Sensory and behavioural responses of the malaria mosquito *Anopheles gambiae* to human odours

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Sensory and behavioural responses of the malaria mosquito *Anopheles gambiae* to human odours

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Contents

Summary

Malaria is one of the most serious human diseases, affecting between 300 and 600 million people per year and killing, on average, two children per minute. In tropical Africa the mosquito *Anopheles gambiae* Giles *sensu stricto* is responsible for much of the transmission of malaria parasites between humans. This mosquito species preferably feeds on human blood, rests inside human houses and breeds close to human dwellings, making it an effective malaria vector. The major cues guiding *Anopheles gambiae* females to their human hosts are volatiles emanating from the human body. The main aim of the present thesis was to investigate the chemical components in human emanations that play a role in the host-seeking behaviour of this mosquito species and how these human odours are perceived by the olfactory system of the mosquito. The knowledge obtained can be applied in developing odour-baited traps that can be used to protect humans from being bitten by mosquitoes or to decrease the chance of being bitten by mosquitoes and to provide an alternative for the traditional but questionable "human landing" method in the investigation of mosquito population size.

Glass beads to which skin emanations from human hands had been transferred elicited a level of attraction similar to a human hand (**Chapter 2**). The attractiveness of these handled glass beads faded away four hours after transfer onto the beads. The headspace of handled glass beads elicited a dose-dependent EAG response. Glass beads provided a suitable neutral substrate for the transfer of human odour to enable the investigation of behavioural and electrophysiological activities of *An. gambiae* exposed to these odours and to allow chemical analysis of the human skin emanations by gas-chromatography-mass spectrometry performed in a twin-project.

To study the chemical basis for the inter-individual differences in human attractiveness to mosquitoes, emanations from 27 human individuals, collected on glass beads, were tested against ammonia in a dual-choice olfactometer to establish the degree of attractiveness to *An. gambiae* (**Chapter 3**). There were clear differences in the trap entry response as well as in the attractiveness relative to that of ammonia between the skin emanations of different volunteers. Consistency of the differences was observed when emanations of the three most and the three least attractive volunteers were tested pair-wise. Electroantennogram responses to skin emanations from volunteers with different behavioural attractiveness were not in all cases positively related to the behavioural response level, suggesting the involvement of repellent components.

Summary

Stockings worn by humans were previously shown to be highly attractive to females of *An. gambiae*. The headspace of nylon stockings was collected and analysed with gas chromatography coupled on-line to electroantennography (EAG). EAG responses were detected consistently at 23 retention times, and 14 compounds that elicited such EAG responses were tentatively identified. These compounds, however, were not of typically human origin (**Chapter 4**).

Ammonia, L-lactic acid and a mixture of carboxylic acids were previously found attractive to *An. gambiae.* These compounds are all present in human skin odours, therefore a mixture of these components was studied in a dual-choice olfactometer (**Chapter 5**). Ammonia was an attractant on itself, whereas lactic acid alone was not attractive. Carboxylic acids, offered as a mixture of 12 compounds, were repellent at the concentration tested. The addition of ammonia to the carboxylic acid mixture overruled the repellent effect of the latter. Combining ammonia with either lactic acid or the carboxylic acid mixture did not enhance the attractiveness of ammonia alone. However, a synergistic effect was found when ammonia, lactic acid and the carboxylic acids were applied as a blend.

Human odour compounds that elicited electrophysiological or behavioural responses were tested in combination with ammonia + L-lactic acid against ammonia alone (**Chapter 6**). The results showed that C3-C8 and C14 carboxylic acids augmented the attractiveness of ammonia + lactic acid at certain concentrations, whereas alcohols, ketones, phenol, 4-ethylphenol and indole only reduced the attractiveness at the concentrations tested. For some compounds, no effect was found at any of the concentrations tested.

Based on the behavioural and electrophysiological findings, a field study in The Gambia (West Africa) was carried out to investigate the efficiency of mosquito traps baited with synthetic odour blends or human odour (**Chapter 7**). This study showed that odours released from counterflow geometry (CFG) traps baited with up to 9 compounds that were mixed during release were in many cases more attractive than odours from a tent occupied by a human. Carbon dioxide substantially increased the catch of the CFG traps for all mosquito species. CFG traps baited with the mixture of ammonia + lactic acid + 3-methyl butanoic acid + CO_2 resulted in the highest catches for most mosquito groups; the mixture is considered to be a promising candidate odour blend in the control of nuisance mosquitoes. Experiments with traps indoors showed that one odour mixture, consisting of ammonia + lactic acid + CO_2 + geranyl acetone + indole + 4-ethyl phenol was more attractive for *An. gambiae* than the control odour; this mixture holds promise for further experiments under conditions of higher *An. gambiae* abundance and for implementation in vector control programs.

Using a single sensillum recording method, an electrophysiological study on the olfac-

tory neuron responses of female *An. gambiae* mosquitoes was undertaken (**Chapter 8**). Six functional types of sensilla trichodea and five functional types of sensilla basiconica (grooved peg sensilla) were identified. "Generalist" ORNs that are tuned to a broad range of odours were found in sensilla trichodea subtype E, whereas "moderate specialist" ORNs that are tuned to a narrow range of odours were found in subtype C and grooved peg sensilla, with two "extreme specialist" ORNs tuned to only one odour. There was overlap in response spectra between sensilla trichodea E and C or grooved peg sensilla, but no overlap was found between sensilla trichodea C and grooved peg sensilla except that both responded to ammonia. Neurons associated with the same sensillum tended to respond to similar odour stimuli but with different sensitivities. Neurons in grooved peg sensilla were tuned to more polar compounds including the important behavioural attractant ammonia and its synergist lactic acid, responses to which were only found in grooved peg sensilla. Phenols were among the most effective stimulants for several neuron types belonging to different functional classes. Across-fibre patterning is the most plausible coding principle operating in the olfactory system of this mosquito species.

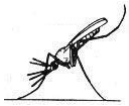
After a blood meal, female mosquitoes minimise host seeking activity and rest during egg maturation. To investigate whether the sensitivity of olfactory neurons changed after a blood meal and whether these changes correlate with the observed behavioural change, we compared the responses of ORNs in sensilla trichodea and grooved peg sensilla 2 - 24 h post blood meal with that of mosquitoes that had not fed on blood (**Chapter 9**). Three instead of two functional types of sensilla trichodea E were found following a blood meal. A functional type that had not been detected in mosquitoes deprived of blood was found repeatedly. The most responsive neuron of the "new" functional type of sensillum showed a high sensitivity to indole. This neuron was also highly responsive to C6-9 carboxylic acids and moderately responsive to the human-specific odour compounds 7-octenoic acid and 3-methyl-2-hexenoic acid. These results indicate that changes in sensitivity and response profile of ORNs as a result of a blood meal are involved in modulating behaviour of *An. gambiae* females.

The main conclusions from this thesis can be summarised as follows. This thesis provides additional evidence that chemical cues play a substantial role in the host attraction of *An. gambiae* (**Chapter 2**) and that skin emanations alone contribute significantly to inter-individual differences in attractiveness of humans to mosquitoes (**Chapter3**). The GC-EAG method can be used in the detection of kairomones used by *An. gambiae*, but a suitable substrate for collecting odours is essential (**Chapter 4**). Synergism was demonstrated to operate between ammonia, lactic acid and a mixture of carboxylic acids in attracting females of *An. gambiae* (**Chapter 5**) and olfactometric studies demonstrated the dose-dependent effects of

human odour components to *An. gambiae* in addition to ammonia and lactic acid (**Chapter 6**). The results of our field study provided evidence that mosquito traps baited with synthetic mixtures were superior to those baited with a human being, suggesting great potential of these traps in future malaria control programs (Chapter 7). Based on the response to several compounds, olfactory receptor neurones were classified into functional groups, providing fundamental information for future studies of these neurons (Chapter 8). Qualitative and quantitative changes were found in olfactory neuron responsiveness before and after a blood meal, suggesting the involvement of the peripheral nervous system in the modulation of mosquito behaviour observed in different physiological stages (Chapter 9).

1

Introduction



Anopheles gambiae sensu stricto, the major human malaria vector

Mosquitoes belonging to the genus *Anopheles* are haematophagous insects that act as vectors for parasites of the genus *Plasmodium*, the causal agents of malaria. Malaria is a prevalent parasitic tropical disease. It is by far the most serious infectious human disease next to HIV/AIDS and tuberculosis and threatens 40% of the world population (Snow *et al.*, 2005). The endemic regions of malaria are predominantly in Africa including more than 33 countries. Each year between 1 and 1.5 million people die from the disease, 90% of the victims being children under five years (Greenwood *et al.*, 2005). The parasite causing the most lethal form of human malaria is *Plasmodium falciparum*, which is transmitted from human to human by female mosquitoes belonging to the genus *Anopheles* (Diptera, Culicidae).

There are approximately 450 species of anophelines, of which 30 are major vectors, and another 30 are minor or local vectors of human malaria (White, 1982). The most effective vectors of malaria belong to the *An. gambiae* Giles complex, which contains four freshwater species (*An. gambiae* Giles *sensu stricto, An. arabiensis* Patton and *An. quadrianulatus* A and B Theobald), one mineral-water species (*An. bwambae* White) and two saltwater species (*An. merus* Dönitz and *An. melas* Theobald) (Hunt *et al.*, 1998; Coetzee *et al.*, 2000). In tropical Africa *An. gambiae s.s.* and *An. arabiensis* are the predominant species. *An. funestus* Patton, sympatric with members of the *An. gambiae* complex, is another efficient and important malaria vector in Africa. This species is not further discussed in this thesis.

To transmit human malaria, a female mosquito must bite a human individual carrying *Plasmodium* sporozoites and subsequently live long enough to allow the parasite to complete its sexual cycle after which it must bite another human being for transmission to occur (MacDonald, 1957; Charlwood, 1996). Thus, those species with greater longevity and with a closer association with humans tend to be more important vectors than short-lived species and/or species that are opportunistic in their host choice. *An. gambiae s.s.* is highly anthropophilic, i.e. it has a strong tendency to feed on humans. Where humans make up the majority of host resources, the human blood index (HBI) of *An. gambiae s.s.* is around 80-90% (White, 1974). Other members of the *An. gambiae* complex are opportunistic (*An. arabiensis*, *An. merus*, *An. melas*, *An. bwambae*) or zoophilic (*An. quadriannulatus* A and B) and of lower importance for malaria transmission. Locally, though, some can be important vectors. *Anopheles gambiae s.s.* is also endophilic, i.e. females tend to feed and rest indoors after a blood meal. Moreover *An. gambiae s.s.* is well adapted to breeding sites created by the activities of humans and the domestic animals close to their settlements (Curtis, 1996) and build up high populations around these places. This species is also highly susceptible to *Plasmodium* parasite infection, notably infections with *P. falciparum*. Furthermore, *An. gambiae s.s.* also has a high biting frequency and overlapping gonotrophic cycles are found both in the laboratory and in the field (Briegel & Horler, 1993; Beier, 1996). All these characteristics make *An. gambiae s.s.* a highly efficient vector of malaria. Because this thesis has *An. gambiae s.s*. as its main subject of study, I will henceforth refer to it as *An. gambiae*, unless otherwise stated.

Semiochemical-mediated behaviour of *Anopheles gambiae*

The life of a female mosquito comprises several major activities: foraging (sugar and blood), mating and ovipositing, which are all regulated by endo- and exogenous factors (Fig. 1). During these activities semiochemicals assist the female mosquitoes to find their sugar sources, blood-hosts, mating partners or oviposition sites (Takken & Knols, 1999).

Mating behaviour

Normally, mating of anopheline mosquitoes starts with a swarm formed by males above certain sites that are believed to act as visual sign-posts (Marchand, 1984; Yuval *et al.*, 1993). Copulation occurs when a virgin female flies into the swarm, and is caught by a conspecific male, which recognises the species–specific wing beat frequency she produces (Spielman & D'Antonio, 2001). Because swarms formed by male *An. gambiae* mosquitoes are rarely observed, it is possible that this species employs other cues for mate finding and recognition

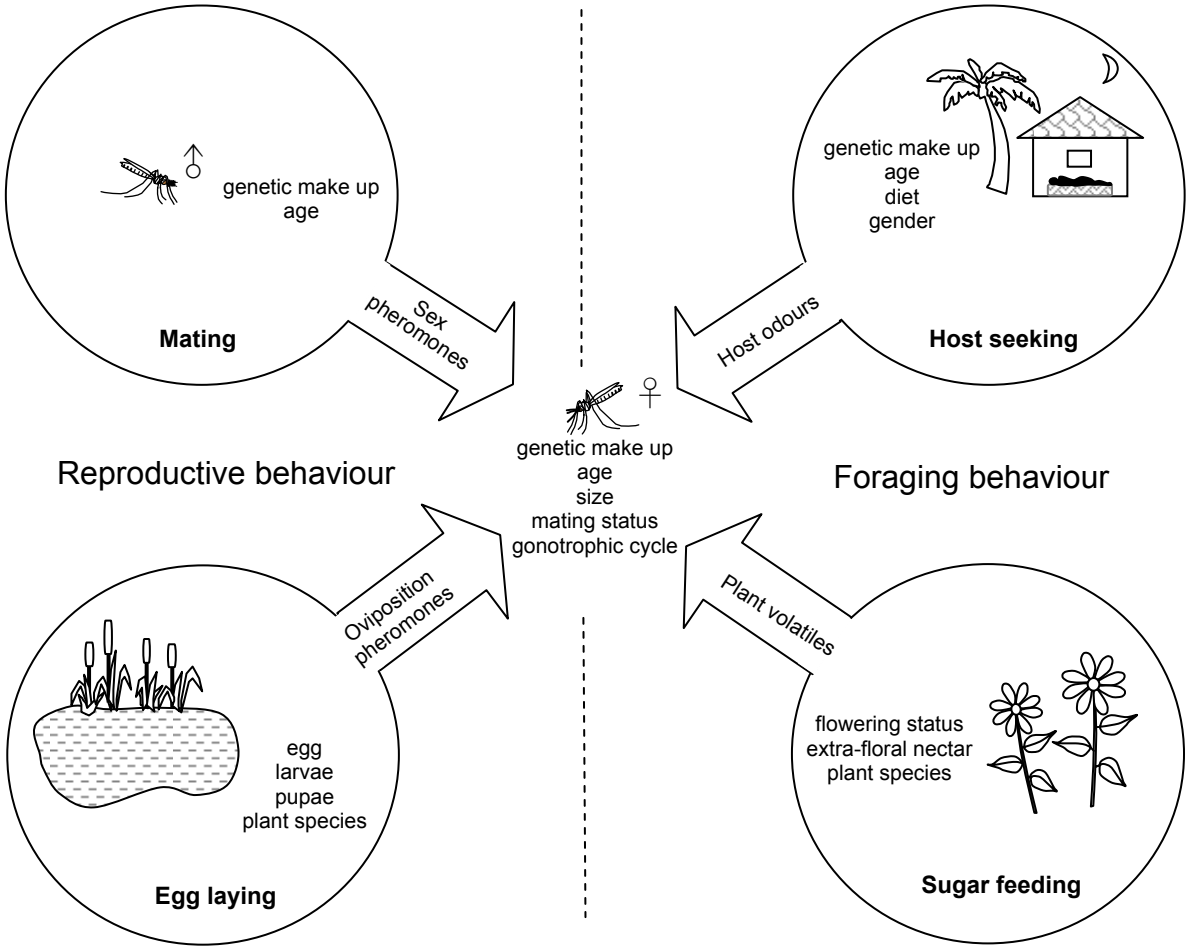


Fig. 1 The four major types of behaviour of adult female *Anopheles gambiae* and the semiochemicals involved. Modified after Takken & Knols (1999).

(Takken & Knols, 1999). Male *An. gambiae* are found attracted to nylon stockings worn by a human (R.C. Smallegange & H.V. Pates, personal communication), suggesting that males might make use of human odour to find females at their blood hosts. This behaviour has also been reported to occur in several culicine species (McIver, 1968). Species-specific contact pheromones were found for several mosquito species, a mechanism for the males to recognise their conspecific female mates upon contact (Kliewer *et al.*, 1966; Nijhout & Graig, 1971; Lang, 1977). At present, however, the role of semiochemicals in mating behaviour of *An. gambiae* remains speculative.

Searching for sugar sources

Both female and male mosquitoes use sugar, obtained mainly from floral and extrafloral nectar and honeydew, as a source of energy (Foster, 1995). Although female *An. gambiae* are seldomly found feeding on sugar sources in nature, there is evidence that females can find and use honeydew and floral nectar effectively. Young female adults (<5 days old) prefer the odour of honey over that of a human-worn sock, indicating a potential for using odours from natural sugar sources as baits (Beier, 1996; Foster & Takken, 2004; Gary & Foster, 2004; Impoinvil *et al.*, 2004).

Mosquitoes locate sugar sources mainly by using the associated visual and chemical cues. Generally, flowers with lighter colour attract more mosquitoes than darker colours and flowers with a stronger fragrance often attract more mosquitoes to visit them (see review by Foster, 1995). Flower extracts and synthetic plant odours were shown to attract mosquitoes (Healy & Jepson, 1988; Jepson & Healy, 1988; Hancock & Foster, 1993; Foster & Hancock, 1994). Information on the compounds in floral fragrances attractive to mosquitoes is scarce. The major compounds of floral fragrances include: aldehydes, alcohols, carboxylic acids, aliphatic esters, terpenes and their ketones, and aromatics, among which phenols. Olfactory neurons in three types of sensilla trichodea of *Aedes aegypti* L. were found to respond to several acyclic and monocyclic monoterpenes (Lacher, 1967). Neurons responsive to terpenes, green leaf volatiles, fatty acid esters and miscellaneous plant-derived compounds were found in various types of sensilla trichodea of *Culex pipiens* L. (Bowen, 1992). These studies suggest that the olfactory system of mosquitoes is able to detect volatiles of plant origin.

Host-seeking behaviour

Females of *An. gambiae* need human blood for the completion of reproduction (Takken *et al.*, 1998). Female mosquitoes are guided to their blood hosts by the physical and chemical cues emanating from the hosts. Heat, moisture and visual cues from blood hosts are perceived by searching female mosquitoes at a close range. (Gillies & Wilkes, 1968) reported that carbon dioxide could attract mosquitoes at distances of 18-36 m and natural odours from a calf at distances of 54-73 m.

Attractiveness of natural human odour sources

Human emanations or human secretions have been found attractive to *An. gambiae* both in laboratory and field studies. Braks and Takken (1999) reported that incubated human sweat

was attractive to *An. gambiae* and that most of this effect could be ascribed to the emission of ammonia produced by microbial activity in sweat. Differences in the abundance of components, such as indole, 1-dodecanol, 6-methyl-5-hepten-2-one and geranyl acetone, were found in the chemical composition of the highly attractive incubated and lowly attractive fresh sweat (Meijerink *et al.*, 2000).

Female mosquitoes were strongly attracted to nylon stockings worn by a human (Pates *et al.*, 2001b). The chemical basis of the high mosquito attractiveness to human-worn nylon stockings is not fully understood. GC-MS analysis of the extracts of Limburger cheese, which resembles, to a human nose, the smell of unwashed human feet, revealed the major components to be carboxylic acids (Knols *et al.*, 1997). A synthetic mixture of twelve of the carboxylic acids found in the cheese odour exerted variable attractiveness in different olfactometer tests (Knols *et al.*, 1997; Braks, 1999; Smallegange *et al.*, 2005). A diethyl-ether extract of human sweat in which 40 compounds were identified, elicited a landing response of *An. gambiae*. A synthetic mixture of 22 carboxylic acids that are major components of the extract did not produce a positive response (Healy & Copland, 2000). Although these results indicate a role of carboxylic acids in the attraction of *An. gambiae* to humans, consistent attractiveness is found only in combination with some other components in human odours. Glass beads that had collected human emanations through handling were shown to be attractive to females of *Ae. aegypti* (Schreck *et al.*, 1981).

Human odour is composed of a complex of volatiles released from human skin and human breath. GC-MS analysis of human skin emanation collected on glass beads revealed 346 compound peaks (Bernier *et al.*, 2000). More than 100 compounds were identified from exhaled human breath (Krotoszynski *et al.*, 1977). Ellin *et al.*, 1974 analysed the composition of total human body effluvia and identified 135 out of more than 300 compounds detected. Several single chemical components of human origin have been documented to attract *An. gambiae* (Takken & Knols 1999).

Attractiveness of individual human odour components

Carbon dioxide is exhaled by all warm-blooded vertebrates. Carbon dioxide partially accounts for the attraction of *An. gambiae* to humans (Snow, 1970; Healy & Copland, 1995; Costantini *et al.*, 1996a; Mboera & Takken, 1997; Dekker *et al.*, 2001). It is not surprising that a highly anthropophilic mosquito species, such as *An. gambiae*, does not totally rely on CO_2, a non-specific odour, to locate human blood hosts. Ammonia is an end product of the nitrogen metabolism of all animals and is used for host location by haematophagous arthropods (Rudolfs, 1922). Incubated human sweat was found to contain a higher concentration of ammonia and consequently had a higher pH value than fresh sweat, which was less at-

tractive to female *An. gambiae* (Braks *et al.*, 2001). The same study demonstrated that ammonia was attractive to female *An. gambiae* in a certain concentration range in the olfactometer when applied either on glass slides or from an air bag. It was hypothesized therefore that the higher attractiveness of incubated human sweat was mainly due to ammonia. For *Ae. aegypti*, ammonia alone was not attractive, but it augmented the attractiveness of L-lactic acid (Geier *et al.*, 1999). Ammonia either acts as host-associated attractant or aggregation pheromone for several other blood-sucking insects such as horseflies *Hybomitra spp.* (Hribar *et al.*, 1992; Kristensen & Sommer, 2000), the human body louse *Pediculus humanus* (Mumcuoglu *et al.*, 1987) and the haematophagous bug *Triatoma infestans* (Taneja & Guerin, 1997; Otalora Luna *et al.*, 2004). Ticks (Acari, Ixodidae) are attracted to ammonia and the receptor neurons for ammonia and other host odours are located in sensilla associated with Haller's organ (Sonenshine *et al.*, 1986).

Human skin emanations contain more lactic acid than emanations from other animals, therefore this compound is considered as a quantitatively characterizing human odour (Steib *et al.*, 2001; Dekker *et al.*, 2002). Lactic acid itself is an attractant for *Ae. aegypti*, and it also acts as a synergist and can increase the attractiveness of other human odours (Acree *et al.*, 1968; Smith *et al.*, 1970; Geier *et al.*, 1996; Steib *et al.*, 2001). In fact, adding lactic acid to animal odours that are originally not or slightly attractive to *Ae. aegypti* or *An. gambiae* can increase the attractiveness of these odours to a level similar to that of human odour (Steib *et al.*, 2001; Dekker *et al.*, 2002). In contrast to *Ae. aegypti*, lactic acid seems to play a less important role in the host seeking behaviour of *An. gambiae*. Braks *et al*. (2001) reported lactic acid to be attractive to *An. gambiae* at one concentration, but the removal of this compound did not affect the attractiveness of incubated human sweat. It has been reported that differences in CO_2 emission rates and L-lactic acid concentration on the human skin may explain differences between human individuals in the extent to which females of *An. gambiae* are attracted (Brady *et al.*, 1997; Dekker *et al.*, 2002).

Oxocarboxylic acids were found in human blood and urine (Chalmers & Lawson, 1982). Six oxocarboxylic acids were reported to stimulate landing responses of *An. gambiae* (Healy *et al.*, 2002). Chemical analysis revealed that the typical human axillary odour is composed of C_6 to C_{11} straight-chain, branched and unsaturated acids (Zeng *et al.*, 1991; Zeng *et al.*, 1996). A field study in Burkina Faso studied two of the axillary odour components, and showed that addition of 7-octenoic acid to carbon dioxide increased the number of *An. gambiae s.l.* attracted to odour-baited entry traps (Costantini *et al.*, 2001).

Single components or blends of kairomones tested thus far were in no case as attractive as the complex odour blend released by a human hand or entire body or skin extracts (Pates *et al*., 2001). Therefore, we do not yet know the key chemical compounds and their correct

blend ratio on which mosquitoes rely for locating human hosts.

Difference of humans in their attractiveness to mosquitoes

It is commonly observed that, when two persons are equally accessible, mosquitoes bite one person more often than the other. The mechanisms underlying such discrimination await elucidation. Many studies have documented the differential attractiveness of human individuals to mosquitoes (Muirhead-Thomson, 1951; Smith, 1956; Brouwer, 1960; Mayer & James, 1969; Carnevale *et al.*, 1978; Curtis *et al.*, 1987; Schreck *et al.*, 1990; Lindsay *et al.*, 1993; Knols *et al.*, 1995; Brady *et al.*, 1997; Mukabana *et al.*, 2004a). The differential attractiveness of human individuals is determined by both physical and chemical characteristics including body colour, body temperature, body moisture, body mass and body odour; these characteristics might in turn be affected by genetic background, gender, age, physical state and diet (Fig. 1).

Smart and Brown (1957) observed a higher landing frequency of *Ae. aegypti* on hands with a darker colour. These authors also reported that when *Ae. aegypti* is offered hands of two individuals with different temperatures it alights more often on the warmer hand. Schreck *et al*. (1990) studied human emanations collected on glass marbles and tested their attractiveness when kept at different temperatures. They demonstrated that although heat is not required to attract mosquitoes, warmed human skin residues attracted higher numbers of *Ae. aegypti*. A recent study showed that skin temperature and relative humidity significantly affect the individual attractiveness for *An. gambiae* with the following rules: when the temperature difference was larger than 0.5°C, the person with warmer skin was preferred by female *An. gambiae* whereas when the temperature differed less than 0.5°C, the person whose skin had a lower relative humidity was preferred by the mosquitoes (Mukabana, 2002). An earlier study with *Ae. aegypti* also showed that when hands or forearms with different surface humidity were offered, the drier one was more frequently visited (Smart & Brown, 1957; Gilbert *et al.*, 1966).

A positive correlation between human body mass or surface area and mosquito catch was found in several studies (Muirhead-Thomson, 1951; Spencer, 1967; Port *et al.*, 1980). Recently, it was reported that there is a positive correlation between pregnancy and attractiveness for malaria mosquitoes, indicating that pregnant women suffer a higher risk of malaria infection (Lindsay *et al.*, 2000; Ansell *et al.*, 2002; Himeidan *et al.*, 2004). Mukabana (2002) compared the attractiveness of two human individuals during a period when one of the individuals suffered from clinical symptoms of *P. falciparum* infection and suggested that malaria infection enhanced the relative attractiveness of a human individual to *An. gambiae*. Recently, this suggestion was proven correct by (Lacroix *et al.*, 2005) who demon-

strated that *Plasmodium*-infected children were significantly more attractive to *An. gambiae* than uninfected children.

Oviposition behaviour

Mosquitoes rely on visual, olfactory and tactile cues for locating oviposition sites (Beehler *et al.*, 1993; Dhileepan, 1997). Substances originating from mosquito larvae, pupae and eggs have been found attractive to gravid females (reviewed by Bentley & Day, 1989; Chadee, 1993; Zahiri *et al.*, 1997; Blackwell & Johnson, 2000).

Heneicosane has been identified as oviposition pheromone of larval origin for *Ae. aegypti* (Mendki *et al.*, 2000). An oviposition pheromone was identified from the egg raft of *Culex quinquefasciatus*, and a more volatile synthetic compound, (5R, 6S)-6-acetoxy-5-hexadecanolide, had an action similar to that of the natural compound (Laurence & Pickett, 1982, 1985). Mosquito breeding sites produce semiochemicals, mainly of microbial origin, that stimulate mosquito oviposition (Hazard *et al.*, 1967; Hasselschwert & Rockett, 1988; Beehler *et al.*, 1994). Infusions from decaying wood were found attractive to gravid *Ae. triseriatus* and an active component was identified as *p*-cresol (4-methylphenol) (Bentley *et al.*, 1979; Bentley *et al.*, 1981). Grass infusions were shown to contain oviposition stimuli for *Culex* mosquitoes, the attractive compounds include, among others, 3-methylindole, 4-methylphenol and indole (Millar *et al.*, 1992; Du & Millar, 1999). Female *C. quinquefasciatus* had a higher electrophysiological sensitivity for 3-methylindole than males (Blackwell *et al.*, 1993). A recent study showed a synergistic effect between (5R, 6S)-6-acetoxy-5-hexadecanolide and 3-methylindole (Olagbemiro *et al.*, 2004). Recent work by Sumba *et al.*, 2004 and Rejmankova *et al.*, 2005 inferred that olfactory cues associated with oviposition substrate affect oviposition site choice and egg-laying behaviour of anopheline mosquitoes.

Sensory physiology of mosquito olfaction

Morphology of olfactory sensilla of female *An. gambiae*

Olfactory receptor neurons (ORN) in insects are contained in sensilla, cuticular extensions of various shapes predominantly present on their antennae and mouthparts. The antenna of female *An. gambiae* mosquitoes carries two types of thick-walled sensilla chaetica and four types of thin-walled sensilla: sensilla trichodea, sensilla basiconica or grooved peg sensilla, sensilla coeloconica and sensilla ampullacea. Innervated by a bipolar neuron, sensilla chaetica are mechanosensilla that are sensitive to touch and air movement. Sensilla coeloconica and sensilla ampullacea are possibly innervated by thermoreceptor neurons (Davis &

Sokolove, 1975; McIver, 1982). Each antenna of female *An. gambiae* mosquitoes bears about 630 sensilla trichodea. Boo (1980) studied the fine structure of sensilla trichodea of *An. stephensi* and identified five types (A, B, C, D and E) according to the hair diameter, hair wall thickness, pore channel density and dendritic branching in the hair lumen. The same author also found that regardless of type, 95% of sensilla trichodea are innervated by two neurons, whereas sensilla with one neuron were found in low numbers on the distal flagellar segments. Each antenna of female *An. gambiae* bears 84 grooved peg sensilla, which are double-walled sensilla (Ismail, 1964; Steinbrecht, 1997). Two types were found in *An. stephensi* and these contain two and three-four olfactory neurons respectively (Boo & McIver, 1976).

Maxillary palps of anophelines posses three types of innervated sensilla: the sensilla chaetica, sensilla campaniforma and sensilla basiconica (capitate pegs); the first two types are mechanosensilla and the last one is an olfactory sensillum responding to CO_2 (McIver & Siemicki, 1975; Grant *et al.*, 1995).

Properties of olfactory sensilla

A typical insect olfactory sensillum is composed of one or several bipolar olfactory receptor neurons (ORNs), three auxiliary cells and the surrounding glia, epidermis and cuticle (Fig. 2). The surface of the insect cuticle is hydrophobic, a property that reduces water evaporation. Odour molecules, often non-polar, first must overcome the barrier of the sensillum wall before reaching the ORNs. The wall of single-walled olfactory sensilla contains pore channels through which the non-polar components are excreted to the epicuticle during the formation of sensilla. Remaning pore channels are later used for the transportation of the odour molecules into the sensillum lymph (Steinbrecht, 1997) (Fig. 3a, c). In contrast, a double-walled olfactory sensillum is composed of hollow cuticular finger-like structures (Fig. 3b), which are fused to each other and form spoke-channels at the fusion points. It is likely that odour molecules enter the sensillum lumen of double-walled sensilla via these channels (Steinbrecht, 1997)(Fig. 3d).

Olfactory transduction

Transduction is the process by which quality (molecular structure) and quantity (concentration) of odorants are converted to the neural code contained in the frequency and temporal patterns of action potentials. The nature of this process is considered to be common to all insects, although most information is derived from studies on Lepidoptera and the fruitfly, *Drosophila melanogaster*. The sensillum lymph of olfactory sensilla contains water

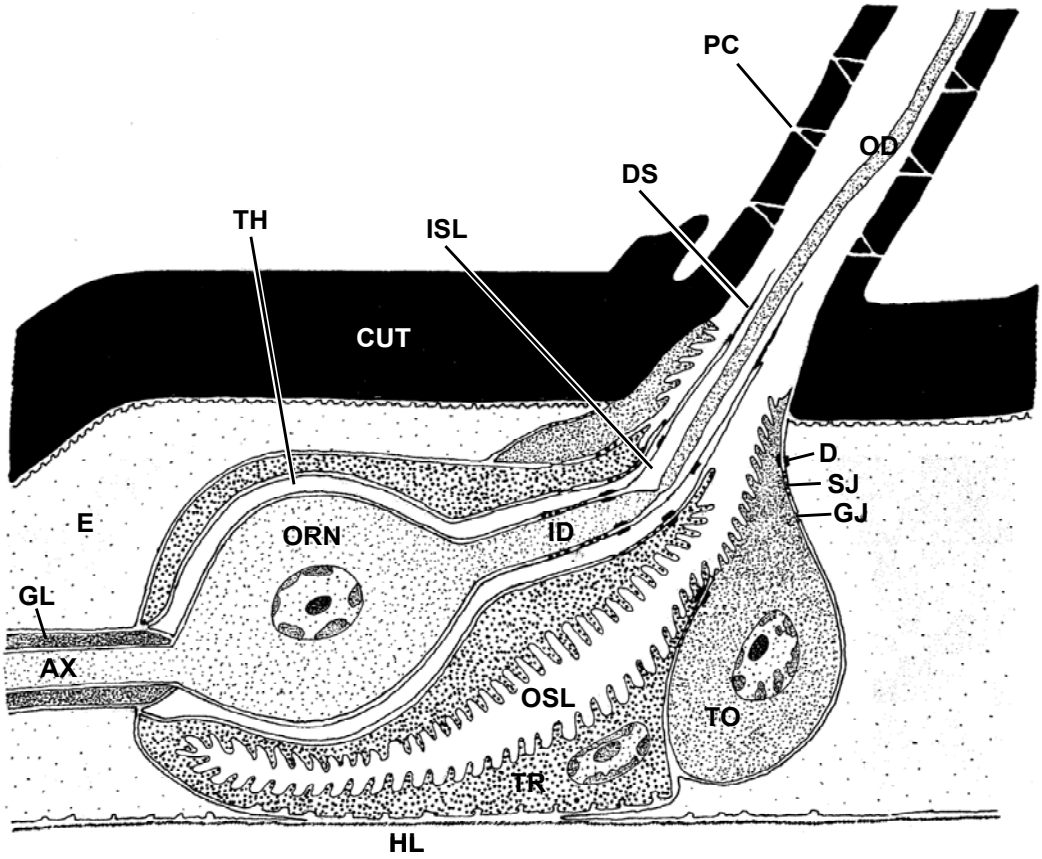


Fig. 2 Schematic drawing of the structure of typical olfactory sensilla. AX :axon CUT: cuticle; D: desmosomes; DS: dendrite sheath; E: epidermis; GJ: gap junctions; GL: glia; HL: haemolymph; ID: inner dendritic segment; ISL: inner sensillum lymph space; OD: outer dendritic segment=ciliary dendrite; ORN: olfactory receptor neuron; OSL: outer sensillum lymph space; PC: pore channel; SJ: septate junction; TH: thecogen cell; TO: tormogen cell; TR: trichogen cell. After Keil & Steinbrecht (1987).

soluble proteins, called odorant binding proteins (OBPs) that combine with odour molecules and transport them to receptor molecules on the dendritic membrane (Vogt & Riddiford, 1981; Klein, 1987). One type of OBPs are pheromone binding proteins (PBPs) that are found in male moths (Lepidoptera: Noctuidae) and bind specifically with sex pheromones (Vogt & Riddiford, 1981). Recently PBPs in some noctuid insects were found in both sexes (Vogt, 2002). Another type of OBPs that do not bind specifically with sex pheromones and that are present in both sexes are called general odorant binding proteins (GOBPs) (Vogt *et al.*, 1991; Laue *et al.*, 1994; Steinbrecht *et al.*, 1995). In moths, PBPs are found in the pheromone-sensitive long sensilla trichodea, whereas GOBPs are found mainly in sensilla basiconica that are sensitive to host-plant odours (Lee & Strausfeld, 1990; Vogt *et al.*, 1991; Laue *et al.*, 1994; Steinbrecht *et al.*, 1995; Kalinova *et al.*, 2001). The possible functions of

OBPs include: selective binding with odour molecules; binding with irrelevant or harmful compounds to reduce their chance of coming into contact with the dendritic membrane (Park *et al.*, 2000); selective transport of odour molecules to specific receptor molecules on the dendritic membrane and selective inactivation of odour molecules (Steinbrecht, 1998).

The completion of the genome sequence of *An. gambiae* (Holt & *et al*., 2002) enables the identification of putative genes encoding OBPs, based on the presence of six conserved cystein residues and a conserved spacing between the cysteins (Xu *et al.*, 2003). Some of the putative OBP genes are specifically expressed in the head of female *An. gambiae* and *An. arabiensis* (Li *et al.*, 2005). A study on OBP expression patterns in the sphingid moth *Man-*

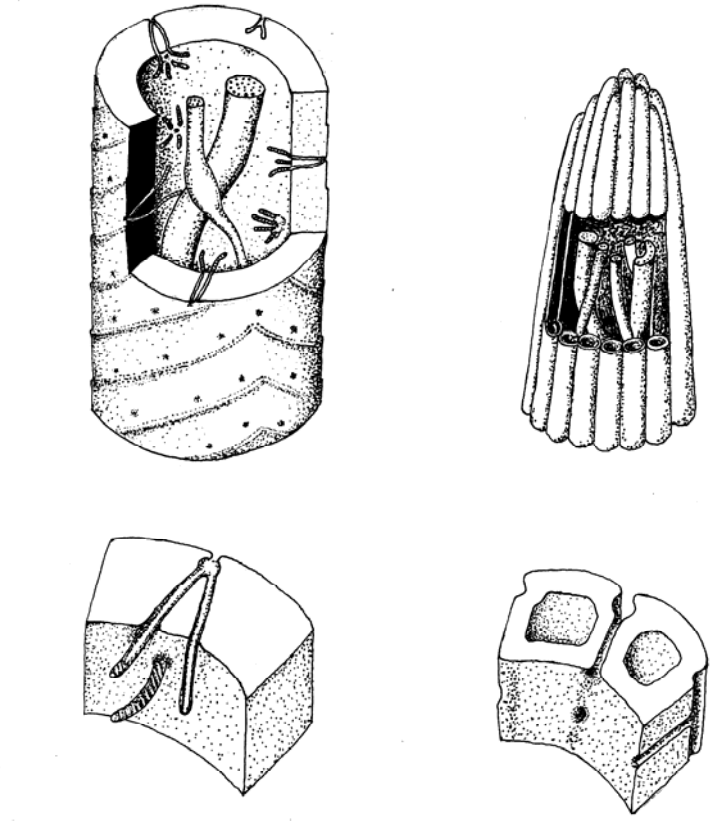


Fig. 3 Schematic diagrams of single- and double-wall structure of olfactory sensilla.A. section through a sensillum trichodeum (single-walled) and pore tubules. HW: hair wall; D: dendrite; P: pores. **b**. Coeloconic sensillum (double-walled), opened to show dendrites therein. The cuticular fingers (F) are hollow and partly fused. **c**. Detail of wall of sensillum trichodeum. PC: pore channel; PT: pore tubules. **d**: Detail of the double wall of a coeloconic sensillum. SC: spoke channels; F: fingers; GR: groove. After Keil (1999).

duca sexta suggested the co-expression of different types of OBPs in the same sensillum (Nardi *et al.*, 2003).

Odorant receptors (OR) are G-protein-coupled receptors with a seven transmembrane domain and are located on the plasma membrane of the olfactory neuron dendrite. When odour ligands bind with an OR, the receptor molecule changes it conformation and releases GTP, which in turn activates special enzymes to catalyse synthesis of second messengers including cyclic AMP and inositol 1,4,5-triphosphate (IP_3) (reviewed by Zwiebel & Takken, 2004). It is known from studies with male moths that pheromone application directly induces the increase of IP_3 concentration, a maximum is reached within 50 ms, followed by a decrease in concentration back to a normal level within 200 ms, the time range corresponding to that of action potential production (Boekhoff *et al.*, 1990; Breer *et al.*, 1990; Kaissling & Boekhoff, 1993). When the concentrations of the second messengers reach a certain level, the cAMP- or IP_3- specific Ca^{2+} channels open, leading to the influx of Ca^{2+} and Na^{+} (Stengl, 1994; Wegener *et al.*, 1997). When the depolarisation of the membrane reaches a threshold, action potentials are generated at the point where the axon exits the perikaryon and travel along the axon, which projects to the antennal lobe in the brain.

From the genome of *An. gambiae*, 276 G-protein-coupled receptors were identified and 79 of these receptors are considered as putative odorant receptors (Hill *et al.*, 2002). Up to 435 gene products are either up- or down-regulated after the ingestion of a blood meal by female *An. gambiae* (Holt & *et al*., 2002; Ribeiro, 2003). One of the putative OR genes (AgOr1) is strongly down regulated 12 h after blood feeding (Fox *et al.*, 2001). When AgOr1 was transferred to a *Drosophila* ORN of which the original OR was deleted, it was found specifically responsive to 4-methylphenol (Hallem *et al.*, 2004a).

Peripheral odour coding

Properties of odour coding

ORNs have been shown to encode odour quality, concentration and its temporal changes and spatial distribution (Heinbockel & Kaissling, 1996; de Bruyne *et al.*, 2001; Mustaparta, 2002). The function of various OR-genes has recently been studied by deleting an OR gene in *D. melanogaster* and replacing it with other OR genes (Dobritsa *et al.*, 2003; Hallem *et al.*, 2004b).

By using the single sensillum recording method, the action potentials of ORNs can be recorded *in situ*; their specificity and sensitivity in response to odour stimuli can be studied by quantifying their production of action potentials. ORNs selectively respond to odour compounds, their tuning being determined by the structural features such as chain length,

electron cloud distribution, the position of double bonds and functional groups of the odour molecule (Boeckh & Ernst, 1983; Liliefors *et al.*, 1985; Liliefors *et al.*, 1987; Hansson, 1995; Shields & Hildebrand, 2001). Not only the differences but also the overlap of the response spectra between different ORNs is important information for the central nervous system to discriminate the quality of an odour compound (Sass, 1978; dan Otter & van der Goes van Naters, 1993; de Bruyne *et al.*, 2001). Based on the response spectra of ORNs to a panel of odorant stimuli, sensilla can be classified into different functional groups (de Bruyne *et al.*, 1999; de Bruyne *et al.*, 2001). The spectrum of ligands that can bind to a certain identified OR can be determined by expressing only one OR in an ORN and recording its electrophysiological responses upon stimulation with the ligands (Hallem *et al.*, 2004b). Although it has been considered a general principle that only one OR is expressed in each ORN, a recent study demonstrated coexpression of two OR genes in one class of ORN, challenging this assumption (Goldman *et al.*, 2005).

In some cases ORNs respond to some odour stimuli by excitation whereas to other odours by inhibition of the spontaneous activity (de Bruyne *et al.*, 2001; Shields & Hildebrand, 2001). This possibility adds one more degree of freedom for odour coding. The process is controlled by the same ORN and can be explained by a simple model (Hallem *et al.*, 2004b). Without odour stimulus, ORs are present as two forms in homeostasis: active and inactive. Once an OR binds with a ligand that elicits an excitation response, the active form is stabilised and the firing frequency is increased; in case an OR binds with a ligand that elicits an inhibition-type response, the inactive form is stabilised and the firing frequency is decreased.

The responses of ORNs to odorants are concentration-dependent, and both absolute concentration and the change of concentration can be detected. However, a particular concentration of an odorant can not be detected without the identification of the quality of the odorant (Todd *et al.*, 1992). It seems that pheromone-sensitive-ORNs in lepidopterans are only sensitive to the concentration change (Kaissling, 1998). Carbon dioxide-sensitive receptor neurons of some insect species can detect both absolute and relative concentrations (Bogner *et al.*, 1986; Bogner, 1990; Bogner, 1992; dan Otter & van der Goes van Naters, 1992; Grant *et al.*, 1995). Grant *et al*. (1995) studied a CO_2-sensitive receptor neuron on the maxillary palps of *Ae. aegypti* and found that the range of the dose-response curve is beyond the naturally occurring CO_2 concentration range. More ORs are expressed when exposed to higher concentrations of odours, which might be a mechanism for insects to detect the absolute odour concentrations (Hallem *et al.*, 2004b).

Insect ORNs are housed in sensilla and in most cases two or more ORNs are co-compartmentalised in the same sensillum This provides the nervous system with one more dimension for odour coding (Todd & Baker, 1999). There is no evidence that co-

compartmentalised neurons interfere with each other in response to odour mixtures (Boeckh & Ernst, 1983; Akers & O' Connell, 1988). The fact that sex pheromone-sensitive receptor neurons in moths, which are especially sensitive to the ratio of often two major components, are commonly housed in the same sensillum, implies a function of co-compartmentalisation in discrimination of ratios between components of odour blends (O' Connell, 1975; Hansson *et al.*, 1987; Baker *et al.*, 1988; Cossé *et al.*, 1995).

Temporal characteristics of ORN responses to odours are another feature of odour coding, which is especially important in the odour discrimination while flying or walking upwind (Kennedy *et al.*, 1981; Baker, 1985; Kramer, 1992). A response is called phasic if the frequency of firing action potentials decreases abruptly shortly after the onset of the excitation response. A tonic response is characterized by an increase in firing frequency that outlasts the duration of stimulation. The temporal response characteristics of an ORN to a certain stimulus is independent of dosage (de Bruyne *et al.*, 2001).

Response of mosquito ORNs to odour stimuli

Relatively few studies have been published that addressed the functions of several types of olfactory neurons in sensilla trichodea and grooved peg sensilla. Subtypes C and/or E (Boo, 1980) of sensilla trichodea respond either by excitation or inhibition to short-chain carboxylic acids (C2-C6), 1-octen-3-ol and 3- and 4-methylphenol (van den Broek & dan Otter, 1999; Meijerink *et al.*, 2001). Subtype C of sensilla trichodea was most sensitive to geranyl acetone but also showed excitation responses to 3-methyl-butanol, 6-methyl-5-hepten-2-one and ammonia. Another subpopulation of sensilla trichodea subtypes C and E showed similar response patterns to the last three compounds but was most sentive to indole, while it was not responsive to geranyl acetone (Meijerink *et al.*, 2001). Grooved peg sensilla were reported to respond to polar compounds such as ammonia, lactic acid, acetone, butyl- and pentyl-amine as well as to complex odours such as incubated sweat, cow odour and Limburger cheese odour, which was perceived to simulate human foot odour (van den Broek & dan Otter, 1999; Meijerink *et al.*, 2001).

Van den Broek and dan Otter (1999) compared the sensitivities of ORNs in anophelines with different host preferences and found differences in the number and sensitivity of ORNs in sensilla trichodea responding to carboxylic acids and 1-octen-3-ol. When comparing the sensitivity of grooved peg sensilla to cow odour or Limburger cheese odour , no differences were found between *An. gambiae* and *An. quadriannulatus* (van den Broek & den Otter, 2000).

ORNs sensitive to L-lactic acid were found to innervate the grooved peg sensilla of *Ae. aegypti* (Davis, 1976). Lactic acid sensitivity was found to be down-regulated after female

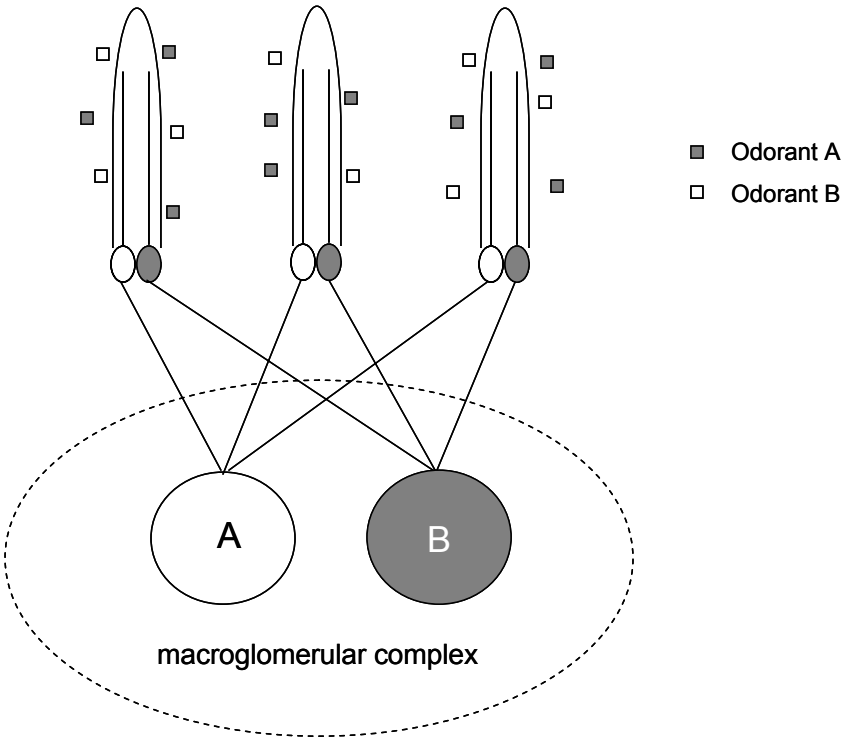


Fig. 4 Schematic diagram of neuron projections in labeled-line coding. Pheromone specific neurons sensitive to two pheromone components project to different areas in the macroglomerular complex.

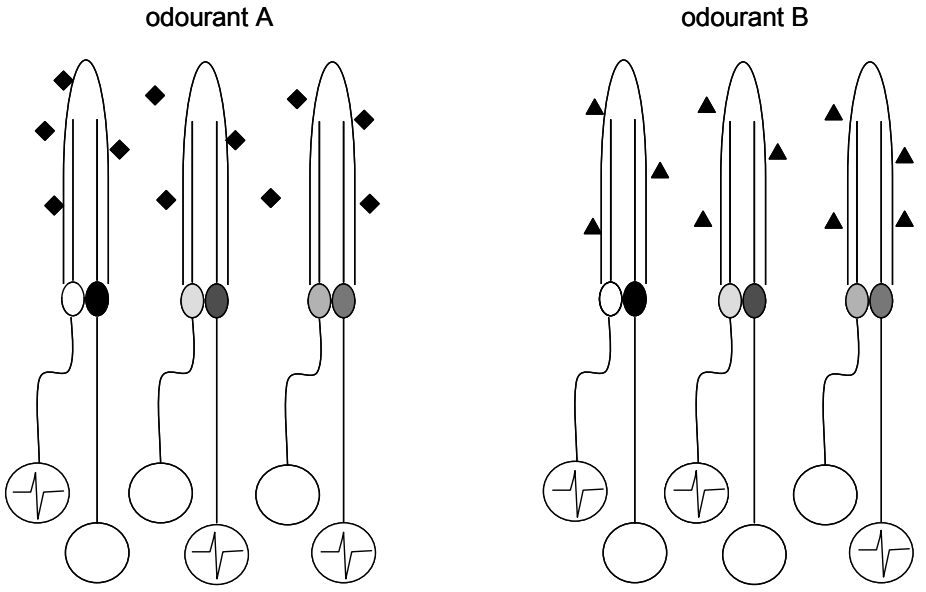


Fig. 5 Schematic diagram of neuron projections in across-fibre coding. Two odours (A and B) are perceived by ORNs and activate glomeruli with overlapping and difference, which enables the central nervous system to discriminate between these odours.

mosquitoes took a blood meal, suggesting an involvement of the peripheral olfactory system in the modulation of foraging behaviour of *Ae. aegypti* (Davis, 1984). Olfactory neurons responsive to carboxylic acids as well as plant- and oviposition site-related compounds have been described in sensilla trichodea of *Ae. aegypti, Ae. triseriatus, Culex pipiens* (Davis, 1977; Bentley *et al.*, 1982; Bowen, 1992).

For mosquitoes and biting midges (Diptera: Ceratopogonidae), a receptor neuron sensitive to CO_2 was found in sensilla basiconica on the maxillary palps (Kellogg, 1970; Grant *et al.*, 1995; Grant & O' Connell, 1996; Grant & Kline, 2003).

Olfactory processing in the antennal lobe

The first integration centre of olfactory information in the insect brain is the antennal lobe, which is composed of species-specific numbers of glomeruli, which are dense spheroidal neuropil structures. ORNs expressing the same olfactory receptor gene project their axons to the same glomerulus. Information about the quality of odorants can be coded using two distinct strategies, designated as labelled line and across-fibre patterning. Typical labelled line coding is found in the neural coding of sex pheromones in male moths (Dethier, 1976; Shepherd, 1985). For example, the sex pheromone of *M. sexta* consists of two major components (A and B). The specific receptor neurons for these components innervate the same sensilla and axons of these two types of neurons project separately to two substructures in the macroglomerular complex (Fig. 4). In labelled-line coding, each odour activates one glomerulus, therefore it is highly specific and extremely sensitive. Labelled-line coding has also been proposed for host volatile perception in some insect species (Anton & Hasson, 1994; Anton & Hansson, 1995; Roche-King *et al.*, 2000). Across-fibre patterning is considered to be the more commonly employed principle in host odour coding. In across-fibre coding, one odour activates more than one type of ORN and consequently activates more than one glomerulus, or different odours activate the same glomeruli resulting in overlapping patterns (Fig. 5) (Dethier, 1976; Shepherd, 1985). Glomerular activation patterns can be visualised with optical imaging techniques. The more similar the molecular structure of odours, the more overlap there is between the glomeruli they activate (Rubin & Katz, 1999; Wachowiak & Cohen, 2001; Sachse & Galizia, 2002; Meijerink *et al.*, 2003). By comparing the different patterns of glomeruli activated, the central nervous system can supposedly discriminate between different odours (Fig. 5).

ORNs in sensilla trichodea and grooved pegs of mosquitoes project to different glomerular areas of the antennal lobe (Anton *et al.*, 2003; Anton & Rospars, 2004). These findings correspond with functional differences between these sensilla. Although the majority of

ORN's are located on the antenna, carbon dioxide sensitive neurons contained in sensilla on the maxillary palp also project to the antennal lobe, but to a distinctly different area (Anton *et al.*, 2003).

Research aims and outline of this thesis

Research aims

The first aim of my research was (a) to develop a sensitive method for collecting human skin emanations, which cause responses by *An. gambiae* in behavioural and electrophysiological bioassays and are suitable for chemical analysis, (b) to investigate the differential attractiveness of human individuals to *An. gambiae* based on chemical components causing differences in attractiveness between human individuals, which might be either attractants or repellents. Chemical analysis of human odours will be the subject of a separate PhD thesis resulting from a twin project in the same research program (A.M. Galimard, in preparation). The second aim was to investigate olfactory neuron responses of *An. gambiae* to human odour components and the effect of a blood meal on olfactory neuron sensitivity. The third aim of this research was to screen for behavioural activity of human odour components and develop attractive odour blends to increase the efficiency of mosquito traps.

Outline of thesis

In order to develop a collection method for human skin odours suitable to demonstrate bioactivity for *An. gambiae* in behavioural and electrophysiological assays and to allow chemical analysis of the same samples, human emanations were collected on glass beads (**Chapter 2**).

Human individuals differ in their attractiveness to mosquitoes (see above) and these differences are rather consistent. It is unknown whether human odour alone causes the consistent individual differences for *An. gambiae* and which chemical components are causing the differences. To answer these questions, emanations from 27 human individuals, collected on glass beads, were tested against ammonia in a dual-choice olfactometer to establish this degree of attractiveness to *An. gambie* (**Chapter 3**). Chemical analysis of skin emanations with different attractiveness to *An. gambiae* has been carried out and will be reported elsewhere (A.M. Galimard, unpublished data)

Stockings worn by humans have previously been shown to be highly attractive to females of *An. gambiae*. The headspace of nylon stockings was collected and analysed with

gas chromatography coupled on-line to electroantennography (EAG)(**Chapter 4**).

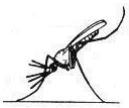
Ammonia, L-lactic acid and a mixture of carboxylic acids were previously found attractive to *An. gambiae.* These compounds are all present in human skin odours. Therefore, combinations of these components were studied in a dual-choice olfactometer (**Chapter 5**). Subsequently, human odour compounds that elicited electrophysiological or behavioural responses were tested in combination with ammonia + L-lactic acid against ammonia alone (**Chapter 6**). Based on the behavioural and electrophysiological findings, a field study in The Gambia was carried out to investigate the attractiveness of candidate odour blends to mosquitoes (**Chapter 7**).

In **Chapter 8**, an electrophysiological study on olfactory neuron responses of female *An. gambiae* mosquitoes is reported. Six functional types of sensilla trichodea and five functional types of sensilla basiconica (grooved peg sensilla) were identified. After a blood meal, female mosquitoes minimise host seeking activity and rest during egg maturation. To investigate whether the sensitivity of olfactory neurons changed after a blood meal and whether these changes correlate with the behavioural change, we compared the responses of ORNs in sensilla trichodea and grooved peg sensilla 2-24 h post blood meal with that of mosquitoes that had not fed on blood (**Chapter 9**).

Finally, all the findings in the experimental studies in this thesis are integrated in **Chapter 10**). Major conclusions are drawn and suggestions for further research are proposed.

2

Behavioural and electrophysiological responses of the malaria mosquito *Anopheles gambiae* to human skin emanations



Qiu, Y.T., Smallegange, R.C., Hoppe, S.,
van Loon, J.J.A., Bakker, E.-J. & Takken, W

Abstract

Behavioural and electrophysiological responses of *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) to human skin emanations collected on glass beads were studied using a dual-port olfactometer and electroantannography. Glass beads to which skin emanations from human hands had been transferred elicited a level of attraction similar to a human hand. The attractiveness of these handled glass beads faded away four hours after transfer onto the beads. Storage at -20°C for up to eight weeks showed a decreased but still attractive effect of the beads. In a choice test between one individual and 4 others, the emanations from the reference individual were significantly more attractive in 3 out of 4 cases. The headspace of handled glass beads elicited a dose-dependent EAG response. The substances

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causing EAG activity could be removed partially by dichloromethane, ethanol and pentane-ether. Glass beads provide a suitable neutral substrate for the transfer of human odour to enable chemical analysis of the human skin emanations for identification of kairomones of anthropophilic mosquitoes.

Introduction

Anopheles gambiae is one of the most important vectors of human malaria in tropical Africa, which is affected by the highest incidence of malaria. The importance of *An. gambiae* as malaria vector is largely due to its high degrees of anthropophily, endophagy and endophily, bringing it into the proximity of a human host (White 1974). This nocturnally active mosquito is guided to its human host predominantly by chemical cues discharged from the human body (Costantini *et al.*, 1996; Takken & Knols, 1999). Human emanations or human secretions have been found attractive to *An. gambiae*. Female mosquitoes were strongly attracted to skin emanations collected on nylon stockings (Pates *et al*., 2001). Braks & Takken (1999) reported that incubated human sweat was attractive to *An. gambiae* and that most of this effect could be ascribed to the emission of ammonia produced by microbial activity in sweat. A diethyl-ether extract of human sweat elicited a landing response of *An. gambiae* (Healy & Copland, 2000). Attraction of *An. gambiae* to several single chemical components of human origin has been documented. Carbon dioxide, which is exhaled by all warm-blooded vertebrates, is considered to contribute partially to the attraction of *An. gambiae* (Snow, 1970; Healy & Copland, 1995; Costantini *et al.,* 1996; Mboera *et al.*, 1997; Dekker *et al.,* 2001). Lactic acid was found to augment the attractiveness of other kairomones to two anthropophilic mosquito species, *Aedes aegypti* (L.) and *An. gambiae* (Acree *et al.*, 1968; Smith *et al.*, 1970; Geier *et al.,* 1996; Steib *et al.*, 2001; Dekker *et al.*, 2002). Differences in CO_2 emission rates and L-lactic acid concentration on the human skin may explain differences between human individuals in the extent to which mosquitoes are attracted (Brady *et al*., 1997; Dekker *et al*., 2002). Analyses of Limburger cheese volatiles, which to the human nose resemble human foot odour, revealed 19 aliphatic carboxylic acids (Knols *et al.*, 1997). A synthetic blend of these acids was attractive for *An. gambiae* when being highly diluted, whereas the undiluted mixture was repellent. Six oxocarboxylic acids were reported to stimulate landing responses of *An. gambiae* (Healy *et al*., 2002). Addition of the human-specific 7-octenoic acid to carbon dioxide attracted greater number of *An. gambiae s. l.* to traps in Burkina Faso (Costantini *et al*., 2001). In spite of these discoveries of *An. gambiae* kairomones, single components or blends of kairomones tested thus far were in no case as attractive as the complex odour blend released by a human hand, entire body or skin extract (Pates *et al*., 2001). Therefore we do not yet know the key chemical compounds and their

correct blend ratio on which mosquitoes rely for locating human hosts.

Human emanations attractive to *Ae. aegypti* can be transferred to glass beads by rubbing these beads in human hands (Bar-Zeev *et al*. 1977, Schreck *et al.,* 1981; 1990, Bernier *et al*. 1999). More than three hundred forty compounds have been identified in human hand emanations (Bernier *et al.*, 2000). Whereas collection of human emanations on glass beads proved to be useful for the study of kairomones of *Ae. aegypti*, it is not known whether such emanations also elicit behavioural responses in *An. gambiae*. In the present study we tested the behavioural and electrophysiological responses of female *An. gambiae* to human skin emanations collected on glass beads.

Materials and Methods

Insects

The *Anopheles gambiae s.s.* colony originated from Suakoko, Liberia (by courtesy of Professor M. Coluzzi, Rome) and was maintained under standard insectary conditions (27 ± 1° C, 80 ± 5% R.H., L:D 12:12h). Adult mosquitoes were held in a mesh cage (30´30´30cm^3) with access to 6% glucose solution. Female mosquitoes were blood fed from a human arm twice a week and eggs were collected on damp filter paper. The larvae were reared in tap water in plastic trays and fed daily with Tetramin® baby fish food. Pupae were collected daily and placed in adult cages. For the experiments 5 to 8-days-old females that had not been blood fed were used. The behavioural experiments were conducted during the last four hours of the scotophase. Experimental female mosquitoes were randomly collected 14 to 18 hours before the start of the experiments by suction tube and placed in a releasing cage with access to water via damp cotton wool placed on top of the cage.

Olfactometer

A dual-port olfactometer was used to study the response of An. gambiae to human emanations on glass beads. The olfactometer consists of a Luxan flight chamber of 1.60 ´0.66´0.43 m. Charcoal-filtered and humidified warm air with a speed of 0.22 ± 0.02 m/s was led through two Perspex mosquito trapping devices that were linked to two ports (diameter 4 cm, 28 cm apart) into the flight chamber (See Pates *et al.*, 2001). Dim light (1 Lux) was produced by one light bulb (75 Watt) and was filtered and scattered through a screen of yellow cloth hanging about 1 m above the flight chamber. The temperature of the experimental room was maintained at 28 ± 1°C and a relative humidity of 62 ± 5%. The temperature in-

side the flight chamber was equal to that of the experimental room and the relative humidity was maintained at 67 ± 4%. The relative humidity at the ports was maintained at 80 ± 4% and the temperature was 28 ± 1°C.

Behavioural experiments

Between 5 and 7 replicates were conducted for each sample tested. In each experiment the behavioural response of 30 mosquitoes over a period of 15 minutes was tested. Mosquitoes left in the olfactometer were removed by vacuum cleaner after each experiment. The mosquitoes trapped in both trapping devices were anaesthetised with CO_2 and counted. The operator wore surgical gloves to avoid contamination of the experimental equipment with skin residues.

Collection of human hand emanations

Clean glass beads with a diameter of 16 mm (behavioural experiments) or 2.9 mm (electrophysiological experiments) were handled for 10 minutes by rolling the beads in human hands which had been rinsed with water and dried with tissue paper 5-10 minutes prior to handling. Prior to treatment glass beads were cleaned by rinsing in non-perfumed soap (CLY-MAX Heavy Duty Cleaner, Rogier Bosman Chemie B.V., Heijningen, The Netherlands), distilled water and then in ethanol (Merck, 99.8% purity). Rinsed beads were dried in an oven at 200ºC.

Dose response to human-handled glass beads

Different numbers (0, 1, 2, 4, 6, 8, 10, 20) of glass beads (see previous section) handled by a human subject were placed in one of the mosquito trapping devices. An equal number of clean beads were placed in the other trap as a control. In addition to the testing of skin residues on beads, four fingers of a human hand were inserted through an entry slit behind one of the trapping devices (see Dekker *et al*., 2001) into the main air stream to test human-skin odour. Contamination of the inner side of the slit was avoided by wearing surgical gloves from which the fingers had been removed.

Residual Effect

1st experiment: Glass beads (6 beads, 16mm) were handled at different intervals (0h, 1h, 2h, 4h and 8h) prior to the experiments and their attractiveness to mosquitoes was compared

with that of clean beads. Handled beads were stored in sealed glass jars between handling and testing. The beads were then placed within one of the mosquito trapping devices and an equal number of clean beads were placed in the other trap.

2nd experiment: Glass beads were handled 0h, 1h and 24h prior to the experiments. Handled beads were stored at room temperature in sealed glass jars between handling and testing. The beads were then placed within one of the mosquito trapping devices and an equal number of clean beads were placed in the other trap. Beads handled 1h and 24h prior to the experiments were also tested directly against newly-handled beads.

Storage of glass beads at –20°C

Glass beads (6 beads, 16mm) handled by a human subject were placed in tightly closed glass jars and stored at –20 ± 1°C for periods of 1, 2, 4 and 8 weeks. The beads were then placed in one of the mosquito trapping devices and an equal number of clean beads, which had been stored under the same conditions, were placed in the other trap as control. Each treatment was repeated twice on the same day. The experiments were repeated over three days. The entire experiment was repeated twice.

Attractiveness of glass beads handled by different subjects

Six glass beads handled by one of four different people were tested directly against beads handled by a reference subject (the experimenter). Beads were handled as in the other experiments and placed in one of the trapping devices. Criterions for the inclusion of human subjects were: non-smoking, free from a chronic illness and not using medication of any kind on a regular basis, and (for women) not being pregnant. A day before the experiments the volunteers were not allowed to drink alcohol. Other dietary variables were not controlled.

Electrophysiological experiments

Human-handled beads and their extracts

A human subject handled small glass beads (2.9mm in diameter) for 10 minutes. Handled beads (8, 16, 24 and 32) were placed in a glass Pasteur pipette and the headspace was blown

directly over a mosquito antenna mounted in an electroantennogram (EAG) measuring circuit (see below). Thirty-two beads with a 2.9-mm diameter have a surface area equal to that of a 16-mm diameter bead. The handled beads were stored in a closed container at room temperature and tested again after 24h. Extracts were obtained from different numbers of handled beads by rinsing beads in 0.5 ml of one of three solvents (ethanol, dichloromethane or pentane-ether (2:1) mixture). Ethanol (99.8% purity), dichloromethane (>99.5% purity), pentane (>99% purity) and ether (>98% purity) were purchased from Merck KGaA, Darmstadt, Germany. Rinsed beads were placed in glass pipettes and tested for a residual effect. The solvent was then evaporated until about 50µl was left. The extracts were then transferred to the original number of clean glass beads and the remaining solvent was allowed to evaporate. They were subsequently put in glass Pasteur pipettes and the headspace was tested (see above). A dose of 0.1% 1-octen-3-ol in ether was used as standard stimulus.

Odour samples from two human subjects with different attractiveness

Two hundred small glass beads (2.9mm in diameter) were handled by two volunteers (SH and AG) with different attractiveness according to the behavioural tests (Table 2). Handled beads were rinsed three times in a mixture of pentane and ether (2:1). Handling and washing was repeated five times to produce about 12ml (total solution) containing skin emanation from each subject. Extracts were concentrated to approximately 200µl total volume by heating on a warm plate at 40^{o}C. Concentrated extracts as well as 1:2 and 1:4 dilutions of the extracts were tested for EAG activity. A standard stimulus of 0.1% indole in pentane/ether (2:1) and a blank of pentane/ether were also tested. To prepare the odour samples for EAG testing, a 25µl aliquot of each stimulus was transferred by a calibrated pipette onto a small strip of filter paper and the solvent was allowed to evaporate for 10 sec before the filter paper was placed inside a Pasteur pipette. Both ends of the Pasteur pipette were sealed with Parafilm® (Pechiney Plastic Packing, Chicago, USA) prior to and in between tests.

EAG recording

Mosquito head preparations excised from female *An. gambiae* (aged 5-8 days) were used for electrophysiological recordings. The excised head was mounted between two glass electrodes filled with 0.1 M KCl (Merck, >99%). AgCl-coated silver wires were inserted into the glass capillary, placed in a holder, and connected to a DC amplifier (10´; Syntech, Hil-

versum, The Netherlands). The indifferent electrode, which was grounded, was inserted through the cervix. The recording electrode was slid over the tip of the antenna, from which the tip segment had been removed. A moistened, charcoal filtered, continuous air stream (1000 ml/min) was led through a glass tube (1 cm diameter) ending 0.5 cm from the preparation. Stimulus puffs (300ml/min.) lasted for 0.5 sec and were applied using a stimulus controller (Syntech, Hilversum, The Netherlands) and were injected into the air stream, 10 cm from the outlet of the tube. The potential differences between the electrodes were imported via an IDAC interface box and an A/D converter (Syntech, Hilversum, The Netherlands) into an Intelâ 486-based personal computer. Recordings were analysed using EAG software version 2.6 (Syntech, Hilversum, The Netherlands).

Statistics

The relative attractiveness of two odours was analysed by comparing the total number of mosquitoes caught in the two trapping devices baited with these odours in 5-7 replicates using a Chi-square test. A Generalized Linear Model with binomial function (GLM; Genstat for Windows, release 4.2, fifth edition) was used to investigate the effect of treatments on two parameters:

1. The relative attractiveness: the effect of a treatment on the percentage of the mosquitoes caught in the treatment trap relative to all mosquitoes responding.

2. The total response: the effect of a treatment on the number of female mosquitoes caught in both trapping devices as percentage of mosquitoes that flew out of the release cage.

The treatments investigated included: number of beads, time between handling and testing, and storage time in freezer on the attractiveness of human-handled beads. Two-sided t-probabilities were calculated to test pair-wise differences of means. Effects were considered to be significant at $P<0.05$.

EAG response was transformed into percentage relative to the standard stimulus. Differences between treatments were analysed by General Linear Model, Univariate procedure (SPSS for Windows, release 10.0.5). The effects of human-handled beads or its elution or the residual after elution are fixed factors in separate linear regression models. The other two factors considered in each model are the number of beads and different mosquito antennae (replicates). Interactions between the fixed factors are excluded from the model when the interaction effect is not significant. The effect of a fixed factor was tested by the F statistic and was considered to be significant when $P<0.05$. The differences between means were

compared pair-wise by Tukey's honestly significant difference test. The effect was considered significant when $P<0.05$.

Results

Behavioural experiments

Dose response to human- handled glass beads

When no odour was present 13% of the mosquitoes flew into the traps without a preference for either of the two traps (chi-square test, $df=1$, $P=0.886$). From this we concluded that the olfactometer was symmetrical.

One glass bead handled by a human subject attracted more mosquitoes than one clean bead (Fig. 1, chi-square test, $df=1$, $P=0.007$). Two handled beads also attracted more mosquitoes than two clean beads (chi-square test, $df=1$, $P=0.0004$), but the relative attractiveness was not higher than that of one handled bead (GLM, $df=1$, $P=0.125$). Four, 6, 8, 10 or 20 handled beads were significantly more attractive than similar numbers of clean beads (chi-square test, $df=1$, $P<0.001$) and the relative attractiveness exhibited by these numbers was greater than that of one bead. The total responses of mosquitoes in experiments with 2, 4, 6, 8, 10 and 20 beads were not significantly different from each other (GLM, $df=1$, $P>0.05$). The odours released from four human fingers attracted more mosquitoes than no odour (chi-square test, $df=1$, $P<0.001$) or one human-handled bead (GLM, $df=1$, $P=0.003$). The odours released from four human fingers were equally attractive as 2, 4, 6, 8, 10 and 20 glass beads handled by the same subject (GLM, $df=1$, $P>0.05$).

To standardise further behavioural tests, six glass beads (16-mm diameter) were used in each olfactometer test.

Residual effects

Human-handled beads tested immediately following collection of skin emanations and those tested 2h later attracted more mosquitoes than clean beads (Fig. 2). No attractive effect was found for handled beads aged 4 and 8h after handling compared with clean beads (chi-square test, $df=1$, $P>0.05$). Handled beads tested at 0 and 2 hours attracted more mosquitoes than handled beads tested 8 hours later (GLM, $df=1$, $P<0.05$) but did not attract more mosquitoes than handled beads tested 4 hours later (GLM, $df=1$, $P>0.05$). In a separate experi-

ment handled beads "aged" for 1 hour attracted more mosquitoes than clean beads (chi-square test, df=1, P<0.001; Fig. 3), whereas the "aged" beads attracted fewer mosquitoes than newly handled beads (chi-square test, df=1, P<0.001). Handled beads "aged" for 24 hours were significant less attractive than newly handled beads (chi-square test, df=1, P<0.001). These same beads were no longer attractive when tested against clean beads (chi-square test, df=1, P=0.046).

***Storage at* –20ºC**

Handled beads that had been stored at -20 °C for 1, 2, 4 and 8 weeks were more attractive than clean beads that had been similarly stored (Table 1, chi-square test, df=1, P<0.05). All stored beads attracted fewer mosquitoes than newly handled beads (GLM, df=1, P<0.005).

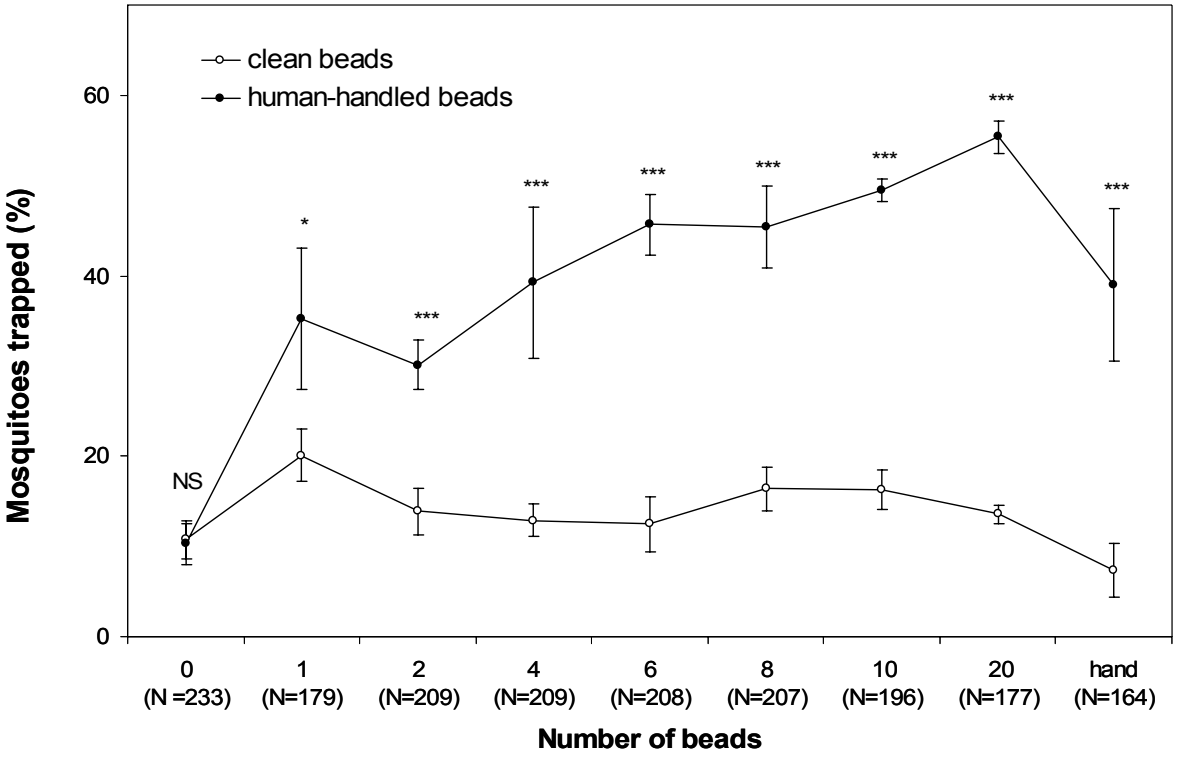

Fig. 1 Dose response of female *An. gambiae* to 16-mm diameter glass beads handled by a human subject and to odours released from four fingers of the same subject. Error bars represent standard errors of the mean; N: total number of mosquitoes that were released in the olfactometer; *: chi-square test P<0.05; ***: chi-square test P<0.001; NS: chi-square test P>0.05.

No differences in relative attractiveness were found between handled beads stored for different weeks (GLM, *df*=1, *P*>0.05). The total response in the experiments with newly treated beads was higher than in all experiments with human-handled beads stored at -20°C (GLM, *df*=1, *P*<0.001). The total responses in experiments with handled beads stored for 2 weeks were higher than in experiments with beads stored for 1 week (GLM, *df*=1, P<0.001). The 2-week storage treatment also evoked a higher total response than beads stored for 4 and 8 weeks (GLM, *df*=1, *P*<0.001). No *differences* were found for the total response in experiments with no odour and handled beads stored for 4 or 8 weeks (GLM, *df*=1, *P*=0.387).

Attractiveness of glass beads handled by different human subjects

Glass beads handled by a reference human subject (SH) were tested against beads handled by four other individuals in a two-choice assay. Three out of four volunteers attracted fewer mosquitoes than the reference subject did (chi-square test, *df*=1, *P*<0.05) (Table 2) and one

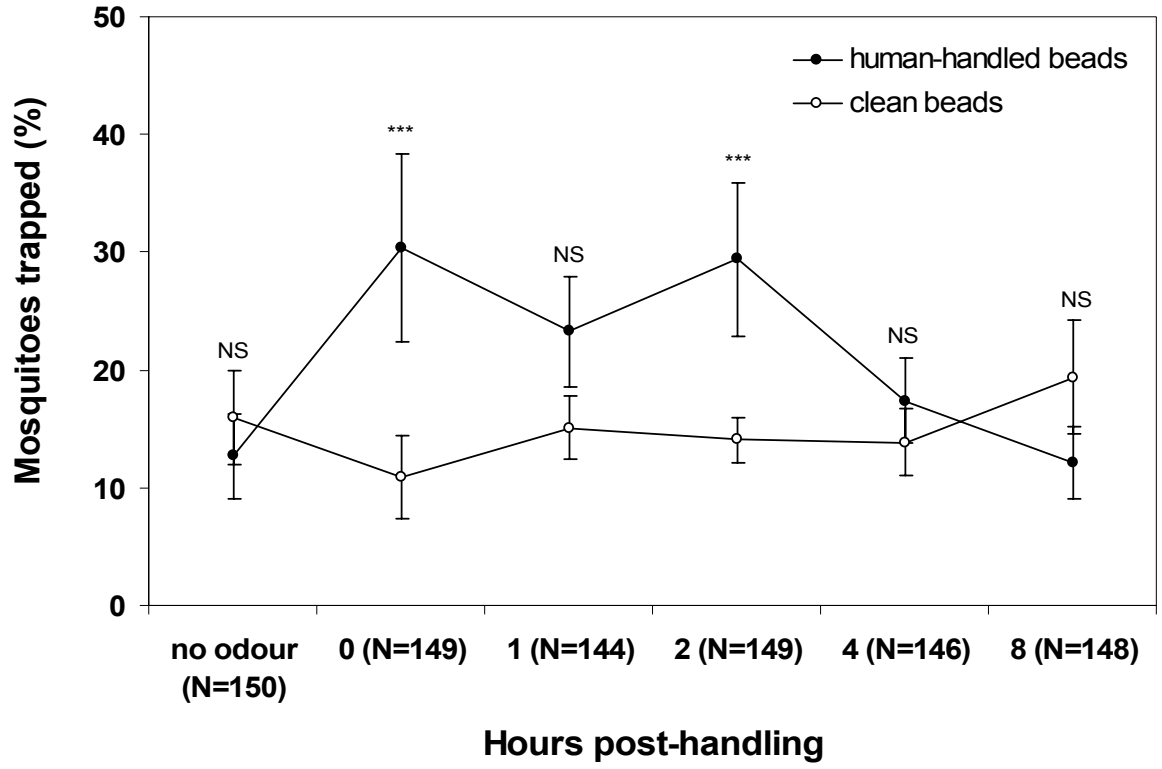

Fig. 2 Response of *An. gambiae* to 16-mm diameter glass beads 0, 1, 2, 4 and 8 h after human handling. Error bars represent standard errors of the mean; N: total number of mosquitoes that were released in the olfactometer; *: chi-square test *P*<0.05; **: chi-square test *P*<0.01; ***: *P*<0.001; NS: chi-square test *P*>0.05.

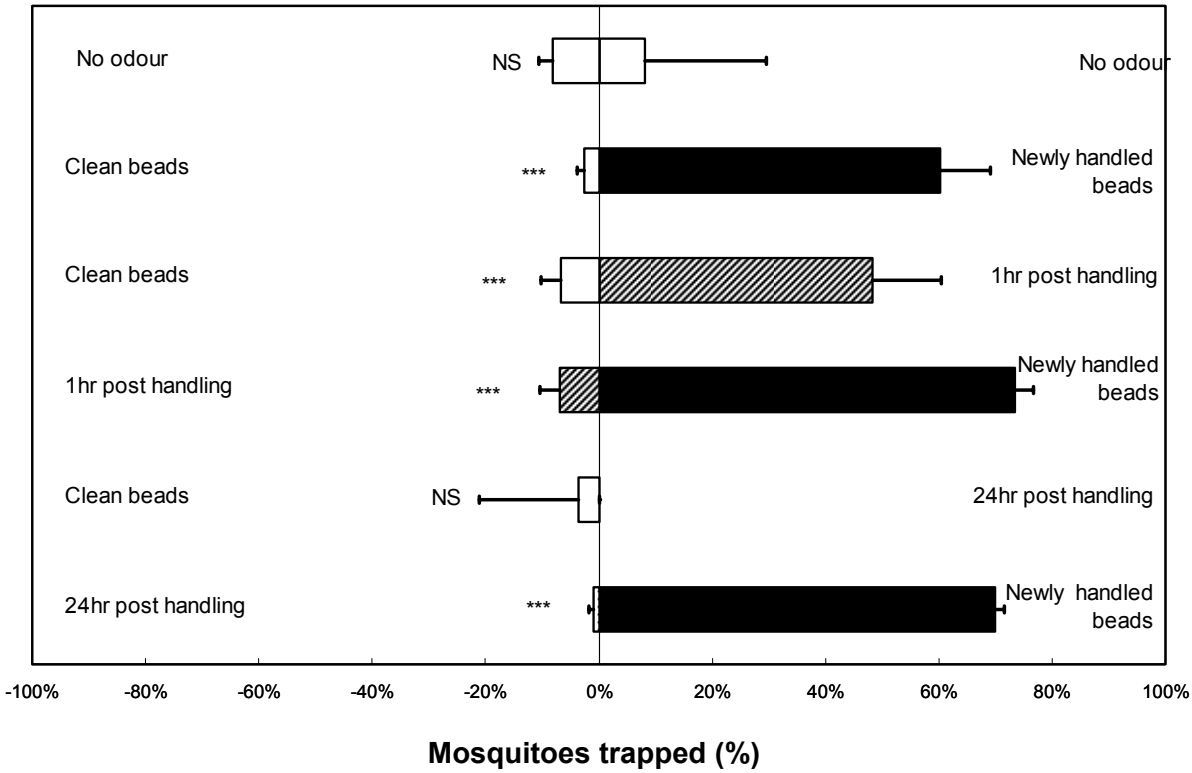

Fig. 3 Response of *An. gambiae* to glass beads 1 and 24 hours after human handling. Error bars represent standard errors of the mean; N: total number of mosquitoes that were released in the olfactometer;*: chi-square test $P<0.05$; ***: chi-square test $P<0.001$; NS: chi-square test $P>0.05$.

individual attracted as many mosquitoes as the reference subject did (chi-square test, $df=1$, $P>0.05$).

Electrophysiological experiments

EAG response to handled beads, extracts and residues

The standard stimulus, 0.1% 1-octen-3-ol, evoked EAG responses of -0.29 ± 0.03 mV (N=23) on average. The headspace of 8 handled glass beads elicited higher EAG responses than did clean beads when blown directly over the antennae of *An. gambiae* (GLM, $df=1$, $P<0.001$). The amplitudes of EAG responses to 16, 24 and 32 handled beads were similar and they were higher than the responses to no odour and 8 handled beads (Fig. 4A).

Although human emanations aged for 24 h on glass beads elicited higher EAG responses than did clean beads (GLM, *df*=1, P<0.001), these responses were lower than the EAG response to newly handled beads (GLM, *df*=1, *P*<0.001). The number of beads did not affect the EAG responses of the aged beads.

Ethanol extracts and the respective residues after extraction elicited lower EAG responses than odours from newly human-handled beads (GLM, *df*=1, *P*<0.001) (Fig.4B). Ethanol extracts of handled beads elicited higher EAG responses than the residues remaining after extraction (GLM, *df*=1, *P*<0.001). Yet EAG responses to the residues remaining

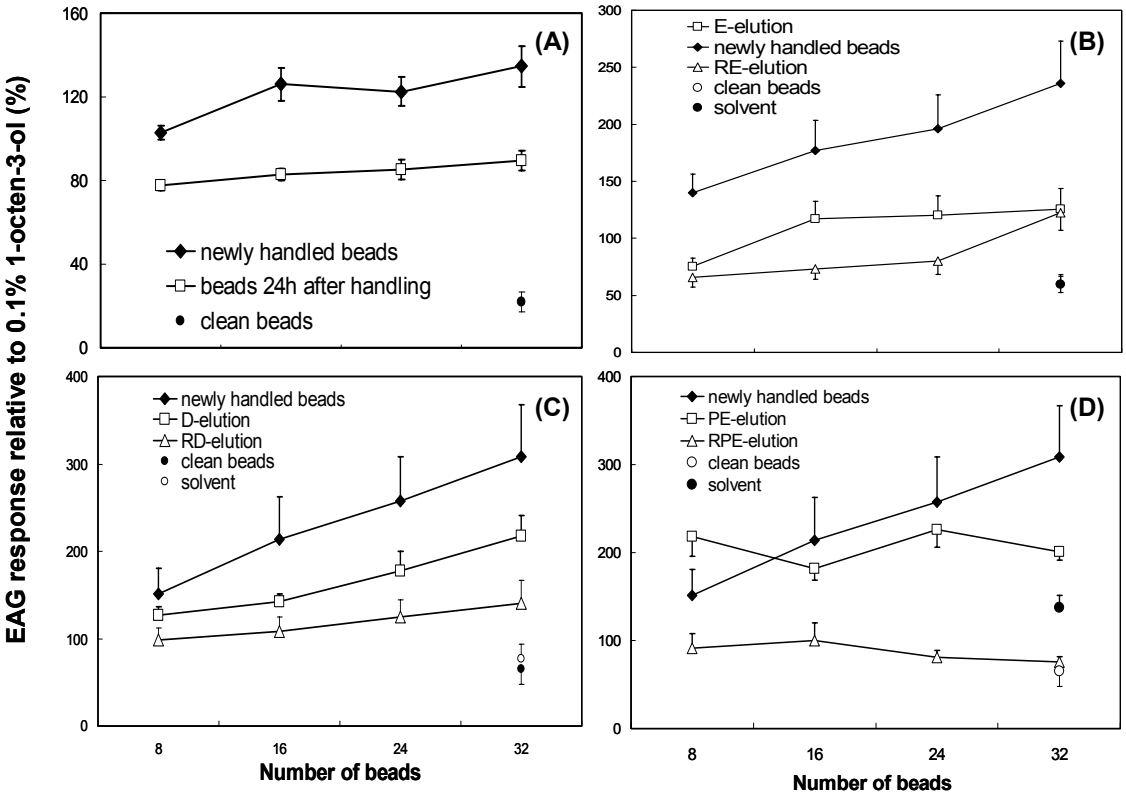

Fig. 4 Normalised EAG response of female *An. gambiae* mosquitoes to: (A) different number of glass beads covered with human skin emanation tested directly after treatment (newly treated beads) and tested 24 h later (24h after handling), (B) glass beads covered with human skin emanation (newly treated beads), ethanol extract of glass beads covered with human emanation (E-elution) and the residual after the extraction (RE-elution), (C) glass beads covered with human skin emanation (newly treated beads), dichloromethane extract of glass beads covered with human emanation (D-elution) and the residual after the extraction (RD-elution) and (D) glass beads covered with human skin emanation (newly treated beads), pentane-ether (2:1 in v:v) extract of glass beads covered with human emanation (PE-elution) and the residual after the extraction (RPE-elution). Error bars represent standard errors of means.

after ethanol extraction were higher than the responses to the control beads (GLM, *df*=1, *P*=0.013). EAG responses to the solvent and the clean beads were similar (GLM, *df*=1, *P*=0.92). Dichloromethane extracts of human-handled beads elicited lower EAG responses than the newly handled beads (GLM, *df*=1, *P*<0.001) (Fig. 4C). The EAG responses to the residues after dichloromethane extraction were considerably lower than the responses to the respective extracts (GLM, *df*=1, *P*<0.001), but the EAG responses were higher than to the solvent (GLM, *df*=1, *P*<0.001). EAG responses to the solvent and the clean beads were similar (GLM, *df*=1, *P*=1.000). Pentane-ether (2:1) extracts resulted in EAG responses similar to those from newly handled beads (GLM, *df*=1, *P*=0.231) (Fig. 4D). Residues of pentane-ether extracts showed notably weaker EAG responses than their corresponding extracts (GLM, *df*=1, *P*<0.001). EAG responses to residues left after pentane-ether extraction were no different than the responses to the solvent (GLM, *df*=1, *P*=0.082) and to clean beads (GLM, *df*=1, *P*=0.807). The pentane-ether solvent elicited slightly higher EAG responses than clean beads did (GLM, *df*=1, *P*=0.037).

EAG responses to extracts of glass beads handled by human subjects

Pentane-ether extracts of handled beads from the more attractive subject SH elicited significantly higher EAG responses than clean air (GLM, *df*=1, *P*<0.001) (Fig. 5). A 1:2 dilution of the extract from beads handled by subject SH evoked a higher EAG response than clean air (GLM, *df*=1, *P*=0.001). Further dilution of the extract by a factor of 2 (to 25% of the original strength) did not evoke an EAG response different from clean air (GLM, *df*=1, *P*=0.688). The extracts and the dilutions of the less attractive subject AG elicited a similar EAG response as clean air (GLM, *df*=1, *P*>0.05). Undiluted and 1:2 diluted extracts from beads handled by subject SH caused a larger EAG response than the corresponding extract and dilution from subject AG (GLM, *df*=1, *P*=0.034 and 0.039, respectively).

Discussion

A suitable odour collection method fulfilling a number of requirements is essential for the identification of mosquito attractants present in human odour. First, the method should produce odour samples with detectable results in behavioural, chemical and preferably also electrophysiological tests. Second, contamination of the odours collected from other sources than human subjects should be minimised. Finally, the method should impose only a small burden on volunteers. Using glass beads to collect human emanations fits these requirements. Our results show that *An. gambiae* females can detect odours released from a single glass bead carrying human hand emanations in the olfactometer. The attraction increased as

additional handled beads were included. These findings resemble those of Schreck *et al.* (1990) for *Ae. aegypti* who showed that this mosquito species was attracted to human-handled beads from a distance. It is remarkable that human emanations on more than two glass beads were as attractive as four human fingers despite the absence of physical cues (moisture and heat), which are inevitably presented simultaneously by human fingers. This indicates that the components attractive to *An. gambiae* were transmitted effectively from human hands onto glass beads. This result also supports the theory that chemical cues are important cues in the host-seeking behaviour of mosquitoes (Takken, 1991).

Human emanations attracting *An. gambiae* mosquitoes in an olfactometer have been well documented (Braks & Takken, 1999; Braks *et al*., 2001; Healy *et al*., 2002; Knols *et al*., 1997; Pates *et al*., 2001; Takken & Knols, 1999). Attractiveness of incubated human sweat and occasionally of fresh sweat was found by Braks & Takken (1999) and 81 components were identified from these two samples (Meijerink *et al.,* 2000). A diethyl ether ex-

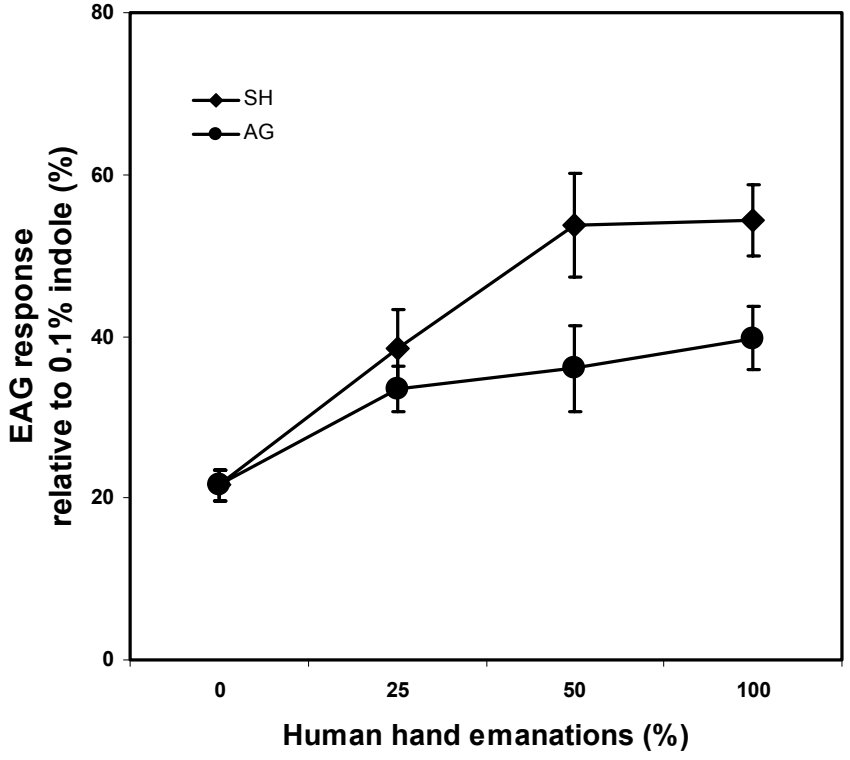

Fig. 5 Normalised EAG response of female *An. gambiae* mosquitoes to extracts and their dilutions from human skin emanation with different behavioural attractiveness. AG: extracts of skin emanations of subject with low attractiveness; SH: extracts of skin emanations of subject with high attractiveness. Error bars represent standard errors of means.

tract of human skin emanations stimulated landing of *An. gambiae* females (Healy & Copland, 2000) and 73 compounds were identified from this sample. An ethanol extract of human skin emanations was also attractive for females of this mosquito species (Pates *et al*., 2001). Human skin emanations collected on nylon stockings were highly attractive for female *An. gambiae* mosquitoes (de Jong & Knols, 1995; Pates *et al.,* 2001). However, using a coupled GC-EAG method to identify bio-active components of human origin from nylon stockings appeared to be unsuitable due to the high amounts of non-human background compounds (Qiu *et al.*, 2004; Chapter 4). Although these various human odour samples caused attraction to the mosquitoes, their chemical composition differed and do not allow a conclusion on which components of these complex blends are essential for attracting *An. gambiae* females.

One hour after handling treated beads were still highly attractive to *An. gambiae* when tested against no odour. However, the mosquitoes showed a clear preference for newly handled beads over one-hour aged beads when these were tested against each other. This indicates the loss of attractive material over time, presumably by evaporation. Schreck *et al.* (1981) reported that the attraction of hand emanations to *Ae. aegypti* decreased by about 50% within one hour and that the attraction was no longer detectable after 6h. Our results showed that the residues of human emanations on glass beads were attractive up to 2h after collection. The complete loss of sample attractiveness after 4h restricts the viability of this method in storing the odour sample at room temperature for later testing. This result also suggests that important attractive components or synergists are highly volatile and may operate on both anthropophilic mosquito species in the same way. A similar conclusion was reached by Braks *et al.* (2000) who reported the loss of attractiveness of incubated human sweat after 20 min. Bernier *et al.* (2002) compared volatile composition of fresh and aged skin emanations under the assumption that differences in compounds might reveal candidate kairomones in mosquito host seeking but no concomitant studies based on these results have been reported. Although the EAG responses to the handled beads aged for 24h were lower than to the newly handled beads, they were higher than to clean air. This suggests that emanations aged for 24h either lacked qualitatively or quantitatively the necessary components for attraction. This is in contrast with results from skin emanations on stockings (Pates *et al*., 2001; R.C. Smallegange, personal observations) for which attraction to the sample remained high for several weeks. We suspect that the different duration of attraction to human emanations on glass beads compared to stockings is due to microbial activity on the latter, releasing essential olfactory components from the stockings. Once these components have evaporated from the beads lacking a viable microbial flora, they are not produced further and the sample looses its attractiveness due to incomplete odour blends.

Our results demonstrate that although the handled beads attracted more mosquitoes than unhandled beads after storage at –20 ºC, the total responsiveness in these experiments was much lower than in the experiments with newly handled beads. This suggests a loss of some of the kairomones during this storage procedure, perhaps during the warming phase of the odour sample prior to each experiment. Others reported that human emanations concentrated in ethanol were still attractive after cold storage for weeks (Schreck *et al*., 1981; H.V. Pates, personal communication). Whereas the glass beads appear thus a good method for the collection and chemical analysis of a representative sample of human skin emanations shortly after sampling (Bernier *et al.*, 1999; 2000), they prove less suitable for residual studies.

Headspace of human-handled beads elicited EAG responses. These EAG responses correspond with our behavioural results, as both the dose-response characteristics as well as the residual effects were similar. EAG responses of *An. gambiae* were found to incubated sweat but not to fresh sweat (Meijerink *et al.*, 2000), which also corresponded with behavioural findings (Braks & Takken, 1999). Unlike ethanol and dichloromethane, a mixture of pentane and ether can dissolve most of the substances evoking EAG responses from the beads. The low EAG responses to ethanol and dichloromethane extracts may not be due to the insufficient removal of substances that elicited EAG responses from the handled beads, because EAG responses to the beads after extraction were low, indicating a thorough removal of the human emanations. Our results suggest that evaporation of active components during a longer time needed for concentrating the extracts due to the higher boiling point of ethanol and dichloromethane might be the cause of the low EAG response to the extracts. Schreck *et al.* (1981) found ethanol to be the most effective solvent, among all the solvents tested, for removing and redepositing the behavioural active substances for *Ae. aegypti*. In their experiments the substances were not concentrated by solvent evaporation. This might explain why they recovered more activity in the ethanol extract for this mosquito species.

Human individuals exhibit considerable variation in physical (such as skin colour, temperature and humidity) and chemical cues (body odour) that are used by mosquitoes for host location causing differential attractiveness to mosquitoes (Schreck *et al.,* 1990; Clements, 1999). By comparing the response of glass beads handled by different human subjects only chemical elements are transferred and compared. Indeed, we were able to demonstrate differences in attractiveness between five individuals. Subsequently we have studied the response to a large group of human individuals and ranked them according to their attractiveness using this method (Smallegange *et al*., 2003; Chapter 3). Chemical analyses are in progress to compare the relative abundance of odour components from individuals with distinctively different degrees of attractiveness. The EAG responses to skin extracts of human subjects with different attractiveness corresponded with the behavioural results. Differences in mosquito attraction between human individuals can be due to qualitatively different compo-

sition of attractants and, possibly, repellents, quantitatively different amounts of attractants and repellents or a combination of these factors (Skinner *et al*., 1965; Maibach *et al*., 1970; Bosch *et al*., 2000; Bernier *et al*., 2002). A preliminary analysis of glass beads rubbed by the two individuals with different attractiveness (SH and AG) by thermodesorption GC-MS showed that the difference between the odour profiles of the two individuals was quantitative rather than qualitative (Y.T. Qiu, unpublished results). Similar results were reported by Bernier *et al*. (2000) when they compared volatile spectra of skin emanations from four different subjects. The emanations of the more attractive individual contained greater amounts of urea and fatty acids. Therefore, for these two persons, the higher attractiveness and stronger EAG responses might have been due to higher concentrations of attractants. Bernier *et al*. (2002) found increased amounts of long-chain carboxylic acids in the human emanations more attractive to *Ae. aegypti*.

The results of this study demonstrate that human odour, attractive to the mosquito *An. gambiae*, can be transferred to glass beads and that these residues are active at the behavioural and electrophysiological level. This method provides a means for the study of airborne human odour while physical parameters like body temperature and moisture are ruled out. This method also has advantages for chemical analyses because the skin compounds can be removed from the glass beads through thermodesorption, avoiding the need of solvent extraction (Bernier *et al*., 1999). The chemical analyses of human emanations on glass beads will be reported in a separate paper (A.M. Galimard, unpublished data).

Acknowledgements

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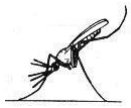
3

Interindividual variation in odour-mediated attractiveness of humans to the malaria mosquito *Anopheles gambiae*



Qiu, Y.T., Smallegange, R.C., van Loon, J.J.A. and Takken, W.

Abstract

The causes of reported differences between human individuals in their attractiveness to female mosquitoes are not well understood. Skin emanations from 27 human individuals, collected on glass marbles, were tested against ammonia in a dual-choice olfactometer to establish the degree of attractiveness to anthropophilic *Anopheles gambiae s.s.* mosquitoes. Trap entry response in tests of a standard dose of ammonia versus clean marbles was lower than in tests of ammonia against marbles handled by all except two of the volunteers. Skin emanations of all volunteers attracted significantly more mosquitoes than ammonia. There were clear differences in the trap entry response as well as in the attractiveness relative to that of

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ammonia between the skin emanations of different volunteers. Consistency of the differences was observed when emanations of the three most and the three least attractive volunteers were tested pair-wise. A gender effect was found for the trap entry response but not for the relative attractiveness. Odours of younger volunteers significantly raised the trap entry response and were preferred over odours from older volunteers. Emanations from volunteers with different behavioural attractiveness elicited different levels of electroantennogram responses, these are not in all cases correlated to the behavioural response level, suggesting the involvement of repellent components.

Introduction

Most female blood-feeding mosquitoes require blood meals for reproduction. The physical and chemical cues emanating from the host guide female mosquitoes to these blood sources. Although optical cues and physical ones such as heat and moisture play a role in host location, chemical cues are considered to be the most important (Takken, 1991). Inorganic chemicals such as water vapour, carbon dioxide, and ammonia are considered to be important for guiding mosquitoes to their human hosts (Price *et al.*, 1979; Braks *et al.*, 2001). In addition, organic chemicals such as a number of carboxylic acids, lactic acid, 1-octen-3-ol, and acetone have been shown to attract anthropophilic mosquitoes (Takken *et al.*, 1997a; Bosch *et al.*, 2000).

It is commonly observed that, when two persons are equally accessible, mosquitoes bite one person more than the other. The mechanisms underlying such discrimination await elucidation. Numerous studies have been published on the differential attractiveness of human individuals to mosquitoes (Muirhead-Thomson, 1951; Smith, 1956; Brouwer, 1960; Mayer & James, 1969; Carnevale *et al.*, 1978; Curtis *et al.*, 1987; Schreck *et al.*, 1990; Lindsay *et al.*, 1993; Knols *et al.*, 1995; Brady *et al.*, 1997; Mukabana *et al.*, 2004b). Smart and Brown (1957) observed a higher landing frequency on hands with a darker colour. These authors also reported that when the highly anthropophilic mosquito *Aedes aegypti* (L.) is offered hands from two individuals with different temperatures it alights more often on the warmer hand. Schreck *et al*. (1990) studied human emanations collected on glass marbles and tested their attractiveness when kept at different temperatures. They demonstrated that although heat is not required to attract mosquitoes, warmed human skin residues attracted higher numbers of *Ae. aegypti*.

When hands or forearms with different surface humidity were offered, the drier one was more frequently visited (Smart & Brown, 1957; Gilbert *et al.*, 1966). Positive correlations between human body mass or surface area and mosquito catch were found in several studies (Muirhead-Thomson, 1951; Spencer, 1967; Port *et al.*, 1980). There are also reports of a

correlation between pregnancy and attractiveness with pregnant women being bitten more often than non-pregnant women (Lindsay *et al.*, 2000; Ansell *et al.*, 2002; Himeidan *et al.*, 2004).

In most of these studies the attractiveness of human bodies or human hands from different people were compared. However, in many studies it was not possible to identify olfactory factor(s) causing any differences found because of factors such as body size, hue, heat, and moisture. Variations in attractiveness between individuals due to the latter confounding factors can be minimised by collecting human skin residues on glass marbles. Skin volatiles on glass marbles are attractive to *Ae. aegypti* and *Anopheles gambiae* Giles *sensu stricto* (Schreck *et al.*, 1981; Schreck *et al.*, 1990; Qiu *et al.*, 2004a; Chapter 2). This method offers the possibility of comparing air-borne human odour from various individuals.

In the present study, we examined the attractiveness of skin emanations from 27 human individuals to females of the malaria mosquito *An. gambiae s.s*. (henceforth in this paper termed *An. gambiae*), which is strongly anthropophilic and the most important malaria vector in Africa (White, 1974) . The experiments were done in a dual-choice olfactometer. The human subjects were ranked according to their index of attraction for *An. gambiae*. Emanations from three individuals with high and three with low indices were then compared pairwise for behavioural and electroantennographic (EAG) responses of the mosquitoes.

Materials and methods

Volunteers

We examined the attractiveness of 27 adult humans (16 male and 11 female) aged between 22 and 53 years. Twenty five were Caucasian, one Asian and one Hispanic. All volunteers were non-smokers, free from chronic illnesses and not using any medication on a regular basis. None of the participating women were pregnant. The volunteers were requested not to drink alcohol on the day before the experiments (Shirai *et al.*, 2002), peel garlic or onions, or eat garlic, onions, or spicy food. On the morning of the experiments, volunteers were requested not to use perfumed cosmetics or peel citrus fruits. One hour before the experiments, hands were washed with a standard perfume-free soap (Dermoline liquid soap-free washing emulsion, Tramedico BV, The Netherlands) and rinsed in tap water and dried on tissue paper. According to their age, the volunteers (Males and Females) were divided into three groups, nine in each group: 24-27 years (“Young”), 28-37 years (“Middle aged”) and 40-55 years (“Old”). The volunteers are coded as follows: starting with a unique number from 1-27 for each volunteer, and M=male; F=female; O=old, M=middle aged; Y=young.

Insects

The *Anopheles gambiae s.s*. colony originated from Suakoko, Liberia and has been reared by blood feeding on humans since 1988. The mosquitoes were maintained at 27 ± 1°C, 80 ± 5% RH, and at a photo-scotophase of LD 12:12 h.

The experiments were performed during the last four hours of the scotophase. Groups of thirty mosquitoes were randomly collected from the rearing cage 14 to 18 hours before the start of the experiments and placed in a releasing cage, with access to water via a piece of moistened cotton wool placed on the top of the cage. For the behavioural experiments, 5 to 8-day-old female mosquitoes that had not received a blood meal were used.

Olfactometer

A dual-port olfactometer (Pates, 2001), consisting of a Perspex flight chamber of 1.60 m x 0.66 m x 0.43 m, was used to study the behavioural response of the female mosquitoes to different odour stimuli. Charcoal-filtered and humidified, warm pressurised air was led through two Perspex mosquito trapping devices, which were linked to two ports (diameter 4 cm, 28 cm apart), into the flight chamber at a speed of 0.22 ± 0.02 m/s. Dim light was produced by one light bulb (75 watt) and was filtered and scattered through a screen of yellow cloth hanging ~ 1 m above the flight chamber, providing a light intensity of approx. 1 Lux. The temperature of the experimental room was maintained at 28 ± 1.0 °C and a relative humidity of 66 ± 4.0%. The temperature inside the flight chamber was similar to that of the room, and the humidity was maintained at 70 ± 5.0%. The humidity of the air streaming through the ports was maintained above 80% and the temperature was 29 ± 1.0 °C.

Odour stimuli

One day before the experiments, 250 µl of a solution of 2.5% ammonia in water (diluted ten times with distilled water from a concentrated ammonia solution, 25% in water, analytical grade, purchased from Merck, Amsterdam, The Netherlands) was injected in a 80-l Teflon air sample bag (SKC Gulf Coast Inc., Houston, TX, USA). Subsequently, the bag was filled with 60 l humidified and filtered warm pressurised air at least 17 hours prior to the experiments to allow the solution to evaporate. This procedure resulted in an ammonia concentration of 136 ppm in the bag. Another 80-l air sample bag filled with 250 µl distilled water and 60 l of air was prepared in a similar way to be used as the control stimulus. During the experiments, the air was pumped at 0.23 l/min (Air pump Model 224-PCXR4, SKC Gulf

Coast Inc., Houston, TX, USA) from the air sample bags through Teflon tubes (diameter 7 mm) into the trapping devices where it merged with the main air stream of 23.5 l/min. Glass marbles 16 mm in diameter were cleaned by rinsing in non-perfumed soap (CLY-MAX Heavy Duty Cleaner, Rogier Bosman Chemie B.V., Heijningen, The Netherlands), distilled water and then in ethanol (Merck, 99.8% purity). Rinsed marbles were dried in an oven at 200 ºC. Skin emanations from the 27 individuals were collected on six of the glass marbles which the subjects had handled for 10 minutes.

Experimental procedure

The marbles were put in one of the trapping devices directly after handling. For testing the attractiveness of the volunteers individually, clean air from an air sample bag was pumped into this trapping device. Gaseous ammonia was pumped into the other trapping device which contained clean glass marbles. For directly comparing the attractiveness of the skin emanations of two volunteers, each trapping device contained six marbles handled by one of the two individuals.

In each trial, a group of 30 mosquitoes was released simultaneously from a release cage that was placed at the downwind end of the flight chamber, 1.60 m from the two ports. The trial duration was 15 minutes. Trapped mosquitoes were counted at the end of the experiments. Odours of each volunteer was tested six times, twice on three different mornings. Direct comparisons of two individuals were done four times on one and the same morning. The experiments were done over an eight-month period, from June 2001 to February 2002. Each trial started with new mosquitoes, clean traps, and freshly prepared stimuli. Test stimuli were alternated between right and left ports to rule out any positional effects. Experiments without any odour source in either port were done to test the symmetry of the trapping system. To avoid contaminating the equipment with human volatiles, the operator wore surgical gloves during all experiments. After use, traps were washed with soapy water (CLY-MAX Heavy Duty Cleaner, Rogier Bosman Chemie B.V., Heijningen, The Netherlands), rinsed with tap water and then cleaned with pure ethanol.

Electrophysiological experiments

Human handled marbles and their extracts

On the mornings when odours of paired individuals were tested against each other in the olfactometer, the same individuals also handled small glass marbles (2.9 mm in diameter)

for 10 min. Different numbers of handled marbles (8, 16, 24, and 32) were placed into a glass Pasteur pipette and the headspace was blown directly over a mosquito antenna mounted in an electroantennogram (EAG) measuring circuit (Qiu *et al.*, 2004a; Chapter 2). The combined surface area of 32 small marbles is equal to that of one large bead (16 mm in diameter) used in the behavioural experiments (see above under "odour stimuli")

EAG recordings

Mosquito head preparations excised from naive female *An. gambiae* (aged 5-8 days) were used for electrophysiological recordings. The excised head was mounted between two glass electrodes filled with 0.1 M KCl. An AgCl-coated silver wire (diameter 1.5 mm) was inserted into a glass capillary (diameter 1.1 mm), placed in a holder, and connected to a DC amplifier (10´; Syntech, Hilversum, The Netherlands). A grounded AgCl-coated silver wire was inserted into a glass capillary and was used as the indifferent electrode. The foramen magnum of a mosquito head was attached to the indifferent electrode. The recording electrode was slid over the tip of the antenna, from which the top segment had been removed. A moistened, charcoal-filtered, continuous air stream (1000 ml/min) was led through a glass tube (1 cm diameter) ending 0.5 cm from the preparation. Stimulus puffs (4.1 ml in volume) lasting for 0.5 s were applied using a stimulus controller (Syntech) and were injected into the air stream 10 cm from the outlet of the tube delivering the continuous air stream. The amplified potential differences between the electrodes were imported via an IDAC interface box and an A/D converter (Syntech) into an Intelâ 486-based personal computer. Recordings were analysed using EAG software version 2.6 (Syntech).

Data analysis

Analysis of behavioural data

For each volunteer, the difference between the number of mosquitoes trapped in the port from which the headspace of the handled marbles was delivered and the number trapped in the port from which ammonia-containing air was delivered were analysed with a chi-square test using the total number of mosquitoes trapped summed over all replicates.

A Generalized Linear Model (GLM; Binomial, linked in logit; Genstat, release 8.11) was used to investigate the effect of test moment and gender or age or individual on two parameters. Two-sided t-probabilities were calculated to test pair-wise differences between

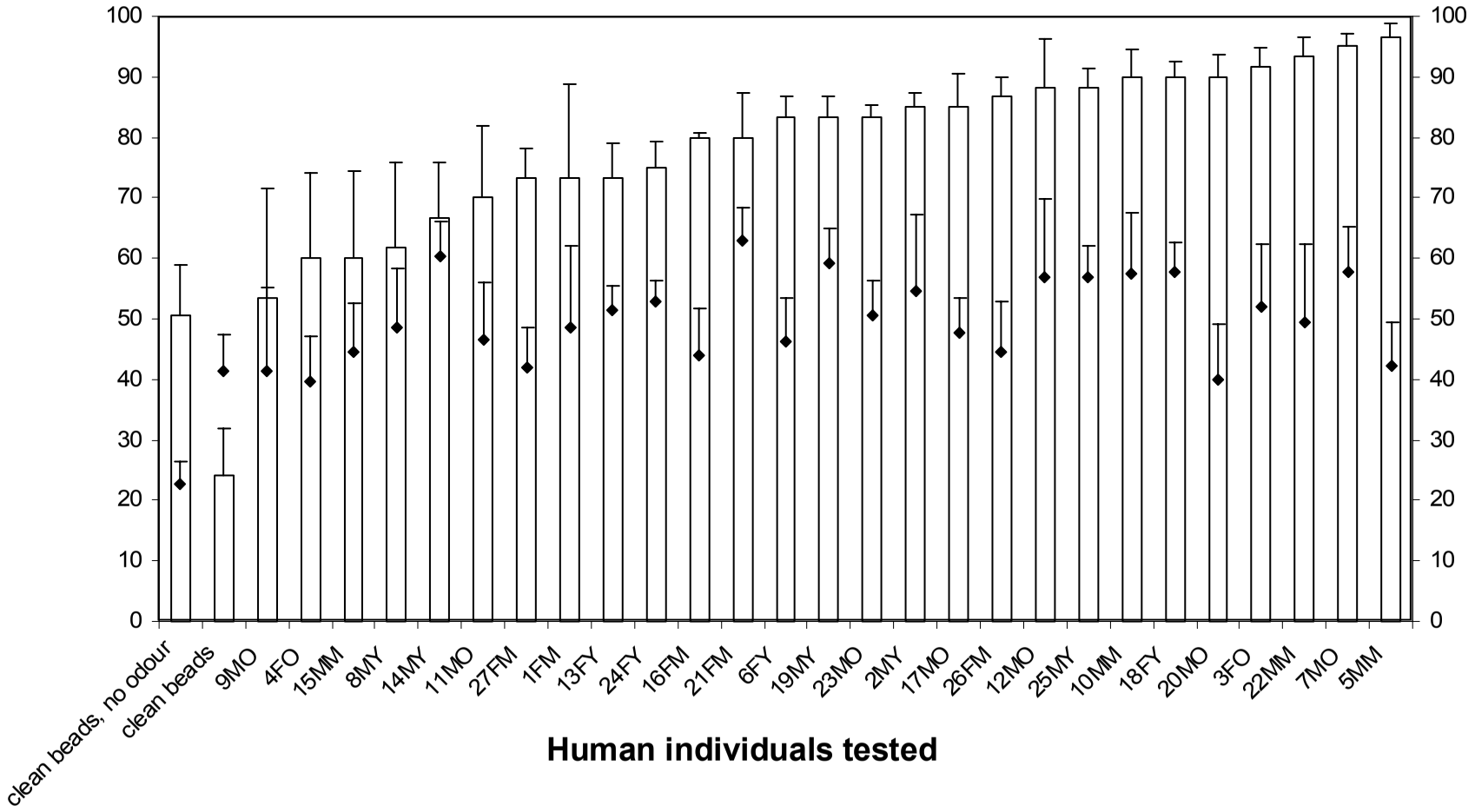

Fig. 1 Results of olfactometer bioassays in which clean beads or beads with human skin emanations of 27 individuals were tested against gaseous ammonia. Black diamonds show the number of mosquitoes which entered the olfactometer traps as a percentage of those which left the release cage (trap entry response). Height of the bars shows the number entering the trap with beads with or without skin emanations as a percentage of those entering either this trap or the one with ammonia (relative attractiveness). The symmetric tests of the olfactometer with clean beads in both trapping devices were labelled "clean beads, no odour". Human individuals are numbered randomly, the first letter behind the number, F or M, stands for female or male respectively; the second letter, Y, M or O, stands for young, middle and old age groups respectively. Error bars represent SE of means from 6 replications.

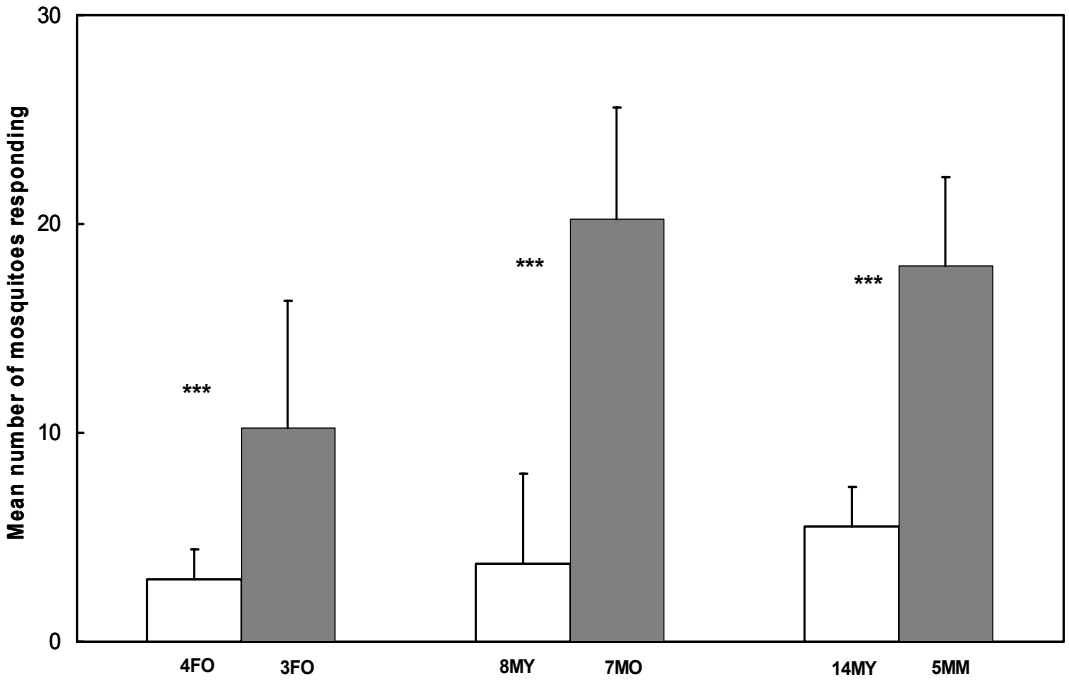

Fig. 2 Mean number of mosquitoes caught in a trapping device baited with odours from three pairs of volunteers when tested against each other. Bars indicate mean values of four experiments. Error bars represent SD of means. ***: significance level (P<0.001) obtained from chi-square test between the number of mosquitoes attracted by odours from two volunteers.

means. Effects were considered to be significant at P<0.05 (Oude Voshaar, 1994; Sokal & Rohlf, 1998). The two parameters were (1) The trap entry response expressed as the number of female mosquitoes caught in both trapping devices as percentage of the number of mosquitoes that flew out of the release cage; and (2) The relative attractiveness, expressed as the number of mosquitoes caught in the trapping device with the treatment under test divided by the total number of mosquitoes trapped in both trapping devices during each experiment (Qiu *et al.*, 2004a; Chapter 2).

Analysis of EAG data

EAG response was transformed into a percentage relative to the standard stimulus, 0.1% 1-octen-3-ol. Differences between treatments were analysed by General Linear Model, Univariate procedure (SPSS for Windows, release 10.0.5). The effect of individual volunteers

was set as fixed factor in the linear regression model. The other two factors considered in each model were the number of marbles and different mosquito antennae (replicates). Interactions between the fixed factors were excluded from the model when the interaction effect was not significant. The effect of a fixed factor was tested by the F statistic and was considered to be significant when $P<0.05$. The differences between means were compared pairwise by Tukey's Honestly Significant Difference Test. Effects was considered significant

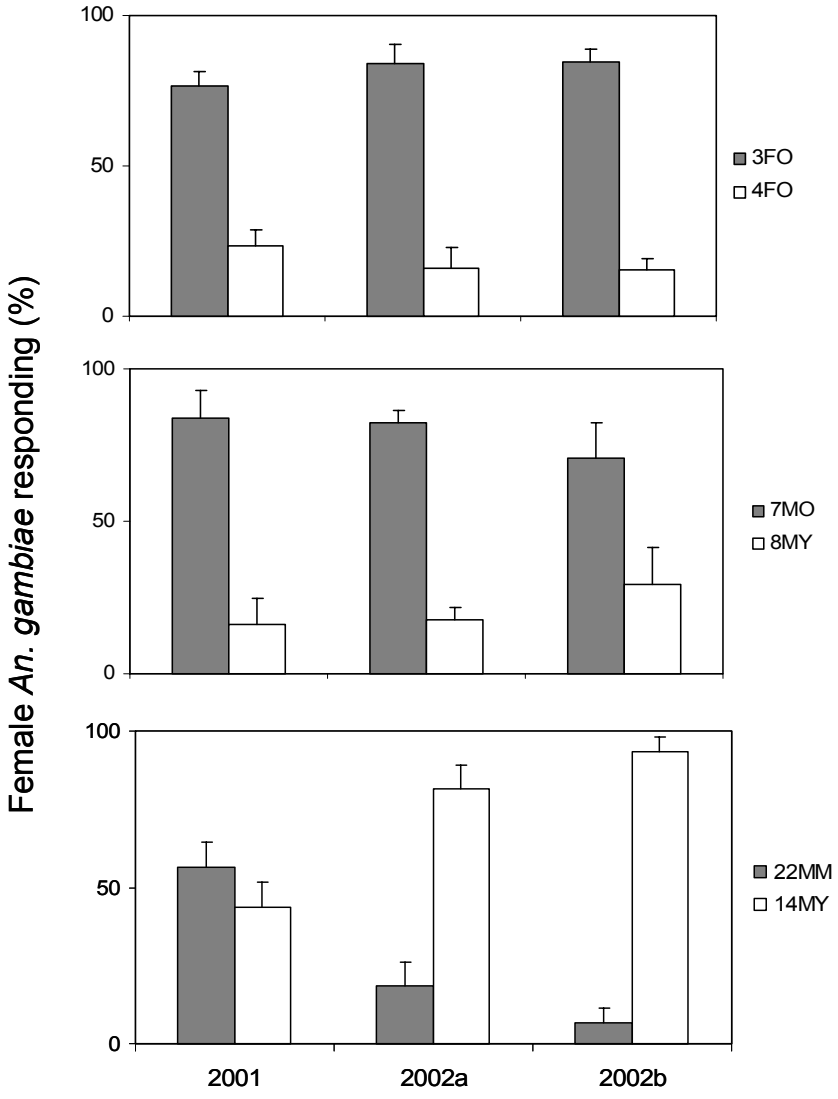

Fig. 3 Mean relative attractiveness, i.e. the number of mosquitoes caught in a trapping device baited with human skin emanations as a percentage of the total number of mosquitoes caught in both trapping devices. Skin emanations from three pairs of volunteers were tested against each other in three experiments over a period of eight months. Error bars represent SE of means of four replicates.

when $P<0.05$.

Results

Skin emanations tested against ammonia

Without odours there was no significant difference in the number of mosquitoes collected in both trapping devices (chi-square test, $P= 0.57$, n=18) showing that the olfactometer was symmetrical. As shown by the black diamonds in Fig. 1, when clean beads were tested against ammonia, 41% of the mosquitoes which left the release cage entered either the ammonia baited trap or the trap with clean beads (i.e. the trap entry response was 41%); and this was significantly higher than in the symmetry test when only clean beads were offered (23%) as was also shown by GLM analysis ($P<0.001$). When skin emanations from the volunteers were tested individually against ammonia, the mean trap entry response of all experiments was 51 ± 21% (Fig. 1, n=162). GLM analysis demonstrated that the trap entry responses in the experiments testing ammonia against clean marbles were lower than in experiments testing ammonia against marbles handled by all except two volunteers (4FO and 5MM). There were differences in the trap entry response between the skin emanations from the volunteers (GLM, $P<0.001$, $df=26$). Skin emanations from the volunteers in the youngest group elicited higher trap entry responses than skin emanations of the volunteers in the two older age groups (GLM, $P<0.001$ and $P<0.001$, $df=2$). A gender effect was also found: odours from the male volunteers elicited higher trap entry responses than the odours from the female volunteers (GLM, $P=0.04$, $df=1$).

Skin emanations from all volunteers attracted more mosquitoes than the standard dose of ammonia (chi-square test, $P< 0.0001$), whereas ammonia attracted more mosquitoes than clean marbles (chi-square test, $P< 0.0001$). There were clear differences in attractiveness between the volunteers when tested individually (GLM, $P<0.001$, $df=26$) (Fig. 1). The differences appeared to be independent of the gender of the volunteers when relative attractiveness is considered (GLM, $P=0.73$, $df=1$). Skin emanations from people in the youngest age group attracted on average a greater number of mosquitoes (GLM, $P=0.016$ and 0.035, $df=1$).

Pair-wise comparisons

The skin emanations of three poorly attractive and three highly attractive persons of the same gender were tested pair-wise in the olfactometer (4FO against 3FO; 8MY against 7MO; 14MY against 5MM). In all three pairs, there were significant differences in trap en-

try responses and relative attractiveness between the two individuals when ammonia was the alternative odour source (Fig. 1). For all three pairs, the skin emanations from the persons that had been found in the previous experiment (skin emanations from 27 humans tested against ammonia) to be the most attractive, attracted more mosquitoes than did the skin emanations from the persons that were the least attractive in those former experiments (chi-square tests, $P<0.001$) (Fig. 2).

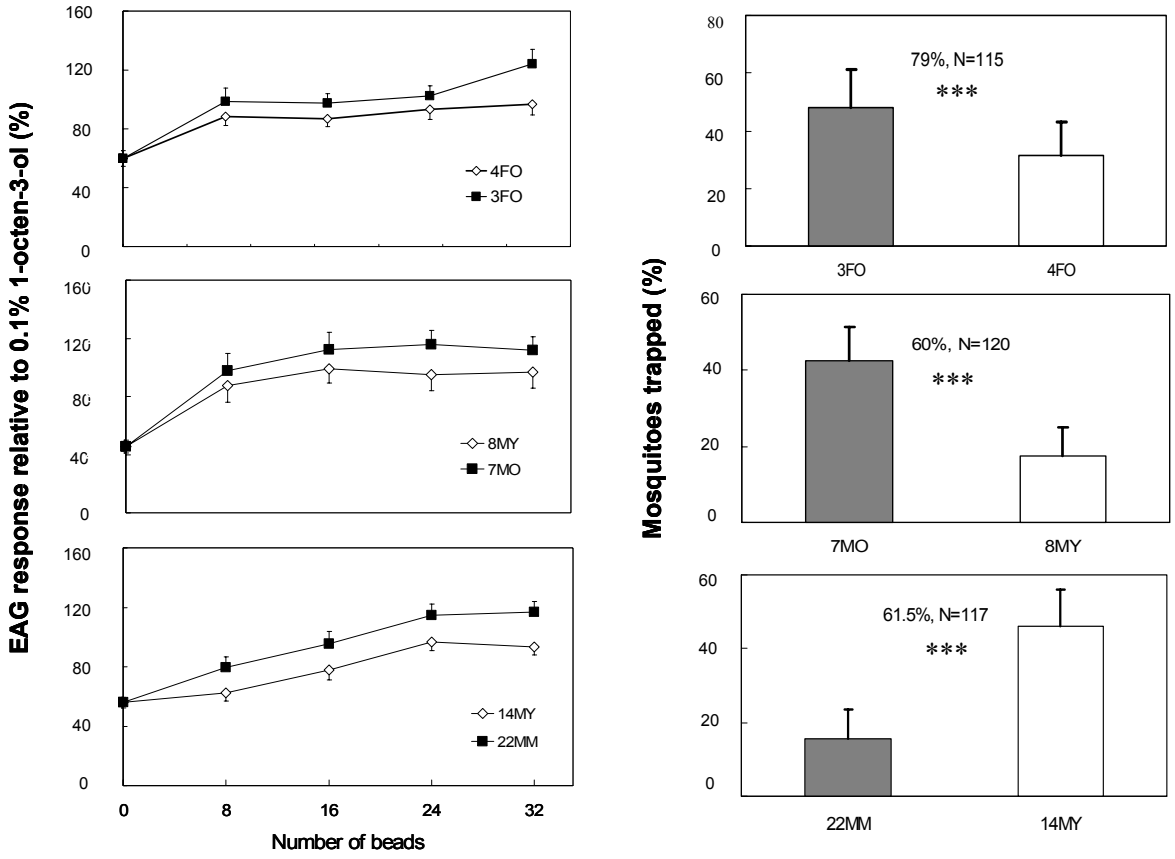

Fig. 4 EAG and corresponding behavioural responses of odours from two human individuals with different mosquito attractiveness. The three figures on the left side are EAG responses elicited by different number of marbles handled by human volunteers relative to a standard stimulus, 0.1% 1-octen-3-ol. The error bars are SE of means (n = 10). The figures on the right side are mean relative attractiveness, i.e. the number of mosquitoes caught in a trapping device baited with an odour stimulus in percentage of the total number of mosquitoes caught in both trapping devices, of odours from three pairs of volunteers when tested against each other. Bars stand for mean values of four experiments. Error bars represent SD of means. ***: significance level ($P<0.001$) obtained from chi-square test between the relative attractiveness of odours from two volunteers.

We conducted three series of pair-wise tests among six volunteers over seven months (Fig. 3). Because volunteer 5MM was not available in our later experiments, we replaced him with 22MM, who showed high attractiveness in the first set of behavioural assays (Fig. 1). Two pairs of the volunteers (4FO-3FO and 8MY-7MO) showed consistent patterns of relative attractiveness, whereas the third pair, 14MY and 22MM did not have results consistent with the results shown in Fig. 1.

EAG responses

For the emanations from the two female volunteers differing widely in attractiveness, the strongly attractive 3FO and weakly attractive 4FO, EAG responses of female *An. gambiae* to the former were higher than the EAG responses to the latter (Fig. 4). For the emanations from one pair of male volunteers 7MO and 8MY, EAG responses to the more attractive skin emanations from 7MO were higher than the EAG responses to the less attractive skin emanations from 8MY. However, the relative attractiveness of the other two male volunteers, 22MM and 14MY was not consistent in the repeated tests (Fig.s 3 and 4). Skin volatiles from 14MY were more attractive than those from 22MM in the bioassay that ran simultaneously with the EAG tests, whereas EAG responses to the skin emanations from 14MY were lower than the EAG responses to the skin emanations from 22MM.

Discussion

In our experiments, skin emanations from individual volunteers differed significantly in attractiveness to female *An. gambiae* mosquitoes. Because the skin emanations were collected on glass marbles that were tested in the olfactometer, the effect of skin odour alone on differential attractiveness was examined, while the physical factors, body heat and humidity, were excluded. Using a similar method, Schreck *et al.*, 1990 demonstrated that skin emanations from different individuals vary in attractiveness to another highly anthropophilic mosquito, *Ae. aegypti.* Nevertheless, the ranking order of the volunteers differed when trap entry response or relative attractiveness was considered. In the dual-choice olfactometer, mosquitoes first needed to be activated by odour stimuli to fly upwind, and when they came close to the two trapping devices they were attracted by one of the two test odours. It is possible that different sets of compounds are responsible for activation and attraction of *An. gambiae* mosquitoes (Dekker *et al.*, 2001).

Our previous research demonstrated that ammonia is attractive to female mosquitoes of *An. gambiae* in an olfactometer (Braks *et al.*, 2001; Smallegange *et al.*, 2005).In the present

study, we found a relative mosquito response (82.3 ± 8%) similar to that previously found for the same concentration of ammonia (82.4 ± 17.5%) (Smallegange *et al.*, 2005). Compared with ammonia, *An. gambiae* preferred skin emanations from all but two of the volunteers. These results indicate that more chemicals than ammonia are involved in guiding female *An. gambiae* to their human hosts.

The results of the pair-wise comparisons confirmed the consistency of the ranking of human individuals in the tests of their odours relative to that of ammonia (Fig. 1 and 2). The order of attractiveness was consistent over time for two out of three pairs of individuals, whereas such consistency was not found for the third pair (22MM and 14MY) (Fig. 4). The more attractive skin emanations from 7MO and 3FO elicited higher EAG responses than did the less attractive skin emanations from 4FO and 8MY, whereas the less attractive skin emanations from 22MM elicited higher EAG responses than did the more attractive skin emanations from 14MY. It is known that both attractants and repellents elicit depolarisation measured as EAG responses (Blackwell *et al.*, 1997). Therefore, a possible explanation for our EAG and/or behavioural results is that a human skin emanation is more attractive to mosquitoes either because it contains higher amounts of attractants or because it contains lower amounts of repellents (Skinner *et al.*, 1965; Maibach *et al.*, 1970; Bernier *et al.*, 2002). In the first case, the more attractive emanations might produce higher EAG response values than the less attractive emanations, whereas in the latter case, the EAGs of the more attractive emanations might be lower than the less attractive emanations. This might mean that volunteers 3FO and 7MO produced higher amounts of attractants to *An. gambiae* than 4FO and 8MY, and 22MM produced greater amounts of repellents to *An. gambiae* than 14MY. These results suggest that the balance between attractive and repellent compounds in the human odour blend determine to an important degree whether mosquitoes are attracted or not. This finding should be further investigated.

We found a significant gender effect for the attractiveness relative to ammonia. Men probably produce a larger quantity of compounds that activate the mosquito host-seeking process than women (Stoddart, 1990). However, the number and nature of chemicals that attract the mosquitoes are likely to be similar for men and women. Men were landed upon and bitten more frequently than women by *An. gambiae* (Carnevale *et al.* 1978). Similar findings were also reported for *Ae. aegypti* (Rahm, 1956; Gilbert *et al.*, 1966)

In addition to gender, we found that age also affected mosquito attractiveness: younger persons (24-27 years old) being, on average, more attractive than older ones. Maibach *et al.*, 1966 reported that younger volunteers (20-49 years) were more attractive for *Ae. aegypti* than older ones (50-59 years). Infants and children, however, have been repeatedly reported to be bitten less frequently by mosquitoes (Smith, 1956; Spencer, 1967; Carnevale *et al.*, 1978; Port *et al.*, 1980), a phenomenon probably caused by differences in body mass or by

physiological differences in this very young age group.

In this study, we demonstrated that chemical cues alone cause consistent differential attractiveness of human subjects to mosquitoes. The differences in odour production by human individuals might be of quantitative or qualitative nature, or a combination of both (Sastry *et al.*, 1980). A comparison of odour profiles of human individuals with different attractiveness to mosquitoes will contribute to elucidating the chemical basis of mosquito host-seeking behaviour, by identifying key host-seeking kairomones. These kairomones could be used to improve the efficiency of the existing odour-baited mosquito traps used for the surveillance and control of the malaria mosquito *An. gambiae*.

The odour profiles of the three pairs of volunteers with different mosquito attractiveness have been compared by chemical analysis and subsequent statistical analysis. These results will be reported elsewhere (A.M. Galimard, unpublished data).

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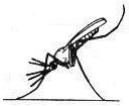
4

GC-EAG analysis of human odours that attract the malaria mosquito *Anopheles gambiae*



Qiu, Y.T., Smallegange, R.C., Smid, H.M., van Loon, J.J.A., Takken, W., Galimard, A.M., van Beek, T.A. & Posthumus, M.A.

Abstract

A coupled gas-chromatography-electroantennography (GC-EAG) method was used to identify kairomones that mediate the host-seeking behaviour of *An. gambiae*. Nylon stockings that had been worn by human volunteers were found to be highly attractive to female *An. gambiae* in olfactometer experiments. Tenax-trapped headspace of 15 pairs of stockings and extracts thereof were analysed with GC-EAG. EAG responses were detected repeatedly at 23 retention times, and 14 compounds were identified that elicited on-line EAG responses. The role of these compounds as kairomones in the host-seeking behaviour of *An. gambiae* is discussed.

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Introduction

Female mosquitoes of *Anopheles gambiae* locate their blood hosts predominantly by human odours (Takken & Knols, 1999). Human odour sources such as aged sweat, skin emanations on glass beads and worn stockings attracted female *An. gambiae* in wind tunnel tests (Braks, 1999; Healy & Copland, 2000; Pates *et al.*, 2001b; Smallegange *et al.*, 2003; Chapter 2 and 3). More than 300 components were identified from human-handled glass beads (Bernier *et al.*, 2000). It will be an almost infinite amount of work to examine the behavioural effects of all human skin volatiles with bioassays at various concentrations and combinations. Gaschromatography linked to electroantennogram recording (GC-EAG) has been widely used for the identification of insect pheromones and kairomones (Weissbecker *et al.*, 2000; Smid *et al.*, 2002). The technique makes it possible to test on-line EAG responses simultaneously with the FID signals; therefore, it is an efficient tool for the detection of bioactive components in odour blends. In this study, we used the GC-EAG method to identify potential kairomones for host-seeking behaviour of the malaria mosquito *An. gambiae*.

Materials and Methods

Insects

Unfed female *An. gambiae* of 5-8 days old were used for all tests. For the rearing process of the mosquito colony we refer to Pates *et al*. (2001).

Human odour collection

Headspace of 15 pairs of nylon stockings, worn by human volunteers for 3 days continuously, were collected with a Tenax trap (Smid, *et al.* 2002). The headspace was then extracted with 2ml distilled pentane and concentrated by evaporation to about 150ml.

GC-EAG analysis

A Carlo-Erba series 8000 gas chromatograph (Rodano, Italy) was equipped with a cold-on-column injector and a 30m DB5 column (0.25m film thickness). Helium carrier with a flow rate of 1.3ml/min was mixed with a 30ml/min make-up helium flow; and the mixture was split to FID and EAG with a ratio of 1:2 (v/v). The GC-EAG interface (Syntech, Hilversum, The Netherlands) was kept at 225º and the outgoing flow was mixed with 600 ml/min char-

coal-filtered, humidified air. The GC column temperature was programmed as follows: injection at 45ºC with secondary cooling for 20 sec, 1 min 45ºC, then the temperature was increased by 3ºC/min to 95ºC, then by 5ºC/min to 165ºC and then by 15ºC/min to 225ºC and held at 225ºC for 5 min. In each run, 2ml of the headspace extract was injected. For EAG responses, an excised head of a female *An. gambiae* was mounted between two glass electrodes filled with 0.1 M KCl. AgCl-coated silver wires were inserted into the glass capillary, placed in a holder, and connected to a DC amplifier (10´; Syntech, Hilversum, The Netherlands). The grounded indifferent electrode was inserted through the cervix. The recording electrode was slid over the tip of the antenna, from which the top segment had been removed. The FID and EAG signals were recorded and displayed on-line on a personal computer using an IDAC-signal acquisition board and GC-EAG software version 2.3, both from Syntech (Hilversum, The Netherlands).

GC-MS analysis

A HP 6890A gas chromatograph (Agilent Technologies) was equipped with the same type of column used in the GC-EAG analysis. Similar chromatographic conditions were used except that the headspace extract was injected in a septum less injection head at 250°C with a split less mode. The selective mass spectrometer (5973 N, Agilent Technologies) was operated in the 70 eV EI ionization mode. Peaks were identified by comparing them with the spectra from the NIST 98 library (National Institute of Standards and Technology, Gaithersburg, Maryland, USA).

Olfactometer tests

The behavioural responses of *An. gambiae* were tested with the same olfactometer as described by Smallegange *et al.* (2003). Fifteen pairs of nylon stockings worn by 15 human volunteers for 3 days continuously were placed in an extended chamber of the trapping device described by Smallegange *et al.* (2003), and were tested against the same number of clean stockings in a similar chamber of the second trapping device. One compound found to elicit EAG responses, benzothiazole (Sigma, purity min. 99%), was applied (100 ml of a 0.01% solution in diethyl ether) on a sandblasted glass slide (5x2 cm) and tested against the same amount of solvent on a similar glass slide.

Results and discussion

Attraction to stockings worn by human volunteers of *An. gambiae*

The total response, *i.e.* the number of mosquitoes caught in the two trapping devices as percentage of the total number of released mosquitoes was 64.3%. Ninety eight percent of the mosquitoes caught entered the trap baited with worn stockings, while the unbaited trap collected 2% of the mosquitoes ($P<0.001$, c^2 test) (Fig. 1).

We have tested different human odour blends (such as those originating from hand-handled glass beads, skin extracts, sweat ect.) and synthetic human odour blends in the olfactometer and although most of these were attractive to *An. gambiae*, worn stockings were always found to be the most attractive (R.C. Smallegange, personal observation). The attractiveness lasted for a long time (up to half a year) when stored at –20°C or at room temperature (Y.T. Qiu and R.C. Smallegange, personal observation). Pates *et al*. (2001) also reported the strong attractiveness of worn nylon stockings.

GC-EAG and GC-MS analysis

The amplitudes of on-line EAG responses of female *An. gambiae* were low. Therefore the eadspace of the worn stockings was tested repeatedly on 12 different antennae of *An. gambiae* to screen for consistent EAG responses at specific retention times. Simultaneous EAG responses were found repeatedly at 23 different retention times; 14 of the components were

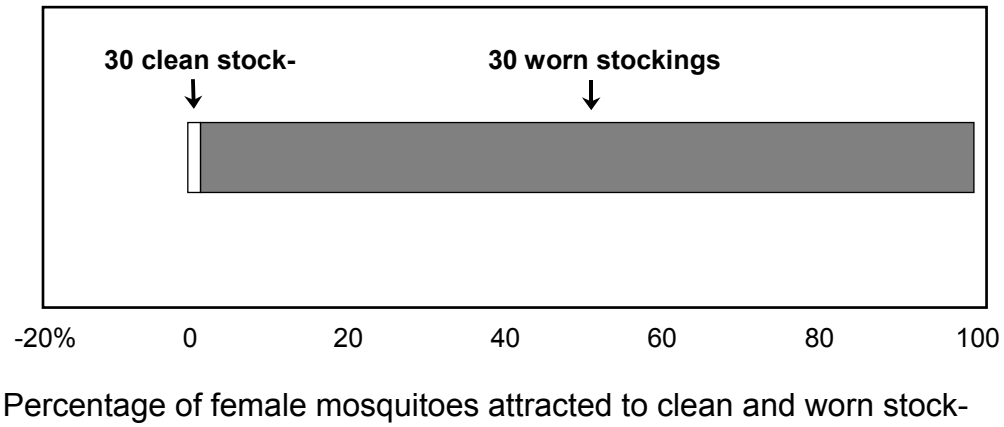

Fig. 1 Attractiveness of stockings worn by human volunteers to *An. gambiae* in a dual-choice olfactometer.

Table 1 Compounds identified by GC-MS that elicited GC-EAG responses by female *An. gambiae* present in the headspace of fifteen pairs of nylon stockings worn for 3 days continuously by human volunteers

Number	Retention time	Chemical identified	No. antennae responding (N=12)
1	9'42"	1,2,4-trimethyl benzene	4
2	10'16"	Siloxanes + octanal (t)	4
3	11'06"	1-methyl-3-propylbenzene or 1-methyl-4-propylbenzene	4
4	11'58"	2-ethyl-1-hexanal	2
5	13'50"	2-nonanone	4
6	15'08"	1,2,4,5-tetramethyl benzene	5
7	15'20"	1,2,3,5-tetramethyl benzene	12
8	16'29"	3-phenylbut-1-ene	9
9	16'54"	1,2,3,4-tetramethyl benzene	4
10	18'10"	naphthalene	5
11	19'32"	decanal	4
12	19'55"	benzothiazole or 1,2-benzisothiazole	4
13	21'12"	2-[(2-ethylhexyl)oxy]-ethanol	8
14	23'05"	[1,1'-bicyclopentyl]-2-one and tridecane	4

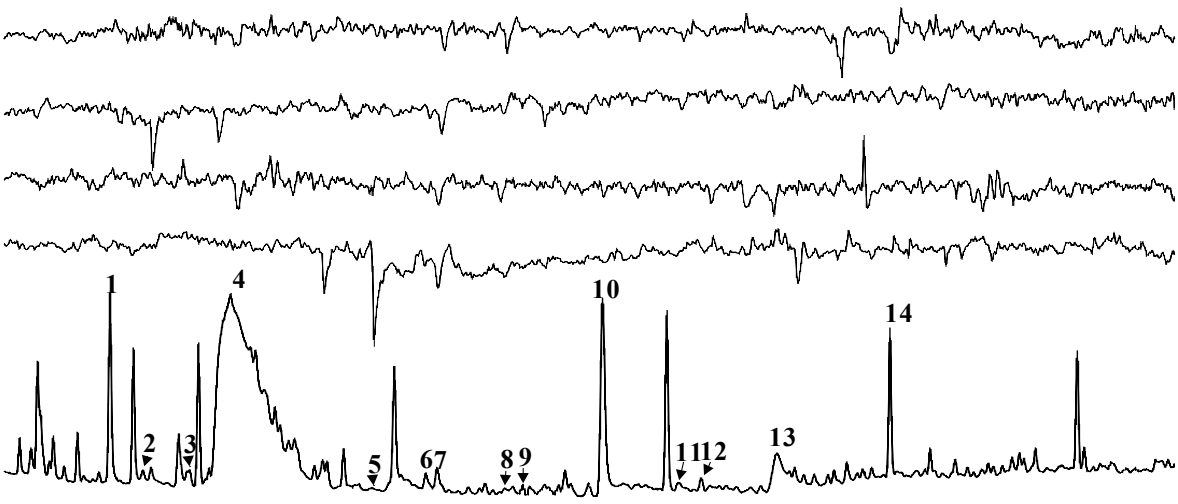

Fig. 2 Four EAG tracks (upper four) and their GC chromatogram (lowest one) recorded from antennae of female *An. gambiae* in response to the headspace of fifteen pairs of nylon stockings worn for three days continuously by human volunteers.

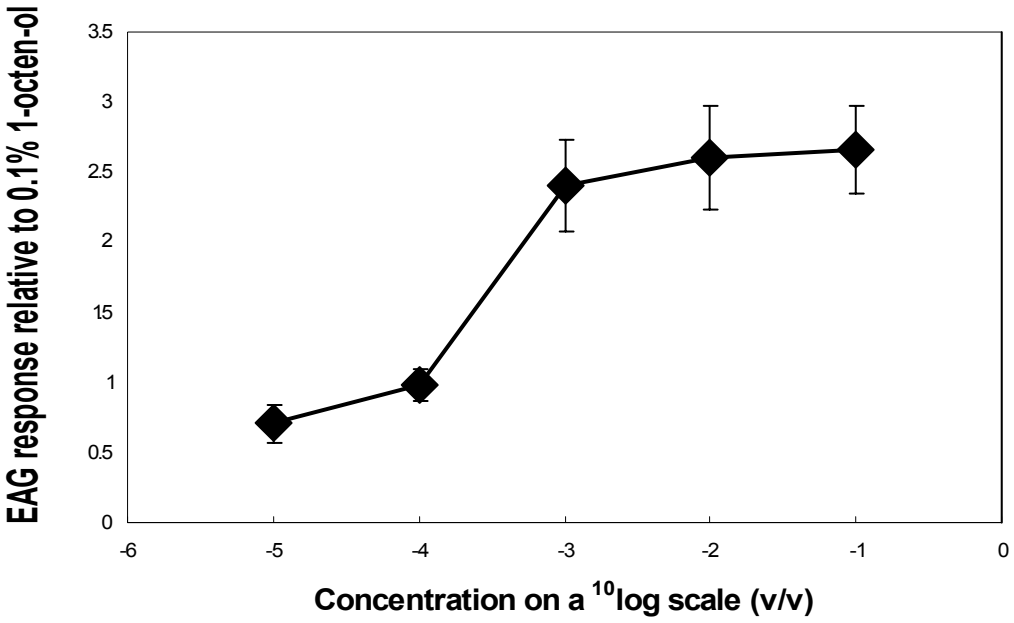

Fig. 3 EAG dose-response curve of female *An. gambiae* to benzothiazole. The diamonds and error bars represent the mean values and the SEs of the standardised EAG responses (N=8).

identified by a separate GC-MS analysis (Fig. 2 and Table 1)

The compounds in Table 2 were not typically of human origin. They were likely to be released from the material of the nylon stockings or were present in the environment where the volunteers had been during the 3 days before the experiment. In order to investigate whether the mosquitoes responded to volatiles associated with human-worn apparel (socks and shoes), we tested off-line EAG responses of one of the identified compounds, benzothiazole, which was also found in odours from unwashed feet (E.J. van der Meent, personal communication)

The EAG dose-response curve of benzothiazole had a classical sigmoidal shape (Fig. 3). The EAG response for 0.1% benzothiazole was 2.5 times higher than that of the 0.1% 1-octen-3-ol positive control. It was confirmed that female *An. gambiae* responded strongly to benzothiazole by EAG.

Would electrophysiological activity of benzothiazole correlate with behavioural attraction to females of *An. gambiae*? To answer this question, we also tested benzothiazole in the

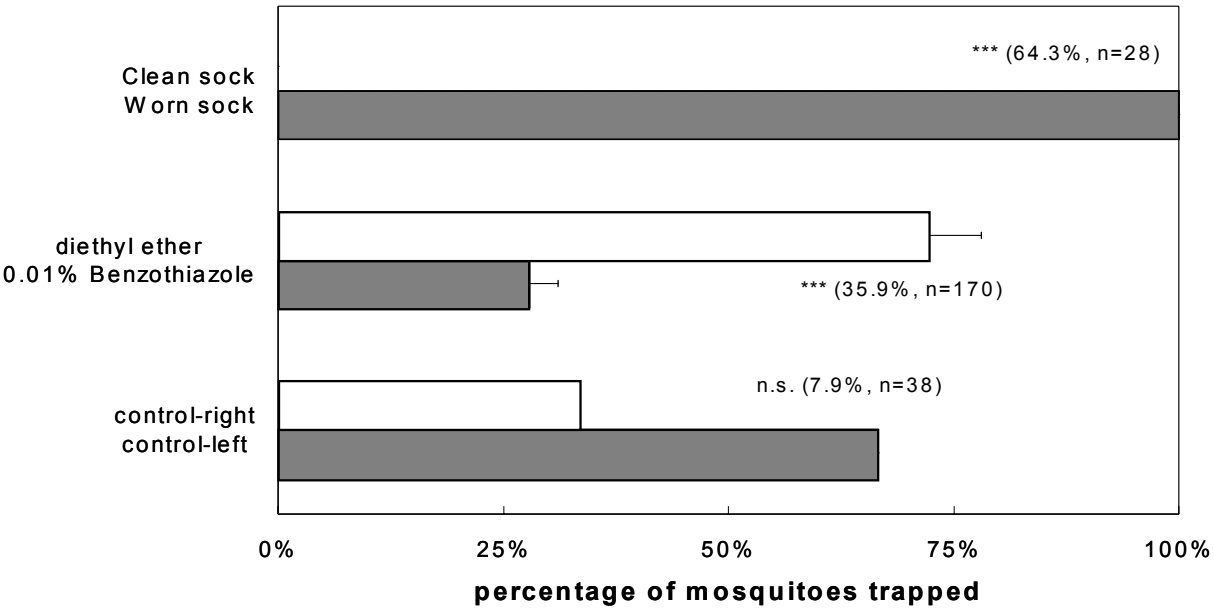

Fig. 4 Responses of female *An. gambiae* to odours tested pair-wise in a dual-choice olfactometer.

olfactometer. As observed before, worn stockings were found to be highly attractive compared with clean stockings (chi-square test, $P<0.001$) (Fig. 4). The two trapping devices caught together 64.3% of the total number of released mosquitoes. When we tested 0.01% benzothiazole against the solvent, diethyl-ether, the benzothiazole-baited trapping device caught significantly fewer mosquitoes than the solvent-baited trapping device (chi-square test, $P<0.001$). However, the total responses in the experiments with benzolthiazole (35.9%) were higher than in the experiments with no odours (7.9%). It seems that benzothiazole stimulated the searching behaviour of the mosquitoes from a distance, but repelled the mosquitoes at close range.

These studies show that an on-line GC-EAG study of volatile host stimuli for investigations of the olfactory behaviour of *An. gambiae* is possible and as such, this is the first report documenting its feasibility. However, the sampling method applied, using nylon stockings as an adsorbing matrix produced a range of benzene derivatives (7 of the 14 compounds listed in Table 1) which we consider to be contaminants of non-host origin which may have obstructed the detection of relevant kairomones for these insects. This conclusion is supported by the occurrence of these same contaminants in headspace samples of clean nylon stockings (data not shown). In this study, 14 of the 23 compounds producing consistent EAG activity could be identified by GC-MS, leaving 9 compounds of potential interest that remain to be identified. Consistent activity was detected in response to a number of al-

dehydes, a ketone and an alcohol, compounds which have been detected previously in human emanations (Meijerink *et al*., 2000), some of which have been shown to produce electrophysiological activities (Chapter 8 and 9). Future studies should employ different methods, such human handled glass beads (Chapter 2 and 3) and Solid Phase Microextraction (SPME), for collecting volatiles emanating from the human skin. The volatiles are subsequently volatilized by thermodesorption, thereby avoiding the use of solvents. Furthermore, the application of an on-line GC-MS-EAG setup would be useful in the identification of possible attractants or repellents for *An. gambiae* (A.M. Galimard, unpublished data).

Acknowledgements

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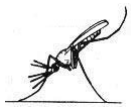
5

Synergism between ammonia, L-lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae*



Smallegange, R.C., Qiu, Y.T., van Loon, J.J.A. & Takken, W.

Abstract

Host odours play a major role in the orientation and host location of blood-feeding mosquitoes. *Anopheles gambiae* Giles *sensu stricto*, which is the most important malaria vector in Africa, is a highly anthropophilic mosquito species and host-seeking behaviour of the females of this mosquito is guided by volatiles of human origin. Ammonia, lactic acid and several carboxylic acids are known to be present in the human odour blend. We investigated the effect of these compounds on naive female mosquitoes using a dual-port olfactometer. Ammonia was an attractant on itself, whereas lactic acid was not attractive. Carboxylic ac-

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ids, offered as a mixture of 12 compounds, were repellent at the concentration tested. The addition of ammonia to the carboxylic acid mixture overruled the repellent effect of the latter. Combining ammonia with either lactic acid or the carboxylic acids did not enhance the attractiveness of ammonia alone. However, a synergistic effect was found when ammonia, lactic acid and the carboxylic acids were applied as a blend. Our findings indicate that *An. gambiae s.s.* relies on the combination of ammonia, lactic acid and carboxylic acids in its orientation to human hosts. The role of lactic acid in this tripartite synergism differs from that reported for the yellow fever mosquito *Aedes aegypti*.

Introduction

Females of the mosquito *Anopheles gambiae* are important vectors of malaria in Africa. The dominance of this mosquito as a malaria vector is largely due to its preference for human blood (White, 1974; Coluzzi *et al.*, 1979; Pates *et al.*, 2001a). Because female mosquitoes use host odours to find their blood-hosts (Takken, 1991; Takken & Knols, 1999) and because *An. gambiae* females are highly anthropophilic, it is likely that the latter use human-specific odour compounds for orientation.

Field studies in Africa showed that carbon dioxide (CO_2), a major component of breath, accounts for only a minor part of the attractiveness of a human host to *An. gambiae* females. Human body odour appeared to play a larger role (Costantini *et al.*, 1996a; Mboera *et al.*, 1997; Costantini *et al.*, 1998). This is in accordance with the hypothesis mentioned above since all warm-blooded vertebrates exhale CO_2 and it is unlikely that *An. gambiae*, which has a preference for humans, will be guided to its host by this compound alone.

Laboratory studies aimed at elucidating the compounds constituting human-produced odour blends that mosquitoes use for host location have yielded several active mixtures and individual substances. Human sweat was found to be attractive to *An. gambiae* (Braks *et al.*, 1997; Braks & Takken, 1999; Healy & Copland, 2000). One of its components is ammonia and it was shown in a dual-port olfactometer that ammonia is a kairomone for *An. gambiae* (Braks *et al.*, 2001). Carboxylic acids make up an important part of human sweat (Cork & Park, 1996). However, an artificial blend of 22 aliphatic carboxylic acids did not elicit landings of *An. gambiae* females whereas 2-oxopentanoic acid did, although the latter compound was not attractive from a distance (Healy & Copland, 2000). In contrast, Knols *et al*. (1997) found that a synthetic mixture of 12 aliphatic carboxylic acids was attractive to females of *An. gambiae* in a dual-port olfactometer. Human sweat also contains lactic acid (Cork & Park, 1996; Healy & Copland, 2000) and humans seem to have uniquely high levels of this compound on their skin compared to animals (Dekker *et al.*, 2002). Although Healy and Copland (2000) found that *An. gambiae* females land on filter papers impregnated with hu-

man sweat, the concentration of lactic acid that was present in sweat did not elicit landing responses. Braks *et al.* (2001) showed that this compound was slightly attractive to *An. gambiae* females in an olfactometer.

Lactic acid is known to play an important role in the host-seeking behaviour of another anthropophilic mosquito species, the yellow fever mosquito, *Aedes aegypti* L. (Acree *et al.*, 1968; Smith *et al.*, 1970; Geier *et al.*, 1996). Ammonia was also identified as an attractant for *Ae. aegypti*; it is not attractive when tested alone, but it enhances the attractiveness of lactic acid (Geier *et al.*, 1999). Fatty acids of chain length C1-C3, C5-C8 or C13-C18 had the same effect when mixed with lactic acid. The combination of lactic acid, ammonia and two fatty acids appeared to be almost as attractive as an extract of human skin residues (Bosch *et al.*, 2000).

Building upon the results of Geier *et al.* (1999) and Bosch *et al.* (2000) with *Ae. aegypti* and with the knowledge that the human sweat compounds ammonia, L-lactic acid and several carboxylic acids were attractive to *An. gambiae*, we examined the effects of blends of these compounds on the behaviour of this mosquito. Initially, because the amount released by humans is to our knowledge unknown, the concentration range within which ammonia is attractive to *An. gambiae* females was examined. Subsequently experiments were done to examine whether ammonia might cause synergism when combined with lactic acid and/or a mixture of carboxylic acids.

Materials and Methods

Mosquitoes

The *Anopheles gambiae* Giles *sensu stricto* colony at Wageningen University, The Netherlands, originated from Suakoko, Liberia. The mosquitoes have been cultured in the laboratory since 1988 with blood meals from a human arm twice a week. The adult mosquitoes were maintained in 30 x 30 x 30 cm gauze cages at 27 ± 1°C, 80 ± 5% relative humidity, and a photo-scotophase of 12:12 LD. They had access to a 6% glucose solution on filter paper. The larvae were reared in tap water in plastic trays and fed daily with Tetramin® baby fish food. Pupae were collected daily and placed in adult cages for emergence.

Olfactometer

A dual-port olfactometer (see Pates *et al.*, 2001b), consisting of a Luxan flight chamber of 1.60 x 0.66 x 0.43 m, was used to study the behavioural responses of female mosquitoes to different odour stimuli. Pressurised air was charcoal filtered, humidified and led through

two perspex mosquito trapping devices, which were linked to two ports (diameter 4 cm, 28 cm apart), into the flight chamber with a speed of 0.22 ± 0.02 m/s. The light of one tungsten light bulb (75 Watt) was filtered and scattered through a screen of yellow cloth hanging ± 1 m above the flight chamber. This resulted in dim light of about 1 Lux in the olfactometer. The experimental room was maintained at a temperature of 28 ± 1.5 °C and a relative humidity of 60 ± 6 %. The temperature inside the flight chamber was equal to that of the room and the relative humidity was maintained at 65 ± 6 %. The relative humidity of the air flowing out of the ports was maintained above 80% and the temperature was 28 ± 1.5 °C.

Odour stimuli

Compounds were tested singly and in combinations with each other. They were tested either against clean air or against one or two of the test stimuli. The bioassay methods described by Braks *et al.* (2001) were used for the preparation of ammonia and lactic acid; the mixture of carboxylic acids was made following the method of Knols *et al.* (1997).

Ammonia

One day before the experiments, a specified amount of an aqueous ammonia solution (Stock: Merck, min. 25%, 1l = 0.91 kg) was injected in a 80 litre dual stainless steel fitted Tedlar air sample bag (SKC Inc., USA) (Table 1). Subsequently, the bag was filled with 60 litre humidified and filtered warm pressurised air at least 17 hours prior to the experiments to allow the ammonia to evaporate. This procedure resulted in ammonia concentrations of 13.6, 136, 1,364, 13,637, 27,274 or 136,371 ppm in different bags (Table 1). Another 80 litre air sample bag filled with 250 µl of distilled water and 60 litre air was prepared in a similar way to be used as the control stimulus. During the experiments, the air was pumped at 230 ml/min (MG-4 Ametek air pump, Ametek, U.S.A.) from the air sample bags through silicon tubes (diameter 7 mm; Rubber b.v., The Netherlands) into one or both trapping devices where it mixed with the main air stream (Braks *et al.*, 2001). Based on the response of the mosquitoes to these six concentrations of ammonia (Fig. 1), an ammonia concentration of 136 ppm was used in subsequent experiments.

Lactic acid

L-(+)-lactic acid sodium salt (98%, L-7022, Sigma, St. Louis, MO, USA) was dissolved in

Table 1. Conversion table used for the calculation of the NH_3

Concentration of NH_3 in watery solution (%)	Volume of NH_3 solution (ml) added to air sample bag	Concentration of NH_3 in air sample bag (ppm)*
0.25	0.25	13.6
2.5	0.25	136.4
25	0.25	1363.7
25	2.5	13,637.1
25	5	27,274.2
25	25	136,370.9

*We calculated the concentration of ammonia as follows:
$[(C \times V \times d \times V_m)/(A \times MW)] \times 10^6$
C: concentration of ammonia solution (%)
V: volume of ammonia solution added to the air sample bag (ml)
d: density of ammonia solution (g/ml)
V_m: molar volume of gas at 25ºC and one atmosphere pressure (24.5 l/mol)
A: volume of the air in the sample bag (60 l in our experiments)
MW: molecular weight of ammonia (17.03 g/mol)

ethanol (Merck, Ethanol absolute, pro analysis) following the method used by Braks *et al*. (2001) and Geier *et al.* (1999) who found certain concentrations of this compound to be attractive to *An. gambiae* and *Ae. aegypti*. For each experiment 100 µl of a lactic acid solution was applied on filter paper (5x2 cm; Whatman 2 or Schleicher & Schuell 595) which was placed in an iron clip. The ethanol was allowed to evaporate before the clip with the filter paper was put in a trapping device. For the control stimulus an equivalent amount of ethanol was applied in a similar way and placed in the other trapping device (Braks *et al.*, 2001).

Initially, five different concentrations of lactic acid (0.1, 0.01, 0.001, 0.0001, and 0.00001 g/ml) were tested in combination with gaseous ammonia (136 ppm) and against gaseous ammonia only (136 ppm) to obtain the highest attractiveness of the mixture of these two compounds together. Based on the results of this experiment and the results previously obtained by Braks *et al.* (2001), the concentration of lactic acid used in the subsequent experiments was 0.001 g/ml

Mixture of carboxylic acids

A mixture of carboxylic acids was dissolved in diethyl ether (Merck, min. 98%) based on the relative amounts of each acid in the acid extracts of Limburger cheese samples, which were shown to be attractive to *An. gambiae* (de Jong and Knols, 1995; Knols *et al*., 1997). A sandblasted glass slide (5x2 cm) with 100 µl of the 10^8 times diluted synthetic mixture of the 12 carboxylic acids (Sigma, min. 99%) was placed in a trapping device. A control glass slide with an equivalent amount of diethyl ether was placed in the opposite trapping device after the diethyl ether had evaporated.

Experimental procedure

Thirty female mosquitoes, 5-8 days-old, which had not received a blood meal, were randomly collected from their cage 14 to 18 hours before the start of the experiments and placed in a cylindrical release cage (diameter 8 cm, height 10 cm) with access to tap water from damp cotton wool placed on top of the cage.

The experiments were performed during the last four hours of the dark period, when *An. gambiae* is normally active. In each trial test odours were released in the airstream before a group of mosquitoes was set free from a cage which was placed at the downwind end of the flight chamber, 1.60 m from the two ports. Mosquitoes were left in the flight chamber for 15 minutes. The female mosquitoes that had entered either trapping device were counted at the end of the experiments, after anaesthetisation with 100% carbon dioxide. Mosquitoes remaining in the flight chamber were removed with a vacuum cleaner.

Each trial started with new mosquitoes, clean trapping devices, and new stimuli. Experiments were repeated at least six times on different days. The sequence of test odours was randomised on the same day and between days. Test stimuli were alternated between right and left ports in different replicates to rule out any positional effects. Experiments with clean air only in either port were done to test the symmetry of the trapping system. Surgical gloves were worn by the experimenter to avoid contamination of the equipment with human volatiles.

Statistical analysis

For each two-choice test a chi-square test was used to analyse whether the total (i.e. sum of all replicates) number of mosquitoes that was trapped in the treatment trapping device and

the total number that was trapped in the control trapping device differed from a 1:1 distribution. A Generalized Linear Model (GLM; Binomial, linked in logit; Genstat, release 4.2) was used to investigate the effect of the different odour stimuli on the total response (i.e. total number of mosquitoes that entered both the treatment and control trapping device as fraction of the total number of mosquitoes that left the release cage). Two-sided t-probabilities were calculated to test pairwise differences between means. Effects were considered to be significant at $P<0.05$ (Oude Voshaar, 1994; Sokal & Rohlf, 1998).

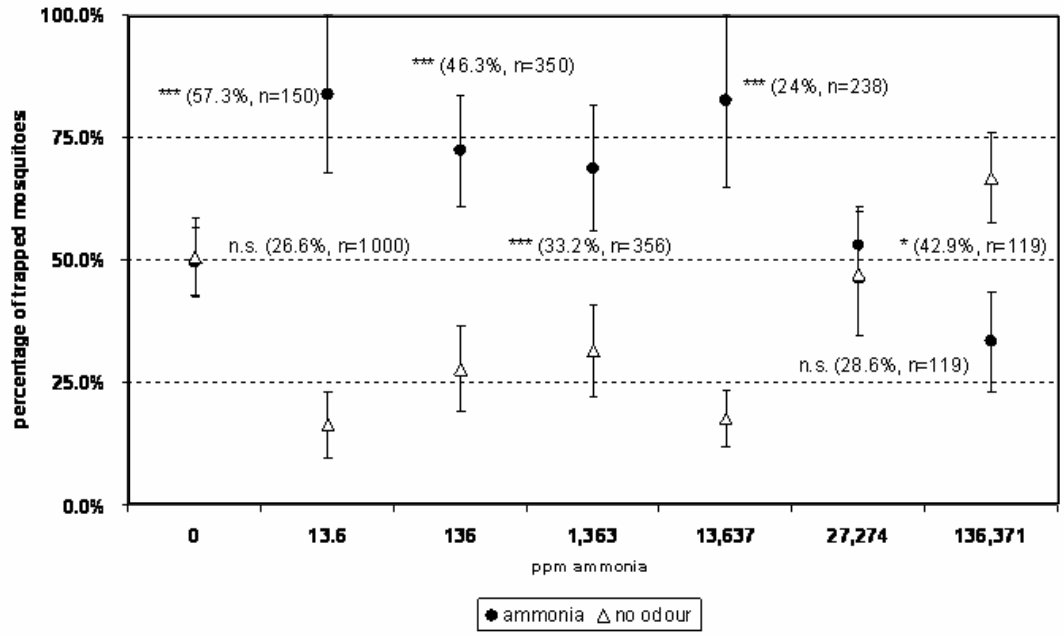


Fig. 1 Responses of *An. gambiae s.s.* females to different concentrations of gaseous ammonia (ppm), applied from sample bags, plotted on a logarithmic scale. The total percentage of mosquitoes that flew into either trap is shown. Vertical bars show standard errors of the mean. Asterisks mark significant differences between the total number of mosquitoes trapped in both trapping devices; with ammonia and no odour, respectively (chi-square test: n.s.: not significant; *: $P<0.05$; ***: $P<0.001$). Between brackets with each datapoint, the total percentage of mosquitoes that entered one or two of the trapping devices is given together with the total number of mosquitoes that left the release cage (n).

Table 2 Responses of *An. gambiae s.s.* females to gaseous ammonia (NH_3, released at 136.4 ppm) combined with lactic acid (LA) in 5 different concentrations (1: lowest concentration; 5: highest concentration tested, see Materials and methods)

Stimuli		N released[a]	Response[b]		Chi-square[c]	Total response[d]
treatment	control		treatment	control		
NH_3	NH_3	145	17	25	ns	29.0
NH_3 + LA conc 1	NH_3	169	29	20	ns	29.0
NH_3 + LA conc 2	NH_3	173	15	32	*	27.2
NH_3 + LA conc 3	NH_3	168	15	13	ns	16.7
NH_3 + LA conc 4	NH_3	173	23	14	ns	21.4
NH_3 + LA conc 5	NH_3	162	19	16	ns	21.6

[a] The total number of mosquitoes that left the release cage.
[b] The response is given as the total number of mosquitoes caught in either the treatment or control trapping device.
[c] Significant differences (*: $P<0.05$) or no significant differences (n.s.: $P>0.05$) between the total number of mosquitoes caught in the treatment and control trapping device (chi-square test).
[d] The total number of mosquitoes that entered both the treatment and control trapping device as fraction of the total number of mosquitoes that left the release cage (%).

Results

Symmetry of trapping system

An. gambiae females responded to a humidified odourless air stream as was shown previously by (Knols *et al.*, 1994). In all our experiments in which we tested clean air coming from both ports, equal numbers of mosquitoes were caught in both trapping devices (chi-square test; P^3 0.50) which demonstrated that the olfactometer was symmetrical (Fig. 1).

Dose-response effects of ammonia

Fig. 1 shows the dose-dependent response of *An. gambiae* to gaseous ammonia. Odorous air from sample bags containing 13.6, 136, 1,364 or 13,637 ppm ammonia attracted significantly more mosquitoes than clean air (chi-square test; $P< 0.05$). Catches with air containing 27,274 ppm ammonia did not differ from the catches in the trapping device with clean air

Table 3 Responses of *An. gambiae* s.s females to combinations of gaseous ammonia (NH_3), lactic acid (LA) and a synthetic mixture of 12 carboxylic acids (CAmix).

	Stimuli		N released[a]	Response[b]		Chi-square[c]	Total response[d]
	treatment	control		treatment	control		
A	clean air	clean air	921	42	44	ns	9.3
	NH_3	clean air	489	59	37	*	19.6
	LA	clean air	732	54	38	ns	12.6
	CAmix	clean air	592	38	79	***	19.8
	NH_3 + LA	clean air	375	57	24	***	21.6
	NH_3 + CAmix	clean air	169	31	16	*	27.8
	LA + CAmix	clean air	378	28	45	*	19.3
	NH_3 + LA + CAmix	clean air	381	57	21	***	20.5
B	NH_3	NH_3	107	7	6	ns	12.1
	NH_3 + LA	NH_3	119	5	8	ns	10.9
	NH_3 + CAmix	NH_3	116	14	17	ns	26.7
	NH_3 + LA + CAmix	NH_3	115	17	6	*	20.0
C	LA	LA	110	2	4	ns	5.5
	NH_3 + LA	LA	139	5	2	ns	5.0
	LA + CAmix	LA	138	6	6	ns	8.7
	NH_3 + LA + CAmix	LA	140	19	5	**	17.1
D	CAmix	CAmix	85	0	2	ns	2.4
	NH_3 + CAmix	CAmix	131	11	3	*	10.7
	LA + CAmix	CAmix	130	7	6	ns	10.0
	NH_3 + LA + CAmix	CAmix	128	17	2	***	14.8
E	NH_3 + LA + CAmix	NH_3 + LA	180	22	25	ns	26.1
	NH_3 + LA + CAmix	NH_3 + CAmix	172	17	12	ns	16.9
	NH_3 + LA + CAmix	LA + CAmix	178	26	8	**	19.1

[abd] See descriptions under Table 1;
[c] Significant differences (*: P<0.05; **: P< 0.01; ***: P<0.001) or no significant differences (n.s.: P>0.05) between the total number of mosquitoes caught in the treatment and control trapping device (chi-square test).
NH_3 = ammonia mixed at 136.4 ppm with main airstream, see Material and methods.
LA = lactic acid released from a solution of 0.001 g/ml in ethanol
CAmix = mixture of 12 carboxylic acids applied at 10^{-8} dilution in diethyl ether. Composition given in Knols *et al.*(1997)

(chi-square test; P³ 0.05). The mosquitoes preferred the clean air to the highest concentration of ammonia tested, 136,371 ppm (chi-square test; P= 0.02), indicating that this concentration is repellent for *An. gambiae*.

The total number of mosquitoes responding in the experiments with 13,637 (24%) and 27,274 (28.6%) ppm ammonia was not significantly different from the control (clean air against clean air) (26.6%), whereas the response (varying from 33.2 - 57.3%) was significantly higher when the other concentrations were tested (GLM; Fig. 1).

Different concentrations of lactic acid in combination with ammonia

None of the concentrations of lactic acid that were tested against ammonia alone (136 ppm) increased the attractiveness of ammonia (chi-square test) (Table 2). The concentration of 0.0001 g/ml of lactic acid combined with ammonia was repellent.

Responses to blends of ammonia, lactic acid and a synthetic mixture of 12 carboxylic acids

Gaseous ammonia at a dose of 136 ppm alone was attractive when tested against clean air (chi-square test; P= 0.02), whereas lactic acid alone was not (chi-square test; P= 0.10). The combination of ammonia and lactic acid attracted significantly more mosquitoes than clean air (chi-square test; P= 0.0003) (Table 3A).

The synthetic mixture of the 12 carboxylic acids was repellent when tested alone and in combination with lactic acid (against the solvents only) (chi-square test; P= 0.0002 and P= 0.047, respectively). However, the combination of the carboxylic acid mixture with ammonia and with both ammonia and lactic acid was significantly more attractive than clean air (chi-square test; P= 0.03 and P= 0.00005, respectively; Table 3A).

The attractiveness of ammonia was not increased by the addition of lactic acid or by he carboxylic acid mixture (chi-square test; P= 0.41 and P= 0.59, respectively; Table 3B). However, the combination of ammonia, lactic acid and carboxylic acids was significantly more attractive than ammonia alone (chi-square test; P= 0.02).

A mixture of carboxylic acids and lactic acid or a mixture of ammonia and lactic acid did not attract more mosquitoes than lactic acid alone (chi-square test; P= 1.000 and P= 0.26, respectively). But as with the previous series of experiments (Table 3B), the combina-

tion of all chemicals together attracted more mosquitoes than only one of the compounds, lactic acid (chi-square test; P= 0.004; Table 3C).

Lactic acid combined with the carboxylic acids was not more attractive than the latter alone (chi-square test; P= 0.78). Adding ammonia only or ammonia together with lactic acid overruled the repellency of the carboxylic acid mixture (chi-square test; P= 0.03 and P= 0.0006, respectively; Table 3D).

No significant differences were found between the number of mosquitoes attracted to the combination of ammonia, lactic acid and the carboxylic acid mixture and the numbers attracted to the mixture of ammonia and lactic acid or the combination of ammonia and the carboxylic acid mixture (chi-square test; P= 0.66 and P= 0.35, respectively; Table 3E). The combination of lactic acid and the carboxylic acid mixture was significantly less attractive than the mixture of all chemicals together (chi-square test; P= 0.002).

Discussion

Ammonia, lactic acid and several carboxylic acids have been detected in human sweat and in human skin emanations (Eiras & Jepson, 1991; Cork & Park, 1996; Bernier *et al.*, 1999; Bernier *et al.*, 2000; Braks *et al.*, 2001). Our experiments clearly show that the combination of these components affects the host-seeking behaviour of the highly anthropophilic females of the malaria mosquito *An. gambiae s.s.*.

As was shown before by Braks *et al.* (2001), ammonia alone is attractive to *An. gambiae* females. Concentrations of 13.6 to 13,637 ppm of ammonia in the sampling bag attracted 25 to 57 percent of the mosquitoes. Higher concentrations produced repellency (Fig. 1). These data confirm that ammonia is an important kairomone derived from incubated sweat, an attractive complex odour source containing higher amounts of ammonia (49.4 mM) than fresh sweat (6.3 mM), which is not or slightly attractive (Braks, 1999; Meijerink *et al.*, 2000; Braks *et al.*, 2001). By our calculations the dilution of ammonia from the air sample bags in the surrounding air stream was 100 fold, but due to the turbulence of the plume, causing heterogeneous mixing of odour components, it is likely that instantaneous peak concentrations of ammonia may have been present, similar to the ammonia concentration in the sample bags (Murlis *et al.*, 1992), to which the mosquitoes were exposed, starting with the undiluted concentrations as released from the bags (Table 1). Assuming a 100 fold dilution, the lowest concentration for which we found attractiveness (136 ppb) is within the range found in human breath (between 40 and 3170 ppb, Larson *et al.* 1979). This concentration is above the threshold for attractiveness reported with *Ae. aegypti* (between 2 and 17 ppb, Geier *et al.*, 1999). Another crucial difference between the two species is that in *Ae. aegypti* ammo-

nia was only active in combination with lactic acid as an essential synergist. Conversely, lactic acid alone attracts *Ae. aegypti* (Geier *et al.*, 1996; Geier *et al.*, 1999), but not *An. gambiae.* In *An. gambiae* the mixture of ammonia and lactic acid does not attract more mosquitoes than ammonia alone (Tables 2 and 3B). The synthetic mixture of the 12 carboxylic acids did not increase the attractiveness of ammonia either (Table 3B). However, only the combination of ammonia, lactic acid and carboxylic acids was significantly more attractive than any of the three components alone (Table 3B-D). Therefore, enhanced attractiveness of the combined components by definition implies a tripartite synergistic effect between these stimuli. This tripartite synergism was not revealed for *Ae. aegypti* as the carboxylic acids have only been tested in combination with lactic acid (Bosch *et al.*, 2000). These data also demonstrate the important role of ammonia in the blend, as the addition of lactic acid or carboxylic acids to binary blends of ammonia and carboxylic acids or ammonia and lactic acid, respectively, did not result in enhanced attractiveness, whereas addition of ammonia to a blend of lactic acid and carboxylic acids clearly caused significantly enhanced effects (Table 3E).

Although in our experiments lactic acid alone was not significantly more attractive than clean air (Table 3A), contrary to what was found by Braks *et al.* (2001) and Smallegange *et al.* (2002), but corresponding to Dekker *et al.* (2002), the data presented here show that lactic acid is necessary to evoke the synergistic effect with ammonia and carboxylic acids that we observed (Table 3).

It was remarkable that ammonia suppressed the repellent effect of the carboxylic acid mixture (Table 3A, B). When tested alone, the carboxylic acid mixture was repellent (Table 3A). This result appears in contrast with the first report of olfactory activity of carboxylic acids to *An. gambiae* from our laboratory, where a similar mixture of carboxylic acids at the same concentration (diluted by a factor of 10^8) was reported attractive (Knols *et al.*, 1997). Impurities in the (commercially) obtained carboxylic acids may be one explanation for the differences in results between those of Knols *et al*. and the present study. The repellent effect of carboxylic acids alone is also illustrated by the very low response (2.4%) of mosquitoes when the acids were released from both odour ports of the olfactometer (Table 3D). Whether only one or several carboxylic acids caused the repellency of the 12-component mixture needs to be investigated. For *Ae. aegypti*, Bosch *et al*. (2000) found that especially C1-C3 and C5-C8 carboxylic acids enhanced attractiveness of lactic acid whereas C9 and C11 reduced attractiveness. We already know from electroantennographic (EAG) studies that *An. gambiae* females can detect saturated carboxylic acids. These studies showed that short-chain carboxylic acids elicit higher EAG responses than less volatile, long-chain, acids (Cork & Park, 1996; Knols *et al.*, 1997). Experiments with *An. gambiae* females that were done in Y-tube olfactometers showed that the synergistic effect could also be achieved when

combining ammonia and lactic acid with only one of the short-chain carboxylic acids that was present in our synthetic mixture: hexanoic acid (Smallegange *et al.*, 2002). In addition, Costantini *et al.* (2001) reported attractiveness of an unsaturated carboxylic acid, 7-octenoic acid, which is a human-specific component secreted from the apocrine sweat glands in the axillary regions (Zeng *et al.*, 1991).

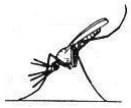
Our results show that a synthetic blend of volatiles, all naturally present in human odour (Bernier *et al.*, 2000; Healy & Copland, 2000; Braks *et al.*, 2001), provides a synergistic effect on olfaction-based trap-entry responses of *An. gambiae* mosquitoes. It is clear from field studies that *An. gambiae* is strongly attracted to natural human odours (Haddow, 1942; Ribbands, 1950; Costantini *et al.*, 1996a; Mboera *et al.*, 1997), and the development of traps baited with highly attractive synthetic blends simulating human odours appears realistic.

Acknowledgements

We thank F. van Aggelen, A. Gidding, and L. Koopman for rearing the mosquitoes. The research was financed by the Technology Foundation of the Netherlands Organisation for Scientific Research (STW-NWO) under project WBI.4834.

6

Host-seeking behaviour of the malaria mosquito *Anopheles gambiae* in response to synthetic odour blends



Qiu, Y.T., Smallegange, R.C., van Loon, J.J.A. & Takken, W.

Abstract

Host-seeking behaviour of the anthropophilic malaria vector *Anopheles gambiae* is mediated predominantly by olfactory cues. Several hundreds of odour components have been identified from human emanations, but only few have been proven to be attractants or synergists for the female mosquitoes. Among these, carboxylic acids were found to be important components of human sweat and synthetic blends have been demonstrated to attract *An. gambiae*. Alcohols and ketones were found to elicit electrophysiological activity in antennal olfactory neurons of female *An. gambiae* mosquitoes but likely behavioural effects of these compounds have not been investigated. In this study, behavioural responses of female *An. gambiae* to several concentrations of 16 carboxylic acids, a phenol, indole, two alcohols and three ketones, in combination with ammonia and lactic acid, were examined when tested against ammonia alone in a dual-choice olfactometer. The results showed that C3-C8 and C14 carboxylic acids augmented the attractiveness of ammonia + lactic acid at certain concentrations; whereas 4-ehthylphenol, indole, alcohols and ketones only reduced the stimulating effect of ammonia + lactic acid. The possible roles of these compounds in the host-

seeking behaviour of *An. gambiae* are discussed.

Introduction

Females of *Anopheles gambiae* Giles *sensu stricto* are effective human malaria vectors because of their strong preference for human blood, high longevity, and close association with human dwellings. Their host-seeking behaviour is predominantly mediated by chemical cues emanating from human bodies (Takken & Knols, 1999).

Although hundreds of components have been identified from human odours, only a few individual compounds were proven to be attractants on their own (Bernier *et al.*, 2000; Braks *et al.*, 2001; Healy *et al.*, 2002; Smallegange *et al.*, 2005). Ammonia concentrations were higher in incubated human sweat, which was more attractive than fresh sweat and ammonia alone was found to be consistently attractive in olfactometer bioassays (Braks *et al.*, 2001; Smallegange *et al.*, 2005). L-lactic acid alone was not or only slightly attractive to *An. gambiae* (Smallegange *et al.*, 2005; Braks *et al.*, 2001). However, synergism between ammonia, lactic acid and a blend of 12 carboxylic acids was found causing greater attraction than the individual components or binary mixtures (Smallegange *et al.*, 2005). Differences in L-lactic acid concentration on human skin may explain differences in attractiveness to mosquitoes between human individuals (Dekker *et al.*, 2002). A similar role was ascribed to CO_2 (Brady *et al.*, 1997). Six oxocarboxylic acids were reported to stimulate landing of *An. gambiae*, the compounds being considered as contact chemosensory cues (Healy *et al.*, 2002). Addition of the human-specific 7-octenoic acid to carbon dioxide attracted a greater number of *An. gambiae s.l.* to traps in Burkina Faso (Vale, 1987; Torr & Hall, 1992; Bosch *et al.*, 2000; Kappmeier, 2000; Costantini *et al.*, 2001). Although none of these studies have revealed an odour mixture that is as attractive as a human host is, it appears that the attraction of anopheline mosquitoes to humans is caused by a blend of volatile components in certain ratios rather than by single components (Vale, 1987; Torr & Hall, 1992; Bosch *et al.*; Kappmeier, 2000; Smallegange *et al*., 2005).

Electrophysiological studies provided evidence that olfactory sensilla on the antennae of female *An. gambiae* are tuned to phenols, indole and geranyl acetone (van den Broek & dan Otter, 1999; Meijerink *et al.*, 2001; chapter 8 & 9). This raised the question whether these compounds would elicit behavioural responses in olfactometer bioassays.

In the present study, the responses of female *An. gambiae* to several human odour components, alone or in combination with ammonia and L-lactic acid, were investigated in a dual-choice olfactometer at several concentrations. The results provide evidence for the role of these odour compounds in the host-seeking behaviour of *An. gambiae*.

Materials and methods

Mosquitoes

The *Anopheles gambiae sensu stricto* (henceforth termed *An. gambiae*) colony at Wageningen University, The Netherlands, originated from Suakoko, Liberia (courtesy Prof. M. Coluzzi, Rome, Italy). The mosquitoes have been cultured in the laboratory since 1988 with blood meals from a human arm twice a week. The adult mosquitoes were maintained in 30 x 30 x 30 cm gauze cages at 27 ± 1°C, 80 ± 5% relative humidity, and a photo-scotophase of 12:12 LD. They had access to a 6% glucose solution on filter paper. The larvae were reared in tap water in plastic trays and fed daily with Tetramin® (Melle, Germany) baby fish food. Pupae were collected daily and placed in adult cages for emergence.

Olfactometer

A dual-port olfactometer (Fig. 1), consisting of a Luxan flight chamber of 1.60 x 0.66 x 0.43 m, was used to study the behavioural responses of female mosquitoes to different odour stimuli. Pressurised air was charcoal filtered, humidified and led through two Perspex mosquito trapping devices into the flight chamber with a speed of 0.22 ± 0.02 m/s. The trapping devices were connected to two ports (diameter 4 cm, 28 cm apart). The light of one tungsten light bulb (75 Watt) was filtered and scattered through a screen of yellow cloth hanging ± 1 m above the flight chamber. This resulted in dim light of about 1 Lux in the olfactometer. The experimental room was maintained at a temperature of 28 ± 1.5 °C and a relative humidity of 60 ± 6 %. The temperature inside the flight chamber was equal to that of the room and the relative humidity was maintained at 65 ± 6 %. The relative humidity of the air flowing out of the ports was maintained above 80% and the temperature was 28 ± 1.5 °C.

Odour stimuli

In previous studies we found that females of *An. gambiae* are attracted to ammonia alone. Adding L-lactic acid did not increase the attractiveness of ammonia. However, a synergist effect was found for the combination of ammonia, L-lactic acid and a mixture of carboxylic acids (Smallegange *et al*., 2005). In this study, single odour compounds were tested in combination with ammonia and L-lactic acid against ammonia, using the ammonia and L-lactic acid concentrations that was present in the attractive mixture as described by Smallegange *et al*. (2005).

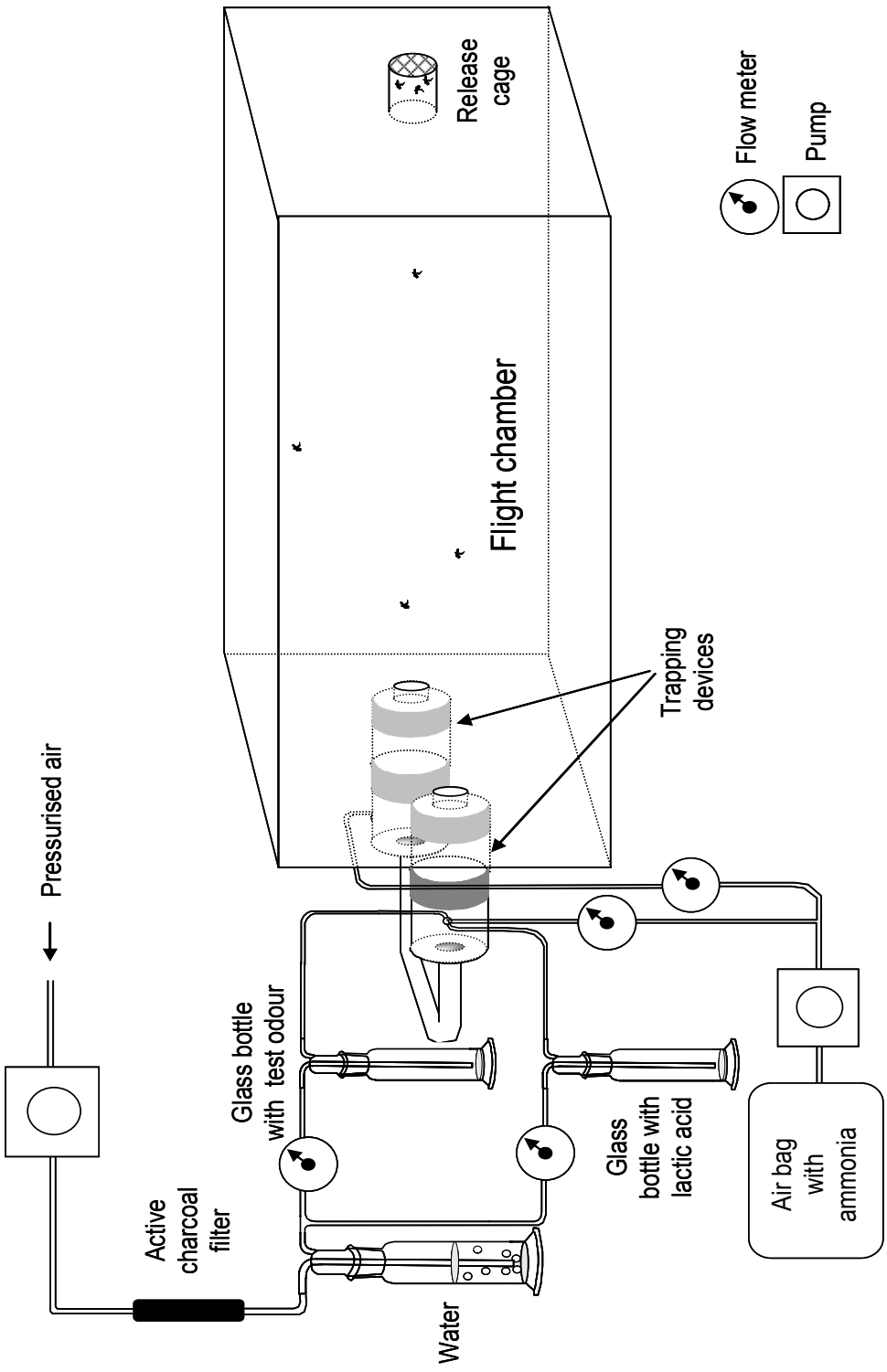


Fig. 1 Diagram of the dual-choice olfactometer.

Ammonia

One day before the experiments, 250 µl of 2.5% ammonia aqueous solution (25% in water, analytical grade, Merck) was injected in a 80 l Teflon air sample bag (SKC Gulf Coast Inc., Houston, TX, USA). Subsequently, the bag was filled with 60 l humidified and filtered warm pressurised air at least 17 hours prior to the experiments to allow evaporation of the solution. This procedure resulted in an ammonia concentration of 136 ppm in the bag. Another 80 l air sample bag filled with 250 µl distilled water and 60 l of air was prepared in a similar way to be used as the control stimulus. During the experiments, the air was pumped (Model 224-PCXR4, SKC Gulf Coast Inc., Houston, TX, USA) from the air sample bags through Teflon tubes (diameter 7 mm) into the trapping devices at 230 ml/min regulated by a mechanical flowmeter (Brooks Instruments, Veenendaal, The Netherlands), where it was mixed with the main air stream of 23.5 l/min.

L-lactic acid

To test the attractiveness of L-lactic acid (90% aqueous solution, analytical grade, Purac Bioquimica) alone, it was injected in the main air stream from a 250 ml glass bottle (Fisher Scientific B.V.,'s Hertogenbosch, The Netherlands) through Teflon tubing. A flowmeter (Gilmont, Fisher Scientific B.V.,'s Hertogenbosch, The Netherlands) regulated? flow rates of 15, 100 or 230 ml/min, respectively. In other experiments a flow rate of 15 ml/min was used, achieving a release rate comparable to that from a human hand (Smith *et al.*, 1970; Geier *et al.*, 1999).

Acetone

One day before the experiments, 9 µl of acetone (≥ 99.5%, Merck) was injected in a 80 l Teflon air sample bag (SKC Gulf Coast Inc., Houston, TX, USA). Subsequently, the bag was filled with 60 l humidified and filtered warm pressurised air at least 17 hours prior to the experiments to allow the evaporation of acetone. During the experiments, gaseous acetone was pumped (Model 224-PCXR4, SKC Gulf Coast Inc., Houston, TX, USA) from the air sample bags through Teflon tubes (diameter 7 mm) into the trapping devices at 230 ml/min through regulation by a mechanical flowmeter. At the outlet of the trapping device, the acetone vapour was mixed with the main air stream of 23.5 l/min.

Table 1 Chemicals used, their purity, supplier and calculated release rates

Compound	Abbreviation used in text	Purity	Supplier	Estimated release rate in the olfactometer (mg/15min)*
Ammonia 25% aqueous solution	NH_3	Analytical grade	Riedel-de Haën	3.3×10^{-1}
L(+)-lactic acid 90% aqueous solution	LA	Analytical grade	Purac Bioquimica	4.7×10^{-6}~9.3×10^{-4}
Acetic acid	C2	> 99%	Sigma	4.1×10^{-3}~0.83
Propanoic acid	C3	± 99%	Sigma	1.6×10^{-3}~0.32
2-Methylpropanoic acid		99%	Sigma	8.0×10^{-4}~0.16
3-Methylbutanoic acid	3MC4	≥ 99%	Sigma	1.5×10^{-3}~1.5×10^{-2}
Butanoic acid	C4	> 99%	Aldrich	4.2×10^{-4}~8.4×10^{-2}
Pentanoic acid	C5	> 99%	Sigma	1.4×10^{-4}~2.8×10^{-2}
Hexanoic acid	C6	≥ 99%	Sigma	2.8×10^{-5}~5.5×10^{-3}
Heptanoic acid	C7	± 98%	Sigma	
Octenoic acid	C8	≥ 99%	Sigma	2.3×10^{-6}~4.6×10^{-4}
Nonanoic acid	C9	≥ 97%	Sigma	
Decanoic acid	C10	99-100%	Sigma	6.8×10^{-9}~1.3×10^{-6}
Dodecanoic acid	C12	≥ 99%	Sigma	
Tridecanoic acid	C13	≥ 98%	Sigma	
Tetradecanoic acid	C14	99-100%	Sigma	
Hexadecanoic acid	C16	≥ 99%	Sigma	
1-dodecanol		≥ 99.5%	Fluka	
6-Methyl-5-hepten-2-one		99%	Aldrich	
3-Methyl-1-butanol		≥ 99.8%	Fluka	
Indole		≥ 99%	Sigma	
Geranylacetone		≥ 98%	Fluka	
4-Ethylphenol		99%	Aldrich	
7-Octenoic acid		> 99%	Dr. Mike Birkett**	
Tertyl-butyl-methyl-ether	TBME			
Diethyl ether	DE			
Ethanol absolute		100%	Riedel-de Haën	

* The amount of odours flowed into the olfactometer in 15 min is calculated according to the flowing formula: 0.053 × mv × P; in which mv is the molecular weight of the compound, and P is the vapour pressure of the compound at certain temperature (28°C) (Ough & Stone, 1961).

** Dr. Mike Birkett and Prof. J. Pickett, IACR-Rothamsted, Harpenden, Hertfordshire, UK.

Chemicals applied from glass bottles

Chemicals with the highest purity available were used in the experiments (Table 1). Single compounds (10 ml or 10 g of pure compound) were applied from a 250 ml glass bottle (Fisher Scientific B.V.,'s Hertogenbosch, The Netherlands). A charcoal filtered and humidified air stream passed through the bottle and carried the odour through Teflon tubing into the main stream in one of the trapping devices at the desired flow rate. Flow rates were regulated by Gilmont flowmeters (Fisher Scientific B.V.,'s Hertogenbosch, The Netherlands): 0.5, 5, 50 and 100 ml/min. The estimated concentrations of some of the compounds in the air stream of the olfactometer are listed in Table 1.

Compounds applied on sand-blasted glass slide

Two compounds, 7-octenoic acid and hexanoic acid, were studied by application on a sand-blasted glass slide. Dilutions of 7-octenoic acid (99%, kindly provided by Dr. M.A. Birkett and Prof. J.A. Pickett) were made using tertyl-butyl methyl ether (99%, Merck). Four concentrations of 7-octenoic acid, 92.5, 9.25, 0.93 and 0.093 ng/µl were tested, 10 µl of each was applied on a sand-blasted glass slide. The solvent was allowed to evaporate before the experiments. All four concentrations of 7-octenoic acid were tested in combination with ammonia and L-lactic acid as described before. These combinations were tested against 10 µl tertyl-butyl methylether on a sand blasted glass slide in combination with ammonia. A comparable protocol was used in the experiments with eight concentrations (0.00001-100 µg/µl) of hexanoic acid (≥ 99%, Sigma). Diethyl ether was used as the solvent and 100 µl of dilutions or solvent were applied on the sand-blasted glass slides.

Human odour

Human skin emanations were collected from a human foot by letting a volunteer wear a nylon sock for 7-14 h during the day before it was used in an experiment (Pates *et al.*, 2001b). The nylon sock was placed in a clean glass jar before use in an experiment. During the experiment, the worn sock was put in one of the trapping devices, whereas a clean sock was placed in the opposite trapping device as a control.

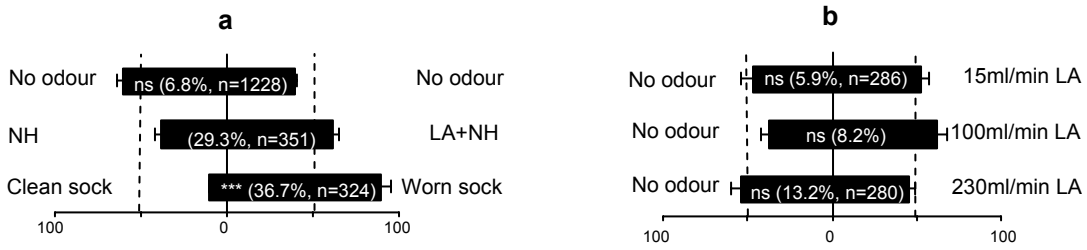


Fig. 2 Behavioural responses of *An. gambiae* females to: a) clean air (no odour), L-lactic acid + ammonia against ammonia and worn sock against clean sock; b) three concentrations of L-lactic acid. Bars are mean mosquito percentages that entered the trapping device baited with a certain odour which is indicated in the figure next to the bar, error bars are standard deviations of means. The number of mosquitoes caught in both trapping devices in percentage of the number of mosquitoes that left the release cage and the total number of mosquitoes in all replicates are given in between brackets. Significant differences (**P*<0.05; ** *P*<0.01; *** *P*<0.001) or no significant differences (NS *P*≥ 0.05) between the total number of mosquitoes caught in the two trapping devices (chi-square test). LA: L-lactic acid; NH3: ammonia.

Experimental procedure

Thirty female mosquitoes, 5-8 days-old, which had not received a blood meal, were randomly collected from their cage 14 to 18 hours before the start of the experiments and placed in a cylindrical release cage (diameter 8 cm, height 10 cm) with access to tap water from damp cotton wool placed on top of the cage.

The experiments were performed during the last four hours of the dark period, when *An. gambiae* is normally active. In each trial, test odours were released in the air stream and a group of mosquitoes was set free from a release cage which was placed at the downwind end of the flight chamber, 1.60 m from the two ports. Mosquitoes were left in the flight chamber for 15 minutes. The female mosquitoes that had entered either trapping device were counted at the end of the experiments, after anaesthetisation with 100% carbon dioxide. Mosquitoes remaining in the flight chamber were removed with a vacuum cleaner. After use, traps were washed with soapy water (CLY-MAX Heavy Duty Cleaner, Rogier Bosman Chemie B.V., Heijningen, The Netherlands), rinsed with tap water and then cleaned with pure ethanol.

Each trial started with new mosquitoes, clean trapping devices, and new stimuli. Experiments were repeated at least six times on different days. The sequence of test odours was randomised on the same day and between days. Test stimuli were alternated between right

and left ports in different replicates to rule out any positional effects. Experiments with clean air only in either port were done to test the symmetry of the trapping system. The operator wore surgical gloves to avoid contamination of the equipment with human volatiles.

Statistical analysis

For each two-choice test a chi-square test was used to analyse whether the total (i.e. sum of all replicates) number of mosquitoes that was trapped in the treatment trapping device and the total number that was trapped in the control trapping device differed from a 1:1 distribution. A generalised linear model (GLM, binomial, linked in logit, Genstat, release 6) was

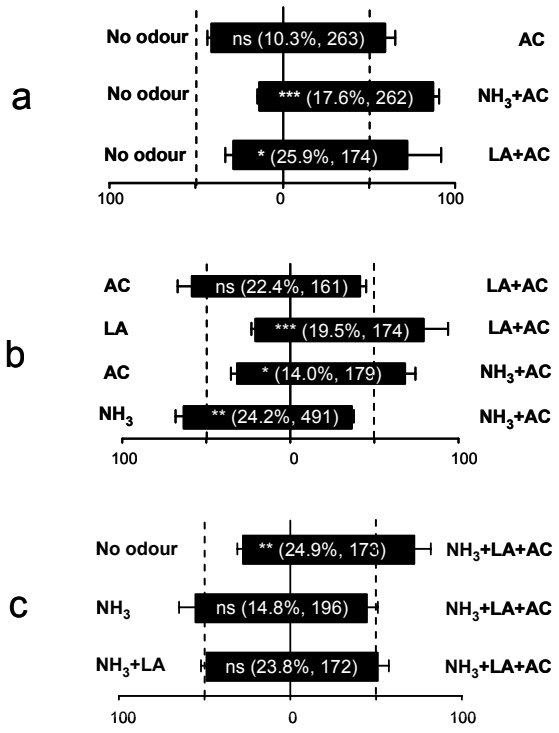


Fig. 3 Responses of *An. gambiae* females to ammonia, L-lactic acid and acetone in different combinations. a) tested against clean air (no odour); b) binary blends tested against single components and c) tripartite blends tested. Bars are mean mosquito percentages that entered the trapping device baited with a certain odour which is indicated in the figure next to the bar, error bars are standard deviations of means. The number of mosquitoes caught in both trapping devices in percentage of the total number of mosquitoes that left the release cage and the total number of mosquitoes in all replicates are given in between brackets. Significant differences (*P<0.05; ** P<0.01; *** P<0.001) or no significant differences (NS $P \geq 0.05$) between the total number of mosquitoes caught in the two trapping devices (chi-square test). LA: L-lactic acid; NH3: ammonia; AC: ace-

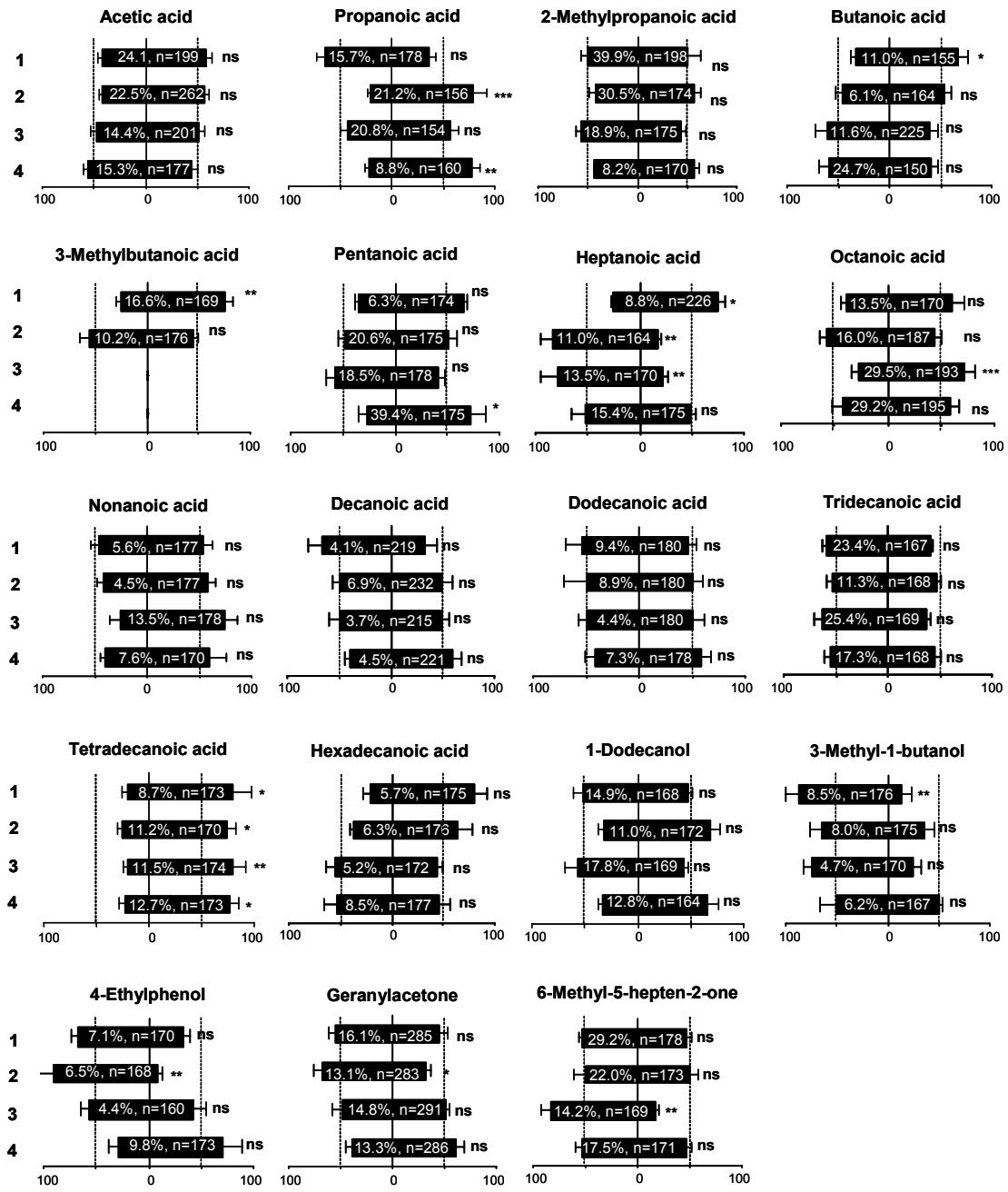


Fig. 4 Mean percentages of *An. gambiae* females that were caught in the two trapping devices, one baited either with a concentration of test compound + ammonia + L-lactic acid (the right side of the bar), the other one baited with ammonia alone (the left side). Error bars are standard deviations of means. The number of mosquitoes caught in both trapping devices in percentage of the total number of mosquitoes that left the release cage and the total number of mosquitoes in all replicates are given in between brackets. Significant differences (*P<0.05; ** P<0.01; *** P<0.001) or no significant differences (NS $P \geq 0.05$) between the total number of mosquitoes caught in the two trapping devices (chi-square test). Flow rates tested are listed at the left-hand side, 1, 2, 3 and 4 are 0.5, 5, 50 and 100 ml/min respectively.

used to investigate whether adding an odour to the combination of ammonia + L-lactic acid enhanced attractiveness when compared to the situation in which ammonia + L-lactic acid were tested against ammonia. Two-sided *t*-probabilities were calculated to test differences between means. Effects were considered to be significant at $P<0.05$.

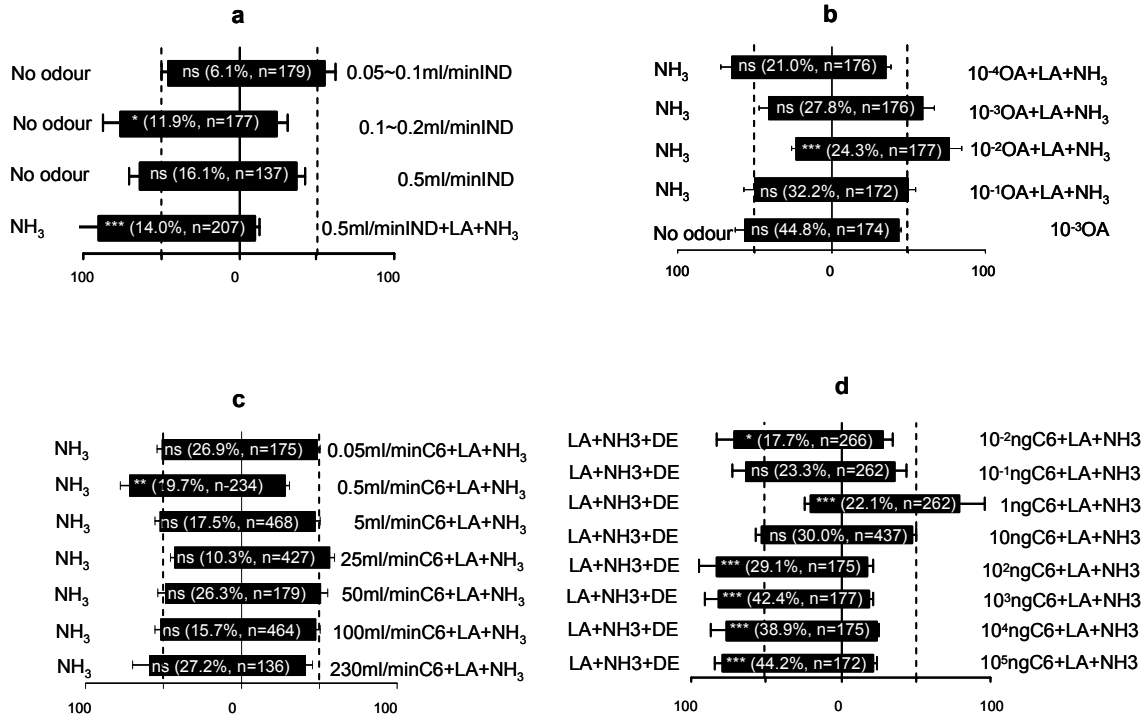


Fig. 5 Mean percentages of *An.s gambiae* females that are caught in the two trapping devices, error bars are standard deviations of means. The odours emanating from each trapping device are given next to the bars a) indole from a glass bottle, b) 7-octenoic acid on a sand-blasted glass slide, c) hexanoic acid from a glass bottle and d) hexanoic acid on a sand-blasted glass slide. The number of mosquitoes caught in both trapping devices in percentage of the total number of mosquitoes that left the release cage and the total number of mosquitoes in all replicates are given in between brackets. Significant differences (**P*<0.05; ** *P*<0.01; *** *P*<0.001) or no significant differences (NS $P \geq 0.05$) between the total number of mosquitoes caught in the two trapping devices are shown (chi-square test). LA: L-lactic acid; NH3: ammonia; IND: indole; OA: 7-octenoic acid; C6: hexanoic acid.

Results

Control odours

In all experiments with clean air coming from both ports, equal numbers of mosquitoes were caught in both trapping devices (chi-square test, P=0.50); this demonstrated that the olfactometer was symmetrical (Fig. 2a). L-lactic acid, tested at three flow rates, did not attract more mosquitoes than clean air (chi-square test, P= 0.50) (Fig. 2). The combination of ammonia and L-lactic acid trapped numbers of mosquitoes similar to those trapped by ammonia alone (chi-square test, P=0.14) (Fig. 2a). Based on these results and the synergism between ammonia, L-lactic acid and a mixture of carboxylic acis on which we reported recently (Smallegange *et al.*, 2005), we decided to test single carboxylic acids and other compounds by adding them to the mixture of ammonia and L-lactic acid and to test this tripartite synthetic blend against ammonia alone.

In order to check the responsiveness of female mosquitoes to a highly attractive odour source, nylon socks worn by a human volunteer were used as a control (Pates *et al.*, 2001b). As expected, human worn socks caught almost 90 percent of the mosquitoes that left the releas cage (Fig. 2a).

Effect of acetone

Acetone alone did not trap more mosquitoes than clean air (chi-square test, P=0.34), but adding acetone to ammonia or L-lactic acid resulted in an attraction of a significantly greater number of mosquitoes than clean air (chi-square test, P< 0.001 and P=0.03) (Fig. 3a).
The combination of acetone with ammonia was less attractive than ammonia alone (chi-square test, P=0.002) but was more attractive than acetone alone (chi-square test, P=0.01) (Fig. 3b), The combination of acetone with L-lactic acid attracted a similar number of *An. gambiae* as acetone alone (chi-square test, P=0.32) but attracted greater numbers than L-lactic acid alone (chi-square test, P<0.001) (Fig. 3b). More *An. gambiae* entered the trapping device baited with the mixture of acetone, ammonia and L-lactic acid than entered the trapping device releasing clean air (chi-square test, P=0.004) (Fig. 3c). However, the tripartite mixture did not attract more mosquitoes than ammonia (chi-square test, P=0.58) or a binary mixture of ammonia and L-lactic acid (chi-square test, P=0.88) (Fig. 3c).

Effects of carboxylic acids

The responses of *An. gambiae* to several (in most cases four) concentrations of 16 single carboxylic acids were tested in the olfactometer, in choice assays between a concentration of a carboxylic acid mixed with L-lactic acid and ammonia passing through one trapping device and ammonia alone passing through the other.

As shown in Fig. 4, 7 carboxylic acids at one or more concentrations mixed with L-lactic acid and ammonia, were found to attract more, or fewer, than ammonia alone. The additional effect by adding these odours to L-lactic acid + ammonia were significant compared with the effect of L-lactic acid + ammonia versus ammonia (GLM, $P<0.05$). Attraction of *An. gambiae* was found in response to C4, 3MC4, C5, C7 and C8 carboxylic acids at the lowest (C4, 3MC4 and C7, GLM, $P<0.001$), the second lowest (C8, GLM, $P<0.001$) or the highest dose (C5, GLM, $P<0.001$) (Fig. 4). The second lowest and the highest dose of propanoic acid enhanced the attraction to ammonia and L-lactic acid (GLM, $P<0.001$) (Fig. 4). All four dose rates of tetradecanoic acid increased the attraction to L-lactic acid and ammonia compared with the attraction to ammonia alone (GLM, $P<0.005$) (Fig. 4).

At the lowest flow rate, heptanoic acid increased the attractiveness of ammonia + L-lactic acid (GLM, $P<0.001$). However, fewer mosquitoes were caught at the two intermediate flow rates of heptanoic acid added to L-lactic acid and ammonia (GLM, $P<0.001$). (Fig. 4). Hexanoic acid at seven different flow rates was tested in combination with L-lactic acid and ammonia against ammonia; none of the flow rates resulted in enhanced attractiveness whereas repellence was found at the second highest flow rate (GLM, $P<0.001$) (Fig. 5c). When eight doses of hexanoic acid were tested using the glass slide method in combination with ammonia + L-lactic acid against ammonia, attraction was found for hexanoic acid at the dose of 1 ng (GLM, $P<0.001$) (Fig. 5d). Four concentrations of 7-octenoic acid were tested in combination with ammonia and L-lactic acid against ammonia. Attraction was found for 7-octenoic acid at 10^{-3} dilution (GLM, $P<0.001$) (Fig. 5b).

Carboxylic acids eliciting no response

Anopheles gambiae females were neither attracted nor repelled to seven carboxylic acids, namely C2, 3MC3, C9, C10, C12, C13 and C16, at any of the concentrations tested (GLM, $P \geq 0.05$) (Fig. 4).

Effects of phenol, alcohols , ketones and indole

Of the two alcohols tested, 3-methyl-1-butanol had a repellent effect at the lowest dose tested (GLM, P<0.001) and 1-dodecanol had no effect at any concentration (GLM, $P \geq 0.05$) (Fig. 4). A repellent effect was found for 4-ethylphenol at the second lowest concentration (GLM, P<0.001) (Fig. 4). Repellent effects were found at the second and third concentrations for geranylacetone and 6-methyl-5-hepten-2-one respectively (GLM, P<0.001) (Fig. 4). Indole acted as a repellent at the second lowest and the highest concentrations (GLM, P<0.001) (Fig. 5a).

Discussion

The effect of acetone

We found that the mixture of L-lactic acid with acetone attracted greater number of mosquitoes than clear air. Because neither L-lactic acid nor acetone alone elicited attraction of *An. gambiae*, the attraction of the mixture of these two compounds might be due to synergism. The mixture of ammonia and acetone elicited an attractive response, but this mixture was less attractive than ammonia alone, indicating that ammonia accounted for the attractiveness of the mixture and that at the dose tested, acetone has a repellent effect when offered against ammonia alone as the alternative. This repellency is neutralised when L-lactic acid is present in the mixture (Fig 3C). The attractiveness of the mixture of L-lactic acid and acetone seems to depend on the alternative offered.

Acetone is a component of human breath (Crofford *et al.*, 1976) and was found in fresh but not in incubated human sweat (Meijerink *et al.*, 2000). The combination of CO_2 + acetone elicited attraction of *An. gambiae* in a wind tunnel assay (Takken *et al.*, 1997a). At the dose tested, our results did not show a contribution of acetone to the attractiveness of L-lactic acid + ammonia. It is possible that acetoneoperates at a different level in *An. gambiae*, attracting the mosquitoes in the presence of CO_2.

The effects of carboxylic acids

The carboxylic acids which enhanced the attractiveness of the mixture of ammonia and L-lactic acid for *An. gambiae* in this study have all been found in human skin emanations (Bernier *et al.*, 2000; Healy & Copland, 2000). Our results suggest that some of the carboxylic acids, depending on their chain length and concentration, possibly contribute to the at-

tractiveness of humans to *An. gambiae*. (Bosch *et al.*, 2000)The acids tested in this study were all included in a synthetic mixture of 12 carboxylic acids that was attractive (Knols *et al.*, 1997) or repellent (Smallegange *et al.*, 2005)to *An. gambiae* on its own, but attractive in combination with ammonia and L-lactic acid (Smallegange *et al.*, 2005). It is possible that the carboxylic acids eliciting attraction of *An. gambiae* in this study account for the attractiveness of the mixture in the previous study.

In another anthropophilic mosquito, *Ae. aegypti* L., it was found that C1-3, C5-8 and C13-18 carboxylic acids enhance the attractiveness of L-lactic acid (Bosch *et al.*, 2000). C3 was found in higher concentrations in fresh sweat than in the more attractive (to *An. gambiae*) incubated human sweat (Meijerink *et al.*, 2000), whereas 3MC4 was found in higher concentrations in incubated human sweat. Levels of butanoic acid were similar in fresh and incubated sweat. The higher attractiveness of incubated sweat to *An. gambiae* might partly be due to the effect of 3MC4. Olfactory neurons tuned to carboxylic acids were found to innervate antennal sensilla trichodea (Meijerink, 2000; chapter 8). Antennal grooved peg sensilla contain neurons sensitive to C5 and C6 carboxylic acids (chapter 8).

At the lowest dose, heptanoic acid increased the attractiveness of ammonia + L-lactic acid. However, fewer mosquitoes were caught at the two intermediate doses of heptanoic acid added to L-lactic acid and ammonia. (Fig. 4). This result demonstrates that not only the molecular structure of the odour but also the dosage determines whether an odour acts as an attractant or a repellent (Vale & Hall, 1985) .

Hexanoic acid was tested at seven different flow rates in combination with L-lactic acid and ammonia against ammonia alone; none of the doses resulted in enhanced attractiveness whereas repellency was found at the second highest dose (Fig. 5). When eight doses of hexanoic acid were tested using the glass slide method in combination with ammonia + L-lactic acid against ammonia alone, attraction was found for hexanoic acid at the dose of 1 ng/µl (Fig. 5). To understand why no attraction was found using the wash bottle method, the SPME (Solid Phase Micro Extraction) method was applied to determine the airborne hexanoic acid concentration in the olfactometer produced by both odour-release systems. Hexanoic acid concentrations measured in the olfactometer when a flow of 0.5 ml/min was used from a glass bottle and when a concentration of 1 µg/µl was applied on a sandblasted glass was compared. The glass bottle method produced about 20 times higher levels of hexanoic acid at the trap openings. Apparently the concentrations tested in the glass bottle experiments were too high to elicit attraction of *An. gambiae*. If the flow rate could be further reduced, which was not realised in our study, as we had no flow regulators with extremely low flow rates to our disposal, attractive response might have been found with the glass bottle method.

Healy and Copland (2000) reported that the amount of carboxylic acids in a human skin

emanation, which stimulated the landing response of *An. gambiae*, ranged from 0.1-5.4 µg/µl, but a blend of 22 carboxylic acids (1 µg/µl each) was not attractive. It is possible that the release rate of a single carboxylic acid from a natural human odour blend is less than for an acid released singly or from a mixture of a few acids because of the presence of other compounds that possibly interact or react with the acids in the natural odour blend.

A synergistic effect of the combination ammonia + lactic + hexanoic acid was found in a Y-tube olfactometer test with *An. gamibae* (Smallegange *et al.*, 2002). Bosch *et al.* (2000) found that several carboxylic acids including hexanoic acid augmented the attractiveness of L-lactic acid to *Ae. aegypti*. Both sensilla trichodea and grooved peg sensilla contain neurons sensitive to hexanoic acid (Chapter 8). Therefore it is likely that hexanoic acid is used by *An. gambiae* as kairomone in its host-seeking behaviour.

Four concentrations of 7-octenoic acid were tested in combination with ammonia and L-lactic acid against ammonia. Attraction of 7-octenoic acid was found at 10^{-3} dilution (Fig. 5b). This compound was identified in human axillary odour and was found to increase the attraction of carbon dioxide to *An. gambiae sensu lato* in a field assay (Costantini *et al.*, 2001). An olfactory receptor neuron sensitive to 7-octenoic acid was found in sensilla trichodea in the antennae of *An. gambiae* (Chapter 8) and a neuron type which we found only after a blood-meal was sensitive to this compound (Chapter 9). Our findings suggest that 7-octenoic acid, like hexanoic acid, might be a cue that is used by *An. gambiae* as a kairomone.

Several carboxylic showed neither an attractive nor a repellent effect to females of *An. gambiae* at any of the concentrations tested. The estimated release rates (in mg/min) of the carboxylic acids tested in our olfactometer are listed in Table 1. We do not know the actual concentration of these acids as encountered by mosquitoes when engaged in host-seeking at varying distances from the host. It is possible that these acids were tested at concentrations outside of the range that naturally occurs in volatile blends released by humans. Further study of the absolute concentration of human-produced volatiles in the odour plume is required to clarify the role of single components as kairomones for blood sucking insects.

Alcohols and ketones in host-seeking behaviour

In incubated human sweat, which was found to be significantly more attractive than fresh sweat (Braks & Takken, 1999), 3-methyl-1-butanol, 1-dodecanol and indole were found at considerably higher levels than in fresh human sweat. Similar amounts of geranyl acetone and 6-methyl-5-hepten-2-one were found in fresh and incubated sweat, but both ketones occurred at relatively high concentrations (Meijerink *et al*., 2000). From the data presented

here, it seems unlikely that they contributed to the documented higher attractiveness of incubated sweat. A recent study on the attraction of host odours to cattle flies, 6-methyl-5-hepten-2-one was found attractive at certain concentrations to *Musca autumnlis* in a wind-tunnel but was repellent at the applied dose in a field study (Birkett *et al.*, 2004). Olfactory neurons sensitive to alcohols, phenols, indoles and ketones were located in sensilla trichodea (Chapter 8). Olfactory neurons highly sensitive to geranyl acetone were found in sensilla trichodea (Chapter 8; van den Broek & den Otter, 1999; Meijerink *et al*. 2001). Central processing of the pattern of response across different olfactory neurons is involved in determining the attractiveness of an odour blend (Boeckh *et al.*, 1996; Voskamp *et al.*, 1999). Evidence is accumulating for the notion that the differential attractiveness between hosts of blood-sucking insects is determined by the presence of attractive odour components on the one hand, and the repellent odour components on the other hand (Bernier *et al.*, 2003; Birkett *et al.*, 2004; A. M. Galimard unpublished results; Chapter 3).

Our results show that carboxylic acids with defined chain lengths (C3-C8 and C14) enhance the attractiveness of ammonia + L-lactic acid , the effect being dose-dependent. These compounds may serve as kairomones in the host-seeking behaviour of *An. gambiae*. Adding alcohols, phenol, indole or ketones to ammonia + L-lactic acid either had no effect or even diminished the attractiveness. Both attractive and repellent compounds might be involved in the differential host preference of this mosquito species. Odours showing behavioural activities in this study have been tested in a field study in which efficiency of mosquito traps baited with these odours was investigated (Chapter 7).

Acknowledgements

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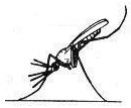
7

Field study on the attractiveness of mosquito traps baited with synthetic odour blends and human odours in The Gambia



Qiu, Y.T.; Smallegange, R.C.; ter Braak, C.J.F.; Spitzen, J.; van Loon, J.J.A; Jawara, M.; Milligan, P.; Galimard, A.M.; van Beek, T.A.; Knols, B.G.J. and Takken, W.

Abstract

Chemical cues play an important role in the host-seeking behaviour of blood feeding mosquitoes. African malaria vectors are anthropophilic and are attracted to human odours from a distance. In laboratory studies odour blends caused greater attraction than single compounds. We carried out a field study in The Gambia to investigate the effects of synthetic odour blends on the attraction of mosquitoes, including species acting as malaria vectors. Counterflow Geometry traps (CFG traps) baited with 16 odour blends to which CO_2 was added were tested in four sets of experiments; in a second set of experiments CFG traps with 14 odour blends without CO_2 were tested. The combination of ammonia and L-lactic acid with or without CO_2 was used as the control mixture. Odours collected from a human host were used as a natural odour source. Six treatments were carried out in each set and this was repeated during 12 nights according to a Latin-square design. CDC traps were placed in a village and an experimental house to monitor the population dynamics of mosquitoes.

The CFG traps caught a total number of 196,756 mosquitoes, with the most abundant species belonging to the genera *Mansonia* spp. (70.6%), *Anopheles* (17.5%) and *Culex* (11.5%). Anophelines trapped were comprised of *Anopheles ziemanni* (16.3%), *An. pharoensis* (0.9%), *An. gambiae s.l.* (0.3%) and *An. wellcomei* (0.02%). The most abundant mosquito species caught by the CDC traps (56,290 in total) belonged to the genus *Mansonia* spp. (59.4%), *Anopheles* (*An. gambiae s.l.* (16.0%) and *An. ziemanni* (11.3%)), and *Culex* spp. (11.6%). CFG traps baited with synthetic mixtures were in many cases more attractive than human odours collected from a human-occupied tent. Carbon dioxide substantially increased the trapping efficiency of the CFG traps for all mosquito species. A mixture of ammonia + lactic acid + 3-methyl butanoic acid + CO_2 was the most attractive odour for most mosquito species; this mixture is suggested to be a promising candidate odour blend in the control of nuisance mosquitoes.

Indoor experiments showed that a blend of ammonia + lactic acid + CO_2 + geranyl acetone + indole + 4-ethyl phenol, was more attractive for *An. gambiae s.l.* than the control odour. The latter mixture warrants further investigation when *An. gambiae* is more abundant, and might fulfil a role in malaria vector control programmes. The application of odour-baited CFG traps for vector management is discussed.

Introduction

The mosquito *Anopheles gambiae* is an effective human malaria vector because of its strong preference for human blood and close association with human dwellings. Host seeking and blood feeding of *An. gambiae* takes place during the night. Physical cues and, more importantly, chemical cues emanating from human bodies mediate its host-seeking behaviour (Takken & Knols, 1999). In this process, physical stimuli such as temperature, humidity gradients and visual cues are effective when the mosquitoes are close to the hosts (Gillies & Wilkes, 1968), whereas volatile chemicals attract mosquitoes from a distance. Such volatiles emanate from human skin and breath (including carbon dioxide) or from waste products (Takken, 1991). Gillies and Wilkes (1968) reported that carbon dioxide (CO_2) can attract mosquitoes to hosts at distances of 18-36 m and skin odours at distances of 54-73 m. The complete human body odour is attractive to *An. gambiae*, and skin odours were demonstrated to account for most of the attractiveness (Costantini *et al.*, 1996a; Costantini *et al.*, 1998), whereas breath was proven to have only a slightly attractive or even a repellent effect (Mboera *et al.*, 1997; Mukabana *et al.*, 2004b). It is considered that the variation of the attractiveness between human individuals to malaria mosquitoes is odour-mediated (Brady *et al.*, 1997; Costantini *et al.*, 1998; Mukabana, 2002; Chapter 3).

Although hundreds of components have been identified from human odours, only a few

compounds were tested and proven to be attractive to mosquitoes (Bernier *et al.*, 2000; Healy *et al.*, 2002). Ammonia, a compound present in incubated human sweat, was repeatedly reported to be attractive in the olfactometer (Braks *et al.*, 2001; Smallegange *et al.*, 2005). L-lactic acid was not or only slightly attractive to *An. gambiae*. However, synergism between ammonia, L-lactic acid and a blend of carboxylic acids was found while binary blends of these compounds were less attractive or even repellent (Smallegange *et al.*, 2005). The L-lactic acid concentration on the human skin may explain differences between human individuals in the level of attractiveness for mosquitoes (Brady *et al.*, 1997; Dekker *et al.*, 2002). Carbon dioxide, which is exhaled by all warm-blooded vertebrates, is considered to contribute partially to the attraction of humans to *An. gambiae* (Snow, 1970; Healy & Copland, 1995; Costantini *et al.*, 1996a; Mboera & Takken, 1997). A study in West Africa demonstrated that CO_2 is more attractive to zoophilic than to anthropophilic mosquito species (Costantini *et al*. 1996), suggesting that anthropophilic mosquito species rely to a greater extent on skin odours in addition to CO_2 (Dekker & Takken, 1998; Takken & Knols, 1999). There is evidence that only a turbulent CO_2 plume elicits a searching response (Dekker *et al.*, 2001); J. Spitzen and R.C. Smallegange, unpublished results). Six oxocarboxylic acids were reported to stimulate landing responses of *An. gambiae*, acting after contact (Healy *et al.*, 2002). Addition of the human-specific 7-octenoic acid to carbon dioxide attracted a greater number of *An. gambiae s.l.* to traps in Burkina Faso (Costantini *et al.*, 2001). Evidence is accumulating that the attraction of mosquitoes to humans is not realised by single components, but by a blend of components in specific ratios.

In olfactometer studies we found several compounds that increase mosquito attraction when being added to ammonia and L-lactic acid (Smallegange *et al.*, 2005); Chapters 5 and 6). Electrophysiological studies provided evidence that olfactory sensilla on the antennae of female *An. gambiae* are strongly tuned to phenols, indole and geranyl acetone (van den Broek & dan Otter, 1999; Meijerink *et al.*, 2001; Chapter 8). We are interested in whether odour mixtures that showed mosquito attractiveness in the laboratory can be used in the field to collect mosquitoes in odour-baited traps and whether combinations of compounds to which olfactory receptor neurons of *An. gambiae* were found to be strongly tuned to, would provide a lure for this mosquito species.

Although many field studies have shown that the efficiency of mosquito traps can be substantially improved by adding odours that are attractive to the mosquitoes (Kline *et al.*, 1991; Costantini *et al.*, 1996a; Mboera *et al.*, 1997; Costantini *et al.*, 1998; Costantini *et al.*, 2001), an odour baited trap that can compete with a human is still to be developed. In *An. gambiae*, CO_2 gave various results according to different field studies. The present paper reports on a field study carried out in The Gambia (West Africa) to investigate the effects of

synthetic odour blends on mosquito catches, using a modified Counterflow Geometry trap, either with or without CO_2 as an additional stimulus.

Materials and methods

Study site

The study was carried out near Wali Kunda in the Central River Division of The Gambia, West Africa (13º28'N, 16º34'W) (Fig. 1). Wali Kunda is a small village located at the south bank of the River Gambia, about 180 km from the coast. The area is a flat Sudan Savanna. Rice fields in the surroundings of the village and temporary pools of rainwater are major breeding sites of anopheline mosquitoes (Fig. 2). The experiments were conducted during the last part of the rainy season, from August 15 till October 4, 2003. Malaria transmission is at its maximum in this period of the year in The Gambia (Lindsay *et al.*, 1991).

Mosquito traps

The Counterflow Geometry (CFG) trap was used to assess the effect of candidate odours (Kline, 1999; Mboera *et al.*, 2000b). The odour delivery system was modified to allow the release of blends of odours (Fig. 3). A sealed airtight container was mounted on top of the CFG trap (Kline, 1999). The container had two plastic plates with twelve holes in each so that glass vials could be held in the holes. Odour compounds were contained individually in 4 ml screw type clear glass vials with hole-cap and a PTFE/silicone septum inside (Supelco, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). A medical injection needle (21G x 5/8", 0.8x16 cm, Terumo, Leuven, Belgium) was stuck through the septum, with one end above the surface of the solution, serving as a vent for the volatiles. The volatiles from the different vials dispersed out of the vial to form a blend in the container (A.M. Galimard & R.C. Smallegange, unpublished results). CO_2 from a gas cylinder passed a flowmeter (250 ml/min) through silicone tubing (diameter 7 mm; Rubber BV, The Netherlands) and was led into the odour container where it mixed with the candidate odours. The headspace of the test compounds in the containers was ventilated to the "attractant plume tube" by one of the two 12V fans. A rechargeable lead-acid battery (12 V; 3A, Super Sona YP3-12, Eijlander Electronics, Ede, The Netherlands) was used to power the electric ventilators. The CFG trap was suspended on a wooden tripod (outdoor experiments); the lowest point of the trap was about 50 cm above the ground. Grease was used to prevent ants from reaching the mosquitoes caught in the CFG trap. In spite of these precautions, we occasionally found ants eating

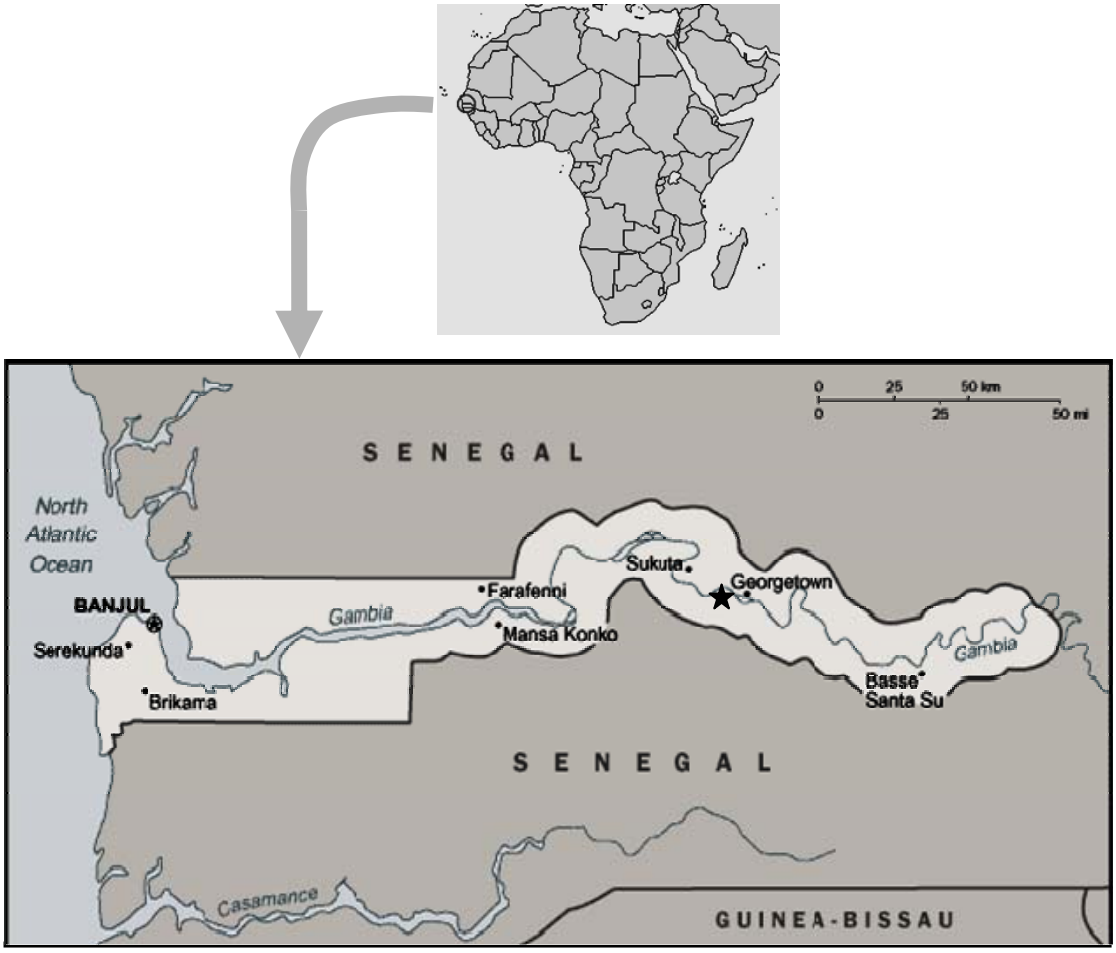


Fig. 1 A map of The Gambia. The asterisk marks the approximate position where the field experiments were carried out.

mosquitoes in our traps. Data from these traps were discarded.

The Centres for Disease Control miniature light trap (CDC trap, Model 512, John W. Hock Company, Gainesville, FL, USA) as described by (Sudia & Chamberlain, 1962; Garrett-Jones, 1964) was used to provide background information on the seasonal biting activity of host-seeking mosquitoes. Male Gambian volunteers slept under bednets next to the CDC traps.

Table 1 Chemicals used in the field study.

Compound	Purity	Supplier	Bioactivity[1]	Reference
CO_2	100%		B	Costantini *et al.,* 1996
Ammonia	25% aqueous solution	Riedel-de Haën	B,E	Broek and Otter, 2000; Meijerink *et al*., 2001
L(+)-lactic acid	85+%	Aldrich	B	Braks *et al.,* 2001; Dekker et al., 2002
Propionic acid	±99%	Sigma	B	Chapter 6
3-Methylbutanoic acid	99%	Aldrich	B	Chapter 6
Butanoic acid	99+%	Aldrich	B	Chapter 6
Hexanoic acid	Min. 99%	Sigma	B	Smallegange *et al.,* 2002; Chapter 6
Heptanoic acid	Min. 99%	Sigma	B	Chapter 6
Octenoic acid	Min. 99%	Sigma	B	Chapter 6
Tridecanoic acid	Min. 99%	Sigma	B	Chapter 6
Tetradecanoic acid	99-100%	Sigma	B	Chapter 6
Hexadecanoic acid	±99%	Sigma	B	Chapter 6
1-Dodecanol	≥ 99.5%	Fluka Chemika	B	Chapter 6
6-Methyl-5-hepten-2-one	99%	Aldrich	E	Meijerink et al., 2001; Chapter 8
3-Methyl-1-butanol	≥ 99.5%	Fluka	E	Meijerink et al., 2001; Chapter 8
Indole	≥ 99%	Fluka	E	Meijerink et al., 2001; Chapter 8
Geranylacetone	≥ 97%	Aldrich	E	Meijerink et al., 2001; Chapter 8
4-Ethylphenol	99%	Aldrich	E	van den Broek and den Otter, 1999; Chapter 8
7-Octenoic acid	> 99%	Dr. Mike Birkett[2]	B,E	Costantini *et al*., 2001; Chapter 6&8
Ethanol absolute	100%	Riedel-de Haën		

[1]: B: behavioral response; E: electrophysiological response

[2]: Dr. Mike Birkett and Prof. J. Pickett, IACR-Rothamsted, Harpenden, Hertfordshire, UK.

Test odours

Synthetic odour compounds

Chemicals used in our experiments, their purity, producer as well as the bioactivity reported for *An. gambiae* are listed in Table 1. One ml or one gram of a pure compound was put in a vial (see above). Carbon dioxide (100%) was applied via a gas regulator (Tornado 2000, Messer Cutting & Welding AG, Frankfurt, Germany) and the flow rate was fine-regulated to 250 ml/min (an amount equivalent to that exhaled by a breathing human being in rest) with a flow meter (Brooks Instruments, Rijswijk, The Netherlands) (Gillies & Wilkes, 1968).

Human odour

In order to add human odour to a CFG trap, a Gambian male individual (22 years old) slept

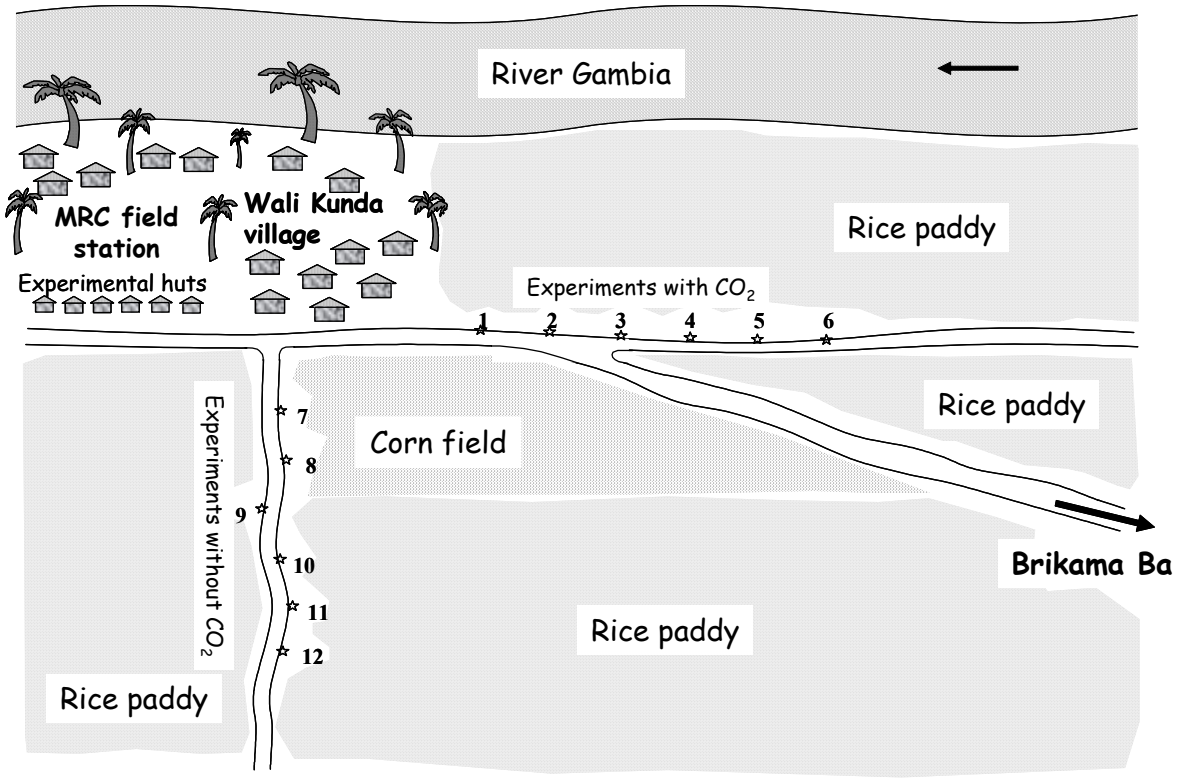


Fig. 2 A schematic map of the experimental site, showing the location of the traps. The objects have been drawn on different scales.

in a nylon tent during each night, from 8 pm to 7 am, of the experiments. A hole was made at one side of the tent in which an electric ventilator was fixed (Sunon, 12 V, 2.8 W). The ventilator constantly blew air from the tent through lay-flat tubing (11 cm in diameter) to the CFG trap 10 meter away from the tent. The outlet of the lay-flat tubing was approximately 5 cm from the attractant plume tube of the CFG trap.

Experimental houses and village houses

Indoor experiments were carried out in six experimental houses within the MRC field station (Lindsay *et al.*, 1993). These houses were designed in traditional African house style with mud walls and a thatched roof. The inside-floor area was ≈ 2.5m^2 and the houses lay ≈ 12 m apart in a straight line. Mosquitoes could enter the houses via an aperture under the two opposite eaves. During all experimental nights a Gambian male individual (27 years old) slept from 8 pm to 7 am in one of the houses under a clean bednet. Every night, a CDC trap was operated next to the bednet, about 50 cm above the ground, for the collection of house-entering mosquitoes.

To monitor the population dynamics of mosquitoes during the experimental period in the nearby village, a CDC trap was suspended every night, from 8 pm to 7 am, during the experimental period in a house in Wali Kunda village. The house was a traditional house with clay walls and a thatched roof. There was no closable door in the house. Each night a Gambian male individual (24 years old) slept under a clean bednet, next to which a CDC trap was placed.

Experimental setup

Outdoor experiments

The effects of candidate odours released from CFG traps were tested following a double 6x6 Latin square design. Six odour blends were tested in each experimental set and repeated for 12 nights. Sixteen odour blends with CO_2 were tested in four sets of experiments (Table 2) and 14 odour blends without CO_2 were tested in 3 sets of experiments (Table 3). The CFG traps baited with CO_2 were placed along a small road parallel to the River Gambia; CFG traps without CO_2 were placed along a small road leading from the River Gambia (Fig. 2). CFG traps were placed 50 m apart and were more than 100 m from the nearest house. Both roads were along the edge of a large rice field. In each set a control odour, ammonia + lactic acid with (odour A) or without CO_2 (odour B) was tested together with other odour blends. A human odour-baited CFG trap was tested in the four sets of experiments with CO_2.

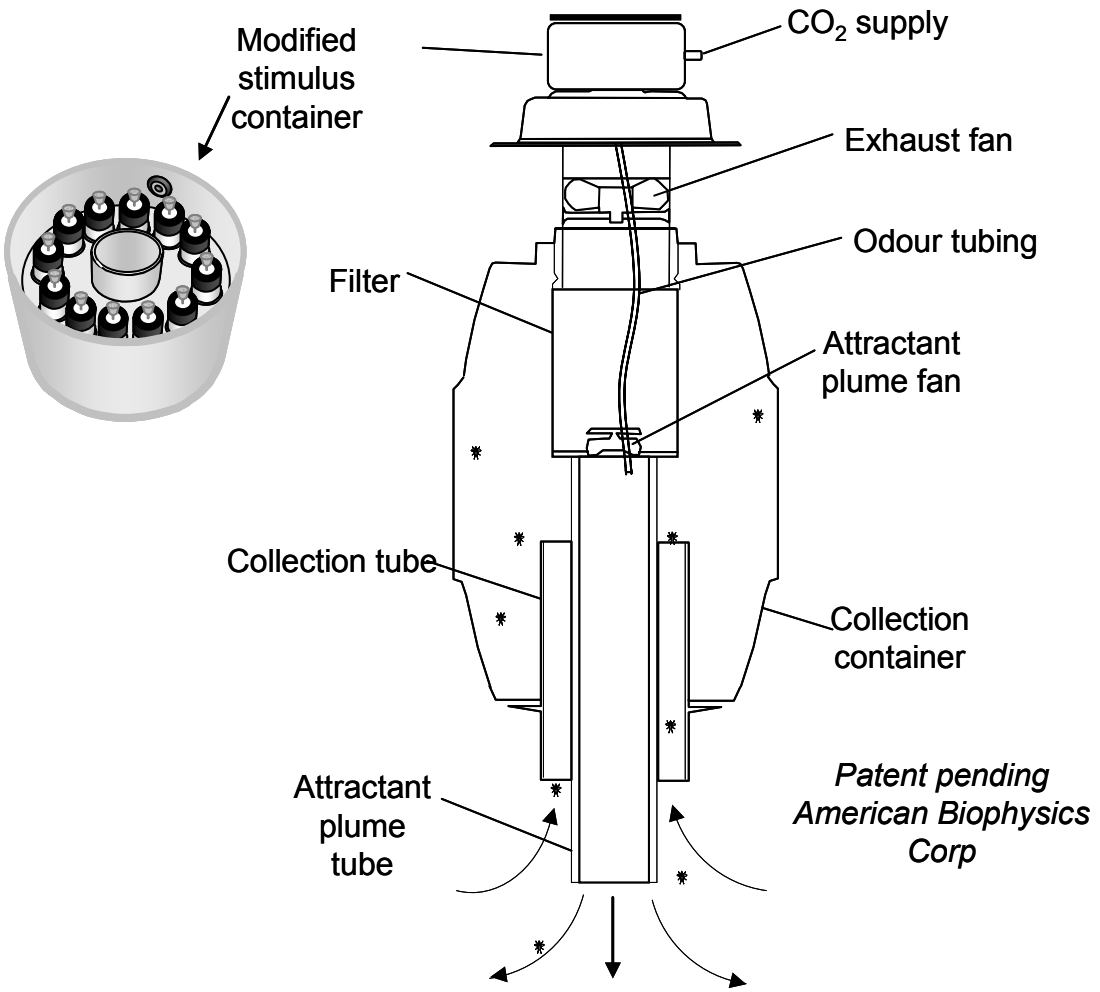


Fig. 3 Schematic drawing of the Counterflow Geometry trap (after Kline, 1999) with modified stimulus container.

Chemicals were placed in the vials 3-4 h before the start of the experiments. The traps were placed at the experimental locations around 7 pm every evening. At around 7 am the next day the traps were taken back to the laboratory and put into a freezer for 1 h to kill the trapped mosquitoes. Subsequently, mosquitoes were collected, identified and counted. The container of the CFG trap was cleaned with 100% ethanol solution before a new odour blend was tested.

Indoor experiments

Based on the results from the outdoor experiments, the three odour blends that caught the

highest number of *Anopheles* mosquitoes were subsequently tested in the experimental houses described above. CFG traps were suspended from the roof with the lowest point about 50 cm from the floor. Odour from the human-occupied tent was pumped via a lay-flat tubing via a window into the house (see above). The lay-flat tubing was surrounded by gauze, so that no mosquito could enter the house through the window. During the experiments the doors of the houses were closed. A window of about 50x35 cm and about 1.5 m from the floor of the house was left ajar so that an opening of 5 cm wide was created.

Identification of mosquitoes

The mosquitoes caught in each trap during one night were morphologically identified and the numbers of each species or genus were counted (Gillies & Wilkes, 1968; Gillies & Coetzee, 1987). Female *An. gambiae s.l.* mosquitoes were placed in a 4 ml Eppendorf tube with dry silica gel. These mosquitoes were transported to the Laboratory of Entomology of Wageningen University (The Netherlands) for PCR analysis.

Species identification of An. gambiae complex

Species identification for mosquitoes of the *An. gambiae* complex was realized by using the DNA-polymerase chain reaction (PCR) (Scott *et al.*, 1993). Head and thorax were used for DNA extraction, from which 1 µl was used for PCR.

Weather data collection

Daily rainfall was registered by a rainmeter (Remote Rain Gauge model RGR122, Oregon Scientific, Berkshire, UK). Wind speed during the outdoor experiments was measured by an anemometer (Windmaster 2®, Kaindl Electronic, Rohrbach, Germany). Temperature and humidity were registered daily by data loggers (Intab, Tiny Tag Plus, Sweden).

Data analysis

For each species or genus, the number of female mosquitoes caught in the traps were analysed by regression analysis using a generalized linear model with logarithmic link function and a negative binomial distribution (McCullagh & Nelder, 1989) using the procedure RNEGBINOMIAL in the statistical package Genstat 7.1 (Genstat Committee, 2004). Preliminary analyses assuming a Poisson distribution showed strong overdispersion for the more abundant mosquitoes. The negative binomial distribution models this overdispersion

with a mean-variance relation of variance = mean + a mean^2, with $a \geq 0$, the aggregation parameter. If $a = 0$, then the Poisson distribution is obtained. The predictors in this loglinear regression were the factor site, a smoothing of day of capture with four degrees of freedom (Hastie & Tibshirani, 1990) and the factor odour mixture. The smooth function of day naturally accounted for the differences in numbers caught between sets as judged by preliminary deviance tests. Therefore, the factor set was not included in further analyses. The factor odour mixture was coded in such a way that the regression coefficients of the mixtures expressed the (natural) logarithm of the relative attractiveness of each mixture with respect to the control odour (odour A for experiments with CO_2 and odour B for experiments without CO_2), where relative attractiveness is defined as the ratio of the expected numbers of mosquitoes caught in traps with the odour mixture and those with the control odour. Approximate 95%-confidence intervals were calculated from the coefficients and their standard errors in the usual way (Jongman *et al.*, 1995), which were then converted to base 10 logarithms. If the interval embraces 0, the odour does not differ significantly in attractiveness from the control odour at the 5% significance level. Additional analyses were carried out in which the factor odour was replaced by a set of 0/1 variables representing the compounds that constituted each mixture. Regression analysis using all possible subsets revealed which compounds influenced the number of mosquitoes caught. For the compounds in the model with minimum Aikailce Information Criterion (AIC) (McCullagh & Nelder, 1989) the relative attractiveness was calculated together with approximate 95%-confidence intervals.

To display patterns in the relative attractiveness of the odour mixtures for the different species, a principal components biplot (Jongman *et al.*, 1995) was produced from the species-by-mixture table containing all estimates of log-attractiveness obtained by the separate regression analyses. The principal component analysis was non-centred and the rows (columns) of the table were weighted inverse with the row (column) average variance of the estimate. Because odour B had in this way such a large weight, it was downweighted by a factor 40, but this downweighting did not change the biplot much.

Redundancy analysis (Jongman *et al.*, 1995) was used to detect the effects of the weather conditions on the number of mosquitoes of the different species caught. The response variables of this analysis were the log(y+1)-transformed numbers caught of the different species and the predictor variables were five weather variables. The analysis was adjusted for the linear and quadratic effect of day (season), site and odour mixture by specifying these variables as covariables. The results are presented in a biplot of weather variables and mosquito species. Redundancy analysis was also used to display in a biplot the difference in species composition of CDC traps in the experimental house and village house. In this analysis day, dM = -(day-24)^2 and the weather variables were entered as supplementary variables. All biplots were produced with Canoco for Windows 4.5 (ter Braak & Šmilauer,

2002).

Results

The temperature in the period of the study varied between 20.5 and 33.3ºC, with an average of 27.6 ºC during the day and 25.5 ºC during the night. The relative humidity varied between 57.5 and 99.9%, with an average of 84.1% (day) and 94.1% (night). Total rainfall during the study period was 1171 mm, varying from 0 to 116 mm per day (Fig. 4).

The CFG traps caught a total number of 196,756 mosquitoes during this study, with the most abundant species being *Mansonia* spp. (70.6%), *Anopheles* spp. (17.5%) and *Culex* spp. (11.5%). Anophelinae trapped comprised of *Anopheles ziemanni* Grünberg (16.3%), An. *pharoensis* Theobald (0.9%), *An. gambiae s.l.* Giles (0.3%) and *An. wellcomei* Theobald (0.02%). In all experiments 94.7% of the mosquitoes trapped were females. Less than one

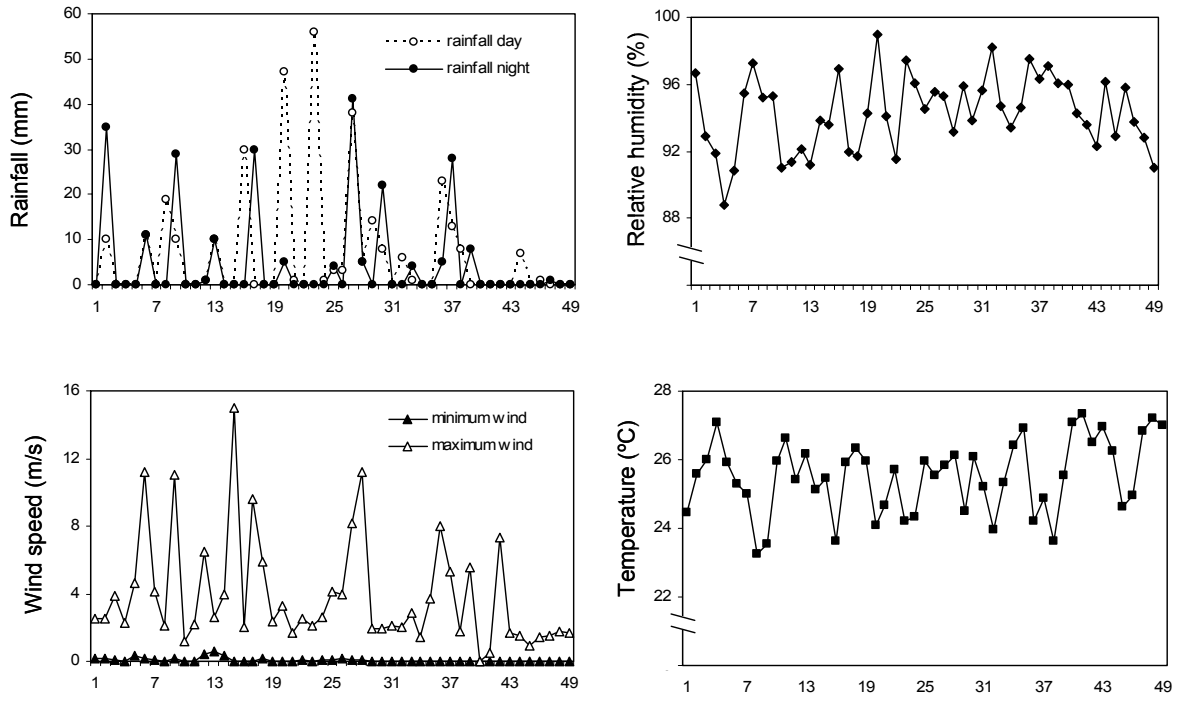


Fig. 4 eather data showing daily average values of rainfall during the day and at night (left upper), minimum and maximum wind speed (left lower), relative humidity (right upper) and temperature (right lower). The x-axis shows the days during the experimental period.

Table 2 Average number (mean ± sd) of mosquitoes trapped by CFG baited with synthetic odours with CO_2 or human odours. Odours in the same sets were tested in the same experimental nights.

Set	Code	Test Odor	N	*An. gambiae*	*An. pharo-ensis*	*An. zie-manni*	*Culex spp.*	*Mansonia spp.*	*Aedes spp.*
1	A	LA+NH_3+CO_2	10	2.2±2.2	4.5±5.9	86.6±106	73.6±84.5	680±734	12.5±11.6
	B	LA+NH_3	10	0±0	0.2±0.4	0.3±0.5	4.7±3.9	11.8±8.8	0.5±1.0
	C	human in tent	5	1.2±1.1	2.2±3.3	6.0±6.4	14.8±10.6	147±100	1.6±2.1
	D	A+3MC4	12	1.8±2.0	8.3±8.8	179±154	127±169	743±711	12.9±13.7
	F	A+3MC4+C3+C4	11	1.7±1.5	5.2±6.8	119±94.0	74.2±59.4	589±415	7.2±6.9
	G	A+C14	10	1.4±2.5	6.3±6.7	102±89.0	65.2±65.2	632±464	12.4±10.5
2	A	LA+NH_3+CO_2	12	4.1±3.9	26.7±61.7	262±401	146±262	549±627	2.6±2.9
	C	human in tent	12	3.3±4.6	1.7±3.4	14.3±27.2	26.5±39.2	316±303	1.1±1.4
	H	F+C14	12	3.7±4.1	10.4±10.5	173±155	93.6±81.0	520±342	5.0±9.0
	I	A+C6	11	4.2±4.8	20.7±24.6	277±289	163±183	709±650	2.8±2.1
	J	A+C6+7OA	12	4.0±3.3	12.1±9.7	91.8±76.8	69.8±52.8	420±259	2.7±3.2
	E	CO_2	12	2.1±3.3	12.7±18.3	200±201	85.3±100	548±485	1.9±2.4
3	A	LA+NH_3+CO_2	12	1.8±1.8	5.2±5.8	340±272	178±197	766±542	3.1±1.8
	C	human in tent	12	2.1±2.7	0.4±0.8	7.6±11.2	17.6±20.7	551±557	0.9±2.0
	K	J+C7+C8	11	1.2±1.4	8.4±6.3	130±116	140±178	757±532	2.1±2.1
	L	J+3MC4+C14	11	2.0±2.6	5.2±7.3	124±97.3	97.1±58.9	624±337	1.3±1.3
	M	A+3MC4+C6	12	1.3±1.4	5.2±6.0	173±151	114±92.8	630±409	1.7±1.3
	N	A+GA+IND+4EP	12	2.0±3.0	5.2±7.0	112±163	132±123	568±521	0.8±1.3
4	A	LA+NH_3+CO_2	11	0.2±0.4	1.6±3.5	83.5±97.9	50.4±96.4	300±398	0.2±0.6
	O	N+3MC4	11	1.0±1.4	0.5±1.0	32.5±40.6	20±10.4	152±82.5	0.5±0.8
	P	N+C6	12	0.8±1.4	0.8±0.8	32.4±45.6	24.3±19.5	179±148	0±0
	Q	N+C6+ 7OA	10	0.6±0.9	1.9±2.5	95.5±145	43.4±62.7	313±356	0.4±0.7
	R	N+3MC4+C6	11	0.1±0.3	0.3±0.9	22.4±24.6	21.3±11.6	194±104	0.1±0.3
	S	N+3MC4+C6+7OA	12	0.2±0.4	1.3±2.1	61.1±86.2	37.0±44.2	281±357	0.4±0.7

percent of the trapped mosquitoes were *Aedes* spp. More mosquitoes were caught in traps with CO_2 than without; and more mosquitoes were trapped outdoors than indoors.

Outdoor experiments

Experiments with CO_2

For all species odour B (L-lactic acid + ammonia) was the least attractive (Fig. 5). The con-

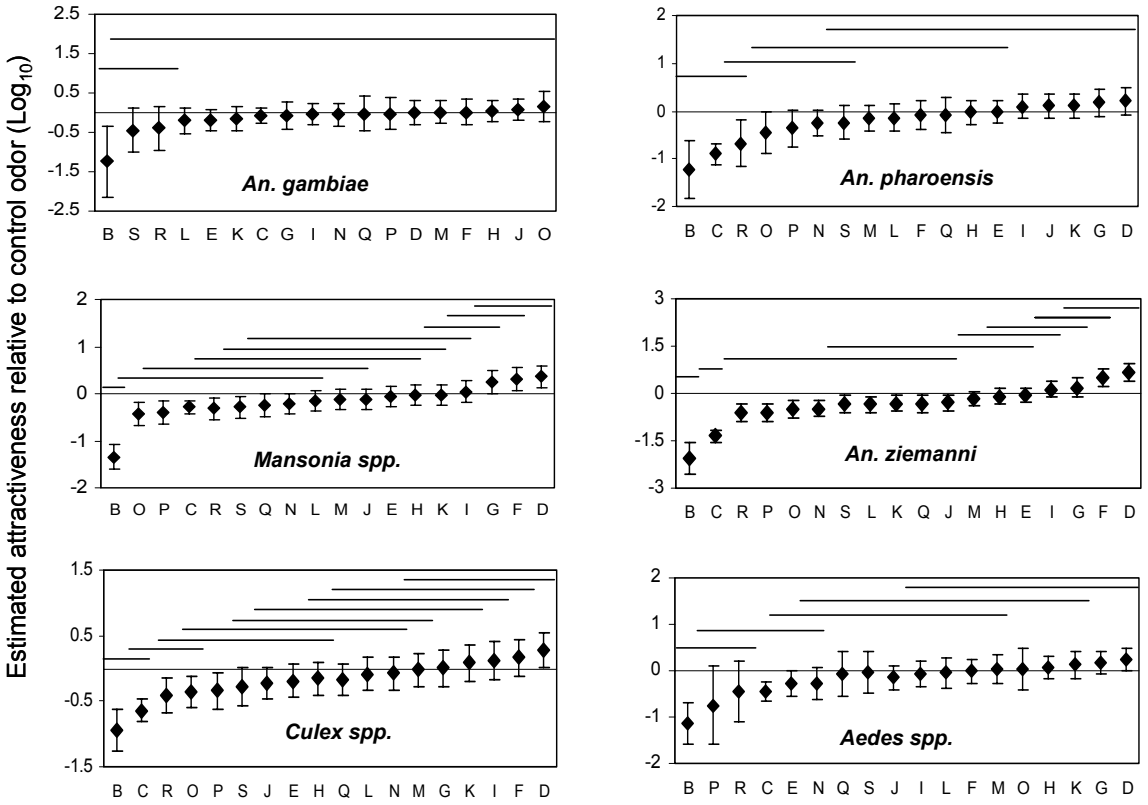


Fig. 5 Attractiveness of odours in the experiment with CO_2 relative to the control odour A, on a ^{10}log scale, as estimated by loglinear regression. The vertical error bars indicate the approximate 95% confidence intervals for the attractiveness. An odour differs significantly ($P<0.05$) in attractiveness from the control if the error bars do not cross the horizontal line at a log-attractiveness of 0. The horizontal lines at the top indicate groups of odours that do not differ significantly as judged by a t-test on the difference of their regression coefficients. See Table 2 for the explanation of the odour codes.

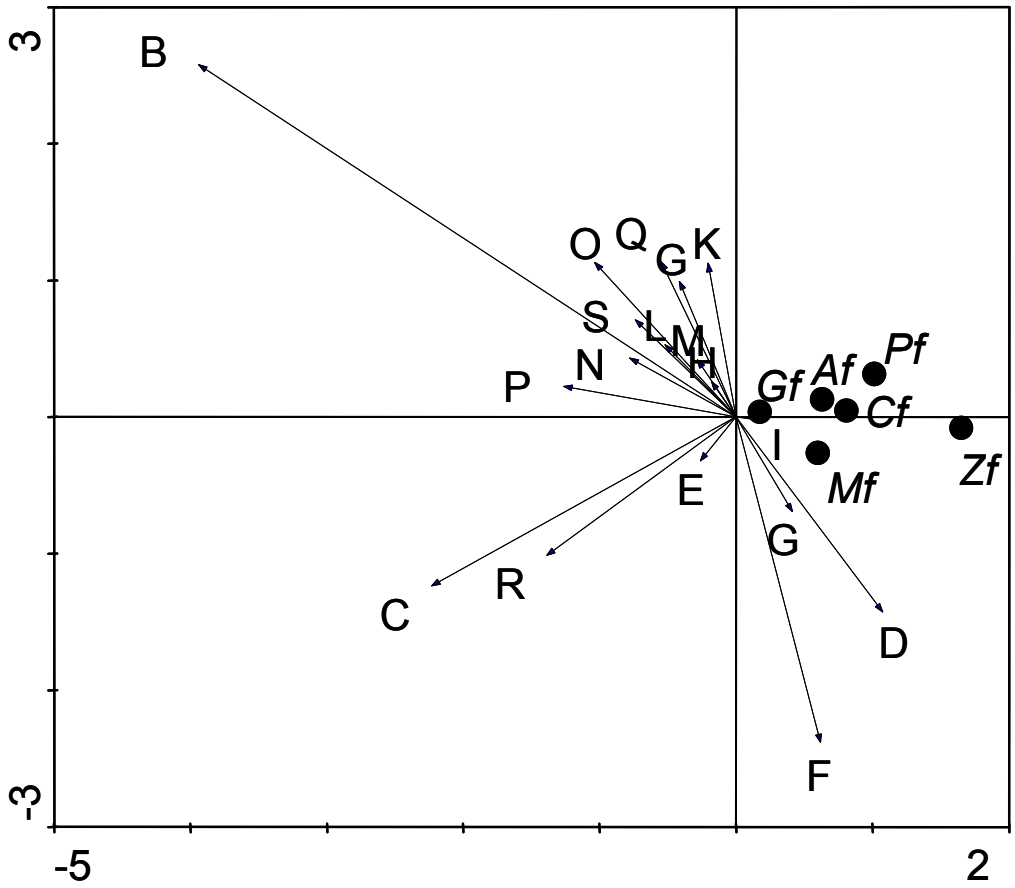


Fig. 6 Biplot display of the log-relative attractiveness of species groups to odours, based on a weighted non-centred principal components analysis, displaying 92% of the sum of squared data values. The scaling is such that distances among species groups display their Pythagorean dissimilarity. Arrows are odours, see Table 2 for the codes of the odours. Black dots stand for females (*f*) of mosquito species *A: Aedes* spp.; *C: Culex* spp.; *G*: *Anopheles gambiae s.l.*; *M: Mansonia* spp.; *P: An. pharoensis*; and *Z: An. ziemanni*.

trol odour A (L-lactic acid + ammonia + CO_2) attracted a greater number of mosquitoes than natural human odour (odour C) for all mosquito species except for *An. gambiae* (Fig. 5). For *Aedes* species, the control odour (A) attracted more mosquitoes than CO_2 alone (odour E); but for the other mosquito groups the control odour attracted similar numbers of mosquitoes as CO_2 alone (Fig. 5).

All mixtures tested attracted few *An. gambiae* (<5 mosquitoes per night on average, Ta-

ble 2). Odour B attracted fewer *An. gambiae* than all other odours, but the difference was not significant for odours S and R (Fig. 5). No differences in attraction to *An. gambiae* were found between other odours.

For *Mansonia* species odours D, F and G were significantly more attractive than the control odour (Fig. 5). Eight odours, including all odours containing the combination of geranyl acetone, indole and 4-ethylphenol (odour N, O, P, Q, R and S) were significantly less attractive than the control odour (Fig. 5). *Culex* species preferred odour D to the control, but showed less attractiveness to odour mixtures O, P and R (Fig. 5). For *An. pharoensis* none of the odours tested were significantly more attractive, and four odours including odour O and R were significantly less attractive than the control odour (Fig. 5). For *An. zie-*

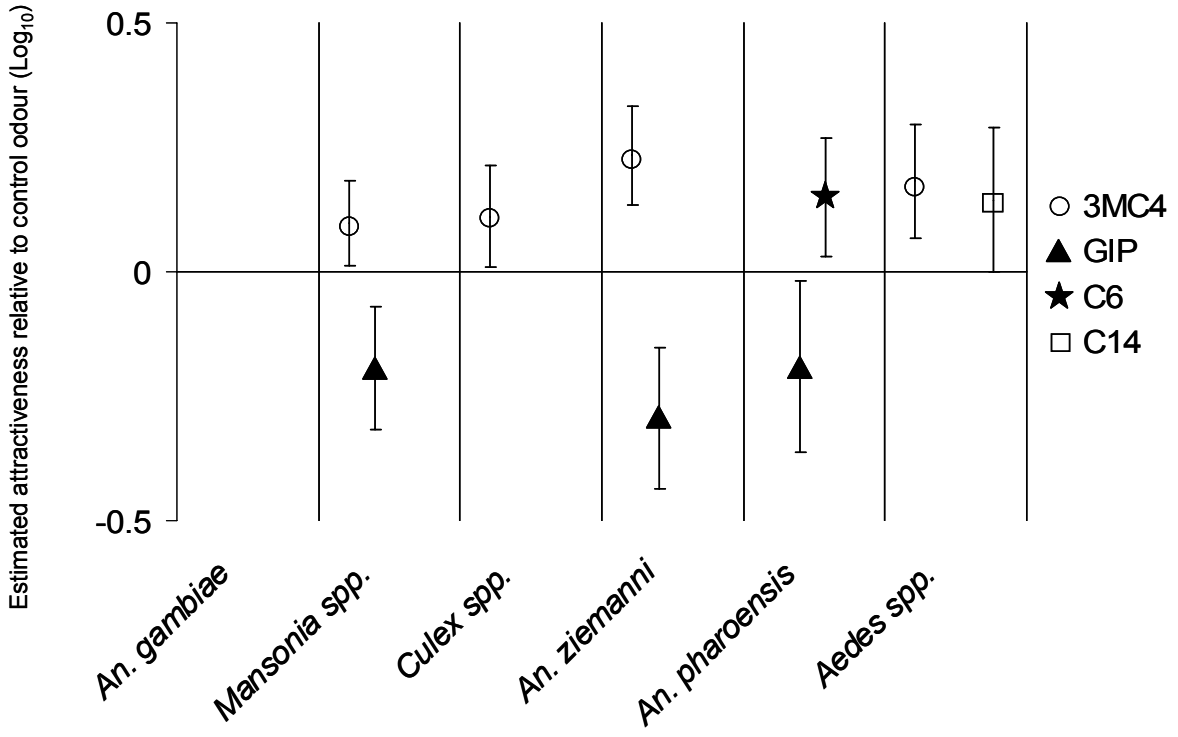


Fig. 7 Attractiveness of odour mixtures in which certain compounds were contained in the experiments employing release of CO_2 relative to the control odour A, on a ^{10}log scale, as estimated by loglinear regression. The vertical error bars indicate the approximate 95% confidence intervals for the attractiveness. An odour mixture had a significant ($P<0.05$) effect on the attraction if the error bars do not cross the horizontal line at a log-attractiveness of 0. 3MC4: odour mixtures containing 3-methylbutanoic acid; GIP: odour mixtures containing geranyl acetone, indole and 4-ethylphenol; C6: odour mixtures containing hexanoic acid; C14: odour mixtures containing tetradecanoic acid.

manni most of the odours tested were less attractive than the control odour; except odour D and F, which were more attractive than the control odour (Fig. 5).

Figure 6 summarizes the pattern of attractiveness graphically. All mosquito spp. were rarely attracted to odour B compared to the control and were not attracted to or to a less extent to all other odours, except odours D, F and G. The horizontal dimension of Fig. 6 shows that *An. gambiae* (Gf) and *An. ziemanni* females (Zf) differed most in their odour preference: the response to odours was much stronger in *An. ziemanni* than in *An. gambiae*. The other species were intermediate in response size. The vertical dimension distinguishes *Mansonia* species from *An. pharoensis* (Pf), with the other groups being intermediate. *An. pharoensis* females (Pf) disliked odours C and R more than *Mansonia* spp. *An. pharoensis* was less attracted to odours G, D and F than *Mansonia* species.

Odour blends with 3-methyl butanoic acid attracted more female mosquitoes of *An. ziemanni*, *Mansonia*, *Culex* and *Aedes* species (Fig. 7). *An. pharoensis* showed a preference for

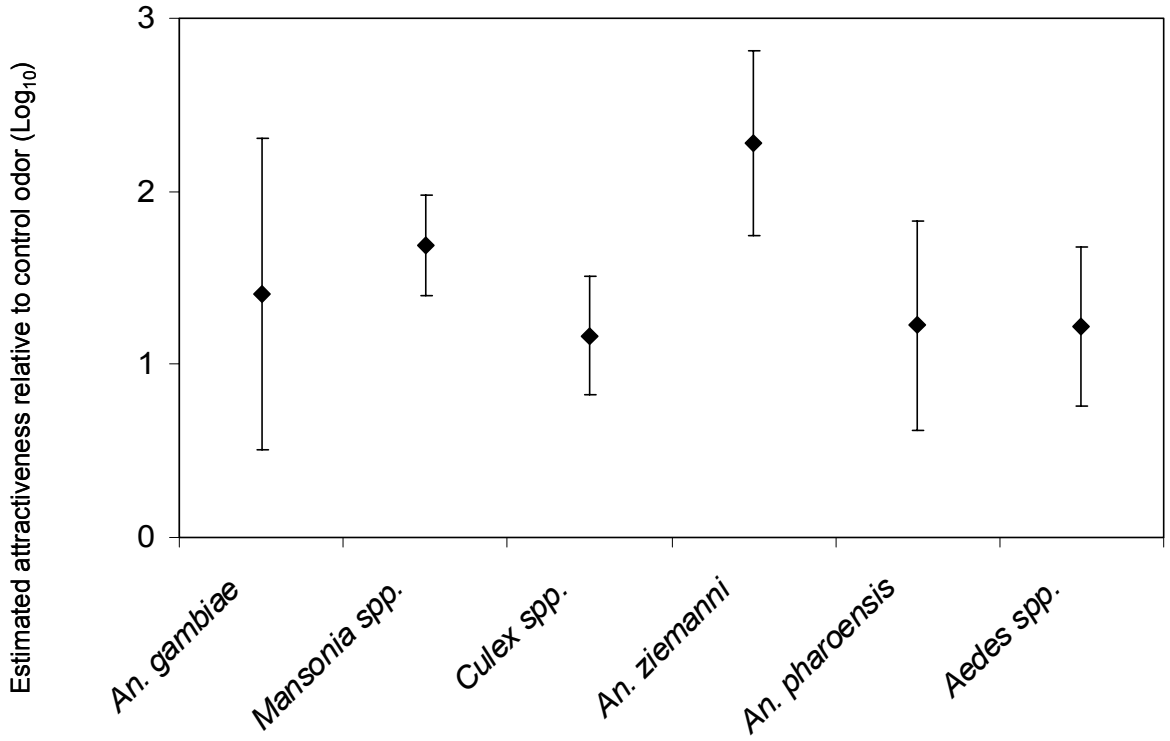


Fig. 8 Attractiveness of odour mixtures in the experiments in which CO_2 was released from traps relative to the same odour mixtures in experiments without CO_2, on a ^{10}log scale, as estimated by loglinear regression. The vertical error bars indicate the approximate 95% confidence intervals for the attractiveness. Odours with and without CO_2 differed significantly ($P<0.05$) in attractiveness if the error bars do not cross the horizontal line at a log-attractiveness of 0. See Table 2 for the codes of the odour mixtures.

odour mixtures containing hexanoic acid; a similar preference was found for *Aedes* mosquitoes to odour mixtures containing tetradecanoic acid. Odour mixtures containing the combination of geranyl acetone, indole and 4-ethylphenol were less attractive than the control odour for *Mansonia* spp., *An. ziemanni*, and *An. pharoensis*.

Experiments without CO_2

The number of mosquitoes caught in CFG traps without CO_2 was lower than the number caught with the same odours to which CO_2 had been added (Table 2, 3). For all species, add-

Table 3 Average number (mean ± sd) of mosquitoes trapped by CFG baited with synthetic odours without CO_2. Odours in the same set were tested in the same experimental nights.

Set	Code	Test Odor	N	*An. gambiae*	*An. pharoensis*	*An. ziemanni*	*Culex spp.*	*Mansonia spp.*	*Aedes spp.*
1	B	LA+NH_3	12	0	0	3.3±5.0	4.4±3.4	19.8±18.6	0.3±0.5
	Y	no odor	12	0	0.2±0.4	1.8±2.8	8.6±17.9	17.6±29.7	0.2±0.4
	D'	B+3MC4	11	0	0	1.5±2.1	7.7±7.5	11.7±8.4	0.5±0.5
	F'	B+3MC4+C3+C4	12	0	0.1±0.3	3.0±3.7	8.3±8.9	24.2±25.9	0.1±0.3
	G'	B+C14	12	0	0.1±0.3	2.2±3.5	4.6±3.5	12.8±10.2	0.3±0.6
	H'	F'+C14	10	0	0.1±0.3	0.6±0.8	5.2±6.3	15.3±17.7	0.1±0.3
2	B	LA+NH_3	12	0.1±0.3	0.1±0.3	0.9±1.4	2.6±5.4	8.2±11.7	0
	I'	B+C6	12	0	0.2±0.4	1.5±2.1	2.1±2.9	13.7±19.3	0.1±0.3
	J'	B+C6+7OA	12	0	0.1±0.3	1.1±0.8	0.9±0.8	5.5±4.5	0
	K'	J'+C7+C8	12	0	0.2±0.6	2.3±3.6	1.8±3.0	15.8±22.2	0.1±0.3
	L'	J'+3MC4+C14	12	0	0.1±0.3	0.9±1.1	1.8±2.5	7.3±10.0	0
	N'	B+GA+IND+4EP	12	0.1±0.3	0.2±0.4	1.3±2.3	1.1±1.4	6.4±7.	0.1±0.3
3	B	LA+NH_3	6	0	0	3.3±4.8	1.2±1.3	10.5±11.4	0.2±0.4
	T	N'+3MC4+C6+C14	6	0	0.2±0.4	1.2±1.2	1.5±1.1	6.3±5.2	0
	U	N'+C13+C16	6	0	0.2±0.4	0.8±1.6	2.2±3.3	11.3±8.1	0.2±0.4
	V	N'+DD	6	0	0.3±0.8	3.2±4.3	4.2±7.4	10.7±12.0	0
	W	N'+6M5H	6	0	0	2.3±4.3	1.8±2.1	10.8±16.2	0
	X	N'+3MB	6	0	0.2±0.4	3.0±2.0	3.5±1.9	14.0±7.1	0

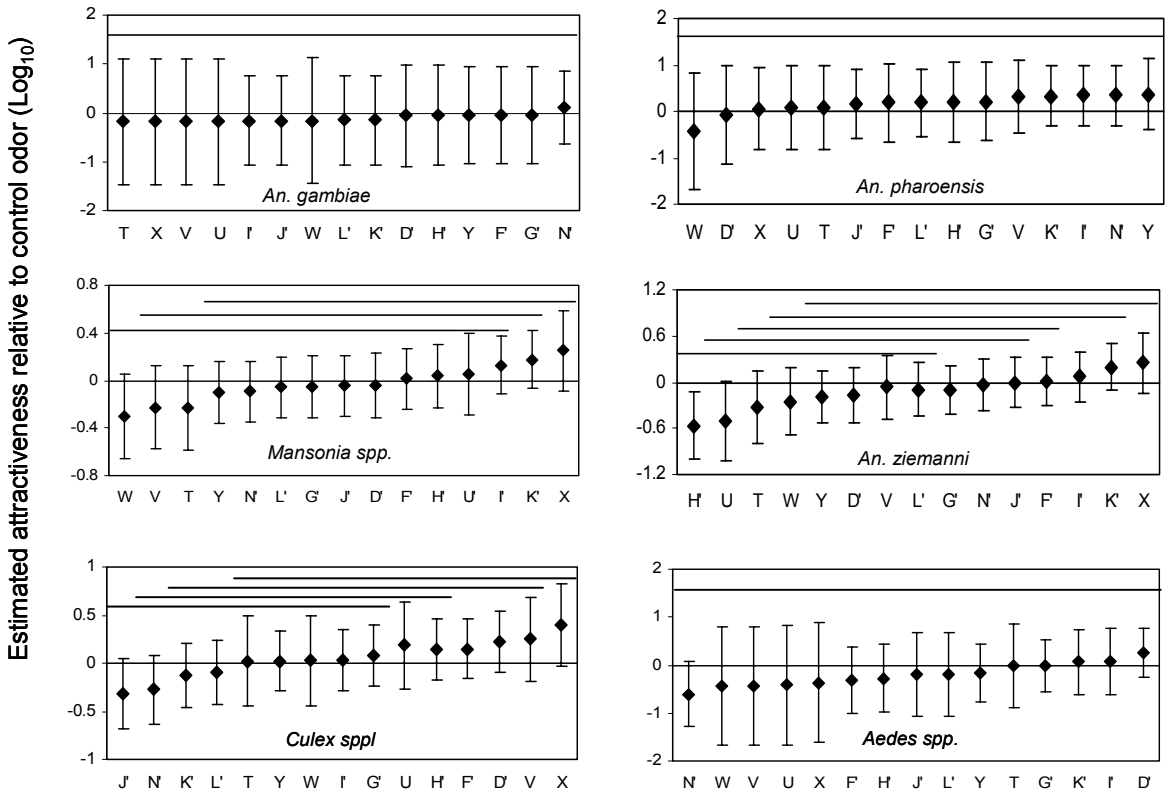


Fig. 9 Attractiveness of odours relative to the control odour B, on a ^{10}log scale, in the experiments without CO_2, as estimated by loglinear regression. The vertical error bars indicate the approximate 95% confidence intervals for the attractiveness. An odour differs significantly ($P<0.05$) in attractiveness from the control if the error bars do not cross the horizontal line at a log-attractiveness of zero. The horizontal lines at the top indicate groups of odours that do not differ significantly as judged by a t-test on the difference of their regression coefficients. See Table 3 for the codes of the odours.

ing CO_2 to any odour mixture increased the catch significantly (GLM, $P<0.001$) (Fig. 8). The effect was independent of the test odour mixtures.

Few *An. gambiae*, *An. pharoensis* and *Aedes* species were trapped by the test odours. The trap catch did not differ significantly between the test odours (Table 3; Fig. 9). For these mosquito species, none of the odours tested caught a greater number of mosquitoes than the control odour; odour H' and U attracted fewer mosquitoes than the control odour B (Fig. 9). For *Mansonia* species, *Culex* species and *An. ziemanni*, odour X showed the highest attractiveness among all the odours tested but this odour was not significantly more attractive than no odour (Fig. 9).

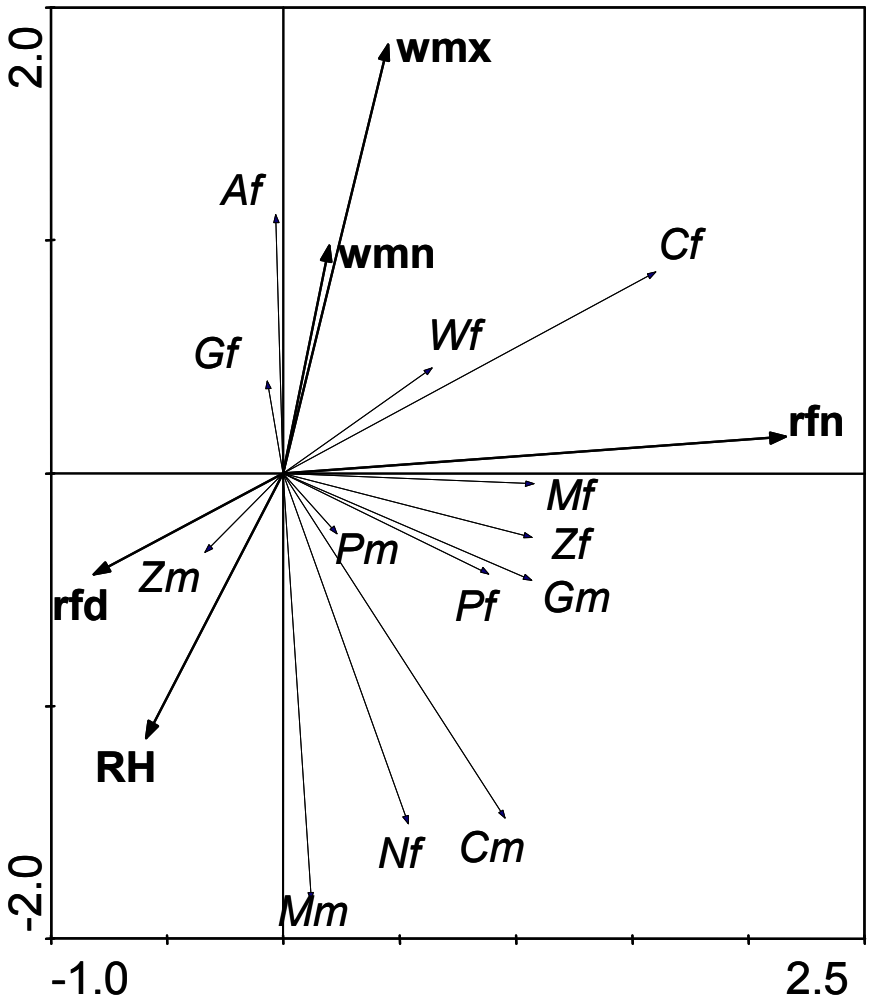


Fig. 10 Biplot of the effects of weather variables on species groups based on a partial redundancy analysis of log-counts on the weather variables (see text), displaying 93% of the variance in the effects. The weather variables are rainfall during the day (rfd) and at night (rfn), minimum (wmn) and maximum wind speed (wmx) and relative humidity (RH). Although the weather variables have a statistically significant effect on the trapping results, they explain only 5% of the variance that remains after accounting for season and site. Codes for females (*f*) and males (*m*) of mosquito species are *A*: *Aedes* spp.; *C*: *Culex* spp.; *G*: *Anopheles gambiae s.l.*; *M*: *Mansonia* spp.; *P*: *An. pharoensis*; *W*: *An. wellcomei*; Z: *An. ziemanni*. *N*: unidentified *Anopheles* spp.

Effect of weather conditions on mosquito catches

Fig. 4 shows the weather data collected during the experimental period. A noticeable decrease in rainfall was observed during the last 10 days of the experimental period, at the end

of the rainy season. The maximum wind speed decreased as well.

The effects of weather variables on the catches for different groups of mosquitoes are shown in Fig. 10. Weather variables had a statistically significant effect on the trap results, but their effects were small and explain only 5% of the variance that remains after accounting for time and site.

Rainfall during the day and night caused an opposite effect (Fig. 10). Night rainfall affected the catch of all mosquito species positively, with exception of male *An. ziemanni* and female *An. gambiae* (Fig. 10). In contrast, rainfall during the day affected the catch of female mosquitoes negatively except for some unidentified *Anopheles* species; to the contrary, the catches of males of *Mansonia* species, *Culex* species and *An. ziemanni* were positively affected by rainfall during the day (Fig. 10).

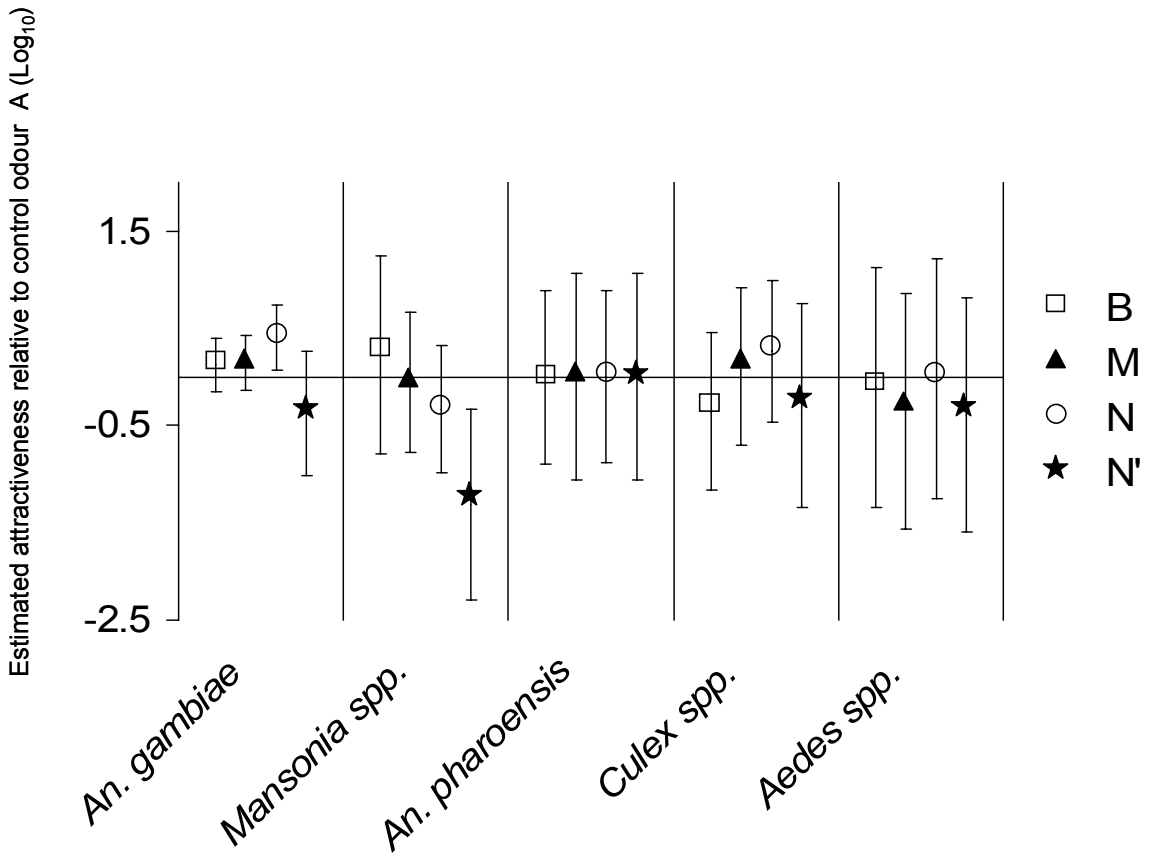


Fig. 11 Attractiveness of odours relative to the control odour A, on a ^{10}log scale, in the indoor experiment, as estimated by loglinear regression. The vertical error bars indicate the approximate 95% confidence intervals for the attractiveness. An odour mixture had significant ($P<0.05$) effect on the attraction if the error bars do not cross the horizontal line at a log-attractiveness of 0. See Table 2 and 3 for the codes of the odours.

Table 4 Average number (mean±sd) of mosquitoes trapped by CFG baited with synthetic or human odours tested inside experimental houses. See Table 2 & 3 for codes of test odours.

Test Odour	N	*An. gambiae*	*An. pharoensis*	*An. ziemanni*	*Culex* spp.	*Mansonia* spp.	*Aedes* spp.
A	10	0.1±0.3	0	0	0.2±0.6	3.6±5.8	0.1±0.3
C	10	0.2±0.4	0	0	0	5.5±6.4	0.1±0.3
M	11	0.2±0.6	0	0	0.2±0.4	3.1±3.0	0
N	10	0.5±1.3	0	0	0.4±0.7	1.8±2.6	0.1±0.3
N'	11	0	0	0	0	0.1±0.3	0

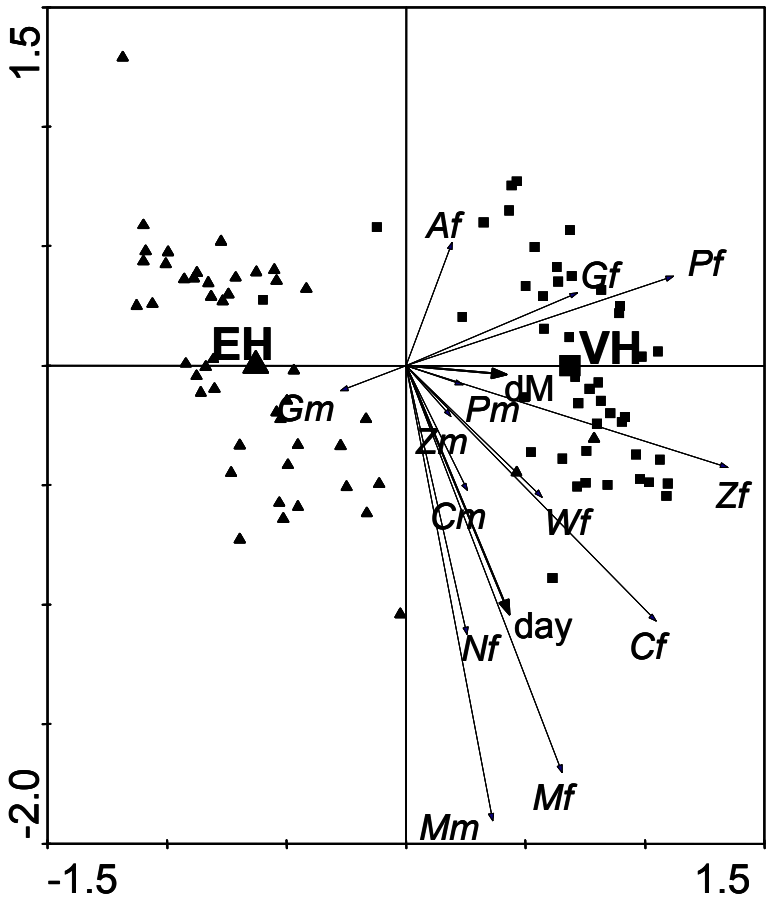


Fig. 12 Triplot showing the difference in the species composition between traps in the experimental house (EH, triangles) and the village house (VH, squares) and the changes in species composition with season based on a redundancy analysis of log-counts on house indicator variables with the season variables (dM and day respectively) as supplementary variables. The constrained horizontal axis (EH versus VH) explains 43% of the variance; the unconstrained vertical axis displays 18% of the variance. Codes for females (*f*) and males (*m*) of mosquito species are *A: Aedes* spp.*; C: Culex* spp.*; G*: *Anopheles gambiae s.l.; M: Mansonia* spp.*; P: An. pharoensis; W*: *An. wellcomei*; Z*: An. ziemanni*. *N*: unidentified *Anopheles* spp.

Table 5 PCR species identification of *An. gambiae* complex

Trap	*An. gambiae s.s.*	*An. arabiensis*
17 August ~ 1 September (CDC)	188 (80%)	48 (20%)
2 September ~ 18 September (CDC)	26 (90%)	3 (10%)
19 September ~ 4 October (CDC)	16 (76%)	5 (24%)
All traps (CDC+CFG)	444 (85%)	79 (15%)

Wind speed, be it measured either as the maximum or minimum wind speed on a day, affected the catches of female *Culex* species, *Aedes* species, *An. gambiae*, *An. wellcomei* and *Mansonia* species positively, whereas they affected the catch of males of all mosquito groups and females of *An. pharoensis* and *An. ziemanni* negatively (Fig. 10). The maximum and minimum wind speed on a day correlated with each other.

High humidity had a negative effect on the catch of females of most mosquito species, but exerted a positive effect on the catch of most male mosquitoes (Fig. 10).

Indoor experiments

The numbers of mosquitoes caught by CFG traps indoors was much lower than outdoors (Table 4). Odour mixture N attracted more female *An. gambiae* than the control odour (odour A), but the same odour mixture without CO_2 (N') attracted similar numbers as the control odour A (Fig. 11). Fewer *Mansonia* mosquitoes were caught by odour N' than the control odour (Fig. 11). Both odour N and N' attracted numbers of mosquitoes of all other species similar to those attracted to the control odour (odour A) (Fig. 11). Mosquito catches with odour M and the odour from a human occupied tent were not different from the control odour A (Fig. 11).

CDC traps and population dynamics

The most abundant mosquito species caught by the CDC traps (56,290 in total) were *Mansonia* spp. (59.4%), *An. gambiae s.l.* (16.0%), *Culex* spp. (11.6%) and *An. ziemanni* (11.3%). Of all mosquitoes caught by CDC traps, 98.4% were female mosquitoes.

The average number of mosquitoes trapped by the CDC light traps suspended next to a sleeping human in the experimental house and in a village house were compared (Fig. 12). The CDC trap in the village house trapped more mosquitoes of all species than the CDC trap

in the experimental house, except for male *An. gambiae* (Fig. 12: horizontal axis). The difference was greatest for female *An. ziemanni*, *An. pharoensis*, and *Culex* spp., less for *An. gambiae* and small for *Mansonia* spp. and *Aedes* spp.

The populations of mosquitoes varied over the experimental period (day), as shown by the vertical axis of Fig. 12. From top to bottom of Fig. 12, *Aedes* spp. were most abundant at the start of the experimental period, *Mansonia* spp., *Culex* spp. and some unidentified *Anopheles* spp. were most abundant at the end and the other species were intermediate.

Species composition of *An. gambiae s.l.*

Results from the DNA polymerase chain reaction showed that of the *An. gambiae* complex only *An. gambiae s.s.* and *An. arabiensis* were recovered from the traps. The average ratio of the two species was 84.9% and 15.1% respectively with a slight variation in species ratio over the experimental period (Table 5).

Discussion

The overall results of the study demonstrate the feasibility of using odour-baited CFG traps for the collection of blood-feeding mosquitoes in the field. It was particularly encouraging that many of the odour blends tested were as attractive as or more attractive than natural odour from a human host. The results show, however, that many of the blends were not more effective than the control odours, possible reasons for this are discussed below.

Experiments with CO_2

Large numbers of mosquitoes from various taxonomic groups were collected in this study, with *Mansonia* and *Culex* spp. being the most abundant. Because of their high abundance and because the main target of the study were anopheline mosquitoes, *Culex* and *Mansonia* mosquitoes were not identified to species, but probably existed of *Culex poicilipes* Theobald, *C. bitaeniorhynchus* Giles, *Mansonia uniformis* Theobald and *M. africana* Theobald (Gillies & Wilkes, 1968). The most abundant anopheline species were *An. ziemanni*, *An. pharoensis* and *An. gambiae s.l.* With PCR analysis we identified the *An. gambiae s.l.* trapped in our experiments to be either *An. gambiae s.s.* or *An. arabiensis*, with slight fluctuations in species composition over the experimental period. Similar seasonal fluctuations of these two species were reported by other authors (Dia *et al.*, 2003; Koenraadt *et al.*, 2004). Since the experiments were carried out during the night, we assume that the majority of the mosquitoes caught were nocturnal species.

For all species except for *Aedes* spp., the reference odour, ammonia + L-lactic acid + CO_2, was not more attractive than CO_2 alone. Previous research had shown that ammonia alone and the combination of ammonia and lactic acid was attractive to *An. gambiae s.s.* in a dual-choice olfactometer when tested against no odour (Smallegange *et al.*, 2005). Furthermore, the combination of ammonia + lactic acid + CO_2 was shown to be more attractive than CO_2 alone (J. Spitzen and R.C. Smallegange, unpublished data). It is possible that the concentration or ratios of the odours released from the CFG traps was not within the range that is most effective for attracting *An. gambiae*.

Due to the low overall abundance of *An. gambiae*, we are rather uncertain about the true relative attractiveness in this field study. From Fig. 8, the confidence interval of the $\log_{10}$ ratio CO_2/Control is [-0.47, 0.08] with P=0.15. In terms of expected numbers caught, this interval implies that the control odour attracted between 3 (= $10^{0.47}$) and 0.8 times as many females as CO_2, with 1.6 times as the most likely estimate. When the statistical analysis is done with set 2 only, the most likely estimate is 2 times as many with 95% confidence bounds of 3.7 and 0.98. In this analysis the effect was close to significance (P = 0.051). Our conclusion is that this experiment does certainly not refute the earlier findings that the control odour was more attractive than CO_2.

The control odour attracted more mosquitoes of all species than the odour from a human staying in a tent. Meanwhile, according to the mean number of mosquitoes trapped with CO_2 alone and with the odour from a human-occupied tent, CO_2 seemed to have accounted for 63% of the attractiveness of the human odour to *An. gambiae*, and CO_2 alone caught more mosquitoes of the other species than the odour from a human-occupied tent (Table 2). The contribution of carbon dioxide to the attractiveness of a human to *An. gambiae s.l.* was estimated to be 9% and 50% by (Costantini *et al.*, 1996a; Mboera *et al.*, 2000a) respectively. The reasons for the relatively higher catches with the control odour compared to the human odour observed in our study might be that the additional attractiveness caused by ammonia and lactic acid made the control odour highly attractive, and therefore a good candidate odour for future study.

Compounds selected for this study were all components of human emanations and were found to cause either behavioural or electrophysiological activities in *An. gambiae s.s.* in the laboratory (Bernier *et al.*, 2000; Meijerink *et al.*, 2000; Costantini *et al.*, 2001; Healy *et al.*, 2002). The most attractive mixture for all mosquito groups except *An. gambiae s.l.* was odour D: control odour with CO_2 + 3-methylbutanoic acid. The latter compound was detected in the volatiles of Limburger cheese and incubated human sweat, and these volatiles were found to be attractive to female *An. gambiae* (Knols *et al.*, 1997; Meijerink *et al.*, 2000; Healy *et al.*, 2002). Electrophysiological responses to 3-methylbutanoic acid were found at the antennal and the single olfactory neuron level in *An. gambiae* (Cork & Park,

1996; Meijerink & van Loon, 1999; Chapters 8 and 9). Using an online gas chromatograph-coupled single sensilla recording method, Dougherty *et al.* (1999) found that neurons in ascoid sensilla of the sandfly *Lutzomyia longipalpis* responded to 3-methylbutanoic acid present in volatiles from foxes. Meanwhile, 3-methylbutanoic acid is also a component of mosquito oviposition sites, pig manure and various plant odours and was considered a potential kairomone for various insects (Kramer *et al.*, 1980; Cossé & Baker, 1996; Larson *et al.*, 2003). Our results suggest that the combination of ammonia, L-lactic acid, CO_2 and 3-methylbutanoic acid (odour D) produces a broad-ranging attractant for many species of nocturnal African mosquitoes. The above mentioned mixture in combination with propanoic acid and butanoic acid (odour F) was the second most attractive mixture for *An. ziemanni*, *Culex* spp. and *Mansonia* spp.; the other compounds reduced the attraction (Fig. 6). The attractiveness decreased further when tetradecanoic acid was added to odour F (odour H) or when hexanoic acid was added to odour D (Fig. 6). These latter compounds (tetradecanoic acid and hexanoic acid), apparently, are causing repellency and/or masking effects at the concentrations tested.

Olfactory receptor neurons in subpopulations of sensilla trichodea were found to be strongly tuned to indole, geranylacetone or 4-ethylphenol (Chapter 8 and 9). Certain concentrations of these compounds, when tested in combination with ammonia and lactic acid against ammonia in a dual-choice olfactometer, showed a repellent effect in *An. gambiae s.s.* (Chapter 6). The results of the outdoor experiments showed that adding these three compounds either did not cause additional attractiveness or reduced the attractiveness of the control odour for all groups of mosquitoes. The process of mosquito host location and host preference is based on chemoreception of both attractive as well as repellent components by the olfactory system. The choice of a certain host is the end result of a complex host-selection process , that might include both kairomones and allomones (Birkett *et al.*, 2004).

Synthetic blends without CO_2

A comparison of the number of mosquitoes trapped by odour blends with and without CO_2 revealed that CO_2 contributes substantially to the trapping efficiency of the CFG traps (Fig. 8). Carbon dioxide accounted for more than 97% of the attraction of the reference odour for all mosquito spp. except for *Culex* spp. For the latter species, CO_2 accounted for 79% of the attraction. Carbon dioxide is considered a general cue employed by almost all blood feeding mosquito species in their host-seeking behaviour (Takken, 1996). For highly anthropophilic species such as *An. gambiae s.s.*, CO_2 was considered to account for only a minor part of the attractiveness of a human (Costantini *et al.*, 1996a; Mboera & Takken, 1997; Takken *et al.*, 1997a). One possible reason that CO_2 plays such a dominant role in the present study might

be due to the design of the CFG odour delivering system, in which CO_2 was an extra driving force that carried the odour mixtures to the odour release point at the bottom of the traps. It is possible that not all other volatiles were effectively carried along with the CO_2-laden air stream. Another possible reason might be because of the activation effect of CO_2, which is essential for the mosquitoes to get close to the traps (Kawada *et al*., 2004; Pates *et al*., 2005).

Indoor collections

The number of mosquitoes of almost all species that were trapped indoors was lower than outdoors. *An. pharoensis* and *An. ziemanni* were not collected indoors. It is remarkable that odour N, ammonia + L-lactic acid + CO_2 + geranyl acetone + indole + 4-ethylphenol, attracted more *An. gambiae s.l.* than the control odour A, although the absolute number of mosquitoes trapped was low. Because odour N did not attract a greater number of mosquitoes than the control odour in the outdoor experiments, the effect is ambiguous.

Mansonia mosquitoes were most abundant in the indoor experiments. (Snow, 1987) studied the house-entering habits of different mosquito species and found that endophilic species such as *An. gambiae* and *Mansonia* spp. were less affected by the increasing wall height than the exophilic mosquitoes such as *An. pharoensis* and *Culex* spp. The design of the experimental houses is such that mosquitoes can only enter via a slit under the two eaves (at a height of about 1.9 m). Therefore only highly endophilic mosquito species may have managed to enter the house and could be caught in the traps, and this may explain the relatively low indoor catches of the exophilic species. Another factor explaining the poor result indoors could have been the lack of convection heat to carry the odours to the eaves. In a natural situation body heat will cause convection currents that will carry volatiles out of the house.

CDC traps

The numbers of *An. gambiae s.l.* caught in the CFG traps were lower than the numbers caught by the CDC traps placed in a man-occupied house. We do not consider this to be an effect of a lower trapping efficiency of the CFG traps, because it was demonstrated that a CFG trap caught more *An. gambiae* and *C. quinquefasciatus* than a CDC trap (Mboera *et al.*, 2000a). Females of *An. gambiae* are highly endophilic, i.e. they rest inside human houses, and therefore the population of *An. gambiae* in the study area is concentrated near human dwellings. In our study, CFG traps were deliberately placed as far as possible away from the village to avoid interference of the human odours from the houses. The hypothesis

is confirmed by results of a pilot study, showing a significant difference in the average number of *An. gambiae s.l.* caught by two CDC traps, one suspended in the MRC research station (2.8±2, n=9) and the other in the field (0.1±0.3, n=9) near the transect where the CFG traps with CO_2 were placed. The results of the latter possibly explains why relatively few *An. gambiae* mosquitoes were trapped in our outdoor CFG experiments. This indicates that odour-baited mosquito traps should be placed near human dwellings in order to obtain a successful control of anthropophilic mosquitoes such as *An. gambiae.*

The average number of mosquitoes trapped in the CDC trap suspended in a village house was higher for all species of mosquitoes than the CDC trap suspended in the experimental house. The difference might have been caused by the differential attractiveness of the two human individuals sleeping under the bednet adjacent to the trap. Another possible reason for the difference is that the population level of blood feeding mosquitoes in the village was higher than in the experimental house because of the availability of more potential hosts. A third explanation might be a structural difference between the two houses: the village house had no door, which was probably the main mosquito entrance; the experimental house had a door that was closed during the experiments, and the main mosquito entrance was the slit under the eaves. When comparing the species composition of the CDC traps in these two different traps, the percentage of the endophilic species *An. gambiae s.l.* and *Mansonia* spp. was higher in the experimental house, whereas the exophilic mosquitoes *An. pharoensis*, *An. ziemanni* and *Culex* spp. were more abundant in the village house. This again can be explained by the "wall-height hypothesis" (Snow, 1987). Another possible reason for the difference in mosquito species composition in the two traps is that a number of goats were kept in the village house at night, which might have contributed to the high proportion of zoophilic mosquitoes.

The present study shows that CFG traps baited with synthetic mixtures were in many cases more attractive than human odours pumped from a tent. Carbon dioxide substantially increased the catches of the CFG traps of all mosquito species. CFG traps baited with the odour mixture of ammonia + lactic acid + CO_2 + 3-methyl butanoic acid was the most attractive odour for most mosquito groups. This mixture might be a promising candidate odour blend for the control of nuisance mosquitoes. Indoor experiments showed that one odour mixture, ammonia + L-lactic acid + CO_2 + geranyl acetone + indole + 4-ethyl phenol, was more attractive for *An. gambiae* than the control odour. The latter mixture warrants further investigation under conditions of higher abundance of *An. gambiae* and might fulfil a role in malaria vector control programs. The CFG traps in our experiments were placed away from human dwellings, where the population of the anthropophilic and endophilic species *An. gambiae* was lower than near or in these dwellings. For the purpose of malaria management, the CFG traps should be placed close to human dwellings to increase the trapping

efficiency.

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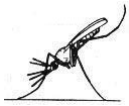
Olfactory coding in antennal neurons of the malaria mosquito, *Anopheles gambiae*



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Abstract

Olfactory receptor neurons in the antenna of insects serve to encode odours and transmit action potentials to the olfactory lobe of the deuterocerebrum. The response characteristics of odorous stimuli provide a basis for a functional classification of olfactory neurons. We performed a functional analysis of olfactory neurons in the antennae of the female malaria mosquito *Anopheles gambiae* s.s by recording responses from single sensilla. We identified six functional types of sensilla trichodea and five functional types of sensilla basiconica (grooved peg sensilla) based on a hierarchical cluster analysis. "Generalist" ORNs that are tuned to a broad range of odours were found in sensilla trichodea subtype E; whereas "moderate specialist" ORNs that are tuned to a narrow range of odours were found in subtype C and grooved peg sensilla, with two "extreme specialist" tuned to only one odour. There was an overlap in the response spectra between sensilla trichodea E and C or grooved peg sensilla, but no overlap was found between sensilla trichodea C and grooved peg sensilla except that both responded to ammonia. Neurons associated with the same sensillum tended to response to similar odour stimuli but with different sensitivities. Different compounds elicited characteristic temporal activity patterns in the same neuron. Neurons in grooved peg sensilla were tuned to more polar compounds including the important behav-

ioural attractant ammonia and its synergist lactic acid responses of which were only found in grooved peg sensilla. Phenols were among the most effective stimulants for several neuron types belonging to different functional classes. Across-fibre patterning is the most plausible coding principle operating in the olfactory system of this mosquito species. Olfactory coding of human odours and its relation with behavioural responses of female malaria mosquitoes is discussed.

Introduction

Insects rely to a large extent on olfactory information to locate food, mating partners, and breeding sites (Hildebrand & Shepherd, 1997). Olfactory receptor neurons (ORN) in insects are contained in sensilla, cuticular extensions of various shapes predominantly present on their antennae and mouthparts. ORNs have been shown to encode odour quality, quantity, temporal changes in odour concentration and spatial distribution (Heinbockel & Kaissling, 1996; de Bruyne *et al.*, 1999; de Bruyne *et al.*, 2001; Mustaparta, 2002). Action potentials of a single neuron can be recorded *in situ* and ORNs can be classified according to their response spectra. Unravelling the mechanisms of odour coding will significantly contribute to our understanding of odour-mediated behaviour.

Anopheles gambiae is the main vector of malaria in Africa causing more than one million victims each year. Several reasons make it highly relevant to study olfactory coding mechanisms operating of this mosquito. The recent identification of the complete genome of *An. gambiae* (Holt & et al., 2002), with more than 79 putative OR genes discovered (Hill *et al.*, 2002), has led to its introduction as a new model-organism for investigating vector-born diseases. It allows functional studies of genes encoding olfactory receptor molecules (Hallem *et al.*, 2004a). Being nocturnal, a female *An. gambiae* mosquito is guided to its human hosts predominantly by olfactory cues (Takken, 1991). This mosquito species is highly anthropophilic (Coluzzi *et al.*, 1979; Pates *et al.*, 2001b), leading to the prediction that the olfactory system is tuned to human odours. Understanding odour coding will benefit the development of new attractants or repellents useful in the control of this mosquito.

The antennae of female *An. gambiae* mosquitoes carries 4 types of sensilla: sensilla trichodea, sensilla basiconica or grooved peg sensilla, sensilla coeloconica and sensilla ampullacea (Fig. 1). The last two types of sensilla are present in low numbers on the antennae and they are possibly innervated by thermoreceptor neurons (Davis & Sokolove, 1975; McIver, 1982). Each antenna of female *An. gambiae* mosquitoes bears about 630 sensilla trichodea and 84 grooved peg sensilla, both types contain olfactory neurons (Ismail, 1964; van den Broek & dan Otter, 1999; Meijerink *et al.*, 2001). Assuming that the number of olfactory neurons innervating sensilla trichodea and grooved peg is 2 and 3~4 respectively (Boo

& McIver, 1976), the total number of olfactory neurons in each antenna is estimated to be about 1512~1596. Subtypes C and/or E of sensilla trichodea (Boo, 1980) respond either by excitation or inhibition to short-chain carboxylic acids (C2-C6), 1-octen-3-ol and 3- and 4-methylphenol (van den Broek & dan Otter, 1999; Meijerink *et al.*, 2001). Subtype C of sensilla trichodea (Boo, 1980) was most sensitive to geranyl acetone but also showed excitation responses to 3-methyl-butanol, 6-methyl-5-hepten-2-one and ammonia. Sensilla trichodea subtypes C and E that were sensitive to indole showed similar response patterns to the last three compounds, whereas they were not responsive to geranyl acetone (Meijerink *et al.*, 2001). Grooved peg sensilla have been reported to respond to polar compounds such as ammonia, lactic acid, acetone, butyl- and pentyl-amine as well as to complex odours emanating from incubated sweat, urine and manure (van den Broek & dan Otter, 1999; Meijerink *et al.*, 2001).

ORNs in sensilla trichodea and grooved pegs project to different glomerular areas of the antennal lobe, the first integration centre in the deuterocerebrum (Anton *et al.*, 2003; Anton & Rospars, 2004). These findings correlate with the functional differences between sensilla trichodea and grooved peg sensilla. Carbon dioxide sensitive neurons contained in sensilla on the maxillary palp also project to the antennal lobe, but to a distinctly different area (Anton *et al.*, 2003).

Behavioural studies have demonstrated that various human skin emanations or skin secretions attract female *An. gambiae* mosquitoes (Braks & Takken, 1999; Healy & Copland, 2000; Pates *et al.*, 2001b). Attraction to several single chemical components of human origin has been documented for *An. gambiae*. Ammonia alone was attractive in the olfactometer (Braks & Takken, 1999; Smallegange *et al.*, 2005). Lactic acid was found to augment the attractiveness of other kairomones. We recently demonstrated a synergistic effect among ammonia, L-lactic acid and a mixture of 12 carboxylic acids which are components of human emanations (Smallegange *et al.*, 2005). Differences in L-lactic acid concentration on the human skin may partly explain differences between human individuals in the extent to which mosquitoes are attracted (Brady *et al.*, 1997; Dekker *et al.*, 2002). Six oxocarboxylic acids were reported to stimulate landing responses of *An. gambiae* (Healy *et al.*, 2002). A component of human axillary odour, 7-octenoic acid, increased the attraction to carbon dioxide of *An. gambiae s. l.* in a field study (Costantini *et al.*, 2001).

In this paper, we describe six functional groups of sensilla trichodea and five functional groups of grooved peg sensilla. The distinction between the functional groups was based on the response spectra of the sensilla to 44 odours from diverse classes of compounds associated with the human body or with mosquito oviposition sites. We included all those pure compounds that have been documented to elicit behavioural responses from to *An. gambiae*.

Materials and methods

Insects

Anopheles gambiae mosquitoes were reared in the laboratory according to a standard protocol (Qiu *et al.*, 2004a). Female mosquitoes used were 5-8 days old and had only access to 6% glucose solution. After the legs had been removed, a female mosquito was attached to a transparent Perspex block (1.1x1.1x1.5cm) by a piece of transparent Scotch® double-sided sticky tape (3M, Leiden, The Netherlands). The wings, mouthparts and each junction between antennal segments were pressed gently against the tape to immobilize the mosquito. The antennae of the mosquito were viewed with an Olympus CK2 inverted microscope at 600x magnification. The length of sensilla trichodea C and E were measured by a calibrated graded scale placed in the ocular of the microscope. Single sensilla recordings were made from short and medium-length sensilla trichodea (subtypes C and E) and grooved peg sensilla on segments 6-13 of the antenna.

Cryo-scanning electron microscopy

Adult female mosquitoes were sedated by cooling on ice, mounted on a specimen holder using carbon adhesive tabs (EMS Washington USA) and quickly frozen in liquid nitrogen. The frozen samples were placed in a dedicated cryo-preparation chamber (Oxford Instruments CT 1500 HF, Eynsham, England). In this cryo-preparation chamber the samples were freeze dried for 3 minutes at -90°C at 1x8-4 Pa to remove water vapor contamination. After 3 minutes the samples were sputter coated with a layer of 15 nm platinum at the same temperature. The sample was cryo-transferred into the field emission scanning microscope (JEOL 6300F, Japan) on a sample stage at -190°C. The analysis was performed at a working distance of 16 mm, with SE detection at 5 kV. All images were recorded digitally (Orion, 6 E.L.I. sprl,, Charleroi Belgium) at a scan rate of 100 seconds (full frame) at a size of 2528 x 2030, 8 bit. The images were optimized and resized with Adobe Photoshop CS.

Single sensilla recording methods

Action potentials were recorded with a tungsten microelectrode (0.1 mm shaft diameter, World Precision Instruments (Berlin, Germany). The microelectrode was electrolytically sharpened to a tip diameter smaller than 1µm by repeated dipping into a saturated KNO_2 solution at a constant voltage of 4V. The recording electrode was positioned at the base of a

sensillum (dan Otter *et al.*, 1980). An electronic micromanipulator (Eppendorf Micromanipulator 5170, Eppendorf-Netheler-Hinz, Hamburg, Germany) was used to move the recording electrode to a position at which electrophysiological activity was recorded. The signals were digitized by a USB-IDAC analog-digital conversion interface (Syntech, Hilversum, The Netherlands) at a sample rate of 11,900 / sec and amplified 1,024x. Autospike software (Syntech) was used for both recording and data analysis.

The response of a neuron to a stimulus is quantified as the average action potential firing frequency in the first 0.5 sec after the onset of a 0.2 sec stimuli puff minus the average firing frequency in the six intervals of 0.5 sec preceding stimulus delivery. Action potentials from different cells were sorted visually based on (1) discrete classes observed in the amplitude histogram produced by Autospike (2) wave form (3) the occurrence of interspike intervals shorter than the refractory period (3.5 msec) (Lewicki, 1998; Meunier *et al.*, 2003). To evaluate the accuracy of our counting method, the spike numbers manually counted for responses of neurons in sensilla trichodea type E and C to indole, 4-methyl phenol and geranyl acetone were compared with those estimated by a recently proposed algorithm to calculate the activity of each single neuron in recordings in which two neurons are active, based on the "silent period" and number of "doublets" (Meunier *et al.*, 2003). The spike frequencies resulting from visual sorting were statistically similar to those estimated by the algorithm (Wilcoxon signed ranks test, $P>0.05$).

An. gambiae has proven to be a difficult insect species for electrophysiological studies due to the low signal-to-noise ratio recorded via the surface contact method employed. The amplitude of the action potentials was 0.2-0.4 mV, a value about 10 times lower than action potentials of ORNs from flies (fruit fly, house fly (Kelling *et al.*, 2002) and tsetse fly (van der Goes van Naters *et al.*, 1996)) or moths (Shields & Hildebrand, 2001). The success rate of extra-cellular recordings from the antennal neurons of this species is concomitantly low (<10%).

Odour stimulation

A charcoal-filtered and humidified air stream (40ml/sec) was passed constantly over the mosquito antenna. Test compounds were dissolved in tertyl-butyl methyl ether (TBME), except for ammonia which was dissolved in water. A piece of filter paper (1x1.5cm) with 25 µl solution of test odour was put into a Pasteur pipette; TBME was allowed to evaporate before use. Water and TBME were used as a blank control. The odour stimulus in the Pasteur pipette was injected into the main air stream using a stimulus controller (C5-01/b, Syntech); the flow of the air stream carrying the stimulus was set at 6.7 ml/sec, producing a stimulus puff of 1.3 ml, while keeping the total flow constant.

Chemicals were of the highest purity grade commercially available: most of them were 95% to >99% pure. Aqueous solutions of L(+)-lactic acid (90% aqueous solution, analytical grade) and ammonia (25% aqueous solution, analytical grade) were used. Most chemicals were purchased from Sigma-Aldrich and Fluka, except the following: L-lactic acid (Purac, Gorinchem, The Netherlands) and 3-methyl butanoic acid (Acros, 's Hertogenbosch, The Netherlands). Two compounds, 7-octenoic acid and 3-methyl-2-hexenoic acid (both >99% in purity) were kindly supplied by Dr. M. Birkett (IACR-Rothamsted, Harpenden, Hertfordshire, UK). Stimuli were tested in a random order; lower concentrations of stimuli were tested first to prevent the possible effect of adaptation at higher dosages. A 10^{-2} (v/v for liquid or w/v for solid) dilution of 44 compounds was used to screen the response spectrum of a sensillum. Test compounds were dissolved and diluted with tertyl-butyl methyl ether (TBME), except for ammonia which was diluted with water. The three oxocarboxylic tested were found in human blood and urine (Healy & Copland, 2000); 7-octenoic acid and 3-methyl-2-hexenoic acid were found in human axillary odours (Zeng *et al.*, 1996; Healy *et al.*, 2002); esters were components of odours from mosquito oviposition sites (Du & Millar, 1999); the other compounds were all found in human skin emanations (Bernier *et al.*, 2000; Meijerink *et al.*, 2000; A.M. Galimard, unpublished data). The compounds for which behavioural activity for *An. gambiae* has been documented are indicated in Table 1.

Statistical analysis

Hierarchical cluster analysis (using SPSS software package (release 11.0.1, SPSS Inc., Chicago, Illinois, USA)) was applied to classify the olfactory neurons based on their responses to a set of compounds. The neurons were clustered based on response frequencies (positive value for excitation, negative value for inhibition) to several compounds. The chosen compounds showed highest heterogeneity in responses by different neurons. For sensilla trichodea four compounds used for the cluster analysis were: indole, geranyl acetone, 4-ethyl phenol and hexanoic acid. The three compounds used for the grooved peg sensilla classification were: lactic acid, 2-oxobutanoic acid and hexanoic acid. The distances between data points were calculated according to Ward's method, individual neurons were grouped based on the distances. The optimal number of groups was related to the largest distance between clusters. The classification at the sensillum level was based on the response spectrum of the most responsive neuron of the co-compartimentalised neurons in the same sensillum.

Dose response relationships were analysed by General Linear Model, Univariate procedure (SPSS for Windows, release 10.0.5). Stimuli and concentration were set as fixed factors. Interactions between the fixed factors were excluded from the model when the interaction effect was not significant. The effect of a fixed factor was tested by the F statistic and

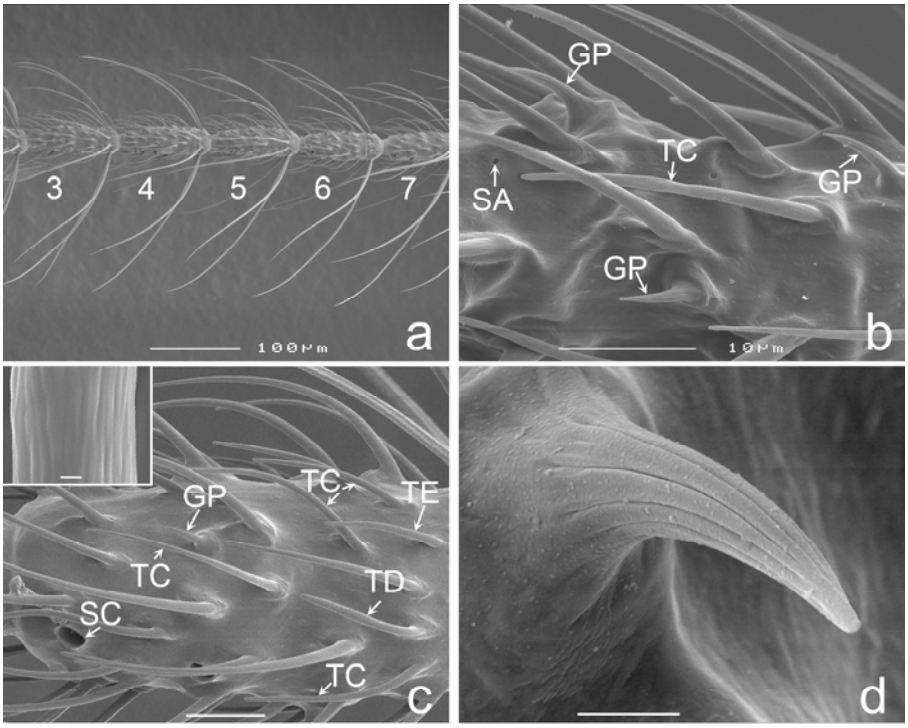

Fig.1 Scanning electron micrographs of antennal sensilla of a female mosquito of *Anopheles gambiae*. (a) the 3-7th antennomeres of a female *An. gambiae*. Bar = 100 μm. (b) detail showing grooved peg sensilla (GP), sensilla trichodea C (TC) and sensilla ampullacea. Bar = 10 μm. (c) sensilla trichodea C (TC), D (TD) and E (TE); sensilla coeloconica (SC). Bar = 10μm; Inset, wall structure of TC showing slit-like structures on the surface. Bar = 0.1μm. (d) sensilla basiconica or grooved peg sensilla (GP). Bar = 1μm.

was considered to be significant when $P<0.05$.

Results

Sensilla trichodea and grooved peg sensilla, as well as the subtypes of sensilla trichodea can be distinguished using their appearance under the light microscope (Fig. 1). Antennal sensilla trichodea E (TE) of *An. gambiae* were the shortest trichoid sensilla, having a sharp tip and a length of 16.9±1.4 μm (Fig. 1c). Sensilla trichodea C (TC) had a similar shape as TE but were slightly longer, with a mean length of 21.3±2.2 μm (Fig. 1b, c). The distinction between sensilla TE and TC was not always unambiguous; some sensilla have intermediate length and were grouped post-hoc based on their response spectra.

We recorded the electrophysiological responses of 45 sensilla trichodea and 20 grooved peg sensilla on segments 6-13 of the antennae of female *An. gambiae* to a panel of 44 odour stimuli (Table 1). The location of these sensilla is given below. Approximately 37.2 msec of the total latency (42 ± 12 msec) between the onset of the stimuli and the start of the neuron response can be attributed to the travel time of the stimulus from its point of injection into the airstream to reach the sensillum, the actual response latency being 5.8 msec. This value was similar for sensilla trichodea and grooved peg sensilla (Mann-Whitney U test, p=0.08). In most cases spontaneous activity of more than one neuron was recorded (Fig. 2). Thirteen compounds as well as the control odours did not elicit a response (as defined above) from any of the sensilla tested (Table 1).

Functional types of sensilla trichodea

Single sensillum recordings from TE and TC displayed spontaneous action potentials from two ORNs, based on differences in their amplitudes and doublets (Fig. 2a-d). We hereafter named the neuron for which we recorded a larger amplitude neuron A and the one with a smaller amplitude neuron B. The levels of spontaneous activity of neurons in TE and TC were similar, the A neuron (18.5±7.4, n=324) showing a higher spontaneous activity than B the neuron (14.0±7.3, n=324) (t test, $P<0.001$). In most cases ORNs innervating TE-sensilla responded to the tested odour stimuli by excitation (Fig. 2a-d). Inhibition of spontaneous activity was found only occasionally (Table 1).

Cluster analysis of single sensillum recording responses of ORNs in 45 sensilla trichodea to four odour stimuli resulted in one of six functional types (Fig. 3). Only one of the sensilla could not be assigned to any of the six groups (Table 1). As shown in Table 1, twenty eight out of 31 compounds elicited a response in ORNs in sensilla trichodea. The responses of ORNs to a set of 13 compounds, to which highest responses were found, are shown in Fig. 3. Two functional types were mainly associated with sensilla trichodea E (TE1 and TE2). Four functional types were mainly associated with TC and were named TC1-4.

The largest proportion of sensilla trichodea we studied belonged to TE1 (15 out of 45). One or both of the ORNs in TE1 showed excitation responses to 14 stimuli. Both TE1-neurons responded to ammonia, C5-7 carboxylic acids, 1-hexen-3-ol, 3-methyl-1-butanol, phenolics and indole (Table 1, Fig. 3a). Only TE1A responded to 2-phenoxy ethanol and 6-methyl-5-hepten-2-one by excitation and responded to C3-C4 carboxylic acids by inhibition. TE1A - neurons showed the strongest responses to 4-methyl- and 4-ethylphenol, and intermediate response intensity to 1-hexen-3-ol, indole and phenol. Ammonia was the strongest stimulant for TE1B- neurons, followed by indole and three phenolic compounds. TE1A-

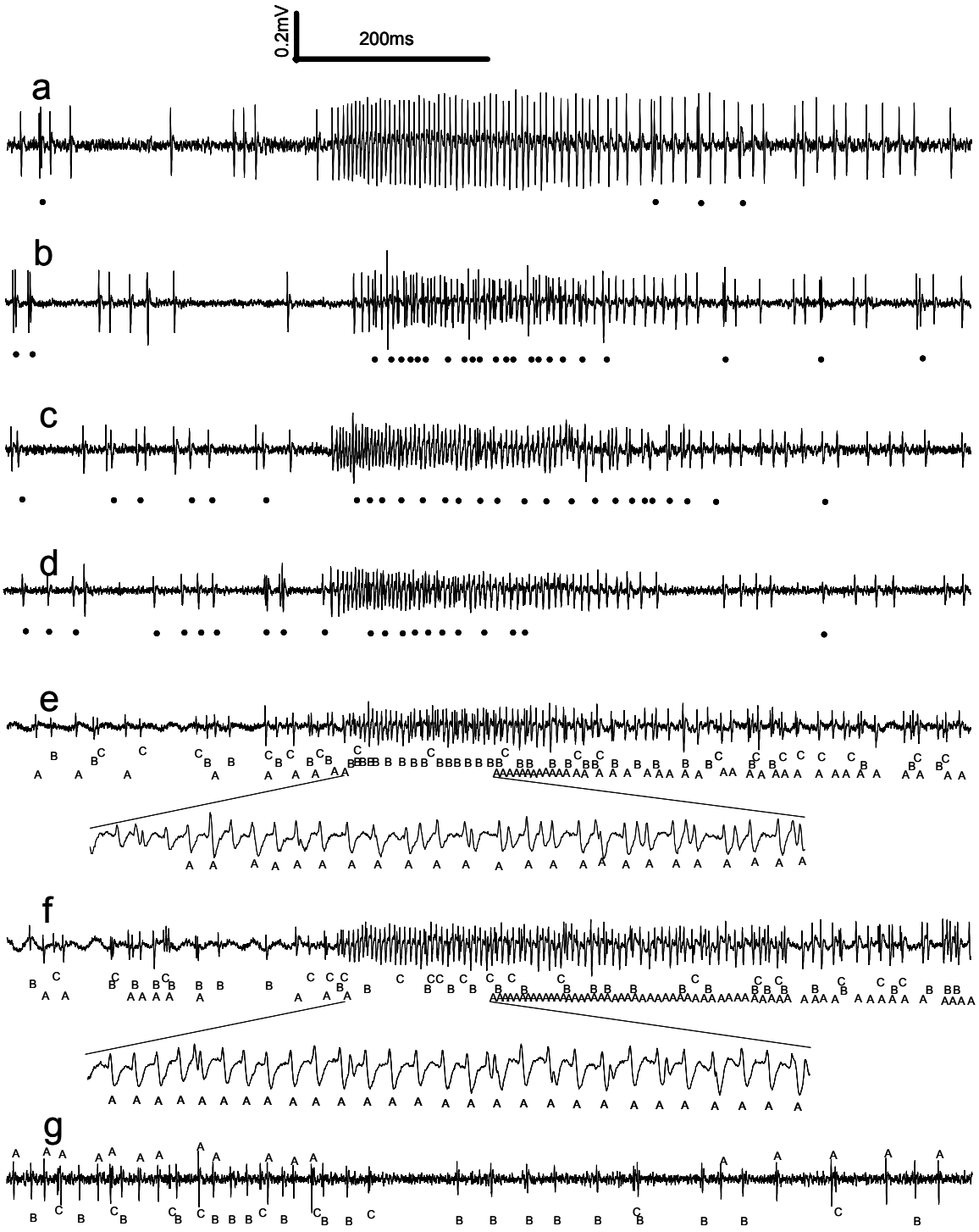

Fig. 2 Examples of action potentials from different neurons during the odour stimulation. Horizontal bar indicates the onset and end of odour delivery. Black dots in a, b, c and d indicate the spikes from neuron B, and the other spikes are from neuron A. The spikes from each neuron was marked with A, B, and C in e to g. Excitation responses include: a: TE1 to 1% 4-ethylphenol; b: TC1 to 1% 4-methylphenol; c: TE2 to 1% geranyl acetone; d: TC2 to1% geranyl acetone; e and f: GP3 to 0.25% ammonia and 1% lactic acid. Inhibition response: g: GP4 to 4-methyl butanoic acid.

Table 1 Response spectra of ORNs to 44 odour compounds (See next page for details).

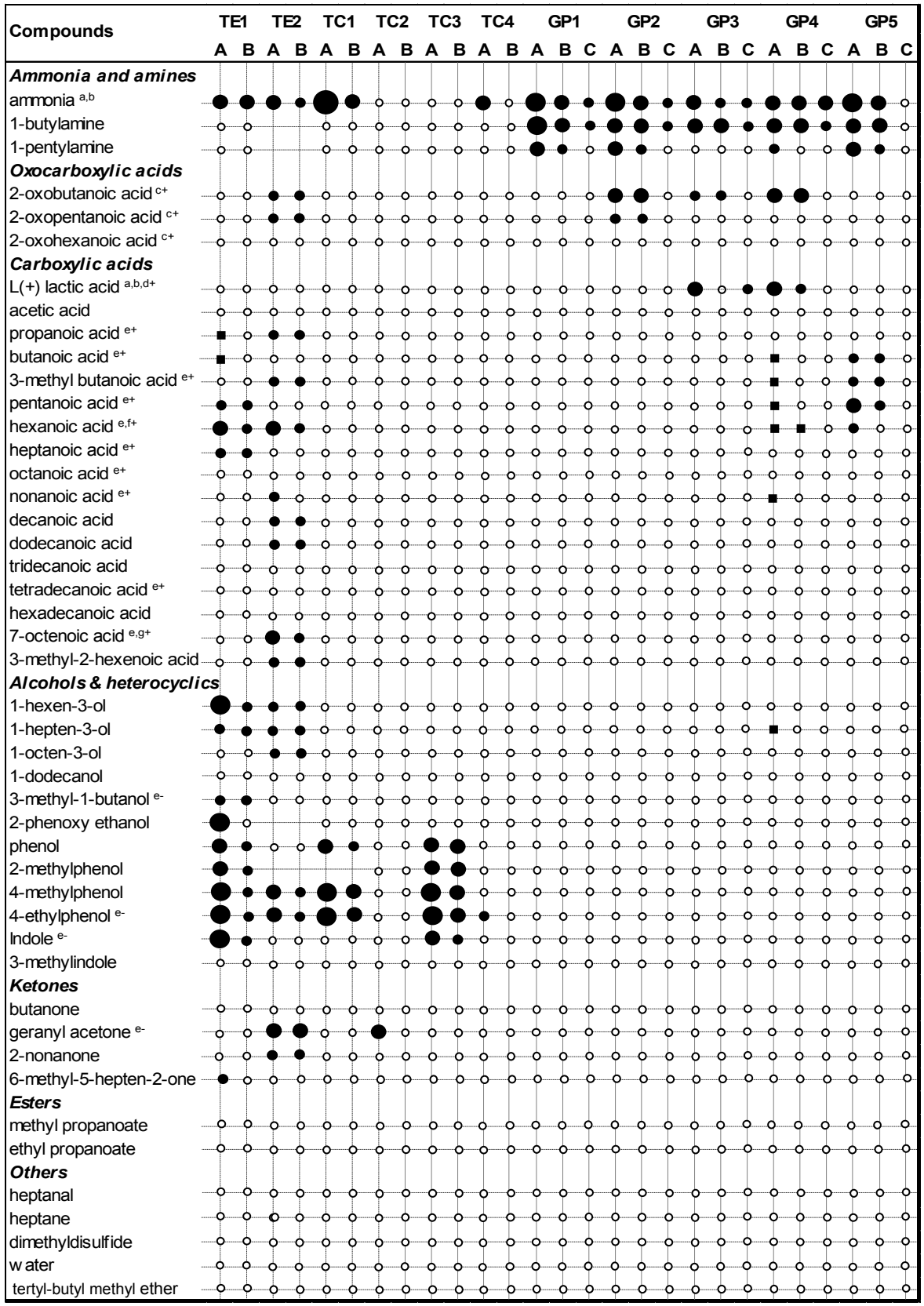

Table 1 Response spectra of each ORN class innervating sensilla trichodea E(TE), sensilla trichodea C(TC) and grooved peg sensilla (GP). The stimuli were test at 10^{-2} dilution, with exception of ammonia tested at 2.5%. An excitation responses were considered when the increase of action potential frequency was higher than 11/s, which was twice the standard deviation of the simultaneous response. The intensity of the responses were in relative to the highest response: 160/s. ○ : no response; ■ :inhibition response; ● : increase of frequency less than 20% of the highest response; ● : increase of frequency between 20-50% relative to the highest response; ● : increase of frequency between 50-80% relative to the highest response; ● : increase of frequency higher than 80% of the highest response; empty: no recording. The number of replicates for each type of sensilla were: TE1: n=15; TE2: n=4; TC1: n=10; TC2: n=6; TC3: n=4; TC4: n=5; GP1: n=8; GP2: n=6; GP3: n=3; GP4: n=2; GP5: n=1. a-g : behavioral activities found for *Anopheles gamibiae* in bioassies. a: Braks *et al*., 2001; b: Smallegange, et al. 2005; c: Healy & Copland 2002; d: Dekker *et al*., 2002; e: chapter 6; f: Smallegange *et al*., 2002; g: Costantini *et al*., 2001. +: attractive responses; -: repellent response.

neurons responded to the majority of stimuli (eight out of 14) more strongly than TE1B-neurons (t-test, $P<0.05$), both neurons responded similarly to the other six odours (paired sample t-test, $P>0.05$).

TE2 formed a less abundant functional type (4 out of 45) containing two neurons with broad response spectra. TE2-neurons were responsive to 18 of the 44 test stimuli (Table 1). TE2A responded most strongly to ammonia and geranyl acetone, whereas hexanoic acid, 4-methyl- and 4-ethylphenol, and 7-octenoic acid were weaker stimulants. TE2A also showed responses to propanoic acid, 3-methyl butanoic acid, C9-10 and C12 carboxylic acids, 3-methyl-2-hexenoic acid, 1-hexen-3-ol, 1-hepten-3-ol and 2-nonanone. TE2B-neurons showed similar response spectra with similar intensity as TE2A (paired sample t-test, $P>0.05$) except that it was not responsive to nonanoic acid and that it responded to hexanoic acid less strongly than TE2A (t-test, $P<0.05$).

Functional type TC1 was relatively common (10 out of 45). TC1A- and TC1B-neurons were responsive to phenols and ammonia; response intensities of these neurons were similar (paired sample t-test, $P>0.05$) (Table 1 and Fig. 3c). The most specific functional type we found was TC2, in which only TC2A was responsive to geranyl acetone (Table 1 and Fig. 3d). A small proportion of sensilla trichodea belong to functional type TC3 (4 out of 45). The two ORNs housed in this functional type were responsive to phenols and indole (Table 1 and Fig. 3e). Another distinct functional type was TC4, of which TC4A neurons responded to ammonia and 4-ethylphenol, TC4B was not responsive to any of the test stimuli

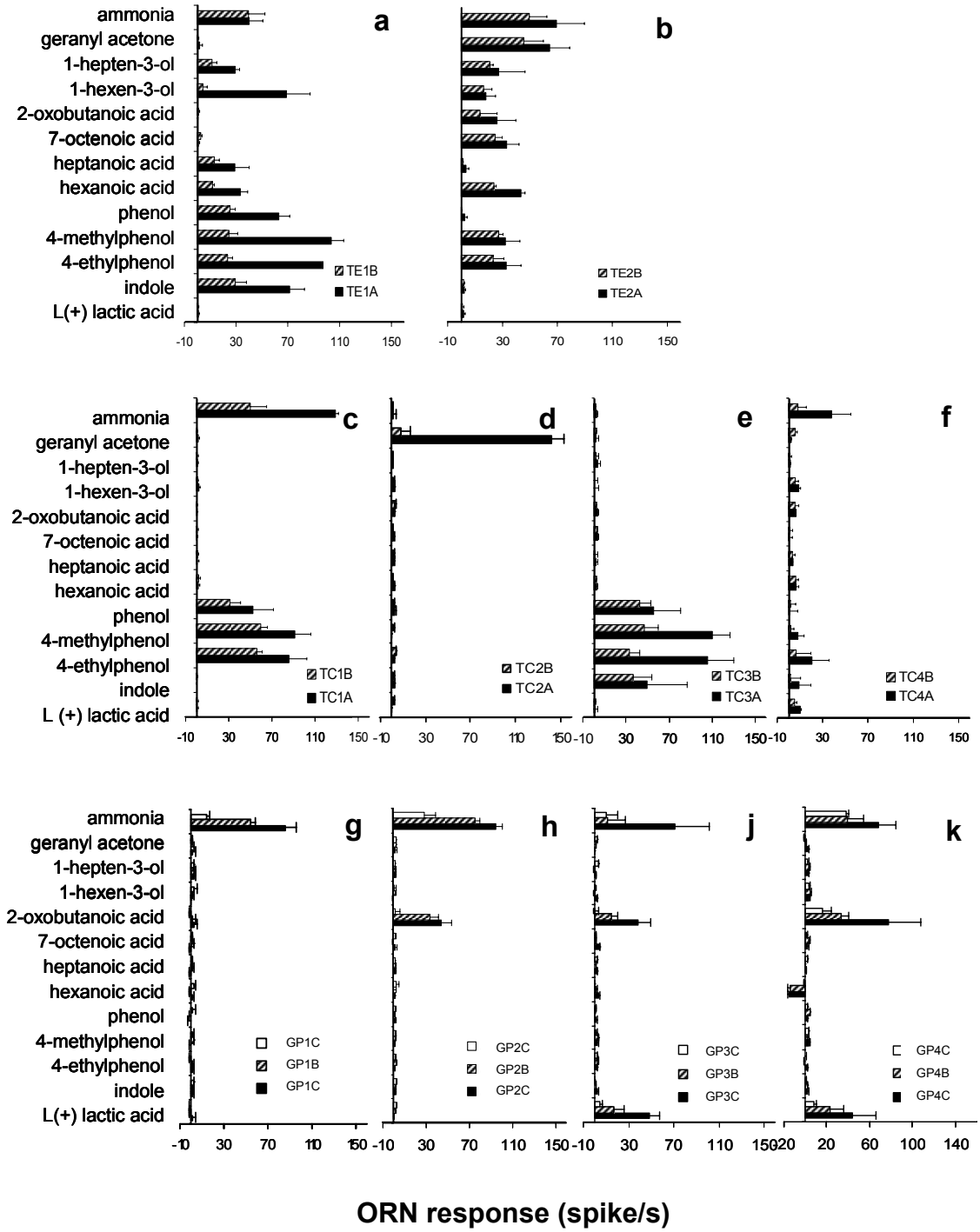

Fig. 3 Response patterns of olfactory receptor neurons innervating sensilla trichodea and grooved peg sensilla of female *Anopheles gambiae* mosquito antennae to thirteen odours at a dilution of 10^{-2} (ammonia at 2.5%) listed along the vertical axis of the bar graphs. Solid bars: neuron A, hatched bar: neuron B, empty bar: neuron C. For number of replicates for different sensilla see Table 1.

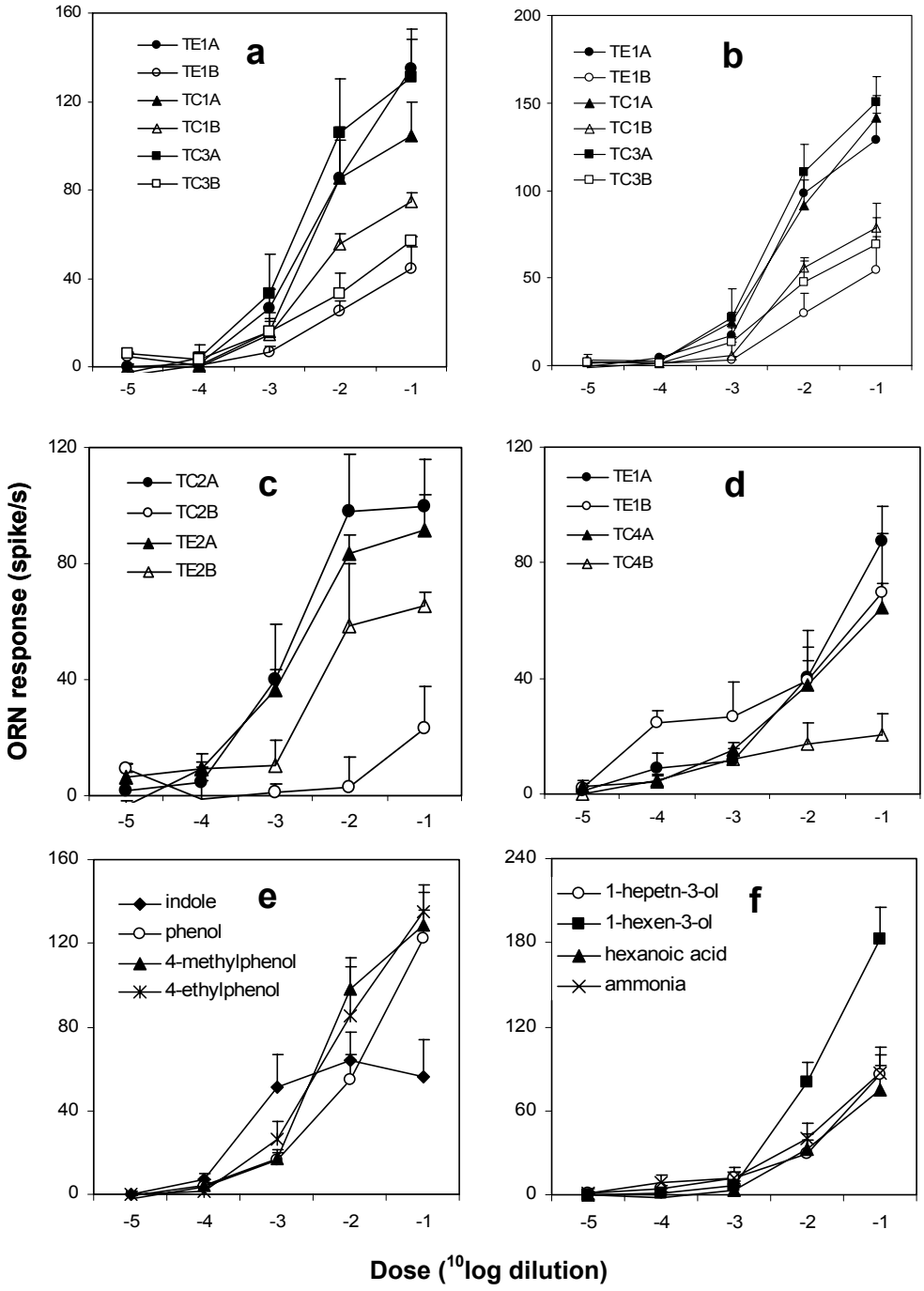

Fig. 4 Dose-response curves of olfactory receptor neurons to odour stimuli at five concentrations. X axis show the logarithm values of the dilutions. Fig. a-d showed dose- response curves of different neurons to the same odourant stimuli. a: neurons in sensilla TE1, TC1 and TC3 (n=5, 6 and 3) to 4-ethylphenol; b: neurons in sensilla TE1, TC1 and TC3 (n= 5, 6 and 3) to 4-methylphenol; c: neurons in sensilla TC2 and TE2 (n=4 and 2) to geranyl acetone; d: two neurons in sensilla TE1 (n=5) and three neurons in GP1 (n=8) to ammonia. Fig. e and f are neurons in TE1 to different odour stimuli (n=5).

(Table 1 and Fig. 3c).

Functional types of grooved peg sensilla

Single sensilla recordings from grooved peg sensilla (Fig. 1e-g) exhibited electrophysiological activity of three neurons. Neurons with large, medium and small spike amplitudes have been designated as A, B and C respectively (Fig. 2e, f). All the sensilla we recorded from contained at least one neuron that responded to ammonia and 1-butylamine. Cluster analysis revealed at least five functional types that could be identified among 19 grooved peg sensilla according to their responses to lactic acid, 2-oxobutanoic acid and hexanoic acid. The spontaneous activity of neurons in different functional types of grooved peg sensilla were similar, the A neuron exhibiting the highest (43±17, n=136), the B neuron intermediate (33±16, n=136) and the C neuron the lowest spontaneous activity (15±13, n=136) (ANOVA, $P<0.001$). Functional type GP1 appeared to be most abundant (8 out of 19). ORNs in this type responded to ammonia but not to the other three stimuli (Table 1, Fig. 3g). ORNs in functional type GP2, to which 3 out of 19 sensilla belonged, were tuned to 2-oxobutanoic acid in addition to ammonia. ORNs in functional type GP3 (6 out of 19 sensilla tested) were tuned to lactic acid in addition to ammonia and 2-oxobutanoic acid (Table 1; Fig. 3h, i). GP4- neurons (2 out of 19 sensilla tested) displayed excitation responses to ammonia, lactic acid and 2-oxobutanoic acid and were inhibited by C4-C6 and C9 carboxylic acids (Table 1; Fig. 3j). One sensillum (GP5) contained ORNs responding to C5-C6 carboxylic acids by excitation.

Dose-response relationships

The dose-response relationships of neurons innervating three functional types of sensilla trichodea TE1, TC1 and TC3 to 4-ethylphenol are shown in Fig. 4a. A-neurons in all three functional types responded more intensely to 4-ethylphenol than B-neurons did and the sensitivity of the A- or B-neurons to 4-ethylphenol in the three types was similar (GLM). The dose-response relationship for 4-methylphenol of these neurons was similar to that for 4-ethylphenol (Fig. 4b). The dose-response curves of TE2 and TC2 neurons to geranyl acetone are shown in Fig. 4c. The A-neurons in these two sensilla types had similar sensitivity to geranyl acetone, whereas TE2B showed much higher sensitivity than TC2B. Fig. 4d presents the ammonia dose-response curves of two neurons in TE1 and TC4. The dose-response relationships for ammonia recorded from TE1A, TE1B and TC4A were similar, and revealed a higher sensitivity than found in TC4B.

Fig. 4e-f illustrates dose-response curves of TE1A neurons to their most effective stimu-

lants. This neuron type had the lowest threshold to indole, but the responses saturated at concentrations higher than the 10^{-3} dilution. The responses of TE1A to the 10^{-3} dilution of phenols were lower than to indole but increased linearly with concentration (Fig. 4e). The sensitivity to 1-hexen-3-ol is clearly higher than to 1-hepten-3-ol. The dose-response curves to hexanoic acid were comparable to that of 1-hepten-3-ol (Fig. 4f).

The sensitivities of neurons innervating grooved peg sensilla to ammonia, lactic acid and

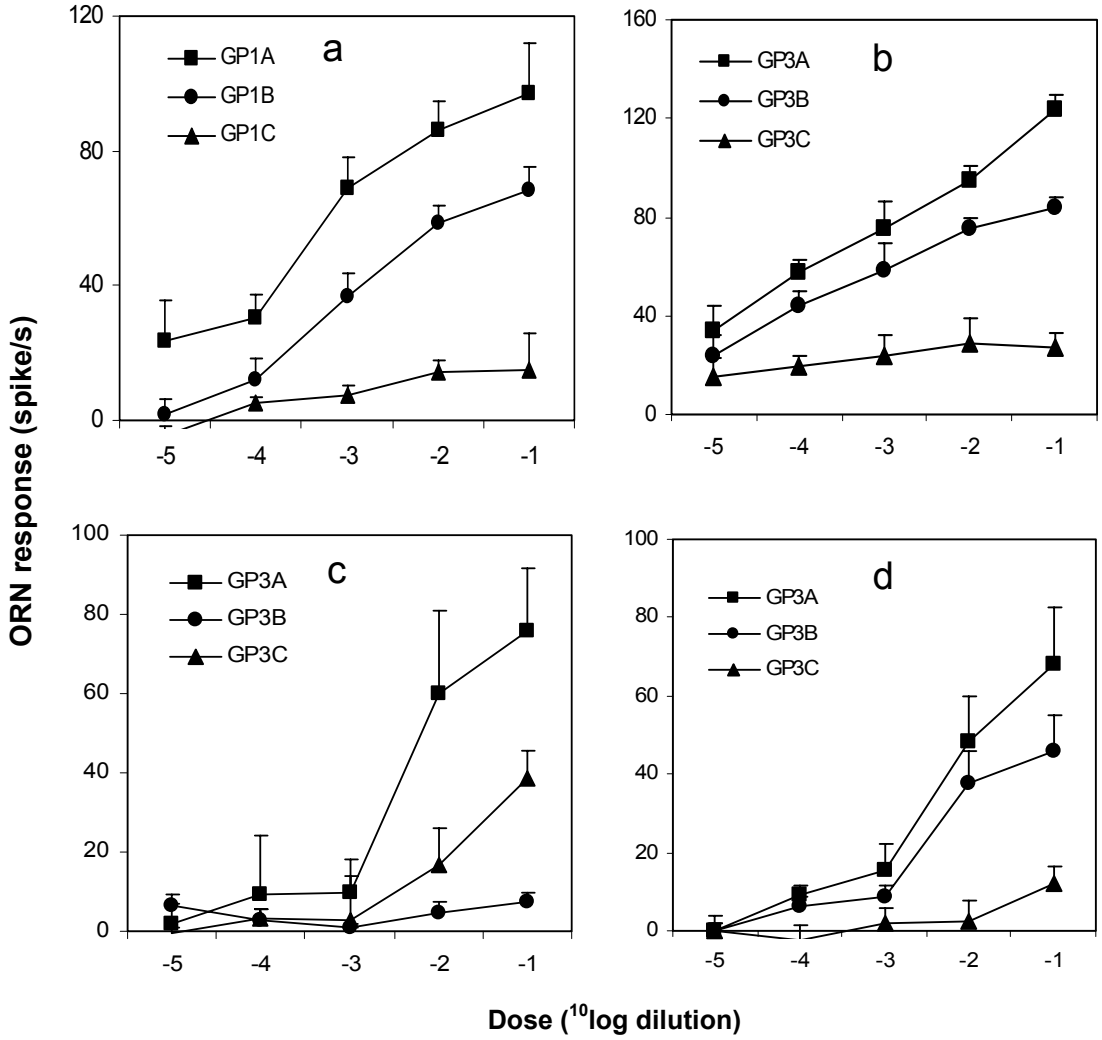

Fig. 5 Dose-response curves of ORNs in grooved peg sensilla to odour stimuli. a: GP1-neurons responding to ammonia. X axis show the logarithm values of the dilutions (for ammonia, 10^{-1} dilution shows 25%). a, b: GP1- and GP3-neurons responding to ammonia; c: GP3-neurons to L-lactic acid; d: GP3-neurons to oxobutanoic acid.

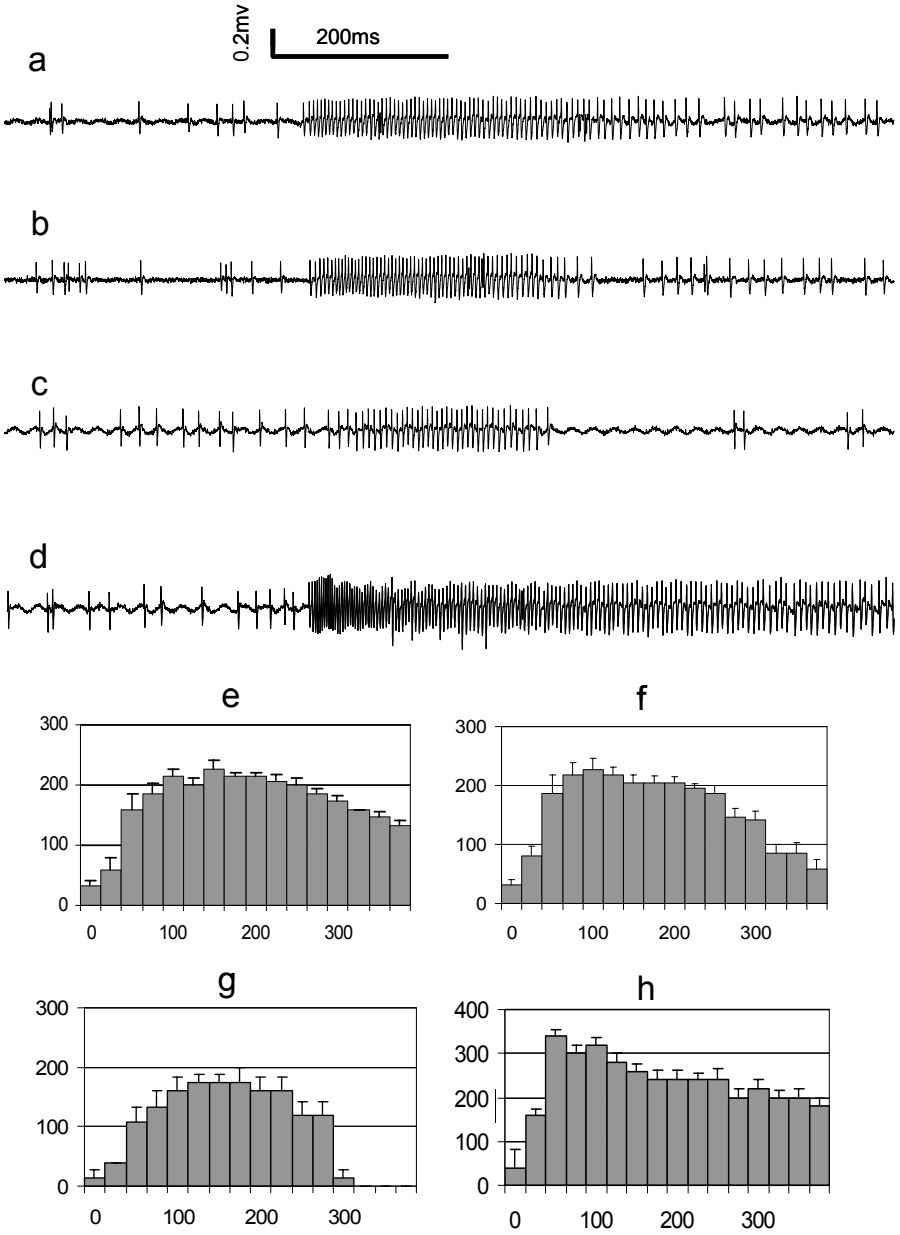

Fig. 6 Temporal characteristics of ORNs in sensilla trichidea TE1 in response to different odour stimuli. Horizontal bar indicate the on-set and end of the stimulations, vertical bar shows the scale of 0.2 mv. a: spike train of TE1 responding to 1% 4-methylphenol; and e: the histogram of the action potentials, showing a typical prolonged phasic- tonic response (n-6). b: spike train of TE1 responding to 1% indole; and f: the histogram of the action potentials, showing abrupt decrease of the firing frequency shortly after the end of the stimulus (n=9). c: spike train of TE1 responding to 1-hexen-3-ol and g: the histogram of the action potentials, showing clear phasic fashion (n=3); the response to 1-hexen-3-ol changed to a phasic- tonic fashion at a higher concentration (10%) (d and h, n=2). Gray bars in e-h are mean frequency (Hz) in intervals of 25 ms during the 400 ms after the on-set of the odour stimuli, error bars are SE of means.

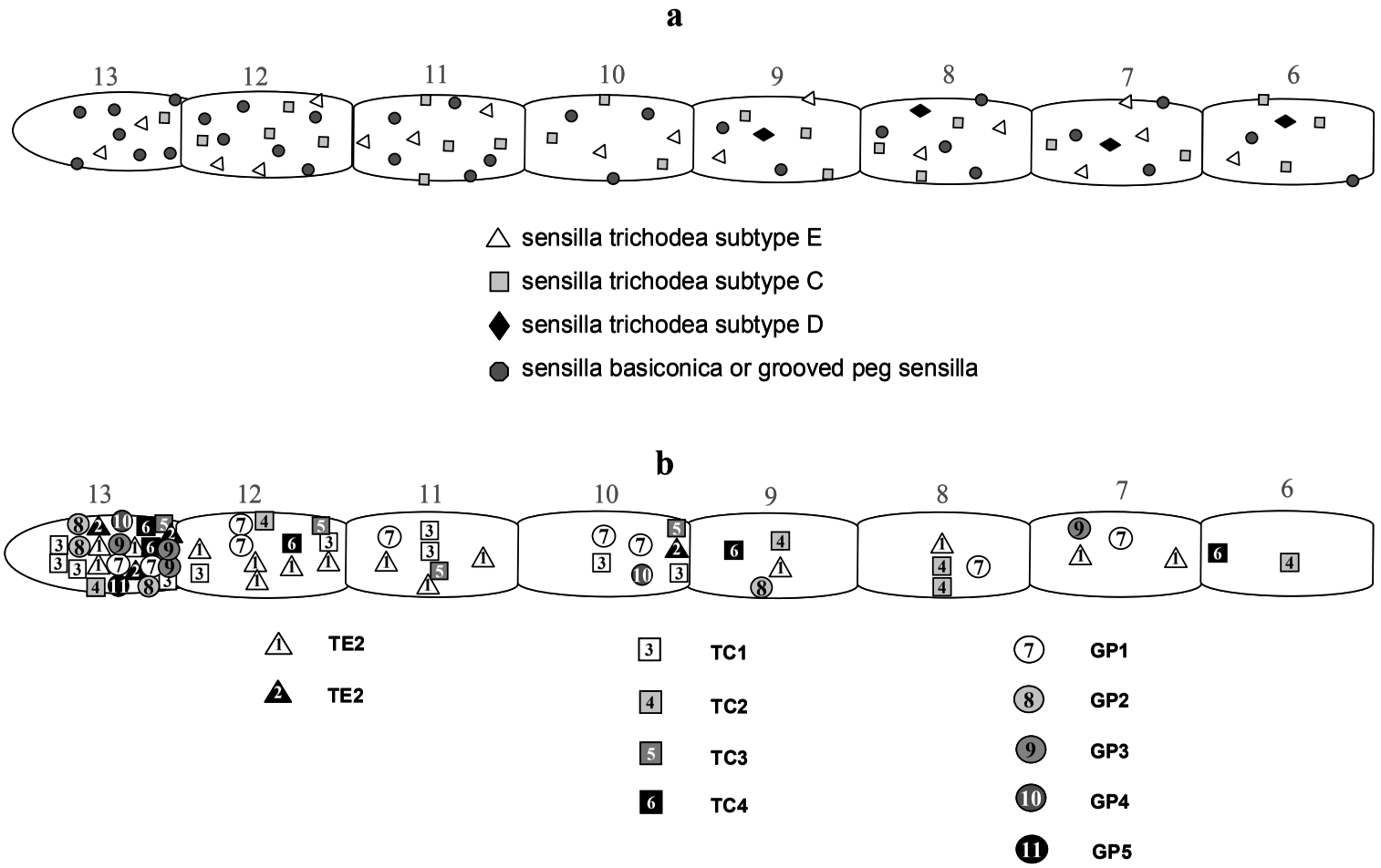

Fig. 7 a: A map of various types of olfactory sensilla located on the 6-13th segment of antennae of female *Anopheles gambiae*, showing one side of the segment. The number of each type was base on half of the average number from seven antennae observed from the dorsal side. b: a functional mapping of six types of sensilla trichodea and five types of grooved peg sensilla according to the real position when the recordings were made. The position on the longitude direction was more reliable than on the latitudinal direction because the possibly twisting of the antennae.

2-oxobutanoic acid are shown in Fig. 5. In both GP1 and GP3 sensilla, the A-neuron had the highest and the C-neuron the lowest sensitivities to ammonia among three neurons (Fig. 5a, b). For GP1A and GP3A the threshold concentration was below the 10^{-5} dilution. Neurons A and B in GP3 were responsive to lactic acid, GP3C was not responsive to any tested concentration (Fig. 5c). Neuron GP3A had similar sensitivity as GP3B to 2-oxobutanoic acid and both were higher than neuron GP3C (Fig. 5d)

Temporal aspects of ORN responses

The temporal patterns of ORN-activity depended on the odour stimulus. As shown in Fig. 6, the excitation response of TE1A showed prolonged responses to phenols (Fig. 6a, e), that persisted beyond the end of stimulus delivery. Responses of this neuron to indole lasted for a shorter period than to phenols. In contrast, the same neuron showed excitation responses to 1-hexen-3-ol (Fig. 6c, g) and 3-methyl-1-butenol (not shown) that stopped abruptly when stimulus delivery ended (post-stimulus quiescence). However, when stimulated with higher concentrations of the compounds last mentioned, a prolonged response pattern was produced (Fig. 6d, h).

A topical map of the distribution of functional sensillum types on the antennae

Approximately 30 TE-sensilla, 42 TC-sensilla and 59 GP-sensilla were located on the 6-13th segment of a female mosquito antenna (Fig. 7a). A topical map was constructed of the six functional types of sensilla trichodea and five types of grooved peg sensilla on the antennae of female mosquitoes (Fig. 7b). More than half of the recordings were made from sensilla located on the first two segments. We saw no evidence of a distinct distribution pattern of different functional sensilla-types across antennal segments.

Discussion

Odour coding and functional neuron classes

This paper provides a systematic study on the function of olfactory neurons in three types of antennal sensilla. Six functional types of sensilla trichodea and five functional types of grooved peg sensilla were identified based on their response spectra to 5 and 3 odour stimuli respectively. “Generalist” ORNs that are tuned to a broad range of odours were found in sensilla trichodea subtype E, whereas “moderate specialist” ORNs that are tuned to a narrow

range of odours were found in subtype C and grooved peg sensilla, with two "extreme specialists" in each tuned to only one odour. There was an overlap in response spectra between trichodea E and trichodea C or grooved peg sensilla, but no overlap was found between response spectra of sensilla trichodea C and grooved peg sensilla except for ammonia. Neurons co-compartmentalized in the same sensillum exhibited similar response spectra overall, but with different sensitivities.

Each organism is surrounded by numerous volatile chemicals that occur in the environment. One of the most important functions of an olfactory system is filtering, i.e. each odourant receptor neuron is only tuned to a limited number of odours that are important for survival and reproduction of the organism. In the olfactory neurons of female *An. gambiae* we found that only in 11% of the neuron-stimuli combinations studied, responses were elicited (Table 1). This proportion is comparable to the 15% found for *Drosophila* (de Bruyne *et al.*, 2001). If lower concentrations were used, ORNs would have shown a higher degree of specificity (de Bruyne *et al.*, 2001; Hallem *et al.*, 2004b). Thirteen from the 44 compounds did not elicit a response from any of the neurons examined. There is no report about the behavioural activity of these compounds to female *An. gambiae*, although these compounds were found in human odours. Due to the fact that we studied a limited sample (153) of the entire ORN-population present in the antenna (estimated to contain 1512~1596 ORNs) we cannot exclude the possibility that other ORNs that were not recorded from in this study are tuned to these compounds.

In this study we identified 6 functional types of sensilla trichodea, each containing two ORNs; and 5 functional types of grooved peg sensilla, each containing 3 ORNs. At the single neuron level, twenty two of these 27 ORNs (81%) responded to more than one ligand, five of them (19%) responded to more than 10 compounds, belonging to different chemical classes. Olfactory neurons that respond to a number of odour molecules with different chemical structures are common in insects (de Bruyne *et al.*, 1999; de Bruyne *et al.*, 2001; Shields & Hildebrand, 2001). The paradigm has been that one olfactory neuron expresses only one odourant receptor gene as a seven-transmembrane receptor molecule, until Goldman *et al.* (2005) found evidence that two genes can be co-expressed in the same ORN. By expressing single olfactory receptor genes in a mutant olfactory neuron and *in vivo* electrophysiological recording, it has been established that one odourant receptor can interact with multiple odourants (Hallem *et al.*, 2004b). Three of the 27 identified neuron types did not respond to any of the test odours suggesting that the odours to which these neurons are tuned were not included in our test set.

Some compounds caused responses in several different classes of ORNs. Overlap in response spectra is considered very important in the odour discrimination performed by the brain (Dethier, 1972; Boeckh & Ernst, 1983). This overlap appears also in recently per-

formed optical imaging studies in the antennal lobe, in which different odours may activate partly the same glomeruli but each activates a unique ensemble of glomeruli (Hansson *et al.*, 2003; Meijerink *et al.*, 2003; Sachse & Galizia, 2003). It is assumed that each neuron class sends its axons to one glomerulus. If an odour evokes activity in several glomeruli, it is expected that several ORN-classes respond**.** The differences in specificity of ORNs for compounds with similar structures are in accordance with these findings and likely enable the brain to distinguish between odours. In the *Drosophila* antennae different classes of ORNs seldom show overlap for the same "best" odour stimuli, which is in contrast to our findings in *An. gambiae* (de Bruyne *et al.*, 2001). This contrast might be explained by the contrast in food specialization. *Drosophila* is a saprophagous generalist that exploits a broad range of food sources, whereas *An. gambiae* and *Ae. aegypti* are carnivorous specialists, both preferring human blood to blood of other animals (McIver, 1968; White, 1974; Mukwaya, 1976).

Ammonia seems to present a special case, as it caused excitation responses in 21 (78%) of the ORN-types studied, and was a potent ligand (eliciting the strongest response) for most types (76%). It should be pointed out that ammonia is the most volatile among all the test compounds. However, the concentration in this study, allowing comparison with previous results (Meijerink *et al.*, 2001), was 2.5 times higher at the source than for the other compounds. This might be a reason why ammonia elicited responses in a broad range of ORNs. Yet, even at a lower concentration (0.25%), still 71% of the ORNs showed responses to ammonia, suggesting that concentration alone is not the only explanation. Davis (1976) found in another highly anthropophilic mosquito, *Ae. aegypti,* that 130 out of 136 neurons in grooved peg sensilla responded to lactic acid either by excitation (59%) or inhibition (37%). Lactic acid is one of the few compounds that on its own elicits attraction of *Ae. aegypti.* In contrast, *An. gambiae,* is attracted by ammonia on its own but not by lactic acid (Smallegange *et al.*, 2005). By having more ORNs tuned to one odour ligand, the olfactory system of the mosquito gains a higher sensitivity to this ligand, comparable to what has been described for pheromone detection by moths (Boeckh & Boeckh, 1979). As olfactory neurons converge onto a smaller number of olfactory first-order interneurons in the glomeruli of the olfactory lobe, sensitivity of detection is further enhanced. Furthermore, the fact that ORNs sensitive to ammonia in the majority of sensilla co-occur with neurons with different specificity spectra allows an accurate measurement of the ratio between ammonia and other ligands, which might contain important information on the producer of the blend (Barata *et al.*, 2002).

Our findings suggest that across-fibre patterning is a plausible odour coding mechanism operating in female *An. gambiae*: each odour was a ligand for more than one neuron and/or each neuron had more than one ligand. In other words, none of the odours was the only ligand to only one ORN. Although in some insect species indications for labelled-line olfac-

tory coding for host related odours have been found (Anton & Hansson, 1995; Roche-King *et al.*, 2000), across-fibre patterning is considered to be most common (Dethier, 1976; Shepherd, 1985). An advantage of across-fibre coding is that a large number of odours can be encoded by a small number of neurons.

Two of the phenols, 4-ethylphenol and 4-methylphenol were potent ligands for 4 (15%) ORNs in sensilla trichodea. Neurons of *Drosophila* engineered with the odour receptor gene AgOr1, which was only found in the antennae of female *An. gambiae* mosquitoes and was down-regulated after a blood meal (Fox *et al.*, 2001) was specifically responsive to 4-methylphenol (Hallem *et al.*, 2004a). The AgOr2 gene was shown to confer a specific response to 2-methylphenol. The responses of the phenol-sensitive ORNs of *An. gambiae* might thus be due to the involvement of AgOr1 or AgOr2. However, we found that ORNs that were tuned to phenols were always responsive to all the phenols tested, although in a different degree depending on the length and position of the aliphatic side group. This is in disagreement with the high specificity as found by Hallem *et al* (Hallem *et al.*, 2004a). One reason for this apparent difference might be that the specificity of certain odourant binding proteins (OBPs) that occur in the receptor lymph and transport and deliver odour molecules to the receptor molecule in the neuronal membrane had different specificity in *Drosophila* compared to analogous OBPs in *An. gambiae* mosquitoes.

Grooved peg sensilla had response spectra distinctly different from sensilla trichodea. These two types of sensilla differ fundamentally in their cuticular wall structure (Steinbrecht, 1997). Our results support the hypothesis that neurons in grooved peg sensilla were tuned to the most polar compounds (Table 1). The difference we found in response spectra between these two types of sensilla is in line with the fact that neurons from sensilla trichodea and grooved peg sensilla project each into two distinct non-overlapping zones in the antennal lobe (Anton & Hasson, 1994). However, both grooved peg sensilla and sensilla trichodea contain neurons tuned to ammonia, oxocarboxylic acids, short-chain carboxylic acids and L-lactid acid. The neurons in grooved peg sensilla had higher sensitivities to ammonia than neurons in sensilla trichodea, as has been reported previously (Meijerink *et al.*, 2000). We found ORNs that responded to some odours by excitation while to other odours by inhibition (Table 1). Meijerink and van Loon (1999) reported inhibition-type responses to carboxylic acids of neurons innervating sensilla trichodea. Different response modes of olfactory neurons adds another degree of freedom for odour coding (de Bruyne *et al.*, 2001).

Coding of intensity

In accordance with previously performed single sensillum recordings in *An. gambiae*, our results show that the neurons co-compartmentalized in the same sensilla often had similar

response spectra (van den Broek & dan Otter, 1999; Meijerink *et al.*, 2001). Similar specificity but different sensitivity (Fig. 4a-d) might help the mosquito to increase the resolution of concentrations encountered in flight. Although one ORN can be tuned to more than one ligand, its sensitivity to these ligands differed. As shown in Fig. 4 e-f, the shape of the dose-response curves for neuron TE1A in response to ligands from different chemical classes was different. The shapes of the curves differed between indole, phenols and alcohols, whereas the curve for 1-hepten-3-ol had a shape similar to that for hexanoic acid and ammonia. Using behavioural bioassays we demonstrated that *An. gambiae* distinguishes ammonia from a mixture of ammonia and hexanoic acid (Smallegange *et al.*, 2002; Chapter 6). This can only be achieved using at least one additional functional neuron type. There was a clear shift to higher sensitivity of TE1A among the phenols: the ones with the aliphatic group at position 4, 4-ethyl- and 4-methylphenol were the best ligand to TE1A, whereas 2-methylphenol and phenol were less effective stimuli to this neuron (Table 1). Among the alcohols, 1-hexen-3-ol was a better ligand than 1-hepten-3-ol, 1-octen-3-ol and 3-methyl-1-butanol to TE1A. Several structure-activity studies at the ORN level employing series of chemical analogs are available in haematophagous insects (Davis, 1988; dan Otter & van der Goes van Naters, 1993) demonstrating that the stereochemical configuration rather than the vapour pressure determine whether a chemical is a suitable ligand of a receptor neuron.

Temporal coding

Concerning temporal features, two types of excitation responses were observed: phasic and phasic-tonic. The former shows an abrupt increase and end of firing frequency; the latter an abrupt increase but with a longer lasting increased firing frequency compared to the spontaneous activity. We observed that one ORN may respond to different odourants at a certain concentration with different temporal characteristics. For example TE1A responded to 1-hexen-3-ol, 1-hepten-3-ol, 3-methyl-1-butanol and indole with a phasic type or moderate phasic-tonic pattern, whereas its responses to phenols were phasic-tonic with a longer lasting train of higher frequency firing. Temporal characteristics of action potentials provide an extra dimension in the coding of odourant stimuli. Olfactory neurons responding to different odour stimuli with different temporal characteristics were reported in *Drosophila* by de Bruyne *et al.* (2001). When the concentration of a stimulus to which a neuron responded in a phasic fashion gets high enough, the response characteristics can change into phasic-tonic. Usually a behavioural attractant becomes repellent when the concentration is above a certain threshold. There is evidence that temporal characteristics of ORN responses provide the brain with information of odour plume structure; and bursts of action potentials result into upwind flight (Kaissling, 1986; Baker *et al.*, 1988; Baker, 1990). The shift of olfactory neu-

ron response from tonic to phasic might correlate with the change of the behavioural response from attraction to avoidance.

ORN activity and behavioural responses

Fourteen of the 44 compounds that were tested in the present study were found to elicit attraction form *An. gambiae* (Table 1)(Takken *et al.*, 1997a; Braks *et al.*, 2001; Costantini *et al.*, 2001; Healy *et al.*, 2002; Smallegange *et al.*, 2002; Smallegange *et al.*, 2005; Chapter 6). Olfactory neurons tuned to 12 out of the 14 compounds were found in the sensilla types we investigated. The correlations between the response characteristics of single ORNs documented here and behavioural responses suggest that both grooved peg sensilla and sensilla trichodea are involved in odour coding that underlies host seeking behaviour.

We found several types of phenol-sensitive ORNs in sensilla trichodea. Because gene expression of olfactory receptors for 4-methylphenol was found to be down- regulated after blood feeding, it is likely that this compound is important in host seeking behaviour (Fox *et al.*, 2001; Hallem *et al.*, 2004a). Although several phenols were detected in human sweat or skin emanations (Cork & Park, 1996; Bernier *et al.*, 2000) these compounds have been considered animal-associated odours and are used by zoophilic insect species for host location (Bursell *et al.*, 1988; Vale *et al.*, 1988; Warnes, 1990). Anthropophilic insect species may use phenols as indicators of non-host animal odour and display an avoidance response. Compounds that elicit strong responses from ORNs in *An. gambiae* such as 4-ethylphenol, 4-methylphenol, 2-phenoxy ethanol, geranyl acetone, indole and 1-hexen-3-ol, are candidate compounds affecting the behaviour of this mosquito either as attractant, repellent or as synergist. Repellence effects were found for 4-ethylphenol, geranyl acetone and indole in a behavioural bioassay (Chapter 6).

We recently demonstrated a synergistic effect among ammonia, lactic acid and a mixture of 12 carboxylic acids as attractants for *An. gambiae* females (Smallegange *et al.*, 2005). Apparently information about a complex odour blend signalling human presence to the mosquito is integrated in the olfactory system, either peripherally or centrally. Synergistic effects of sex pheromone and plant odours were found at the peripheral level in *Helicoverpa zea* (Dickens *et al.*, 1993; Ochieng *et al.*, 2002; Said *et al.*, 2005). How odour mixtures affect the responses of the olfactory neurons of *An. gambiae* and whether synergistic effects occur either at the peripheral level or centrally or both, needs further investigation.

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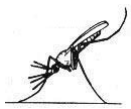
9

Influence of feeding status on responsiveness of olfactory neurons in the antennae of *Anopheles gambiae*



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Abstract

The human malaria mosquito A*nopheles gambiae sensu stricto* is a highly anthropophilic nocturnal insect, and its host- and oviposition site-seeking behaviour are mediated mainly by olfaction. The olfactory sensilla trichodea and grooved peg sensilla on the antennae contain neurons that are sensitive to putative kairomones of this species. After a blood meal, large female mosquitoes experience a stationary period for about 48 h. Using a single sensillum recording method, we compared the odour processing in the E type of sensilla trichodea and grooved peg sensilla 2-24 h post blood meal (pbm) with that before the blood meal (bbm). Three in stead of two functional types of sensilla trichodea E were found pbm. The response spectra and intensities of neurons in sensilla pTE1 pbm were similar to those in TE1 bbm, although the sensitivities to ammonia, short-chain carboxylic acids and certain alcohols were lower in pTE1. The response spectra of neuron pTE2A pbm showed a specific response to geranyl acetone, whereas pTE2B expressed similar response spectra as TE2A and TE2B. Interestingly, a functional type pTE3 that was never registered bbm was found repeatedly pbm. The most responsive neuron pTE3A showed higher sensitivity to in-

dole, an odour associated with mosquito oviposition sites, than neuron TE1A and TE1B bbm. Neuron pTE3A was also highly responsive to C6-9 carboxylic acids and moderately responsive to mosquito oviposition stimulant 3-methylindole and human specific odour compounds 7-octenoic acid and 3-methyl-hexenoic acid. These results indicate that changes in sensitivity and response profile of ORNs as a result of a blood meal are involved in modulating behaviour of *An. gambiae* females.

Introduction

Anopheles gambiae is the main vector of human malaria in sub-Saharan Africa. The species is highly anthropophilic and strongly associated with human dwellings. Being nocturnal, the behaviour of *An. gambiae* is predominantly mediated by olfaction (Takken, 1991; Takken & Knols, 1999).

The life of a female mosquito consists of several distinct activities during most if not all of which olfaction plays a crucial role. Both male and female mosquitoes feed on plant nectars which provide energy for survival and adult activities (Healy & Jepson, 1988; Foster, 1995). Odours produced by plants attract mosquitoes to these extra food sources (Foster & Takken, 2004). Females of *An. gambiae* preferred honey odour to human odour during the first days of adulthood, whereas the preference was reversed when they were more than 5 days old.

Female *An. gambiae* are guided to the human blood host predominantly by chemical cues discharged from the human body (Takken, 1991; Costantini *et al.*, 1996b). Human emanations or human secretions have been found attractive to *An. gambiae* (Braks & Takken, 1999; Healy & Copland, 2000; Pates *et al.*, 2001b; Qiu *et al.*, 2004a). Attraction of *An. gambiae* to several single chemical components of human origin has been documented. For example, carbon dioxide, which is exhaled by all vertebrates, is considered to contribute partially to the attraction of humans to *An. gambiae* (Snow, 1970; Healy & Copland, 1995; Costantini *et al.*, 1996b; Mboera & Takken, 1997; Dekker *et al.*, 2001). Besides, CO_2 has been demonstrated to account for the inter-individual differences between humans (Brady *et al.*, 1997; Mukabana *et al.*, 2004b). Similarly, difference in L-lactic acid concentration on the human skin was found to be correlated with the differences between human individuals in the extent to which mosquitoes are attracted (Dekker *et al.*, 2002). Healy *et al.* (2002) reported that six oxocarboxylic acids stimulated landing responses of *An. gambiae*. A field study in Burkina Faso demonstrated that addition of the human-specific 7-octenoic acid to carbon dioxide attracted a greater number of *An. gambiae s. l.* to traps (Costantini *et al.*, 2001).

The antennae of *An. gambiae* females bear two types of major olfactory sensilla: sensilla trichodea and grooved peg sensilla. Based on the response spectra of neurons housed in olfactory sensilla to odour stimuli, distinct functional groups of sensilla can be identified (Chapter 8). Our previous studies suggest that sensilla trichodea subtypes E and C (Boo, 1980), housing two olfactory receptor neurons (ORN) each, include two and four functional types respectively. Five functional types of grooved peg sensilla were distinguished (Chapter 8). The E-type of sensilla trichodea had broader response spectra than the C type. The majority of ORNs in either sensilla trichodea or grooved peg sensilla were sensitive to ammonia. ORNs in sensilla trichodea were sensitive to phenols, indoles, carboxylic acids, alcohols and ketones, whereas ORNs in grooved peg sensilla were especially sensitive to ammonia, amines, oxocarboxylic acids, lactic acid and headspace of incubated human sweat (van den Broek & dan Otter, 1999; Meijerink *et al.*, 2001; Chapter 8).

Female mosquitoes need to take blood meals for egg development. Blood feeding of *An. gambiae* takes place during the night. For mosquitoes having a large body size, one blood meal is sufficient to complete egg development within one gonotrophic cycle, but for small-sized mosquitoes a second blood meal is often needed (Takken *et al.*, 1998). Takken *et al*. (2001) found that host-seeking behaviour of large-sized mosquitoes was suppressed 3-24 h post blood meal (pbm) and restored at 48 h pbm. It is possible that inhibition of behavioural responses to odour cues from hosts at this stage is associated with oviposition behaviour. Recently it was demonstrated that *An. gambiae* was attracted to oviposition sites by odour cues (Sumba *et al.*, 2004; Rejmankova *et al.*, 2005). Davis *et al.* (1984) reported that the sensitivity of an olfactory neuron sensitive to L-lactic acid, an attractant for *Aedes aegypti*, decreased after a blood meal, suggesting the involvement of the peripheral nervous system in the modulation of host-seeking behaviour of this species. More than 79 putative olfactory receptor genes (OR genes) have been identified in *An. gambiae* (Hill *et al.*, 2002). One of the odour receptor genes in *An. gambiae*, *AgOr*1, was expressed specifically in olfactory tissue and was down-regulated after a blood meal, and therefore was considered to be involved in host-seeking behaviour. When *AgOr*1 was expressed in a *Drosophila* olfactory neuron from which endogenous OR genes were deleted, the neuron was specifically responsive to 4-methylphenol (Hallem *et al.*, 2004a), a component of human and animal odours (Kyorku *et al.*, 1990; Cork & Park, 1996), and a mosquito oviposition stimulant (Bentley *et al.*, 1979).

The aim of the present study is to investigate the effect of the feeding status on the sensitivity of olfactory receptor neurons to human-, nectar- and oviposition site-related odours in order to understand the regulation of the sensitivity of the peripheral nervous system on the switching between host- and oviposition site-seeking behaviour. We compared the response

spectra and sensitivities of ORNs innervating sensilla trichodea E to various odour stimuli before and after a blood meal.

Materials and methods

Mosquitoes

The *An. gambiae s.s.* colony originated from Liberia and has been reared on blood from a human arm since 1988. The mosquito culture was reared in the laboratory along standard methods (Qiu *et al.*, 2004a). To make sure that mosquitoes were not responsive to host odours in the first 24 hrs post blood meal (pbm), only mosquitoes of a large body size were used (Takken *et al.*, 2001). Large mosquitoes were obtained by using the rearing protocol described by Takken *et al.* (1998). In the first 5 days, the larvae were fed with 0.2 mg baby fish food (Tetramin, Tetra Werke, Melle, Germany) per larva; thereafter they obtained 0.3 mg/larva. The density of the larvae was kept at 1 larva/25 ml. Each day the emerged adults were kept separately in gauze cages and had access to a 6% sucrose solution. Five to eight-days old female mosquitoes were allowed to take a full blood meal, indicated by droplets appearing at the end of the abdomen, from a human arm. Adults that did not take a blood meal were removed from the cage. The blood-fed mosquitoes were kept in the climate room under the same conditions as the mosquito colony before they were used. Single sensilla recordings were made from mosquitoes 2-24 h pbm. Unfed females of similar age were used as controls.

Single sensilla recording methods

A female mosquito, with legs removed, was stuck to a transparent Perspex block (1.1x1.1x1.5cm) with a piece of transparent Scotch® double-sided sticky tape (3M, Leiden, The Netherlands). The wings, mouthparts and each junction between antennal segments were pressed gently against the tape to immobilize the mosquito. The antennae of the mosquito were viewed with an Olympus CK2 inverted microscope at 600 x magnification. Recordings were made from short and medium-length sensilla trichodea subtypes (E and C) on segments 6-13 of the antenna.

Action potentials were recorded with a tungsten microelectrode (0.1 mm shaft diameter, World Precision Instruments, Harry Fein, Berlin, Germany). The microelectrode was electrolytically sharpened to a tip diameter smaller than 1 µm by repeated dipping into a saturated KNO_2 solution. The recording electrode was positioned at the base of a sensillum. An electronic micromanipulator (Eppendorf Micromanipulator 5170, Eppendorf-Netheler-Hinz,

Hamburg, Germany) was used to move the recording electrode to a position at which electrophysiological activity was recorded. The signals were digitized by a USB-IDAC analog-digital conversion interface (Syntech, Hilversum, The Netherlands) at a sample rate of 11,900/sec and amplified 1024x. Autospike software (Syntech) was used for recording and data analysis.

The response of a neuron to a stimulus was quantified as the mean action potential firing frequency during the first 0.5 sec after the onset of a 0.2 sec stimuli puff minus the average firing frequency in the 0.5 sec previous to the onset of a stimulus. Action potentials from different cells were sorted and counted based on the amplitude histogram of Autospike and the occurrence of interspike intervals shorter than the refractory period (3.5 msec) (Lewicki, 1998; Meunier *et al.*, 2003).

Odour stimulation

A charcoal-filtered and humidified air stream (40 ml/sec) passed constantly over the mosquito antenna. Test compounds were dissolved and diluted with tertyl-butyl-methyl ether (TBME), except for ammonia which was diluted with water. A piece of filter paper (1x1.5 cm) with 25 µl solution of a test odour was put into a Pasteur pipette; TBME was allowed to evaporate before use. The odour stimulus in the Pasteur pipette was injected into the main stream using a stimulus controller (C5-01/b, Syntech); the flow was set at 500 ml/min, producing a stimulus puff of 0.2 s x 6.7 ml/sec = 1.3 ml.

Chemicals were of the highest purity grade commercially available: most of them were 95% to >99% in purity. For L-lactic acid and ammonia, 90% and 25% aqueous solution (analytical grade) were used respectively. Most chemicals were purchased from Sigma, Sigma-Aldrich and Fluka, except the following: L-lactic acid (Purac, Gorinchem, The Netherlands) and 3-methyl butanoic acid (Acros,'s Hertogenbosch, The Netherlands). Two compounds, 7-octenoic acid and 3-methyl-2-hexenoic acid (both >99% in purity) were kindly supplied by Dr. Mike Birkett (IACR-Rothamsted, Harpenden, Hertfordshire, UK). Stimuli were tested in a random order; lower concentrations of a stimulus were tested first to prevent the possible effect of adaptation at higher dosages. A 10^{-2} (v/v for liquid or w/v for solid) dilution was used to screen the response spectra of a sensillum. A panel of 44 compounds was composed based on their reported occurrence in human skin emanations or volatiles reported to be present at mosquito oviposition sites (Zeng *et al.*, 1996; Du & Millar, 1999; Bernier *et al.*, 2000; Healy & Copland, 2000; Braks *et al.*, 2001; A. Galimard, unpublished data).

Table 1 Response spectra of each ORNs innervating sensilla trichodea E(TE) after a blood meal. The stimuli were test at a 10^{-2} dilution. An excitation response were considered when the increase of action potential frequency was higher than 11/s, which was twice the standard deviation of the simultaneous response. The intensity of the responses were in relative to the highest response: 160/s. O: no response; +: increase of frequency less than 20%; ++: increase of frequency between 20-50% ; +++: increase of frequency between 50-80% ; ++++increase of frequency higher than 80%; empty: no recording. The number of replicates for each type of sensilla were: pTE1: n=8; pTE2: n=4; pTE3: n=4; TE1: n=15; TE2: n=4.

Compounds	pTE1		pTE2		pTE3		TE1		TE2	
	A	B	A	B	A	B	A	B	A	B
Ammonia and amines										
ammonia	++	o	+	o	+	o	++	++	++	+
1-butylamine					o	o	o	o		
1-pentylamine					++	-	o	o		
Oxocarboxylic acids										
2-oxobutanoic acid	o	o	o	o	o	o	o	o	+	+
2-oxopentanoic acid	o	o	o	o	o	o	o	o	+	+
2-oxohexanoic acid	o	o			o	o	o	o	o	o
Carboxylic acids										
L(+) lactic acid	o	o	o	o	o	o	o	o	o	o
acetic acid	o	o	o	o	o	o	o	o	o	o
propanoic acid	o	o	o	o	o	o	-	o	o	+
butanoic acid	o	o	o	o	o	o	-	o	o	o
3-methyl butanoic acid	o	o	o	o	+	o	o	o	+	+
pentanoic acid	+	o	o	o	+	o	+	+	o	o
hexanoic acid	++	o	o	+	++	+	++	+	++	+
heptanoic acid	+	o	o	o	++	o	+	+	o	o
octanoic acid	o	o	o	o	+++	o	o	o	o	o
nonanoic acid	o	o	o	o	++	o	o	o	+	o
decanoic acid	o	o	o	o	o	o	o	o	+	o
dodecanoic acid	o	o	o	o	o	o	o	o	+	+
tridecanoic acid	o	o			o	o	o	o	o	o
tetradecanoic acid	o	o	o	o	o	o	o	o	o	o
hexadecanoic acid	o	o	o	o	o	o	o	o	o	o
7-octenoic acid	o	o	o	+	++	o	o	o	++	+
3-methyl-2-hexenoic acid	+	o	o	o	+	o	o	o	+	+
Alcohols & heterocyclics										
1-hexen-3-ol	+++	o	o	+	o	o	++	+	++	+
1-hepten-3-ol	o	o	o	o	o	o	+	o	+	+
1-octen-3-ol	o	o	o	o	o	o	o	o	+	o
3-methyl-1-butanol	o	o	o	o	o	o	+	+	o	o
1-dodecanol	o	o	o	o	o	o	o	o	o	o
2-phenoxy ethanol	+++	o	o	o	o	o	+++	o		
phenol	+++	+	o	+	+	+	++	+	o	o
2-methylphenol	+++	+			++	+	++	+		
4-methylphenol	++++	+	o	++	+	o	+++	+	++	+
4-ethylphenol	++++	+	o	++	+	o	+++	+	++	+
Indole	+++	+	o	o	+++	o	++	+	o	o
3-methylindole	+	o	-	o	+	+	o	o	o	o
Ketones										
geranyl acetone	o	o	+++	+	o	o	o	o	++	++
butanone	o	o	o	-	o	o	o	o	o	o
2-nonanone	o	o	o	-	o	o	o	o	+	+
6-methyl-5-hepten-2-one	+	o	o	o	o	o	+	o	o	o
Esters										
methyl propanoate	o	o	o	o	o	o	o	o	o	o
ethyl propanoate	o	o	o	o	o	o	o	o	o	o
Others										
dimethyldisulfide	o	o	o	o	o	o	o	o	o	o
heptane	o	o	o	o	o	o	o	o	o	o
heptanal	o	o	o	o	+	o	o	o	o	o
water	o	o	o	o	o	o	o	o	o	o
tertyl-butyl methyl ether	o	o	o	o	o	o	o	o	o	o

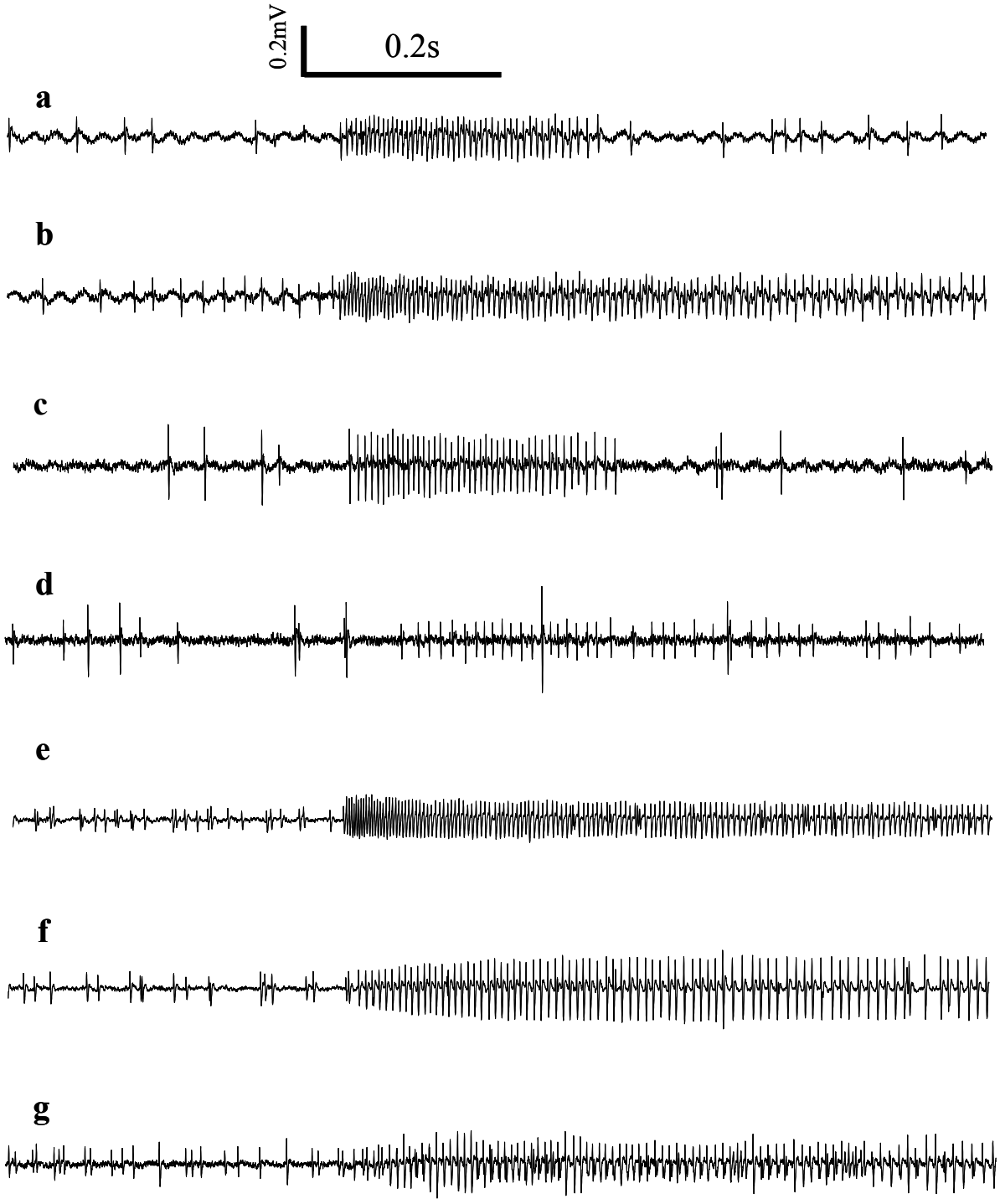

Fig. 1 Examples of action potentials from different neurons during odour stimulation. Horizontal bar indicates the onset and end of odour delivery. Excitation response of a: pTE1A to 1% indole; b: pTE1A to 1% 4-ethylphenol; c: pTE2A to 1% geranyl acetone; d: pTE2B to phenol; e: pTE3A to 0.1% indole; f: pTE3A to heptanoic acid; g: pGP1A to 2.5% ammonia.

Statistical analysis

Hierarchical cluster analysis was applied to classify the olfactory neurons based on their responses to a set of compounds by using SPSS software package (release 11.0.1, SPSS Inc., Chicago, Illinois, USA). The neurons were clustered based on response frequencies (positive value for excitation, negative value for inhibition) to several compounds. The chosen compounds showed highest heterogeneity in responses by different neurons. For sensilla trichodea five compounds used for the cluster analysis were: indole, geranyl acetone, 4-ethyl phenol, heptanal and 7-octenoic acid.

Dose response relationships were analysed by General Linear Model, Univariate procedure (SPSS for Windows, release 10.0.5). Stimuli and concentration were set as fixed factors. Interactions between the fixed factors were excluded from the model when the interaction effect was not significant. The effect of a fixed factor was tested by the F statistic and was considered to be significant when $P<0.05$.

Results

Response spectra of ORNs in sensilla trichodea E to a panel of 44 compounds 2-24 h after a blood meal is shown in Table 1. In most cases, spontaneous activity of more than one neuron was recorded; the neurons can be distinguished from each other based on their differences in amplitude (Fig. 1). We hereafter name the neuron with larger amplitude neuron A and the one with smaller amplitude neuron B (mark examples of A and B in Fig. 1). Individual neurons differed in their response spectra. Sensilla trichodea E can be classified into three functional groups based on the response spectra of the ORNs therein to five diagnostic compounds.

After a blood meal, ORNs in sensilla trichodea E responded to 25 out of 44 test stimuli (Table 1). Fig. 2 shows the responses of ORNs in different functional groups of sensilla trichodea E to 13 compounds, to which highest responses and inter-neuron heterogeneities were found, before and after a blood meal. The first functional group (Fig. 2a) had a similar response spectrum as TE1, which was the most abundant functional type in sensilla trichodea E before blood feeding (Fig. 2d). Eight out of 16 of the sensilla studied belong to this type. We name this functional type pTE1 (“p” for post blood feeding). One or both of the ORNs of this type showed excitation responses to in total 14 stimuli. Neuron A (pTE1A) was responsive to ammonia, C5-7 carboxylic acids, 3-methyl-2-hexenoic acid, 1-hexen-3-ol, phenolics, indoles and 6-methyl-5-hepten-2-one (Table 1, Fig. 2a). The responses of pTE1A to these compounds were not different from TE1A except for phenol (t-test, $P=0.032$),

which was higher in pTE1A. In contrast to TE1A, pTE1A was not excited by hepten-3-ol and 3-methyl-1-butanol and not inhibited by C3-4 carboxylic acids. However, it was excited by 3-methyl-2-hexenoic acid and 3-methylindole which did not excite TE1A neurons (Table 1).

The B neuron in the pTE1 sensilla was less responsive to stimulation than TE1B. As

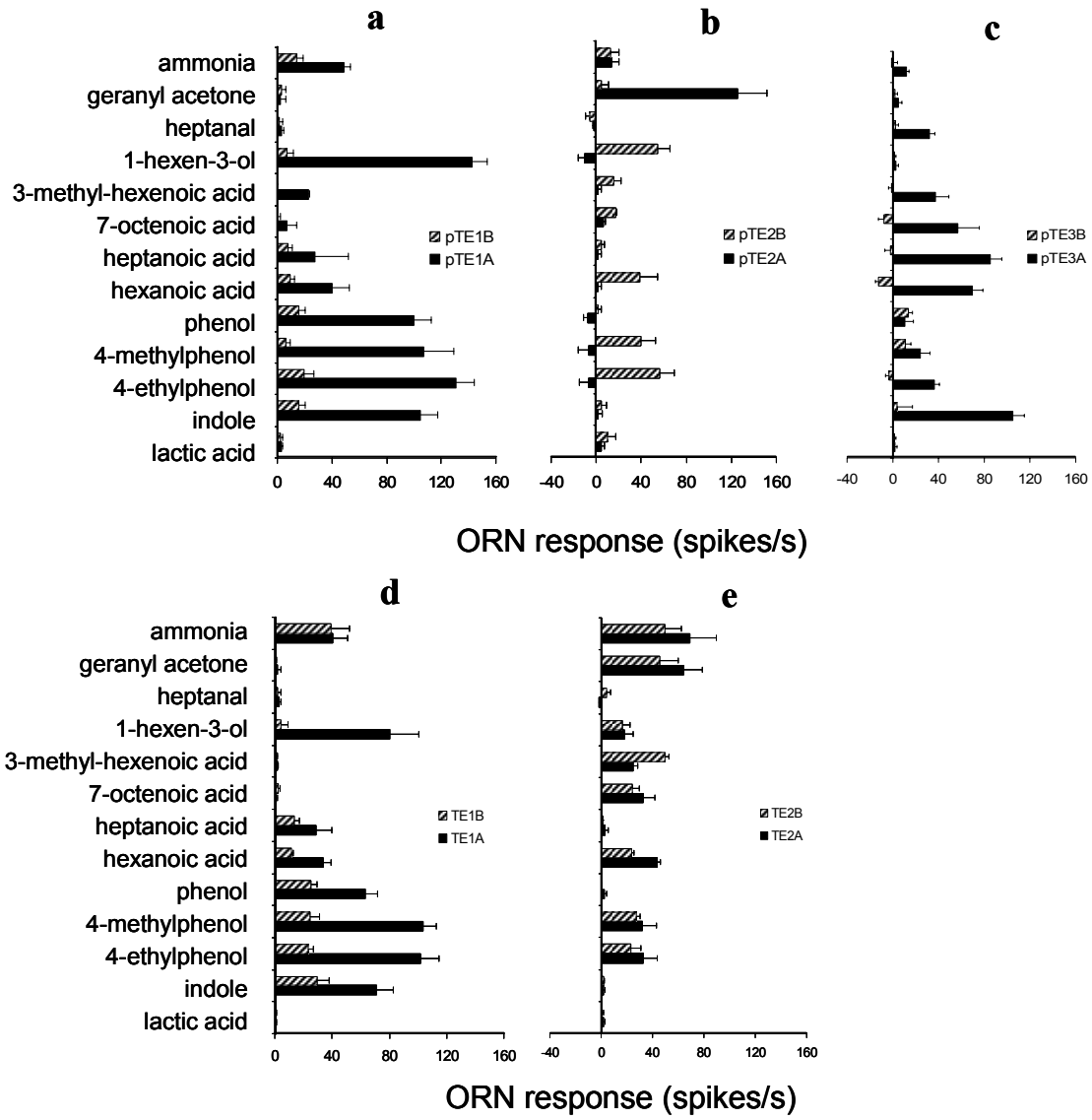

Fig. 2 Response patterns of olfactory receptor neurons innervating sensilla trichodea and grooved peg sensilla on female *Anopheles gambiae* mosquito antennae to thirteen odours at a dilution of 10^{-2} listed along the vertical axis of the bar graphs. Solid bars: neuron A, hatched bar: neuron B. a: pTE1; b: pTE2; c: pTE3; d: TE1; e: TE2. For number of replicates for different sensilla see Table 1.

shown in Table 1 and Fig. 2b, this neuron was in some cases responsive to phenols and indole but not to other compounds. Unlike TE1B, neuron pTE1B was not responsive to ammonia, C5-7 carboxylic acids, 1-hexen-3-ol, 1-hepten-3-ol and 3-methyl-1-butanol. Variability in responses between individual neurons occurred.

Before blood feeding one of the two functional types of sensilla trichodea E, TE2, contains ORNs responsive to geranyl acetone. We found a functional type of TE in mosquitoes after blood feeding, which contained geranyl acetone-sensitive neurons. We name this type pTE2. Four out of 16 sensilla trichodea E we studied belonged to this type. There was overlap of response spectra between this functional type and TE2, e.g., both types contained ORNs responsive to ammonia, hexanoic acid, 7-octenoic acid, 3-methyl-2-hexenoic acid, 1-hexen-3-ol, 1-octen-3-ol, phenols and geranyl acetone. A substantial difference between TE2 and pTE2 was the clear differentiation of neuron A and B in the latter type. Unlike neuron TE2A and TE2B, which had similar response spectra, pTE2A and pTE2B had different response spectra: neuron pTE2A was specifically tuned to geranyl acetone, whereas neuron pTE2B was tuned to a broader range of compounds (Table 1, Fig. 1, 2b).

A third functional type of sensilla trichodea E, pTE3, was found only after blood feeding. Four out of 16 sensilla trichodea E belonged to this type. Although this type contains one neuron tuned to indole, the overall pattern of response spectra was dissimilar to that of TE1 neurons (Table 1, Fig. 2c). Neurons in pTE3 displayed a higher total spontaneous action potential activity (37 ±8/sec) than the neurons in pTE1 (16±7/sec) (t-test, $P<0.001$). Neuron pTE3A responded by excitation to 16 compounds (Table 1). This neuron responded most strongly to indole followed by responses to C7-8 carboxylic acids. Neuron pTE3A was the only one that responded to heptanal among all the neurons we studied before or after a blood meal. The two human-specific odours, 7-octenoic acid and 3-methyl-2-hexenoic acid elicited excitation responses in neuron pTE3A. Neuron pTE3B was less responsive than pTE3A; it responded only occasionally to hexanoic acid, phenols and 3-methylindole.

Neuron pTE3A was compared with TE1A for the sensitivity to phenols and carboxylic acids (Fig. 3a, b). The sensitivities of neuron pTE3A to C6-9 carboxylic acids were higher than those of neuron TE1A, whereas the latter had higher sensitivities to three phenols than the former.

The dose-response curves of TE1A, pTE1A and pTE3A in response to indole are compared in Fig. 3c. Neuron pTE3A was more sensitive to indole than the other two neurons at lower doses (dilution $10^{-4} \sim 10^{-6}$); the response decreased at the two highest doses. A GLM analysis showed that neuron pTE3A was most sensitive to indole, pTE1A was intermediate and TE1A was the least sensitive.

Action potentials from three neurons were registered from grooved peg sensilla. Similar as before blood feeding, grooved peg sensilla contain neurons sensitive to ammonia. The

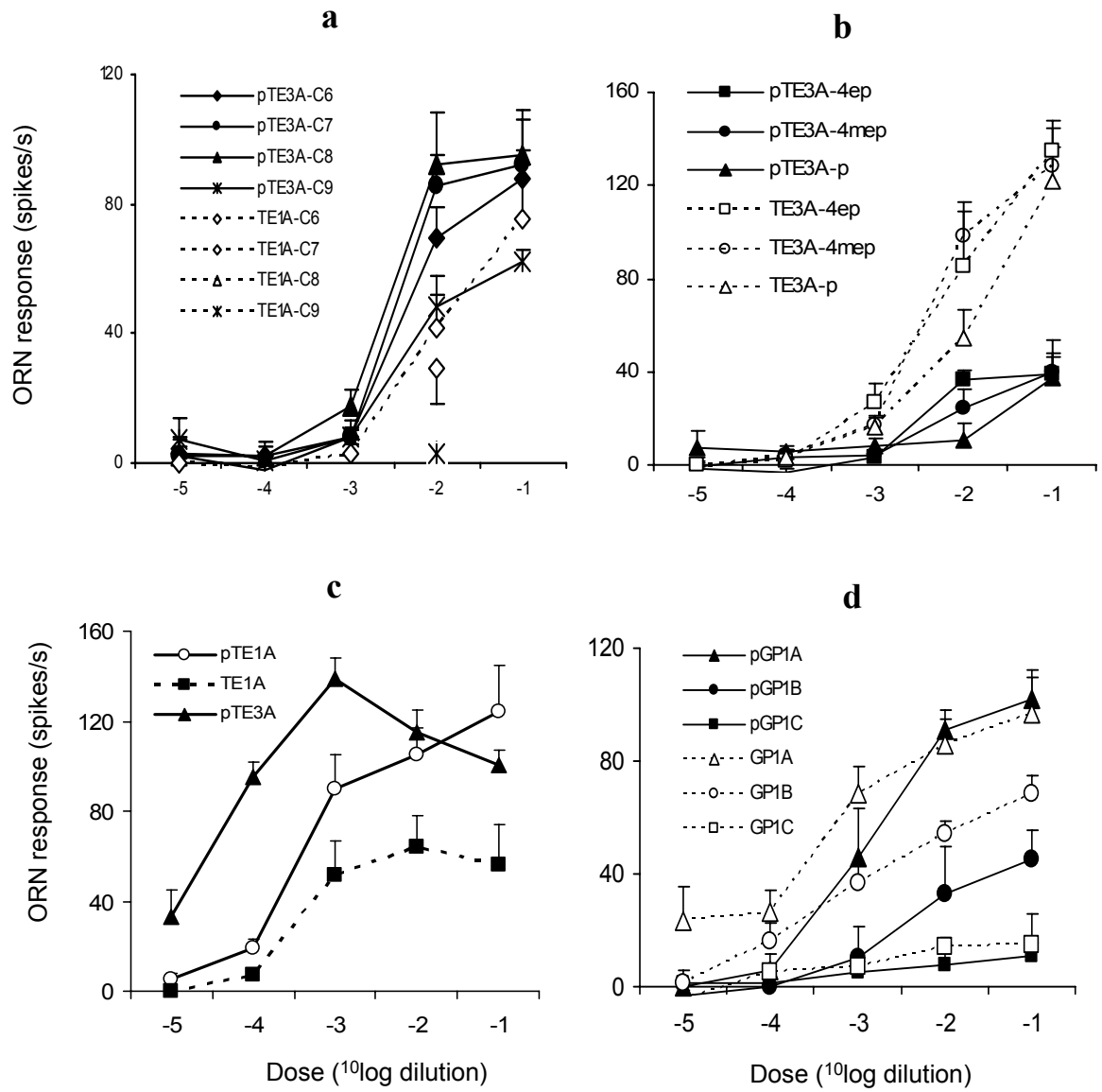

Fig. 3 Dose-response curves of olfactory receptor neurons to odour stimuli at five concentrations. X axis show the logarithm values of the dilutions. a: responses of pTE3A and TE1A to C6-9 carboxylic acids; b: responses of pTE3A and TE1A to three phenols, "4ep" : 4-ethylphenol, "4mep": 4-methylphenol, "p": phenol; c: responses of pTE1A, pTE3A and TE1A to indole; d: responses of pGP1- and GP1- ORNs to ammonia.

response threshold of the two most responsive neurons GP1A and GP1B were higher before blood feeding (Fig. 3d). Neuron pGP1B was less sensitive to ammonia after blood feeding than GP1B (GLM, $P<0.05$).

Discussion

In unfed female *An. gambiae* two functional types of sensilla trichodea E were classified. Shortly after blood feeding we identified three functional types of this sensillum type. Functional type pTE1 was similar to TE1. We consider these functional types as the same population of sensilla trichodea E. The proportion of pTE1 was lower (50%) compared to the proportion of TE1 in sensilla trichodea E bbm (79%). Neurons in pTE1 were responsive to fewer compounds than neurons in TE1. The reduction of sensitivity of pTE1 to several compounds including ammonia, carboxylic acids and alcohols might contribute to the inhibition of host-seeking behaviour following a blood meal.

The response spectra of ORNs in functional type pTE2 were different from those of TE2. Neuron pTE2A was responsive only to geranyl acetone, whereas neurons in TE2A were responsive to 17 stimuli in addition to geranyl acetone. There are similarities in the response spectra of neuron pTE2B and TE2B, although the response spectrum of the former neuron was narrower. The proportion of pTE2 in E type sensilla trichodea (21%) was similar compared to the proportion of TE2 (25%). It is likely that pTE2 sensilla are from the same population as TE2 and the blood meal caused the shift of response spectra observed. Because sensillum TE2 was the only type that contained neurons that were responsive to two human specific odours, 7-octenoic acid and 3-methyl-2-hexenoic acid (Zeng *et al.*, 1991), in addition to other human related odours, we speculated that these neurons were correlated with host-seeking behaviour (Chapter 6). In this study, we also found that the sensitivity to 7-octenoic acid and 3-methyl-2-hexenoic acid of both neurons in pTE2 was lower than before a blood meal; this supports our suggestion about the role of this type of sensillum in the regulation of host-seeking behaviour. The drastic change in the response spectrum in neuron pTE2A might be caused by neuromodulators produced as a result of the blood meal. On the one hand, the response of pTE2B to geranyl acetone decreased compared with TE2B. On the other hand, the response of pTE2A to geranyl acetone increased compared with TE2A. Possibly pTE2A evolved into a more narrowly tuned receptor neuron following a blood meal. We found a geranyl acetone-specific neuron TC2A in sensilla trichodea type C in unfed mosquitoes (Chapter 8). We did not make recordings from sensilla trichodea C in this study, so the effect of a blood meal on the activity of these neurons remains unknown. It seems that geranyl acetone, which was found in human sweat, plays an important role in the odour coding of female *An. gambiae* (Meijerink *et al.*, 2000; Chapter 8).

We also found a third functional type pTE3 in sensilla trichodea E (25%), of which the neuron response spectrum was dissimilar from any of the identified functional types we

found in unfed mosquitoes (Chapter 8). The response of the neuron pTE3A to indole was higher than that observed in neuron TE1A or pTE1A, especially at lower dosages. The sensitivity of pTE3A to C6-9 carboxylic acids and heptanal was also higher than any neuron class we studied with unfed mosquitoes. It appears that pTE3 is a third sensillum type that contains neurons that were sensitive to the two human specific odours, 7-octenoic acid and 3-methyl-hexenoic acid. We did not encounter this type of sensillum in unfed mosquitoes (Chapter 8). It is possible that some of the sensilla type TE1 changed their response spectra to type pTE3 after a blood meal, because both types are indole-sensitive and the sum of percentages of pTE1 and pTE3 was close to that of TE1 before blood feeding. The "new" type could also have evolved from a population of sensilla trichodea E that were not responding before a blood meal. We found the existence of this functional type 48 hrs after a blood meal before the eggs were laid (data not shown). Although the compounds to which pTE3 sensilla responded are all compounds that have been found in human sweat or skin emanations (Cork & Park, 1996; Bernier *et al.*, 2000; Meijerink *et al.*, 2000), some of them, such as indoles, phenols and carboxylic acids, were also found to affect oviposition behaviour in mosquitoes (Bentley *et al.*, 1979; Kyorku *et al.*, 1990; Millar *et al.*, 1994; Blackwell & Johnson, 2000). Therefore, it is difficult to speculate about the role of these neurons in producing behavioural responses.

It is notable that we found both pTE1 and pTE3 neurons responding to 3-methyl-indole, whereas none of the neurons we recorded from before a blood meal were responsive to this compound. Using electroantennography coupled to gas chromatography (GC-EAG), (Du & Millar, 1999) identified 3-methylindole to be present in the mosquito oviposition site odour, and found a concentration of this compound to stimulate oviposition of *Culex tarsalis* and *Cu. quinquefasciatus*. Blackwell & Johnson (2000) reported EAG responses of *An. gambiae* to compounds derived from larval water, including 3-methylindole. The increase of sensitivity of neurons in gravid *An. gambiae* might assist the location of an oviposition site later in the gonotrophic cycle (Sumba *et al.*, 2004).

We found a decreased sensitivity of neurons to ammonia in grooved peg sensilla after a blood meal. Ammonia was proven to be an important attractant to *An. gambiae* (Braks *et al.*, 2001; Smallegange *et al.*, 2005). Grooved peg sensilla were found responsive to polar compound such as ammonia, amines, oxocarboxylic acids, lactic acid and short-chain carboxylic acids (Davis, 1976; van den Broek & den Otter, 2000; Meijerink *et al.*, 2001; Diehl *et al.*, 2003; Chapter 8) which are all host-seeking kairomones. The reduction of neuron sensitivity to ammonia following a blood meal might be involved in the suppression of the host-seeking behaviour.

Davis (1984) found that the sensitivity of lactic acid-sensitive neurons was suppressed after a blood meal and considered that the reduction of neuron sensitivity regulated the be-

haviour via a haemolymph-born factor. This author did not study the effect of blood meals on other neurons.

Although the sensitivity of neurons in pTE1 showed similar sensitivity to phenols, neurons in pTE2 and pTE3 showed lower sensitivity to phenols after a blood meal. Therefore, the overall sensitivity of sensilla trichodea E to phenols decreased after a blood meal. A female-specific putative olfactory receptor gene *AgOr*1 and *AgOr*2 was reported to be down-regulated after a blood meal and, when this gene was expressed in an engineered neuron of *Drosophila*, the neurons showed specific response to 4-methylphenol and 2-methylphenol respectively (Fox *et al.*, 2001; Hallem *et al.*, 2004a). The overall decrease of sensitivity to phenols we found might be caused by the down-regulation of these genes. The reduction of neuron sensitivities to these compounds correlated with the suppression of host-seeking behaviour as reported by Takken *et al*. (2001). Meanwhile we also found increased sensitivities of neuron pTE2A to geranyl acetone and pTE3A to indole, C6-9 carboxylic acids, 7-octenoic acid and 3-methyl-hexenoic acid. As far as we know, the increase of ORN sensitivity during a behaviourally inactive state in a gonotrophic cycle of haemophagous insects has not been reported before. It is possible that some olfactory receptor genes are only turned on with the stimulation from the first blood meal. The expression of these receptors might be useful to the mosquitoes for the location of oviposition sites and for more efficient host-seeking behaviour in the following gonotrophic cycles.

In conclusion, blood feeding induced changes in responses of olfactory receptor neurons to putative kairomones of female *An. gambiae*. The overall sensitivity to ammonia and phenols was decreased after a blood meal, supporting the important role of these compounds in the modulation of host-seeking behaviour. Some types of neurons display a consistent response pattern to odour stimulants before and after a blood meal. However, blood feeding results in the occurrence of a new functional type of sensilla trichodea E, which might be involved in more successful host-, nectar- or oviposition-site- location in successive foraging activities of these mosquitoes.

Acknowledgements

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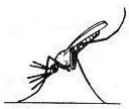
10

General discussion



Chemical communication plays a crucial role for female *Anopheles gambiae* in locating conspecific male mates, blood hosts, sugar sources and breeding sites (Takken & Knols, 1999). Female *An. gambiae* are highly anthropophilic and can discriminate human odour from odours of other mammals (Coluzzi *et al.*, 1979; Pates *et al.*, 2001b). Understanding which components of human odours cause chemosensory and behavioural responses in females of *An. gambiae* will enable us to develop synthetic human odours that can be used for monitoring and control of this malaria vector. In this chapter the findings from the behavioural and electrophysiological studies described in this thesis will be integrated with existing knowledge to get a better picture of the role odours might play in the behaviour of female *An. gambiae* and to indicate in which areas further work is needed.

Chemical cues in host-seeking behaviour of *Anopheles gambiae*

Female mosquitoes are guided to their blood hosts by optical cues, physical cues such as heat and moisture, as well as by chemical cues. Although optical cues are necessary for upwind anemotaxis (Cardé, 1996), they are less important in the searching behaviour of nocturnal than of diurnal mosquito species. Heat alone does not elicit mosquito attraction (Schreck *et al.*, 1990) whereas moisturised air induces a slight response in *An. gambiae*

(Takken *et al.*, 1997b). Human skin odours cause the strongest behavioural response (Takken *et al.*, 1997b; Pates *et al.*, 2001b). Therefore it is fair to state that host-seeking behaviour is mediated predominantly by chemical cues. The results we achieved by releasing human emanations from glass beads showed that 6-20 beads caused more than 45% of the test mosquitoes to respond, which was similar to the response to emanations from a human hand (Chapter 2). Behavioural responses correlated with the EAG responses elicited by similarly treated glass beads. These results provide again evidence that chemical cues play a substantial role in the host attraction of *An. gambiae*.

Differential attractiveness: a useful approach for kairomone identification

A useful method for the identification of kairomones in the host-seeking behaviour of mosquitoes is to find two similar odour sources of which one has a higher attractiveness to mosquitoes than the other. Through comparing the chemical composition if the sources, those compounds that are more abundant in the more attractive odour are putative attractants and those more abundant in the less attractive odour might be repellents. Ideally, the two odour sources should cause as large as possible differences in attractiveness and a relatively small difference in chemical composition, so that the components responsible for the difference are easily identified. An example of such a system is the lowly attractive fresh sweat and the highly attractive incubated sweat. By comparing the chemical compositions of these two odour sources, an important attractant for *An. gambiae*, ammonia, was identified (Braks & Takken, 1999).

A differential attractiveness approach was applied in the present research (Chapters 2 and 3). In Chapter 2, glass beads with skin emanations from four different human beings were found to elicit differential attractiveness. Subsequently, skin emanations from 27 humans were ranked based on their attractiveness relative to ammonia, and significant differences among the individuals were found (Chapter 3). The skin emanations from the most and least attractive individuals were subjected to chemical analysis. Five compounds (2-phenoxyethanol, 2-pentadecyloxyethanol, 1-dodecanol, dodecanethiol and hexadecanoic acid) were identified as potential attractants and two alkanes (C26 and C27) were possible repellents (A.M.S Galimard, personal communication). The behavioural effect of these compounds remains to be tested.

The attraction exerted by human-handled glass beads completely disappeared four hours after treatment (Chapter 2). The change of chemical composition over time was examined and both quantitative and qualitative changes were found (A.M.S Galimard, personal communication). The disappearance of attractiveness was probably due to the decrease in total amount of the odour and/or due to the change of the composition over time. Because the

beads were handled by the same person, the variance between different samples was small, i.e. the method is a "cleaner" method with less "noise" compared with collecting samples from different individuals. This method would be less informative when it would turn out that the change in attraction was mainly caused by a gradual reduction of odour quantity by evaporation over time.

Natural blends versus synthetic blends

Although several authors demonstrated that natural human skin emanations, extracts of human skin emanations or Limburger cheese odours were highly attractive to *An. gambiae* (Knols & de Jong, 1996; Braks & Takken, 1999; Healy & Copland, 2000; Pates *et al.*, 2001b), attempts to make synthetic mixtures mimicking the attractiveness of human skin odour have not been successful up to the present time (Knols *et al.*, 1997; Healy & Copland, 2000; Smallegange *et al.*, 2005). Whereas synthetic blends alone attracted large proportions of *An. gambiae* in an olfactometer, when tested against natural odours from a human hand or foot, synthetic blends were considerably less attractive than the natural volatile blends. Possible explanations are: (1) some key compounds are missing in the synthetic mixtures, (2) the relative abundance of compounds in the mixtures is not optimal, (3) the concentration tested is different from that occurring in the natural blends.

Based on the chemical composition in the headspace of Limburger cheese, Knols *et al*. (1997) made a synthetic mixture of 12 carboxylic acids, in similar proportions as in the cheese headspace. Although the synthetic mixture was initially found to cause 21% of mosquito response at one concentration, it did not show any attractive effect in later tests (Knols *et al.*, 1997; Braks, 1999; Smallegange *et al.*, 2005; Chapter 5). Because the proportions of the carboxylic acids in the mixture were similar to those found in the cheese headspace, and because they have been tested over a wide range of concentrations, it is most likely that the synthetic mixture was lacking key compounds necessary for causing attraction as high as the cheese headspace. In addition to aliphatic carboxylic acids, dimethyldisulphide and dimethyltrisulphide were present in the headspace of the cheese odour (Knols *et al.*, 1997). Other odours may have been present in the cheese headspace but were possibly not detected by the GC-MS analysis. Our experiments in Chapter 5 show that the attractiveness was increased by adding ammonia to the carboxylic acid mixture; and a strong synergistic effect was found for the mixture of ammonia, lactic acid and carboxylic acids.

An example in which a synthetic mixture successfully mimics a natural odour blend is the mixture of 1-octen-3-ol, acetone and CO_2, which causes similar attractiveness as ox odour for *Glossina morsitans morsitans* (Hall *et al.*, 1984). The mixture was less attractive to another tsetse fly species, *G. pallidipes*. Subsequent studies showed that *G. pallidipes* was

highly attracted to blends of 1-octen-3-ol, acetone, two phenols and CO_2 (Willemse & Takken, 1994). Analogous to these findings, we believe it is possible to develop a synthetic odour blend that is as attractive to *An. gambiae* as natural human odour. The results of our field study in The Gambia, in which a synthetic blend of ammonia, L-lactic acid and CO_2 attracted the same number of mosquitoes as the natural odours from a human volunteer (Chapter 7) indicate that the principle of collecting mosquitoes with synthetic odour baits is realistic. Although at present the development of such a synthetic blend is not quite accomplished, we have already collected much information about the ingredients therein, which will be discussed below.

Single compounds that might be involved in the host-seeking behaviour

Ammonia

Our results in Chapter 5 confirmed the attractiveness of ammonia for *An. gambiae*. The ammonia concentration eliciting an attractive response of *An. gambiae* ranged from 13.6 to 13,637 p.p.m. in our bioassay. The concentration that was used in all the subsequent experiments carried out (136 p.p.b. in the olfactometer) fell in the range of ammonia concentrations (40~3170 p.p.b.) measured in human breath (Larson *et al.*, 1979). Braks *et al.* (2001) found that the slightly attractive fresh sweat samples contained 0.315 µmol ammonia, and this was below the ammonia threshold eliciting attraction (between 1.34 and 13.4 µmol). The amount of ammonia in incubated sweat was 2.47 µmol, thus within the range of attractive concentrations. It is therefore concluded that ammonia accounts substantially for the attractiveness of incubated human sweat for *An. gambiae*.

Unlike the fresh and incubated sweat samples, human skin emanations collected on glass beads contained only about 0.97 nmol ammonia (A.M.S. Galimard, personal communication), an amount far too low to attract *An. gambiae* (Braks *et al.*, 2001; Chapter 5). This finding suggests that the attraction of human skin emanations on glass beads to *An. gambiae* was not caused by ammonia. The quantity of ammonia in skin emanations on the glass beads was even found to increase during aging, while the mosquito attractiveness decreased. Interestingly, we found that human-handled beads were in almost all cases more attractive than the standard concentration of ammonia (Chapter 3). Possibly, except for those compounds that differed significantly in their abundance in more and less attractive individuals, the skin emanations on the glass beads contain other, hitherto unknown, chemical components that contribute to the general attraction of mosquitoes to humans. Useful techniques for further identification of kairomones for *An. gambiae* are the GC-EAD (gas chromatography coupled electroantennographic detection) or GC-SSR (GC-coupled single sensillum re-

cording) methods. GC-EAD has been used in Chapter 4 for analysing human skin residues collected on nylon socks and the method as such appears to be feasible for *An. gambiae*. Further improvements include the analysis of human odour samples with lower background "noise" odours such as odours collected on glass beads, or with Solid Phase Micro Extraction (SPME). Different sampling methods in combination with different types of GC columns might result in the identification of different types of active compounds. To increase the sensitivity of GC-EAD analysis, more mosquito antennae can be tested simultaneously, and a method in which odour molecules leaving the GC-column are first adsorbed to the wall of the GC-EAG transfer tube by cooling and subsequently desorbed by quick heating (termed 'flash desorption', J.N.C. van der Pers, personal communication) can be applied to enable pulsed delivery of odour molecules which will enhance the amplitude of the EAG signal.

Once we knew that ammonia is an important kairomone for mosquitoes and a common component of human odour, we considered it appropriate to use ammonia as a background odour in the identification of active compounds like those we studied in Chapters 5 and 6. The benefit of such an approach is to bring the selection one step closer to the natural odour blend. Odours that cause additional effects or augment the effect of ammonia are discriminated from odours that decrease or counteract the effect of ammonia. Ammonia plays a different role in the host-seeking behaviour of *An. gambiae* than in *Ae. aegypti*: ammonia alone attracted *An. gambiae* but not *Ae. aegypti* (Geier *et al.*, 1999; Braks *et al.*, 2001; Chapter 5), eventhough ammonia was found to augment the effect of lactic acid in *Ae. aegypti* (Geier *et al.*, 1999). The latter compound also functions differently in the behaviour of these two mosquitoes, which will be discussed below.

Lactic acid

L-lactic acid has been identified as a key compound in the attraction of *Ae. aegypti* to humans. It is not only an attractant on its own, but also a synergist for other components (Smith *et al.*, 1970; Bosch *et al.*, 2000; Steib *et al.*, 2001; Dekker *et al.*, 2002). The compound was also found responsible for the higher attractiveness of humans for *Ae. aegypti* compared with other mammals (Steib *et al.*, 2001). Nevertheless, lactic acid alone was found not or only slightly attractive to *An. gambiae* (Braks *et al.*, 2001; Smallegange *et al.*, 2005; Chapter 5) and removal of lactic acid from human sweat did not affect the attractiveness of the sweat (Braks *et al.*, 2001). However, a synergistic effect was found when lactic acid was blended with ammonia and a mixture of 12 carboxylic acids (Chapter 6), underscoring the essential role of this compound in the behaviour of *An. gambiae*. The importance of lactic acid in the behaviour of anthropophilic mosquitoes is further shown by the presence

of lactic acid-sensitive olfactory neurons located on the antennae (Davis, 1976; Chapter 8).

Carboxylic acids

A number of carboxylic acids showed attractive effects in addition to ammonia (Chapter 6). Carboxylic acids are common constituents of human odour and were considered to account for the attraction of anthropophilic mosquitoes such as *An. gambiae* and *Ae. aegypti* to humans (Cork & Park, 1996; Knols *et al.*, 1997; Bernier *et al.*, 2000; Bosch *et al.*, 2000; Healy & Copland, 2000). Our results also showed that the effect of carboxylic acids was highly dose-dependent; the attractive effect was found mostly at one test concentration only (Chapter 6). In future studies, compounds with an attractive effect should be added to synthetic mixtures one by one, at the effective concentration, and the respective mixtures should be tested in a bioassay. Such studies will provide information on whether the effects of the components are additive, synergistic or suppressive.

Other chemical components

Females of *An. gambiae* were repelled by several pure alcohols, phenols, heterocyclic compounds and ketones, although these compounds were found at higher concentrations in the highly attractive incubated human sweat. Whether these compounds were contributing to the attractiveness of the incubated sweat needs further investigation. Apparently, their presence at least did not completely suppress the activity of the attractive compounds, e.g. ammonia and carboxylic acids, if they themselves did not contribute to the attractiveness of the whole mixture. We cannot exclude the possibility that these compounds act as synergists or attractants in a different mixture, even though in combination with ammonia and L-lactic acid they caused a repellent effect under the conditions in our bioassay. These compounds were found in human skin emanations collected on glass beads, and it did not become clear whether they play a role in the attraction of *An. gambiae* to human skin residues. Indole is not only a major component in incubated human sweat, but also a common component of the odours released by mosquito oviposition sites and flowers (Millar *et al.*, 1992; Du & Millar, 1999; Meijerink *et al.*, 2000; Knudsen *et al.*, 2001; Dötterl *et al.*, 2005). There is overlap between components of human odour, plant scent and odours from mosquito oviposition sites.

Not single components but the combination of the key components in the odour blend inform mosquitoes about the quality of the odour source. It is likely that not only attractants but also repellents are important in host discrimination (Birkett *et al.*, 2004). It could also be that the physiological or behavioural effect of attractants and repellents is limited to a cer-

tain physiological stage of the insect (Takken *et al.*, 2001; Zwiebel & Takken, 2004). Whether the compounds that are repellent to unfed females are also repellent to gravid females needs further investigation.

Application of odour baited traps

In our field experiments, odour mixtures with or without CO_2 were compared for their attractiveness to mosquitoes based on trap catches. Counterflow Geometry (CFG) traps releasing synthetic odours with CO_2 trapped considerably more mosquitoes than those without odours; CO_2 was found to account for most of the attraction. However, adding odours did not produce a statistically significant effect in addition to CO_2 on the catch of the malaria mosquito *An. gambiae* (Chapter 7). Our results showed the significant impact of odours on the catching rate of mosquito traps, suggesting the potential of odour-baited mosquito traps in population manipulation and control of mosquitoes. The reason that our experiments failed to show an effect of odours when added to CO_2 might be explained as follows: (1) CO_2 functioned as a driving gas in the odour delivery system, therefore the effect of CO_2 was over-estimated because the CO_2-effect was confounded with the effect of other odours, (2) the odour composition in the mixtures was not optimal: either lacking essential components or having the wrong ratio, (3) the odour concentration was not optimal. These factors could not be controlled under the experimental conditions, because the odours were being blended from pure compounds by leading air over the vessels containing the odorous compounds (Chapter 7). In future research, a better odour delivery system should be established and more effective mixtures should be designed and properly formulated. The emission rate from the release system should be measured with chemical methods or by bio-sensory analysis beforehand (Färbert *et al.*, 1997; van der Pers & Minks, 1997; Hillbur *et al.*, 2000). Instead of gaseous CO_2, aqueous ammonia and lactic acid, salt compounds such as ammonium carbonate, ammonium bicarbonate, urea or ammonia lactate might be used. Formulated odour mixtures might be released from synthetic or natural adsorbents, as used for tsetse fly attractants, insect sex pheromones or other semiochemicals (Hall *et al.*, 1984; Vale, 1993; Staten *et al.*, 1997).

Sensory physiology and odour coding in female *An. gambiae*

The sensitivities of three morphological types of sensilla on the antennae of *An. gambiae* were tested with a panel of 44 potential mosquito kairomones, and the sensilla were classified into functional groups based on the response spectra of the olfactory receptor neurons (ORN) therein (Chapter 8). ORNs in the two functional types of the short sharp tipped sen-

silla trichodea (type E) were found to be generalists responding to a broad range of odours including carboxylic acids, alcohols, and ammonia. By contrast, sensilla trichodea C, of medium length and with a sharp tip, were either "moderate specialist" ORNs tuned to a narrow range of odours (alcohols, phenols, heterocyclics and ketones) or "extreme specialist" ORNs tuned to only one odour (geranyl acetone). Neurons in grooved peg sensilla were not responsive to alcohols, heterocyclics and ketones, they were either "moderate specialist" ORNs sensitive to more polar compounds such as ammonia, amines and carboxylic acids or "extreme specialist" ORNs only responding to ammonia and amines (Chapter 8). Olfactory coding of general odours in the antennae of *An. gambiae* mainly follows the across-fibre pattern principle: one neuron responds to more than one odorant and/or one odorant activates more than one neuron. None of the odours tested up to now is the unique ligand of one neuron only, so no indication for labelled-line coding has been found.

Compounds that are proven to have attractive or repellent activities for *An. gambiae* (Chapter 6) were found eliciting electrophysiological responses from at least one ORN-type (Chapter 8). Interestingly, odorants that are proven to be attractants or landing stimulants on their own for *An. gambiae*, such as ammonia and 2-oxocarboxylic acids (with the exception of 2-oxohexanoic acid) elicited a response of ORNs in both grooved peg sensilla and a subpopulation of sensilla trichodea E. From this, it appears that not only grooved peg sensilla but also sensilla trichodea are involved in host-seeking behaviour (Meijerink *et al.*, 2001). In future studies when single sensilla recording (SSR) or GC-SSR is applied for the identification of attractants for *An. gambiae*, both grooved peg sensilla (especially GP4) and the sensilla trichodea E (TE2) that were sensitive to various host odours should be investigated (Chapter 8). Several aliphatic carboxylic acids activating sensilla trichodea (but not grooved peg sensilla) were attractive to *An. gambiae* in addition to ammonia. By contrast, some alcohols, heterocyclics and ketones that activate sensilla trichodea showed a repellent effect. Therefore, there is no simple correlation between electrophysiological responses of certain types of sensilla and behavioural activities. ORNs send information about the quality and quantity of an odour to the central nervous system, where the information is integrated and the behavioural activity is modulated (de Belle & Kanzaki, 1999).

After a blood meal, the sensitivity of grooved peg sensilla to ammonia decreased, which might be a mechanism for the temporary suppression of host-seeking behaviour (Fox *et al.*, 2001). It would be interesting to test whether ammonia sensitivity of these sensilla recovers at the start of the second gonotrophic cycle, after the eggs have been laid. In addition, a new functional type of sensilla trichodea was found after blood ingestion. One olfactory neuron in the new type was highly sensitive to indole and 3-methyl indole, potential kairomones for oviposition behaviour (Millar *et al.*, 1992; Du & Millar, 1999). Interestingly, the same neuron was also sensitive to C5-9 carboxylic acids and, especially 7-octenoic acid and 3-

methyl-2-hexenoic acid, two so-called "human specific" compounds (Zeng *et al.*, 1996). The biological relevance of the changes in neuron sensitivity to various compounds deserves further investigation.

Anton and Rospars (2004) found that neurons from sensilla trichodea and grooved peg sensilla each projected to two distinct zones in the antennal lobe. Although neurons sensitive to indole were never found responding to geranyl acetone, and *vice versa*, yet both zones corresponding with sensilla trichodea contain arborisation of neurons sensitive to indole and geranyl acetone. Whether these two zones receive arborisations of neurons from morphologically or functionally different sensilla trichodea is an interesting question for future research. One neuron in sensilla trichodea C that was specifically responding to geranyl acetone and neurons in a functional type of sensilla trichodea E responsive to geranyl acetone were also sensitive to various other compounds, and the arborisation of these neurons are therefore of special interest (Anton & Rospars, 2004). There are no obvious morphological sub-types of grooved peg sensilla on the antennae of female *An. gambiae*. The arborisation patterns for different functional types of grooved peg sensilla are to be elucidated.

Recently, odorant receptor (OR) genes of *An. gambiae* were expressed in a *Drosophila* olfactory neuron, from which the original OR had been deleted, and the function of the ORs were studied by extracellular recordings (Hallem *et al.*, 2004a). The method provides a powerful tool for the functional examination of putative ORs. However, the results from such a study might not reflect the function of these ORs in the donor, female *An. gambiae*, because odour perception is also dependent on odour binding proteins. Therefore, next to transgenic approaches, using an extracellular recording method to study the function of the olfactory neurons in different types of olfactory sensilla in *An. gambiae* is still essential in the understanding of odour coding in this mosquito.

Carbon dioxide has been shown to play an important role in the host seeking behaviour of *An. gambiae* (Snow, 1970; Healy & Copland, 1995; Costantini *et al.*, 1996a; Mboera & Takken, 1997; Dekker *et al.*, 2001; Chapter 7). Olfactory receptor neurons for CO_2 were found in sensilla basiconica on the maxillary palps of mosquitoes (Kellogg, 1970; Grant *et al.*, 1995; Grant & O' Connell, 1996). For *Drosophila*, neurons sensitive to CO_2 were found in the large sensilla basiconica on the antennae. It would be interesting to examine the potential sensitivity of maxillary olfactory organs of *An. gambiae* for CO_2 and other compounds.

An important aspect for future study is the physiological responses to odour blends. In a natural situation, mosquitoes will encounter complex odour mixtures. The ORNs might respond differently to single odours and a mixture of odours or there might be interactions between odours at the peripheral level (Ochieng *et al.*, 2002). We found a synergistic effect of ammonia, L-lactic acid and carboxylic acids; it would be of interest to investigate whether

such synergism happens at the ORN level or at the level of the central nervous system.

Future outlook

This thesis provides evidence that the attractiveness of mosquito traps is increased substantially by baiting them with odours of human origin, indicating that using odour- baited traps in the manipulation of population dynamics and in the control of the malaria mosquito *An. gambiae* is feasible. Although our results suggested several promising odour mixtures, future research ought to further optimise the combination and release ratios of the attractive components with behavioural and electrophysiological measures, and these mixtures should be tested in the vicinity of human dwellings where populations of *An. gambiae* are often high. Our results also suggested that components that attract and repel *An. gambiae* are both present in human odours; the differences in odour profiles between individuals determine their differential attractiveness to the mosquitoes and can possibly reveal important active components. In further studies on the identification of these components, GC-EAD and GC-SSR methods can be applied. Different sampling methods and different types of GC-columns should be explored to identify various types of active odour components. For GC-SSR analysis, certain functional types of sensilla grooved peg (GP5) and sensilla trichodea (TE2) should be explored with priority. Compounds with electrophysiological activities are worth to be investigated further for their behavioural activities. Single components as well as synthetic odour blends, with ratios similar to naturally occurred, highly attractive or repellent odours, should be tested with bioassays in the laboratory and in the field. The repellents and attractants can be used in combination to establish a more effective push-pull strategy to manipulate the behaviour *An. gambiae* and therefore reduce the transmission of malaria.

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Nederlandse Samenvatting

Malaria, een van de ernstigste menselijke ziekten, treft elk jaar 300 – 600 miljoen mensen en veroorzaakt elke minuut de dood van twee kinderen. In tropisch Afrika is de mug *Anopheles gambiae* Giles *sensu stricto* de voornaamste overdrager van de malariaparasiet van mens tot mens. Deze muggensoort voedt zich bij voorkeur met menselijk bloed, rust binnenshuis en plant zich voort dicht bij menselijke nederzettingen, drie eigenschappen die deze soort een effectieve malaria-vector maken. De belangrijkste signalen die vrouwtjes van *An. gambiae* naar hun menselijke gastheer leiden zijn vluchtige stoffen afkomstig van het menselijk lichaam. Het hoofddoel van dit proefschrift was het onderzoeken van de chemische verbindingen welke een rol spelen in het gastheerzoekgedrag van deze muggensoort en van de wijze waarop deze verbindingen waargenomen worden door het olfactorisch systeem van de mug. De verkregen kennis kan toegepast worden in de ontwikkeling van geurvallen die kunnen worden gebruikt om mensen te beschermen tegen muggenbeten of om de kans om door muggen gebeten worden te verkleinen en kan een alternatief verschaffen voor de traditionele maar betwijfelde methode om muggen op menselijke vrijwilligers te laten landen als een methode om de omvang van muggenpopulaties te onderzoeken.

Glazen knikkers waarop vluchtige stoffen afgegeven door de huid van mensenhanden waren aangebracht, leverden een vergelijkbaar niveau van aantrekking op als een menselijke hand (**Hoofdstuk 2**). De aantrekkelijkheid van deze behandelde glazen knikkers was 4 uur na behandeling van de knikkers afgenomen. Luchtmonsters die over de behandelde glazen knikkers waren geleid wekten een dosis-afhankelijke electroantennogram reactie op. Glazen knikkers bleken een geschikt neutraal substraat te zijn om menselijke geur op over te brengen en dit maakte het mogelijk om de gedrags- en electrofysiologische activiteit van *An. gambiae* die aan deze geuren werden blootgesteld te onderzoeken en ook om de vluchtige stoffen afgegeven door de menselijke huid chemisch te analyseren middels gaschromatografie-massaspectrometrie welke in een tweeling-project werd uitgevoerd.

Om de chemische basis van de verschillen tussen mensen in aantrekkelijkheid voor muggen te bestuderen, werden monsters van vluchtige stoffen afgegeven door de huid van 27 menselijke individuen verzameld op glazen knikkers en getest tegen ammonia in een twee-keuze-olfactometer om de mate van aantrekkelijkheid voor *An. gambiae* vast te stellen (**Hoofdstuk 3**). Er waren duidelijke verschillen tussen de monsters van huidstoffen van

verschillende vrijwilligers wat betreft de kans dat de muggen de vangpotten in de olfactometer binnenvlogen en wat betreft de relatieve aantrekkelijkheid ten opzichte van ammonia. Consistente verschillen werden waargenomen wanneer monsters van vluchtige huidstoffen afkomstig van de drie meest en de drie minst aantrekkelijke vrijwilligers paarsgewijs werden getest. Monsters van huidstoffen van mannen gaven een hogere kans te zien dat de muggen de vangpotten binnenvlogen dan monsters van vrouwen. Geurstoffen van jongere vrijwilligers verhoogden de vangkans significant en werden door de muggen verkozen boven de geurstoffen afkomstig van oudere vrijwilligers. Electroantennogram reacties op monsters van vluchtige huidstoffen van vrijwilligers welke op gedragsniveau een verschil in aantrekkelijkheid lieten zien waren niet in alle gevallen positief gerelateerd aan de sterkte van de gedragsreactie, hetgeen een rol suggereert voor afstotende verbindingen.

Kousen die waren gedragen door mensen hebben voorheen een hoge aantrekkelijkheid laten zien voor *An. gambiae*. Lucht die over nylon kousen was geleid werd verzameld en geanalyseerd met behulp van gaschromatografie welke direct was gekoppeld aan electroantennografie (EAG). EAG reacties werden consistent aangetoond op 23 retentie-tijden en 14 van deze verbindingen werden voorlopig geïdentificeerd. Deze verbindingen bleken echter niet van typsich menselijke oorsprong te zijn (**Hoofdstuk 4**).

In vorig onderzoek was de aantrekkelijkheid van ammonia, L-melkzuur en een mengsel van carboxylzuren voor *An. gambiae* aangetoond. Deze verbindingen zijn alle aanwezig in menselijke huidgeuren, hetgeen reden was om een mengsel van deze verbindingen in een twee-keuze olfactometer te bestuderen (**Hoofdstuk 5**). Ammonia alleen veroorzaakte aantrekking, terwijl melkzuur alleen niet aantrekkelijk bleek. Carboxylzuren, aangeboden als een mengsel van 12 verbindingen, veroorzaakten afstoting bij de geteste concentratie. De toevoeging van ammonia aan het mengsel van carboxylzuren overstemde het afstotende effect van dit mengsel. De combinatie van ammonia met ofwel melkzuur ofwel het mengsel van carboxylzuren liet geen verhoging zien van de aantrekkelijkheid van ammonia alleen. Er werd echter een synergistisch effect gevonden wanneer ammonia, melkzuur en carboxylzuren als een mengsel werden aangeboden.

Componenten van menselijke geuren welke electrofysiologische - of gedragsreacties opwekten werden getest in combinatie met ammonia en melkzuur tegen ammonia alleen (**Hoofdstuk 6**). De resultaten lieten zien dat C3-C8 and C14 carboxylzuren de aantrekkelijkheid van ammonia en melkzuur verhoogden bij sommige concentraties, terwijl alcoholen en ketonen de aantrekkelijkheid alleen verlaagden bij de geteste concentraties.
Gebaseerd op de gedragsmatige en electrofysiologische bevindingen, werd een veldstudie uitgevoerd in The Gambia (West Afrika) om de efficiëntie te onderzoeken van

muggenvallen waarin kunstmatig samengestelde geurmengsels of menselijke geur waren aangebracht (**Hoofdstuk 7**). Deze studie liet zien dat combinaties van 4 - 9 geuren, gemengd terwijl ze vrijkwamen uit vallen werkend volgens het 'counterflow geometry'(CFG)-principe, in vele gevallen sterkere aantrekking te zien gaven dan geuren uit een tent waarin een mens verbleef. Kooldioxide liet een aanzienlijke verhoging zien van de vangsten met de CFG-vallen voor alle muggensoorten. CFG-vallen waarin een mengsel van ammonia + melkzuur + 3-methylbutaanzuur + CO_2 was aangebracht lieten de grootste vangsten zien voor de meeste groepen muggen; dit mengsel wordt beschouwd als een veelbelovend kandidaat-geurmengsel in de bestrijding van muggen die overlast veroorzaken. Experimenten met vallen die binnenshuis waren geplaatst, toonden aan dat een geurmengsel bestaande uit ammonia + melkzuur + CO_2 + geranyl-aceton + indool + 4-ethylfenol, aantrekkelijker was voor *An. gambiae* dan de controle-geur; dit mengsel lijkt veelbelovend voor voortgezette experimenten onder omstandigheden waarin de dichtheden van *An. gambiae* hoger zijn en voor toepassing in vectorbestrijdingsprogramma's.

Gebruikmakend van een methode waarin de activiteit van afzonderlijke sensillen wordt geregistreerd, werd een electrofysiologische studie ondernomen van de geurzintuigcellen van vrouwelijke *An. gambiae* (**Hoofdstuk 8**). Zes functionele typen sensilla trichodea en vijf functionele typen sensilla basiconica ('grooved peg' sensilla) werden geïdentificeerd. 'Generalistische' olfactorische receptor-neuronen (ORN's), welke reageren op een breed spectrum van geurstoffen, werden gevonden in sensilla trichodea subtype E, terwijl 'middelmatig specialistische' ORN's, welke afgestemd zijn op een klein spectrum van geuren, werden aangetroffen in subtype C en grooved peg sensilla, met twee 'extreem specialistische' ORN's die slechts op één van de geteste geurstoffen reageerden. Er was overlap in de reactiespectra tussen sensilla trichodea E en C of grooved peg sensilla, maar deze overlap was afwezig tussen sensilla trichodea C en grooved peg sensilla, met uitzondering van ammonia waarvoor beide typen sensilla gevoelig waren. Neuronen die geassocieerd zijn met dezelfde sensillen vertoonden een tendens om te reageren op dezelfde geurstimuli maar met verschillende gevoeligheden. Neuronen in grooved peg sensilla reageerden vooral op meer polaire verbindingen, zoals het belangrijke attractans ammonia en de synergist melkzuur, waarop alleen reacties werden gevonden in grooved peg sensilla. Fenolen behoorden tot de meest effectieve stimuli voor verscheidene neurontypen, behorend tot uiteenlopende functionele klassen. 'Across-fibre patterning' is het meest plausibele coderingsprincipe dat functioneert in het olfactorisch systeem van deze muggensoort.

Na een bloedmaaltijd minimaliseren vrouwelijke muggen hun gastheerzoekgedrag en

rusten tijdens rijping van hun eieren. Om te onderzoeken of de gevoeligheid van olfactorische neuronen veranderde na een bloedmaaltijd en of deze veranderingen correleerden met de waargenomen gedragsverandering, vergeleken we de reacties van ORN's in sensilla trichodea en grooved peg sensilla 2 – 24 uur na een bloedmaaltijd met die van muggen die geen bloedmaaltijd hadden gehad. (**Hoofdstuk 9**). Na een bloedmaaltijd werden drie in plaats van twee functionele typen sensilla trichodea E gevonden. Een functioneel type dat niet was ontdekt in muggen die van bloed gedepriveerd waren, werd herhaaldelijk ontdekt. Het meest gevoelige neuron van het 'nieuwe' functionele sensillum-type liet een hoge gevoeligheid zien in reactie op indool. Dit neuron vertoonde ook sterke reacties op C6-C9 carboxylzuren en was middelmatig gevoelig voor de verbindingen 7-octeenzuur en 3-methyl-2-hexeenzuur. Deze resultaten leveren een aanwijzing dat veranderingen in gevoeligheid en reactiespectrum van ORN's als gevolg van een bloedmaaltijd een rol spelen in de modulatie van gedrag in vrouwtjes van *An. gambiae*.

De belangrijkste conclusies van dit proefschrift kunnen als volgt worden samengevat. Het proefschrift verschaft aanvullende bewijzen dat chemische stimuli een aanzienlijke rol spelen in de aantrekkelijkheid van gastheren voor *An. gambiae* (Hoofdstuk 2) en dat vluchtige stoffen afkomstig van de huid op zich een significante bijdrage leveren aan de verschillen in aantrekkelijkheid tussen individuele mensen (Hoofdstuk 3). The GC-EAG methode kan ingezet worden in de detectie van kairomonen die *An. gambiae* gebruikt, maar een geschikt substraat om de geuren te verzamelen is essentieel (Hoofdstuk 4). Synergisme tussen ammonia, melkzuur en een mengsel van carboxylzuren in de aantrekking van vrouwtjes van *An. gambiae* werd aangetoond (Hoofdstuk 5) en olfactometrische studies lieten de dosis-afhankelijke effecten op *An. gambiae* zien van andere menselijke geurcomponenten dan ammonia en melkzuur (Hoofdstuk 6). De resultaten van onze veldstudie leverden bewijs dat muggenvallen waarin mengsels van 4 - 9 verbindingen waren aangebracht aanzienlijk beter werkten dan vallen waarin een mens geurstoffen produceerde, hetgeen een groot potentieel van deze vallen in toekomstige malaria-bestrijdingsprogramma's suggereert (Hoofdstuk 7). Gebaseerd op de reactie op verschillende verbindingen werden olfactorische receptor neuronen geclassificeerd in verschillende functionele groepen, hetgeen fundamentele informatie verschaft voor toekomstig onderzoek aan deze neuronen (Hoofdstuk 8). Kwalitatieve en kwantitatieve veranderingen werden gevonden in de reacties van olfactorische neuronen voor en na een bloedmaaltijd, hetgeen doet vermoeden dat het perifere zenuwstelsel een rol vervult in de modulatie van het gedrag van muggen die in verschillende fysiologische fasen verkeren (Hoofdstuk 9).

中文摘要

疟疾是危害人类生命和健康的最严重疾病之一。 至今，每年仍有三到六亿人受疟疾感染，而死于疟疾的儿童人数则高达一百万以上，全世界百分之九十以上的病例发生在非洲。由于非洲疟蚊(*Anopheles gambiae* Giles *sensu stricto*) 具有专嗜人血、常栖息在人类的住所内并适于孳生和繁衍于房屋附近等习性，使其成为疟疾在非洲传播的主要虫媒。非洲疟蚊雌蚊主要依靠人体释放的化学气味来寻找血源寄主。本论文的主要内容是关于人体释放的化学成分在非洲疟蚊寻找寄主行为中所起的作用，以及这些气味物质如何被雌蚊嗅觉系统感受的。所获得的知识可设计含气味诱饵的诱蚊器，用于避免或减少人受蚊虫的叮咬；并取代传统的“人饵”诱捕调查种群数量的方法。

通过揉搓玻璃珠收集的人手皮肤分泌物对雌蚊具有与人手相似的引诱作用（第二章）。此分泌物挥发的气味能诱导雌蚊产生触角电位。四小时后，收集在玻璃珠上的分泌物对雌蚊的引诱作用完全消失。用玻璃珠作为收集人体气味的介质适合于对所收集的气味物质进行行为学、电生理以及化学成分分析的研究。

为研究人类个体间对疟蚊吸引力差别的化学基础，将分别从27名志愿者收集于玻璃珠上的皮肤分泌物相对于氨气于嗅觉测量器中进行二项选择测试，结果显示，不同个体的分泌物对非洲疟蚊雌蚊的吸引力存在着显著的差异（第三章）。将对疟蚊吸引力最强的三名志愿者的皮肤分泌物分别与对疟蚊吸引力最弱的三名志愿者的皮肤分泌物在嗅觉测量器中进行成对测试的结果与前一试验的结果相吻合。并非所有对疟蚊吸引力较强的皮肤分泌物都引发较强的触角电位，其可能的原因是某些对疟蚊吸引力较弱的皮肤分泌物中含有驱避物质。

实验表明，经穿着的尼龙丝袜对疟蚊雌蚊具有极强的引诱作用，应用气相色谱与触角电位联用技术对经穿着的尼龙丝袜气味进行分析。在气相色谱的23 个特定的保留时间处测量到触角电位，利用质谱分析对其中的14 个化合物进行了初步分析，发现这些化合物并非典型的人体气味 （第四章）。

过去的研究表明氨气、乳酸和十二种脂肪酸的混合物分别对非洲疟蚊具有引诱作用。因为上述化合物均存在于人体气味中，我们对这些气味的混合物在嗅觉测量器中进行了测试 （第五章）。我们的实验结果显示，氨气本身对雌疟蚊具有引诱作用；单独测定乳酸并未发现引诱作用； 而脂肪酸混合物则具有驱避作用，加入氨气能将其驱避作用抵消；乳酸和脂肪酸混合物并不能提高氨气的引诱作用；但氨气、乳酸和混合脂肪酸三者间存在增效作用。

本论文的第六章报导了应用嗅觉测量器对22种气味化合物对氨气和乳酸的增效作用进行的测试研究，所选择的化合物已被证明能引发非洲疟蚊的电生理或行为反应。结果表明三至八碳和十四碳脂肪酸在一定的浓度下能提高氨气和乳酸对疟蚊的引诱作用； 而加入醇类和酮类化合物以及吲哚则会降低氨气和乳酸的引诱作用。

基于行为学和电生理的研究结果，我们在西非的冈比亚对一系列的合成与天然的人体气味混合物的诱蚊效果进行了田间试验 （第七章）。这项试验表明，对流型诱蚊器 (CFG) 加入合成气味诱饵（最多达九种气味）在室

外试验的大多数情况下较从住人帐篷中散发出的气味诱集到更多的蚊虫。其中一种合成气味：氨气+ 乳酸 + 二氧化碳 + 3-甲基丁酸，对几乎所有种类蚊虫均在十六种供试气味中具有最强的引诱作用，该合成气味在蚊虫治理工程中具有很大的应用潜力。加入二氧化碳能明显提高CFG 诱蚊器的诱蚊效果。室内实验结果表明一种合成气味（包含：氨气+ 乳酸 + 二氧化碳 + 犄牛儿基丙酮 + 吲哚 + 4-乙基酚）对非洲疟蚊较对照气味（氨气+ 乳酸 + 二氧化碳）具有更强的引诱效果。该气味值得在疟蚊种群密度更高的条件下（如靠近村庄）进行进一步的测试研究。

本论文的第八章报导了利用单细胞纪录技术对非洲疟蚊雌蚊嗅觉系统对人体气味感受进行的研究。 根据嗅感神经对44 种气味的反应， 可将毛形感器分为六种功能类型，将锥形感器分为五种功能类型。对多种气味产生反应的“广谱型”的嗅感神经分布于E 型毛形感器内；而反应谱较窄的嗅感神经则分布于C 型毛形感器或锥形感器内，其中包括两类“专一型”的嗅感神经仅对一种供试气味产生反应。E 型毛形感器与C 型毛形感器和锥形感器的气味反应谱之间存在部分交迭；但C 型毛形感器和锥形感器的气味反应谱除氨气外不存在交迭。在同一嗅感器内的不同嗅感神经一般具有相似的气味反应谱但反应敏感度不同。分布于锥形感器内的嗅感神经均调谐于极性较强的气味化合物， 包括能引发非洲疟蚊寻觅行为的氨气及其增效化合物——乳酸， 后者的受体神经仅在锥形感器内发现。酚类化合物是几种嗅感神经类型的最有效的刺激气味。非洲疟蚊雌蚊嗅觉系统对人体气味感受主要通过“交叉纤维编码”的编码方式来完成。

雌蚊吸血后便停止觅食行为静待卵的成熟。为了调查雌蚊嗅感神经在吸血后对气味敏感度的变化，以及这些变化是否与 雌蚊行为的变化相关联，我们比较了雌蚊吸血前后毛形感器和锥形感器内的嗅感神经对气味的反应（第九章）。不同于吸血前雌蚊的E 型毛形感器包含两种功能类型，吸血后的雌蚊同类嗅感器包含三种功能类型， 其中一种新的功能类型在未吸血雌蚊中从未被发现过。新的功能类型的嗅感神经对吲哚极其敏感，而且对六至九碳脂肪酸也产生很强的反应。 值得注意的是，新的功能类型的嗅感神经对人体“特异”气味7-辛烯酸和3-甲基-2-己烯酸也产生反应。这些结果指示嗅感神经在吸血前后对气味反应谱和敏感度的变化参与对雌蚊行为的调控。

本论文的主要结论可总结如下：本论文中所提供的证据进一步证明化学气味在非洲疟蚊寻找血源寄主行为中起着重要的作用（第二章），而且皮肤分泌物对人与人个体之间对疟蚊吸引力的差别起着重要作用（第三章）。气相色谱与触角电位联用技术可用于探测非洲疟蚊寻找寄主行为所利用的种间信息素，但更为适宜的收集气味的方法有待进一步探索（第四章）。氨气、乳酸和混合脂肪酸对非洲疟蚊的引诱具有增效作用 （第五章）；嗅觉测量器的研究显示某些脂肪酸在一定的浓度下能提高氨气和乳酸对疟蚊的引诱作用； 而加入醇类、酮类、酚类化合物以及吲哚则会降低氨气和乳酸的引诱作用（第六章）。田间试验结果显示，对流型诱蚊器 (CFG) 加入合成气味诱饵较从人体散发出的气味诱集到更多的蚊虫，表明了这类诱蚊器 在控制疟疾传播中的巨大潜力（第七章）。根据嗅感神经对气味的反应谱，将嗅感器分为不同的功能类型，为对非洲疟蚊嗅感神经进行进一步的研究打下了基础 （第八章）。雌蚊嗅感神经在吸血前后对气味敏感度有着质与量的变化，提示周边神经系统在疟蚊调控不同生理阶段中的行为反应中可能起着重要的作用 （第九章）。

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Qiu, Y.T., Smallegange, R.C., van Loon, J.J.A. & Takken, W. Interindividual variation in odour-mediated attractiveness of humans to the malaria mosquito *Anopheles gambiae sensu stricto* (conditionally accepted).

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Publications to be submitted

Qiu, Y.T., Loon J.J.A. van, Takken, W., Meijerink, J. Olfactory coding in antennal neurons of the malaria mosquito, *Anopheles gambiae*.

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Qiu, Y.T., Smallegange, R.C., van Loon, J.J.A. & Takken, W. Host seeking behaviour of the malaria mosquito *Anopheles gambiae* in response to synthetic odour blends.

Qiu, Y.T., Smallegange, R.C., ter Braak, C.J.F., Spitzen, J., Jawara, M., Milligan, P., van Loon, Galimard, A.,J.J.A., van Beek T., Takken, W. & Field studies on the response of *Anopheles gambiae* to natural and synthetic host volatiles in The Gambia.

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Curriculum Vitae

On 12 July 1966 I, Yu Tong Qiu (邱宇彤), was born in Xilinhot, Nei Monggol, China. I finished my high school education in 1982 and went to study Plant Protection in Hebei Agricultural University. I was inspired by the lecture of Prof. Chiu Shin-Foon in 1985 and went to South China Agricultural University (SCAU) for MSc and PhD study, after my graduation in 1986. The subject of my study supervised by Prof. Chiu Shin-Foon was about the mechanism of insecticide resistance of diamondback moth (*Plutella xylostella*) and the effectiveness of botanical insecticides and insect growth regulators. During my PhD study I was a part time teaching assistant in the Laboratory of Insect Toxicology. After receiving my PhD degree in 1991, I was appointed lecturer in Plant Protection at SCAU. I taught the course "Rice insect pest management" and "Botanical insecticides and their mode of action". Representing the Plant Protection Department, I was appointed as technical manager in Dongguan Shilong Aseptic Cultured Plant Co. LTD for half a year in 1992.

In October 1994, with kind help of Prof. Louis Schoonhoven, I came to the Laboratory of Entomology, Wageningen University, as a visiting scholar funded by the Royal Netherlands Academy of Arts and Sciences. Dr. Joop van Loon guided me through the study of chemosensory receptors for oviposition deterrents of diamondback moth. From 1996-1999, I joined the research project on the evaluation of criteria for the selection of natural enemies of the whitefly, *Bemisia tabaci*, under leadership by Prof. Joop van Lenteren.

My acquaintance with medical entomology started in February 2000, when I began with my second PhD fellowship under the supervision of Dr. Joop van Loon, Dr. Willem Takken and Prof. Joop van Lenteren. In the five-year period, I studied human odours that are attractive to the malaria mosquito *Anopheles gambiae*. I investigated the behavioural and electrophysiological activities of compounds that were found in highly attractive human odours. Behavioural studies were first carried out in an olfactometer in the laboratory and then in the field in The Gambia to evaluate the effectiveness of these odours as lure to increase the effectiveness of mosquito traps. In order to understand the odour perception in the peripheral nervous system, I used the single sensilla recording method to study the neuron coding of olfactory receptor neurons to human odours.

From August to October 2005, I studied the electrophysiological activities of putative pheromone components of the malaria mosquitoes; the project was funded by the International Atomic Energy Agency, under leadership of Dr. Bart Knols.

Since November 2005, I work as a Post-doc for neurobiology in the Laboratory of Entomology within the Grand Challenge project funded by Bill-Melinda Gates foundation.

PE&RC PhD Education Statement Form

With the educational activities listed below the PhD candidate
has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 22 credits (= 32 ECTS = 22 weeks of activities)

Review of Literature (3 credits)

The role of olfaction in the behaviour of the malaria mosquito *Anopheles gambiae* (2005)

Post-Graduate Courses (7 credits)

Sensory ecology (2000)
Advanced statistics (2001)
Multivariate analysis (2005)

Deficiency, Refresh, Brush-up and General Courses (5 credits)

Basic statistics (2000)
Techniques for scientific writing and presentation (2001)
Written English (2001)
Scientific writing (2003)
Career orientation (2004)

PhD Discussion Groups (5 credits)

AiO discussion group Entomology (2000-2005)

PE&RC Annual Meetings, Seminars and Introduction Days (0.75 credits)

PE&RC annual meeting: "Ethics in Science" (2002)

International Symposia, Workshops and Conferences (5 credits)

3rd International congress of vector Ecomology (2001)
19th Annual meeting of the ISCE (2002)
8th European symposium for insect taste and olfaction (2003)
14e Entomologendag (2003)
15e Entomologendag (2004)

Laboratory Training and Working Visits (1 credit)

Laboratory training. Chemical Ecology Group, SLU, Alnarp, Sweden (2003)

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