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The Behavioral Response of Mosquitoes to Different Snake Odors

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Biology Department.

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ABSTRACT

Due to it causing high mortality rates, Eastern Equine Encephalitis (EEEV) is considered to be one of the most medically important encephalitic viruses in the Eastern United States. In order to be able to control the transmission of this virus, understanding of vector behavior and feeding preferences is necessary. In this study, the response of *Culex nigripalpus* to different snake skin odors was tested to determine if this species of mosquito responded to particular snake species. *Culex nigripalpus* showed the greatest response to Copperhead (*Agkistrodon contortrix*) and Cottonmouth (*A. piscivorus*) snakes, as compared to other venomous snakes or non-venomous snakes, suggesting that odorants of these snakes associated with EEEV overwintering are more attractive to known vector mosquitoes. Future studies should additionally examine the response of this mosquito, and other mosquito species related to the transmission of EEEV, to live snakes. Efforts should also be made to identify which components of snake odorants are attractive to mosquitoes.

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Introduction

In the 1930s, Western Equine Encephalitis Virus, Eastern Equine Encephalitis Virus, and Venezuelan Equine Encephalitis Virus were isolated from the brains of infected horses in California, New Jersey, Virginia, and Venezuela. Of these viruses, Eastern Equine Encephalitis Virus (EEEV) causes the greatest mortality for both humans and horses (Zacks & Paessler 2010). Between the years of 2003-2017, there have been 3,096 equine cases of EEEV reported in the United States, with 257 of these cases, almost all in equines occurring in Georgia, which equates to 8.3% of the total (APHIS 2018).

Human cases are rarer than equine cases; on average, 7 human cases of EEEV are reported each year. Infection by this virus can result in a systemic or encephalitic infection. Systemic infections are characterized by chills, fever, joint pain, muscle pain, and malaise. Encephalitic infections are much more severe, as they involve inflammation of the brain. This type of infection is characterized by fever, headache, restlessness, irritability, anorexia, diarrhea, vomiting, cyanosis, convulsions, and coma. Approximately 1 in 3 patients who develop an encephalitic infection dies, and most survivors will have permanent mild to severe nervous system damage, which could still lead to death within the span of a few years. There is no readily available vaccine or antiviral medication for humans with EEEV; patients ordinarily must be treated symptomatically (CDC 2017).

The transmission cycle of EEEV primarily occurs via a mosquito-avian cycle, with *Culiseta melanura* being the primary enzootic vector. However, *Cs. melanura* is not considered an important bridge vector in the transmission to horses, humans, and other

hosts, as this mosquito species very rarely feeds on mammals (Bingham et al. 2015). Typically, during outbreak years EEEV infections build up in passerine bird populations in woodland habitats, largely as a result of transmission by *Cs. melanura* early in the season (Spring and early Summer). Transmission to humans, horses and other mammals is achieved by "bridge vectors", i.e., mosquitoes that will feed on both birds and mammals at high rates. Well documented bridge vectors include *Aedes vexans* and *Coquilletidia perturbans*, and these mosquitoes are the ones primarily implicated when infections occur in horses and humans.

Overwintering mechanisms for EEEV have long been a mystery, because virus does not overwinter in mosquitoes, birds or mammals, but recent studies have indicated that long-lived reptiles, including venomous snakes, may serve as sources of EEEV for introduction into bird populations in the Spring (Cupp et al., 2004; Bingham et al. 2015. For this to occur, a different type of bridge vector that readily feeds on reptiles and birds must be involved. Of particular note is that in central Alabama, two snake species, *Agkistrodon piscivorus* and *Agkistrodon contortrix* were found to be positive for EEEV RNA, which led researchers to suggest that these two snakes serve as the main over-wintering hosts (Bingham et al. 2012).

One mosquito species that may be an important bridge vector is *Culex erraticus.* This is due to its high abundance at epizootic sites of EEEV, the fact that viral development is a frequent occurrence in this species, and because of its feeding patterns, which includes feeding on mammals, birds, and ectotherms (Irby and Apperson, 1988; Robertson et al., 1990, Bingham et al. 2015). Two other mosquito species that are

potential bridge vectors are *Aedes albopictus* and *Culex nigripalpus*, for reasons similar to *Cx. erraticus* (Day 1997, Gratz 2004).

EEEV is considered one of the most medically important encephalitic viruses in the eastern part of the United States due to its high virulence and high mortality rate. In order to completely understand the virus, and hopefully control its spread, we must first gain understanding of the behavior and feeding choices of the vectors, the reservoir hosts, and the interactions between the two species (Graham et al. 2012). Much of the research centered around EEEV has not been conducted in Georgia, thus the contributory roles of locally abundant vectors and potential vertebrate hosts are poorly documented. In this study, the goal was to further the understanding of the transmission cycle of EEEV by testing the response of mosquitoes to different snake odors, with the hopes of determining if these mosquitoes prefer certain snake odors. To do so, we compared the attractiveness of snake odorants, including establishing the attractiveness of snake skins and comparing the attractiveness of non-venomous to venomous snakes, and venomous snakes to each other. We hypothesized that odorants produced by snakes from the genus *Agkistrodon* (Copperheads and Water Moccasins [Cottonmouths]) would attract more mosquitoes than other snakes or controls.

Materials and Methods

Initially, mosquitoes were sampled with a vacuum aspirator (John Hock Co.) underneath a bridge located on Akins Pond Road in Statesboro, Georgia, with the hopes of collecting *Cx. erraticus*. Unfortunately, collection attempts were never adequately successful. These attempts were stopped, and the focus was moved to *Ae. albopictus.*

Eggs of this species were obtained from a colony originating in Raleigh, NC (from Charles Apperson, North Carolina State University) and placed in an enamel pan with room temperature water to hatch, along with sufficient slurries of liver powder (ICN Nutritional Biochemicals) in water to promote larval growth but which did not produce bacterial overgrowth. After maturation to pupae, they were collected using a disposable pipet, transferred into small beakers, and placed into 12x12x12 inch collapsible mosquito cages (Bioquip) kept at 80% RH, 25ºC, and 16:8 L:D photoperiod. Behavioral assays using the olfactometer with these mosquitoes were unsuccessful (Table 3), so additional mosquito larvae were collected from stagnant water in a wheelbarrow in Clito, Georgia during February and April 2018, and reared under similar conditions until emergence. These were subsequently identified as *Cx. nigripalpus.* These larvae were also placed into a 12x12x12 inch collapsible cage, and allowed to develop into adults used for assasys.

Snake skins were obtained from the Georgia Southern University Wildlife Education Center (courtesy Scott Courdin). Skins were collected immediately after shedding and stored frozen in individual plastic bags. Snake skins used included the Eastern king snake (*Lampropeltis getula*), corn snake (*Pantherophis guttatus*), Eastern indigo snake (*Drymarchon couperi*), Florida pine snake (*Pituophis melanoleucus mugitis*), Eastern hognose snake (*Heterodon platirhinos*), timber rattlesnake (*Crotalus horridus*), pygmy rattlesnake (*Sistrurus miliarius*), Eastern diamondback rattlesnake (*Crotalus adamanteus*), water moccasin (*Agkistrodon piscivorus*), and copperhead snake (*Agkistrodon contortrix*). All snake skins were stored frozen until use in olfactometer assays.

To determine if *Cx. nigripalpus* showed a preference for particular snake odors, bioassays were completed, and the behavioral response of each mosquito was recorded. These bioassays were completed using a 1x1x2 (HxWxL) feet dual choice olfactometer containing a vertical barrier half the olfactometer into two separate chambers (Figure 3). The olfactometer had 10 1/4 in. holes drilled into the top of each side of the back to improve air flow, and the holes were covered with mesh to prevent mosquito egress. The bioassays were completed at 25^{o}C, with the olfactometer placed 20 cm above the table top in order to improve air flow. A coin flip was used to randomly assign the snake skins to one of the two sides of the olfactometer. Snake skin were placed in 100 mL beakers for assays; for controls, empty beakers were placed in the identical position on the opposite side of the olfactometer. Small groups of *Cx. nigripalpus* (3-5 mosquitoes) were transferred to the olfactometer and released at a designated "start line," which was approximately halfway between the entrance of the olfactometer and the dividing panel (about 6 inches). The initial landing locations of the mosquitoes were recorded, and the locations were recorded once again after 5 minutes. The locations were recorded as chamber 1, which contained the snake skin, or chamber 2, which was empty. The mosquitoes could also have what was considered "no response," in which the mosquito did not choose a chamber, but instead chose to land in other areasc of the olfactometer (see Figure 3). Trials were repeated a total of 3 times for each bioassay. Between assays, the olfactometer was aired out by setting it vertically with the upper end open.

Bioassays were first completed to determine if *Cx. nigripalpus* showed a response to each of the individual snake species. Each snake skin was tested against an empty chamber (Table 1). For assays testing attractiveness of individual skins versus empty

controls, whole snake skins were used (range: 0.5 g -10.5 g). Based on the results from these bioassays, additional bioassays were completed to determine the response of *Cx. nigripalpus* to two different snake odors, with 0.5 g samples of snake skins used for each type of snake (Table 2). Calculations were made to determine the proportion of mosquitoes that chose chamber 1, 2, or neither. The results of these were analyzed using Chi-square tests with VassarStats (http://vassarstats.net/).

Results

A total of 158 mosquitoes were tested in 13 separate bioassays. The proportions of mosquitoes that chose each chamber are shown in Table 1. After analyzing the total number of mosquitoes across the bioassays, more mosquitoes were more attracted to the chamber with the snakeskin in it (proportion of total = 0.32) than the empty chamber (0.25), but the difference in response was not statistically significant ($n=116$, $X^2(2)=3.74$, $p>.05$) (Figure 1). Also, more mosquitoes were more attracted to the snake skin chamber when there was a venomous species present (0.35) compared to a nonvenomous snake's skin (0.31).

In the Eastern diamondback vs. empty, cottonmouth vs. empty, and copperhead vs. empty bioassays, after 5 minutes the mosquitoes were more attracted to the chamber with snake skin in it at higher proportions than the other bioassays (0.42, 0.33, and 0.50 respectively). Based on these results, Eastern diamondback, cottonmouth, and copperhead were tested against each other (Table 2). When comparing the results, the mosquitoes were more attracted to the copperhead than the Eastern diamondback (0.42

vs. 0), the cottonmouth than the Eastern diamondback (0.53 vs. 0.13), and the copperhead than the cottonmouth (0.27 vs 0.20). None of these results were statistically significant ($n=12$, X^2 $(2)=2.5$, $p>0.05$; $n=15$, X^2 $(2)=3.6$, $p>0.05$; $n=15$, X^2 $(2)=2.8$, $p>0.05$, respectively).

Discussion

When analyzing the snakeskin v. empty bioassays, in general more mosquitoes had chosen a chamber at the end of 5 minutes as compared to the initial response, and mosquitoes responded more frequently to the chamber that contained a snake skin rather than the empty chamber. Also, mosquitoes responded more strongly to the venomous snake skins as compared to the nonvenomous snake skins, likely due to both the quantity and composition of the scent of the snake skins themselves; the venomous snake skins had a noticeably stronger odor than their nonvenomous counterparts.

Cx. nigripalpus had a relatively strong response to the Eastern diamondback, copperhead, and cottonmouth snake skins when tested individually. However, when the Eastern Diamondback snake skin was tested against the copperhead and cottonmouth skins, the mosquitoes responded more strongly to both copperhead and cottonmouth snakes compared to the Eastern diamondback. When the copperhead and cottonmouth were compared against each other, the difference in response was minor (0.27 and 0.20, respectively), but the copperhead was the more attractive snake skin. These responses suggest that *Cx. nigripalpus* mosquitoes are more attracted to venomous snakes from the genus *Agkistrodon* than they are to venomous snakes of the genus *Crotalus* (rattlesnakes).

Approximately 58% of the mosquitoes chose one of the chambers, rather than failing to enter one of the two chambers; this percentage was greater than the results observed by Wells (2015), who had approximately a 49% response rate when studying the response of *Cx. erraticus* mosquitoes to different snake odors. The *Cx. erraticus* mosquitoes used in Wells's study were more attracted to the chamber with the snake skin in it 23% of the time and were more attracted to the empty chamber 25%, as compared to the *Cx. nigripalpus* mosquitoes used in this study, which were more attracted to the snake skin chamber 32% of the time and were more attracted to the empty chamber 25% of the time (Wells, 2015). The general improved response of mosquitoes could have been due to modifications of the olfactometer; in Wells's study, the olfactometer did not have holes in it, so there was little or no air flow. Multiple efforts were made to ensure adequate air flow inside of the olfactometer for this study. Holes were drilled into the back panel, the bioassays were completed inside of the incubator, which has a fan in it, and the olfactometer was also placed 20cm above table height because the air flow from the fan was stronger. Additionally, Wells did not have access to snake skins from *Akgistrodon* species, the two snakes whose skins were most attractive in this study.

Out of the 10 different snake skins tested, *Cx. nigripalpus* showed the best response to the cottonmouth and copperhead snakes. These findings support the data found from blood samples of snakes in Alabama, which suggests that snakes from the genus *Agkistrodon* may serve as the over-wintering hosts for EEEV (Bingham 2012). In the Tuskegee National Forest, cottonmouth snakes represented the majority of the reptilian biomass sampled in studies completed by Bingham et al. (2012) and Graham et al. (2012). Studies suggest that snakes of the genus *Agkistridon* are commonly exposed to

EEEV in their environment, and the hibernation and emergence patterns of female mosquitoes and cottonmouths are similar enough that cottonmouths are most likely very common hosts for mosquitoes exiting diapause (Graham et al., 2012). Furthermore, cottonmouth and copperhead snakes were both found to have detectable levels of EEE virus (not just antibodies) in blood samples collected in another study in the Tuskegee National Forest (Bingham et al., 2012).

This study indicates that *Cx. nigripalpus,* a mosquito thought to be involved in the transmission of EEEV, is attracted to snakes that have been suggested as the over-wintering hosts for EEEV (cottonmouth and copperhead snakes). To improve this study, larger sample sizes could have been used. Although proportions of mosquitoes attracted to copperhead and cottonmouth snakes were suggestive, when calculating chi-square values, the small sample sizes did not provide adequate statistical power to provide confidence in statistical inference. For example, for the comparison between the response to cottonmouth snakes and eastern diamondback rattlesnakes, for sample sizes 10 times larger than what were used, but maintained the same ratio of responses (i.e., if the results were 80 and 20 instead of 8 and 2), the resulting chi square value would have indicated highly significant results ($p<0.0001$).

In the future, additional studies should be completed in order to improve the quality of the results. A larger sample size should be used, as the data will be more representative of the entire population. In this study, the response of mosquitoes was recorded after 5 minutes. A longer response time could be used in future studies, but according to Wells, who completed bioassays for a total of 20 minutes, roughly 80% of the mosquito activity occurred within the first 5 minutes (Wells, 2015). In order to better

control for response rates, bioassays could be completed in the same time period each day. Additionally, air flow can be created using a fan, which would provide for better air flow than the fans inside of the incubator. Live snakes should also be used in future studies in addition to snake skins, to control for attractiveness of live animals relative to the odor relicts represented by snake skins, and additional snake species, particularly non-venomous water snakes such as the banded water snake (*Nerodia fasciata*), a common aquatic snake throughout southeast Georgia, should also be included. Overall, this would help clarify the relative importance of odorants compared to the contributions of motion, carbon dioxide output, bacterial communities found on the skins of live snakes and the thermal image provided by a live organism. Additionally, chemical analysis of volatile compounds is likely a productive avenue of investigation; chemical separation techniques could enable the isolation of specific attractant compounds associated with *Agkistrodon* snakes in particular from the variety of volatile compounds typically associated with the surface of organisms.

Future studies should include different species of mosquitoes, as *Cx. nigripalpus* is not the only mosquito species associated with the transmission of EEEV. As stated previously, a potential bridge vector for EEEV is a mosquito that is in high abundance at epizootic sites of the virus that does not have selective feeding patterns (a mosquito that feeds on mammals, birds, and ectotherms). *Cx. erraticus* is a species that fits this description (Bingham et al. 2015), along with *Ae. albopictus* and *Cx. nigripalpus* (Day 1997, Gratz 2004). *Cx. territans* is also a mosquito species that has been found to seek blood from multiple vertebrate classes (Irby and Apperson, 1988; Shephard et al., 2016) and feeds significantly on both reptiles and birds, suggesting it may also play a role in

movement of EEEV from reptile to bird populations. It is important for future studies to consider all potential vectors of EEEV, in order to determine if these species are actually attracted to the snake species that are considered to be the over-wintering reservoirs for the virus.

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Tables and Figures:

Table 1. The response of *Cx. nigripalpus* to various snake odors vs. an empty chamber over 5 minutes (presented as proportions).

Bioassay	Initial Response		Response After 5 Minutes	
	Chamber 1	Chamber 2	Chamber 1	Chamber 2
Hognose v. Empty	0.22	0.00	0.22	0.22
King v. Empty	0.11	0.33	0.22	0.56
Corn v. Empty	0.33	0.17	0.50	0.33
Indigo v. Empty	0.25	0.33	0.25	0.33
Florida Pine v. Empty	0.33	0.33	0.33	0.25
Timber v. Empty	0.33	0.25	0.47	0.08
Pygmy v. Empty	0.08	0.33	0.08	0.42
E. Diamondback v. Empty	0.50	0.00	0.42	0.08
Cottonmouth v. Empty	0.42	0.25	0.33	0.17
Copperhead v. Empty	0.57	0.21	0.50	0.29

Table 2. The response of *Cx. nigripalpus* to two different snake odors (presented as proportions, chamber number listed as a subscript).

Bioassay	Initial Response		Response After 5 Minutes	
	Chamber 1	Chamber 2	Chamber 1	Chamber 2
$Copperhead_1$ v. $Cottonmouth_2$	0.33	0.20	0.27	0.20
E. $Diamondback_1$ v. $Copperhead_2$	0.00	0.58	0.00	0.42
E. $Diamondback_1$ v. $Cottonmouth_2$	0.20	0.47	0.13	0.53

Table 3. The response of *Ae. albopictus* to various snake odors vs. an empty chamber over 5 minutes (presented as proportions).

Bioassay	Initial Response		Response After 5 Minutes	
	Chamber 1	Chamber 2	Chamber 1	Chamber 2
King v. Empty	0.00	0.08	0.00	0.08
Corn v. Empty	0.08	0.17	0.08	0.17
Indigo v. Empty	0.08	0.00	0.08	0.00

Figure 1. Proportions of mosquitoes to choose a chamber across all snake vs. empty bioassays. (n=116, X^2 (2)=3.74, p>0.05).

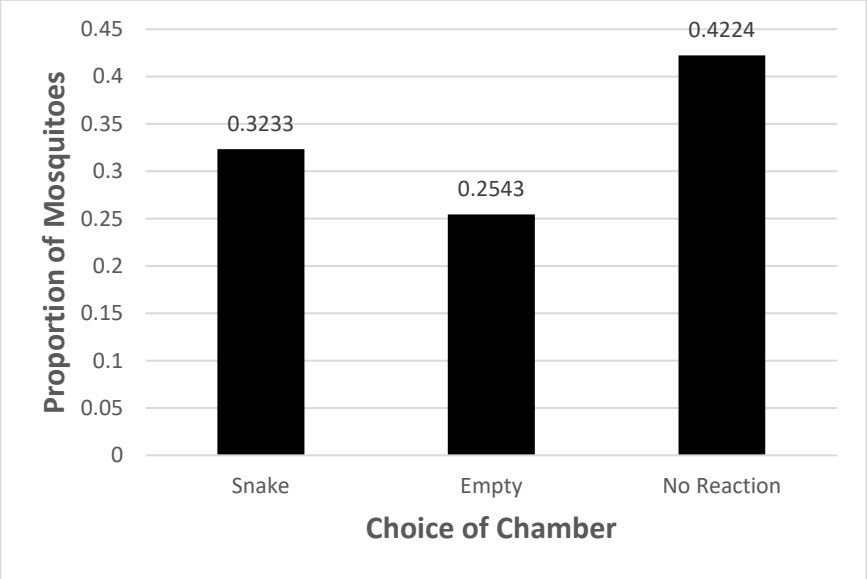
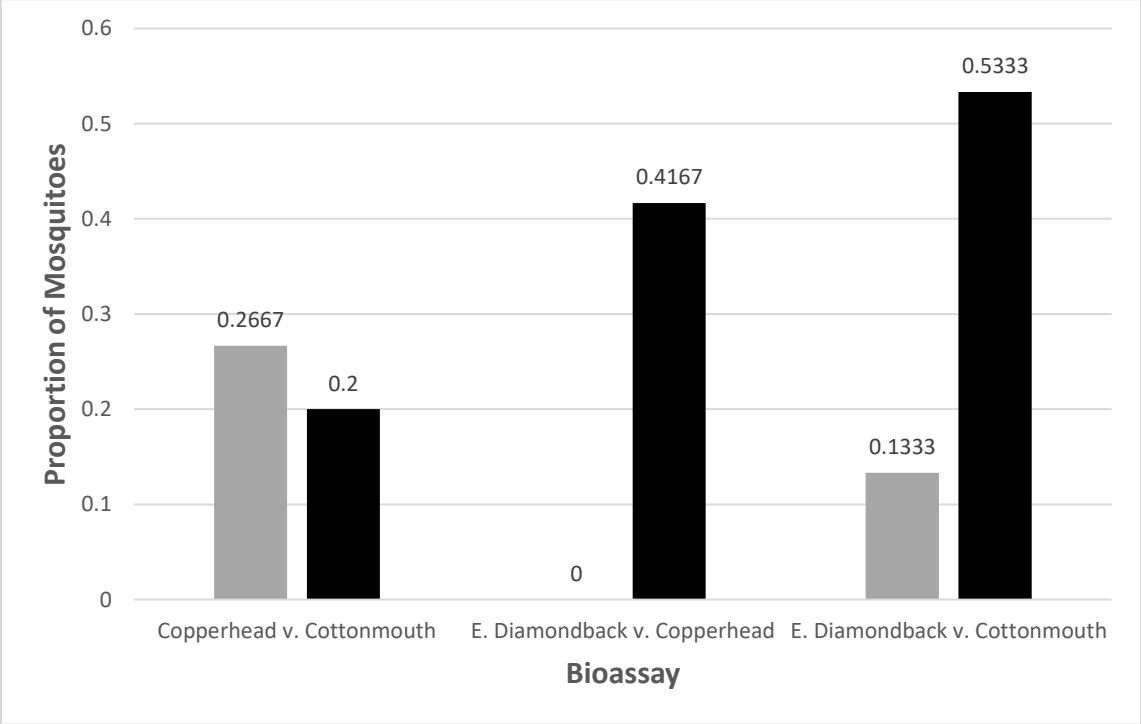
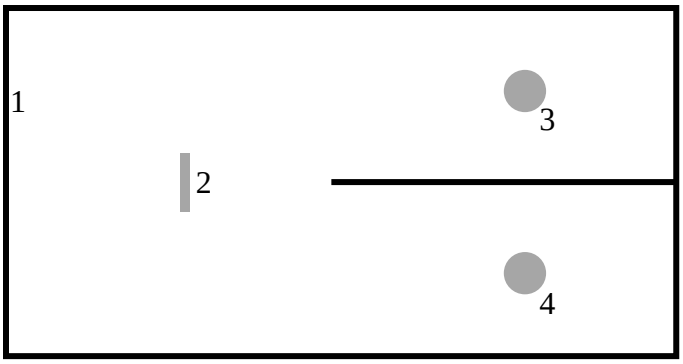


Figure 2. The response of mosquitoes when presented with two choices of snake skins.



Copperhead v. Cottonmouth: (n=15, $X^2(2)=2.8$, $p>0.05$)
E.Diamondback v. Copperhead: (n=12, $X^2(2)=2.5$, $p>0.05$)
E. Diamondback v. Cottonmouth: (n=15, $X^2(2)=2.5$, $p>0.05$)

Figure 3. A labeled diagram of the box olfactometer. Mosquitoes could choose a chamber (fly into the left or right side of the olfactometer), or they could have no response, in which they fly around the entrance of the olfactometer instead of choosing a side.



1. The entrance of the olfactometer
2. The "start line," approximately 6 inches from the entrance
3. The left "chamber"
4. The right "chamber"