Short Communication

Orientation of *Anopheles gambiae* (Diptera: Culicidae) to Plant-Host Volatiles in a Novel Diffusion-Cage Olfactometer

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Received 4 June 2015; Accepted 28 September 2015

Abstract

A novel diffusion-cage olfactometer tested the responses of *Anopheles gambiae* Giles to plant volatiles. Green-leaf volatiles are often released from cut or injured plant tissue and may alter the headspace of plants used in olfactometer assays. The diffusion-cage olfactometer is designed for use with whole, intact plants, hence giving a more realistic behavioral assay. Its simple plastic construction, ease of assembly, and accommodation to whole plants makes it a useful tool for measuring mosquito orientation to plant volatiles within large enclosures. We compared its performance to that of the more commonly used T-tube wind-tunnel olfactometer, by testing the orientation of mosquitoes to volatiles of a few prevalent plants of eastern Africa reportedly utilized by *An. gambiae* for sugar: *Parthenium hysterophorus* (Asteraceae), *Ricinus communis* (Euphorbiaceae), *Lantana camara* (Verbenaceae), and *Senna occidentalis* (Fabaceae). Results indicate that the diffusion-cage olfactometer is an effective alternative to conventional wind-tunnel olfactometers, to test mosquito orientation to plant volatiles under seminatural conditions.

Key words: olfactometer, plant attractant, *Anopheles gambiae*, plant volatile, screenhouse

Disease-vectoring mosquitoes often rely on olfactory cues to locate and infect vertebrate hosts, and are therefore important in determining their vectorial capacity (Foster, 2008). Mosquito orientation and response to volatiles is most accurately measured under conditions that simulate nature as closely as possible. Laboratory-based experiments that utilize olfactometers face the challenge of recreating these conditions, because the mosquitoes are confined to small spaces. Studies that employ large indoor mesocosms or outdoor screenhouses for studying the behavior of *Anopheles gambiae* s.s. Giles and *An. arabiensis* Patton have gained prominence (Knols et al. 2002, Impoinvil et al. 2004, Ferguson et al. 2008, Helinski et al. 2008, Ng'habi et al. 2010, Stone et al. 2011, 2012a,b, Jackson et al. 2015), because they allow these malaria vectors to behave closer to the way they might in a natural setting. We describe a cage-based olfactometer that is simple in design and ideal for use with *An. gambiae* inside semifield environments such as mesocosms and large screenhouse enclosures. We used this olfactometer with whole plants to test the orientation of mosquitoes to their volatile organic chemicals that help guide them toward nectar sources. This olfactometer saves time and effort spent on direct observation and circumvents unintended and currently unknown effects of cut-plant volatiles that cannot be avoided when plant cuttings must be used in smaller olfactometers.

Materials and Methods

Mosquito Source and Production

The *An. gambiae* s.s. strain used here had been colonized by Mario Coluzzi in 1987, originating from Suakoko, Bong, Liberia, and maintained at The Ohio State University (OSU) for >10 yr. Colony adults received human bloodmeals and were maintained in accordance with OSU's Biosafety protocol No. 2005R0020 and Biomedical protocol No. 200440193. Groups of 100 first-instar larvae were transferred to pans (33 by 24.1 by 5.1 cm³) and fed a standardized regimen (Gary and Foster 2001) of powdered Tetramin fish food until pupation. Wing-length means $\pm$ SE raised under this regimen were 2.92 ± 0.014 mm for females ($n = 30$) and 2.78 ± 0.013 mm for males ($n = 30$).

Diffusion Olfactometer: Description and Procedure

The olfactometer was a modified mosquito cage of clear acrylic plastic, 66 by 50 by 67 cm³ (L by W by H; Fig. 1), serving as a trap. It was placed within a climate-controlled walk-in enclosure in a greenhouse 2.4 by 1.8 by 2.1 m (=9.1 m³), as described by Stone et al. (2009), but without feeders or dark resting sites. The ceiling of the cage consisted of two equal pieces (66 by 25 cm²), the front one

Fig. 1. Schematic diagram of diffusion-cage olfactometer, showing full assembly and plant placement. Inset is the window section showing Styrofoam barrier placement.

attached only by three 2-cm hinges to the back piece and forming an access door. The front wall had two sliding windows (20 by 22 cm²), 8 cm apart. The two windows provided mosquito entry into the cage. They were covered by solid 25- by 25-cm Styrofoam panels 2 cm thick, mounted inside the cage on four 5-cm-long spacers, attached at the corners with Plasticine modeling clay, so that there was a 5-cm gap between the front wall of the cage and all four sides of each panel. These window gaps served as the only openings through which mosquitoes gained entry to the cage interior.

Within the cage trap, humidity was raised above ambient by a pan of water (50 by 30 cm²), covered by netting to prevent mosquitoes from becoming mired and drowning. Air was circulated within the cage by a Sprite model SP2A2L 115 V 50/60 Hz 9 W PC-fan (Rotron, Woodstock, NY) with an air speed of 1.2 m s⁻¹ measured 5 cm above the fan. The fan was held on its side on top of the netting over the water pan, to facilitate movement of plant volatiles toward the gaps around the window panels. Four cylindrical cups were also positioned on their sides to provide resting sites for the mosquitoes, and two 2-dram vials with protruding wicks provided 10% sucrose solution to keep trapped mosquitoes alive. Mosquito response was recorded for four plant species common in western Kenya and previously reported to be preferred by *An. gambiae* (Manda et al. 2007): *Parthenium hysterophorus* (Asteraceae) with flowers, *Ricinus communis* (Euphorbiaceae) lacking flowers, *Lantana camara* (Verbenaceae) with flowers, and *Senna occidentalis* (Fabaceae) with flowers. A cage with high humidity but lacking a plant (i.e., a blank) served as a control. The diffusion olfactometer tests required two trap cages: one containing similar-sized whole potted test plants and the other left blank. The treatment and control cages were placed back-to-back, with a 10-cm space between the cages and with the entry windows facing in opposite directions. Two hundred *An.*

gambiae pupae of both sexes were allowed to emerge in small 7.2-liter acrylic plastic cages. Adults were supplied ad libitum with water on soaked cotton wicks placed within the cage. Mosquitoes (~50:50 sex ratio) were released directly into the enclosure 24 ± 12 h after emergence, 2 h prior to scotophase and collected from the trap cage the next morning about 15 h later and counted to determine the response to the plant and to the blank control.

T-Tube Olfactometer Used for Comparison: Description and Procedure

We compared the performance of the diffusion olfactometer to that of a modified T-tube wind-tunnel-type olfactometer (Geier et al. 1999), also used in our laboratory at the time. It was made of clear acrylic plastic, 49.6 cm in total length, consisting of four sections, starting from the downwind end: a 7.2-liter holding chamber, a 20.1- by 6.1-cm-diameter flight tube, a 16- by 36.1- by 12.7-cm stimulus-mixing choice chamber, and two cylindrical choice arms (13.5 cm long, 6.1 cm i.d.), connected to cylindrical trap compartments that contained cuttings of the plant species used as baits and held the mosquitoes until they were removed in the morning. Further olfactometer details are described by Nikbakhtzadeh et al. (2014). In addition to design and environment, tests with this T-tube wind-tunnel design differed from the diffusion olfactometer in that the grass *Dactylis glomerata* (Poaceae) was used in the control trap, instead of a blank.

T-tube olfactometer experiments were conducted in an indoor bioassay room, with a controlled air flow at 70 ml s⁻¹, 27 ± 1°C, and 68% relative humidity. The olfactometer system was connected to an exhaust system that led the olfactometer effluent into a fume hood. Between 150 and 250 mosquitoes of both sexes (~50:50 sex ratio) were released into the holding chamber for T-tube

Fig. 2. Side-by-side comparisons of diffusion-cage and T-tube olfactometers when tested with four African plants. Legend indicates treatment responses to *Lantana camara*, *Senna occidentalis*, *Parthenium hysterophorus*, and *Ricinus communis* in noncompetitive tests. Error bars represent standard error of measurement (SEM).

experimental tests 12–24 h after emergence, 2 h prior to scotophase. Mosquitoes were counted in the trap compartments, choice arms, and flight chamber the next morning, 12 h after they were released, to measure the response to the two plants.

Other Experimental Details

The positions of treatment and control choices were alternated between replications during the experiments. To confirm the symmetry of each system prior to comparing a test plant with a blank, double-blank runs were conducted. In the T-tube olfactometer, verification was performed by tests with attractants on both sides. Raw honey was used as the attractant for these tests. All plants and plant cuttings used in these experiments were maintained at a greenhouse at OSU, with no contact whatsoever with pesticides.

Statistical Analysis

Olfactometer performance was evaluated by three parameters: total response ($(T + C)/N$); treatment response, T/N ; and discrimination $T/(T + C)$. The total response was the total number in both treatment and control traps ($T + C$) as a proportion of all mosquitoes released (N) into the T-tube holding chamber or mesocosm enclosure. The treatment response was the number caught in the treatment (test-plant-baited) trap (T) as a proportion of all mosquitoes released (N). Discrimination was the number of mosquitoes in the treatment trap (T) as a proportion of mosquitoes trapped in both treatment and control traps ($T + C$), that is, only mosquitoes that made a choice. Normal distributions were established by Kolmogorov–Smirnov tests, and differences were detected by ANOVA. The effects of alternative

positions of the olfactometer traps, when both were unbaited (blank) and when both were baited with honey, were detected with paired-sample t -tests. All data were analyzed using IBM SPSS Software version 19 (SPSS Inc., Chicago, IL).

Results

With all four plant species, the diffusion-cage olfactometer performed as well or better than the modified Geier T-tube olfactometer (Fig. 2). In all cases, the total response was higher in the diffusion-cage olfactometer than in the modified T-tube olfactometer. Percent caught in the treatment trap of the diffusion and T-tube olfactometers, respectively, were as follows: *Lantana* 48 versus 8%, *Senna* 31 versus 15%, *Parthenium* 28 versus 24%, and *Ricinus* 13 versus 6% ($F = 12.857$; $df = 1, 54$; $P < 0.0001$). For each olfactometer, the treatment response was affected by the type of plant used to bait the olfactometer ($F = 4.112$; $df = 3, 55$; $P < 0.01$), with the highest responses predicted for *Lantana* and *Parthenium* and lower responses for *Senna* and *Ricinus*, in that order. There also was a significant interaction between the olfactometer used and the plant tested, in determining the treatment response ($F = 5.069$; $df = 3, 55$; $P < 0.01$).

Though the treatment response had a significant bearing on olfactometer discrimination ($F = 12.857$; $df = 1, 54$; $P < 0.0001$), the two olfactometers reported similar levels of discrimination ($F = 0.551$; $df = 1, 54$; $P = 0.461$), despite the use of a plant in the control trap of the T-tube. Additionally, we found that the species of plant tested significantly affected the discrimination levels reported

($F=2.823$; $df=3, 54$; $P<0.05$), and the significant interaction between the olfactometer used and the plant tested (see above) affected the discrimination levels reported ($F=3.983$; $df=3, 54$; $P<0.05$). There was no difference in responses between the alternative positions of the traps, for either the T-tube olfactometer ($t=1.26$, $df=6$, $P=0.256$) or the diffusion-cage olfactometer ($t=0.805$, $df=6$, $P=0.452$). When blanks or identical attractants were compared between the two choices, they likewise produced insignificant differences.

Discussion

This study demonstrates the high performance of a new simple tool for measuring mosquito orientation to plant-derived volatile organic compounds in semifield enclosures. Gaining significant prominence in behavioral and ecological studies (Knols et al. 2002; Okech et al. 2003; Helinski et al. 2008; Stone et al. 2011, 2012a,b), semifield systems provide a reliable intermediate to field trials of these compounds, and they may circumvent the challenges inherent in standardized laboratory methods, whose artifacts may lead to shortfalls in field efficacy (Seyoum et al. 2002). The diffusion-cage olfactometer provides a simple design and setup, and given its par performance compared with a more precisely controlled and standardized system, it offers a pragmatic and reliable alternative. Furthermore, the higher proportion of mosquitoes responding in the diffusion-cage setup indicates that a more natural situation, where mosquitoes are less restricted, is more likely to give reliable behavioral information. A notable advantage of this system over the T-tube or other dual-port olfactometers is the high throughput potential, especially when screening a large number of plants. We acknowledge the use of a lab-adapted colony in these experiments and advise caution in the interpretation of the results for the field context. In conclusion, the diffusion-cage olfactometer will not replace wind-tunnel olfactometer systems when precisely controlled conditions are needed, but it will complement them and will be especially useful at the semifield stage of experimentation with plant-related attractants.

Acknowledgments

We would like to thank Willem Takken for originally supplying us with the Suakoko strain of *Anopheles gambiae* and Ashley Jackson for her help in rearing mosquitoes used in this research. The work was supported by the National Institutes (NIH) grant number R01-AI077722 from the National Institute of Allergy & Infectious Diseases (NIAID) to W.A.F. Its content is solely the responsibility of the authors and does not represent the official views of NIAID or NIH.

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