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A synthetic lure for *Anopheles gambiae* (Diptera: Culicidae) based on the attractive plant *Parthenium hysterophorus*

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Sugar is the sole diet for male mosquitoes and a complementary meal for females. Searching for natural sources of sugar is mediated by semiochemicals. Floral nectars, extra floral nectaries, damaged tissues of plants and rotten fruits are the most common sources of sugar in nature. I provide laboratory evidence of the high attraction of *Parthenium hysterophorus* L., a weed that grows in tropical climates, to *Anopheles gambiae* Giles. This study has tried to identify the chemicals which might be involved in the chemical attraction of *A. gambiae* to this plant. Using quantitative GC-MS analysis, α-pinene, camphene, 1-octen-3-ol, β-pinene, cis-β-ocimene, bornyl acetate, α-caryophyllene, hexadecanoic acid, and α-linolenic acid were identified as the main constituents of *P. hysterophorus* volatiles. Successive olfactory assays helped a better understanding of the more attractive chemicals of *P. hysterophorus* to *A. gambiae* which was the basis for testing a possible synthetic blend. Olfactory experiments proved this synthetic blend to be as attractive as *Parthenium* intact plants for *A. gambiae*. A minimal blend, consisting of only α-pinene, camphene, and cis-β-ocimene, was also produced and laboratory experiments indicated its relative attraction for *A. gambiae*. This blend can be tested in the attractive toxic sugar bait stations for sampling, surveillance, or control programs of mosquitoes in tropical Africa, where *A. gambiae sensu stricto* transfer malaria among residents.

Key words: sugar feeding, phytochemicals, toxic sugar-bait stations, mosquito surveillance

Introduction

Mosquitoes have evolved many specializations in order to find and utilize sugar as their source of energy (Foster 1995, Peach and Gries 2020). In nature, male mosquitoes solely feed on natural sources of sugar (Yuval 1992), such as floral nectar, honeydew, rotten fruits, extrafloral nectaries, and damaged tissues (Foster 1995).

The required energy for flight, survival, and fecundity of female mosquitoes is obtained by both sugar and blood meals (Nayar and Van Handel 1971, Van Handel 1972, Reisen and Emory 1976). Previous studies indicated that sugar could be the single energy source of flight for host-seeking female *Anopheles* (Takken and Knols 1999), while newer research revealed that different species of mosquitoes could utilize different sources to obtain their required energy. For example, in *A. gambiae*, sugar and blood have the same contribution in providing the required flight energy, while *A. atroparvus* and *Ae. aegypti* mainly utilize carbohydrates to gain energy (Kaufmann and Briegel 2004).

Much of the research indicates sugar feeding in *A. gambiae* is more common than what was once thought (Muirhead-Thompson 1951, Gillies and De Meillon 1968, McCrae 1989). Nyasembe et al. (2018) reported that 24% of the females *A. gambiae* caught in different habitats of Kenya were positive for fructose. While most of the older literature name plant nectars, extrafloral nectaries, and honeydew as the sources of sugar for female mosquitoes, the more recent studies have provided evidence of plant tissue feeding as well (Nyasembe et al. 2018).

Mosquitoes use olfactory stimuli shared among vertebrates, plants, and honeydew (Dekel et al. 2019, Ignell and Hill 2020, Peach and Gries 2020). They detect such chemicals using olfactory receptors in their antennal olfactory receptor neurons. Such information is further integrated with the senses of temperature and

humidity, as well as vision, processed in the brain into a behavioral output, leading to host finding (Coutinho-Abreu et al. 2022). In addition to innate olfaction-mediated behaviors, mosquitoes rely on olfactory learning which increases their individual fitness by selecting advantageous choices (Lutz et al. 2017). Long-range attraction to a stimulus is presumably steered by olfaction, while visual cues are involved at shorter range (Thorsteinson and Brust 1962, Healy and Jepson 1988, Jepson and Healy 1988).

Until recently, the information on specific sugar meal sources was mostly narrative and came from few observations, despite this phenomenon's importance (Foster 1995). *Anopheles gambiae* appears to show some preferences among vegetational communities of tropical Africa (Gary and Foster 2004, Impoinvil et al. 2004, Manda et al. 2007a, Gouagna et al. 2010, Müller et al. 2010a, Beier at al. 2012, Nyasembe et al. 2018). Using molecular techniques, Nyasembe et al. (2018) indicated that *Senna alata* (Fabaceae), *Senna tora* (Fabaceae), *Ricinus communis* (Euphorbiaceae), *Parthenium hysterophorus* (Asteraceae), and *Leonotis nepetifolia* (Lamiaceae) were the preferred host plants of *A. gambiae* in Kenya. In laboratory settings during cage studies and olfactometry experiments, *A. gambiae* was selective in plant choice, which indicates a preference for particular plant species as sources of sugar in the field (Manda et al. 2007a, Nikbakhtzadeh et al. 2014). Furthermore, studies on plant feeding demonstrated that some plant species promote the survival of *A. gambiae* males and females (Gary and Foster 2004, Impoinvil et al. 2004, Manda et al. 2007b, Nikbakhtzadeh et al. 2016).

Mosquitoes depend on their ability to respond to chemical cues for feeding, host preference, and mate location/selection (Liu et al. 2010). Chemosensory cues have a major role in directing *A. gambiae* to the hosts and are a significant determinant in vectorial capacity of the female individuals (Liu et al. 2010). Olfactory cues mediate a variety of behaviors in females, i.e., sugar feeding, host-seeking, and oviposition (Takken and Knol 1999).

Availability of sugar sources is a major factor, in regulating the dynamics of mosquito populations and their vectorial potential. Therefore, control interventions targeting natural sources of sugar are a promising strategy to lower mosquito populations, and thus the local transmission of *Plasmodium* and other mosquito-borne pathogens (Kline 2007, Gu et al. 2011). There are only a few studies that have investigated the most attractive plants to *A. gambiae* in either the field (Müller et al. 2010a) or laboratory (Gary and Foster 2004, Gouagna et al. 2010, Impoinvil et al. 2004, Manda et al. 2007a, Nyasembe et al. 2012, Nikbakhtzadeh et al. 2014). *Parthenium hysterophorus* L. (Asterales: Asteraceae) has been identified as a plant that is likely attractive to *A. gambiae* (Manda et al. 2007a, Nikbakhtzadeh et al. 2014, Nyasembe et al. 2018). It is native to South-central America, from the Gulf of Mexico and West Indies to central Argentina (Picman and Towers 1982) and has been widely introduced to Africa, Australia, and southeastern Asia as an aggressive, invasive weed (Picman and Picman 1984). There have been contradictory results concerning sugar providing preference of *P. hysterophorus* for *A. gambiae* (Manda et al. 2007a, Nyasembe et al. 2012, Nikbakhtzadeh et al. 2016).

Nikbakhtzadeh et al. (2014) studied the most abundant volatile organic compounds (VOCs) in the floral and foliar scent of *P. hysterophorus*. The current research builds on that study by investigating what exact combination of compounds *A. gambiae* s.s. might respond to. Therefore, this work will first provide a robust laboratory proof regarding the attraction of *A. gambiae* to *P. hysterophorus* and then add more to the recent findings of *P. hysterophorus* chemical profile. Various parts of *Parthenium* shrubs were chemically analyzed to have a better understanding of its floral and foliar volatile compounds. The attractive components for *A. gambiae* were subsequently screened in a dual-port olfactometer, and a synthetic blend was developed in the laboratory.

Materials and Methods

Mosquito Rearing

Anopheles gambiae utilized in this study were from the Mbita Strain, originating from a local population in Mbita point (00° 26–27' S, 34° 12–13' E), Suba District, Nyanza, and western Kenya. This strain was identified by staff of the International Centre of Insect Physiology and Ecology in 2001 and its genetic allocation was verified by molecular techniques (Scott et al. 1993, Stone at al. 2011). Specimens for behavioral assays were taken from the Mbita colony at The Ohio State University.

Female individuals were blood fed for 15 min from a human arm twice per week, conducted in accordance with The Ohio State University's Biosafety Protocol No. 2005R0020 and Biomedical protocol No. 200440193. Adults were maintained in small acrylic cages (26.7 × 20.3 × 14 cm) in a mosquito insectary (27 ± 1°C, 75 ± 5% RH, L:D 12:12 h) and had access to aged tap water and 10% (v/v) sucrose-soaked cotton wicks. The clock time of light-dark and dark-light transitions (19:00, and 07:00, with 1.5-h crepuscular periods at each end of scotophase) approximately coincided with the light cycle during olfactometer tests (see below). The eggs, which were laid in half-filled water dishes (3.3 cm high, 9 cm diam), hatched 2 days later. Groups of 200 first instar larvae were transferred to a pan (33 × 24.1 × 5.1 cm) and fed a regimen of powdered Tetramin® fish food (Tropical Flakes, Tetra Holding, Inc. Blacksburg, VA, USA) until pupation. The mean of wing lengths ± SE was 3.313 ± 0.016 mm for females (n = 30) and 3.12 ± 0.014 mm for males (n = 30). For olfactometer tests, pupae were transferred in cups to the same acrylic cages (26.7 × 20.3 × 14 cm), provided with two water wicks, for adult emergence. Each cage had a wide-sleeved opening at one end and a wide-screened opening at the other, so that it could serve as a releasing chamber, connecting directly to the downwind end of an olfactometer without being handled prior to each test.

Experimental Plants

Seedlings of *P. hysterophorus* and *Senna didymobotrya* (Fabales: Fabaceae) were grown, pesticide-free, in the Biological Sciences Greenhouse facility at The Ohio State University. *Parthenium hysterophorus* was not only used to measure its relative olfactory attractiveness to *A. gambiae* but used for chemical analysis and developing a synthetic blend as well. *Parthenium* is a fast-growing annual weed. Its leaves are branched and covered with soft fine hairs. The small white flowers (4 mm across) have five distinct corners and grow on the stem tips. Each flower produces four or five black wedge-shaped seeds that are 2 mm long with thin white scales (Australian CRC weed management 2003, Stamps 2011). This plant was observed to be stimulating to *A. gambiae* in experimental cages (Manda et al. 2007a) and olfactometer (Nyasembe et al. 2012, Nikbakhtzadeh et al. 2014).

Leaves of orchardgrass, *Dactylis glomerata* L. (Cyperales: Poaceae), served as a simultaneously tested neutral control. This common grass, native throughout most of Europe, temperate Asia, and northern Africa (Shu et al. 2006), and common in North America (Kartesz 2011), lacks nectaries (Drabble and Drabble 1927) and was considered likely to elicit no more than a general attraction to a background of green-plant volatiles. Preliminary experiments demonstrated that it was more attractive than a blank (i.e., no plant)

(paired *t*-test, $t = 16.1$, $df = 5$, $P < 0.001$), but did not support mosquito survival longer than water alone (paired *t*-test, $t = 0.81$, $df = 3$, $P > 0.05$). Prior to tests, plants were inspected for infesting insects, e.g., ants, aphids, scales, and spiders, that might interfere with a plant's volatiles.

Olfactometer

Six, dual-port, wind-tunnel olfactometers were used simultaneously, all being modified T-tube versions of Geier's improved Y-tube design (Geier et al. 1999), to measure the oriented flight response of mosquitoes to the VOCs of *Parthenium* plant, *Parthenium* extract or synthetic blends in an air current (Fig. 1). The olfactometer tests were conducted in a room maintained at ambient 27°C. Suspended 135 cm above the olfactometers was a 183 × 122-cm network of miniature incandescent white lights. Prior to and at the outset of the test, the overhead light intensity was 10 lux. Between 19:00 and 20:30, the light automatically and gradually dimmed to 1 lux and remained so for the night. Between 06:30 and 08:00, it gradually returned to 10 lux.

Olfactometer Structure

Two air-pressurized diaphragm air pumps were independently connected to three olfactometers. The airstream of each pump passed through a granular activated charcoal filter, humidified to 70% RH by being bubbled through warm water, and split equally among six tubes with separate flow meters. This arrangement provided stable air speed and physical conditions throughout the testing period. In each olfactometer, pressurized conditioned air streams passed into two polypropylene bags (50.8 × 76.2 cm; ULINE, Pleasant Prairie, WI), one holding the test plant, plant extracts, or synthetic blends, and the other one holding the control plant, a solvent, or a different blend. The exiting airflow (20 ml/s) from each polypropylene bag entered three cylindrical trap compartments (23.5 cm long, 10 cm diam.) that retained only trapped mosquitoes. Each chamber was fitted with a conical polyethylene terephthalate baffle piece (10.5 cm long, 5.8–7.5 cm diameter) (Verhulst et al. 2008), projecting less than halfway into the trap from the port. This piece, netted at one side, allowed air to flow around its baffle at the margin of its narrower upwind end, and it allowed attracted mosquitoes to enter the port from the choice chamber, pass around the baffle, and be retained in the trap compartment (Fig. 1).

The trap compartment was enlarged, mainly based on my experience with a previous prototype where the trap was much smaller (Nikbakhtzadeh et al. 2014). This design provided a larger trap space and was found to be very useful in attracting a higher number of mosquitoes. Because of its depth, the baffle was significantly farther from the port and trapped mosquitoes had little chance to find their way and return downwind to the choice chamber. This new setting is bigger both in length and diameter versus the previous model (23.5 cm vs 13.5 cm length and 10 cm vs 6.1 cm diameter). The length of baffle trap itself was increased from 6 cm in the previous model to 10.5 cm in the present setting. While only 31.3% of the released mosquitoes flew upwind in the presence of an attractive source odor in the previous model of my olfactometer (Nikbakhtzadeh et al. 2014), more than 60% of mosquitoes were activated and responded to the same attractant in my new olfactometer.

This olfactometer is divided into parts, each made of clear acrylic plastic: (i) a choice chamber (36.1 × 16.0 × 12.7 cm, l,w,h), (ii) a cylindrical upwind flight section (20.1 cm long, 6 cm diam.), and (iii) a release chamber (26.7 × 20.3 × 14 cm). The choice chamber,

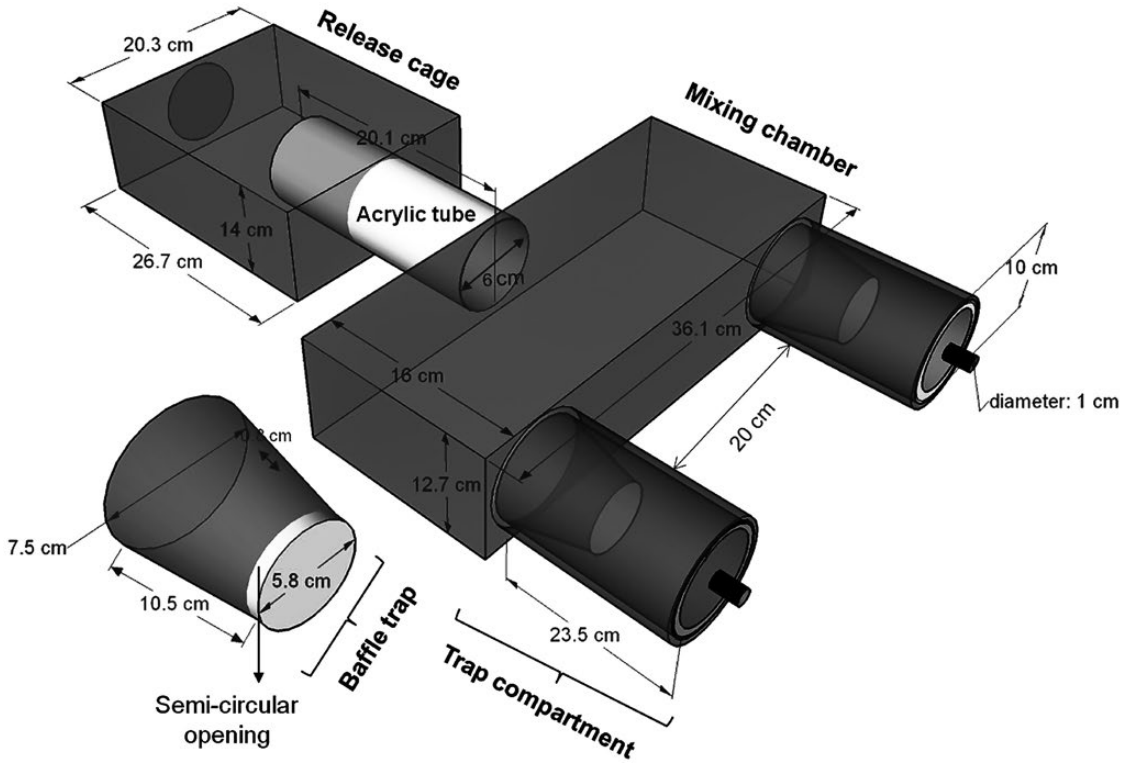

Fig. 1. Schematic design of one double-port olfactometer with choice chamber. Baffle trap, part of trap compartment, is enlarged to indicate details. Light-colored band on baffle trap refers to semi-circular opening. Terminal part of baffle trap (diameter 5.7 cm) is covered by netting.

Table 1. Compounds detected and identified in a hexane-base extract of *Parthenium hysterophorus*. Compounds are ordered based on their elution time in a GC column. The last two compounds were unattractive for *A. gambiae* in a dual-port olfactometer, and thus excluded from the *Parthenium*-base synthetic blend.

Components in *Parthenium* extract	Molecular weight (g/mol)	Boiling point (˚C)	Diagnostic EI-mass fragments	Concentration in *Parthenium* extract (mean ± SE), ng/µl	Required mass in a synthetic blend (ng/µl)
α-pinene	136.23	155	93, 91, 77, 39	0.76 ± 0.325	11.4
Camphene	136.23	159	93, 121, 79, 39	2.56 ± 0.266	38.4
1-octen-3-ol	128.21	174	57, 43, 72	9.36 ± 0.383	140.4
β-pinene	136.2	165	93, 43, 69	19.24 ± 0.536	288.6
cis-β-ocimene	136.23	175	93, 91, 79, 77	13.83 ± 0.278	207.45
bornyl acetate	196.29	223	95, 43, 93, 121	2.11 ± 0.173	31.65
α-caryophyllene	204.35	266	93, 133, 91, 41	2.21 ± 0.22	33.15
Hexadecanoic acid	256.42	351	43, 73, 60, 41	1.68 ± 0.206	-
α-linolenic acid	278.42	443	79, 67, 93, 95	6.9 ± 0.349	-

perpendicular to the flight section, served to combine the airstreams from the two 6-cm ports, the inter-margins 20 cm apart, entering the chamber from each port at 0.7 m/s, as measured by anemometer (VelociCalc®, Model 9545, TSI Inc., Shoreview, MN). Within the chamber, between the ports, a HOBO® datalogger (Onset®, Bourne, MA) recorded temperature and humidity as follows: 27 ± 0.5˚C and RH 70 ± 5% (n = 50). At the centrally positioned exhaust, air exited the choice chamber and entered the flight section at 0.2 m/s. When mosquitoes were to be tested, the downwind end of the flight section was connected directly to the sleeved end of the mosquito-containing release chamber. From there, the air was conducted via a dryer vent hose from the release chamber's down-wind screened end, assisted by an AC-CPU fan, into a fume hood, drawing at a speed of about 0.2 m/s. To detect possible intrinsic bias between the two ports, I used 4,930 mosquitoes to conduct five tests with both ports blank, seven tests with both ports baited with honey and 18 tests with honey-baited ports versus blank. No difference was found by t-test with dual blanks (t = 0.66, df = 4, P = 0.55) and dual honey (t = 0.8, df = 6, P = 0.45), but a highly significant difference was observed when tested honey versus blank (t = 4.59, df = 9, P < 0.001).

Olfactometry Procedure

The release chamber of mosquitoes was transferred from the mosquito insectary to the testing laboratory at least 1 h prior to its attachment to the flight section. The release chamber contained a mean of about 200 (range ~180–220) untested mixed male and female adults, which at the time of testing had emerged about 24 h earlier, having had continuous access to water on wicks but no access to blood or sugar. Intact potted plants were also transferred from the greenhouse to the lab 2–3 h before testing. Intact *P. hysterophorus*, *D. glomerata* (carefully inspected to be pest-free) and sachets containing *Parthenium* extracts, or synthetic blends were placed on their sides within polypropylene bags. These bags are connected to in- and out-pressurized air tubes (ID: 6.4 mm; Tygon tubing, Saint-Gobain) via two metal connectors.

Each test ran overnight, from 19:00 to 08:00. Mosquitoes in each trap and in the downwind sections were collected, separated by sex, and counted at 08:00 the following morning. Mosquitoes that had left the release chamber and were found in the choice chamber were categorized as "activated." Those in the treatment trap were in fact both activated and "attracted," but here I call them attracted to be clearly differentiated from the other groups. Each experiment consisted of at least 10 replicates, unless otherwise stated, conducted on consecutive nights, and the test and control ports were alternated between right and left in successive replications to cancel any positional effect. Pieces of Olfactometers were washed thoroughly with hot water for 3 min. All pieces were then placed for 15 min in the oven at 120℃.

Extract Preparation

Leaf and floral extracts of *P. hysterophorus* were made by dipping 2.13 and 0.64 g fresh leaves and flowers into 12 and 4 ml 99% n-hexane (Alfa Aesar, Ward Hill, MA) for 3 h, respectively. The whole-plant extract was prepared using 3.9 g sprigs containing both leaves and flowers (1:1 w/w), immersed in 15 ml n-hexane for the same exposure time. In order to have a higher precision, five extracts of *P. hysterophorus* shrubs were prepared and the amount of each volatile compound was measured (n = 5). The final concentration of each compound in a synthetic blend was calculated based on the average weight of a small-size *Parthenium* shrub (mean ± SE = 60.1 ± 1.83 g, n = 5). Since the average weight of a small *Parthenium* shrub was approximately 15 times higher than that of sprigs (3.9 g), I prepared a 15-time higher concentration of each volatile (Table 1) in order to reach the same competence level with a *Parthenium* shrub in an olfactometer. For instance, when 0.76 ng α-pinene was found in 1 µl of *Parthenium* extract, the final dose of this compound in synthetic blend would be 15 folds higher, i.e., 11.4 ng (Table 1).

Chemical Analysis of Volatiles (Quantitative GC-MS)

One microliter of each compound, plant extract or a chemical blend was injected, in non-split mode, into an Agilent 6890 GC instrument, connected to a 5973 quadrupole mass selective detector (Agilent technologies, USA). Regular negative controls (n-hexane blanks) were injected between runs to ensure that none of the detected records was due to a prior contamination. Vials of plant extracts, authentic compounds and synthetic blends were vortexed for 30 s before any injection. The Agilent gas chromatograph was equipped with a Zebron™ ZB-5 (Phenomenex, Torrance, CA) bonded-phase fused-silica capillary column (30 m, 250 µm ID, 0.25 µm FT). The oven temperature was held at 25˚C for 3 min, first ramped at 10˚C/min to 80˚C and then at 25˚C/min to 250˚C where it was held for 5 min. Helium was used as the carrier gas at 36 cm/s (flow rate: 1 ml/min). The injector, ion source, and transfer line temperatures were set at 250, 240, and 250˚C, respectively. The detector was set off for 2.00 min; mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1 scan/s from m/z 20–350. Autointegration was achieved by Agilent Enhanced Chemstation® (version D.00.38, 2001). Chemical components of leaf, floral, and the whole plant extracts were identified by matching their mass spectra to NIST Mass Spectral Library 2005 and comparison of retention indices to

published values (Adams 2007). Decisive identification was made by comparison of spectra and retention times of major components to the authentic standards. Electron impact mass fragments of compounds identified in hexane-base extract of *P. hysterophorus* are presented in Table 1. Standards of all chemicals were provided from Aldrich (Milwaukee, WI), Sigma-Alrich Co. (St. Louis, MO), and Aldrich (Steinheim, Germany).

In order to calculate the amount of each compound within the *Parthenium* chemical mixture, three regression curves were developed based on allocation of each compound to a chemical group. For terpenes, (-)-β-pinene (99%, Aldrich, St. Louis, MO) was serially diluted in 99% n-hexane and 1 µl of five ascending concentrations (0.24–61.8 ng/µl) of (-)-β-pinene was injected to GC-MS instrument as external standards. This provided a linear regression equation (Y = 434148X), in which X and Y axes represent mass of (-)-β-pinene versus peak area respectively (R^2: 0.96). The same procedure was followed for alcohols and acids, which are the other main components of a *Parthenium* whole-plant extract. 1-octen-3-ol (98%, Aldrich, St. Louis, MO) and hexadecanoic acid (99%, Sigma-Aldrich, Milwaukee, WI) were also serially diluted and used for quantitation of alcohols and acids, respectively. GC peaks of 1-µl injections of 1-octen-3-ol, and hexadecanoic acid in ascending concentrations (0.2–61.8 ng/µl and 0.3–323 ng/µl for the two compounds, respectively) helped me develop two relevant regression curves with high accuracy for alcohols (Y = 57744X + 15015, R^2: 0.997) and acids (Y = 38070X + 237175, R^2: 0.952). These regression equations were used to measure the mass of each volatile component in *Parthenium* extracts.

Volatile Concentration- and the Release Rate Optimization

Low-density polyethylene (LDPE) sachets (8 × 3.85 cm, ULINE, Pleasant Prairie, WI) were used to test the liquid extracts or blends. LDPE materials have proved very useful, because they let the VOCs release at a constant low rate, and do not need to be refilled for a long period of time (Torr, et al. 1997). Another advantage of using LDPE sachets is the easier adjustment of release rate, while release rate optimization of nylon strips, despite their effectiveness (Mukabana et al. 2012), is more challenging. The release rate of a chemical blend in a sachet was optimized via series of olfactometry experiments until the best mosquito attraction, non-significantly different from the plant itself, was recorded.

Before being used, the headspace of empty LDPE sachets and polypropylene bags were collected by SPME gray fibers (Supelco, 2 cm-50/30 µm DVB/carboxen™/PDMS StableFlex™) and analyzed by GC-MS to exclude any volatile compound which could be observed in the chemical analyses of plants or synthetic blends.

In order to measure the attraction rate of a *Parthenium* extract, an LDPE sachet was filled with *P. hysterophorus* extract, heat sealed, and placed in a polypropylene bag. The same volume of a hexane-filled LDPE sachet (control) was similarly placed in another polypropylene bag, and the two bags were connected to an olfactometer. When testing a blend, each component was individually packed in LDPE sachets of the same dimensions and thickness and placed in a single polypropylene bag. In this way, possible unknown effects of these compounds on each other were avoided.

Subtractive olfactometry bioassays were consecutively conducted on the full synthetic blend to determine the relative attraction of each ingredient for *A. gambiae*. The release rate was controlled by the volume of mineral oil, changing the surface of the LDPE sachet, or putting sachet in another one (double layered).

A seven-component blend (including the most abundant compounds in GC profile) was placed in one olfactometer port while a reduced six-compound blend, in which one of the components was missing, was placed in the other port. Each reduced blend was tested 10 times. After figuring out the significance of each chemical on mosquito attraction, I compared the attraction of the finalized minimal blend against *Parthenium* intact plant and the full blend.

Statistical Analysis

To compare the attractiveness of *Parthenium* plant, *P. hysterophorus* extract, full synthetic blend or subtractive blends versus my control plant, n-hexane and *P. hysterophorus* extract, the number of mosquitoes caught in their respective ports was counted for each of the 10 replications. Those numbers were divided by total number of released mosquitoes in order to have a proportional value. Distribution of these values was determined by a Kolmogorov–Smirnov test. If a normal distribution existed, a paired-comparison *t*-test was used to detect any significant difference between the numbers attracted to the tested plant/extract/synthetic blend and the control; otherwise, the non-parametric Mann–Whitney *U*-test was used. Analyses were performed with SPSS statistical package Ver. 28.00 (Chicago, Illinois, USA) and graphics were drawn by Microsoft Excel® (version 18.2110.13110.0, Redmond, WA).

Results

Attraction of *P. hysterophorus* for *A. gambiae*

In the present study, intact *P. hysterophorus* plant was compared with the sugar-poor plant, *Dactylus glomerata*, in an olfactometer to provide quantitative evidence for the olfactory attraction of *A. gambiae* to this plant. The series of experiments strongly indicated that *P. hysterophorus* was much more attractive than the baseline control plant, *D. glomerata*. While over 48% (mean value) of mosquitoes attracted to *P. hysterophorus*, only 15% of them responded to *D. glomerata* potted shrubs (Fig. 3a); therefore, *P. hysterophorus* is confirmed as a highly attractive plant for *A. gambiae* males and females (paired *t*-test, $t = 8.2$, $df = 10$, $P < 0.001$). My side-by-side olfactometry experiments did not

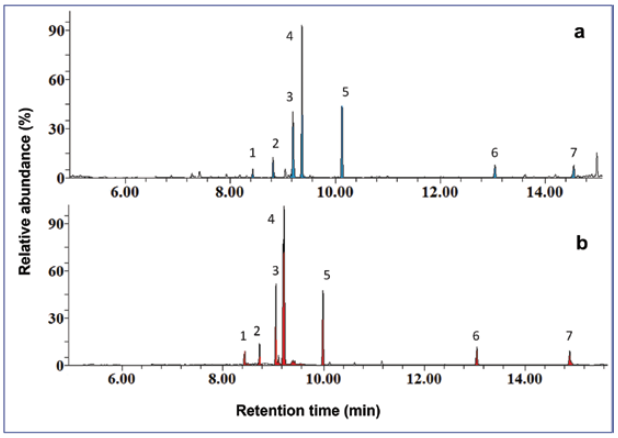

Fig. 2. Total ion chromatogram of (a) *Parthenium hysterophorus* L. (Asterales: Asteraceae) whole-plant extract in 99% n-hexane and (b) synthetic blend. Detected and quantified compounds are shown in numbers based on their elution time. 1: α-pinene (8.5E, 8.43B), 2: camphene (8.8E, 8.81B), 3: 1-octen-3-ol (9.1E, 9.19B), 4: β-pinene (9.36E, 9.21B), 5: cis-β-ocimene (10.1E, 9.99B), 6: bornyl acetate (13.04 E,B), 7: caryophyllene (14.6E, 14.8B). E: extract, B: blend.

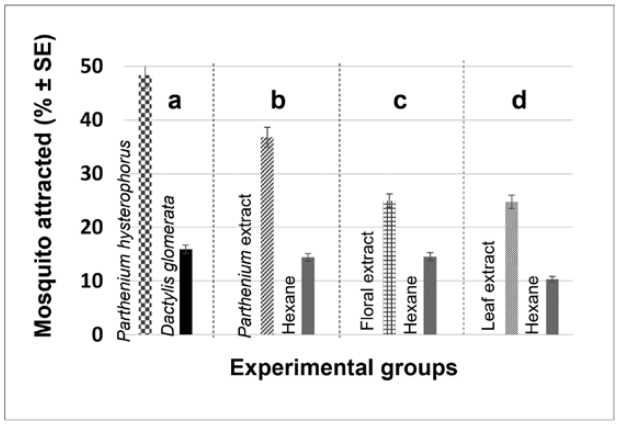

Fig. 3. Percentage of mean proportions (± SE) of *Anopheles gambiae* attracted to (a) *Parthenium hysterophorus* L. (Asterales: Asteraceae) and *Dactylis glomerata* L. (Poales: Poaceae), (b) *P. hysterophorus* extract, (c) *Parthenium* floral extract and (d) *Parthenium* foliar extract versus 99% n-hexane in a dual-port olfactometer. Data had a normal distribution. $n = 11$, $df = 10$, Paired sample *t*-test; (a): $t = 8.217$, $P < 0.001$ (b): $t = 4.1$, $P < 0.01$ (c): $t = 3.1$, $P < 0.05$ (d): $t = 3.6$, $P < 0.01$.

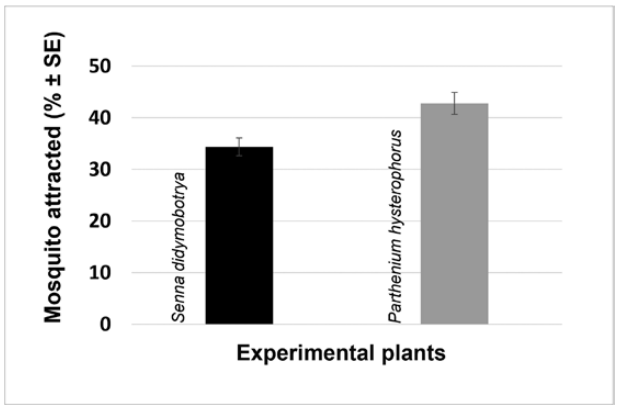

Fig. 4. Percentage of mean proportions (± SE) of *Anopheles gambiae* responded to *Senna didymobotrya* (Fresen.) Irwin & Barneby (Fabales: Fabaceae) and *Parthenium hysterophorus* L. (Asterales: Asteraceae) in a dual-port olfactometer. $n = 9$, data had a normal distribution. Paired sample *t*-test ($t = -2.24$, $df = 8$, $P > 0.05$).

indicate any significant difference in total attraction response between *P. hysterophorus* and *S. didymobotrya* (paired *t*-test, $t = 2.24$, $df = 8$, $P > 0.05$); however, a higher number of mosquitoes were attracted to *P. hysterophorus* (Fig. 4). These side-by-side experiments provided an unambiguous conclusion on the relative preference of these very attractive plants for *A. gambiae*.

In another experiment, 15 µl of *Parthenium* extract in 99% n-hexane was tested versus the same volume of blank (99% hexane) to investigate its attractive level for mosquitoes (Fig. 3b). Data collected clearly indicated a significant difference between the two groups (Paired *t*-test, $t = 4.1$, $df = 9$, $P < 0.01$). Similar olfactometry experiments were arranged between *Parthenium* floral and foliar extracts on one hand, and the n-hexane blank on the other hand (Fig. 3c, d). Both experiments confirmed *Parthenium* superiority in attracting *A. gambiae* over blank (paired *t*-test, floral test: $t = 3.1$, $df = 9$, $P < 0.05$; foliar test: $t = 3.6$, $df = 9$, $P < 0.01$). The experiments with 99% n-hexane versus blank (empty LDPE sachets) under the same experimental conditions showed no significant difference. Therefore hexane, as a solvent, did not attract *A. gambiae* better than an empty LDPE sachet (paired *t*-test, $t = 1.05$, $df = 7$, $P = 0.32$).

Chemical Characterization of *P. hysterophorus* Volatiles

Volatile compounds of *P. hysterophorus* plant, floral, and foliar extracts were chemically characterized and quantified by gas chromatography-mass spectrometry (Fig. 5a–c). Terpenes, such as α-pinene and cis-β-ocimene are the dominant compounds in *P. hysterophorus* hexane-based extract (Fig. 3a). All these compounds, except for hexadecanoic- and α-linolenic acid, which are not very volatile due to their high molecular weights and boiling points, were quantified and used for making a synthetic blend (Table 1). None of hexadecanoic- and α-linolenic acid stimulated *A. gambiae* individuals in my olfactometry experiments, either as a single compound or in mixture, compared to a hexane blank sachet (*Mann-Whitney U* test, $df = 9$, $Z = 1.14$, $P > 0.05$). Furthermore, adding them to the rest of *Parthenium*-identified volatiles within a blend reduced mosquito response.

The chemical profile of *P. hysterophorus* whole plant (Fig. 5a) was to some extent different from those of flowers (Fig. 5b) and leaves (Fig. 5c); however, terpenes were still the most abundant volatile compounds in the plant's foliar and floral extracts. Floral scent of *P. hysterophorus* was overwhelmed by terpenes. For example, β-pinene, cis-β-ocimene, and β-vatirenene constitute about 70% of the flowers' volatile compounds (Fig. 5b). Foliar scent, on the other hand, shows a different pattern, in which α-linolenic acid, with over 60% relative abundance, is the predominant compound (Fig. 5c). Mean ± SE of concentration of detected compounds within a *P. hysterophorus* extract has been summarized in Table 1.

Synthetic Blend

Total ion chromatogram of the synthetic blend, consisting of the seven most abundant compounds has been shown in comparison with the chromatogram of *P. hysterophorus* whole plant extract in 99% hexane (Fig. 2). These two chromatograms indicate characteristic peaks of the most abundant compounds in whole plant extract and the blend which have been eluted at the same retention times. This provides evidence that the most abundant chemicals of the two chromatograms are identical. The full blend was tested in olfactometer comparing an intact *Parthenium* plant (Fig. 6a), a *Parthenium* extract (Fig. 6b), and a hexane blank (Fig. 6c). The first two served as positive controls, hexane as the negative control. Paired *t*-test analysis ($df = 9$) of olfactometry data demonstrated that there was no significant difference between attraction response of my full blend and that of the plant ($t = 1.02$, $P > 0.05$), or its extract ($t = 0.38$, $P > 0.05$). I also tested my blend against n-hexane in LDPE sachets, and found it highly attractive ($t = 3.08$, $P < 0.01$). Therefore, the blend attracted the mosquitoes as effectively as the plant itself under laboratory conditions.

Successive olfactometry experiments determined the relative role of each component of my full synthetic blend in attracting mosquitoes. One component was excluded at a time and the attraction response of mosquitoes was measured in its absence. If eliminating a single compound had no significant impact on the mosquito response to the rest of blend, then it was considered as a non-major component. Two hundred olfactometry experiments were carried out, each time by excluding one or more compounds from the full blend, to recognize the most attractive compounds for *A. gambiae*. The final minimal blend contained only α-pinene,

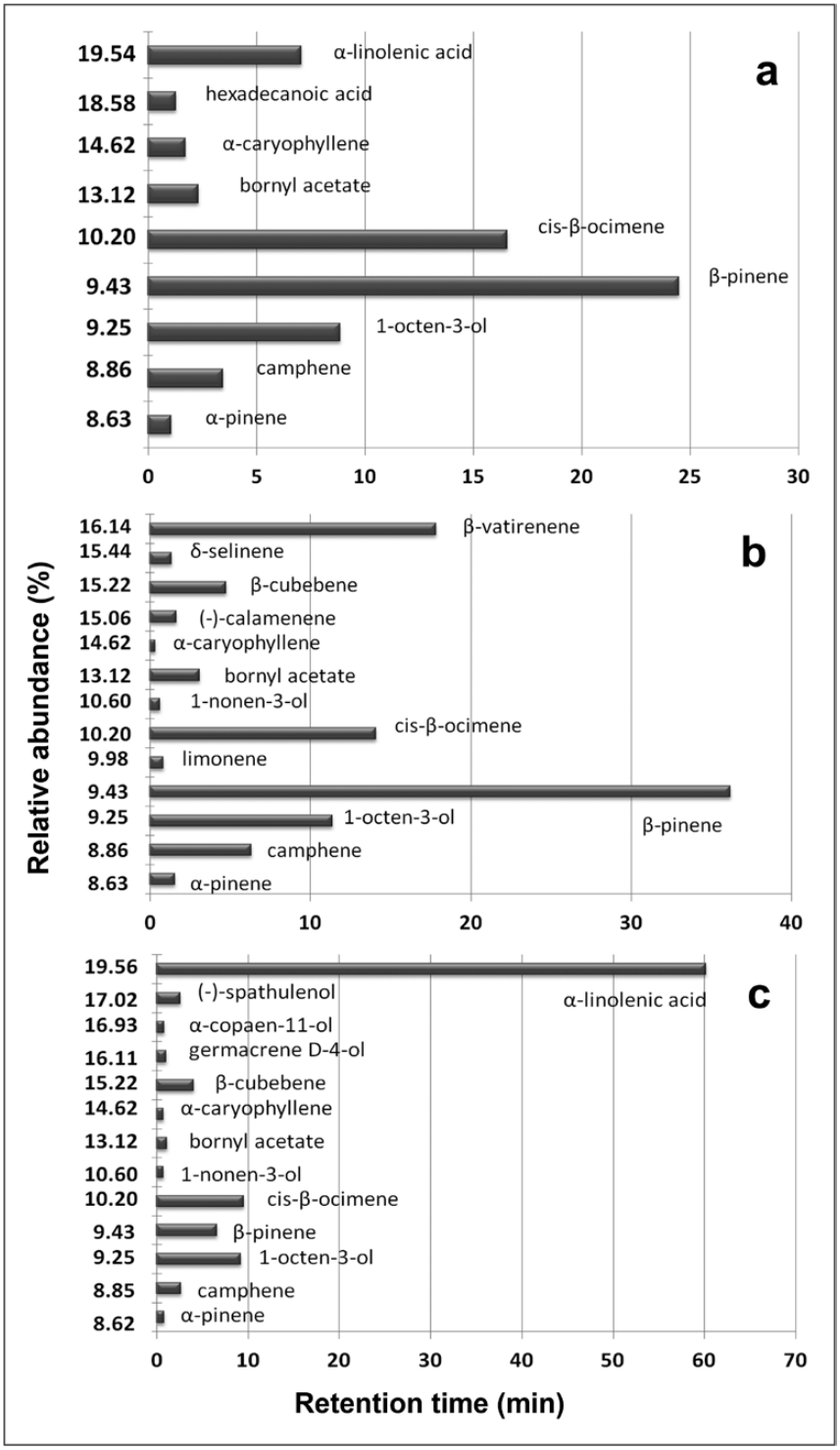

Fig. 5. Most abundant VOCs in hexane-based extract of *Parthenium hysterophorus* L. (Asterales: Asteraceae) (a): whole plant, (b): flowers, and (c): leaves; Detected and quantified by an Agilent 6890 gas chromatograph, connected to a 5973 Agilent mass detector.

camphene, and cis-β-ocimene, which could not be removed from the blend without a significant negative effect on the mosquito response. This reduced blend was tested versus *Parthenium* plant (Fig. 7a), and the olfactometry data confirmed its successful competence with a live intact plant (paired *t*-test, $df = 9$, $t = 0.52$, $P > 0.05$). The minimal blend was similarly tested versus the full blend (Fig. 7b) and attracted about the same proportion of mosquitoes (paired *t*-test, $df = 9$, $t = 2.14$, $P > 0.05$). The required concentration of each of these compounds remained the same as the full blend and has been indicated in Table 1.

Discussion

Attraction response of mosquitoes to plant volatiles and honey has been represented in many behavioral assays, either with extracts (Thorsteinson and Brust 1962, Vargo and Foster 1982, Jepson and

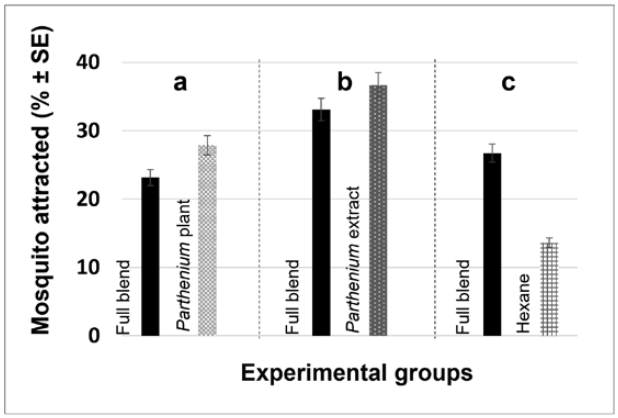

Fig. 6. Percentage of mean proportions (± SE) of *Anopheles gambiae* attracted to (a): full synthetic blend versus *Parthenium hysterophorus* L. (Asterales: Asteraceae) intact plant, (b): full synthetic blend versus *Parthenium* hexane-base extract, and (c): full synthetic blend versus 99% n-hexane in a dual-port olfactometer. Data had a normal distribution. Paired sample *t*-test. (a): $df = 13$, $t = 1.02$, $P > 0.05$; (b): $df = 9$, $t = 0.38$, $P > 0.05$; (c): $df = 9$, $t = 3.08$, $P < 0.01$.

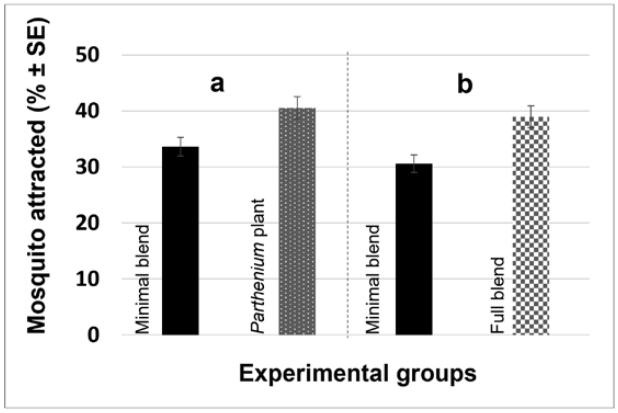

Fig. 7. Percentage of mean proportions (± SE) of *Anopheles gambiae* attracted to (a): minimal blend versus *Parthenium hysterophorus* L. (Asterales: Asteraceae) intact plant and (b): minimal versus full blend in a dual-port olfactometer. Data had a normal distribution. Paired sample *t*-test. $n = 10$, $df = 9$, $P > 0.05$ (a): $t = 0.52$; (b): $t = 2.14$.

Healy 1988, Hancock and Foster 1997, Mauer and Rowley 1999, Foster and Takken 2004) or a single floral component (Jhumur et al. 2006).

Healy and Jepson (1988) reported responses of *A. arabiensis* to inflorescences of yarrow, *A. millefolium*, which mainly consisted of monoterpenes. Female *A. aegypti* and *C. pipiens* also responded to inflorescences of yarrow, *Achillea millefolium* L. (Asterales: Asteraceae) (Peach et al. 2019a). Males and females, and also all gonotrophic stages and prediapausing females of *A. messeae* Falleroni, *C. pipiens* L., and *C. torrentium* Martini not only are attracted to *A. millefolium* but have been observed to feed on its nectar (Andersson and Jaenson 1987, Jaenson and Ameneshewa 1991). *Culex pipiens* females were reported being attracted to thujone, a terpene often found in essential oils of various plants. Similarly, ocimene has been shown to be detected by the antennae of *Aedes aegypti* (Dekker et al. 2011). (E)-β-ocimene was also found in the headspace odorants of tansy (*Tanacetum vulgare* L.) inflorescences (Peach et al. 2019a).

Azeem et al. (2019) showed that the essential oil of *P. hysterophorus* induces repellent activity in *Aedes aegypti* which does not last more than a few minutes. They attributed this short-term repellency to the presence of monoterpenes β-myrcene and trans-β-ocimene in the *Parthenium* essential oil. β-myrcene has also been reported as a repellent against *Culex* mosquitoes (Park et al. 2005). It should be noted that even though olfaction has a prominent role in the mosquito search for the natural resources of sugar, they eventually find the sugar-rich plants by a combination of visual and chemical cues (Foster and Hancock 1994). Behavioral bioassays have also indicated that the odor attraction is enhanced by the UV inflorescence cues (Peach et al. 2019b).

In series of olfactometry experiments, Nikbakhtzadeh et al. (2014) indicated that both sexes of *A. gambiae* are attracted towards some plants, including *S. didymobotrya* and *P. hysterophorus*, presumably by releasing attractive volatiles. A comparison of the headspace composition of these attractive plants revealed the high importance of some VOCs, such as enantiomers of caryophyllene, phellandrene, pinene, and ocimene to *A. gambiae*.

Mosquitoes apparently detect only a selected number of a plant's volatile compounds (Nyasembe et al. 2012, Nikbakhtzadeh et al. 2014). In addition to the role of each component in a volatile plume, concentration of each compound within the mixture is also of high importance in locating host plants by *A. gambiae* (Nyasembe et al. 2012, Nikbakhtzadeh et al. 2014). It is likely that these compounds function as mosquito attractant stimuli at relatively low concentrations, but as repellent at higher concentrations (Jaenson, et al. 2006, Nyasembe et al. 2012). Many studies indicate that the antennal sensillae of an insect only detect some components of a volatile blend and the most abundant components are not necessarily the most notable ones in terms of behavior (Cha et al. 2008, Riffell et al. 2009). It should be noted that sensory transduction, neural encoding, and plasticity are different among mosquito species. These factors determine mosquito responses to attractants or repellents (Wolff and Riffell 2018). Many chemicals have so far been developed to attract mosquitoes (Benelli et al. 2016), however, mosquitoes endemic to different zoogeographical regions do not necessarily respond to a single chemical the same. A chemical compound attractive to a species may be a repellent to others. Therefore, any effort in developing new attractants or repellents should take into consideration the sensory and behavioral specifics of each target vector (Potter 2014, Xu et al. 2015).

Parthenium hysterophorus is specifically discussed in this context because not only this study, but many preceding works have proved it to be a very attractive plant for *A. gambiae* (Manda et al. 2007b, Nyasembe et al. 2012, Nikbakhtzadeh et al. 2014). Chemical components of this plant have been analyzed in a few studies (Chen et al. 2011, Nikbakhtzadeh et al. 2014), and used in olfactometry assays (Nyasembe et al. 2012, Nikbakhtzadeh et al. 2014). The current study focused on *Parthenium* and its relative attraction and chemical analysis of its volatiles. The purpose was to confirm some previous observations/studies, reveal other aspects of its chemistry, and, most importantly, develop an efficient synthetic lure.

There are a few studies on *P. hysterophorus* palatability for *A. gambiae* (Manda et al. 2007b, Nyasembe et al. 2012, Nikbakhtzadeh et al. 2014) and on the volatile compounds which might be involved (Nyasembe et al. 2012, Nikbakhtzadeh et al. 2014). *Parthenium hysterophorus* was first noticed to be attractive for *A. gambiae* when a high rate of feeding was observed in a multiple-choice assay (Manda et al. 2007b). My quantitative olfactometry experiments with cuttings of *P. hysterophorus* and *S. didymobotrya* demonstrated both plants as attractive for *A. gambiae* (Nikbakhtzadeh et al. 2014). Those experiments were

achieved with plant cuttings compared to orchard grass; therefore, this time *S. didymobotrya* and *Parthenium* intact plants were compared side by side in a series of olfactometry experiments. This new set of data once again confirmed that these two plants evoke the same positive response in *A. gambiae* (Fig. 3).

Parthenium intact plant was considerably more attractive than the sugar-poor orchard grass, which is routinely used in my olfactometry assays as a base line of comparison (Fig. 2a). Floral and foliar, as well as the whole-plant extracts, indicated an outstanding attraction compared to the hexane blank. Hexane-filled sachets, which were tested versus empty sachets, did not do any better than the polyethylene material (paired *t*-test, df = 7, $t = 1.05$, $P > 0.05$).

Here a question arises whether *Parthenium* is a good source of sugar for *A. gambiae*. Manda et al. (2007b) reported no sugar in *Parthenium*'s flowers, and only a low volume of sucrose and galactose in leaves. Nyasembe et al. (2012) claims remarkable sugar content for *P. hysterophorus*. In another study, Nyasembe et al. (2015) detected four types of sugar in the gut of *A. gambiae* which had previously fed on *P. hysterophorus*, *Bidens pilosa* and *Ricinus communis*. Manda et al. (2007b) interestingly found that even though a substantial number of *A. gambiae* could be sugar positive, their ingested-sugar profile was not matched with that of *P. hysterophorus*. Furthermore, it was perceived that most of *A. gambiae*, already fed on *Parthenium* shrubs, had a considerably shorter life span (Manda et al. 2007b). Nyasembe et al. (2015), on the other hand, have reported 10.61 ± 0.17 (mean ± SE) days survival for *A. gambiae* who fed on *Parthenium* shrubs, which does not look considerable when compared with the survival of a pooled population of *A. gambiae* on *S. occidentalis* (13.69 ± 1.00 d) and *S. didymobotrya* (19.00 ± 1.13 d) which are also abundant in the same localities in Eastern Africa (Nikbakhtzadeh et al. 2016). However, Nikbakhtzadeh et al. (2016) proved very low survival rates for mosquitoes feeding on *Parthenium* (5.9 ± 0.67 d). They found that mosquitoes on *P. hysterophorus* showed a sharp decline in survival within the first 5 days, but a few survivors lived up to 25 days. Female *A. gambiae* were even able to survive for more than 15 days (Nikbakhtzadeh et al. 2016). Therefore, it seems that depending on how different studies have defined a long survival, they have concluded that *Parthenium* may or may not support high *A. gambiae* survival rates.

Parthenium hysterophorus is an exception to the attraction-value rule, since it is highly attractive according to olfactometer experiments (Nikbakhtzadeh et al. 2014) and the multiple-choice cage assays yet having little survival value (Nikbakhtzadeh et al. 2016). In a cage study with intact *Parthenium* shrubs, cold anthrone tests detected very low numbers of fructose-positive *A. gambiae* of both sexes, and testing those few positive individuals revealed only a low volume of fructose (Nikbakhtzadeh et al. 2016).

Nyasembe et al. (2018) found discrepancy in the number of fructose positive mosquitoes and those with plant DNA which might suggest that mosquitoes can pierce through plant tissue to feed on nutrients other than just nectar. Similar observations have been made by other research groups confirming direct piercing of plant tissues (Junnila et al. 2010). These observations can to some extent explain why measuring fructose might not be the best way to determine the benefit of an attractive plant to mosquitoes. While a specimen might be devoid of any fructose (or other sugars in general), it can still be in close association with a specific plant species to obtain other necessary compounds. Many studies confirm the important role of sugar in the survival and vectorial capacity of mosquitoes; however, it is not yet known what role the other compounds may play in the natural fitness of these insects. The fact that *A. gambiae* should spend energy to detoxify the cytotoxic compound parthenin while feeding on *P. hysterophorus* (Nyasembe et al. 2015) may indicate that sugar reward might not be the only reason for a vector-plant association.

Some studies have shown the disruptive effect of *P. hysterophorus* foliar extract on behavior and reproduction of *Aedes aegypti* (Kumar et al. 2011), but it seems that *A. gambiae* survives relatively well on *Parthenium* (6–10 days) despite ingesting the toxic compound parthenin (Nyasembe et al. 2015, Nikbakhtzadeh et al. 2016). Overall, it can be said that *Anopheles gambiae's* strong preference for *P. hysterophorus* seems to be anomalous (Manda et al. 2007a).

Analyzing *P. hysterophorus* using high-performance liquid chromatography, Azeem et al. (2019) found β-myrcene and trans-β-ocimene as the main compounds in the plant essential oil. My previous analyses also confirm the presence of these two compounds in the headspace of *P. hysterophorus* flowers (Nikbakhtzadeh et al. 2014). Chen, et al. (2011) could isolate 17 and 18 compounds from the floral and foliar extracts of *Parthenium*, respectively, in which terpenoids were predominant and constituted over 90% of the volatiles. This is to some extent different from my liquid-extract analysis of *Parthenium* (Fig. 4b, c). These differences have been frequently observed in the literature and could be explained by variation in the components or their abundances in the plants themselves according to growth phase and geographical origin, to the ease of transformation among enantiomers for various reasons, or to differences in the techniques used, including which parts of the plant were specifically excluded (Nelson et al. 2012).

Some important aspects of any control approach in the field are the ease of use, low cost, and safety to people, animals, and environment. Using a synthetic lure in the field has been shown to be effective, easy, and safe (Müller et al. 2008, Müller et al. 2010b), and the cost can be kept low in many ways. Not a lot of the ingredients are usually needed to make an efficient mosquito lure. Subtractive testing is a method to identify the non- or low chemical efficacy, by dropping select ingredients until an ideal minimal blend is found which is still highly effective. Though the minimal blend introduced in this study attracted a relatively lower number of mosquitoes, it remains effective and comparable to the full blend (Fig. 7). The minimized one has only the three main components of *Parthenium* volatiles and LDPE sachets functioned well in the slow releasing of the compounds over a 12-h time frame in my olfactory experiments. Preliminary tests indicate that with some adjustments, LDPE sachets will be able to deliver the chemicals over an even longer period. A test in a semi-field provision, where mosquitoes have easy access to competitive plants, will demonstrate whether this developed blend has the same success outside laboratory conditions.

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References

Adams, RP. *Identification of essential oil components by gas chromatography/ mass spectroscopy*, 4th ed. Carol Stream, IL: Allured Publishing; **2007**.

Andersson IH, Jaenson TGT. Nectar feeding by mosquitoes in Sweden, with special reference to *Culex pipiens* and *Cx torrentium*. *Med Vet Entomol*. **1987**:1(1):59–64. https://doi.org/10.1111/j.1365-2915.1987.tb00323.x

Australian CRC Weed Management. Weed Management Guide: *Parthenium* Weed, *Parthenium hysterophorus*. **2003**. https://doi.org/http://www.weeds.org.au/docs/parthenium_weed_mgt_guide.pdf

Azeem M, Zaman T, Tahir M, Haris A, Iqbal Z, Binyameen M, Nazir A, Shad SA, Majeed S, Mozūraitis R. Chemical composition and repellent activity of native plants essential oils against dengue mosquito, *Aedes aegypti*. *Ind Crops Prod*. **2019**:111609. doi:https://doi.org/10.1016/j.indcrop.2019.111609

Beier JC, Müller GC, Gu W, Arheart KL, Schlein Y. Attractive toxic sugar bait (ATSB) methods decimate populations of *Anopheles* malaria vectors in arid environments regardless of the local availability of favoured sugar-source blossoms. *Malar J*. **2012**:11:31. https://doi.org/10.1186/1475-2875-11-31

Benelli G, Jeffries CL, Walker T. Biological control of mosquito vectors: past, present, and future. *Insects*. **2016**:7(4):52. https://doi.org/10.3390/insects7040052

Cha DH, Nojima S, Hesler SP, Zhang A, Linn CE Jr, Roelofs WL, Loeb GM. Identification and field evaluation of grape shoot volatiles attractive to female grape berry moth (*Paralobesia viteana*). *J Chem Ecol*. **2008**:34(9):1180–1189. https://doi.org/10.1007/s10886-008-9517-0

Chen Y, Wang J, Wu X, Sun J, Yang N. Allelopathic effects of *Parthenium hysterophorus* L. volatiles and its chemical components. *Allelopathy J*. **2011**:27:217–224.

Coutinho-Abreu LV, Riffell JA, Akbari OS. Human attractive cues and mosquito host-seeking behavior. *Trends Parasitol*. **2022**:38:3. https://doi.org/10.1016/j.pt.2021.09.012

Dekel A, Yakir E, Bohbot JD. The sulcatone receptor of the strict nectar-feeding mosquito *Toxorhynchites amboinensis*. *Insect Biochem Mol Biol*. **2019**:111:103174. https://doi.org/10.1016/j.ibmb.2019.05.009

Dekker T, Ignell R, Glinwood R, Hopkins R. Identification of repellent odours from *Ocimum forkolei*. *Parasite Vector*. **2011**:4:183.

Drabble E, Drabble H. Some flowers and their dipteran visitors. *New Phytol*. **1927**:26(2):115–123. https://doi.org/10.1111/j.1469-8137.1927.tb06711.x

Foster WA. Mosquito sugar feeding and reproductive energetics. *Annu Rev Entomol*. **1995**:40:443–474. https://doi.org/10.1146/annurev.en.40.010195.002303

Foster WA, Hancock RG. Nectar-related olfactory and visual attractants for mosquitoes. *J Am Mosq Control Assoc*. **1994**:10(2 Pt 2):288–296.

Foster WA, Takken W. Nectar-related vs. human- related volatiles: behavioural response and choice by female and male Anopheles gambiae (Diptera: Culicidae) between emergence and first feeding. *Bull Entomol Res* **2004**:94: 145–157.

Gary RE, Foster WA. *Anopheles gambiae* feeding and survival on honeydew and extrafloral nectar of peridomestic plants. *Med Vet Entomol*. **2004**:18(2):102–107. https://doi.org/10.1111/j.0269-283X.2004.00483.x

Geier M, Bosch OJ, Boeckh J. Ammonia as an attractive component of host odour for the yellow fever mosquito, *Aedes aegypti*. *Chem Senses*. **1999**:24(6):647–653. https://doi.org/10.1093/chemse/24.6.647

Gillies MT, De Meillon B. *The Anophelinae of Africa south of the Sahara (Ethiopian Zoogeographical Region)*. Johannesburg, South Africa: South African Institute for Medical Research; **1968**.

Gouagna LC, Poueme RS, Dabiré KR, Ouédraogo JB, Fontenille D, Simard F. Patterns of sugar feeding and host plant preferences in adult males of *A. gambiae* (Diptera: Culicidae). *J Vector Ecol*. **2010**:35(2):267–276. https://doi.org/10.1111/j.1948-7134.2010.00082.x

Gu W, Müller G, Schlein Y, Novak RJ, Beier JC. Natural plant sugar sources of *Anopheles* mosquitoes strongly impact malaria transmission potential. *PLoS One*. **2011**:6(1):e15996. https://doi.org/10.1371/journal.pone.0015996

Hancock RG, Foster WA. Larval and adult nutrition effects on the blood/nectar choice of *Culex nigripalpus* mosquitoes. *Med Vet Entomol*. **1997**:11(2):112–122. https://doi.org/10.1111/j.1365-2915.1997.tb00299.x

Healy TP, Jepson PC. The location of floral nectar sources by mosquitoes: the long-range responses of *Anopheles arabiensis* Patton (Diptera: Culicidae) to *Achillea millefolium* flowers and isolated floral odour. *Bull Entomol Res*. **1988**:78(4):651–657. https://doi.org/10.1017/s0007485300015509

Ignell R, Hill SR. Malaria mosquito chemical ecology. *Curr Opin Insect Sci*. **2020**:40:6–10. https://doi.org/10.1016/j.cois.2020.03.008

Impoinvil DE, Kongere JO, Foster WA, Njiru BN, Killeen GF, Githure JI, Beier JC, Hassanali A, Knols BJG. Feeding and survival of the malaria vector *Anopheles gambiae* on plants growing in Kenya. *Med Vet Entomol*. **2004**:18:108–115.

Jaenson TGT, Ameneshewa B. Prehibernation diet and reproductive condition of female *Anopheles messeae* in Sweden. *Med Vet Entomol*. **1991**:5:243–252.

Jaenson TGT, Pålsson K, Borgkarlson A. Evaluation of extracts and oils of mosquito (Diptera: Culicidae) repellent plants from Sweden and Guinea-Bissau. *J Med Entomol*. **2006**:43(1):113–119. https://doi.org/10.1093/jmedent/43.1.113

Jepson PC, Healy TP. The location of floral nectar sources by mosquitoes: an advanced bioassay for volatile plant odours and initial studies with *Aedes aegypti* (L.) (Diptera: Culicidae). *Bull Entomol Res*. **1988**:78(4):641–650. https://doi.org/10.1017/s0007485300015492

Jhumur US, DötterL S, Jürgens A. Naïve and conditioned responses of *Culex pipiens pipiens* Biotype *molestus* (Diptera: Culicidae) to flower odors. *J Med Entomol*. **2006**:43(6):1164–1170.

Junnila A, Müller GC, Schlein Y. Species identification of plant tissues from the gut of *An. sergentii* by DNA analysis. *Acta Trop*. **2010**:115(3):227–233. https://doi.org/10.1016/j.actatropica.2010.04.002

Kartesz JT. *Dactylis glomerata* L. ssp. *glomerata*. In: *Biota of North America Program*. United States Department of Agriculture; **2011**. http://plants.usda.gov/java/profile?symbol=DAGLG.

Kaufmann C, Briegel H. Flight performance of the malaria vectors *Anopheles gambiae* and *Anopheles atroparvus*. *J Vect Ecol*. **2004**:29:140–153.

Kline DL. Semiochemicals, traps/targets and mass trapping technology for mosquito management. *J Am Mosq Control Assoc*. **2007**:23(2 Suppl):241–251. https://doi.org/10.2987/8756-971X(2007)23[241:STAMTT]2.0.CO;2

Kumar S, Singh A, Nair G, Batra S, Seth A, Wahab N, Warikoo R. Impact of *Parthenium hysterophorus* leaf extracts on the fecundity, fertility and behavioural response of *Aedes aegypti* L. *Parasitol Res*. **2011**:108:853–859.

Liu C, Pitts RJ, Bohbot JD, Jones PL, Wang G, Zwiebel LJ. Distinct olfactory signaling mechanisms in the malaria vector mosquito *Anopheles gambiae*. *PLoS Biol*. **2010**:8(8):e1000467. https://doi.org/10.1371/journal.pbio.1000467

Lutz EK, Lahondère C, Vinauger C, Riffell JA. Olfactory learning and chemical ecology of olfaction in disease vector mosquitoes: a life history perspective. *Curr Opin Insect Sci*. **2017**:20:75–83. https://doi.org/10.1016/j.cois.2017.03.002

Manda H, Gouagna LC, Foster WA, Jackson RR, Beier JC, Githure JI, Hassanali A. Effect of discriminative plant-sugar feeding on the survival and fecundity of *Anopheles gambiae*. *Malar J*. **2007a**:6:113. https://doi.org/10.1186/1475-2875-6-113

Manda H, Gouagna LC, Nyandat E, Kabiru W, Jackson RR, Foster WA, Githure JI, Beier JC, Hassanali A. Discriminative feeding behaviour of *Anopheles gambiae* s.s. on endemic plants in Western Kenya. *Med Vet Entomol*. **2007b**:21:103–111.

Mauer DJ, Rowley WA. Attraction of *Culex pipiens pipiens* (Diptera: Culicidae) to flower volatiles. *J Med Entomol*. **1999**:36(4):503–507. https://doi.org/10.1093/jmedent/36.4.503

Mccrae AWR. Differences in sugar-feeding activity between tropical and temperate mosquitoes: field observations and their implications. *Vector Ecol Newsl*. **1989**:20:16.

Muirhead-Thompson, RC. *Mosquito behavior in relation to malaria transmission in the tropics*. London, UK: E. Arnold; **1951**.

Mukabana WR, Mweresa CK, Otieno B, Omusula P, Smallegange RC, Van Loon J, Takken W. A novel synthetic odorant blend for trapping of malaria and other African mosquito species. *J Chem Ecol*. **2012**:38:235–244.

Müller GC, Beier JC, Traore SF, Toure MB, Traore MM, Bah S, Doumbia S, Schlein Y. Field experiments of *Anopheles gambiae* attraction to local fruits/seedpods and flowering plants in Mali to optimize strategies for malaria vector control in Africa using attractive toxic sugar bait methods. *Malar J*. **2010a**:9(1):1–11. https://doi.org/10.1186/1475-2875-9-262

Müller GC, Junnila A, Qualls W, Revay EF, Kline DL, Allan S, Schlein SY, Xue RD. Control of *Culex quinquefasciatus* in a storm drain system in Florida using attractive toxic sugar baits. *Med Vet Entomol*. **2010b**:24:346–351. https://doi.org/10.1111/j.1365-2915.2010.00876.x

Müller GC, Kravchenko VD, Schlein Y. Decline of *Anopheles sergentii* and *Aedes caspius* populations following presentation of attractive toxic (spinosad) sugar bait stations in an oasis. *J Am Mosq Control Assoc*. **2008**:24(1):147–149. https://doi.org/10.2987/8756-971X(2008)24[147:DOASAA]2.0.CO;2

Nayar JK, Van Handel E. The fuel for sustained mosquito flight. *J Insect Physiol*. **1971**:17:471–481.

Nelson XJ, Pratt AJ, Cheseto X, Torto B, Jackson RR. Mediation of a plant-spider association by specific volatile compounds. *J Chem Ecol*. **2012**:38:1081–1092. https://doi.org/10.1007/s10886-012-0175-x

Nikbakhtzadeh MR, Terbot JW II, Otienoburu PE, Foster WA. Olfactory basis of floral preference of the malaria vector *Anopheles gambiae* (Diptera: Culicidae) among common African plants. *J Vec Ecol*. **2014**:39:372–383.

Nikbakhtzadeh MR, Terbot II JW, Foster WA. Survival value and sugar access of four east African plant species attractive to a laboratory strain of sympatric *Anopheles gambiae* (Diptera: Culicidae). *J Med Entomol*. **2016**:53:1105–1111.

Nyasembe VO, Cheseto X, Kaplan F, Foster WA, Teal PE, Tumlinson JH, Borgemeister C, Torto B. The invasive American weed *Parthenium hysterophorus* can negatively impact malaria control in Africa. *PLoS One*. **2015**:10(9):e0137836. https://doi.org/10.1371/journal.pone.0137836

Nyasembe VO, Tchouassi DP, Pirk CWW, Sole CL, Torto B. Host plant forensics and olfactory-based detection in Afro-tropical mosquito disease vectors. *PLoS NeglTrop Dis*. **2018**:12(2):e0006185. https://doi.org/10.1371/journal.pntd.0006185

Nyasembe VO, Teal PE, Mukabana WR, Tumlinson JH, Torto B. 2012. Behavioural response of the malaria vector *Anopheles gambiae* to host plant volatiles and synthetic blends. *Parasite Vector*. *5*:234. https://doi.org/10.1186/1756-3305-5-234

Park BS, Choi WS, Kim JH, Kim KH, Lee SE. Monoterpenes from thyme (*Thymus vulgaris*) as potential mosquito repellents. *J Am Mosq Control Assoc*. **2005**:21(1):80–83. https://doi.org/10.2987/8756-971X(2005)21[80:MFTTVA]2.0.CO;2

Peach DAH, Gries G. Mosquito phytophagy—sources exploited, ecological function, and evolutionary transition to haematophagy. *Entomol Exp Appl*. **2020**:168(2):120–136. https://doi.org/10.1111/eea.12852

Peach DAH, Gries R, Zhai H, Young N, Gries G. Multimodal floral cues guide mosquitoes to tansy inflorescences. *Sci Rep*. **2019a**:9(1):3908. https://doi.org/10.1038/s41598-019-39748-4

Peach DAH, Ko E, Blake AJ, Gries G. Ultraviolet inflorescence cues enhance attractiveness of inflorescence odour to *Culex pipiens* mosquitoes. *PLoS One*. **2019b**:14(6):e0217484. https://doi.org/10.1371/journal.pone.0217484

Picman AK, Towers GHN. Sesquiterpene lactones in various populations of *Parthenium hysterophorus*. *Biochem Syst Ecol*. **1982**:10:145–153.

Picman J, Picman AK. Autotoxicity in *Parthenium hysterophorus* and its possible role in control of germination. *Biochem Syst Ecol*. **1984**:12(3):287–292. https://doi.org/10.1016/0305-1978(84)90051-6

Potter CJ. Stop the biting: targeting a mosquito's sense of smell. *Cell*. **2014**:156(5):878–881. https://doi.org/10.1016/j.cell.2014.02.003

Reisen WK, Emory RW. Blood-feeding of *Anopheles stephensi*. *Ann Entomol Soc Am*. **1976**:69(2):293–298. https://doi.org/10.1093/aesa/69.2.293

Riffell JA, Lei H, Christensen TA, Hildebrand JG. Characterization and coding of behaviorally significant odor mixtures. *Curr Biol*. **2009**:19:335–340.

Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *Am J Trop Med Hyg*. **1993**:49(4):520–529. https://doi.org/10.4269/ajtmh.1993.49.520

Shu YM, Shenglian L, Phillips SM. Dactylis linnaeus. *Fauna China*. **2006**:22:309–310.

Stamps RH. Identification, impacts, and control of Ragweed Parthenium *(Parthenium hysterophorus* L.). *ENH1187 in Environmental Horticulture*. UF/IFAS Extension. **2011**: 10 pp. https://edis.ifas.ufl.edu/pdf/EP/EP44800.pdf

Stone CM, Hamilton IM, Foster WA. A survival and reproduction trade-off is resolved in accordance with resource availability by virgin female mosquitoes. *Anim Behav*. **2011**:81(4):765–774. https://doi.org/10.1016/j.anbehav.2011.01.008

Takken W, Knols BGJ. Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annu Rev Entomol*. **1999**:44(1):131–157. https://doi.org/10.1146/annurev.ento.44.1.131

Thorsteinson AJ, Brust RA. The influence of flower scents on aggregations of caged adult *Aedes aegypti*. *Mosq News*. **1962**:22:349–352.

Torr SJ, Halla DR, Phelpsa RJ, Vale GA. Methods for dispensing odour attractants for tsetse flies (Diptera: Glossinidae). *Bull Entomol Res*. **1997**:87:299–311.

Van Handel E. The detection of nectar in mosquitoes. *Mosq News*. **1972**:32:458.

Vargo AM, Foster WA. Responsiveness of female *Aedes aegypti* (Diptera: Culicidae) to flower extracts. *J Med Entomol*. **1982**:19(6):710–718. https://doi.org/10.1093/jmedent/19.6.710

Verhulst NO, Takken W, Smallegange RC. Structural design affects entry response of mosquitoes in olfactometers. *Proc Neth Entomol Soc Meet*. **2008**:19:129–136.

Wolff GH, Riffell JA. Olfaction, experience and neural mechanisms underlying mosquito host preference. *J Exp Biol*. **2018**:221(Pt 4):jeb157131. https://doi.org/10.1242/jeb.157131

Xu P, Zhu F, Buss GK, Leal WS. 1-Octen-3-ol—the attractant that repels. *F1000Res* **2015**:4:156. https://doi.org/10.12688/f1000research.6646.1

Yuval B. The other habit: sugar feeding by mosquitoes. *Bull Soc Vector Ecol*. **1992**:17:150–156.

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DETERRENT EFFECTS OF GLYPHOSATE ON OVIPOSITION AND LARVAL DEVELOPMENT OF *CULEX QUINQUEFASCIATUS*

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ABSTRACT. Glyphosate is one of the most common herbicides used to control weeds in the USA. This herbicide can impact the mosquito life cycle through being carried to mosquito habitats by runoff. This study investigates the effects of glyphosate on the larval development and oviposition of a laboratory colony of *Culex quinquefasciatus*. Our experiments indicated that a concentration of 1 g/liter glyphosate was lethal to larval instars or the larvae impacted were either unable to molt to pupae or never emerged as adults. Larval instars exposed to 0.5 g/liter glyphosate experienced a similar impact; however, the larval stage was extended and pupation was considerably delayed. Mosquitoes oviposited in 0.5 g/liter glyphosate laid the same number of egg rafts as in water, but a considerably lower number of eggs exposed to glyphosate hatched as first instars. If gravid females laid their eggs in 1 g/liter glyphosate versus water, the difference between the 2 groups would be statistically significant and a very low number of eggs exposed to glyphosate could ever hatch into larvae. In an oviposition assay carried out in a cage with 3 choices of water, 0.5 g/liter, and 1 g/liter glyphosate, a higher number of egg rafts were laid in water, followed by 0.5 g/liter and 1 g/liter glyphosate concentrations, respectively. Our study indicated such a severe effect of glyphosate on all immature stages of *Cx. quinquefasciatus* that no adult could ever emerge.

KEY WORDS *Culex quinquefasciatus*, glyphosate, herbicide contamination, larval development, mosquito oviposition deterrent

INTRODUCTION

Glyphosate [N-(phosphonomethyl) glycine, CAS No. 1071-83-6], the world's most widely used herbicide (Bataillard et al. 2020), is an isopropyl amine salt classified as an organophosphorus broad-spectrum herbicide (Salazar Lopez et al. 2016). Glyphosate has nonselective and systemic foliar action (Prata et al. 2003, Gimsing et al. 2007, Sanchís et al. 2012). Most of the herbicides applied in agriculture do not reach their target sites (Nguyen et al. 2016) and are therefore found in considerable levels beyond agricultural fields (Peruzzo et al. 2008, Struger et al. 2008, Bai and Ogbourne 2016). Glyphosate similarly leaves a target site through surface runoff, where it has been detected in aquatic systems frequently (Morrissey et al. 2015, Kibuthu et. al. 2016, Van Bruggen et al. 2018). In addition to surface runoff, direct overspray and drift during herbicide application may also lead to high levels of glyphosate that infiltrate the aquatic ecosystems (Solomon and Thompson 2003).

Many studies have shown that glyphosate can induce biochemical, physiological, and behavioral changes in nontarget species (Miller 2004, Annett et al. 2014, Gill et al. 2018, Daam et al. 2019). For example, honeybees that received very low doses of glyphosate developed neurotoxic effects (Herbert et al. 2014, Balbuena et al. 2015). Because of its presence in nontarget sites, the effects of glyphosate on nontarget species, including mosquitoes, have attracted attention (Baglan et al. 2018). Immature stages of mosquitoes, including eggs, larvae, and pupae, are vulnerable to water contaminants.

Culex quinquefasciatus Say, more commonly known as the southern house mosquito, is one of the most widespread mosquitoes in southern California. This species is responsible for transmitting arboviruses such as West Nile virus (WNV), St. Louis encephalitis (SLE), and western equine encephalomyelitis (WEE) to the local residents. *Culex* species are known for laying their eggs in stagnant water, making them vulnerable to glyphosate exposure. Such exposure may change mosquito behavior, life history traits such as larval survival rate or reproduction (Bara et al. 2014), and resistance to pesticides (Riaz et al. 2009, Bataillard et al. 2020), which in turn can influence the mosquito vectorial capacity.

Most of the previous studies on herbicides have focused on the field realistic doses (Herbert et al. 2014, Baglan et al. 2018, Bataillard et al. 2020) or residual (Mohamed 2011, Bara et al. 2014, de Saraiva et al. 2016, Mehdizadeh et al. 2021), which have often been shown to be nonlethal to mosquito immature stages. The current study was conducted to investigate the effect of higher levels of glyphosate on *Cx. quinquefasciatus* oviposition, eggs, larvae, and pupae. In other words, we wanted to better understand how the various stages of *Cx. quinquefasciatus* may react to higher doses of glyphosate, a nonselective herbicide. Glyphosate formulations (mixed with adjuvants) have been reported to be more toxic than pure glyphosate (Gill et al., 2018, Nagy et al. 2019, Pochron et al. 2020). Available glyphosate-based herbicides on the market have several compounds with variable toxicity. Surfac-

tants, which are added to an herbicide formulation to improve its performance, are mostly responsible for disrupting physiological or behavioral mechanisms of nontarget organisms (Annett et al. 2014). In the current study, we have reported some of these reactions ranging from avoiding egg laying in a glyphosate substrate and toxic effects on the egg viability to deterring larval growth and molting, prolonging larval stages, and toxic effects on pupae, which might prevent them from emerging as adults. All such effects can impact the fecundity, population size, and adult fitness and eventually reduce the vectorial capacity. Therefore, even though herbicides help weed management in modern agriculture and urban landscape, their side effects on other organisms should be also taken into consideration.

MATERIAL AND METHODS

Mosquito rearing

Egg rafts of *Cx. quinquefasciatus* were hatched to first-stage larvae in environmental chambers under 65 ± 5% RH, 25 ± 2°C temperature, and 12:12 (L:D) photoperiod. Groups of 120 first instars were transferred to shallow plastic pans (29.3 × 19.7 × 6 cm), filled with 750 ml of aged tap water. All larval instars were fed daily with grinded TetraMin® tropical fish food based on a larval diet protocol (Gary and Foster 2001) until pupation. Pupae were transferred to 9-cm-diam Petri dishes, and a total of 400 pupae were placed in each mosquito field cage (40 × 40 × 40 cm, BioQuip Products, Inc., Compton, CA). Emerging adults had access to aged tap water and 10% (w/v) sucrose-soaked cotton wicks.

Two-to-three-day-old adults were blood fed by an artificial feeding apparatus for 2 consecutive nights using Na citrate defibrinated bovine blood (LAMPIRE Biological Laboratories, Pipersville, PA). Four days after the second blood meal, half-filled water dishes (9 cm in diam) were placed in each cage for oviposition.

Glyphosate concentration

Roundup® Weed and Grass Killer, Super Concentrate, was used as a commercially available glyphosate product (50.2% glyphosate isopropylamine salt; Monsanto, Marysville, OH). Concentrations of 0.5 and 1 g/liter glyphosate were made from this super concentrate formulation.

Larval experiment

Twenty larvae of each instar were placed in small containers (400 ml volume, 10.8 cm diam) with 200 ml aged tap water. Glyphosate concentration was kept at 0.5 or 1 g/liter depending on the experiment, while control larvae were added to the same quantity of aged tap water. Larval experiments were replicated 10 times ($n = 10$) versus control ($n = 10$). Larvae of treatment and control replicates were fed daily with 0.02 g of TetraMin® fish food, and their mortality, pupation, or emergence as adults was recorded daily to prepare the survival curves for each experimental group. If larvae were molted to pupae, the number of live pupae was considered in the calculation of survival ratio and survival rate. Those pupae were observed daily to check whether they emerged as adults. If they did so, the number of adults was also added to the total number of live individuals. In this way, comparison of treatment and control groups was possible even if the pupation of treated larvae would have been prolonged.

Oviposition experiment

To observe the mosquito oviposition, 9-cm-diameter Petri dishes (ovicups) with 20 ml water were used as control, whereas treatments contained 0.5 or 1 g/liter glyphosate concentrations at the same volume of water. Those ovicups were placed in cages with 400 adult mosquitoes overnight, 4 days after the second blood meal. Upon removal from the cage, the number of egg rafts was counted for each ovicup. These experiments were repeated 5 times ($n = 5$) for both treatment and control groups.

Egg viability experiment

Egg rafts of each ovicup were individually transferred to Petri dishes, and the number of hatched first instars was counted for 3 days. The total number of first instars was then compared between treatment and control replicates ($n = 5$).

Triple-choice oviposition experiment

Three ovicups (each containing 20 ml liquid), one with 0.5 g/liter glyphosate, one with 1 g/liter glyphosate, and one with aged tap water, were placed in 3 corners of a Bioquip adult cage for a night with 400 adult mosquitoes 4 days after the second blood meal. The number of egg rafts was then counted for the 2 glyphosate solutions and control (water) to find the oviposition preference of gravid females when they had access to various choices ($n = 5$). The corner location of the 3 choices was randomly changed in each replication to avoid a systemic bias.

Statistical analyses

Survival curves were constructed with the Kaplan-Meier estimator and analyzed with Wilcoxon tests (Nikbakhtzadeh et al. 2016). ANOVA, followed by a Waller-Duncan post-hoc test, was used to compare the means of surviving larvae in consecutive days and determine the significantly different groups (Timmermann and Briegel 1996, Manda et al. 2007, Baz 2017). To normalize the distribution of our oviposition data between treatment and control, data in each of the two groups were transformed by arc sin $\sqrt{a/(a+b)}$ and arc sin $\sqrt{b/(a+b)}$, respectively, and their mean value were calculated. This transfor-

mation enabled us to use the parametric *t*-test for comparing the number of egg rafts between treatment and control groups (Manda et al. 2007, Nikbakhtzadeh et al. 2016). The distributions of transformed data were tested with a Shapiro-Wilk test for normality at $P > 0.05$, followed by a paired-comparisons *t*-test (Nikbakhtzadeh et al. 2016). All analyses were performed with SPSS statistical package version 28.00 (Chicago, Illinois, USA), and graphics were generated with Microsoft Excel (version 18.2110.13110.0, Redmond, WA).

RESULTS

These sets of experiments focused on the impact of glyphosate at 0.5 and 1 g/liter concentrations on different larval instars of *Cx. quinquefasciatus* from hatching time until pupation and eventually emergence as adults.

Larval instars exposed to 0.5 g/liter of glyphosate

When exposed to 0.5 g/liter glyphosate, the survival ratio of the first instars sharply dropped to less than 0.2 in 2 days and continued to decrease until all larvae died in 9 days. A slower trend was observed for the second instars in which survival ratio dropped to 0.205 in day 5 and continued its gradual reduction, finally reaching zero on day 13. The dose response for the third larval instar overall followed a less sharp curve, in which the survival ratio reached 0.215 on day 7 and reached zero in 10 days. Although it was expected that the fourth instars would react to the same dose even more slowly than the second or third instars, their survival ratio dropped to zero in 8 days (Fig. 1a). Control replicates (water) of each of those instars did not show a drastic change and kept their high survival ratio throughout the experiments (Fig. 1a). Analysis of variance (ANOVA) on the mean value of the survived larvae also indicated that control larvae within all 4 instars had more or less a constant survival rate, while larvae under glyphosate treatment began their sharp reduction from day 2. Presumably, as larvae grow and molt into a higher instar, they can tolerate the same dose of glyphosate for a longer period (Fig. 2).

Larval instars exposed to 1 g/liter glyphosate

The pattern of dose response to 1 g/liter glyphosate was very similar to 0.5 g/liter; however, the distinction among larval instars was more readily observable. The survival ratio of larval instars 1–4 reached zero on day 3, day 5, day 11, and day 14, respectfully. Therefore, younger larvae could not tolerate being exposed to a higher dose of glyphosate for a long time, but older instars could resist the higher dose for up to 2 wk (Fig. 1b). Compared to the control (water), all larval instars exposed to 1 g/liter glyphosate showed very low survival rates, as seen in Fig. 3. While the survival rate of control replicates remained constant over several days from the beginning of the experiments, those of treated larvae were reduced significantly. This drop was so rapid for the first larval instar (Fig. 3a) that the survival rate came to less than 1 just in 2 days. As larvae grew, this drop was delayed; therefore, in the case of larval instars 2–4, the very low survival rate, similar to that of first instars, was observed at days 3, 6, and 8, respectively (Fig. 3b–3d).

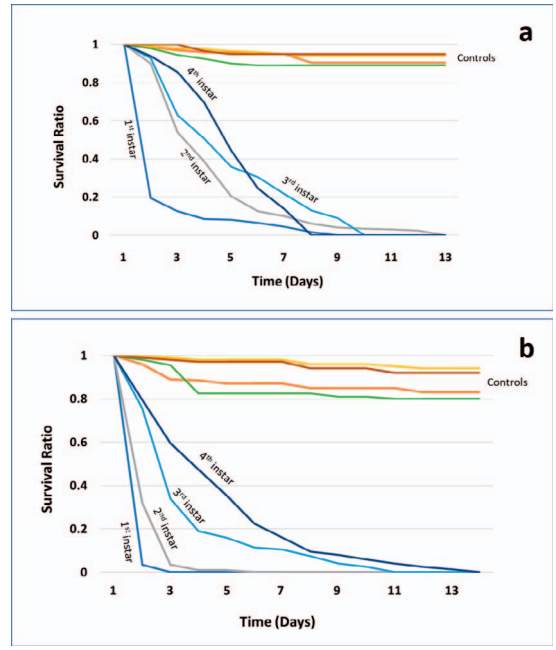

Fig. 1. Survival curve of *Culex quinquefasciatus* larval instars exposed to (a) 0.5 g/liter and (b) 1 g/liter glyphosate. Kaplan-Meier survival estimator, followed by Wilcoxon test.

Oviposition experiment

When female *Cx. quinquefasciatus* were offered an ovicup containing 0.5 g/liter glyphosate, no significant difference (Fig. 4a) was observed between the number of egg rafts deposited in glyphosate and water containers (paired-sample *t*-test, $n = 5$, $df = 4$, $t = 0.276$, $P = 0.796$). Mean value for laying eggs in water was 5.00 versus 6.4 for glyphosate. Oviposition of *Cx. quinquefasciatus* gravid females in 1 g/liter glyphosate solution indicated a significant difference (paired sample *t*-test, $n = 5$, $df = 4$, $t = 3.515$, $P < 0.05$) in the number of egg rafts between treatment and control (water only) groups. The glyphosate-treated ovicups showed a very low number of egg rafts (mean = 3.6) laid, while control ovicups had a higher number of eggs laid (mean = 13.2), as seen in Fig. 4b. Most of the eggs in glyphosate solutions turned to a dark color soon after being laid, which might be an indication of losing their viability.

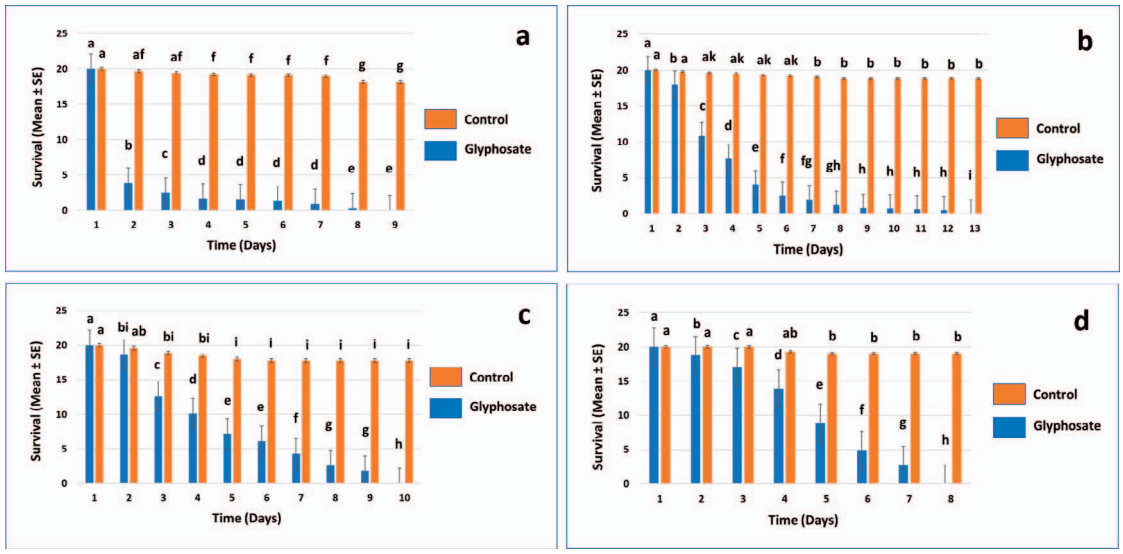

Fig. 2. Effect of 0.5 g/liter glyphosate (mean ± SEM) on the survival of *the Culex quinquefasciatus* (a) first, (b) second, (c) third, and (d) fourth instars. Different letters indicate statistically significant groups, $P < 0.001$, $n = 10$. Waller-Duncan post-hoc test.

Egg viability experiment

Although the number of eggs rafts deposited by female mosquitoes gives us some information on their preferred substrate for oviposition, we did not know if eggs deposited in a glyphosate solution had been affected or not; therefore, right after the oviposition experiment, we transferred the egg rafts into separate Petri dishes and counted the number of eggs hatched into first instars. This is a good indicator of whether the eggs were actually alive. In other words, we wanted to measure the viability percentage of eggs laid in a glyphosate solution versus the plain water solution. As seen in Fig. 5a, our findings indicate that when comparing 0.5 g/liter glyphosate with water (control), the percentage of hatched eggs was significantly higher for water (69%) versus glyphosate (12.7%) (paired-sample *t*-test, $n = 5$, df $= 4$, $t = 3.589$, $P < 0.05$). When the same assay was arranged for 1 g/liter glyphosate versus water, the number of eggs hatched to first

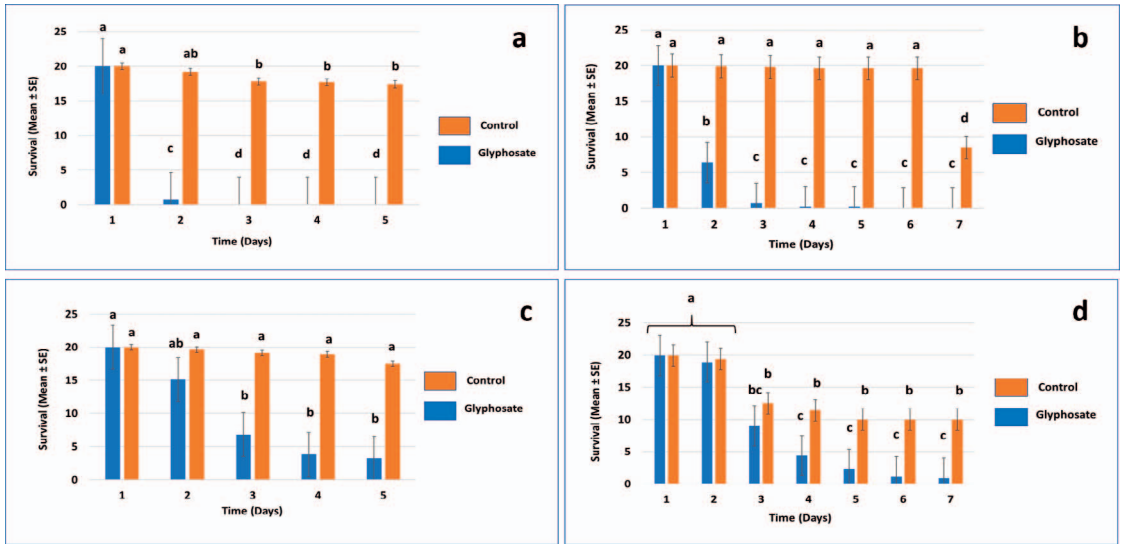

Fig. 3. Effect of 1 g/liter glyphosate (mean ± SEM) on the survival of the *Culex quinquefasciatus* (a) first, (b) second, (c) third, and (d) fourth instars. Different letters indicate statistically significant groups, $P < 0.001$, $n = 10$; Waller-Duncan post-hoc test.

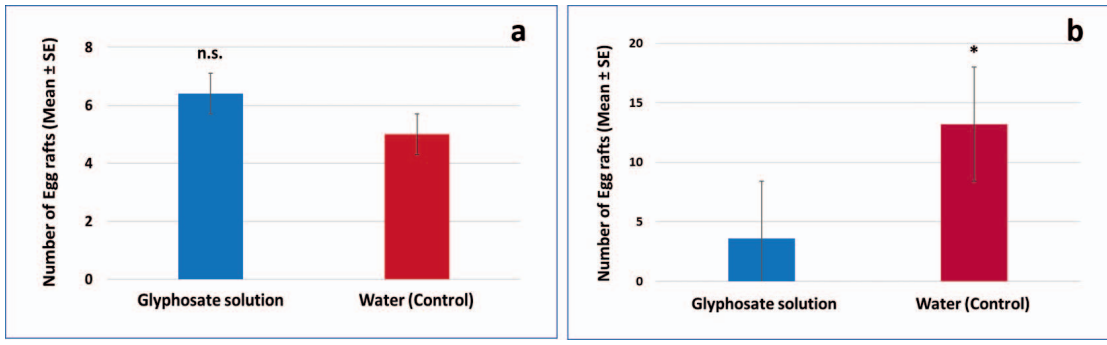

Fig. 4. *Culex quinquefasciatus* oviposition in (a) 0.5 g/liter (b) 1 g/liter glyphosate versus water (control). (a) Paired-sample *t*-test, $P = 0.796$; ns: nonsignificant. (b) Paired-sample *t*-test, $P = 0.0245$. Asterisk on a column shows a statistically significant difference.

instar was significantly higher for water (paired-sample *t*-test, $n = 5$, $df = 4$, $t = 17.759$, $P < 0.001$; Fig. 5b). This time the mean percentage of eggs hatched in water was 85.5% versus 7.6% for 1 g/liter glyphosate. It looks like as we increase the glyphosate concentration, fewer eggs remain viable in that solution.

Triple-choice assay

While the two aforementioned oviposition experiments provided very useful information about the impact of glyphosate on *Cx. quinquefasciatus* oviposition and egg viability, we still did not know what the females' choice would be if they had access to contaminated water with various levels of glyphosate versus plain water. Therefore, triple-choice assays were therefore designed to observe the females' choice (Fig. 6). According to this assay, female *Cx. quinquefasciatus* could discriminate between water and glyphosate concentrates and mostly chose to lay their eggs in water (mean value = 11.4), while significantly lower numbers of egg rafts were deposited in 0.5 g/liter glyphosate (mean value = 4.6) and 1 g/liter glyphosate (mean value = 2.2). The ANOVA showed significantly different groups with these parameters: $F = 33.165$, $df = 14$, $P < 0.001$, $n = 5$ (Fig. 6).

DISCUSSION

Glyphosate, the most widely used agricultural herbicide in the world with approximately 150,000 tons applied annually in the USA alone (Atwood and Paisley-Jones 2017), can easily leave its target site and enter stagnant water bodies in different concentrations (Bataillard et al. 2020). The enormous use of glyphosate for decades has led to its widespread presence in the environment, which threatens ecosystems and the life of many organisms (Zhan et al. 2018). Improper application practices and overspray have also resulted in its extensive leaking into aquatic and terrestrial environments (Hanke et al. 2010).

According to the Weed Science Society of America (WSSA), glyphosate is technically nontoxic to mammals and birds (oral LD_{50} for mice, rabbits, and goats is greater than 10,000 mg/kg, US National Library of Medicine 1995), but invertebrates, including insects, are highly sensitive to this herbicide (WSSA 2014). Studies have indicated that mosquitoes can be easily exposed to glyphosate concentra-

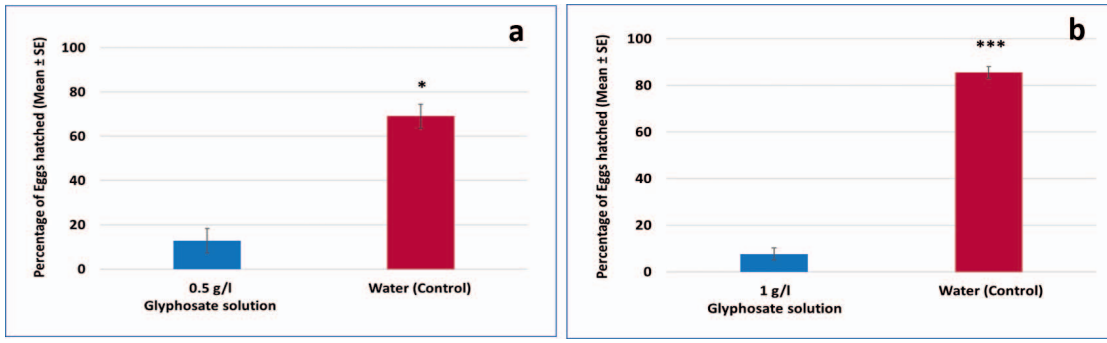

Fig. 5. Percentage of *Culex quinquefasciatus* eggs hatched to first instars in (a) 0.5 g/liter and (b) 1 g/liter glyphosate versus water (control). Paired-sample *t*-test, (a) $P < 0.05$ and (b) $P < 0.001$. Asterisk on a column shows a statistically significant difference at (a) 95% and (b) 99.9% confidence levels.

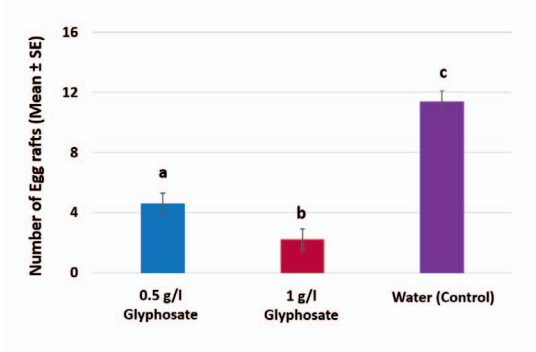

Fig. 6. Number of *Culex quinquefasciatus* egg rafts oviposited in the triple-choice cage assay [0.5 g/liter and 1 g/liter glyphosate versus water (control)]. Different letters indicate statistically significant groups, $P < 0.001$, $n = 5$; Waller-Duncan post-hoc test.

tions, which in turn can impact their development, reproduction, and behavior (Kibuthu et al. 2016, Morris et al. 2016). Although many studies have been conducted on the impacts of chemicals on different insects (Kibuthu et al. 2016, Oliver and Brooke 2018), no study has ever focused on the impact of glyphosate on *Cx. quinquefasciatus* in southern California. On the other hand, previous studies have focused on the exposure of mosquito larvae to very low doses of glyphosate, while we know that glyphosate in water, particularly in water polluted with organic materials, has a considerably shorter life span with a rapid breakdown into its metabolites (Zaranyika and Nyandoro 1993, Mallat and Barceló 1998). Glyphosate is degraded through either biotic pathway (microbial activity) or abiotic approaches such as adsorption and thermolysis (Lund-Høie and Friestad 1986). Zaranyika and Nyandoro (1993) studied glyphosate degradation in rivers with high sediments and found that the degradation mechanism was mostly microbial. They reported an immediate 35% glyphosate reduction in such rivers due to adsorption to suspended particles and deposition in the bottom of river. In another study, Mallat and Barceló (1998) found that half-life of glyphosate in river water with pH 7 was about 4 days. In their review, Zhan et al. (2018) concluded that the main degradation mechanism of glyphosate in water, especially organic-polluted water, was the microbial activity.

We performed a laboratory study in which larvae of *Cx. quinquefasciatus* reared in water containing a high volume of organic material (larval food diet) under higher temperatures (approx. 26–27°C); therefore, single use of a higher dose of glyphosate could compensate for the rapid degradation so that all immature stages could come into contact with a sufficient dose of glyphosate. In most previous studies, the rearing substrate was refreshed for every instar/life stage, and therefore a specific dose of glyphosate was added again to retain the concentration throughout the experiment (Baglan et al. 2018). We did not refresh the rearing water in our experiments to simulate what really happens in the field.

Although other studies have mostly focused on the residual impacts of glyphosate on the behavior and physiology of freshwater invertebrates (Annett et al. 2014, Baglan et al. 2018), we were more interested in the lethal or deterrent effects of glyphosate on the eggs and larvae *of Cx. quinquefasciatus*. Exposure to 1 g/liter of glyphosate led to a rapid reduction of survival ratios over the course of 3–13 days, depending on the larval stage. The survival ratio of first and second instars dropped to zero in 3 and 6 days, respectively, whereas third and fourth instars showed a slow reduction of survival ratio and reached a zero point in 11 and 13 days, respectively (Fig. 6). The slower decline of the survival ratio in third and fourth larval instars can be attributed to their lower rate of feeding. When mosquitoes reach the last larval instars, they stop feeding (AMCA 2022), and this ceases the glyphosate ingestion. A lower concentration of glyphosate (0.5 g/liter) also reduced the survival ratio, but with a much slower pace. The survival ratio of larval instars 1–4 dropped to zero over a period of 9–13 days (Fig. 1). This time, younger and older instars survived longer, had a prolonged larval stage, and molted into pupae with a considerable delay. Even though glyphosate is highly toxic to invertebrates (WSSA 2014), similar studies also suggest that mosquito larvae are more tolerant to glyphosate than many other insects (Bataillard et al. 2020), and that is why they can still develop in a high level of glyphosate solution and even molt to pupae. In both the 0.5 g/liter and 1 g/liter glyphosate experiments, none of the exposed larvae ever emerged as adults, while a very high percentage of control larvae eventually left the puparium as adults. Studying sublethal doses of glyphosate, Kibuthu et al. (2016) also noticed that glyphosate-caused mortality in larvae and pupae of *Cx. quinquefasciatus* impacted the adult emergence rate.

In addition to the induced larval mortality, glyphosate impacted the life cycle of mosquitoes by significantly extending the larval period and deterring larvae from molting into pupae. Those effects were repeatedly observed for both concentrations of glyphosate. While control fourth-stage larvae entirely molted into pupae in 3–4 days, the treated larvae molted in 7–8 days. Very similar findings have been recorded for *Aedes aegypti* (L.), where pupation was considerably delayed because of glyphosate exposure (Morris et al. 2016). We will continue these series of experiments to figure out the impact of lower concentrations of glyphosate on the larval development. The effect of glyphosate concentrations on the oviposition of *Cx. quinquefasciatus* is also very interesting. When 0.5 g/liter glyphosate was compared with water in single-choice experiments, the egg rafts laid in those media did not show any significant difference (Fig. 4a), but when 1 g/liter

glyphosate was used in the same experimental setting, a significantly higher number of egg rafts were laid in water (Fig. 4b). Gravid females take advantage of visual and olfactory cues to find suitable sites for their oviposition. As a site is approached, visual, olfactory, tactile, and gustatory cues are used to figure out the site appropriateness for oviposition (Day 2016). These cues originate from plant infusions, microbes, mosquito immature stages, and predators (Afify and Galizia 2015). We do not know what cues gravid females used to detect glyphosate in water, but it appears regardless of the mechanism involved, they were unable to discriminate between a site with a lower concentration of glyphosate and water (Fig. 4a). When 1 g/liter glyphosate was used as the oviposition site, females could differentiate it from plain water very well (Fig. 4b). Similarly, Kibuthu et al. (2016) found that female *Cx. quinquefasciatus* preferred to oviposit in nitrogenous fertilizers and not in a glyphosate solution. They also concluded that agrochemicals not only affected mosquito oviposition site selection, but impacted offspring survival as well. Gravid females of *Ae. aegypti* also avoided temephos-treated substrates (Quiroz-Martinez et al. 2012). Overall, herbicides and other pesticides can modify mosquito oviposition behavior, but the direction of the response is dependent upon pesticide and its concentration (Kibuthu et al. 2016).

Most of the eggs deposited in glyphosate solutions soon changed to a dark color, which can be considered as a sign of decay. When the egg rafts in both treatments and control groups were watched until hatching time, it was observed that a significantly higher percentage of eggs deposited in water (69%) hatched versus those in 0.5 g/liter glyphosate solution (12.7%) (Fig. 5a). The percentages for eggs hatched in 1 g/liter glyphosate solution versus water were 7.6% and 85.5%, respectively (Fig. 5b). Therefore, when the glyphosate solution is more concentrated, fewer eggs ever hatched. In a similar study, Schneider et al. (2009) found that the eggs of *Chrysoperla externa* Steinmann exposed to glyphosate were abnormal and appeared smaller than control eggs; they were dehydrated and eventually perished 2 days after oviposition. In another research study, Avigliano et al. (2014) concluded that being exposed to glyphosate, a considerable number of estuarine crab eggs, *Neohelice granulata* (Nana), could not hatch into larvae. Even if they successfully hatch to larvae, several larval abnormalities potentially develop that deter them from emerging as adults. Although these findings are, per se, interesting, they are single-choice experiments where gravid females had no choice other than laying eggs in a chemically contaminated water substrate. Therefore, a question is raised as to how females would react if they had access to more diverse types of oviposition sites. In other words, if freshwater sites are also accessible, would females still oviposit in glyphosate-contaminated water? To answer this question, multiple-choice cage assays were designed, where 3 ovicups with aged tap water (control) and the 2 varying concentrations of glyphosate were simultaneously placed in adult BioQuip cages. In this way, we could figure out whether females exhibited any site preference. Analysis of our data indicated that most gravid females preferred to oviposit in a water-only ovicup, and the number of egg rafts laid there was significantly higher than any glyphosate concentration (Fig. 6). The second choice for oviposition was the 0.5 g/liter glyphosate solution. Of course, avoiding a glyphosate substrate is understandable because females try to avoid any unfavorable oviposition site which might put their offspring at risk. For successful reproduction, female mosquitoes must find and evaluate potential oviposition sites (Navarro et al. 2003). To select a suitable oviposition site, gravid females have to first respond to visual or olfactory (attractant or repellent) stimuli, resulting in orientation toward oviposition sites. Secondarily, they should respond to chemotactile cues or deterrents, which may eventually result in the egg deposition (Bentley and Day 1989). In an evolutionary trend females have developed a site selection capability that lets them optimize the selected breeding site to increase the suitability for their larval development. For example, female *Ae. aegypti* do not lay eggs in high-salinity water to avoid exposing their larvae with a lethal salt concentration (Bentley and Day 1989). *Aedes aegypti* also avoid oviposition in a chlorine-treated water (Sherman et al. 1998). Factors involved in choosing a suitable breeding site are chemicals, salinity, pH, and a predictable/reliable food content of water (Merrit et al. 1992).

Our lab experiments clearly indicated that higher concentrations of glyphosate could be lethal to eggs, larvae, and pupae of *Cx. quinquefasciatus*. Furthermore, they prolong the larval development and delay pupation. Glyphosate at both 0.5 g/liter and 1 g/liter concentrations caused 100% mortality in pupae and therefore prevented the mosquito immature stages from emerging as adults. Our data with a high concentration of glyphosate demonstrated the lethal effect on all immature stages of *Cx. quinquefasciatus*, but even in sublethal concentrations, herbicides can affect mosquito life histories and thus impact their vectorial capacity. For example, exposure to the herbicide Atrazine caused longer aquatic stage development in *Ae. aegypti* and *Ae. albopictus* (Skuse), resulting in smaller adult females (Bara et al. 2014), and glyphosate has been found to prolong larval development at high concentrations and decrease proportional survivorship to adulthood in *Ae. aegypti* (Morris et al. 2016). Because of the vast application of herbicides in agriculture, more research is needed to find the effects of various concentrations of glyphosate on the natural fitness and vectorial capacity of major mosquito vectors. Different mechanisms have been suggested for the toxicity of all glyphosate-based herbicides in nontar-

get organisms, such as inducing oxidative stress damage, acetylcholinesterase inhibition, and genotoxicity. It is plausible that several mechanisms are involved, depending on the herbicide formulation and the living organism. Since exposure of aquatic organisms to glyphosate will be increased as a result of more herbicides being used globally, studying the mode of action of glyphosate-based herbicides across various taxa is another area to be explored (Annett et al. 2014).

It is also important to accomplish more single-dose exposure studies to understand how a one-time exposure may affect adult mosquitoes. Many mosquito vectors around us can be found in suburban agricultural fields, and such studies will increase our knowledge of the vector-environment interactions and thus enable us to find more effective control approaches. At the same time, we should notice that although glyphosate has helped farmers to reduce various weeds on their farms, sustainable farming practices are required (Gill et al. 2018) to preserve the balance between commercial monoculture and fragile surrounding ecosystems which that our food webs.

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REFERENCES CITED

Afify A, Galizia CG. 2015. Chemosensory cues for mosquito oviposition site selection. *J Med Entomol* 52:120–130. https://doi.org/10.1093/jme/tju024

AMCA [American Mosquito Control Association]. 2022. *Mosquito larvae* [Internet]. Sacramento, CA: AMCA [accessed January 18, 2022]. Available from: https://www.mosquito.org/page/biology.

Annett R, Habibi HR, Hontela A. 2014. Impact of glyphosate and glyphosate-based herbicides on the freshwater environment. *J Appl Toxicol* 34:458–479. https://doi.org/10.1002/jat.2997

Atwood D, Paisley-Jones C. 2017. *Pesticides industry sales and usage: 2008–2012 market estimates* [Internet]. Washington, DC: EPA [accessed January 18, 2022]. Available from: https://www.epa.gov/sites/production/files/2017-01/documents/pesticides-industry-sales-usage-2016_0.pdf.

Avigliano L, Alvarez N, MacLoughlin C, Rodríguez EM. 2014. Effects of glyphosate on egg incubation, larvae hatching, and ovarian rematuration in the estuarine crab *Neohelice granulate*. *Environ Toxicol Chem* 33:1879–1884.

Baglan H, Lazzari CR, Guerrieri FJ. 2018. Glyphosate impairs learning in *Aedes aegypti* mosquito larvae at field-realistic doses. *J Exp Biol*221:jeb187518. https://doi.org/10.1242/jeb.187518

Bai SH, Ogbourne SM. 2016. Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. *Environ Sci Pollut Res* 23:18988–19001. https://doi.org/10.1007/s11356-016-7425-3

Balbuena MS, Tison L, Hahn ML, Greggers U, Menzel R, Farina WM. 2015. Effects of sublethal doses of glyphosate on honeybee navigation. *J Exp Biol* 218:2799–2805.

Bara JJ, Montgomery A, Muturi EJ. 2014. Sublethal effects of atrazine and glyphosate on life history traits of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae). *Parasitol Res* 113:2879–2886.

Bataillard D, Christe P, Pigeault R. 2020. Impact of field-realistic doses of glyphosate and nutritional stress on mosquito life history traits and susceptibility to malaria parasite infection. *Ecol Evol* 10:5079–5088.

Baz MM. 2017. Influence of the aquatic plant, *Lemna minor* on the development and survival of *Culex pipiens* mosquito immature. *Egypt Acad J Biolog Sci* 10:87–96.

Bentley MD, Day JF. 1989. Chemical ecology and behavioral aspects of mosquito oviposition. *Ann Rev Entomol* 43:401–421.

Daam MA, Moutinho MF, Espíndola ELG, Schiesari L. 2019. Lethal toxicity of the herbicides acetochlor, ametryn, glyphosate and metribuzin to tropical frog larvae. *Ecotoxicology* 28:707–715. https://doi.org/10.1007/s10646-019-02067-5

Day JF. 2016. Mosquito oviposition behavior and vector control. *Insects* 7:65. https://doi.org/10.3390/insects7040065.

de Saraiva AS, Sarmento RA, Pedro-Neto M, Teodoro AV, Erasmo EAL, Belchior DCV, de Azevedo EB. 2016. Glyphosate sub-lethal toxicity to non-target organisms occurring in *Jatropha curcas* plantations in Brazil. *Exp Appl Acarol* 70:179–187. DOI 10.1007/s10493-016-0078-6

Gary RE, Foster WA. 2001. Effects of available sugar on the reproductive fitness and vectorial capacity of the malaria vector *Anopheles gambiae* (Diptera: Culicidae). *J Med Entomol* 38:22–28.

Gill JPK, Sethi N, Mohan A, Datta S, Girdhar M. 2018. Glyphosate toxicity for animals. *Environ Chem Lett* 16:401–426. https://doi.org/10.1007/s10311-017-0689-0

Gimsing AL, Szilas C, Borggaard OK. 2007. Sorption of glyphosate and phosphate by variable-charge tropical soils from Tanzania. *Geoderma* 138:127–132. https://doi.org/10.1016/j.geoderma.2006.11.001

Hanke I, Wittmer I, Bischofberger S, Stamm C, Singer H. 2010. Relevance of urban glyphosate use for surface water quality. *Chemosphere* 81:422–429.

Herbert LT, Vazquez DE, Arenas A, Farina WM. 2014. Effects of field-realistic doses of glyphosate on honeybee appetitive behaviour. *J Exp Biol* 217:3457–3464.

Kibuthu TW, Njenga SM, Mbugua AK, Muturi EJ. 2016. Agricultural chemicals: life changer for mosquito vectors

in agricultural landscapes? *Parasites Vectors* 9:500. DOI10.1186/s13071-016-1788-7

Lund-Høie K, Friestad HO. 1986. Photodegradation of the herbicide glyphosate in water. *Bull Environ Contam Toxicol* 36:723–729.

Mallat E, Barceló D. 1998. Analysis and degradation study of glyphosate and of aminomethylphosphonic acid in natural waters by means of polymeric and ion-exchange solid-phase extraction columns followed by ion chromatography–post-column derivatization with fluorescence detection. *J Chromatogr A* 823:129–136.

Manda H, Gouagna LC, Foster WA, Jackson RR, Beier JC, Githure JI, Hassanali A. 2007. Effect of discriminative plant-sugar feeding on the survival and fecundity of *Anopheles gambiae*. *Malar J* 6:113. https://doi.org/10.1186/1475-2875-6-113

Mehdizadeh M, Mushtaq W, Siddiqui SA, Ayadi S, Kaur P, Yeboah S, Mazraedoost S, AL-Taey DKA, Tampubolon K. 2021. Herbicide residues in agroecosystems: fate, detection, and effect on non-target plants. *Rev Agric Sci* 9:157–167. https://dx.doi.org/10.7831/ras.9.0_157

Merrit RW, Dadd RH, Walker ED. 1992. Feeding behaviour, natural food, and nutritional relationships of larval mosquitoes. *Annu Rev Entomol* 37:349–376.

Miller GT. 2004. Water resources and water pollution. In: *Sustaining the earth: an integrated approach*. 6th edition. Pacific Grove, CA: Brooks/Cole. p. 211–216.

Mohamed AH. 2011. Sublethal toxicity of Roundup to immunological and molecular aspects of *Biomphalaria alexandrina* to *Schistosoma mansoni* infection. *Ecotoxicol Environ Safety* 74:754–760. https://doi.org/10.1016/j.ecoenv.2010.10.037

Morris A, Murrell EG, Klein T, Noden BH. 2016. Effect of two commercial herbicides on life history traits of a human disease vector, *Aedes aegypti*, in the laboratory setting. *Ecotoxicology* 25:863–870. https://doi.org/10.1007/s1064 6-016-1643-923

Morrissey CA, Mineau P, Devries JH, Sanchez-Bayo F, Liess M, Cavallaro MC, Liber K. 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. *Environ Int* 74:291–303. https://doi.org/10.1016/j.envint.2014.10.024

Nagy K, Duca RC, Lovas S, Creta M, Scheepers PTJ, Godderis L, Ádám B. 2019. Systematic review of comparative studies assessing the toxicity of pesticide active ingredients and their product formulations. *Environ Res* 181:108926. https://doi.org/10.1016/j.envres.2019.108926

Navarro DMAF, de Oliveira PES, Potting RPJ, Brito AC, Fital SJF, Goulart Sant'Ana AE. 2003. The potential attractant or repellent effects of different water types on oviposition in *Aedes aegypti* L. (Dipt., Culicidae). *J Appl Ent* 127:46–50.

Nguyen MH, Nguyen THN, Hwang IC, Bui CB, Park HJ. 2016. Effects of the physical state of nanocarriers on their penetration into the root and upward transportation to the stem of soybean plants using confocal laser scanning microscopy. *Crop Prot* 87:25–30. https://doi.org/10.1016/j.cropro.2016.04.012

Nikbakhtzadeh MR, Buss GK, Leal WS. 2016. Toxic effect of blood feeding in male mosquitoes. *Front Physiol* 7:4. https://doi.org/10.3389/fphys.2016.00004

NLM [National Library of Medicine]. 1995. *Hazardous substances databank* [Internet]. Bethesda, MD: NLM [accessed May 23, 2022]. Available from: https://www.nlm.nih.gov/toxnet/index.html.

Oliver SV, Brooke BD. 2018. The effect of commercial herbicide exposure on the life history and insecticide resistance phenotypes of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). *Acta Trop* 188:152–160.

Peruzzo PJ, Porta AA, Ronco AE. 2008. Levels of glyphosate in surface waters, sediments and soils associated with direct sowing soybean cultivation in north pampasic region of Argentina. *Environ Pollut* 156:61–66. https://doi.org/10.1016/j.envpol.2008.01.015

Pochron S, Simon L, Mirza A, Littleton A, Sahebzada F, Yudell M. 2020. Glyphosate but not Roundup® harms earthworms (*Eisenia fetida*). *Chemosphere* 241:125017. https://doi.org/10.1016/j.chemosphere.2019.125017

Prata F, Cardinali VCB, Lavorenti A, Tornisielo VL, Regitano JB. 2003. Glyphosate sorption and desorption in soils with distinct phosphorus levels. *Sci Agric* 60:175–180.

Quiroz-Martinez H, Garza-Rodriguez MI, Trujillo-Gonzalez MI, Zapeda-Cavazos IG, Siller-Aguillon I, Martinez-Perales JF, Rodriguez-Castro VA. 2012. Selection of oviposition sites by female *Aedes aegypti* exposed to two larvicides. *J Am Mosq Control Assoc* 28:47–49.

Riaz MA, Poupardin R, Reynaud S, Strode C, Ranson H, David JP. 2009. Impact of glyphosate and benzo[a]pyrene on the tolerance of mosquito larvae to chemical insecticides. Role of detoxification genes in response to xenobiotics. *Aquat Toxicol* 93:61–69.

Salazar Lopez NJ, Norma J, Silveira Gramont MI, Zuno Floriano FG, Rodriguez Olibarria G, Hengel M, Aldana Madrid ML. 2016. Dissipation of glyphosate from grapevine soils in Sonora, Mexico. *Terra Latinoam* 34:385–391.

Sanchís J, Kantiani L, Llorca M, Rubio F, Ginebreda A, Fraile J, Garrido T, Farré M. 2012. Determination of glyphosate in groundwater samples using an ultrasensitive immunoassay and confirmation by on-line solid-phase extraction followed by liquid chromatography coupled to tandem mass spectrometry. *Anal Bioanal Chem* 402:2335–2345. https://doi.org/10.1007/s00216-011-5541-y

Schneider MI, Sanchez N, Pineda S, Chi H, Ronco A. 2009. Impact of glyphosate on the development, fertility and demography of *Chrysoperla externa* (Neuroptera: Chrysopidae): ecological approach. *Chemosphere* 76:1451–1455.

Sherman C, Feandez EAChAS, Lozano RC, Leontsi E, Winch PJ. 1998. La Untadita: a procedure for maintaining washbasins and drums free of *Aedes aegypti* based on modification of existing practices. *Am J Trop Med Hygiene* 58:257–262.

Solomon KR, Thompson DG. 2003. Ecological risk assessment for aquatic organisms from over-water uses of glyphosate. *J Toxicol Environ Health* 6:289–324.

Struger J, Thompson D, Staznik B, Martin P, McDaniel T, Marvin C. 2008. Occurrence of glyphosate in surface waters of southern Ontario. *Bull Environ Contam Toxicol* 80:378–384. https://doi.org/10.1007/s0012 8-008-9373-1

Timmermann SE, Briegel H. 1996. Effect of plant, fungal and animal diets on mosquito development. *Entomol Exp Appl* 80: 173–176. https://doi.org/10.1007/978-94-009-1720-0_41

Van Bruggen AHC, He MM, Shin K, Mai V, Jeong KC, Finckh MR, Morris JG. 2018. Environmental and health effects of the herbicide glyphosate. *Sci Total* 616–617:255–268. https://doi.org/10.1016/j.scitotenv.2017.10.309

WSSA [Weed Science Society of America]. 2014. *Herbicide handbook*. Champaign, IL: WSSA.

Zaranyika MF, Nyandoro GN. 1993. Degradation of glyphosate in the aquatic environment: an enzymatic kinetic model that takes into account microbial degradation of both free and colloidal (or sediment) particle adsorbed glyphosate. *J Agric Food Chem* 41:838–842.

Zhan H, Feng Y, Fan X, Chen S. 2018. Recent advances in glyphosate biodegradation. *Appl Microbiol Biotechnol* 102:5033–5043. https://doi.org/10.1007/s00253-018-9035-0