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The main component of the scent of *Senecio madagascariensis* flowers is an attractant for *Aedes aegypti* (L.) (Diptera: Culicidae) mosquitoes

Research Paper

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Abstract

Aedes aegypti (L.) (Diptera: Culicidae) is one of the main vectors of arboviruses, including dengue, Zika, and chikungunya. It almost exclusively inhabits urban areas. Both sexes feed on plant carbohydrates, although for males, this is their only food source. In the case of floral nectars, mosquitoes locate plant sugar sources assisted by volatile compounds. In this work, we found that the floral scent of *Senecio madagascariensis* elicited a behavioral response in males; therefore, we focused on identifying the volatiles emitted by these flowers. The terpenes (±)-α-pinene, β-pinene, sabinene, and phellandrene and 1-alkenes 1-undecene, and 1-nonene were identified. To determine which compounds are bioactive, pure synthetic lures were assessed using an olfactometer. Only the main compound 1-nonene was an attractant for males. Since our goal was the introduction of synthetic floral-based attractants in toxic sugar-baited traps, we formulated 1-nonene in solid paraffin and stearin matrices to obtain a controlled release system. The bioassay with a toxicological end point showed that the incorporation of a feeding attractant to the toxic sugar trap increased overall mortality. These results suggest that it is possible to use plant volatile compounds or flower cuttings as male *Ae. aegypti* attractants to improve the efficacy of baited traps.

Introduction

Aedes aegypti (L.) (Diptera: Culicidae) mosquitoes almost exclusively inhabit urban areas (Valença *et al.*, 2013; Ye *et al.*, 2016). The marked feeding preference of females for human blood makes them efficient vectors of mosquito-borne viruses, such as dengue fever, yellow fever, chikungunya, and Zika. Male and female mosquitoes also feed on plant sugars, with sugar being the only food for male mosquitoes, regardless of the species (Yee and Foster, 1992; Foster and Hancock, 1994; Gary and Foster, 2004). Sugar consumption has a significant influence on vector dispersion capacity (Stone and Foster, 2013), and both males and females need sugar throughout their adult life (Foster, 1995). Initially, both sexes usually visit a plant for the first time after emergence. Afterward, males require sugar at frequent intervals to maintain their energy reserves (Yuval, 1992), while females consume sugar between blood samples when they are digesting blood or when they are gravid.

Neither the relationship of sugar feeding with blood feeding nor the behavioral outcomes associated with food source selection have been elucidated for *Ae. aegypti*. The relevance and frequency of each type of intake depends on the mosquito species and physiological state.

The common sugar sources in nature are floral and extrafloral nectar (Patterson *et al.*, 1969; Foster and Hancock, 1994), honeydew (Gary and Foster, 2004), and ripe fruits (Joseph, 1970). Several factors help attract and orient mosquitoes toward vascular plants (e.g., floral scents and nectar) (Raguso, 2004). Cues such as flower color and nectar provide mosquitoes with information regarding the location, abundance, quality of food, and pollen, thus influencing their attraction toward the plant (Magnarelli, 1977).

The positive response of mosquitoes to floral volatiles was observed in the absence of visual stimuli (Jepson and Healy, 1988), suggesting that volatiles act as long-range attractants, even before visual contact. Floral scents seem to drive long-range host localizing, while visual signals might play a role in short-range detection (Jepson and Healy, 1988). Mosquito feeding field studies have shown that they feed on a very limited number of plant species (Abdel-Malek and Baldwin, 1961; Abdel-Malek, 1964; Müller and Schlein, 2006), which may explain the importance of these volatile cues. In the case of *Ae. aegypti*, laboratory behavioral bioassays and electroantennograms have demonstrated their ability to recognize volatile compounds commonly associated with specific species as a way of locating nectar sources (Jhumur *et al.*, 2007; von Oppen *et al.*, 2015).

Because both sexes rely on sugar sources, attractive toxic sugar baits have been proposed as lower impact mosquito trap. Traditional mosquito control methods are pesticide-intensive which can lead to resistant mosquito populations (Smith *et al.*, 2016), and negatively impact ecosystem or human health. Therefore, efforts to find methods with low environmental impact are worthwhile. However, the results from field traps that use sugar baits are still controversial (Xue *et al.*, 2008; Fikrig *et al.*, 2017). A study carried out in Australia suggests that female mosquitoes seldom feed on sugar making it in an unreliable bait, while recent work in South America found that sugar feeding is in fact common (Fikrig *et al.*, 2017). Our area of interest is also in South America, where *Ae. aegypti* is extensively spread and has been responsible for several dengue epidemics. These circumstances could make South America an ideal candidate region for testing sugar trap efficacy.

This research examined how plant volatile compounds affect mosquito behavior generally in order to gain insight into native *Aedes* feeding behavior. An initial screening assessing adult mosquito preferences for domestic plants found that *Lobularia maritima*, *Plectranthus neochilus*, *Euryops pectinatus*, and *Tagetes patula* were preferred (von Oppen *et al.*, 2015). As a continuation of this project, we screened mosquitoes' preference for *Senecio madagascariensis* as it is largely distributed in the Argentine Pampa Region, including their breeding sites located in an urban area around our campus. No information is currently available regarding the role of *S. madagascariensis* scents in the chemical ecology of insect pests. This prompted us to study the attraction properties and chemical composition of its volatile compounds. Once preference for *S. madagascariensis* was established, behavioral bioassays were carried out, the chemical composition of volatiles was assessed, and the active components of flowers were identified and incorporated in controlled release formulations. Ultimately, the goal of this project was to use these insights in the application of field traps.

Materials and methods

Mosquitoes

A CIPEIN insecticide-susceptible strain of *Ae. aegypti* mosquito was used. This colony was derived from a Rockefeller strain introduced from Venezuela in 1996 and was reared in our laboratory. Eggs were laid on wet cotton for 2 days and then dehydrated at ambient temperature and stored for at least 30 days. Before the experiments, the eggs were placed in distilled water (250 eggs l^{-1}) at 25 ± 2°C (Seccacini *et al.*, 2006). After hatching, the larvae were placed in 250-ml plastic containers and transferred to $20 \times 20 \times 20$ cm^3 acrylic boxes for molting. Larvae were fed with a mixture of rabbit pellets and yeast powder (80:20 wt:wt). Adults were fed *ad libitum* with water and 10% sucrose. The mosquito breeding room was set at 25 ± 2°C and 70 ± 5% R.H., with a L12:D12 photoperiod. To enhance the responses of adult mosquitoes toward volatile cues from food sources, they were subjected to starvation (24 h) in separate cages before tests were conducted (Vargo and Foster, 1982; Jhumur *et al.*, 2007). All adults were tested between 2 and 7 days old.

Plant material

S. madagascariensis plants found in bloom around the breeding sites of mosquitos were collected at our institute campus. They were examined to check whether they were undamaged by herbivory. Whole plants were extracted from the ground, transferred to polypropylene pots, brought to the laboratory, and rinsed with clean water. They were left outdoors for 3 days before being used in the experiments to reduce any putative cross contamination. The material was carefully manipulated with latex gloves to avoid contamination with skin volatiles.

For the landing preference bioassay, the flowers were cut short at their stems within approximately an hour of the experiment. The cut ends were carefully covered with moistened cotton and placed into containers without damaging the flower.

For the olfactometer behavioral assay, the flowers were cut at their stems and placed in vases with clean water during the day of the experiment to prevent them from wilting. Cut flowers were maintained under light to avoid closing. During the experiment, entire vases were insulated with an oven bag (30×45 cm^2; B.P. Premium, Argentina) to avoid interference from volatiles released by the cut area.

Chemicals

Acetone (>99.8%) was obtained from Merck (Darmstadt, Germany). Paraffin wax (mp 53–58°C, ASTM D 87) and chemical standards 1-nonene (>99%), (+)-α-pinene (>98%), (−)-α-pinene (>98%), β-pinene (>98%), and alkane standards (C5–C30) (>99%) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Stearin wax of technical grade was obtained from Parafarm® (Saporiti, Argentina). Dichloromethane (DCM) (HPLC grade, >99.5%) was acquired from Sintorgan S.A. (Argentina). Sucrose (>99%) was purchased from Anedra (Tigre, BA, Argentina). Imidacloprid (94.3%) was supplied by Bayer (Leverkusen, Germany).

Are Ae. aegypti *attracted to the floral scent of* S. madagascariensis? *Using imidacloprid to assess sugar feeding*

Landing preference bioassay

To assess the attraction to a sugar bait, we incorporated the insecticide imidacloprid into sucralose solutions in both the control (clean vessel) and treatment (flowers) boxes. According to our established protocol (von Oppen *et al.*, 2015), cotton plugs soaked at 10 p.p.m. imidacloprid in 10% sucrose solution was placed next to the plant stimulus as a phagostimulant for mosquitoes (Xue *et al.*, 2003; Müller *et al.*, 2010; Allan, 2011). Imidacloprid was added as feeding marker to facilitate the counting of mosquitoes, which would be knocked down after ingesting the toxic sugar baits. Imidacloprid is an insecticide that acts though oral ingestion and has little contact or inhalation toxicity, and it does not elicit spatial repellency or attraction *per se* toward *Ae. aegypti* (Antonio-Arreola *et al.*, 2011). In this way, we could determine if the number of mosquitoes selecting the enhanced bait was significantly more than if they selected it at random.

In detail, the experimental setup was polyacrylate boxes ($40 \times 30 \times 40$ cm^3) in a controlled environment at 27 ± 1°C, 60–70% R.H., and L12:D12. Both boxes, i.e., test and control, contained three plastic vessels, as described in fig. 1. The only water-containing vessel (125 ml) was placed in the middle of the cage, and it contained water-soaked cotton on the top of a permeable nylon over an empty vessel. The second vessel (250 ml) was placed in one corner and had cotton soaked in 10% sucrose on top of the nylon cloth. This container was left empty to offer the option of food without scent stimulus. The third vessel (250 ml) was placed in the other corner and included cotton

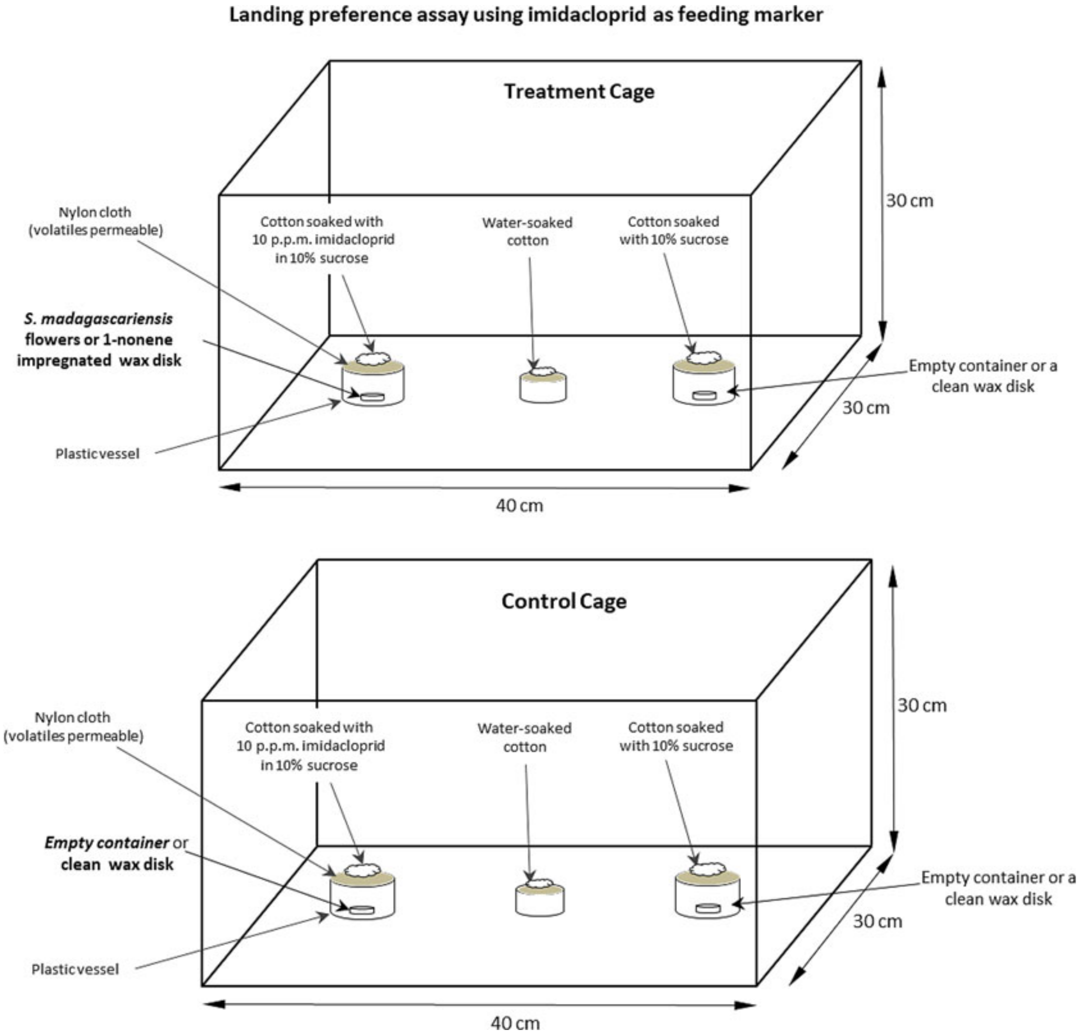

Figure 1. Treatment and control cage used in the landing preference bioassay on cut *S. madagascariensis* flowers or on impregnated disks.

soaked in sucrose and imidacloprid at 10 p.p.m. and 30 g of cut *S. madagascariensis* flowers as the scent stimulus. For the control experiment, the same setup with the three vessels was used but without plant material (i.e., no scent stimulus). Seven to twelve starved mosquitoes of each sex were released into separated boxes and left for 24 h. After this period, mortality was surveyed by counting knocked down mosquitoes and living mosquitoes. Four replicates of bioassays were carried out. Mortality was corrected with Abbott's formula (Abbott, 1925).

In a tracking experiment for environmental conditions, solutions were provided simultaneously in each replicate by putting a plastic vessel with 0.25 g of cotton soaked in a 10% sucrose solution at the bottom of the acrylic cage and releasing ten mosquitoes inside. This experiment indicated whether the dead mosquitoes in the treatment and control cages (groups) were caused by the ingestion of imidacloprid alone. If nonzero mortality was observed in this experiment, then the bioassay was discarded.

Olfactometer behavioral assay

The Y-tube olfactometer setup was the same as that used in von Oppen *et al.* (2015) and designed according to Geier and Broeckh (1999). The air flowing through the olfactometer was provided by a central supply at 80 liters min^{-1}, filtered through activated charcoal, and adjusted to 75% ± 10 R.H. and 26°C. After air flow splits inside the apparatus, the linear speeds were 0.5 and 0.25 m s^{-1} at the central arm and each lateral arm, respectively. The olfactometer was cleaned with 96% ethanol between replicates, and latex gloves were worn at all times.

Flowers with covered stems were put into a polypropylene bottle connected to one arm and were changed with fresh ones every hour. The bottles had two connections for air entry and exit. At the beginning of the experiment, ten starved mosquitoes of the same sex were loaded through the acclimatization chamber in the olfactometer base with the airflow in the closed position. After 5 min of acclimatization, the airflow ran for 30 s before opening the door to release the insects, which were allowed to freely move through the arms for 5 min. After this time, the doors were closed and the mosquitos in each sector of the olfactometer were surveyed. Eight replicates for each sex were performed. Between experiments, the mosquitoes were removed with a vacuum cleaner.

Identification of natural components present in the floral scent

Headspace of S. madagascariensis

Flowers were cut at the stem and put into a 500 ml Erlenmeyer tube containing purified water to maintain freshness.

Inflorescences, including bracts, were covered with an oven bag (30×45 cm^2; B.P. Premium, Argentina). The system was equilibrated for 30 min at 30°C in a temperature-controlled system, and afterward, a solid phase microextraction (SPME) divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA, USA) was punctured through the bag for adsorption of volatiles. Then, the fiber was exposed for 30 min to the sample headspace and subsequently injected in a Shimadzu QP-5050 gas chromatograph-mass spectrometer (GC-MS) for desorption at 240°C for 1 min with the injector in splitless mode. A blank with the same setup as the fiber in an empty bag was performed before collection of each volatile. DB-5MS ($30 \text{ m} \times 0.25$ mm, 0.25 µm film thickness) and DB-Wax ($30 \text{ m} \times 0.32$ mm, 0.25 µm film thickness) columns were used, and for chiral analysis, a CYCLOSIL-B ($30 \text{ m} \times 0.25$ mm, 0.25 µm film thickness) column was used (Agilent Technologies, Santa Clara, CA, USA). The detector operated in electron ionization mode at 70 eV, and masses were scanned from 45 to 280 m/z, with an interface at 245°C. For the chemical identification of single compounds, according to the availability, standard references were used. Comparisons of RI values were performed with the literature data and/or by matching the mass spectrum (MS) against the Wiley 7 library.

Flight preference bioassay of the main components of S. madagascariensis

Olfactometer behavioral assay

The same Y-tube olfactometer, setup, and procedure applied in the previous section were followed. To test pure compounds found in the *S. madagascariensis* headspace, 100 µl of a 1 mg ml^{-1} acetone solution of each compound was placed into one arm with 100 µl of pure acetone in the opposite arm. Once the chemical was in place, the mosquitoes were put into the olfactometer through the base and then exposed to airflow inside the acclimation camera for 2 min before being released. The first arm chosen by each mosquito was registered. If the mosquitoes were unresponsive after 3 min, the trial was discarded. Overall, 71 ± 6 responsive mosquitoes were tested, and a control was performed by measuring the response of the mosquito to acetone in both arms using 67 ± 3 responsive mosquitoes. The sample was randomly placed in the right or left arms of the olfactometer.

Formulation of attractant components in solid matrices for controlled release and evaluation of their biological activity

Preparation of solid disks containing 1-nonene: molded disks composed of 10 ml of paraffin or stearin were impregnated with 10, 1, and 0.5 mg of 1-nonene. For this, waxes were melted at their fusion temperature in a water bath and poured in a 25 ml flask. Then the active component was added to a DCM solution (5 mg ml^{-1}) under stirring to achieve the final doses of 10, 1, and 0.5 mg per disk. DCM was chosen because it easily solubilizes alkenes and waxes, and because its boiling point (40°C) is lower than the melting points of stearin (54–75°C) and paraffin (52–54°C); thus, it evaporates during the preparation of disks. The boiling point of 1-nonene is 146.9°C, which is higher than the stearin and paraffin melting points. 1-Nonene solutions were poured into an open aluminum mold 6 cm in diameter. The disks were allowed to solidify, and the mold was used to perform a bioassay on the same day of preparation.

Landing preference bioassay on impregnated disks

The experimental design described in the landing preference bioassay (fig. 1) was followed, although impregnated disks were used instead of flowers to evaluate the influence of the individual attractant-impregnated disks on mortality caused by imidacloprid.

The disk was set inside at the base of the 250 ml receptacle, covered with a nylon cloth, and a 0.25 g piece of cotton embedded in a 10% sucrose and 10 ppm imidacloprid solution. For the control experiment, prepared disks of paraffin or stearin containing no attractants were simultaneously used.

Once the cages were setup, 12 starved mosquitoes of each sex were transferred from the stock cage to the experimental or control cage by very careful aspiration. Four to six replicates were carried out, and mortality was evaluated after 24 h. Simultaneous tracking experiments with the same setup detailed in the landing preference bioassay were performed.

Statistical analysis

Statistical analyses were performed using the InfoStat program package (InfoStat group, FCA, Universidad Nacional de Córdoba, Argentina). In landing preference bioassays, the mortality rates (after correction) were compared with the Kruskal–Wallis test, which is a nonparametric method adequate for nonnormality datasets. To evaluate the flight preference response in the olfactometer bioassay, the chi-square test was used. In all cases, $P \leq 0.05$ was considered statistically significant.

Results

Landing preference bioassay

As observed in fig. 2, the presence of *S. madagascariensis* led to an increase in mortality in males ($n = 4$, $P = 0.0286$) but not in females ($n = 4$, $P = 0.8286$) compared to the control conditions (absence of *S. madagascariensis*). Based on this result, we tested the potential attractant effect of *S. madagascariensis* in a two-way olfactometer. No dead mosquitoes were found in the tracking cage during the experiments.

Olfactometer bioassay

From the observations of the landing preference test, the potential attractant effect of *S. madagascariensis* flowers on male *Ae. aegypti* was confirmed in the two-way olfactometer. Both males and females were evaluated, and only male mosquitoes were observed to prefer the airstream containing volatiles of *S. madagascariensis* inflorescences ($P < 0.05$) (fig. 3), which is consistent with previous results. Female mosquitoes showed no preference ($P > 0.10$), which is also consistent with the results of the landing preference bioassay. Less than 20% of the mosquitoes were nonresponsive.

Plant volatile identification

The observed attractant effect of *S. madagascariensis* flowers on males led us to identify the volatiles presumably responsible for that behavior. From the list of compounds collected from the flowers, the volatiles found in the oven bag and the components from column bleeding, e.g., alkyl siloxanes, were eliminated. After subtraction, terpenes and 1-alkenes were found to be the main components of floral scent (table 1). All the terpenes found corresponded to monoterpenes, including (±)-α-pinene,

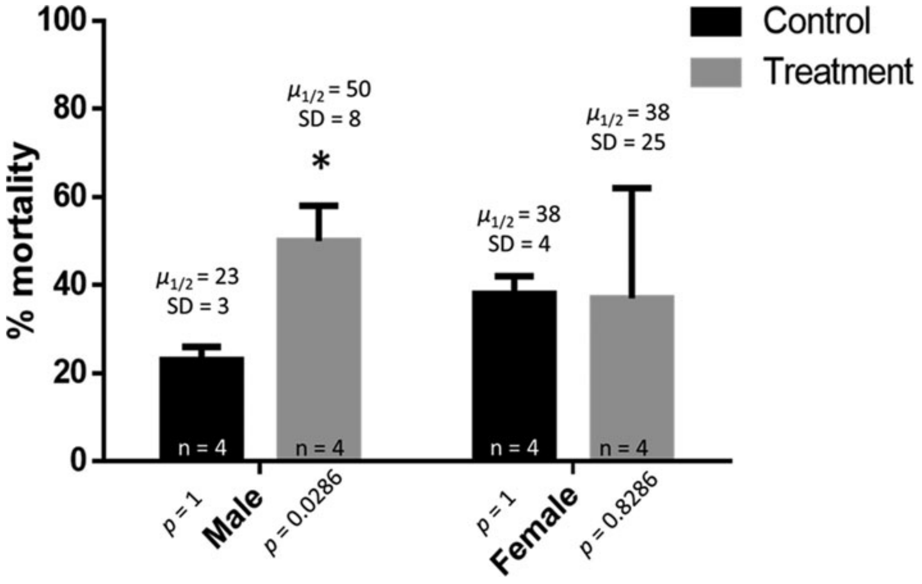

Figure 2. Landing preference bioassay on cut *S. madagascariensis* flowers. Asterisks express significant differences in response vs. the acetone control in the K–W test (*$P < 0.05$). $\mu_{1/2}$, median; SD, standard deviation.

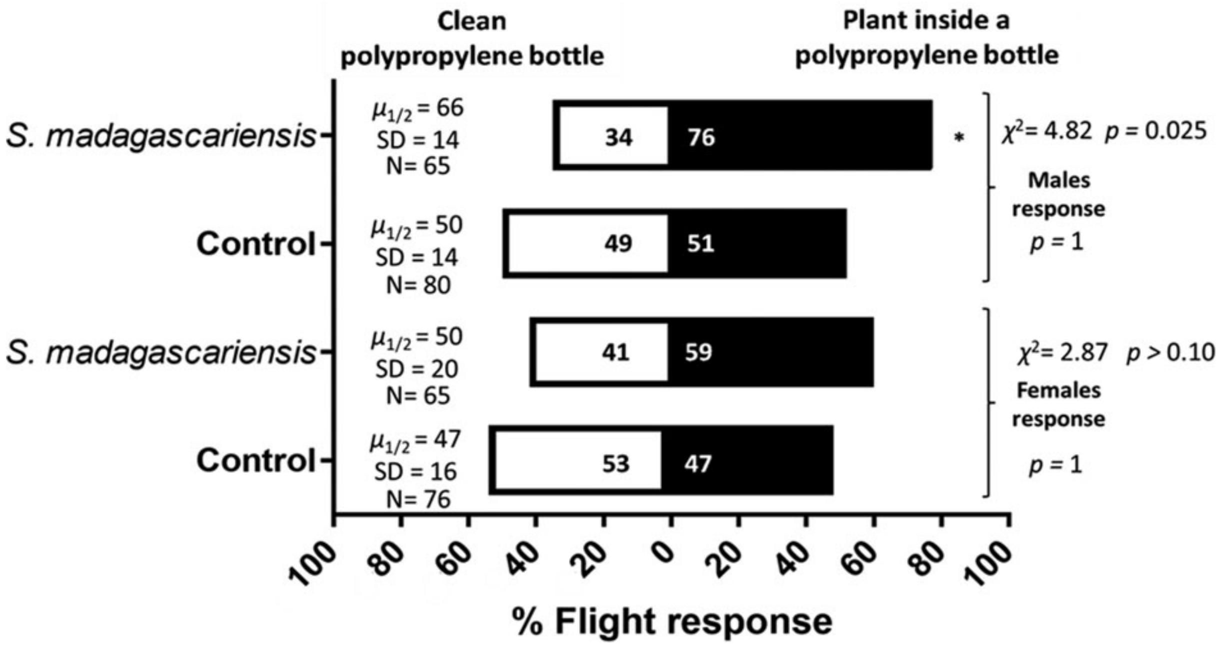

Figure 3. Two-way olfactometer behavioral assay of *S. madagascariensis* toward male and female *Ae. aegypti*. $\mu_{1/2}$, median; SD, standard deviation. Asterisks express a significant difference in response vs. the acetone control in the chi square test (*$P < 0.05$, $df = 1$, critical value $\chi^2_{(1,\ 0.05)} = 3.84$).

β-pinene, sabinene, and phellandrene. The 1-alkenes corresponded to 1-nonene and 1-undecene.

The identities of the monoterpenes and alkenes were confirmed by co-injection of authentic standards. The chirality of pure enantiomers was confirmed using a chiral column. 1-Nonene was by far the most abundant component, suggesting its crucial role as an attractant of male mosquitoes.

Flight orientation bioassay with S. madagascariensis *volatiles*

The volatiles found in *S. madagascariensis* inflorescences and available as commercial standards were subsequently evaluated in an olfactometer.

Females showed a significant positive response only to (+)-α-pinene at a 10 µg dose ($P < 0.01$, fig. 4). Males showed a positive flight response only toward the main component 1-nonene at a 10 µg dose ($P < 0.005$, fig. 5). Less than 20% of the mosquitoes were nonresponsive.

Landing preference bioassay on attractant impregnated disks

The landing preference bioassay was performed on stearin and paraffin disks impregnated with 1-nonene (figs 6 and 7). For male mosquitoes, the mortality caused by imidacloprid in the presence of stearin disks impregnated with 1-nonene was significantly higher than that in the control test without 1-nonene at all

Table 1. Headspace analysis of *S. madagascariensis* plants with flowers collected by SPME in a DVB/CAR/PDMS fiber and analyzed by GC-MS in a DB-5 column

Retention time	Area (%)	Volatile compound	RI in DB-5MS	Similarity (%) of MS[a]	Reported RI in DB-5MS
4.89	75.7	1-Nonene	891	97	889
5.81	9.2	(±)-α-Pinene	933	98	933
6.65	0.3	Sabinene	967	92	969
6.79	5.19	β-Pinene	976	96	976
7.40	4.77	4-Hexen-1-ol acetate	1003	97	–
8.00	1.34	β-Phellandrene	1028	88	1028
9.40	0.25	1-Undecene	1088	90	1091

[a] Compared to the Wiley database.

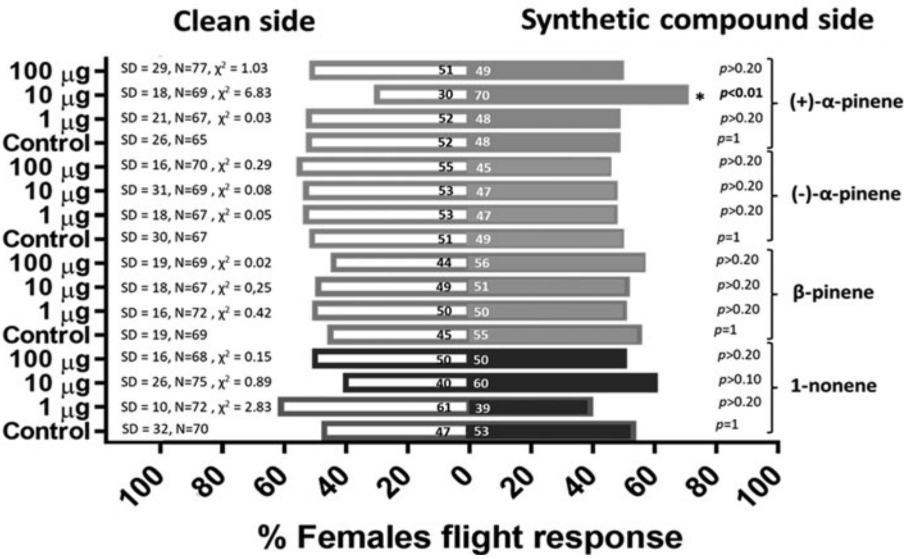

Figure 4. Behavioral responses of female *Ae. aegypti* to four *S. madagascariensis* volatiles using a dual-port flight olfactometer. Responding mosquitoes that crossed the mark at the end of the arm were counted. Asterisks express significant differences in response vs. the acetone control in the chi-square test with a significance threshold of 0.05 (*$P < 0.05$, critical value $\chi^2_{(1,\ 0.05)} = 3.84$). SD, standard deviation.

tested doses ($P < 0.019$, fig. 7). For females, only the highest dose of 1-nonene (1 mg ml^{-1}) produced significantly more landings ($P = 0.033$), and it also corresponded to more deaths than the other doses used (fig. 6). For paraffin disks (figs 6 and 7), the 0.05 and 0.1 mg ml^{-1} doses of 1-nonene did not increase landing preference for females or males. However, the 1 mg ml^{-1} dose increased overall mortality for both males ($P = 0.024$) and females ($P = 0.033$). No dead mosquitoes were found in the tracking cage during the experiments.

Discussion

This work found that the volatiles of *S. madagascariensis* flowers elicit a differential behavioral attraction response in male and female *Ae. aegypti* mosquitoes. *S. madagascariensis* flowers attracted males but not females under the tested conditions, and this differential effect between sexes was observed in both experimental setups, namely, the two-way olfactometer and the landing preference bioassay. Once the attractant effect of *S. madagascariensis* was characterized, the volatiles emitted by flowers were identified and the main components were individually tested in laboratory behavioral bioassays. The terpenes identified in the *S. madagascariensis* headspace were (+)-α-pinene, (−)-α-pinene, β-pinene, sabinene, phellandrene, and 1-alkenes; 1-nonene and 1-undecene, with 1-nonene representing the main compound. There are no previous reports on the composition of *S. madagascariensis*, although 1-nonene has been reported in volatiles of *Ruta chalepensis*, *Arabidopsis thaliana*, *Larrea tridentata*, and *Vigna unguiculata* (Lwande *et al.*, 1989; Jardine *et al.*, 2010; Haddouchi *et al.*, 2013; Kegge *et al.*, 2013). However, to our knowledge, it has not been reported as an attractant for insects.

In the behavioral evaluation of individual attraction to pure compounds, across all the tested compounds and doses, only 10 μg of 1-nonene was an attractant for males, while 10 μg of (+)-α-pinene was an attractant for females. In both cases, higher or lower doses did not show a significant attractant effect, thus indicating a clear optimal concentration. It is worth mentioning that (+)-α-pinene vapor pressures elicited a knockdown effect on *Aedes* (Lucia *et al.*, 2013).

This differential attraction of males and females to volatile cues could be attributed to several factors, two of which seem to us most probable. First, the physiological status of females may influence their sugar seeking and flower attraction behavior. In our experiments, both male and female adults were kept in the same cage with free sugar access post-eclosion up until 24 prior to bioassays. Thereby, before isolation adults have free access to potential mates, meaning some females could have started their gonotrophic cycle. Their potentially nulliparous or gravid conditions could be impacting over their sugar-seeking impulses or response to floral scents. Second is the experimental design and

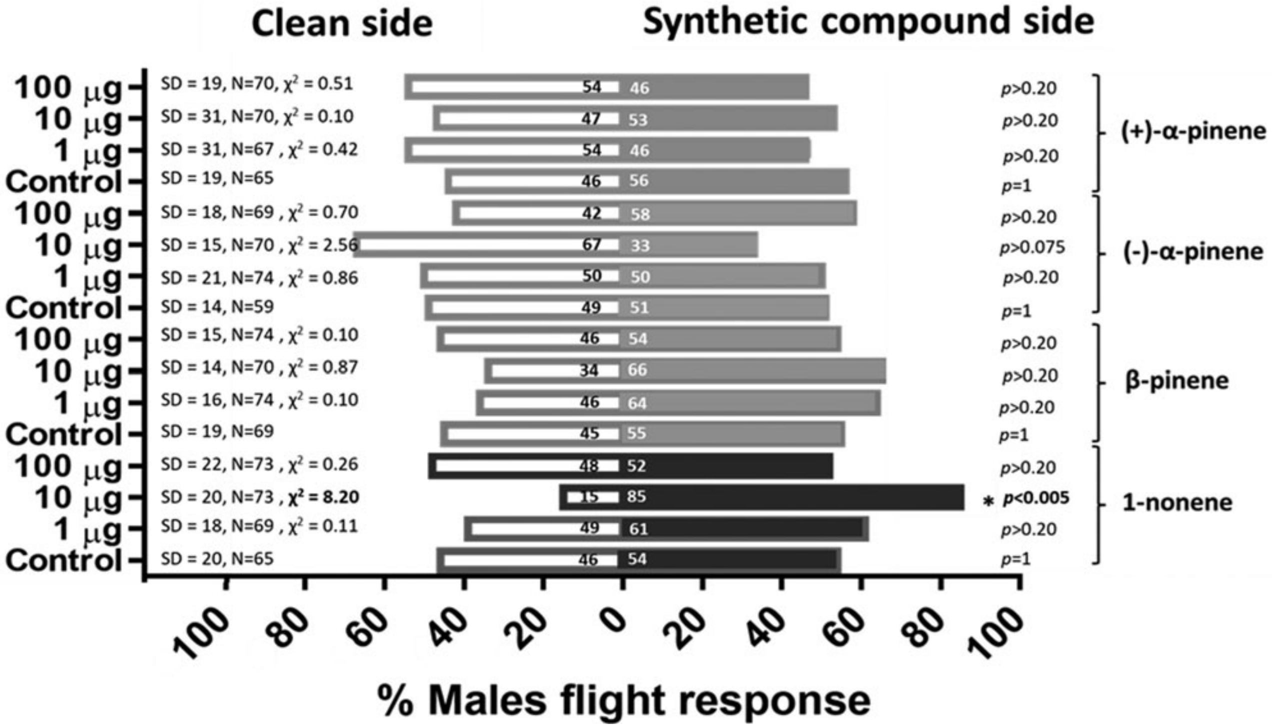

Figure 5. Behavioral responses of male *Ae. aegypti* to four identified *S. madagascariensis* volatiles using a dual-port flight olfactometer. Responding mosquitoes that crossed the mark at the end of the arm were counted. Asterisks express significant differences in response vs. the acetone control in the chi-squared test ($*P < 0.05$; $df = 1$; critical value $\chi^2_{(1,\ 0.05)} = 3.84$). SD, standard deviation.

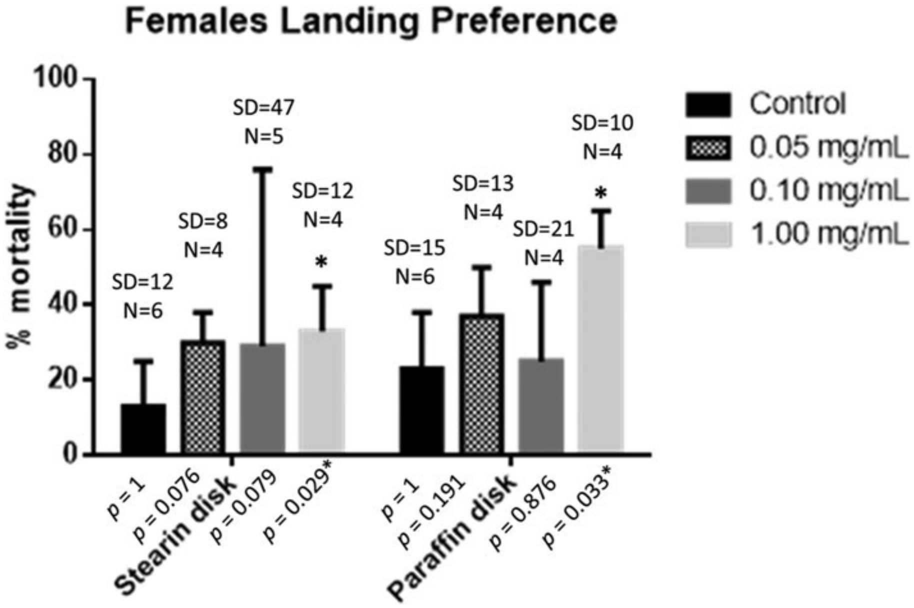

Figure 6. Mortality of male *Ae. aegypti* in the presence of toxic sugar food baited with stearin and paraffin disks containing imidacloprid with the addition of 1-nonene at different concentrations. Asterisks express significant differences in response vs. the acetone control in the K–W test ($*P < 0.05$).

mosquito biology. *Ae. aegypti* often live in urban habitats (Braks *et al*., 2003; Tsuda *et al*., 2006) meaning that may have less access to carbohydrates. In our design, adults had free access to sugar feeding post-eclosion, being starved 24 h prior the bioassays. The continuous sugar provision could have led to multiple feeding instances and high sugar intakes, impacting a sugar seeking impetus (Wensler, 1972; Vargo and Foster, 1982). For a deeper understanding of the influence of these factors, more parameters need to be controlled.

Regardless, the existence of a plant or chemical that only attracts males is quite relevant because it can be exploited in sterile male programs. In this context, traps baited with flowers or with formulated bioactive chemicals could be displayed in a potentially infested environment and withdrawn before the release of sterile males, thereby increasing the efficacy of management by reducing the reproductive competition (Lacroix *et al*., 2012).

Regarding traps baited with flowers, Müller *et al*. (2008) collected up to 75 times more *Anopheles sergentii* (Theobald) in

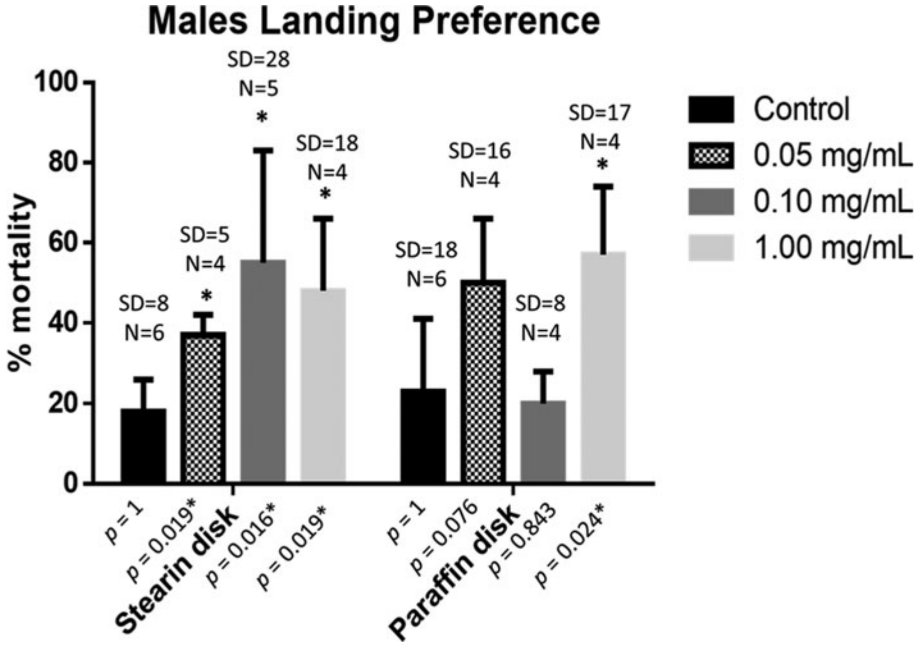

Figure 7. Mortality of female *Ae. aegypti* in the presence of toxic sugar food baited with stearin and paraffin disks containing imidacloprid with the addition of 1-nonene at different concentrations. Asterisks express significant differences in response vs. the acetone control in the K–W test (*$P < 0.05$).

traps baited with *Acacia raddiana* flowers than in traps baited with the non-flower part of the plant. Moreover, they sprayed *Acacia raddiana* flowers with a sucrose solution containing a colorant and an insecticide and found a significant decrease in the mosquito population, thus supporting the use of flowers combined with insecticides as toxic baits in IPM for mosquito control (Müller and Schlein, 2006; Mathew *et al.*, 2013; Xue *et al.*, 2013).

Differential responses to field traps scented with *Psidium guajava* (guayaba) and *Mangifera indica* (mango) in males and females of *Ae. aegypti* were reported by Fikrig *et al.* (2017), with only males collected in the traps. In this study conducted in Australia, the overall mortality was modest, suggesting that the impetus to feed on sugar might not be strong enough to merit its use as a lure for passive trapping. These authors correlated their findings with a field test performed in Thailand that showed that females did not consume sugar in the field for 2 or 3 days. Although this background might not support our final goal, another recent work carried out in Ecuador (Qualls *et al.*, 2016) showed that outdoor sugar feeding is a common behavior of *Ae. aegypti* and can be targeted as a control strategy in urban landscapes of Latin America. In another study, Revay *et al.* (2014) demonstrated the potential use of attractive sugar baits for *Ae. albopictus* Skuse (Diptera: Culicidae) pest control. These findings support the potential of our laboratory-derived results to drive field testing.

Since the main goal of this study was to introduce a male attractant in toxic baited traps, 1-nonene was formulated in a solid matrix for its controlled release using paraffin and stearin wax, which were selected because they are economic, nontoxic, environmentally friendly, chemically stable, easy to handle, and chemically compatible with 1-nonene due to their similar partition coefficient (Kow) and their low water solubility. The reasonable solubility of the active principle in the matrix is expected to favor its slow release and prevent a burst effect during release.

Mortality caused by imidacloprid in the presence of stearin disks impregnated with 1-nonene at all evaluated doses was significantly higher than that in the control test without 1-nonene for male mosquitoes. Although for females, high mortality was only observed for the highest dose. For paraffin disks, only the highest dose (1 mg ml^{-1}) of 1-nonene showed increased mortality for both males and females.

The increased mortality of males in the presence of disks impregnated with 1-nonene was expected and has promising implications for its use in field traps baited with toxic substances. The differences in mortality between paraffin and stearin disks (with the stearin disk being effective in a wider range of doses) could be attributed to the higher solubility of 1-nonene in paraffin compared to stearin, which would lead to a lower release rate and consequently to lower bioavailability.

The mortality of females, although not expected based on the olfactometer results, could be attributed to the higher dose of 1-nonene in the disks compared to the concentration present in flowers. A bioassay with toxicological end points performed with 1-nonene impregnated on solid disks showed that the incorporation into a solid matrix of a volatile attractant increased the overall mortality of the toxic sugar bait.

The results obtained thus far could be improved by using synthetic blends or combinations of 1-nonene with individually nonattractive volatiles since it is known that stronger behavioral responses can be elicited in insects exposed to appropriate blends compared to insects exposed to single compounds (Bruce and Pickett, 2011).

In summary, in this work, the *S. madagascariensis* flower scent was found to act as an attractant for male mosquitoes. Potential volatile attractants were identified and independently tested in an olfactometer, and one was found to affect males. It was then incorporated in a solid matrix together with a toxin as a successful toxic sugar bait. Beyond describing the floral scent that acts as an attractant for *Ae. aegypti*, the responsible compound was identified, biologically evaluated, and incorporated in a solid controlled release formulation. Due failures in current control programs, the WHO has prioritized the search for new tools to be incorporated in IPMs for mosquitoes. Among these new tools, the use of attractive toxic baits is strongly suggested (WHO, 2008; Galili, 2017). This shows the importance of our study that lays the groundwork for designing a new type of baited trap for mosquito control or surveillance.

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