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Specific hydrocarbons associated with interspecific larvae shape niche segregation and competitive exclusion in *Aedes aegypti* and *Culex quinquefasciatus*

Zaid Khan[1], Betelehem Wondwosen[2], Björn Bohman[1], Rickard Ignell[1], Sharon R. Hill[1,*]

[1] Disease Vector Group, Department of Plant Protection Biology, Swedish University of Agricultural Sciences, Box 190, 234 22 Lomma, Sweden
[2] Department of Zoological Sciences, Addis Ababa University, Addis Ababa, Ethiopia
* Corresponding author: sharon.hill@slu.se

With 2 figures and 2 tables

Abstract: Interspecific competition at breeding sites regulates oviposition site choice and egg laying in mosquitoes. The aim of this study was to identify and assess the role of odour cues associated with interspecific larval presence at oviposition sites in shaping niche separation, and competitive exclusion between the sympatric species, *Aedes aegypti* (L.) and *Culex quinquefasciatus* (Say) (Insecta: Culicidae). Gravid *Ae. aegypti* preferred to oviposit at sites in response to odours associated with low densities of 4th instar *Cx. quinquefasciatus* larvae, whereas *Cx. quinquefasciatus* preferred to oviposit at sites without heterospecific odours, in both multi- and dual-choice assays. Specific biologically active hydrocarbons and ketones, associated with the heterospecific larvae, were identified through combined gas chromatography and electroantennographic detection (GC-EAD), as well as GC and mass spectrometry (MS) analyses. Oviposition site selection in response to synthetic blends of these compounds generally reflected that of the natural odour in the behavioural assays. This study demonstrates that select interspecific-related VOCs directly regulate oviposition site selection and thus indirectly regulate larval competition, while also providing functional evidence that the frequently overlooked hydrocarbons play a critical role in regulating a key behaviour of mosquitoes.

Keywords: Culicidae, oviposition, mosquito, semiochemicals, competition

1 Introduction

Olfaction plays a significant role in the assessment and selection of oviposition sites, and thus mosquito fitness, as breeding site quality is a determining factor for the growth, development, and survival of offspring (Khan et al. 2022). Gravid mosquitoes rely on volatile organic compounds (VOCs) to assess habitat quality (Suh et al. 2016), predation risk (Kiflawi et al. 2003), as well as intra- and interspecific competition (Gonzalez et al. 2016; Shteindel et al. 2024). While gravid mosquitoes detect and respond to VOCs released from water that contains, or has recently contained, heterospecific immature stages (Khan et al. 2022), the mechanism is not yet described. Such a mechanism may shape niche separation in sympatric species, such as the yellow fever mosquito, *Aedes aegypti*, and the southern house mosquito, *Culex quinquefasciatus* (Santana-Martínez et al. 2017).

Interspecific competition is a major factor determining species distribution and community structure (Juliano 2009). Among mosquitoes, interspecific competition affects the duration of larval development and survival rate, as well as adult emergence, body size, sex ratio, and fecundity (Juliano 2009). Limited space and food within the aquatic environment generate competition and predation among larvae, and the physical interaction leads to stress, which may prevent larvae of the subordinate species from successful feeding resulting in competitive exclusion over time (Costanzo et al. 2005; Santana-Martínez et al. 2017). The emission from such sites of organic compounds associated with heterospecific aquatic stages, volatile and otherwise, may be used as true signals to avoid the negative effects of interspecific interactions (Ikeshoji & Mulla 1974).

Semiochemicals involved in oviposition site selection have been identified from biotic sources in and around larval habitats, and the VOCs to which a gravid mosquito responds are species- and genera-specific (Khan et al. 2022). Most behaviourally active VOCs from mosquito breeding habitats have been identified from living or decaying vegetation,

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E. Schweizerbart'sche Verlagsbuchhandlung, 70176 Stuttgart, Germany, www.schweizerbart.de

and include mono- and sesquiterpenes, phenol- and indole derivatives, and C_{20}–C_{26} long chain hydrocarbons (Khan et al. 2022). C_4–C_{20} alcohols, C_4–C_{20} fatty acid derivatives, phenol- and indole derivatives, as well as S_2–S_4 dimethyl sulphides have been identified from microorganisms in breeding habitats (Khan et al. 2022). Terpenes, as well as C_{21} and C_{23} alkanes, are the most prevalent VOCs identified from non-mosquito resource competitors and predators at mosquito breeding sites (Khan et al. 2022). C_4–C_{20} fatty acid derivatives, as well as C_9 and C_{21} unbranched alkanes, and dimethyldisulphide and dimethyltrisulphide are associated predominantly with the aquatic stages of conspecific and heterospecific mosquitoes (Khan et al. 2022, 2023).

While hydrocarbons have been demonstrated to be of ecological relevance for insects (Blomquist & Bagnères 2010), their role as semiochemicals for mosquitoes is less well understood (Khan et al. 2022). Short-to-medium range (C_9–C_{16}) saturated or mono-unsaturated hydrocarbons are known semiochemicals involved in many aspects of chemical communication in insects (Blomquist & Bagnères 2010). Furthermore, gravid insects respond to such hydrocarbons from potential breeding sites, e.g., pentadecane acts as a larviposition pheromone in tsetse flies (Saini et al. 1996), and it was recently shown that 2,4-dimethylheptane, 4-methyloctane and 2-methylnonane together with myrcene deter oviposition by the diamondback moth (Yan et al. 2023). Mosquitoes also use hydrocarbons as semiochemicals while assessing oviposition sites (Khan et al. 2023; Wooding et al. 2020). However, most of the alkanes and alkenes identified with roles as semiochemicals are reported as either "other" or unknown branched compounds, without confirmed regiochemistry (Khan et al. 2023; Zhao et al. 2022). An exception is *n*-heneicosane (C_{21}), identified from mosquito larvae, which is important for assessing conspecific occupation of sites during oviposition site selection by *Ae. aegypti* and the Asian tiger mosquito, *Aedes albopictus* (Gonzalez et al. 2016). Hydrocarbons associated with vertebrate hosts and the epicuticle of mosquitoes have been identified as variably abundant and structurally diverse (C_9 to $>C_{40}$) hydrocarbons (Wooding et al. 2020), and thus represent a rich pool of potential semiochemicals for female mosquitoes. These compounds have been largely omitted from physiological and behavioural analyses due to the intrinsic problem of identification.

The behavioural response of gravid mosquitoes to heterospecific aquatic stages, and associated odours, is dynamic and dependent on the aquatic stage and their density, as well as taxon- and species-specific interactions (Khan et al. 2022; Shteindel et al. 2024). For example, in semi-field conditions, *Ae. albopictus* are attracted to, and prefer to oviposit in, breeding sites in which there is a low density of *Ae. aegypti* larvae (Shragai et al. 2019). While the olfactory cues are implicated in shaping niche displacement and competition between heterospecific aquatic stages (Gonzalez et al. 2016), the nature of the odour cues remains unidentified. The purpose of this study was to investigate odour-mediated oviposition site selection as a mechanism shaping niche separation and competitive exclusion between *Ae. aegypti* and *Cx. quinquefasciatus*. For this purpose, dose-dependent behavioural assays were conducted using water conditioned with heterospecific larvae. Through combined gas chromatography and electroantennographic detection (GC-EAD), as well as GC-mass spectrometry (MS), blends of bioactive VOCs were identified and subsequently evaluated behaviourally. Findings from this study describe the density-dependent ecological mechanism regulating the interaction between egg-laying *Ae. aegypti* and *Cx. quinquefasciatus* with heterospecific aquatic stages, demonstrating the importance of hydrocarbons in driving this behaviour.

2 Materials and methods

2.1 Mosquito rearing

Aedes aegypti (Rockefeller) and *Culex quinquefasciatus* (Johannesburg), common laboratory strains, were reared according to standard protocols (Khan et al. 2023). Briefly, larvae were reared in distilled water (1 l) contained in plastic trays (3 l; L: 24.5 × W: 18.5 × H: 7.5 cm; Emballator Lagan AB, Ljungby, Sweden) at 27 ± 1 °C, 65 ± 5% relative humidity, 12: 12 h light: dark cycle. Larvae were fed with Tetramin® fish food (Tetra GmbH, Melle, Germany). Pupae were collected in clear plastic cups (30 ml; Essentra components, Malmö, Sweden), and then placed in a Bugdorm-1 cage (L: 30 × W: 30 × H: 30 cm; Mega View Science, Taichung, Taiwan) for adult emergence. Thereafter, adults were provided with access to 10% sucrose solution *ad libitum*. For experiments, adult mosquitoes (5–7 days post-emergence), which are sufficiently mature to be capable of blood feeding during the first gonotrophic cycle, were provided defibrinated sheep's blood (Håtunalab, Bro, Sweden) using a Hemotek membrane feeding system (Hemotek Ltd, Blackburn, UK) heated to 37 °C, an approximation of host body temperature, for 2 h. In oviposition experiments, fully engorged *Ae. aegypti* and *Cx. quinquefasciatus* were used 5 and 7 days after blood feeding, respectively, to increase the probability of gravid mosquitoes developing and laying the majority of their eggs within the duration of the experiments.

2.2 Conditioning water with 4th instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus*

To obtain the 4th instar larval-conditioned water (LCW), 1st instar larvae were transferred to rearing trays containing different larval densities, and reared to 4th instar without changing the water. Larvae densities were chosen based on Khan et al. (2023). Each larva was fed Tetramin fish food (ca. 10 mg) during development: 0.6 mg of food larva^{-1} d^{-1} was given to 1st to early 3rd instar larvae and 2 mg of food larva^{-1} d^{-1} to late 3rd and 4th instar larvae. Distilled water, serving as the control, was treated with equivalent amount

of food, under the same rearing conditions and over the same timeframe as above. Food particles were strained out of the water through nylon mesh and removed daily, prior to adding new food. The amount of food added to the controls daily was equivalent to the amount added to the highest density of larvae in the treatment, in order to match the experimental conditions as closely as possible, and focus on the effect of the presence of the larvae on the water and associated VOCs. The larvae, along with any remnants of previous moults and excess food, were removed from the conditioned water using a nylon mesh net and a folded filter paper (18.5 cm diameter; Whatman Int ltd, Maidstone, England). The LCW and control water were used immediately in subsequent assays.

2.3 Multi-choice oviposition assay

The effect of VOCs emitted from LCW on the egg-laying choice of gravid *Ae. aegypti* and *Cx. quinquefasciatus* was assessed using a multi-choice oviposition assay (Khan et al. 2023; Additional file 1, Fig. S1A). The artificial oviposition site containers were randomly distributed among five artificial oviposition sites in Bugdorm-1 cages (Mega View Science) placed in each corner, 8–10 cm away from the cage wall, and one in the centre of the cage (Additional file 1, Fig. S1A). *Aedes spp.* and *Culex spp.* use different oviposition strategies to adapt to less than favourable conditions within egg-laying habitats, i.e., skip oviposition to spread the risk among sites (Harrington & Edman 2001), and egg retention to delay oviposition until habitat conditions improve (Bentley & Day 1989), respectively. A pilot study was conducted to determine the time within which ≥75% of eggs or egg rafts were laid and to assess the effect of access to sugar prior to and during egg laying. *Aedes aegypti* and *Cx. quinquefasciatus* surpassed 75% of eggs laid by 18–20 h and 44–46 h, respectively, after provided access to oviposition sites with LCW, which reflects the timeline of egg deposition found by other researchers (Joint, FAO/IAEA 2018; Harrington & Edman 2001; Nayak et al. 2024). Moreover, when provided with energy resources (i.e., 10% sucrose), *Cx. quinquefasciatus* retained eggs for ≥4 d after the LCW oviposition sites were introduced, whereas deprivation of sugar resulted in high levels of mortality in gravid *Ae. aegypti*. Therefore, single *Ae. aegypti* and *Cx. quinquefasciatus* were released in Bugdorm-1 cages 2 h before scotophase, and thereafter allowed to select oviposition sites for 19 ± 1 h and 45 ± 1 h, respectively. While *Ae. aegypti* were provided access to 10% sucrose solution throughout the duration of the assay, *Cx. quinquefasciatus* were denied sugar 24 h prior to and during the bioassay to avoid delayed egg-laying. The mosquito egg-laying response (number of eggs or rafts per female in each artificial oviposition site) was recorded following the completion of the experiment. For *Ae. aegypti,* the oviposition assay was repeated three to four times, with each repetition containing at least 30 replicates, whereas for *Cx. quinquefasciatus*, the egg-laying assay was performed five times, with each replicate comprising 25 mosquitoes, since *Cx. quinquefasciatus* retained eggs, and thus fewer females laid eggs than in *Ae. aegypti*.

2.4 Solid phase extraction (SPE)

VOCs in LCW (150 ml) were trapped on a Chromafix C18 cartridge (VWR, Stockholm, Sweden), which was then dried by passing nitrogen gas over the column for 2 min. Subsequently, the trapped VOCs were eluted with dichloromethane (0.5 ml; 99.9%; Merck, Stockholm, Sweden). Subsamples of each of three to five replicates were pooled and then concentrated 10 times, to ca. 0.2 ml, for subsequent electrophysiological and behavioural assays, or stored at −80 °C for future analysis.

The oviposition response of *Ae. aegypti* and *Cx. quinquefasciatus* was assessed in a dual-choice assay comparing water conditioned with interspecific SPE extracts (10 µl in 20 ml water) with water containing dichloromethane (10 µl in 20 ml water) as a control. Single gravid *Ae. aegypti* were allowed 19 ± 1 h and *Cx. quinquefasciatus* 45 ± 1 h to choose between potential egg-laying sites in Bugdorm-1 cages, the rationale for which can be found above. The number of eggs or egg rafts deposited in each of the two artificial oviposition sites was counted.

2.5 Combined gas chromatography and electroantennographic detection analysis

SPE extracts were collected from five larvae-LCW from both species, while extracts were also collected from 20 *Cx. quinquefasciatus* larvae, using 3–5 replicates. Extracts from the same density treatments were pooled, and then antennal responses of gravid *Ae. aegypti* (5 d post-blood meal) and *Cx. quinquefasciatus* (7 d post-blood meal) recorded using GC-EAD analysis.

The GC (6890, Agilent Technologies, Santa Clara, USA) was fitted with a fused silica capillary J&W HP-5 column (30 m length × 0.25 mm i.d. × 0.25 µm film thickness (Agilent Technologies). Hydrogen was used as a carrier gas at a linear flow rate of 45 cm s^{-1}. Each sample (2 µl) was injected into the GC in splitless mode (30 s, inlet temperature 225 °C). The GC oven temperature was programmed from 40 °C (hold for 1 min) to 275 °C (10 min hold), at an incremental rate of 8 °C min^{-1}. At the GC effluent splitter, nitrogen was added and the GC effluent flow split 1:1 in a 3D/2 low dead volume four-way-across splitter (Gerstel, Mülheim, Germany) between the flame ionization detector (FID) and EAD (Ockenfels Syntech GmbH, Buchenbach, Germany). The GC effluent capillary passed towards the EAD through a Gerstel ODP-2 transfer line (Gerstel) with its temperature synchronized to that of the GC oven before it was directed into a glass tube (10 cm × 8 mm), where it was mixed with charcoal-filtered humidified air (1.5 l min^{-1}) and then passed over the antennal preparation, placed ca. 0.5 cm from the outlet of the tube.

The preparation for the EAD analysis consisted of the excised head of a cold-anesthetized female mosquito with the distal antennal flagellomere severed. Both electrodes were made from pulled glass microcapillaries filled with Beadle-Ephrussi Ringer solution. The reference electrode was inserted into the head capsule through the foramen, while the recording electrode was placed over the cut tip of the antenna. The recording electrode was connected to a pre-amplifier (10×), a high impedance DC amplifier interface box (IDAC-2; Ockenfels Syntech GmbH) and to a computer for visualization, analysis and storage of data. Three stable recordings from each species were used to detect the physiologically active VOCs in each heterospecific LCW extract. The GC-EAD data were analysed using the software GC-EAD 2011 (v.1.2.3, Ockenfels Syntech GmbH).

2.6 Chemical analysis

The bioactive compounds from the pooled extracts (2 µl) of the heterospecific LCW and controls were analysed by GC-MS. Extracts (2 µl) of the heterospecific LCW and controls were injected into a GC-MS (6890–5975, Agilent Technologies, EI, 70 eV) fitted with an HP-5MS column (60 m length × 0.25 mm i.d. × 0.25 µm film thickness) and the oven temperature program was the same for the GC-MS and GC-EAD analyses. Helium was used as the carrier gas at a constant flow rate of 34 cm s^{-1}. VOCs, detected using GC-EAD analysis, were identified based on retention data and mass spectra, in comparison with the NIST-17 library, and confirmed with authentic standards, with the exception of 2,6-dimethylnonane and 3-octadecene (unknown *E*/*Z*-isomer), which were only tentatively identified by comparisons of retention indices and mass spectra, with those from reference compounds in the literature (NIST-17; Shlyakhov et al. 1975; Soják 2004). Each standard was verified through GC-EAD analysis (Tables 1–2). The relative abundance of each bioactive VOC in the pooled extracts was estimated based on each respective peak area in the total ion chromatogram. The synthetic blends were prepared based on the estimated ratios of the bioactive VOCs. The purity of each synthetic compound was confirmed to be ≥ 97% by GC-MS.

2.7 Dual-choice oviposition bioassay

The dose-dependent behavioural effect of the synthetic odour blends of the 4th instar heterospecific LCW was tested in a dual-choice assay (Khan et al. 2023; Additional file 1, Fig. S2). Several doses were tested for each of the three synthetic blends (of *Cx. quinquefasciatus* five-LCW blend: 10^{-4}, $10^{-3.5}$, 10^{-3}, $10^{-2.5}$, 10^{-2}; *Cx. quinquefasciatus* 20-LCW-blend: 10^{-3}, $10^{-2.5}$, 10^{-2}, $10^{-1.5}$, 10^{-1}; and *Ae. aegypti* five-LCW blend: 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}). Each dose of the synthetic blend (6 ml) was tested against the solvent control (6 ml), which both were delivered through wick dispensers (Karlsson et al. 2017; Additional file 1, Fig. S2). Each wick dispenser was placed in individual 250 ml glass wash bottles (VWR).

Table 1. Bioactive VOCs in *Culex quinquefasciatus* 4th instar conditioned water detected by *Aedes aegypti* using GC-EAD and GC-MS. [1]The estimated relative abundance (%) is calculated based on the peak area of each compound divided by the total peak area of compounds in the pooled extract of water conditioned with 20 larvae.

Retention time	Retention index	Compound	Relative abundance[1]
6.97	818	2,4-Dimethylheptane	0.24
7.75	861	4-Methyloctane	0.19
10.90	1024	2,6-Dimethylnonane (tent. id.)	0.18
11.70	1063	4-Methyldecane	0.60
12.48	1101	Undecane	0.26
13.70	1166	2-Methylundecane	0.30
14.66	1217	2,6-Dimethylundecane	0.97
15.77	1282	3-Methyldodecane	0.26
17.89	1402	Tetradecane	1.53
20.86	1594	1-Hexadecene	0.92

Table 2. Bioactive VOCs in *Aedes aegypti* 4th instar conditioned water detected by *Culex quinquefasciatus* using GC-EAD and GC-MS. *Likely a contaminant. [1]The estimated relative abundance (%) is calculated based on the peak area of each compound divided by the total peak area of compounds in the pooled extract of water conditioned with five larvae.

Retention time	Retention index	Compounds	Relative abundance[1]
13.92	1174	Decan-5-one	0.10
14.28	1192	Decan-2-one	0.22
23.55	1786	3-Octadecene (tent. id.)	0.53
25.57	1943	7,9-Ditert-butyl-1-oxaspiro [4.5]deca-6,9-diene-2,8-dione*	1.87

Charcoal-filtered air was passed through the glass bottles via Teflon tubing (6 mm o.d.) using an air pump (Model V-20, Hailea, China), into two 12-channel flow meters (Kytola Instruments, Muurame, Finland), at a flow rate of 0.1 l min^{-1}. Teflon tubing connected the flow meters to the artificial oviposition sites (Additional file 1, Fig. S2; Mosquera et al. 2023a).

Individual post-blood fed *Ae. aegypti* and *Cx. quinquefasciatus* were treated and introduced into the bioassay as described above. Subsequently, the number of eggs or egg rafts were counted, and oviposition choice indices calculated: C / (C + T) and T / (T + C), in which C is the number of eggs/rafts laid in the control and T is the number of eggs/rafts laid in the treatment. No significant positional bias was observed in the assay. Females not laying eggs were deemed to have retained eggs, and their proportion was calculated as those retaining eggs/total number of blood fed females.

2.8 Statistical analysis

A Shapiro-Wilk test demonstrated that the datasets fulfilled the assumption of normality (JMP Pro v. 16, SAS Institute Inc., Cary, NC, 1989–2021). During the dual- and multi-choice assays, the oviposition response of *Ae. aegypti*, which lays eggs individually and displays skip oviposition (i.e., lays eggs from the same batch in multiple sites), was analysed using a Student t-test and an ANOVA followed by Tukey's post hoc test, respectively, to account for the quantitative nature of the data (a set of positive, continuous whole numbers). In contrast, the oviposition response of *Cx. quinquefasciatus*, which lays egg rafts and does not demonstrate skip oviposition, was tested by a multinomial general regression using maximum likelihood in the multi-choice test, and a binomial regression in the dual-choice assay (JMP Pro v. 16), to account for the presence/absence nature of the data collected. Dual-choice oviposition assays, comparing the odour extracts against the solvent control, were analysed by Student t-test and binomial logistic regression analysis for gravid *Ae. aegypti* and *Cx. quinquefasciatus*, respectively, whereas the behavioural response to the synthetic odour blend was analysed using binary logistic regression followed by an odds ratio comparison (JMP Pro v. 16). The oviposition response in the bioassay was the dependent variable, and dose the independent fixed parameter.

3 Results

3.1 Larval conditioned water affects oviposition of heterospecific mosquitoes

Multi- and dual-choice assays were used to determine the influence of LCW of either *Cx. quinquefasciatus* or *Ae. aegypti* on the oviposition response of their heterospecific gravid counterparts (Fig. 1A–L). In multi-choice assays, individual gravid *Ae. aegypti* demonstrated skip oviposition, i.e., they distributed eggs in multiple oviposition containers during one gonotrophic cycle, ovipositing more in LCW with lower densities of *Cx. quinquefasciatus* larvae compared to higher larval densities and the controls (Fig. 1A, $F_{4,465} = 4.97$, $P = 0.0006$; Fig. 1B, $F_{4,630} = 5.94$, $P = 0.0001$; Additional file 1, Table S1AB). In contrast, gravid *Cx. quinquefasciatus* generally preferred to oviposit in the control rather than *Ae. aegypti* LCW ($\chi^2 = 7.10$, $df = 4$, $P = 0.13$, Fig. 1C; Additional file 1, Table S1C). These results were confirmed in dual-choice assays with LCW (Fig. 1E, *Ae. aegypti* ovipositing on *Cx. quinquefasciatus*: $t = 4.28$, $P < 0.0001$; Fig. 1F, $t = 3.51$, $P = 0.0006$; Fig. 1G, *Cx. quinquefasciatus* ovipositing on *Ae. aegypti*: $\chi^2 = 12.80$, $P = 0.0003$; Additional file 1, Table S1EFG). Moreover, the corresponding SPE extracts elicited behavioural activity in both species (*Ae. aegypti* ovipositing on *Cx. quinquefasciatus*: Fig. 1I, $t = 4.04$, $P < 0.0001$; Fig. 1J, $t = 3.96$, $P < 0.0001$; Fig. 1K, *Cx. quinquefasciatus* ovipositing on *Ae. aegypti*: $\chi^2 = 9.33$, $P = 0.0022$; Additional file 1, Table S1). The choice to not lay eggs was observed in 13–20% of *Ae. aegypti* females (data not shown) and 30–40% of *Cx. quinquefasciatus* females (Fig. 1D, H, L) in response to heterospecific LCW and the solvent controls (Additional file 1, Fig. S3B, Fig. S4B).

3.2 Bioactive compounds identified in larval conditioned water

Combined GC-EAD and GC-MS analyses were used to identify physiologically active compounds in LCW. The GC-EAD analyses demonstrated antennal responses of gravid *Ae. aegypti* to the SPE extract of *Cx. quinquefasciatus* larvae with five and ten bioactive VOCs identified from the LCW extracts conditioned with 5 and 20 larvae, respectively (Fig. 1MN). *Aedes aegypti* responded specifically to a total of ten hydrocarbons, including C_9–C_{14} alkanes and 1-hexadecene, with five VOCs being below the detection level of the mosquito antenna in the lower density LCW. In addition to the electrophysiologically active hydrocarbons, more than 20 C_9–C_{21}-alkanes were found in *Cx. quinquefasciatus* LCW (data not shown) at relative levels of abundance higher than that of undecane, the least abundant of the electrophysiologically active hydrocarbons (Table 1). Apart from hydrocarbons, *Culex quinquefasciatus* responded to 2-decanone, 5-decanone and 3-octadecene (the latter tentatively identified).

While all compounds used in bioassays were confirmed using GC-MS and GC-EAD analyses with authentic standards, the possibility of other naturally occurring regioisomers of some branched alkanes and alkenes cannot be ruled out due to their very similar GC-MS properties. Of the seven bioactive VOCs present in the *Ae. aegypti* LCW extract, three were also found in the extracts from the water controls.

3.3 Synthetic odour blends reflect the oviposition response of gravid mosquitoes

To determine the behavioural response of gravid mosquitoes to synthetic odour blends, based on the heterospecific LCW VOCs, dual-choice assays were conducted (Additional file 1, Fig. S2). Synthetic blends were constructed reflecting the identified ratio of VOCs in water conditioned with five *Cx. quinquefasciatus* larvae (2,4-dimethylheptane, 4-methyloctane, 4-methyldecane, undecane, 2:1:4:2) and 20 *Cx. quinquefasciatus* larvae (2,4-dimethylheptane, 4-methyloctane, 4-methyldecane, undecane, 2-methylundecane, 2,6-dimethylundecane, 4-methyldodecane, tetradecane, 1-hexadecene, 2:1:3:1:3:3:2:2:5). 2,6-Dimethylnonane, which was not commercially available, was not included. Similarly, a synthetic blend reflecting the estimated ratio of VOCs in water conditioned with five *Ae. aegypti* larvae (5-decanone, 2-decanone, 7,9-di-tert-butyl-1-oxaspiro(4.5)deca-6,9-diene-2,8-dione, 1:2:19) was prepared. 3-Octadecene was not commercially available, and thus not included. While 7,9-di-tert-butyl-1-oxaspiro(4.5)deca-6,9-diene-2,8-dione is a known plasti-

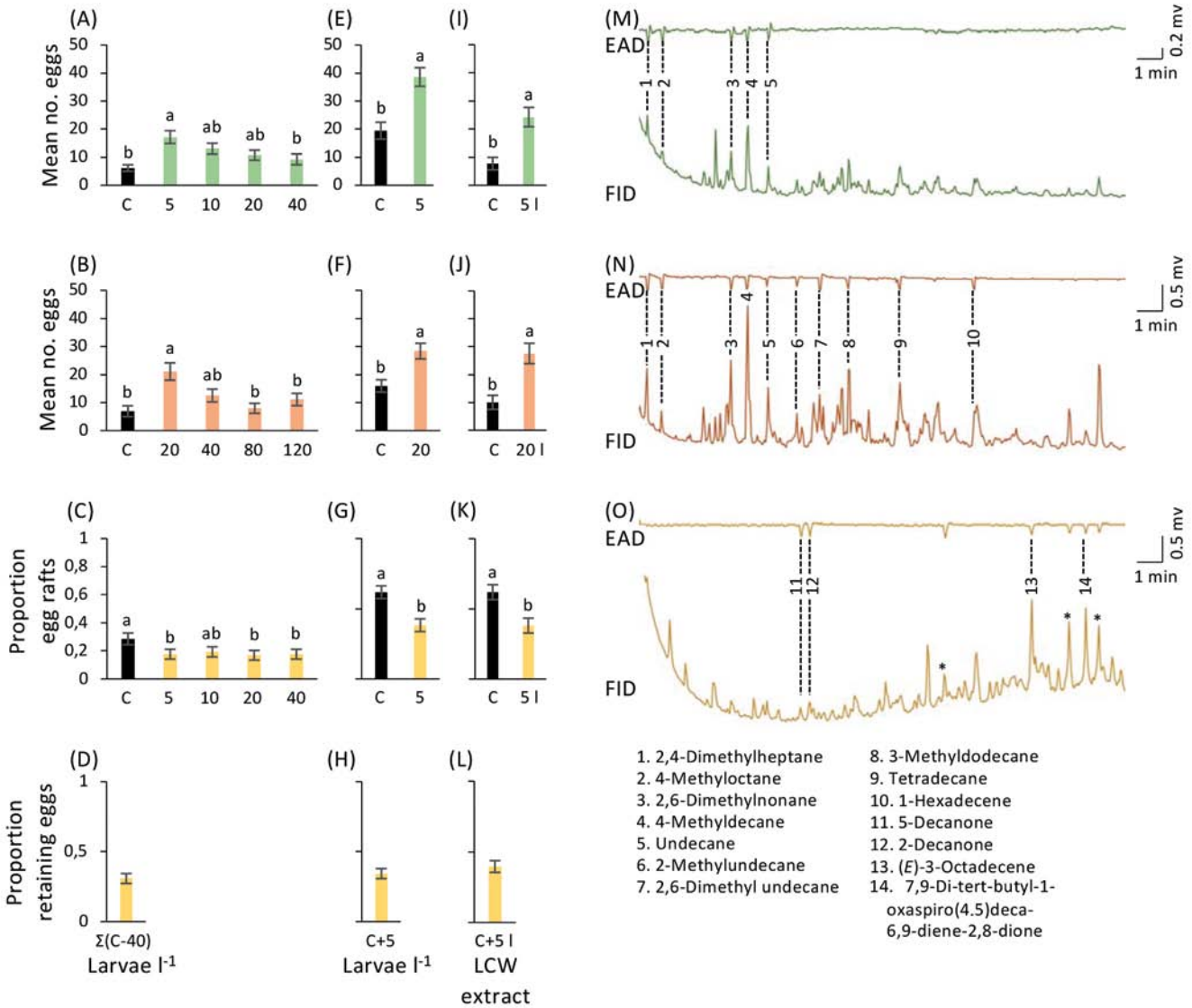

Fig. 1. Oviposition site selection by *Aedes aegypti* (green, orange), and *Cx. quinquefasciatus* (yellow) in multi- (A–D) and dual-choice (E–L) assays in response to water conditioned with various densities (larvae l^{-1}) of heterospecific 4th instar larvae (A–H) and solid phase extracts of the larval-conditioned water (LCW) (I–L). Green and yellow: low density LCW and extract-conditioned water. Orange: high density LCW and extract-conditioned water. Black: concomitant controls. Different letters indicate significant differences. A significant proportion of *Cx. quinquefasciatus* females retained eggs in response to the LCW and the extract for the duration of the experiment (D, H, L). Error bars represent the standard error of the mean (*Ae. aegypti*) or proportion (*Cx. quinquefasciatus*) (A–L). Combined gas chromatography and electroantennographic detection (EAD) analyses (M–O) demonstrate antennal responses of *Ae. aegypti* (M, N) and *Cx. quinquefasciatus* (O) to the bioactive compounds from LCW extracts of 5 larvae l^{-1} (green and yellow) or 20 larvae l^{-1} (orange), as recorded by the flame ionization detector (FID). Asterisks in the FID trace (O) represent bioactive compounds that were also present in the control extracts.

ciser (Félix et al. 2012), this compound was only found in trace amounts in a single control, and was thus still included in the bioassays.

Gravid *Ae. aegypti* and *Cx. quinquefasciatus* oviposited in response to the synthetic blends in a dose-dependent manner, consistent with the behavioural response to the heterospecific LCW (Fig. 2A, 5 *Cx. quinquefasciatus* larvae synthetic blend: $\chi^2 = 10.76$, $df = 4$, $P = 0.029$; Fig. 2B, 20 *Cx. quinquefasciatus* larvae synthetic blend: $\chi^2 = 6.74$, $df = 4$, $P = 0.15$; Fig. 2C, 5 *Ae. aegypti* larvae synthetic blend: $\chi^2 = 6.50$, $df = 3$, $P = 0.090$, with post hoc analysis revealing dose-dependence). Gravid *Ae. aegypti* were stimulated to lay eggs in response to the 20 larvae synthetic blend ($F = 3.57$, $P = 0.0082$, Fig. 2E). While gravid *Ae. aegypti* did not retain eggs in response to either *Cx. quinquefasciatus* larvae synthetic blend (5, $\chi^2 = 0.040$, $df = 4$, $P = 0.99$; 20, $\chi^2 = 0.22$, $df = 4$, $P = 0.99$), gravid *Cx. quinquefasciatus* retained eggs in a dose-dependent manner in response to the synthetic

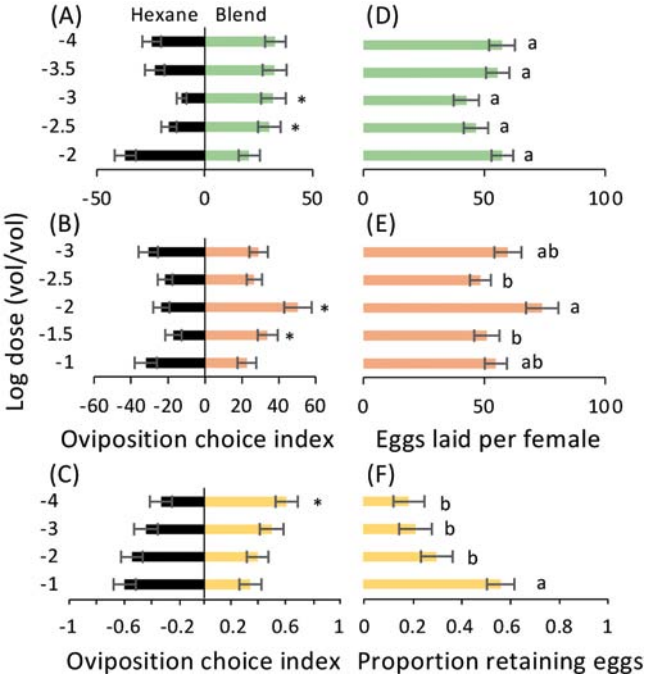

Fig. 2. Synthetic odour blends of 4th stage-conditioned water affect oviposition site-selection and egg-laying in *Aedes aegypti* and *Culex quinquefasciatus*. The dose-dependent oviposition site-choice by gravid mosquitoes to individual heterospecific synthetic odour blends identified as associated with water conditioned with (A) five *Cx. quinquefasciatus* 4th instar larvae, (B) 20 *Cx. quinquefasciatus* 4th instar larvae, and (C) five *Ae. aegypti* 4th instar larvae in comparison with a solvent control (hexane). *: $P < 0.05$ (binomial/nominal logistic general regression analysis followed by an odds ratio comparison). Gravid *Ae. aegypti* and *Cx. quinquefasciatus* were differentially attracted to lay eggs in response to these treatments (D–F). Different letters indicate significant differences (ANOVA followed by posthoc test). Error bars represent the standard error of the mean (A, B, D, E) and standard error of proportion (C, F). The sample size is >30 for all comparisons.

blend of 5 *Ae. aegypti* larvae ($\chi^2 = 21.33$, $df = 3$, $P < 0.0001$, Fig. 2F). The proportion of females retaining eggs in response to the lower doses of the synthetic blend was similar to those found in the control experiment (Additional file 1, Fig S3B).

4 Discussion

Cues associated with heterospecific aquatic stages regulate oviposition site choice and egg laying by mosquitoes in a density- and species-specific manner (this study; Khan et al. 2022). In this study, gravid *Ae. aegypti* and *Cx. quinquefasciatus* selected oviposition sites differentially in a density-dependent manner when encountering odour cues emanating from heterospecific LCW. Qualitative and quantitative differences in the bioactive VOC blends, especially with respect to hydrocarbons, regulate oviposition site selection, egg-laying and the decision to retain eggs species-dependently. These results indicate that odour cues are sufficient to shape niche separation and competitive exclusion in breeding sites shared by sympatric mosquito species.

To optimise fitness, gravid mosquitoes are required to assess the presence of intra- and inter-specific competition at breeding sites (Khan et al. 2022). *Aedes aegypti* lay more eggs in sites with low densities of *Cx. quinquefasciatus* larvae, indicating that *Ae. aegypti* actively assess the relative level of interspecific competition on the basis of VOCs. The mechanism by which this is regulated could be due to a density-dependent change in the relative abundance of VOCs, as observed in this study, or a combination of quantitative and qualitative changes in blend composition, resulting in aversion at higher larval densities (Suh et al. 2016). This would indicate that *Ae. aegypti* offspring benefit from co-habitation with low densities of *Cx. quinquefasciatus* larvae, as has been observed in both culicines and anophelines (Shragai et al. 2019; Wachira et al. 2010). One reason for this may be that breeding sites containing mosquito larvae are associated with microbes, comprising a significant food resource for larvae (Mosquera et al. 2023b) with the capacity to compete for these food resources within the limited degrees of interspecific pressure. While *Ae. aegypti* and *Cx. quinquefasciatus* use and share breeding sites (Santana-Martínez et al. 2017), each species uses the resources within the habitat differentially (Leisnham et al. 2014), with VOCs associated with low interspecific larval competition shaping resource partitioning, or niche separation, in *Ae. aegypti*. In contrast, *Cx. quinquefasciatus* avoids VOCs from *Ae. aegypti* larvae, which may lead to competitive exclusion (this study; Santana-Martínez et al. 2017). These findings emphasise that mosquitoes are under natural selection to respond to distinct VOC profiles (odours) to decrease competition among the offspring, in line with the "mother knows best" theory for oviposition site selection, originally posited for herbivorous insects (García-Robledo & Horvitz 2012), and here extended to mosquitoes.

Gravid *Cx. quinquefasciatus* retained eggs dose-dependently in response to the synthetic *Ae. aegypti* LCW blend suggesting that these females are under strong selection pressure to avoid VOCs associated with heterospecific larvae (this study), and hence interspecific competition in breeding sites (Costanzo et al. 2011; Santana-Martínez et al. 2017). This competitive exclusion is likely a result of asymmetrical interspecific competition for resources between heterospecific larvae (Santana-Martínez et al. 2017). Both *Ae. aegypti* and *Ae. albopictus* forage and convert food resources to biomass more efficiently than *Culex* mosquitoes resulting in more rapid growth and development, which may lead to the out-competition of members of the *Culex* species from the breeding sites (Carrieri et al. 2003; Santana-Martínez et al. 2017). *Culex* mosquitoes may escape this competition using temporal and spatial avoidance, as well as differential resource use strategies. For example, compared with *Aedes spp., Culex* species colonise breeding sites earlier in the season (Costanzo et al. 2005; Leisnham et al. 2014),

prefer different niche-related characteristics within cohabited breeding sites (Carrieri et al. 2003; Leisnham et al. 2014), and make use of different food resources within the same microhabitat (Costanzo et al. 2011). By eavesdropping on volatiles associated with sites containing heterospecific larvae, these gravid mosquitoes are taking advantage of these cues to improve their own fitness. Taken together, the results of this study emphasize that specific VOCs associated with breeding sites regulate oviposition site selection of mosquitoes by signalling the level of heterospecific competition, following the principles of niche separation and competitive exclusion.

VOCs associated with heterospecific larvae regulate oviposition site choice (this study; Khan et al. 2022) and stimulate egg laying (this study) in a species-specific and density-dependent manner. The specific nature of these VOCs, however, has only been described in this study. We demonstrate that gravid mosquitoes detect and select VOCs emanating from heterospecific LCW and are able to regulate discrimination among oviposition sites. While there are technical limitations with the methodology used, the demonstrated behavioural responses to the extracts and synthetic blends reflect that of the natural breeding water. This indicates that the identified VOCs are sufficient for driving oviposition site selection and egg laying by gravid mosquitoes. The response to an odour blend including a medium length (C_{16-18}) alkene is conserved between the two mosquito species, albeit that the alkenes in question are of different lengths in the emanations of the two species (i.e., 1-hexadecene, 3-octadecene), indicating the value of detecting these alkenes as part of a reliable signal of breeding site quality. Other than this, the species diverged from one another in which VOCs constituted the odour blends. *Aedes aegypti* responded to alkanes, specifically to C_{9-14} straight and methyl-branched alkanes, while *Cx. quinquefasciatus* responded to ketones (i.e., 2-decanone, 5-decanone) and 7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione. While 7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione has been found in mushrooms, plants, bark and algae, it is a known contaminant from plastic containers and pipes (Félix et al. 2012), which are common oviposition sites for the container breeding *Ae. aegypti*. Among the identified compounds, only the C_{11-14} alkanes (i.e., 4-methyldecane, undecane, 2,6-dimethylundecane, an unidentified methyl dodecane regioisomer, and tetradecane) have previously been tentatively confirmed to be associated with the LCW of *Ae. aegypti* (Xia et al. 2021), and their associated microbial communities (Heenan-Daly et al. 2021; Mosquera et al. 2023b). Although the branched alkane regioisomers were not unambiguously confirmed in this study, the methyl-branched alkanes tested were electrophysiologically and behaviourally active. Future studies are thus required to conclusively determine the isomers of these compounds, as the position and nature of the side chain may have a significant impact on oviposition (e.g., Ikeshoji & Mulla 1974).

Since short-to-medium length hydrocarbons have not been identified in detail in previous studies on mosquitoes, few such compounds have been tested for behavioural function, including, e.g., nonane and heneicosane (Khan et al. 2022). More broadly, hydrocarbons of diverse length, branching and unsaturation levels, have diverse roles in insect chemical communication (Blomquist & Bagnères 2010). Best known are probably cuticular hydrocarbons, which are generally long-chain alkanes or alkenes with limited branching (Blomquist & Bagnères 2010), while short-chain, more volatile hydrocarbons have been reported less frequently. Closely related to the context of our work, several studies have reported similar hydrocarbons to affect larviposition and oviposition in other insects. In the tsetse fly, *Glossina morsitans*, gravid females are strongly attracted to oviposition sites containing dodecane and pentadecane, which are also present in conspecific larvae (Saini et al. 1996). Gravid cabbage loopers, *Trichoplusia ni*, on the other hand, are strongly repelled by 3-tetradecene and 1-dodecene, at natural concentrations from soybeans (Liu et al. 1988), compounds which are analogous with those identified in this study as oviposition deterrents for *Cx. quinquefasciatus*. Moreover, in diamondback moths, *Plutella xylostella*, a blend of 2,4-dimethylheptane, 4-methyloctane, 2-methylnonane and myrcene induced oviposition repellence (Yan et al. 2023). Of these compounds, 2,4-dimethylheptane and 4-methyloctane were identified as used by *Ae. aegypti* to discriminate among VOCs associated with water conditioned with different densities of the *Cx. quinquefasciatus* larvae, their extracts and as part of the synthetic blend (this study). Thus, available data suggests that the olfactory systems of highly divergent insect species have evolved to detect hydrocarbons, which are used to attract, repel or deter gravid insects at different distances from the oviposition site.

Gravid *Ae. aegypti* and *Cx. quinquefasciatus* detect semiochemicals as blends, using combinatorial coding, which enables mosquitoes to select and discriminate among resources at release rates significantly lower than that of individual VOCs (Ignell & Hill 2020). While the preference of *Ae. aegypti* for low densities of heterospecific LCW and their associated blends did not differ, an increase in larval density increased the relative abundance of predominantly the C_{11-14} alkanes above the physiological detection level of the gravid female antennae. Whether the blend composition changes further with ever increasing densities of larvae, as observed for *An. gambiae* (Suh et al. 2016), remains to be investigated. The overall result of this study demonstrates that VOCs regulate the choice of oviposition site in *Ae. aegypti* and *Cx. quinquefasciatus.*

Both *Ae. aegypti* and *Cx. quinquefasciatus* regulate oviposition site selection and egg-laying in response to odours associated with heterospecific larvae in a density-dependent and species-specific manner in accordance with the theories of niche differentiation and competitive exclusion, respectively. The composition of the detected blends regulating

oviposition in a heterospecific context depends on larval density, and are composed predominantly of $C_{9\text{-}14}$ methylalkanes, in addition to $C_{16\text{-}18}$ alkenes and C_{10} ketones, which had not been described previously to regulate odour-mediated behaviours in mosquitoes. The choice by a gravid mosquito to avoid heterospecific aquatic stages in a breeding site or mitigate the risks associated with ovipositing in such sites is complex and likely regulated by the time spent in the gravid state and environmental cues, in addition to larval associated odours. Further investigations using more complex systems, e.g., additional sympatric species and semi-field assays, are needed to address the observed interspecific interaction along with the mechanisms regulating detection of the novel oviposition cues. Moreover, the development of reliable and robust synthetic odour blends, including the determination of the regiochemistry of the branched hydrocarbons that induce long-lasting avoidance of potential oviposition sites by mosquito disease vectors, may provide the basis for a tool missing from current control strategies, particularly as the "push" in a "push-pull" system.

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Table S1ab–k, Fig. S1–S4