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Nutritional Ecology of the Western Cottonmouth (Agkistrodon piscivorus leucostoma)

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Nutritional Ecology of the Western Cottonmouth (*Agkistrodon piscivorus leucostoma*)

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by

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Abstract

Nutritional ecology aims to understand the factors that shape the diets of animals, how these ingested meals are processed, and how the assimilated nutrients are used to shape an organism's interactions with its environment. As environmental parameters are altered due to anthropogenic changes, the availability and quality of prey may be altered. The alteration of the nutritional landscape can be devastating to nutritional specialists, yet generalist predators may be able to cope with these changes. The western cottonmouth (*Agkistrodon piscivorus leucostoma*) is a unique semiaquatic pitviper that can both forage along the land-water interface and in upland habitat when faced with drought conditions. Given the potentially wide diet breath of this species, how would it respond to shifts in prey base? Is there a relationship between prey preference and digestive performance of the cottonmouth? I explored this question using stable isotope dietary analysis, behavioral prey preference trials, and measurement of the metabolic response to feeding (specific dynamic action). The notoriously wide diet breath of the cottonmouth was verified through stable isotope analysis, where animals were consuming various fish and frog species, and potentially crayfish. Prey preference trials conducted using an X maze showed that these snakes would readily eat fish, frog, and mouse, yet they showed a very strong aversion to crayfish. Of the prey types that were readily eaten by cottonmouths, mouse meals generated the highest overall specific dynamic action response while fish and frog meals were comparable to one another.

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Contents

Dissertation Introduction

Nutritional ecology aims to understand the nutrition-mediated interactions between an organism and its environment (Raubenheimer et al., 2012). Specifically what factors shape the diets of animals, how these ingested meals are processed, and how the assimilated nutrients are used to shape an organism's interactions with its environment and other organisms (Karasov and Martínez del Rio, 2007). As environmental parameters are altered due to anthropogenic changes, the availability and quality of prey may be altered (Birnie-Gauvin et al., 2017). The alteration of the nutritional landscape can be devastating to nutritional specialists, yet generalist predators may be able to cope with these changes (Raubenheimer et al., 2012). The western cottonmouth (*Agkistrodon piscivorus leucostoma*) is a unique semiaquatic pitviper that can both forage along the land-water interface and in upland habitat when faced with drought conditions (Eskew et al., 2009; Hill, 2004; Hill and Beaupre, 2008; Savitzky, 1992). Given the potentially wide diet breath of this species, how would it respond to shifts in prey base? Is there a relationship between prey preference and digestive performance of the cottonmouth? I explored this question using a variety of techniques where each chapter was informed by each subsequent one.

In chapter one I set out to determine what prey free-ranging cottonmouths were consuming in a small section of the Buffalo National River. Stable isotope analysis was chosen over other methods of diet determination due to its ability to detect soft-bodied prey items and its potential resistance to opportunistic meals outside of what an organism typically eats (Nielsen et al., 2017; Willson et al., 2010).

In chapter two I used the results from chapter one to determine if there was a preference between prey types. In squamate reptiles, a long-standing approach to determining prey preference is the use of scented cotton swab to determine predator interest based on tongue flick

intensity (Sheffield et al., 1967; Wilde, 1938). The reductionist nature of the cotton swab techniques does not provide a clear indication that a predator would consume the prey species being tested. To overcome this, I used a modified radial arm maze (an X maze) to determine prey preference in cottonmouths when offered the choice of crayfish, fish, frog or mouse.

In chapter three I used specific dynamic action (SDA) response to feeding as a measure of digestive performance (Secor, 2009) in cottonmouths fed different prey types (fish, frog, or mouse) and prey forms (homogenized or whole). Open-flow respirometry was used to measure resting and post-feeding metabolic rates (Zaidan and Beaupre, 2003).

Literature cited

Birnie-Gauvin, K., Peiman, K.S., Raubenheimer, D., Cooke, S.J., 2017. Nutritional physiology and ecology of wildlife in a changing world. Conserv Physiol 5, cox030.

Eskew, E.A., Willson, J.D., Winne, C.T., 2009. Ambush site selection and ontogenetic shifts in foraging strategy in a semi-aquatic pit viper, the Eastern cottonmouth. Journal of Zoology (London) 277, 179-186.

Hill, J.G., 2004. Natural history of the western cottonmouth (*Agkistrodon piscivorus leucostoma*) from an upland lotic population in the Ozark mountains of northwest Arkansas, University of Arkansas.

Hill, J.G., Beaupre, S.J., 2008. Body size, growth, and reproduction in a population of western cottonmouths (*Agkistrodon piscivorus leucostoma*) in the Ozark mountains of northwest Arkansas. Copeia 2008, 105-114.

Karasov, W.H., Martínez del Rio, C., 2007. Physiological Ecology: How Animals Process Energy, Nutrients, and Toxins. Princeton University Press, Princeton, NJ.

Nielsen, J.M., Clare, E.L., Hayden, B., Brett, M.T., Kratina, P., Gilbert, M.T.P., 2017. Diet tracing in ecology: Method comparison and selection. Methods in Ecology and Evolution 9, 278-291.

Raubenheimer, D., Simpson, S.J., Tait, A.H., 2012. Match and mismatch: conservation physiology, nutritional ecology and the timescales of biological adaptation. Philos Trans R Soc Lond B Biol Sci 367, 1628-1646.

Savitzky, B.A.C., 1992. Laboratory studies on piscivory in an opportunistic pitviper, the cottonmouth, Agkistrodon piscivorus. Biology of the pitvipers, 347-368.

Secor, S.M., 2009. Specific dynamic action: a review of the postprandial metabolic response. Journal of Comparative Physiology 179B, 1-56.

Sheffield, L.P., Law, J.H., Burghardt, G., 1967. On the nature of chemical food sign stimuli for newborn snakes. Communications in Behavioral Biology 2, 7-12.

Wilde, W.S., 1938. The role of Jacobson's organ in the feeding reaction of the common garter snake, *Thamnophis sirtalis sirtalis* (Linn.). Journal of Experimental Zoology 77, 445-465.

Willson, J.D., Winne, C.T., Pilgrim, M.A., Romanek, C.S., Gibbons, J.W., 2010. Seasonal variation in terrestrial resource subsidies influences trophic niche width and overlap in two aquatic snake species: a stable isotope approach. Oikos 119, 1161-1171.

Zaidan, F., III, Beaupre, S.J., 2003. Effects of body mass, meal size, fast length, and temperature on specific dynamic action in the timber rattlesnake (*Crotalus horridus*). Physiological and Biochemical Zoology 76, 447-458.

Chapter 1: Evaluation of stable isotope analysis for determining diet in the Western Cottonmouth (*Agkistrodon piscivorus leucostoma*)

Jason Ortega, Joseph A. Baecher, Steven J. Beaupre

Abstract

Nutritional ecology aims to understand the factors that shape the diets of animals, how these ingested meals are processed, and how the assimilated nutrients are used to shape an organism's interactions with its environment. A vital step in this process is determining the diet of the focal consumer. The western cottonmouth (*Agkistrodon piscivorus leucostoma*) is a semi aquatic pitviper that has been classified as a generalist predator due to its ability to forage in a variety of habitat types. Stable isotope dietary analysis was used to determine the diet breadth of cottonmouths in a small section of the Buffalo National River. Cottonmouth scale clips and whole potential prey species were analyzed to determine isotopic ratios ($\delta^{13}C$ and $\delta^{15}N$). Potential prey included various species of fish, frog, and one species of crayfish. The results of this study suggest that these snakes are eating a wide array of fish and frog species. Through our results it is unclear whether or not crayfish are being consumed.

Introduction

Events that alter the energy budget of an animal have the potential to impair growth, reproduction, or even the likelihood of an individual's survival (Congdon, 1989). Variation of these processes in individuals scale up to impacting entire populations of organisms and may ultimately lead to local extirpation (Dunham et al., 1989). The diet of an organism directly

impacts its energy budget (total energy assimilated) while also imparting physiological limitations due to diet composition (variation in essential nutrients) (Carter et al., 2020; McKenzie, 2005). Beyond compositional variation, different prey types may require varying effort to encounter, handle, consume, and digest (Karasov and Martínez del Rio, 2007). The identity of prey consumed can greatly impact a predator's energy budget, nutrient budget, life history, and population dynamics.

The western cottonmouth (*Agkistrodon piscivorus leucostoma*) is a North American pitviper that is unique because of its semiaquatic nature, allowing it to forage in a wide range of habitats from within a body of water to the forest floor (Hill and Beaupre, 2008). With such a wide range of habitat types, this species has been characterized as a generalist, eating a massive litany of prey items throughout its range. Stomach contents and behavioral observations have shown that this species has consumed invertebrates, mammals, birds, fish, amphibians, reptiles, and can be cannibalistic (Gloyd and Conant, 1990; Savitzky, 1992).

Nutritional ecology is the study of the mechanisms that influence diet and in turn, how resulting assimilated nutrients are allocated to competing functions such as activity, growth, maintenance, and reproduction (Karasov and Martínez del Rio, 2007). To understand if there is an interaction between prey preference and digestive performance in this generalist predator, the first necessary step is to determine what prey are eaten by cottonmouths. Direct observations of stomach contents have been used for a variety of snake species. The advent of forcing regurgitation has allowed stomach content analysis to move beyond lethal snake dissections (Brown et al., 2014; Dorcas and Willson, 2009; Fitch, 1987). Stomach content analysis only provides a snapshot into the snake's diet by only representing that immediate observed meal, while also being limited in its ability to detect small meals and soft-bodied prey (such as

tadpoles). Fecal analysis has also been used to identify prey based on indigestible (exoskeletons) or undigested components (feathers or fur) (Schalk et al., 2018). As with stomach content analysis, this method can have difficulty identifying soft-bodied species and/or those that are highly digestible (Dorcas and Willson, 2009; Fitch, 1987; Klare et al., 2011; Nielsen et al., 2017). The use of molecular techniques to determine prey species from fecal material and stomach contents can detect highly digestible prey (that lack indigestible or resilient anatomical structures), but as with stomach content analysis it is temporally limited to the meal(s) that were present at time of sampling (Egeter et al., 2015; Nielsen et al., 2017). The temporal limitations of gastric and fecal diet determination are intensified when coupled with an animal that will routinely eat carrion, such as the cottonmouth (Gloyd and Conant, 1990; Lillywhite et al., 2002; Savitzky, 1992). Opportunistic carrion meals of a novel prey type would add error to diet analysis due to the inability of the gastric and fecal methods to limit the impact of sporadic carrion meals (Nielsen et al., 2017).

Diet determination via stable isotope analysis is more resistant to errant opportunistic meals due to it's nature as a measure of the record of prey eaten and assimilated by the predator, not just glimpses into what has been recently consumed (Karasov and Martínez del Rio, 2007). The isotopic signature of a consumer's tissues will not be significantly impacted by errant meals that do not represent the animal's base diet. The use of stable isotope analysis for determining diet in snakes has been applied to a variety of species (Durso and Mullin, 2017; Pilgrim, 2005; Rebelato et al., 2020; Rush et al., 2014; Smith et al., 2002; Willson et al., 2010), but the success of its application depends on the inherent variation in stable isotope values within and among the predator and potential prey for a given location (Bearhop et al.; Karasov and Martínez del Rio, 2007; Newsome et al., 2007; Post, 2002).

Herein, I aimed to determine the efficacy of stable isotope analysis in identifying the diet of western cottonmouths within the Buffalo National River, in North-Central Arkansas. The ability of stable isotope analysis to determine snake diet depended on the following: 1) That predator and potential prey stable isotope ratios are significantly different from one another. 2) That there is significant variation in stable isotope ratios between potential prey types. 3) There is significant variation in stable isotope ratios between adult and juvenile cottonmouths, while not pivotal, the ability to make the distinction between adult and juvenile diets would expand the breadth of future work on the nutritional ecology of this snake.

Methods

Sample collection:

All specimens were collected from Big Creek within the Buffalo National River over the course of two months (May to June 2013); all sampling was conducted under NPS permit (BUFF-00120, issued to JO). Scale clip samples were collected from cottonmouth snakes (*Agkistrodon piscivorus,* adults and juveniles). Whole potential prey consisted of: one species of crayfish (Northern crayfish, *Faxonius virilis*); two species of frog (American Bullfrog, *Lithobates catesbeianus*, adults and tadpoles; Blanchard's Cricket frog, Acris blanchardi, adults only); and three species of fish (Stippled darter, *Etheostoma punctulatum*; Brook silverside, *Labidesthes sicculus*; Longear sunfish, *Lepomis megalotis*). Six individuals were used per each sample type.

Snake scale clips were collected from snakes that were captured during nighttime visual encounter surveys. Juvenile cottonmouths were categorized as being less than 150 g and still exhibited high-contrast tail tips (Gloyd and Conant, 1990). A squeeze box was used to take scale

clips from six adult and six juvenile cottonmouths (Beaupre and Greene, 2012; Quinn and Jones, 1974). Scale clips were taken from ventral scales via the use of flamed forceps and scissors. Approximately 3 mm X 1 mm of total scale material was collected from each snake (Pilgrim, 2005). Snakes were treated with a liquid bandage (New-skin®) at the site where scale clip(s) were taken. Each individual scale clip was stored in a screw-top microcentrifuge tube and kept frozen until processing.

Adult frogs were captured by hand or by small net and were kept moist in ventilated containers until euthanasia, all animals were euthanized not later than 24 hours after being captured. The frogs were euthanized via overdose of buffered tricaine mesylate (MS-222), by being placed in the MS-222 solution until ventilation ceased (Beaupre et al., 2004) and frozen. Tadpoles were captured via net and maintained in buckets (filled with creek water and with a battery powered aerator) until euthanized via overdose of buffered MS-222. Tadpoles were euthanized not later than 6 hours after capture and were frozen until analysis. Fish were captured via seining and maintained in buckets (filled with creek water and with a battery powered aerator) until euthanized via overdose of buffered MS-222 at site of capture (UFR (Use of Fishes in Research) Committee, 2013). Fish were euthanized no later than one hour after capture and were placed on ice until frozen for later analysis. Crayfish were captured by seining or hand nets and were maintained in buckets filled with creek water and battery powered aerators. Crayfish were euthanized no later than 6 hours after capture by being initially chilled with an ice slurry and then frozen. All prey euthanized for analysis were small enough to be consumed by an adult cottonmouth, any prey that were too large were released at site of capture.

Sample processing:

Whole animals and scale clips were lyophilized while frozen for at least 24 hours or until mass loss plateaued. All samples were individually homogenized and were not pooled. Scale clips were diced into small pieces using scissors and then where homogenized using a Wig-L-Bug Mill®. To ensure proper drying of whole animals, each was cut into several transverse sections. Crayfish were not cut into sections as they were separated at the junction of the tail and cephalothorax prior to lyophilization. All prey except for crayfish were individually homogenized by use of a Wiley Mill followed by a Wig-L-Bug Mill®. Crayfish internal organs were removed from the carapace prior to homogenization via a Wig-L-Bug Mill®. The carapaces of crayfish were not analyzed due to the apparent inability of cottonmouths to digest chitin. One of the authors (JO) has observed several instances of crayfish claws passing through the gastrointestinal tract of cottonmouths without any apparent degradation of the carapace, this is most likely due to the absence of endogenous chitinase production in this snake species (Micha et al., 1973). All homogenized samples were lyophilized again for 24 hours post-homogenization prior to isotopic analysis. Lipids were not extracted prior to isotope analysis (Willson et al., 2010).

Sample analysis:

Each sample was loaded into 3 x 5.5 mm tin capsules and weighed for isotopic analysis at the University of Arkansas Stable Isotope Laboratory (UASIL). All samples were analyzed at the UASIL for carbon and nitrogen isotope ratios using an Elemental Analyzer and a Delta Plus continuous flow mass spectrometer. The isotopic content of the samples (A_{sample}) were compared to specific standards (A_{std}) to determine both ^{13}C:^{12}C and ^{15}N:^{14}N for each sample; the following

standards were used, Pee Dee Cretaceous Belemite (PDB) as the carbon standard and atmospheric air was used as the nitrogen standard (Fry, 2006; Pilgrim, 2007). Sample isotope compositions were reported in conventional delta notation (δ), per mill (‰) units; using the formula $\delta I_{std} = (A_{sample}/A_{std} -1)(1000)$ (Fry, 2006; Martinez del Rio and Wolf, 2005).

Statistical analysis:

All statistical analyses were conducted using R version 4.1.0 (R Core Team, 2021). Comparisons of isotopic ratios ($\delta^{13}C$ and $\delta^{15}N$) among potential whole prey and snake scale clips were analyzed though use of multivariate analysis of variance (MANOVA). The "car" package was used to conduct a MANOVA and post-hoc pairwise Tukey tests (Fox and Weisberg, 2019). Levene's test was used to verify the assumption of homoscedasticity. Post-hoc pair wise interactions were tested through ANOVA for the interaction between sample type and mean $\delta^{13}C$ or $\delta^{15}N$ to examine which isotope ratio led to significant differences in MANOVA results. The normality of both ANOVAs was verified by examining the residuals for each analysis (mean = 0 and variance = 1) using a Shapiro-Wilk test.

Results

A total of 54 samples were analyzed for isotopic ratios. Mean $\delta^{13}C$ and $\delta^{15}N$ values (± 1 SE, Figure 1) showed varying patterns of clustering among prey types and snake scales. The mean ratios for frog species (adults and tadpole) and Northern crayfish were highly clustered for both mean $\delta^{13}C$ and $\delta^{15}N$, ranging ~0.8 ‰ for both carbon and nitrogen. Fish species showed a looser aggregation with $\delta^{13}C$ ranging ~1.5 ‰ and $\delta^{15}N$ ~0.3‰. Snake scales clips were also loosely clustered with $\delta^{13}C$ ranging ~0.4 ‰ and $\delta^{15}N$ ~1.4‰.

There was an overall significant difference between samples for the interaction between mean $\delta^{13}C$ and $\delta^{15}N$ (MANOVA, $F_{8,16} = 7.3105$, $P < 0.0001$). Levene's test indicated that the data used in the MANOVA were homoscedastic ($P = 0.3867$). Post-hoc pairwise Tukey tests followed many of the trends seen in Figure 1. Amphibians and the Northern crayfish were not significantly different from each other (lowest $P = 0.6492$) and all fish species were not significantly different from one another (lowest $P = 0.4722$). Overall, there were some prey species that were significantly different in mean $\delta^{13}C$ and $\delta^{15}N$ values when compared to others, but these were species that were outliers due to being highly depleted or enriched. Adult cottonmouth scale clips were not significantly different from juvenile scale clips ($P = 0.6541$). When compared to prey, adult cottonmouths were significantly different for the interaction between mean $\delta^{13}C$ and $\delta^{15}N$ for all potential prey (highest $P = 0.0349$), except for the Brook silverside ($P = 0.7403$).

Discussion

Overall, stable isotope analysis is not a viable method for determining prey type for cottonmouths in the Buffalo National River. While there were some significant differences between adult snakes and potential prey for the interaction between average $\delta^{13}C$ and $\delta^{15}N$ values, there was significant overlap in isotope ratios between potential prey types, making it impossible to identify specific species within clusters (Figure 1). The most troublesome of the clusters being the grouping of all amphibians and crayfish. At this site snakes have been observed predating upon amphibians (adult and tadpole) and crayfish have been found in snake fecal material. Within the fish cluster, the only species of fish that been observed being predated

upon are Brook silversides, which were shown to not be significantly different from snake samples (both adult and juvenile), indicating that they may not be the predominate fish eaten.

Use of ANOVA analysis for comparing mean $\delta^{13}C$ and $\delta^{15}N$ individually between all samples (snake and potential prey) showed very little variation in $\delta^{13}C$. The $\delta^{15}N$ values are most responsible for significant interactions seen between mean $\delta^{13}C$ and $\delta^{15}N$ (MANOVA). Carbon isotope ratios were only significantly different for Blanchard's cricket frog (ANOVA, $F_{8,45} = 2.653$, $P < 0.01$), Northern crayfish (ANOVA, $F_{8,45} = 2.653$, $P = 0.0105$), Stippled darter (ANOVA, $F_{8,45} = 2.653$, $P < 0.001$), where the only significant post-hoc pair-wise interaction seen was between adult snakes and darters ($P = 0.0113$). Nitrogen isotope ratios were all significantly different between samples (ANOVA, $F_{8,45} = 30.44$, highest significant value $P = 0.02$) except for Brook silverside (ANOVA, $F_{8,45} = 2.653$, $P = 0.1819$), the post-hoc pair-wise interactions were significant between clusters (as seen in Figure 1) but not within.
The clustering of samples for both $\delta^{13}C$ and $\delta^{15}N$ dictate the patterns shown in the post-hoc analyses described above. Longear sunfish and Brook silverside where similar in $\delta^{13}C$, but Longear sunfish were more depleted in ^{15}N when compared to Brook silverside. Stippled darters and Brook silverside were similar in $\delta^{15}N$, while Stippled darters were more depleted in ^{13}C than Brook silverside.

The general relationship between isotopic ratios of adult and juvenile cottonmouths was as expected (Pilgrim, 2005), with adults being more enriched for both ^{13}C and ^{15}N (Figure 1), yet this difference was not statistically significant. Following this trend, adult American bullfrogs were slightly more enriched than tadpoles for both ^{13}C and ^{15}N, but not at a statistically significant level or as significantly enriched as seen by others (Willson et al., 2010).

An increase in sampling to include other active seasons could show a spreading of $\delta^{13}C$ and $\delta^{15}N$ values, in particular ^{13}C (Dekar et al., 2009; Willson et al., 2010). The relatively low spread of $\delta^{13}C$ among the samples could be due to the timing of our sampling. But the relatively wide SEs for bullfrogs (both tadpole and adult), crayfish, and darters may already represent the potential for the spreading of the clusters, in particular the highest variation in prey $\delta^{13}C$ may occur during the winter when snakes are not active or feeding (Dekar et al., 2009).

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Literature Cited

Bearhop, S., Adams, C.E., Waldron, S., Fuller, R.A., Macleod, H., Determining trophic niche width a novel approach using stable isotope. Journal of Animal Ecology 73, 1007-1012.

Beaupre, S.J., Greene, H.W., 2012. Handling Hazardous Live Reptiles, in: R.W. McDiarmid, M.S. Foster, C. Guyer, J.W. Gibbons, N. Chernoff (Eds.), Reptiles: Standard Methods of Inventory and Monitoring. University of California Press, Los Angeles, CA, 130-134.

Beaupre, S.J., Jacobson, E.R., Lillywhite, H.B., Zamudio, K., 2004. Guidelines for use of live amphibians and reptiles in field and laboratory research, second edition., Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists, 1-43.

Brown, D.S., Ebenezer, K.L., Symondson, W.O., 2014. Molecular analysis of the diets of snakes: changes in prey exploitation during development of the rare smooth snake Coronella austriaca. Mol Ecol 23, 3734-3743.

Carter, W.A., DeMoranville, K.J., Pierce, B.J., McWilliams, S.R., 2020. The effects of dietary linoleic acid and hydrophilic antioxidants on basal, peak, and sustained metabolism in flight-trained European starlings. Ecol Evol 10, 1552-1566.

Congdon, J.D., 1989. Proximate and Evolutionary Constraints on Energy Relations of Reptiles. Physiological Zoology 62, 356-373.

Dekar, M.P., Magoulick, D.D., Huxel, G.R., 2009. Shifts in the trophic base of intermittent stream food webs. Hydrobiologia 635, 263-277.

Dorcas, M.E., Willson, J.D., 2009. Innovative Methods for Studies of Snake Ecology and Conservation, in: S.J. Mullin, R.A. Seigel (Eds.), Snakes: Ecology and Conservation. Cornell University Press, USA, 5-37.

Dunham, A.E., Grant, B.W., Overall, K.L., 1989. Interfaces between biophysical ecology and the population ecology of terrestrial vertebrate ectotherms. Physiological Zoology 62, 335-355.

Durso, A.M., Mullin, S.J., 2017. Ontogenetic shifts in the diet of plains hog-nosed snakes (Heterodon nasicus) revealed by stable isotope analysis. Zoology (Jena) 120, 83-91.

Egeter, B., Bishop, P.J., Robertson, B.C., 2015. Detecting frogs as prey in the diets of introduced mammals: a comparison between morphological and DNA-based diet analyses. Mol Ecol Resour 15, 306-316.

Fitch, H.S., 1987. Collecting and life-history techniques, in: R.A. Seigel, J.T. Collins, S.S. Novak (Eds.), Snakes: Ecology and Evolutionary Biology. Macmillan Publishing Company, New York, NY, 143-164.

Fox, J., Weisberg, S., 2019. An {R} Companion to Applied Regression, Third ed. Sage, Thousand Oaks, CA.

Fry, B., 2006. Stable Isotope Ecology. Springer, New York.

Gloyd, H.K., Conant, R., 1990. Snakes of the Agkistrodon Complex: A Monographic Review. Society for the Study of Amphibians and Reptiles, Athens, GA.

Hill, J.G., Beaupre, S.J., 2008. Body size, growth, and reproduction in a population of western cottonmouths (*Agkistrodon piscivorus leucostoma*) in the Ozark mountains of northwest Arkansas. Copeia 2008, 105-114.

Karasov, W.H., Martínez del Rio, C., 2007. Physiological Ecology: How Animals Process Energy, Nutrients, and Toxins. Princeton University Press, Princeton, NJ.

Klare, U., Kamler, J.F., Macdonald, D.W., 2011. A comparison and critique of different scat-analysis methods for determining carnivore diet. Mammal Review 41, 294-312.

Lillywhite, H.B., Sheehy, C.M., McCue, M.D., 2002. Scavenging behaviors of cottonmouth snakes at island bird rookeries. Herpetological Review 33, 259.

Martinez del Rio, C., Wolf, B.O., 2005. Mass-Blance Models for Animal Isotopic Ecology in: J.M. Starck, T. Wang (Eds.), Physiological and Ecological Adpatations to Feeding in Vertebrates. Science Publishers, Enfield, NH, 141.

McKenzie, D.J., 2005. Effects of DIetary Fatty Acids on the Physiology of Environmental Adaptation in Fish in: J.M. Starck, T. Wang (Eds.), Physiological and Ecological Adpatations to Feeding in Vertebrates. Science Publishers, Enfield, NH, 363-388.

Micha, J.C., Dandrifosse, G., Jeuniaux, C., 1973. Activity of Gastric Chitinases in Reptiles as a Function of pH. Archives Internationales de Physiologie et de Biochimie 81, 629-637.

Newsome, S.D., Martinez del Rio, C., Bearhop, S., Phillips, D.L., 2007. A niche for isotopic ecology. Frontiers in Ecology and the Environment 5, 429-436.

Nielsen, J.M., Clare, E.L., Hayden, B., Brett, M.T., Kratina, P., Gilbert, M.T.P., 2017. Diet tracing in ecology: Method comparison and selection. Methods in Ecology and Evolution 9, 278-291.

Pilgrim, M.A., 2005. Linking microgeographic variation in pigmy rattlesnake (Sistrurus miliarius) life history and demography with diet composition: a stable isotope approach. University of Arkansas.

Pilgrim, M.A., 2007. Expression of maternal isotopes in offspring: implications for interpreting ontogenetic shifts in isotopic composition of consumer tissues. Isotopes Environ Health Stud 43, 155-163.

Post, D.M., 2002. Using stable isotopes to estimate trophic position models methods and assumptions. Ecology 83, 703-718.

Quinn, H., Jones, J.P., 1974. A squeeze box technique for measuring snakes. Herpetological Review 5, 35.

R Core Team, 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rebelato, M.M., Winemiller, K.O., Durso, A.M., Tozetti, A.M., de Camargo, P.B., Verrastro, L., 2020. What do stable isotopes tell us about the trophic ecology of Thamnodynastes hypoconia (Serpentes: Dipsadidae) in southern Brazil? Zoology (Jena) 141, 125812.

Rush, S.A., Sash, K., Carroll, J., Palmer, B., Fisk, A.T., 2014. Feeding Ecology of the Snake Community of the Red Hills Region Relative to Management for Northern Bobwhite: Assessing the Diet of Snakes Using Stable Isotopes. Copeia 2014, 288-296.

Savitzky, B.C., 1992. Laboratory studies on piscivoury in an opportunistic pitviper, the cottonmouth, *Agkistrodon piscivorus*. Biology of the Pitvipers, 347-368.

Schalk, C.M., Trees, T., Pierce, J.B., Rudolph, D.C., 2018. Food Habits of Sympatric Pitvipers from the West Gulf
Coastal Plain, USA. Herpetological Review 49, 1-5.

Smith, K.F., Sharp, Z.D., Brown, J.H., 2002. Isotopic composition of carbon and oxygen in desert fauna: investigations into the effects of diet, physiology, and seasonality. Journal of Arid Environments 52, 419-430.

UFR (Use of Fishes in Research) Committee, 2013. Guidelines for the use of fishes in research. American Fisheries Society, Bethesda, Maryland.

Willson, J.D., Winne, C.T., Pilgrim, M.A., Romanek, C.S., Gibbons, J.W., 2010. Seasonal variation in terrestrial resource subsidies influences trophic niche width and overlap in two aquatic snake species: a stable isotope approach. Oikos 119, 1161-1171.

Figure

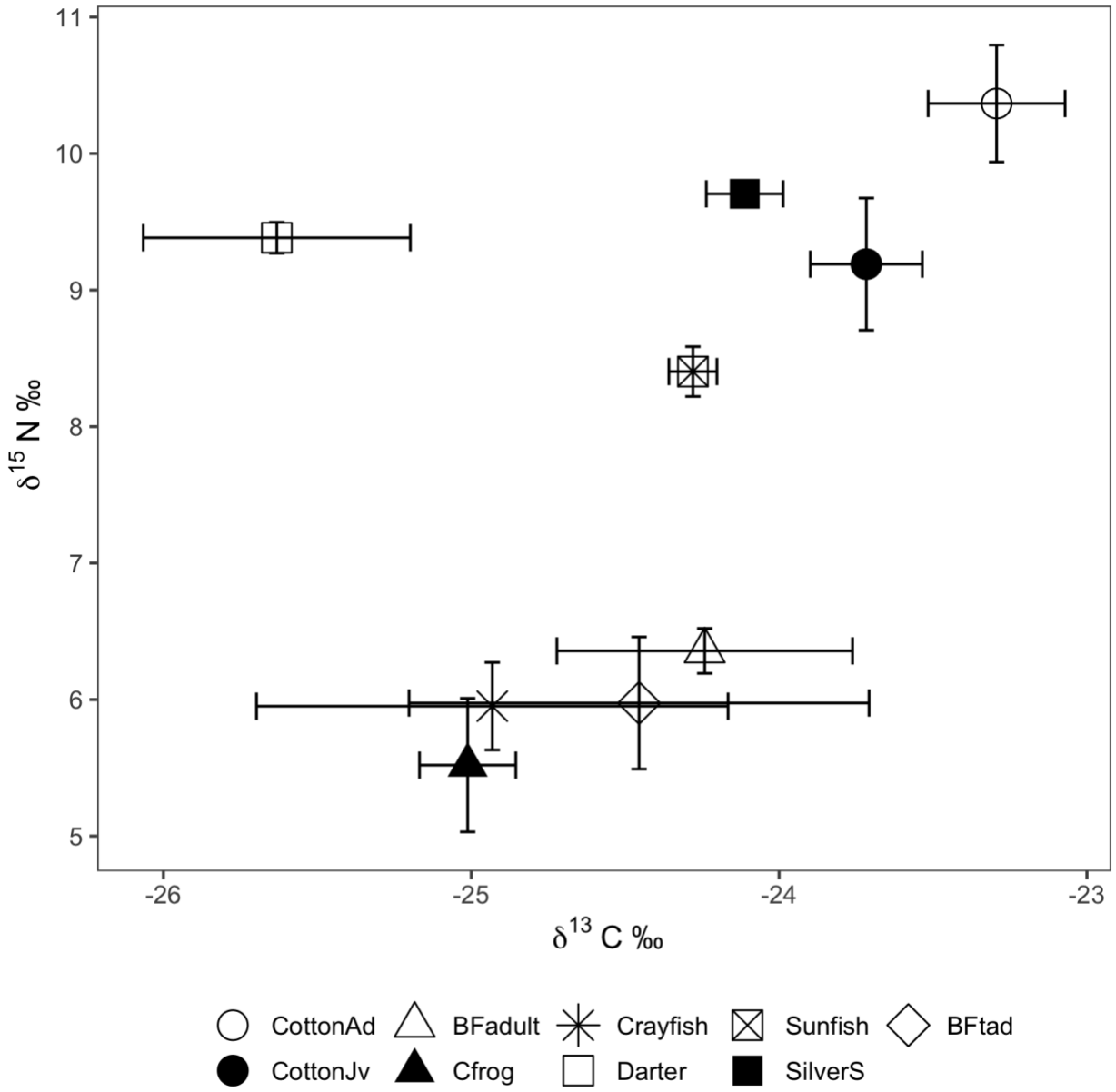

Figure 1. Comparison of adult and juvenile cottonmouth scale clips to seven types of potential prey. Each point represents average $\delta^{13}C$ and $\delta^{15}N$ values (± 1 SE) of all samples analyzed in this study, n = 6 for all points. CottonAd = Adult cottonmouth; CottonJv = Juvenile cottonmouth; BFadult = Adult American bullfrog; Cfrog = Blanchard's cricket frog; Crayfish = Northern crayfish; Darter = Stippled darter; Sunfish = Longear sunfish; SilverS =Brook silverside; BFtad = American bullfrog tadpole.

Chapter 2: Prey preference in the Western Cottonmouth (*Agkistrodon piscivorus leucostoma*)

Jason Ortega

Abstract

The determination of preferred prey is vital to understanding the nutritional ecology of any species. Some species have been found to have a surprisingly wide diet breadth, yet many of those food items may not represent the animal's core diet. To determine the prey preference of the western cottonmouth (*Agkistrodon piscivorus leucostoma*), snakes were placed in an X maze and were offered various items to choose from. The X maze consisted of four terminal boxes where each one was linked to a center box by PVC tubing. Each terminal box had a small fan that would blow air towards the center box. The center box had sliding doors that allowed the test animals to acclimate to the arena before the doors were remotely opened. Maze runs consisted of placing four items in each of the terminal boxes, placing a snake in the center box with the doors lowered and with the fans running. Cottonmouths were allowed to acclimate to the maze for one hour before the doors were lifted; the snake was given 4 hours to make prey choices. All runs were recorded using a video camera; the resulting footage was watched and scored for arm choice order and item consumption order. Snakes (N = 37) were offered the following items: crayfish, fish, frog, and mouse A total of 74 trials were conducted wherein each snake was run two times through the maze. Cottonmouths showed a preference for vertebrate prey while almost entirely ignoring crayfish. Snakes did not show a significant overall preference for fish versus frog versus mouse.

Introduction

Identifying the diet of an animal is an essential part of understanding an organism's nutritional ecology. An organism's diet is the product of the interaction of phylogenetic constraint (Van Soest, 1994), behavior (Glaudas et al., 2019), anatomy (Sherbrooke and Schwenk, 2008), physiology (Beaupre and Montgomery, 2007), and ecology (Raubenheimer et al., 2009). Because the energetic cost and gain of procurement and processing of meals can vary greatly due to prey type, a first step in bioenergetic analysis requires identification of prey type and preference (Karasov and Martínez del Rio, 2007).

When investigating the diet of an animal it is important to differentiate between an animal's observed diet and its preferred diet. Observed diet and preferred diet may misalign due to a variety of factors such as availability of preferred prey (seasonality) (Cooper and Vitt, 2002), changes in the physiological state of the organism that necessitates an altered profile of nutrients due to reproductive needs (Wilder and Rypstra, 2008), migration (Brower et al., 2006), ontogenetic shifts in preference (Durso and Mullin, 2017), or ontogenetic changes in foraging technique (Wikelski et al., 1993). Characterizing differences between preferred and observed diets can illuminate the foraging ecology and digestive physiology of the consumer, as well as reveal tradeoffs and constraints in the expression of life history (Dunham et al., 1989, Congdon 1989).

Herein, I studied the prey preference of the western cottonmouth (*Agkistrodon piscivorus luecostoma*) to understand the interplay between prey preference and digestive performance. The cottonmouth is a semiaquatic pitviper that can forage in the water (Savitzky, 1992), at the land-water interface (Eskew et al., 2009), and terrestrially, away from water (Hill, 2004; Hill and Beaupre, 2008). The high diversity in habitats used by this species allows them to be generalist

predators with the wideset diet breadth of any north American pitviper (Burkett, 1966; Himes, 2004; Vincent et al., 2004). Whereas, identifying the prey eaten (gut content) by cottonmouths is valuable for designing experiments to investigate prey preference, these presence records alone cannot definitively determine preference of one ingested prey species over another. Stomach content analysis and fecal analysis only provide a snapshot into the snake's diet by only representing that immediate observed meal, while also being limited in its ability to detect small meals and soft-bodied prey (such as tadpoles) (Dorcas and Willson, 2009; Schalk et al., 2018).

Due to the heightened chemosensory capabilities of squamate reptiles (amphisbaenians, lizards, and snakes) imparted by a highly sensitive vomeronasal organ and forked tongue (Burghardt, 1967, 1968; Burghardt and Pruitt, 1975; Cundall and Greene, 2000), prey preference work in these taxa have relied on measuring chemosensory behavior (tongue flicking and post-strike searching). A longstanding method for determining prey preference in squamate reptiles has been examining tongue flicking rates when presented with cotton swabs perfused with scents of potential prey (Cooper and Burghardt, 1990; Wilde, 1938), wherein high tongue flick rates or even attacking of the cotton swab is scored as preference towards that prey type. Cottonmouths have not been directly used in these types of studies, but tongue flicking rates have been examined in the context of studying prey searching behavior (Chiszar et al., 1979), where a slight preference was shown for fish versus mouse scents. Despite the extensive use of cottonmouths in studying strike-induced chemosensory searching, where vipers follow prey movements post envenomation and release (Chiszar et al., 1982; Chiszar et al., 1986; Chiszar et al., 1985; Chiszar et al., 1979), only one study investigated variation in this behavior due to prey type (Chiszar et al., 1986) and found no significant difference between two types of prey (fish versus mouse). To date only one study has examined prey preference in cottonmouths (Lillywhite et al., 2015),

where the impact of prior meal type on prey odor preference in captive reared neonates was examined using movement within a Y maze (a maze where an initial corridor splits into two terminal branches). Lillywhite et al.'s (2015) study showed that the neonates preferentially moved towards the meal type on which they had previously fed (fish versus mouse).

The reliance on chemosensory behavior (tongue flicking rates and maze movement) versus actual counts of prey ingestion can offer clear indications of prey preference (Jackrel and Reinert, 2011). However, these methods are limited in the number of prey types that are tested at a single time, where cotton swabs must be presented one at a time to individual animals and Y mazes are limited to only two selections. To improve on the testing of prey preference, I developed and used a modified radial arm maze, which will be referred to as the X maze (Olton, 1979). The X maze allowed for each snake to choose from four prey options per maze run (behavioral trial), where the snake was free to eat as many prey items as it wished to (Figure 1); the prey types used were crayfish, fish, frog, and mouse. During the course of maze runs: 1) Prey preference was determined by the order that prey species were consumed in the maze. 2) The tendency of snakes to strike at different prey types was determined. 3) The handling and ingestion times of prey items were determined.

Methods

Animals:

Western cottonmouths (*Agkistrodon piscivorus*, n=35) were captured by use of nighttime visual encounter surveys from various locations in northwestern Arkansas. All animals used were adults and none of the females were reproductively active, which was determined by palpating for follicles/embryos. Each animal was individually maintained in ventilated lockable

plastic tubs that were lined with newspaper, rooms were kept at a temperature of 25.0 ± 1.0 ˚C with a light cycle of 12:12 (L:D), and water was provided *ad libitum*. All snakes used in prey preference trials were new captures and naïve to captive conditions. Snakes were fasted for a minimum of 14 days prior to preference testing.

Prey items:

The prey items used in the preference trials were all sourced from commercial vendors; crayfish (*Procambarus clarkia*, Kyle LeBlanc Crawfish Farms, Raceland, LA), fish ("Black salty", *Carassius auratus*, Anderson Minnow Farms, Lonoke, AR), frogs (*Lithobates sphenocephalus*, Carolina Biological Supply, Burlington, NC, and mice (*Mus musculus*, Rodent Pro, Inglefield, IN). Mice were purchased pre-frozen, crayfish were frozen upon delivery, while fish and frogs were euthanized prior to freezing. Crayfish were chilled using an ice water slurry prior to freezing. Both fish and frogs were euthanized by exposure to CO_2 gas and then frozen. Due to logistical issues dead prey were used for the preference trials, which is in line with the work done by Burghardt and others, where many prey preference and scent trailing studies used dead prey items (Burghardt, 1967; Burghardt and Pruitt, 1975; Chiszar et al., 1982; Chiszar et al., 1985; Cooper and Burghardt, 1990; Wilde, 1938). Additionally, cottonmouths are well known scavengers that will readily consume freshly deceased animals or carrion (Devault and Krochmal, 2002; Lillywhite et al., 2008; Lillywhite et al., 2002; Lillywhite and McCleary, 2008; Lillywhite et al., 2015).

Maze construction:

The maze used in these behavioral trials is an X maze which consisted of four terminal chambers linked to a single central chamber via four arms of PVC tubing (Figure 1). All five chambers were originally opaque lockable plastic storage boxes with lids (#1925N, 27 L, 43.18 cm x 28.26 cm x 32.39 cm; Sterilite, Townsend, MA). All four vertical sides of the central chamber had holes cut out to accommodate the attachment of PVC closet flanges to enable connection to PVC pipes (7.62 cm in diameter) to the exterior of the chamber (Figure 1 & 2). The walls of the chamber were sandwiched between an external closet flange and an internal flange spacer; a ring of polyethylene weatherstripping was used on the external wall to promote a draft-proof seal. Each inner flange spacer allowed for a gate made of perforated Lexan plastic to sit on the internal side of the flange though the use of large washers (3.81 cm in diameter) (Figure 2B). Each terminal box had one hole made to accommodate the attachment of a PVC flange and another hole to accommodate the attachment of a computer fan (Figure 2). The holes made on the terminal chambers were on opposite sides on the two narrow walls. All flanges and fans were bolted to the chambers using bolts, washers, and butterfly nuts for quick disassembly and cleaning. Each terminal chamber was attached to the central chamber via a length of PVC and a 45-degree elbow, which created a total travel path of 34 cm from the central chamber to each terminal chamber. The use of the 45-degree elbow prevented animals from having a clear line of sight into each terminal chamber from the central chamber (Figure 1)

To allow for the viewing of activity within each terminal chamber, each lid had a central portion removed and the resulting hole was overlaid with a piece of clear plexiglass. The plexiglass was permanently bolted to a lid and the seam was sealed with silicone caulking. The latches of each chamber would secure the lid in place during preference trials. The central

chamber had a lid made of hardware cloth that allowed for viewing and for the passage of monofilament lines that were connected to the inner gates. The central lid was secured to the chamber using bolts and butterfly nuts (Figure 1).

The inner gates of the central chamber were raised and lowered via individual attachment to a clear monofilament line. Each gate had a non-lead egg sinker strung though the attached monofilament to weigh down each gate (Figure 2B). The sinker and monofilament line were attached to the center of the top edge of each gate. The monofilament lines were run up and through the metal shelving that also served as a mount for the video camera (Figure 2A). All four lines converged into a single line that was anchored to the wall allowing for the raising and lowering of the gates from behind an opaque screen. When the gates were lifted, it allowed a snake in the central chamber to enter any of the radial arms.

The computer fans were mounted using rubber washers to minimize vibration of the chamber during their operation and oriented to pull air into their respective chamber. Snakes were protected from fan blades by fine aluminum window screen. All four fans were wired in parallel to a DC power source that provided 0.4 ± 0.1 A and 4.0 ± 0.5 V. Fans were removed from each terminal chamber before cleaning between maze runs.

Maze runs were recorded using a using a digital video camera (HDR-CX7; Sony, New York, NY) fitted with a wide-angle adapter (VCL-HA07A; Sony). The footage from the camera was simultaneously recorded onto DVD and digital format. Digital files were downloaded and labeled for later scoring; DVDs were labeled with only the run ID# to serve as a backup.

Snake and Prey item selection:

At least 12 hours before each maze run, the snake and its food items were selected. The snake to be used was selected randomly from the pool of available individuals that had been fasted for at least 14 days and had not yet been used for that run number. The chosen snake was weighed and watered before prey were selected. Prey items were chosen and weighed while still frozen. The mass of each individual prey item did not exceed 20 % of snake body mass for the snake chosen for that trial. The collective masses of all prey items offered during a single run did not exceed 50% of the snake's mass, which was done to ensure that each snake could in theory eat all four prey items before becoming satiated. Pitvipers have the capacity to eat very large meal sizes in relation to body size (more than 100%) wherein a total meal of 50% body mass is well within their capacity (Greene, 1992). Prey were thawed in individual cups with 200 mL of cold tap water and were placed in a refrigerator (~4.0 ˚C) for overnight thawing.

Maze runs:

Each snake had two prey preference trials in the maze (two maze runs), each snake was naïve to the maze and the prey items for the first run. Prior to each maze run, all sections of the maze that touched snakes and/or prey items were washed with hot water and 10% dilute bleach. After each cleaning the maze was reassembled, gates and fans were tested for proper function. All maze runs started between the hours of 10:00 and 13:00. The room where the maze was located was kept at 24.0 ± 0.5 ˚C, the HVAC system operated on positive pressure, and the room was lit by one bank of florescent lights. Each maze run was given a unique, non-repeating randomly assigned three-digit ID number. The placement of each prey item in their respective terminal chamber was randomly determined by use of a four-sided die. The arrangement of prey

items was never repeated for an individual snake between maze runs. Once the terminal prey items were assigned, they were placed on a ceramic plate with 60 mL of water in which they were thawed. The added water prevented the prey items from drying out and adhering to the plate during the runs (Figure 1). Before placing the snake in the central chamber, all prey items were placed in their assigned terminal chambers, the terminal chamber lids were secured, and each fan was turned on. The snake was placed in the central chamber and the central lid was secured (Figures 1 & 2). Once the snake was secured, the recording of the run started. Each snake was allowed to acclimate to the maze for one hour, at the end of the acclimation period, the gates were lifted, and the snake was given four hours to make gate and diet selections and feed.

At the end of the four hours the snake was removed from the maze and the maze was dissembled for cleaning. If the animal was in the process of feeding at the end of the trial, it was allowed to finish without being disturbed. Any uneaten prey items were offered to that individual snake for one hour after being removed from the maze.

Scoring maze footage:

Scoring of the maze run footage was conducted in a double-blind fashion, the movie files were given a non-repeating randomly generated ID number. The files were randomly distributed to the trained reviewers. Most recordings were only scored by one reviewer, the exception to this were two recordings that were given to all the reviewers to assess consistency in their scoring, wherein these recordings were mixed in with their batches of videos. The reviewers did not know the identity of the animal in each movie file nor if the video was of the snake's first or second run. Reviewers used a provided excel worksheet to score each video. The worksheet contained

drop down menus for selecting each behavior and entering the corresponding timestamp for each behavior. The worksheet automatically calculated elapsed time from the lifting of the gates for each entered observed event. The completed scoring sheets were codified for analysis.

Behavior categories:

The scored behaviors focused on describing the snake as it moved through the X maze and how the animal interacted with the offered prey items. The following behaviors were recorded if they occurred during a video: gate choice, snake movement, striking a prey item, initiation of ingestion (with or without striking), end of ingestion, order of ingestion, which items were not ingested, return to a chamber after feeding, and attempt to eat an empty plate. The orientation of a snake's head was used to score gate choice before the gates were raised, if a snake's head was clearly facing a gate immediately prior to the lifting of the gates, that gate was scored as its gate choice. Snake movement was scored in reference to the location of a snake's head in the maze, this was necessary because a snake could visit another chamber without completely leaving the central chamber. Once a snake's head was visible in a chamber it was scored that it visited that chamber. Striking of offered prey items was scored when a snake would quickly lunge at a prey item with an open mouth and bite it (Kardong, 1975). If the snake held onto the prey item after the initial bite this was noted as well as if a snake struck without holding onto the prey item. The start of ingestion was characterized by alternating unilateral movements of the sides of a snake's head, where one side of the head moves, then the other (Cundall and Greene, 2000), this may or may not be preceded by striking. The end of ingestion was characterized by the end of unilateral head motions, active tongue flicking, and/or yawning. The order that offered prey items were ingested as well as which prey were not ingested per run were

noted. Return to an empty chamber was characterized by a snake that returned to a chamber where they had already consumed the offered prey item. Attempt to eat an empty plate was characterized by a snake that tried to eat a plate that had previously held a food item. All the behaviors listed above were coded as discrete counts (frequency of occurrence). From the completed scoring sheets, three continuous variables based on elapsed time were extracted. These time measurements were time to first chamber visit, handling time, and ingestion time. Time to first visit was the amount of time a snake took to leave the central chamber and enter one of the terminal chambers after the gates were lifted. Handling time is the amount of time a snake took from an initial strike and hold of a prey item until the end of ingestion. Ingestion time is the amount of time a snake took between the initiation of ingestion until the end of ingestion, this measurement excludes any time that lapsed if a snake struck and held a prey item before starting ingestion.

Data analysis:

All statistical analyses were conducted using R version 4.1.0 (R Core Team, 2021). Comparisons between the masses of prey offered versus eaten and prey mass to snake mass were done using Two Way ANOVAs via the "car" package (Fox and Weisberg, 2019) with post-hoc comparison of least-square means. Counts of scored snake behaviors (discrete variables) were transferred from the main data set into smaller contingency tables for analysis. Due to the presence of zero counts in many categories, statistical testing of the contingency tables was conducted by Fisher's Exact Tests, followed by post-hoc pairwise comparisons. Time measurements (continuous variables) were analyzed by mixed effect models using the "nlme" package (Pinheiro et al., 2021) and were followed by post-hoc Tukey tests. All statistical tests

recognized statistical significance at $P \leq 0.05$. The normality of ANOVA and repeated measure mixed models were verified by examining the residuals for each analysis (mean=0 and variance=1) through use of Shapiro-Wilks tests.

Results

Summary of snakes and offered prey:

In total 35 snakes were subjected to two prey preference maze trials (two runs per snake); 22 male snakes (mean mass 373.54 g, SD ± 61.87) and 13 female snakes (mean mass 326.51 g, SD ± 37.66). The prey offered during maze runs are summarized as follows: crayfish average mass 27.79 g (SD ± 14.09), fish average mass 13.24 g (SD ± 3.73), frog average mass 29.32 g (SD ± 7.69), and mouse average mass 21.48 g (SD ± 3.01). The masses of prey offered were significantly different between prey types (ANOVA, $F_{3,276} = 54.61$, $P < 0.0001$) (Figure 3). Prey masses offered were not significantly different within each prey type when compared between runs, nor were overall prey masses offered per run.

Overall prey choices and snake movement:

Of the 35 snakes used, five snakes (all males) refused to feed at all during their first maze run and all snakes fed on at least one item during the second maze run. No crayfish were consumed during a first maze run and only a single crayfish was consumed by a male during a second maze run (Figure 4). A Fischer's exact test of the total counts of all prey eaten or not eaten within each run is strongly associated ($P < 0.0001$, for both runs), yet once crayfish values are removed from the counts there is no longer any significant association between prey type and if was eaten for either run.

The effect of prey size on prey choice (prey eaten), was examined using ANCOVA, where prey mass was compared between prey types and maze runs where snake mass was used a covariate (Figure 5). Due to the low palatability of crayfish, those values were removed and only fish, frog, and mouse values were analyzed. Eaten prey masses were significantly different between prey types (ANCOVA, $F_{2,166} = 140.7646$, $P < 0.0001$), but there was no significant effect of run number (ANCOVA, $F_{1,166} = 1.7080$, $P = 0.1931$) nor snake mass (ANCOVA, $F_{1,166} = 0.2550$, $P = 0.6142$). The effect of snake sex on mass of prey eaten was also examined (Figure 6) via ANCOVA with the exclusion the single crayfish feeding event. For this analysis snake mass was again used as a covariate. Prey masses eaten were significantly different between prey types (ANCOVA, $F_{2,166} = 132.0880$, $P < 0.0001$), but there was no significant effect of sex (ANCOVA, $F_{1,166} = 0.0422$, $P = 0.8375$) nor snake mass (ANCOVA, $F_{1,166} = 1.3735$, $P = 0.2429$). Both ANCOVAs passed assumptions of slope homogeneity.

Counts of gate choice and first chamber visited were compared between maze runs (Figure 7) using Fischer's exact test. There was no significant association between gate choice and first chamber visit for either maze run.

The time that each snake took to make its first chamber visit was compared between prey types and maze runs (Figure 8), a repeated measure mixed model was used where prey type and run number were fixed effects and snake ID was the random effect. The time to first visit was significantly impacted by run number ($F_{1,132} = 34.8635$, $P<0.001$), but not due to type of prey ($F_{2,132} = 0.1610$, $P=0.8515$).

Prey choice order:

While snakes were allowed to eat as many prey items as they wished, there was variation in the total number of items that they consumed per maze run. For maze run 1: 6 snakes ate 1 item, 5 snakes ate 2 items, 19 snakes ate 3 items, none of the snakes ate 4 items. For maze run 2: 1 snake ate 1 item, 5 snakes ate 2 items, 28 snakes ate 3 items, 1 snake ate 4 items. Counts of feeding order (1^{st}, 2^{nd}, 3^{rd}, or 4^{th}) per prey type by maze run were aggregated for analysis (Figure 9). With or without including the single crayfish feeding event of run 2, no significant association was found between feed order, prey type, and maze run (Fischer's exact test).

The relationship between snake gate choice and first prey type eaten per maze run were analyzed (Figure 10). Within run 1 there was a significant association between gate choice and first meal type (Fischer's exact test, $P = 0.01025$), but this association was not significant for run 2 (Fischer's exact test, $P = 0.3445$).

The relationship between first chamber visited and first prey type eaten per maze run were analyzed (Figure 11). Within both run 1 and 2 there was a significant association between first chamber visited and first meal type (Fischer's exact test, $P < 0.0001$, for both).

Other snake and prey interactions:

During isolated feeding events a snake would strike and sometimes hold onto a prey item before initiating feeding. Counts of incidents of prey strikes were aggregated and compared per prey type between runs (Figure 12). For run 1 there was no significant association (Fischer's exact test, $P = 0.2162$), but for run 2 there was a significant association (Fischer's exact test, $P < 0.0001$) between striking and prey type, where frogs were struck ~60% of the time when compare to single striking events for fish and mouse .

Handling time and ingestion time were calculated for all scorable occurrences and compared per prey type by maze run (Figure 13). For both handling time and ingestion time the single crayfish meal was omitted. For handling time and ingestion time two repeated measure mixed models were used where prey type and run number were fixed effects and snake ID was the random effect. Handling time was significantly impacted by prey type ($F_{2,103}$ = 24.7158, P<0.0001), but not run ($F_{1,103}$ = 3.0047, P=0.08302). Frog handling time was significantly different (P<0.001, for both) from that of fish and mouse. Fish and mouse handling times were not significantly different from each other (P = 0.127). Overall mean handlings times (run 1 and 2 combined) were: fish 2.26 s (N = 54, SE ± 0.25), frog 9.74 s (N = 48, SE ± 0.93), and mouse 4.59 s (N=42, SE ± 0.29). Ingestion time was significantly impacted by prey type ($F_{2,103}$ = 16.8270, P<0.0001) and by run ($F_{1,103}$ = 4.4798, P=0.0343), but there was no significant interaction effect between prey type and run ($F_{2,103}$ = 0.1049, P=0.9004). Frog ingestion time was significantly different (P<0.01, for both) from that of fish and mouse. Fish and mouse ingestion times were not significantly different from each other (P=0.0530). Overall mean ingestion times (run 1 and 2 combined) were: fish 1.83 s (N = 54, SE ± 0.21), frog 6.89 s (N = 48, SE ± 0.63), and mouse 3.78 s (N=42, SE ± 0.22).

Some snakes would revisit chambers after ingesting the prey item that was assigned to that chamber. Counts of chambers revisits were aggregated for each prey type by maze run. There was no significant association (Fischer's exact test) between prey type and chamber return for either maze run. During some chamber revisits, a subset of snakes would attempt to eat the empty ceramic plate. Counts of incidents of plate attacks were compiled for each prey type by maze run (Figure 14). There was a significant association between prey type and the empty plate being attacked for run 1 (Fischer's exact test, P = .03255) where ~75% of fish plates, 50% of

frog plates, and ~30% of mouse plates were attacked. A significant association between prey type and the empty plate being attacked was also found for run 2 (Fischer's exact test, $P < 0.0001$) where ~50% of fish plates, ~50% of frog plates, and a single mouse plate were attacked.

Discussion

Suitability of X maze and offered prey:

Overall, use of the X maze allowed for determination of prey preference in the western cottonmouth, where snakes showed a general preference for fish, frog, and mouse. There was a high level of participation by snakes, where 86% made a choice and fed in run 1, and 100% of snakes did so in run 2. The high participation rates were surprising given the issues that Lillywhite et al. (2015) reported that cottonmouths had "irritable behavior" that necessitated re-running animals in their study.

Except for a single feeding event, crayfish was shown to be unpalatable to these animals (Figure 4). The relative size of the crayfish offered were larger than the fish and mice used but were not larger than the frogs offered (Figure 3). Therefore, the size of the crayfish offered was most likely not a factor in their unpalatability to these snakes. Except for the single feeding event, no other snakes even attempted to eat a crayfish, additionally no snakes abandoned any feeding attempts due to the size of prey. Yet, crayfish have been shown to be consumed by cottonmouths (Gloyd and Conant, 1990) and the author has seen wild snakes regurgitate and defecate portions of crayfish carapaces. There is the possibility that cottonmouths may prefer to consume crayfish that have recently molted, as seen in *Regina septemvittata* (Jackrel and Reinert, 2011), yet this was not tested by the present study. There were no obvious differences

between male and female snakes in the type of prey eaten (Figure 6), except for the single crayfish that was eaten by a male.

Prey choice:

In general, there were no statistically significant differences in total numbers of prey eaten when comparing between prey types (Figure 4), although more prey were eaten in total during run 2. When comparing masses of prey items eaten or refused there was no significant difference when crayfish were excluded from that analysis (Figure 5). The overall order in which prey were eaten was not significant, but qualitatively frogs were slightly favored as the first meal eaten (by four instances) when both runs are combined, followed by fish and then mouse (Figure 9). There was no significant association between gate choice and first chamber visited (Figure 7). The association between gate choice and first meal was stronger (Figure 10). Within run 1 there was a significant association between gate choice and first meal type, particularly for mouse and frog, but this association was not significant for run 2. The strong relationship between a mouse gate choice and a mouse first meal for run 1 may be attributed to the novelty of a domestic mouse, yet this pattern does not hold as true for run 2.

When compared to gate choice, first chamber visited was a much better predictor for first meal type (Figure 11). Within both run 1 and 2 there was a very strong association between first chamber visited and first meal type for fish, frog, and mouse. While some snakes did enter the crayfish chamber first, the only snake to eat a crayfish did so for its fourth and last meal.

Snakes did show a significant decrease in the time they took to visit their first chamber between run 1 and run 2 (Figure 8). The strong overall decrease in first chamber visit time for all

prey types in run 2 is indicative that the snakes learned that the maze was a source of food and/or were bolder in their decision making (Kellogg and Pomeroy, 1936).

In some metrics frogs were slightly ahead of mice, but none of these measurements were statistically significant. The novelty of domestic mice was not enough to completely skew the results solely towards mouse meals. One aspect to consider is that this study only investigated wide differences between prey types with one representative species per meal type. Others have suggested that snakes can show species specific preferences among frogs (McKnight et al., 2014) and or in the course of their work they freely mixed different species together to represent a specific ecotype, fresh water versus marine fish (Lillywhite et al., 2015).

Other snake and prey interactions:

Prey handling time and ingestion time were greatly impacted by prey type (Figure 13). Frog meals had the highest times for both values, which was most likely due to their larger size relative to offered fish and mouse meals (Figures 3 and 5) and lack of a streamlined (fusiform) body shape (Mori, 2006). Comparatively, fish and mouse meals were smaller and more fusiform in their body shape (Vincent et al., 2006; Willson and Hopkins, 2011).

The frequency of striking prey decreased in run 2 when compared to run 1 for fish and mice while frequency of striking frogs slightly increased during run 2 (Figure 12). The high sustained levels of this activity may be due to ingrained behavior where frogs are preferentially struck at even if they are deceased, out of fear of losing the item. Yet if the fear of losing a prey item was the driving factor, fish would also have sustained strike rates.

The tendency of some snakes to attempt to eat empty plates that had contained prey items is a testament to the cottonmouth's high tendency to eat carrion (Figure 14) (Devault and

Krochmal, 2002). The sustained levels of plate eating in run 2 can be interpreted as their higher preference for fish and frogs as compared to mouse. Cottonmouths have been documented to purposely ingest a variety of objects (sticks, leaves, and seaweed) that were scented with fish (Lillywhite and McCleary, 2008). The return to a site that had a fish or frog may be tied to behavior or individual snake's experiences of feeding on prey at vernal pools that are drying up, where the snake is looking for signs of other individuals to eat at that location (McKnight et al., 2014; Savitzky, 1992)

In summary the cottonmouths in this study were true to their designation of being a generalist predator where animals did not clearly favor one palatable prey (fish, frog, and mouse) over another. The almost outright rejection of crayfish will lead to other questions concerning whether only freshly molted crayfish are eaten or if most records of crayfish consumption were due to secondary ingestion of prey that readily consume crayfish. The use of an X maze and the recording of actual consumption of prey offered is a useful method that should be expanded to prey preference questions in other squamate taxa.

Acknowledgments

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Literature Cited

Beaupre, S.J., Montgomery, C.E., 2007. The meaning and consequences of foraging mode in snakes, in: S.M. Reilly, L.B. McBrayer, D.B. Miles (Eds.), Lizard Ecology. Cambridge University Press, 334-368.

Brower, L.P., Fink, L.S., Walford, P., 2006. Fueling the fall migration of the monarch butterfly. Integr Comp Biol 46, 1123-1142.

Burghardt, G.M., 1967. Chemical-Cue Preferences of Inexperienced Snakes: Comparative Aspects. Science 157, 718-721.

Burghardt, G.M., 1968. Chemical Preference Studies on Newborn Snakes of Three Sympatric Species of Natrix. Copeia 1968, 732-737.

Burghardt, G.M., Pruitt, C.H., 1975. Role of the tongue and senses in feeding of naive and experienced garter snakes. Physiology and Behavior 14, 185-194.

Burkett, R.D., 1966. Natural history of the cottonmouth moccasin, *Agkistrodon piscivorous* (Reptilia). University of Kansas Publications of the Museum of Natural History 17, 435-491.

Chiszar, D., Andren, C., Nilson, G., O'connell, B., Mestas, J.S., Jr, Smith, H.M., Radcliffe, C.W., 1982. Strike induced chemo sensory searching in old-world vipers and new-world pit vipers. Animal Learning and Behavior 10, 121-125.

Chiszar, D., Radcliffe, C.W., Boyd, R., Radcliffe, A., Yun, H., Smith, H.M., Boyer, T., Atkins, B., Feiler, F., 1986. Trailing behavior in cottonmouths (*Agkistrodon piscivorus*). Journal of Herpetology 20, 269-272.

Chiszar, D., Radcliffe, C.W., Overstreet, R., Poole, T., Byers, T., 1985. Duration and Strike-Induced Chemosensory Searching In Cottonmouths *Agkistrodon piscivorus* and a Test of the Hypothesis That Striking Prey Creates a Specific Search Image. Canadian Journal of Zoology 63, 1057-1061.

Chiszar, D., Simonsen, L., Radcliffe, C.W., Smith, H.M., 1979. Rate of tongue flicking by cottonmouths (*Agkistrodon piscivorus*) during prolonged exposure to various food odors, and strike-induced chemosensory searching by the cantil (*Agkistrodon bilineatus*). Transactions of the Kansas Academy of Science 82, 49-54.

Cooper, W.E., Burghardt, G.M., 1990. A Comparative Analysis Of Scoring Methods For Chemical Discrimination Of Prey By Squamate Reptiles. Journal of Chemical Ecology 16, 45-65.

Cooper, W.E.J., Vitt, L.J., 2002. Distribution, extent, and evolution of plant consumption by lizards. Journal of Zoology 257, 487-517.

Cundall, D., Greene, H.W., 2000. Feeding in snakes, in: K. Schwenk (Ed.), Feeding: Form, Function, and Evolution in Tetrapod Vertebrates. Academic Press, San Diego, 293-333.

Devault, T.L., Krochmal, A.R., 2002. Scavenging By Snakes: An Examination Of The Literature. Herpetologica 58, 429-436.

Dorcas, M.E., Willson, J.D., 2009. Innovative Methods for Studies of Snake Ecology and Conservation, in: S.J. Mullin, R.A. Seigel (Eds.), Snakes: Ecology and Conservation. Cornell University Press, USA, 5-37.

Durso, A.M., Mullin, S.J., 2017. Ontogenetic shifts in the diet of plains hog-nosed snakes (Heterodon nasicus) revealed by stable isotope analysis. Zoology (Jena) 120, 83-91.

Eskew, E.A., Willson, J.D., Winne, C.T., 2009. Ambush site selection and ontogenetic shifts in foraging strategy in a semi-aquatic pit viper, the Eastern cottonmouth. Journal of Zoology (London) 277, 179-186.

Fox, J., Weisberg, S., 2019. An {R} Companion to Applied Regression, Third ed. Sage, Thousand Oaks, CA.

Glaudas, X., Glennon, K.L., Martins, M., Luiselli, L., Fearn, S., Trembath, D.F., Jelic, D., Alexander, G.J., 2019. Foraging mode, relative prey size and diet breadth: A phylogenetically explicit analysis of snake feeding ecology. J Anim Ecol 88, 757-767.

Gloyd, H.K., Conant, R., 1990. Snakes of the Agkistrodon Complex: A Monographic Review. Society for the Study of Amphibians and Reptiles, Athens, GA.

Greene, H.W., 1992. The ecological and behavioral context for pitviper evolution, in: J.A. Campbell, E.D. Brodie (Eds.), Biology of the Pivipers. Selva, Tyler, TX, 107-118.

Hill, J.G., 2004. Natural history of the western cottonmouth (*Agkistrodon piscivorus leucostoma*) from an upland lotic population in the Ozark mountains of northwest Arkansas, University of Arkansas.

Hill, J.G., Beaupre, S.J., 2008. Body size, growth, and reproduction in a population of western cottonmouths (*Agkistrodon piscivorus leucostoma*) in the Ozark mountains of northwest Arkansas. Copeia 2008, 105-114.

Himes, J.G., 2004. The non-fish, vertebrate diet of sympatric populations of the cottonmouth (*Agkistrodon piscivorus*) and northern watersnake (*Nerodia sipedon*). Herpetological Review 35, 123-128.

Jackrel, S.L., Reinert, H.K., 2011. Behavioral Responses of a Dietary Specialist, the Queen Snake (*Regina septemvittata*), to Potential Chemoattractants Released by Its Prey. Journal of Herpetology 45, 272-276.

Karasov, W.H., Martínez del Rio, C., 2007. Physiological Ecology: How Animals Process Energy, Nutrients, and Toxins. Princeton University Press, Princeton, NJ.

Kardong, K.V., 1975. Prey Capture in the Cottonmouth Snake (*Agkistrodon piscivorus*). Journal of Herpetology 9, 169-175.

Kellogg, W.N., Pomeroy, W.B., 1936. Maze learning in water snakes. Journal of Comparative Psychology 21, 275-295.

Lillywhite, H.B., III, C.M.S., III, F.Z., 2008. Pitviper Scavenging at the Intertidal Zone: An Evolutionary Scenario for Invasion of the Sea. BioScience 58, 947-955.

Lillywhite, H.B., III, C.M.S., McCue, M.D., 2002. Scavenging behaviors of cottonmouth snakes at island bird rookeries. Herpetogical Review 33, 259-261.

Lillywhite, H.B., McCleary, R.J.R., 2008. Trophic Ecology of Insular Cottonmouth Snakes: Review and Perspective. South American Journal of Herpetology 3, 175-185.

Lillywhite, H.B., Pfaller, J.B., Sheehy, C.M., 2015. Feeding preferences and responses to prey in insular neonatal Florida cottonmouth snakes. Journal of Zoology 297, 156-163.

McKnight, D.T., Harmon, J.R., McKnight, J.L., Ligon, D.B., 2014. Notes on the diets of seven sympatric snakes in the genera *Agkistrodon*, *Nerodia*, *Sistrurus*, and *Thamnophis*. Herpetology Notes 7, 171-177.

Mori, A., 2006. Is headfirst ingestion essential in gape-limited predators? Prey-handling behavior of the anurophagous snake Rhabdophis tigrinus (Colubridae). Canadian Journal of Zoology 84, 954-963.

Olton, D.S., 1979. Mazes, maps, and memory. American Psychologist 34, 583-596.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Team, R.C., 2021. nlme: Linear and Nonlinear Mixed Effects Models.

R Core Team, 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Raubenheimer, D., Simpson, S.J., Mayntz, D., 2009. Nutrition, ecology and nutritional ecology: toward an integrated framework. Functional Ecology 23, 4-16.

Savitzky, B.C., 1992. Laboratory studies on piscivoury in an opportunistic pitviper, the cottonmouth, *Agkistrodon piscivorus*. Biology of the Pitvipers, 347-368.

Schalk, C.M., Trees, T., Pierce, J.B., Rudolph, D.C., 2018. Food Habits of Sympatric Pitvipers from the West Gulf Coastal Plain, USA. Herpetological Review 49, 1-5.

Sherbrooke, W.C., Schwenk, K., 2008. Horned lizards (Phrynosoma) incapacitate dangerous ant prey with mucus. Journal of Experimental Zoology 309A, 447-459.

Van Soest, P.J., 1994. Nutritional Ecology of the Ruminant, 2nd ed. Comstock Publishing Associates, Ithaca.

Vincent, S.E., Herrel, A., Irschick, D.J., 2004. Sexual dimorphism in head shape and diet in the cottonmouth snake (*Agkistrodon piscivorus*). Journal of Zoology 264, 53-59.

Vincent, S.E., Moon, B.R., Shine, R., Herrel, A., 2006. The functional meaning of "prey size" in water snakes (Nerodia fasciata, Colubridae). Oecologica 147, 204-211.

Wikelski, M., Gall, B., Trillmich, F., 1993. Ontogenetic changes in food intake and digestion rate of the herbivorous marine iguana (Amblyrhynchus cristatus, Bell). Oecologia 94, 373-379.

Wilde, W.S., 1938. The role of Jacobson's organ in the feeding reaction of the common garter snake, *Thamnophis sirtalis sirtalis* (Linn.). Journal of Experimental Zoology 77, 445-465.

Wilder, S.M., Rypstra, A.L., 2008. Diet quality affects mating behaviour and egg production in a wolf spider. Animal Behaviour 76, 439-445.

Willson, J.D., Hopkins, W.A., 2011. Prey morphology constrains the feeding ecology of an aquatic generalist predator. Ecology 92, 744-754.

Figures

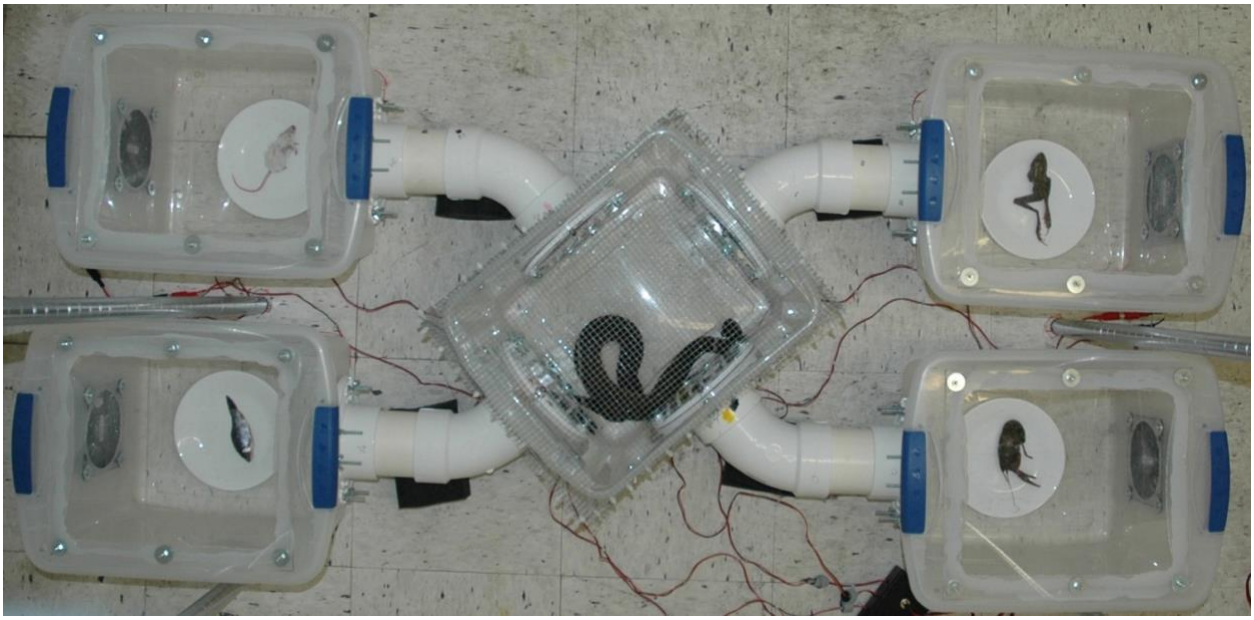

Figure 1. Overhead view of the X maze showing a snake in center chamber and the four terminal chambers with offered prey. In this iteration a mouse is in chamber one (top left), a frog in chamber two (top right), a fish in chamber three (bottom left), and a crayfish in chamber four (bottom right). The view represented in this figure also represents the framing of all the recorded footage.

A

B

Figure 2. Images of maze setup. A) Camera and fan placement. B) Snake in central chamber with lowered gates.

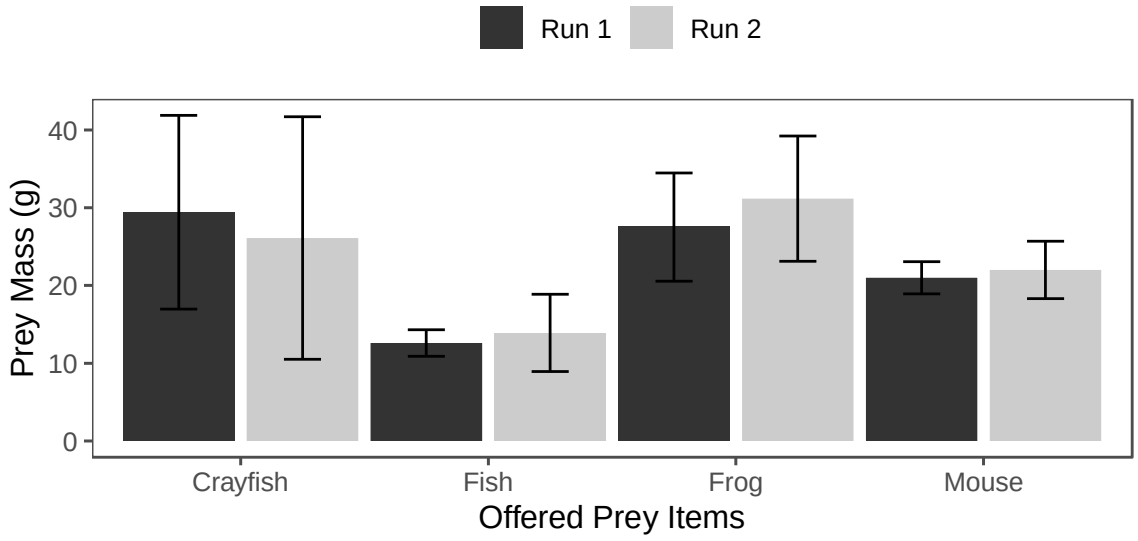

Figure 3. Comparison of masses of offered prey items. Mean masses of prey offered by run number. Error bars are ± 1 standard deviation

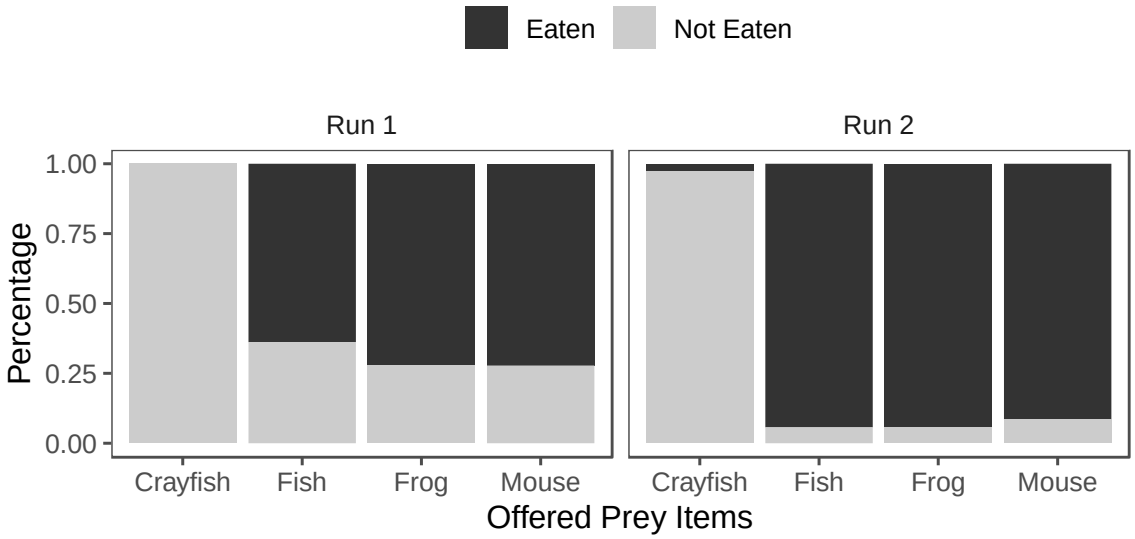

Figure 4. Percentages of counts of prey eaten or not eaten per maze run. Crayfish were not eaten in run one and only a single crayfish was eaten in run two.

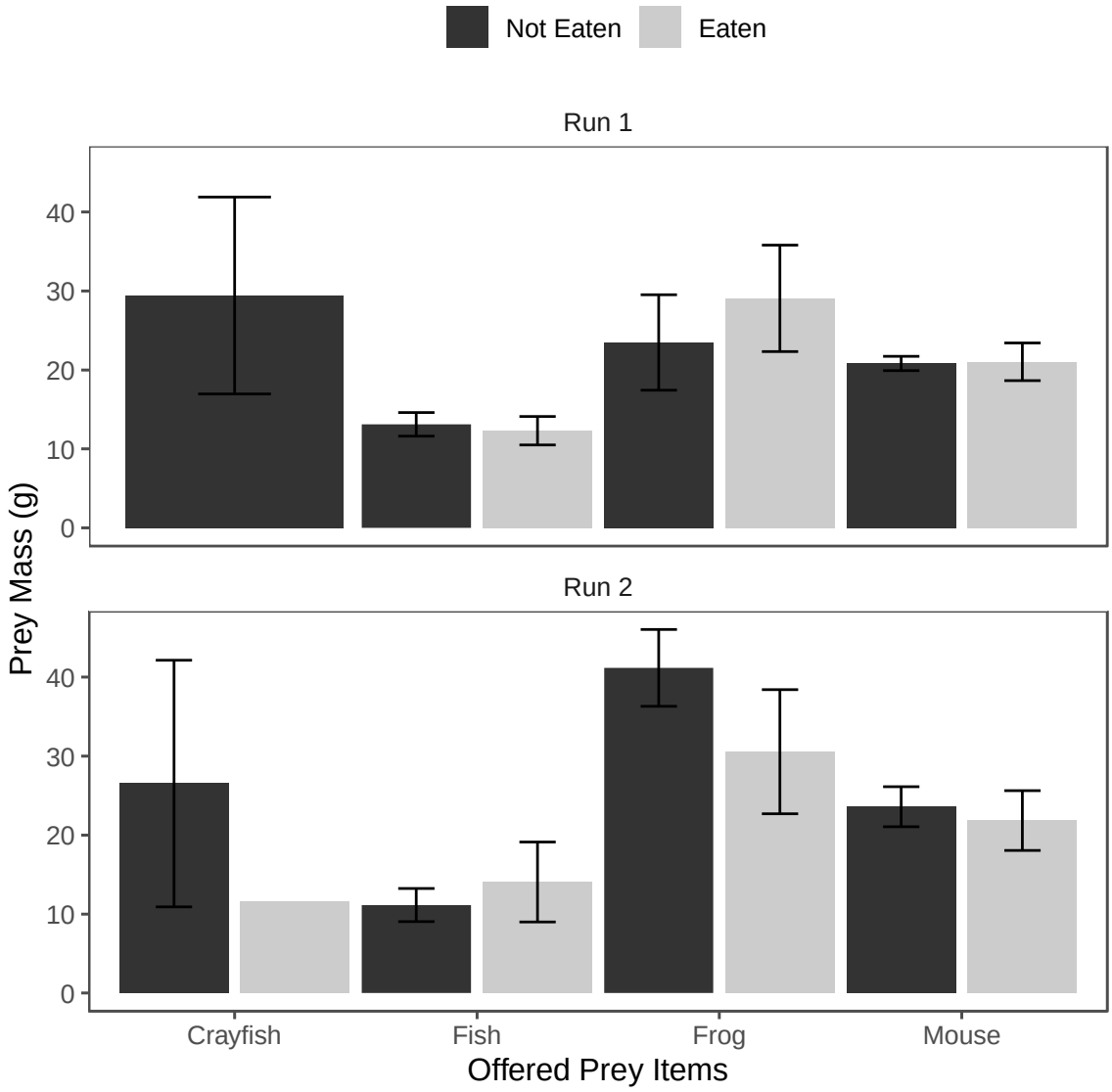

Figure 5. Comparison of mean prey masses of items eaten or refused per run. Crayfish was not eaten in run one and only a single crayfish was eaten in run two. Error bars are ± 1 standard deviation.

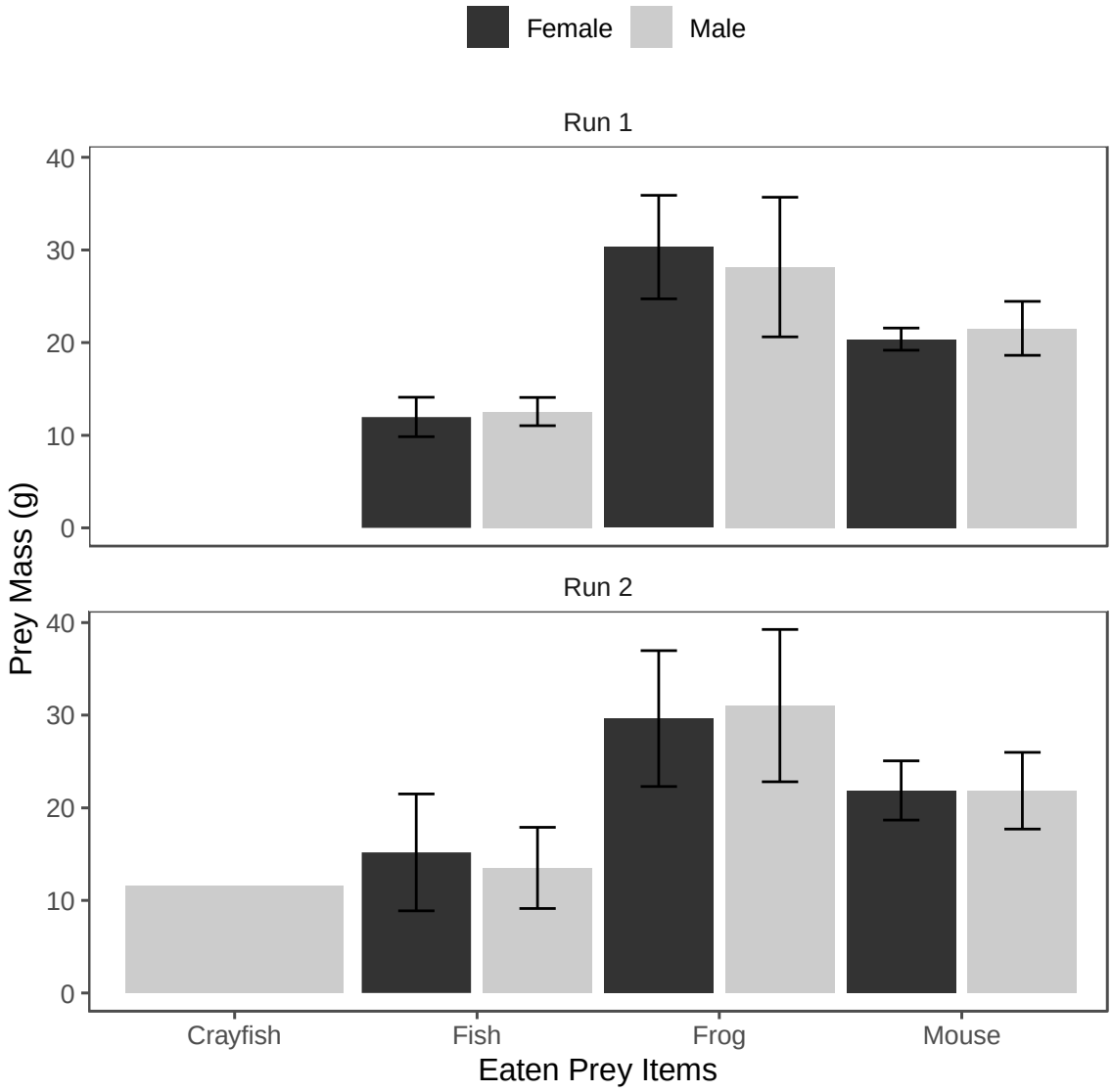

Figure 6. Comparison of mean prey mass of eaten items by snake sex per run. Crayfish was not eaten in run one and only a single crayfish was eaten in run two by a male snake. Error bars are ± 1 standard deviation.

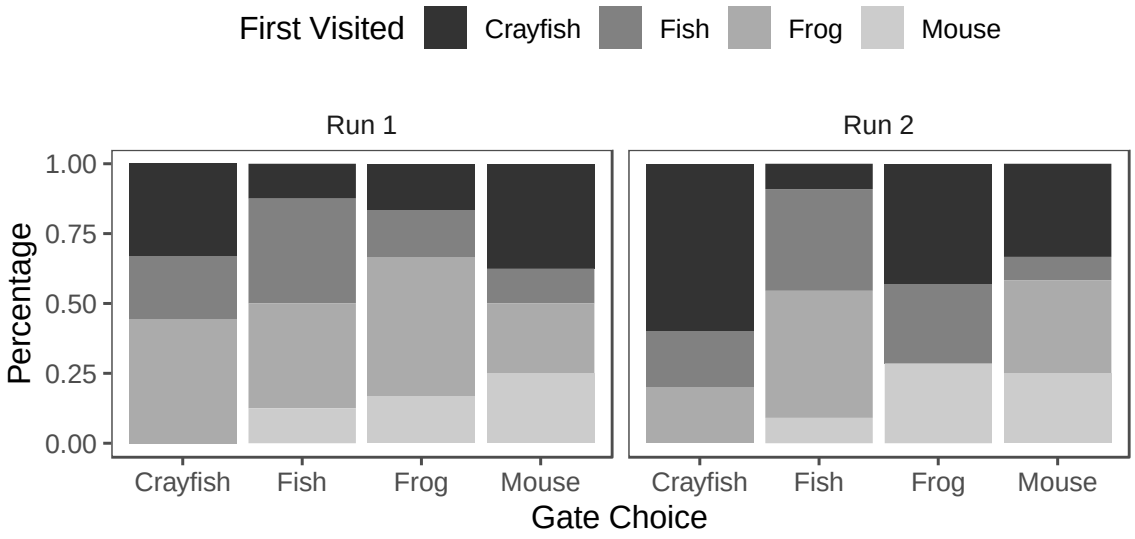

Figure 7. Percentages of counts of gate choice compared to first chamber visited between maze runs. The horizontal axis represents gate choice, and the shading of each bar represents which chamber was first visited. For example, during run 1, snakes that chose the crayfish gate did not visit the mouse chamber first at all yet visited the frog chamber first nearly 50% of the time.

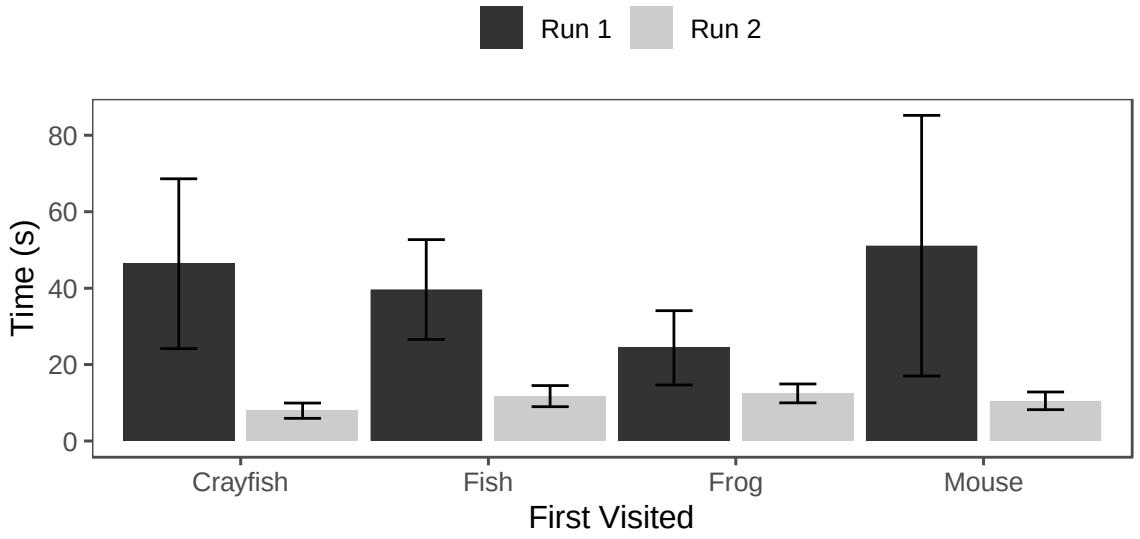

Figure 8. Mean elapsed time for first chamber choice by prey type and run number. Error bars are ± 1 standard error.

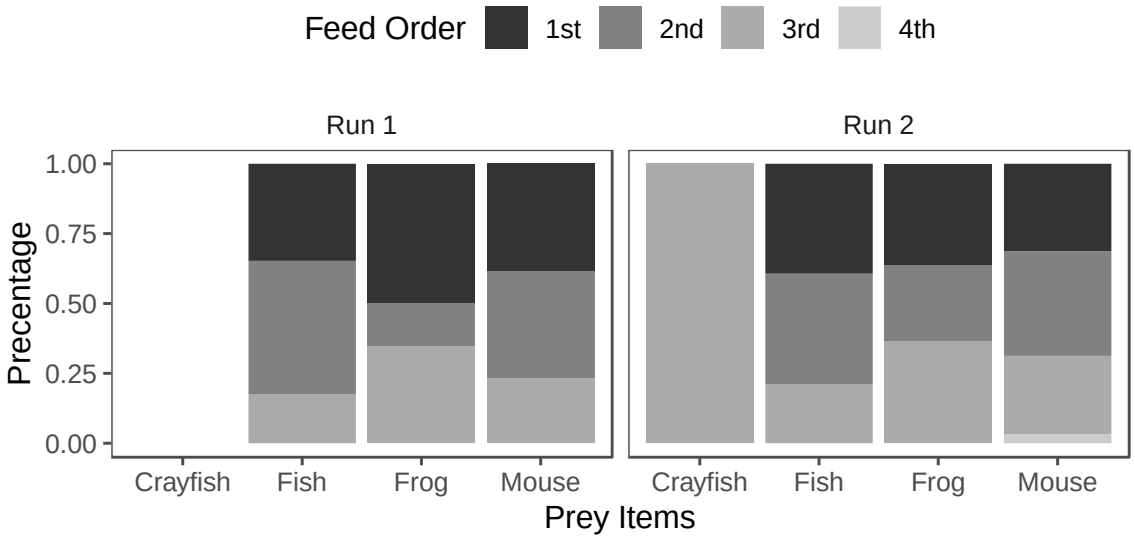

Figure 9. Percentages of counts of the order that prey was eaten between maze runs. The horizontal axis represents each prey type, and the shading of each bar represents the feeding order for each prey type. For example, during run 1 crayfish were not eaten and only a single crayfish was eaten in run 2 as a third item. Additionally, the only 4th item eaten was a mouse in run 2.

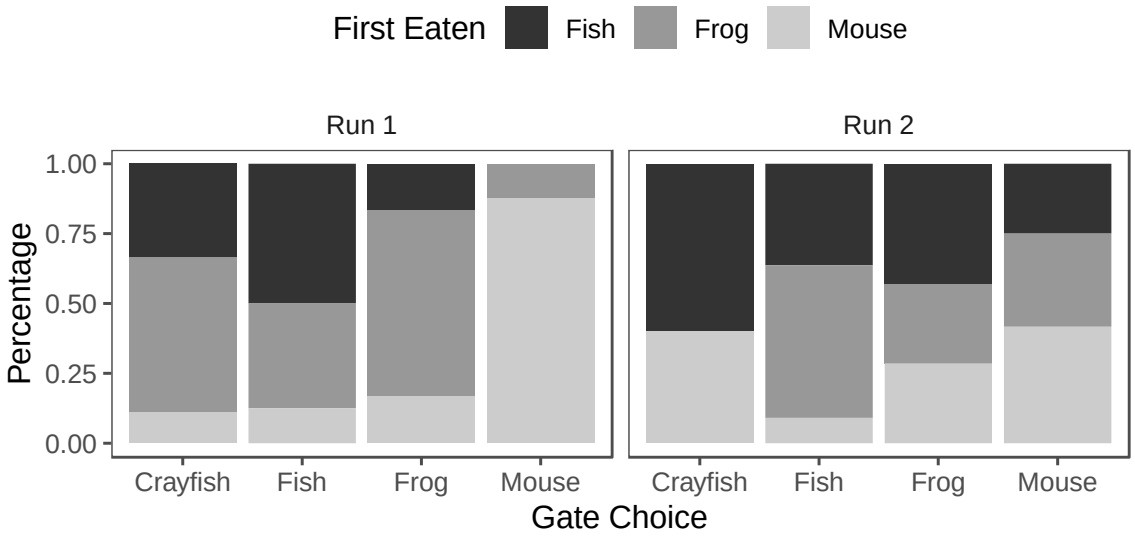

Figure 10. Percentages of counts of gate choice compared to first prey item eaten between maze runs. The horizontal axis represents gate choice, and the shading of each bar represents which prey item was first eaten. For example, during run 1 approximately 90% of the snakes that chose the mouse gate, ate the mouse as their first meal. When compared to run 2 where approximately 30% of snakes that chose the mouse gate, ate the mouse as their first meal.

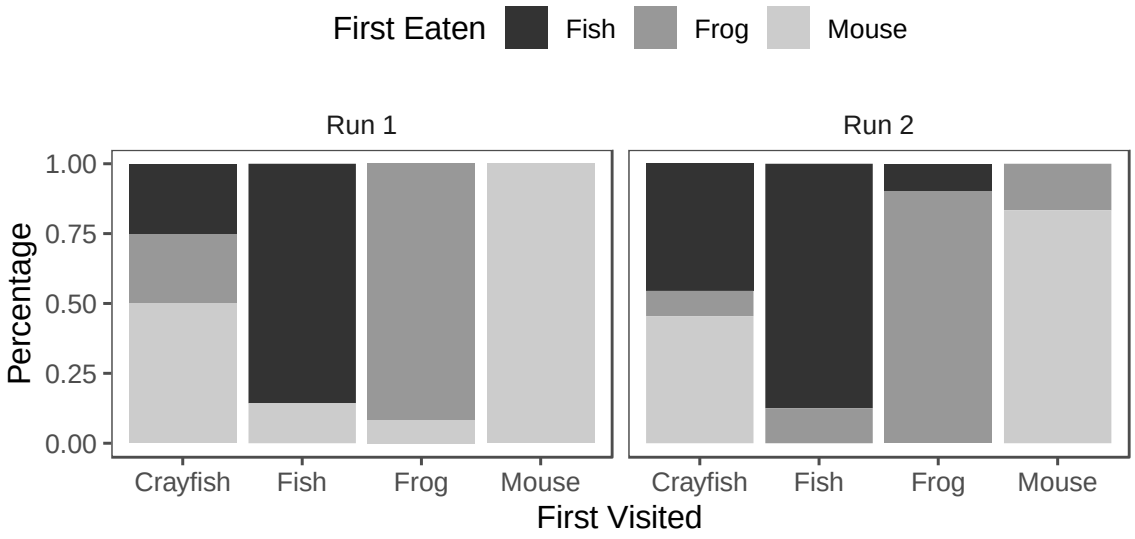

Figure 11. Percentages of counts of first chamber visited compared to first prey item eaten by maze run. The horizontal axis represents first chamber visited, and the shading of each bar represents which prey item was first eaten. For example, in run 1, 100% of the snakes that visited the mouse chamber first, ate that prey item first. Note that the only crayfish consumed during run 2 was not the first item consumed by that snake during that maze run.

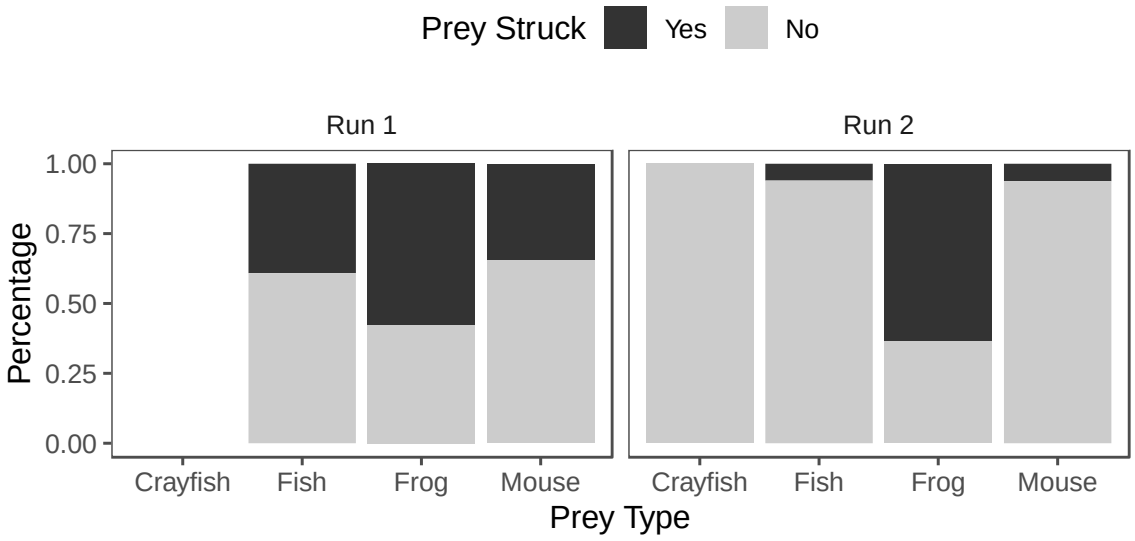

Figure 12. Percentages of counts of prey items struck during feeding compared by prey type between runs. Note that no crayfish were consumed during the first maze run.

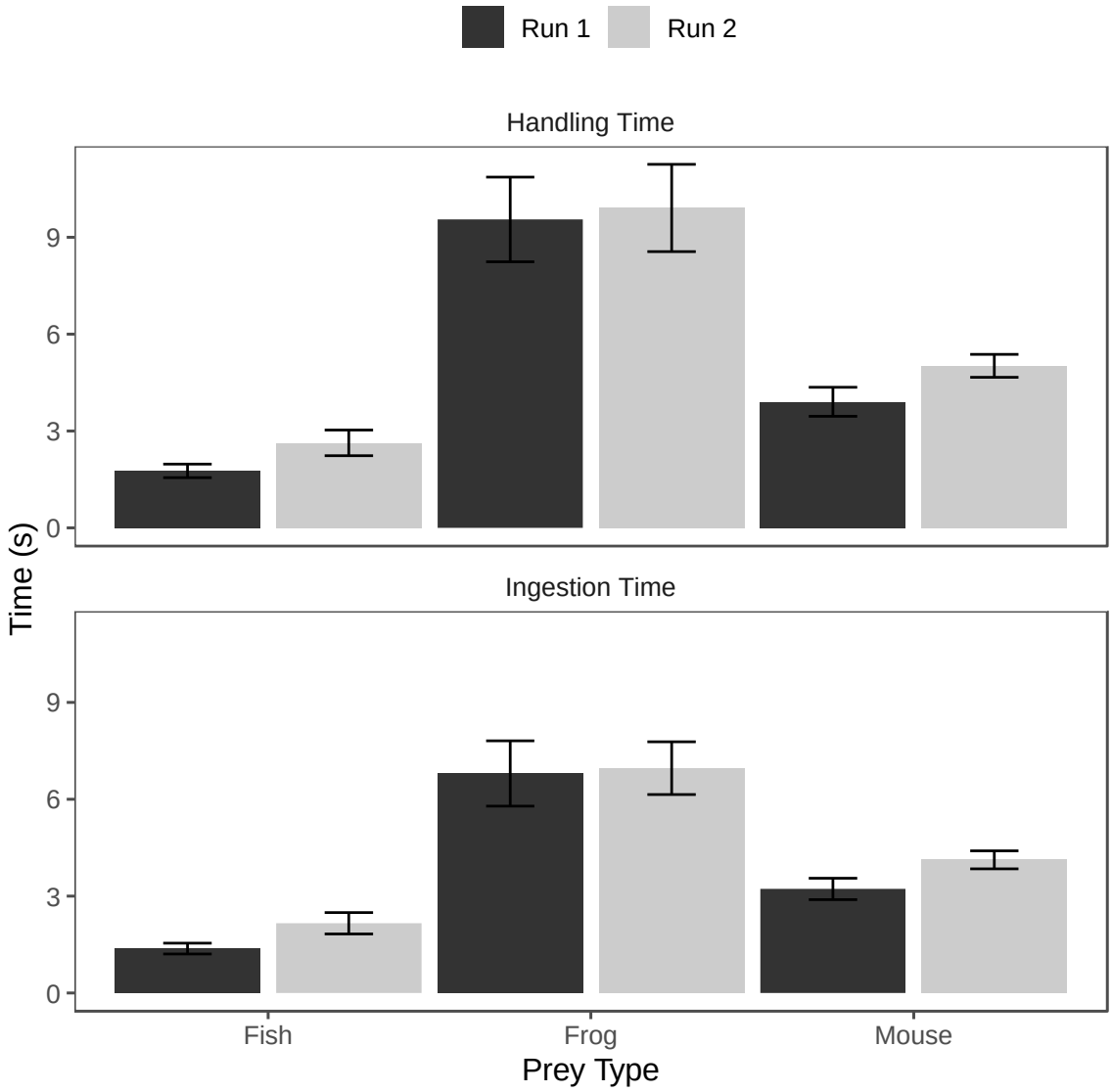

Figure 13. Mean Handling time and mean Ingestion time per prey type by run number. Error bars are ± 1 standard error.

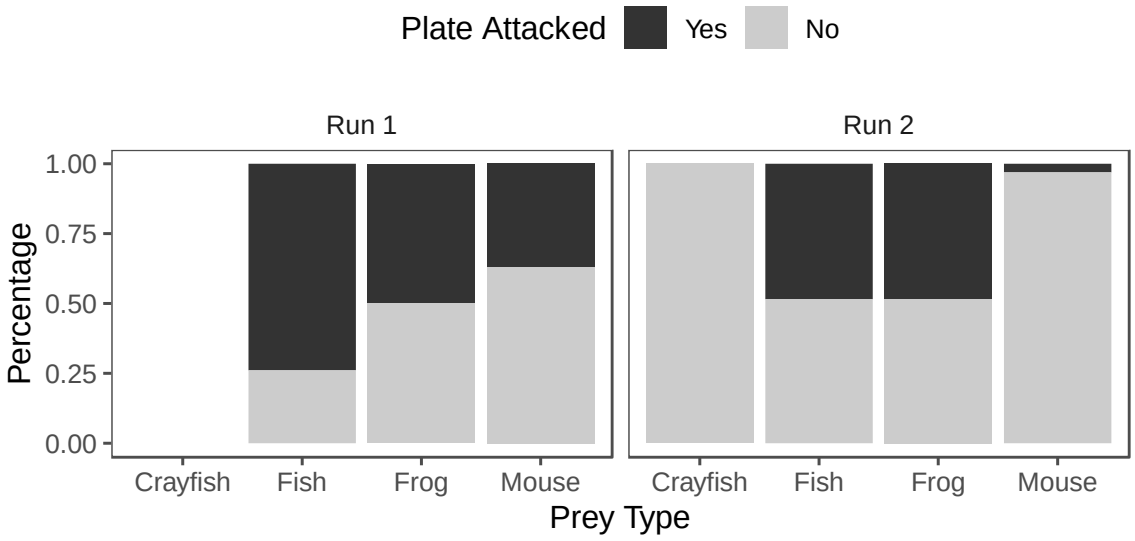

Figure 14. Percentages of counts of snakes that attempted to eat empty plates compared by prey type and maze run. Note that no crayfish were consumed during the first maze run.

Video links

Video 1. Sped up footage of a prey selection maze trial. The footage for this maze run was trimmed down to one hour and sped up (20X). The footage begins during the snake's acclimation period and ends with its last prey selection.
https://youtu.be/5iQzZd8hQa4

Video 2. Footage of a snake attempting to eat an empty plate that once held a food item (bottom right corner).
https://youtu.be/QdZG8qrU9Ok

Chapter 3: The effect of meal type on Specific Dynamic Action in the Western Cottonmouth (*Agkistrodon piscivorus leucostoma*)

Jason Ortega and Steven J. Beaupre

Abstract

A predator's seemingly wide diet breadth may not coincide with the scope of its digestive performance when fed a wide array of prey. In our continued efforts to examine the interplay between prey preference and digestive performance, specific dynamic action (SDA) was examined in the western cottonmouth (*Agkistrodon piscivorus leucostoma*). Snakes were fed meals of varying prey types (fish, frog, and mouse) and prey forms (homogenized and whole) and their SDA responses were measured. Resting and post-feeding metabolic rates (VCO_2 and VO_2) were measured using open-flow respirometry. The SDA response to meal type (the interaction of prey type and form) was overall highest for homogenized mouse meals and lowest for whole frog meals. Within each prey form (homogenized versus whole) mouse meals had the highest SDA response, while homogenized meals had collectively higher values. Our results suggest that mouse meals have a higher processing cost when compared to fish / frog meals.

Introduction

Examining how an animal's diet impacts the other facets of its life is a major tenet of nutritional ecology (Karasov and Martínez del Rio, 2007). Food intake may be one of the most critical variables affecting life histories of reptiles (Congdon 198 Beaupre, 2002). Diet preference can be reflected in many aspects of an animal's biology: anatomically (e.g., specialized dentition (Davis et al., 2010) or modified organs (Godoy-Vitorino et al., 2008)), behaviorally (e.g., grooming (Pauli et al., 2014)), and physiologically (e.g., eating toxic prey (Van Soest, 1994)).

An important facet of nutritional ecology aims to understand how well organisms process specific meal types (Raubenheimer et al., 2012). Often, studies have focused on digestive efficiency to answer how much energy is extracted by the consumer when fed a particular diet (Karasov and Martínez del Rio, 2007). While these studies have been invaluable in understanding the amount of energy a consumer can extract from a meal, they do not offer insight to the energetic cost of digesting the meals themselves. The cost of digestion is not captured in estimates of digestive efficiency.

Specific dynamic action (SDA) is the increase in metabolic rate due to feeding and processing a meal (Brody, 1945; Coulson and Hernandez, 1979; Kleiber, 1961; Secor, 2009). The SDA response encompasses the energetic expenditures associated with digestion of a meal and processing of the absorbed nutrients from that meal, resulting in tissue growth (Waas et al., 2010). Due to the extreme feeding biology of snakes (Cundall and Greene, 2000), these animals have served as models for understanding how SDA response is impacted by fasting (Starck and Beese, 2001; Zaidan and Beaupre, 2003), meal size (Andrade et al., 1997), meal composition (Enok et al., 2013), and meal type (Willson and Hopkins, 2011). Under the scope of SDA, work

has been conducted examining the impact of feeding on tissue growth (Henriksen et al., 2015; Starck and Beese, 2001) and repair (Riquelme et al., 2011). It has even been suggested that the SDA response of snakes plays a role in addressing questions about human evolution (Boback et al., 2007). Whereas continued work that investigates the mechanisms of SDA is needed (Secor, 2009), a fairly small subset of SDA work has posed questions about how SDA response is factored into snake energetics (Andrade et al., 1997; Dorcas et al., 2004; McCue and Lillywhite, 2002; Zaidan and Beaupre, 2003) and snake feeding biology (Britt et al., 2006; Spencer et al., 2020; Willson and Hopkins, 2011). Even fewer studies have examined SDA response due to prey type (Britt et al., 2006; Willson and Hopkins, 2011), but some have examined the effect of meal nutritional composition on SDA through the use of artificial diets (Enok et al., 2013; Henriksen et al., 2015; McCue et al., 2005).

To better understand the interplay between prey preference and digestive performance we present our work examining effect of meal type on SDA response. The objective of this study was to determine the effect of prey type (species) and form (homogenized or whole) on the SDA response of a generalist predator. The western cottonmouth (*Agkistrodon piscivorus leucostoma*) is a unique semi-aquatic pitviper that can forage along the land-water interface (Eskew et al., 2009; Savitzky, 1992) allowing this species to encounter a wide potential prey breadth. During times of drought in northwest Arkansas, cottonmouths in upland habitats have been observed foraging for rodents using foraging behavior seen in other pitvipers such as rattlesnakes (Cundall and Greene, 2000; Hill, 2004; Hill and Beaupre, 2008). To examine the effect of prey type on the SDA response in the cottonmouth, snakes were fed three different types of prey (fish, frog, and mouse) in two different forms (homogenized and whole). The prey types chosen are vertebrate prey that were favored by cottonmouths during the course of prey preference trials, where snakes

showed no distinct preference between these three prey types (Chapter 2). The use of varying prey forms (homogenate versus whole) was an effort to reduce non-feeding events during the course of the study and to provide data on the impact of prey form on SDA response in snakes. We also present data investigating the impact of the act of gavage on SDA response.

Methods

General experimental layout:

The measurement of specific dynamic action (SDA) in response to feeding requires that metabolic rates prior to feeding be measured to properly capture the scope and duration of the specific dynamic action response (Secor, 2009). The open-flow respirometry system used allowed for the measurement of metabolic rates through indirect calorimetry, where seven animals were measured within each block of measurements, wherein snakes were randomly assigned to one of two blocks that alternated being fasted and fed during the course of the study (Lighton, 2008). Prior to being fed each meal snakes were fasted to ensure that their pre-feeding metabolic rates represented an animal that is not undergoing the act of digestion. After the minimum fasting period of 14 days, snakes were placed in respirometry chambers, and their carbon dioxide production (VCO_2) and oxygen consumption (VO_2) was measured for at least 48 hours prior to feeding (Zaidan and Beaupre, 2003). The pre-feeding or baseline measurement represents the approximation of each animal's resting metabolic rate over a 24 hour period, which will encompass any daily circadian variation in metabolic activity (Bennet and Dawson, 1976). After measurement of its baseline, each snake was fed its predetermined prey type (fish, frog, or mouse) that was presented as one of the two prey forms (homogenized or whole). For clarity, the combination of prey type and prey form is referred to as meal type. Once fed its

assigned meal type metabolic measurements resumed for each snake for a minimum for 240 hours after feeding. Once post-feeding metabolic measurements ended animals were returned to their enclosures and the other block of seven animals were placed in chambers to begin their measurements. Baseline and post feeding metabolic rates (VCO_2 and VO_2) were used to determine total SDA responses, CO_2 SDA and O_2 SDA due to meal type.

Animals:

Western cottonmouths (*Agkistrodon piscivorus*, N = 37, female = 17; male = 20) were captured by use of nighttime visual encounter surveys from various locations in northwestern Arkansas. All animals used were adults (female mean mass = 253.69 (g) ± 73.59 SD; male mean mass = 218.00 (g) ± 54.68 SD) and none of the females were either vitellogenic nor pregnant, which was determined by palpating for follicles/embryos (Van Dyke and Beaupre, 2011). Each animal was individually maintained in ventilated lockable plastic tubs that were lined with newspaper. Rooms were kept at a temperature of 25.0 ± 1.0 ˚C with a light cycle of 12:12 (L:D), and water was provided *ad libitum*. Snakes were fasted for a minimum of 14 days prior to metabolic measurements.

Respirometry:

Metabolic measurements were made using a Sable Systems (TR-3, Las Vegas, NV) open flow system that measured O_2 consumption (ppm) and CO_2 production (ppm) (Figure 1). All tubing used in the following setup was either Bev-A-line (Thermoplastic Processes, Georgetown, DE) or Tygon FEP lined tubing (Avantor, Radnor, PA) with a 3.18 mm (1/8 inch) inner diameter;

these materials are selected due to their ability to prevent the infiltration of atmospheric gases into the sample air streams (Lighton, 2008).

A purge gas generator (FT-IR 75-45, Cytiva, Marlborough, MA) was used to produce CO_2 and water free air, this feed was pushed through (positive pressure) a manifold and split into 8 channels. One channel served as baseline air and the other seven were sent to ported respirometry chambers that housed live animals during measurement. Respirometry chambers were gas-tight except for the presence of two bulk-head fittings representing incurrent and excurrent air flow. The excurrent air flow from each chamber lead to a computer-controlled multiplexer (V2, Sable Systems) which would switch between lines as dictated by the Expedata software (V1.9.14, Sable Systems) to send the excurrent airflow to a battery of sensors. The multiplexer was set to sample each line for 7.5 minutes each hour, with the baseline measurements bookending the other channels and was sampled for 3.75 minutes at the top and bottom of every hour; each hour produced 720 lines of data per hour. Once the airflow left the multiplexer it was sent to a Sub-sampler (SS4, Sable Systems) that measured mass flow rate (mL min^{-1} @ STP). The airflow was then sent to a subsampling manifold that sampled at a flow rate less than 45% of the incoming airstream via negative pressure. The resulting subsampled air was pulled through a Nafion drying column (MRD110-072TKV1111-0, Perma-Pure, Lakewood, NJ) and one 30 mL Drierite (6 mesh, WA Hammond Drierite, Xenia, OH) column before entering a paramagnetic O_2 analyzer (FC-1, Sable Systems). Excurrent air from the O_2 analyzer was pulled through a single 15 mL Drierite column and then entered an infrared CO_2 analyzer (LI 6251, Licor, Lincoln, NE). Excurrent air from the CO_2 meter was pulled through a second Sub-sampler (SS4, Sable Systems) wherein the air passed through a mass flowmeter, then past a needle valve, and finally left the system through the subsampler pump that generated the negative pressure for

all components downstream of the subsampling manifold. Control of the multiplexer and recording of all sensor outputs was done by a computer-controlled Universal interface (UI-2, Sable Sytems) using the program Expedata.

During measurements all respirometry chambers were contained in an environmental chamber that had a 12:12 (L:D) light cycle and was kept at 25.0 ± 1.0 ˚C. Respirometry chambers used ranged from 1600 mL to 2300 mL and were constructed from modified plastic Lock&Lock containers (HPL824 and HPL825, Lock&Lock, Cerritos, CA) that were fitted with two plastic bulkhead fittings (6149-0002, Nalgene, Thermo Fisher Scientific, Waltham, MA) with 6.35 mm sized barb fittings.

Due to the use of a subsampling manifold the incurrent flow rate of each respirometry chamber could be set independently of one another, with the lower limit being 2.5X higher than the subsampling flow rate used for that block of measurements; subsampling flow rates used ranged from 260 to 350 mL min^{-1}. With this lower limit in mind, the incurrent flowrate and respirometry chamber size used was determined by the size of each individual animal to achieve a headspace turnover rate of ~1.5 min. Assuming that animal mass (mg) closely approximates animal volume (mL), the following equation was used (Lighton, 2008):

$$\text{Headspace turnover rate (min)} = \frac{\text{Chamber volume (mL)} - \text{Animal volume(mL)}}{\text{Flow Rate (mL min}^{-1}\text{)}}$$

Chamber flow rates used ranged from 580 to 820 mL min^{-1}.

Prey items and processing:

The prey items used in the SDA trials were all sourced from commercial vendors; fish ("Black salty", *Carassius auratus*, Anderson Minnow Farms, Lonoke, AR), frogs (*Lithobates sphenocephalus*, Carolina Biological Supply, Burlington, NC), and mice (*Mus musculus*, Rodent

Pro, Inglefield, IN). Mice were purchased pre-frozen, while fish and frogs were euthanized prior to freezing. Both fish and frogs were euthanized by exposure to CO_2 gas and then frozen.

Homogenized diets were prepared by manually griding whole frozen animals in a hand meat grinder (#10 stainless steel meat, The Sausage Maker, Buffalo, NY). Each batch of frozen food items were passed through the grinder using a #10 plate (4.5 mm pore size, The Sausage Maker, Buffalo, NY) three times before being aliquoted into smaller containers, weighed, and refrozen.

Gavage and whole prey feeding:

Prey and meal type were assigned to each snake prior to the start of metabolic measurements. When possible, each prey type (fish, frog, or mouse) was present within each set of metabolic measurements (seven animals at a time), wherein each set had at least two individuals receiving the same prey type, the seventh individual in each set had a randomly assigned prey type. Meal type (homogenized or whole prey) was typically uniform within each set of measurements. Feeding was done by either gavage of homogenized prey or the offering of whole prey. The target meal size was 15±1% of the snake's body mass for both meal types. For gavage meals, frozen prey homogenate was thawed to room temperature (~23.0 ˚C) in a water bath before being loaded into the gavage rig. The gavage rig was based on a modified 60 mL plastic syringe, where the barrel was fitted with a brass bulkhead fitting and a Buna gasket that connected to a brass 3/8 in (10.25 mm) barb fitting. A vinyl plastic tube (rated for potable water) with an outer diameter of 9.0 mm and a length of 38.0 cm was fixed to the barb fitting and used as the gavage tube (Figure 2); each prey type had its own dedicated gavage rig. Prior to force feeding each snake, the gavage rig was filled with the specified prey type, weighed, and the tube

was lubricated with K-Y jelly (RB Health, Mount Vernon, IN). Before gavage, metabolic measurements were paused and each respirometry chamber was removed from the environmental chamber.

To safely gavage these venomous snakes each individual animal was removed from its respirometry chamber and gently coxed into a series of clear plastic restraint tubes that matched the snake's girth. The initial tube had a length of ~60 cm and was the tightest fitting on an individual snake. The animal was coaxed up through the first tube until no less than the first 1/3 of its length was within and the snake was manually restrained in the tube. A second slightly wider and shorter tube (~30 cm) was placed over the first tube, this allowed the first tube to be slid off the animal while keeping the snake within the second tube. The second tube was adjusted to allow the snake's head to emerge from one end of the tube while its posterior section was controlled at the other tube end (Figure 2). With the animal's head exposed, the gavage tube was gently inserted down the snake's esophagus, and the plunger was slowly depressed until the target volume was dispensed. Homogenized prey had a density of approximately 1.0 g L^{-1}. After gavage the snake was returned to its respirometry chamber through the use of a series of additional restraint tubes, which allowed the animal to "back into" the chamber. Once all snakes were placed back in their respective chambers metabolic measurements were resumed.

For whole prey meals, one or two individual food items were chosen while frozen to equal the target meal size for a particular snake. Once specific prey items were chosen, they were thawed in water overnight in a refrigerator and were brought to room temperature (~23.0˚C) prior to being offered to each snake. Before feeding, each snake's respirometry chamber was removed from the environmental chamber. Whole meals were offered to each animal within its respirometry chamber. Snakes were left undisturbed for two hours to allow for ingestion to

occur. After the two hours passed, any uneaten food items were removed and metabolic measurements were resumed. If a snake only ate one of two items offered, the refused item was weighed and the snake was used for post feeding metabolic measurements. When a snake completely refused a meal, it was offered a whole mouse meal (or alternate if a mouse meal was initial refused) of the target mass and given 15 minutes to ingest it. If the backup meal was refused, the snake was removed from that series of measurements, returned to its enclosure, watered, and given a maintenance mouse meal 24 hours later.

Post-feeding metabolic measurements:

After feeding, each animal's respirometry chamber was reconnected, checked for leaks, and measurements resumed. Post feeding measurements continued for at least 240 h after feeding (but no more than 250 h). If a snake regurgitated after feeding it was removed from its respirometry chamber and removed from that set of measurements. Once post-feeding measurements ended each animal was returned to its enclosure and watered.

Sham feeding:

Force feeding snakes via gavage has been used for snake maintenance (Panizzutti et al., 2001), therapeutic care (Sykes and Greenacre, 2006), and as a technique for digestion studies (Boback et al., 2007; Enok et al., 2013; Henriksen et al., 2015; McCue et al., 2005). Despite the use of this technique, the explicit impact of the act of gavage on snake SDA response has not been reported. If the act of gavage significantly effects SDA, then this increase in metabolic expenditure must be considered when calculating total SDA response. To measure the effect of the act of force feeding on metabolic rate, 14 animals, in two blocks of measurements were force

fed water via gavage in place of food (sham feeding). The procedures for this experiment followed the same general protocols as the feeding experiments described above except that each animal was given 10 mL of water via gavage and post sham feeding metabolic rates were measured for no less than 36 hours, but no more than 48 hours.

Processing of Metabolic Data:

Metabolic data were initially processed using Expedata. Each hour of measurement produced a single raw file where respirometry chamber flow rate (mL min $^{-}$1), CO_2 concentration (ppm), and O2 concentration (ppm) were recorded every 5 seconds for 7.5 minutes; generating 90 data points every hour per chamber (animal). A macro was used within Expedata to correct for time lag between gas sensors, conduct baseline drift corrections, correct for washout lag in switching between chambers, and calculate VCO_2 and VO_2. A lag correction of 1 second was applied to CO_2 concentrations (ppm) to account for the time delay in gas samples traveling between O_2 and CO_2 sensors (Figure 1), this ensured that O_2 and CO_2 peaks were aligned. Baseline drift corrections used the top and bottom 3.5 min of each hour, which measured unused baseline incurrent air to correct gas concentrations for potential drift due to changing atmospheric conditions and drift in the paramagnetic O_2 sensor. To account for any washout lag in the system caused by the multiplexer switching between chambers the macro only exported the last 70 out of 90 rows of data for each chamber (animal). The reduction in the number of samples resulted in 350 seconds (5.833 min) of each chamber's data per hour being used to calculate metabolic rates.

Gas concentrations (ppm) were converted to fractional concentrations and used to calculate VCO_2 and VO_2. Carbon dioxide production rate (mL h $^{-1}$) was calculated as:

$$\dot{V}_{CO_2} = (F_e - F_i) \times FR \times 60$$

where F_e is the fractional concentration of CO_2 in the chamber excurrent air, F_i is the fractional concentration of CO_2 in the chamber incurrent air, FR is the flow rate (mL min $^{-1}$) of air travelling through each chamber, and 60 converts the rate (mL min $^{-1}$) to (mL h $^{-1}$) (Lighton, 2008). Oxygen consumption rate (mL h^{-1}) was calculated as:

$$\dot{V}_{O_2} = \frac{FR(F_iO_2 - F_eO_2) - F_iO_2(F_eCO_2 - F_iCO_2)}{(1 - F_iO_2)} \times 60$$

where F_eCO_2 is the fractional concentration of CO_2 in the chamber excurrent air, F_iCO_2 is the fractional concentration of CO_2 in the chamber incurrent air, F_iO_2 is the fractional concentration of O_2 in the chamber incurrent air, F_eO_2 is the fractional concentration of O_2 in the chamber excurrent air, FR is the flow rate (mL min $^{-1}$) of air travelling through each chamber, and 60 converts the rate (mL min $^{-1}$) to (mL h $^{-1}$) (Lighton, 2008). The resulting data produced by the Expedata macro was imported into R version 4.1.0 (R Core Team, 2021) for aggregation and transformation to facilitate statistical analysis.

To reduce the impact of snake physical activity within the chamber on metabolic rates, both VCO_2 and VO_2 were smoothed by taking a 5 hour running average for each variable for each individual's set of measurements (Zaidan and Beaupre, 2003). The smoothed metabolic rates were used for all following calculations and analyses. Pre-feeding baseline metabolic rates (or Resting Metabolic Rates, RMR) were trimmed down to the 24 hour period prior to feeding (hours -24 to -1). Total SDA responses, CO_2 SDA (mL) and O_2 SDA (mL) were determined by correcting post feeding totals (hours 0 to 239, 240 hours total post feeding) for baseline totals (24 hour baseline totals that were extrapolated to 240 hours) (Beaupre, 2005). Which resulted in all post feeding totals having an extrapolated baseline total subtracted from them. Baseline and post feeding totals were determined using the "pracma" package in R that summed the step-wise trapezoidal integrals of VCO_2 by hour and VO_2 by hour.

Sham feeding data were processed through Expedata just as feeding data were. Post Expedata processing did differ, wherein baseline metabolic rates were trimmed to 36 hours before sham feeding (hours -36 to -1) and 36 hours post sham feeding (hours 0 to 35). Hourly average VCO_2 and VO_2 values were calculated. No integrated total CO_2 or O_2 values were determined for sham feeding data because no differences were observed.

Data analysis:

All data analyses were conducted within R. The effect of the act of gavage on the metabolic rates of snakes was evaluated using a Welch two sample t-test to compare the difference between log_{10} transformed pre and post gavage average VCO_2 and VO_2 rates (mL h^{-1}) for each hour (Figure 3).

To illustrate the pattern seen in hourly averaged VCO_2 and VO_2 rates (mL h^{-1}) of all individuals in the study, metabolic rates were corrected for meal size (g) to generate Figure 4, yet ratio-based values were not used for analyses (Beaupre, 2005). Mixed model linear ANCOVAs were used to examine the effect of prey form (homogenized or whole) and prey type (fish, frog, or mouse) on the relationship between meal mass and CO_2 SDA and O_2 SDA, using the package "nlme" (Bates et al., 2015). The models used prey type, prey form, CO_2 SDA, and O_2 SDA as fixed effects, while meal mass was the covariate, and Snake ID was the random effect used for both models. Meal mass, snake mass, CO_2 SDA and O_2 SDA were linearized via log_{10} transformation. The covariance structures used had the following AIC scores, -88.771 for CO_2 SDA and -12.6737 for O_2 SDA (Bozdogan, 1987). For both models, assumptions of normality and homogeneity of slopes were met. The package "nlme" was used for these comparisons

Results

General summary:

Despite outright and partial refusals of whole meals, samples sizes per treatment varied little along with average meal sizes. Sample size was similar for most treatments (N = 14), except for whole frog (N = 11) and whole mouse (N = 20). Average meal sizes when corrected for snake size generally fell within the target 15 ± 1% body mass meal size, except for whole mice whose average meal size was 13.17% ± 0.02 SD; all analyses used non-ratio meal sizes (Beaupre, 2005).

Sham feeding:

Pre-gavage and post-gavage metabolic rates were not significantly different for VCO_2 (T= -1.5981, DF=25.77, P = 0.1222) nor VO_2 (T=-0.74853, DF = 24.938, P = 0.4611), thus it was not possible to estimate an "SDA" response for gavage alone.

Meal type:

Meal type (interaction of prey type and prey form) did not have a significant effect on SDA response for CO_2, but was significant for O_2. Carbon dioxide SDA response was significantly impacted by prey type ($F_{2/44}$=37.0337, P <0.0001) and prey form ($F_{1/44}$=22.8248, P <0.0001), but not meal type (interaction of prey type and form) ($F_{2/44}$=4.2722, P =0.1181). Snake ID only accounted for less than 1% of total variance for the model. Oxygen SDA response was significantly impacted by prey type ($F_{2/44}$=15.1911, P <0.0001), prey form ($F_{1/44}$=13.3173, P <0.001), and meal type (interaction of prey type and form) ($F_{2/44}$=4.0903, P =0.02079). Snake ID only accounted for less than 1% of total variance for the model. Between meal types and for both

CO_2 and O_2 whole frog had the lowest response while homogenized mouse had the highest (Figure 5). Within CO_2 SDA there was no significant difference between homogenized fish, whole fish, and homogenized frog, yet whole frog was significantly different from homogenized frog ($P = 0.0338$). Homogenized mouse was significantly different from all other meal types with p-values < 0.001. Whole mouse was not significantly different from homogenized fish ($P = 0.0818$), and homogenized frog ($P = 0.3331$), yet was significantly different from whole fish ($P = 0.0127$) and whole frog ($P = 0.0001$). Within O_2 SDA response there is no significant difference between homogenized fish, whole fish, homogenized frog, whole frog, and whole mouse; except for the significant difference between whole frog and whole mouse ($P=0.0121$) (Figure 5). Homogenized mouse was significantly different from all the other meal types with a maximum P value of 0.0031.

Discussion

General feeding patterns:

In general, the snakes responded quite well to the process of gavage, only two individuals had to have their homogenized meal SDA response measurements redone due to regurgitation. There was a surprising amount of whole prey refusal during this study, where whole frogs were the most refused of the prey items (N = 11 eaten). Yet, snakes also refused whole mouse and whole fish meals as well. The high number of whole mouse meals (N = 20) was a by-product of snakes refusing a fish or frog meal but eating an offered mouse; some animals refused to eat at all.

Sham gavage:

As seen in Figure 3, the effect of sham gavage on metabolic rate is very short lived (4 hour increase), this small spike in metabolic rate is similar in scope to increased activity due to animal handling and initial placement into a respirometry chamber (Ortega, personal observation). The short lived, increase is most likely due to small bouts of activity and stress-induced increases in metabolic rate due to elevated corticosterone levels from handling stress (Durant et al., 2008; Schuett et al., 2004; Wack et al., 2012). The lack of a significant effect of sham gavage on metabolic rate is supported by the work of Enok et al. (2013) where they compared whole mouse SDA response to homogenate SDA response, yet they did not test the act of gavage itself, furthermore their lack of a significant difference between whole and homogenized prey is contrary to what this and two other studies found (Britt et al., 2006; McCue et al., 2005). Britt et al. (2006) used gelatin capsules as a diet delivery vector and did not account for the impact of gelatin on SDA response however, in another study gelatin was fed to snakes and elicited no SDA response (McCue et al., 2005).

Meal type and SDA response:

When visually comparing meal size corrected average metabolic rates (Figure 4) the general shape of each curve telegraphs the differences seen amongst the CO_2 SDA and O_2 SDA measurements (Figure 5). Examining these smoothed rates which still clearly show bouts of activity exemplifies how important it is to apply a smoothing function to long term metabolic measurements (Zaidan and Beaupre, 2003), while also showing how much "nosier" O_2 consumption measurements are when compared to CO_2 production measurements (Lighton, 2008).

There are two interesting patterns that can be seen in the CO_2 SDA and O_2 SDA values (Figure 5), in general the homogenized meals are higher than the whole meals and within each meal form (homogenized versus whole) mouse is much higher than frog or fish. Higher homogenized SDA responses have been documented, yet this is from a small pool of work (Britt et al., 2006; McCue et al., 2005). Unfortunately an equal amount of studies have shown either no difference due to form (Enok et al., 2013) or that homogenized meals have a lower SDA response (Boback et al., 2007) (note that this was in reference to ground beef versus pieces of steak). The pattern observed in our work may be due to the homogenized meals spending less time undergoing gastric processing and having a longer exposure time to intestinal processes which have been shown to contribute more to overall SDA response (Enok et al., 2013). The initial purpose of feeding homogenized meals was to reduce incidences of animals refusing to feed during an SDA experiment and to provide clearer data to elucidate the pattern of homogenized versus whole meals. The stark differences shown in prey form were not expected.

The higher overall mouse SDA response is unexpected due to its relative nutritional composition. The current understanding of SDA response is that it is proportional to the protein content of the meal being processed (Secor, 2009). Relative to the other prey types the mouse has a lower protein content (~55% of dry matter (DM)) and higher fat content (~23% DM) while the fish and frog have similar protein content (~74% DM) and fat content (~10% DM) (Bernard and Allen, 2002; Dierenfeld et al., 2002). Taking their relative dry matter content into consideration, based on these values the nutrient content of a theoretical 10 g (~20% DM) fish/frog would be 1.48 g of protein and 0.2 g of fat in contrast to a theoretical 10 g (~32% DM) mouse would be 1.76 g of protein and 0.736 g of fat. Despite the mouse having a higher protein content on a dry matter basis this difference is most likely not the direct cause of such a dramatically higher

observed SDA response, especially considering a snake's inability to completely digest a mouse, where a portion of the mouse pelage passes through the gastrointestinal tract intact. Willson and Hopkins (2011) reported no significant difference in SDA response when snakes were fed prey with slightly varying nutritional composition which is in line with our observations of fish and frog meals.

In summary our results suggest that the act of gavage does not cause a significant change in metabolic rate and its effect can be ignored for any study that uses it as a method for administering meals to snakes. We also conclude that SDA response does vary due to prey type (fish / frog versus mouse) and form (whole versus homogenate). The increased SDA cost associated with rodent meals may impact the frequency at which these snakes engage in rodent-focused foraging behavior and may explain why it is had been observed in Ozark cottonmouths only during times of dramatic environmental change, such as drought (Hill, 2004; Hill and Beaupre, 2008). The lower SDA cost of fish / frog meals when coupled with the relatively higher abundance of these prey types may dictate foraging preferences in cottonmouths (Beaupre and Montgomery, 2007). Yet the ability to forage away from water may allow these snakes to be resistant to changes in their prey landscape. Further work needs to be conducted to elucidate the explicit mechanisms that are responsible for the trends that we observed in comparisons between whole and homogenized meals.

Literature Cited

Andrade, D.V., Cruz-Neto, A.P., Abe, A.S., 1997. Meal size and specific dynamic action in the rattlesnake Crotalus durissus (Serpentes : Viperidae). Herpetologica 53, 485-493.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software 67.

Beaupre, S.J., 2005. Ratio representations of specific dynamic action (mass-specific sda and sda coefficient) do not standardize for body mass and meal size. Physiological and Biochemical Zoology 78, 126-131.

Beaupre, S.J., Montgomery, C.E., 2007. The meaning and consequences of foraging mode in snakes, in: S.M. Reilly, L.B. McBrayer, D.B. Miles (Eds.), Lizard Ecology. Cambridge University Press, 334-368.

Bennet, A.F., Dawson, W.R., 1976. Metabolism, in: C. Gans, W.R. Dawson (Eds.), Biology of the Reptilia. Academic Press, New York, 127-223.

Bernard, J.B., Allen, M.E., 2002. Feeding captive piscivorous animals: nutritional aspects of fish as food, Nutrition Advisory Group Handbook Fact Sheet, 1-12.

Boback, S.M., Cox, C.L., Ott, B.D., Carmody, R., Wrangham, R.W., Secor, S.M., 2007. Cooking and grinding reduces the cost of meat digestion. Comparative Biochemistry and Physiology 148A, 651-656.

Bozdogan, H., 1987. Model selection and Akaike's Information Criterion (AIC): The general theory and analytical extensions. Psychometrika 52, 345-370.

Britt, E.J., Hicks, J.W., Bennett, A.F., 2006. The energetic consequences of dietary specialization in populations of the garter snake, Thamnophis elegans. Journal of Experimental Biology 209, 3164-3169.

Brody, S., 1945. Bioenergetics and growth. Harner, New York, New York.

Coulson, R.A., Hernandez, T., 1979. Increase in metabolic rate of the alligator fed proteins or amino acids. Journal of Nutrition 109, 538-550.

Cundall, D., Greene, H.W., 2000. Feeding in snakes, in: K. Schwenk (Ed.), Feeding: Form, Function, and Evolution in Tetrapod Vertebrates. Academic Press, San Diego, 293-333.

Davis, J.S., Nicolay, C.W., Williams, S.H., 2010. A comparative study of incisor procumbency and mandibular morphology in vampire bats. J Morphol 271, 853-862.

Dierenfeld, E.S., Alcorn, H.L., Jacobsen, K.L., 2002. Nutrient composition of whole vertebrate prey (excluding fish) fed in zoos, National Agricultural Library.

Dorcas, M.E., Hopkins, W.A., Roe, J.H., Douglas, M.E., 2004. Effects of Body Mass and Temperature on Standard Metabolic Rate in the Eastern Diamondback Rattlesnake (Crotalus adamanteus). Copeia 2004, 145-151.

Durant, S.E., Romero, L.M., Talent, L.G., Hopkins, W.A., 2008. Effect of exogenous corticosterone on respiration in a reptile. Gen Comp Endocrinol 156, 126-133.

Enok, S., Simonsen, L.S., Wang, T., 2013. The contribution of gastric digestion and ingestion of amino acids on the postprandial rise in oxygen consumption, heart rate and growth of visceral organs in pythons. Comparative Biochemistry and Physiology Part A 165, 46-53.

Eskew, E.A., Willson, J.D., Winne, C.T., 2009. Ambush site selection and ontogenetic shifts in foraging strategy in a semi-aquatic pit viper, the Eastern cottonmouth. Journal of Zoology (London) 277, 179-186.

Godoy-Vitorino, F., Ley, R.E., Gao, Z., Pei, Z., Ortiz-Zuazaga, H., Pericchi, L.R., Garcia-Amado, M.A., Michelangeli, F., Blaser, M.J., Gordon, J.I., Dominguez-Bello, M.G., 2008. Bacterial community in the crop of the hoatzin, a neotropical folivorous flying bird. Appl Environ Microbiol 74, 5905-5912.

Henriksen, P.S., Enok, S., Overgaard, J., Wang, T., 2015. Food composition influences metabolism, heart rate and organ growth during digestion in Python regius. Comp Biochem Physiol A Mol Integr Physiol 183, 36-44.

Hill, J.G., 2004. Natural history of the western cottonmouth (*Agkistrodon piscivorus leucostoma*) from an upland lotic population in the Ozark mountains of northwest Arkansas, University of Arkansas.

Hill, J.G., Beaupre, S.J., 2008. Body size, growth, and reproduction in a population of western cottonmouths (*Agkistrodon piscivorus leucostoma*) in the Ozark mountains of northwest Arkansas. Copeia 2008, 105-114.

Karasov, W.H., Martínez del Rio, C., 2007. Physiological Ecology: How Animals Process Energy, Nutrients, and Toxins. Princeton University Press, Princeton, NJ.

Kleiber, M., 1961. The fire of life. An introduction to Animal Energetics. Wiley, New York, New York

Lighton, J.R.B., 2008. Measuring Metabolic Rates: A Manual for Scientists. Oxford University Press, New York.

McCue, M.D., Bennett, A.F., Hicks, J.W., 2005. The effect of meal composition on specific dynamic action in Burmese pythons (*Python molurus*). Physiological and Biochemical Zoology 78, 182-192.

McCue, M.D., Lillywhite, H.B., 2002. Oxygen Consumption and the Energetics of Island-Dwelling Florida Cottonmouth Snakes. Physiological and Biochemical Zoology 75, 165-178.

Panizzutti, M.H.M., Mendoça de Olivera, M., Barbosa, J.L., Cavalcanti, P.L.X., Rocha-Barbosa, O., 2001. Evaluation of a balanced fresh paste diet for maintenance of captive neotropical rattlesnakes used for venom production. Journal of the American Veterinary Medical Association 218, 912-914.

Pauli, J.N., Mendoza, J.E., Steffan, S.A., Carey, C.C., Weimer, P.J., Peery, M.Z., 2014. A syndrome of mutualism reinforces the lifestyle of a sloth. Proc Biol Sci 281, 20133006.

R Core Team, 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Raubenheimer, D., Simpson, S.J., Tait, A.H., 2012. Match and mismatch: conservation physiology, nutritional ecology and the timescales of biological adaptation. Philos Trans R Soc Lond B Biol Sci 367, 1628-1646.

Riquelme, C.A., Magida, J.A., Harrison, B.C., Wall, C.E., Marr, T.G., Secor, S.M., Leinwand, L.A., 2011. Fatty Acids Identified in the Burmese Python Promote Beneficial Cardiac Growth. Science 334, 528-531.

Savitzky, B.C., 1992. Laboratory studies on piscivoury in an opportunistic pitviper, the cottonmouth, *Agkistrodon piscivorus*. Biology of the Pitvipers, 347-368.

Schuett, G.W., Taylor, E.N., Kirk, E.A.V., Murdoch, W.J., 2004. Handling Stress and Plasma Corticosterone Levels in Captive Male Western Diamond-backed Rattlesnakes (*Crotalus atrox*). Herpetological Review 35, 229-233.

Secor, S.M., 2009. Specific dynamic action: a review of the postprandial metabolic response. Journal of Comparative Physiology 179B, 1-56.

Spencer, M.M., Pierson, M.T., Gienger, C.M., 2020. Comparative energetics and thermal responses to feeding in allied Agkistrodon snakes with contrasting diet and habitat use. J Comp Physiol B 190, 329-339.

Starck, J.M., Beese, K., 2001. Structural flexibility of the intestine of Burmese python in response to feeding. Journal of Experimental Biology 204, 325-335.

Sykes, J.M., Greenacre, C.B., 2006. Techniques for Drug Delivery in Reptiles and Amphibians. Journal of Exotic Pet Medicine 15, 210-217.

Van Dyke, J.U., Beaupre, S.J., 2011. Bioenergetic components of reproductive effort in viviparous snakes: Costs of vitellogenesis exceed costs of pregnancy. Comparative Biochemistry and Physiology 160A, 504-515.

Van Soest, P.J., 1994. Nutritional Ecology of the Ruminant, 2nd ed. Comstock Publishing Associates, Ithaca.

Waas, S., Werner, R.A., Starck, J.M., 2010. Fuel switching and energy partitioning during the postprandial metabolic response in the ball python (Python regius). J Exp Biol 213, 1266-1271.

Wack, C.L., DuRant, S.E., Hopkins, W.A., Lovern, M.B., Feldhoff, R.C., Woodley, S.K., 2012. Elevated plasma corticosterone increases metabolic rate in a terrestrial salamander. Comparative Biochemistry and Physiology A 161, 153-158.

Willson, J.D., Hopkins, W.A., 2011. Prey morphology constrains the feeding ecology of an aquatic generalist predator. Ecology 92, 744-754.

Zaidan, F., III, Beaupre, S.J., 2003. Effects of body mass, meal size, fast length, and temperature on specific dynamic action in the timber rattlesnake (*Crotalus horridus*). Physiological and Biochemical Zoology 76, 447-458.

Figures

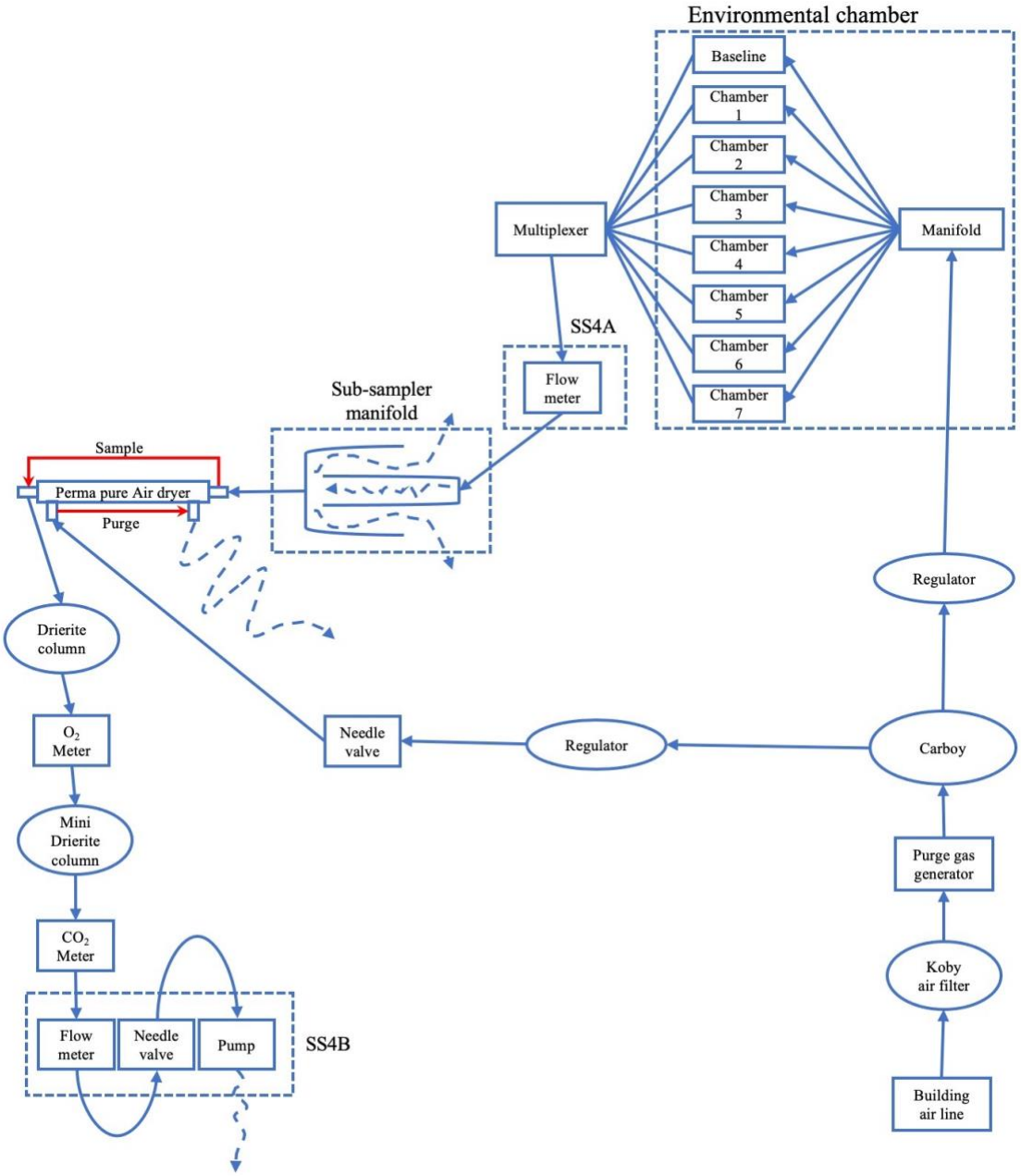

Figure 1. Diagram of flow through respirometry equipment and connections used for measuring metabolic rates of snakes pre and post feeding.

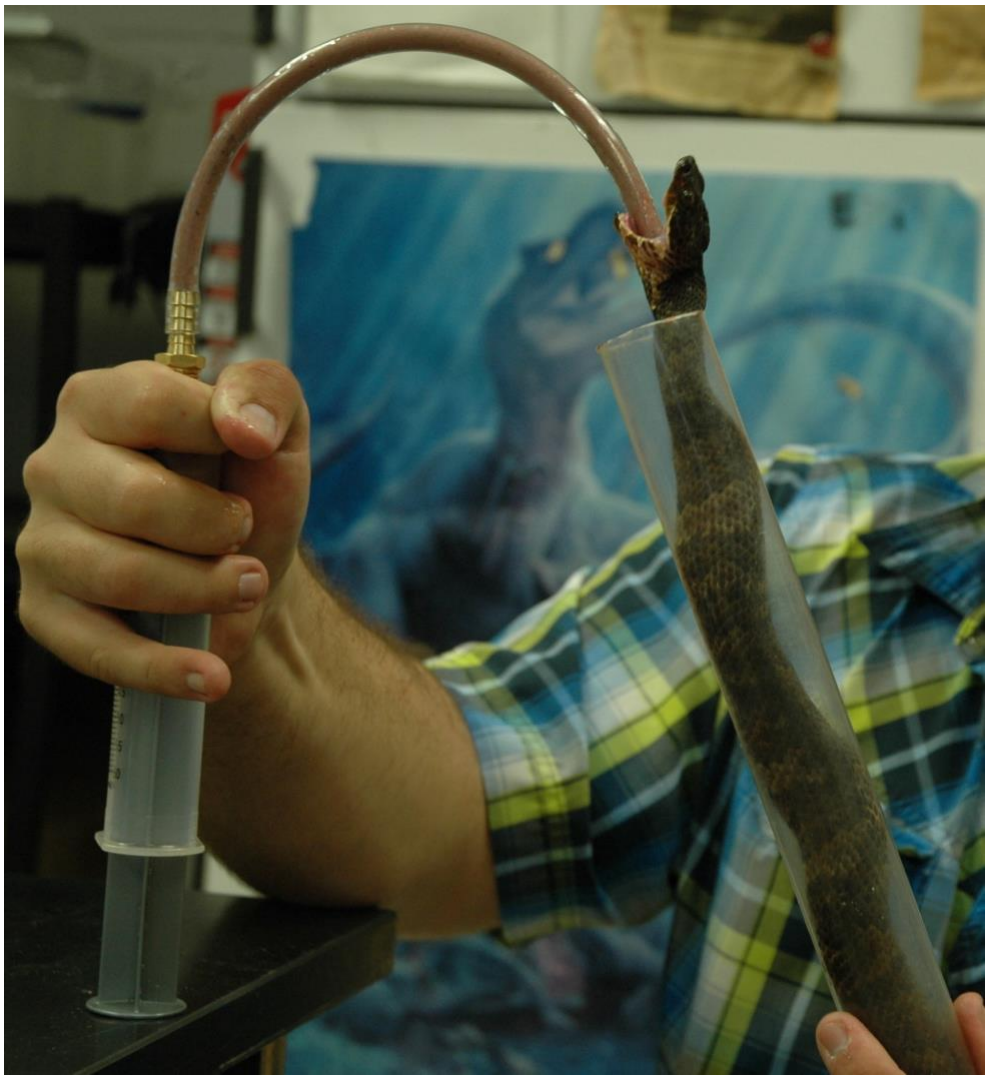

Figure 2. Process of feeding a snake via gavage.

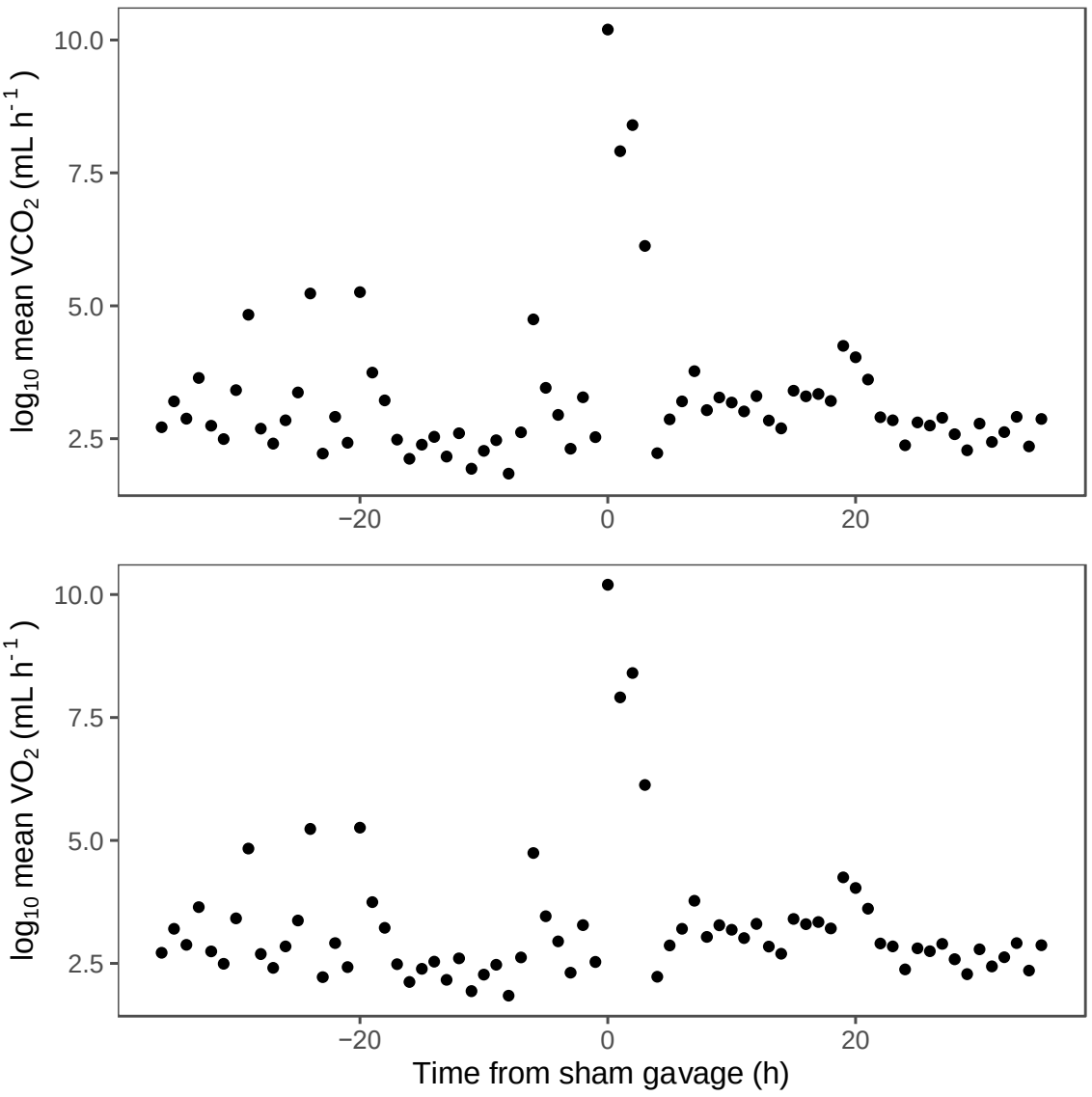

Figure 3. Pre and post sham gavage metabolic rates, representing 36 hours before and 36 hours after animals were sham force fed via gavage 10 mL of water.

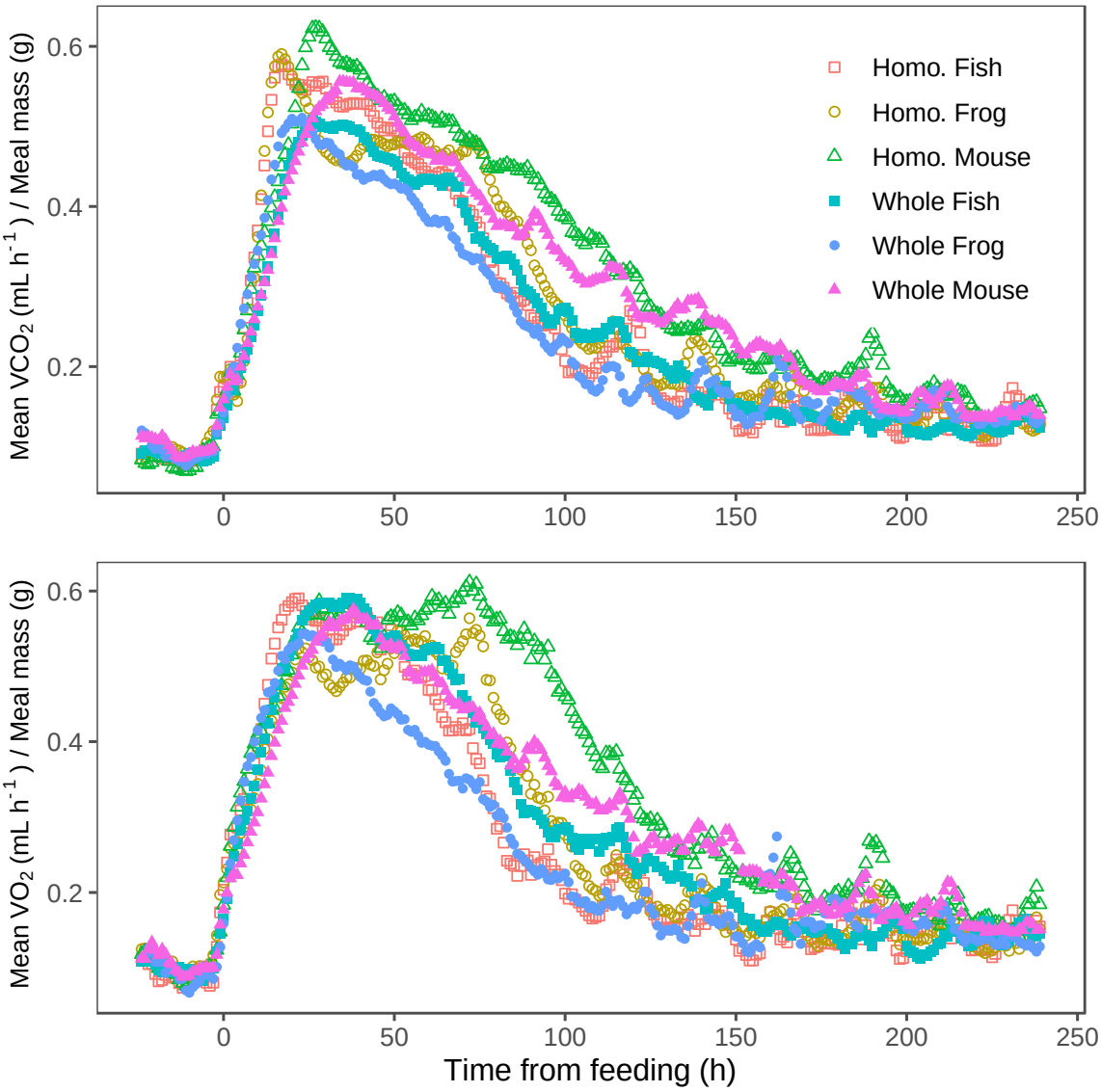

Figure 4. Average metabolic rates (VCO_2 and VO_2) per meal type per hour (average responses of all individuals). The two traces show 24 hours prior to feeding and 240 hours after feeding. Hollow markers correspond to homogenate meals and solid markers correspond to whole meals. Fish meals are represented by squares, frog meals are represented by circles, and mouse meals are represented by triangles. Metabolic rates were corrected for meal mass for the purpose of summarizing metabolic rates of all individuals used for analysis. Ratio-based values were not used for analyses.

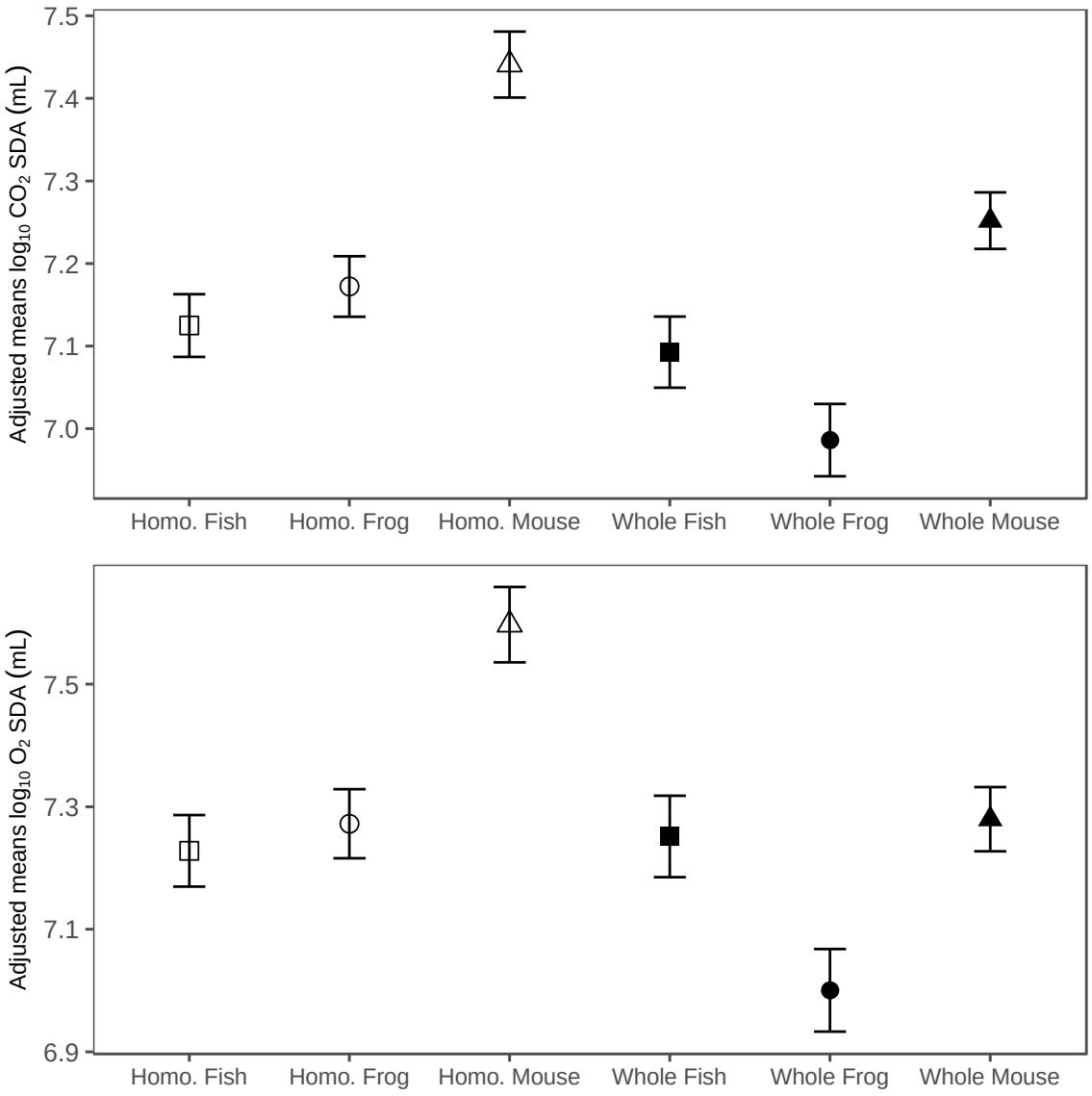

Figure 5. Adjusted (mixed model ANCOVA) means of CO_2 SDA and O_2 SDA values per meal type. Error bars = ± 1 SE.

Dissertation conclusions

The focus of my dissertation was to investigate the relationship between prey preference and digestive performance in the western cottonmouth (*Agkistrodon piscivorus leucostoma*). My overall approach to this question was to determine prey preference and measure digestive performance of the snakes when fed their preferred prey types. Because this snake species is thought as a generalist predator it was vital to determine what types of prey animals from this region were being consumed, as this would dictate what types of prey would be used for the other portions of my work. Results of the stable isotope dietary analysis from chapter one showed that these animals were eating fish and frogs, with crayfish being a potential prey item. In chapter two prey preference trails were conducted using the results from chapter one. The prey preference trials used an X maze where snakes were offered crayfish, fish, frog, and mouse as items to chose from. The results from chapter two supported the view of cottonmouths being generalist predators wherein snakes readily ate fish, frog, and moue, while almost completely ignoring crayfish. The type of prey that were given to snakes in chapter three were determined by the results of the prey preference trials. Specific dynamic action response (SDA) was used as a metric of digestive performance for snakes fed different combinations of prey types (fish, frog, or mouse) and prey form (homogenized or whole). The results of chapter three showed a significant elevated SDA response when snakes were fed whole and homogenized mouse when compared the other meal types suggesting that mouse meals may be disadvantageous due to the high metabolic cost of processing that prey type. Overall while cottonmouths showed no clear distinction between vertebrate prey offered, the snakes did show varying digestive performance between fish/frog and mouse meals. Which may be why cottonmouths in Arkansas switch to foraging for rodent prey during times of drought.

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MEMORANDUM

TO: Steven Beaupre

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: May 12, 2010

SUBJECT: IACUC PROTOCOL **APPROVAL**
Expiration date : May 14, 2013

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#10040- "ISOTOPIC AND NUTRITIONAL COMPOSITION OF COTTONMOUTH (*AGKISTRODON PISCIVORUS LEUCOSTOMA*) PREY**". You may begin this study immediately.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes in the protocol during the research, please notify the IACUC in writing **prior** to initiating the changes. If the study period is expected to extend beyond **05-14-2013,** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

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Research Compliance
Office of the Director

MEMORANDUM

TO: Steven Beaupre

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: May 11, 2011

SUBJECT: IACUC PROTOCOL **APPROVAL**
Expiration date : **July 1, 2014**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#11043- "NUTRITIONAL ECOLOGY OF THE WESTERN COTTONMOUTH (AGKISTRODON PISCIVORUS LEUCOSTOMA) IN THE BUFFALO NATIONAL RIVER**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes in the protocol during the research, please notify the IACUC in writing [Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **08-31-2012,** you may request an extension via the Modification Request form and can extend the approval period up to **05-05-2014** (3 years from original approval date). By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

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cc: Animal Welfare Veterinarian

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UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Dr. Steven J. Beaupre

FROM: Craig N. Coon, Chairman
Institutional Animal Care and Use Committee (IACUC)

DATE: 12-5-2014

SUBJECT: IACUC APPROVAL
Expiration date: December 4, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol 15025: *'Prey preference, digestive efficiency, and specific dynamic action in the western cottonmouth (Agkistrodon piscivorus leucostoma)"* to begin immediately

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing(via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond December 4, 2017, you must submit a new protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

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