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# The Behavioral Response of *Culex erraticus* to Different Snake Odors

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## **The Behavioral Response of *Culex erraticus* to Different Snake Odors**

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in the  
Biology Department.

By  
Lindsey Wells

Under the mentorship of Dr. William Irby

### **ABSTRACT**

Eastern equine encephalitis virus (EEEV) is an arbovirus that can cause fatal infections in humans and horses. Unfortunately, the transmission mechanisms of this virus are still largely unknown. *Culex erraticus* displays a strong potential for serving as a vector of EEEV because of its indiscriminate feeding pattern and abundance in areas with the highest prevalence of infection. However, *Culex erraticus* is incapable of over-wintering the virus, yet EEE recurs each spring. Snakes may play an important role in over-wintering the virus, and certain snake species may be infected more frequently than others. This study was conducted to determine if *Culex erraticus* showed a behavioral response to odors released by snake skins of different species. *Culex erraticus* mosquitoes were collected in Bulloch County, Georgia, and bioassays were completed comparing the response of the mosquitoes to each snake skin alone and then comparing the response between two snake species. *Culex erraticus* did not show a strong behavioral response to any of the snake skins tested in this study. This may suggest that *Culex erraticus* is not attracted to the odor released by these snake species. It may also suggest that if odor does attract *Culex erraticus* to snakes, the attracting odor is not solely produced by the skin. Future studies should be conducted using skins of other snake species, especially those shown previously to have active viruses or antibodies against EEEV. Also, bioassays conducted on intact snakes would help indicate if factors other than snake skin odor attract *Culex erraticus*.

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## INTRODUCTION

Eastern equine encephalitis virus (EEEV) is an arbovirus that is transmitted by mosquito vectors to a variety of hosts including birds, small mammals, and, more recently discovered, reptiles (Calisher 1994, Bingham et al. 2012). Since first isolating the microbe in 1933, eastern equine encephalitis virus has been of particular concern to those living along the eastern coast of the United States and can cause severe illness in both horses and humans (summarized in Clements 2012, Calisher 1994). Common signs of an infectious horse include fever, loss of appetite, weakness, and eventually more serious symptoms such as lack of coordination, blindness, and convulsions. In 75-90% of cases, death occurs, sometimes before any symptoms arise (APHIS 2008). When a human becomes infected with EEEV, the outcomes of the disease highly vary. If the infection remains systemic, the individual may show symptoms such as chills, a low fever, and malaise for a couple of weeks and then recover. However, if the infection becomes encephalitic, symptoms worsen to include a high fever, headaches, vomiting, and diarrhea. Central nervous system damage frequently occurs and tremors, muscle spasms, paralysis, brain lesions, and death often result. Among those that recover from the infection, intellectual disabilities and nervous system dysfunctions are common (Calisher 1994). An average of 8 human cases is reported each year in the United States with the majority of cases occurring in Massachusetts and Florida (Figure 1). While the prevalence of EEE is much lower among humans than horses, EEEV is considered to be one of the deadliest arbovirus transmitted by mosquitoes to humans with a mortality rate of 33% (CDC 2015; summarized in Clements 2012).

As shown in previous studies, EEEV is primarily transmitted by *Culiseta melanura* in North America (Armstrong & Andreadis 2010, Cohen et al. 2009; summarized in Clements 2012). However, this mosquito species feeds almost exclusively on birds and is therefore unlikely to serve as a vector between avian and mammalian species (Calisher 1994). In order to identify vectors capable of interspecific transmission, blood meals of various mosquito species have been determined. As recent studies conducted in Tennessee suggest (Cohen et al. 2009), one mosquito species, *Culex erraticus*, displays a strong competency of serving as a vector between species. *Culex erraticus* is an indiscriminate feeder with its blood meals originating from mammalian, avian, and reptilian species (summarized in Clements 2012). According to Cohen et al. (2009), from a sample collected in Tennessee, *Culex erraticus* acquired only 16% of its blood meals from avian species. The remaining blood meals were acquired from non-avian species, including 7% from reptiles and snakes and the majority from mammals. The diversity of its blood meal hosts indicates that *Culex erraticus* may be responsible for transmitting EEEV across species. Additionally, *Culex erraticus* is one of the most abundant species in many areas of the southeastern United States where EEEV cases are commonly reported (Cupp et al. 2003, Cohen et al. 2009).

In the *Culex erraticus* species, only inseminated females enter diapause in November (Breeland et al. 1961). Because these mosquitoes have never consumed a blood meal, the individuals should not be infected with EEEV. Therefore, *Culex erraticus* is incapable of over-wintering the virus and causing the reoccurrences of EEEV noted each spring. In response, research has been conducted to determine the competency of commonly infected animals to serve as the over-wintering host for EEEV. Birds are not

likely over-wintering the virus because their immune systems show a strong response to the pathogen, clearing it from the bird's bloodstream and building antibodies to prevent future infections (summarized in Clements 2012). Therefore, birds do not remain viremic for the length of time necessary to serve as an over-wintering host.

Recent studies have shown that ectotherms, specifically snakes, may play an important role in over-wintering the virus. When inoculated with EEEV (White et al. 2011), snakes not only show susceptibility to the virus but can remain viremic throughout hibernation. Recently, other studies have shown that snakes are also naturally infected with EEEV in the wild and suggest that certain species are infected more frequently than others (Graham et al. 2012, Bingham et al. 2012). The presence of antibodies against EEEV, indicating prior exposure to the virus, was detected in 35% of all serum samples taken from 9 snake species in Alabama (Graham et al. 2012). The following year, in the serum samples of two snake species *Agkistrodon piscivorus* and *Agkistrodon contortrix*—commonly referred to as the cottonmouth and copperhead, respectively—active infections were detected for the first time in snakes (Bingham et al. 2012). The detection of antibodies against EEEV and the virus itself in these two wild snake species suggests that the animals play an important role in the over-wintering of EEEV.

Previous studies have shown multiple host-feeding strategies that mosquitoes have adapted to help locate their next blood meal. One important strategy identified is the dependence of mosquitoes on odors secreted by the host (summarized in Clements 1999). The purpose of this study was to determine if *Culex erraticus* showed a behavioral response when exposed to odors released by snake skins of various species. A behavioral response to snake skins would support the previous studies suggesting that snakes may be

serving as the over-wintering host of EEEV. If *Culex erraticus* shows a stronger response to certain snake skin odors over others, this would help identify what snake species show the strongest potential of over-wintering the virus.

## **MATERIALS AND METHODS**

Mosquitoes were collected from beneath two bridges in Bulloch County, Georgia. One bridge was located on Akins Pond Road and the other on Lakeview Road, and both bridges overpass Mill Creek. The mosquitoes were collected from mid-August to early November 2015 using a backpack vacuum and were transferred into a 12x12x12 inch collapsible cage purchased from BioQuip. *Culex erraticus* mosquitoes were then identified based on morphological characteristics such as alternating dark brown and light tan coloration on the abdomen and 3 white scales present on the thorax. Those identified as *Culex erraticus* were transferred to a separate cage for use in future bioassays. All mosquitoes were fed 10% sugar water and were stored in an incubator at 27°C and 80% relative humidity.

Fresh snake skins were obtained from the Wildlife Education Center at Georgia Southern University (Statesboro, Georgia). The snake skins originated from a variety of species including the corn snake, hognose snake, timber rattlesnake, Eastern kingsnake, Eastern indigo snake, and Florida pine snake. All of these species are currently present in Georgia (SRELHERP 2015). All snake skins were stored frozen.

In order to determine if *Culex erraticus* showed a response to the odor released by the skins of different snake species, multiple bioassays were completed and the behavioral response of each mosquito recorded. Bioassays were conducted using a dual-choice box olfactometer (1x1x2 feet). For each trial, the placement of the snake skin(s)

was randomly assigned (coin flip) to one of the two chambers (sides) in the box olfactometer. Ten *Culex erraticus* mosquitoes were transferred into a small jar. The jar was then placed at a designated “starting line” in the box olfactometer, and the lid was removed. The mosquitoes were allowed twenty minutes to choose a chamber in the box olfactometer, and the location of each mosquito was recorded after every 5 minutes. The locations were recorded as chamber 1, chamber 2, or neither chamber (mosquitoes remained near the start line or flew to the back of the box). Trials were replicated five times for each bioassay, resulting in a total of 50 mosquitoes tested in each bioassay.

The first set of bioassays served as a control to determine if *Culex erraticus* showed a behavioral response to each of the snake species. For these bioassays, each snake skin was tested against an empty chamber (air) (i.e., corn snake skin v. empty; Table 1). Using the results of the first set of bioassays, a second set was performed to determine if *Culex erraticus* showed a stronger response to a certain species of snakes over another. For these bioassays, two snake skins were tested against one another (i.e., corn snake skin v. timber rattlesnake skin; Table 2). An additional bioassay was completed following the first bioassay (corn snake skin v. empty). This bioassay compared the behavioral response of *Culex erraticus* to corn snake skin odors at different temperatures and humidity. Identical bioassays were conducted (using corn snake skins versus an empty chamber) at room temperature and in the incubator. Results of this bioassay were used to determine the temperature and humidity of subsequent bioassays.

The proportion of mosquitoes that chose chamber 1, chamber 2, and neither chamber was calculated for each bioassay. The results were then analyzed using Chi-square tests.

## RESULTS

A total of 439 mosquitoes were tested in 9 separate bioassays (number less than 450 because some mosquitoes were killed in the transferring process). The percentages of mosquitoes that chose each chamber during the various bioassays are shown in Table 1.

The intermediate bioassay performed on corn snakes showed that a higher percentage of mosquitoes chose one of the two chambers (corn snake skin or empty) when performed at room temperature (0.469) rather than in the incubator (0.277) (Figure 2).

When analyzing the total number of mosquitoes across all bioassays, a slightly greater percentage chose the empty chamber (0.252) than the snake skin chamber (0.235) by the completion of the trial. However, this difference was not statistically significant ( $n=294$ ,  $\chi^2=0.175$ ;  $p=0.676$ ) (Figure 3).

As time progressed, the overall percentage of mosquitoes choosing both chambers increased, from 0.197 and 0.204 to 0.235 and 0.252 for the snake skin chamber and empty chamber, respectively. However, roughly 80% of the mosquitoes that chose a chamber did so within the first five minutes of the trials (Figure 4).

The timber rattlesnake skin v. empty bioassay was the only individual bioassay that showed results differing from those seen when analyzing the overall response across all bioassays. In the timber rattlesnake skin v. empty bioassay, a greater percentage of mosquitoes chose the snake skin chamber (0.300) than the empty chamber (0.120) by the completion of the trials. This difference was significant ( $n=50$ ;  $\chi^2=3.857$ ;  $p=0.0495$ ) (Tables 1 and 2; Figure 5).

Based on this finding, a second set of bioassays was conducted comparing the response of *Culex erraticus* to the timber rattlesnake skin versus other species of snake skins. The timber rattlesnake skin was tested against the corn snake skin and then the king snake skin because these two showed the lowest percentage of response during the snake skin v. air bioassays (only 0.163 and 0.220 for the corn and king snake skin, respectively) (Table 1). However, when the timber rattlesnake skin was tested against these two “other” snake skins, a greater percentage of mosquitoes chose the “other” skin (timber v. corn: 0.28, timber v. king: 0.313) than the timber rattlesnake skin (timber v. corn: 0.22, timber v. king: 0.188) by the completion of the trials (Table 2). However, neither bioassay showed a statistically significant difference (timber v. corn: n=50;  $\chi^2=0.36$ ; p=0.5485, timber v. king: n=48;  $\chi^2=1.5$ ; p=0.2207) (Figure 5).

## DISCUSSION

After the first bioassay was complete (corn snake skin v. empty), it was clearly evident that the mosquitoes were not responding as expected to the presence of a snake odor. With only 46% of the mosquitoes choosing either chamber in this bioassay, the majority of the mosquitoes were quite inactive for the full twenty minutes. Based off the results of this first bioassay, an intermediate bioassay was added to determine if temperature and humidity were affecting the activity of the mosquitoes. Because the incubator more closely mimicked the conditions of the months that mosquitoes are more abundant and active in nature (27°C and 80% humidity), the mosquitoes were expected to show a greater response to the odor in the conditions of the incubator when compared to room temperature (Breeland et al. 1961). After running an identical bioassay (corn snake skin v. air) in the incubator, the results contradicted the prediction. *Culex erraticus* was

even less active in the incubator than at room temperature, and therefore, all subsequent bioassays were conducted at room temperature. This discrepancy may be explained by a complication experienced during the transfer process. When transferring the mosquitoes in the incubator, moisture collected within the transfer tube because of the high humidity. Because of this, the mosquitoes came into direct contact with the moisture and often stuck to the sides of the tube, killing some mosquitoes and possibly stunning others. Also, the temperature of the room was still relatively warm (around 21°C) and would mimic temperatures near dusk and dawn during spring and summer months, the times *Culex erraticus* has been shown to engage in host-seeking behavior (summarized in Clements 1999; Breeland et al. 1961).

Analysis of all the snake skin v. empty bioassays showed a similar trend to that observed in the initial corn snake skin v. empty bioassay. Overall, less than half of all mosquitoes had chosen a chamber by the completion of the trials. As time progressed, the mosquitoes did become more active and chose a chamber. Therefore, allowing mosquitoes more time to choose a chamber may provide different results. However, roughly 80% of this activity occurred within the first five minutes of the trials. This finding suggests that time most likely did not strongly influence the lack of response observed.

When evaluating the overall results of the snake skin v. empty bioassays, no statistically significant difference was seen between the responses of *Culex erraticus* to snake skin odors versus ambient air. This trend was apparent for each individual bioassay as well with the exception of the timber rattlesnake skin. However, when the skin from this species was tested against that of other snake species, the difference in behavioral

response of mosquitoes to the timber rattlesnake was no longer apparent. The findings of these bioassays can be explained by previous studies. One study (Graham et al. 2012) sampled various reptiles and amphibians and found timber rattlesnakes that were seropositive for EEEV antibodies, indicating exposure to the virus. However, a second study (Bingham et al. 2012) sampled different snake species and detected active viruses in some of the serum samples. Although the timber rattlesnake was sampled, it did not test positive for active virus like other species. Together, these findings suggest that the timber rattlesnake does not show the strongest potential of serving as an over-wintering host of EEEV, and the mixed results of the present study further support this idea.

Rather than the timber rattlesnake, the studies previously mentioned indicate that the cottonmouth (*Agkistrodon piscivorus*) and the copperhead (*Agkistrodon contortrix*) should be investigated further as potential over-wintering host since a relatively large percentage of the sampled snakes of these species had antibodies against EEEV and active virus was detected in serum samples of these species only (Graham et al. 2012; Bingham et al. 2012). Unfortunately, skins of *Agkistrodon piscivorus* and *Agkistrodon contortrix* were not available while completing this study. However, future work will include repeating the bioassays using skins from these two species. If snake skin odor is playing a primary role in attracting *Culex erraticus* to an over-wintering host, the odor released by the skins of *Agkistrodon piscivorus* and *Agkistrodon contortrix* will likely elicit a behavioral response of the mosquitoes.

Out of the six species of snakes that this study tested, *Culex erraticus* did not show a strong behavioral response to any. This finding suggests that *Culex erraticus* is not attracted to the odors released by the skins of the species used. Future bioassays

testing the skins of *Agkistrodon piscivorus* and *Agkistrodon contortrix* may further validate this assumption if behavioral responses are seen when using the skins of these two species. However, the lack of behavioral response to any species of snake skin may also suggest that *Culex erraticus* is not attracted to an odor released from snake skins alone. Various odors have been shown to influence host-feeding behaviors including epidermal secretions, flatus, and urinal or fecal scents (summarized in Clements 1999). Therefore, any of these odors or others released by snakes may play a more important or complementary role with odors released by the skin. In addition, the lack of behavioral response to snake skin odors could also suggest that an odor is not the primary source of attraction. Other factors such as amount of carbon dioxide produced, body size, and even color have shown correlations to behavioral response of different mosquito species (summarized in Clements 1999). Future bioassays should also be conducted on intact snakes (rather than just skins) to determine which of these hypotheses may be applicable to *Culex erraticus*.

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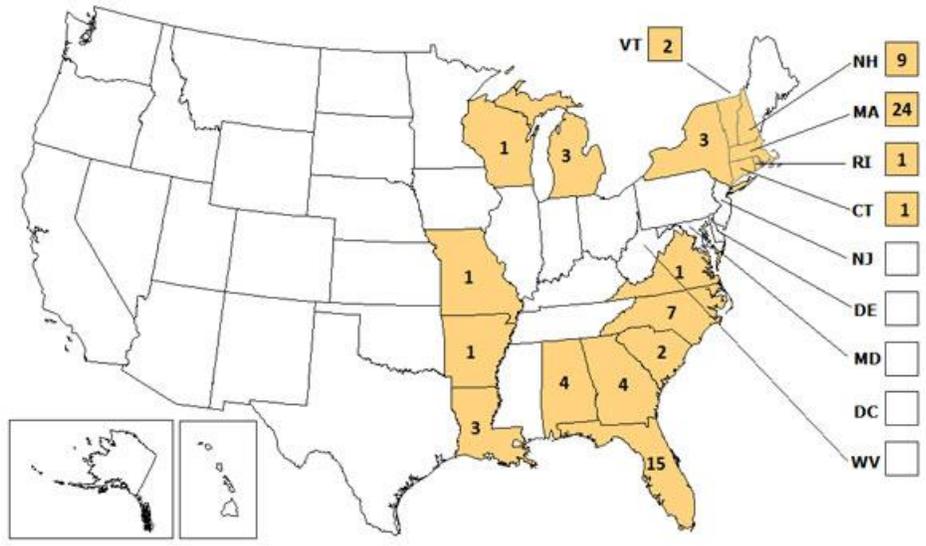
## APPENDIX

**Table 1.** First set of bioassays conducted to determine the response of *Culex erraticus* mosquitoes to different snake skin odors over a 20 minute interval (presented as percentages). Bioassay titles formatted as “Chamber 1 v. Chamber 2.”

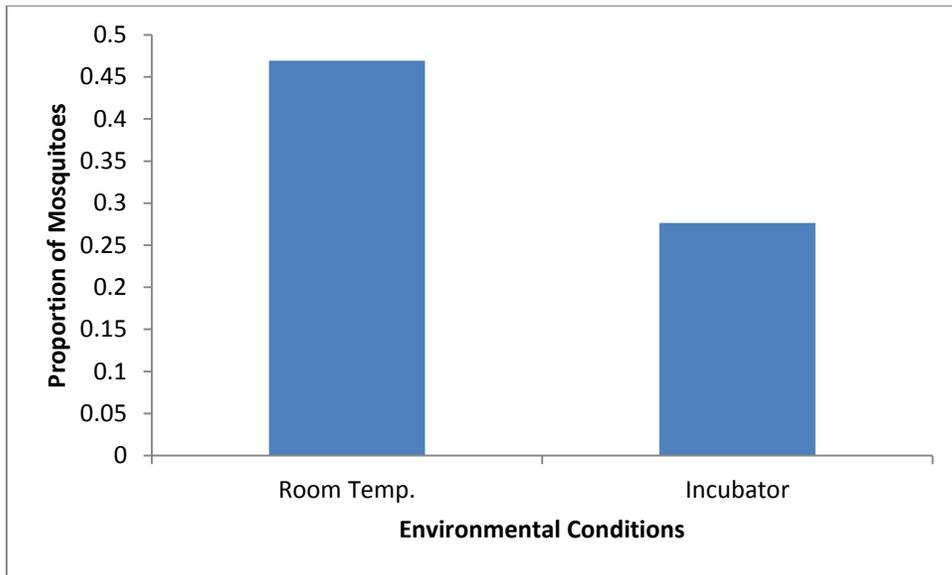
Bioassay	Time (minutes)							
	5		10		15		20	
	Chamber 1	Chamber 2	Chamber 1	Chamber 2	Chamber 1	Chamber 2	Chamber 1	Chamber 2
<b>Corn v. Empty</b>	0.143	0.204	0.163	0.245	0.184	0.286	0.163	0.306
<b>Hognose v. Empty</b>	0.271	0.229	0.208	0.250	0.250	0.250	0.271	0.271
<b>Timber v. Empty</b>	0.200	0.120	0.320	0.120	0.360	0.120	0.300	0.120
<b>King v. Empty</b>	0.200	0.240	0.140	0.260	0.160	0.260	0.220	0.260
<b>Indigo v. Empty</b>	0.188	0.208	0.229	0.250	0.229	0.229	0.250	0.271
<b>Florida Pine v. Empty</b>	0.184	0.224	0.204	0.204	0.163	0.204	0.204	0.286

**Table 2.** Second set of bioassays conducted to compare the response of *Culex erraticus* mosquitoes to timber rattlesnake skin versus other species skin over a 20 minute interval (presented as percentages). Bioassay titles formatted as “Chamber 1 v. Chamber 2.”

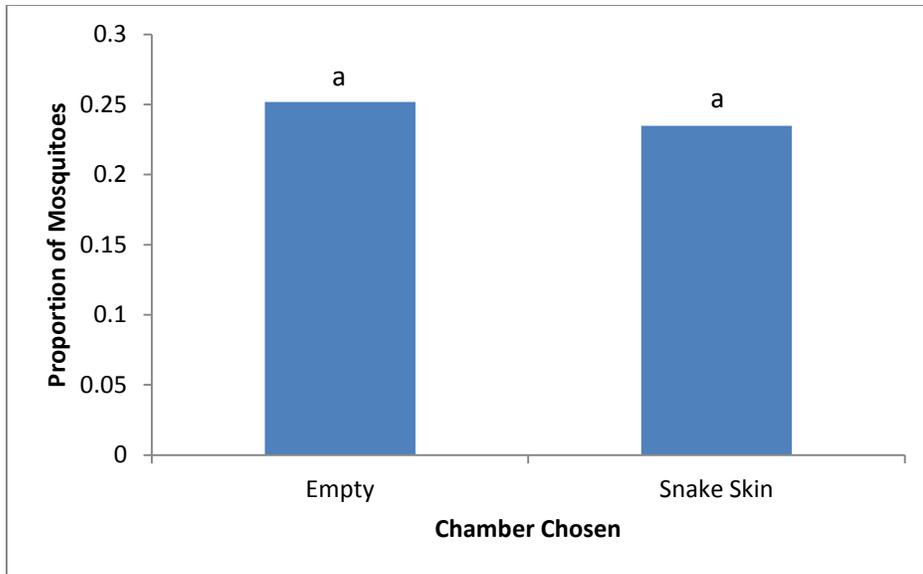
Bioassay	Time (minutes)							
	5		10		15		20	
	Chamber 1	Chamber 2	Chamber 1	Chamber 2	Chamber 1	Chamber 2	Chamber 1	Chamber 2
Timber v. Empty	0.200	0.120	0.320	0.120	0.360	0.120	0.300	0.120
Timber v. Corn	0.220	0.200	0.220	0.220	0.140	0.280	0.220	0.280
Timber v. King	0.167	0.271	0.188	0.313	0.208	0.313	0.188	0.313



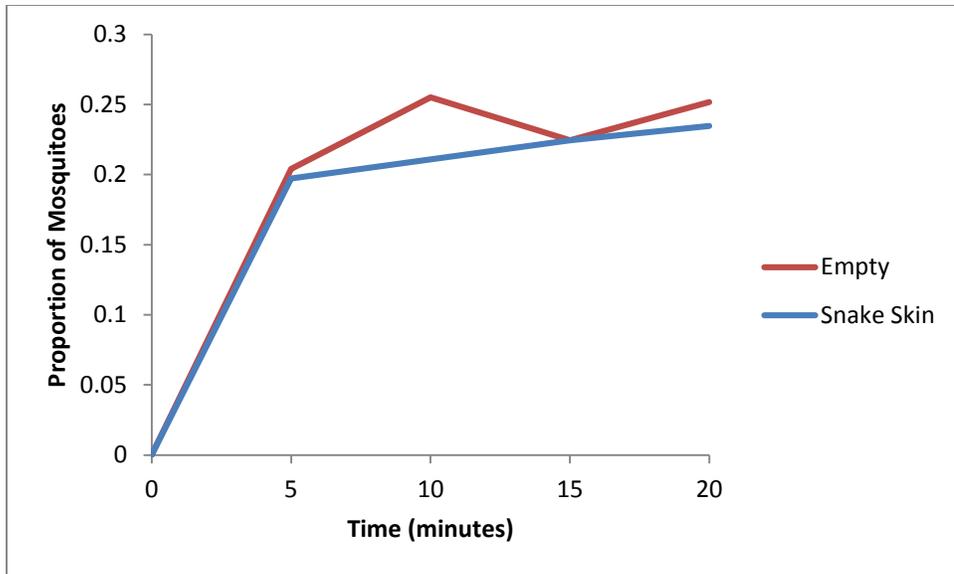
**Figure 1.** Eastern equine encephalitis human cases reported by states from 2004-2013 (retrieved from CDC)



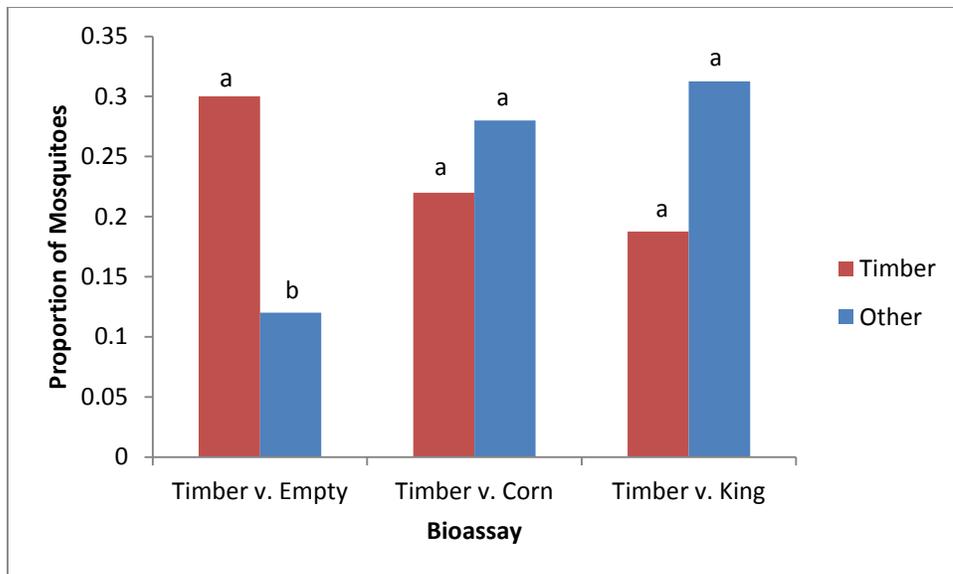
**Figure 2.** Corn snake skin v. empty bioassay completed at room temperature (n=49) and in the incubator set at 27<sup>0</sup>C and 80% humidity (n=47).



**Figure 3.** Comparison of overall proportion of mosquitoes choosing a chamber across all bioassays (n=294). No significant difference existed between chambers ( $\chi^2=0.175$ ;  $p=0.676$ ).



**Figure 4.** Change in overall proportion of mosquitoes choosing chamber over length of trial (n=294).



**Figure 5.** Three bioassays comparing the response of mosquitoes to timber rattlesnake skin vs. air and two other snake skins. Timber v. empty showed a significant difference (n=50;  $\chi^2=3.857$ ; p=0.0495) while the other two bioassays showed no difference (n=50;  $\chi^2=0.36$ ; p=0.5485 and n=48;  $\chi^2=1.5$ ; p=0.2207, respectively).