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# Identification of Chemicals Associated *Gambusia affinis* (Cyprinodontiformes: Poeciliidae), and Their Effect on Oviposition Behavior of *Culex tarsalis* (Diptera: Culicidae) in the Laboratory

**Adena M. Why,[1,2,3] Dong-Hwan Choe, [2] and William E. Walton[†]**

[1]Current address: Department of Environmental Health, Alameda County Vector Control Services District, 1131 Harbor Bay Parkway, Suite 166, Alameda, CA 94502, USA, [2]Department of Entomology, University of California, Riverside, CA 92521, USA [†]Author is deceased and [3]Corresponding author, email: adena.why@acgov.org

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## Abstract

The western mosquitofish, *Gambusia affinis* (Baird & Girard), has been used worldwide for the control of larval mosquitoes for more than 100 yr. We found that the western encephalitis mosquito, *Culex tarsalis* Coquillett (Diptera: Culicidae), can detect the presence of *G. affinis* in oviposition sites based on associated chemicals, leading to a decrease in the number of egg rafts laid. Three volatile chemical compounds were identified in the headspace above the water where *G. affinis* had been held for 24 h. Oviposition bioassays conducted using standards of the volatile compounds identified (dimethyl disulfide [DMDS], dimethyl trisulfide [DMTS], and S-methyl methanethiosulphonate) found that females reduced oviposition only when low concentrations of DMTS were present, but this response was not consistent across all trials and concentrations tested. DMDS, DMTS, and S-methyl methanethiosulphonate are known bacterial metabolic waste products and may be the source of the compounds. Two nonvolatile compounds of interest were found to be present in the *Gambusia*-exudate water. After tasting *Cx. tarsalis* were deterred from ovipositing onto *Gambusia*-treated water from which the bacteria had been removed by filtration, indicating that the kairomone may consist of nonvolatile compound(s). One of the nonvolatile compounds isolated from the *Gambusia*-treated water has a benzene ring structure similar to that of cholesterol but the structure of the two nonvolatile deterrents remains to be fully characterized. Our research shows that three volatile compounds and two nonvolatile compounds are present in water associated with *G. affinis* (Poeciliidae: Gambusia) and affect the oviposition behavior of *Cx. tarsalis* in laboratory bioassays.

**Key words:** mosquito, mosquitofish, semiochemical, deterrent

The western mosquitofish, *Gambusia affinis* (Baird & Girard), has been introduced worldwide for the biological control of mosquitoes beginning in the early 1900s (Walton et al. 2012). Many studies done over the subsequent decades have tested the efficacy of *G. affinis* as a biological control agent. However, the exploration of semiochemical(s) associated with *G. affinis*, as well as other species, and their potential role in affecting mosquito oviposition is an important area of basic and applied research that has only recently begun to be investigated (Bentley and Day 1989, Angelon and Petranka 2002, Navarro-Silva et al. 2009, Walton et al. 2009, Afify and Galizia 2015, Why et al. 2016).

*Culex tarsalis* the western encephalitis mosquito, is native to North America and much of its geographic range currently overlaps with that of *G. affinis*. It is the most important vector of arboviruses in western North America, responsible for the maintenance, amplification, and epidemic transmission of Western Equine Encephalomyelitis virus (WEEV) and St. Louis Encephalitis virus (SLEV). It also vectors California Encephalitis virus and is currently the main vector of West Nile virus (WNV) in the western U.S. (CDC 2013). It has been shown that female *Cx. tarsalis* can detect the presence of *Gambusia*-associated semiochemicals in oviposition sites and are deterred from ovipositing (Why and Walton 2020).

*Culex* oviposition behavior consists of several consecutive steps and is likely to be influenced by both volatile and nonvolatile semiochemicals that function as attractants, stimulants, arrestants, repellents, or deterrents to egg-laying. The long-range orientation to a potential oviposition site, hovering at the site (arrestment), landing on the aqueous substrate, sampling the substrate, and finally, deposition of an egg raft (Klowden 1990, Du and Millar 1999) are influenced by chemicals in the atmosphere and in the water. Although significant research has been done in this area, with trying to determine which semiochemicals influence oviposition behavior in various mosquito species, we still lack studies looking at how semiochemicals associated with *Gambusia* might influence oviposition behavior of *Cx. tarsalis*. The air that is present over a given oviposition site (the headspace) is likely to contain a mixture of chemicals emanating from various organisms and may contain chemicals associated with food resources, other aquatic macroinvertebrates, as well as potential predators and competitors.

Females of several *Culex* species "taste" the water in oviposition sites before laying egg rafts responding to salt concentration as well as other factors (Clements 1999). The presence of a nonvolatile cue associated with conspecifics in the water enhanced oviposition by *Cx. tarsalis* (Hudson and McLintock 1967). Isoe and Millar (1995) cautioned that results from the egg raft counting bioassays, which has been used frequently to screen for oviposition attractants, should be interpreted with caution because high oviposition rates may be due to factors such as nonvolatile, contact oviposition stimulants rather than to volatile attractants. Why and Walton (2020) suggested that egg-laying by *Cx. tarsalis* is reduced by nonvolatile compounds more than by volatile compounds in oviposition sites conditioned by mosquitofish. The putative semiochemicals deterring mosquito oviposition were still active following dilution and were not removed by filtration. The increased use of pyrethroids and other compounds for mosquito control has led to resistance developing in wild populations of Culex across the world (Scott et al. 2015, Sugiura et al. 2021). If environmentally-friendly alternatives can be developed, which can alter a female mosquitoes oviposition behavior and rate, this would help to decrease subsequent populations and the need to apply more insecticides for mosquito control and to prevent disease transmission.

The objectives of this study were 1) to determine what volatile compounds were present in the headspace of the *Gambusia*-treated water using solid-phase microextraction (SPME) and gas chromatography–mass spectrometry (GC/MS), 2) determine how long the chemicals persist in the headspace after the fish have been removed from the water, 3) determine if nonvolatile chemical compounds are present in the *Gambusia*-treated water using a variety of analytical techniques, including solid-phase extraction (SPE), gas chromatography–mass spectrometry (GC/MS), GC/MS Time of Flight (GC/MS-TOF) and liquid chromatography–mass spectrometry (LC/MS), and 4) using standards of the identified compounds, determine their effect on the oviposition behavior of female *Cx. tarsalis*.

## Materials and Methods

### Mosquito Colony and Rearing

*Culex tarsalis* adults were reared from a colony derived from wild individuals collected at the Eastern Municipal Water District's demonstration constructed treatment wetland (San Jacinto, CA) in 2001. The mosquito colony was supplemented with (~200) individuals collected by carbon dioxide-baited suction traps from several sites in Riverside, CA at the start of the experiments in 2013.

*Culex tarsalis* larvae were reared in enamel pans under standard laboratory conditions (27°C, 16:8 h light:dark [LD] cycle with 1 h dusk/dawn periods) and fed ad libitum on a mixture of ground rodent chow and Brewer's yeast (3:1, v:v). Pupae were collected into 200-ml wax-lined cups (Solo Cup Co., Chicago, IL) and placed into emergence cages (46 cm × 31 cm wire frame covered with a sleeve of organza) placed onto a plastic tray. Cages were kept in a 3.7 m × 1 m humidified chamber in a rearing room in the lab. Temperatures and relative humidity ranged from 21 to 26°C and 50 to 70% RH. The adult mosquitoes were allowed to feed ad libitum on a 10% sucrose and water mixture, as well as a cup containing 3–4 raisins that had been soaked in water and sprinkled with 5 ml of granulated sugar. Food was replaced weekly. Once each week, female mosquitoes were fed overnight on a 2–5 d-old restrained chick.

Within 18 h of a bloodmeal, 30 blood-fed female *Cx. tarsalis* were aspirated into 30 × 30 × 30 cm cages (Model # 1450B; Bioquip Products, Rancho Dominguez, CA). Females were determined to have successfully blood-fed by the appearance of blood in the abdomen. Females were provided a 10% sucrose and water mixture and allowed to feed ad libitum. Blood-fed females were kept in assay cages in a humidified chamber in the rearing room.

### *Gambusia* Colony

Mosquitofish, *G. affinis*, were kept in a 4 × 7 $m^2$ earthen pond at the University of California, Riverside (UCR) Aquatic Research Facility in Riverside, CA. Fish were trapped using minnow traps (Cuba Specialty Mfg., Fillmore, NY) lined with window screen (mesh opening: 1.5 mm) and baited with dog food. Fish were transported back to the lab in 19-liter plastic buckets (Home Depot, Atlanta, GA). Water was continuously oxygenated using a battery-operated aquarium pump. In the lab, fish were kept in a 75-liter aquarium and maintained with a standard aquarium filter and pump. Fish were fed daily on Tetra Pond fish flakes (Spectrum Brands Inc., Melle, Germany).

### *Gambusia*-exudate Water

A 3-day protocol was used to make *Gambusia*-exudate water. During the first 24 h, a mix of adult and young-of-the-year fish were allowed to acclimate and feed ad libitum on Tetra Pond flaked fish food in an aquarium containing 75 liters of tap water that had been continuously oxygenated with a standard aquarium pump (Petco Animal Supplies Inc., San Diego, CA) and aged for 24 h. During the second 24 h, the fish were moved to a 19-liter aquarium containing aged tap water and allowed to empty their guts. Water was continuously oxygenated using a standard aquarium pump. On day 3, 10 fish: 1 liter aged tap water (30 fish total) were moved to an 11-liter round glass container, with a corresponding amount of aged tap water, that was continuously oxygenated with a standard aquarium pump. The fish were removed after 24 h and the *Gambusia*-exudate water was used to test the ovipositional responses of the mosquitoes.

The control water consisted of an equivalent amount of tap water (3 liters) that had been aged and oxygenated using a standard aquarium pump for 24 h.

### Identification of Volatile Compounds Associated with *Gambusia*-exudate Water Using Solid Phase Microextraction (SPME) and GC/MS Analyses

Volatiles emanating from *Gambusia*-exudate water were collected in the headspace using solid phase microextraction (SPME) fibers with a carboxen/polydimethylsiloxane (PDMS; 75 µm) coating (Sigma–Aldrich Corp., St. Louis, MO) and analyzed on an Agilent GC/MS (Model: 7890A, 5975C VL MSD with triple-axis detector;

Agilent Technologies, Santa Clara, CA). *Gambusia*-exudate water was prepared according to the previously stated protocol at a ratio of 10 fish: 1 liter aged tap water. *Gambusia*-exudate water was prepared in a 20.32 cm × 20.32 cm (height × diameter) round glass cylinder (Fisher Scientific, Waltham, MA) and air was provided using a standard aquarium pump. At the end of the 3-day process, the fish were removed and the top of the glass container was covered with aluminum foil to minimize loss of the volatile compounds emanating from the water's surface. A piece of cardboard, with a small hole punched in the center, was placed on top of the container in order to provide a platform onto which the SPME fiber holder could sit. Volatiles were collected for 24 h.

At the end of the collection time, the SPME fiber was removed and immediately desorbed in the GC/MS using the following parameters: electron impact mass spectra (70 eV) were analyzed on an Agilent 5975C mass selective detector coupled to Agilent 7890A gas chromatograph equipped with a DB-5 column (30 m × 0.32 mm inner diameter). Samples were run on the following temperature program: 50°C for 1 min, then 10°C $min^{-1}$ to 280°C for 5 min. Samples were injected in splitless mode with helium as the carrier gas. The temperature of the injector and transfer line were 250°C. Five replicates were analyzed.

Four replicates were also run on the GC/MS using the following parameters in order to gain better resolution of the various analytes: 10°C for 1 min, then 5°C $min^{-1}$ until 280°C and held at 280°C for 5 min in splitless mode on a DB5 column. Dry ice was pulverized and placed in the GC oven to cool the column down to the desired start temperature, i.e., 10°C. A new batch of *Gambusia*-exudate water was made for each sample. Control samples consisted of 3 liters of aged tap water, which had been aged for 24 h, and aerated with a standard aquarium pump in an equivalent 20.32 cm × 20.32 cm round glass cylinder. The control samples were collected at the same time as the treatment samples using an equivalent SPME fiber with a carboxen/PDMS coating. Five replicate control samples were analyzed using the same parameters on the GC/MS. Samples were injected in splitless mode on a DB5 column with helium as the carrier gas. The temperature of the injector and transfer line were 250°C.

## Time of Persistence of Volatile Compounds from Aged *Gambusia*-exudate Water Using SPME Analysis

To determine the persistence of volatile compounds in the headspace of the *Gambusia*-exudate water under normal atmospheric conditions, SPME collections were performed at three different time intervals: 24 h, 36 h, and 48 h. The timing of the collections began immediately after the removal of the fish from the water. The amount of water used to determine the persistence of the headspace volatiles corresponded to the same amount of water (150 ml) that was used for the two treatments (*Gambusia*-exudate water or 24-h aged tap water) in the oviposition bioassays, but was placed into separate 600-ml glass beakers (Fisher Scientific, Waltham, MA). The diameter of the 600-ml glass beaker was approximately equivalent to the diameter of the waxed cups used in the oviposition bioassays. Samples were left uncovered at ambient room temperature until the corresponding time-period had elapsed (24 h, 36 h, and 48 h) then the tops of the beakers were covered with aluminum foil and a SPME fiber, with a carboxen/PDMS coating, was used to collect the volatile compounds for approximately 1 h. Samples were only collected for one hour in order to determine the presence of the compounds at the exact time points being measured (24 h, 36 h, and 48 h). This was done in order to compare the presence versus absence, i.e., off-gassing of chemical compounds from the *Gambusia*-exudate water over time, i.e., persistence of the compounds in the headspace after fish were removed. Samples were not taken at the zero time point, i.e., immediately after fish had been removed, as $n = 9$ replicates had been previously run to identify the volatile compounds present in the *Gambusia*-exudate water at the zero time point.

Samples were analyzed as described above using the following parameters: 50°C for 1 min, then 10°C $min^{-1}$ to 280°C for 5 min. Samples were injected in splitless mode with helium as the carrier gas. Temperature of the injector and transfer line were 250°C.

## Volatile Analyses of Filtered *Gambusia*-exudate Water

Volatile compounds were collected and analyzed from filtered *Gambusia*-exudate water to determine whether removal of the bacteria from the water would also remove the source of the volatile compounds identified from the natural blend of *Gambusia*-exudate water. *Gambusia*-treated water prepared as described above was filter-sterilized by passing fish-conditioned water through a membrane filter (pore size either 0.2 µm or 0.45 µm; Fisher Scientific, Waltham, MA) under low vacuum (10–15 psi) (Hobbie et al. 1977, Meyer-Reil 1978). The control consisted of aged tap water that had been filtered through a filter with the same pore size as that used for the *Gambusia*-exudate water; 150 ml of the filtered water was placed into a 600-ml glass beaker. The top of the beaker was covered with aluminum foil and a SPME fiber with a carboxen/PDMS coating (75 µm) was used to collect the volatile compounds. The samples were analyzed by GC/MS using the following parameters: 50°C for 1 min, then 10°C $min^{-1}$ to 280°C for 5 min. Samples were injected in splitless mode with helium as the carrier gas. Temperature of the injector and transfer line were 250°C. Volatiles were collected for the following time periods for 0.2 µm-filtered *Gambusia*-exudate water: 18 h ($n = 2$), 24 h ($n = 4$), 36 h ($n = 1$); 0.45 µm-filtered *Gambusia*-exudate water: 24 h ($n = 8$). The time points indicated represent the number of total hours after filtration, of the *Gambusia*-exudate water, when the headspace collection of volatiles took place. Collection of the headspace volatiles samples, in some cases, was run concurrently with oviposition bioassays, i.e., the same batch of water was used in order to determine the volatile compounds present and if they had any biological significance to the female mosquitoes. The control sample consisted of 150 ml aged tap water, which had been aged for 24 h with a standard aquarium pump and filtered in the same manner ($n = 1$).

## Nonvolatile Chemical Analyses

Solid phase extraction (SPE) was used to examine the less-volatile constituents of *Gambusia*-exudate water. The fish-exudate water was prepared according to the previously stated protocol and extracted according to protocols based on EPA guidelines for method 1694 (USEPA 2007). Waters HLB glass SPE columns (Milford, MA) were preconditioned using 6 ml of HPLC grade methanol (Fisher Scientific, Hanover Park, IL) and 12 ml of deionized water, and eluted under gravity. *Gambusia*-exudate water (300 ml) was then pulled through each column by vacuum (10–15 psi) using a vacuum manifold (Restek Inc., Bellefonte, PA). The control sample consisted of 300 ml tap water that had been aerated for 24 h and was extracted using the same protocol.

After all the *Gambusia*-exudate water had been extracted, the column was left under vacuum to dry for a minimum of 1 h. After drying, the columns were extracted using 20 ml of HPLC grade

methanol, under gravity, into a 20 ml glass vial. The vials were stored, capped, in the refrigerator until further analysis. Ten replicate extractions were performed.

For liquid chromatography–mass spectrometry, the solid phase extract was dried completely under $N_2$ gas prior to analysis. Samples were analyzed on an Agilent 6210 Time of Flight GC/MS and a C18 reverse phase column (Thermo Scientific Hypersil Gold, column diameter 100 mm/2.1 mm, 3.5 µm particle size) was used for separation. A 10 µl sample was loaded and a linear gradient was applied. Buffer A was $H_2O$/0.1% formic acid and buffer B was Acetonitrile (ACN)/0.1% formic acid. An 18 min LC gradient started from 5% B to 100% B.

For gas chromatography–mass spectrometry time of flight (GC/MS-TOF), the solid phase extract was dried under $N_2$ gas prior to analysis and analyzed on a Micromass GCT Premier instrument coupled to an Agilent 7890A GC. The column used was an Agilent VF-5ms, diameter 30 m × 0.5 µm. Samples were run using the following temperature program: 50°C for 1 min to 320°C for 20 min. A 1 µl sample was injected and electron bombardment ionization was used. A standard of cholesterol (obtained from J. Millar, UC, Riverside,CA) was analyzed on GC/MS to confirm the structural similarity between cholesterol and the unknown compound based on comparisons of their mass spectral fragmentation patterns. The following run parameters were used: 50°C for 1 min, then 10°C $min^{-1}$ to 280°C for 5 min on a DB5 column.

## Oviposition Bioassays

### Oviposition Bioassays with *Gambusia*-exudate Water

For all bioassays, mixed aged female mosquitoes from the same parent colony were used. The majority of females used were 1–2 wk old, but females were chosen randomly from the parent colony at the time of each assay and previously blood-fed females (which had not been used in prior experiments) were not excluded from use in the experiments. Females were blood-fed at the same day and time, using above stated protocols. All females had the opportunity to mate postblood feeding and prior to the start of the bioassay. All assays began 3 d postblood feeding in order to provide enough time for females to fully maturate a batch of eggs.

Binary choice bioassays were conducted to determine whether female *Cx. tarsalis* could detect the presence of semiochemicals associated with *G. affinis* in oviposition sites (treatment water) when compared to water containing no *Gambusia* semiochemicals (control). The treatment water was prepared with either live *Gambusia* or chemical standards corresponding to the volatile chemicals identified from the *Gambusia*-exudate water. Thirty female mosquitoes (all in the same cage) were presented with two white 200-ml wax-lined cups (Solo Cup Co., Chicago, IL) containing either 150 ml of treatment water or the control. The cups were placed into the mesh cages measuring 30 × 30 × 30 cm (Model # 1450B; Bioquip Products, Rancho Dominguez, CA) at 16: 30 h, 30 min before the 1-h dusk period in the L:D cycle begins. The placement of the cups within each replicate cage was randomized among the four corners of the cage. Cups were removed from the cages no sooner than 24 h the following day and the numbers of egg rafts in each cup were counted. A fresh cup containing either the control or the treatment water was placed into the cages. While we could not control the rate at which the volatile compounds dissipated from the treatment cups, we replaced the cups every 24 h to replicate the same level of semiochemicals in the cups over the three consecutive days of the trial. Each female normally lays a single egg raft and each cage was monitored over three successive nights. At the end of each trial, the number of egg rafts in each treatment within a replicate cage was pooled across dates (3 d) to obtain an overall number of rafts for each treatment.

For each trial, three to eight replicate cages of blood fed females were used. This variation in number of replicate cages was based on the number or females that had successfully blood-fed prior to the experiment. Only one trial was conducted at a time to ensure that any volatiles emanating from the semiochemical-laden water, or chemical standards, had time to completely dissipate from the area prior to the start of the next trial. This was done to ensure that any differences seen in oviposition could only be attributable to the compound(s) being tested. The ventilation system of the insectary provided complete turnover of the air approximately every 7 min, providing adequate airflow to minimize contamination from volatile chemicals between bioassays. Assays were run in an insectary at the University of California, Riverside, under the same room conditions as the parent colony of *Cx. tarsalis* was maintained.

No-choice bioassays were conducted to determine whether female *Cx. tarsalis* were deterred from ovipositing onto water containing *G. affinis* semiochemicals, leading to an overall decrease in oviposition rate when no alternative oviposition site (i.e., aged clean tap water) was presented. The no-choice bioassays were conducted using the same protocol as described above for the binary choice bioassays except female mosquitoes were presented with two cups of either the control (aged tap water) or treatment water (*Gambusia*-exudate water or water containing chemical standards) in each cage. Treatment and control were run in separate rooms to prevent any possible contamination of the control by volatile compounds emanating from the treatment water.

To ascertain the responses of gravid mosquitoes to the olfactory cues emanating from potential oviposition sites, sticky-screen bioassays were carried out following the methods of Isoe et al. (1995). Briefly, 30 g of insect glue (Tanglefoot, Grand Rapids, MI) was diluted with 70 ml hexane in a petri dish. Galvanized hardware cloth (6.5-mm mesh) was dipped in the diluted insect glue and placed in a fume hood for 3 h to evaporate the hexane. Thirty female mosquitoes were presented with two white 120-ml wax-lined cups (Solo Cup Co., Chicago, IL) containing either 100 ml of *Gambusia*-exudate water or the control in each of the aforementioned bioassay cages. The oviposition cups were covered by sticky screens. The level of the water was below the sticky screens so the mosquitoes could not contact the water. Mosquitoes were placed in the cages in the late afternoon and the numbers of mosquitoes on the sticky trap screens were counted the following morning. The density of mosquitofish used to condition the water was 0.5 fish/liter. Seven trials using 3–5 replicate cages per trial were run under the same conditions as the binary choice assays.

Clipped-wing bioassays were also conducted to examine females' oviposition responses to various treatments where they were forced to remain in contact with the water. For all bioassays, mixed age females from the same parent colony were used. The majority of females used were 1–2 wk old, but females were chosen randomly from the parent colony at the time of each assay, and previously blood-fed females were not excluded from use in the experiments. Females were blood-fed at the same day and time, using the above stated protocol. All females had an opportunity to mate postblood feeding and prior to the start of the bioassay. Blood-fed females were aspirated into a holding cage and given a 10% sucrose solution and allowed to feed ad libitum until needed. After 3 d, the females were removed from the cage and anesthetized under $CO_2$ at a rate of 10 psi for approximately 15–20 s, or until movement had ceased. After anesthetization, females were placed onto a work surface and one, or

both, wings were partially amputated. After their wings had been amputated, mosquitoes were placed on a small piece of lumite screening that had been placed in a 100 × 15 mm (diameter × height) plastic petri dish (Fisher Scientific, Waltham, MA) containing 40–45 ml of either the control (24-h aged tap water) or treatment water. This was done to supply the female with a platform onto which she could recover safely after being anesthetized. The petri dishes were covered with a plastic lid and females were allowed to oviposit. Assays were started between 17:00–19:00 h to correspond to the "dusk" oviposition period. If any female did not recover from having her wing(s) amputated (i.e., was not upright and standing on the lumite screening, side of the petri dish, or water surface) after 10 min she was replaced with a new female prior to the start of the experiment. Some females died after the initial 10-min recovery period and were not included in the final data analysis. The dishes were checked for egg rafts after 1 h had elapsed and, if an egg raft was not observed, again after 5–6 h.

### Oviposition Bioassays with Water Containing Volatile Chemical Standards

To determine if the volatile chemicals collected and identified in the headspace above fish-conditioned water are responsible for the female mosquito's behavioral response to the *Gambusia*-exudate water, the oviposition assays were conducted with water samples spiked with known amounts of chemical standards. For dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and S-methyl methanethiosulphonate, chemical standards (Sigma–Aldrich Corp., St. Louis, MO) were used.

Concentrated standard solutions were prepared by first creating a 1 M stock solution by diluting 9.4 g DMDS (or 12.6 g of DMTS or S-methyl methanethiosulphonate) in 100 ml of ethanol. In order to make the serial dilutions, 0.1 ml of the 1 M stock solution was diluted in 100 ml of ethanol to obtain the 0.1% solution. The process was repeated to obtain the 0.1%, 0.001%, and 0.0001% solutions (for DMDS only). The treatment water was prepared by adding 10 µl of each chemical standard preparation (in ethanol) into 150 ml of 24-h aged tap water and mixing it. For the binary choice assays, the control consisted of 10 µl 95% EtOH pipetted onto the surface of 150 ml 24-h aged tap water. 200-ml wax-lined cups (Solo Cup Co., Chicago, IL) cups were used and replaced every 24 h for 3 d.

The following replicates were run for each concentration of DMDS used: 67 nM/liter (0.1% in 150 ml of aged tap water; $n = 1$), 6.7 nM/liter ($n = 4$), and 0.67 nM/liter ($n = 4$). The following replicates were run for each concentration of DMTS used: 6.7 nM/liter ($n = 6$), 0.67 nM/liter ($n = 4$). The following replicates were run for each concentration of S-methyl methanethiosulphonate used: 6.7 nM/liter ($n = 5$) and 0.67 nM/liter ($n = 2$).

For the no-choice bioassays, the following concentrations were tested for DMDS: 67 nM/liter ($n = 1$), 6.7 nM/liter ($n = 4$); DMTS: 6.7 nM/liter ($n = 2$), and S-methyl methanethiosulphonate: 6.7 nM/liter ($n = 6$).

### Oviposition Bioassays with Volatiles Placed Next to Water Source

Additional assays were conducted with DMDS where the standard was presented separately from the water source. Suh et al. (2016) ran similar experiments with *Anopheles coluzzii* (Coetzee and Wilkerson) and found that the presence of DMDS next to an oviposition site led to a decrease in oviposition. A $1 \times 10^{-4}$ % (0.067 nM) solution of DMDS in 95% EtOH was used as the treatment and $1 \times 10^{-4}$ % (0.067 nM) solution of DMSO in 95% EtOH (dimethyl sulfoxide) was used as the control. One ml of each solution was pipetted into a glass GC vial (Agilent Co., Santa Clara, CA) and the vial was attached with masking tape to the side of a 200-ml waxed-lined cup (Solo Cup Co., Chicago, IL) containing 150 ml of aged tap water. The mosquitoes could not physically contact the chemical standards in the GC vials; therefore oviposition preference was based on the presence or absence of volatile cues emanating from the GC vials attached to the sides of the cups. New chemical standards were made every 24 h, and the cups were replaced every 24 h. At the end of the 3 d, the number of egg rafts laid per treatment was pooled and analyzed.

### Semi-quantitative Comparisons of Headspace Volatiles between *Gambusia*-exudate Water and Water Samples Containing Standards

To obtain a measure of the concentrations of the standard compounds in the headspace used in the oviposition bioassays, semiquantitative comparisons were conducted between *Gambusia*-exudate water and the water samples spiked with standards. For the standard samples (dimethyl disulfide [DMDS], dimethyl trisulfide [DMTS] and S-methyl methanethiosulphonate standards) the lowest concentration (0.67 nM/liter) was chosen for comparison. For each bioassay, 150 ml of the water sample (*Gambusia*-exudate water [made as stated previously] or aged tap water) spiked with 10 µl of the standard preparation (in ethanol) containing the three standards was placed into a 600-ml glass beaker and covered with foil for 24 h. The concentration of each standard in the final solution was: 0.67 nM/liter DMDS, 0.67 nM/liter DMTS, and 0.67 nM/liter S-methyl methanethiosulphonate. A SPME fiber with a carboxen/PDMS coating was used to collect volatiles from the headspace for 24 h. The SPME fiber samples were subsequently analyzed on GC/MS using the same run parameters that were used for compound identification: 50°C for 1 min, then 10°C $min^{-1}$ to 280°C for 5 min. To make semiquantitative comparisons of DMDS, DMTS, and S-methyl methanethiosulphonate between different samples, the areas of the corresponding peaks in the chromatograms were obtained (GC/MSD ChemStation Software, Agilent Technologies) and compared (Romeo 2009). Due to the small replication size ($n = 2$ for each group), formal statistical tests were not conducted for the peak areas.

### Oviposition Bioassays with Water Containing Nonvolatile Chemical Extracts

Oviposition bioassays were conducted to determine whether the nonvolatile constituents of the *Gambusia*-exudate water obtained through SPE elicited a deterrent response in the female mosquitoes. The MeOH HLB column extracts, derived from the *Gambusia*-exudate water, were concentrated down from 20 ml to approximately 1.5 ml of solution using a rotary evaporator. HPLC grade methanol was added back to the concentrated extract to make a final solution of 1.5 ml. The treatment consisted of 0.5 ml of the extract pipetted into 150 ml of aged tap water, equivalent to 100 ml of *Gambusia*-exudate water. HPLC grade methanol (0.5 ml) pipetted into 150 ml of aged tap water was used for the control. Binary choice assays were conducted comparing 237 ml of aged tap water to 237 ml of the HLB extract containing the equivalent of 100 ml *Gambusia*-treated water per cage. Three cages of 30 blood-fed females were used in each of three trials ($n = 3$).

## Results

### Identification of Volatile Compounds Associated with *Gambusia*-exudate Water Using Solid Phase Microextraction (SPME) and GC/MS Analyses

Dimethyl disulfide (DMDS: $C_2H_6S_2$), dimethyl trisulfide (DMTS: $C_2H_6S_3$), and S-methyl methanethiosulphonate ($CH_3SO_2SCH_3$) were detected in the headspace of the *Gambusia*-exudate water but were absent from the aged tap water control samples (Fig. 1). Each compound was tentatively identified using the NIST Standard Reference Database. The retention time of DMDS was 2.54–2.601 min (Fig. 2a) under the aforementioned GC/MS conditions. The retention time of DMTS was 6.80–7.10 min in all samples tested. The retention time of S-methyl methanethiosulphonate was 16.0–16.25 min. The identities of DMDS, DMTS, and S-methyl methanethiosulphonate were confirmed by running the chemical standards at the same parameters as the samples (Figs. 2c–e).

### Time of Persistence of Volatile Compounds from Aged *Gambusia*-exudate Water Using SPME Analysis

Dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and S-methyl methanethiosulphonate were present in the headspace samples taken at 24 h after removal of the fish from the *Gambusia*-exudate water. This water contained the full complement of microorganisms. However, only DMDS and DMTS were detectable in the headspace samples taken at 36 h and 48 h (Figs. 3a and b).

### Volatile Analyses of Filtered *Gambusia*-exudate Water

GC/MS analysis showed that after filtration through a 0.2 or 0.45 µm pore Whatman filter to remove any bacteria present, DMDS, DMTS, and S-methyl methanethiosulphonate were not present at detectable levels in the headspace of the filtered *Gambusia*-exudate water for any time period tested (18, 24, or 36 h).

### Nonvolatile Chemical Analyses

From GC/MS and LC/MS analyses, two unknown compounds were present in the *Gambusia*-exudate water solid phase extracts that were absent from the control samples (Figs. 4 and 5), but the full chemical structures of the compounds based on the analyses completed to date remains undetermined. One of the chemical compounds has a possible ring structure and mass spectrum that resembles cholesterol (Fig. 6). The structure of the second compound present in the *Gambusia*-exudate water was not determined.

GC/MS analysis showed that DMDS, DMTS, and S-methyl methanethiosulphonate were absent from the solid phase extracts, indicating that these more volatile chemicals had been removed during the extraction process (Fig. 7).

### Binary Choice Assays with Aged *Gambusia*-exudate Water

Female *Cx. tarsalis* were significantly deterred from ovipositing onto 150 ml *Gambusia*-exudate water that had been aged for 24 h prior to use in the assay ($t$ = 5.795, d.f. = 10, $P$ < 0.001). The number of egg rafts laid in each of the two treatments was consistent among the replicate cages ($X^2$ = 18.307, d.f. = 10, $P$ > 0.1); on average 12 egg rafts were laid on the control water and 3 egg rafts were laid on the *Gambusia*-exudate water (Table 1).

### Sticky Screen Bioassays

Significantly more mosquitoes were attached to screens above water conditioned with *Gambusia* than were attached to screens above the control (24-h aged tap water; $\chi^2$ = 5.04, df = 1, $P$ < 0.025). On average, 57% of collections were on sticky screens above *Gambusia*-exudate water versus 43% of gravid females on sticky screens above the control treatment (Fig. 8).

### Binary Choice and No-choice Bioassays with DMDS

*Culex tarsalis* females were not deterred from ovipositing onto sites that contained a 0.1% standard solution of DMDS (67 nM) when compared to control cups containing aged tap water in a no-choice assay ($t$ = –0.394, d.f. = 2, $P$ = 0.732). On average females laid 16 egg rafts on the controls and 17 egg rafts on the treatment water. Females were also not deterred from ovipositing onto sites containing a standard of 0.01% DMDS (6.7 nM) when compared to aged tap water in no choice assays ($t$ = –2.124, d.f. = 11, $P$ = 0.057); on average females laid 18 egg rafts on control water and 20 egg rafts on the treatment water. Females were not deterred from ovipositing onto sites containing a standard of 0.001% DMDS (0.67 nM) when compared to aged tap water ($t$ = 0.295, d.f. = 16, $P$ = 0.772) in choice assays. The responses of egg-laying mosquitoes were significantly heterogeneous among the replicate cages ($X^2$ = 26.296, d.f. = 16, $P$ < 0.001) but females laid an average of 5 egg rafts in both the control and treatment cups (Table 1).

### Oviposition Bioassays with Volatiles Placed Next to Water Samples

In trials conducted using a 1 × $10^{-4}$ % (0.067 nM) standard concentration of DMDS as the treatment and a 1 × $10^{-4}$ % (0.067 nM) standard concentration of dimethyl sulfoxide (DMSO) as the control, *Cx. tarsalis* demonstrated no difference in oviposition between the two treatments ($t$ = –1.546, d.f. = 13, $P$ = 0.146). The responses of egg-laying mosquitoes to the two treatments were heterogeneous among the replicate cages ($X^2$ = 22.362, d.f. = 13, $P$ < 0.001). An average of 2 egg rafts were laid in the control cups and 3 egg rafts on the treatment water (Table 1).

### Individual Female Clipped-wing Bioassays with DMDS

No statistically significant difference in *Cx. tarsalis* oviposition rate was seen between cups containing aged tap water and aged tap water spiked with a 1 × $10^{-4}$ % (0.067 nM) standard of DMDS after 1 h ($F$ = 1.761; d.f. = 1, 49; $P$ < 0.1845). There was also no significant difference in oviposition seen after 24 h ($F$ = 0.412; d.f. = 2,49; $P$ < 0.521; Table 1). The average number of females that laid an egg raft in the control was 83%, while 70% of females laid an egg raft on the treatment water. The number of females used per trial varied and was dependent on the number of blood-fed females available, as well as the number of females that survived the amputation procedure.

### Binary Choice and No-choice Bioassays with DMTS

*Culex tarsalis* females were not deterred from ovipositing onto water that contained a 0.01% standard (6.7 nM) of DMTS when compared to aged tap water in binary choice assays ($t$ = –0.677, d.f. = 19, $P$ = 0.506). An average of 8 egg rafts were laid in the control cups and 8 egg rafts in the treatment cups.

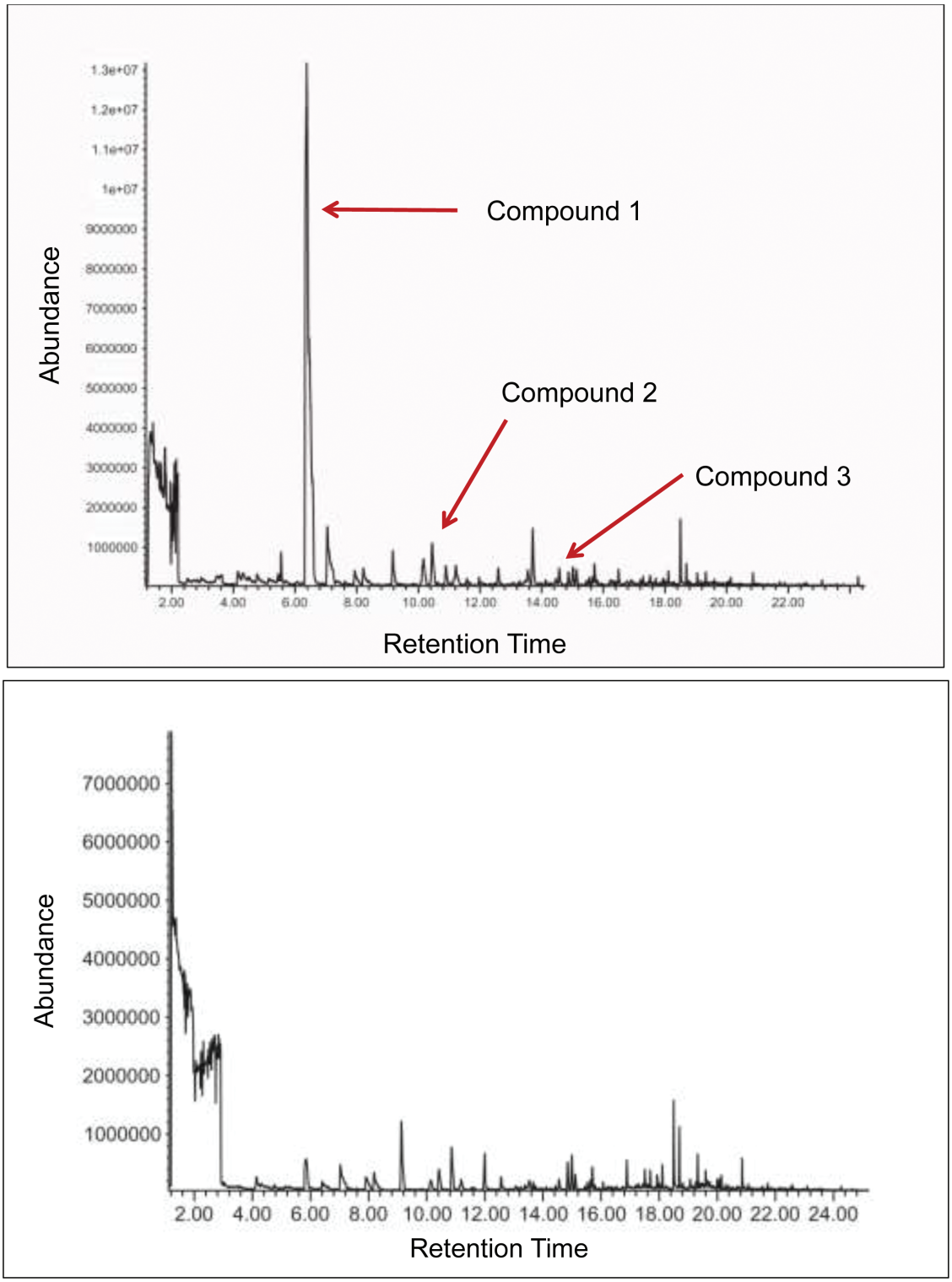



**Fig. 1.** GC/MS chromatogram showing the headspace profile collected from *Gambusia*-exudate water (upper panel) and control (aged tap water: lower panel). Three compounds of interest were present in the fish-conditioned water that were not present in the aged tap water: Compound 1: dimethyl disulfide ($C_2H_6S_2$), Compound 2: dimethyl trisulfide ($C_2H_6S_3$), Compound 3: S-methyl methanethiosulphonate ($CH_3SO_2SCH_3$).

*Culex tarsalis* was significantly deterred from ovipositing onto water that contained a 0.01% standard (6.7 nM) of DMTS in no-choice assays ($t$ = 3.3428, d.f. = 4, $P$ = 0.028). The responses of egg-laying mosquitoes to the two treatments were consistent across replicate cages ($X^2$ = 9, d.f. = 4, $P$ > 0.1). Females laid an average of 14 egg rafts on the control and 10 egg rafts on the treatment water

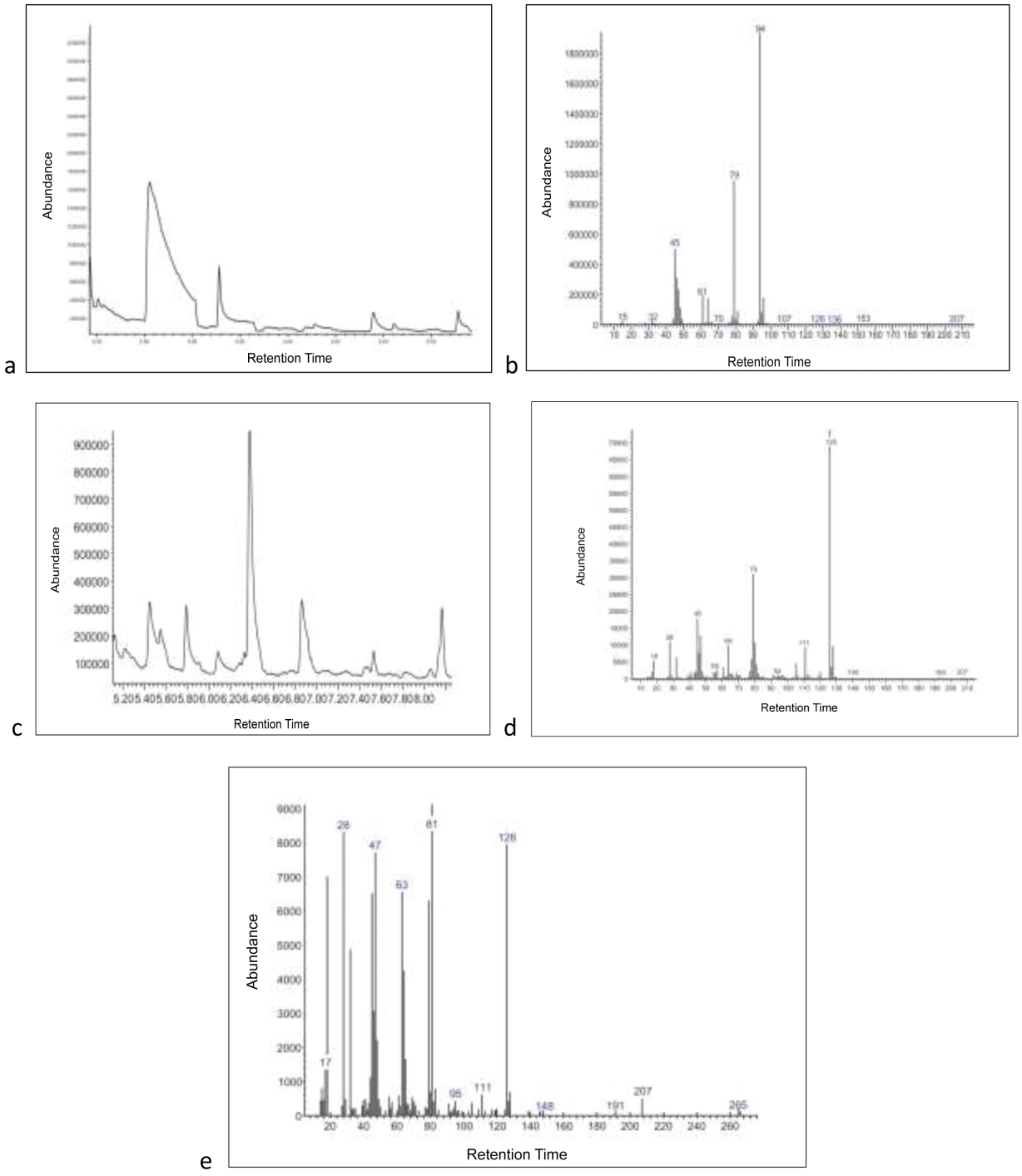
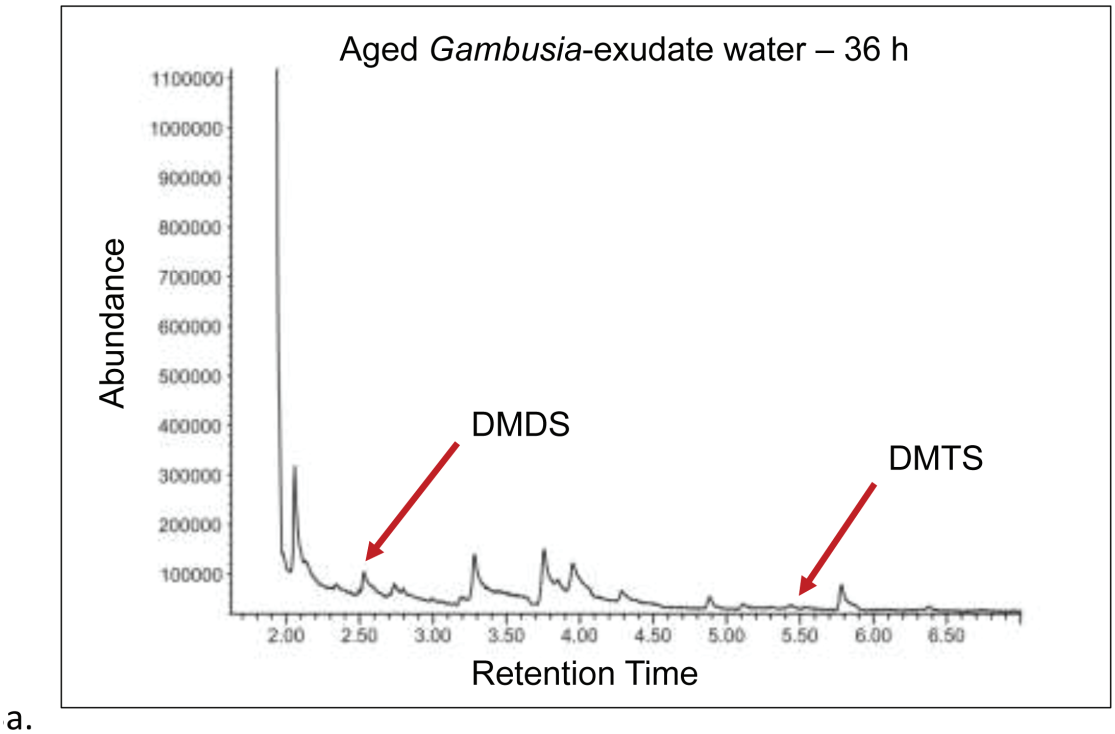



**Fig. 2.** Chromatogram (a) and mass spectrum (b) of dimethyl disulfide (DMDS) from *Gambusia*-exudate water. Chromatogram (c) and mass spectrum (d) of dimethyl trisulfide (DMTS) from *Gambusia*-exudate water and (e) mass spectrum of S-methyl methanethiosulphonate from *Gambusia*-exudate water.

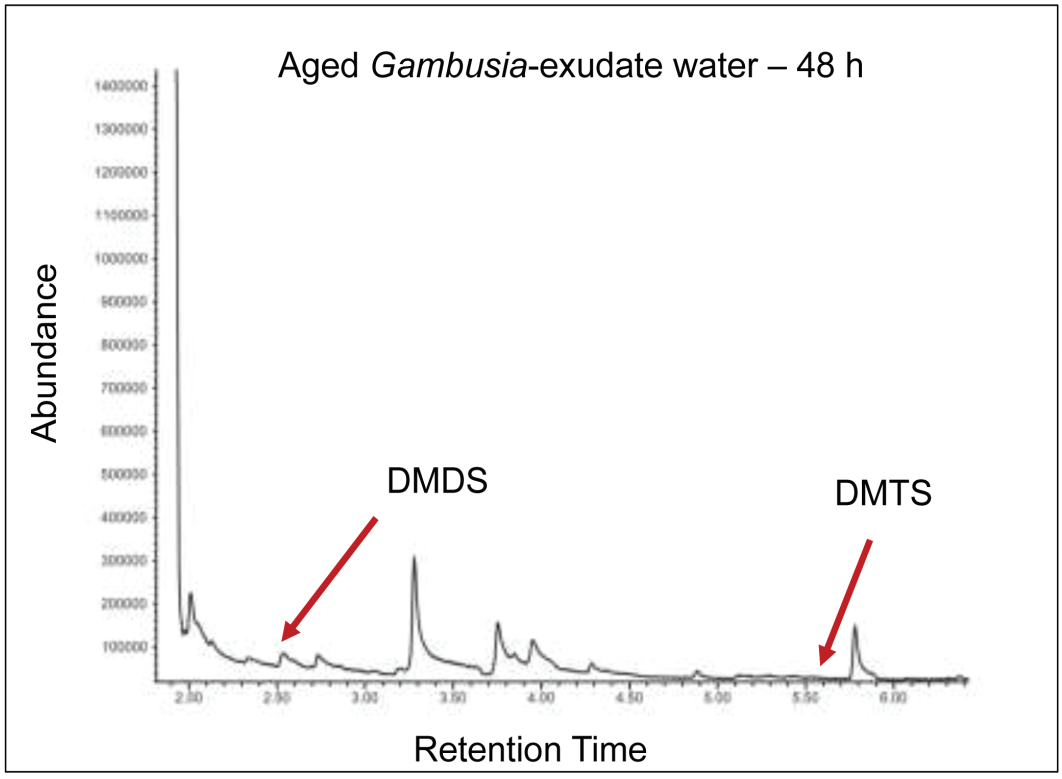
(Table 1). Female *Cx. tarsalis* were also deterred from ovipositing onto water containing 0.001% standard (0.67 nM) of DMTS when compared to aged tap water ($t$ = 2.549, d.f. = 12, $P$ = 0.025) in binary choice assays and the response of mosquitoes was consistent among the replicate cages ($X^2$ = 21, d.f. = 12, $P$ > 0.1). On average 6 egg rafts were laid on controls and 4 egg rafts on treatment water (Table 1).

## Binary Choice and No-choice Bioassays with S-methyl Methanethiosulphonate

*Culex tarsalis* was not deterred from ovipositing onto water that contained a 0.01% standard (6.7 nM) of S-methyl methanethiosulphonate when compared to aged tap water in binary choice assays ($t$ = –0.853, d.f. = 16, $P$ = 0.406). The responses of egg-laying mosquitoes to the two treatments were heterogeneous among




a.




b.

**Fig. 3.** a Chromatogram of *Gambusia*-exudate water after 36 h. Dimethyl disulfide and dimethyl trisulfide (highlighted with arrows) were still present in the headspace. The third compound, S-methyl methanethiosulphonate was not present at detectable levels. b Chromatogram of *Gambusia*-exudate water after 48 h. Dimethyl disulfide and dimethyl trisulfide (highlighted by arrows) were still present in the headspace of the *Gambusia*-exudate water. The third compound, S-methyl methanethiosulphonate was not present at detectable levels.

the replicate cages ($X^2$ = 26, d.f. = 16, $P$ < 0.01) but on average 6 egg rafts were laid in both the control and treatment cups. *Culex tarsalis* was also not deterred from ovipositing onto water that contained a 0.01% standard (6.7 nM) of S-methyl methanethiosulphonate in no-choice assays ($t$ = –0.347, d.f. = 20, $P$ = 0.732). The responses of egg-laying mosquitoes were heterogeneous among the replicate cages ($X^2$ = 31, d.f. = 20, $P$ < 0.025), where on average 12 egg rafts were laid in both the control and the treatment cups (Table 1).

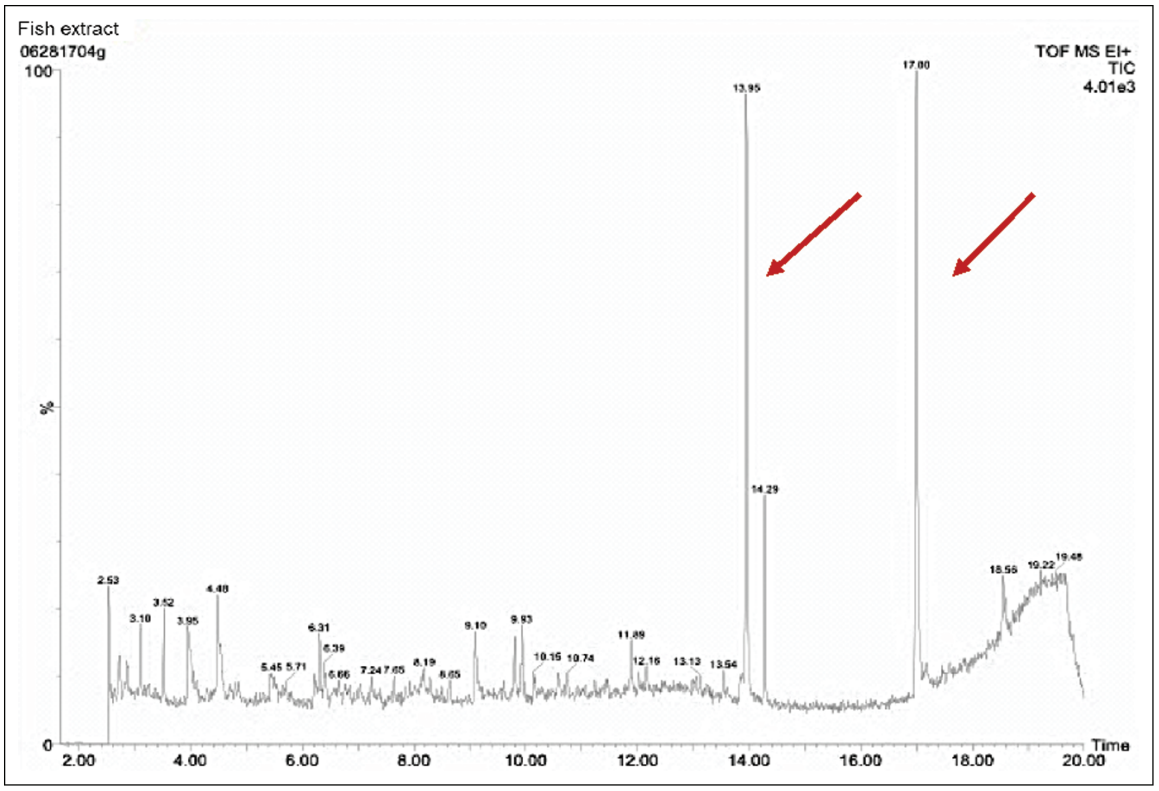



**Fig. 4.** GC/MS Time of Flight chromatogram of the *Gambusia*-exudate water.

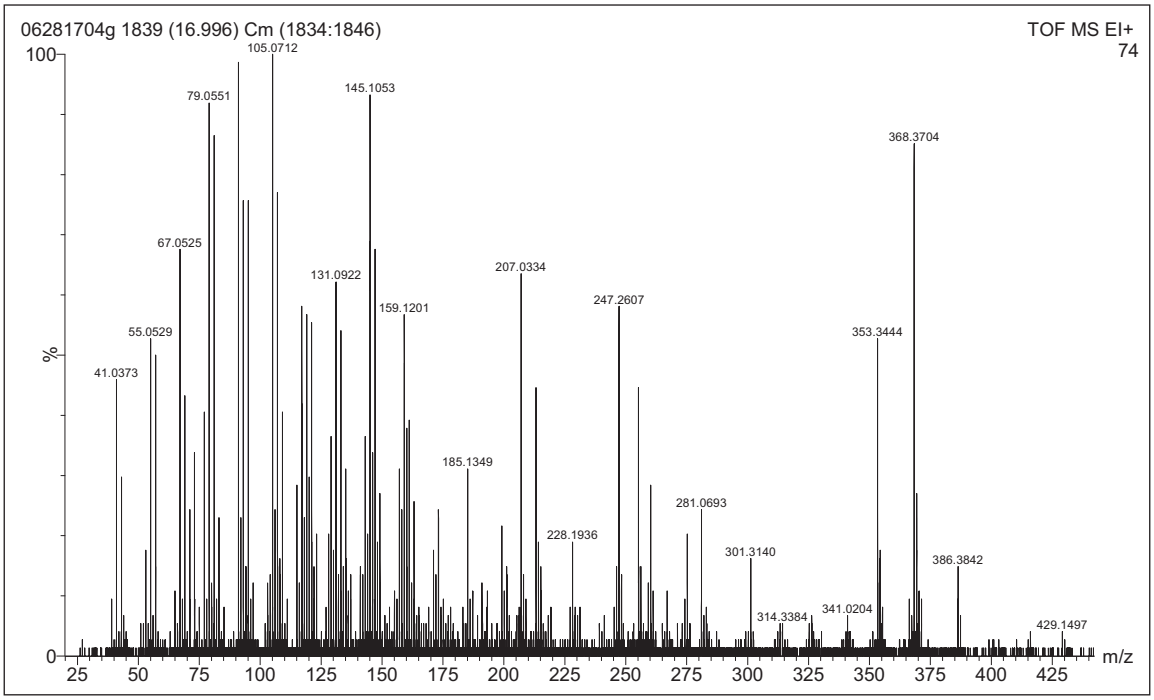



**Fig. 5.** Mass spectrum of the largest peak found in the *Gambusia*-exudate water analyzed by GC/MS-TOF.

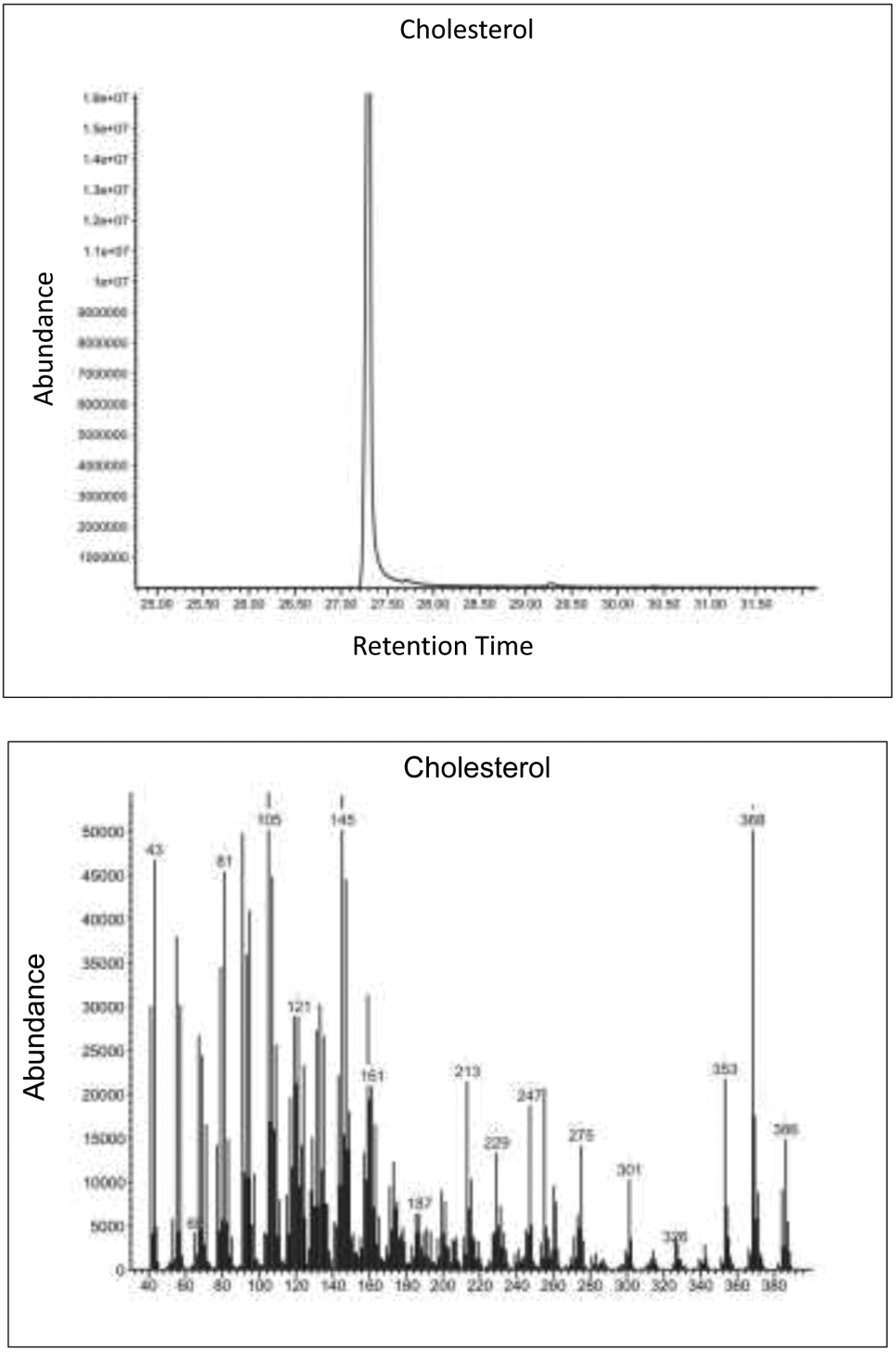



**Fig. 6.** Chromatogram (upper panel) and mass spectrum (lower panel) of cholesterol standard. The spectrum resembles that of the mass spectrum of the non-volatile compound detected in *Gambusia*-exudate water.

Female mosquitoes showed no difference in oviposition when presented with sites containing a 0.001% standard (0.67 nM) of S-methyl methanethiosulphonate and aged tap water in binary choice assays ($t$ = –0.891, d.f. = 19, $P$ = 0.384). The responses of egg-laying mosquitoes were heterogeneous among the replicate cages ($X^2$ = 30, d.f. = 19, $P$ < 0.025). On average, females laid 8 egg rafts on controls and 9 egg rafts in treatment cups (Table 1).

## Binary Choice Assays with 0.001% DMDS, DMTS, and S-methyl Methanethiosulphonate

*Culex tarsalis* females were not deterred from ovipositing onto water that contained a 0.001% standard (0.67 nM) of DMDS, DMTS, and S-methyl methanethiosulphonate when compared to aged tap water in binary choice assays ($t$ = 0.493, d.f. = 15, $P$ = 0.629). The responses of egg-laying mosquitoes were significantly heterogeneous among the replicate cages ($X^2$ = 25, d.f. = 15, $P$ < 0.025). Females

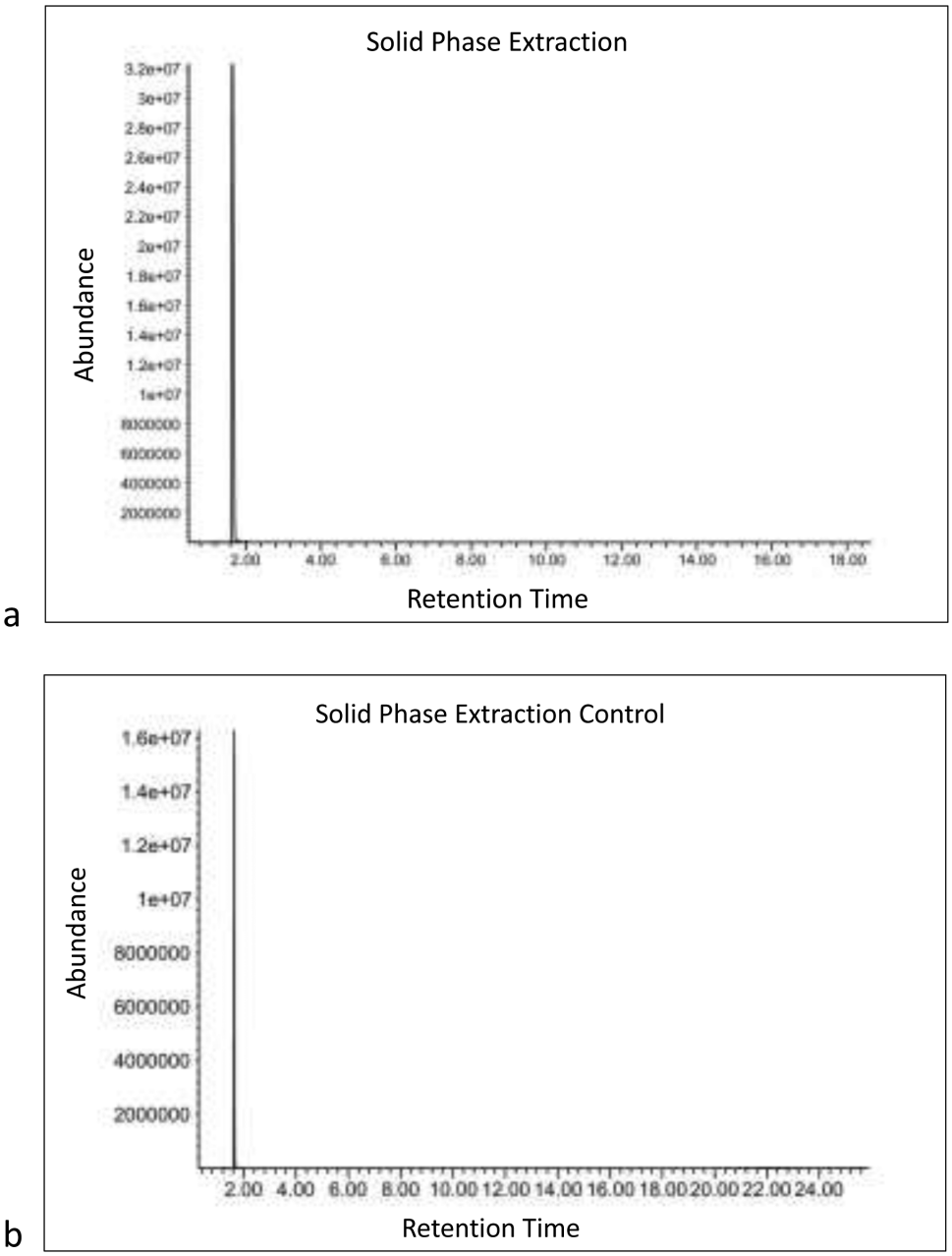



**Fig. 7.** Chromatogram of the headspace of the *Gambusia*-exudate water (upper panel) and control water (lower panel) after solid phase extraction. The three volatile compounds, dimethyl disulfide, dimethyl trisulfiide, and S-methyl methanethiosulphonate, are not present in the headspace of *Gambusia*-exudate water following solid phase extraction.

laid an average of 6 egg rafts on control water and 5 egg rafts on the treatment water (Table 1).

### Semiquantitative Comparisons of Volatile Compounds Present in the Headspace between *Gambusia*-exudate Water and Water Samples Containing Standards

Two replications of 150 ml *Gambusia*-exudate water resulted in the following integration values: 77,826,524 (trial 1) and 71,061,570 (trial 2) for DMDS; 28,819,794 (trial 1) and 12,347,169 (trial 2) for DMTS. No detectable amount of S-methyl methanethiosulphonate was seen in either sample. Two replications with aged tap water samples containing standards of DMDS, DMTS, and S-methyl methanethiosulphonate (at 0.67 nM concentration for each) showed the following integration values: 5,470,562,993 (trial 1) and 5,187,704,860 (trial 2) for DMDS; 4,549,735 (trial 1) and 7,830,116,695 (trial 2) for DMTS; 48,162,638 (trial 1) and 64,070,183 (trial 2) for S-methyl methanethiosulphonate. Based on data for DMDS and DMTS, the integration values obtained from the water samples spiked with standard compounds were about 55–74 times larger when compared to those from the *Gambusia*-exudate water samples.

It was subsequently found upon further analysis that although the standards were of analytical quality, all three standards contained significant amounts of the other two sulfur compounds. The

**Table 1.** Compiled data for all bioassays conducted

| Bioassay type: Control vs *Gambusia*-exudate water | Mean no. egg rafts laid per trial | | Females responding amongst replicate cages, % | Concentration tested (nM) | Replicates |
|---|---|---|---|---|---|
| | Control | *Gambusia*-exudate water | | | Trials (cages) |
| Binary choice: aged tap $H_2O$ vs aged *Gambusia*-conditioned $H_2O$ | 12 | 3*** | 20–70% | N/A | 2 (11) |
| No choice: aged tap $H_2O$ vs 0.1% DMDS | 16 | 17 | 33–70% | 67 | 1 (3) |
| No choice: aged tap $H_2O$ vs 0.01% DMDS | 18 | 20 | 30–93% | 6.7 | 4 (12) |
| Binary choice: aged tap $H_2O$ vs 0.001% DMDS | 5 | 5 | 20–63% | 0.67 | 3 (14)††† |
| Binary choice: aged tap $H_2O$ vs $1 \times 10^{-4}$% DMDS | 2 | 3 | 7–50% | 0.067 | 4 (14)††† |
| Binary choice: aged tap $H_2O$ vs 0.01% DMTS | 8 | 8 | 10–73% | 6.7 | 6 (20) |
| No choice: aged tap $H_2O$ vs 0.01% DMTS | 14 | 10* | 67–100% | 6.7 | 2 (5) |
| Binary choice: aged tap $H_2O$ vs 0.001% DMTS | 6 | 4* | 17–40% | 0.67 | 4 (13) |
| Binary choice: aged tap $H_2O$ vs 0.01% S-methyl methanethiosulphonate | 6 | 6 | 27–73% | 6.7 | 4 (17)†† |
| No choice: aged tap $H_2O$ vs 0.01% S-methyl methanethiosulphonate | 12 | 12 | 10–57% | 6.7 | 6 (21)† |
| Binary choice: aged tap $H_2O$ vs 0.001% S-methyl methanethiosulphonate | 8 | 9 | 17–97% | 0.67 | 3 (20)† |

**Table 1.** Continued

| Bioassay type: Control vs *Gambusia*-exudate water | Mean no. egg rafts laid per trial | | Females responding amongst replicate cages, % | Concentration tested (nM) | Replicates |
|---|---|---|---|---|---|
| | Control | *Gambusia*-exudate water | | | Trials (cages) |
| Binary choice: aged tap $H_2O$ vs 0.001% DMDS, DMTS, S-methyl methanthiosulphonate | 6 | 5 | 13–73% | 0.67 | 2 (16)† |
| Binary choice: aged tap $H_2O$ vs HLB extract | 11 | 5* | 37–77% | N/A | (9)††† |

Difference between treatments by *t*-test: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.
Goodness of fit among replicates by $\chi^2$ test: † $P \leq 0.05$, †† $P \leq 0.01$, ††† $P \leq 0.001$.

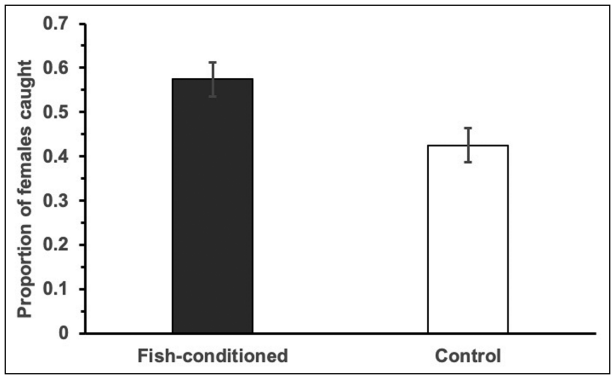



**Fig. 8.** The proportion (mean ± SE) of female *Culex tarsalis* collected overnight on sticky screens in binary choice bioassays using water conditioned with *Gambusia affinis* versus control (aged tap water). Results are for seven trials using 3–5 replicate cages per trial.

DMDS standard contained DMTS, the DMTS standard contained both DMDS and S-methyl methanethiosulphonate, and the S-methyl methanethiosulphonate standard contained both DMDS and DMTS. Due to the impurities present in the standards in relatively large quantities, any further quantitative estimation between these compounds (e.g., ratio) in the headspace was not possible. However, based on the data we were able to obtain, the samples containing the analytical standards were roughly two orders of magnitude higher than the *Gambusia* water. Kristiana et al. (2010) detail the issues associated with trying to quantify various volatile organic sulfur compounds in aqueous systems using SPME and GC/MS due to their "low concentrations, thermal instability and their susceptibility to undergo oxidation and disproportion reactions."

### Binary Choice Bioassays with Water Containing Compounds Extracted by HLB Column

*Culex tarsalis* was deterred from ovipositing onto the water in cups that contained compounds extracted from *Gambusia*-exudate water via SPE when compared to aged tap water ($t = 3.06$, d.f. = 8, $P = 0.0156$). The responses of gravid mosquitoes to the two treatments were heterogeneous among the replicate cages ($X^2 = 16$, d.f. = 8, $P < 0.001$). An average of 11 egg rafts was laid on the control water and 5 egg rafts on the treatment (Table 1).

## Discussion

Water conditioned with the western mosquitofish, *G. affinis*, contained two nonvolatile chemicals that deterred oviposition by gravid *Cx. tarsalis* mosquitoes. Three volatile compounds were identified in the headspace of the *Gambusia*-exudate water which were not present in the control samples: dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and S-methyl methanethiosulphonate.

Results from three different bioassays (binary choice, no-choice, and clipped-wing) indicated that *Cx. tarsalis* females reduced oviposition when low concentrations of DMTS were present in oviposition water, but this response was not consistent across all trials and concentrations tested. It is currently unknown why female *Cx. tarsalis* only responded to the presence of DMTS, and not to the related compounds of DMDS and S-methyl methanethiosulphonate in oviposition sites.

In sticky screen bioassays where gravid mosquitoes could respond to a blend of volatile chemicals emanating from oviposition sites but could not taste the water, water conditioned with fishes was more attractive to gravid females than was water in the control treatment. The presence of DMDS above a water body may act as a cue to a gravid female mosquito that she has found a desirable body of water in which to lay her eggs and that bacteria are present and available as a food source for her offspring. DMDS and DMTS are common bacterial metabolites (Schulz and Dickschat 2007) and as such may be ubiquitous in aquatic systems. Suleman and Shirin (1981) noted that the presence of organic matter associated with bacteria cause an aquatic environment to be more attractive to ovipositing mosquitoes. Rockett (1987) concluded that Culex *pipiens* (L.) can discriminate between different bacterial species commonly encountered in natural breeding sites. This may help explain why female *Cx. tarsalis* responded to DMTS but not DMDS in oviposition sites, as DMTS is a byproduct of DMDS and is generated from a large amount of DMDS being present (Schulz and Dickschat 2007). If the DMDS is being generated by bacteria, then a high concentration of DMTS above an oviposition site may indicate a higher bacterial load in the water column and signal to the female that the water is too polluted for her offspring. *Cx. tarsalis* usually oviposit

in water bodies that are larger and less polluted when compared with the breeding sites of *Cx. pipiens* and *Culex quinquefasciatus* Say (Diptera: Culicidae) (Clements 1999). However, when bacteria were filtered out of the *Gambusia*-exudate water, and the most likely source of these volatile cues was eliminated, this level of attractiveness diminished and there was a reduced rate of oviposition by female *Cx. tarsalis* (Why and Walton, 2020). Furthermore, after filtering the bacteria from the *Gambusia*-exudate water, DMDS, DMTS, and S-methyl methanethiosulphonate were no longer detected in the headspace of the oviposition sites tested.

Suh et al. (2016) found that DMDS and DMTS were present in the headspace above larval habitats of *An. coluzzii* (Coetzee) and at a concentration of $1 \times 10^{-7}$ % (9.419 µg/liter) DMDS, and when added to water elicited a decrease in oviposition behavior. However, no difference in oviposition between cups containing a standard of $1 \times 10^{-4}$ % (0.067 nM) DMDS in 95% EtOH and aged tap water was seen in the trials conducted with *Cx. tarsalis*. Suh et al. (2016) used an enclosed plastic chamber and much smaller diameter oviposition sites to test the responses of the females in contrast to the mesh-sided cages and larger cups used in our experiments. Moreover, gravid female mosquitoes exposed to *Gambusia*-exudate water in small, enclosed experimental chambers (plastic vials) behaved differently from females exposed in chambers with screened caps, i.e., allowing the volatile chemicals present to dissipate (Why and Walton 2020).

Additionally, the sequence of behaviors that anophelines exhibit during oviposition is different from those of *Cx. tarsalis* (Clements 1999). *Anopheles coluzzii* lay their eggs using skip oviposition behavior, where they hover over a body of water and drop their eggs singly on the edge of water bodies, while *Cx. tarsalis* must sit on the water surface for approximately 15–20 min in order to lay the full complement of eggs found in a normal-sized egg raft (Clements 1999). It may be that female *Cx. tarsalis* have different stimuli that must be met in order for oviposition behaviors to be aborted. Their entire genetic compliment is found in each egg raft and therefore they must try to ensure the survival of the majority of offspring, where *Anopheles* spread their eggs over a much wider area and if a particular oviposition site is poorly chosen, it will not cause a significant loss to her overall genetic contribution as other eggs may hatch.

The three volatile compounds identified from the headspace of the *Gambusia*-exudate water potentially stem from one parent compound, methanethiol, which is then oxidized into DMDS, DMTS, and S-methyl methanethiosulphonate. The biogenesis of these methionine-derived volatiles is often mediated by bacteria (Schulz and Dickschat 2007). DMDS and DMTS are produced by bacteria in several genera, including *Brevibacterium*, *Corynebacterium*, *Micrococcus*, *Staphylococcus*, *Arthrobacter*, *Lactococcus*, and *Lactobacillus*. These compounds are derived from two different L-methionine catabolism pathways which most likely coexist in the bacteria: direct cleavage of L-methionine or transamination to α-keto-γ-methylthiobutyric acid and reductive demethiolation (Schulz and Dickschat 2007). If a similar pathway exists involving the bacteria associated with *G. affinis*, this would explain why a greater amount of DMDS, relative to S-methyl methanethiosulfonate was captured in the headspace volatiles of the *Gambusia*-exudate water, as DMDS and DMTS are the first reaction products produced from the parent compound and are highly volatile. Further analysis needs to be completed in order to determine whether the parent compound is methionine.

It is not clear if the microbiome associated with the fish mucus, gills or the gut is the potential source of the compounds detected by *Cx. tarsalis* and whether the microbiome associated with the fish changes seasonally. Leonard et al. (2014) characterized the skin microbiome of *G. affinis* using a combination of 16S rRNA profile pyrosequencing and bacterial cultures to identify the bacteria present in the fish mucus. Over half of the bacterial sequences were classified into 5 genera: *Acinetobacter, Sphingomonas, Acidovorax*, *Enhydrobacter*, and *Aquabacterium*. If researchers can confirm the origin of the sulfur compounds from among the bacteria present, they can then focus on determining the origin of other compounds composing the true "blend" of semiochemicals leading to deterrence in ovipositing mosquito females.

Different populations of *G. affinis* may have a different microbiome associated with their mucus. Cahill (1990) suggested that stress factors including temperature fluctuations, poor water quality, overcrowding, nutritional deficiencies, parasitism, viral infections, and trauma could affect the microbiota composition of the gills and mucus. Researchers have demonstrated that seasonality (Al-Harbi and Uddin 2004) and poor water quality (Masouleh et al. 2006) are factors that influence the composition of gill microbiota. If the different cohorts of fish that were used over the course of the experiments had different microbiota fauna, this could also help to explain the differences seen in oviposition rates throughout the course of the trials.

In addition to the three volatile compounds found, two nonvolatile compounds were found in *Gambusia*-exudate water and these latter compounds were deterrent to ovipositing *Cx. tarsalis* in laboratory bioassays. Females were significantly deterred from ovipositing onto water that contained the HLB column extract of the *Gambusia*-exudate water when compared to aged tap water. The two unknown compounds were not found in the control water. GC/MS analysis of the headspace profile of the extracted water also confirmed that DMDS, DMTS, and S-methyl methanethiosulphonate were not present in the headspace after extraction. While more experimentation needs to be done in order to confirm how the mosquitoes are reacting behaviorally, i.e., deterrence or repellence, it may be that when a *Cx. tarsalis* female alights on the water surface the DMDS, DMTS, and S-methyl methanethiosulphonate no longer "mask" the presence of the less volatile compounds present in a given water body. And it may be the presence of these less volatile compounds in an oviposition site that signal the presence of a predator.

Many interorganismal interactions in the natural world are mediated by semiochemicals in a dose-dependent manner (Brönmark and Hansson 2012). It is possible that the concentrations of chemicals tested in the laboratory assays may not closely mimic "real-world" conditions, leading them to affect the oviposition behavior of female *Cx. tarsalis* differently than a natural blend of semiochemicals would have. In the current study, some of the assays were conducted with a single standard compound tested by itself, but a blend of all three compounds was tested to see if it had an effect on oviposition behavior. It has been shown that blends of chemicals are what organisms react to in nature, and especially in aquatic systems (Brönmark and Hansson 2012). Rarely does one compound elicit a response on its own without being presented in the "context" of the other chemical compounds present (Bentley and Day 1989). Considering these factors, findings from the current reports should be interpreted with caution as additional studies still need to be done.

Our studies have shown that *Cx. tarsalis* can detect the presence of semiochemicals associated with *G. affinis* in oviposition sites, leading to a change in oviposition behavior. It appears that DMDS and S-methyl methanethiosulphonate do not deter oviposition by *Cx. tarsalis* in the laboratory. DMTS deterred oviposition by *Cx. tarsalis* at the two lowest concentrations tested in binary choice assays. The presence of these sulfur-containing compounds may indicate to the female mosquito that a potential oviposition site exists with adequate food resources for her larvae, i.e., the presence of bacteria in the water. Two unknown chemical compounds were detected

using SPE and GC/MS analysis in the *Gambusia*-exudate water. The crude extract, when added to water led to a decrease in oviposition rates in binary choice assays. Full chemical characterization of the compounds present in the SPE extract needs to be conducted and their effect, if any, on mosquito oviposition behavior evaluated to determine whether they truly act as repellents or deterrents to ovipositing female *Cx. tarsalis*.

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## Dedication

This paper is dedicated to the memory of Dr. W.E.W. A.M.W. will forever be grateful for your support, wisdom, guidance and for all that you have taught me about science and entomology. Your memory will live on in those who you have guided over the years and our careers will forever be a tribute to your mentorship.

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