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Venom Expelled by Cottonmouths (*Agkistrodon Piscivorus*) Across Different Prey Sizes, Prey Taxa, and Snake Body Temperatures

Kari Lynn Spivey

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**VENOM EXPELLED BY COTTONMOUTHS (AGKISTRODON PISCIVORUS)
ACROSS DIFFERENT PREY SIZES, PREY TAXA, AND SNAKE BODY
TEMPERATURES**

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

Kari Spivey

May 2016

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Biology

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Master of Science

Kari Spivey

ABSTRACT

Pit vipers possess a sophisticated venom delivery system enabling them to efficiently disable prey. To avoid retaliatory countermeasures, pit vipers typically release envenomated prey which are then trailed and consumed after succumbing to venom effects. Successful retrieval of released prey should vary with venom resistance and trail ability of prey types. The effects of prey size and prey type (mice, lizards, and frogs) on foraging behavior and venom expenditure in a cohort of juvenile cottonmouths was examined. Venom expenditure did not vary significantly among prey sizes or prey types. However, lizard prey were held significantly more often than mice. The effect of snake body temperature on foraging response variables across a range of ecologically relevant temperatures (18°C-30°C) was also examined. Cottonmouths injected significantly less venom, and held prey significantly more often, at 18°C than at 25°C or 30°C. These results are consistent with a thermal constraint on envenomation performance at lower operant temperatures. Overall, these results suggest that cottonmouths modify foraging behavior to compensate for both venom resistance in ectothermic prey and decreased performance at lower temperatures.

KEYWORDS: cottonmouths, *Agkistrodon piscivorus*, venom expelled, skinks, frogs, snake body temperature

This abstract is approved as to form and content

Brian D. Greene, PhD
Chairperson, Advisory Committee
Missouri State University

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INTRODUCTION

Venoms and venom delivery systems are key adaptations associated with the evolutionary success and recent radiation of advanced snakes (Greene, 1997), with pitvipers (Viperidae) possessing the most highly derived venom delivery systems (Fry et al., 2008). Given the obvious medical relevance of venom properties to drug discovery and human health (see Pal et al., 2002 for review) it is unsurprising that venom composition and biochemical properties are the most commonly researched subjects in venomous snake biology, accounting for at least 85% of the publications on pitvipers in the last 25 years (Krochmal, 2014). With predator-prey interactions primarily driving venom evolution (Casewell et al., 2013), the use of venom by snakes in ecologically relevant contexts is essential for understanding the adaptive significance of venom delivery systems but has received far less attention. Recent studies of snake genomics revealed that venom components have evolved under strong selection pressure to provide a mechanism for rapid prey immobilization, convincingly demonstrating a link between toxicity and foraging success (Vonk et al., 2013). Furthermore, intraspecific variation in venom composition has been suggested to be adaptively linked to diet variation (Barlow et al., 2009; Gibbs and MacKessy, 2009).

Prey envenomated by vipers are typically released, reducing the potential for retaliatory injuries that can be inflicted by dangerous prey (Kardong, 1986). The act of striking and releasing prey triggers a phenomenon known as strike-induced chemosensory searching (SICS) which is characterized by elevated tongue-flick rates used to locate the chemical trail left by fleeing prey (Chiszar et al., 1977). The

biomolecular composition of venom changes the scent of envenomated prey, permitting snakes to subsequently trail prey away from the initial attack location (Chiszar et al., 1983). Disintegrins, a non-enzymatic protein component of venom, have been reported to provide the primary chemical cue used by snakes to trail envenomated prey (Saviola et al., 2013). Strike-induced chemosensory searching (SICS) has been documented in nearly all pitvipers (Chiszar et al., 1982), colubrids (Cooper et al., 1989; Withgott 1996), varanid lizards (Cooper, 1989), and even helodermatid lizards (Cooper and Arnett, 1995).

The recovery of released prey is likely improved by rapid immobilization of prey such that prey dispersal distances are minimized. House mice (*Mus musculus*) envenomated by prairie rattlesnakes (*Crotalus viridis*) typically died within 78 s while travelling a distance of 600 cm (Estep et al., 1981). Foraging success using the SICS strategy requires recovery of prey before chemical cues degrade. Snakes have been documented to search for 24 hours post-envenomation; however prey recovery success decreases over time with increasing dissipation of chemical cues (Smith et al., 2000). To compensate for variation in tracking difficulties and venom susceptibility among prey individuals, adjustments in venom expenditure have been documented for pitvipers.

A variety of experimental studies have reported specific sources of variation in venom expenditure in foraging vipers (reviewed by Hayes et al., 2002). For example, young, naïve vipers inject relatively more venom per bite than experienced adult conspecifics (Hayes et al., 2002; Pe and Cho, 1986). In addition, individual prairie rattlesnakes (*Crotalus viridis*) inject larger quantities of venom into large mice than smaller mice (Hayes, 1995; Hayes et al., 1995, 2002). The amount of venom injected by *C. viridis* was also reported to be greater for both birds and lizards than mice (Hayes,

1992; Hayes et al., 2002). Given the central relationship of venom to foraging success, venom expenditure and other behavioral components of SICS should be under strong selection pressure to accommodate variation in prey size (Hayes et al., 1995), venom susceptibility (Gibbs and Mackessy, 2009), and trailing potential (Hayes, 1992).

Snake body temperature is another potentially important proximate influence on SICS that has yet to be investigated. Virtually all body movements of snakes are temperature-dependent because of the thermal sensitivity of muscle performance in ectotherms (Peterson et al., 1993). Cottonmouths (*Agkistrodon piscivorus*) foraging across an ecologically relevant range of body temperatures released envenomated prey at warm body temperatures but held prey at colder body temperatures (Benbow, 2008). Explanations for such a change in behavior of cold snakes include performance constraints on the kinematics of striking (and envenomating) prey and possible difficulty trailing released prey at cold temperatures. Currently, the thermal consequences on venom expenditure or other behavioral aspects of prey capture have not been described for any pitviper.

Interpretations of studies reporting consistent patterns of variation in venom expenditure across prey sizes and taxa categories are typically that snakes actively adjust the amount of venom delivered during a bite (see Hayes et al., 2002). Such a “venom-metering” pattern has been demonstrated not only in pitvipers but in scorpions (Nisani and Hayes, 2011) and spiders (Cooper et al., 2015) as well. The venom metering hypothesis is described as an optimization strategy (Morgenstern and King, 2013) that is consistent with the high energetic cost to venom production (McCue, 2006). Contrasting views hold that variation in venom expenditure results from physical factors surrounding

the bite rather than any active decision by snakes (e.g. Young et al., 2002). In either case, patterns of venom expenditure in pitvipers are generally consistent with hypotheses relating venom quantities to foraging success.

The research addressing ecologically relevant aspects of venom use in pitvipers is almost entirely derived from a limited number of rattlesnake species. Conducting parallel studies on other pitviper taxa would provide broader phylogenetic and ecological perspectives. For example, the cottonmouth (*Agkistrodon piscivorus*), a semi-aquatic pitviper with a broad diet, would represent an interesting ecological contrast to rattlesnakes and add a potentially important contribution to the existing knowledge of foraging ecology in pitvipers. The cottonmouth is also an attractive target for studies of venom use because other relevant aspects of its feeding ecology are well known, including diet (Burkett, 1966; Himes, 2003; Lillywhite and McCleary, 2008), feeding behavior (Kardong, 1982; Savitsky, 1992; Lillywhite et al., 2008; Young et al., 2008; Lillywhite et al., 2015), foraging mode (Eskew et al., 2009), and functional morphology of feeding (Vincent et al., 2004, 2005). Currently, there is minimal information on the use of venom by cottonmouths in foraging contexts. A widely-cited study by Gennaro et al. (1961) evaluated prey size influence on venom expenditure in large cottonmouths feeding on guinea pigs and mice, which represent neither the dietary diversity inherent in most cottonmouth populations or, in the case of guinea pigs, ecologically relevant prey.

This study examined venom use by cottonmouths in predatory contexts comparative to similar studies in other pitvipers in an attempt to expand the understanding of pitviper foraging behavior. Specifically, I examined variation in venom expenditure as a function of prey size and prey type. The prey types chosen (a mouse,

skink, and frog) are commonly consumed by cottonmouths (Gloyd and Conant, 1990) and represent a diverse array of prey types that may vary in venom susceptibility. In addition, I examined the effect of temperature, a variable impacting snake performance, which is an unexplored source of variation on venom expenditure. Consistent with previous studies on venom expenditure in pitvipers, I hypothesized that cottonmouths would increase venom expenditure with increasing prey size and expend greater venom quantities on ectothermic than endothermic prey. Finally, I hypothesized that venom expenditure would negatively correlate with snake body temperature due to thermal constraints on muscle performance of cold snakes.

METHODS

Animal Collection and Maintenance

Test subjects were 10 juvenile cottonmouths (*Agkistrodon piscivorus*) selected from a lab-reared cohort of offspring from three gravid females collected in Stone County, MO. These snakes were born in September, 2013 and were approximately 21 months old with a mean body mass of 67.8 ± 3.44 g when testing began. All were individually housed in polycarbonate cages (42 x 30 x 22.5 cm) with a paper substrate, a shelter made of a halved section of pvc pipe (20 cm long), and a water dish. Cages were placed in a cabinet with a heat source along the back edge of each shelf, providing a thermal gradient in each cage so that snakes could thermoregulate. The room was maintained on a 12:12 light:dark cycle at 25°C. During the first 20 months of life, snakes were fed minnows (mostly *Pimephales* sp. and *Cyprinella* sp.) every 8-10 days and water was provided *ad libidum*. To minimize any experience-related effects on feeding performance, I began feeding snakes live mice approximately one month prior to trials. Once foraging trials began, envenomated prey items were harvested for analysis and replaced with pre-killed mice brought from a commercial supplier (RodentPro.com, Inglefield, IN).

Five-lined skinks (*Plestiodon fasciatus*) were collected in southwestern Missouri (Benton, Greene, and Webster Counties) and maintained in a 2.25 L plastic container with moistened moss as a substrate, a shelter made of a halved section of pvc pipe (20 cm long), and a water dish. Skinks were fed crickets weekly until used. Southern leopard frogs (*Lithobates sphenoccephala*) were also collected in southwestern Missouri in July

(Taney County) and October 2015 (Webster County). Frogs were maintained in 2.25 L plastic containers supplied with 1 L of aerated, de-chlorinated tap water and fed blood worms (Omega One) twice weekly. Water was drained and fresh water was supplied twice a week. Live mice (*Mus musculus*) were obtained from a local supplier (Finley Valley Serpenterium near Ozark, MO) once a week and maintained in filtered cages supplied with pellets (OxBow Essentials) and tap water until used. Animal use was approved by the Missouri State University Institutional Animal Care and Use Committee (March 2015, protocol #15-023).

Experimental Protocol

I used a repeated-measures design where each snake was tested in each of three treatments in three separate foraging experiments. Each experiment addressed a different foraging question involving variation in snake responses due to either prey size, prey type, or snake body temperature. The order of prey categories was randomized for each snake across all prey categories. Trials were repeated for each snake every nine to ten days at 25°C, except for trials evaluating snake body temperature. All snakes were tested twice for each experimental condition and response variables were averaged for analysis.

At the beginning of each foraging trial, a previously weighed prey item was introduced within the snake's housing chamber in the corner opposite the snake. After envenomation, prey were removed, re-weighed after death, coded, and stored at -20°C in 1 L Zip-Loc plastic bags. Death of prey was determined by lack of response to a light pinch at the tail base (mice and skinks) or a hind limb (frogs). Following removal of envenomated prey, a thawed mouse was placed in the snake cage as a meal. Every trial

was video recorded (Sony HDR CX240) to ensure snakes used foraging strikes versus defensive strikes, and to later review for further data collection. Data recorded from video trials included snake's latency to strike, duration of contact (i.e. struck and released versus struck and held), and prey's time to death. Envenomated prey were later thawed and homogenized (see homogenate protocol below) to quantify amount of venom expelled by the snake (see quantification protocol below) for each trial.

For Experiment 1, I investigated the influence of prey size on venom expenditure using house mice (*Mus musculus*) of three different size classes at 25°C. Mice were weighed and placed into relative body mass categories for each snake, representing <10% (small), 10 to 15% (medium), and >15% (large mice), respectively.

For Experiment 2, I investigated the influence of prey type on venom expenditure for house mice (*Mus musculus*), five-lined skinks (*Plestiodon fasciatus*), and southern leopard frogs (*Lithobates sphenoccephala*) at 25°C. The data from Experiment 1 provided the mouse sample for comparison with skinks and frogs. Skinks (mean \pm SE; 7.0 ± 0.45 g; $n = 18$) and frogs (6.2 ± 1.56 g; $n = 16$) were compared against combined data for mouse sizes from Experiment 1.

For Experiment 3, I investigated the influence of snake body temperature on venom expenditure. To adjust snake body temperature, I placed snake cages in an environmental chamber set to either 18°C or 30°C and allowed snakes to acclimate for 4 hours prior to experimentation. Mice greater than 15% snake body mass were used for both 18°C and 30°C treatments and compared against data for large mice from Experiment 1 where trials were run at 25°C.

Venom Extraction

To create a standard curve of known venom concentrations, venom was extracted from wild-caught cottonmouths ($n = 9$; mean mass \pm SE; 210.8 ± 24.79 g), collected from the same population as the test subjects, using a standard protocol (Steve Mackessy, personal communication). Briefly, snakes were introduced into an induction chamber and anesthetized with vaporized isoflurane until they ceased voluntary movements (especially tongue flicks). After removal from the chamber, a 100mL capillary tube was positioned over each fang, and a 1.7 mL microcentrifuge tube was placed beneath the open end of each capillary tube to catch the venom. Both venom glands were then manually massaged until venom stopped flowing. Venom left in the capillary tube was manually expelled into the microcentrifuge tube. Extracted venom was initially stored at -20°C and lyophilized within three days for long-term storage at -20°C .

Homogenate Protocol

An envenomated prey item was thawed and placed in a 0.15 L plastic container (85 mm diameter). A homogenizer (Sper Scientific 460003) was used to homogenize the prey item with 2 mL of 1X ELISA wash buffer (ImmunoChemistry). The homogenate was then added to a 30 mL Oak Ridge centrifuge tube (Thermo Scientific) and centrifuged at 15,000 rpm for 5 minutes. The supernatant was collected in 1 mL aliquots in 1.7 mL microcentrifuge tubes and a total of 3 mL was stored at -20°C . The homogenizer, plastic container, and centrifuge tube were rinsed three times with warm water, submerged in a dilute bleach solution, and rinsed three times again with warm

water. This process was repeated for each prey item and for an individual of each prey type that was not envenomated to serve as a background control.

Quantification of Envenomation by ELISA

Samples were thawed and a Bradford assay (Bradford, 1976) was performed to normalize each homogenate to 1 mg/mL of protein by diluting it in coating buffer (1X antigen coating buffer; ImmunoChemistry). Following normalization, 50 μ L was loaded into a well of a 96-well plate (Micolon® 600 high-binding, Greiner Bio-One) in triplicate and then incubated for 24 hours at 4°C for binding. The plate was washed three times with 150 μ L of 1X ELISA wash buffer solution (ImmunoChemistry) and blocked for 2 hours (200 μ L BSA blocking buffer, ChemCruz) at room temperature. The plate was then washed three times with 150 μ L of 1X wash buffer and 100 μ L of polyvalent crotalid anti-venom (20 μ g/mL diluted in blocking buffer; VenomVet™) was added to each well. The plate was incubated for 2 hours at room temperature and then washed four times with 150 μ L of 1X wash buffer. Goat anti-horse IgG HRP conjugate (100 μ L, 0.02 μ g/mL diluted in blocking buffer) was added to each well and incubated for 2 hours at room temperature. The wells were emptied and washed five times with 150 μ L of 1X wash buffer. To each well 100 μ L of One-step Ultra TMB was added and incubated for 15 minutes at room temperature. The reaction was stopped with 50 μ L of 2M sulfuric acid and absorbance was measured at 450 nm. A standard curve was generated by diluting lyophilized venom and adding 50 μ L of the resulting solution to each well with final concentrations ranging from 100 μ g/ μ L to 10 pg/ μ L and then used to calculate the relative concentration of venom per μ L. The final relative concentration of venom expelled into the prey item was

then multiplied by the total quantity of homogenate added into each well (50 μ L) and finally multiplied by the initial dilution factor (determined by Bradford assay, see above), resulting the μ g of venom injected.

Data Analysis

Data were evaluated for violation of parametric assumptions prior to analyses and appropriately transformed where necessary to improve normality and heterogeneous variances. However, given the small sample sizes, non-parametric Wilcoxon signed rank tests were used to compare pre-envenomation and post-death mass of prey in all experiments. The paired replicated measurements for each snake and sampling situation were evaluated with paired t-tests for possible order effects. Data points for first and second encounters were averaged for each snake before analysis.

One-way ANOVAs were used to test for differences in mean response variables across prey sizes, prey type, and snake body temperatures. Significant ANOVAs were followed by Tukey post-hoc tests to clarify differences among means. Chi-square tests were used to compare the frequency of prey struck and released and prey struck and held across prey sizes, prey types, and snake body temperatures.

Given the modest sample sizes in my study, I calculated effect sizes to aid in the interpretation of non-significant p-values and facilitate comparison across studies (Nakagawa and Cuthill, 2007). ANOVA effect sizes were estimated with η^2 which I loosely interpreted as low (0.01), medium (0.06) and large (0.14). Effect sizes for chi-square tests were estimated with Cramer's V with values of 0.3 considered moderate and >0.5 considered large (Cohen, 1988). All effect sizes used provide a general indication

of the proportion of variation in a dependent variable explained by an independent variable (Cohen, 1988).

RESULTS

There was substantial variation in time to death due to the presence of several outliers as revealed by box plots of this variable for all prey factors (Figure 1). In an attempt to discern the validity of outlying values I plotted prey time to death as a function of venom concentration and determined that most outliers apparently involved no or very small amounts of venom. Because these likely represent failed strikes by snakes, I treated these points as experimental errors and excluded them from analyses.

There were no significant differences between replicate encounters for snake latency to strike (Table 1), prey time to death (Table 2), or venom expelled (Table 3) for most factors. The only exceptions were an increased time to death for frogs during the second encounter and for mice exposed to 30°C snakes in the second encounter (see Table 2).

Experiment 1: Prey Size

The mean masses mouse size classes (mean \pm SE; small, 4.2 ± 0.19 g; medium, 8.4 ± 0.32 g; large, 17.1 ± 0.83 g) were all significantly different from each other ($F = 158.8$; $P < 0.001$; Tukey Test $P < 0.05$; Figure 2). There was no significant difference in snake latency to strike across prey sizes ($F = 0.63$; $P = 0.54$; $\eta^2 = 0.04$; Figure 3). However, there was a significant difference in prey time to death across prey sizes ($F = 3.67$; $P = 0.04$; $\eta^2 = 0.21$; Figure 4), in that medium and large mice died significantly faster than small mice (Tukey Test $P < 0.05$). There was not a significant difference in venom expenditure across mouse size classes ($F = 0.43$; $P = 0.65$; $\eta^2 = 0.03$; Figure 5).

Although small mice were struck and held more frequently than other sizes, the difference was not significant ($\chi^2 = 5.33$; $P = 0.07$; $V = 0.22$; Figure 6). Pre-envenomation masses were significantly greater than post-death masses for small (Wilcoxon signed rank, $Z = -2.18$; $P = 0.03$, $n = 19$), medium ($Z = 2.23$; $P = 0.02$, $n = 19$), and large mice ($Z = -3.29$; $P < 0.001$, $n = 17$).

Experiment 2: Prey Type

Snakes generally took longer to strike skinks but the difference in latency to strike was not significantly different across prey types ($F = 2.77$; $P = 0.07$; $\eta^2 = 0.11$; Figure 7). The amount of venom expended did not differ significantly across prey types ($F = 1.82$; $P = 0.17$; $\eta^2 = 0.07$; Figure 8). However, there was a significant difference in time to death across prey type ($F = 9.69$; $P < 0.001$; $\eta^2 = 0.30$; Figure 9), in that mice succumbed more rapidly than skinks and frogs (Tukey Test $P < 0.05$). In addition, skinks and frogs were held significantly more often than mice ($\chi^2 = 8.82$; $P = 0.01$; $V = 0.22$; Figure 10). Pre-envenomation and post-death masses were not significantly different for skinks ($Z = -0.37$; $P = 0.73$, $n = 18$) but frogs were significantly lighter after death than prior to being envenomated ($Z = -3.52$; $P < 0.001$, $n = 16$). Because of difficulty obtaining frogs in the summer, there was a three month gap in frog samples used for each replicate, which resulted in frogs for sample 1 (mean = 1.67 ± 0.09 g) being significantly larger than those used for sample 2 (mean = 11.91 ± 2.0 g; Mann-Whitney U Test, $P < 0.001$).

Experiment 3: Snake Body Temperature

There was no significant difference in snake latency to strike across snake body temperatures ($F = 0.32$; $P = 0.73$; $\eta^2 = 0.02$; Figure 11). However, time to death varied significantly across snake body temperatures ($F = 22.84$; $P < 0.001$; $\eta^2 = 0.63$; Figure 12) in that mice envenomated by snakes at 18°C took longer to die than mice envenomated by warmer snakes (Tukey Test $P < 0.05$). The amount of venom expended by snakes was also significantly related to their body temperatures ($F = 6.37$; $P = 0.005$; $\eta^2 = 0.32$; Figure 13) in that mice envenomated by snakes at 18°C and 30°C contained less venom than mice envenomated by snakes at 25°C (Tukey Test $P < 0.05$). In addition, snakes held mice more often at 18°C than at 25°C or 30°C although the difference was not significant ($\chi^2 = 5.71$; $P = 0.06$; $V = 0.23$; Figure 14).

The mean masses of mice used in temperature trials (18°C, 20.5 ± 1.17 g; 25°C, 17.1 ± 0.83 ; and 30°C 19.3 ± 1.17 g) were not significantly different ($F = 158.8$; $P < 0.001$; Figure 2). Pre-envenomation masses were significantly greater than post-death masses for mice exposed to snakes with a body temperature of 18°C ($Z = -1.98$; $P = 0.05$, $n = 20$) but not for mice exposed to 30°C snakes ($Z = -1.90$; $P = 0.06$, $n = 19$).

DISCUSSION

My data suggest that cottonmouths modify foraging behavior in response to differences in prey size and type, and with changes in body temperature, but not in the ways originally predicted. I initially predicted that cottonmouths would (1) increase venom expenditure with increasing prey mass, (2) expend greater venom quantities on ectothermic than endothermic prey, and (3) increase venom expenditure with decreasing snake body temperature. Cottonmouths did not conform to any of the above hypotheses. Venom expenditure was the same across prey sizes and prey types. However, venom expenditure substantially declined at lower body temperatures. While a change in foraging strategy from striking and releasing to striking and holding with decreasing body temperature was expected, differences in prey handling among prey types was not.

Pre-envenomation masses exceeded post-death masses for nearly all prey categories and these differences appeared to result from urination and/or defecation that occurred after envenomation. The excretion of urine and feces following envenomation was particularly obvious for mice and resulted in significant post-death mass reductions for all mouse treatment categories. Despite a lack of urination and defecation during trials, frogs also exhibited significant mass reductions which were likely a consequence of desiccation in a dry experimental environment. Skinks did not urinate or defecate following envenomation and maintained consistent body masses between measurements. These results indicate a significant deficiency in the estimation of venom expenditure through changes in mass, which was done prior to the development of molecular techniques, such as ELISAs, that can directly measure the quantity of venom in a sample.

Cottonmouths exhibited similar strike latencies for all prey types presented to them, corroborating the broad diet composition typically reported for wild populations. Response variables were also highly repeatable between replicate trials for each individual, except for those involving frogs and for mice in the 30°C snake treatment. In both cases, mean times to death for the second trial were significantly greater than the first. For frogs, this result could be explained by the increase in mass for the sample obtained later in the year. As previously mentioned, the individuals used in the first trial were recent metamorphs collected in June, while the second trial sample was composed of adults collected in early October.

On the other hand, the elevated latencies observed for mice envenomated by 30°C snakes in replicate trials could not be explained by differences in mouse body size or by systematic differences in quantity of venom injected (see Table 3). I observed a decrease in snake hunger levels by the end of the study in early fall when experiment 3 was concluding. For Experiments 1 and 2, only two thawed mice fed to snakes following trials were not consumed, one of which was explained by ecdysis of a particular snake. For Experiment 3, eight thawed mice were not consumed by six of the ten total cottonmouths (two of these six snakes did not eat twice). Video recordings of the second trials for mice envenomated by 30°C snakes did not reveal anything atypical about the bites. However, if snake hunger levels influence bite mechanics, changes in the accuracy of strikes or depth of fang penetration could impact how rapidly venom takes effect.

Envenomation strategies of snakes feeding on mice vary with the age and size of prey. In some venomous snake species, such as the red spitting cobra (*Naja mossambica pallida*), snakes can strike and hold small mice without ever injecting any venom,

perhaps because small mice do not require envenomation to be captured (Radcliffe et al., 1984). In my study, small mice were struck and held more often than other size classes and but did not contain significantly more or less venom than larger mice. Interestingly, small mice exhibited the greatest latency to death despite receiving the same amount of venom relative to the other size classes. These results are consistent with an increased sensitivity to venom with age which has also been reported for mice injected with scorpion venom (Padilla et al., 2003; Pucca et al., 2011).

That cottonmouths in my study expended the same quantities of venom across mouse sizes contradicts a widely-cited study by Gennaro et al. (1961) reporting that venom quantities were positively correlated with prey size across several mammalian prey species. However, the snakes used by Gennaro et al. (1961) were experienced, adult cottonmouths whereas the snakes used in my study were inexperienced, juvenile cottonmouths. Perhaps cottonmouths require a learned response from prey time to death via SICS in order to adjust quantities of venom. This discrepancy could also suggest that snake responses to a particular prey category may be complex, potentially varying with ontogeny and species of prey. Additional studies designed to evaluate specific influences of both snake and prey-specific factors would be of interest.

Skinks and frogs survived longer following envenomation than any mice despite receiving mean venom quantities that were statistically the same as mice (Figure 9). Although the differences that I observed in venom quantities injected into mice and ectotherms were not significant, the sample size was small. Furthermore, my results are consistent with other studies reporting higher venom resistance for ectotherms compared to endotherms. For example, leopard frogs (*Lithobates pipiens*) have significantly higher

resistance than mice to venom of *Sistrurus* rattlesnakes (Gibbs and Mackessy, 2009). Adaptive adjustments by vipers to improve foraging success on venom-resistant prey may include the injection of larger venom quantities (Hayes et al., 2002) or holding rather than releasing prey (Radcliffe et al., 1984). Cottonmouths in my study also held ectothermic prey significantly more frequently than mice.

Holding rather than releasing prey could convey multiple advantages to foraging snakes. First, holding ectothermic prey could ensure that venom has penetrated deep in the body or holding could allow for more venom to be expelled into the prey body (Morgenstern and King, 2013). Another contributing factor could be that skinks and frogs are less able to inflict retaliatory injury than mice, thereby increasing the benefit of holding prey (Kardong, 1986). For example, Stiles et al. (2002) reported that copperheads (*Agkistrodon contortrix*) struck and held lizards but struck and released mice. Lastly, holding ectotherms could be advantageous for increasing the retrieval potential if venom resistance would allow the prey to travel further than endothermic prey before succumbing to venom effects. In other words, cottonmouths could be exhibiting a trade-off for holding prey instead of risking a meal loss.

Experiment 3 revealed two important patterns in response variables associated with snakes foraging at 18°C that contrasted with responses of warmer snakes. First, snakes at 18°C injected on average about 10% as much venom as warmer snakes, which apparently explains why prey death latencies were significantly longer compared to mice struck by snakes foraging at other body temperatures. A small number of large mice were also held at 18°C while none were held at 25°C and 30°C. This pattern was distinctly different from that observed at 25°C (Experiment 1) where only small mice

were held, indicating that snakes changed their foraging strategy and attempted to hold some large mice at 18°C. My results parallel those of Benbow (2008) who reported that cottonmouths took significantly longer to complete foraging trials at 15°C than at higher temperatures and held most mice at cold body temperatures but released all of them at 30°C. My venom injection data have helped clarify the mechanism of this temperature-dependency and suggest that thermal constraints on muscle performance are interfering with the ability of the snakes to inject adequate quantities of venom at cold body temperatures. Therefore, cottonmouths may be holding prey at cooler ambient temperatures to compensate for a thermal performance handicap by preventing prey from escaping while also possibly allowing more time for venom to be injected.

My results tend to suggest that snakes could hold or release prey for at least two possible reasons. Ectothermic prey (particularly lizards) are struck and held because of their relatively greater venom resistance than mice. However, it appears that mice are facultatively held, either because of small body size (which is correlated with increased venom resistance) or because of a thermal handicap interfering with venom injection at cold body temperatures. Neither of these results support my initial predictions that venom quantities would increase with prey size and increase at colder snake body temperatures. Both of these results are, to my knowledge, sources of variation in viperid snake foraging behavior that have not been previously reported. Additional studies of thermal influences on foraging in other pit viper species across ecologically relevant temperatures would provide important information about a potentially important but relatively unexplored aspect of snake foraging ecology.

In conclusion, my study provides new information evaluating sources of variation in cottonmouth foraging behavior. My data does not support the notion that cottonmouths adjust venom expenditure in response to prey type and size by injecting greater quantities of venom into prey categories exhibiting the highest venom resistance. This directly contrasts consistent studies on rattlesnakes (Hayes et al. 2002). However, my data was obtained with inexperienced juveniles and perhaps experience is required for cottonmouths to show adjustments in venom expenditure. Snakes also showed evidence of adjusting prey handling behavior by increasing the frequency of holding venom-resistant prey types. These changes in snake foraging behavior related to prey size and type appear to be innate as all test subjects were born in captivity and had no prior foraging experience with any of the prey used in my experiments. In addition, venom expenditure by cottonmouths and prey capture method varied across body temperatures such that snakes at 18°C injected substantially less venom and held prey more often than snakes foraging at temperatures in their preferred body temperature range. Combined, these results show that cottonmouths modify foraging behavior to compensate for both variation in venom resistance of prey and decreased performance at lower foraging temperatures.

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Table 1. Comparing snake latency to strike between first and second encounters.

Prey Type	N	Mean First Encounter \pm SE (s)	Mean Second Encounter \pm SE (s)	T-value	P-value
Small Mouse	9	139.1 \pm 78.1	176.6 \pm 52.4	-0.64	0.540
Medium Mouse	9	333.8 \pm 203.2	75.3 \pm 42.6	0.31	0.766
Large Mouse	7	238.4 \pm 65.3	149.0 \pm 129.6	2.14	0.076
Skink	8	339.6 \pm 135.9	302.6 \pm 96.6	0.21	0.842
Frog	7	257.6 \pm 102.0	394.1 \pm 137.0	-0.82	0.442
18°C	10	294.7 \pm 167.8	259.0 \pm 97.9	0.18	0.863
30°C	9	221.6 \pm 128.6	104.3 \pm 28.0	-0.58	0.576

Table 2. Comparing prey time to death between first and second encounters.
Significance is denoted by an asterisk (*).

Prey Type	N	Mean First Encounter \pm SE (s)	Mean Second Encounter \pm SE (s)	T-value	P-value
Small Mouse	9	201.7 \pm 82.9	279.9 \pm 69.7	-0.80	0.446
Medium Mouse	9	76.1 \pm 10.7	159.9 \pm 40.5	-1.89	0.096
Large Mouse	7	181.4 \pm 57.8	98.6 \pm 33.3	1.60	0.160
Skink	8	614.6 \pm 70.1	590.8 \pm 79.7	0.34	0.741
Frog	7	112.4 \pm 33.5	761.7 \pm 239.6	-3.03	0.023*
18°C	10	1438.6 \pm 375.6	1188.6 \pm 488.7	0.33	0.746
30°C	9	79.7 \pm 9.9	288.1 \pm 46.3	-3.97	0.004*

Table 3. Comparing amount of venom expelled between first and second encounters.

Prey Type	N	Mean First Encounter \pm SE (μ g)	Mean Second Encounter \pm SE (μ g)	T-value	P-value
Small Mouse	9	3923.1 \pm 1227.6	6007.5 \pm 2101.7	-1.20	0.265
Medium Mouse	9	4033.6 \pm 1404.2	3537.3 \pm 973.4	0.33	0.753
Large Mouse	7	5610.8 \pm 1854.9	4386.6 \pm 2439.9	0.52	0.622
Skink	8	6931.1 \pm 1653.5	9734.2 \pm 1670.0	-0.92	0.386
Frog	7	2287.7 \pm 1079.2	6618.9 \pm 2859.1	-1.50	0.186
18°C	10	391.4 \pm 276.5	594.1 \pm 210.7	-1.55	0.155
30°C	9	2680.0 \pm 1656.3	1599.5 \pm 716.8	0.87	0.408

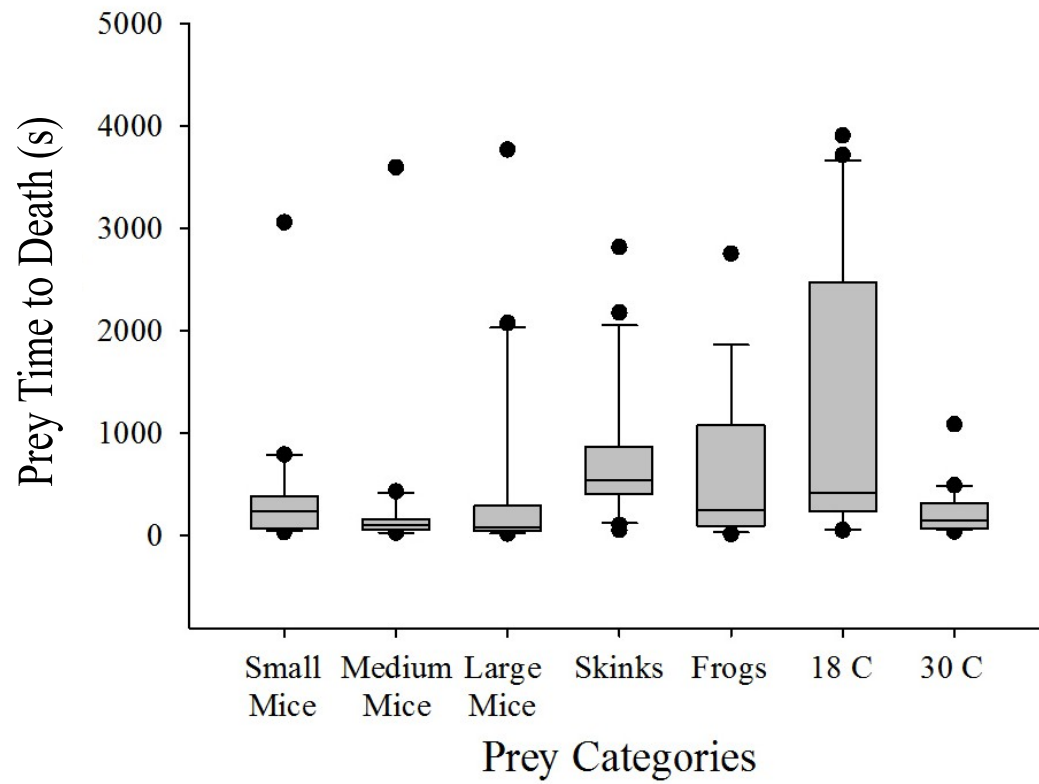


Figure 1. Box plots of prey time to death across all prey categories.

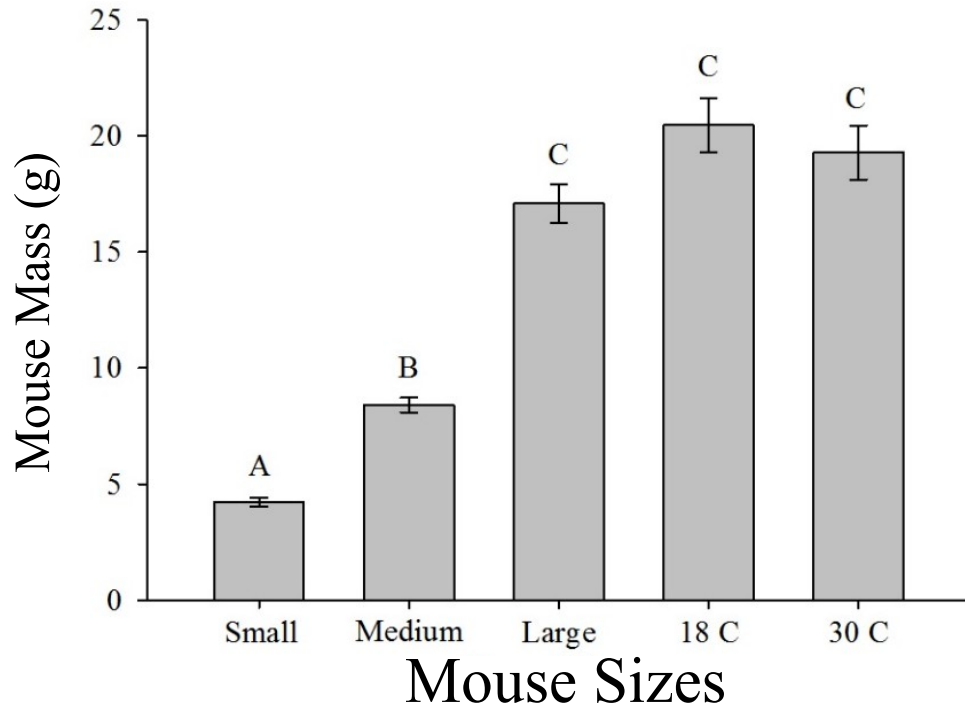


Figure 2. Pre-envenomation masses (mean \pm SE; small, 4.2 ± 0.19 g; medium, 8.4 ± 0.32 g; large, 17.1 ± 0.83 g; 18°C , 20.5 ± 1.17 g; 30°C , 19.3 ± 1.17 g) of mouse prey across size categories and snake body temperatures. Bars labelled with the same letter are not significantly different (ANOVA $F = 158.8$; $P < 0.001$). Sample sizes for each group are as follows: small $n = 19$, medium $n = 19$, large $n = 17$, 18°C $n = 20$, and 30°C $n = 19$.

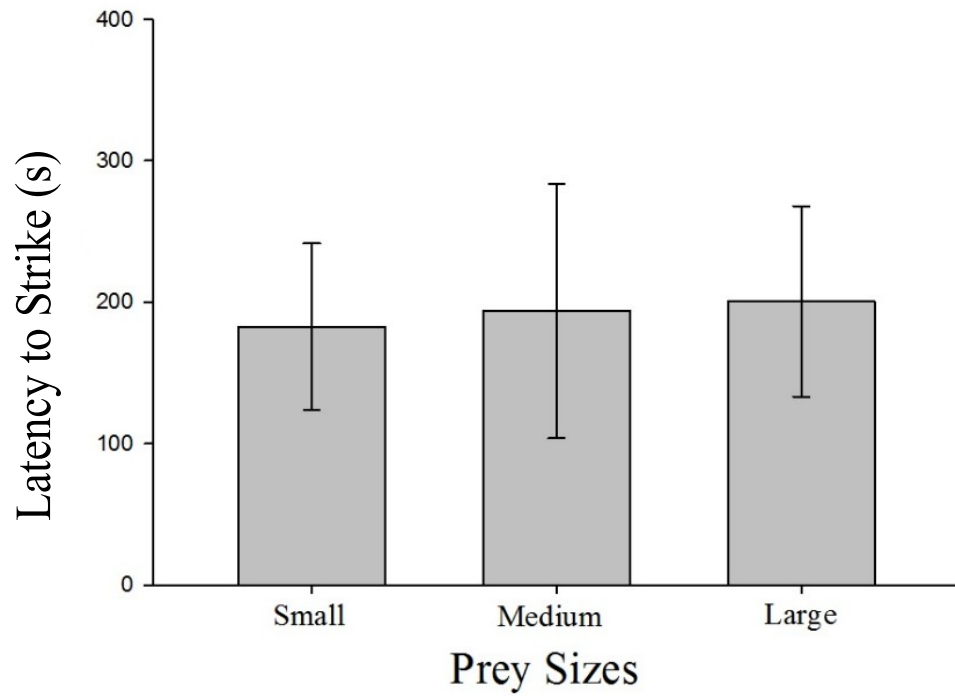


Figure 3. Snake latency to strike (mean \pm SE; small, 182.6 ± 58.85 s; medium, 193.8 ± 89.93 s; large, 200.4 ± 67.46 s) across mouse prey sizes. No significance was determined (ANOVA $F = 0.63$; $P = 0.54$; $\eta^2 = 0.04$). Data points for first and second foraging trials of each snake were averaged, resulting in a sample size of 10 for all size categories.

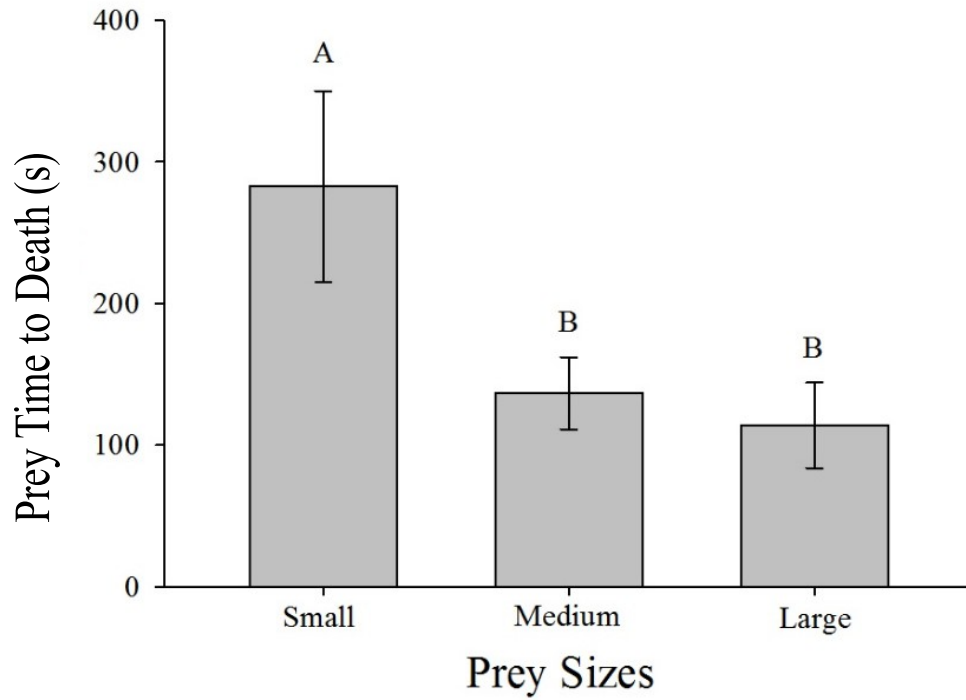


Figure 4. Prey time to death (mean \pm SE; small, 282.7 ± 67.44 s; medium, 136.6 ± 25.55 s; large, 113.9 ± 30.38 s) across mouse prey sizes. Bars with different letters denote significant differences (ANOVA $F = 3.67$; $P = 0.04$; $\eta^2 = 0.21$). Data points for first and second foraging trials for each snake were averaged, resulting in a sample size of 10 for all size categories.

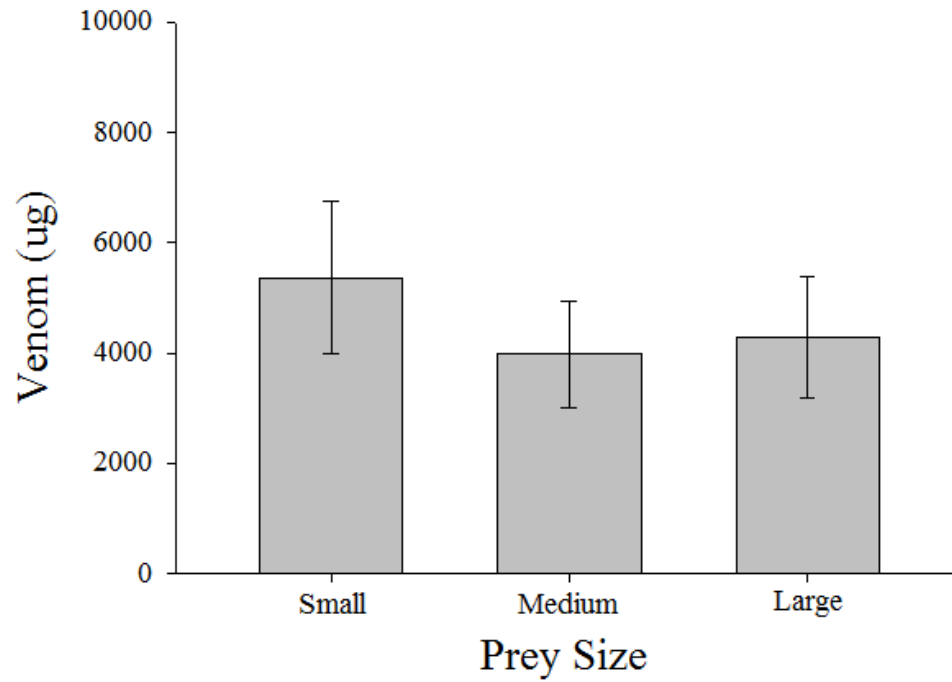


Figure 5. Amount of venom expelled (mean \pm SE; small, 5366.90 \pm 1387.68 μ g venom; medium 3974.02 \pm 957.30 μ g; large, 4275.81 \pm 1098.64 μ g) across prey sizes was not significantly different (ANOVA $F = 0.43$; $P = 0.65$; $\eta^2 = 0.03$). Data points for first and second foraging trials for each snake were averaged, resulting in a sample size of 10 for all size categories.

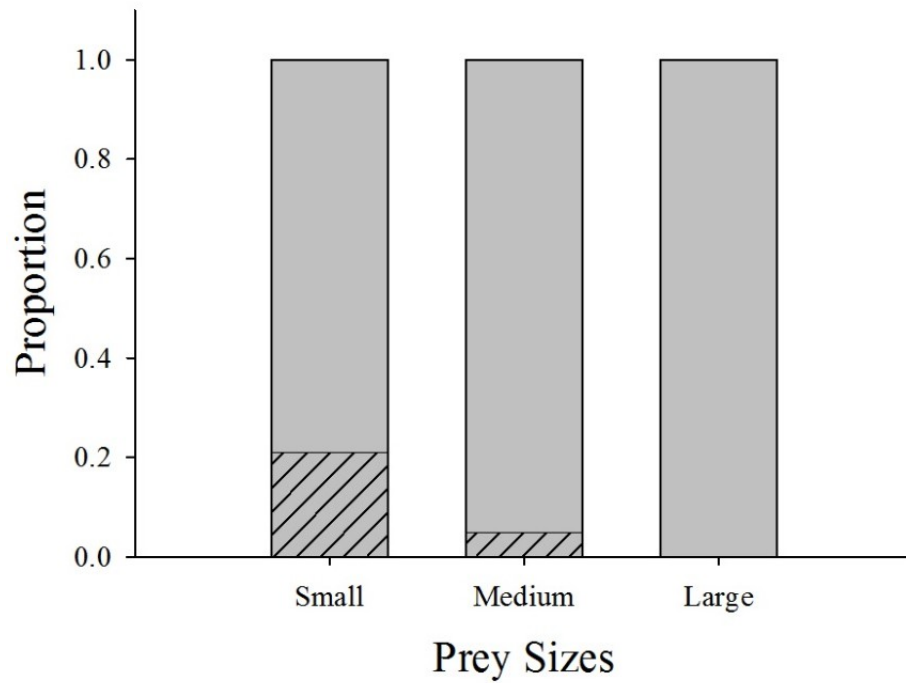


Figure 6. Proportion of mice struck and released (solid) and held (dashed) across three prey size categories. While small mice were struck and held more often than other sizes, the difference was not significant ($\chi^2 = 5.33$; $P = 0.07$; $V = 0.22$).

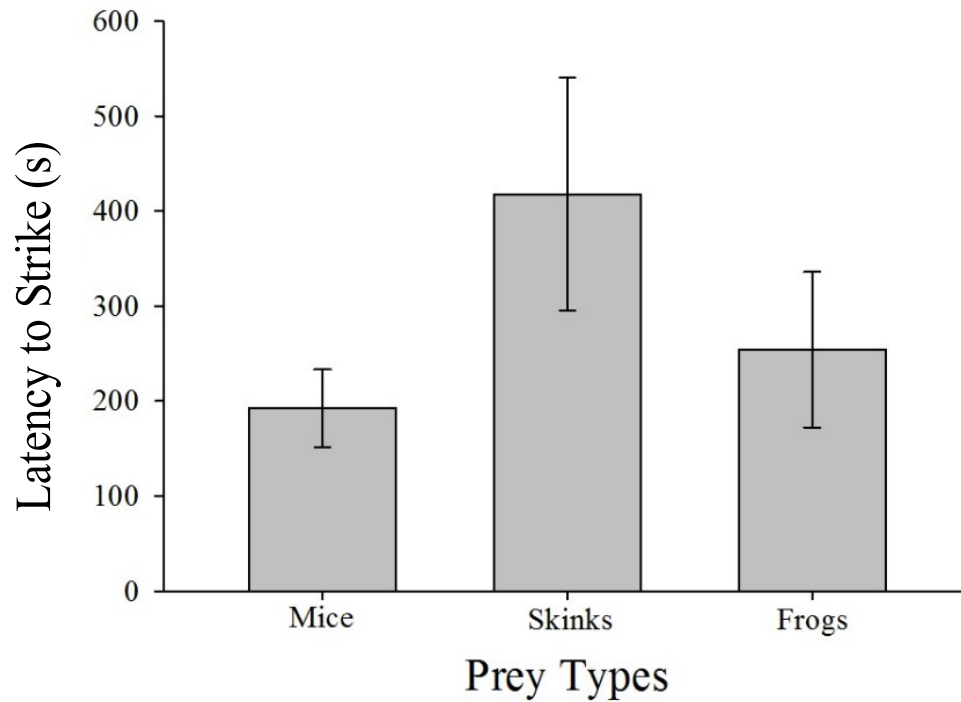


Figure 7. Snake latency to strike (mean \pm SE; mice, 192.3 ± 40.84 s; skinks, 417.9 ± 122.67 s; frogs, 254.1 ± 82.10 s) across prey types. No significance was determined across prey types (ANOVA $F = 2.77$; $P = 0.07$; $\eta^2 = 0.11$). Data points for first and second encounters of each snake were averaged, resulting in a sample size of 10 for skinks, 9 for frogs, and 30 for mice, where all three size categories were combined.

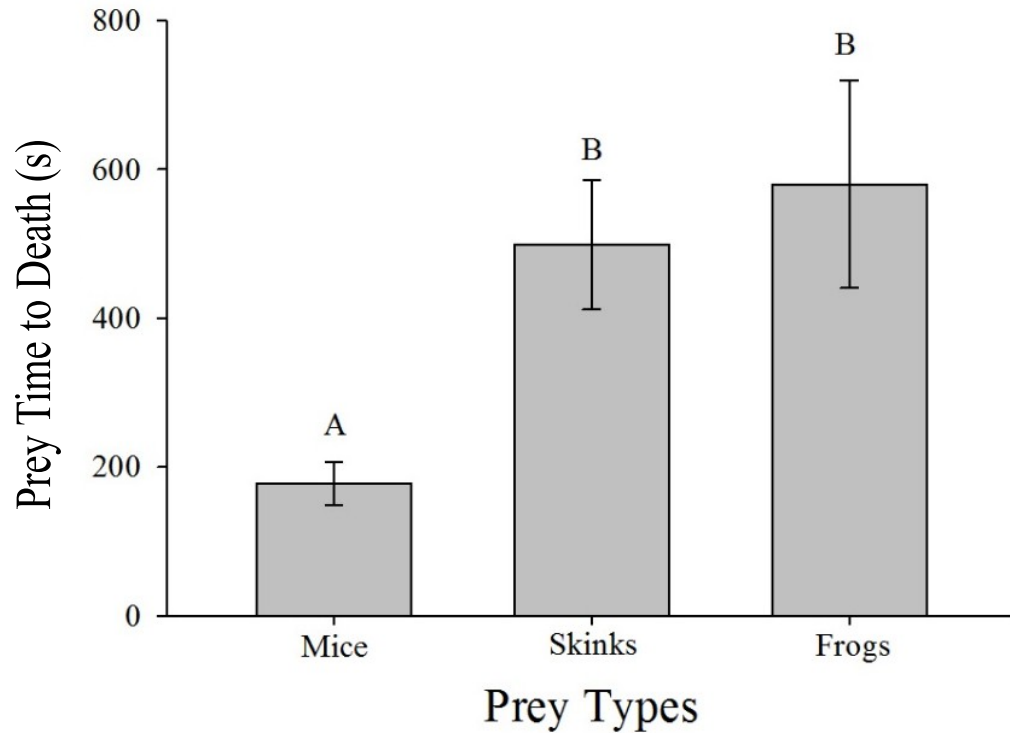


Figure 8. Prey time to death (mean \pm SE; mice, 177.3 ± 28.75 s; skinks, 498.5 ± 87.20 s; frogs, 580.1 ± 139.29 s) across prey types with letters denoting significance (ANOVA $F = 9.69$; $P < 0.001$; $\eta^2 = 0.30$) between groups. Data points for first and second foraging trials for each snake were averaged, resulting in a sample size of 10 for skinks, 9 for frogs, and 30 for mice, where all three size categories were combined.

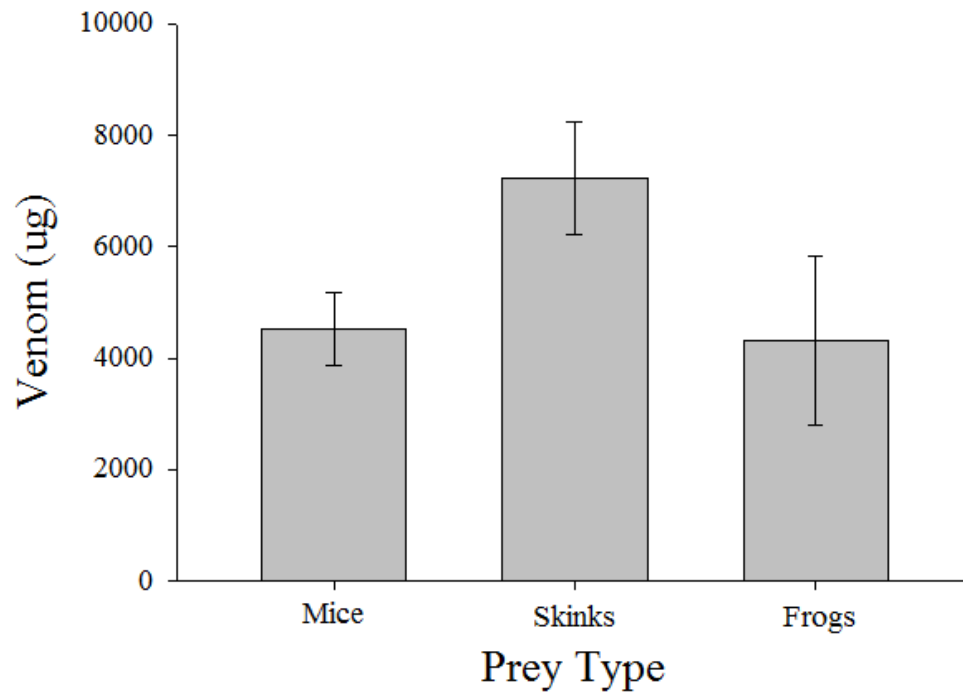


Figure 9. Amount of venom expelled (mean \pm SE; mice, 4538.91 \pm 656.67 μ g venom; skinks, 7236.75 \pm 1000.98 μ g; frogs, 4304.72 \pm 1521.12 μ g) across prey types. No significance was determined (ANOVA $F = 1.82$; $P = 0.17$; $\eta^2 = 0.07$). Data for first and second foraging trials for each snake were averaged, resulting in a sample size of 10 for skinks, 9 for frogs, and 30 for mice where all three size categories were combined.

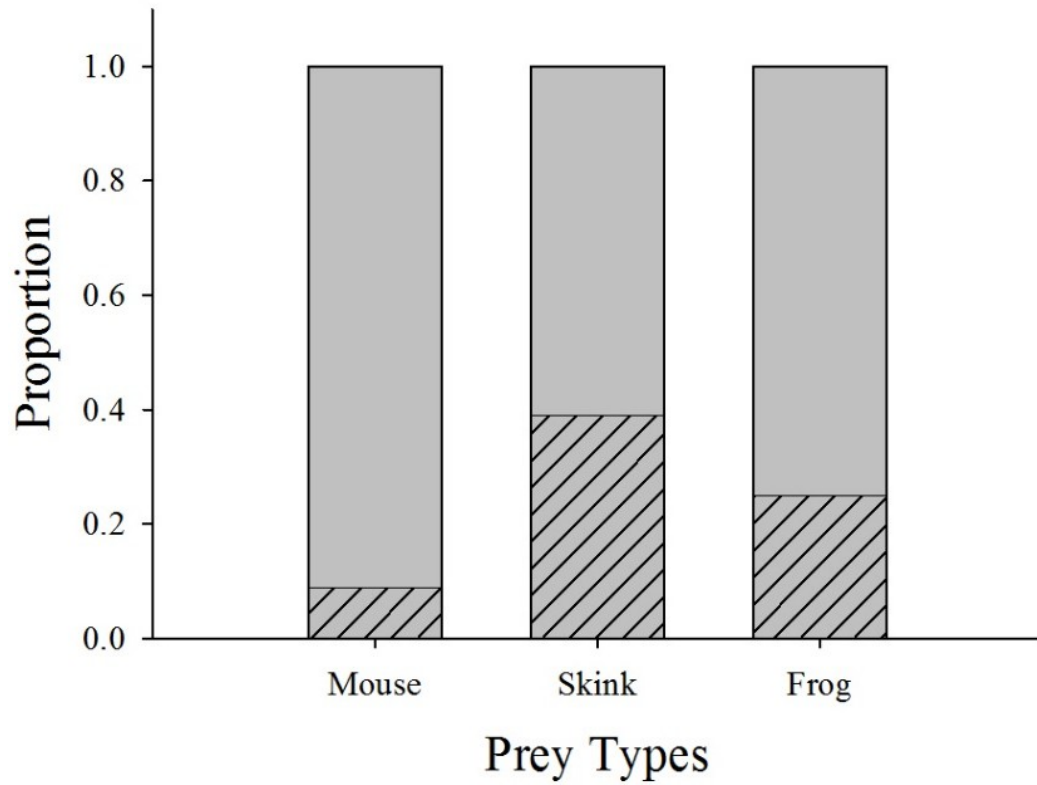


Figure 10. Proportion of prey struck and released (solid) and prey struck and held (dashed) across prey types. Skinks and frogs were struck and held significantly more often than mice ($\chi^2 = 8.82$; $P = 0.01$; $V = 0.22$).

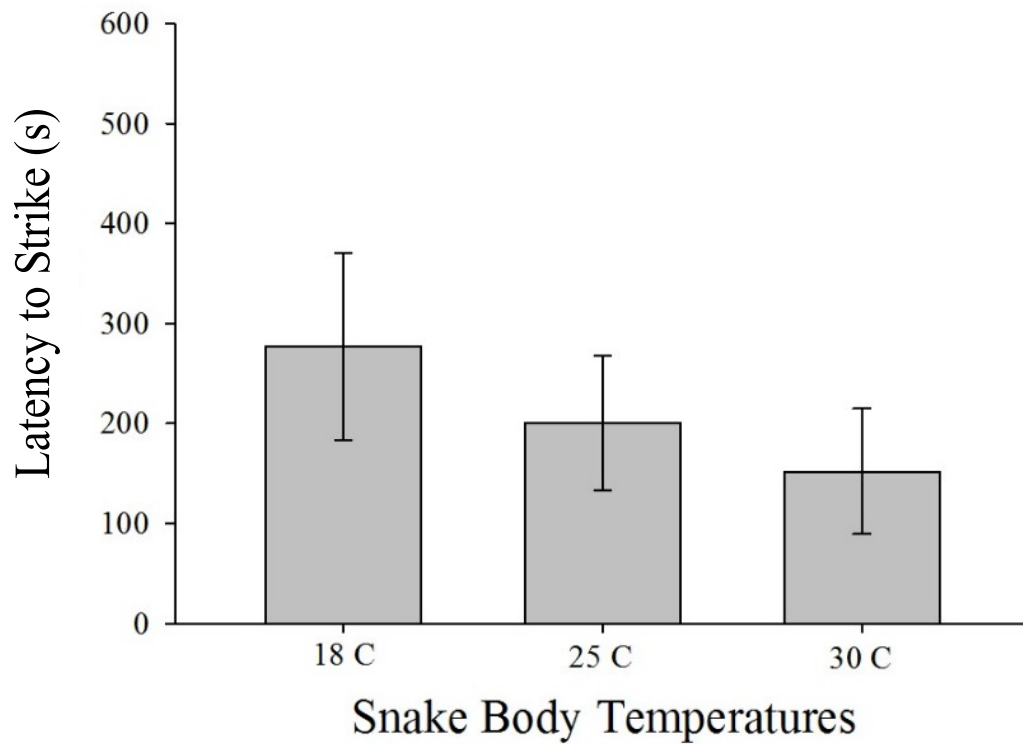


Figure 11. Snake latency to strike (mean \pm SE; 18°C, 276.9 \pm 93.53 s; 25°C, 200.4 \pm 67.46 s; 30°C, 152.1 \pm 62.86 s) across snake body temperatures. No significance was determined (ANOVA $F = 0.32$; $P = 0.73$; $\eta^2 = 0.02$). Data points for first and second foraging trials for each snake were averaged, resulting in a sample size of 10 for all categories.

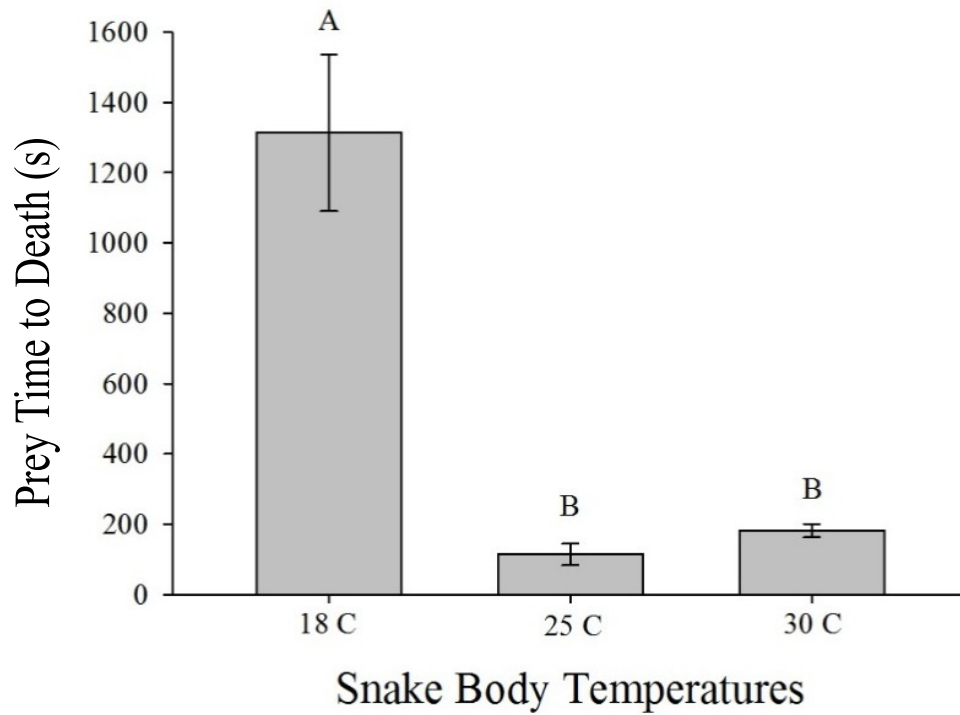


Figure 12. Prey time to death (mean \pm SE; 18°C, 1313.6 \pm 223.00 s; 25°C, 113.9 \pm 30.38 s; 30°C, 181.1 \pm 18.8 s) across snake body temperatures. Bars with different letters are significantly different (ANOVA $F = 22.84$; $P < 0.001$; $\eta^2 = 0.63$). Data points for first and second foraging trials for each snake were averaged, resulting in a sample size of 10 for all categories.

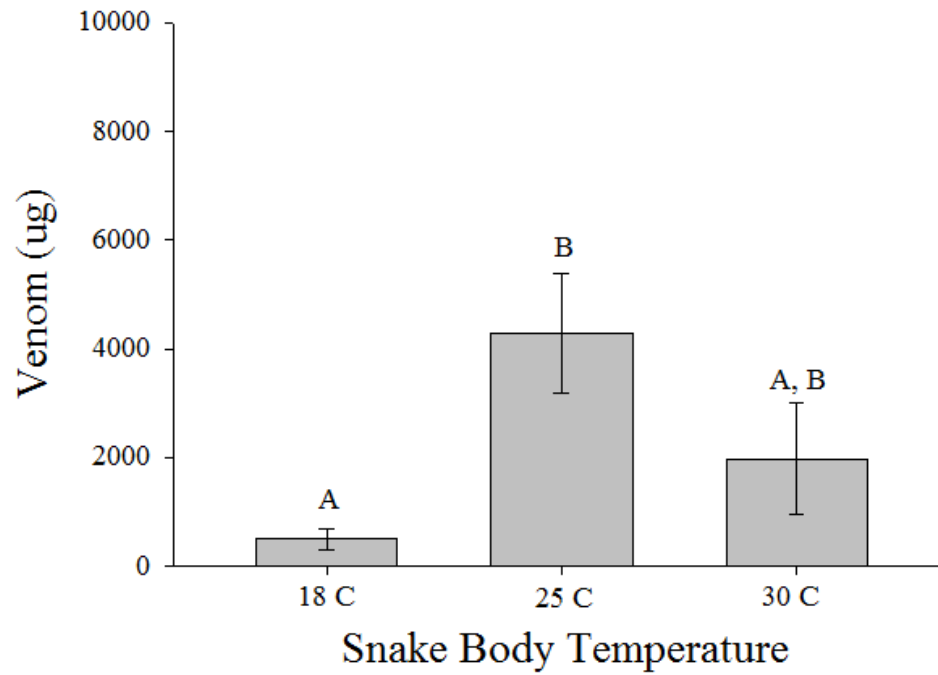


Figure 13. Amount of venom expelled (mean \pm SE; 18°C, 492.76 \pm 201.72 μ g venom; 25°C, 4275.81 \pm 1098.64 μ g; 30°C, 1968.63 \pm 1023.72 μ g) across snake body temperature categories. Bars with different letters are significantly different (ANOVA $F = 6.37$; $P = 0.005$; $\eta^2 = 0.32$). Data points for first and second foraging trials for each snake were averaged, resulting in a sample size of 10 for all categories.

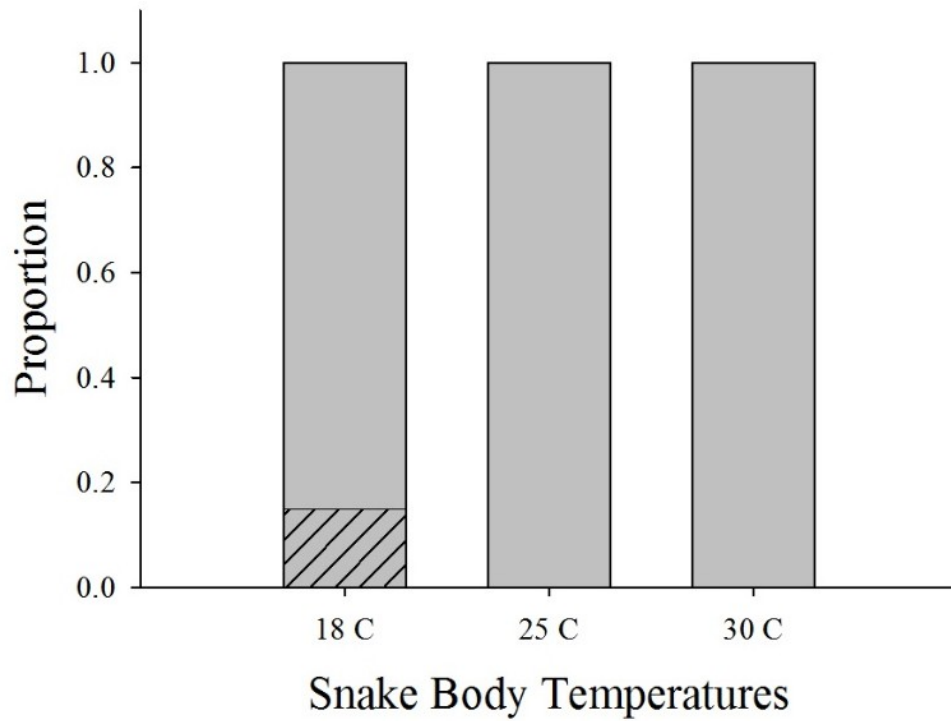


Figure 14. Proportion of prey struck and released (solid) and prey struck and held (dashed) across snake body temperatures. While mice were struck and held more often by cottonmouths at 18 C, the difference was not significant ($\chi^2 = 5.71$; $P = 0.06$; $V = 0.23$).