Behavioral Response of *Aedes aegypti* (Diptera: Culicidae) Larvae to Synthetic and Natural Attractants and Repellents

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ABSTRACT *Aedes aegypti* (L.) (Diptera: Culicidae) is the key vector of three important arboviral diseases: dengue, yellow fever, and chikungunya. Immature stages of this species inhabit human-made containers placed in residential landscapes. In this study, we evaluated a few compounds in a sensitive behavioral assay with *Ae. aegypti* larvae. The orientation of larvae to different compounds was surveyed using a performance index (PI). The PI represents the response to each odorant, where a value of +1 is indicative of full attraction and −1 represents complete repulsion. The widely used insect repellent *N*, *N*-diethyl-*m*-toluamide elicited a significantly negative PI, as did acetophenone and indole. A yeast extract, a known food source, elicited a significantly positive PI, as did 2-methylphenol, 1-octen-3-ol, 3-methylphenol, and fish food. On the other hand, no response was observed for the essential oil of *Eucalyptus grandis x Eucalyptus camaldulensis* at the concentration evaluated. Pretreatment of larvae with *N*-ethylmaleimide and ablation of the antennae resulted in a suppression of behavioral responses. The overall mobility of ablated larvae was indistinguishable from unablated controls, and absence of any visible locomotor dysfunction was observed. This work is a contribution to the study of the chemical ecology of disease vectors with the aim of developing more efficient tools for surveillance and control.

Natural and synthetic compounds attractive to *Ae. aegypti* larvae should be incorporated into integrated pest management programs through the use of baited traps or by improving the efficacy of larvicides commonly used in control campaigns.

KEY WORDS   mosquito, larva, attractant, repellent

Aedes aegypti (L.) is a container-breeding mosquito that commonly inhabits urban and suburban areas throughout the world. They are diurnally active, highly anthropophilic, and potential vectors of the dengue, yellow fever, and chikungunya viruses in humans. Developmental stages of *Ae. aegypti* can be found in artificial containers and natural sites close to human dwellings (Barrera 1996, Scott et al. 2000). In such situations, the likely breeding sites can be treated with larvicides and oviposition repellents, as a component of the integrated approach to mosquito population management (Hwang et al. 1980, Curtis and Hill 1988, Rapley et al. 2009, Xue et al. 2001).

The ability to detect a wide range of sensory cues is essential for the survival of mosquitoes. Of these sensory stimuli, chemosensory inputs, especially olfactory cues, are crucial in food detection, oviposition, mating, predator avoidance, and other behaviors (Sharma et al. 2008, Ignell et al. 2010, Gonzalez et al. 2014). Furthermore, these olfactory cues are essential for many behavioral processes that mediate the capacity of *Ae. aegypti* as a vector and other disease-carrying mosquitoes (Klowden 1996, Lacey et al. 2014).

The behavior of the immature stages of holometabolous insects principally relates to short-range orientation to food sources, feeding behavior, and defensive responses. The sensory requirements of the larvae are more limited than those of the adults, and this is reflected in the smaller number of integumental sensilla and the lower capabilities of certain larval sense organs.

Aqueous larval habitats inherently represent a confined and therefore a more easily targeted site for mosquito control strategies. Although a lot of efforts have been put into developing novel repellents and attractants with low toxicity toward other organisms to control adult mosquitoes, very few similar studies have been performed in larvae. Coupled with certain larval attractants, larvicides may have a greater chance to kill their larvae targets. This approach requires further studies regarding the behavior of *Ae. aegypti* larvae, especially olfactory-driven behavior.

Chemically induced behavioral responses are inhibited by various chemicals that may interact in different ways with an insect chemoreceptor (Kaissling 1977, Dye 1982, Frazier 1985). Some authors suggest that insect chemoreception is affected by the intracellular concentration of reduced glutathione (Kosower and

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Kosower, 1969). *N*-ethylmaleimide (NEM) is one of a number of N-substituted maleimides that readily reacts with thiols because of its $C=C$ double bond; however, NEM is not very stable, and in aqueous solution the pyrrole ring opens, leading to the formation of *N*-ethyl maleamate (Webb 1966). Previous work in our laboratory demonstrated that NEM and other sulfhydryl groups (-SH) reagents, when topically applied on fifth-instar nymphs and adults of the reduviid bug *Triatoma infestans* (Klug) resulted in a suppression of food intake attributed to a chemoreceptor blockage produced by -SH reagents (Picollo et al. 1993, Gonzalez Audino et al. 1997).

In this work, few compounds already evaluated in *Anopheles gambiae* (Giles) were tested in *Ae. aegypti* using the methodology described by Xia et al. (2008) with minor modifications. We also analyzed if these behavioral responses were directly associated with the olfactory system by antennal ablation and treatment with NEM.

This is a first approach to study the response of *Ae. aegypti* larvae to natural and synthetic compounds. The results obtained could allow the development of novel vector-control strategies targeting larval feeding and other behaviors to enhance their effectiveness; for example, the use of larval attractants coupled with larvicides. These approaches require further study regarding the olfactory-driven behavior of *Ae. aegypti* larvae.

Materials and Methods

Chemicals. 1-octen-3-ol (98%), 2-methylphenol (98%), 3-methyl-phenol (>97%), *N*, *N*-diethyl-*m*-toluamide (DEET; 97%), and *n*-ethylmaleimide (NEM; 98%) were purchased from Sigma-Aldrich (St. Louis). Indole and acetone (>99.8%) was purchased from Merck (Germany). Acetophenone (>99%) was purchased from Carlo Erba Reagenti (Italy). Fish food, Full Balanced Basic Food for Tropical Fish, was supplied by Shulet Tropical (Buenos Aires, Argentina), composed of a mix of flours made with fish, cereals, meat, liver, soybean and algae; wheat, casein, yeast, and fish oil and enriched with vitamins A, D3, and E, ascorbic acid, folic acid, pantotenic acid, menadione, thiamin, riboflavin, pyridoxin, biotin, niacin, cyanocobalamin, and selenium, iodine, copper, manganese, iron, cobalt, and zinc as oligoelements. Yeast was purchased from Levadura de Cerveza Virgen (Tucumán, Argentina); agar-agar was purchased from Parafarm (Saporiti, Buenos Aires, Argentina); *Eucalyptus grandis x Eucalyptus camaldulensis* essential oil was provided by CIPEIN (Lucia et al. 2008) and kept dry under −4°C until use.

Biological Material. A susceptible strain of *Ae. aegypti* (CIPEIN) was used. This strain originated from the Rockefeller strain in Venezuela and had been kept in the laboratory since 1996, reared at $25 \pm 2°C$ under a photoperiod of 12:12 (L:D) h according to previous reports from the authors' laboratory (Seccacini et al. 2006). For this study, late third-instar or early fourth-instar larvae and 2–3-d-old adults of both sexes were used.

Larval Behavior Assay and Data Analysis. One hundred third-instar *Ae. aegypti* larvae were selected and washed carefully in dechlorinated water to eliminate any food particles, kept at 27°C and starved for 2 h. Odorant stock solutions were prepared by dissolving a specific amount of the odorants in a preheated 5% agar-agar solution. Bioassays were performed in 39.5- by 23.5- by 5.2-cm Pyrex dishes containing 500 ml of dechlorinated water and maintained at 27°C. A test zone and a control zone were determined and outlined according to Xia et al. (2008), with minor modifications. The larvae were released in the center of the dish and allowed to swim freely for 1 h. The odorant or control solution was inserted into a mesh bag and placed in the middle of the corresponding zone. Real-time images were acquired every 30 s over a 60-min period. The number of larvae in both test and control zones were counted at all time points.

In all cases, a performance index (PI; Heimbeck et al. 1999) at a discrete 40-min time point, time where maximum behavioral responses were observed, was calculated as follows:

$$PI = \frac{Odorant^A - Control^B}{Odorant^A + Control^B}$$

where, Odorant^A indicates the number of larvae in the test zone and Control^B indicates the number of larvae in the control zone. PI values of +1 indicate full attraction while −1 represents complete repulsion. Accordingly, tested chemicals with a PI of +0.30 and above were considered as possible larvae attractants or stimulants, while those with −0.30 and below were considered as possible repellent or deterrent compounds.

Concentrations used in this study were 95 mg/ml of DEET; 0.01 and 10 mg/ml of indole; 1 and 10^{-3} mg/ml of acetophenone; 10^{-3} mg/ml of 3-methylphenol, 2-methylphenol and 1-octen-3-ol; 10 mg/ml of yeast; and 1 mg/ml of fish food. These concentrations were chosen, as they evoke the maximum attractant or repellent responses in *An. gambiae* larvae as stated by Xia et al. (2008). The behavioral responses of *Ae. aegypti* larvae to these concentrations were examined along with the appropriate nonodorant control. When we used acetophenone and indole, an additional concentration was tested because those that exhibit the strongest response in *An. gambiae* (Xia et al. 2008) did not produce the same result as in *Ae. aegypti* larvae.

Eucalyptus essential oils are easily extractable, eco-friendly, biodegradable, with low or no toxicity against mammals and include a wide variety of compounds that can act as semiochemicals and is effective against a wide spectrum of insect pests (Isman 2000). The behavioral responses of *Ae. aegypti* larvae to 0.1 mg/ml of *Eucalyptus grandis x Eucalyptus camaldulensis* essential oil were examined.

Ablation of the Larval Antennae. Both antennae were carefully removed using entomological forceps. Larvae were allowed to recover under rearing conditions for 1 d before behavioral analyses.
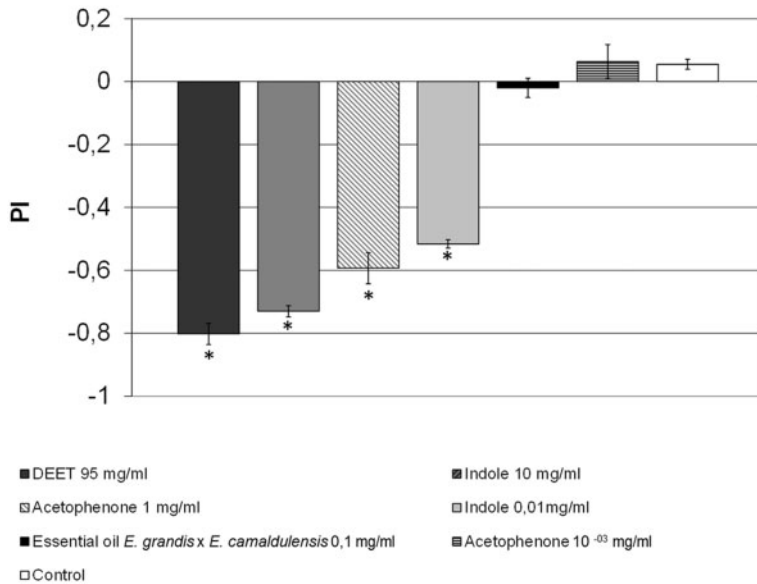
Fig. 1. Behavioral responses to compounds eliciting repellent responses of different odorants (DEET, indole, acetophenone, and essential oil of *Eucalyptus grandis x Eucalyptus camaldulensis*) in *Ae. aegypti.* Error bar stands for SEM. * indicates a statistically significant difference by a single-group one-sample t-test ($P \leq 0.05$). $n \leq 5$.

Behavioral responses for two compounds that normally show strong and opposite reactions (10^{-3} mg/ml of 1-octen-3-ol and 95 mg/ml of DEET) were then examined and the respective PIs calculated as indicated before.

Effect of NEM on Larvae Behavior. In order to establish the effect of NEM on *Ae. aegypti* larval behavior, 1 ml of 0.005 µg/ml of NEM in acetone was added to 100 ml of water in a 10- by 10- by 4-cm container. Acetone was allowed to evaporate for 15 min and 100 third-instar *Ae. aegypti* larvae were added for 5 min. Later, larvae were carefully washed and the assay was continued as described above.

In addition, behavioral responses to two compounds that normally show strong opposite reactions (10^{-3} mg/ml of 1-octen-3-ol and 95 mg/ml of DEET) were then examined and the respective PI calculated as indicated.

Statistical Analysis. The significances of PI values were analyzed for statistical significance by single-group one-sample t-test included in Sigmaplot 11.0 (Systat Software Inc., San Jose, CA).

Results

Larval Behavior Assay to Synthetic or Natural Odorant. Larval behavioral response of *Ae. aegypti* to synthetic or natural odorant stimulus was evaluated. As seen in Figs. 1 and 3, DEET evokes highly significant repellency at 95 mg/ml with a PI of −0.80 ($t = -23.600$, df = 3, $P = < 0.001$).

When using indole dilutions of 0.01 and 0.1 mg/ml, larvae respond with a significant negative PI of −0.51 ($t = -39.335$, df = 3, $P \leq 0.001$) and −0.73, respectively ($t = -40.820$, df = 3, $p \leq 0.001$; Fig. 1).

Acetophenone did not evoke statistically significant behavioral effects in larvae at 10^{-3} mg/ml (PI = 0.06, $t = 1.170$, df = 3, $P = 0.327$). However, it was strongly repellent to larvae at 1 mg/ml with a PI of −0.59 ($t = -11.943$, df = 3, $P = 0.001$; Fig. 1).

Eucalyptus grandis x Eucalyptus camaldulensis essential oil did not evoke statistically significant behavioral effects in larvae at 0.1 mg/ml (PI = −0.02, $t = -0.656$, df = 3, $P = 0.559$; Fig. 1).

We also found that *Ae. aegypti* larvae showed attraction to fish food (1 mg/ml) as well as to yeast extract (10 mg/ml) with a PI + 0.84 ($t = 30.189$, df = 3, $P \leq 0.001$) and +0.83, respectively ($t = 13.844$, df = 3, $P \leq 0.001$; Fig. 2). The monounsaturated eight-carbon alcohol, 1-octen-3-ol, was attractive at 10^{-3} mg/ml with a PI + 0.72 ($t = 19.575$, df = 3, $P < 0.001$). For the cresols tested, 2-methylphenol (*o*-cresol) and 3-methylphenol (*m*-cresol), a strongly attraction of larvae was obtained at 10^{-3} mg/ml with a PI + 0.51 ($t = 8.826$, df = 4, $P \leq 0.001$) and +0.52, respectively ($t = 9.431$, df = 3; $P = 0.003$; Fig. 2).

Elimination of Behavioral Response. With regard to the antennae ablation assay, it can be seen that the overall mobility of ablated larvae was indistinguishable from the unablated controls, indicating an absence of any visible locomotor dysfunction (data not shown). Behavioral responses of 1-octen-3-ol and DEET after ablation of the larval antennae resulted in a dramatic loss of odorant-driven behavioral responses (Fig. 3). Larvae distributed randomly in the plate and their PI was not significantly different from zero for 1-octen-3ol (PI = 0.09, $t = 1.904$, df = 3, $P = 0.153$) and for DEET (PI = −0.09, $t = -2.321$, df = 3, $P = 0.103$).

The response to 1-octen-3-ol and DEET by *Ae. aegypti* larvae was inhibited by pre-exposure to
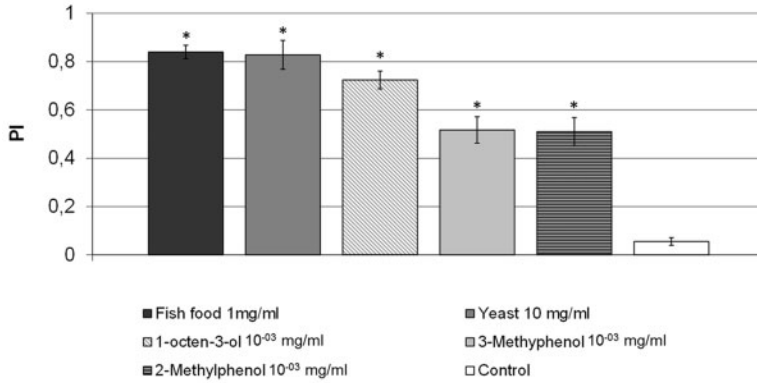
Fig. 2. Behavioral responses to compounds eliciting attractive responses of different odorants (fish food, 2-methylphenol, 3-methylphenol, acetophenone, 1-octen-3-ol, yeast, and acetophenone) in *Ae. aegypti*. Error bar stands for SEM. * indicates a statistically significant difference by a single-group one-sample t-test ($P \leq 0.05$). $n \leq 5$.
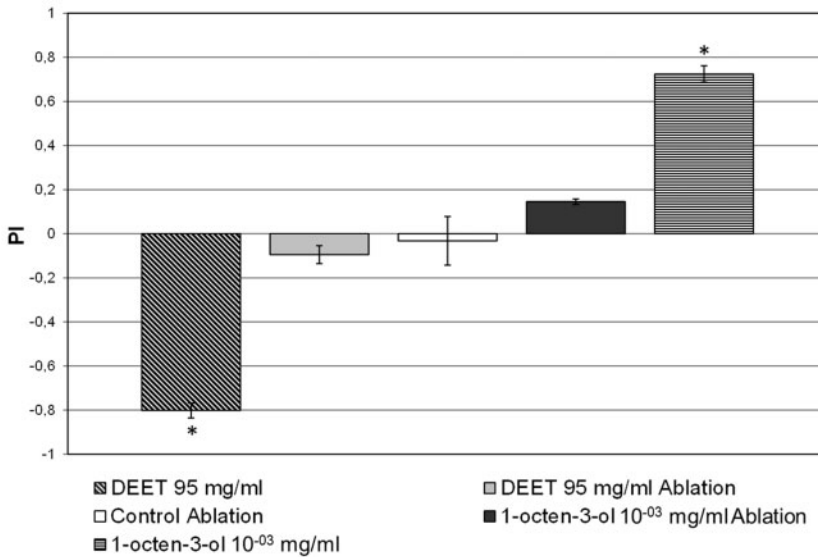

Fig. 3. Olfactory responses to 1-octen-3-ol and DEET in *Ae. aegypti* larvae with antennal ablation. Error bar indicates SEM. * indicates a statistically significant difference by a single-group one-sample t-test ($P \leq 0.05$). $n \leq 5$.

NEM (Fig. 4). In the case of 1-octen-3-ol, PI was -0.07 ($t = -0.522$, df $= 2$, $P = 0.654$) and for DEET, PI was 0.11 ($t = 0.830$, df $= 2$, $P = 0.494$).

Discussion

Mosquito larvae express a number of behavioral responses toward different types of stimuli such as light, food, color, etc. They accumulate in regions where food is found, as the result of orthokinetic responses with the involvement of their olfactory systems toward soluble constituents diffusing from the food (Merritt et al. 1992).

In this study, *Ae. aegypti* larvae displayed significant responses to the nine odorants tested (Figs. 1 and 2). Of these odorants, several show strong attractant-like characters (fish food, yeast, 1-octen-3-ol, 3 methylphenol, and 2-methylphenol), while the others showed a repellent-like response (DEET, indole, and acetophenone) at the concentrations assayed. The cresols, 2-methylphenol (*o*-cresol) and 3-methylphenol (*m*-cresol), showed positive PIs at 10^{-3} mg/ml in *Ae. aegypti* as well as in *An. gambiae* (Xia et al. 2008). These compounds are considered as products of organic decay, which constitute a major food source for mosquito larvae (Thiery et al. 1991).

In our study, 1-octen-3-ol evoked positive PIs for *Ae. aegypti* larvae at 10^{-3} mg/ml. This compound is known for being present in the sweat of several vertebrates, including humans and is also an attractant for many insect species including *Ae. aegypti* adults (Majeed 2013).
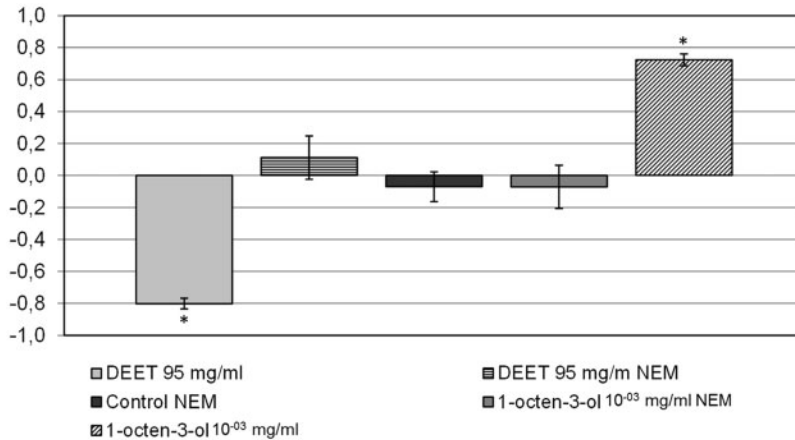
Fig. 4. Reduction of olfactory responses to 1-octen-3-ol and DEET in *Ae. aegypti* larvae exposed to NEM. Error bar indicates SEM. * indicates a statistically significant difference by a single-group one-sample t-test ($P \leq 0.05$). $n \leq 5$.

To assess responses to potential larval food sources, yeast extract and fish food previously reported as attractants for *An. gambiae* larvae (Xia et al. 2008) were used in our behavioral assay. We found the same attractive behavior both towards yeast and fish food with a significantly positive PI for *Ae. Aegypti*. At the other end of the behavioral spectrum, acetophenone, indole, and DEET provoked negative PIs consistent with potentially repellent effects. Acetophenone induced a different response depending on the concentration; it was repellent at 1 mg/ml, while it did not evoke a statistically significant behavior at 10^{-3} mg/ml, indicating no differences in the number of larvae found in the odorant zone and control zone. A similar pattern of attraction–repulsion responses with different concentrations was reported in *An. gambiae* by Xia et al. (2008). The same odorant can induce attractive or repulsive responses depending on its concentration in various animals. However, how information of the odor concentration is encoded in a neural network and how changes of odor concentrations lead to an olfactory preference switch are still poorly understood (Yoshida et al. 2012).

Indole, another aromatic compound, evoked significant negative PIs for *Ae. aegypti* larvae at 0.01 and 10 mg/ml. However *An. gambiae* larvae were strongly attracted by indole at 0.01 mg/ml (Xia 2008, Xia et al. 2008).

To provide additional evidence that the behavioral response is indeed mediated by the larval olfactory system, an antennal ablation study was carried out. As a result, the absence of larval antennae completely removed the responses toward DEET and 1-octen-3-ol, further indicating that DEET and 1-octen-3-ol directly targets specific larval olfactory receptors that are expressed on the larval antennae. These results were consistent with other studies in *An. gambiae*, where ablation of the larval antenna reduced olfactory responses (Xia et al. 2008). Similarly, when larvae were pretreated with NEM, we obtained an inhibition of the behavioral response. NEM reacts with the SH groups that have an essential role in chemoreception in *Ae. aegypti* larvae. Kaissling (1977) and Mayer and Mankin (1985) have already reported that applying SH reagents to different insect species inhibited behavioral responses to odorants. It must be borne in mind that the lack of specificity of these agents results in a range of biological effects (Webb 1966, Cohen 1968).

Several works have shown that these odorants that resulted larval behavior modifiers in our study also affect adults. We could suggest that the receptors involved in the mechanism of olfaction in the adult stage mosquito are already expressed in the immature stages. It is known that indole, 3-methylphenol and acetophenone evoke strong electrophysiological activity on the antennae of female *Ae. aegypti* (Jhumur et al. 2007, Siju et al. 2010). 1-octen-3-ol has also shown enantiomeric selectivity in behavioral and electrophysiological responses of adult *Ae. aegypti* (Cook et al. 2011).

Since larvicides for *Ae. aegypti* control are mainly used in drinking water, not all the known compounds can be used as management tools. WHO allows only the use temephos, *Bti*, spinosad, and some IGRs such as methoprene, pyriproxyfen, and novaluron (WHO 2007a,b). In addition, some synthetic pyrethroids are very effective but care must be taken when used as larvicides due to their toxicity to aquatic nontarget organisms (WHO 2006). The use of larvicides is limited by issues such as the emergence of resistance, already known for temephos in almost all Latin-American countries, including Argentina (Coosemans and Carnevale 1995, Braga et al. 2004, Ocampo et al. 2011). The efficacy of larvicides relies on several factors including formulation, water quality, and the susceptibility of the targeted larvae (Walker and Lynch 2007, Harburguer et al. 2009). If a slow release formulation could be modified by the addition of a strong attractant, it would be possible to increase the larval density proximate to insecticides and thereby, greatly enhance their effectiveness. Pickett et al. (2010) emphasized the importance of investigating the chemical ecology on disease

vectors with the aim of developing more efficient tools for surveillance and control.

The detection and measurement of the mosquito population has often proved easier by routine light-trap catches and by biting collections than by larval sampling. Despite all inherent difficulties in sampling mosquito larval populations, there remains the need to develop better sampling procedures (Service 1993). Harrison et al. (1982) developed a floating-mosquito larval trap for medium to large size artificial containers that eliminates many biases commonly associated with human collection techniques. In addition, Nam et al. (2003) worked in a method to sample *Aedes* in large containers. The introduction of natural and synthetic compounds in baited traps for *Ae. aegypti* larvae will lead to the improvement of integrated pest management programs.

Our data suggest that *Ae. aegypti* larvae are attracted or repelled by certain synthetic and natural compounds at a particular concentration. However, as behavioral responses vary according to the concentration used, further studies on the mechanism of olfactory-driven behavior using compounds at different concentrations will be needed (Bohbot et al. 2010).

Acknowledgments

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