Synergism between nonane and emanations from soil as cues in oviposition-site selection of natural populations of *Anopheles gambiae* and *Culex quinquefasciatus*

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Abstract

**Background:** Olfactory cues have been shown to have an important role in guiding gravid mosquito females to selected sites for egg laying. The objective of this study was to determine the influence of emanations from soil from a breeding site and the putative oviposition pheromone nonane on oviposition-site selection of natural populations of *Anopheles gambiae* s.l. and *Culex quinquefasciatus*.

**Methods:** This field-based study was conducted in Mvomero District in East-central Tanzania. In a dual-choice experimental set up, clay bowls were dug into the ground and filled with one of the following treatments: (i) distilled water + autoclaved soil (control), (ii) distilled water + soil from a natural mosquito breeding site, (iii) distilled water + nonane and (iv) distilled water + nonane + soil from a natural breeding site. Soil was either left untreated or was autoclaved before use. The number of larvae present in the bowls after 10 d was used as outcome measure.

**Results:** Autoclaved soil had a significant effect on oviposition behaviour of *Cx. quinquefasciatus* ($P<0.005$) but no effect on *An. gambiae* ($P=0.18$). Nonane and emanations from untreated soil significantly influenced the selection of oviposition sites by both *An. gambiae* ($P<0.0001$) and *Cx. quinquefasciatus* ($P<0.0001$). A mixture of nonane and untreated soil caused a synergistic effect on oviposition behaviour compared to either nonane or untreated soil alone, in both *An. gambiae* (Oviposition Activity Index (OAI) = 0.56; $P<0.001$) and *Cx. quinquefasciatus* (OAI =0.59; $P<0.0001$).

**Conclusion:** The larval pheromone nonane and emanations from breeding-site soil both induced oviposition in wild *An. gambiae* s.l. and *Cx. quinquefasciatus*, with a synergistic effect when both stimuli were present simultaneously. This is the first study in which nonane is shown to cause oviposition under natural conditions, suggesting that this compound can potentially be exploited for the management of mosquito vectors.

**Background**

Vector control is a fundamental element of the existing global strategy to fight mosquito-borne diseases [1]. Existing mosquito control programmes have been developed based on understanding of the behaviour and lifecycle of the vectors. The use of insecticide-treated nets (ITNs) and the recently developed toxic sugar bait technique resulted from the exploitation of blood and plant host-seeking
behaviour of mosquitoes, respectively [2-5]. Additionally, indoor residual spraying with insecticides (IRS) is based on the observation that mosquitoes rest on walls after feeding and thereby come into contact with insecticides [6]. Larviciding and other environmentally-based interventions [7], on the other hand, are based on observations that mosquito eggs are laid on water and immature stages develop in water. Oviposition has been considered to be an important target to exploit for the control of mosquito-borne diseases [8-10].

Currently, the main effective methods of mosquito-borne disease control are the use of ITNs and IRS [11]. However, these methods are challenged by the wide-spread development of insecticide resistance [12-14] and the observed behavioural adaptation of mosquitoes to avoid insecticides [15]. Mosquitoes can detect the presence of insecticides from treated surfaces and divert their biting activities in time and space to exploit untreated resources [16-18].

Recently it was reported that malaria mosquitoes have altered their blood host-seeking behaviour by shifting biting time [19-22] and place, i.e. outdoors vs. indoors [23, 24] in response to the wide coverage of ITNs. Moreover, in certain areas malaria vectors have shifted their blood-host preference to other vertebrate species as a result of insecticide use [25]. In such circumstances, the core interventions of ITNs and IRS need to be supplemented by larval source management, which includes vector habitat modification, habitat manipulation, larviciding and biological control [11].

One of the possible options is habitat manipulation by using infochemicals derived from mosquito breeding sites to lure vectors into death traps [26-28]. Already habitat-derived infochemicals have been used to enhance the trapping of gravid Culex quinquefasciatus [29-31] and Aedes aegypti [32, 33] mosquitoes in autocidal oviposition traps. The use of infochemicals from breeding sites to lure gravid mosquitoes has the potential to target egg-laying adults [26, 34]. Thus, infochemicals that direct gravid mosquitoes to lay eggs in selected habitats are likely to be the focus of future vector control strategies.

These strategies are relevant because most insects express a preference for oviposition habitats that improve survival, growth and reproductive potential of their offspring, especially for species in which juveniles are incapable of migrating away from poor-quality habitats sky[35]. Sites selected for
oviposition by mosquitoes are usually few and, once identified, they can easily be targeted for control measures. Oviposition-habitat selection is particularly relevant in insect vectors of medical importance as it determines the localities to which larvicidal control measures need to be targeted [36].

The search for oviposition attractants is aimed at discovering a chemical compound or blends of compounds that attract selected species [37]. Skatole and (5R,6S)-6-acetoxy-5-hexadecanolide were discovered to be oviposition cues for *Cx. quinquefasciatus*, and a blend of these compounds caused a synergistic response in gravid mosquitoes [38]. Recently, it was reported that gravid mosquitoes of *Anopheles gambiae* are attracted to cedrol, a compound identified from a natural breeding site, as oviposition cue [39]. Cedrol was found to be derived from grass species found in breeding sites of *An. gambiae* s.s. [28]. Additionally, nonane, a compound identified in the headspace from mosquito larval habitats in the laboratory, was found to be attractive to gravid *An. gambiae* [40]. Nonane is a volatile chemical compound with nine carbon atoms. A related chemical compound with nine carbon atoms similar to nonane and which acts as an attractant to mosquitoes is nonanol, which is known to attract *Cx. quinquefasciatus* [41]. Current evidence suggests that species-specific as well as habitat-derived chemicals affect oviposition behaviour of mosquitoes.

The objective of the present study was to explore the influence of habitat-derived infochemicals and nonane on the selection of oviposition sites by *An. gambiae* and *Cx. quinquefasciatus* under field conditions. Studies were done i) to establish the most effective (artificial) oviposition device for field use, ii) to examine the effect of soil-derived infochemicals and of the putative oviposition cue nonane and iii) to investigate the interaction between emanations from breeding-site soil [42] and nonane on oviposition behaviour of wild *An. gambiae* and *Cx. quinquefasciatus*.

**Methods**

**Study area**

The study was carried out in Mvomero District in east-central Tanzania (latitude 5°47′09″-7°23′40″S, longitude 37°11′09″- 38°01′33″E), between March and June 2012. This area has typical tropical characteristics: temperatures oscillating between 19 and 31°C, RH >80%, and annual rainfall of
1,146mm (based on data collected from Mtibwa meteorological station, 2008-2013). The area has a bi-modal type of rainfall with long rains from March to June and short rains from October to December, with a relatively short dry spell between July and September.

Digoma village was selected for the field experiments; the village borders the Nguu mountains and receives water from rivers which flood the valleys. This enables irrigated rice production in the river basin throughout the year. In addition to rice production, therefore, the area has favourable environmental conditions for mosquito production. Malaria and lymphatic filariasis are the most common mosquito-borne diseases in the area [43]. The most abundant mosquito vectors in the area include *An. gambiae* Giles s.s., *An. arabiensis* Patton, *An. funestus* Giles and *Cx. quinquefasciatus* Say. *Anopheles gambiae* s.s. and *An. arabiensis* are genetically related and morphologically indistinguishable, and are here grouped as *An. gambiae* unless otherwise mentioned.

**Oviposition containers**

Containers used for oviposition in this experiment included clay pots, plastic bowls, aluminium pans and plates which were either blue or transparent in colour (Fig. 1). With the exception of aluminium plates, which had a diameter of 27 cm and a depth of 4 cm, all other containers were of similar size (average diameter of 25 cm and a depth of 7 cm).

**Distilled water**

Distilled water was used in the experiment to dilute chemicals and obtain the desired dosages, and also to dissolve oviposition substrates before setting up the experiments. Distilled water was also used for rinsing all washable items used in the experiments. It was produced and packed by LAL Laboratories, Tanga, Tanzania. Distilled water was used alone in the early experiments as oviposition substrate and in the control arm. For experiments involving nonane, a control solution was used, which consisted of 55% v/v distilled + 40% v/v methanol + 5% v/v tween20. Previous studies had not found any behavioural or larvicidal effect of this mixture [40].

**Soil**

Clay soil originated from a natural breeding site in Mvomero that contained early-stage larvae of *An. gambiae*. Two hundred gram of dry soil was added to each container to simulate natural conditions of
breeding sites. Previous studies have shown that volatile emissions associated with microbial organisms in the soil mediate the location of potential mosquito habitats [44]. Therefore, a fraction of soil samples from known breeding sites was dried, autoclaved twice for 15 min. at 130°C and 1.4 kg/cm² pressure and allowed to cool down to kill any organisms that might be involved in the production of volatile chemicals [44, 45].

**Chemical cues**

Nonane (Lot and filling code: 132995235107188, ≥ 99.0%; Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands) was selected as chemical cue to lure gravid mosquitoes (Schoelitz et al In Press). Nonane is insoluble in water and therefore it was dissolved in methanol and tween20 in the following ratio: 55% v/v of nonane + 40% v/v methanol + 5% v/v tween20. The mixture was further diluted in distilled water with one magnitude per dilution. In experiments 3 & 4, nonane was tested at a concentration of $5.5 \times 10^{-5}$M and it was paired with a control solution of distilled water + methanol + tween20 (see above).

**Selection of artificial oviposition containers**

In our effort to simulate natural breeding sites, we conducted experiments in order to search for the most preferred artificial breeding site for a natural population of mosquitoes. We therefore evaluated a range of man-made liquid receptacles in the field in order to identify the most suitable oviposition bowl for mosquitoes in the area. These included plastic bowls (blue and transparent), aluminium plates and pans, and clay pots. To explore possible colonization of artificial habitats by wild mosquitoes, 25 containers were placed randomly in an open sunlit field. Five lines, each composed of five containers were placed 3 m from each other, in sub-soil with the top of the container being at soil surface level. Containers were filled with distilled water to capacity and were checked daily for the presence of larvae/pupae for a period of 10 days. Evaporated water was replenished with an equal amount of water in each container daily. Distilled water had already been successfully used as oviposition substrate for mosquitoes in the laboratory and semi-field environment [40]. Therefore, the aim of this experiment was to test if a natural population of mosquitoes would oviposit in our
simulated breeding sites containing distilled water. The container that produced the highest number of larvae was selected for the behavioural experiments.

**Site selection for oviposition trial**

Four sites (north, south, east and west) were selected for the dual-choice oviposition trial in an area covering a total of 4 ha. on both sides of a river near Digoma village, N.E. Tanzania. This area was chosen based on the following criteria: proximity to the river basin, presence of rice fields, absence of flooding, open to sun and proximity to human settlements. Rice growing is the main economic activity. All sites were surrounded by a wire mesh to prevent humans, animals or frogs from interfering with the experiments. In addition, a local field worker was hired to oversee the site during the entire study period.

**Clay pots**

As clay pots gave the best result as oviposition container (see above), there were selected for the remainder of the study. The pots had an average diameter of 200 mm and a depth of 100 mm was used as artificial breeding sites for the field trial. They were made locally from clay soil, moulded by hand to make a pot shape and left to dry, where after they were cured by fire. In our study, clay pots were positioned in the ground so that the margins of the pots were level with the surrounding ground. The pots were placed in the valley plain, within a rice field in the vicinity of a village.

**Design of oviposition experiments**

Clay pots were placed at selected sites in the field 72 h before the start of the experiment and filled till capacity with distilled water; water was replenished until the clay reached saturation. Prior to the start of the experiment, the clay pots were emptied and immediately filled with 1L of the oviposition substrate (treatment or control) one h before sunset. Oviposition pots were left undisturbed for five days and from the 6th day, pots were inspected every morning and larvae were collected and recorded daily from 06:30 am for the next 10 days. Whenever the water level decreased in the pots, distilled water was added to maintain the water level. Collected larvae were then transferred to a temporarily established local laboratory together with the water from the pot and reared under controlled conditions. This water was used as rearing substrate in the laboratory for the first 24 h.
After that time, larvae from each oviposition pot were transferred to mosquito rearing bowls which contained distilled water. Rearing bowls were placed below light bulbs and larvae were fed Tetramin® fish food twice daily. Larval growth and development were observed and recorded until pupation and adult formation. Oviposition pots containing experimental substrates remained in the field for 15 d thereafter the substrates were removed; the pots were cleaned and replaced. For each pair of treatment and control, the pots were oriented facing East and West positions, and these positions were exchanged for each replicate.

**Dual-choice tests**

The effect of substrate on oviposition choice of wild mosquitoes was tested in a dual choice test, where one clay pot contained the treatment substrate and the other pot the control substrate (Table 1). Treatment and control were placed 3 m from each other. Each treatment pair was replicated 40 times, 10 pairs at four different sites (see site selection above); pots with substrate were incubated in the field for five days, and then examined for the presence of larvae for 10 days. Newly emerged larvae were collected daily. For each replicate, the positions of the treatment and control were exchanged each time to counterbalance the effects of wind direction.

**Influence of autoclaved soil from a natural breeding site**

We placed 200 g of autoclaved soil from a natural anopheline breeding site + distilled water in a clay pot and tested against distilled water only. Pots were each filled with 1250 mm distilled water.

**Influence of untreated soil from a natural breeding site**

Clay pots were filled with 200 g of dried soil from a natural anopheline breeding site. To test whether soil produces chemical cues or acts only as a visual cue for gravid mosquitoes [26], the soil was tested against autoclaved soil. The pots were each filled with 1250 ml of distilled water.

**Influence of nonane**

1250 ml of the nonane solution + autoclaved soil was tested against 1250 ml of distilled water + autoclaved soil. Pots were filled with either 200 g autoclaved soil and distilled water or 200 g autoclaved soil and a nonane solution.

**Influence of soil from a natural breeding site and nonane**
To investigate the interactive effects of breeding-site soil and nonane, combinations of both candidate stimuli were tested alone or as a mixture: (a) nonane + breeding-site soil against nonane + autoclaved breeding soil and (b) nonane + breeding-site soil against distilled water + breeding-site soil. Pots were filled with 200 g of soil and distilled water or a nonane solution until capacity.

**Mosquito species composition**

All larvae collected were transferred to the insectary and reared until adult emergence. Newly emerged anopheline adults were identified to species level using morphological keys [46]. *Anopheles gambiae* specimens were preserved in Eppendorf tubes which contained silica gel for further identification to distinguish between sibling species in the *An. gambiae* complex. Genotypic identification was conducted by using the ribosomal DNA-polymerase chain reaction (PCR) to separate *An. gambiae* s.s. from *An. arabiensis* [47]. Culicine mosquitoes were identified as *Cx. quinquefasciatus* or other culicines.

**Data analysis**

SPSS 14 for Windows® was used to conduct Wilcoxon signed-rank tests for paired samples in order to determine the difference in the number of larvae in each oviposition bowl as an indicator of number of eggs laid. A Friedman test for multiple samples was used to determine the oviposition preference among several containers. The preferences of mosquitoes for ovipositing on different treatments were evaluated based on container index (CI) (% bowls harbouring larva). All statistical tests were conducted by using absolute numbers of larvae in pots as a proxy for the number of eggs laid in the pot.

The larval density index (LDI) was defined as the total number of larvae found divided by the total number of oviposition containers with larvae.

The oviposition active index (OAI) was used to determine the attractiveness to the treated substrate compared to control. It was calculated according to the formula; \( OAI = \frac{N_t - N_c}{N_t + N_c} \) [48]. Where \( N_t \) = number of larvae on the test substrate and \( N_c \) = number of larvae on the control substrate. In this study, we found that anopheline eggs, which are black in colour, tend to stick to the surface of the clay pot which is also black. This poses a challenge to accurately score the number of eggs as a
measure of oviposition activity of gravid females. Therefore, the number of larvae was scored as a proxy for the eggs that were laid in respective pots.

Results

**Mosquito species composition**

During the study on oviposition site selection and containers (see below), a total of 1,349 anopheline larvae and culicine 2,815 larvae were collected. All anopheline larvae collected in the containers consisted of *An. gambiae*. Molecular analysis (by PCR) of a subsample of 200 *An. gambiae* larvae revealed that 86% were *An. gambiae* s.s. and 16% *An. arabiensis*. Culicine larvae were identified as *Cx. quinquefasciatus*.

**Mosquito oviposition-site selection between different substrates and containers**

Preliminary experiments showed that natural populations of *Cx. quinquefasciatus* oviposited in containers that were filled with distilled water. By contrast, a natural population of *An. gambiae* mosquitoes did not oviposit in containers filled with distilled water only. After adding 200 g of soil from a known anopheline breeding site to each container, we observed significantly more larvae in clay pots than in other containers. On average, 80.4 ± 5.7 ($\chi^2 = 14.97, p=0.005$) larvae of *Cx. quinquefasciatus* and 33.4 ± 5.4 ($\chi^2 = 9.92, p= 0.042$) larvae of *An. gambiae* were found in clay pots containing soil (Fig. 2). It was therefore decided to use clay pots for all successive experiments as proxies for natural breeding sites.

**Influence of chemical cues from soil**

In a choice assay between distilled water with breeding-site soil and distilled water with autoclaved soil, the accumulated total number of *An. gambiae* larvae found in pots containing breeding-site soil left for 10 d was 254, with an average of 6.35 ± 1.16 larvae per pot. The number of *Cx. quinquefasciatus* larvae was 644, with an average of 16.1 ± 1.6 larvae per pot. The number of larvae found in pots containing breeding-site soil was significantly higher than that in pots with autoclaved soil for both *An. gambiae* and *Cx. quinquefasciatus* ($z = -4.016, P< 0.0001$ and $z = -4.32, P< 0.0001$ respectively). There were only few larvae of *An. gambiae* found in pots containing autoclaved breeding-site soil (Table 2).
Influence of nonane

In a choice assay between control + autoclaved soil + nonane and control + autoclaved soil only, a total of 503 larvae of *An. gambiae* were found in 32 out of 40 clay pots containing nonane, while a total of 825 larvae of *Cx. quinquefasciatus* were found in all 40 clay pots containing nonane. Larvae of *An. gambiae* were mainly found in pots containing nonane, with an average number of 12.6 ± 1.6 larvae per pot, whereas larvae of *Cx. quinquefasciatus* were found in both treated and control pots with an average of 20.6 ± 1.1 found in nonane pots and 5.7 ± 1.0 in control pots. The number of larvae found in pots containing nonane was significantly higher than the number of larvae found in the control pots for both *An. gambiae* and *Cx. quinquefasciatus* (z = -4.978, P < 0.0001 and z = -3.846, p < 0.0001, respectively) (Tables 2 and 3).

The influence of nonane and soil from a breeding site

In a choice test between distilled water with autoclaved soil + nonane against distilled water with breeding-site soil, a total of 751 *An. gambiae* larvae were found. Of these, 57.1% were found in pots containing autoclaved soil + nonane and 42.9% were found in pots containing breeding-site soil (Table 2 and Fig. 2). Also, a total of 1,364 larvae of *Cx. quinquefasciatus* were found; 62.1% of these were found in pots containing autoclaved soil + nonane and 37.9% were found in pots containing breeding-site soil (Table 3). There was no significant difference between the number of larvae found in pots containing autoclaved soil + nonane and pots with breeding-site soil for *An. gambiae* (z = -1.658, P < 0.097). However, for *Cx. quinquefasciatus*, there was a significantly higher number of larvae in pots containing a mixture of autoclaved soil and nonane compared to breeding-site soil (z = -4.179, P < 0.0001).

Influence of a mixture of nonane and soil from a natural breeding site

In a choice test between distilled water + breeding-site soil + nonane against distilled water + breeding-site soil, a total of 1,248 *An. gambiae* larvae were found. Of these, 77.9% were found in pots containing distilled water + soil + nonane while 22.1% were found in pots containing distilled water + soil (Table 2 and Fig. 2). Additionally, a total of 2,013 larvae of *Cx. quinquefasciatus* were found; 79.4% of these were found in pots containing distilled water + soil + nonane and 20.6% were found in
pots containing distilled water + soil only (Table 3). The number of larvae found in pots containing distilled water with distilled water + soil + nonane was significantly higher than larvae found in distilled water + soil for both *An. gambiae* and *Cx. quinquefasciatus* \((z = -5.046, P < 0.0001\) and \(z = -5.512, P < 0.0001\), respectively).

**Discussion**

The oviposition pheromone nonane and emanations from breeding-site soil both attracted wild females of *An. gambiae* and *Cx. quinquefasciatus* to oviposit in the oviposition containers under field conditions. When both stimuli were present simultaneously, they acted synergistically. These results demonstrate the role that natural products play in the oviposition behaviour of wild populations of mosquitoes. In this study, *An. gambiae* selected clay pots above plastic or aluminium bowls for oviposition. It is likely that clay pots simulate the natural conditions that mosquitoes prefer for oviposition.

Similar to our study, Herrera et al. [26], while studying oviposition behaviour in Kenya, reported that oviposition substrates containing soil from a known breeding site produced significantly more larvae than a substrate without soil. This suggests that soil from a known breeding site contains and emits a chemical signal associated with microbial activity that attracts gravid mosquitoes and induces oviposition [49-53]. The cue produced by the breeding-site soil is likely to be inter-specific because it originates from microbial interactions and attracted gravid *An. gambiae* s.l. and *Cx. quinquefasciatus* to lay their eggs in pots containing such soil. In this study, autoclaved soil, in which all microorganisms had been killed, did not affect the oviposition behaviour of *An. gambiae*. This is consistent with other studies of mediation of oviposition-site selection by infochemicals of microbial origin [45, 54].

The effect of cues from micro-organisms present in breeding-site soil appears to be much stronger on *An. gambiae* than on *Cx. quinquefasciatus*, as the latter also laid eggs in pots containing autoclaved soil and even distilled water only, and thus behaved as a generalist species, in contrast to *An. gambiae*. Nevertheless, when untreated soil from a natural breeding site was used, the number of larvae of *Cx. quinquefasciatus* collected was significantly enhanced as with *An. gambiae* s.l. (Table 2),
suggesting the production of chemical oviposition cues by the soil/water substrate affecting both mosquito species. This was recently supported by work from Eneh et al. [28] and Herrera et al. [26], who demonstrated the emission of such cues from breeding-site soil and grasses. The latter authors suggest that these cues are derived from micro-organisms present in natural breeding sites. This suggestion is supported by our observation that with autoclaved soil few *An. gambiae* s.l. females were attracted to the oviposition bowls, while with natural, untreated, soil many females laid eggs in the bowls.

The assay with nonane as a single cue (Fig. 2) indicated the mediation of oviposition behaviour of a natural population of *An. gambiae* s.l. by an intra-specific cue, as the volatile compound is produced by larvae and it attracts conspecific gravid mosquitoes to oviposit. As described previously [40], nonane is a pheromone that originates from *An. gambiae* larvae and attracts conspecific gravid mosquitoes. The response elicited by nonane in this study is consistent with our previous findings on the effects of infochemicals emitted by early instars of *An. coluzzii* on the oviposition behaviour of conspecific gravid females in the laboratory [55] and in the semi-field system with *An. gambiae* s.s. [40]. A similar response was observed with *Cx. quinquefasciatus* despite the fact that nonane originated from *An. gambiae*. This strongly suggests that the two species use the same chemical cues to locate suitable breeding sites. The presence of eggs or larvae of one species can thus act as an oviposition attractant for gravid mosquitoes of another species. It has previously been found that the oviposition pheromone of *Cx. quinquefasciatus* also attracted other culicine species, and this suggests that mosquitoes use a wide range of chemical cues in their oviposition behaviour [29, 39]. The finding that two different mosquito species, which are not genetically related, have evolved to respond to the same oviposition cues, is an interesting topic for future research, and suggests that olfactory receptors associated with oviposition are widely shared between different mosquito genera.

Mosquitoes use both inter- and intra-specific cues in locating suitable breeding sites. Previous studies on the role of the two cues reported conflicting findings. Some scholars thought that the intra-specific cue (pheromone) emitted by larvae from suitable breeding sites augments the attraction to inter-specific volatiles associated with microbial activity in natural anopheline pools [39, 54, 56]. Other
authors reported that the presence of larvae in distilled water, even at low density, does not increase oviposition compared to distilled water without larvae [57, 58]. Similarly, in our previous studies [55], we found that the presence of late-stage larvae, even at a low density, did not increase the oviposition response when compared to distilled water alone. However, we found that the presence of early instars increases oviposition compared to distilled water alone, suggesting that early instars emit intra-specific cues that are attractive to gravid mosquitoes. Nonane was identified from headspace volatiles collected from both early and late instars [40], and the present study shows that wild *An. gambiae* s.l. females prefer to oviposit in breeding sites emitting this compound, which, therefore, should be considered a cue signifying a suitable site for larval development.

Previous studies on the role of infochemicals emitted by larvae have cleared earlier doubts on whether the larval pheromone is stimulatory by itself (i.e., in the absence of the kairomone) or that the production of this pheromone occurs only in *An. gambiae* habitats containing suitable organic matter, microbes and algae [52, 54, 59]. Furthermore, in order to understand the interaction between an intra-specific signal (pheromone) and an inter-specific cue to the behaviour of *An. gambiae*, we combined nonane and soil from a natural breeding site in a choice assay and tested this combination against soil or pheromone alone. The number of larvae that were found in water with nonane and breeding-site soil was higher than the number of larvae found in water with either nonane or soil alone. This indicates that inter- and intra-specific cues act synergistically when attracting gravid mosquitoes to lay eggs. Recently, cedrol was reported as an oviposition stimulant for *An. gambiae*, derived from breeding-site soil [39]. This compound was found to originate from grass present in the breeding site [26, 60]. Additionally, gravid *An. coluzzii* and *An. arabiensis* were found to be attracted to grass volatiles [61]. It is therefore possible that in our study, plant- or soil-derived chemicals were responsible for the behavioural effect of the soil, of which cedrol to date is the only identified compound with proven activity in the field. The observed synergistic response on *An. gambiae* and *Cx. quinquefasciatus* may thus have been caused by the interaction of nonane and cedrol. The discovery of these chemical cues opens the way to the development of oviposition-based mediation/manipulation of populations of these harmful mosquito species.
Our results resemble the effect of the *Cx. quinquefasciatus* oviposition pheromone (5R,6S)-6-acetoxy-5-hexadecanolide and infochemicals derived from hay infusions, where a similar synergistic effect of both stimuli on gravid females was found [38]. This study suggests that there are several mechanisms ensuring that gravid females are guided effectively to sites that are suitable for egg laying and that the observed additive behavioural responses are a result of the perception of several interacting stimuli.

Being an infochemical of *An. gambiae* origin, nonane was expected to affect only gravid *An. gambiae* mosquitoes. In this study, significantly more culicine larvae were also found in pots containing nonane. This suggests that this compound can be used for the surveillance of other mosquito species as well. This finding is consistent with other studies which suggest that *An. gambiae* and *Cx. quinquefasciatus* share breeding sites in many cases [62-66]. Further studies on the interaction of inter-and intra-specific cues of *An. gambiae* in the behaviour of other species will help to understand the universal role of infochemicals among various species of mosquitoes.

The findings from our study suggest that breeding-site derived infochemicals can be used for surveillance and control of mosquito vectors. In order for ovitraps to become effective as control agents in situations of multiple alternative oviposition sites (such as rice fields), the ovitrap should be at least as attractive, preferably more attractive than existing oviposition sites [67]. In the present study, nonane has been as attractive as breeding-site soil and the mixture of nonane and breeding-site soil induced a synergistic response. Therefore, if various signals are combined and emitted from specified breeding sites, gravid mosquitoes can be manipulated to lay their eggs on designated sites, which can easily be targeted for larvicide application. Therefore, the study reveals that the above attractants have the potential for use in developing a lure-and-kill system for the control of disease vectors.

**Conclusions**

This study shows that nonane and emanations from natural breeding-site soil attract gravid females of *An. gambiae* and *Cx. quinquefasciatus* to sites containing these cues, and that both stimuli, once combined, act synergistically.
Declarations

Ethics approval and consent to participate

The study was conducted according to Standard Operating Procedures approved by the Medical Research Coordinating Committee (MRCC) of the National Institute for Medical Research (NIMR), Tanzania. It received a research permit from MRCC with reference number NIMR/HQ/R.8a/Vol. IX/573 and a permit from the Tanzania Commission for Science and Technology with reference number CST/RCA 138/225/2008.

Consent for publication

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Availability of data and material

Original data collected during the study are available with the authors upon request

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

VSM, LEGM and WT conceived the idea and designed the study. VSM conducted the experiments, analysed the data and drafted the manuscript. LEGM and WT commented on the manuscript. All authors read and approved the final manuscript.

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References

1. WHO. Global Vector Control Response 2017-2030. Geneva: World Health Organization; 2017:53.

2. Malima RC, Oxborough RM, Tungu PK, Maxwell C, Lyimo I, Mwingira V, Mosha FW, Matowo J, Magesa SM, Rowland MW. Behavioural and insecticidal effects of organophosphate-, carbamate- and pyrethroid-treated mosquito nets against African malaria vectors. Med Vet Entomol. 2009;23:317-325.

3. Marshall JM, White MT, Ghani AC, Schlein Y, Muller GC, Beier JC. Quantifying the mosquito's sweet tooth: modelling the effectiveness of attractive toxic sugar baits (ATSB) for malaria vector control. Mal J. 2013;12:291

4. Qualls WA, Muller GC, Revay EE, Allan SA, Arheart KL, Beier JC, Smith ML, Scott JM, Kravchenko VD, Hausmann A, et al. Evaluation of attractive toxic sugar bait (ATSB)-Barrier for control of vector and nuisance mosquitoes and its effect on non-target organisms in sub-tropical environments in Florida. Acta Trop. 2014;131:104-110.

5. Revay EE, Muller GC, Qualls WA, Kline DL, Naranjo DP, Arheart KL, Kravchenko VD, Yefremova Z, Hausmann A, Beier JC, et al. Control of Aedes albopictus with attractive toxic sugar baits (ATSB) and potential impact on non-target organisms in St. Augustine, Florida. Parasitol Res. 2014;113:73-79.

6. Curtis CF, Lines JD: Impregnated fabrics against malaria mosquitoes. Parasitol Today. 1985;1:147.

7. Curtis CF, Malecela-Lazaro M, Reuben R, Maxwell CA. Use of floating layers of polystyrene beads to control populations of the filaria vector Culex quinquefasciatus. Ann Trop Med Parasit 2002;96:S97-S104.
8. Bentley MD, Day JF. Chemical ecology and behavioral aspects of mosquito oviposition. Annu Rev Entomol. 1989;34:401-421.

9. Dugassa S, Lindh JM, Oyieke F, Mukabana WR, Lindsay SW, Fillinger U. Development of a Gravid Trap for Collecting Live Malaria Vectors Anopheles gambiae s.l. Plos One. 2013;8(7):e68948.

10. Killeen GF, Kiware SS, Seyoum A, Gimnig JE, Corliss GF, Stevenson J, Drakeley CJ, Chitnis N. Comparative assessment of diverse strategies for malaria vector population control based on measured rates at which mosquitoes utilize targeted resource subsets. Mal J. 2014;13:338.

11. WHO. World Malaria Report 2018. Geneva: World Health Organization; 2018:166.

12. Norris LC, Norris DE. Efficacy of long-lasting insecticidal nets in use in Macha, Zambia, against the local Anopheles arabiensis population. Mal J. 2011;10:254.

13. Ranson H, N'Guessan R, Llines J, Moiroux N, Nkuni Z, Corbel V. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends Parasit. 2011;27:91-98.

14. Hemingway J, Ranson H, Magill A, Kolaczinski J, Fornadel C, Gimnig J, Coetzee M, Simard F, Roch DK, Hinzoumbe CK, et al. Averting a malaria disaster: will insecticide resistance derail malaria control? Lancet. 2016;387:1785-1788.

15. Govella NJ, Chaki PP, Killeen GF: Entomological surveillance of behavioural resilience and resistance in residual malaria vector populations. Mal J. 2013,12:124.

16. Takken W. Do insecticide-treated bednets have an effect on malaria vectors? Trop Med Int Hlth. 2002;7:1022-1030.

17. Lindblad KA, Eisele TP, Gimnig JE, Alaii JA, Odhiambo F, ter Kuile FO, Hawley WA, Wannemuehler KA, Phillips-Howard PA, Rosen DH, et al. Sustainability of reductions in malaria transmission and infant mortality in western Kenya with use of insecticide-
treated bednets: 4 to 6 years of follow-up. JAMA. 2004;291:2571-2580.

18. Grieco JP, Achee NL, Chareonviriyaphap T, Suwonkerd W, Chauhan K, Sardelis MR, Roberts DR. A new classification system for the actions of IRS chemicals traditionally used for malaria control. PLoS ONE. 2007;2:e716.

19. Moiroux N, Gomez MB, Pennetier C, Elanga E, Djenontin A, Chandre F, Djegbe I, Guis H, Corbel V. Changes in Anopheles funestus biting behavior following universal coverage of long-lasting insecticidal nets in Benin. J Infect Dis. 2012;206:1622-1629.

20. Yohannes M, Boelee E. Early biting rhythm in the afro-tropical vector of malaria, Anopheles arabiensis, and challenges for its control in Ethiopia. Med Vet Entomol. 2012;26:103-105.

21. Sougoufara S, Diedhiou SM, Doucoure S, Diagne N, Sembene PM, Harry M, Trape JF, Sokhna C, Ndiath MO. Biting by Anopheles funestus in broad daylight after use of long-lasting insecticidal nets: a new challenge to malaria elimination. Mal J. 2014;13:125.

22. Thomsen EK, Koimbu G, Pulford J, Jamea-Maiasa S, Ura Y, Keven JB, Siba PM, Mueller I, Hetzel MW, Reimer LJ. Mosquito behavior change after distribution of bednets results in decreased protection against malaria exposure. J Infect Dis. 2017;215:790-797.

23. Govella NJ, Okumu FO, Killeen GF. Short Report: Insecticide-treated nets can reduce malaria transmission by mosquitoes which feed outdoors. Am J Trop Med Hyg. 2010;82:415-419.

24. Russell TL, Govella NJ, Azizi S, Drakeley CJ, Kachur SP, Killeen GF. Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania. Mal J. 2011;10:80.

25. Mwangangi JM, Mbogo CM, Orindi BO, Muturi EJ, Midega JT, Nzovu J, Gatakaa H,
Githure J, Borgemeister C, Keating J, Beier JC. Shifts in malaria vector species composition and transmission dynamics along the Kenyan coast over the past 20 years. Malaria J. 2013;12:13.

26. Herrera-Varela M, Lindh J, Lindsay SW, Fillinger U. Habitat discrimination by gravid *Anopheles gambiae* sensu lato-a push-pull system. Malar J. 2014; 13:133.

27. Okal MN, Herrera-Varela M, Ouma P, Torto B, Lindsay SW, Lindh JM, Fillinger U. Analysing chemical attraction of gravid *Anopheles gambiae* sensu stricto with modified BG-Sentinel traps. Parasit Vector. 2015;8:301.

28. Eneh LK, Okal MN, Borg-Karlson AK, Fillinger U, Lindh JM. Gravid *Anopheles gambiae* sensu stricto avoid ovipositing in Bermuda grass hay infusion and its volatiles in two choice egg-count bioassays. Malaria J. 2016;15:276.

29. Mboera LEG, Mdira KY, Salum FM, Takken W, Pickett JA: Influence of synthetic oviposition pheromone and volatiles from soakage pits and grass infusions upon oviposition site-selection of *Culex* mosquitoes in Tanzania. J Chem Ecol. 1999;25:1855-1865.

30. Barbosa RMR, Regis L, Vasconcelos R, Leal WS. *Culex* mosquitoes (Diptera: Culicidae) egg laying in traps loaded with bacillus thuringiensis variety israelensis and baited with skatole. J Med Entomol. 2010;47:345-348.

31. Barbosa RMR, Regis LN. Monitoring temporal fluctuations of *Culex quinquefasciatus* using oviposition traps containing attractant and larvicide in an urban environment in Recife, Brazil. Mem I Oswaldo Cruz. 2011;106:451-455.

32. Barrera R, Mackay AJ, Amador M. An improved trap to capture adult container-inhabiting mosquitoes. J Am Mosq Control Assoc. 2013, 29:358-368.

33. Barrera R, Amador M, Acevedo V, Caban B, Felix G, Mackay AJ. Use of the cdc autocidal gravid ovitrap to control and prevent outbreaks of *Aedes aegypti* (Diptera:
34. Zeichner BC, Perich MJ. Laboratory testing of a lethal ovitrap for *Aedes aegypti*. Med Vet Entomol. 1999;13:234-238.

35. Peckarsky BL, Taylor BW, Caudill CC. Hydrologic and behavioral constraints on oviposition of stream insects: implications for adult dispersal. Oecologia. 2000;125:186-200.

36. Service MW. *Mosquito Ecology - Field Sampling Methods*. second edn. London: Elsevier Applied Science; 1993.

37. Bernier UR, Kline DL, Posey KH, Booth MM, Yost RA, Barnard DR. Synergistic attraction of *Aedes aegypti* (L.) to binary blends of L-Lactic acid and acetone, dichloromethane, or dimethyl disulfide. J Med Entomol. 2003;40:653-656.

38. Mboera LE, Takken W, Mdira KY, Pickett JA. Sampling gravid *Culex quinquefasciatus* (Diptera: Culicidae) in Tanzania with traps baited with synthetic oviposition pheromone and grass infusions. J Med Entomol. 2000;37:172-176.

39. Lindh JM, Okal MN, Herrera-Varela M, Borg-Karlson A-K, Torto B, Lindsay SW, Fillinger U. Discovery of an oviposition attractant for gravid malaria vectors of the *Anopheles gambiae* species complex. Malaria J. 2015;14:119.

40. Schoelitsz B, Mwingira VS, Mboera LEG, Beijleveld H, Koenraadt CJM, Spitzen J, van Loon JJA, Takken W. Chemical mediation of oviposition by *Anopheles* mosquitoes: a push-pull system driven by volatiles associated with larval stages. J Chem Ecol. 2020:In Press

41. Syed Z, Leal WS. Acute olfactory response of *Culex* mosquitoes to a human- and bird-derived attractant. Proc Natl Acad Sci USA. 2009;106:18803-18808.

42. Herrera-Varela M. Larval habitat discrimination by the African malaria vector *Anopheles gambiae* sensu lato: observations from standardized experiments and field
43. Mboera LEG, Senkoro KP, Rumisha SF, Mayala BK, Shayo EH, Mlozi MRS. *Plasmodium falciparum* and helminth coinfections among schoolchildren in relation to agro-ecosystems in Mvomero District, Tanzania. *Acta Trop.* 2011;120:95-102.

44. Knols BGJ, Sumba A, Guda TO, Deng AL, Hassanali A, Beier JC. Mediation of oviposition site selection in the African malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) by semiochemicals of microbial origin. *Int J Trop Insect Sci.* 2004;24:260-265.

45. Trexler JD, Apperson CS, Zurek L, Gemeno C, Schal C, Kaufman M, Walker E, Wesley Watson D, Wallace L. Role of bacteria in mediating the oviposition responses of *Aedes albopictus* (Diptera: Culicidae). *J Med Entomol.* 2003;40 841-848.

46. Gillies MT, Coetzee M. *A supplement to the Anophelinae of Africa South of the Sahara.* Johannesburg: The south African Institute for Medical Research; 1987.

47. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *Am J Trop Med Hyg.* 1993;4:520-529.

48. Kramer WL, Mulla MS. Oviposition attractants and repellents of mosquitoes - oviposition responses of culex (Diptera, Culicidae) mosquitoes to organic infusions. *Environ Entomol.* 1979;8:1111-1117.

49. Ikeshoji T, Saito K, Yano A. Bacterial production of the ovipositional attractants for mosquitoes on fatty acid substrates. *Appl Ent Zool.* 1975;10:239-242.

50. Benzon GL, Apperson CS. Reexamination of chemically mediated oviposition behavior in *Aedes aegypti* (l) (Diptera, Culicidae). *J Med Entomol.* 1988;25:158-164.

51. Takken W, Knols BGJ. Odor-mediated behavior of afrotropical malaria mosquitoes.
Annu Rev Entomol. 1999;44:131-157.

52. Gimnig JE, Ombok M, Kamau L, Hawley WA. Characteristics of larval anopheline (Diptera: Cilicidae) habitats in western Kenya. J Med Entomol. 2001;38(2):282-288.

53. Ponnusamy L, Xu N, Nojima S, Wesson DM, Schal C, Apperson CS. Identification of bacteria and bacteria-associated chemical cues that mediate oviposition site preferences by Aedes aegypti. Proc Natl Acad Sci USA. 2008;105:9262-9267.

54. Sumba LA, Guda TO, Deng AL, Hassanali A, Beier JC, Knols BGJ. Mediation of oviposition site selection in the African malaria mosquito Anopheles gambiae (Diptera: Culicidae) by semiochemicals of microbial origin. Int J Trop Insect Sci. 2004;24:260-295.

55. Mwingira VS, Spitzen, J, Mboera LEG, Torres-Estrada JL, Takken W. The influence of larval stage and density on oviposition site-selection behavior of the Afro-tropical malaria mosquito Anopheles coluzzii (Diptera: Culicidae). J Med Entomol. 2019: In Press.

56. Rejmankova E, Higashi R, Grieco J, Achee N, Roberts D. Volatile substances from larval habitats mediate species-specific oviposition in Anopheles mosquitoes. J Med Entomol. 2005;42:95-103.

57. Munga S, Minakawa N, Zhou GF, Mushinzimana E, Barrack OOJ, Githeko AK, Yan GY. Association between land cover and habitat productivity of malaria vectors in western Kenyan highlands. Am J Trop Med Hyg. 2006;74:69-75.

58. Sumba LA, Ogbunugafor CB, Deng AL, Hassanali A. Regulation of oviposition in Anopheles gambiae s.s.: role of inter- and intra-specific signals. J Chem Ecol. 2008;34:1430-1436.

59. Merritt RW, Dadd RH, Walker ED. Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. Annu Rev Entomol. 1992;37:349-376.
60. Eneh LK, Saijo H, Borg-Karlson AK, Lindh JM, Rajarao GK. Cedrol, a malaria mosquito oviposition attractant is produced by fungi isolated from rhizomes of the grass *Cyperus rotundus*. Mal J. 2016;15:478.

61. Asmare Y, Hill SR, Hopkins RJ, Tekie H, Ignell R; The role of grass volatiles on oviposition site selection by *Anopheles arabiensis* and *Anopheles coluzzii*. Mal J. 2017;16:65.

62. Minakawa N, Sonye G, Mogi M, Yan G. Habitat characteristics of *Anopheles gambiae* s.s. larvae in a Kenyan highland. Med Vet Entomol. 2004;18:301-305.

63. Munga S, Minakawa N, Zhou G, Barrack OJO, Githeko AK, Yan G. Effects of larval competitors and predators on oviposition site selection of *Anopheles gambiae sensu stricto*. J Med Entomol. 2006;43:221-224.

64. Mutuku FM, Bayoh MN, Gimnig JE, Vulule JM, Kama L, Walker ED, Kabiru E, Hawley WA. Pupal habitat productivity of *Anopheles gambiae* complex mosquitoes in a rural village in western Kenya. Am J Trop Med Hyg. 2006;74:54-61.

65. Mwangangi JM, Muturi EJ, Shililu J, Muriu SM, Jacob B, Kabiru EW, Mbogo CA, Githure J, Novak R. Contribution of different aquatic habitats to adult *Anopheles arabiensis* and *Culex quinquefasciatus* (Diptera : Culicidae) production in a rice agroecosystem in Mwea, Kenya. J Vector Ecol. 2008;33:129-138.

66. Mwingira VS, Mayala BK, Senkoro KP, Rumisha SF, Shayo EH, Mlozi MR, Mboera LE. Mosquito larval productivity in rice-fields infested with Azolla in Mvomero District, Tanzania. Tanz J Hlth Res. 2009;11:17-22.

67. Trexler JD, Apperson CS, Schal C. Laboratory and field evaluations of oviposition responses of *Aedes albopictus* and *Aedes triseriatus* (Diptera: Culicidae) to oak leaf infusion. J Med Entomol. 1998;35:967-976.

Tables
Table 1 - Experimental treatments for the dual-choice tests

| Treatment series | Substrate A                                  | Substrate B                                  | No of replicates |
|------------------|----------------------------------------------|----------------------------------------------|------------------|
| 1                | Distilled water + autoclaved soil            | Distilled water                              | 40               |
| 2                | Distilled water + untreated soil             | Distilled water + autoclaved soil            | 40               |
| 3                | Distilled water + autoclaved soil + nonane   | Distilled water + autoclaved soil            | 40               |
| 4                | Distilled water + autoclaved soil + nonane   | Distilled water + untreated soil             | 40               |
| 5                | Distilled water + untreated soil + nonane    | Distilled water + untreated soil             | 40               |

Table 2 - Mean number of larvae from a natural population of *Anopheles gambiae* s.l. that oviposited in clay pots filled with distilled water (DW), and distilled water + autoclaved soil (AC), distilled water + untreated soil (BS) and distilled water + nonane in a dual choice set-up in the field. Larval density index (LDI), oviposition activity index (OAI) and *p*-values are shown.

| Oviposition substrate     | Pot positivity | Quantity of larvae in pots | OAI |
|---------------------------|----------------|----------------------------|-----|
|                           | No.            | LDI | No. (%)     | Mean ± SE |     |
| DW + AC soil              | 2              | 3.5 | 7 (1.7)     | 0.2 ± 0.8 | 1   |
| DW                        | 0              | 0   | 0 (0)       | 0         |     |
| DW + AC soil              | 2              | 5.5 | 11 (0)      | 0.3 ± 0.8 | 0.9 |
| DW + BS soil              | 22             | 11.6| 254 (100)   | 6.4 ± 1.2 |     |
| DW + AC soil + nonane     | 32             | 15.7| 503 (100)   | 12.6 ± 1.6| 0.9 |
| DW + AC soil              | 3              | 5.3 | 16 (0)      | 0.4 ± 0.2 |     |
| DW + AC soil + nonane     | 22             | 19.5| 429 (57.2)  | 10.7 ± 1.7 | 0.1 |
| DW + BS soil              | 14             | 23  | 322 (42.8)  | 8.1 ± 1.8  |     |
| DW + BS soil + nonane     | 38             | 25.6| 972 (78.3)  | 24.3 ± 2.0 | 0.6 |
| DW + BS soil              | 24             | 11.5| 276 (21.7)  | 6.9 ± 1.1  |     |

*No. of pots per treatment: n=40

Table 3 - Mean number of larvae from a natural population of *Culex quinquefasciatus* that oviposited in clay pots filled with distilled water (DW), and distilled water + autoclaved soil (AC), distilled water + untreated soil (BS) and distilled water + nonane in a dual choice set-up in the field. Larval density
index (LDI), oviposition activity index (OAI) and $p$-values are shown.

| Oviposition substrate                | Pot positivity | Quantity of larvae in pots | OAI |
|--------------------------------------|----------------|---------------------------|-----|
|                                      | No. | LDI | No. (%) | Mean ± SE |
| DW + AC soil                         | 24  | 10.5| 252 (61.6)| 6.3 ± 0.9 | 0.2 |
| DW                                   | 20  | 7.9 | 157 (38.4)| 3.9 ± 0.7 |
| DW + AC soil                         | 22  | 9.6 | 210 (24.6)| 5.3 ± 0.9 | 0.5 |
| DW + BS soil                         | 32  | 20.1| 644 (75.4)| 16.1 ± 1.6|
| DW + AC soil + nonane                 | 40  | 20.6| 825 (78.3)| 20.6 ± 1.1| 0.6 |
| DW + AC soil                         | 20  | 14.9| 229 (21.7)| 5.7 ± 1.0 |
| DW + AC soil + nonane                 | 40  | 21.2| 847 (62.1)| 21.2 ± 1.3| 0.2 |
| DW + BS soil                         | 36  | 14.4| 517 (37.9)| 12.9 ± 1.0|
| DW + BS soil + nonane                 | 40  | 39.9| 1599 (79.4)| 40.0 ± 1.7| 0.6 |
| DW + BS soil                         | 34  | 12.2| 414 (20.6)| 10.4 ± 0.9|

*No. of pots per treatment: n=40

Figures
The mean number ± S.E. of larvae resulting from oviposition by a natural population of mosquitoes in a multiple choice set-up of oviposition containers. Asterisks indicate statistical differences from other treatments for a given species (*: p < 0.05, Friedman test). The blue bars represent anophelines while the red bars represent culicines.
Figure 2

Oviposition response of Anopheles gambiae s.l. to a substrate containing soil, nonane or soil + nonane in a dual-choice field study. Distilled water + autoclaved soil was used as control. Median and quartiles are given; asterisks indicate statistical differences between treatment and control for a given pair (*: p<0.05, Wilcoxon signed rank test).
Figure 3

Oviposition response of Culex quinquefasciatus to a substrate containing soil, nonane or soil + nonane in a dual-choice field study. Distilled water + autoclaved soil was used as control. Median and quartiles are given; asterisks indicate statistical differences between treatment and control for a given pair (*: p<0.05, Wilcoxon signed rank test).