test, $P=0.298$-0.689). On day one,

among trials, the males' latencies, overall, were significantly greater than the females (RMANOVA $F_{3, 18}$=14.513, P=0.002, η^2=0.304; F5). The sex x trial interactions were not significantly different (RMANOVA $F_{9, 208}$=1.708, P=0.183, η^2=0.038; F5). However, males took significantly longer to utilize the cover object during trial one (Tukey Test, P<0.001) and trial four (Tukey Test, P=0.002).

The mean distances travelled by males to shelters decreased significantly over consecutive days (RMANOVA $F_{3, 18}$=18.619, P<0.001, η^2=0.281; F6). Distance travelled for day one was significantly greater than each successive day (Tukey Test, all P<0.001), but differences among subsequent days were not significant (Tukey Test, P=0.810-0.986). There was no significant interaction of day and trial ($F_{9, 208}$=0.923, P=0.512, η^2=0.054). However, on day one, the mean distance travelled was significantly greater than trial four (Tukey Test, P=0.021). Comparisons for all other trials were not significant (Tukey Test, P=0.121-1.000).

There was a non-significant decline in mean daily distances travelled by females (RMANOVA $F_{3, 18}$=3.195, P=0.054, η^2=0.087; F6), but there was no significant day x trial interaction (RMANOVA $F_{9, 208}$=1.228, P=0.302, η^2=0.108).

Overall, mean daily distances travelled by males and females were significantly different (RMANOVA $F_{9, 208}$=3.020, P=0.031, η^2=0.032; F6); this is due to the males travelling 62% farther than females on day one (Tukey Test, P<0.001). Differences among subsequent days were not significantly different (Tukey Test, P=0.729-0.986). Males travelled greater distances than females on day one (RMANOVA $F_{3, 18}$=4.781, P=0.049, η^2=0.081; F6), but there was no significant sex x trial interaction (RMANOVA $F_{9, 208}$=0.279, P=0.840, η^2=0.014; F6).

Experiment Two: Foraging Responses to Chemical Cues

In mock foraging trials snakes selected locations with fish cues significantly more often than sites associated with tap water (x^2=11.045, P=0.0008, n=33, V=0.409; F7). A majority of the snakes (24), chose rat cues relative to tap water, although the difference was non-significant (x^2=3.409, P=0.064, n=33; V=0.227; F7). There was no significant difference in snake responses between the mint cue and tap water, with a minority of the snakes (13) choosing the mint cue (x^2=0.742, P=0.389, n=33, V=0.106; F7).

Experiment Three: Conditioning Using Visual Stimuli

Fourteen of 16 snakes (87.5%) conditioned to forage with the index card established foraging postures in association with this visual cue when prey stimuli were controlled. This result was significantly different from what was expected by chance (x^2=4.5, P=0.009, φ=0.530). In the untrained group, equal numbers of snakes established foraging postures at the index card and unmarked bowl (F8). However, after two feeding cycles without reinforcement (25 days later), the trained snakes (x^2=0.444, P=0.505, φ=0.167) scored similarly to the untrained group in follow-up trials (x^2=0.571, P=0.449, φ=0.189; F9).
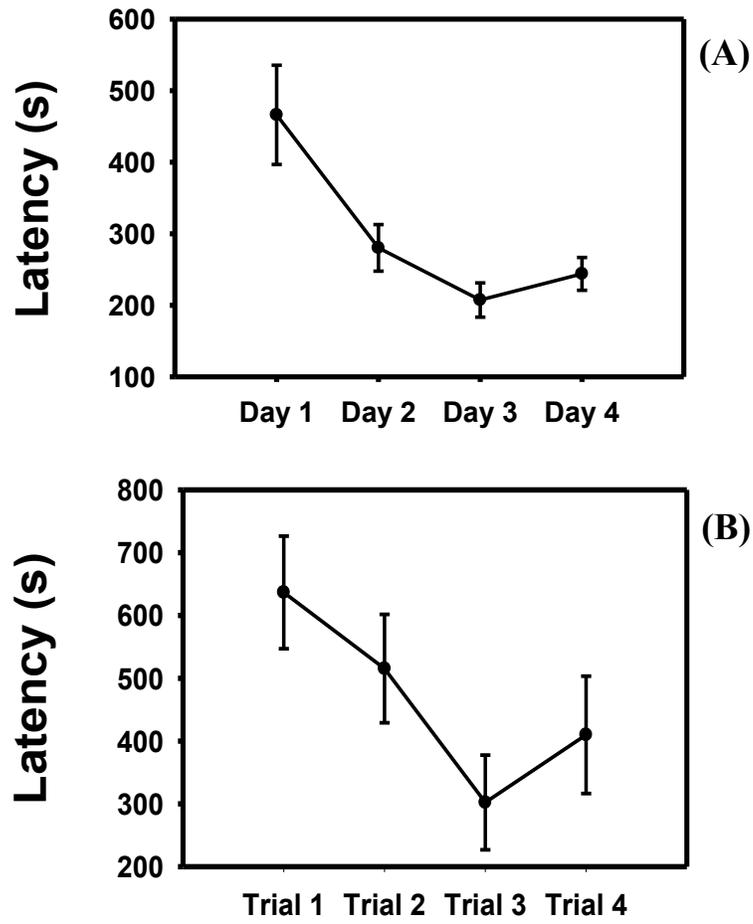
Figure 3. Mean latency to enter cover object by 14 *A. piscivorus* by (A) day and (B) trials on day one. Error bars represent 1 SE.
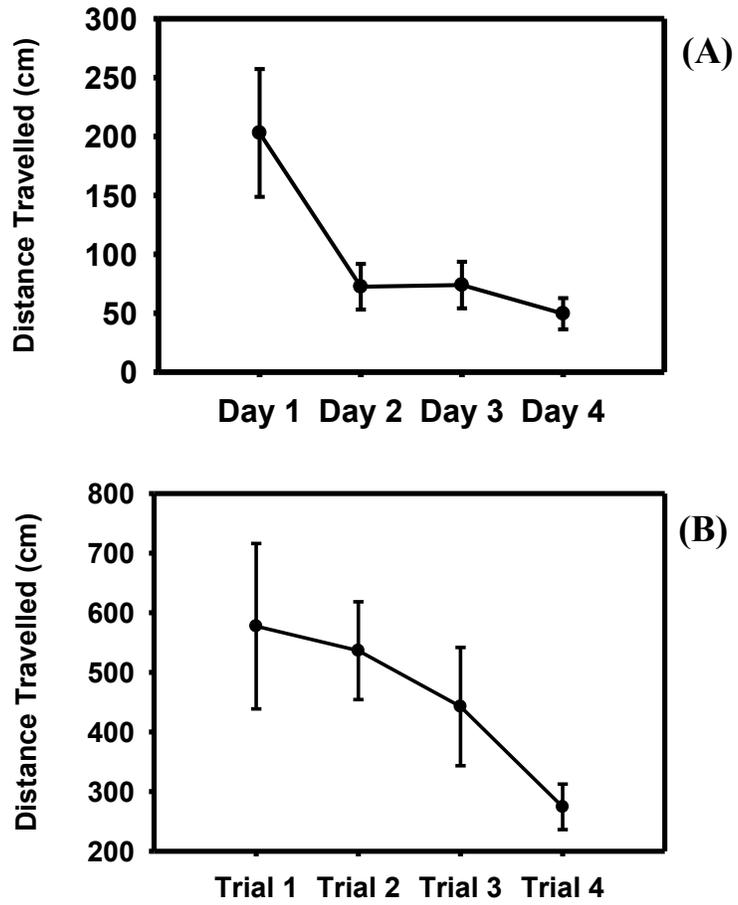
Figure 4. Mean distance travelled to cover object by 14 *A. piscivorus* by (A) day and (B) trials on day one. Error bars represent 1 SE.
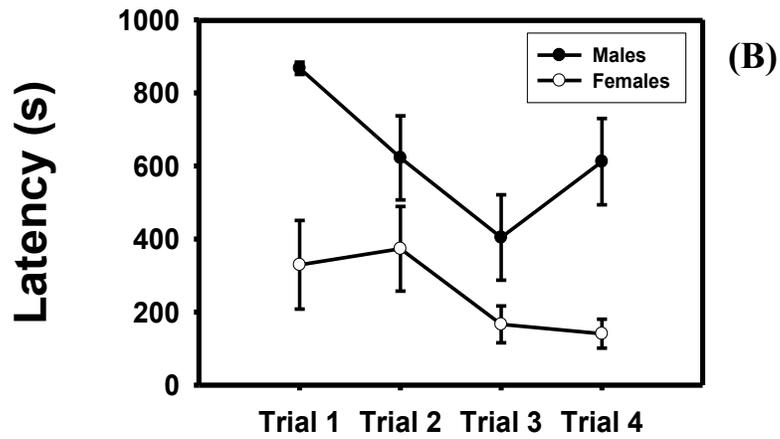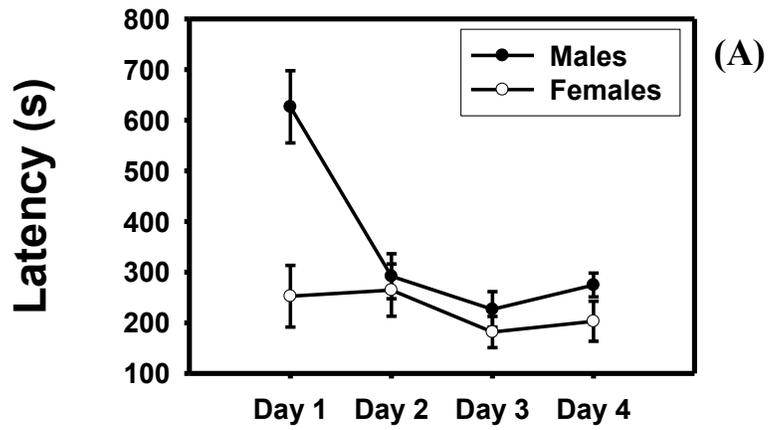
Figure 5. Mean latency to enter cover object in spatial learning trials by (A) day over four consecutive days for eight male and six female *A. piscivorus,* and (B) trial on day one. Error bars represent 1 SE.
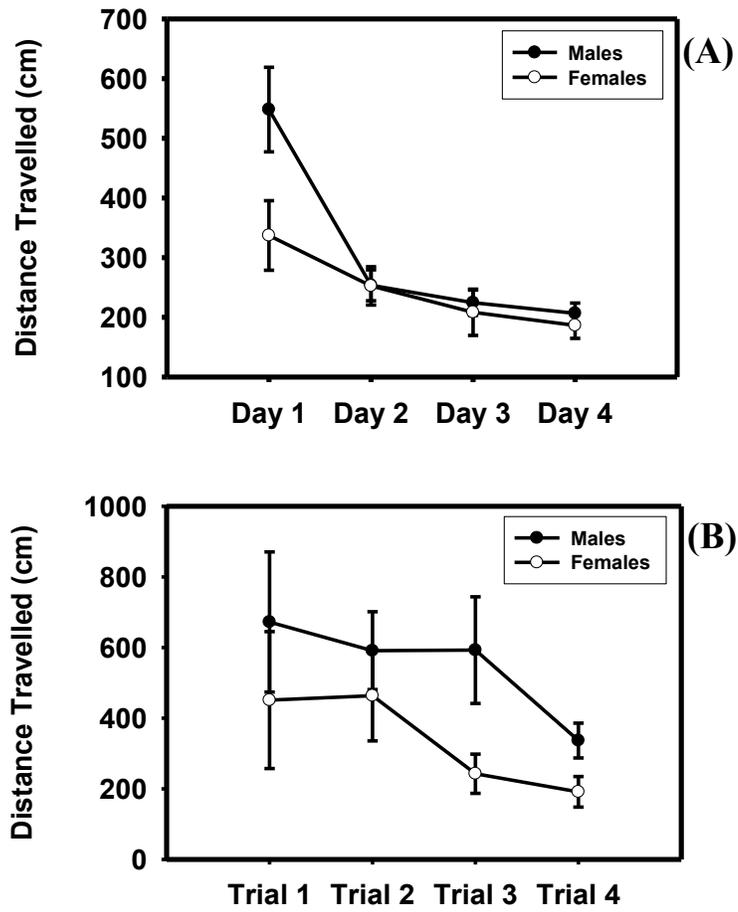
Figure 6. Mean distance travelled per (A) day by eight male and six female *A. piscivorus* to cover objects during spatial learning trials over four consecutive days, and (B) trials for day one. Error bars represent 1 SE.
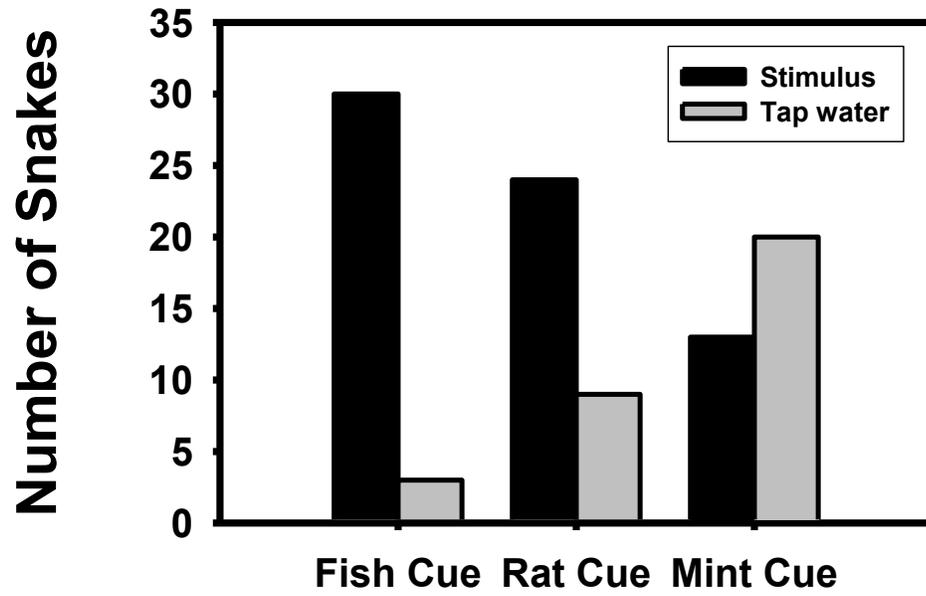
Figure 7. Number of snakes (n=33) associating with three chemical stimuli (dark bars) compared to tap water control (light bars) in dichotomous choice foraging experiment.
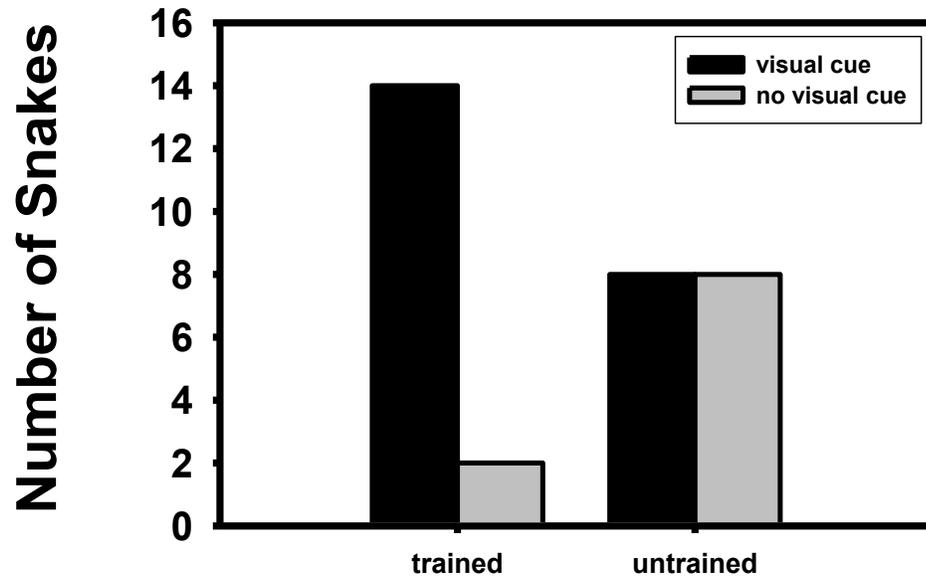
Figure 8. Results of associative learning experiment where snakes were conditioned to forage with or without a visual cue for eight feeding trials. Number of snakes adopting a foraging posture at a water bowl adjacent to a visual cue (black bars) vs. a water bowl with no visual cue (gray bars) in dichotomous choice foraging experiment.
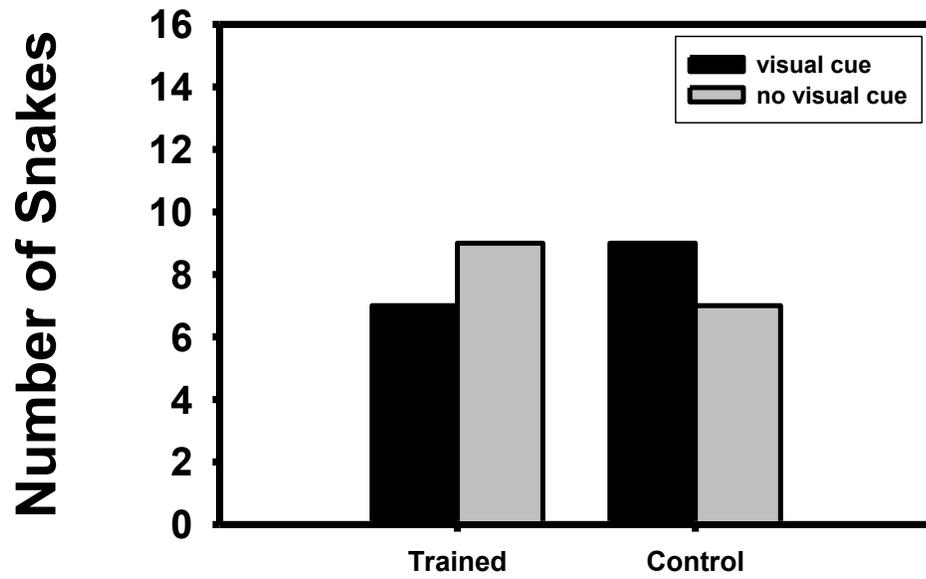
Figure 9. Results of associative learning experiment after trained snakes completed two feeding cycles without reinforcement. Dark bars represent number of snakes selecting foraging postures at a water bowl adjacent to a visual cue. Gray bars indicate number of snakes establishing foraging postures at a bowl lacking a visual cue.

DISCUSSION

Experiment One: Spatial Learning

Juvenile cottonmouths placed in a novel environment rapidly learned to locate cover and did so in a pattern that was consistent with a previous study involving corn snakes (*Elapha guttata guttata*). The mean latency reduction of 56% and mean distance travelled reduction of 57% for cottonmouths were similar to corresponding values of 64% and 50% for corn snakes under comparable testing circumstances (Holtzman et al. 1999). In contrast, spotted pythons (*Anteresia maculosus*) facing a comparable spatial challenge were reluctant to explore the environment and did not exhibit any change in latency to locate the shelter. Instead, they demonstrated a significant reduction in distance travelled to the shelter across trials, suggesting that pythons changed their direction of movement but not their rate of movement. It was speculated that nocturnal pythons may have been inherently less willing than the crepuscular corn snakes to explore novel environments in the highly illuminated testing conditions employed in the study (Stone et al. 2000). However, cottonmouths tested in a bright environment performed similarly to corn snakes rather than pythons, despite their predominantly nocturnal nature.

Cottonmouths appeared to learn shelter locations in the arena rapidly because the largest decrease in time and distance occurred between trial one and three on day one, with comparatively minimal changes in response variables after day two (F3). Despite a generally similar result, latencies for corn snakes declined significantly for three consecutive days instead of just the first two (Holtzman et al. 1999). One possible explanation for this difference is that corn snakes were trained with eight hide structures

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instead of the four used in my study. Thus, cottonmouths may have learned the same spatial task more quickly than corn snakes because they were tested in a less complex environment. Alternatively, these dissimilarities in performance could be a result of differences in learning abilities.

Another similarity between cottonmouth and corn snake spatial learning trials was the tendency toward increased exploratory behavior across consecutive trials on the same day. In later trials, some cottonmouths initially bypassed the shelter to investigate the edge of the arena before deciding to hide. Holtzman et al. (1999) interpreted this pattern of behavior in corn snakes to indicate a motivational change from hiding to attempted escape of the arena over time.

The size and age of snakes used in the study appeared to influence the willingness of snakes to perform spatial learning tasks. My results were derived from two-year-old juveniles that were considerably larger than neonates but still well short of sexual maturity. My early attempts to conduct spatial learning trials with one-year-old snakes were completely unsuccessful because these younger individuals were highly sensitive to disturbance and preferred to engage in defensive behaviors rather than explore the arena. I made the decision to switch to two-year-old snakes because they were less prone to defensive behaviors toward human handlers (Glaudas 2004). Whether the younger cohort of animals in my study did not explore the test environment because they had not habituated to human handling or because of an ontogenetic difference in cognitive development affecting their willingness to explore novel environments (Ford 1995) is unknown. The age-related contrasts in behavior that I observed add to an emerging body of knowledge of age-related differences in spatial learning in snakes and suggest that

ontogenetic influences on learning may be an interesting topic for further study (Holtzman 1998).

Sex appeared to be an important source of variation in spatial learning performance of cottonmouths. Females commonly went directly to a cover object whereas males would frequently circle the arena multiple times before entering the shelter. This difference contributed to greater latencies and distances travelled for males relative to females, but only on the first day. The tendency for males to engage in greater exploratory movements than females is consistent with several lines of evidence for gender-specific spatial differences in cottonmouths, including male-biased home range sizes (Roth 2005), a relatively larger medial cortex (a brain region associated with spatial processing) in males (Roth et al. 2006), and social differences that result in wider spacing in males than females (Roth & Lutterschmidt 2011). My results for movement latencies are opposite those for spotted pythons, where females, due to their reluctance to explore the testing arena, took longer than males to find shelters despite statistically similar travel distances for each sex (Stone et al. 2000). Essentially, female pythons took longer than males to move similar distances to shelters, whereas male and female cottonmouths both moved quickly away from their release points, but males engaged in more exploratory behavior than females. Thus, males of both species were relatively more active than conspecific females either in their rates of movement (pythons) or distance travelled (cottonmouths) and the apparent sexual difference among cottonmouths and pythons was due to a dissimilarity in female behavior. Given the importance of mate searching on reproductive success of male snakes (Duvall & Schuett 1997; Brown & Weatherhead

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1999; Shine 2003), a male-biased propensity for exploratory behavior may be common in snakes; testing this hypothesis will require research on many additional taxa.

Overall, the improving performance across consecutive spatial trials suggests that cottonmouths learned the landmarks to locate a shelter within an array of choices in the test arena. The study design did not completely control for the influence of other possible cues and even though the arena contained a number of visual cues, the specific cues utilized are still unclear. Since the arena floor was always arranged in the same way, it is possible that tactile cues were also used by the snakes. Despite my efforts to minimize landmark visibility outside the testing arena, it is also possible that snakes used visual information on the testing blind or ceiling for navigation because these variables were consistent across trials. Although the floor of the testing arena was cleaned between trials, it is also possible the snakes could have used additional chemosensory cues that were not controlled, such as a chemical associated with the white plastic card used as a landmark in the experiment. Therefore, I can only conclude that snakes potentially learned shelter locations from a variety of possible cues.

Experiment Two: Foraging Responses to Chemical Cues

Juvenile cottonmouths chose ambush sites near minnow chemical cues. While this pattern has been observed in natural settings (Roth et al. 1999; Theodoratus & Chiszar 2000), it has not, to my knowledge, been shown in a captive environment as a bioassay for selection. There was no significant difference between the rat cue, mint cue, and tap water. However, rat cue selection was marginally non-significant, suggesting that hungry juvenile cottonmouths responded positively to chemical stimuli derived from

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prey items and not non-prey controls. Responses were particularly strong for the fish

stimulus, where 30 out of 33 snakes (91%) chose the fish cue over the blank control.

Two of the three snakes that did not choose the fish cue were in the process of shedding,

which likely inhibited their feeding responses (Ford 1995). In contrast, only 24 of the 33

snakes (74%) chose the rodent cue over the control, suggesting a weaker foraging

response to small mammal cues than to fish.

Although fish are typically an important dietary component in cottonmouth

populations (e.g. Himes 2003; Vincent et al. 2005) the diet of the population of origin for

my snakes is dominated by mammals and does not include fish (Greene & Meyer, in

prep). Juvenile cottonmouths reared in captivity have been reported to exhibit

preferences for cues associated with prey that they have become conditioned to feed

upon, while showing less interest in natural prey types for which they have no feeding

experience (Lillywhite et al. 2015).

Experiment Three: Conditioning Using Visual Stimuli

Juvenile cottonmouths in my study learned to associate a novel visual stimulus

with foraging experience and modify their behavior to increase foraging success. After

training, 87.5% of the cottonmouths chose to setup ambush postures near the visual

stimulus (red index card). The two snakes that did not set up postures at the visual cue

were beginning to shed, when snakes are expected to reduce feeding responses (Ford

1995). Additional evidence of learning through foraging experience in pitvipers was

provided by Clark (2004a), who reported that juvenile timber rattlesnakes (*Crotalus

horridus*) learned to associate energetically valuable prey items treated with a chemical

cue (Clark 2004a). While snakes are well known for modifying their feeding responses due to feeding experience (Gans & Liner 1969), my study is the first, to my knowledge, to demonstrate such a learned response using a visual stimulus.

Interestingly, the removal of the visual cue resulted in rapid loss of the learned response in trained cottonmouth snakes. After just two feeding cycles, the responses of trained and control snakes were statistically similar, suggesting that consistent reinforcement is needed to retain the learned response. This result is consistent with learned patterns of defensive behavior in cottonmouths where individuals that were not consistently handled exhibited higher defensive scores compared to their habituated conspecifics (Glaudas 2004). However, timber rattlesnakes (*C. horridus*) showed learning retention without reinforcement over similar timeframes, when the associated cue was chemosensory (Clark 2004a), which seems to provide support for the suggestion that chemosensation is a dominant perceptual mechanism in snakes (Ford & Burghardt 1993).

In conclusion, my study provides evidence of learning by cottonmouths in two ecologically relevant contexts: spatial memory and foraging. In experiment one, the spatial learning abilities of cottonmouths were evaluated for comparison with parallel studies on spatial learning in colubrids and pythons. All three studies provided some evidence that snakes can learn an important spatial task, the location of a shelter in an unfamiliar environment. In addition, my study, along with Stone et al. (2000), provides evidence that males are travelling larger distances than females to complete the same spatial learning tasks. In experiment two, I provided evidence that juvenile cottonmouths will establish ambush foraging postures in a captive environment and, in

experiment three, I showed that they can be conditioned to associate visual landmarks

with successful foraging sites. These findings show that cottonmouths are able to use

experience with visual cues to enhance foraging success.

COTTONMOUTHS AS TEST SUBJECTS

In many ways, the cottonmouths made excellent research organisms. They were easily maintained in captivity, they are prey generalists, and they display obvious antipredator responses to aversive stimuli. Despite the positive qualities, they did present some challenges. This study required the snakes to setup foraging postures, in which the cottonmouths seemed to be particular. The best results were obtained in the snakes' home enclosures. The juvenile cottonmouths did not readily setup ambush postures in new areas, and they needed the environment to be dark for some time in order to setup postures. This study also reinforces the statement made by Clark (2004a) that ambush foraging postures can be used as a bioassay for foraging experiments in pitvipers.

I was able to obtain meaningful data because the subjects were juveniles and were small enough to move around with minimal disturbance to the animal. All the snakes were born in the lab, and they had been habituated to humans over the course of one to two years. I was also able to control for all past experiences with prey items and cover objects. I do not believe that wild caught adults would have been suitable for this study.

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