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ABSTRACT

Olfactory cues emanating from oviposition sites have strong potential as management tools for insect pests. The mosquito *Culiseta melanura* is the principal vector of eastern equine encephalitis (EEE) and utilizes regionally specific oviposition sites. In the Northeast, these are usually red maple 'crypts.' Volatile organic compounds were collected from red-maple crypts occupied by *C. melanura*. In the laboratory, these volatiles were then assessed in a behavioral bioassay to determine attraction of female *C. melanura*. Thirty-seven percent of *C. melanura* test subjects responded positively to volatiles, none responded to controls, and the other eighty-percent were unresponsive. These results suggest an olfactory role in oviposition site selection in *C. melanura*; however further testing with more robust sample sizes should be conducted. The threat of EEE transmission from this vector to mammalian hosts including humans is serious and control measures based on environmentally benign semiochemicals rather than insecticides should be developed.

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INTRODUCTION

Exploitation of olfactory attractants has long been a valuable tool in insect pest management (Navarro et al. 2009). Semiochemicals have proven their worth in the control of significant human pests such as *Aedes aegypti* (Dekker et al. 2001) and *Anopheles* species (Braks et al. 2001). As detailed by Navarro et al. (2009), the number of olfactory attractants and host-cue semiochemicals has grown significantly in recent years, and they have seen much usage across many genera within the Culicidae. However, a surprisingly small amount of work has been done with *Culiseta melanura*, which Molaei et al. (2006) showed to be the principal vector of eastern equine encephalitis (EEE). Villari et al. (1995) estimated that EEE places an annual economic burden of $1.5 million USD from medical and educational costs as well as lost revenue from the workplace. This figure does not include veterinary costs to infected horses, or lost show-horse revenue. Losses have no doubt increased since 1995.

Oviposition for *C. melanura* occurs in water-filled crypts of cypress, white cedar and/or red maple trees (Buckner et al. 2012), with red maple being the principal oviposition site in Central New York due to its relative abundance. The objective of this research was to determine if *C. melanura* females use olfactory cues associated with red maple crypts to locate oviposition sites.

METHODS

Field collection of crypt volatiles was done in Toad Harbor Swamp near Oswego, NY. Air from within the crypt was drawn through Porapak-Q and activated charcoal traps in series with an electric air pump (Demand spray pump, Flojet, Santa Ana, CA). powered by a twelve-volt deep cycle battery (Energizer, Inc., St. Louis, MO). The adsorbents (350 mg) were packed

between glass wool in separate glass tubes and washed with methylene chloride (J.T. Baker, Ultra Resi-analyzed grade, Center Valley, P.A.) before use. A small section of Teflon tubing was used to connect the two adsorbent traps. The apparatus (Fig. 1) was placed in the field on a red maple crypt in which *C. melanura* adults were observed. Sampling began at 10:00 AM on 4 October, 2013 and ended at 09:00 AM on 5 October, 2013. Only one crypt was sampled, and it was sampled once. The adsorbent tubes were sealed with Teflon tape and transported to the laboratory for extraction. The activated charcoal trap was extracted with dichloromethane (DCM 2.0 ml) and the Porapak-Q was extracted with hexane (2.0 ml) (J.T. Baker, Ultra Resi-analyzed grade, Center Valley, P.A.).

A y-tube olfactometer with adult *C. melanura* was used to assay the extracted volatiles. As the method was being developed, the initial two trials utilized a smaller olfactometer (stem length of 21 cm, arm length of 10 cm, and diameter of 3 cm). In the first trial, a mosquito breeder container (Bioquip, Inc., Rancho Dominguez, CA) was used as a holding chamber at the end of the olfactometer stem so the mosquitoes could freely enter the olfactometer stem. However, this methodology was abandoned, because the mosquitoes remained in the holding chamber. Charcoal-filtered, compressed air was humidified by bubbling in de-ionized water in an Erlenmeyer flask and passed to a flow-control splitter and then to the olfactometer arms at a rate of 6 ml/min (Geier et al. 2003). The y-tube itself was of glass construction, six centimeter diameter, twenty-four centimeter stem length, and twenty-one centimeter arm length, each arm (trials three and four). (Fig. 2). The y-tube was rinsed with DCM and hexane and then dried prior to use.

Field-collected, adult female *C. melanura* were provided by Dr. Joanne Oliver (New York State Department of Health) and used within 24 hr. Due to collection technique, a small

number of *Culex spp.* were collected alongside the *C. melanura* females. Those not used immediately were held in an environmental chamber at 12 °C and warmed to ambient temperature for two hours prior to use. Assays were performed in a dark room at ambient temperature (~20 °C). Darkness was required because *C. melanura* is most active for the first two hours following sunset (Buckner et al. 2012). Mosquitoes were tested in groups of four to sixteen as available. Before mosquitoes were introduced to the olfactometer, aliquots of volatile samples (50 µl) each from the activated charcoal and the Porapak-Q extracts were placed onto a 1 cm^2 piece of filter paper and the solvent was allowed to dry before the paper was placed in an olfactometer arm. Dichloromethane and hexane (50 µl each) were used as a control in the opposing arm. Mosquitoes were lightly anesthetized with carbon-dioxide, and then quickly transferred to the common base of the y-tube, which was then closed with fine-mesh to prevent escape. Due to the virulence of the EEE virus potentially carried by the test mosquitoes, head counts were performed before and after each assay. Mosquitoes were left in the olfactometer for two hours; their y-tube choice was recorded, and then identified to species. Individuals were used once, then euthanized with CO_2 in the olfactometer. The olfactometer was rinsed with both DCM and hexane between assays. The stimulus and control positions were changed between assays and the test stimulus was tested in different olfactometer arms to eliminate potential position effects.

RESULTS

The first trial was inconclusive. The methodology of the first trial involved the mosquito breeder container holding chamber, which prevented the mosquitoes from entering the y-tube.

Because the sample size of each trial was small, data from trials two, three and four were combined for statistical analysis.

Altogether, nineteen *C. melanura* and eight *Culex* spp. were used in the behavioral assays. Of the *C. melanura*, seven responded positively to the crypt volatiles (36.8%), none responded negatively, and twelve were unresponsive (63.2%). None of the *Culex spp.* responded positively to the crypt volatiles, six responded negatively (75%), and two were unresponsive (25%) (Table 1).

The results of the individual, non-abandoned trials were as follows. The second trial involved five *C. melanura* and two *Culex* spp. Two of the five *C. melanura* at the end of the two hour trial were found in the volatile-containing arm, and were recorded as positive responses. The other three *C. melanura* remained in the stem of the y-tube and were scored as unresponsive. Both of the *Culex* spp. chose the control arm and were recorded as negative responses. These assays were the only two trials to involve the smaller y-tube.

The third trial involved eleven *C. melanura* and five *Culex* spp. Three of the eleven *C. melanura* at the end of the two hour trial were found in the volatile-containing arm and were scored as positive responses. Eight of the eleven *C. melanura* were found in the stem of the y-tube at the end of the assay and were as unresponsive. Four of the five *Culex* spp. at the end of the assay were found in the control arm and were recorded as negative responses. The other *Culex* spp. was found in the stem of the y-tube and was recorded as unresponsive.

The fourth trial involved three *C. melanura* and one *Culex* spp.. Two of the three *C. melanura* specimens were found in the volatile-containing arm at the end of the assay, and were scored as positive responses. One of the three *C. melanura* specimens was found in the stem of

the y-tube and was scored as a neutral response. At the end of the assay the one individual *Culex* spp. was found in the stem of the y-tube and scored as a neutral response.

Statistical analysis was performed on the compiled data of trials two, three and four. A chi-square test applied to the responsive *C. melanura* (i.e., omitting the unresponsive individuals) indicates that the number responding to the crypt volatiles (7) was not significantly different from the number choosing the control (0) (χ^2=2; P=0.16).

DISCUSSION

The results of my assays reveal a trend that is indicative of olfactory cues attracting *C. melanura* to oviposition sites. Even though thirty-seven percent of the *C. melanura* specimens responded positively to the olfactants in question, it is interesting that none of the *Culex* spp. responded positively. This supports the suggestion that olfactory cues are used for oviposition site selection in *C. melanura*, and not *Culex*. As these two species do not share ovipositional sites, they would not have identical ovipositional cues, which could be derived from a variety of sources within specific sites, including microbial flora specific to red maple crypts. (Ponnusamy et al. 2010).

These data should be interpreted cautiously due to the small sample size. It is worthy to note that control for blooded vs. unblooded specimens was not conducted prior to trials due to limitations in anesthetic procedures. Any bias in regards to having had a blood meal, however, will have been limited, as only two specimens appeared to have not recently had a blood meal. Further testing with larger sample sizes and consistent methodology should be pursued.

CONCLUSIONS

The potential threat that *C. melanura* poses as a vector for EEE is staggering. It is common knowledge that *C. melanura* is the principle vector for EEE, however its precise carry-rate is still being evaluated. Recent evaluations estimate a minimum infection rate as high as 40/1,000 with sample pools testing positive for EEE in 1/7 cases (Hassan, et al. 2003). Andreadis, et al. (1998) showed that of thirty-six cases of confirmed EEE infected pools, all but one of them were populated by confirmed EEE infected *C. melanura* mosquitoes. These figures paint an ominous picture for EEE outbreaks, if populations of *C. melanura* are forced to feed on non-avian hosts. It is well documented that *C. melanura* has a strong preference for avian hosts, but will opportunistically feed on mammals, most notably horses (Molaei, et al. 2006). These opportunistic feedings have grim prognoses and are usually met with swift protective action against further incidences of mosquito biting, such as anti-insect mesh covers for the animals, which are usually successful. Such measures are a temporary solution to a problem of growing concern: lack of effective management strategies for *C. melanura*. Research on more prominent vectors of human disease such as *A. aegypti* have paved the way for research on other mosquito vectors of more regional concern. Semiochemicals, including ovposition and host attractants, promise to lead to the development of effective control measures that can be put into use to mitigate the risks of disease transmission to human populations as well as those of other non-avian hosts.

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APPENDICES

Figure 1, Extraction apparatus in field.

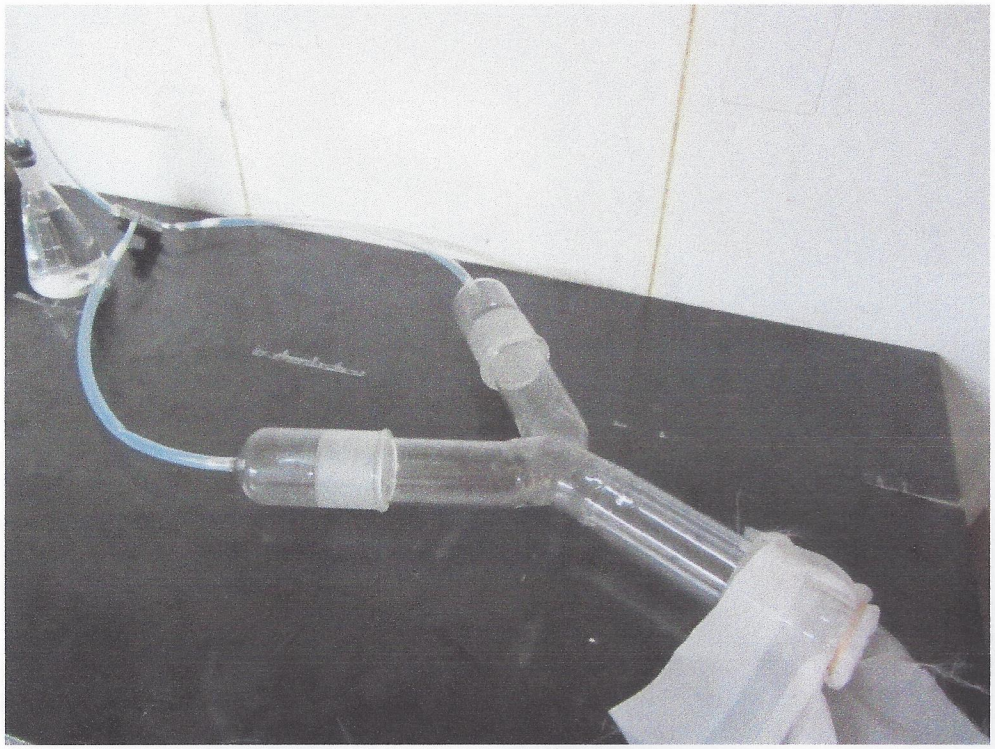

Figure 2, Behavioral bioassay apparatus (large y-tube pictured).

Table 1. Responses of *C. melanura* and *Culex* spp. to volatiles collected from red maple crypts near Oswego, NY (combined results of trials two, three, four).

Specimen type	Response		
	+	-	Null
C. melanura	7	0	12
Culex spp.	0	6	2