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Electrophysiological and behavioural response of *Aedes albopictus* to *n*-heinecosane, an ovipositional pheromone of *Aedes aegypti*

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Abstract

Aedes aegypti (L.) and *Aedes albopictus* (Skuse) (Diptera: Culicidae) are highly anthropophilic mosquito species and potential vectors of dengue and yellow fever. The location of suitable sites for oviposition requires a set of visual, tactile, and olfactory cues that influence females before they lay their eggs. In this study, the effect of *n*-heneicosane, a recognized oviposition pheromone of *Ae. aegypti*, on the olfactory receptors of the antennae of *Ae. aegypti* and *Ae. albopictus* was studied using electroantennographic detection coupled to gas chromatography (GC-EAD). A significant electroantennographic response to *n*-heneicosane in adult females of both mosquito species was observed. In addition, gravid *Ae. albopictus* females laid more eggs in substrate treated with *n*-heneicosane at 0.1, 1, or 10 p.p.m. than in the control, denoting oviposition attractancy. Conversely, at 30, 50, 100, and 200 p.p.m., more eggs were laid in the control substrate, indicating oviposition repellency. Analysis of the larval cuticle by GC and mass spectrometry confirmed the presence of *n*-heneicosane in the cuticles of *Ae. albopictus* larvae. The species-specific role of *n*-heneicosane as an oviposition pheromone in *Ae. aegypti* and its significance as a behaviour modifier of *Ae. albopictus* in breeding sites is discussed.

Introduction

The decision where to oviposit is essential to maternal fitness in species of which the immature stages are unable to move to a suitable habitat if conditions become adverse (Onyabe & Roitberg, 1997; Spencer et al., 2002). Oviposition is one of the most important events in the life cycle of mosquitoes, requiring the integration of internal and external stimuli. Pre-oviposition and oviposition behaviours are controlled by various factors, including endogenous and exogenous factors, as well as inherent circadian rhythms. As such, mating and egg maturation are associated with endogenous factors, whereas selection of oviposition sites is associated with exogenous factors (Kennedy, 1978; Klowden, 1990). Moreover, in anautogenous mosquito species, the taking of a blood meal and subsequent maturation of eggs suppresses host-seeking behaviour and stimulates pre-oviposition behaviour such as searching for an oviposition site.

Most mosquito behaviours associated with feeding and reproduction/oviposition are mediated by chemical cues of a variety of origins (Takken, 1999). The way in which mosquitoes respond to behavioural cues depends on their age, size, and physiological status with regard to nutrition, digestion, and gonotrophic state (Klowden, 1996). The role of insect attractants and parapheromones in pest management was reviewed extensively by Renou & Guerrero (2000) and Plimmer et al. (1982), respectively, who placed emphasis on synthetic compounds. These authors found that oviposition aggregation pheromones could influence many insect females to lay their eggs in the same site, resulting in greater egg deposition. In mosquitoes, the first unequivocal evidence for an oviposition pheromone was found in *Culex tarsalis* Coquillett by Osgood & Kempster (1971); these researchers elucidated the difference between an oviposition attractant and an oviposition stimulant. The recognition of this difference is very important

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in bioassays of chemicals for possible use in mosquito control, as the usefulness of a compound to which mosquitoes orient from a distance (attractant) may be quite different from one to which mosquitoes respond only on contact (stimulant).

If oviposition is prevented at the individual level, the mosquito life cycle is disrupted and population growth reduced. In situations in which the number of mosquito oviposition sites is limited, oviposition repellents could be used to shield them against gravid females, which could then be attracted towards and induced to oviposit in lethal ovitraps (Xue et al., 2001). Pickett et al. (2010) emphasized the importance of investigating the chemical ecology of disease vectors, with the aim of developing more efficient tools for surveillance and control. These tools could lead to protective measures against virus transmission, and potentially be included in future strategies of integrated vector control.

Aedes aegypti (L.) and *Aedes albopictus* (Skuse) (Diptera: Culicidae) are container-breeding mosquitoes that commonly inhabit wooded suburban areas throughout the world. Both species are diurnally active, highly anthropophilic, and potential vectors of the dengue viruses to humans. Developmental stages of these mosquitoes inhabit artificial containers and natural sites close to human dwellings. In such situations, breeding sites can be treated with effective larvicides or oviposition repellents, as a component of an integrated approach to mosquito population management (Hwang et al., 1980; Schultz et al., 1982).

Previous studies have identified *n*-heneicosane, a C_{21} straight-chain hydrocarbon, in larval stages of *Ae. aegypti* and it was found to be highly attractive for gravid females to oviposit (Mendki et al., 2000; Seenivasagan et al., 2009). To understand whether the presence of *Ae. aegypti* larvae could influence oviposition behaviour of *Ae. albopictus* females (Allan & Kline, 1998), we wanted to study the role of *n*-heneicosane in the oviposition behaviour of *Ae. albopictus*.

In the present study, we compared the effect of *n*-heneicosane on olfactory receptors on the antennae of female *Ae. aegypti* and *Ae. albopictus*, using the electroantennograph (EAG) technique and gas chromatography coupled to electroantennographic detection (GC-EAD). Moreover, behavioural tests were performed to evaluate how the *Ae. aegypti* pheromone influences the oviposition of *Ae. albopictus*.

Materials and methods

Chemicals

The *n*-heinecosane used in the present study was purchased from Sigma Aldrich, St. Louis, MO, USA. For oviposition assays, all stock solutions of the chemical tested were dissolved in high-performance liquid chromatography (HPLC) grade *n*-hexane (Merck, Darmstat, Germany) and stored at −4 °C after each experiment. Dichloromethane (Cl_2CH_2) (Merck) and *n*-dodecane (C_{12}) (Sigma Chemical, Sydney, Australia) were used for chemical analysis of cuticular lipids as solvent and standard, respectively.

Mosquitoes

Aedes aegypti (derived from the Rockefeller strain from Venezuela) and *Ae. albopictus* (derived from the USDA strain from Gainesville, FL, USA) were used as susceptible reference strains. These colonies were reared since 1996 and 2010, respectively, in our insectary at 25 ± 2 °C, 80–90% r.h., and L12:D12 photoperiod and have been free of exposure to pathogens, insecticides, or repellents (Seccacini et al., 2006; Gomez et al., 2011). Larvae of both species were fed on a mixture of rabbit pellets and yeast. *Aedes albopictus* eggs were collected on a wet cardboard or paperboard and stored for at least 30 days in a Ziploc® bag, with low moisture conditions, in a chamber at 18 °C. Eggs of *Ae. aegypti* were collected on a wet filter paper, dried at room temperature, and stored under ambient conditions for at least 30 days. Stored eggs were submerged in dechlorinated water (500 eggs per 2 l water) at 25 ± 2 °C, and first instars were observed 24 h later. Pupae were transferred to 250-ml plastic containers and allowed to hatch in 20 × 20 × 20 cm acrylic cages. Adults were offered ad libitum water and raisins and fed on pigeons. The larval instars and the age of females used in each experiment are described below.

Gas chromatography coupled to electroantennographic detection (GC-EAD)

A Shimadzu GC-17A gas chromatograph (Kyoto, Japan) with flame ionization detector (FID) and manual injector was used. The carrier was high purity N_2, with a total flow of 9.0 ml per min and column flow of 1 ml per min, purge at 3 ml per min, and a split ratio of 5.

The column used was a Cyclosil-B [0.25 mm inner diameter (i.d.) × 0.25 μm thick film × 30 m long] (J & W Scientific Brand Agilent Technologies, Santa Clara, CA, USA). The chromatograph was modified internally with the addition of a needle valve (OSS-2, SGE), which split the flow from the column to the detector and the electroantennographic detector (EAD). This valve closed the front duct, thus regulating the ratio of sample directed to the FID and the EAG. The capillary tubes leading to the FID and the antenna were 35 cm long, 0.25 mm diameter, and coated on their inner walls with silica gel, deactivated to avoid undesired adsorption problems. In this way, the

compounds reached the antennae and the GC detector at the same time. Splitter valve was set so as to maximize the percentage of the flow output of the spine to the FID when GC experiments were conducted. To register GC-EAD signals on *Ae. aegypti* and *Ae. albopictus*, GC-EAD software (version 4.6 for PC; Syntech®, Kirchzarten, Germany) was used, which allowed us to acquire, analyse, process, and export the data. The volume injected into the column was 2 µl of a 1 mg ml^{-1} solution of *n*-heneicosane in *n*-hexane.

Electroantennogram

Females were immobilized on their dorsal surface in a notch on Plasticine® block using U-shaped copper wires. Glass microelectrodes were made from borosilicate glass tubing (2 mm outer diameter, 1.16 mm i.d.; Clark Electromedical Instruments, Reading, UK) using a microelectrode puller (PULL-I WPI®; Sarasota, FL, USA) and filled with Ringer solution (Roelofs & Comeau, 1971). The microelectrodes were held with micromanipulators (Leica, Wild Heerbrugg, Heerbrugg, Switzerland) and connected to high input impedance (10^{12} Ω) AC/DC micro-amplifier (Syntech) using Ag/AgCl junctions. The distal end of a cut antennal flagellum was inserted into the recording electrode and the reference electrode into the eye close to the other antenna. Amplified EAG responses were digitized using an IDAC-2 board and displayed and processed on a PC using EAG software (Syntech).

A pulse generator CS-01 (Syntech) connected to an air valve allowed the stimulation of the antenna using a 2-s pulse at an analytical airflow of 300 ml per min through a Pasteur pipette containing a filter paper soaked with a 0.04-µM solution of the test compound. A continuous flow of 500 ml per min of humidified air was directed to the antenna to prevent its drying out during the measurements. The system was connected to a grounding wire to reduce low-frequency electronic interferences. The sample was tested at least twice for each EAG preparation, and controls (25 µl *n*-hexane) were run before and after each sample. Mean EAG responses were divided by the mean control response taken before and after the analysis.

The peripheral olfactory response of 3- to 4-day-old female *Ae. aegypti* and *Ae. albopictus* mosquitoes was tested for *n*-heneicosane. Seven replicates on different individuals of each species were performed, based on the method described by Takken et al. (2001) and Puri et al. (2006), with a few modifications.

Oviposition experiments

Two coated wire frame voile cages (750 × 600 × 600 mm) were used for each replicate of the oviposition bioassays. For each dose tested, 3–4 replicates were done on different dates. One control jar and one treated jar (125 ml plastic jar, 10 cm diameter), each filled with 60 ml of de-chlorinated water, were used as oviposition substrates. Choice assays were performed, in which mosquitoes were allowed to choose between treated and untreated (control) oviposition substrates according to Ganesan et al. (2006), with a few modifications. Gravid *Ae. albopictus* females of 5–7 days old were used. The females were fed on pigeons twice: 2–3 days before and on the day when the assay started.

One ml of the stock solution containing the desired quantity of *n*-heneicosane for 0.1, 1, 10, 30, 50, 100, and 200 p.p.m. concentrations in hexane was placed in a 125-ml plastic jar, 60 ml tap water was added together with a piece of cardboard for oviposition. The eggs laid in the control and treated oviposition substrates were counted manually to assess the oviposition preference of the mosquitoes. The oviposition attractant/repellent property of the compound was expressed as the oviposition activity index (OAI) (Kramer & Mulla, 1979) calculated as: OAI = (Nt − Ns)/(Nt + Ns), where N denotes the mean number of eggs laid, in treated water (t) or in the control (s). Index values fall within the range of +1 to −1, with 0 indicating a neutral response. A positive value indicates that more eggs were laid in the treated substrate than in the control. Conversely, more eggs laid in the control than in the treated substrate result in a negative OAI value.

Chemical analysis of larval cuticle

Qualitative analysis was performed on a Shimadzu QP5000 equipped with a DB-WAX column (Agilent Technologies) (30 m × 0.32 mm i.d. × 0.25 µm film thickness). It was programmed from 60 to 240 °C at a rate of 5 °C per min. The initial and final temperatures were held for 2 min.

n-Heneicosane was quantified using a Shimadzu GC-17A with FID and autoinjector, using N_2 as carrier gas, with a total flow of 17.5 ml per min, column flow of 1 ml per min, and purge at 3 ml per min. The column was a 30-m VF-5 ms (Varian) wide bore glass capillary column (0.25 mm i.d. × 0.25 µm film thickness). It was programmed from an initial temperature of 60 °C (2 min) to reach 240 °C (2 min) at a rate of 10 °C per min. Samples were injected in splitless mode. For both Shimadzu chromatographs, the software for acquisition and data processing was GC Solution (version 13.10 for PC).

Third or fourth instar *Ae. albopictus* were used to perform the qualitative analysis of the cuticular wax. For extraction, we followed the technique of Phillips et al. (1988), with minor modifications. Fifty larvae were placed in a 2-ml glass vial, with 200 µl of Cl_2CH_2 at room temperature for 10 min to extract enough cuticular wax for

hydrocarbon analysis without contamination by the insect's internal lipids. The solution was concentrated by evaporating the solvent almost to dryness under a N_2 flow. The cuticular residue was resuspended in 2 µl dichloromethane for GC-mass spectrometry (MS) analysis. Pure *n*-heneicosane was used as standard for its identification.

Third or fourth instar *Ae. albopictus* and *Ae. aegypti* were used to perform a quantitative analysis of the cuticular lipids according to Kittayapong et al. (1990). In total, 800 mosquito larvae of each species were extracted with 5 ml of Cl_2CH_2 at room temperature for 10 min in a glass vial. The extract was quantitatively transferred to another glass vial. An internal standard of 20 µl of 400 ng μl^{-1} of *n*-dodecane was included in all samples and vortexed for 1–2 min. The solution was concentrated by evaporating twice to near dryness under a N_2 flow. The cuticular lipidic residue was resuspended in 0.5 ml of Cl_2CH_2 for GC analysis. The hydrocarbon peak corresponding to *n*-heneicosane was recognized by comparison with the standard. The concentration of *n*-heneicosane was calculated against a standard solution of 1 mg ml^{-1} in methylene chloride, using *n*-dodecane as internal standard.

Statistical analysis

Statistical analyses were conducted with STATISTICA 1999 edition for PC (StatSoft, Tulsa, OK, USA). OAI values were square root transformed and subjected to one-way ANOVA, followed by Tukey's test for post-hoc comparisons if significant effects were indicated.

Results

Electroantennogram response to *n*-heneicosane in *Aedes albopictus* and *Ae. aegypti*

Electroantennographic signal amplitude recorded for *n*-heneicosane at 1 mg ml^{-1} was 120 µV for *Ae. albopictus* and 200 µV for *Ae. aegypti* (Figure 1). On the other hand, the mean electrophysiological response to *n*-heneicosane was stronger in *Ae. aegypti* females than in *Ae. albopictus* (1.366 ± 0.567 vs. 0.342 ± 0.105 mV; t-test for independent samples: $t = -2.46$, d.f. = 7, $P<0.05$).

Oviposition response of *Aedes albopictus*

Oviposition response of *Ae. albopictus* was influenced by the concentration of *n*-heneicosane ($F_{6,21} = 199.6$, $P<0.05$; Figure 2). In the range of 30–200 p.p.m., OAI values were negative, that is, more eggs were laid in the control than in the treated substrate. When concentrations of 10, 1, and 0.1 p.p.m. were used, the OAI was positive, indicating that more eggs were laid in the treated than in the control substrates (Figure 2).

Chemical analysis of cuticular lipids

n-Heneicosane was identified in cuticles of *Ae. albopictus* by GC-MS comparison against a pure *n*-heneicosane standard (Figure 3). The total amount was 48.39 ± 27.78 pg per *Ae. albopictus* larva and 47.86 ± 11.30 pg per *Ae. aegypti* larva.

Discussion

Oviposition behaviour of gravid female mosquitoes can be influenced by volatile cues, both while the mosquitoes are orienting to a site and when they are resting on the water surface. Chemicals cues mediating attraction to a stimulus source must be volatile to act over a distance, whereas contact chemicals mediating behaviour, such as egg laying, can be either volatile or non-volatile (Sharma et al., 2008). Considerable evidence has accumulated that larvae can act as a stimulus source; some larval-produced oviposition attractants and stimulants may be pheromones (Bentley, 1989).

Aedes atropalpus (Coquillett) prefer to oviposit in water that previously held conspecific larvae (Kalpage & Brust, 1973) and *Ae. albopictus* females deposited significantly more eggs in cups containing larval water from either *Ae. aegypti* or *Ae. albopictus* compared with controls (Allan & Kline, 1998). *n*-Heneicosane, an oviposition attractant of *Ae. aegypti*, has also been identified in larval-conditioned water (LCW) and in the cuticles of this mosquito (Mendki et al., 2000). The presence of an oviposition attractant suggests an adaptive value in assembling females for oviposition at particularly suitable breeding sites. If the attractant is relatively stable, its concentration may increase as succeeding generations are reared successfully at the same site and females return to it for oviposition. *Aedes albopictus* may use the stimulus provided by its oviposition attractant to the exclusion of most other stimuli for selection of an oviposition site (Bentley, 1989).

A significant EAG response to *n*-heneicosane in 3- to 4-day-old *Ae. aegypti* and *Ae. albopictus* females was observed. For a concentration of 1 mg ml^{-1}, the response was higher in *Ae. aegypti* than in *Ae. albopictus*, although this difference in antennal sensitivity cannot be directly linked to a difference in behavioural effects.

Concerning the behavioural oviposition response of *Ae. albopictus* to *n*-heneicosane, compounds with an OAI of +0.30 and above are considered as attractants (Kramer & Mulla, 1979), whereas those with an OAI of −0.30 and below are considered as repellents. For *n*-heneicosane concentrations of 0.1, 1, and 10 p.p.m., positive mean OAI values of 0.2 and below were found, indicating that the oviposition activity of *Ae. albopictus* females was slightly influenced. Ten p.p.m. of *n*-heneicosane has been reported

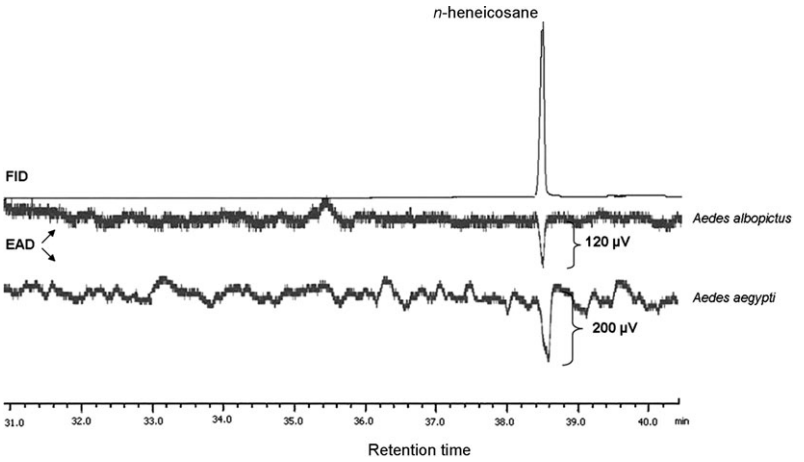

Figure 1 Gas chromatography coupled to electroantennographic detection (GC-EAD) of *n*-heneicosane in *Aedes aegypti* and *Ae. albopictus* females. The EAD signal (response of the antennae) and detector signal FID (flame ionization detector) of the gas chromatograph are shown.

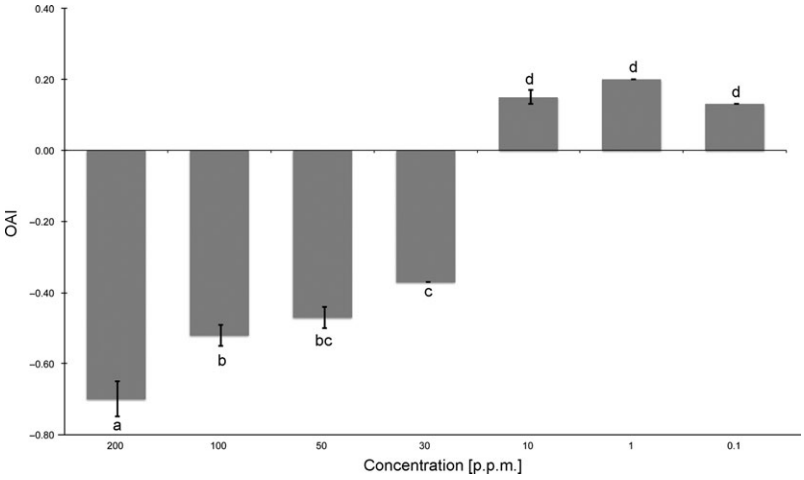

Figure 2 Mean (± SE) oviposition activity index (OAI) of gravid *Aedes albopictus* females in response to various concentrations of *n*-heneicosane. Means capped with the same letter are not significantly different (Tukey's test: $P>0.05$).

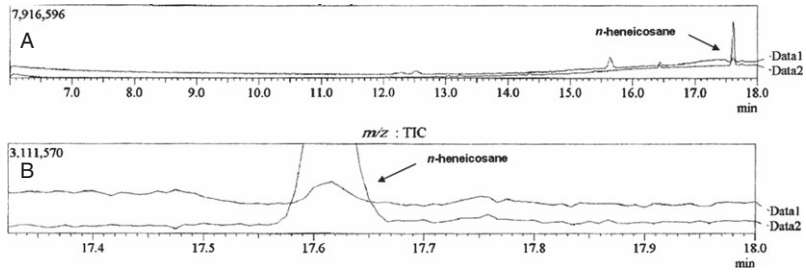

Figure 3 Chromatographic identification of *n*-heneicosane in the cuticle of *Aedes albopictus* larvae. (A) GC trace of larvae extract and standard. (B) Magnification at peak retention time. Data 1: GC trace of cuticle larvae. Data 2: GC trace of standard.

as an attractant for *Ae. aegypti* females (Seenivasagan et al., 2009). These findings agree with the EAG response of *Ae. albopictus* being lower than the *Ae. aegypti* response to the same concentration of *n*-heneicosane.

At concentrations above 30 p.p.m., egg laying decreased compared with controls, obtaining values of OAI between –0.37 and –0.71. This value suggests a repellent effect by *n*-heneicosane, as gravid females may perceive high concentrations of this compound as an indicator of larval crowding, and thus avoid oviposition in a less-viable breeding site; high intraspecific larval density and limited availability of food in the container causes a negative effect on individuals (Lord, 1998), consequently affecting the population growth of mosquitoes. Larval density also affects site selectivity. Rearing water with high larval density (900 larvae l^{-1}) was repellent to ovipositing *Ae. atropalpus* females reared under axenic conditions (Maire, 1985). In these cases, a chemical that acts as an inhibitor and is produced by larvae could have evolved as a mechanism regulating oviposition, making it less favour-

able and/or indicating that the site is overcrowded (Bentley, 1989).

Several C_{21} fatty acid esters have shown oviposition response of *Ae. aegypti* and *Ae. albopictus*. Hexadecyl pentanoate, tetradecyl heptanoate, and tridecyl octanoate showed significant oviposition-repellent activity against both mosquito species, whereas propyl octadecanoate was found to attract *Ae. aegypti* to the treated oviposition substrate at 1 and 10 p.p.m. (Sharma et al., 2008). We established that *Ae. albopictus* has a sensory antennal response to *n*-heneicosane and that this compound influences its oviposition behaviour. In addition, *n*-heneicosane was identified in the cuticles of larvae of this mosquito species, as has been previously reported for *Ae. aegypti* larval cuticle (Seenivasagan et al., 2009). Quantification in both *Ae. aegypti* and *Ae. albopictus*, however, showed great variability between samples. *n*-Heneicosane has been reported to act as a pheromone for *Ae. aegypti*; now, we found that *Ae. albopictus* also responds to it, but differently.

Aedes aegypti and *Ae. albopictus* are common vectors of human diseases and have similar ecological niches. The pheromone *n*-heneicosane could be involved in interspecific competition for oviposition sites, acting as a repellent to *Ae. albopictus* at certain concentrations.

Oviposition attractants or repellents have considerable potential for enhancing the sensitivity of ovitraps to control these mosquito species. The influence of *n*-heneicosane on the oviposition mechanism of *Ae. albopictus* could be a very useful tool to provide better ovitraps for control or surveillance of this vector.

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