Natural odor ligands for olfactory receptor neurons of the female mosquito Aedes aegypti: use of gas chromatography-linked single sensillum recordings

Majid Ghaninia1,2,*, Mattias Larsson1, Bill S. Hansson1,3 and Rickard Ignell1
1SLU, Department of Plant Protection Biology, 230 53 Alnarp, Sweden, 2Department of Plant Protection, College of Agriculture, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran and 3Max Plank Institute for Chemical Ecology, Department of Evolutionary Neuroethology, DE-07745 Jena, Germany

*Author for correspondence (e-mail: majid.ghaninia@vv.slu.se)

Accepted 16 July 2008

INTRODUCTION

Female Aedes aegypti are vectors of dengue and yellow fever. Odor volatiles are the predominant cues that drive the host-seeking behavior of Ae. aegypti. Odorant molecules are detected and discriminated by olfactory receptor neurons (ORNs) housed in sensory hairs, sensilla, located on the antennae and maxillary palps. In a previous study, we used odor volatiles that are behaviorally and/or electrophysiologically active for Ae. aegypti and other mosquito species to show that antennal ORNs of female Ae. aegypti are divided into functionally different classes. In the present study, we have, for the first time, conducted gas chromatography-coupled single sensillum recordings (GC–SSR) from antennal trichoid and intermediate sensilla of female Ae. aegypti in order to screen for additional putative host attractants and repellents. We used headspace collections from biologically relevant sources, such as different human body parts (including feet, trunk regions and armpit), as well as a plant species used as a mosquito repellent, Nepeta faassenii. We found that a number of ORN types strongly responded to one or more of the biological extracts. GC–SSR recordings revealed several active components, which were subsequently identified through GC-linked mass spectrometry (GC–MS). Electrophysiologically active volatiles from human skin included heptanal, octanal, nonanal and decanal.

Key words: Aedes aegypti, biologically active volatiles, electrophysiology, olfactory receptor neurons.
from the plant (Takken and Knols, 1999). A few host plant-related compounds have been shown to be detected by ORNs of mosquitoes (Bowen, 1992; Davis, 1977). Some plant species are, however, repellent to mosquitoes (Curtiss et al., 1991). Olfactory receptor neurons responsible for the detection of the active component(s) of these plants have not been reported.

In the present study, we have explored the specificity and sensitivity of gas chromatography-linked single sensillum recordings (GC–SSRs) from female *Ae. aegypti* in order to identify novel biologically active volatile compounds. Apart from the previously characterized trichoid sensilla (Ghaninia et al., 2007) we performed GC–SSRs from intermediate sensilla in order to expand our knowledge concerning olfactory coding in this species. In order to identify compounds potentially used by *Ae. aegypti* for orientation towards their human host, we collected volatile samples from feet, trunk (chest and urogenital) regions, armpits and urine. We also collected volatiles from catnip, *Nepeta faassenii* (Lamiaceae). Species within the genus *Nepeta* contain volatile compounds that act as strong attraction inhibitors to mosquitoes (Amer and Mehlhorn, 2006a; Amer and Mehlhorn, 2006b).

**MATERIALS AND METHODS**

**Mosquitoes**

Four- to 8-day-old, non-bloodfed female *Aedes aegypti* L. mosquitoes of the Rockefeller strain were used in our experiments. Larvae were reared in plastic containers (20×18×7 cm) and were fed with Tetramin fish food. Pupae were put in a small plastic cup and were transferred to cylindrical buckets (20 cm diameter × 30 cm height) in which 200–300 adults were kept under 28°C, 75% relative humidity and at a 12 h:12 h L:D photoperiod. Adults had access to 10% sugar water presented on a filter paper.

**Headspace samples**

Headspace samples were collected by placing odor sources in 31 polycarbonate oven bags, through which charcoal-purified air was circulated by means of an electric pump (KNF Neuberger, Stockholm, Sweden). Volatiles were trapped on filters with two compartments, containing 150+75 mg Porapak Q (Supelco) situated at the exhaust of the bag. Volatile collections lasted between 24 and 48 h. Extracts were prepared by rinsing filters with 800 μl of distilled hexane and concentrated to approximately one-third of the volume before use.

**Armpit odor sampling**

The method that we used for armpit sampling has been provided by Curran et al. (Curran et al., 2005). Ten volunteers (eight males and two females, 29–39 years old) were given two double-layer sterile gauze pads (7×10 cm) to attach under their armpits for two consecutive days. The volunteers were also instructed to follow their usual hygiene routine as B (Fig. 3A,B).

**Foot and trunk odor sampling**

Fifteen male and five female volunteers aged 25–45 years were subject to foot odor sampling. All volunteers were given fresh socks to wear for 48 h as they do in their daily life. Some of the volunteers performed physical exercise. To collect volatiles from trunk regions three males and one female volunteer gave us their undergarments. Headspace collections and extractions of the volatiles from feet (through the pooled socks) and trunk regions (through the pooled undergarments) were performed as described above.

**Urine odor sampling**

Urine from two male volunteers collected in a glass bowl was put into polyacrylate food bag for headspace collection.

**Plant volatiles sampling**

Whole, potted, *Nepeta faassenii* plants were placed inside the collection bags for plant odor collection.

**Mud volatiles sampling**

Mud samples were collected from two small standing water lakes located in the vicinity of the institute, in a plastic tray (20×18×7 cm). The tray was then conveyed to the institute and placed in collection bags.

**Electrophysiology**

**Mosquito preparation**

A female mosquito was cooled by placing it in a −5°C freezer for ~1-2 min and then glued to a piece of double-sided sticky tape on a microscope slide (76×26 mm). The animal was secured by covering half of the thorax and the abdomen by tape. The antenna was lifted and placed on a small coverslip (18×18 mm) bearing a piece of double-sided sticky tape. The antenna of the mounted animal was viewed through an Olympus light microscope (BX51W1), which allowed for a highly magnified (750×) view of the sensilla on all antennal segments.

**Single-sensillum recordings (SSR) and gas chromatography (GC)-linked SSRs**

Single sensillum recordings and GC–SSRs were performed according to standard protocols described by Stensmyr et al. (Stensmyr et al., 2003) and Ghaninia et al. (Ghaninia et al., 2007). Briefly, a sharpened tungsten microelectrode with a ~1 μm tip diameter was inserted into the eye. A second tungsten microelectrode was positioned at the base of a sensillum until electrical contact with the sensillum was established (Fig. 1). Action potentials of the ORNs housed in the sensillum were amplified through a USB-IDAC interface amplifier (Syntech, Kirchzarten, Germany), displayed on a computer screen and recorded for further investigations. SSRs were performed on previously characterized functional classes of trichoid sensilla (Ghaninia et al., 2007), as well as intermediate sensilla (Fig. 2). In order to identify the functional type of trichoid sensilla, we delivered a set of diagnostc compounds (see Ghaninia et al., 2007). After characterization, the activity of each biological extract was determined by stimulating the sensillum with 10 μl of each extract, pipetted on a piece of filter paper (5×20 mm) placed inside a Pasteur pipette. When an extract elicited responses from the ORNs, 2 μl of the extract was subsequently injected into a GC linked to the SSR recording setup via a heated transfer line (see below; Figs 1 and 3). Occasionally, contacts were lost before running the GC–SSR owing to inevitable environmental vibrations or animal muscle contractions, which may cause damage to the receptor neurons. Successful electrophysiological data were recorded and processed by means of Autospike 3 (Syntech). Spikes from neurons present in single sensilla were differentiated based on spike amplitude, where the larger amplitudes were denoted as A and the smaller amplitudes as B (Fig. 3A,B).

**Injections of the extracts**

Injections of the extracts were conducted on a HP 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) fitted with a splitless injector (220°C) and flame ionization detector (FID) (220°C). Compounds were separated on a polar capillary column DB-WAX (30 m×0.25 mm inner diameter coated with chromatographic film with 0.25 μm film thickness). Carrier gas was...
obtained.

injection with synthetic reference compounds when these could be

Technologies). Final confirmation of identity was achieved by co-

with references from mass spectral libraries (NIST05, Agilent

10 min.

temperature was held at 40°C for 2 min and then increased at 10°C min –1 to a final temperature

of 230°C, which was held for 10 min. The GC was fitted with a

split at the end of the column, delivering half the effluent to the

FID and the other half to the air stream flushing over the antenna
via a heated transfer line (230°C).

Chemical identification

Identification of active compounds in the extracts was performed
by means of coupled gas chromatography–mass spectrometry
(GC–MS). Each extract (2μl) was injected into a 6890N gas
chromatograph (Agilent Technologies) coupled to a 5975 mass
spectrometer (Agilent Technologies). Compounds were separated
on a polar capillary column DB-WAX (30 m×0.25 mm inner
diameter coated with chromatographic film with 0.25μm film
thickness). Carrier gas was helium (speed 36 cm s –1). The oven

temperature was held at 40°C for 2 min and then increased at 10°C min –1 to a final temperature of 230°C, which was held for

10 min.

The identity of active compounds was determined by comparison
with references from mass spectral libraries (NIST05, Agilent
Technologies). Final confirmation of identity was achieved by co-
injection with synthetic reference compounds when these could be obtained.

Dose–response relationships

For verification of the physiological activity of chemicals identified
through GC–MS, dose–response experiments were performed on
the responding cells with synthetic reference chemicals when these
could be obtained. The net response to a stimulus was quantified
as the number of spikes 0.5 s after stimulation minus 0.5 s before
stimulation. The outcome was then multiplied by two. Concentration of each synthetic compound ranged from 0.001 to 10% (v/v),
dissolved in paraffin oil. Delivery of the compounds and analysis
of the responses are described by Ghaninia et al. (Ghaninia et al.,
2007).

Synthetic compounds

Compounds used for physiological characterization of sensilla were
obtained from commercial suppliers (Ghaninia et al., 2007).

Synthetic references for confirmation of chemical identity and
dose–response experiments in this study were obtained from SAFC
(heptanal, +92%), Fluka (octanal, ≥98%; nonanal, ≥95%) and Sigma
decanal, 99%).

RESULTS

In the present study, we encountered nine of the 11 functional types
of antennal trichoid sensilla previously identified by Ghaninia et al.
(Ghaninia et al., 2007) (Table 1). A schematic drawing of all trichoid
sensillum types together with the approximate distribution of their
various functional types are shown in Fig. 2 (Ghaninia et al., 2007).

Most neurons had spontaneous activity ranging from 20 to 30Hz.

There appeared to be no consistent differences in spontaneous
activity between sensillum types; we would rather attribute
differences between individual sensilla to an effect of electrode
penetration discussed by Meijerink et al. (Meijerink et al., 1999).

Of the sensilla encountered in the present study, we managed to
perform 25 successful GC–SSR runs on the nine previously defined
trichoid sensillum types (Ghaninia et al., 2007) as well as on three
novel types of intermediate sensilla, which we term i-1, i-2 and i-3
(Table 1).

Based on the number of FID peaks, all extracts contained roughly
between 30 and 70 compounds (data not shown). Only four extract
types (feet, trunk, armpit and Nepeta) elicited a response from
antennal ORNs (Table 1), to a total of 12 FID peaks (components)
(Table 2). Examples of chromatograms produced from different
extract types, along with ORN responses corresponding to the peaks,
are shown in Fig. 4. Overall, eight responding compounds, i.e.
heptanal, octanal, nonanal, decanal, dodecanal, 2,6-dimethyl-2,6-
octadien, geranylacetone (6,10-dimethyl-5,9-undecadien-2-one) and
nepetalactone, were identified through GC–MS analyses (Table 2).

Four of these compounds were verified by commercially available
synthetic standards and their biological activity was confirmed by
dose–response experiments (Fig. 5). The mass spectra of four
physiologically active compounds could not be matched to any
reference mass spectrum and are listed as ‘unknown’ (Table 2).

Neither urine nor mud headspace extracts elicited a response in any
of the ORN types tested (Table 1). These extracts contained the same
complexity of peaks as seen in, for example, feet and trunk extracts
(data not shown).

THE JOURNAL OF EXPERIMENTAL BIOLOGY
Overall, ORNs were narrowly tuned to one or a few components present in the extracts. Of the short sharp trichoid (sst) sensilla, only sst-4 responded to one of the extracts tested (trunk extract).

However, damage to this sensillum during the recording process did not allow us to run a GC–SSR experiment, and this type was not found again during the experiments (Table 1). The ‘A’ neuron of the short blunt trichoid sensillum type I (sbtI-1-A) responded only to the Nepeta extract, with the active compound identified as nepetalactone (Table 2). The sbtII-2A cell detected the highest number of extract components (six in total): heptanal, octanal, nonanal, decanal, 2,6-dimethyl-2,6-octadien and geranylacetone (Table 2). None of the extracts elicited a response in long sharp (ls) trichoid sensilla (Table 1).

Based on GC–SSR analysis, we were able to define three novel functional classes of intermediate sensilla. These sensilla resemble the four distinct morphological types of the sensilla trichodea but vary in length (Davis and Rebert, 1972) (M.G., unpublished) and displayed unique responses to the tested extracts (Table 1). One of the intermediate sensillum types, i-1, responded to trunk volatiles, decanal and ‘unknown 2’ (Table 2). Five components, found in extracts of feet, trunk and Nepeta, activated the i-2A cell. We were able to identify two of these compounds as dodecanal and geranylacetone (Table 2). We observed a response of the i-2A neuron to the armpit extract but were unable to perform a GC–SSR run.

---

**Fig. 2.** (A-D) Schematic drawing of four morphologically distinct antennal trichoid sensilla of *Ae. aegypti* and (E) the approximate distribution, however, not the exact location, of their various functional types (Ghaninia et al., 2007) between the antennal segments. For the scanning electron micrograph of the sensilla, refer to Ghaninia et al. (Ghaninia et al., 2007). sst, short sharp-tipped; lst, long sharp-tipped; sbtI, short blunt-tipped I; sbtII, short blunt-tipped II; i, intermediate.

**Fig. 3.** Examples of recordings from receptor neurons showing spontaneous activity and responses of olfactory stimuli. (A) Spontaneous activity of two ORNs co-located in a short blunt type II trichoid sensillum, sbtII-2. (B) Inset showing 0.1 s of the spontaneous activity at higher resolution. Differences in spike amplitudes allow separation of two neurons, i.e. A (larger spikes) and B (smaller spikes). (C) Sensitivity of the neurons to the feet headspace extract was first tested by puffing it over the sensillum. Differences in spike amplitudes allow separation of two neurons, i.e. A (larger spikes) and B (smaller spikes). (D) Sensitivity of the neurons to the feet headspace extract was first tested by puffing it over the sensillum. Differences in spike amplitudes allow separation of two neurons, i.e. A (larger spikes) and B (smaller spikes). (E) Stimulation of the sbtII-2A cell with 0.1% octanal, identified through GC–MS analyses of the feet headspace extract, elicited an excitatory response. (E) Expanded view of the response to octanal. Horizontal scale bars: 0.5 s odor stimulation. For the blank test we used paraffin oil only.
The A-neuron of the third intermediate sensillum type, i-3A, responded to a single compound in the trunk headspace extract (Table 1), later identified as geranylacetone (Table 2).

Dose–response experiments
In order to evaluate the sensitivity of the identified ORNs to the novel ligands, we obtained dose–response relationships for two of the functional classes of sensilla, sbtII-2 and i-1 (Fig. 5A,B). This was conducted by exposing the sensilla to different concentrations of the synthetic compounds (Table 2, Fig. 5C-L; see also Materials and Methods). The most potent stimulus, nonanal, elicited a significant response at 0.01% (Fig. 5A). The sensitivity threshold for nonanal was close to 0.001%, whereas the thresholds for octanal, heptanal and decanal were 10- or 100-fold higher. The responses to nonanal and octanal peaked at concentrations of 0.1% and 1%, respectively, and thereafter a reduction or no change in the response to higher concentrations was observed (Fig. 5A). The 'A' neuron of the intermediate sensillum, i-1A, exhibited a dose-dependent response to decanal with a response threshold of 0.1% (Fig. 5B).

DISCUSSION
In the present study, we have, for the first time, investigated the applicability of the GC–SSR technique to identify biologically relevant ligands for ORNs of female Ae. aegypti. One of the strengths of this technique is that it does not rely on a priori assumptions about components of odor blends when selecting candidates for subsequent electrophysiological or behavioral evaluations. The GC–SSR technique has a higher resolution and sensitivity compared with GC-coupled electroantennographic detection (GC–EAD), another method commonly used to screen for biologically active compounds (Logan et al., 2008; Qiu, 2005).

Through systematic GC–SSRs from physiologically characterized sensilla, we have been able to identify eight natural odor ligands from different headspace extracts that are detected by the ORNs of female Ae. aegypti. All the compounds identified in this study, except

Table 2. Physiologically active compounds identified from the different extract types

<table>
<thead>
<tr>
<th>Extract type</th>
<th>Sensillum type</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feet</td>
<td>sbtI-2</td>
<td>Octanal*</td>
</tr>
<tr>
<td></td>
<td>i-2</td>
<td>Nonanal*</td>
</tr>
<tr>
<td></td>
<td>sbtII-2</td>
<td>Unknown 1</td>
</tr>
<tr>
<td>Trunk</td>
<td>sbtI-2</td>
<td>Octanal*</td>
</tr>
<tr>
<td></td>
<td>i-2</td>
<td>Nonanal*</td>
</tr>
<tr>
<td></td>
<td>sbtII-2</td>
<td>Unknown 1</td>
</tr>
<tr>
<td></td>
<td>sbtII-2</td>
<td>2,6-Dimethyl-2,6-octadien†</td>
</tr>
<tr>
<td></td>
<td>sbtII-2</td>
<td>Heptanal*</td>
</tr>
<tr>
<td></td>
<td>i-1</td>
<td>Decanal*</td>
</tr>
<tr>
<td></td>
<td>i-1</td>
<td>Known 2</td>
</tr>
<tr>
<td></td>
<td>i-2</td>
<td>Dodecanel†</td>
</tr>
<tr>
<td></td>
<td>i-2</td>
<td>Geranylacetone†</td>
</tr>
<tr>
<td></td>
<td>i-3</td>
<td>Geranylacetone†</td>
</tr>
<tr>
<td>Armpit</td>
<td>sbtI-2</td>
<td>Octanal*</td>
</tr>
<tr>
<td></td>
<td>sbtI-2</td>
<td>Nonanal*</td>
</tr>
<tr>
<td>Nepeta</td>
<td>sbtI-2</td>
<td>Nepetalactone†</td>
</tr>
<tr>
<td></td>
<td>i-2</td>
<td>Unknown 3</td>
</tr>
<tr>
<td></td>
<td>i-2</td>
<td>Unknown 4</td>
</tr>
</tbody>
</table>

sbtI, short blunt-tipped I; sbtII, short blunt-tipped II; i, intermediate sensilla.
*Identified by means of comparison with synthetic standard (mass spectrum, co-injection).
†Identified by means of comparison with mass spectral database.
for 2,6-dimethyl-2,6-octadien, which, to our knowledge, represents a novel component of human skin, have previously been reported to be present in human skin emanations or in Nepeta species volatiles (Bernier et al., 2000; Bernier et al., 2002; Curran et al., 2005; McElvain et al., 1941). An interesting observation is that heptanal, octanal, nonanal and decanal, which are present in either fresh and/or incubated human sweat (Meijerink et al., 2000), are detected by ORNs of Ae. aegypti (present study) but were not found to elicit a response in female An. gambiae antennal ORNs using the EAG technique (Meijerink et al., 2000). This observation may be due to low resolution of the latter technique and/or it might be linked to the partial divergence of the Ae. aegypti and An. gambiae olfactory receptor repertoire (Bohbot et al., 2007). Future studies, including heterologous expression and behavioral studies will have to be designed to address this issue. Although some weak electrophysiological responses of the maxillary palp-associated ORNs to the above-mentioned aldehydes were reported in An. gambiae and Culex quinquefasciatus (Lu et al., 2007; Syed and Leal, 2007), until recently almost nothing was known about the behavioral importance of these compounds in mosquito life. Recently, GC–EAD studies of human-derived headspace have revealed some compounds identical to those found in the present study. The compounds included octanal, nonanal, decanal, dodecanal and geranylacetone, to which mosquitoes responded behaviorally (Logan et al., 2008).

The origin of human-specific volatiles emanating from different body regions has been attributed to the aggregation of diverse communities of microbiota (Braks et al., 1999). It has therefore been suggested that differences in microbiota on the human skin play an important role in generating individual body odors, driving the attraction of mosquitoes to different host individuals and even different body regions (Braks et al., 1999). Quantitative as well as qualitative differences of specific body odors have been suggested to underlie this differential attraction (Bernier et al., 2002; Penn et al., 2006). In the present study, GC–SSRs revealed that Ae. aegypti ORNs responded to octanal, nonanal and decanal. These compounds have previously been reported to be present in differing ratio patterns between individuals, indicating qualitative similarities among individuals with quantitative differences (Bernier et al., 2002; Curran et al., 2005). By contrast, 2,6-dimethyl-2,6 octadien and 6,10-dimethyl-5,9-undecadien-2-one were found at physiologically active levels exclusively in trunk headspace extracts, indicating a qualitative difference between body regions. The latter compound has previously been reported to be present in most but not all human individuals (Bernier et al., 2005). In conclusion, the peripheral olfactory system of female Ae. aegypti contains ORNs capable of detecting compounds that could be used to differentiate between individual hosts and even body regions. Behavioral studies have to be conducted to verify the role of these compounds in the complete volatile blend that mediates host attraction.
In addition to responses to human volatiles, we observed responses to nepetalactone in sbtI1 sensilla. Nepetalactone is the primary component of catnip oil, the vapors of which have been shown to be repellent to a diverse number of insect species, including mosquitoes (Amer and Mehlhorn, 2006a; Eisner, 1964; Peterson and Coats, 2001; Peterson et al., 2002). In behavioral tests, nepetalactone acts as a 'spatial repellent', inhibiting the landing rate of *Ae. aegypti* and other mosquito species more than the commonly used synthetic mosquito repellent DEET (Bernier et al., 2005; Hui-Ling et al., 2006; Peterson and Coats, 2001).

Overall, very few ORNs associated with trichoid sensilla responded to the extracts tested. We assume that other sensillum types, i.e. grooved pegs as well as intermediate sensilla (as our study shows), might be involved in the detection of the current extract-associated components. Problems with odor collection/extraction of some human related compounds have also been reported (Bernier et al., 2000; Cork and Park, 1996).

To this date, laboratory and field studies indicate that the use of CO₂ is one of the few environmentally safe procedures to suppress mosquito densities (Knols et al., 1994; Knols et al., 1998). Although CO₂ plays an important role in attracting mosquitoes in the field, this compound is non-specific. CO₂-baited traps predominantly catch zoophilic mosquitoes whereas highly anthropophilic mosquitoes, which seem to require additional attractants, show limited attraction to the traps (Constantini et al., 1993; Knols et al., 1998; Mboera et al., 2000). Furthermore, application of CO₂ in the field is costly; it needs to be transported into the field in pressurized gas cylinders or as dry ice (Bernier et al., 2003; Curtis, 1996; Knols et al., 1994; Knols et al., 1998; Mboera et al., 2000). By contrast, the use of human-associated kairomones is considered as a good alternative method for collecting, monitoring or controlling host-seeking
mosquitoes, as these in a series of behavioral tests in the laboratory and field have shown to elicit high levels of attraction without the presence of CO₂ (Bernier et al., 2003; Edman, 1979; Eiras and Jepson, 1991; Eiras and Jepson, 1994; Gillies and Wilkes, 1974; Silva et al., 2005). The use of GC–SSRs and other analytical methods will be valuable for selecting additional kairomone compounds to optimize an attractive bait.

We are grateful to Gőran Bigraszer, Elisabeth Marling and Satoshi Okawa for technical assistance. We also thank anonymous volunteers of our experiments.

REFERENCES


